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ABSTRACT

Purines 2018 Basic and Translational Science on Purinergic Signaling and its Components for a Healthy and Better World

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The Brazilian Purine Club and Introduction of the Event:

The Brazilian Purine Club founded in 2009 with the objective of gathering scientists interested in purinergic signaling and of organizing congresses focuses on the latest developments in this area. Seven congresses were held in the period from 2010 to 2017, with the congresses in 2010, 2013 and 2015 being international with the presence of a great number of renowned speakers from abroad. The Brazilian Purine Club in view of many new memberships, scientific publications and Ph.D. theses developed in this area, has developed to international recognition and was nominated for the organization of the world congress Purines 2018.
Purines 2018 International - Basic and Translational Science on Purinergic Signaling and its Components for a Healthy and Better World was held from June 19 - 22, 2018 in the Brazilian town of Foz do Iguaçu in the State of Paraná. The program was developed by the board of the Society (the President Dr. Henning Ulrich, University of São Paulo, the Vice-President Ana Maria O. Battastini, Federal University of Porto Alegre and the General Secretary Ana L. M. Ventura, Fluminese Federal University and the Past-President Dr. Robson Coutinho Silva, Federal University of Rio de Janeiro, with the support of the council, the local organizing committee and the international appointed Scientific Committee.
Plenary lectures focused on biology and signaling of ectonucleotidases and P1 and P2 receptors and structural properties of these proteins. The opening session honored Prof. Geoffrey Burnstock, who was unable to attend the conference due to health reasons.
The Opening Conference held by Prof. Christa Müller, University of Bonn, Germany, was followed by seven plenary lectures, the Closing Conference held by Prof. Henning Ulrich, University of São Paulo, 38 symposia and a workshop on publishing in scientific journals, a session of oral communication and 112 presented posters. The participants came from 25 countries and 4 continents. Around one third of the attendees were undergraduate and graduate students, demonstrating the importance of the congress for the formation of researchers in the field of purinergic signaling.

ABSTRACTS

OPENING LECTURE:

Drugs for purinergic targets

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Purine and pyrimidine derivatives, such as the nucleotides ATP, ADP, UTP, and UDP, the nucleoside adenosine and the nucleobase adenine, are important signaling molecules, which activate membrane receptors termed P0 (adenine receptors), P1 (adenosine receptors), P2Y and P2X (nucleotide receptors). P0, P1 and P2Y receptors are G protein-coupled, while P2X receptors are ATP-gated ion channels.[1] There is a metabolic link between P1 and P2 receptor agonists since the nucleotides ATP and ADP (P2 agonists) are hydrolyzed by various ectonucleotidases producing the P1 agonist adenosine. While ATP is a danger signal mediating pro-inflammatory effects, adenosine acts as a stop signal inducing anti-inflammatory and immunosuppressive activities. Despite decades of research, only few drugs have been approved that interact with purine receptors, most prominently the $P2Y_{12}$ receptor antagonists (clopidogrel, prasugrel, cangrelol, ticagrelor) which have become an important class of antithrombotic drugs.[2] Recently, new hypes and hopes have been created in the field, due to (1) the successful clinical trials for the P2X2 receptor antagonist gefapixant in chronic cough, an inflammatory condition, (2) the advancement of the partial A_1 adenosine receptor agonist neladonoson bialanate into phase III clinical trials for heart failure, and, most imporantly, (3) the gold rush fever in immuno-oncology. Blockade of A_{2A} and A_{2B} adenosine receptors and/or inhibition of adenosine formation by blocking ectonucleotidases, such as CD39 or CD73, are being pursued as novel principles that activate the immune system to defeat cancer. In this context, our group has recently shown that the A_{2B} adenosine receptor, which is typically upregulated under hypoxic conditions, i.e. in inflammation and cancer, forms stable heteromeric complexes with the A_{2A} receptor subtypes and thereby completely blocks A_{2A} receptor signaling.[3] This finding will likely have implications for the development of drugs.

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PLENARY LECTURE 1
Purinergic Optopharmacology: Beyond the light

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G protein-coupled adenosine receptors are promising therapeutic targets for a wide range of pathological conditions. However, the ubiquity of adenosine receptors and the ultimate lack of selectivity of certain adenosine-based drugs have frequently diminished their therapeutic use. Optopharmacology is a novel approach that allows the spatiotemporal control of receptor function, thus circumventing some of these limitations. Accordingly, we developed light-sensitive adenosine receptor-based drugs to photocontrol receptor's function both in vitro and in vivo. For instance, MRS7145, the first photo-controlled A2AR antagonist, can bind and block A2AR in a light-dependent fashion in living cells. Interestingly, upon local brain irradiation, MRS7145 allows the fine control of spontaneous locomotor activity and reversal of pharmacologically-induced Parkinsonian-like behavior. Thus, we demonstrated that this compound can be effectively photo-delivered in the striatum of rodents, increasing locomotor activity while reverting pharmacologically-induced parkinsonian-like symptoms. Collectively, we show here a proof of concept to design novel optopharmacological approaches for the management of adenosine receptor related disorders.
Keywords: Adenosine receptors; Optopharmacology; Parkinson's disease.

PLENARY LECTURE 2
Glial P2X7 receptors are indispensable mediators of the necrotic/apoptotic effect of ATP on neurons

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P2X7 receptors (Rs) have a widespread distribution in the peripheral and central nervous system. It is generally accepted that they occur on Schwann cells in the PNS and astrocytes/microglia in the CNS, however, recently there is increasing evidence that they are absent on neurons. Their stimulation by high local concentrations of ATP induce apoptosis in glial cells via the activation of the caspase pathway, and necrosis via opening of membrane pores and the subsequent loss of cell constituents of vital significance. There is also strong evidence that P2X7Rs release gliotransmitters such as glutamate and ATP itself, and inflammatory mediators such as interleukin-1β, reactive oxygen intermediates and endocannabinoids. All these astrocytic/microglial mediators cause indiscriminate neuronal damage, in a similar manner as the neurotoxic glutamate (and probably ATP) of glial origin does. Experiments with two classic P2X7R-deleted mice did not provide unequivocal arguments for or against neuronal P2X7Rs, because several splice variants and small nucleotide polymorphisms of the receptor escaped inactivation by genetic deletion. New approaches such as the use of a transgenic Tg(P2X7-EGFP) mouse expressing EGFP under the control of the P2X7R promoter, and a conditional P2X7-knockout mouse in which the receptor can be selectively switched off in neurons, also fail to supply convincing arguments. Neuronal effects can be also initiated indirectly by the stimulation of P2X7Rs situated at adult neural progenitor cells (NPCs). In the subventricular zone of the lateral ventricle and the subgranular zone (SGZ) of the hippocampus, radial glia-like NPCs reside which produce mature neurons during the whole life of an individual. P2X7Rs at the SVZ NPCs are targets of a massive release of ATP occurring during status epilepticus. This may interfere on the one hand with hippocampal learning processes and on the other hand may promote the ectopic settlement of granule cells in the hilus hippocampi, considered to be a reason for the manifestation of chronic seizures after a one time status epilepticus in childhood. Thus, necrosis/apoptosis of NPCs may be both deleterious and beneficial, respectively. Pathological neuronal activity of granule cells in the dentate gyrus may result in the release of glutamate, in addition to that of ATP, and could kill overtly produced NPCs by the simultaneous activation of P2X7- and NMDA/AMPA-Rs. At a more protracted time axis, the release of the neuroinflammatory interleukin-1β may add up to the effects of ATP/glutamate. In conclusion, apoptotic/necrotic P2X7Rs are probably situated at glial rather than neuronal cells and thereby secondarily damage neurons during various neurodegenerative illnesses. However, their location at NPCs may have beneficial effects in the case of limbic epilepsy.
Keywords: Glial cells; P2X7 receptors.

PLENARY LECTURE 3
Regulation of cellular energy balance by the P2X7 receptor

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The P2X7 receptor (P2X7R) is emerging as one of the key plasma membrane receptors involved in the promotion of inflammation (Di Virgilio et al., 2017, Di Virgilio et al., 2018a). Lately, it has also become clear that this receptor has a strong trophic effect on cell metabolism, promotes growth and support tumor progression di virgilio (Di Virgilio and Adinolfi, 2017, Di Virgilio et al 2018b). This is in striking contrast with the established opinion built up over the years that held the view that the P2X7R was/is a cytotoxic receptor. We previously reported that basal/tonic activation of the P2X7R by locally-released ATP had a strong effect on mitochondrial metabolism as well as on aerobic glycolysyis (Adinolfi et al., 2005, Amoroso et al., 2012), thus providing a metabolic basis for the trophic/growth-promoting effect. Our data show that the P2X7R localizes to the mitochondria and deeply affect mitochondrial matrix Ca^{2+} concentration, Respiratory Complex I (NADH Coenzyme Q oxidoreductase) expression and function. These findings open an entirely novel perspective on its pathophysiological function and give new impetus to the development of novel drugs targeting the P2X7R.

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Keywords: P2X7; Cancer; Inflammation; Mitochondria.

PLENARY LECTURE 4
NTPDase8 prevents intestinal inflammation by blocking P2Y receptor activation

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We have recently identified, cloned and characterized NTPDase8 as the last member of the E-NTPDase family. We found here that it was expressed at the luminal surface of intestinal epithelial cells in both human and mouse where it is responsible for the ectonucleotidase activity of these cells. As extracellular nucleotides, the substrates of NTPDase8 are generally proinflammatory and as the product of nucleotide hydrolysis by NTPDase8 and CD73, adenosine, is a potent anti-inflammatory compound we hypothesized that NTPDase8 could protect the intestine from inflammation in murine models of colitis. To this end we have generated mice deficient for its expression. Entpd8-/- mice showed exacerbated intestinal inflammation in the DSS model of colitis which correlated with increased levels of proinflammatory cytokines, increased intestinal permeability and increased histopathological damage. The increase of inflammation correlated with increased activation of a P2Y receptor. Bone marrow transplantation studies demonstrated that this receptor expressed in non-hematopoietic cells, namely in intestinal epithelial cells, was responsible for the increased inflammation. Furthermore, the administration of either NTPDase8 activity or a P2Y receptor antagonist by intra-rectal administration totally protected inflammation in the DSS model of colitis. In conclusion, these data show that NTPDase8 protects the intestine from inflammation by preventing nucleotide receptor activation. In addition, the implementation of either NTPDase8 activity or P2Y receptor blockers can be used to prevent inflammation induced by endogenous nucleotide secreted in the lumen of the intestine in inflammatory conditions. This treatment can be potentially applied to patients that suffer from inflammatory bowel diseases which we are actually investigating.

Keywords: NTPDase; P2Y receptors; inflammatory bowel diseases.

PLENARY LECTURE 5
P2X7 receptor in inflammatory diseases: Angel or demon?

Robson Coutinho Silva
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P2X7 receptor has been the most studied P2 receptor in immune cells, being involved in both innate and adaptive immune responses. Depending on its level of activation and cell type studied, P2X7 receptor activation induces cell death, proliferation and activation, as well as, phagocytosis of apoptotic bodies, cytokine secretion, and parasite control. P2X7 receptor activation can induces large-scale ATP release via its intrinsic ability to form a membrane pore, or in association with other membrane channels, amplifying immune responses. In innate immune cells, this purinergic receptor is associated with inflammasome activation, maturation and secretion of pro-inflammatory cytokines and production of reactive oxygen and nitrogen species. During adaptive immune response, P2X7 receptor is required for T cell activation by mediating calcium influx and IL-2 production. In addition, it favors the generation of Th17 lymphocytes in detriment of T regulatory ones. Therefore, the participation of P2X7 receptor in inflammatory and parasitic diseases is a kind of puzzle. Depending on the inflammatory context, the activation of P2X7 receptor can culminate in different and contrasting effects from beneficial to detrimental responses. The outcome that results from P2X7 receptor activation seems to depend on its level of activation, cell studied, and type of pathogen, virulence, or severity of infection. It will be discussed the P2X7 receptor dual function, and its pharmacological modulation in the context of inflammatory and parasite diseases. Funds. CNPq, FAPERJ

Keywords: danger signal; IL-1b; inflammation; parasite control.

PLENARY LECTURE 6
In the Eye of the Storm: Adenosine, Cartilage and Arthritis

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Adenosine is generated extracellularly from ATP released from cells and adenosine can engage its cell surface receptors to regulate the function and health of many different tissues and organs. With age and inflammation cells have lower levels of ATP and, in some tissues, release lower levels of ATP (inflammaging) resulting in lower extracellular adenosine levels. We have recently discovered the role that inflammaging plays in chondrocyte and cartilage aging and degeneration by reducing extracellular adenosine levels, leading to diminished adenosine A2A receptor occupation resulting in cartilage and chondrocyte degeneration.

PLENATY LECTURE 7
Dynamics of purinergic signalling in the hearing organ

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The cochlea is one of the most highly differentiated tissues in the body and the sense of hearing it provides spans the broadest dynamic range of our sensory systems. Purinergic signalling is integral to the establishment of the hearing organ, the regulation of the exquisite sensitivity of sound transduction and auditory coding, and preservation of hearing function across a lifetime. This occurs through the precise spatiotemporal regulation of expression of the purinergic signalling elements, the P1 and P2 receptors, and the ectonucleotidases that regulate the local signalling via extracellular purines and pyrimidines. The localization of these proteins to specific regions of the sensory hair cells, supporting cells in the organ of Corti, the cochlear partition, and to the spiral ganglion auditory neurons, are integral to the complementarity of P1 (A1, A2a/b, A3) and P2X / P2Y receptor signalling. Studies with transgenic mice have yielded considerable insight into the roles of these signalling effectors. A major "theme" of purinergic signalling in hearing function is oto-protection, where activation of the A1 receptors, for example, using ADAC – adenosine amine congener - after noise trauma rescues the sensory hair cells (Vlajkovic et al. Biomed Res Int, 2014, 841489). Similarly, while P2X receptor subunit diversity is broad in the cochlea, P2X2 receptor expression in the cochlear partition is central to purinergic hearing adaptation. These P2X2 receptor – type ATP-gated ion channels are activated by sustained elevation of sound levels and act to preserve hearing function which would otherwise be irreversibly lost; P2rx2 null mice fail to adapt to moderately loud sound (Housley et al., 2013, PNAS 110:7494) and these mice, and humans with loss of function mutations, are vulnerable to noise-induced hearing loss, and accelerated hearing loss with ageing (Yan et al., 2013, PNAS 110:2228). This pronounced pathophysiology associated with disruption of purinergic signalling across mice and humans is indicative of the significance of purinergic regulation of hearing for maintenance of normal hearing. Indeed, the adaptation profile of the outer hair cell - derived 'cochlear amplifier' as sound levels rise, may well reflect the innate capacity of cochlear purinergic signalling to confer resistance of a person's ears to damage from acoustic over-stimulation. Supported by National Health & Medical Research Council project APP1089838.
Keywords: Cochlea; hearing loss; P1 & P2 receptors; ATP-gated ion channels.

PLENARY LECTURE 8
Therapeutic potential of CD39

Simon C. Robson
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Purinergic signaling is a central component of inflammation, and involves responses to extracellular nucleotides and adenosine, which are tightly regulated by ectonucleotidases. Since the P2-receptors, which bind ATP and ADP and the receptors of adenosine often transduce opposite effects, resulting cellular responses are attributable to both the ratio of ADP and ATP to adenosine concentrations and the relative levels of expression and signaling intensity of the specific receptors. Major molecular pathways of extracellular nucleotide phosphohydrolysis involve two-step enzymatic processes, which are regulated by ectoenzymes. In the first steps of the major canonical pathway, ATP and ADP are converted to AMP through the ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and family members. An alternative non-canonical pathway centered upon CD38 has been also proposed, which converts NAD+ to ADPR by the linked ADP ribosyl-cyclase catalytic function. This product of ADPR then generates AMP following catalysis by ectonucleotide pyrophosphatase/phosphodiesterase-1 CD203a/PC-1. In both pathways, the extracellular generation of adenosine requires ecto-5′-nucleotidase (CD73), which converts extracellular AMP to adenosine. Aberrant purinergic signaling pathways have been implicated in disordered thromboregulation and immune dysregulation, as noted in both transplant vascular injury and the pathogenesis of inflammatory bowel disease and other gastrointestinal/hepatic autoimmune diseases. In these settings, genetic inheritance and environmental factors closely regulate the levels of expression and phosphohydrolytic activity of CD39, both on immune cells and released microparticles or exosomes. We have recently shown that upregulation of CD39 is dependent upon ligation of the aryl hydrocarbon receptor (AHR), as with xenobiotics or natural ligands such as bilirubin and 2-(1′ H-indole-3′-carbonyl)-thiazole-4- carboxylic acid methyl ester (ITE.) Boosting CD39 ectonucleotidases expression by such treatment modalities or administration of soluble ectonucleotidases has the potential to improve inflammatory vascular and immunological disorders, particularly in transplanted grafts subject to ischemia reperfusion and in patients with low level, endogenous expression of these crucial ectonucleotidases. In contrast, blocking CD39 can augment host cellular responses and alter vascular homeostasis. This has the impact of bolstering immunostimulatory effects, as in overcoming immune exhaustion in chronic viral infection and augmenting responses to the release of ATP as in the setting of chemotherapy of cancer. This presentation will cover novel strategies for manipulating the tumor microenvironment by the inhibition of ectonucleotidases of CD39 family, which provide promise for potential future cancer treatments. New developments in this arena of purinergic signaling will open up several new avenues for the treatment of patients with inflammatory diseases and cancer.

CLOSING LECTURE

Purinergic Signalling in neuronal differentiation and neurodegeneration: From in vitro studies towards therapeutic applications

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Purinergic receptors have been shown to be important for development and tissue regeneration. Here, we have explored the participation of purinergic receptors in neural differentiation. In pluripotent stem cells, P2Y1, P2Y2 and P2X7 receptors participate in the maintenance of pluripotency and proliferation as well as in the increase of the number of glial cells. P2Y2, P2Y6 and P2X2 receptor expression and activity participated in stem cell differentiation into neuronal lineages. P2X7 receptor expression and activity diminished in cells undergoing neurogenesis. On the other hand, P2X7 receptor expression was important for gliogenesis. Ascl1 transcription factor expression levels, important for neuronal fate determination, declined when neural stem cells (NSC) had been exposed to the P2X7 receptor agonist Bz-ATP, as revealed by time-lapse imaging. P2Y2 receptor stimulation by 2-thio-UTP, on the other hand, induced continuous Ascl1 expression. The increase in Ascl1/Neurog2 expression ration by P2Y2 receptor activation was important for GABAergic fate specification. The P2X7 receptor is believed to be involved in neurodegenerative diseases, and its inhibition presents a promising strategy for prevention of neuroinflammation and neuronal cell death. Further, it is suggested that P2X7 receptor activity could prevent endogenous NSC from mobilization and differentiation. An animal model of Parkinson's disease was used to further address this question. For this purpose, unilateral hemisphere lesions of the nigrostriatal pathway of adult male Sprague-Dawley rats were induced by stereotactic injection of 6-hydroxydopamine (6-OHDA). One week after lesion, the animals presented rotational behavior when challenged with apomorphine. Treatment with Brilliant Blue-G (BBG), an antagonist of P2X7 receptor, had beneficial functional effects. Animals injected with 6-OHDA, which in the following received BBG during 7 days at a 50mg/kg dose, showed a statistically significant decrease in the number of rotations per minute, whereas animals receiving only saline did not reveal any significant improvements in rotational tests. In agreement, levels of regeneration of dopaminergic neurons in BBG-treated animals, as revealed by anti-tyrosine hydroxylase staining of the substantia nigra, were prominent when compared to those observed for the saline-treated control group. Therapeutic effects remained following cessation of BBG treatment. Antagonism of P2Y6 receptors partially prevented dopaminergic deficit in the substantia nigra, defining a new target for combating Parkinson's disease. Summing up, the here shown results of in vitro and in vivo studies point at the importance and various functions of purinergic receptors in neurogenesis and neurodegeneration with novel therapeutic applications. Support: Grants and student fellowships awarded by FAPESP, CNPq and CAPES (Brazil).

DIAMOND SPONSOR LECTURE

TissueFAXS Cytometry – Applications in Science and Diagnosis

Rupert Ecker
TissueGnostics, Vienna, Austria

Determining the in-situ immune status of diseased organs or quantify coexpression of molecules on the single-cell level has mostly been subject to visual estimation, or – at best – to manual counting for decades. Hence, experts usually had the choice of the "least of evils" between *guessing* and *endless (manual) counting*. In tumor immunology, infiltrating inflammatory cells need to be phenotypically characterized on a quantitative basis. To better understand the function of inflammatory cells in tumor development, type and number of inflammatory cells and their proximity to glandular/tumor structures have to be analyzed in-situ and correlated with disease state. Using TissueFAXS™ Cytometry the time-consuming and error-prone human evaluation of stained histological sections can be approached with an observer-independent and reproducible technology platform, offering a high degree of automation, paired with user interaction at relevant points of the analytical workflow. This platform can be applied as a means of tissue cytometry for both immunofluorescence and immunohistochemistry and thus constitutes the microscopic equivalent to flow cytometry (FACS). Likewise FACS, TissueFAXS™ can quantify any type of molecular marker in any type of cell – but in tissue context or in adherent cell culture monolayers without the need to solubilise the cells (i.e. TissueFAXS permits analyses *in-situ*)! The TissueFAXS Cytometry platform can be used in clinical multi-center studies to determine the immune response to certain drugs *in-situ*, measure proliferation, apoptosis, cytokine expression, signalling molecules, and others. It can do end-point assays as well as live-cell imaging and time-kinetic experiments. But TissueFAXS Cytometry also promotes tissue cytometry to a new level of quality, where complex cellular interactions can be addressed on the single-cell level but still in histological context.

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SYMPOSIUM 1: Purinergic signaling in parasite-caused diseases

1. Role of purinergic signaling in human and experimental Chagas disease

Maria Pilar Aoki
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Background and Objective: Chagas disease, caused by *Trypanosoma cruzi* infection, persists as the major infectious heart disease worldwide. After infection innate and adaptive immune response control parasite replication but fail to completely clear the infection, and most individuals remain infected for life. One potential regulatory system that could counteract microbicidal effector functions is the extracellular level of CD73-derived adenosine (ADO). Thus, we focused on the impact of CD39/CD73/ADO pathway on experimental Chagas cardiomyopathy. Furthermore, we aimed to explore ATP catabolic machinery in human pathology.Methods and Results: To this aim, infected BALB/c mice were treated with CD73-specific inhibitor APCP for 3 consecutive days starting 4 days post-infection (dpi). CD73 inhibition lengthen the microbicidal M1 macrophage (Ma) profile within infected myocardium by avoiding the premature shift from M1 phenotype (CD11b+F4/80+CD86+CD206-) toward M2 Ma (CD11b+F4/80+ CD86-CD206+) observed in non-treated mice at 7dpi. Accordingly, APCP treatment induces a significant increment in cardiac M1-related cytokines (IL-1β, IL-6 and TNF), nitric oxide production and diminishes the frequency of IL-10-producing CD4+ T cells compared with non-treated mice. This potent microbicidal response accounted for decreased parasite load and the improvement of cardiomyopathy (Ponce et al. J Immunol 197; 814, 2016). Strikingly, infected CD73-deficient mice exhibited an enhanced cardiac anti-parasite immune response but increased parasitemia in comparison C57BL/6 (WT) mice, indicating that there would be a niche for parasites outside myocardium. Indeed, purinergic signaling balance (ATP/ADO ratio) had a very low impact on liver and adipose tissue immune response and as such, these tissues constitute permissive targets for *T. cruzi* replication in CD73KO mice which have increased basal VAT/body weight. In order to found key cytokines able to modulate the ATP catabolic enzymes, we observed a significant diminution in the frequency of CD39+ leukocytes infiltrating myocardium in IL-6KO mice compared to WT mice. In agreement, the percentage of CD39+ bone marrow derived Ma and the frequency of CD39+ human monocytes from infected patients increase upon IL-6 stimulation (Sanmarco et al. Biochim Biophys Acta 1863, 857, 2017). Regarding adaptive immune response, in Chagas disease patients (age 25–60, n=22) the frequency of CD39+ and CD73+ CD8+ T-cells is significantly diminished in comparison to control donors (age 25-48, n=24) (Sanmarco et al. Front Immunol 7; 626, 2016) (Ethics Committee approval 194/2014 acta). This is associated with a decreased cytotoxic T-cell functions and increased inflammatory state.Conclusions: The results suggest that ATP catabolic machinery is functionally involved in innate and adaptive immune response against *T. cruzi*, and the balance among purinergic metabolites drive the outcome of cardiomyopathy. Fundings: SECyT-UNC; ANPCyT-FONCyT; CONICET.
Keywords: CHAGAS CARDIOMYOPATHY; CD39; CD73; IMMUNE RESPONSE

2. Endothelial P2Y receptors and NTPDases 2 and 3 contribute to leukocyte adhesion during schistosomiasis

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Schistosomiasis is a neglected disease. Currently, more than 200 million people worldwide suffer from chronic schistosomiasis. It is caused by an intravascular parasite (Schistosoma mansoni) and therefore, endothelial cells are an early target of the disease. Endothelial cells are sources of extracellular purines and express P2 purinoceptors and ectonucleotidases (NTPDases) which, in turn, modulate endothelial cell phenotype. Mesenteric endothelial cells from infected mice showed an increased expression of NTPDases 2 and 3 as compared to controls, and a higher extracellular ATP hydrolysis and ADP formation, an endogenous agonist of P2Y1 receptors (P2Y1R). Leukocyte adhesion to mesenteric endothelial cells in vitro and leukocyte transmigration in vivo were higher in the infected than in the control group. In vitro, the pharmacological stimulation of control endothelial P2Y1R with 2-MeSATP mimicked the data observed in the infected group. The knockdown of endothelial P2Y1R or blockage with MRS2179 prevented the agonist effect, and also reduced basal monocyte adhesion in the infected group, suggesting an autocrine modulation of endothelial cells by ADP. Additionally, preliminary data also pointed to a pro-inflammatory role of UTP acting on endothelial P2Y2R, but not of UDP acting on P2Y6R. In conclusion, current data add to the understanding of the endothelial purinergic signaling contribution to schistosomiasis pathogenesis. Mesenteric endothelial cells are primed by disease to a pro-inflammatory phenotype characterized by an increased expression of NTPDases 2 and 3. This condition favors ADP accumulation, endothelial P2Y1R activation and leukocyte adhesion, possibly contributing to mesenteric inflammation and schistosomiasis morbidity.
Financial support: CNPq (Brazil).
Keywords: purinoceptor; NTPDase; endothelial cell; inflammation.

3. Purinergic signaling and the PfSR25 parasite GPCR-like for sensing changes in *Plasmodium* environment

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Plasmodium falciparum, the most important etiological agent of human malaria, is endowed with a highly complex cell cycle that is essential for its successful replication within the host. A number of evidence suggest that changes in parasite Ca^{2+} levels occur during the intracellular cycle of the parasites and play a role in modulating its functions within the RBC. Nucleosides and nucleotides (adenosine, ADP, ATP, UDP and UTP) bind to the so-called purinergic receptors and mediate several biological processes in most eukaryotic cells. Here we show that ATP elicits cytosolic [Ca^{2+}] increases when added to isolated P. falciparum parasites both at the trophozoite and segmented schizont stages. Addition of the purinoreceptor antagonists, KN62 and Ip5I, on parasites blocked the ATP-induced Ca^{2+} rise. Besides the compounds, the hydrolysis of ATP with apyrase added in culture drastically reduce RBC infection by parasites, suggesting a role of extracellular ATP during RBC invasion and the presence of putative purinoceptor in P. falciparum. Additionally, we show for the first time that *P. falciparum* E-NTPDase (an enzyme that hydrolyzes extracellular nucleotides and participates in invasion and as a virulence factor in many pathogenic protozoa) is relevant for parasite lifecycle as inhibition of this enzyme impairs the development of *P. falciparum* within RBCs. Serpentine receptors comprise a large family of membrane receptors distributed over diverse organisms, such as bacteria, fungi, plants and all metazoans. However, the presence of serpentine receptors in protozoan parasites is largely unknown so far. The *P. falciparum* serpentine-like receptor PfSR25 is a monovalent cation sensor capable of modulating Ca^{2+} signaling in the parasites. Deletion of PfSR25 had no effect on [Ca^{2+}]cyt in

response to changing KCl concentration in the knocked out (PfSR25−) parasites, indicating SR25 role in perceiving ionic change during erythrocyte rupture. Other possible functional roles of PfSR25 have been investigated in PfSR25− parasites during stress condition. We observed PfSR25− parasites were more susceptible to stress induced by NO production with a much larger increase in metacaspase expression when compared to wt parasites. Moreover, PfSR25− parasites appear to be more sensitive than wt cells to chloroquine induced death and to stress induced by albumax deprived medium. Finally, we observed that PfSR25− parasites show higher inhibition in hemozoin formation after piperaquine treatment in comparison to wt parasites.
Keywords: Plasmodium; Purinergic signaling; Apyrase; GPCR.

4. P2X7 drives Th1 vs Tfh cell differentiation in experimental malaria

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Malaria still causes the death of approximately half a million people yearly despite efforts to develop vaccines. The ability of *Plasmodium* parasites to survive the immune effector mechanisms indicates how suitable the immune response must be to eliminate the infection. CD4 T cells have a dual role in protection against blood-stage malaria by producing IFNγ and helping B cells to secrete antibodies. Infected erythrocytes release adenosine triphosphate (ATP), a damage signal that can be recognized by purinergic receptors. Among them, the P2X7 senses extracellular ATP and induces CD4 T cell activation and death. We have evaluated the role of P2X7 in the CD4 T cell response during blood-stage *Plasmodium chabaudi* (*Pc*) malaria. We observed that P2X7 was activated in CD4 T cells following the rupture of infected erythrocytes and these cells became highly responsive to ATP during acute infection. Moreover, P2rx7-/- mice had increased susceptibility to infection, which correlated with impaired T helper 1 (Th1) cell differentiation. In contrast, an increase in follicular T helper (Tfh) cell population, germinal center reaction and anti-parasite antibody production was found in infected P2rx7-/- mice. The selective expression of P2X7 in CD4 T cells was required for Th1 cell differentiation. The IL-2 and IFNγ secretion, as well as T-bet expression, critically depended on P2X7 signaling in CD4 T cells. This effect was shown in mice transferred with either P2rx7-/- or B6 CD4 T cells, as well as in mice co-transferred with both CD4 T populations.The P2X7 signaling can influence Th1/Tfh cell differentiation by inducing the T-bet-controlled Th1 cell program, which hinders the development of the Bcl6-controlled Tfh cell program. In fact, P2X7 deficiency led to lower expression of Blimp-1 in CD4 T cells and this transcription factor is a known antagonist of Bcl6. It is generally accepted that P2X7 signaling amplifies T cell receptor (TCR)-induced calcium influx and thus increases IL- 2 secretion. Accordingly, calcium influx and IL-2 secretion were dependent on P2X7 expression in CD4 T cells from acute *Pc* malaria. An alternative non-exclusive molecular mechanism by which the P2X7 can change the Th1/Tfh balance relies on the high susceptibility of Tfh cells to ATP-induced cell death. Suggesting that this mechanism operates during *Pc* infection, P2X7 deficiency reduced the apoptotic cell death in germinal centers and phosphatidylserine exposure in Tfh cells. Furthermore, Tfh cells are particularly prone to die spontaneously through P2X7. The relatively low CD39 expression may be insufficient to degrade extracellular ATP rapidly and thus prevent ATP-induced Tfh cell death, which can be accelerated by the extremely high levels of P2X7 in these cells. Our findings provide mechanistic insights into malaria pathogenesis by demonstrating the importance of damage signals for the fine-tuning between Th1 and Tfh cell populations and thus for the outcome of the disease.
Keywords: P2X7; malaria; Plasmodium chabaudi; CD4 T cells.

5. Oral Communication: Recruitment of Adenosine A2b receptor is important for dendritic cell inhibition by metacyclic promastigotes of Leishmania amazonensis

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Leishmania amazonensis infection is associated with a lack of antigen-specific T-cell responses. Dendritic cells direct the differentiation of T-helper 1 lymphocytes that contribute to the control of *Leishmania* infection. In a previous work, we showed that infection by *L. amazonensis*, but not by *L. braziliensis* or *L. major*, impairs dendritic cells responses by activating adenosine A2B receptors. Here, we evaluated the expression of adenosine receptors in infected cells. With this aim, bone marrow-derived dendritic cells from C57BL/6 mice were infected with promastigotes of either *L. amazonensis*, *L. braziliensis* or *L. major*. Fluorescence microscopy revealed that *L. amazonensis* infection stimulates the recruitment of A2B receptor to the surface of infected dendritic cells, without altering the amount of mRNA evaluated by RT-qPCR or the total A2B receptor protein density, as gauged by Western blotting. This effect is unique to metacyclic promastigotes of *L. amazonensis*, since log-phase promastigotes or axenic amastigotes of *L. amazonensis* or metacyclic promastigotes of *L. braziliensis* or *L. major* do not stimulate A2B receptor recruitment. On the other hand, the distribution and density of A1, A2A and A3 receptors are similar between uninfected and infected dendritic cells. A2B receptor clusters are localized in lipid rafts and the disruption of these membrane domains by cyclodextrin treatment impairs the recruitment of A2B receptor. Lipophosphoglycan, a virulent factor of *Leishmania* parasites, is important for the recruitment of A2B receptor, since the incubation of parasites with an anti-lipophosphoglycan antibody before infection decrease the recruitment of this receptor in infected cells. In addition, we report that *L. amazonensis* increases cAMP production and ERK1/2 phosphorylation in infected dendritic cells by through and A2B receptor-mediated mechanism, which decrease CD40 expression and IL-12p70 production by dendritic cells. The treatment with cyclodextrin also impairs the activation of A2B receptor. We conclude that *L. amazonensis* metacyclic promastigotes stimulate the recruitment of A2B receptors to the surface of infected dendritic cells and this

recruitment is essential for the ability of this parasite to inhibit dendritic cells response. Supported by: CNPq, CAPES, FAPEMIG, Ciência sem Fronteiras, FCT.
Keywords: Adenosine A2B receptor; Leishmania amazonensis, dendritic cells.

SYMPOSIUM 2 - Pharmacology of Purinoreceptors

1. Functional selectivity and biased agonism in individual receptors and in receptor heteromers

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Adenosine receptors have been instrumental in demonstrating the occurrence of receptor heteromers and in showing that receptor heteromers are therapeutic targets for a variety of diseases. On the one hand, this talk will address the particular properties derived from the occurrence in natural sources of receptor-receptor interactions, i.e. of heteroreceptor complexes. We will provide examples of the pharmacological and functional consequences of heteromer formation for dopaminergic neurotransmission and for cancer progression. On the other hand, structural basis of functional selectivity will be addressed also using adenosine receptors as models. The inter-relationships between functional selectivity and biased agonism will be also commented.
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Keywords: adenosine A1 and A2A receptors; receptor heteromers; signaling; neuroscience.

2. FRET-based biosensors reveal dynamic receptor activation of the A1 adenosine receptor

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The publication of the antagonist bound structure of the adenosine A1 receptor in combination with the published structures of the adenosine A2A receptor has revealed details about the conformational changes associated with the receptor activation process. To study receptor activation in living cells, we used dynamic fluorescence resonance energy transfer (FRET) measurements of a modified A1-receptor construct. The receptor contained the amino acid motif CCPGCC in the 3rd intracellular loop and was fused to the cyan fluorescent protein (CFP) at the C-terminus. The CCPGCC motif can selectively bind the small soluble fluorophore FlAsH, which together with CFP is suitable for FRET measurements. Ten optical sensors were created with individually inserted mutations that were predicted from the crystal structure to interact with agonist or antagonists. Each of these constructs was stably expressed in HEK293 cells. Due to ligand dependent conformational changes upon receptor activation, we could observe dynamic ligand binding of adenosine, NECA, CPA or inhibition thereof by theophylline as changes in the FRET-signal. We identified three different effects of these mutations. One class causes problems in membrane localization of the A1-receptor but not the A2A-receptor. The 2nd group is involved in binding of the ribose moiety and has stronger effects in the A1-receptor compared to the A2A-receptor. Last, but not least we identified mutations that exhibit differential effects depending on the ligand used for receptor activation. In summary, we could demonstrate that this novel technology can be used to study the endogenous ligand and mutation dependent effects in living cells.
Keywords: A1 receptor; FRET-sensor.

3. Pharmacology of the platelet P2Y12 receptor

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Background: The platelet P2Y12 receptor is an important target in pharmacotherapy. The receptor is activated by ADP and couples to Gαi2 mediating an inhibition of cyclic AMP accumulation and additional downstream events including the activation of phosphatidylinositol-3-kinase and Rap1b proteins. In addition to their role in platelet aggregation P2Y12 receptors are expressed on a number of cells including vascular smooth muscle cells and microglial cells. The nucleoside analogue ticagrelor and active metabolites of the thienopyridine compounds ticlopidine, clopidogrel and prasugrel block P2Y12 receptors and decrease, thereby, ADP-induced platelet aggregation. These drugs are used for the prevention and therapy of cardiovascular events such as myocardial infarction. Objective: In the present study, we analyzed the molecular modes and sites of action of ticagrelor, of the endogenous antagonist farnesyl pyrophosphate (FPP) and of the cangrelor analogue PSB-0413. Methods: Recombinant wild-type or mutant human P2Y12-receptors were stably expressed in Chinese Hamster Ovary Flp-In cells. Receptor function was assessed by quantification of ADP- and 2-methylthio-ADP-mediated inhibition of forskolin-induced cellular cAMP production either using a [3H]cAMP-radioaffinity assay or a cAMP response element (CRE)-driven luciferase reporter gene assay. Results: ADP inhibited forskolin-induced cAMP formation at the wild-type P2Y12 receptor with a lower potency compared to 2-methylthio-ADP. Ticagrelor shifted the concentration-response curves of both agonists in a parallel and surmountable manner to the right. Increasing concentrations of ticagrelor caused increasing shifts. Schild-plot analysis revealed pA2 values of 8.8 for ticagrelor against ADP, and 8.7 against 2-methylthio-ADP, respectively, and slopes of the regression lines not different from unity. In cells expressing a recombinant C194A-mutant P2Y12 receptor construct, ticagrelor lost antagonistic potency when tested against ADP or 2-methylthio-ADP. FPP antagonized the effect of the agonist 2-methylthio-ADP in a surmountable manner with an apparent pKB value of 5.2 at wild-type P2Y12 receptors, whereas PSB-0413 was more potent with an apparent pKB value of 8.3 at wild-type P2Y12 receptors. The antagonistic potency was of FPP increased at C194A-mutant receptors, whereas at

K280A-mutant receptors fused on the C-terminus to enhanced cyan fluorescent protein (ECFP) and at K173A/K174A- and K174A-mutant receptors, the potency of FPP was decreased. The same was true for PSB-0413. Conclusions: The experiments reveal a surmountable and competitive mode of antagonism of ticagrelor at P2Y12-receptors activated by either the natural agonist ADP or the synthetic agonist 2-methylthio-ADP. Cys194 (located within transmembrane region 5) is likely to be involved in the interaction of ticagrelor. FPP and PSB-0413 also act as antagonists with possible interactions with the residues Lys174 and Lys280.
Keywords: P2Y12 receptor; ticagrelor; farnesyl pyrophosphate.

4. Avermectins, Alcohol use disorder and P2X4 receptors

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The deleterious effects of alcohol use disorders (AUDs) on human health have been documented worldwide. The enormous socioeconomic burden coupled with lack of efficacious pharmacotherapies underlies the need for improved treatment strategies. Ligand-gated ion channels (LGICs) have been widely implicated to play important roles in ethanol behavior. In particular, the group of P2X receptors (P2XRs) is becoming the focus of investigation in ethanol studies. To this end, recent preclinical evidence demonstrates the potential of avermectins [ivermectin (IVM), abamectin (ABM), moxidectin (MOX) but not selamectin (SEL)] as potential candidates for the treatment of AUDs. Avermectins are derived by fermentation of soil micro-organism, Streptomyces avermitilis and have been extensively used for treatment of parasitic infections. From the mechanistic standpoint, avermectins are positive modulators of purinergic P2X4Rs that belong to the P2X superfamily of cation permeable ion channels gated by adenosine-5'-triphosphate (ATP). Building evidence has implicated a role for P2X4Rs in regulation of ethanol intake and that ethanol can inhibit ATP-gated currents in P2X4Rs. Investigations using recombinant cell models and animal models of alcohol drinking have reported that IVM, ABM, MOX but not SEL, were able to antagonize the inhibitory effects of ethanol on P2X4Rs in vitro and reduce ethanol intake in vivo. Furthermore, IVM was shown to reduce ethanol consumption via P2X4R potentiation in vivo; supporting involvement of P2X4Rs in IVM's anti-alcohol effects and that P2X4Rs can be used as a platform for developing novel anti-alcohol compounds. In further support of P2X4Rs' role in ethanol intake in vivo and its utility as platform for drug development for AUDs, shRNA-mediated knockdown of P2X4Rs in the mesolimbic circuity has been reported to alter ethanol drinking behavior in rodent models of alcohol drinking. The presence of P2X4Rs in the mesolimbic circuitry and its reported role in regulating dopamine (DA) neurotransmission (a critical neurotransmitter for drug reward) may underlie the mechanism by which P2X4Rs regulate ethanol intake. In total, the findings provide new insights regarding pathway(s) and mechanism(s) through which P2X4Rs play a role in the regulation of ethanol intake and illustrate the utility of avermectins as a novel class of drug candidates for treatment of AUDs. Support: AA022448 (DLD) and USC School of Pharmacy.
Keywords: Alcoholism; Avermectins; pharmacotherapy; P2X4Rs.

5. Oral Communication: P2X7 receptor is a major modulator of extracellular ATP concentration and related immune response in oncogenesis

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Objectives/background: P2X7 receptor is an ATP gated ion channel that is recognized as a mediator of ATP activity in oncogenesis and it is involved in cancer cell proliferation, vascularization, migration and metastatization. It has been demonstrated that P2X7 blocking drugs act, in vivo, as efficacious anti-tumoral agents in different murine models. However, paradoxically tumor growth is increased in P2X7 null mice due to lack of immune infiltration. To clarify this issue, we analyze the effect of P2X7 on eATP and immune response modulation in tumoral microenvironment. Methods and results: The role of P2X7 in ATP secretion has been explored thanks to a luciferase probe detecting eATP (PmeLUC), which was engineered to be expressed on the outer facet of the plasma membrane and emits photons in an ATP dependent-manner. We obtained clones of B16 melanoma cells and WEHI-3B leukemic cells stably expressing PmeLUC. These cells allowed us to measure eATP levels in tumor microenvironment of two different P2X7 null strains: C57 bl/6 and Balb/cJ. P2X7 null mice show reduced eATP in the tumor milieu as compared to wild type counterpart. Reduced eATP is accompanied by increased tumor growth. On the contrary eATP levels are unaltered following P2X7 pharmacologic blockade causing tumor growth arrest. Interestingly, host P2X7 receptor is able to modulate tumoral levels of eATP. Moreover, data suggest that the alteration of eATP could be dependent upon microvesicles release of nucleotide by both tumor and host immune cells. Animal experimentation were authorized by the ethics committees of the Universitiy of Ferrara, and from the Italian Ministry of Health. Conclusions: Taken together we demonstrate that P2X7 expression or blockade differentially affects tumor growth. P2X7 receptor can also affects the tumor microenvironment by eATP and immune system modulation.
Keywords: P2X7; ATP; oncogenesis; immune system.

SYMPOSIUM 3 - Adenosine control of synaptic activity & neuropsychiatric diseases

1. Sex differences in the effects of caffeine in the Attention Deficit and Hyperactivity Disorder

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Epidemiological studies suggest sex differences in attention deficit and hyperactivity disorder (ADHD) symptomatology. The potential benefits of caffeine have been reported in the management of ADHD, but its effects were not properly addressed with respect to sex differences. The present study examined the effects of caffeine (0.3 g/L) administered since childhood in the behavior and brain-derived neurotrophic factor (BDNF) and its related proteins in both sexes of a rat model of ADHD (spontaneously hypertensive rats—SHR). Hyperlocomotion, recognition, and spatial memory disturbances were observed in

adolescent SHR rats from both sexes. However, females showed lack of habituation and worsened spatial memory. Although caffeine was effective against recognition memory impairment in both sexes, spatial memory was recovered only in female SHR rats. Besides, female SHR rats showed exacerbated hyperlocomotion after caffeine treatment. SHR rats from both sexes presented increases in theBDNF, truncated and phospho-TrkB receptors and also phospho-CREB levels in the hippocampus. Caffeine normalized BDNF in males and truncated TrkB receptor at both sexes. These findings provide insight into the potential of caffeine against fully displayed by females in the ADHD model. Besides, our data revealed that caffeine intake since childhood attenuated behavioral alterations in the ADHD model associated with changes in BDNF and TrkB receptors in the hippocampus.
Keywords: Caffeine; ADHD; neurodevelopment; sex differences.

2. Adenosine A2A receptor: a neuroglial effector in Alzheimer's Disease

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Consumption of caffeine, a non-selective antagonist of adenosine A2A receptor (A2AR), mitigates cognitive decline during ageing, reduces Alzheimer's disease (AD) risk in humans, and also decreases amyloid and Tau pathologies in AD transgenic mouse models (Flaten et al., 2014). In line, selective A2AR blockade improves memory and pathology in transgenic models mimicking Tau and amyloid lesions (Laurent et al., 2016; Viana da Silva et al., 2016; Orr et al., 2017; Faivre et al., submitted). These data not only support A2A receptor as a valuable target in AD but also suggest that A2A receptors are involved in the pathophysiological development of the disease. Beneficial effects of caffeine and A2AR antagonists are presumably ascribed to the normalization of dysregulated A2AR activity. Indeed, brains of aged and AD individuals but also AD models are characterized by an abnormal upsurge of A2ARs (Albasanz et al., 2008), being of neuronal and astrocytic origin (Faivre et al., unpublished; Lopes et al., unpublished; Viana da Silva et al., 2016; Orr et al., 2015). However, respective impact of neuronal and astrocytic A2AR dysregulations towards development of cognitive deficits and AD lesions remains largely unknown. To investigate the role of neuronal and glial A2A dysregulation, we have developed new tools allowing conditional overexpression of A2A adenosine receptors. At the meeting, we will provide new insights on the impact of neuronal and astroglial gain of A2AR function towards cognition and brain lesions in transgenic models of AD.
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This project is supported by LabEx DISTALZ,ANR ADONTAGE, Région Hauts de France (PARTENAIRR COGNADORA), LECMA, FCT, and Université de Lille (AAP Internationalisation).
Keywords: A2A receptors; Alzheimer's Disease; transgenic models; Tau.

3. Adenosine, stress & aging

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Caffeine is associated with procognitive effects in humans by counteracting overactivation of the adenosine A2A receptor (A2AR), which is upregulated in the human forebrain of aged and Alzheimer's disease (AD) patients. Interestingly, cognitive impairments also occur when excessive levels of corticosteroids are attained due to disease or in response to a stressor. In one of our studies we revealed that an anti- A2AR therapy in an early-life stress model (Maternal separation) reverts age-like memory deficits, hippocampal dendritic atrophy and synaptic impairments by reestablishment of the hypothalamic pituitary-adrenal (HPA) axis feedback, namely glucocorticoid receptor expression and corticosterone circadian levels. These observations suggest that A2AR over-activation and glucocorticoid dysfunction are key events in age-related hippocampal deficits; but their direct connection had never been explored. We tested the hypothesis that A2AR over-expression in cortical areas is sufficient to trigger AD-related hippocampal deficits via disruption of the HPA axis, by modulating GR actions. We generated a model of A2AR overexpression in forebrain neurons - in an aging-like profile – and shown that it is sufficient to trigger HPA-axis dysfunction. We also demonstrated that GR-dependent plasticity deficits are amplified by A2AR over-activation and rescued by anti- A2AR therapy. Finally, we provided data supporting that A2AR acts on GR nuclear translocation and GR-dependent transcriptional regulation. The expansion of this interaction to the immune response, cell proliferation, tumor response and other cellular functions that imply GR or corticosteroids use in therapeutics, could have an enormous clinical impact.
Funding: Mind-Brain College Universidade de Lisboa; LVL is an Investigator FCT.
Keywords: A2A; caffeine.

4. Adenosine, mood & fear reactivity

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Adenosine assists encoding information salience through a combined activation of inhibitory A1 and facilitatory A2A receptors (A2AR). In hippocampus, A2AR selectively affect synaptic plasticity and spatial reference memory: their activation is sufficient to deteriorate memory and their overactivation is necessary for alteration of synaptic plasticity and memory in models of early Alzheimer's disease. This matches the decreased incidence of memory deficits in aging and Alzheimer's disease in individuals consuming caffeine (adenosine receptor antagonist). A2AR blockade also controls fear memory and amygdala synaptic plasticity, as gauged by the ability of A2AR antagonists (SCH58261), global A2AR knockout mice or bilateral intra-amygdala injection of lentivirus encoding short hairpin RNAs to downregulate A2AR (shA2AR), to reduced the acquisition and expression of conditioned fear. Furthermore, thinkering with A2AR formats the extinction of fear memories. This paves the way to foster the therapeutic impact of A2AR to manage pathologies associated with abnormal encoding of aversive memory, in accordance with the association between A2AR polymorphisms and phobia or panic attacks in humans. Using a mouse model of chronic unpredictable stress, selective A2AR antagonists (KW6002, 3 mg/kg, p.o.), global and forebrain neuron-selective A2AR knockout mice dampen mood and memory deficits though a normalization of synaptic plasticity and A2AR blockade can therapeutically reverts these installed aberrant phenotypes upon repeated stress. This matches the lower incidence of depression and suicide ideation in caffeine consumers. Repeated restraint stress (4h daily for 2 weeks) increased the density of A2AR in amygdala, mainly in glutamatergic synapses, and also bolstered synaptic plasticity in excitatory synapses of the lateral amygdala. The bilateral amygdala injection of shA2AR attenuated stress-induced aberrant synaptic plasticity in amygdala and also attenuated the stress-induced increase of plasma corticosterone levels and prevented the anxiogenic-like and helpless-like behaviors caused by repeated stress, thus indicating that ablating amygdala A2AR is sufficient to prevent stress-induced behavioral and functional modifications associated with depressive-like conditions. Overall, this prompts targeting amygdala A2AR as a promising strategy to manage mood-related disorders. (Supported by DARPA, FCT, QREN, NARSAD)
Keywords: adenosine; mood.

SYMPOSIUM 4 - Purinergic Signaling in the cardiovascular system

1. P2Y-mediated signaling in lung vascular endothelial barrier strengthening

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Endothelial cells (EC) are a specialized cell type that line the lumen of the blood vessels serving every major organ system and provide a semi-selective barrier between the blood and the interstitial space. In acute lung injury (ALI) the EC barrier is weakened leading to increased permeability (1). The mechanisms involved in the preservation of barrier integrity are largerly unknown. We have demonstrated that extracellular ATP can strengthen EC barrier via activation of P2Y receptors (2). To further evaluate the role of P2Y-mediated signalling in EC barrier enhancement we employed a stable non-hydrolysable ATP analog, ATPγS. Unlike ATP, ATPγS is not involved in inflammatory responses (3), therefore, it may present an attractive agonist for treatment of ALI. Using Transendothelial Electrical Resistance (TER) assay, we demonstrated that similar to ATP, ATPγS increased TER of human lung microvascular EC monolayers reflecting EC barrier strengthening. Selective depletion of P2Y receptors using a small interference RNA (siRNA) technique defined the specific involvement of Gi-coupled P2Y 4 and 12 receptors. P2Y/Gi-mediated signalling events involved unconventional cAMP-independent protein kinase A (PKA) activation and decreases in myosin light chain 20 (MLC20) phosphorylation secondary to the involvement of MLC phosphatase (MLCP). Importantly, depletion of Gαi2 attenuated ATPγS-induced TER increase and reversed ATPγS-induced MLC dephosphorylation suggesting the contribution of G_{i2}-mediating signaling in MLCP activity regulation. PKA and MLCP activation may be coordinated through the actions of GAB1/Shp2 (also known as PTPN11, a non-receptor type 11 Tyr phosphatase) and AKAP2 (PKA anchoring protein 2)-mediated signaling. AKAP2 was found in immune complexes with PKA and G_{i2} and directly interacts with MLCP suggesting that the AKAP2/MLCP axis is a novel regulator of P2Y/Gi-mediated EC barrier enhancement. Further, our data show the involvement of a regulatory molecule ELMO1 (Engulfment and cell motility protein 1), in P2Y/G_i-mediated EC barrier strengthening, which together with the adapter protein Dock180 formed a bipartite GEF for Rac1. Collectively, our data suggested that ATPγS-induced P2Y-mediated EC barrier strengthening requires G_{i2}-mediated, coordinated activation of GAB1/Shp2 and Dock180/ELMO1 leading to activation of PKA and Rac1 pathways, respectively. We hypothesize that cAMP-independent activation of PKA involves G_{i2}/AKAP2/PKA interaction which activates MLCP which dephosphorylates MLC20 resulting in reduced EC contraction and preservation. Support: P01 HL101902. Matthay MA, Ware LB, Zimmerman GA. J Clin Invest. 2012; 122(8):2731-40. Kolosova IA, Mirzapoiazova T, Adyshev D, Usatyuk P, Romer LH, Jacobson JR, Natarajan V, Pearse DB, Garcia JG, Verin AD. Circ Res. 2005; 97:115-24. Kolosova IA, Mirzapoiazova T, Moreno-Vinasco L, Sammani S, Garcia JG, Verin AD. Am J Physiol Lung Cell Mol Physiol. 2008; 294:L319-24.

2. HDAC inhibitor Butyrate cooperates with A1 and A2B receptors to attenuate pulmonary artery vasa vasorum remodeling in hypoxia

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Vasa vasorum (VV) is a microcirculatory network that provides oxygen and nutrients and maintains the integrity of large blood vessels. In a neonatal model of hypoxia-induced pulmonary arterial hypertension (PAH), we have shown that VV neovascularization is associated with impaired endothelial barrier function and perivascular inflammation, indicating that VV may be a contributing factor to the pathogenesis of PAH (1). We have shown that Adenosine has a barrier-protective role in VV endothelial cells (VVECs), and this effect is mediated by A1 receptors (A1R) (2). A1R is expressed in rat pulmonary artery (PA) VVEC, but not in luminal PA EC, indicating a specific role for A1R in VV function. HDAC inhibitors have beneficial effects in cardiovascular diseases, including hypertension, by exerting an anti-proliferative effect in vascular cells (3-5). In this study we investigated whether HDAC inhibitor, sodium butyrate (SB) attenuates hypoxia-induced PA VV remodeling and potentiates a barrier protective effect of adenosine. Using a Sprague Dawley Rat model of hypoxic pulmonary hypertension

(4 weeks, simulated altitude 5,000m), we showed that A1R/A2AR agonist, NECA (65 micrograms/kg) and SB (260 milligrams/kg) decreased heart Fulton index (RV/LV+S), right ventricular systolic pressure (RVSP), pulmonary vascular remodeling, and infiltration of inflammatory cells into the lung and PA wall. The observed effects of NECA were potentiated by A2BR antagonist, PSB603 (15 milligrams/kg). Furthermore, treatment with SB (2 mM, 2 h) enhanced VVEC barrier function (measured as Trans Endothelial Resistance (TER)) and increased a barrier-protective effect of Adenosine. We also found that SB (1 mM, 24 h) increased protein acetylation, stimulated glycolytic and oxidative cellular energy pathways, and enlarged mitochondrial reticulum in VVEC. In addition, SB upregulated the expression of Ga13 protein and p120 catenin, suggesting a potential positive cooperation between metabolic and signaling pathways in VVEC. Thus, our data suggest that purinergic receptor- and HDAC-based therapies may represent novel treatments for PAH and other cardiovascular diseases with vascular barrier dysfunction. Support: R01 HL086783 (E.V. Gerasimovskaya); P01 HL101902 (A.D. Verin)

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Keywords: A1 receptors; vasa vasorum permeability; hypoxia; sodium butyrate.

3. Protective effects of adenosine deaminase inhibition on tumor development and metastases

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Objectives/background: The activity of cell-surface ecto-adenosine deaminase is markedly increased in vascular pathologies that involve endothelial activation and vascular inflammation. We previously demonstrated that this activity originates mainly from endothelial cells and it decreases extracellular adenosine concentration leading to alterations in receptor mediated adenosine signaling. The aim of this work was to test the effects of adenosine deaminase inhibition on tumor development and metastases. Methods and results: Female BALB/c mice were injected orthotopically with saline (control group, n=7) or 4T1 cancer cells (mouse breast cancer cell line, study group, n=7), following the approval of the local ethics committee. Adenosine deaminase inhibitor (2'-deoxycoformycin, dCF) was injected intraperitoneally (0.2mg/kg) twice per week starting from a day of tumor cell implantation. This administration pattern maintained reduced vascular eADA activity to less than 20%. dCF counteracted immune system activation caused by 4T1 cell implantation (increased spleen mass and white blood cell count). Moreover, dCF decreased a tumor growth and a final tumor mass measured 28 days after 4T1 cell implantation. Mice injected with tumor cells revealed increased plasma concentration of endogenous eNOS inhibitor and endothelial damage marker: dimethylo-L-arginine (ADMA) from 0.5 ± 0.05 μM (mean ± SEM) observed in a saline-injected group to 0.9 ± 0.1 μM in 4T1-injected mice. dCF treatment reduced ADMA in 4T1-injected mice to 0.55 ± 0.06 μM. Moreover, dCF counteracted a development of hemorrhage sites in lungs of tumor mice. In vitro studies, using transmigration assay revealed that 200 nM dCF suppressed migration of tumor 4T1 cells through the endothelial cell layer (H5V cell line) by about 30%. It was a mostly endothelial cell-dependent effect since pre-incubation of endothelial cells with dCF also reduced transmigration of tumor cells by 30%. Permeability assay on H5V cells revealed that 200 nM dCF increased a barrier function of endothelial cells decreasing its permeability. Conclusion: This study highlights a key role of increased activity of endothelial cell ecto-adenosine deaminase in tumor development and metastases. The inhibition of eADA by deoxycoformycin reduced tumor progression and metastases that was closely related to endothelial protection and improvement of endothelial barrier function. This study was supported by The National Centre for Research and Development of Poland (STRATEGMED1/233226/11/NCBR/2015).

Keywords: adenosine deaminase; deoxycoformycin; tumor; metastases.

4. Oral Communication: Purinergic system: Platelets and illness

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Platelets can mediate leucocyte recruitment in cardiovascular disease and in inflammatory conditions. Due to their proinflammatory role, they are involved in the pathogenesis of several diseases. Platelets contain high concentrations of ATP that can be broken down to ADP by ectoenzymes. They express the P2Y1, P2Y12 and P2X1 purinergic receptors, which are involved in platelet aggregation. ATP acts in the central sympathetic drive, increasing the systemic arterial blood pressure. Adenosine, produced by nucleotide degradation, inhibits platelet aggregation, and acts as a vasodilator and cardioprotector agent. P2X7 receptor also participates in the development of hypertension via inflammation. Our research group has investigated the purinergic signaling in some of these conditions, which resulted in several publications. We demonstrated altered ectoenzymes activities in platelets of hypertensive rats. Since elevated maternal blood pressure occurs in preeclampsia, and high adenosine levels are found in the fetal-placental circulation, we recently observed a higher adenosine deamination in preeclamptic pregnancies. Elevated levels of circulating ATP and ADP are associated with atherosclerosis and smoking. We observed higher CD39 expression and ATP/ADP hydrolysis in platelets of hypercholesterolemic patients. In an experimental model of hyperlipidemia it was demonstrated a lower ATP, ADP and AMP hydrolysis and an increase in adenosine deamination and platelet aggregation. Purinergic signaling in vascular smooth muscle and endothelial cell is involved in atherosclerosis, but adenosine is protective of ischemic injury. Our group demonstrated increased ATP and ADP hydrolysis in platelets from acute myocardial infarction patients, probably to enhance the adenosine level. High levels of circulating nucleotides in inflammatory diseases may promoting an injury response in vascular tissues. We observed alterations in purinergic signaling in platelets of patients with HIV infection, Chagas disease, sepsis and autoimmune diseases. Adenosine is a potentially important therapeutic target of sickle cell disease, a hematological disease. Then we observed alterations in the ectoenzymes in platelets of sickle cell patients. Cell-platelet emboli formation contribute to the development of metastases. Primary tumors produce molecules that promote angiogenesis; platelets recognize and respond to them, initiating thrombotic events that facilitate cancer progression. Changes were observed in ectoenzymes activities

in platelets of patients with uterine cancer, lung cancer, prostate cancer, breast cancer, thyroid cancer and melanoma. It is known that purinergic signaling is involved in both the physiology and pathophysiology of the blood vessels. Moreover, all cells in the vascular system express one or more types of purine receptors. This demonstrates the importance of purinergic system and platelet participation in vascular disturbs that occur in several diseases.
Keywords: Platelets; cardiovascular disease; inflammation; purinergic system.

SYMPOSIUM 5 - Purinergic signaling in bone cell formation and disease

1. P2X7 receptor in prostate cancer bone metastasis initiating cells

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Background/Objectives: Prostate cancer (PCa) most commonly metastasizes to the bone, causing considerable morbidity and mortality. Bone metastases could occur several years after the removal of primary tumours, indicating a longer tumour dormancy during PCa metastasis. Our recent studies have suggested that the expression of P2X7 receptor (P2X7R) is transcriptionally up-regulated in the dormant, metastasis initiating cells, while others suggested that P2X7R mediates the status of invasiveness/EMT of PCa cells. Therefore, we hypothesize that P2X7R plays a pivotal role in PCa dormancy. To test this hypothesis, two objectives will be achieved via examining whether the P2X7R is functionally up-regulated in dormant PCa cells and whether genetically knocking out P2X7R will affect tumour dormancy. Methodology: Dormant PCa cells (PC3 and C4 2B4 cells) were isolated using a method developed previously based on their ability to retain fluorescent lipophilic dyes when not dividing in vitro. The functional expression of P2X7R was assessed using an ethidium uptake assay (membrane pore formation) under stimuli of exogenous P2X7R agonist BzATP with/without the presence of P2X7R antagonist-A740003. After genetically depleting P2X7R in PCa cells with CRISPR/Cas9 technique (targeting exon 13, a C-terminal domain critical for function), alterations in frequency of dormant population, function of P2X7R and proliferation/viability of PCa cells were then examined in vitro, using FACS, ethidium uptake assay, MTS and Glo MT assay, respectively. Results: Dormant populations of both PCa cell lines showed increased pore formation in response to exogenous BzATP (Linear regression comparison of 30 minutes, $n=3$, $p<0.001$), suggesting enhanced functional expression of P2X7R in dormant PCa cells. Disrupting the P2X7R by CRISPR/Cas9 led to complete abolishment of pore formation in both cell lines and altered dormant cell frequency (t-test, $n=3$, $p<0.05$), without affecting the proliferation and viability (t-test, $n=3$, $p>0.05$). Conclusion: Our data supports the hypothesis that the P2X7R may play an important functional role in the dormant, metastasis initiating PCa subpopulations and warrants further studies to exploit its pharmaceutical potentials for fighting PCa bone metastasis.
Keywords: P2X7; prostate cancer; bone metastasis.

2. P2X7 and multiple myeloma

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Objectives / Background: Bones are dynamic organs, where a constant amount of bone destruction and formation work in balance to maintain optimal bone health. Ageing, or pathologies such as primary or secondary cancers, skew this balance causing higher bone destruction and an accelerated loss of bone. Multiple myeloma is a primary cancer originating within the bone marrow, and consequently, causes a severe bone loss due to aggravated bone destruction. Given that the loss of function single nucleotide polymorphisms (SNPs) in the gene for the P2X7 receptor have been associated with an accelerated bone loss in various cohorts of postmenopausal women and have additionally, been associated with an increased risk of myeloma; we predicted that loss of function alleles in the P2X7 receptor encourages myeloma and potentially, aggravates the myeloma- induced bone disease. Methods: We used 3 bona fide human myeloma cell lines and after genotyping for non-synonymous P2X7 receptor SNPs, expression of P2X7 receptor transcripts, expression of total and surface P2X7 receptor protein, pore formation and calcium influx via P2X7 receptor ion channel; we categorized these 3 myeloma cell lines (RPMI-8226, U266, NCI-H929) in order of P2X7 receptor functionality. Treatment with Bz- ATP (P2X7 receptor agonist) significantly inhibited cell viability and even induced cell cycle arrest but not to the same extent in cells with a reduced P2X7 receptor function. Secondly, a co- culture method involving myeloma cells, bone forming osteoblasts and bone resorbing osteoclasts allowed creation of in vitro conditions that mimic the complex in vivo environment. Modulating P2X7 receptor function in this co- culture system rescued, to an extent, myeloma -induced bone destruction. Conclusion: Understanding the role of P2X7 receptor in myeloma growth and -induced bone loss will further our knowledge on how P2X7 receptor can be modulated to positively activate bone remodeling in patients with primary and secondary cancers.
Keywords: P2X7; bone cancer; Purinergic signaling.

3. P2X7R in primary bone cancer

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Osteosarcoma is the most common type of primary bone cancer affecting adolescents attributed to rapid bone growth and turnover. The 5-year survival rate remains 65% and with metastasis this decreases to 20%. This highlights the need for development of novel therapeutics to treat osteosarcoma. In this study we have investigated the role of purinergic signalling in osteosarcoma, as this is particularly pertinent in the bone tumour microenvironment which could contribute towards disease progression. When Te85 osteosarcoma cells were transfected with P2X7R variants in vitro, we observed increased growth compared to naïve cells ($p<0.05$) which was attenuated upon treatment with P2X7R inhibitors ($P<0.0001$). Te85 cell adhesion to collagen was

significantly reduced (P<0.0001) and migration was significantly increased when cells were treated with BzATP (P<0.0001). As Te85 cells have historically not formed osteosarcoma tumours in mice, we next looked at the effect of P2X7RB expression in an MNNG-HOS cell line, an aggressive tumorigenic derivative of Te85 cells. As with the parental Te85 cells, P2X7RB expression decreased cell adhesion (P<0.0001) and migration was increased when treated with BzATP (P<0.0025). Next, we injected 250,000 MNNG-HOS+P2X7RB cells paratibially into 7-week old female BALB/CJ mice. Two-days after cell inoculation treatment with 50 μg/kg of A740003 or PBS vehicle control was administered by IP injection 3 times a week for 3 weeks. All groups developed palpable tumours which did not vary in size as measured by basic calliper measurement at sacrifice. Analysis of the tibia using micro-CT scanning demonstrated that the MNNG-HOS+P2X7RB cells resulted in a significant 12% increase in total bone volume in the tumour bearing tibia when compared to its corresponding leg (P<0.0123). This increased bone volume, which consists of ectopic bone formation, was ablated when mice were treated with A740003 (P<0.0183). In conclusion, P2X7R variants were found to influence osteosarcoma cell behaviour in vitro. P2X7RB expression in the tumour inducing MNNG-HOS model resulted in a significant increase in total bone volume from ectopic bone formation typical of osteosarcoma tumours. P2X7R antagonism reduced this bone disease. This study provides promising data to support the use of P2X7R inhibitors as a novel therapy for osteosarcoma, however, future studies are required to identify the exact role P2X7RB has in tumour growth and metastasis.

Keywords: Primary Bone Cancer; P2X7R.

4. Positive allosteric modulation of parathyroid hormone 1 receptor function by extracellular nucleotides

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Objectives/background: Parathyroid hormone (PTH) activates the PTH/PTH-related peptide receptor (PTH1R) on osteoblasts and other target cells. Mechanical stimulation of cells, including osteoblasts, causes release of nucleotides such as ATP into the extracellular fluid. In addition to its role as an energy source, ATP serves as an agonist at P2 receptors and an allosteric regulator of many intracellular proteins. Our objective was to investigate the effects of extracellular ATP, at concentrations comparable to those that activate low affinity P2X7 receptors, on PTH1R signaling. Methods: Bioluminescence-based assays were used to monitor concentration-dependent agonist and nucleotide effects on cyclic AMP levels and β-arrestin binding to PTH1R in real time. Activation of cAMP response element binding protein (CREB) was measured using a luciferase reporter. Results: ATP produced no measurable effects on its own, but markedly enhanced PTH-induced cyclic AMP signaling in UMR-106 rat osteoblastic cells, which endogenously express PTH1R. PTH (1-34) alone yielded a sigmoidal concentration dependence curve with pEC50 of 8.75 ± 0.08. In the presence of ATP (1.5 mM), the concentration dependence curve was strikingly shifted to the left (pEC50 = 10.51 ± 0.09). ATP also enhanced agonist-promoted β-arrestin recruitment as measured via a complementation-based luciferase assay in HEK293H cells transiently transfected with human PTH1R. Similarly, ATP promoted PTH (1-34)-induced downstream activation of CREB in UMR-106 cells. CMP – a nucleotide that lacks a high energy phosphate bond and is not generally considered a P2 agonist – mimicked these effects of ATP. Moreover, potentiation was not inhibited by P2 receptor antagonists. Thus, nucleotide-induced potentiation of signaling pathways was independent of P2 receptor signaling. ATP and CMP reduced the concentration of PTH (1-34) required to produce a half-maximal cyclic AMP or β-arrestin response, with no evident change in maximal receptor activity. Increased potency was similarly apparent with PTH1R agonists PTH (1-14) and PTH-related peptide (1-34). As in UMR-106 cells, ATP potentiated the effects of PTH (1-34) via endogenous receptors in the murine osteoblast-like cell line MC3T3-E1. Conclusions: These observations suggest that extracellular nucleotides increase agonist affinity, efficacy or both, and are consistent with modulation of signaling at the level of the receptor or a closely associated protein. Taken together, our findings establish that ATP enhances PTH1R signaling through a heretofore unrecognized allosteric mechanism. These studies were supported by the Canadian Institutes of Health Research (CIHR).
Keywords: ATP; positive allosteric modulator; parathyroid hormone; PTH1R.

5. Oral Communication: NTPDase3 enzyme inhibition or gene silencing rescues the osteogenic potential of post-menopausal mesenchymal stem cells (MSCs) derived from bone marrow

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Introduction: ATP-sensitive P2X7 and UDP-sensitive P2Y6 receptors are critical players in osteogenesis. Tonic activation of P2X7 and P2Y6 is significantly impaired in MSCs from post-menopausal (Pm) compared to younger women due to rapid breakdown of nucleotides by overexpressed NTPDase3 (Noronha-Matos et al. J Cell Physiol. 227: 2694-709, 2012; FASEB J. 28: 5208-22, 2014). Here, we tested whether the osteogenic differentiation potential of MSCs from Pm women could be rescued by NTPDase3 enzyme inhibition and/or gene silencing. Materials and Methods: Bone marrow MSCs were isolated from 19 Pm women (69±2 years old) undergoing total hip arthroplasty and from 5 younger female patients submitted to scoliosis correction (18±4 years old); all procedures were approved by the ethics committees of ICBAS-UP and CHVNG. Cells were kept for 35 days in an osteogenic-inducing medium in the absence and in the presence of NTPDase3 inhibitors (PSB 06126 and hN3-B3S antibody) or of a lentiviral short hairpin RNA (Lenti-shRNA) designed to silence NTPDase3 gene expression (TL313202VD, OriGene). Alkaline phosphatase (ALP) activity, osteogenic transcription factor Osterix (WB assay) and bone nodule formation (alizarin red) were evaluated to indicate MSCs osteogenic commitment. The kinetics of the extracellular ATP and UDP catabolism was assessed by HPLC. Statistical analysis was performed by one-way or two-way ANOVA (Bonferroni´s method). Results: NTPDase3 levels are 1.5 (day 7) to 5.6 (day 21) fold higher in MSCs of Pm women compared to younger females (n=94-234 cells); this results in a 2.5 (ATP) and 6.0 (UDP) fold higher nucleotide dephosphorylation ability of MSCs from Pm vs. young females at culture day 21 (n=3-9). Inhibition of NTPDase3 activity with the hN3-B3s antibody (0.5 μg/ml) and PSB 06126 (3 μM) significantly increased ATP accumulation in the medium. The hN3-B3S antibody enhanced the ALP activity by 345±104% and 525±215 % at culture days 7 and 21 (n=14), respectively. Bone nodule formation was also increased by hN3-B3S and PSB 06126 to 1159±362 % (n=9) and 267±40% (n=18) of control levels, respectively, at culture day 35.

Promotion of osteogenesis by hN3-B3S and PSB 06126 was significantly (P<0.05) attenuated by blockade of P2X7 and P2Y6 receptors with A438079 (3 μM) and MRS 2578 (100 nM), respectively. Lenti-shRNA (MOI 3) gene silencing in Pm MSCs decreased NTPDase3 protein amounts boosting both ALP activity to 480±162% (day 7, n=9) and 613±258% (day 21, n=9), and mineralization to 412±118% (day 35, n=9). Both PSB 06126 (3 μM) and NTPDase3 gene silencing with Lenti-shRNA (MOI 3) increased Osterix levels by about 200% in Pm MSCs. Conclusions: Data show that NTPDase3 enzyme inhibition or gene silencing rescues the osteogenic potential of MSCs in Pm women by facilitating the P2X7 and P2Y6 receptor tonus, which might be therapeutically useful to prevent Pm bone mass loss. Work supported by FCT (PEst-OE/SAU/UI0215/2014 and UID/BIM/4308/2016).
Keywords: Osteogenic differentiation of mesenchymal stem cells; Postmenopausal women; NTPDases; P2 purinoceptor.

SYMPOSIUM 6 - Purinergic signaling in neuroregeneration and neuroprotection

1. Regulation of P2X7 receptor by Sp1 nuclear factor: role in neuroregeneration

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In the nervous system P2X7 receptors (P2X7R) are involved not only in physiological functions (cell growth, differentiation, apoptosis), but also in brain pathologies. Although an increasing number of findings indicate that altered receptor expression has a causative role in neurodegenerative and post-traumatic diseases, little was known about how expression of *P2rx7* gene is controlled. We have demonstrated the first molecular and functional evidence that specificity protein 1 (Sp1) transcription factor plays a pivotal role in the transcriptional regulation of P2X7R in neural cells. We delimited a minimal region in the murine *P2rx7* promoter containing four SP1 sites, two of them being highly conserved in mammals. The functionality of this SP1 sites was confirmed by site-directed mutagenesis, and Sp1 overexpression/downregulation. Using *P2rx7*-EGFP transgenic mice, which express EGFP under the control of proximal *P2rx7* promoter (including SP1 sites), we found a high correlation between reporter expression and Sp1 brain levels. Noticeable, Sp1 is a multifunctional protein expressed constitutively that directly binds with high affinity to GC-rich motifs located in the DNA to modify the expression of a wide variety of genes. At the transcriptional level, Sp1 is not induced in injured neurons, but functions as a scaffolding protein to recruit injury-inducible transcription factors to promote the expression of regeneration-associated genes (RAGs) according to the regeneration program. One of the major causes of incapacity worldwide produced by SNC lesion is the spinal cord injury (SCI), which involves an irreversible loss of function distal to the lesion. Following SCI, large amounts of ATP are released by the traumatized tissue leading to the activation of P2X7R and others that, in coordination with growth factors, induce lesion remodeling and repair. Although no effective treatment currently exists for the mayor neurological deficits after SCI, a promising strategy consists on ependymal stem/progenitor cells (epSPCs) transplantation for promoting spinal cord healing. Transplantation of adult spinal-cord derived neurospheres from spinal cord injured donors (epSPCis) is able to efficiently reverse the paralysis associated with SCI in rats. We have demonstrated that epSPCis express functional ionotropic P2X4 and P2X7, and metabotropic P2Y1 and P2Y4 receptors. Furthermore, severe traumatic SCI early and persistent increases in the expression of P2X7R around the injury in rats, which can be completely reversed by acute transplantation of undifferentiated epSPCis. In conclusion, we have demonstrated that Sp1 is a key element in the transcriptional regulation of P2X7R, suggesting that nerve-injury transcription factors could be capable of enhance P2X7R expression under pathological situations, similarly to what happens with RAGs.
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Keywords: P2X7 receptor; Sp1; spinal cord injury.

2. Impairment of P2Y2 receptor signaling by PGE2. Beneficial or detrimental in neuroinflammation?

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Astrocytes are the most abundant glia cell type of the central nervous system and are essential for brain physiology. Astrocytes and microglia constitute the resident immune cells of the nervous system. They are able to detect danger signals and activate immune responses. Prostaglandin E2 (PGE2) is one of the main bioactive lipids that accumulates after tissue damage or inflammation due to the rapid expression of cyclooxygenase 2. In these situations, nucleotides can also be released and they even contribute to PGE2 production. In previous studies we described that PGE2 selectively impaired P2Y nucleotide signaling in macrophages and fibroblasts, two cellular types implied in inflammation and tissue remodeling. The effect of the prostaglandin was independent of EP receptors and involved PKC and PKD activation. Considering that a similar mechanism involving P2Y signaling could occur in astrocytes in response to neuroinflammation and brain repair, we analyzed the modulation of cellular responses involving P2Y2/P2Y4 receptors by PGE2 in rat cerebellar astrocytes. Using Fura-2 microfluorimetry and calcium imaging we demonstrated that PGE2 inhibits UTP calcium responses. The inhibition was observed in all the cells tested and the magnitude of the initial calcium transients was reduced by a 40%. In addition, the EP3 agonist sulprostone reproduced the effect of PGE2. Stimulation of astrocytes with UTP after preincubation with sulprostone dampened calcium responses, which reached 57.44% of the control responses. EP3 receptors were detected by Western blot. Activation of EP3 receptors by PGE2 or sulprostone not only impaired the calcium responses but also, the extracellular regulated kinases (ERK) and Akt phosphorylation induced by UTP. We proved that PGE2 required EGF receptor transactivation to impair P2Y signaling. PGE2 also attenuated the astrocyte migration elicited by the nucleotide. The effects of PGE2 also occurred in a pro-inflammatory context, as evident in astrocytes stimulated with bacterial lipopolysaccharide (LPS). Overall these findings we found that PGE2 negatively modulated UTP signaling in rat cerebellar astrocytes, as reported in macrophages and fibroblasts. However, there were two differences inasmuch as the effect of PGE2 appeared to be dependent on EP3 receptors, and it was also observed in LPS treated cells. The negative actions of the prostaglandin on UTP signaling could contribute to limit excessive astrogliosis, which could represent part of the adaptive mechanism to neuroinflammation. We are developing studies to know whether P2Y2/P2Y4 receptors are upregulated after prolonged exposure to the endotoxin and whether they can be down-regulated by PGE2. This work was funded by the Spanish Ministerio de Economía y Competitividad (MINECO, BFU 2014-53654-P) and by a Fundación Ramón Areces Grant (PR2018/16-02).
Keywords: astrocytes; PGE2; P2Y2 receptors.

3. Regulation of the celular proteostasis by the purinergic signaling. Relevance in neurological disorders

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The disturbances of cellular proteostasis caused by the alteration in the Ubiquitin-Proteasome System (UPS) have been proposed as a common mechanism underlying several neural pathologies which involve a neuroinflammatory process. After we found that the nucleotide receptor P2Y2 (P2Y2R) regulates the proteasomal catalytic activities in neuroblastoma cell linage N2A cells, we wonder whether this receptor is involved in the UPS disturbances associated with the neuroinflammation process. Using mice expressing an UPS reporter (UbGFP-mice), we found that lipopolysaccharide (LPS)-induced acute neuroinflammation status causes an UPS impairment in astrocytes and microglial cells by a mechanism dependent of P2Y2R. In this line, LPS-treated UbGFP; P2Y2R-/- mice did not present an UPS impairment in astrocytes or a social interaction deficit as severe as that observed in LPS-treated UbGFP mice. In vivo administration of selective P2Y2R agonist, Up4U, reversed the UPS impairment, completely in astrocytes and partially in microglial cells, promoting increased expression of the proteasomal β5 subunit by a mechanism dependent on Src/PI3K/ERK pathway. Altogether, our results suggest that LPS induces unbalanced proteostasis in astrocytes by blocking P2Y2R. Our findings point to the design of selective P2Y2R agonist drugs may be a new therapeutic approach to treat the neuroinflammatory status associated with neurodegenerative diseases.
Keywords: P2Y2; LPS; astrocytes; Ubiquitin-Proteasome System.

4. The role of P2Y12 receptors in animal models of Parkinson's disease

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Objectives / background: Parkinson's disease (PD) is a chronic neurodegenerative disorder, characterized by the progressive degeneration of the nigrostriatal dopaminergic pathway. Neuroinflammation has been recognized as an important mechanism involved in PD pathogenesis. P2Y12 receptor (P2Y12R) is an ADP-sensitive Gi protein coupled receptor expressed by platelets and microglia in rodents and human and regulate different forms of pain as well as local and systemic inflammation. We have recently shown that both genetic deletion and pharmacological inhibition of P2Y12 receptors alleviate acute and chronic inflammatory pain and P2Y12 receptors expressed by platelets contribute to the chronic phase of hyperalgesia and local inflammation (Horvath et al., Neurobiol Dis. 2014 70:162-78., Bekő et al. J Thromb Haemost. 2017 15(6):1223-1235.). In this study, we examined how genetic deficiency and pharmacological inhibition of P2Y12 receptors affect dopaminergic neurodegeneration in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced mouse PD model. Methods and results: Adult (2-3 month old, 30 g) male P2Y12R wild-type (p2ry12 +/+) and knockout (p2ry12 -/-) C57/Bl6 mice were injected with MPTP (4x20 mg/kg i.p) 2h apart, and 72h after the last MPTP treatment, tyrosine hydroxylase (TH) immunohistochemistry was performed and biogenic amine, nucleotide, endocannabinoid and amino acid content of the striatum and substantia nigra was determined by HPLC-EC. The applied MPTP treatment protocol elicited a remarkable depletion in the endogenous dopamine content in the substantia nigra and striatum and the intensity of TH immunostaining was also prominently decreased suggesting the ongoing degeneration of the nigrostriatal dopaminergic pathway. In addition, the levels of 4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA) and that of serotonin (5-HT) were also decreased in the striatum by MPTP. In P2Y12R deficient (p2ry12 -/-) mice, the depletion of endogenous dopamine content in the striatum and the decrease in TH intensity in both the substantia nigra and striatum were significantly alleviated. These effects were replicated by systemic treatment with the P2Y12/13 antagonist cangrelor (3 mg/kg i.p.) in the striata of the wild-type mice. In wild-type animals MPTP treatment decreased endogenous ATP content and elevated the level of the endocannabinoid anandamide, whilst amino acid transmitters (glutamate, GABA) remained unchanged. The induction of anandamide level by MPTP was not observed in the striata of p2ry12 -/- mice. Conclusion: Collectively, our data suggest that genetic deficiency of P2Y12R has a mild, but consistent protective effect in the MPTP-induced subacute PD model in mice, which implies the potential utilization of clinically used P2Y12 receptor blockers in PD.
Keywords: P2Y12 receptor; Parkinson's disease, neurodegeneration, dopamine.

5. Oral Communication: P2X7 receptors in ventral hippocampus are involved in stress response and antidepressant effect

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Introduction: P2X7 receptors (P2X7R) play a central role in stress-related processes such as activation of neuroimmune response, glutamate release, and reactive oxygen species formation. P2X7R polymorphisms have been associated to the severity of depressive symptoms. Moreover, P2X7R inhibition prevents the stress-induced consequences in forced swimming test (FST) and tail suspension test. Based on that, the involvement of P2X7R in the neurobiology of depression and in stress response has been suggested. However, the effects of stress and antidepressant treatment on P2X7R expression was unknown. Additionally, the effect of P2X7R blockade and the mechanisms underlining this response in the Flinders Sensitive Line (FSL) rats, an animal model of depression based on the selective breeding, had not been studied until now. Aims: 1. To investigate the effects of stress and antidepressant treatment on P2X7R levels on frontal cortex (FC) and hippocampus (HIP) of rats submitted to the learned helplessness (LH). 2. To determine the effect of P2X7R blockade on the behaviour and brain-derived neurotrophic factor (BDNF) signalling in FC and HIP of FSL rats. Methods: 1. Male Wistar rats were submitted to pretest session of LH (40 inescapable foot shocks, 0.8mA, 10s, 60s ± 30s interval) or habituation (40 min in the same context without shocks). Animals of each group were treated with antidepressants (desipramine 25mg/Kg/day or imipramine 15 mg/Kg/day) or vehicle for one (acute) or seven (repeated) days. One hour after the last injection, animals were submitted to the test session of LH (30 escapable foot shocks, 0.8mA, 10s, 60s ± 30s interval) or had FC and HIP dissected for posterior evaluation of the P2X7R levels by western blotting (WB). 2. FSL and its control counter partner flinders resistant line (FRL) rats were treated with vehicle or P2X7 receptor antagonist (A-804598 3, 10 or 30 mg/Kg/day) for one or seven days. One hour after the last injection, animals were exposed to FST. Following, FC and HIP were dissected for evaluation of

BDNF, Akt, Erk, mTor and p70 S6 kinase levels by WB. Results: 1. Repeated but not acute antidepressant treatment reverted the stress-induced consequences in the LH model. Stress increased while repeated treatment with antidepressants decreased P2X7R levels on ventral HIP. 2. Repeated but not acute treatment with A-804598 (30 mg/Kg/day) induced antidepressant-like effect on FSL rats. FSL rats presented decreased BDNF and p70S6 kinase levels on vHIP while repeated treatment with A-804598 (30 mg/Kg/day) attenuated this feature. P2X7R blockade increased Akt activation in ventral HIP. Conclusion: Antidepressant effect may involve attenuation of stress-induced P2X7R expression in ventral HIP. P2X7R blockade induces antidepressant-like effects in FSL rats, which is associated with BDNF signalling activation in this structure. Altogether, our data suggest that P2X7R in ventral HIP is involved in stress response and antidepressant effect.
Keywords: P2X7 receptor; stress; antidepressant.

SYMPOSIUM 7 - Purinergic signaling in brain disease

1. A1 adenosine receptors in the striatum play a role in the impairment caused by sleep deprivation through downregulation of the PKA pathway

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Sleep deprivation is known to affect memory formation, but how it interacts with different memory systems is not completely understood. Adenosine, a homeostatic regulator of sleep, is also involved in memory formation. To test if the A1 receptor, PKA and EPAC are associated with memory impairment caused by sleep deprivation, we tested the effect of sleep deprivation (SD) on the performance of two different memory tasks: the hippocampus-independent multiple trial inhibitory avoidance (MTIA) task and the hippocampus-dependent contextual fear conditioning (CFC) task. We also evaluated the effect of SD and the MTIA training session on the protein expression levels of the A1 receptor, PKA phosphorylation and EPAC activity in both the hippocampus and the striatum. We administered DPCPX, an A1 receptor antagonist, to prevent the memory impairment caused by SD. Sleep deprivation impaired the performance in the test sessions of both tasks; DPCPX was able to prevent the impairment in the MTIA test but not in the CFC test. SD increased A1 receptor protein expression levels in the striatum but not in the hippocampus and also decreased PKA phosphorylation in both structures; DPCPX prevented this decrease in the striatum, but not in the hippocampus. Finally, SD had no effect on EPAC activity in either of the structures. These results indicate that the A1 adenosine receptors in the striatum play a role in the impaired performance of hippocampus-independent memory tasks caused by sleep deprivation through modulation of the PKA pathway.
Keywords: Memory; learning; sleep; hippocampus.

2. Antidepressive actions of inosine

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Depression is currently defined only on the basis of behavioral modifications found in patients and the heterogeneity in clinical presentation might suggest the involvement of different neurochemical and genetic substrates. Variants in genes involved in adenosine metabolism and adenosine receptors were associated with increased risk for psychiatric disorders, including anxiety, depression and schizophrenia. In this study, we examined associations between a single nucleotide polymorphism (SNP) in A2A receptor gene (ADORA2A, rs2298383), current depressive episode and symptoms profile. In a cross-sectional population-based study, 1,253 individuals were analyzed by the Mini International Neuropsychiatric Interview 5.0. Our data showed that the TT genotype of ADORA2A rs2298383 SNP was associated with reduced risk for major depression when compared to the CC/CT genotypes (p=0.020). This association remained significant after adjusting for confounding variables as smoking, gender, socioeconomic class and ethnicity [OR=0.631 (95% CI 0.425-0.937); p=0.022]. Regarding the symptoms associated with major depression, we evaluated the impact of the ADORA2A SNP in the occurrence of sad/ discouraged mood, anhedonia, appetite changes, sleep disturbances, motion changes, loss of energy, feelings of worthless or guilty, difficulty in concentrating and presence of bad thoughts. Notably, the TT genotype was independently associated with reduced sleep disturbances [OR=0.438 (95% CI 0.258-0.743); p=0.002] and less difficulty in concentrating [OR=0.534 (95% CI 0.316-0.901; p=0.019]. Hence, our data support an important role for ADORA2A variants in clinical heterogeneity associated with major depression. The presence of at TT genotype was associated with decrease risk for major depression and protection against disturbances in sleep and attention, two of the most common symptoms associated with this disorder.
Keywords: adenosine; A2A; depression; polymorphisms

3. Purinergic Signaling in models of neurological disorders in zebrafish

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Objectives/background: Zebrafish (Danio rerio) has become a promising model in biomedical research, primarily due to its ease of maintenance and genetic manipulation. Its external and visually accessible development, its complex behavioral repertoire, and its genomic, morphological and physiological similarities to mammals have increased interest in this model, especially in relation to neuroscience. P2 and P1 purinoceptors, ectonucleotidases and adenosine deaminase have already been described in the central nervous system of zebrafish with similar characteristics to those observed in mammals. The modulation of purinergic signaling has been demonstrated in different models of neurological disorders in zebrafish, such as Alzheimer's disease, epilepsy, and autism. This study demonstrated the influence of purinergic signaling in models of neurological disorders using zebrafish as an animal model. Methods and results: We established a memory-deficit model in adult zebrafish using scopolamine, a muscarinic cholinergic receptor antagonist, which causes important amnestic effects. Acute pretreatments with caffeine, ZM 241385 (selective antagonists of A2AR) and DPCPX (8-cyclopentyl-1,3-dipropylxanthine, selective antagonists of A1R) prevented scopolamine-induced memory deficits. The inhibition of nucleoside transporters or ADA, using dipyridamole and

EHNA (erythro-9-(2-hydroxy-3-nonyl)-adenine), respectively, also prevented scopolamine-induced memory impairment. Using agonists and antagonists of the adenosine receptor, we observed the anticonvulsant effect induced by the activation of A1R in zebrafish. Cyclopentyladenosine (CPA), an A1R agonist, was able to increase the latency to reach the tonic-clonic seizure stage, and pretreatment with DPCPX, an A1R antagonist, induced a lower latency, suggesting anti- and pro-convulsant effects, respectively. When the study evaluated the effects on A2AR, through an agonist and an antagonist (CGS 21680 and ZM 241385, respectively), no changes were seen in seizure parameters. Zebrafish treated with an ecto-5'-nucleotidase inhibitor (AMPCP –adenosine 5'-(α,β-methylene) diphosphate) were able to achieve epileptic status faster than animals without this pretreatment. In a model of social interaction deficit induced by embryological exposure to valproic acid, there was an increase in AMP hydrolysis whereas the ecto-ADA activity was decreased in adult zebrafish exposed to VPA in early stages of zebrafish development. Conclusion: This animal model has a wide repertoire of pharmacological responses and may be a useful tool for drug screening and preclinical assays to test purinergic drugs as candidates for therapies for neurological disorders. Acknowledgment: FAPERGS, CNPq, CAPES.
Keywords: adenosine; purinergic signaling, neurological disorders, zebrafish.

4. Oral Communication: Purinergic signaling in bipolar disorder

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Background and aims: The physiopathology of Bipolar Disorder (BD) shows the involvement of different neurotransmission systems, including the purinergic signaling pathway as participant of depressive and manic behavior. An important feature of BD pathophysiology is a neuroinflammatory status, which has been associated to both onset and progression of the disorder. In this work, we characterized the inflammatory status in an animal model of mania induced by GBR12909 during different time points and the expression of purinergic receptors associated to neuroinflammation responses. Methods: Young adult C57Bl/6 mice (female and male) were subjected to a single (n = 7) or multiple (n = 7) intraperitoneal injections of 12,5mg/kg GBR 12909 (Sigma Aldrich) or vehicle (NaCl 0,9%; n = 10). Open field test was performed 1 hour after last injection, and animals were euthanized 1 hour after behavioral test. Brain structures were collected and 1. immediately frozen in dry ice and stored in -80°C until the further gene expression experiments (n=4); or 2. immediately digested in trypsin and fixed in paraformaldehyde 4% for further flow cytometry analysis. Results: Open field test analysis shows higher locomotor response from mice that received multiple injections than those that received a single injection. Gene expression data shows increased expression of IL-1b in both striatum and hippocampus of mice chronically induced in comparison to control group, while no significant changes were observed between control and single-injection groups. No alterations were observed in NF-kB gene expression. Among purinergic targets, A2B receptor expression was significantly decreased in both brain regions, and this condition is cell-type dependent, as confirmed by flow cytometry assay. Conclusions: GBR 12909 is a selective dopamine reuptake inhibitor that induces mania-like phenotype by increasing locomotor activity. Our results indicate a better response when injected chronically in comparison to single injections. Chronic administration induces neuroinflammation, which can be modulated by A2b adenosine receptor in both striatum and hippocampus of mice showing manic-like phenotype.
Keywords: inflammation; a2b; mania.

5. Oral Communication: New P2X7 receptor function highlighted in a mouse model of Alzheimer's disease.

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Alzheimer's disease (AD) is the most common form of dementia. This neurodegenerative disease is characterized by two main lesions: neurofibrillary tangles and senile plaques, composed of extracellular aggregates of amyloid β (Aβ) peptides. These peptides might act as an essential trigger for glial cell activation and the release of ATP, leading to purinergic receptors stimulation. Among purinergic receptors, the P2X7 receptor (P2X7R) was reported to be upregulated near Aβ plaques in animal models of AD and in patients with AD (Parvathenani LK et al. J Biol Chem. 2003; McLarnon JG et al. J Neuropathol Exp Neurol. 2006). However, the involvement of P2X7R in the development of AD is still ill defined regarding the dual properties of this receptor. Particularly, P2X7R activates the NLRP3 inflammasome leading to the release of the pro-inflammatory cytokine IL-1β (Ferrari D et al. J Immunol. 2006; Sanz JM et al. J Immunol. 2009) but is also involved in APP processing (Delarasse C et al. J Biol Chem. 2011; Darmellah A et la. J Biol Chem. 2012; Diaz-Hernandez JI et al. Neurobiol Aging. 2012). Moreover, the effects of P2X7R inhibition on cognitive deficits during AD stay an unanswered question, which stress the need for further in-depth study of the role of P2X7R in AD. In the present work, we evaluated the impact of the lack of P2X7R expression in APPPS1 mice, a well-characterized mouse model of AD that develops Aβ brain lesions (Radde R et al. EMBO Rep. 2006). We examined, in detail, the potential role of P2X7R in AD pathological processes: memory and synaptic impairment, neuropathological alterations, APP processing and immune response. We found that P2X7R deficiency decreased Aβ load and also rescued cognitive deficits in mouse model of AD. In addition, we found that lack of P2X7R did not significantly affect the release of IL-1β and the APP processing in the AD mouse model. Furthermore, we highlight a novel function of P2X7R in chemokine release associated with pathogenic T-cells recruitment during Aβ pathology. In conclusion, our work supports the notion that P2X7R may be a promising therapeutic target for AD. This work was supported by grants from Agence Nationale pour la Recherche (ANR-12-MALZ-0003-02-P2X7RAD), Association France Alzheimer and Bpifrance. Our laboratory is also supported by Inserm, CNRS, Sorbonne Université and the "Investissements d'avenir" program ANR-10-IAIHU-06 (IHU-A-ICM).
Keywords: P2X7R; Alzheimer's disease; Chemokines.

SYMPOSIUM 8 - Purinergic signaling in chronic diseases

1. Targeting P2Y2 and P2X7 Receptors in Salivary Glands in the Autoimmune Disease Sjögren's Syndrome

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Objectives/background: Chronic inflammation is typical in many human diseases where sustained accumulation of immune cells exacerbates tissue degeneration and leads to chronic disorders, including Sjögren's syndrome (SS), an autoimmune exocrinopathy of the salivary and lacrimal glands resulting in dry mouth and dry eye, respectively. Salivary and lacrimal gland dysfunction affects millions of patients whose quality of life is severely impacted by oral infections, poor nutrition, vision disorders and ocular pain. Our research objective is to define the role of P2 receptors for extracellular nucleotides as inflammatory mediators and potential therapeutic targets in the treatment of salivary and lacrimal gland dysfunction, since drugs targeting P2 receptors have already undergone clinical trials to treat human diseases, including rheumatoid arthritis, cystic fibrosis and Crohn's disease. Methods and results: Our studies utilize selective antagonists and gene ablation to demonstrate that extracellular ATP-gated ion channel P2X7 receptors (P2X7Rs) and G protein-coupled P2Y2 receptors (P2Y2Rs) regulate cellular responses relevant to the autoimmune phenotype in mouse models of SS. We previously demonstrated that P2X7R activation in salivary epithelium enhances salivary gland inflammation by promoting cell apoptosis, reactive oxygen species production and cytokine release. Utilizing the NOD.H-2h4 SS mouse model that develops hyposalivation, dry eye and lymphocytic infiltration of the submandibular gland (SMG) and lacrimal gland (LG), we found that administration of the selective P2X7R antagonist A-438079 significantly reduces immune cell infiltration of the SMG and LG and increases carbachol-induced saliva and tear secretion. Additionally, we have determined that the P2Y2R is upregulated during salivary gland inflammation where it contributes to proliferation and migration of immune cells through transactivation of growth factor receptors via metalloprotease-mediated growth factor shedding, cytokine release and activation of integrin signaling. In the IL-14α-transgenic mouse model of SS that develops lymphocytic infiltration of the SMG, autoantibody production and hyposalivation, global knockout of the P2Y2R significantly reduces B and T cell infiltration of the SMG. Similarly, administration of the P2Y2R antagonist AR-C118925 attenuated SS-like pathologies in NOD.H-2h4 mice. Conclusion: Taken together, our data suggest that P2X7Rs and P2Y2Rs are novel therapeutic targets for the treatment of SS. This study was supported by National Institutes of Health USA R01 grants DE007389 and DE0223342.
Keywords: autoimmune disease; P2Y2 receptor; P2X7 receptor; inflammation.

2. Fructose 1,6-bisphosphate, a glycolytic intermediate, regulates macrophage activation through adenosinergic-signaling pathway

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Fructose 1,6-bisphosphate (FBP) is an intermediate of glycolysis that, when administered exogenously, has anti-inflammatory properties. However, the mechanisms underlying these effects remain poorly understood. Here, we demonstrate that FBP tunes the metabolic reprogramming of LPS-activated macrophages enhancing production of IL-10, which act as an autocrine signal that self-limit macrophage activation state. Exogenous FBP comes into the glycolysis pathway, increasing the synthesis and secretion of ATP, which is rapidly catabolized into adenosine. Blocking glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an FBP downstream enzyme of the glycolytic pathway, but not hexokinase (HK) or Glut1, inhibit the enhanced production of IL-10 induced by FBP. Moreover, blocking pannexin1, an ATP-releasing channel, or the ectonucleotidases CD39 and CD73 also inhibit the enhanced production of IL-10 by FBP. Also, the inhibition of adenosine receptor A2a (A2aR), but not A1R, A2bR, or A3R, blocked the enhanced production of IL-10 by FBP. In line, FBP failed to enhance IL-10 production in LPS-activated macrophages from A2AR KO mice or in the presence of adenosine deaminase (ADA). Finally, in a model of colitis with DSS in drinking water, treatment with FBP was able to reduce the score of the disease and concentration of pro-inflammatory cytokines, besides increased IL-10 in the colon. Taken together, these data implicate FBP as a key molecule that calibrates the metabolic reprograming of pro-inflammatory macrophages by enhancing the production of IL-10, self-limiting their activation state.
Keywords: Adenosine; ectonucleotidases; macrophage; IL-10.

3. Purinergic Signalling in Inflammatory Bowel Diseases

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Inflammatory bowel disease (IBD), comprising Crohn's disease and ulcerative colitis, represent chronic inflammatory disorders of unknown cause, and unmet therapeutic needs. Damage-associated molecular patterns (DAMPs) constitute endogenous stress molecules released as a result of cell or tissue damage, and have been regarded as pro-inflammatory mediators, and associated with acute and chronic inflammatory disorders, including IBD. Extracellular ATP is an endogenous signaling molecule released by diverse cell types under different stimuli, and has been considered as a DAMP. High concentrations of ATP released into the extracellular medium activate the P2X7 receptor (P2X7-R) in most inflammatory conditions. We previously demonstrated that the activation of P2X7-R by ATP induces apoptosis and autophagy in human epithelial cells, possibly via ROS production. In experimental models, we showed that P2X7-R has a key role during inflammation by triggering the death and retention in the mesenteric lymph nodes of regulatory T cells, and that prophylactic systemic P2X7-R blockade is effective in the prevention of experimental colitis. Moreover, P2X7-R deficient animals did not develop chemically induced colitis. In addition, the overexpression of P2X7 receptor in the inflamed mucosa of patients with IBD is consistent with the involvement of purinoceptors in cell death and inflammation. Together, these observations implicate purinergic signaling in the pathogenesis of intestinal inflammation, and the P2X7-R as a potential novel target for the treatment of IBD.
Keywords: inflammatory bowel disease; P2X7;ATP;purinergic signaling.

4. P2X7 receptor antagonist recovers ileum myenteric neurons after experimental ulcerative colitis
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Objectives/background: The P2X7 receptor is expressed by enteric neurons and enteric glial cells (Da Silva et al. Histochem. Cell Biol. 142; 171, 2015). Studies have demonstrated that administration of antagonist of P2X7receptor, Brilliant Blue G (BBG), prevents neuronal loss. We report the effects of the BBG in ileum enteric neurons immunoreactive (ir) for nitric oxide synthase neuronal (NOSn), acetyl choline transferase (ChAT), pan neuronal (Hu) and glial fibrillary acidic protein (GFAP, marker for glial cells) after ulcerative colitis. Methods and Results: Male rats were used (Rattus novergicus albinus). 2,4,6-trinitrobenzene sulfonic acid (TNBS group, n=5) was injected at a dose of 30 mg/kg in 1 mL of 30% ethanol in the distal colon through the intra-rectal insertion of a polypropylene 8 cm cannula. The BBG (50 mg/kg, BBG group, n=5) or vehicle (Sham group, n=5) was given subcutaneous 1 hour after TNBS. The animals were euthanized after 24 hours and ileum was removed. This study was accordance with the Ethics Committee on Animal Use of the University of São Paulo, Brazil. Tissues were prepared by immunohistochemical methods with double labelling of the NOSn, ChAT, Hu, and GFAP with P2X7 receptor and histological methods. Quantitative analyses of the neurons and glial cells were identified by confocal scanning laser microscope and optical microscope. The number of neurons (cm2) and profile area (μm2) of neurons NOSn-, ChAT-, Hu-ir and, glial cells GFAP-ir were obtained. Data were compared using ANOVA and Tukey's test, $p<0.05$ was statistically significant. The results: there were double labelling of neurons and glial cells with P2X7 receptor. The neurons NOSn-ir/cm2 of sham, TNBS and BBG groups were 7190±393, 5541±240, 7124±107, respectively ($p<0.05$); the neurons ChAT-ir/cm2 of sham, TNBS and BBG groups were 19706±738, 13001±314 and 15112±499, respectively ($p<0.05$); the neurons Hu-ir/cm2 of sham, TNBS and BBG groups were 32916±890, 27836±890 and 34565±664, respectively ($p<0.05$). The glial cells GFAP-ir/cm2 of sham, TNBS and BBG groups were 22180±965, 18981±1253 and, 23071±1092, respectively ($p<0.05$). The neuronal profile area (μm2) demonstrated that neurons NOSn-ir of sham, TNBS and BBG groups was 353±7, 301±4 and 329±7, respectively ($p<0.05$); the neurons ChAT-ir of sham, TNBS and BBG groups were 250±4, 212±4 and 243±4, respectively ($p<0.05$); the neurons Hu-ir of sham, TNBS and BBG groups were 259±4, 263±4 and, 258±3, respectively. Histological studies revealed that the mucosa, lamina propria and submucosal ganglia in the Sham and BBG groups were normal appearance. The lamina propria of the TNBS group displayed increased thickness. Conclusion: Our data conclude that ileum myenteric neurons and glial cells were affected by ulcerative colitis and, treatment with BBG was efficient in neuroprotection. Thus, these results demonstrate that P2X7 receptor may be an important target in the therapeutic strategy. Financial support: FAPESP, CAPES, PIBIC/CNPq
Keywords: antagonist of P2X7 receptor; myenteric neurons; enteric glial cells; inflammation.

5. Oral Communication: Inhibition of AMP Deaminase is Cardioprotective in Acute Oxygen Deprivation

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Background: Clinical analysis of the effect of genetic polymorphisms known to affect cardiac AMP deaminase (AMPD) activity in cardiovascular disease demonstrated diverse results with uncertain mechanisms indicating need for experimental studies. This study evaluated effects of a genetic alteration of AMPD activity in mice hearts subjected to oxygen deprivation. Methods and Results: Double knock-outs for Apolipoprotein E (ApoE) and Low Density Lipoprotein Receptor (LDLR) mice were crossed with AMPD-/- CRE+ strain. Target genetic pattern was confirmed by genotyping for ApoE, LDLR, AMPD, and CRE. Functional confirmation of the genetic alterations was performed by analysis of the heart homogenates of ApoE-/-LDLR-/-AMPD-/-CRE+ (3KO), ApoE-/-LDLR-/- (2KO), AMPD-/-CRE+ (DKO) and wild type (WT) male mice strains (n=7). Activities of AMPD, adenosine deaminase (ADA), ecto-5'nucleotidase (e5NT) and purine nucleoside phosphorylase (PNP) in the mouse hearts were measured by monitoring the conversion of substrates into products by HPLC as described previously. AMPD activity decreased to 25% in 3KO when compared to 2KO strain. Activities of PNP, ADA and e5NT were similar in all groups of animals. Analysis of mRNA expression revealed absence of AMPD1 mRNA in all group, detectable mRNA for AMPD2 and depressed expression of the AMPD3 in KO and 3KO mice as compared to 2KO. When subjected to hypoxia (breathing 7% O2 for 7.5 min), inhibition of AMPD in 3KO male mice resulted in attenuation of ECG STU area changes and in decrease of troponin T concentration in the serum 6h after hypoxia when compared to 2KO male mice- from 188.5±17.2 pg/ml in 2KO to 147.7±8.4 pg/ml in 3KO, n=7, $p<0.001$. In addition, we found that phosphorylation status of AMP regulated protein kinase was significantly 30% elevated in the 3KO mice hearts. Conclusion: This study shows that reduced AMPD activity is protective in acute oxygen deprivation and that activation of AMPK cascade is likely to be the mechanism. This study is consistent with potential use of AMPD inhibitors in cardiac acute oxygen deprivation. Acknowledgements: This research was supported by National Science Centre of Poland (2016/22/M/NZ4/00678) and Foundation for Polish Science (TEAM/2011-8/7).
Keywords: AMP deaminase; purine metabolism; hypoxia; AMP-activated protein kinase.

SYMPOSIUM 9 - Purinergic Signalling in Cancer I

1. CD73 as biomarker of advanced melanoma response to immunotherapy

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Objectives/background: Anti-PD1 agents are successfully used in therapy to treat patients with advanced melanoma. However, a significant percentage of melanoma patients experienced low response rate or being refractory to anti-PD1 therapy, highlighting the need to have valid predictive factors of response to therapy and/or new therapeutic options with a significant impact on patients survival. We hypothesized that the identification of new biomarkers/targets, based on serum CD73 profiling, is an alternative option that responds to such urgent need. CD73 is a membrane-bounded ectonucleotidase able to hydrolyse extracellular ATP into adenosine, that potently suppresses anti-tumor T cells responses

within tumor microenvironment. A soluble form of CD73 (sCD73) has been also found in serum and its levels are significantly increased in serum of cancer patients compared with healthy individuals. In this study we analysed the serum activity of sCD73 and the expression of CD73 in frozen peripheral blood mononuclear cells (PBMCs) from melanoma patients undergoing treatment with nivolumab. Methods and results: Basal levels of CD73 activity were retrospectively analysed in the serum of 94 patients with metastatic melanoma stage IV, collected before starting nivolumab treatment from a single center. The flow cytometry analysis was performed in the first cohort of patients (36 patients) to analyse the relative expression of CD73 on myeloid-derived suppressor cells (MDSCs: CD14+ CD33+ CD11b+ HLA-DR-/low) and CD8+T cells, alone or in association with PD-1. The CD73 levels were correlated with clinical outcomes of patients. Results on sCD73 activity show that the disease control rate to nivolumab was significantly associated with low pre-treatment CD73 enzymatic activity compared with patients with progressive disease [median CD73 activity 13.93 (95%CI: 15.57-35.17) versus 46.29 (95%CI: 54.32-95.43); p=0.0004). At univariate and multivariate analyses, serum CD73 activity was significantly associated with both overall survival (OS) and progression-free survival (PFS). Patients who do not respond to nivolumab therapy presented higher levels of CD73 enzyme activity in the blood (p=0.005). Data on CD73 expression on PBMC revealed that patients with higher baseline frequency of CD8+/PD-1+/CD73+ T cells had lower OS and PFS (9.5 and 2.8 months, respectively; OS $p < 0.003$, PFS $p < 0.007$). At the same time, we found that the percentage of MDSC-CD73+ was significantly higher in patients who do not respond to therapy compared with patients who respond. This study was approved by the ethics committee of the National Cancer Institute "Pascale" of Naples. Conclusions: Taken together our data suggest that, prospectively, CD73 could be used as immunologic biomarker in the peripheral blood, useful to select patients for anti-PD1 therapy. These results also strengthen the therapeutic potential of anti-CD73 drugs
Keywords: CD73; Biomarker; Immunotherapy; Melanoma.

2. P2 receptors are involved in glioma progression

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Objectives / background: Gliomas are tumors of central nervous system, and account approximately 30% of all primary brain tumors. Glioblastoma multiform (GBM), the most common type of glioma, has a bad prognosis and a very limited range of treatments. In recent years, occurred solid progress in understanding the molecular pathogenesis of gliomas.In fact, the Cancer Genome Atlas (TCGA) divided this type of tumor into different subclasses grounded on genetic alterations, mainly IDH. In tumor microenvironment, due to inflammation and the presence of hypoxia in solid tumors, both adenosine triphosphate (ATP) and adenosine may be kept elevated for extended periods. ATP and other nucleotides and nucleosides, such as ADP and UTP, can induce proliferation and migration in esophageal cancer cells, suggesting the involvement of different P2R subtypes (Santos et al. Purinergic Signal. 13(3);279-292, 2017; Zaparte et al., 2018, submited). Previously, we showed that gliomas co-injected with apyrase had a significant reduction in the tumor size when compared with rats injected with gliomas (Morrone et al. BMC Cancer 6; 226, 2006). Another study showed that NTPDase2 overexpression in C6 glioma cells dramatically increased tumor growth and malignant characteristics in vivo (Braganhol et al. Cancer Sci.100(8); 1434-1442, 2009). Since the hydrolysis of ATP lead to a decrease of tumor progression and ADP promoted glioma growth, we aimed to investigate the effect of P2R pharmacological modulation on gliomas progression. Methods and results: Our data showed that ATP-P2RX7 can lead to gliomas cell death and is an important pathway to radiotherapy success (Gehring et al. Purinergic Signal. 8;729–739, 2012). We also showed that high P2X7R expression is a good prognostic factor for glioma radiosensitivity and survival probability in humans (Gehring et al. Int J Biochem Cell Biol. 68; 92–100, 2015). On the other hand, we showed that ATP and its derivates have effects on glioma cells proliferation by stimulation of some of P2Y receptors (Morrone et al. J Neurooncol. 64;211–218, 2003). We, then, evaluated the role of ADP and P2Y12 receptor in the proliferation and survival capacity of glioma cells. The treatment with the P2Y12 antagonist clopidogrel lead to a significant reduction in the number of cells and viability, in C6 rat cell line and human glioblastoma U251-MG cells. P2Y12R blockage diminished the number of colonies after 24 h treatment and led to significant arrest in the G1 phase cell cycle in U251-MG cell line. Therapy with the blocker P2Y12, clopidogrel, triggered autophagy in glioma cell lines. Furthermore, P2Y12R activation can increased the invasion ability of glioma cells. Conclusion: Our data suggest that ATP and ADP, can activate P2R receptors and induce GBM progression. The use of specific P2R antagonists can be a therapeutic alternative to control proliferation and invasion in this type of cancer.
Financial support: FAPERGS, CNPq, CAPES and FINEP.
Keywords: P2R; ATP; ADP; gliomas.

3. P2X7 receptor splice variants A and B as oncogenes and predictors of response to chemotherapy: evidence from patients and murine experimental models

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Objectives/background: P2X7 receptor for eATP recently emerged as a player in oncogenesis, due to its involvement in tumor growth, vascularization, metastasis, and cancer-immune cells crosstalk. The human gene for P2X7 can give rise to nine different protein variants among which only the P2X7A and B are functional ion channels. While several studies associated P2X7A expression to cancer development and progression a characterization of the role of P2X7B in oncogenic patients and mice models are still missing. For these reasons, we analyzed expression of P2X7A and B in acute myeloid leukemia (AML) and melanoma patients in an effort to relate it to disease progression and response to therapy.Methods and results: P2X7A and B mRNA levels were investigated by real-time PCR in two patient's cohorts. The first group included people affected with either AML or myelodysplastic syndrome, a pre-cancerous condition often leading to AML, for a total of 93 individuals. Both P2X7A and B were overexpressed in AML firstly diagnosed as compared to healthy controls and MDS patients, supporting the hypothesis that P2X7 expression positively correlates with disease progression. Of interest, we retrieved a differential expression of receptor's isoforms in AML relapsing patients, which are presented with a return of the pathology after the first therapeutic intervention. Indeed, in relapsing patients, while P2X7A tends to decrease, P2X7B is significantly increased. On the contrary, comparing de novo with remitting patients both P2X7A and B decrease. These data suggest that individuals expressing high levels of P2X7B could be resistant to chemotherapy and prone to relapse. The second cohort analyzed included 68 advanced stage melanoma patients. In this

group of individuals, we were able to associate an increase of both P2XA and B levels with the degree of dissemination at distant sites. To further test the hypothesis that P2X7B could be up modulated by chemotherapy, we set up an AML xenograft model injecting HL-60 cells in nude mice. Tumor-bearing mice were administered the main therapeutic agent received by AML patients: daunorubicin, alone or with P2X7 antagonist AZ10606120. In accordance with patients' data, daunorubicin caused an increase in P2X7B tumor levels, which was reduced by P2X7 pharmacological blockade. Interestingly, both daunorubicin AZ10606120 significantly reduced tumor cell growth and the co-administration of the two drugs proved more efficacious than the single agent treatment. Human and animal experimentation were authorized by the ethics committees of the Universities of Bologna and Ferrara, and from the Italian Ministry of Health. Conclusions: Taken together our data suggest that both P2X7A and P2X7B isoforms could be useful prognostic markers and attractive therapeutic targets in AML and melanoma.
Keywords: P2X7 isoforms; chemotherapy; acute myeloid leukemia; melanoma.

4. Novel evidence that extracellular nucleotides and purinergic signaling induce innate immunity-mediated mobilization of hematopoietic stem/progenitor cells

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Background/Objectives: Pharmacological mobilization of hematopoietic stem progenitor cells (HSPCs) from bone marrow (BM) into peripheral blood (PB) is a result of mobilizing agent-induced "sterile inflammation" in the BM microenvironment due to complement cascade (ComC) activation. Methods/Results: ATP level has been measured by ELISA in the BM of mobilized mice as well in conditioned media from neutrophils exposed to G-CSF or AMD3100. Pathogen-free, 4–6-week-old C57BL/6J wild-type (WT), B6.129P2-P2rx7tm1Gab/J (P2X7–/–), and B6.129S1-Nt5etm1Lft/J (CD73–/–) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Following mobilization, we measured i) the total number of white blood cells (WBCs), ii) the number of circulating clonogenic colony-forming unit granulocyte/macrophage (CFU-GM) progenitors, and iii) the number of Sca-1+c-kit+lineage– (SKL) cells in PB. We provide evidence that ATP, as an extracellular nucleotide secreted in a pannexin-1-dependent manner from BM cells, triggers activation of the ComC and initiates the mobilization process. This process is augmented in a P2X7 receptor-dependent manner, and P2X7-KO mice are poor mobilizers. Furthermore, after its release into the extracellular space, ATP is processed by ectonucleotidases: CD39 converts ATP to AMP, and CD73 converts AMP to adenosine. We observed that CD73-deficient mice mobilize more HSPCs than do wild type mice due to a decrease in adenosine concentration in the extracellular space, indicating a negative role for adenosine in the mobilization process. This finding has been confirmed by injecting mice with adenosine along with pro-mobilizing agents. In sum, we demonstrate for the first time that purinergic signaling involving ATP and its metabolite adenosine regulate the mobilization of HSPCs. While ATP triggers and promotes this process, adenosine has an inhibitory effect. Conclusion: Administration of ATP together with G-CSF or AMD3100 or inhibition of CD73 by small molecule antagonists may provide the basis for more efficient mobilization strategies.
This work was supported by NIH grants 2R01 DK074720 and R01HL112788.
Keywords: "Sterile inflammation"; "bone marrow"; "ATP".

5. Oral Communication: Methotrexate reduces the glioblastoma growth by interfering with the adenosinergic system

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Background and Objectives: Glioblastoma multiforme (GBM) is a deadly cancer of the central nervous system characterized by a pro-tumoral immune response. T regulatory (Treg) lymphocytes suppress effector immune cells through cytokine secretion and the adenosinergic system. Ecto-5'-nucleotidase/CD73 plays a crucial role in Treg-mediated immunosuppression in the GBM microenvironment (GME). Methotrexate (MTX) is an antimetabolite and immunosuppressive drug that can increase the extracellular concentration of adenosine. The present work sought to understand the mechanism by which the MTX reduces the GBM growth. Methods and Results: C6 GBM cells were treated with 1.0 μM MTX for 24h, and ecto-5'-nucleotidase/CD73 expression and extracellular AMP metabolism were analyzed in vitro. To understand MTX mechanism, GBM cells were treated with APCP, dipyridamole, ABT-702 and caffeine before treatment with MTX. For in vivo analysis, rats with implanted GBM were treated for 10 days with MTX-loaded lipid-core nanocapsules (MTX-LNCs, 1 mg/kg/day). The activity and the expression of NTPDase1/CD39 and ecto-5'-nucleotidase/CD73 were measured. The frequencies of T lymphocytes (CD3+CD4+, CD3+CD8+ and CD4+CD25highCD39+) were quantified. In addition, the measurement of the tumor size and tumoral apoptosis were carried out. In vitro, MTX increased CD73 expression (from 209.9 ± 7.9 to 298.8 ± 24.9) and activity (from 136.9 ± 3.9 to 166.8 ± 3.9 nmol Pi/min/mg of protein) in C6 cells, which is in agreement with higher levels of extracellular adenosine (from 808.5 ± 14.57 to 1146.0 ± 110.6 nmol/mg of protein). In GBM cells, inhibitors and antagonist treatment did not alter the anti-GBM effect of MTX. In vivo, with the exception of the CD3+CD8+ subset, none of the studied cells in the GME displayed a change in ADP/ATP hydrolysis or CD39 expression following treatment with MTX-LNC. The treatment with MTX-LNC up-regulated CD73 expression in tissue isolated from GBM (from 56.7 ± 2.5% to 72.1 ± 3.3% of positive cells), in agreement with the higher activity of this enzyme (from 1.2 ± 0.7 to 4.7 ± 1.3 nmol Pi/min/106 cells). More specifically, the treatment increased CD73 expression on CD3+CD4+ (from 34.8 ± 3.5% to 69.7 ± 10.6% of positive cells) and CD3+CD8+ (from 36.0 ± 3.0% to 51.9 ± 2.4% of positive cells). Treatment with MTX-LNCs decreased the frequencies of T-cytotoxic (from 7.0 ± 0.5% to 3.7 ± 0.8%), T-helper (from 19.9 ± 1.3% to 6.3 ± 1.6%) and Treg (from 1.21 ± 0.33% to 0.11 ± 0.04%) lymphocytes in the GME. We also observed a decrease in the tumor size (from 223 ± 47 mm3 to 98 ± 32 mm3) and an increase in apoptosis to 55% in the tumor microenvironment following the treatment. Conclusion: Although more studies are necessary, MTX reduces the GBM growth by modulate the immune system, but not GBM cells itself. MTX-LNC treatment modulates immunosuppressive capability, reduces the tumor size and increases apoptotic death in a pre-clinical glioblastoma model.

Support: CNPq, CAPES
Keywords: Ecto-5′-nucleotidase/CD73; adenosine; glioblastoma; methotrexate.

SYMPOSIUM 10 - Purinergic Signalling in Cancer II

1. Ubiquitination of tumor suppressor PML regulates CD73 to foster an immunosuppressive tumor microenvironment

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Objectives/background: The tumor microenvironment plays an important role in tumor growth and metastasis. However, the mechanism by which tumor cells regulate the cell and non-cell constituents of surrounding stroma remains incompletely understood. PML is a pleiotropic tumor suppressor but its role in tumor microenvironment regulation is poorly characterized. Methods and results: We identify a novel PML ubiquitination/destruction pathway mediated by ubiquitin ligase CRL4WDR4. Clinically, this PML destruction pathway is hyperactivated in lung cancer and correlates with poor prognosis. The WDR4/PML axis induces the expression of CD73, which is known to promote immunosuppression. In both xenograft and genetically engineered mouse models, the WDR4/PML axis elevates intratumoral Tregs and M2-like macrophages and reduces CD8+ T cells to promote lung tumor growth and these immunosuppressive effects are all reversed by CD73 blockade. Conclusion: Our study identifies WDR4 as a novel oncoprotein which negatively regulates PML via ubiquitination to promote lung cancer progression by fostering an immunosuppressive and pro-metastatic tumor microenvironment and suggests a potential of immune-modulatory approaches for treating lung cancer with aberrant PML degradation.
Keywords: CD73; PML; Immunosuppression.

2. Purinergic signalling, zombie Tregs and cancer

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Tumors grow by recruiting a range of protective immune suppressive pathways that prevent the cancer cells being recognized as foreign or dangerous and allow evasion of immune elimination. There are multiple means by which tumors subvert host responses, which can limit inflammatory reactions and induce tolerance towards tumor-antigens. Immunotherapies are newly developing interventions that modify the patient's immune system to fight cancer, by either directly stimulating rejection-type processes or by blocking suppressive pathways. These innovative approaches include chimeric antigen receptor (CAR) T cell therapy and checkpoint blockade with immunomodulatory antibodies (e.g. anti-CTLA-4, anti-PD-1), which have brought hope to many cancer patients. Standard cancer therapies, including radiotherapy, also seem to require the active involvement of the patient's immune system for efficacy. Our focus has been on the regulation of purinergic signaling by the ectonucleotidases of CD39 family, which catalyze phosphohydrolysis of extracellular nucleotides. These ecto enzymes, in tandem with CD73, generate adenosine. This nucleoside is functional as an "immune checkpoint mediator" that interferes with anti-tumor immune responses and may cause resistance to anti-PD1 therapy. This presentation will focus on CD39 and related ecto enzymes of the ENTPD family and encompass novel biochemistry and functionality of these, when expressed on immune, stromal and vascular cells. Effects of CD39 expression on activated and apoptotic Treg (Zombie) and practical inhibition in preclinical, experimental studies will be discussed. The proposed clinical application of purinergic therapies with a focus on this canonical pathway of nucleotide phosphohydrolysis will be addressed. The presentation will be inclusive of suggestions as to how these new approaches might develop in combination with other, more established anti-cancer modalities.
Keywords: CD39; T regulatory cells; adenosine; cancer.

3. Disruption of adenosine A2a receptor signaling in mice impairs immune and metabolic homeostasis to promote liver carcinogenesis

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The targeted blockade of adenosine A2a receptors has recently emerged as a promising approach to promote anti-tumor immunity and enhance the therapeutic activity of immune-checkpoint inhibitors. In this regard, convincing results have been obtained in preclinical tumor models using A2a-deficient mice or short-term treatment with selective A2a inhibitors. However, considering the quasi-ubiquitous expression of A2a receptors and its pivotal role in the regulation of metabolic and immunologic homeostasis, long-term blockade of this pathway might induce deleterious side effects. Using A2a-deficient mice, our results demonstrate that disruption of A2a signaling promotes the development of spontaneous and carcinogen-induced liver cancers. In fact, we observed that adult A2a-deficient mice rapidly developed morbid obesity with age, associated with insulin resistance, increased visceral adiposity, systemic inflammation and non-alcoholic fatty-liver disease. Using bone marrow chimeric mice, we observed that A2a-signaling on hematopoietic cells was required but not sufficient to phenocopy the metabolic alterations observed in full knockout animals. Interestingly, in young A2a-deficient animals, metabolic alterations identified in older animals were absent but a significant reduction in energy expenditure compared to WT mice was detected. While this difference could not be explained by a defect in thermogenesis, we observed a dramatic reduction of locomotor activity in young A2a-deficient mice compared to WT animals. In conclusion, while several A2a receptors inhibitors are currently being tested in phase I clinical trial in cancer patients, our results indicate that long-term blockade of A2a-signaling pathway in mice profoundly perturbs immune and metabolic homeostasis ultimately leading to enhanced liver carcinogenesis
Keywords: CD73; obesity; A2A; liver.

4. Oral Communication: Potential therapeutic application of anti-CD39 monoclonal antibodies

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Objectives / Background: In combination with conventional therapies (e.g. targeted therapy, chemotherapy, and angiogenesis inhibitors etc.), immunotherapies targeting checkpoint molecules have shown promise in the treatment of solid tumors. However, apoptotic regulatory T cells (Treg) induced by such therapies often become more suppressive in the tumor microenvironment (TME), through increased generation of adenosine tightly controlled by ectonucleotidases, viz. CD39 and CD73. CD39/ENTPD1, a novel checkpoint molecule, is highly expressed and activated on the tumor vasculature and infiltrating immune cells, promoting tumor growth. Others and we have shown that deletion or blockade of CD39 enhances anti-tumor activity by augmenting anti-tumor immune responses and inhibiting tumor angiogenesis. Methods and results: Herein, we will discuss the recent development of anti-CD39 monoclonal antibodies and the utility if these in blocking tumor growth with minimal side effects in pre-clinical models. We will also provide novel molecular insights into the mechanism of action of these monoclonal antibodies. Conclusion: Recent data suggests potential of anti-CD39 monoclonal antibodies as therapeutic tools in cancer. Acknowledgment and Financial support: This work is supported by NIH (R21) CA221702 and (R01) DK108894, Department of Defense W81XWH-16-1-0464 and Helmsley 281574.5069091.0010.
Keywords: Cancer Therapy; Immunotherapy; CD39; Monoclonal Antibody.

5. Oral Communication: Role of the Adenosine A2B Receptors in behavior and maturation of normal and malignant stem cells

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Background: The Adenosine A2B Receptor (A2BR) has been implicated in the behavior of normal and malignant stem cell (SC) function. However, the field is somewhat unclear regarding its role(s) in mesenchymal SC (MSC) and cancer SC (CSC). Inhibition of A2BR prevents mesenchymal SC (MSC) differentiation but its presence is critical during the proliferation and expansion processes in hematopoietic SC. The role of A2BR is less clear in CSC, whereby its overexpression has been associated with the tumor microenvironment, but there have been conflicting reports on its role in proliferation. These differences may be explained by host species variation, human donor variations, tissue of origin, and isolation and expansion methods of MSC; as well as the ongoing defining of CSC. More importantly, the literature mostly reports on isolated cells, rather than SC within their natural niche. Further, most groups investigated the role of A2BR in one aspect of SC function without considering other aspects of behavior, such as differentiation and immunomodulatory activity. Together this has contributed to a lack of clarity in the overall role of A2BR in SC. Objective: The purpose of this study is to dissect the role of A2BR in different subsets of SC. Methods: Human MSC (n=3) were isolated from fresh bone marrow aspirates and cultured in monolayer (2D) or three-dimensional (3D) culture conditions. MSCs were exposed to the secretome of MDA-MB-231 triple negative, highly aggressive breast cancer cells (BCC) in co-culture. A similar system was performed by exposing the BCCs to the MSC secretome. Different BCC subsets were used based on a working hierarchy of BCCs, beginning with CSCs. Changes in A2BR expression in each the MSC and BCC were determined using NextGen sequencing and RT-PCR. Results: A2BR is highly expressed in MSCs grown under 2D and 3D culture conditions, and is upregulated following exposure to the BCC secretome. A dissection of A2BR in various CSC and non-CSC BCC populations shows that A2BR expression does not change during the development of BCC progenitor cells. Conclusion: A2BR is critical for maintenance of the SC state, beyond any singular SC-associated function. Interestingly, 3D culture is believed to enhance the SC behaviors of MSC yet does not dramatically increase A2BR expression of MSC. Similarly, CSC maturation into BCC progenitor and tumor cells – a loss of SC state – did not result in decreased A2BR expression. Together, this suggests that the role of A2BR in SC behavior is more complex than the literature suggests, requiring further study.
Keywords: mesenchymal stem cell; cancer stem cell; adenosine A2B receptor; bone marrow.

SYMPOSIUM 11 - Involvement of the Purinergic System in Pain

1. Microglial ATP receptors in neuropathic pain

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Neuropathic pain is a debilitating pain state, which is often caused by peripheral nerve injury (PNI) by autoimmune disease, diabetes, cancer or physical injury. The hallmark symptom of neuropathic pain is tactile allodynia (pain hypersensitivity to innocuous light touching). Tactile allodynia is refractory to available medicines such as non-steroidal anti-inflammatory drugs and opioids. The mechanism of the neuropathic pain was still largely unknown. We found that activated spinal microglia of dorsal horn over-expressed P2X4 receptors (P2X4R) which cause to release brain-derived neurotrophic factor (BDNF) after the stimulation of ATP. BDNF evokes a collapse of their transmembrane anion gradient in the secondary neurons of dorsal hone resulting in tactile allodynia (Tsuda et al. Nature, 424, 778-783, 2003; Coull et al. Nature, 438, 1017-1021, 2005). Now we know that various molecules in microglia play important roles in the relation to the neuropathic pain. We examined what regulates the expression of these molecules and found that the transcription factor interferon-regulatory factor 8 (IRF8) is a critical regulator of the gene expression of these molecules in microglia. Recently, we also have found the important role of IRF5-IRF8 for the expression of P2X4R in microglia (Masuda et al. Nat. Commun, 5:3771, 2014). ATP is released from primary afferent neurons of spinal dorsal horn (SDH) and various glia cells. We found that not microglia nor astrocytes but SDH inhibitory interneurons

that express vesicular nucleotide transporter (VNUT) are a candidate source of the ATP (Masuda, et al. Nat. Commun, 7:12529, 2016; Inoue & Tsuda Nature Rev Neurosci, 19, 138-152, 2018). The role of purinergic P2X4R function in microglia in the mechanisms of neuropathic pain provides exciting insights in its pathogenesis, and suggests P2X4R inhibitors may be potential candidates for new medicines against neuropathic pain. We are finding such inhibitors from already approved medicines as the drug-developing system namely "Eco-Pharma" for providing benefits of science to patients as soon as possible. We will present some examples of Eco-Pharma in the symposium.
Keywords: microglia; neuropathic pain; P2X4 receptors.

2. Acupuncture-induced analgesia

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Acupuncture has been used in China from ancient times since more than 2,000 years ago. A variety of disorders can be treated effectively by inserting long, fine needles into specific acupuncture points (acupoints) on the skin of the patient's body. Since acupuncture was proposed by National Institutes of Health (NIH) consensus in 1997 as a therapeutic intervention of complementary medicine, acupuncture efficacy has become more and more accepted in the Western world. Among acupuncture therapies, the acupuncture-induced analgesic effect has been used widely to alleviate diverse types of pain, particularly chronic pain. To date, acupuncture analgesia has drawn the attention of many investigators and become an important research subject of international interest around the world. Numerous studies have also demonstrated that acupuncture analgesia has physiological, anatomical and neurochemical basis despite that there is still an ongoing debate about the mechanism by which acupuncture alleviates pain. Since Professor Geoffrey Burnstock proposed that purinergic signalling, rather than a mystical subepidermal energy, may explain how acupuncture works in an article in Medical Hypotheses in 2009, the role of purinergic signalling in acupuncture research has gained much attention. So far, more scientists have got started to study the role of purinergic signalling in acupuncture-induced analgesia. In this talk, the work have been done by our group and other scientists will be summarized and where we are going and how we are going to get there in this amazing field will be described.
Keywords: Purinergic signaling; Pain; Acupuncture therapy.

3. Purinergic signalling in migraine pain

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ATP is released in different parts of the trigeminal nociceptive system implicated in generation of migraine pain. Extracellular ATP can control all main actors involved in nociception such as trigeminal nerve endings, dural vessels and meningeal mast cells. Moreover, purinergic signalling is likely involved in migraine-associated neuroinflammatory processes including involvement of peripheral immune cells. We recently showed, both in rats and mice, a robust activation of trigeminal nerve endings in meninges by extracellular ATP. This pro-nociceptive effect was significantly less presented in mice lacking meningeal mast cells suggesting their contribution to ATP-driven pain signalling. Indeed, ATP and BzATP produced an essential degranulation of mast cells, primarily via P2X7 receptor subtype. In contrast, the direct action of ATP on meningeal afferents was mainly mediated by activation of P2X3 receptors expressed on trigeminal nerve endings in dura mater. The migraine mediator calcitonin gene-related peptide (CGRP) increased the meningeal level of ATP and ADP but reduced adenosine concentration in trigeminal cells. Our computer ‘spatial’ model of purinergic signalling provided a rationale for the apparent paradox on how the prolonged activity of ATP in situ is consistent with the phenomenon of fast and profound desensitization of P2X3 receptors. In summary, I will overview both central and peripheral mechanisms of purinergic mechanisms of migraine pain. Supported by the Finnish Academy (grant 277442)
Keywords: ATP; P2X7; P2X3; pain.

4. Oral Communication: Bug off pain: probing P2X channels with venoms

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Today, one fifth of the human population suffer from chronic pain – a figure that will increase to one half for those older than 65 years old.1 Currently there is inadequate treatment for chronic neuropathic pain due to the fact that it is often resistant to opioids.2 A number of ion channels are critical players in pain pathophysiology with some of the P2X receptors implicated in having important roles3. In many cases the most potent and selective blockers of ion channels are toxins from venoms. Purotoxin-1, isolated from spider venom, is a potent and selective peptide inhibitor of P2X3 receptors.4 Our aim is to investigate whether crude spider, cone snail, centipede, or snake venoms contain peptide modulators of key P2X receptors including P2X3, P2X4 and P2X7. In preliminary experiments with either HEK-293 or astrocytoma 1321N1 cells stably expressing human P2X receptors, >300 crude venoms were screened for an inhibitory/potentiating effect. We measured responses using a variety of fluorescent assays such as YOPRO-1 dye uptake, Calcium 6 and fura-2AM calcium indicator dyes on a Flexstation 3 plate reader with P2X receptor agonists ATP or alpha, beta-methylene ATP. Crude spider venoms were found to have inhibitory effect on human P2X4 using HEK-293 cells and YOPRO-1 uptake and this was validated using a second stable cell line and calcium influx assay. Fractionation of crude spider venoms was performed using RP-HPLC. Molecular identification of these inhibitory fractions (compounds 1 and 2) was performed by MALDI-TOF, LC-MS and MS-MS indicate the inhibitory toxins to be structurally uncharacterized small molecules, found in a number of spider species. Spider venoms contain small molecular components in abundance including nucleotides and poly-amines. We tested nucleotides (e.g. ADP) and polyamines (e.g. spermine, spermidine, putrescine, cadaverine) on human P2X receptor responses. We found no inhibitory effect on human P2X4 in YOPRO-1 dye uptake and calcium influx assays. In addition to small molecules, spider venoms mostly contain other components such as peptides. Our screening assay showed some potential inhibitory peptides that might play a role in blocking the human P2X4 receptor. Further characterisation and validation is required to understand whether these novel compounds could be useful in pain pharmacology. This work is supported by BBSRC NRPDTP and the Royal Society.

1 Baliki et al. Neuron 87.3; 474, 2015
2 Finnerup et al. Lancet Neurology 14.2; 162, 2015
3 Stokes et al. Frontiers in Pharmacology 8, 2017
4 Grishin et al. Annals of neurology 67.5; 2010
Keywords: P2X4; spider venoms; drug discovery; pain.

SYMPOSIUM 12 - Adenosine Neuromodulation: Implications for Epilepsy Control

1. Adenosine kinase - target for the prevention of epilepsy

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Adenosine is a well-characterized endogenous anticonvulsant and seizure terminator of the brain. New findings from our laboratory show that epilepsy and its progression can be prevented by the transient therapeutic augmentation of adenosine, which can most effectively be achieved by pharmacologically blocking the major adenosine metabolizing enzyme adenosine kinase (ADK). ADK exists in a cytoplasmic isoform ADK-S, which regulates extracellular levels of adenosine and adenosine receptor activation, and a nuclear isoform ADK-L, which acts as a regulator of DNA and histone methylation. We have initiated medicinal chemistry efforts with the goal to move an ADK-L inhibitor into therapeutic development. Our clinical target is to prevent temporal lobe epilepsy (TLE) and its progression. Our approach is based on solid evidence (i) that overexpression of ADK within an epileptogenic brain area is a biomarker and pathological hallmark for epileptogenesis, and (ii) that a transient dose of adenosine delivered to the brain either directly, via brain implants, or pharmacologically, via an ADK inhibitor, effectively prevents epileptogenesis and disease progression long-term. As regulators of DNA methylation, ADK inhibitors are uniquely suited to reprogram the DNA methylome and thereby to interrupt molecular processes implicated in epileptogenesis. ADK inhibitors designed to enhance adenosine receptor activation via augmentation of extracellular adenosine (based on inhibiting ADK-S) in brain tissue were previously considered for anticonvulsive therapy, but eventually failed due to lack of specificity and adverse events. We propose to develop novel ADK inhibitors that block the specific nuclear isoform ADK-L to capitalize on adenosine's epigenetic effects for the new indication 'antiepileptogenesis'. We have initiated iterative processes to pursue our overarching goal to identify and rigorously test an antiepileptogenic ADK-L inhibitor as a candidate for clinical trials. We developed a lead compound, MRS4203, with demonstrated ADK-L inhibition and epigenetic efficacy in vitro and in the brain; importantly, and in contrast to inhibitors that affect ADK-S, a systemic dose of MRS4203 is not associated with any overt adverse events. Because ADK is overexpressed in the epileptic brain and associated with epileptogenesis, there is a strong rationale for the clinical development of ADK inhibitors that restore normal adenosine function. The development and validation of new ADK inhibitors that mobilize long-lasting antiepileptogenic epigenetic effects through transient ADK-L inhibition will enhance the contribution of desirable epigenetically-based antiepileptogenic effects of adenosine.
Keywords: adenosine kinase; transmethylation; epigenetics; epileptogenesis.

2. Synaptic targets for adenosine to control excitability and plasticity

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Adenosine is considered an endogenous anticonvulsant due to its ability to control excitability. At the hippocampus, a brain area particularly vulnerable to seizures, the main targets for adenosine to control excitability are: Glutamatergic transmission, with adenosine A1 receptors (A1Rs) inhibiting and adenosine A2A receptors (A2AR) facilitating transmission The A1R-mediated inhibition prevail in young adults. In Young adults the A1R-mediated actions prevail and are mostly pre-synaptic, whereas A2ARs-mediated actions in to be mostly directed towards control of AMPA and NMDA-receptors. GABAergic transmission, where A1Rs inhibit tonic inhibition of a sub-set of GABAergic neurons, those containing the cannabinoid tipe-1 receptor (Rombo et al., 2016a - Cereb Cortex 26:1081-95) and the A2ARs facilitate GABA release from the parvalbumin containing GABAergic neurons that project to other GABAergic neurons, thus disinhibiting pyramidal cells (Rombo et al., 2015 - Hippocampus, 25:566-80). In astrocytes and GABAergic nerve terminals, adenosine receptors also control GABA transport (see Rombo et al., 2016b –J Neurochem. 139:1056-70. Control of the synaptic actions of brain-derived neurotrophic factor (BDNF), an action mediated by A2ARs. This interaction between A2AR and BDNF at the hippocampus has relevant implications for the ability of extracellular adenosine to control seizures, as revealed in studies in mice with different levels of adenosine kinase (ADK) in the brain. ADK is the key metabolic regulator of the intra-and consequently extra-cellular levels of adenosine. Mice underexpressing ADK in the brain have enhanced levels of extracellular adenosine, and exacerbated tonic A1R-mediated inhibition (Diógenes et al., 2014 - Cereb Cortex. 24:67-80), which is compatible with a reduced risk of seizures (Boison, 2016 – Neuropharmacology 104:131-9). However, ADK loss of function mutations in humans present a genetic risk factor for the development of epilepsy and learning impairments. In a recent study (Sandau et al., 2016 - J Neurosci. 36:12117-28) we found out that ADK deficiency in the brain triggers neuronal adaptation processes that lead to dysregulated synaptic plasticity, cognitive deficits and seizure risk. These maladaptive synaptic plasticity could be normalized by either blockade of BDNF signaling or A2AR blockade, allowing to conclude that they were originated by an exacerbated A2AR/BDNF receptor cross talk. In conclusion, while adenosine augmentation therapies are a promising therapeutic strategy for the treatment of epilepsy, they should be complemented by A2AR antagonists, to reduce the risk of occurrence of maladaptative plasticity events that may aggravate seizures. The author's work is receiving support from LISBOA-01-0145-FEDER-007391, Fundação para a Ciência e Tecnologia and from EU (H2020 Twinning action; project number: 692340).
Keywords: "Adenosine A1 receptor"; "Adenosine A2A receptor"; "Brain Derived Neurotrophic Factor"; "Synaptic si.

3. Excitability: Models and Mechanisms

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A dynamic link between metabolism and excitability is reflected in the success of metabolic therapy in treating epilepsy. For nearly 100 years a high-fat, low carbohydrate ketogenic diet has been shown to control seizures – even in pharmacoresistant and refractory epilepsies. Alongside the efficacy of this metabolic therapy in treating seizures is the increasing appreciation that dysfunctional metabolism is common among neurological disorders. A ketogenic diet increases mitochondrial profiles and ATP - providing a foundation for stabilizing and maintaining cell energy. However key therapeutic mechanism(s) of the diet that impact neuronal excitability – both during diet administration, and after its discontinuation - are not fully understood. Metabolic hallmarks of increased blood ketones and reduced blood glucose (similar to metabolic changes that occur during fasting) do not appear to be sufficient or consistent enough to produce beneficial effects in reducing excitability. Approximately 10 years ago we proposed that a key mechanism underlying the efficacy of the ketogenic diet is increased adenosine – also a well-established link between metabolism and excitability, and a neuromodulator also able to stop refractory seizures. Evidence supporting this hypothesis in vivo and in vitro, and in a variety of model systems, has revealed new opportunities for endogenous regulation of adenosine as well as new insights into mechanisms underlying and applications for metabolic therapies. This presentation will review briefly the complement of models and mechanisms explored thus far and outline emerging new directions.
Keywords: Adenosine; metabolic therapy; ketogenic diet; seizures.

4. Enhancing adenosine release for seizure control

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Objectives/background: Injury to the brain results in a depletion of cellular ATP and hence a reduction in the reservoir of the endogenous anticonvulsant, adenosine (Frenguelli, Neurochem. Res. 2017 DOI: 10.1007/s11064-017-2386-6). This leaves the brain vulnerable to seizure activity in the post-injury period and may predispose to epileptogenesis. Our objective has been to restore ATP levels in the injured brain by targeting the purine salvage pathway, the primary means by which the mammalian brain makes adenine nucleotides. The importance of this approach stems from the fact that the substrates for the purine salvage pathway are lost from the brain, either via nucleoside transport into the blood supply, or through the formation of xanthine, which is not a substrate for the purine salvage pathway. Methods and results: All animal studies occurred under the appropriate UK Home Licence and with institutional approval. Using brain slices (~3 wk old male SD rats), which suffer from ischemia- and trauma-induced ATP depletion during their preparation, we were able to restore reduced cellular ATP (~17 nmol/mg protein) to physiological levels (~27 nmol/mg protein) using 2-3 hr preincubation with ribose (1 mM) and adenine (50 μM; "RibAde"). This elevation in cellular ATP, as measured with HPLC, resulted in greater adenosine release (measured with adenosine biosensors) in response to: i) physiological stimulation of the hippocampal Schaffer pathway; ii) oxygen-glucose deprivation, and iii) epileptiform activity induced by Mg2+-free/4AP. Under all conditions, the greater release of adenosine caused greater inhibition of glutamatergic synaptic activity as detected with extracellular electrophysiological recording techniques. Moreover, in an in vivo stroke model (60 min occlusion of the middle cerebral artery; male ~300 g Wistar rats), 6 hr intravenous infusion of RibAde (200 and 10 mg/kg/hr, respectively) reduced lesion volume (T2-weighted MRI) by 38 % compared to saline-treated animals (18% reduction). Addition of allopurinol to RibAde (10 mg/kg IP injection; "RibAdeAll") to prevent formation of unsalvageable xanthine resulted in 50% reduction in lesion volume. Conclusion: These observations suggest that the depleted ATP reservoir after brain injury can be restored with compounds (ribose, adenine and allopurinol) which are in use in man, and which could be rapidly deployed at the point of injury. By restoring cellular ATP, injured brain tissue may be able to activate restorative and reparative mechanisms, such as ionic homeostasis and protein synthesis, respectively, and enhance the release of adenosine to mitigate against secondary insults such as spreading depolarisations and seizure activity. Conflict of Interest: Bruno Frenguelli is a Director and share-holder in Sarissa Biomedical, which manufactures the adenosine biosensors used in these studies.
Keywords: adenosine; ATP; injury; epilepsy.

5. Oral Communication: Adenosine as potential modulator of olfactory sensory information processing

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Objectives / background: The neuromodulatory effect of adenosine has been demonstrated in brain function such as regulation of memory formation, sleep, arousal and locomotor control. The olfactory bulb, the first relay station in the olfactory pathway, is a brain area with high activity of adenosine-producing enzymes. Nevertheless, whether adenosinergic neuromodulation affects the processing of sensory information has not been investigated so far. Methods/results: By in situ hybridization we demonstrated the expression of A1 and A2A receptors in mitral cells, projection neurons of the olfactory bulb. Using whole cell patch-clamp recordings in acute slices of the olfactory bulb, we showed that application of adenosine hyperpolarizes mitral cells and decreases spontaneous firing. By use of A1 receptor knock out animals and application of specific antagonists for K+-currents we demonstrated that these effects are mediated by A1 receptors activating two-pore domain K+-channels, which have not been described before as tagets of adenosine receptor signaling in neurons. In contrast to the reduction of spontaneous firing (noise), adenosine does not affect firing evoked by stimulation of sensory neurons (signal). Therefore, adenosine is able to increase the signal-to-noise ratio of the input-output relationship of mitral cells. Mitral cells integrate the incoming sensory information and synaptic excitation and inhibition derived from local interneurons. The dendro-dendritic reciprocal inhibition mitral cells receive by different subtypes of GABAergic interneurons is an important part of odor information processing and is thought to sharpen the mitral cell output. We showed that A1 receptors inhibit N-type and P/Q-type calcium currents and thereby reduce glutamate release at mitral cell dendrites, thus reducing dendro-dendritic inhibition. This indicates a significant impact of adenosine on the inhibitory drive on mitral cells. In line with this, in behavioral tests we could show that A1 receptor knock-out animals were able to detect a hidden piece of food significantly faster than wild-type littermates. Conclusion: Our results show for the first time that A1 receptor signaling affects excitability and synaptic efficacy in a sensory system, namely the olfactory bulb, thereby tuning odor information processing and behavior.
This work has been supported by DFG HI 1288/3-1
Keywords: adenosine; A1 receptor; neuromodulation; neuron.

SYMPOSIUM 13 - Medicinal Chemistry of Purinergic Signalling

1. A2B adenosine receptors: novel tools and new roles

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Adenosine acts as a signaling molecule by activating G protein-coupled membrane receptors (GPCRs) termed A1, A2A, A2B and A3 adenosine receptors (ARs) (Fredholm et al. Pharmacol. Rev. 63; 1, 2011). These receptors are ubiquitously expressed, but their expression levels and subtype distribution is organ- and tissue-specific. Moreover, AR expression is regulated under pathological conditions, e.g. upregulation of A2BARs is observed under inflammatory, ischemic and hypoxic conditions. The A2BAR has remained enigmatic since it is typically activated by high adenosine concentrations only, in the micromolar range, and such high concentrations are only found under extreme conditions, e.g. in certain disease states. The specific blockade of A2BARs has been proposed as a novel therapeutic strategy, e.g. in asthma, inflammatory bowel disease, pain, diabetes, sickle cell disease, and cancer. In particular, the effectiveness of A2BAR antagonists in preclinical cancer models has recently attracted much attention since they do not only prevent suppression of immune cells by adenosine in the microenvironment of cancer tissues, similarly as A2AAR antagonists, but they additionally display direct anti-proliferative effects on cancer cells and inhibit angiogenesis. We recently showed that A2BARs can form homomeric assemblies, as well as heteromeric complexes with the closely related A2AAR subtype. The A2BAR is thereby able to completely block A2AAR signaling, and this effect may constitute a new physiological role for the low-affinity AR subtype (Hinz et al. Oncotarget 9; 13593, 2018). We have recently developed methods and tool compounds for studying A2BARs, including fluorescent ligands and fluorescence-based assays (Köse et al. J. Med. Chem. 2018, in revision), which will be useful for validating A2BARs as drug targets.
Keywords: Adenosine A2B receptors; fluorescent labeling; medicinal chemistry; receptor heteromers.

2. Virtual screening search of bioactive compounds for CD73: Importance of experimental validation and conformational flexibility

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Human ecto-5'-nucleotidase (ecto-5'NT, CD73) is a GPI membrane-anchored protein, which plays a pivotal role in purinergic signaling pathways (1). Ecto-5'NT hydrolyzes AMP to adenosine, acting as a major control point for the extracellular provision of this signal molecule (1,2). Recent studies have shown that ecto-5'NT is upregulated in tumor cells from several types of cancer (2). Indeed, CD73-generated adenosine has been shown to accumulate in the tumor microenvironment, triggering immunosuppressive responses that favours neoplastic progression (3). Accumulated adenosine is also known to regulate tumor angiogenesis, as well as proliferation, differentiation and apoptosis of cancerous cells (2,3). Despite its relevance as a potential target for cancer and even for many other disorders, so far only few ecto-5'NT inhibitors have been reported, and most of them are not suitable as drug candidates. In this report we discuss the Virtual Screening procedures applied to select novel potential inhibitors for some recognized valid targets (4-8), including novel potential human ecto-5'NT inhibitors(4,5). In all cases stressing the importance of the experimental validation. Acknowledgements. FAPESP (Proc. 2014/07248-0, 2012/06633-2, 2016/12392-9); CNPq; CEPID Redoxoma; Openeye Scientific Software.
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*Results under patent pending.
Keywords: Virtual screening, Bioactive compounds; Experimental validation.

3. A new fast, selective and highly sensitive fluorescence-based assay for monitoring nucleoside triphosphate diphosphohydrolase1 (NTPDase1, CD39)

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Objectives / background: Nucleoside triphosphate diphosphohydrolase1 (NTPDase1, CD39) is a major ectonucleotidase that catalyzes – together with ecto-5'-nucleotidase (CD73) – the extracellular hydrolysis of proinflammatory ATP to immunosuppressive adenosine (Zimmermann et al. Purinergic Signal. 8; 437, 2012). Activation of CD39 may be a novel strategy to treat inflammatory conditions while CD39 inhibitors may be useful for the immunotherapy of cancer. Despite its high therapeutic potential, only few CD39 modulators have been identified so far. One reason could be that the low sensitivity of current analytical methods often requires high substrate concentrations, which makes it difficult to identify less potent hit compounds. A CD39 assay based on fluorescence polarization (FPIA) recently developed by our group is the most sensitive one that has been described so far (Fiene et al. Analyst 140; 140, 2015), but it is rather expensive due to the requirement of specific antibodies. Therefore we have set out to develop a novel assay. Methods / Results: The new capillary electrophoresis (CE)–based assay utilizes a fluorescein-labelled ATP derivative (PSB-170621A) as a CD39 substrate, whereby the enzymatic product (fluorescein-labelled AMP) is quantified. To accelerate the CE assays, a two-directional (forward and reverse) system was implemented using 96-well plates. The determined Z'-factor of 0.7 indicated a high assay quality suitable for screening of compound

libraries. The detection limit was as low as 11.7 pM for the forward operation and 2.00 pM for the reverse operation. This means a large increase in sensitivity as compared to previous assays (e.g. malachite-green assay: 8000-fold, CE-UV assay: 4000-fold, FPIA: 120-fold). Michaelis-Menten kinetic analyses resulted in a Km value of 19.6 ± 3.7 μM and a kcat of 119 ± 12 x10-3s-1 for PSB-170621A, indicating similar substrate properties as determined for ATP (11.4 ± 5.1 μM and 165 ± 11 x 10-3s-1) under the same conditions. PSB-170621A was found to be preferably hydrolyzed by CD39 rather than other ectonucleotidases. Subsequent docking studies into a homology model of human CD39 revealed a hydrophobic pocket in which the fluorescein tag is accommodated. The new assay was validated by investigating several standard CD39 inhibitors. Conclusion: The new fluorescence-based CD39 assay has multiple advantages as compared to the previous assays, including picomolar sensitivity, fast operation, easy handling, low costs, and CD39 selectivity.
Keywords: Capillary electrophoresis; CD39; ectonucleotidases; NTPDase1.

4. Oral Communication: A rapid method to probe P2X7 receptor heterologous expression in a plate reader and the discovery of a new entity ATP responsive in HEK293T Cells

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The ATP is an important molecule for living cells. It can be produced during the cellular metabolism, but it plays a role either as a source of energy or as the agonist for some biomolecules. It might be found intracellularly in the range of mM concentration, in spite of extracellularly it is tightly regulated and maintained in the range of nM, unless an environmental disturbance occurs, as in the case of a inflammatory processing that might increase the concentration by a factor of 1000. At high ATP extracellular concentration a nanometer sized dyes transport through plasma membrane might be observed for some cell types. This phenomenon is commonly related to P2X7 receptor activation whose the physiological agonist is the ATP molecule. However, those enlarged channel, that is called as pore, is triggered by a mechanism poorly understood. There are two major explanations for that pore formation: (1) the large molecules above mentioned passes through the pore itself or (2) the channel activation led another protein to promote the permeabilization. Recent data on the literature shed light on the first explanation. However, there is a plenty data on literature indicating the feasibility of the second explanation, in which pannexin1 has been one of the candidates to be the accessory protein to open the pore. For those reasons this issue remains unclear. In this work, we developed, a "within well" control plate reader scan method to probe plasma membrane permeabilization with reduced wash steps and high experimental reproducibility. The J774 (rat macrophage) was the constitutive P2X7 expression model and the HEK293T (human) cell line was applied for P2X7 heterologous expression. First of all, we optimized the best P2X7 heterologous expression in HEK293T cells based on the highest and the lowest dye uptake, induced in the presence and in the absence of extracellular ATP, respectively. Curiously, the non-transfected HEK293T cells (that lack P2X7 receptor) were ATP responsive to dye uptake in spite of no cell death had been observed as indicated by LDH experiment that indicates the presence of a new ATP sensitive entity. In order to explore the identity of this new entity we performed a patch-clamp analysis (whole-cell configuration) on non-transfected HEK293T cells in the presence of mM ATP concentration. The results showed a channel activity that produced a desensitizing current that cannot be blocked by the P2X7 specific antagonist A740003. In addition, a Pannexin inhibitor, Probenecid, was able to block partially the propidium uptake in non-transfected HEK293T cells. Our data suggest that, at least, one new ATP activated molecular entity is present in HEK293T cells and promote cellular permeabilization to propidium dyes. In this way, more studies are still in progress in our group to identify all molecules involved in this phenomenon.
Keywords: plate reader; ATP; P2X7; Pore.

5. Oral Communication: Mapping the binding site for the P2X7 receptor antagonist AZ11645373

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Objectives / background: The P2X7 receptor (P2X7R) is a ligand gated ion channel opened by the binding of extracellular ATP. P2X7R antagonists are promising drug targets for disease states including treatment of inflammation, pain and depression. AZ11645373 (AZ116) is a selective antagonist for the human P2X7R (hP2X7R) (IC_{50} ~ 90 nM) and ineffective at the hP2X1R at 10 μM. However the site of action of the P2X7R antagonist AZ116 remain to be detected. We have used a chimeras between P2X7 and P2X1 (insensitive) to identify region important for AZ116 action and followed the up work with a series of point mutations to map the binding pocket for AZ116. Crystal structures of the panda P2X7R with five different antagonists have been produced based to an inter-subunit allosteric site at the apex of the receptor (Karasawa et al. eLife 5; e22153, 2016). Methods: Xenopus laevis oocytes were injected with cRNA. After 3-7 days two electrode voltage clamp (TEVC) was used to measure membrane currents at a holding potential of -60 mV. ATP was applied via a U-tube perfusion system for 10 seconds at 5-7 minute intervals and evoked reproducible responses. AZ116 was tested against an EC90 concentration of ATP to standardize any shift in ATP potency at chimeras/mutants. Data points were presented as the mean ± standard error of the mean (SEM) (n≥3). The significant differences from the wild type hP2X7 was determined by one-way ANOVA analysis of variance followed by Dennett's test. Results: Compared with hP2X7R, there was no significant difference between AZ116 action at hP2X7-105-114, hP2X7-122-128, hP2X7-164-168 and hP2X7-295-310 chimeras. However, the hP2X7-112-118 chimera was less sensitive to AZ116 antagonist. Point mutations around these chimeras were tested to determine which residues contribute to high affinity antagonist binding. The point mutation at K110Y which removed a positive charge of lysine to tyrosine was more sensitive to AZ116 than the hP2X7R, while the F103A and F108C mutants were less sensitive to AZ116 antagonist. Conclusion: This study shows AZ116 binds at the allosteric site of the hP2X7R. Compared with hP2X7R, the chimera 112-118 was less sensitive to AZ116, indicating variations in this region are important for antagonist action. Mutation at position 103, 108 and 110 have a major role in terms of AZ116 potency.
Acknowledgment: I would like to thank Saudi government for financial support.
Keywords: Electrophysiology; pharmacology.

SYMPOSIUM 14 - Adenosine Signaling in the Retina

1. Adenosine signaling and the daily control of photoreceptor electrical coupling

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Background: Extracellular adenosine in the retina is produced by degradation of released ATP and its level is further regulated by activity of equilibrative transporters and intracellular adenosine metabolism so that adenosine levels are high in darkness and subjective night and lower in light and subjective day (Ribelayga & Mangel, J. Neurosci. 25:215-222, 2005). Thus, changes in concentration of extracellular adenosine occur in opposite phase to extracellular dopamine. Both dopamine and adenosine receptors modulate many ion channels in the retina and dopamine regulates photoreceptor gap junction coupling. Our purpose was to determine whether adenosine regulates photoreceptor coupling. Methods and Results: Phosphorylation of Connexin36 (Cx36) correlates quantitatively with neuronal coupling (Kothmann et al. J. Neurosci. 29:14903-14911, 2009). Photoreceptor Cx36 phosphorylation was assayed by the ratio of immunofluorescence labeling of phospho-Ser293 to total Cx36 on individual gap junctions in the outer plexiform layer using confocal microscopy. Levels of dopamine and DOPAC were measured by HPLC. Photoreceptor coupling was measured by cut-loading using Neurobiotin. Transcript levels were measured by q-rtPCR. Experiments were performed in intact eyes (HPLC, rtPCR) or isolated eyecups of AB strain zebrafish, C57Bl/6 mice, and adenosine A2a receptor knockout mice. Animals of both sexes were used. The median phosphorylation level of Cx36 was higher at night in darkness than at day in light in both zebrafish and mouse and corresponded to higher photoreceptor coupling at night. Application of 10 μM adenosine in the day increased Cx36 phosphorylation, as did the adenosine receptor agonist CGS21680. At night, the selective A2a antagonist 8-chlorostyryl-caffeine reduced Cx36 phosphorylation. The selective A1 receptor antagonist DPCPX increased Cx36 phosphorylation in the day, similar to inhibition of dopamine D4 receptor with L74870. In mice, Cx36 phosphorylation showed a clear daily rhythm, with a peak near midnight and a minimum in darkness 30 minutes before light onset, a time when dopamine secretion remained at its nocturnal low. Transcript levels of D4, A2a and A1 receptors and adenylate cyclase 1 all showed daily rhythms with differing peaks. A2a receptor knockout disrupted the daily rhythm of Cx36 phosphorylation and the rhythms of D4 receptor and adenylate cyclase 1 expression. Conclusion: Adenosine signaling plays a key role in control of photoreceptor gap junctional coupling and is conserved in mice and zebrafish. Circadian and light-driven changes in extracellular adenosine drive increased coupling at night via A2a receptor activity, and low coupling in the day via adenosine A1 receptor activity in concert with dopamine D4 receptor activity. Cyclic changes in expression level of these receptors contribute to regulation and set preferred states for junctional coupling at different times of day. Supported by NIH grants R01EY12857 and R01EY18640.
Keywords: Photoreceptor; gap junction; circadian; dopamine.

2. Neuromodulation by adenosine in the developing retina

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Adenosine modulates cyclic AMP accumulation in the retina of several species including the avian retina, through activation of A1 or A2a receptors which are developmentally-regulated during chick retina embryogenesis. Our previous work showed that a long-term treatment of retinal neuronal cultures with adenosine, A2a receptor agonists, but not A1 agonists, or cyclic AMP analogs is able to promote neuroprotection from glutamate excitotoxicity. Since cell death induced by glutamate in the cultures was blocked by NMDA receptor antagonists, our hypothesis was that the long-term activation of A2a receptors would modulate the expression of NMDA receptors in retinal cultures. In order to test this hypothesis we incubated cultures obtained with dissociated retinal cells from 8-day-old chick embryos and incubated for one day (E8C1) with the A2a agonists DPMA or CGS21680 or the adenylyl cyclase stimulator forskolin up to C3 (48 hours) and measured NMDA receptor activity through (3H) MK801 binding or calcium influx, as well as the expression of NMDA receptor subunits by western blotting or real time PCR analysis. (3H) MK801 binding to retinal cells in culture was stimulated by glutamate showing the presence of functional NMDA receptors. Furthermore, 45Ca++ influx was stimulated with NMDA and blocked by MK801. Preincubation for 48 hours with A2A agonists promoted a dramatic decrease of both glutamate-stimulated (3H) MK801 binding and NMDA-stimulated 45Ca++ influx in the cultures. In agreement with these findings, we found that chronic treatment of cultures with CGS21680 promoted a decrease in both protein and RNA expression of N1 NMDA receptor subunits. Surprisingly, treatment with CGS21680 promoted an increase in N2B subunit RNA and a decrease in N2A RNA expression. The effects by CGS21680 were blocked by the A2a adenosine receptor antagonist ZM241385. Interestingly, long-term treatment with forskolin promoted a decrease in the expression of N1 subunit protein and a decrease in RNA of all 3 subunits. However, the PKA inhibitors H89 or KT5720, or the EPAC inhibitor HJC, blocked the decrease of the N2B RNA expression promoted by forskolin, but not the decrease of N1 or N2A RNAs. The results show that chronic activation of A2A adenosine receptors modulates NMDA receptor subunits expression and strengthens the hypothesis that neuroprotection from glutamate excitotoxicity promoted by adenosine in retinal cultures is a consequence of A2A receptor-mediated modulation of NMDA receptor expression.
Financial support by CNPq, Capes, Faperj and INNT
Keywords: A2A receptor; neuroprotection; NMDA subunit; retinal culture.

3. Caffeine effects in the developing retina

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Background/objective: Caffeine is a worldly consumed psychostimulant that acts mainly by antagonizing adenosine receptors. During developmental stages, high doses of caffeine induce changes in the CNS. In this study, we investigated the effects of a 48-hour exposure of moderate concentration of

caffeine on chick embryo retinal development in normal or ischemic conditions. Methods/Results: Caffeine (30 mg/mL) or vehicle (control) was injected in eggs of Gallus domesticus at 14 embryonic days (E14). Two days later, at E16, retinas were collected and submitted to western blot (WB) and immunohistochemistry (IH), and data analyzed by Mann Whitney test or test T. We also induced ischemia in caffeine-exposed retina by using glucose oxygen deprivation (OGD) protocol for 50 min and evaluated cell death by LDH release. Statistical analyses were performed using GraphPad Prism 6 One way anova for comparison of more than two groups. OGD induced NMDA-dependent retinal cell death since the blockage of NMDA by MK801 prevents the damage. Interestingly, caffeine reduced cell death promoted by OGD. In order to investigate the mechanism of neuroprotection, we evaluate the excitability status of the retina by looking for 3H-MK801 binding, chloride transporters and signaling pathways. We establish the ontogenesis of KCC2 and NKCC1 and shown that caffeine decreased KCC2 retinal content. To test the possible increase in excitability by the reduction of KCC2 content, NMDA activity was measured. The basal activity of NMDA receptor was higher in caffeine, indicating that NMDA receptor activity maybe contribute to the protective effects. Finally, we tested whether the increase in excitability could induce signaling pathways related to calcium increase. Caffeine increased BDNF levels and the inhibition of tyrosine receptors family by K252a, injected in ovo, prevents caffeine-induced neuroprotection. Conclusion: We conclude that, under conditions used here, caffeine affect several neurochemical features of the retina. The exposure to caffeine also decreased retinal susceptibility to ischemia. One of the mechanisms could be a chemical pre-conditioning by caffeine by changing in chloride transporter, NMDA receptor activity and BDNF signaling. Financial support: CAPES, Faperj, CNPq, UFF.
Keywords: KCC2; neuroprotection; BDNF; ischemia

4. Targeting adenosine receptors for the treatment of retinal degenerative diseases

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Retinal degenerative diseases (glaucoma, diabetic retinopathy and age-related macular degeneration) affect more than 200 million people and are leading causes of vision loss and blindness worldwide. These diseases have no cure, and with the exception of glaucoma, the treatments available are scarce, invasive, targeted for the later stages, not effective in many patients and may cause adverse effects. Therefore, new therapies are needed. Early and chronic neuroinflammatory processes in the retina are a common feature in these diseases and it has been shown that microglia plays a major role. Our main goal is to understand whether we can efficiently target adenosine receptors, and particularly adenosine A2A receptors (A2AR), to halt neuroinflammation mediated by microglia in order to prevent retinal neural degeneration. We have been using different antagonists of A2AR, as well as several animal models of disease (ischemia-reperfusion injury and ocular hypertension induced by laser photocoagulation) and in vitro models (cultures of retinal microglial cells, retinal neural cell cultures and retinal organotypic cultures). Neuroinflammation, microglia activation and retinal cell degeneration have been evaluated by molecular and cell biology techniques and bioimaging.
We found that a pro-inflammatory stimulus, such as exposure to lypopolysaccharide (LPS) and elevated hydrostatic pressure (EHP), increases the expression of A2AR in microglial cells. Moreover, these insults, as well as increased intraocular pressure or ischemia-reperfusion injury activate microglial cells, which become less ramified and increase their phagocytic efficiency and migration. There is also an increase in the expression and/or production of pro-inflammatory cytokines (TNF and IL-1beta), the inducible nitric oxide synthase (iNOS) and nitric oxide (NO). Treatment of in vitro and animal models of disease with antagonists of A2AR (SCH 58261 and/or KW 6002) and caffeine efficiently inhibited the expression of A2AR in microglial cells, their morphologic alterations and the phagocytic efficiency, the expression and/or production of pro-inflammatory cytokines, and iNOS and NO. Moreover, the antagonists significantly inhibited retinal cell death, including retinal ganglion cell death. Antibodies against TNF and IL-1beta also prevented retinal ganglion cell death. These findings show that neuroinflammation has a key role in retinal degeneration and the blockade of A2AR significantly inhibits neuroinflammation mediated by microglial cells in the retina and retinal cell death. Therefore, antagonists of A2AR can be envisaged as new pharmacological tools to treat retinal degenerative diseases. Support: Foundation for Science and Technology (FCT), Portugal, COMPETE-FEDER (PTDC/BIM-MEC/0913/2012 and FCOMP-01-0124-FEDER-028417; UID/NEU/04539/2013 and POCI-01-0145-FEDER-007440), Centro 2020 Regional Operational Programme (CENTRO-01-0145-FEDER-000008: BRAINHEALTH 2020).
Keywords: Retina; Microglia; A2A.

5. Oral Communication: New mechanisms of regulation of adenosine transporters in the retina

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Nucleoside Transporters (NTs) are membrane proteins involved in regulation of nucleoside flux across cellular membranes. The SLC29 family comprises the equilibrative nucleoside transporters (ENTs), which are widely distributed in tissues. Adenosine (Ado) is an important neuromodulator in the central nervous system (CNS), regulating synaptic transmission and plasticity, cell proliferation, differentiation, among others. ENT1 and ENT2 play key roles in the rapid removal of extracellular Ado, which can be found in neurons and astrocytes. The contribution of ENTs to purinergic signaling in the brain is critically important but our understanding of the regulation of ENT1 is still limited. Lipid rafts are specialized membrane microdomains enriched in cholesterol, glycosphingolipids and specific proteins. These microdomains act as platforms for the assembly of signaling molecules, and in regulation of different cellular processes such as intracellular trafficking and cellular signaling pathways. MicroRNAs belong a group of noncoding RNAs, that has been demonstrating an important regulatory role in several functions. miR-124 is one of the most abundant microRNAs found in CNS, especially in neurons.Our aim in this work was to evaluate if ENTs function is regulated by a lipid rafts disruptor, methyl-beta-cyclodextrin (MβCD), in mixed cultures of chick retinal cells and also to identify a possible regulation of miR-124 on ENT1 in these same cultures. We measured ENTs activity through [3H]-adenosine uptake assays. Treatment of mixed cultures with MβCD for 45 minutes significantly decreased adenosine uptake in a concentration-dependent fashion (68.8 ± 6.7% of control levels using MβCD 5mM, $p<0.01$, n=3). These initial findings reveal a role for lipid rafts in ENTs activity, which suggests that changes in plasma membrane cholesterol could modulate purinergic transmission in the brain. In terms of regulation of Ado uptake by miR-124, we did not observe a relevant reduction in the conditions treated with miR124 (25 nM) and (50 nM) for 24h. However, in the chronic treatment for 48h with miR-124, we noted a reduction of approximately 40% in the adenosine uptake, especially in miR-124 (50 nM) and (100 nM) conditions. These results suggest that the miR-124 may be modulating the activity of ENT1. We also analyzed the ENT1 protein levels by Western

Blot and our preliminaries results indicate a slight tendency of increasing levels of ENT1 in 50 nM and 100nM concentrations in the treatment for 24h. However, in the cultures kept for 48h after the transfection with miR-124 (100nM), the preliminary results suggest a decrease of ENT1 levels of approximately 50% in comparison to control condition. These data indicate the hypothesis that miR-124 regulates the expression of ENT1.
Acknowledgments: CAPES, Faperj and CNPq
Keywords: Adenosine; lipid rafts; microRNAs.

SYMPOSIUM 15 - Purinergic Receptors and Calcium Signaling

1. Differential actions of purinergic receptors on calcium signaling in the liver

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Objectives/background: Cytosolic Ca^{2+} functions as a key intracellular signal in many cell types, from unicellular organisms to humans. In liver cells, various extracellular agonists acting on G-Protein Coupled Receptors linked to Gq and phospholipase C (PLC) elicit Ca^{2+} signals through the IP3 second messenger system. These hormones, which include catecholamines, vasopressin and purine nucleotides, regulate cytosolic and mitochondrial metabolic pathways, as well as gene expression. The spatiotemporal pattern of Ca^{2+} signals is an important determinant of the downstream targets and overall cellular response. Methods and results: Ca^{2+} signals in acutely isolated rodent hepatocytes and intact perfused liver were monitored using digital imaging fluorescence microscopy with chemical and genetically-encoded fluorescent probes. The agonist-induced Ca^{2+} signals in hepatocytes occur as Ca^{2+} oscillations, with the dose of agonist determining the spike frequency. At the subcellular level, each Ca^{2+} transient initiates at a discrete apical locus and propagates through the cell as a Ca^{2+} wave. Significantly, different agonists give rise to distinct and characteristic Ca^{2+} oscillation patterns, showing a progression from brief baseline-separated Ca^{2+} transients with catecholamines, to more prolonged complex Ca^{2+} spikes with ATP. Other purine nucleotides elicit intermediate Ca^{2+} signal dynamics. The mechanisms underlying this complex behavior depends on cross-coupling between IP3 and $Ca^{2+,}$ and is modulated by multiple inputs including protein kinases and modulation of the enzymes metabolizing inositol phosphates. These agonist-specific Ca^{2+} signaling patterns are also observed in the intact liver, where there is also multicellular coordination of Ca^{2+}signals by propagation between hepatocytes over entire lobules. Conclusions: The functional consequences of these discrete patterns of Ca^{2+} signaling allow different agonists to yield distinct downstream responses, including differences in metabolic output and control of mitochondrial function. Moreover, the rich diversity of purinergic receptors in hepatocytes contributes an extensive repertoire of Ca^{2+} signaling patterns, with potential consequences for signal integration and adaptation.
Keywords: Calcium; IP3; hepatocyte; mitochondria.

2. P2X4-dependent calcium signals and cytokine secretion in human macrophage

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Leukocytes sense extracellular ATP, a danger-associated molecular pattern, released during cellular stress and injury, via the activation of cell surface P2X and P2Y receptors. Though P2X4 is highly expressed in leukocytes and activated at micromolar concentrations of ATP, much attention has been focused on P2X7 – a receptor activated by millimolar ATP. The objective of this study was to understand the role of P2X4 in controlling the shape of intracellular Ca^{2+} signals evoked by ATP, and its role in controlling cytokine secretion.
Monocytes where isolated from human peripheral whole blood with ethical permission of the NHS and University Research Ethics committee. Monocytes were differentiated to macrophage in primary culture with GM-CSF. Expression of P2X1, P2X4, P2X5, P2X7, P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, and P2Y13 was confirmed by quantitative RT-PCR and immunocytochemistry. ATP elicited intracellular Ca^{2+} responses in a concentration-dependent fashion (EC50= 11.4 ± 2.9 μM). P2Y11 and P2Y13 activations mediated the amplitude of $[Ca^{2+}]$i response, whereas P2X4 activation, but not P2X1or P2X7, determined the duration of Ca^{2+} response during a sustained phase. ATP mediated gene induction of CXCL5, a pro-inflammatory chemokine. P2X4 antagonism (PSB-12062 or BX430) inhibited ATP-mediated induction of CXCL5 gene expression and secretion of CXCL5 by primary macrophage. Inhibition of CXCL5 secretion by P2X4 antagonists was lost in the absence of extracellular Ca^{2+} Reciprocally, positive allosteric modulation of P2X4 (ivermectin) augmented ATP-mediated CXCL5 secretion. P2X7, P2Y11, or P2Y13 receptor did not contribute to CXCL5 secretion. Together, the data reveals a role for P2X4 in determining the duration of ATP-evoked Ca^{2+} responses and CXCL5 secretion in human primary macrophage. We thank the British Biotechnology & Bioscience Research Council and British Heart Foundation for continued support.
Layhadi JA, Turner J, Crossman D, Fountain SJ (2018). ATP Evokes Ca^{2+} Responses and CXCL5 Secretion via P2X4 Receptor Activation in Human Monocyte-Derived Macrophages. Journal of Immunology.
Layhadi JA, Fountain SJ (2017). P2X4 Receptor-Dependent Ca^{2+} Influx in Model Human Monocytes and Macrophages. International Journal of Molecular Sciences.
Keywords: P2X4; macrophage; cytokine; calcium.

3. Oral Communication: Adenosine A3 receptor stimulation inhibits pro-nociceptive voltage-dependent Ca^{2+} currents in rat dorsal root ganglion neurons

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Interest has been focused in recent years on the analgesic effects exerted by adenosine and its receptors, A1, A2A, A2B and A3 subtypes, in different in vivo models of acute and chronic pain. A1 agonists are well known analgesic agents, but their therapeutic utility is limited by adverse side effects.

Recent preclinical observations also indicate that A3 receptors (A3R), which are known to be free from cardiovascular side effects, exerts a powerful analgesic action in in vivo rodent models of experimental neuropathic pain, such as spinal nerve ligation or chemotherapy-induced peripheral neuropathy (Little et al., Brain 138:28-35). However, the cellular and molecular basis of A3R-mediated antinociception are still unknown. In this study we investigate whether the A3R agonist Cl-IB-MECA modulates excitability in dorsal root ganglion (DRG) neurons, which are primary sensory peripheral afferences for pain. Dissociated rat DRG neurons were tested for their expression of adenosine A3 receptors (by RT-PCR experiments) and responsiveness (by patch-clamp recordings) to the selective A3R agonist Cl-IBMECA and to the endogenous ligand adenosine (Ado) in the absence or presence of different purinergic antagonists. Patch clamp recordings from primary cultures of rat DRG neurons were performed. Exogenous application of Cl-IB-MECA concentration dependently (0.1-100 nM) inhibited voltage-gated outward currents evoked by a ramp protocol (from +80 mV to -120 mV, 800 ms duration) in medium- and small-sized DRG neurons sensitive to the nociceptive agent capsaicin (CPS: 1 μM). Cl-IB-MECA effect was mimicked by a newly synthesized A3 agonist, MRS5980 (0.1-1000 nM) [4,5], and by adenosine (Ado: 30 μM). Selective A3 antagonists MRS1523, MRS1220 and VUF5574 (100 nM) prevented either Cl-IB-MECA or Ado effects. When tested in the presence of the Ca^{2+}-channel blocker Cd^{2+} (1 mM), Cl-IB-MECA did not modify ramp-evoked currents, thus indicating that A3R activation inhibits Ca^{2+}-K+ cunductances, as confirmed by the lack of effect in the presence of small-conductance (SK) Ca^{2+}-K+ channel blockers apamin (100 nM). In order to verify whether A3R agonist directly inhibits Ca^{2+} entry from VOCCs, we isolated Cd^{2+}-sensitive inward Ca^{2+} currents activated by a 0 mV step depolarization were significantly decreased by 30 nM Cl-IB-MECA ($P<0.0001$, Paired Student's t-test, n=9) and the effect was blocked by the A3 antagonist MRS1523 (100 nM, n=4) and by the N-type VOCC blocker PD173232 (1 μM, n=6), but not by the L-type VOCC inhibitor lacidipin (1 μM, n=4). Present data demonstrate that adenosine A3R activation inhibits N-type Ca^{2+} currents in rat DRG neurons. This effect might represent the molecular basis of A3-mediated antinociceptive action observed in vivo, since N-type Ca+2 current inhibition would result in hampering neurotransmitter release from DRG neurons and in reduction of nociceptive neurotransmission from DRG to CNS.

Keywords: A3 receptors; pain; dorsal root ganglion neurons; calcium currents.

4. Oral Communication: P2X7 receptor is a key modulator of energy cell metabolism

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Objectives/background- The P2X7 Receptor (P2X7R or P2RX7) is widely considered a cytotoxic nucleotide receptor, but is also increasingly recognized to play a central role in cell survival and proliferation. Recently, there has been an increase in efforts to understand the mechanism by which P2X7R supports cells growth or survival. We previously showed that P2X7R expression increases mitochondrial Ca^{2+} concentration, mitochondrial membrane potential, density of the mitochondrial network and overall intracellular ATP content, while on the contrary pharmacological P2X7R stimulation causes mitochondrial potential collapse and fragmentation. These findings point to major role for P2X7R in the modulation of mitochondrial metabolism.Methods/results- To clarify the molecular basis of this effect we investigated mitochondrial oxygen consumption, ATP synthesis and P2X7 mitochondrial localization in WT and P2X7-deleted cells. Our data show that lack of P2X7R decreases basal respiratory rate, ATP-coupled respiration, maximal (FCCP)-uncoupled respiration, resting mitochondrial potential and mitochondrial matrix Ca^{2+} level. This activity depends on P2X7R ability to localize to the mitochondrial membrane, as shown by confocal microscopy analysis and fractionation studies. Reduced respiratory rate in mitochondria from P2X7R-less cells is specifically dependent on block at Site I of the respiratory chain since supplementation of succinate restores near normal oxygen consumption. Furthermore we investigated structure and function of heart mitochondria, a tissue strongly dependent on oxidative phosphorylation, isolated from P2X7-KO and WT mice. Mitochondria from heart of P2X7-KO mice were not apparently altered, but morphometric analysis revealed that they were smaller and showed lower basal and stimulated respiratory rates than mitochondria from P2X7-WT mice. Based on this indication, we exposed P2X7R-KO and WT mice to physical fitness rotarod test, and observed that P2X7R-KO mice have a statistically significant worse performance compared to WT. Conclusion- These data suggest that the P2X7R localizes to the mitochondria, where it plays a key role in energy metabolism. The presence or absence of P2X7R in different cells leads a decreased mitochondrial potential and to reduction of all key mitochondrial metabolic parameters. Reduced mitochondrial metabolism in conditions of lower P2X7R expression, or reduced activity (pharmacological inhibition?), might have so far unanticipated untoward effects on cardiac performance.

Keywords: "P2X7 Receptor; Metabolism; Mitochondria.

SYMPOSIUM 16 - Purinergic Transmission in Glial Cells

1. Purinergic neuron-glia communication in the olfactory bulb

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Objectives / background: It has become increasingly clear that astrocytes are far more than supportive cells, but active elements of the nervous system that contribute to information processing and brain function. Astrocytes respond to physiological stimuli with increases in the free cytosolic calcium concentration, and astrocyte calcium signals trigger a plethora of cell functions, including release of ATP, that mediate neuronal excitation, synaptic plasticity and neurovascular coupling. Methods and results: Astrocytes in the olfactory bulb, the first relay station of odor information processing in the vertebrate brain, respond to ATP released from olfactory sensory neurons with increases in the calcium concentration. Calcium signaling in olfactory bulb astrocytes mediate neurovascular coupling and, thereby, adjust blood flow and supply of neurons with oxygen and glucose. In addition, astrocytes themselves provide a source of ATP in the olfactory bulb. ATP released from astrocytes via gap junction hemichannels stimulates mitral cells, principal neurons of the olfactory bulb. In addition, ATP is degraded to adenosine that affects adjacent neurons. Adenosine A1 receptors inhibit calcium channels in mitral cells and thus decrease synaptic efficacy. Furthermore, adenosine activates background potassium channels of mitral cells, leading to hyperpolarization and reduced spontaneous action

potential firing (noise), whereas synaptic input from sensory neurons (signal) is unchanged. This results in an improved signal-to-noise ratio. Conclusion: Our studies show that astrocytes in the olfactory bulb affect neuronal performance by regulating metabolic support, synaptic transmission and neuronal information processing.
Supported by the DFG (LO779/6 and LO779/10).
Keywords: Astrocytes; ATP release; neuromodulation; synaptic plasticity.

2. Ischemic tolerance and glial purinergic signaling

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Background: Brain ischemic tolerance is an endogenous neuroprotective mechanism, whereby an experience of mild ischemic episode (preconditioning; PC) produces resilience to subsequent much severe ischemic injury. We previously showed that PC-induced activation of astrocytes, and their subsequent expression of HIF-1α is responsible for ischemic tolerance (J Neurosci, 2015). Although PC also increased HIF-1α in neurons, this was not involved in ischemic tolerance. Here, we show the difference in mechanism of HIF-1α increase between neurons and astrocytes, and answer why astrocytic HIF-1α is more important. Methods and Results: We used mouse middle cerebral occlusion (MCAO) for all throughout the studies. Mice received 15 min MCAO as PC (J Neurosci 2015; Glia 2017). It is well-known that an increase in HIF-1α in neurons was dependent on hypoxia/PHD2. In fact, neurons in vitro caused a transient HIF-1α increase in response to hypoxia, but, interestingly, astrocytes did not. Astrocytes did not express even PHD2, an oxygen-dependent HIF-1α degrading enzyme or constitutive HIF-1α. Instead, they showed persistent increase in P2X7 receptor by PC, which was a main mechanism for upregulation of HIF-1α in astrocytes. Conclusion and future perspectives: We have demonstrated that astrocytes, unlike neurons, have a novel mechanism for HIF-1α increase, which is independent of hypoxia but dependent on upregulation of P2X7 receptor. Such machinery allows astrocytes to cause persistent HIF-1α and subsequent strong ischemic tolerance. We also discuss a source of ATP in response to PC, and a possible mechanism of P2X7 receptor activation in this phenomenon.
Acknowledgements: This work was supported by KAKENHI/JSPS and CREST/JST.
Keywords: P2X7 receptor; ischemic tolerance; astrocyte.

3. Diversity of purinergic Ca^{2+} signals in oligodendrocytes and astrocytes

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Astrocytes express neurotransmitter receptors that form microdomains for signaling pathways along their perisynaptic and perivascular processes. The purinergic metabotropic P2Y1 receptor is involved in long-range intercellular signaling of astrocytes including gliotransmitter release. To investigate and temporally control the expression of the P2Y1 receptors more specifically in cerebellar Bergmann glia as well as in cortical astrocytes, we took advantage of GLAST-CreERT2 x floxed P2Y1 receptor mice. For precise temporal control of gene recombination, we explored the pharmacokinetic properties of tamoxifen when injected intraperitoneally using HPLC-MS. In addition, successful astroglial recombination was determined by quantitative real time PCR (qRT-PCR) of genomic DNA in conditional knockout (cKO) mice. For analysis we used adolescent mice of 8 to 12 weeks. Our HPLC-MS analysis showed a fast uptake of TAM and its most active metabolite 4-hydroxytamoxifen (4-OH-TAM) in the brain peaking already at 8 hours post injection. Similarly fast was the clearance of TAM and 4-OH-TAM: both were undetectable already 48 hours after injection. The efficiency of TAM-induced recombination was determined by qRT-PCR of genomic DNA purified from brain homogenates of cKO mice and by quantifying reporter-positive cells in GLAST-CreERT2 x floxedR26-tdTomato mice, revealing the percentage of astrocytes among all cells: 20 % (brainstem), 8 % (cerebellum), 22 % (cortex), 30 % (hippocampus) and 31 % (optic nerve). The astroglial p2ry1 gene deletion resulted then in significant reductions of P2Y1 receptor mRNA of 61 % in the cerebellum and 43 % in the cortex. We are now in a position to study the impact of astroglial P2Y1 receptors, e.g. in motor behavior or brain trauma.
Keywords: astrocytes; P2Y1 receptor; knockout; Bergmann glia.

4. P2X7 receptor signaling in Muller glia: role of glutathione and GABA

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Neuroglia interactions are essential for the nervous system and in the retina Müller cells interact with most of the neurons in a symbiotic manner. Glutathione (GSH) is a low-molecular weight compound that undertakes major antioxidant roles in neurons and glia, however, whether this compound could act as a signaling molecule in neurons and/or glia is currently unknown. Here we used embryonic avian retina to obtain mixed retinal cells or purified Müller glia cells in culture to evaluate calcium shifts induced by GSH. A dose response curve (0.1–10mM) showed that 5–10mM GSH, induced calcium shifts exclusively in glial cells (later labeled and identified as 2M6 positive cells), while neurons responded to 50mM KCl (labeled as βIII tubulin positive cells). BBG 100nM, a P2X7 blocker, inhibited the effects of GSH on Müller glia. However, addition of DNQX 70μM and MK-801 20μM, non-NMDA and NMDA blockers, had no effect on GSH calcium induced shift. Oxidized glutathione (GSSG) at 5mM failed to induce calcium mobilization in glia cells, indicating that the antioxidant and/or structural features of GSH are essential to promote elevations in cytoplasmic calcium levels. Indeed, a short GSH pulse (60s) protects Müller glia from oxidative damage after 30 min of incubation with 0.1% H2O2. Finally, GSH induced GABA release from chick embryonic retina, mixed neuron-glia or from Müller cell cultures, which were inhibited by BBG or in the absence of sodium. GSH also induced propidium

iodide uptake in Müller cells in culture in a P2X7 receptor dependent manner. Our data suggest that GSH, in addition to antioxidant effects, could act signaling calcium shifts at the millimolar range particularly in Müller glia, and could regulate the release of GABA, with additional protective effects on retinal neuron-glial circuit

5. Oral Communication: Inhibition of E-NTPDases regulates proliferation by P2Y1 receptor and modulates cell death in rat retinal progenitors

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The ectonucleotidases (E-NTPDases) are enzymes of the plasma membrane that rapidly break nucleotides into nucleosides. Nucleosides, such as adenine, have an important role in the retina development, performing several functions through P2 receptors. Preliminary results from our group demonstrated that inhibition of E-NTPDases by ARL 67156 for 24 hours increased the number of proliferating cells in rat retina with four postnatal days (P4), an age that cellular proliferation rate is low. Based on this, our aim was to analyze the function of E-NTPDases in vivo during the retina development. This project obtained approval by Ethics Committee on Animal Use under protocol number 547/2014. Lister hooded rats at P4 were anaesthetized by hypothermia and intravitreal injection of ARL 67156 200 μM (E-NTPDases inhibitor) alone or combination with MRS 2179 100 μM (selective antagonist of P2Y1 receptor) was performed. The proliferation was assessed by Ki-67 immunolabeling and cell death by TUNEL assay. The expression of E-NTPDases was evaluated by Real-Time PCR at P0, P3 and P5 rat retina. The treatment with ARL at P4 rats for 24 hours increased proliferating cell number by approximately 30% compared to control but P2Y1 blockage reversed this effect (control= 4734.4 ± 71.4; ARL= 6096 ± 150.9; ARL+MRS= 5113.4 ± 182.2). Considering that retina differentiation occurs from center to periphery we analyzed if this effect was similar at both regions. This increase in proliferation rate was observed both in the periphery and center of the retina, although P2Y1 blockage could not reversed this effect in the center (control: periphery= 5721.8 ± 131.5, center= 4074.2 ± 190.3; ARL: periphery= 7350.4 ± 233.9, center= 4933.2 ± 113.7; ARL+MRS: periphery= 5484.6 ± 227.7; center= 4829.2 ± 295). However, the cellular proliferation induction was not sustained 48 and 72 hours after ARL injection compared to control (control: 48 hours= 4549 ± 141; 72 hours= 3937 ± 125.9; ARL: 48 hours= 4826.7 ± 245.9; 72 hours= 3890.7 ± 209.1). We further analyzed if ectonucleotidases blockade was inducing cell death. The results showed that, after 24 hours, ARL decreased cell death by 40%, but after 48 hours of treatment, there was an increase by approximately 70% on TUNEL positive cells compared to control (control: 24 hours= 100 ± 11.24; 48 hours= 100 ± 17; 72 hours= 100 ± 18.3; ARL: 24 hours= 59 ± 17; 48 hours= 113.4 ± 18; 72 hours= 170.3 ± 19.7). Furthermore, we identified a higher expression of E-NTPDase 1 in P3 and P5 rat retina (P0= 2.97 ± 1.3; P3= 11.38 ± 3; P5= 12.01 ± 3.6), but not for NTPDase 2, 3 or 8, suggesting that it is a possible candidate for the action of adenine nucleotides on neuroblasts proliferation. Our data suggest that ectonucleotidases blockage increased cellular proliferation of rat retinal progenitors dependently of P2Y1 receptor and this raise was counterbalanced through cell death program.
Financial support: Capes, FAPERJ, CNPq and Proppi-UFF. Keywords: E-NTPDases; development; retina; P2Y1 receptor.

SYMPOSIUM 17 - Purines in the Eye

1. Release of lysosomal ATP from astrocytes and epithelial cells triggered by TLR3 activation

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Objectives: While the vesicular release of ATP from non-neural cells is widely acknowledged, the mechanistic pathways involved are not well understood. Here we investigate the role of lysosomal ATP release and investigate the ability of Toll-like receptors to initiate release. Methods: Primary cultures of rat optic nerve head astrocytes, mouse retinal pigment epithelial cells, and the ARPE-19 cell line were used. ATP was determined using the luciferin/luciferase assay, calcium measured with Fura-2, acid phosphatase and beta- hexosaminidase with colormetric assays. Results: ATP release from astrocytes and epithelial cells was triggered by the TLR3 agonist poly(I:C). This released ATP raised intracellular calcium, as the rise was prevented by apyrase. The ATP efflux triggered by poly(I:C) was accompanied by release of lysosomal enzymes lysosomal acid phosphatase and beta hexosaminidase. ATP release was also triggered by lysosomal alkalization with bafilomycin or chloroquine, while poly(I:C) alkalinized lysosomes. ATP and acid phosphatase release was inhibited following lysosomal rupture with glycyl-L-phenylalanine-2-naphthylamide (GPN). Secretory lysosome marker LAMP3 colocalized with VNUT, while MANT-ATP colocalized with LysoTracker. Together, this strongly supports the lysosomal origin of the ATP. Unmodified membrane-impermeant 21-nt and “non-targeting” scrambled 21-nt siRNA triggered ATP and acid phosphatase release, while smaller 16-nt RNA was ineffective. Poly(I:C)-dependent ATP release was reduced by TBK-1 block and in TRPML1-/- cells, while TRPML activation with ML-SA1 was sufficient to release both ATP and acid phosphatase. The ability of poly(I:C) to raise cytoplasmic Ca^{2+} was abolished by removing extracellular ATP with apyrase, suggesting ATP release by poly(I:C) increased cellular signaling. Starvation but not rapamycin prevented lysosomal ATP release. Conclusions: In summary, release of lysosomal ATP follows stimulation of TLR3 in astrocytes and epithelial cells. This links innate immunity to purinergic signaling via lysosomal physiology, and suggests even scrambled siRNA can influence these pathways.
Grant: NIH R01EY013434; R01EY015537
Keywords: ATP release; lysosome; TLR; retina.

2. Regulation of retinal progenitors proliferation by P2 receptors

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The retina is a tissue belonging to the Central Nervous System, in which its histogenesis is completed in the first postnatal days. Some studies in the literature have described purinergic signaling in retinal progenitors proliferation in vitro. Based on this data, the aim of this work was to evaluate the role of P2Y1, 12 and 13 receptors in the proliferation of retinal progenitors from rats at two postnatal days (P2). In P2 rat, hydrolysis of endogenous nucleotides, by intravitreal injection of apyrase, decreased the number of cells ins S-phase labeled for BrdU, however this effect was reversed in the presence of ADPβ-S and ATPγ- S. During the first five postnatal days there was an increase in ATP and ADP hydrolysis and also an increase in the expression of E-NTPDases, in particular the expression of E-NTPDase 1 and 2, suggesting that the availability of these molecules in the extracellular environment became reduced with development. The intravitreal injection of the P2Y1 selective antagonist, MRS 2179, showed a reduction in proliferating cells labeled for Ki-67 after 20 hours of treatment and no cell death was detected in the retina. However, after 48 and 72 hours, the number of proliferating cells was similar to the control. Treatment with MRS 2179 increased the expression of p57Kip2 and reduced the phosphorylation of serine residue 780 of the protein RB, followed by a decrease in cyclin E expression. Interestingly, there was no change in the transition from S phase to G2 and in mitosis, suggesting that the blockade of the P2Y1 receptor caused an arrest in the G1 phase. Unlike P2Y1, intravitreal injection of the P2Y12 receptor antagonists, PSB 0739 or Clopidogrel, increased the Ki-67 positive cell number after 24 hours of the treatment, although the proliferating cell number was restored at 48 hours. The blockage of P2Y12 receptor increased cyclin D1 expression and reduced p57Kip2 expression. There was also an increase in the number of cyclin D1 positive cells, without change the number of cells in S phase or in mitosis, but those cells died by apoptosis. P2Y13 receptor expression was low during the first postnatal week in the retina, and intravitreal injection of antagonist, MRS 2211, did not change the progenitor proliferation. In conclusion, we suggest that the P2Y1 and P2Y12 receptors act during the G1 phase, with P2Y1 being responsible for the maintenance of the cells in the cell cycle, while P2Y12 must be responsible for stimulating the cell cycle exit and postmitotic cells formation.
Keywords: P2Y1 receptor; P2Y12 receptor; development; cell cycle.

3. Role of ATP and P2X7 receptor in the regulation of the regenerative response in the injured retina of adult zebrafish

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Zebrafish can regenerate retinal neurons to replace those lost by damage or disease. Injury triggers cell cycle reprogramming of multipotent progenitor Müller glia to repair the retina. The P2X7 receptor (P2X7R) has been involved in the genesis of several retinopathies (Sanderson et al. Exp. Eye. Res. 127; 270, 2014) as well as in the control of embryonic progenitor cell proliferation in mammals (Glazer et al. PLoS One 9; e9628, 2014). We have generated and characterized an injury model with mainly cytotoxic and possible hypoxia-like components that chiefly kills all photoreceptors in the adult zebrafish retina. By using this injury paradigm we examined the in vivo retina regenerative response in the presence or absence of a specific P2X7R antagonist (A740003) or an excess of apyrase within the vitreous cavity. We quantified the number of proliferative progenitor and microglial cells. We labeled endothelial cells and assessed GFAP immunoreactivity. Quantitative mRNA expression of different hypoxia-induced and cell proliferation-related genes was also analyzed by RT-qPCR. Results: lesioned retinas treated with 25 μM A740003 showed a significant increase in the number of proliferative progenitors including a larger number of dividing nuclei of the GFAP-positive Müller glia. The number of microglial cells around vessels and proliferation-related gene expression were also significantly enhanced. Vascular endothelial growth factor and its receptors and hypoxia-induced factor gene expression was also modified in the antagonist-treated injured retinas. Likewise, 6 U/ml apyrase-treated injured retinas exhibited a larger number of proliferative progenitor cells while the number of apoptotic cells increased in all retina layers whereas that of bipolar cells displayed a significant decrease. On the other hand, in vivo treatments with 1 mM ATP S and 500 μM Bz-ATP of uninjured mature retinas provoked slight activation or injury-induced-like effects, respectively, on multipotent progenitor cell proliferative activity in the retina layers. In conclusion, the antagonist of P2X7R enhanced overall injury severity. Similar results were observed when extracellular nucleotides were eliminated by an excess of intraocular apyrase. These findings suggest that the P2X7R plays a crucial neuroprotective role in the injured environment of the zebrafish retina since the selective blockade of its activity had a deleterious impact on the previously damaged tissue. In contrast, P2X7R activation with a potent agonist (Bz-ATP) in uninjured retinas provoked a damage-like effect with strong activation of progenitor Müller glia activity.
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Keywords: zebrafish retina injury paradigm; photoreceptor cell death; P2X7R antagonist treatment; progenitor.

4. Inosine and nucleotide signaling in the developing avian retina

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In the developing retina, nucleotides regulate cell proliferation, adhesion, migration and survival. While UTP-sensitive P2Y2/4 receptors are associated with the proliferation of neuronal progenitors, ADP-sensitive P2Y receptors are implicated in the proliferation of glial progenitors. ADP-induced cell proliferation requires the activity of phospholipase C, PKC, ERK/CREB and PI3K/Akt signaling pathways. Both P2Y1 and P2Y13 receptor antagonists are able to inhibit ADP-mediated increase in [3H]-thymidine incorporation in retinal cell cultures, suggesting that activation of both ADP-sensitive receptor subtypes is required for ADP-induced proliferation of retinal glial progenitors. In the present study, we show that besides ADP, adenosine and inosine are also able to stimulate the incorporation of [3H]-thymidine in the cultures, showing EC50 values of 16 µM. [3H]-thymidine incorporation induced by adenosine is inhibited by EHNA, an inhibitor of adenosine deaminase that catalyzes the conversion of adenosine to inosine, suggesting that previous conversion of adenosine to inosine is necessary for the adenosine-dependent increase in [3H]-thymidine incorporation in the cultures. Addition of this enzyme to the cultures induces an increase in [3H]-thymidine incorporation, suggesting the presence of endogenous adenosine that can be converted to inosine and induce the incorporation of [3H]-thymidine in the cells. No increase in the amount of cells incorporating [3H]-thymidine prior to treatment with inosine is observed, suggesting that inosine-dependent [3H]-thymidine incorporation is not due to an increase in the survival of glial progenitors in culture. No decrease in inosine-mediated increase in [3H]-thymidine incorporation is observed by incubating the cultures in the presence of adenosine P1 receptor antagonists. However, both MRS2179 and MRS2211, selective antagonists for P2Y1 and P2Y13 nucleotide receptors, respectively, decrease significantly inosine-dependent increase in [3H]-thymidine incorporation, suggesting that both P2Y receptors are required for inosine-

dependent proliferation of glial progenitors. While adenosine has no effect, inosine significantly stimulates intracellular Ca^{2+} increases in cultured glial progenitors, a response that is blocked by the P2Y13 receptor antagonist MRS2211, but not by the P2Y1 receptor antagonist MRS2179, suggesting that inosine raises intracellular Ca^{2+} in glial progenitors by activating P2Y13 receptors. Collectively, our results suggest that inosine sitmulates the proliferation of retinal progenitors in culture in a P2Y1 and P2Y13 receptor dependent manner.
Supported by: CNPq, FAPERJ, CAPES, Proppi-UFF.
Keywords: P2Y1 receptor; P2Y13 receptor; proliferation; glia progenitors.

5. Oral communication: Cannabinoids induce cell death and modulates P2X7 receptor-mediated calcium responses in developing retinal cell cultures

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P2Y1 and 13 receptors are required to late retinal progenitors proliferation (Jacques et al. Cell Signal. 35:95-106, 2017). P2X7 receptors are involved with retinal new born neurons cell death (Anccasi et al. Purinergic Signal. 9(1):15-29, 2013). CB1 and CB2 receptors are important for a wide range of physiological phenomena in the brain, between them calcium currents, neurotransmission, neuroplasticity and neuroprotection (Schwitzer et al, Neural Plast. 2016; 2016). This work aims to investigate the actions of cannabinoids on the proliferation, death and calcium signal on chick retinal cells in culture treated with ATP and ADP. Cultures of retinal cells were obtained from the White-Leghorn chicken embryos at E7 (Approval in ethics committee: CEUA-UNIRIO 2016.02). Cells were seeded on culture dishes (3100 cells/mm2) and cultured for 24 hours at 37°C in a humidified atmosphere of 95% air / 5% CO2. Immunofluorescence microscopy revels that both CB1 and CB2 receptors are expressed on nestine, β-tubulin III and 2M6 positive cells. In order to verify cell proliferation, incorporation of [3H]-thymidine assay was realized. Treatment with 0.5 μM WIN 55,212-2, a non selective CB1 and CB2 agonist, for 24 hours inhibited ~ 84.14% (n=5) of ATP-induced cell proliferation. In the same way, 50 μM URB 602, a MAGL inhibitor, an enzyme that hydrolyze 2-arachidonoylglicerol, inhibited ~ 75% (n=03) of 100 μM ADP-induced cell proliferation. To evaluate cell viability, cultures in E7C1 were treated with increasing concentrations of WIN 55.212-2 (0.5; 1.0 and 5.0 μM) for 24 hours, and submitted to the cell viability assay (MTT). WIN 55,212-2 reduced cell viability (% of effect vs. control ± S.E. Control = 100 ± 2.3; 0.5 μM; 0.5 μM WIN = 98 ± 7.0; 1 μM WIN = 64 ± 2.3; 5.0 μM WIN = 40 ± 2.6. n = 4; $p<0.001$). Cell death induced by WIN 55,212-2 was completely reverted by 1 μM AM251 and 1μM AM630, CB1 and CB2 antagonist receptor, respectively. Using 5 mM fura-2 as calcium probe, the addition of 50 mM KCl induced ~77% of calcium increase only in neurons, while 1 mM ATP has no effect in E7C1 retinal cultures. However, KCl was not able to increase calcium signal in cultures treated with 0.5 μM WIN 55,212-2 for 24 hours, while 1 mM ATP induced ~37.5% of calcium increase only in Müller glial cells. At least 2600 cells were analyzed (n = 3). Finally, cultures in E7C1 were treated with 100 nM A438079, a selective antagonist of P2X7 receptors, plus 1 μM WIN 55,212-2 for 24 hours, and submitted to the cell viability assay (MTT). A438079 completely inhibited the cell death induced by WIN 55,212-2 (% of effect vs. control ± S.E. Control = 100 ± 2.1; WIN 1.0 μM = 68.4 ± 1.6; A438079 100nM = 103.1 ± 3.6; A438079 100 nM + WIN 1.0 μM = 106.7 ± 5.9. n = 3). These data together suggest that cannabinoids, through CB1 and CB2 receptors, inhibits ATP/ADP-induced cell proliferation, induce Müller glial cell response to ATP and promotes P2X7 dependent cell death.
Keywords: Retina; Cell Death; P2X7 receptor; Cannabinoid.

SYMPOSIUM 18 - Purinergic Signaling in Metabolic and Degenerative Diseases

1. ADP accelerates chronic wound healing in diabetic mice

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Background/Objectives: Chronic wounds are a public health problem worldwide, which affect 5.7 millions patients in US and are a challenge to wound care professionals, besides being a significant burden to patients. Such problem, in association with high global prevalence of diabetes, reflects the increase in diabetic ulcers. Considering the absence of an effective and accessible treatment for chronic wounds, our group demonstrated the beneficial role of adenosine diphosphate (ADP) in tissue repair of chronic wounds in diabetic mice. Our previous data demonstrated that ADP accelerated the wound healing and improved the new tissue formation. Therefore, our present aim is to understand the mechanisms involved in the ADP effects. Methods and Results: Diabetes was induced by aloxan (75 mg/Kg i.v.) and seven days later mice were anesthetized and a full-thickness wound was induced surgically using a punch biopsy (1 cm of diameter). ADP (30 uM) was topically applied once a day for 5 days consecutively on the wound. We observed that ADP positively modulated its own receptors, VEGF and TGF-α in the wound. These effects were only observed in diabetic mice. Also, ADP induced the migration of neutrophils, eosinophils and mast cells to the lesion 7 days after lesion. In some in vitro assays, human neutrophils stimulated with ADP produced less reactive oxygen species (ROS) and neutrophil extracellular traps (NET). In order to investigate the role of endothelial cells (EC - hBMEC) in the ADP effects, EC were stimulated with ADP and the supernatant was used to induce neutrophil migration in transwell migration assay. The supernatant from ADP-stimulated EC was more effective to recruit neutrophils. ADP per se did not increase neutrophil migration in our in vitro model, suggesting the involvement of EC in the positive effects of ADP in cell recruitment event. ADP also increased fibroblasts proliferation and migration, which corroborates with our previous in vivo data. Finally, ADP also accelerated the S. aureus-infected wound healing. Clopidogrel treatment, a P2Y12 receptor antagonist, prevented all parameters evaluated, confirming the role of the P2Y12 in ADP effects. It is important to highlight that the chronic treatment of ADP did not cause any systemic toxicity. Conclusion: ADP seems to accelerate the wound healing via cell recruitment and activation, which provides an adequate scenario for wound healing in diabetic mice.
Acknowledgement: CAPES, FAPERJ and CNPq.
Keywords: ADP; cell migration; wound healing; skin.

2. Role of purinergic signaling in insulin secretion and insulin sensitivity

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Objectives: The worldwide epidemic increase in incidence of obesity and type 2 diabetes can be attributed to, among other causes, increased food consumption and decreased energy expenditure. Intracellular nucleotides play an essential role in a balanced energy metabolism, but they are also released from cells and potentially excessive purinergic signaling can contribute to altering function of tissues/organs involved in regulation of body metabolism (1). In our recent studies, we investigate role of purinergic signaling in insulin secreting cells, pancreatic beta-cells, and in insulin sensitive cells, adipocytes. Methods and Results: Pancreatic beta-cell function is regulated by several P2 receptors, but the role of the P2X7 receptor was not clearly established. We show that the P2X7 receptor is expressed in rodent beta-cells, and in rat INS1E cells it regulates cell survival, calcium signaling and insulin secretion. Moreover, high glucose leads to fast ATP release from beta-cells that involves pannexin-1, in addition to exocytotic release from insulin granules. Adipocyte function is regulated by adenosine and P2 receptors (2). It is well established that adipocytes are regulated by sympathetic nerves that presumably also release ATP. Our recent study shows that in addition, adrenergic stimulation leads to significant release of ATP from adipocytes themselves and this depends on pannexin-1, and the process is sensitive to glucose and insulin. Released ATP exerts autocrine effect on adipocytes, e.g. lipolysis, but at high extracellular concentrations it may exert paracrine effects, e.g. on marcophages. Conclusions: Our findings provide novel insights into the role of ATP and purinergic signaling in beta-cells and adipose tissue, and thereby give us novel platform to understand metabolic diseases. References: 1. Novak I & Solini A. Curr Opin Immunol 52; 1, 2018; 2. Tozzi M & Novak I. Front Pharmacol. 8, doi:103389, 2017. Acknowledgements: The studies were supported by The Danish Council for Independent Research | Natural Sciences; Grant number: DFF 4002-00162.
Keywords: beta-cell; adipocytes; pannexin-1;P2X7.

3. Evaluation of nucleotide hydrolysis of obese patients given clinical treatment and bariatric surgery

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Obesity is a chronic multifactorial disease that has reached pandemic proportions throughout the world, as well as a relevant risk factor for diseases such as type 2 diabetes, cardiopathies, hypertension, cerebral vascular accident, polycystic ovary syndrome and certain types of cancer. Bariatric surgery is the most effective treatment for severe obesity and for some of the obesity-associated co-morbidities. In this work we investigated biochemical and inflammatory parameters, biomarkers of oxidative stress and nucleotide hydrolysis in platelets and lymphocytes of morbidly obese patients before and after bariatric surgery and clinical treatment. This study was conducted with 40 individuals distributed into 2 groups: the control group, and the bariatric group. Measurements were made before and 1, 3, 6, and 12 months after surgery. All subjects gave written informed consent to participate in the study. We showed a significant decrease in body weight and body mass index accompanied by a decrease in the lipid profile and glucose and glycated hemoglobin concentrations in the groups that received bariatric surgery. The concentrations of lipid peroxidation, carbonyl protein and NPSH, as well as superoxide dismutase and catalase activity, significantly decreased in both groups after surgery. The concentrations of inteleukin-6, inteleukin-1, TNF-α and resistin were also significantly lower, while adiponectin concentrations significantly increased 12 months after bariatric surgery. No significant alterations were observed in the biochemical, inflammatory or oxidative parameters of the control group after the same period. No significant alterations in ATP, ADP and AMP hydrolysis in control patients were observed, before and after the clinical treatment in platelets. However, bariatric patients presented a significant decrease in ATP, ADP and AMP hydrolysis 3, 6 and 12 months after the surgery when compared with pre-surgical period. The NTPDase activity using ATP and ADP as substrate, as well as ADA activity presented no significant alterations in lymphocytes of control patients, before and after the clinical treatment. However, bariatric patients presented a significant decrease in ATP and ADP hydrolysis and in the ADA activity 1, 3, 6 and 12 months after the surgery when compared with pre-surgical period. Our findings demonstrate a decrease in body mass and a subsequent improvement in biochemical, metabolic and anthropometric parameters in patients given bariatric surgery. This may contribute to the reduction of oxidative damage in these patients and consequently a reduction in the risk of the development and progression of multiple co-morbidities. Furthermore, we can suggest that the decrease in the ATP, ADP and AMP hydrolysis and ADA activity in bariatric patients reflect the accentuated loss weight after surgery and consequently an improvement of metabolic parameters, in special diminution in platelet reactivity and immune response in these patients.
Keywords: Bariatric surgery; nucleotide hydrolysis; inflammation; oxidative stress.

4. Oral Communication: P2X7 receptor and Klotho expressions in diabetic nephropathy progression
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Introduction: Diabetes mellitus (DM) is characterized by increased reactive oxygen species (ROS), leading to high levels of adenosine triphosphate (ATP) and the activation of purinergic receptors (P2X7), which result in cell death. Klotho was recently described as a modulator of oxidative stress, and as having anti-apoptotic properties, among others. However, the roles of P2X7 and klotho in the progression of diabetic nephropathy are still unclear. In this context, the aim of the present study was to characterize P2X7 and klotho in several stages of diabetes in rats. Methods: DM was induced in Wistar rats by streptozotocin, while the control group received the drug vehicle. From the 1st to 8th weeks after the diabetes induction, the animals were placed in metabolic cages on the first day of each week for 24 hours, to analyze metabolic parameters and for the urine collection. Then blood samples and the kidneys were collected for biochemical analysis, including Western blotting and qPCR for P2X7 and klotho. Results: Diabetic rats presented a

progressive loss of renal function, with reduced nitric oxide and increased lipid peroxidation which initiated at the 6th week of the protocol. The P2X7 mRNA expressions were similar up to the 5th, but at the 6th week, they increased when compared to the 5th week (12.02 ± 3.4 vs 0.52 ± 0.2). However, klotho mRNA expression presented an opposite behavior, reducing at 6th when compared to 5th week (1.6 ± 0.3 vs 0.17 ± 0.07), the latter being observed until the 8th week. Conclusion: Our data show an inverse correlation between P2X7 and klotho expressions through the development of DM, which suggests that the management of these molecules could be useful for controlling the progression of diabetic nephropathy.
Keywords: Diabetes mellitus; purinergic receptor; klotho; kidneys.

5. Oral Communication: The effect of adenosine A2A receptor stimulation on mitochondrial metabolism in the pathogenesis and treatment of osteoarthritis

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Osteoarthritis (OA) is the most common form of arthritis, affecting nearly 10% of the US population. There is no therapy to prevent the progression of or reverse OA pathology. Endogenous adenosine 2A receptor (A2AR) stimulation is crucial for chondrocyte viability and cartilage homeostasis, as its downstream signaling mediates inflammation. We have published that mice lacking the A2AR or ecto-5'nucleotidase (CD73) develop spontaneous OA, suggesting that diminished extracellular adenosine levels promote the development of OA. Since human OA chondrocytes have been found to diminish mitochondrial content, we proposed to test the hypothesis that OA pathogenesis deregulates A2AR signaling at least in part by affecting the cell's capacity for ATP production via reduced content or functionality of mitochondria. Primary neonatal WT and A2ARKO chondrocytes were subjected to Seahorse Mito Stress Kit Assays, which revealed reduced oxygen consumption rates (OCR) at baseline and after mitochondrial uncoupling (corresponding to reduced coupling capacity and ATP production). RNA sequencing DESEQ analysis showed increased metalloproteinases expression along with OA-associated pro-inflammatory pathways. We also saw upregulation of genes involved with aging and the production of nitric oxide (NO) and reactive oxygen species (ROS). Histologic staining for 8hydroxyguanosine (8OH-G) residues as a marker for ROS is also markedly increased in A2ARKO mice as early as 8 weeks. A human chondrocyte cell line, T/C28-a2, was used to determine the effect of IL-1β-induced inflammation and A2AR stimulation in vitro. Mitochondrial health and functionality was assessed by mean pixel intensity (MPI) of a fluorescent probe for monitoring mitochondrial membrane potential, TMRM, and by measuring OCR. IL-1β (5ng/mL) incubation for 3 hours followed by A2AR ligation (CGS21680 (CGS; 1uM)) increases mitochondrial membrane potential compared to control, IL-1β alone and CGS alone as measured by TMRM staining. IL-1β incubation for 4 hours with a last hour of A2AR stimulation increases basal OCR and maximal respiratory rate. IL-1β+CGS treated cells had significantly increased ATP production than the control, IL-1β and CGS treated cells as measured by one-way ANOVA. A2AR ligation improves mitochondrial functionality during inflammation and OA progression. Lack of A2AR signaling not only promotes inflammation and catabolism of cartilage matrix, it also damages the cell's mitochondrial metabolic capacity.
Keywords: osteoarthritis (OA); adenosine; adenosine A2 receptor (A2AR); mitochondria.

SYMPOSIUM 19 - Endocrine Purinergic Signaling

1. The multi-faceted role of ATP in the control of melatonin synthesis – New insights for onset and progression of diseases

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Melatonin is a multitasking molecule involved in the chronobiotic and non-chronobiotic control of defense responses. Daily melatonin rhythm is dependent on pineal synthesis, while the non-chronobiotic effect is exerted by melatonin synthesized on demand by tissues and immunocompetent cells. The switch in melatonin synthesis from pineal to immune cells is relevant to favor chemoattraction of neutrophils and monocytes to the lesion site and to adjust the immune response, increasing, for example, the phagocytic activity of macrophages (Markus et al., 2017; Br J Pharmacol. doi: 10.1111/bph.14083). A critical step in melatonin synthesis is the conversion of serotonin into N-acetylserotonin by the enzyme arylalkylamine N-acetyltransferase (AA-NAT). N-acetylserotonin is further converted to melatonin by the enzyme acetyl serotonin O-methyltransferase (ASMT). Regarding the pineal’s melatonergic system, ATP/P2Y1 receptor-mediates the potentiation of the beta;1-adrenoceptor-induced N-acetylserotonin synthesis, but conversely, inhibits the endocrine production of melatonin both in vitro and in vivo by non-identified purinergic signaling. The P2Y1 receptor-mediated response does not involve Aa-nat transcription. Increases in intracellular calcium concentration mediate a PLC-induced enhance in N-acetylserotonin synthesis. By contrast, melatonin reduction is related to an ASMT inhibition at both, the gene transcription and protein levels. This opposite regulation in melatonin and its precursor N-acetylserotonin suggests independent functional roles. In some conditions, N-acetylserotonin synthesis may be preferred over melatonin synthesis. Accordingly, N-acetylserotonin, but not melatonin, was previously shown to activate brain-derived neurotrophic factor and tyrosine kinase receptors type 2 which interaction shows neuroprotective properties by reducing caspase 3 activation in the brain (Iuvone et al., 2014; Adv Exp Med Biol. 801:765). Considering immunocompetent cells, we demonstrated that ATP-induced expression of AA-NAT and its phosphorylation results in the production of melatonin in RAW 264.7 cells. This effect of ATP is mediated by triggering NF-kappa;B pathway through P2X7 receptors activation. The functional response of macrophage-synthesized melatonin was the potentiation of the phagocytosis since it was blocked in the presence of the melatonin receptor antagonist. Altogether, our data raise new insights for the role of purinergic signaling into development and progression of neurological and inflammatory diseases through the modulation of central and local production of melatonin. Support: CAPES, FAPESP (13/13691-1; 12/06110-0; 11/12649-6; 09/12307-8; 07/07871), CNPq (130705/2014-4).
Keywords: P2 receptors; N-acetylserotonin; melatonin; defense response

2. Distribution of purinergic receptors in the mouse suprachiasmatic nucleus and hippocampus

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Objectives/background: P2 purinergic receptors have been implicated in a variety of brain functions, which follow circadian rhythms controlled by the master circadian oscillator, the suprachiasmatic nucleus (SCN) of the hypothalamus. Recently, we analyzed the time-of-day dependent expression of seven P2X subtypes (P2X1-7) and eight P2Y (P2Y1-2,4,6,11-14) receptors in the mouse SCN by immunohistochemistry and real-time PCR. Seven P2 receptors showed a time-of-day dependent variation in the SCN. Especially P2X4-Ir showed a strong increase at mid-dark phase in soma cells in the core region of the SCN and in a dense network of fibres in the shell region of the SCN. Lommen et al. Cell Tissue Res. doi: 10.1007/s00441-017-2634-8, 2017. There is increasing evidence that P2R signaling plays a prominent role in learning and memory and thus in hippocampal neuronal plasticity. However, systematic analyses on spatial distribution of PRs in the hippocampal neuronal network are missing. Therefore, we have now systematically mapped the above listed P2 receptors in the mouse hippocampus. Methods and results. P2X subtypes (P2X1-7) and P2Y (P2Y1-2,4,6,11-14) were analyzed in the hippocampus of male 12-15 week old C57BL mice (n=3) by means of immunohistochemistry. The experimental procedure was approved by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection, Germany (case number: 84–02.04.2013.A358) and conform to international guidelines for the care and use of animals. We found a distinct spatial distribution pattern of the different P2 receptors in the different hippocampal regions and layers. Conclusion: This study provides an important basis for understanding purinergic signalling in the hippocampal neuronal network.
Keywords: hippocampus; P2; mouse.

3. Oral Communication: The ergogenic effects of caffeine depend on A2A receptors

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Objectives/background: Ergogenic aid is a substance or method used for enhancing exercise and sports performance. The efficacy of many of these techniques is controversial. Caffeine is the most used ergogenic aid for amateur and professional athletes; the ergogenic effects of caffeine are clear and very well documented. However, the mechanisms of action have not yet been clarified, currently limited to three hypotheses. The increased (1) intracellular Ca+2 mobilization and (2) cAMP activity were only demonstrated at toxic mM concentrations. At non-toxic concentrations (uM), caffeine acts as an antagonist of adenosine receptors. First, we evaluated the ergogenic and thermogenic effect of caffeine (a non-selective antagonist of A2AR) and SCH-58261 (a selective antagonist of A2AR) on female wildtype mice. Then we confirm the role of A2AR in knockout mice.Methods and results: 42 adult female mice (19±0.6 g body weight, 10-12 weeks old) from a global A2AR knockout colony (FMUC, University of Coimbra) were used. The animals underwent a period of familiarization on the treadmill (3 days x treadmill 10 min, 15 cm/s, inclination 5°, saline i.p) for ergospirometry evaluation (running power and respiratory gases – O2 and CO2) on the 5th day. Caffeine (15 mg/kg, i.p., -15 min) and SCH 58261 (1 mg/kg, i.p., -15 min) were administered on the 4th day of open field behavioral task (15 min) and on the 5th day of ergospirometry test. The animals ran at increasing speeds (incremental test) until they reached exhaustion. The temperature at rest and after exercise was evaluated by infrared thermography. The estrous cycle of the females was determined by vaginal lavage and microscopy evaluation. The animals were perfused for immunohistochemistry of prefrontal cortex. Caffeine was psychostimulant (distance and average speed) for wildtype animals in the open field, but not for SCH-58261-treated or A2AR-KO animals. Caffeine and SCH-58261 were ergogenic for wildtype mice, that is, they increased 68±9% and 82±19% the running performance (vertical power) on the incremental treadmill test. In addition, caffeine and SCH-58261 also increased 36±11% and 47±8% maximal O2 consumption (V̇O2max) in wildtype mice, respectively. CO2 production (V̇CO2) had similar kinetics. But caffeine unchanged running power, V̇O2max and V̇CO2 of A2AR-KO animals. Acute physical activity increased cFos density in the prefrontal cortex of the animals. The different genotypes and treatments did not modify the body and tail temperature at rest and exercise-induced hyperthermia. The estrous cycle of the females did not influence the ergogenic effects of caffeine. Conclusion: Our results suggest that the ergogenic effects of caffeine are mediated by A2AR, possibly in the central nervous system.
Financial support: Maratona da Saúde, CAPES-FCT, CNPq
Keywords: A2AR; caffeine; exercise; fatigue

4. Oral Communication: Adenosine A1 receptors inhibit ejaculation at multiple sites

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Background/Objectives. Adenosine modulates different aspects of male sexual function such as sperm capacitation, penile erection and contractility of male sexual accessory organs. Adenosine A1 receptors (A1R) are widely expressed in brain and spinal cord regions implicated in the modulation of ejaculatory reflex but a regulatory role for the A1R on ejaculation reflex was not investigated before. This study evaluates the role of A1R on ejaculation through a pharmacological analysis of selective A1R ligands effects on different in vitro and in vivo models of ejaculation reflex in rats. Methods. All the experimental procedures were approved by the Institutional Ethics Committee for the Use of Experimental Animals of UNICAMP (process: 4074-1). Adult male (120-180 days old) and female (60-120 days old) Wistar rats were used in the different experiments. In vitro contraction studies. The seminal vesicles (SV), vas deferens (VD) and cauda epididymis (CE) were mounted in 10 ml organ baths to evaluation of isometric contractions and the effects of A1R agonist N6-cyclopentyladenosine (CPA) and antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) on VD, SV and CE contractions induced by electrical field stimulation were evaluated. Additionaly, the effects of in vivo administration of CPA and DPCPX on ejaculation induced by the dopaminergic D3 receptor agonist 7-hydroxy-dipropylaminotetralin (7-OH-DPAT) in urethane-anesthetized rats and on ejaculation in copula were investigated. Data are presented as mean ± sem. Results. In vitro CPA administration inhibited the neurogenic contractions of SV, CE and VD smooth muscle. The CPA inhibitory effects on SV, VD and CE neurogenic contractions were antagonized with high potency by DPCPX (pA2SV: 8.82±0.10, n=4; pA2VD: 8.92±0.30, n=4; pA2CE: 9.16±0.07, n=4) showing A1R-mediated effects of CPA. In vivo administration of CPA (3.0 and 10 ug/kg, iv) reduced the VD contractions, seminal emissions and ejaculations induced by 7-OH-DPAT (100 ug/kg, iv) administration to anesthetized rats and these effects were prevented by DPCPX (30 ug/kg, iv). Administration of CPA (1.0 and 3.0 ug/kg, iv) have no effect on copulatory behavior of male rats but the A1R antagonist DPCPX (30 ug/kg, iv) facilitated the ejaculation decreasing by 53% the ejaculatory latency and by 42% the number of intromissions required for the ejaculation. The DPCPX effects on in copula ejaculation were prevented by the co-administration of CPA (3.0 ug/kg, iv). Conclusions.

Altogether, our results show that A1R activation at multiple sites has inhibitory effects on ejaculation highlighting the A1R as a new player in the physiological control of ejaculation reflex.
Finantial Support: FAPESP (2015/19677-6).
Keywords: A1R; ejaculation; fertility; smooth muscle.

SYMPOSIUM 20 - Purinergic Signaling in Stem Cell Proliferation and Differentiation

1. Purinergic signaling in mesenchymal stem cell differentiation

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Objectives / background: In an ageing population there is an increased need for tissue replacement which cannot be met satisfactorily with the current methods in tissue engineering. Although (differentiated) stem cells combined with a tailored biomaterial might be a reasonable good approach, quite a lot of hurdles still have to be overcome to provide adequate results in the future. Here we focus on patient-derived mesenchymal stem cells (MSCs) from various body parts and induced pluripotent stem cells (iPSC) cells and compare their suitability during differentiation into several lineages with emphasis on osteogenesis. Methods and results: Human-derived iPSCs, and MSCs isolated from liposuction material (approved by the ethic committee of the University of Bonn, No. 209/04), have been characterized by respective markers on the gene and protein level and differentiated towards osteoblasts, smooth muscle cells and endothelial cells in vitro. Their respective purinergic receptor pattern has been determined and the result has been used to improve the outcome of the individual lineage differentiations. Interestingly the signal transduction pathways leading to osteogenesis are different in iPSCs and iPS–derived MSCs, if compared to MSCs. This is also reflected in the time period needed for these differentiations. On the other hand the signaling pathways are basically the same in fat-derived MSCs from different body parts. Nevertheless there are huge variations in the potential of these MSCs to differentiate towards osteoblasts. Conclusion: MSCs are present in various tissues and body parts of the human body. However they never differentiate all towards the desired lineages and the potential to do so varies. Here we unraveled the best body part to isolate MSCs for in vitro osteogenesis and compared the results with osteogenesis in human-derived iPS. Next to this we improved the outcome of this differentiation using artificial ligands for those purinergic receptors involved in the lineage commitment. Taken together the easy accessibility of the mentioned cells enables bone grafting in case of fractures with autologous stem cells differentiated towards osteoblasts and combined with artificial scaffolds. The role of purinergic signaling during bone metabolism in vitro provides promising results for future in vivo applications in patients as wells as for in vitro generation of autologous bone grafts in the future.Acknowledgment: This work was supported by BMBF FHprofUnt [03FH012PB2]; FH-Extra, “Europäischer Fonds für regionale Entwicklung”, EFRE co-financed NRW Ziel 2: “Regionale Wettbewerbsfähigkeit und Beschäftigung” [z1112fh012]; BMBF IngenieurNachwuchs, [13FH019IX5]; NRW/Zeit für Forschung [1609fhz027].
Keywords: iPS cells; MSCs; differentiation; osteogenesis.

2. Extracellular Nucleotide Hydrolysis in Dermal and Limbal Mesenchymal Stem Cells: A Source of Adenosine Production

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Objectives/background: Mesenchymal stem cells (MSCs) have shown a great potential for cell-based therapy and many different therapeutic purposes. Despite the recent advances in the knowledge of MSCs biology, their biochemical and molecular properties are still poorly defined. Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) and ecto-5’-nucleotidase (eNT/CD73) are widely expressed enzymes that hydrolyze extracellular nucleotides, generating an important cellular signaling cascade. Currently, studies have evidenced the relationship between the purinergic system and the development, maintenance, and differentiation of stem cells. The objective of this study is to identify the NTPDases and eNT/CD73 and compare the levels of nucleotide hydrolysis on MSCs isolated from different murine tissues (bone marrow, lung, vena cava, kidney, pancreas, spleen, skin, and adipose tissue) and in MSCs isolated from Limbal (L-MSCs) and Dermal (D-MSCs), two human tissues of wide interest for cell therapies. Methods and results: For this study discarded tissues of human skin (from abdominoplasty) and sclerocorneal rims (from cadaveric donors) were donated by the Skin Bank and Cornea Bank of Santa Casa de Misericordia de Porto Alegre with the acceptance of Research Ethics committee e on Human Beings (CEP) (N°. 54473316.5.0000.5345). While MSCs isolated from different mouse tissues hydrolyze the nucleotides distinctly, MSCs from skin and limbal tissues presented a similar hydrolysis rate. In addition, MSCs from both human tissues hydrolyze AMP at higher rates than ATP and ADP, while the MSCs from mouse tissues presented a wide variation. Conclusion: Thus, considering the degradation of ATP and adenosine production, limbal MSCs are very similar to dermal MSCs, indicating that from the aspect of extracellular nucleotide metabolism L-MSCs are very similar to the characterized D-MSCs. Overall MSCs are an attractive adult-derived cell population for therapies, however, the fact that ecto-nucleotide metabolism can affect the microenvironment, modulating important events, such as immune response, makes the assessment of this metabolism an important part of the characterization of MSCs to be applied therapeutically. Acknowledgment: this work was supported by CAPES, CNPq and FAPERGS.
Keywords: MESENCHYMAL STEM CELLS; PURINERGIC SIGNALING; NTPDases; CD73

3. Purinergic Signaling in the maintenance of cancer stem cells and neuroblastoma metastasis

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Cancer is the third most frequent cause of death in the world, and metastasis is responsible for over 90% of tumor-associated mortality. The cancer stem cells (CSC) theory assumes that cells with normal stem cell properties are radio and chemoresistant and may stay in a latent state for prolonged periods, therefore they would be responsible for local recurrence and metastasis. Conditions of chronic inflammation or tissue damage attracts circulating stem cells to the site of injury. We hypothesize that similar conditions might attract CSC directed by inflammation-

related molecules and thereby induce metastatic behavior of tumor cells. ATP, the main agonist of purinergic system is known to be involved in hematopoiesis and inflammation-related molecules. Our group has obtained evidences of the important role of the purinergic system in the metastatic process, especially through the P2X7 receptor activation. Paradoxically, this receptor can induce both pro- and anti-tumoral responses, which depend on expression balance of alternative splicing isoforms of p2rx7 gene. We are focused to understand the role of isoforms A and B in the metastasis. To achieve this goal we have used wild type human neuroblastoma cells and cancer cells subjected to RNA interference for down-regulating P2X7 receptor isoforms A and/or B expression. We evaluated the in vitro invasiveness of these cells by the chemotaxis assay as well as their resistance to chemotherapy vincristine and characterized them in relation to EMT. Moreover, evaluation of morphology and enrichment of CSC in neuroblastoma cell culture in the presence of different concentrations of ATP was performed. We have also studied histological slides of xenograft tumors from animal treated chronically with BBG, a P2X7 receptor antagonist and evaluated metastasis in these mice. The results showed that P2X7B plays an important role in the in vitro invasion of neuroblastoma cells and the absence of the complete isoform A is important to revert the invasive feature. Our findings have also indicated P2X7B as a main character in EMT and chemoresistance. In addition, differences in the morphology and pluripotency markers were observed in ATP-treated tumor cells. Of great importance, xenotransplanted animals receiving BBG i.p. had drastically decreased the spread of neuroblastoma cells to the metastatic niches that may be explained by the difference observed in histological slides of these xenograft tumors. Our findings are pioneer and enrich the understanding of purinergic system role in the metastasis of neuroblastoma cells, raising novel perspectives for tumor therapy. Supported by: FAPESP, CNPq and CAPES
Keywords: metastasis; cancer-stem cell; neuroblastoma.

4. Why do mesenchymal stem cells sense the nucleotide signal in a different way?

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Background: An increasing number of both in vitro and in vivo studies shows that mesenchymal stem cells (MSCs) release ATP and other nucleotides constitutively or in response to mechanical or chemical stimulation. On the other hand, due to variety of purinergic receptors expressed on the MSCs surface, these cells themselves are sensitive to ecto-nucleotide- and ecto-nucleoside-mediated signaling. Taken together, the extracellular nucleotides can impose significant regulation of MSCs functions and mediate the cross-talk between MSCs and cells residing in the neighborhood. Results: The results of our in vitro studies indicate that MSCs exhibit different sensitivity to purinergic ligands as well as distinct activity and expression profiles of ecto-nucleotidases than cells induced to differentiate and mature cells. The undifferentiated human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) respond to micromolar ATP concentrations in the extracellular environment by increasing the proliferation rate (Czarnecka et al., 2017). The same effect was observed with regard to human MSCs from bone marrow (Riddle et al. 2007) and canine MSCs isolated from the adipose tissue (Roszek et al., 2017). A similar stimulatory influence of BzATP, which is a potent agonist of the P2X receptors, provides evidence that the observed effect is attributable to P2XR-mediated signaling (Roszek et al., 2017). The proliferation- and differentiation-promoting effect of nucleotide signaling is particularly evident in the neurogenically induced cells (Czarnecka et al., 2017). There is also growing evidence supporting the involvement of extracellular ATP in the regulation of MSCs migration under in vitro conditions and also their homing capability in vivo (Ferrari et al., 2011; Peng et al., 2016). In general, the results indicate that ATP exerts a cytotoxic effect and inhibits the proliferation of differentiated and mature cells, whereas in mesenchymal stem cells ATP increases their proliferation rate and/or differentiation capacity, and hence, the regenerative potential. Conclusions: It is expected that the perception and understanding of ATP-mediated signaling will change in the foreseeable future. In the case of multipotent MSCs, the ATP signal is interpreted as the microenvironmental requirement for regenerative processes rather than for cytotoxicity. Therefore, ATP seems to be one of the key regulators in cell and tissue repair and have potent implications in regenerative medicine. We also postulate for a critical role of ecto-nucleotidases and kinases which, by orchestrating a fine-tune regulation of nucleotides concentrations, are integrally involved in modulation and diversification of purinergic signals. This specific hallmark of the MSCs purinome should be linked with cell-specific biological potential and capacity for tissue regeneration.
Keywords: ecto-nucleotidases; mesenchymal stem cells; ATP.

5. Oral Communication: ATP and calcium oscillations in Huntington's disease: targeting neural stem cells

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Background: Purinergic receptors have been attributed with developmental functions including gastrulation and neural differentiation. Upon activation, P2 purinergic receptors trigger intracellular calcium transients controlling cellular processes. Huntington's disease (HD) is a genetic neurodegenerative disease caused by the loss of GABAergic neurons (GABANs) from the basal ganglia and the symptoms onset start around the third decade of life spam. Thus recent studies propose that the disease actually starts much earlier, and HD is a neurodevelopmental disease. For clarifying this correlation, we induced GABAN differentiation of neural stem cells derived from a HD patient and recorded the intracellular calcium oscillations during the process of differentiation and compared to ATP-promoted response and cell death rates. Methods and results: First of all, P2Y2 receptor stimulation along differentiation of mouse embryonic stem cells increases the efficacy of GABANs differentiation (27±5 vs 42±2.2, n=3) by boosting the frequency of spike-like calcium oscillations. Using a technique that couples the imaging of alterations in cytosolic calcium concentration by Fluo4-AM (calcium imaging) and Ascl-1 or Neurogenin 2 by luciferase activity (stable transfected cells with Ascl-1 or Ngn2 promoter-protein fusion to luciferase reporter construct), we observed the effectiveness improvement was due to prolonged expression of Ascl-1. In view of that, we investigated HD-NPC and control NPC differentiation patterns. HD-NPCs differentiated from patient iPS cells did not reveal any spike-like oscillations, while they showed increased cell death / apoptosis rates when compared to the healthy donor (30±8.3 vs 8.6±4.1, n=3), despite of caspase3/7 activation. Moreover, HD NPCs challenged with ATP showed higher amplitude of cytosolic calcium transients if compared to the healthy donor (4.2±0.4 n=51 vs 2.4±0.06 n=35). Blockade of intracellular calcium mobilization by P2Y2 receptors using thapsigargin could not prevent cell death. Thus, NPC differentiation status was changed, by decreasing the pool of undifferentiated NPCs when calcium

oscillations were blocked, indicated by nestin expression detected by flow cytometry (90.7±1.1vs 48.4±10.4, n=3). Conclusion: Altogether these data suggest that P2Y2 receptor activation or inhibition modulates spontaneous calcium oscillations during neural differentiation and consequently changes the expression pattern of Ascl-1, thus controlling the cell fate decision to GABAergic neurons. This process is altered in HD patients' cells, compromising the pool of NPCs during early nervous system development. Acknowledgment: This research is financially supported by FAPESP and CNPQ.
Keywords: P2Y2; spike-like oscillations.

SYMPOSIUM 21 - Guanine-based Purines in Brain Physiology and Pathology

1. Neuroprotective effects of guanosine in experimental protocols of brain diseases

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Glutamate is an excitatory neurotransmitter in mammalian CNS, essential for most brain activities. However, hyper activation of the glutamatergic system may be potentially neurotoxic, involved in the pathogenesis of various relevant acute and chronic brain injuries. The main process responsible by maintaining the extracellular glutamate levels below toxic concentration, thus favoring the physiological glutamatergic tonus, is the glutamate uptake activity of glutamate transporters located in neural cell membranes, mainly in astrocytes. Our group has given strong evidence that systemic guanosine (Guo) administration is effectively neuroprotective against glutamate toxicity, animal models of brain diseases, both in vitro and in vivo studies. In in vivo studies, Guo (i.c.v., i.p. or orally administered) protect against seizures (induced by QA), stroke, hepatic encephalopathy and Alzheimer. Searching for mechanisms implicated in these neuroprotective effects, it was demonstrated that Guo in vitro stimulates the glutamate uptake by cultured astrocytes (from newborn, adult and old rats). Additionally, in some in vivo models of brain injury that is accompanied by a decrease in brain glutamate uptake (measured in brain slices obtained after injury), Guo simultaneously exerts neuroprotective effects and avoids this decrease in glutamate uptake. These results point to a potential applicability of neuroprotective effects of Guo in putative translational studies. In this talk we will discuss the relevance of these results. Financial Support: CNPq, CAPES, FAPERGS
Keywords: guanosine; glutamate; neuroprotection; brain diseases.

2. Guanosine promotes proliferation in neural stem cells and neurogenesis in hippocampus and subventricular zone from adult mice

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Objectives/background: Neural stem cells (NSCs) in the hippocampus of adult brain mice originate mainly from discrete germinal regions of subgranular layer (SGL) of the dentate gyrus (DG). Research on stem cells in adults has shown promising discoveries that may contribute to the maintenance, functioning and improvement of regenerative responses throughout life. In the mice adult brain, stem cells are capable of differentiating generating new neurons in a process of several steps called neurogenesis. This process is regulated by neurotransmitters, hormones, neurotrophic factors, pharmacological agents and environmental factors. Guanosine is a guanine nucleoside that acts as an intercellular messenger in the central nervous system (CNS). Extracellular actions of guanosine include regulation of glutamate excitotoxicity, increased release of trophic factors and astrocytic cells proliferation. We aim to investigate whether in vitro guanosine treatment triggers proliferation of NSCs obtained from DG of adult mice and whether in vivo guanosine treatment triggers proliferation in the mice hippocampus and promotes behavioral alterations. Methods and results: NSCs obtained from DG of adult mice were treated with guanosine (100 μM) for 7 days. Guanosine treatment promotes proliferation ($P<0.05$) of NSC forming neurospheres in suspension cultures, without having a significant effect in neurospheres diameter. This effect was not observed following adenosine treatment. In addition, guanosine promotes NSCs differentiation ($P<0.05$) to neuronal cells, identified due to beta-tubulin III labeling. We wonder where guanosine would have similar effects in vivo. To test the proliferation, adult mice (C57BL/6) were treated with guanosine (8 mg/kg, i.p, for 26 days) and 5-bromo-2'-deoxyuridine (BrdU 50 mg/kg, i.p) was administered during 5 days. Guanosine treatment increases ($P<0.05$) the number of BrdU+ cells in the SGL of DG. Moreover, it increases ($P<0.05$) neuronal differentiation assessed by the percentage of BrdU+, doublecourtin (DCX)+ and NeuN+ cells co-localization. Guanosine treatment also exerts an antidepressant-like behavior in the tail suspension test ($P<0.05$), but it does not alter mice performance in the open field and object location tests. Conclusion: Our findings suggest that guanosine plays an important role in regulating neural stem/progenitor cells in vitro and in vivo, promoting neurogenesis in the hippocampus. These results prompted ongoing investigation of mechanisms of purinergic signaling involved on neural stem cell function. Supported by: CAPES (CSF-PAJT 2014), CNPq, INCT-EN.
Keywords: Guanosine; neural stem cells; neurogenesis; hippocampus.

3. The integrated activity of the Guanosine-Guanine and their respective converting enzymes system opens new pharmacological perspectives

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Background: The system of the guanine-based purines is functionally similar to that of adenine-based counterparts. The released GTP is converted into GDP/GMP by the same enzymes that metabolize extracellular ATP, and extracellular guanosine (GUO), escaped to the uptake, is metabolized up to uric acid by the purine nucleoside phosphorylase (PNP) and guanine (GUA)-deaminase (GDA), released in the extracellular fluid. The functional interplay between the two families of signaling compounds depends on their affinity and competitive activity for their common metabolizing enzymes and on the expression/activity of these enzymes that are influenced by different types of patho-physiological and environmental stimuli. Some of these enzymes (e.g. NTase or GDA) elicit other functions beyond their enzymatic activity. Indeed, it has been reported

that: i) the nucleotidase, namely the cN-II isoform, regulates cell proliferation and cell responsiveness to glucose deprivation, thus contributing, especially in the CNS, to affect cancer cells to anti-cancer drugs, and; ii) the GDA, also named cypin, promotes the microtubule assembly and regulates the dendritic branching and neurite elongation, by directly binding tubulin heterodimers. Results: In this complex scenario, GUO and GUA, in tight and functional connection with the enzymes deputed to their formation and metabolism, play relevant and often opposite pathophysiological roles in the CNS. The anti-apoptotic and neuroprotective effects of GUO have been widely documented. They imply the nucleoside ability to activate both the glial-derived production of neurotrophins, leading to the stimulation of the cAMP system, and the PI3-kinase/PKB/heme-oxygenase/cGMPpathway that mediates neurite/dendrite outgrowth. Less known are the effects caused by extracellular GUA that acts by supporting learning and memory, an effect mediated by the NO-induced cGMP formation. This effect is opposite to the amnesic effect caused by GUO that, through the neurotrophin-induced cAMP formation, causes the over-expression of GDA, whose enzymatic activity reduces the levels of GUA. Therefore, the balance/imbalance between the different signaling pathways associated to cAMP and cGMP activation, both involved in the effects caused by GUO, GUA and their metabolizing enzymes, seems to be crucial for the activities of these compounds. Some promising results indicate that extracellular GUO and GUA may act through new putative G protein-coupled receptors, although it has been recently reported that GUA derivatives may reduce amyloid and tau pathologies and improve cognitive functions by stimulating some microglial Toll Like receptors.Conclusions: These findings represent a starting point to consider GUO, GUA, their putative receptors and their metabolizing enzymes druggable targets for new potential clinical applications.
Keywords: Guanosine; Guanine.

4. Oral Communication: Guanosine treatment improves the long-term behavioral changes induced by olfactory bulbectomy an animal model of depression

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Major depressive disorder (MDD) is the most prevalent mental disorder and the leading cause of disability worldwide. Patients with recurrent episodes, present a notable intellectual (learning and memory abilities) compromising, which was at the same time an early and long-lasting prodromal sign, being an important focus to antidepressant drug development. Recently, we have proposed that Olfactory Bulbectomy (OBX) in mice is a suitable model to investigate long-lasting effects associated to depressive symptomatology, including some strictly parameters related to cognitive functions. Several lines of evidences have suggested that the purinergic signaling could be dysregulated in patients with MDD, and guanosine (GUO) signaling seems to be a promising target. Taken all these evidences, the present study aimed to investigate the potential antidepressant effect of chronic GUO treatment in mice submitted to OBX model of depression. Our results show that chronic GUO treatment, for 45 days, was able to improve the long-term behavioral performance impairment induced by OBX, specifically improving parameters that require cognitive functions. Additionally, these behavioral changes were accompanied by important neurochemical hippocampal modulation promoted by GUO. Thus, considering that our main findings, for the first time, pointed an improvement in memory components promoted by GUO, our results reinforce the GUO neuroprotective effect and establish news perspective in MDD therapeutic developments.
Keywords: Depression; Guanosine; Cognition.

5. Oral Communication: Guanosine Antiparkinsonian Efficacy in Rodent Models of movement Disorders

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Guanosine (GUO) is a non-adenine-based purine nucleoside with important trophic functions and promising neuroprotective properties. Indeed, the neuroprotective effects of guanosine have been corroborated in cellular models of Parkinson's disease (PD). On the other hand, the efficacy of GUO as an antiparkinsonian agent has not been fully explored in PD animal models. Accordingly, we evaluated the effectiveness of GUO in reversing motor impairments in several rodent models of movement disorders, including catalepsy, tremor and hemiparkinsonism. Our results showed that GUO (orally administered) antagonized reserpine-mediated catalepsy, reduced reserpine-induced tremulous jaw movements and potentiated the number of contralateral rotations induced by L-3,4-dihydroxyphenylalanine (L-DOPA) in unilaterally 6-hydroxidopamine- (6-OHDA)-lesioned rats. In addition, GUO (at 5 and 7.5 mg/kg) was able to inhibit L-DOPA-induced dyskinesia (LID) in rats chronically treated with the former pro-dopaminergic agent. Overall, we describe the potential of treatment with GUO, which may be effective not only for reversing parkinsonian motor impairments but also for reducing dyskinesia induced by PD-treatment.
Keywords: Guanosine; Parkinson's disease; Dyskinesia.

SYMPOSIUM 22 - Coffee: Caffeine and Beyond

1. Mechanisms of the psychostimulant effects of caffeine

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Caffeine produces psychostimulant effects by its ability to disinhibit the brake that endogenous adenosine imposes on the ascending dopaminergic system, mainly by its ability to target adenosine A2A receptor (A2AR)-dopamine D2 receptor (D2R) heteromers localized in the striatopalidal neuron. Recent studies using several in vitro and in vivo approaches, including radioligand binding experiments in transfected cells and human tissue, biophysical techniques in transfected cells, electrophysiological experiments in rodent tissue and PET in humans, have provided new information about the quaternary structure and biochemical properties of the striatal A2AR-D2R heteromers. They have a heterotetrameric structure, formed by A2R and D2R homodimers pre-coupled to their respective cognate Gs/olf and Gi/o proteins and also pre-coupled to adenylyl cyclase (AC) subtype AC5. The

A2AR-D2R heterotetramer provides the framework for homomeric and heteromeric allosteric interactions between orthosteric A2AR and D2R ligands. We have revisited the previously demonstrated ability of A2AR agonists to allosterically decrease the affinity and efficacy of D2R agonists. We found that also orthosteric A2AR antagonists, including caffeine, induce the same negative heteromeric allosteric modulation. This represented a challenge to the previously formulated hypothesis of the key involvement of allosteric interactions between A2AR and D2R agonists in the motor activating effects of caffeine and A2AR antagonists. Nevertheless, when co-applied, A2AR agonists and antagonists counteracted each other's effects, which depended on a negative allosteric modulation within the A2AR homodimer of the A2AR-D2R heterotetramer. The functional significance of this homomeric allosteric modulation within the A2AR-D2R heterotetramer was demonstrated in signaling experiments in transfected cells, electrophysiological experiments in striatopallidal neurons and rodent locomotor activity experiments. Our results demonstrate that caffeine and selective A2AR antagonists produce psychostimulant effects, not just by competing with adenosine for its binding to the A2AR, but by exerting a negative homomeric allosteric modulation within the A2AR-D2R heterotetramer. The Gs-Gi-coupled GPCR heterotetramer also provides the framework for the canonical antagonistic interaction at the AC level, by which activation of a Gi-coupled receptor antagonizes AC activation by a Gs-coupled receptor. We could demonstrate that this canonical interaction is a pharmacological property of the A2AR-D2R heterotetramer, since it can be disrupted by synthetic peptides that destabilize the heteromeric interface. Both the allosteric and the canonical interactions are biochemical properties of the A2AR-D2R heterotetramer, which acts as a molecular device that integrates the adenosinergic and dopaminergic signals in the striatopallidal neuron and constitutes a main target for caffeine and its interactions with other psychostimulants.
Keywords: Caffeine; adenosine A2A receptor; dopamine D2 receptor; heteromer.

2. Caffeine targets adenosine A1-dopamine D1 receptor heteromers that modulate the excitability of the spinal motoneuron

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Background & Objectives: Adenosine is a ubiquitous neuromodulator in the central nervous system (CNS), which is involved in numerous functions. More general functions include the regulation of arousal and its role in neuroprotection. The modulatory role of adenosine on dopaminergic transmission depends largely on the existence of antagonistic interactions mediated by specific subtypes of adenosine and dopamine receptors, the so-called A2AR-dopamine D2 receptor (D2R) and A1R-dopamine D1 receptor (D1R) interactions. These specific adenosine-dopamine receptor interactions seem to be involved in the central effects of caffeine, a non-selective A1R-A2AR competitive antagonist. We have recently found a significant antagonistic interaction between A1R and D1R ligands in the mouse spinal cord that mediates the ability of caffeine to produce locomotor enhancement by acting on spinal circuits, although the molecular mechanisms and cellular localization remained to be determined. Methods: We used lumbar slices of the mouse spinal cord to conduct electrophysiological, immunohistochemical and biochemical techniques to detect the presence of A1R-D1R heteromers. Results: In the present study, A1R-D1R heteromerization is first demonstrated in mammalian transfected cells using biophysical techniques. Synthetic peptides with the amino acid sequence of specific transmembrane domains (TMs) of the D1R provided the tool to demonstrate that the antagonistic interaction between A1R and D1R ligands depends on A1R-D1R heteromerization and allowed the specific identification of A1R-D1R heteromers in spinal motoneuron, where they mediate the spinal modulatory control by adenosine and dopamine and the strong spinal pharmacological effects of caffeine. Conclusion: These results can have important implications for the pharmacotherapy of spinal cord injury (SCI) and other neurodegenerative diseases.
Keywords: adenosine; dopamine; heteromer; motorneuron.

3. Caffeine and selective adenosine antagonists reverse the effort-related motivational effects of impaired dopamine transmission: potential relevance for psychiatric and neurological disorders

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Objectives / background: Mesolimbic dopamine (DA) is a critical component of the brain circuitry regulating behavioral activation and effort-related processes. Rats with impaired DA transmission reallocate their instrumental behavior away from food-reinforced tasks with high response requirements, and instead select less effortful food-seeking behaviors. Such tasks are useful as animal models of some of the motivational symptoms that are seen in people with depression, schizophrenia, Parkinson's disease, and other disorders. Previous work showed that caffeine and selective adenosine A2A antagonists, but not A1 antagonists, can reverse the effects of DA D2 antagonists on effort-related choice. Methods and results: Pharmacological conditions that can alter effort-based choice have been developed to serve as models for depression-related symptoms (e.g., the vesicular monoamine transport-2 inhibitor tetrabenazine, and pro-inflammatory cytokines). The effort-related effects of tetrabenazine and cytokines can be reversed by the A2A antagonist MSX-3 in rats tested on operant and T-maze effort-based choice tasks. The highly selective A2A antagonist preladenant is very potent at reversing the effects of tetrabenazine, and also can increase selection of high effort lever pressing when administered alone. The behaviorally effective doses of adenosine A2A antagonists also can reverse signal transduction markers of reduced D2 receptor transmission in the nucleus accumbens. Conclusion: Adenosine A2A and DA D2 receptors interact to regulate effort-related choice behavior, which may have implications for the treatment of psychiatric symptoms such as psychomotor slowing, fatigue or anergia that can be observed in depression and other disorders.
Keywords: motivation; depression.

4. Caffeine improves cognitive and emotional impairments in Attention Deficit Hyperactivity Disorder (ADHD)

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Attention deficit hyperactivity disorder (ADHD) is one of the most common childhood psychiatric disorders affecting between 1.3 and 5 % of primary-school children. In childhood, the disorder is more common in boys than in girls and at least 75% will continue to suffer from the disorder after they have

grown up. ADHD is characterized by the presence of three primary symptoms: hyperactivity, inattention and impulsivity. Additionally, ADHD children have problems with cognitive impulsiveness that may be defined as planning deficits, forgetful- ness, poor use of time and impetuous behavior. The spontaneously hypertensive rat (SHR) is generally considered to be a suitable genetic model for the study of attention deficit hyperactivity disorder (ADHD), since it displays hyperactivity, impulsivity, poorly sustained attention, and deficits in learning and memory processes. Converging evidence suggests a primary role of disturbance in the dopaminergic neurotransmission in ADHD patients and in SHR, and in addition, some studies have also demonstrated alterations in adenosinergic neurotransmission in SHR. In the present presentation we will discuss recent data from our laboratory and other groups showing the benefits of caffeine treatment, physical exercise and their combination in behavioral (olfactory, cognitive and mood) and neurochemical (neurogenesis and neurotrophic factors) deficits displayed by SHR animals. Finally, we will discuss the relevance of a better evaluation of the potential of adenosine receptor antagonists in ADHD therapy.
Financial support: CNPq, CAPES-FCT, FAPESC
Keywords: Attention deficit hyperactivity disorder (ADHD); spontaneously hypertensive rat (SHR); caffeine.

5. Oral communication: The role of coffee constituents other than caffeine on CSK/Src pathway in the Central Nervous System

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Objectives / background: Chlorogenic acids (CGAs) are a group of phenolic compounds broadly found in coffee. They are a esterification product between cinnamic acids, including caffeic (CFA), ferulic and p-coumaric with quinic acid (QA), forming several mono- and di-esterified isomers. The most prevalent and studied compounds are 3-O-caffeoylquinic acid (3-CQA), 4-O-caffeoylquinic acid (4-CQA) and 5-O-caffeoylquinic acid (5-CQA) widely described as having antioxidant and cell protection effects. 3-CQA has been shown to regulate lipopolysaccharide-induced tumor necrosis factor production in microglia. Whereas overactivation of microglia is associated with neuronal loss in brain diseases via reactive oxygen species (ROS) production and glutamate excitotoxicity, naïve (nonactivated) microglia are believed to generate little ROS under basal conditions, contributing to the modulation of synaptic activity and nerve tissue repair. However, the signaling pathways controlling basal ROS homeostasis in microglial cells are still poorly understood. Methods and results: Here we used time-lapse microscopy coupled with highly sensitive FRET biosensors (for detecting c-Src activation, ROS generation, and glutamate release) and lentivirus-mediated shRNA delivery to study the pathways involved in antioxidant-regulated ROS generation and how this associates with microglia-induced neuronal cell death. We report that 3-CQA abrogates the acquisition of an amoeboid morphology in microglia triggered by Aβ oligomers or the HIV Tat peptide. Moreover, 3-CQA deactivates c-Src tyrosine kinase and abrogates c-Src activation during proinflammatory microglia stimulation, which shuts off ROS production in these cells. Moreover, forced increment of c-Src catalytic activity by overexpressing an inducible c-Src heteromerization construct in microglia increases ROS production, abrogating the 3-CQA effects. Conclusion: Overall, we provide further mechanistic insight into the modulation of ROS production in cortical microglia, indicating antioxidant-regulated c-Src function as a pathway for controlling microglia-triggered oxidative damage. Acknowledgment: MC and RPC was supported by CNPq and FAPERJ, Brazil, JBR was supported by FEDER and FCT, Portugal. We thank IDOR, especially Dr. Jorge Moll for support. We thank Dr.Shu Chien (University of California) for providing KRas Src YPet and KRas Src (RV) YPet FRET probes, Dr. Ana P. Silva (IBILI, University of Coimbra, Portugal) for supplying wild-type N9 microglia, Dr. Isabel Cardoso (IBMC) for providing Aβ oligomers.
Keywords: Chlorogenic acids; 3-O-caffeoylquinic acid; microglia; reactive oxygen species.

SYMPOSIUM 23 - Purinergic Signaling in Plants

1. Ectopic expression of an apyrase from Pisum sativum enhances root system architecture and drought tolerance in plants

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A pea apyrase, psNTP9, which was originally purified from nuclei of Pisum sativum, was among the first structurally characterized NTPDases in plants. Earlier results indicated that ectopic expression of psNTP9 in Arabidopsis improved the phosphate uptake and leaf growth of young seedlings, but they did not reveal whether these early effects led to improved root growth or increased seed yield in adult plants, or whether the growth effects of psNTP9 were restricted to Arabidopsis. Here we describe results that address these questions. Ectopic expression of psNTP9 in Arabidopsis thaliana and in soybean (Glycine max) significantly expanded the root system architecture (RSA) of both plants compared with wild-type plants. Regarding RSA, psNTP9 expression increased the total root lengths and surface area, root thickness, and the number of lateral roots. These root changes, taken together, would be expected to increase the growth, nutrient uptake, drought tolerance, and seed productivity of the transgenic plants. Consistent with this prediction, the Arabidopsis and soybean plants expressing psNTP9 were larger, took up phosphate better, and had a higher seed yield than wild-type plants. The transgenic Arabidopsis plants were also more drought tolerant, as judged by survival after three weeks (Arabidopsis) or 11 days (soybean) of water deprivation. The statistical significance of all phenotypic differences reported was verified by two-way ANOVA at α = 0.05 with Tukey’s post-hoc test. Immunofluorescence and biochemical assays indicated that the psNTP9 protein is localized both in nuclei and in the extracellular matrix, where it would limit the concentration of the potent signaling agent, extracellular ATP. In eiher locale, signaling changes induced by the pea apyrase, which is regulated by calcium-activated calmodulin, could lead to gene expression changes. To test this hypothesis, we obtained RNA-sequencing data, and the results indicated that the expression of psNTP9 in Arabidopsis promotes an increased transcriptional expression of genes that induce expanded RSA and drought tolerance in Arabidopsis. We conclude that ectopic expression of psNTP9 in plants can enhance their growth, drought tolerance, and seed productivity.
Supported by grants from NSF and Texas Crop Science to SJR and GC.
Keywords: calmodulin; nuclei; pea.

2. Populus euphratica apyrase2 enhances cold tolerance in Arabidopsis plants

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Apyrase and extracellular ATP (eATP) play crucial roles in mediating plant growth and defense responses. Cold stress causes release of ATP through disrupted membranes and the buildup of ATP in the ECM may cause a reduction in cell viability (Sun et al. Plant, Cell & Environment 35; 893, 2012). Extracellular apyrases (or ectoapyrases) are the principal enzymes that limit eATP accumulation in plants (Wu et al., Plant Physiol 144; 961, 2007). In the cold-tolerant poplar, Populus euphratica, low temperatures up-regulate apyrase (PeAPY2) expression in callus cells. However, the link between apyrase, eATP and cold tolerance has not been fully established. We used confocal microscopy, and quantitative real time PCR analysis to evaluate the physiological significance of PeAPY2 and its role in cold tolerance. PeAPY2 exhibited broad substrate specificity, but it most efficiently hydrolyzed purine nucleotides, particularly ATP. PeAPY2 preferred Mg^{2+} as a cofactor, and it was insensitive to various, specific ATPase inhibitors. When PeAPY2 was ectopically expressed in Arabidopsis, cold tolerance was enhanced, based on root growth measurements and survival rates. Moreover, under cold stress, PeAPY2-transgenic plants maintained plasma membrane integrity and showed reduced cold-elicited electrolyte leakage compared to wild type plants. These responses probably resulted from efficient plasma membrane repair via vesicular trafficking. Indeed, transgenic plants showed accelerated endocytosis and exocytosis during cold stress and recovery. We found that low doses of extracellular ATP accelerated vesicular trafficking, but high extracellular ATP inhibited trafficking and reduced cell viability. Cold stress caused significant increases in root medium extracellular ATP. However, under these conditions, PeAPY2-transgenic lines showed greater control of extracellular ATP levels than wild type plants. We concluded that Arabidopsis plants that overexpressed PeAPY2 could increase membrane repair by accelerating vesicular trafficking and hydrolyzing extracellular ATP to avoid excessive, cold-elicited ATP accumulation in the root medium, thus reduced ATP-induced inhibition of vesicular trafficking. ACKNOWLEDGMENTS: The research was supported jointly by the National Natural Science Foundation of China (31270654, 31570587, 31770643), Beijing National Natural Science Foundation (6182030). We wish to thank the Functional Genomics Unit, Plant Systems Biology (VIB-Ghent University, Ghent, Belgium) for providing the Gateway destination vectors. We are grateful to Dr. Stanley J. Roux (University of Texas) for kindly providing the apyrase inhibitor, NGXT191.
Keywords: Poplar; cold stress; extracellular ATP; vesicular trafficking.

3. Extracellular pyridine nucleotides in plant immunity

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Objectives/background: The pyridine nucleotides nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP) are universal coenzymes, which not only participate in metabolic reactions and intracellular signaling, but also function in the extracellular space (ECS). The mechanisms of perceiving extracellular NAD(P) [eNAD(P)] in animal cells have been extensively studies. However, eNAD(P) has remained unknown in plants till we recently found its existence in the model plant Arabidopsis. Methods and results: eNAD(P) and its function in plants were fortuitously discovered. We previously showed that a key plant immune regulator is regulated by cellular redox potential changes. Since NADPH is the reducing power for most anabolic reactions, we thought that exogenous NADPH might activate immune responses in plants. Indeed, exogenous addition of NADPH induces strong immune responses in Arabidopsis. Surprisingly, other pyridine nucleotides, including NADP+, NADH, and NAD+, all similarly induce immune responses, indicating that the immunity-inducing activity of NADPH is not owing to its reducing power. Furthermore, exogenously added NAD(P) appears not to perturb intracellular NAD(P) homeostasis, suggesting that it may act in the ECS to trigger immune signaling. Importantly, during plant-microbe interaction, NAD(P) leaks out into the ECS at concentrations sufficient to induce immune responses, and removal of eNAD(P) by expressing the human NAD(P)-metabolizing ectoenzyme CD38 suppresses immune responses. To further uncover the underlying mechanisms, we employed genetic approaches to identify signaling components of the eNAD(P) signaling pathway in Arabidopsis. In a forward genetic screen aimed at identifying mutants insensitive to exogenous NAD+ treatment, we have found that the Mediator complex subunits MED14 and MED16 as well as the Elongator complex function downstream of eNAD(P). Moreover, a reverse genetic approach based on microarray analysis of exogenous NAD+-induced transcriptome changes in Arabidopsis has identified a lectin receptor kinase (LecRK), LecRK-I.8, as a potential eNAD-binding receptor. Conclusion: eNAD(P) is a novel damage-associated molecular pattern (DAMP) in plants; during plant-microbe interaction, NAD(P) is released from dead or dying cells into the ECS where it interacts with the adjacent healthy cells' surface receptors/targets, which in turn activate downstream specific immune signaling pathways. Further identification of cell surface eNAD(P) receptors/targets and their downstream signaling components in Arabidopsis as well as determination of the generality of eNAD(P) signaling in crops will help establish eNAD(P) as a conserved DAMP in plants. This work was partially supported by a grant from the USA National Science Foundation awarded to Z.M.
Keywords: Extracellular NAD(P); plant immunity; Arabidopsis; extracellular NAD-binding receptor.

4. Oral Communication: Extracellular ATP DAMPens plant disease by boosting immunity

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Extracellular ATP, a damage-associated molecular pattern (DAMP) released upon exposure to cellular stresses, acts as an important signal for cellular responses to abiotic and biotic stresses. In plants, extracellular ATP is perceived by a purinoceptor, P2 receptor kinase 1 (P2K1), that causes downstream signaling for defense responses. Our recent study (Tripathi et al., Plant Physiology, 176: 511-523, 2018) revealed a synergistic interaction between extracellular ATP and other plant stress hormones, especially jasmonates (JAs), for the activation of plant defense responses. This synergistic response provided strong resistance against a necrotrophic fungus, Botrytis cinerea, which has a broad host range and considerable economic impact. Interestingly, extracellular ATP directly changes the formation of the JA receptor complex, thereby enhancing JA signaling. This signaling crosstalk was increased in a P2K1 overexpression line, whereas there was no crosstalk observed in a P2K1 knockout mutant, suggesting that the functional P2K1 receptor is required for maximizing plant defense responses. The signaling crosstalk requires the formation of the secondary messengers, i.e., cytosolic calcium, reactive oxygen species, and nitric oxide. This finding has given a new direction to understanding defense signaling pathways activated by DAMPs in plants. We

discussed possible insights into how extracellular ATP signaling interacts with other hormonal signaling pathways for plant defense responses. This work was supported by NSF (IOS-1557813).
Keywords: DAMP; plant defense responses; P2 receptor kinase; jasmonates

SYMPOSIUM 24 - Purines in Glial Regulation of Brain Synapses and Networks

1. New P2X4mCherryIN knockin transgenic mice expressing non-internalized P2X4 receptors revealed alteration in hippocampal plasticity and memory

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Extracellular adenosine triphosphate (ATP) is released by neurons and glia and modulates synaptic transmission and plasticity via activation of ionotropic P2X receptors in the CNS. Importantly, ATP signaling and surface P2X4 receptors (P2X4) in microglia and/or neurons are upregulated under chronic pain or neurodegenerative conditions such as Alzheimer’s disease (AD) or amyotrophic lateral sclerosis (ALS). The increase of surface expression of P2X4 in spinal cord microglia drives neuropathic pain (P2X4R+ state). The increase of P2X4 in neurons and/or glia may contribute to ALS pathogenesis or to learning and memory dysfunction observed in AD. To elucidate the cell-specific P2X4 function in a pathological context, we created innovative conditional transgenic knockin P2X4 mice expressing the non-internalized P2X4 gene (Floxed P2X4mCherryIN) in order to mimic the increase of surface P2X4 expression in a defined population of cells. The Cre/Lox based strategy consisted in the conditional substitution of the internalization motif present within the C-terminal tail of P2X4 by the fluorescent mCherry protein in specific cell populations expressing the Cre recombinase. I will present results showing the role of P2X4 in the alteration of synaptic plasticity and memory using P2X4mCherryIN mice expressing non-internalized P2X4 respectively in excitatory neurons (CaMK2Cre-P2X4mCherryIN) or in all P2X4-expressing cells (CMVCre-P2X4mCherryIN). By combining RT-PCR, western blots, immunofluorescent and electron microscopies from different brain structures or peripheral tissues (macrophages), we show that the expression of P2X4mCherryIN occurs in the expected tissues or cell types for both knockin mice and leads to an increased number of surface P2X4. The primary screen of the behavioral phenotype of CaMK2Cre-P2X4mCherryIN, CMVCre-P2X4mCherryIN and control littermates reveals that all transgenic mice are viable and normal in size or in terms of locomotor/exploratory activity. Using a dedicated test battery (Y-maze, novel object recognition and 8-arm radial maze) enabling to dissect different memory forms and stages of memory processing, our results show that the increase of P2X4 at the surface of neurons impaired the spatial memory formation and retrieval of knockin P2X4mCherryIN mice. Moreover, these changes parallel alterations of hippocampal long-term depression and potentiation by field potential recordings of hippocampal neurons in sliced brain tissue. The production of conditional P2X4mCherryIN and P2X4KO mice targeting well-defined cell types will allow us to unravel precisely P2X4 function in physiological or pathological contexts.
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2. Glial mechanisms of central respiratory chemosensing

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Breathing is a robust, highly adaptable behaviour that results from the precise spatiotemporal coordination of activity in multiple respiratory muscles to produce continuous (in most mammals), efficient ventilation. The overall output of the motor network that controls breathing is adjusted to match metabolic demand and maintain homeostatic control of arterial and brain PCO2, pH and PO2 via feedback from chemosensory systems of the peripheral and central nervous systems (PNS, CNS). The ventilatory response to high CO2 consists of a robust increase in ventilation that is mediated primarily by multiple chemosensitive sites within the CNS, including the retrotrapezoid nucleus (RTN), which is also implicated as a key chemosensory integration site. In contrast, the ventilatory response to hypoxia (reduced PO2 levels) is biphasic comprising an initial rapid increase in ventilation mediated by peripheral carotid body chemoreceptors followed by a secondary depressive phase during which breathing falls to a lower steady-state level that is determined by an interaction between central inhibitory and excitatory mechanisms. Disruptions in the function of these homeostatic respiratory control systems is implicated in the pathophysiology of a variety of neurorespiratory disorders, including congenital central hypoventilation syndrome, obstructive sleep apnea, apnea of prematurity, Rett syndrome, sudden unexplained death in epilepsy (SUDEP) and sudden infant death syndrome. Astrocytes are emerging as important contributors to central respiratory chemoreflexes. In the RTN, astrocytes that line the ventral surface of the medulla respond to changes in CO2/pH with elevations in intracellular calcium, and the exocytotic (and hemichannel-mediated) release of ATP that increases the excitability of Phox2B, CO2-sensitive neurons to enhance the hypercapnic ventilatory response (Science 329(5991):571-575, 2010; J Neurosci. 36(42):10750-58, 2016; Nat. Comm., 9:370, 2018). Astrocytes in the preBötzinger Complex, a critical site for inspiratory rhythm generation, sense decreases in O2 through a mitochondrial mechanism that triggers an increase in intracellular calcium (J. Neurosci. 35:10460–73, 2015; J. Physiol. 10.1113/JP274727, 2017; Nat. Comm., 9:370, 2018) and the vesicular release of ATP that is hypothesized to attenuate the secondary hypoxic depression of ventilation. I will discuss recent advances in our understanding of astrocytic mechanisms of pH, CO2, and O2 chemosensing in the central respiratory network. Research supported by Canadian Institutes of Health Research, Natural Science and Engineering Research Council, Canadian Foundation for Innovation, the Women and Children's Health Research Institute (University of Alberta), and Alberta Innovates Health Solutions.
Keywords: retrotrapezoid nucleus; preBötzinger Complex; glia; chemosensitivity.

3. Role for astroglia-derived ATP in gliotransmission and control of synaptic plasticity in neocortex

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Communication between neuronal and glial cells is thought to be very important for many brain functions. Acting via release of gliotransmitters, astrocytes can modulate synaptic strength. Still, the mechanisms of gliotransmission remain uncertain with SNARE-dependent exocytosis being the most

intriguing and debated pathway. Previously, we showed that SNARE-dependent exocytosis of ATP from neocortical and hippocampal astrocytes can be triggered by Ca^{2+} -elevation via direct UV-uncaging or via glia- specific receptors (PAR-1, CB1 or a1-adrenoceptors). We also showed that activation of astrocytes initiated a burst of ATP receptor-mediated currents in adjacent pyramidal neurons. These purinergic currents can be inhibited by intracellular perfusion of astrocytes with Tetanus Toxin light chain (TeNTx), or by astroglia-specific expression of dn-SNARE protein, verifying their origin via astroglial exocytosis.
We found out that astrocyte-derived ATP can down-regulate phasic and tonic GABAergic currents, acting via Ca^{2+}-permeable P2X receptors on pyramidal neurons. Also, P2X receptors activated by astrocyte-derived ATP can facilitate trafficking of AMPA receptor into synapse. Hence, astroglia-derived ATP can strongly influence the balance between excitation and inhibition in neural networks and facilitate the long-term synaptic plasticity. Our data show that synergetic action of astrocyte-derived ATP and D-serine is essential for synaptic plasticity. The LTP was impaired in the neocortex of dn-SNARE mice but could be rescued by application of exogenous D-Serine or non-hydrolysable ATP analogs. We also have found out that weak sub-threshold theta-burst stimulation can induce LTP when astrocytes are additionally activated via endocannabinoid or noradrenaline receptors. This facilitation is dependent on the activation of neuronal ATP receptors and can be significantly reduced by perfusion of astrocytes with TeNTx. Moreover, we have found out the deficit in working memory of dn-SNARE mice; this is a first evidence of physiological relevance of glial exocytosis in vivo. There is growing evidence that impairment of glial function can be related to the pathogenesis of many neurological disorders. We observed the considerable decrease in the astrocytic Ca^{2+}-signaling and release of ATP in the aged wild-type and Alzheimer's disease model mice. Impairment of glia-derived regulation altered the balance between excitation and inhibition leading to age- and AD-related deficit in the synaptic plasticity. Importantly, environmental enrichment (EE) or additional activation of astrocytes via a1 adrenoceptors was able to ameliorate the age-related decline rescue purinergic glia-derived modulation of synaptic plasticity. Combined, our data strongly support the importance of glia-derived ATP for astrocyte-neuron communication and show that age-related decline in gliotransmitter release can bring significant contribution to pathogenesis of neurodegenerative diseases.
Keywords: P2X receptors; synaptic plasticity; ATP release; astrocyte.

SYMPOSIUM 25 - Purinergic Signaling and Immunomodulation

1. LASSBio – 897 Reduces Lung Injury Induced by Silica Particles in Mice: Potential Interaction with the A2A Receptor

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Objective/Background: Silicosis is a lethal fibro-granulomatous pulmonary disease highly prevalent in developing countries, for which no proper therapy is available. Among a small series of N-acylhydrazones, the safrole-derived compound LASSBio-897 (3-thienylidene-3, 4-methylenedioxybenzoylhydrazide) raised interest due to its ability to bind to the adenosine A2A receptor. Here, we evaluated the anti-inflammatory and anti-fibrotic potential of LASSBio-897, exploring translation to a mouse model of silicosis and the A2A receptor as a site of action. Methods: Pulmonary mechanics, inflammatory and fibrotic changes were assessed 28 days after intranasal instillation of silica particles in Swiss-Webster mice. Glosensor cAMP HEK293G cells, CHO cells stably expressing human adenosine receptors and ligand binding assay were used to evaluate the pharmacological properties of LASSBio-897 in vitro. Molecular docking studies of LASSBio-897 were performed using the genetic algorithm software GOLD 5.2. Results: We found that the interventional treatment with the A2A receptor agonist CGS 21680 reversed silica particle-induced airway hyper-reactivity as revealed by increased responses of airway resistance and lung elastance following aerolized methacholine. LASSBio-897 (2 and 5 mg/kg, oral) similarly reversed pivotal lung pathological features of silicosis in this model, reducing levels of airway resistance and lung elastance, granuloma formation and collagen deposition. In competition assays, LASSBio-897 decreased the binding of the selective A2A receptor agonist [3H]-CGS21680 (IC50= 9.3 μM). LASSBio-897 (50 μM) induced modest cAMP production in HEK293G cells, but it clearly synergized the cAMP production by adenosine in a mechanism sensitive to the A2A antagonist SCH 58261. This synergism was also seen in CHO cells expressing the A2A, but not those expressing A2B, A1 or A3 receptors. Based on the evidence that LASSBio-897 binds to A2A receptor, molecular docking studies were performed using the A2A receptor crystal structure and revealed possible binding modes of LASSBio-897 at the orthosteric and allosteric sites. Conclusion: These findings highlight LASSBio-897 as a lead compound in drug development for silicosis, emphasizing the role of the A2A receptor as its putative site of action.
Keywords: Silicosis; A2A receptor; LASSBio-897; Fibrosis.

2. Nanobodies that block gating of the P2X7 ion channel ameliorate inflammation

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Objectives / background: The ATP-gated P2X7 ion channel is a potential therapeutic target in inflammation. Nanobodies - single domain antibody fragments derived from camelid heavy chain antibodies - often display better selectivity and fewer side effects than small molecule drugs [1]. The objective of our work was to develop nanobody-based biologics to modulate the function of the P2X7 ion channel during inflammation.
Methods and results: We immunized llamas with P2X7-encoding cDNA expression constructs [2]. Using phage display technology, we selected nanobodies from the immunized llamas that block gating of mouse P2X7 (13A7) or human P2X7 (Dano1) [2,3]. These nanobodies are highly specific and do not bind to P2X4 or P2X1. Using genetic engineering with flexible peptide linkers we fused a dimeric 13A7 nanobody to an albumin-specific nanobody. The albumin-specific nanobody provides marked half-life extension (HLE) in vivo by lowering renal filtration of the P2X7-antagonizing nanobodies. At the same time, the albumin-specific nanobody provides a means to detect both, soluble unbound 13A7-HLE nanobodies as well as nanobodies bound to P2X7 on the cell surface of leukocytes with the aid of a specific monoclonal antibody. Intravenous injection of a standard

therapeutic dose (2 mg/kg) of dimeric 13A7-HLE resulted in nearly instantenous occupancy of P2X7 on vascular leukocytes, and within minutes to complete occupancy of P2X7 on splenic, liver and renal leucocytes. Full occupancy and functional modulation of P2X7 by the injected nanobodies endured for more than 3 days. In mouse models of antibody-induced glomerulonephritis and delayed type hypersensitivity, systemically injected P2X7-blocking nanobody 13A7 ameliorated disease. In a surragate human inflammation model, Dano1 blocked ATP-induced release of IL-1β from endotoxin treated blood monocytes with 10.000 higher potency than benchmark small molecule inhibitors. Conclusions: Our results confirm P2X7 as a therapeutic target for inflammatory diseases such as antibody-induced glomerulonephritis. Moreover, our results demonstrate the feasibility of targeting an ATP-gated ion channel in inflammation with potent, highly specific nanobody-based biologics. Acknowledgments: This work was supported by grants from the Deutsche Forschungsgemeinschaft No310/11-1 and SFB1192/B5 to FKN.
[1] Eden et al. Front Immunol. 8; 989, 2018.
[2] Menzel et al. Front Pharmacol, 2018.
[3] Danquah et al. Sci Transl Med 8; 366ra162, 2016.
Keywords: P2X7; Nanobodies; Glomerulonephritis.

3. ADORA2A-mediated protection of ischemia-reperfusion injury. Role of protein kinase N1: A new player in the pathophysiology of experimental stroke

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Stroke, a global cause of death and neurological disability, still remains one of the least treatable diseases, making the search for new treatments imperative. In response to hypoxia/ischemia, elevated levels of purine nucleosides are produced and released. Purine nucleosides, such as adenosine act as powerful endogenous neuroprotectants and intra- and intercellular messengers during ischemia-induced energy failure (1). Adenosine further supports the prevention of additional ischemic injury through inhibition of activated immune cells (2,3). During our analysis of the role of Adenosine A2A Receptor (ADORA2A) in hypoxia/ischemia, we discovered a link between this purine-nucleoside signaling cascade and the serine/threonine protein kinase N1 (PKN1/PRK1)(4,5). Here, we identify PKN1 as an important gatekeeper of ADORA2A signaling in neurons and demonstrate the in vitro and in vivo significance of this interaction during experimental stroke-injury (6). Fredholm BB, et al. Annu. Rev. Pharmacol. Toxicol. 45; 385, 2005. Pedata F, et al. Mediators Inflamm. 2014; 805198, 2014. 3. Sitkovsky MV. Trends in immunology. 30; 102, 2009. 4. Thauerer B, et al. Protein Kinase C-Related Kinase (PKN/PRK). Curr Neuropharmacol. 12; 213, 2014. 5. zur Nedden S, et al. Journal of Neurochem. 105; 1901, 2008. 6. zur Nedden et al. manuscript in preparation.
Keywords: Adenosine A2A Receptor;Stroke;Protein Kinase.

4. Oral Communication: P2X7 receptor and metabolism of CD4+ T cell during experimental malaria

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Introduction: The molecular pathways involved in activation and regulation of the immune response are important targets for studies aiming to produce vaccines and to develop new therapeutic approaches. The cells of the immune system recognize not only pathogen-associated molecular patterns (PAMPs), but also intracellular molecules named DAMPs (damage-associated molecular patterns), such as ATP, that are released in the extracellular milieu during cellular damage or stress. The detection of extracellular ATP (eATP) by purinergic receptors (P2X1-7) alerts immunological cells that trigger the inflammatory response. Some studies show that recognition of ATP by P2X7 receptors on CD4+ T lymphocytes is important for cell activation and death. Furthermore, enzymes that cleave eATP participate in the control of tissue damage and inflammation. In malaria, an intense activation of CD4+ T cells is observed, which contributes to IFNγ production (Th1 cells), B cell activation (Tfh cells) and regulation (Treg and Tr1 cells). Methods and Results: To evaluate whether the P2X7 receptor contributes to protection against blood stages of Plasmodium chabaudi AS (PcAS), the disease progression was analyzed in C57BL/6 (B6) and P2rx7-/- mice. Our research group found that the P2X7 receptor promotes Th1 cell differentiation and controls the Tfh cell population during PcAS infection. Additional experiments are being performed to understand the mechanisms involved in P2X7 receptor-dependent differentiation of CD4+ T cells during experimental malaria. A proteomic analysis of CD4+ T cells showed glycolytic pathways activated during the peak of parasitemia with an increase of Th1 cells. On day 4 post infection, we observed an increase in the expression of Tbet (transcriptional factor), IFNγ and glucose transporter-1 (Glut-1) mRNA in CD4+ cells.Conclusion: The engagement of specific metabolic pathways profoundly affects cell differentiation and function. In addition, P2X7 receptors can modulate energy metabolism and T cell growth. Altogether, the main objective of this study is to provide new insight into purinergic signaling and immune system by showing the importance of P2X7 receptor in CD4+ T cells during experimental malaria.
Financial support: FAPESP and CNPq.
Keywords: P2X7 receptor; CD4 T cell; metabolism; malaria.

5. Oral Communication: The A2aR signaling pathway as candidate Immune checkpoint in the T cell compartment

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Objective/background: Blockade of immune checkpoints CTLA-4 and PD-1 has emerged as promising cancer immune therapy. In contrast to these cell surface checkpoints, however, there are cancer therapeutic targets that are located inside the immune cells and are amenable to pharmacological modulation.

Methods and results: A2aR-deficient mice are able to immunologically reject otherwise lethal tumor burdens. We have preclinically validated key A2aR signaling intermediates as bona fide immune checkpoint candidates. We show that genetic ablation of A2aR as well as its key effector target gene NR2F6 similarly improves survival in murine cancer models. Mechanistically, a subset-selective upregulation of the A2aR/NR2F6 axis limits effector (but not regulatory) T cell function at the tumor site.
Conclusion: This represents the conceptional validation of the T cell-intrinsic A2aR/NR2F6 axis as an alternative and potentially druggable cancer immune checkpoint pathway.
Keywords: "immune oncology"; "innovative therapy".

SYMPOSIUM 26 - Structure-Function Aspects of Purine Receptors

1. Structure-guided discovery of adenosine receptor ligands

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G protein-coupled receptors (GPCRs) are intensely studied as drug targets and for their role in signaling. With the determination of crystal structures of GPCRs, interest in structure-based drug discovery has increased. Several atomic resolution structures of the A2A adenosine receptor (A2AAR) have now been determined. Examples of structure-based ligand discovery efforts with the goal to identify agonists and antagonists of the A2AAR will be presented. We have identified novel ligands of the A2AAR based on virtual screens of large chemical libraries against antagonist-bound crystal structures and demonstrated how molecular dynamics simulations can be used to optimize these. All these compounds were antagonists, in agreement with the efficacy of the co-crystallized ligand and the inactive conformational state of the receptor structure. Computational design of novel agonists has been more challenging. A2AAR agonists have several potential therapeutic applications, e.g. as drugs for inflammation, but there are very few available ligand scaffolds that have been shown to activate adenosine receptors. We have utilized agonist-bound structures of the A2AAR to identify novel compounds with the ability to activate adenosine receptors and this work will be the main focus of the presentation.
Relevant publications:
Matricon P, Ranganathan A, Warnick E, Gao ZG, Rudling A, Lambertucci C, Marucci G, Ezzati A, Jaiteh M, Dal Ben D, Jacobson KA, Carlsson J (2017) Fragment optimization for GPCRs by molecular dynamics free energy calculations: Probing druggable subpockets of the A2A adenosine receptor binding site. Sci. Rep. 7, 6398
Rodrdríguez D, Chakraborty S, Warnick E, Crane S, Gao ZG, O’Connor R, Jacobson KA and Carlsson J (2016) Structure-based screening of uncharted chemical space for atypical adenosine receptor agonists. ACS Chem. Biol. 11, 2763.
Ranganathan A, Stoddart LA, Hill SJ and Carlsson J (2015) Fragment-based discovery of subtype selective adenosine receptor ligands from homology models. J. Med. Chem. 58, 9578.
Rodríguez D, Gao ZG, Moss SM, Jacobson KA., and Carlsson J (2015) Molecular docking screening using agonist-bound GPCR structures: Probing the A2A adenosine receptor. J. Chem. Inf. Model. 54, 2004.
Chen D, Ranganathan A, Ijzerman AP, Siegal G, Carlsson J (2013) Complementarity between in silico and biophysical screening approaches in fragment-based lead discovery against the A2A adenosine receptor. J. Chem. Inf. Model. 53, 2701.
Keywords: Adenosine receptors; Virtual screening; Structure-based drug design.

2. Structure-based drug discovery of P2Y receptor ligands

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Objectives / background: We discover and characterize new receptor ligands to modulate purinergic signaling using chemical, pharmacological, and structural approaches. Purine receptors encompass four G protein-coupled receptors (GPCRs) for adenosine, eight GPCRs activated by nucleotides (P2YRs), and seven ATP-gated P2X ion channels.Methods and results: High-resolution X-ray P2Y1R and 2Y12R structures, determined through our collaboration with Ray Stevens and colleagues (Zhang et al. Nature 520; 317, 2015), facilitate the rational design of ligands, either by modification of known agonists and antagonists or by virtual screening to discover novel chemotypes. An extrahelical, allosteric P2Y1R antagonist, diaryl-urea BPTU, displayed both surmountable and insurmountable antagonism (Gao et al. Mol. Pharmacol. 92; 613, 2017). The P2Y1R interactions of allosteric and orthosteric ligands were predicted using docking and molecular dynamics. We introduced sterically constrained rings to mimic native ribose in nucleosides and nucleotides, to determine their preferred conformation when bound to P2Y1R, P2Y6R and other P2YRs. We discovered new bitopic nucleotide agonists of the P2Y6R, which is a potential drug target for inflammation, diabetes and neurodegeneration. Chemical tools for the inflammation-related P2Y14R, such as fluorescent probes, were designed with the aid of molecular modeling based on P2Y12R structures and applied to discovery of novel heterocyclic antagonists (Junker et al. J. Med. Chem. 59; 6149, 2016). We have explored the structure activity relationship of a 3-(4-phenyl-1H-1,2,3-triazol-1-yl)-5-(aryl)benzoic acid antagonist scaffold, assisted by docking and molecular dynamics (MD) simulation at a P2Y14R homology model. A computational pipeline using the High Throughput MD Python environment guided the analogue design. Selection of candidates was based upon ligand-protein shape and complementarity and the persistence of ligand-protein interactions over time. Predictions were largely consistent with empirical results. Conclusion: Purine receptor structures have enabled novel ligand discovery, the elucidation of their biological role and the conceptualization of future therapeutics. Financial support from the NIDDK Intramural Research Program is acknowledged.
Keywords: drug discovery; modeling; P2Y1; P2Y14.

3. Structure and functional reconstitution of the P2X7 receptor

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The P2X7 receptor forms a characteristic membrane pore permeable to molecules up to ~900 Da, which is implicated in the development of chronic pain and cancer. However, how this subtype-specific pore is formed remains unclear. Here I present that the P2X7 receptor, when purified and reconstituted into liposomes, forms an intrinsic pore in the absence of other cellular components. Opening of this pore is independent of its unique C-terminal domain and is facilitated by phosphatidylglycerol and sphingomyelin, but dominantly inhibited by cholesterol. Our cell-based functional studies suggest that the P2X7 receptor itself constitutes a lipid-composition dependent pore, whose opening is facilitated by palmitoylated cysteines near the pore-lining helix. In combination with the recent single channel studies, our data suggest that the P2X7 receptor forms a non-dilating membrane pore readily permeable to a large molecule. This work was supported by the National Institutes of Health (GM114379 and NS072869).
Keywords: P2X7 receptors; Structure; Reconstitution.

4. Integrating mutagenesis, biochemical and modelling approaches to understand P2X receptor properties

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The crystallization of a range of P2X receptor subunits in different states with a variety of ligands has provided a major structural insight into the molecular basis of channel properties (1). These have substantiated many predictions made from mutagenesis based studies. The structures provide snapshots of stable conformations of the receptor obtained under the crystallization conditions and there are several states of the receptor that remain elusive. We have used a cysteine mutagenesis based approach to determine the contribution of defined residues to P2X receptor properties and in combination with computational methods provided validated models of P2X receptor states. MTSEA-biotinylation, pharmacological characterization and voltage clamp fluorometry combined with molecular docking has been used to determine the location of the binding site for the general P2X receptor antagonist PPADS at the hP2X1 receptor. The hP2X3 receptor structure (2) highlighted the interactions of the amino and carboxy termini to form an intracellular cap in a state of the receptor where the transmembrane gate was open. However, under the crystallization conditions used the intracellular termini in apo and desensitized states could not be resolved. The membrane environment plays an important role in channel regulation e.g. through the interactions with cholesterol. Therefore cysteine mutant hP2X1 receptors were expressed in a native membrane environment to apply biochemical approaches to collect information on the intracellular regions. To achieve this we used cysteine reactive cross-linkers as molecular calipers to obtain inter-subunit distance data. The pattern of cross-linking found in the regions close to the TMs indicates that this region is not fully disordered in a native membrane environment. Computational modelling of these data suggests asymmetric movement in the apo state and provides insight to a intracellular "pre-cap" state.
Pasqualetto G, Brancale A, & Young MT (2018) The Molecular Determinants of Small-Molecule Ligand Binding at P2X Receptors. Frontiers in pharmacology 9:58.
Mansoor SE, et al. (2016) X-ray structures define human P2X3 receptor gating cycle and antagonist action. Nature 538(7623):66-71.
Keywords: P2X; modelling.

5. Oral Communication: Harnessing herbs to potentiate P2X7: elucidating the binding site of ginsenoside CK on P2X7

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Objectives/Background: Recent work highlighted that the metabolite compound K (CK), from the traditional Chinese herb Panax ginseng, functions as a positive allosteric modulator of P2X7 (1). This evoked sustained intracellular calcium release and enhanced cell death in macrophages; a mechanism which could be exploited for removal of intracellular pathogens. Therefore, we aimed to delineate the binding site for CK within P2X7 to understand the mechanism of potentiation to ultimately exploit its antimicrobial potential. Methods: A molecular modelling approach was utilized to identify potential binding regions within human P2X7 (hP2X7) using a homology model based on zebrafish P2X4. Amino acid residues predicted to interact with CK were mutated using site-directed mutagenesis and stably-transfected HEK293 cell lines expressing the mutant receptors were generated (HEK-hP2X7). YOPRO-1 assays and whole-cell patch clamp electrophysiology were conducted, as described previously (1). Data were analysed for statistical significance using either unpaired t-tests or one-way ANOVA with post-tests as appropriate. Results: Molecular docking predicted CK to associate with residues found within the central vestibule of P2X7. Consequently, HEK293 cells were transfected with receptors containing mutations in these residues. The dose response to ATP was left-shifted in the D318L, L320A and S60A mutants, indicating increased sensitivity to ATP (EC50 of <50 μM for all the mutants, compared to 266 μM for WT). D318L and L320A could not be potentiated by CK (10 μM). This was demonstrated by decreased uptake of the cell impermeable dye YOPRO-1 (fold change in response to a combination of ATP and CK versus ATP alone, of 0.24 and 0.55 for D318L and L320A, respectively, compared to 3.95 for WT; $p<0.05$; n=3). These observations were further supported by whole-cell patch clamp recordings, which additionally highlighted a significant decrease in the magnitude of potentiation by CK for the N100A and S101A mutations when compared to WT hP2X7 (Fig. 1; *$p<0.05$; n=5-9 cells). HEK293 cells containing mutations in all predicted binding site residues were protected against ATP-dependent cell death in the presence of CK. Conclusions: To conclude, we have identified promising amino acid residues that contribute to the positive allosteric effects of CK on ATP-induced responses by hP2X7 and described a novel binding site within hP2X7. Funding was provided by the Biotechnology and Biological Sciences Research Council (BBSRC).
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Helliwell, R.M., et al. Br J Pharmacol, 172(13); 3326-40, 2015.
Keywords: P2X7; Ginseng; Positive allosteric modulators; Binding sites.

SYMPOSIUM 27 - Oral Communications

1. A2AR stimulation suppresses CD8+ T cells activity by inhibition of Notch1 signalling

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Background: A2AR stimulation is able to determine T cell anergy in a cAMP-dependent manner (Linnemann et al. Immunology. 128; 728–737, 2009). One of the crucial signalling pathway involved in T-cell development, lineage selection and activation is Notch that is activated after TCR engagement and is required for the cytotoxic activity of $CD8^+$ T cells (Dongre et al. Cells. Front Immunol. 12;5:54, 2014). Here we investigated the mechanisms of adenosine/A2AR-Notch crosstalk pathway in $CD8^+$ T cells. Methods and results: We observed that A2AR stimulation (with CGS-21680, 1μM) is able to down-regulate Notch1 receptor expression in CD8+ T cells ($p<0.01$, n=6-10), isolated from C57Bl6 mice (female, 6-8 weeks), upon TCR stimulation. Activation of A2AR impairs IFN-γ and Granzyme B release from activated CD8+ T cells and these effects were enhanced by blocking the Notch1 signalling with a γ-secretase inhibitor PF-03084014. The adenylate cyclase stimulator, forskolin (10μM), mimed the effects of CGS-21680 on Notch1 expression. Notch1 mRNA expression was not significantly different between control cells and CGS21680-stimulated $CD8^+$ T cells. Instead, the mRNA expression of Notch-targeted genes, HES1 and Myc, were significantly reduced after A2AR stimulation, suggesting that it could regulate Notch1 expression at post-transcriptional level and thus its signalling pathway. The stimulation of A2AR, 24h later TCR activation, did not influence Notch1 expression or the production of cytokines. Conversely, Notch1 inhibition reduced IFN-γ e Granzyme B production. These results strongly suggest that stimulation of A2AR impairs the TCR-induced Notch1 expression in a cAMP-depend manner. The suppressive activity of CGS-21680 was lost in $CD8^+$T cells over-expressing the active intracellular domain of Notch1 (N1IC) compared to N1ICf/f $CD8^+$ T cells. As expected, Notch1 inhibitor blocked the cytokines release in N1ICf/f cells, while N1IC cells were completely resistant. Importantly, combination of Notch1 inhibition and A2AR stimulation induced a significantly inhibitory effect on effector functions of N1ICf/f cells, which was reversed after combination with the A2AR antagonist, but not in N1IC cells. In cells deficient of A2AR the inhibitory effects of the Notch1 inhibitor still occurred, suggesting that Notch signalling is not affected by A2AR in $CD8^+$T cells. Conclusion: Altogether, our data, reveal a novel role for A2AR which regulates the T cells effector response, at least in part, by repressing the signalling pathway of Notch1 in CD8+T cells.
Keywords: A2AR; CD8+ T cells; Notch1; immunosuppression.

2. An altered nucleotide metabolism as a novel mechanism leading to Huntington disease related cardiomyopathy

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Huntington's disease (HD) is a neurodegenerative disorder with a significant peripheral component to the disease pathology. This includes an HD-related cardiomyopathy,with an unknown pathological mechanism. In this study,we characterized changes in cardiac nucleotides metabolism in the HD mouse models. Moreover, we aimed to assess the concentrations of adenine nucleotides catabolites in plasma of HD patients.We examined R6/2 mice (n=5,male,12 weeks of age),HdhQ150 mice (n=5,male,22 months of age) and their WT littermates. Plasma samples from HD and control patients (n=5 per group) were obtained from the European Huntington's Disease Network. To investigate changes in the nucleotides metabolism, the concentration of adenine and guanine nucleotides, creatine, NAD and NADH were measured. Activity of eNTPD,AMPD,e5'NT,ADA and PNP as well as serum concentration of nucleotides catabolites were measured with HPLC. We evaluated cardiac substrate preferences using 13C glucose and LC-MS method. Analysis of genes transcripts were performed using RT- qPCR. Moreover, level of AMPK phosphorylation was measured with ELISA KIT. We observed a notable energy metabolism deterioration in hearts of HD mice (ATP/ADP ratio=6.39±0.46 in R6/2 and 3.57±0.57 in WT,*$p<0.05$;8.56 ±1.84 in HdhQ150 and 5.98±0.55 in WT,*$p<0.05$). We demonstrated AMPK over-activation in hearts of HD mice that was accompanied by shift in a cardiac substrate preference from glucose to fatty acids. We found a reduced activity of AMPD and e5'NT, while the activity of ADA was increased. Moreover, we found a significant down-regulation of genes involved in purine de novo biosynthesis and up-regulation of transcripts of genes involved in adenosine degradation.This was accompanied by an increases in concentration of nucleotide catabolites in HD mouse model serum, in comparison to their wild type littermates. Interestingly, we observed prominently increased levels of hypoxanthine and uridine also in HD patients plasma, in comparison to their healthy controls. Moreover, hypoxanthine and uridine levels strongly correlated with HD disease progression parameters. This study highlights a profound deregulation in cardiac energy and nucleotides metabolism in HD mouse models. We suggest that mutant huntingtin disrupts coupling of cardiac energy metabolism with its regulatory pathways that despite its activation is unable to ensure recovery. Consequently, hearts and possibly other organs remains energy depleted that translate into elevated nucleotide catabolites concentration and suppression of nucleotide synthetic pathways. Furthermore, for the first time, our study identified biomarkers that might be linked to HD progression both in pre-clinical and clinical settings. Restoration of energy equilibrium in HD hearts may be important therapeutic target in HD. This work was supported by the National Science Center of Poland (2015/17/N/NZ4/028410) and Polish Ministry of Science and Higher Education (MN – 01-0243/08/256).
Keywords: "nucleotide metabolism"; "Huntington disease", "heart".

3. The first transmembrane domain residues regulate rat P2X7 receptor trafficking and gating

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Objectives: ATP-gated P2X receptors consist of three subunits, each of which contains two transmembrane domains (TM1 and TM2), a cysteine-rich ectodomain, and cytoplasmic N- and C-terminal domains. Seven distinct subunits (P2X1–7) have been cloned in mammalian cells. In contrast to other P2X subunits, the P2X7 subunit has been only found in homomeric receptors. The P2X7 receptor (P2X7R) also differs from other receptors by its low sensitivity to ATP, its potentiation of the effects of ATP and the higher affinity agonist 2′,3′-O-(4-benzoyl-4-benzoyl)-ATP (Bz-ATP) by reducing concentrations of divalent cations, its sensitization during the prolonged agonist stimulation, and by its long C-terminal domain, which has been implicated to have significant effects on channel gating. The gate and ion-selectivity filter of the human P2X7R has been recently identified at and around the intersection of the pore-forming TM2 helices, whereas the possible role of TM1 in gating has not yet been systematically investigated in P2X7R. Methods and Results: To probe TM1 structure, we substituted one by one residues in the rat P2X7-TM1 domain, from G27 to D48, with alanine, expressed mutants in HEK-293T cells, and examined the pore permeability by recording both currents and fluorescent dye uptake, in response to application of BzATP. Membrane currents were recorded from single cells using patch clamp recordings in a whole-cell configuration and cellular accumulation of fluorescent dye was examined by changes in fluorescence EtBr (20 μM). We identified two types of mutants affecting permeability of the channel pore. The TM1 mutants G27A, H34A, Y40A, F43A, L45A, and M46A showed significantly reduced trafficking to the cell surface, accompanied with reduced BzATP-stimulated dye uptake and membrane current amplitude. Two mutants, K30A and D48A, also showed reduced dye uptake and membrane current in response to BzATP application, but without changing significantly the plasma membrane expression. Substitution of K30 with arginine, but not glutamate, fully restored the receptor function. Homology model of P2X7R revealed that the K30 residue could interact with β-1 tail fin forming the cytoplasmic cap in receptor open state. The D48A mutant, located at the interface between TM1 and extracellular domain, lacked the ability to generate the secondary current growth, indicating that this residue could play a role in receptor sensitization after prolonged agonist application. Conclusion: Our results indicate a critical role for the TM1 domain of P2X7R in receptor function, and identify P2X7 channel TM1 residues, which could provide a structural base for specific gating properties of P2X7R.
Acknowledgment: Supported by the Czech Science Foundation (grant 16-12695S), MEYS (the LQ1604 National Sustainability Program II, Project BIOCEV-FAR), and an intramural NIH grant.
Keywords: "ATP-gated receptor-channels";"P2X7 receptor";"transmembrane domains";"alanine scanning mutagenesis".

4. Combined in silico plus electrophysiological studies identify molecular determinants of ivermectin and zinc P2X4R allosterism

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Objectives/background: We aimed at understanding the molecular basis of ivermectin (IVM) and Zn(II) as P2X4 receptor (P2X4R) allosteric modulators, in silico bioinformatic protocols coupled to electrophysiological protocols were conducted. Methods and results: Docking studies, molecular dynamics simulations and non-bonded free energy calculations for the P2X4 receptor in the apo and holo states either alone or in the presence of 3 μM IVM and 10 μM Zn(II) were calculated. Based on the crystallized Danio rerio P2X4 receptor, the rat P2X4, P2X2 and P2X7 receptors were modelled to determine IVM and Zn(II) docking sites. Calculations revealed that the IVM allosteric site in the P2X4R is restricted to transmembrane domains 1 and 2 where the role of Y42 and W46 plus S341 are critical for IVM binding. Parallel studies did not find these amino acids in the correspondent transmembrane domains of the P2X2 and P2X7 receptors, a finding that was confirmed by preferred binding conformations to equivalent sites. Consonant, electrophysiological protocols fully confirmed IVM specificity as a selective P2X4 receptor modulator. 40-50 nanoseconds molecular dynamic calculations in the apo and holo P2X4R states showed the stability of the allosteric receptor binding in agreement with previous publications. HOLE studies plus lateral fenestration determinations of the P2X4R conformational changes in the presence of each or both modulators are compatible with a larger opening of the extracellular vestibule. This finding is in full correspondence with electrophysiological studies indicating additive effects for IVM plus Zn(II) on the 1 μM ATP- induced currents. The P2X4R mutant, C132A, was resistant to Zn(II) but not to IVM, although a slightly reduced 4.9 ± 0.7-fold increase in the ATP-evoked current was recorded as compared to the wild type (wt) P2X4R. Likewise, the simultaneous application of both modulators elicited a 7.1 ± 1.7-fold increase in the ATP-gated current, a value significantly reduced as compared to P2X4R wt. In contrast, both modulators evoked similar ATP-gated currents in the C126A receptor mutant as compared to the P2X4R wt. Finally, a P2X4/2R chimera was IVM insensitive but conserved a 2.7± 0.6-fold increase in the presence of Zn(II). The joint metal application plus IVM in the chimera also evoked a 2.7 ± 0.9-fold increase in the ATP-currents. Conclusions: IVM and Zn(II) acting as P2X4R allosteric modulators, interact at different but complementary sites, increasing the gating of the P2X4R. Allosteric molecular determinants for these ligands were revealed.
Acknowledgements: Funded by FONDECYT 1170842; CEDENNA contributed with additional funds
Keywords: P2X4R; Allosterism; Ivermectin; molecular dynamics.

5. The extracellular NAD+ and NMN metabolism on the surface of human aortic valves

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Nicotinamide adenine dinucleotide (NAD+) plays a crucial role in the energy metabolism as a redox carrier and an important coenzyme. Furthermore, NAD+ is a substrate for enzymes responsible for intra- and extracellular signaling pathways. Previous studies have pointed to an important role of extracellular enzymes in controlling adenine nucleotide levels in pathological conditions, for instance, aortic stenosis (AS). The aim of this study was to investigate the catabolic pathways of NAD+, mononucleotide nicotinamide (NMN), and nicotinamide (Nam) on the surface of human aortic valves in patients with AS. Stenotic aortic valves have been obtained from patients undergoing aortic valve replacement (AVR, study group, n=50), while non-stenotic valves after Bentall surgery (control group, n=10) followed by the approval of the local Bioethical Committee. The fragments of aortic valves were immediately placed in a specially designed 24-well plate, which allowed for

the exposure of the well-known surface. A buffer and the corresponding substrate: NAD+, NMN or Nam, respectively, were then added to wells and 2 hours incubation was carried out. Additionally, an experiment with inhibitors for CD73 (ecto-5'nucleotidase), NPP (nucleotide pyrophosphatase/phosphodiesterase), ALP (alkaline phosphatase), and CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase) was performed to demonstrate, which of the enzymes could be responsible for the catabolism of NAD+ and its derivatives. The analysis of the products concentrations was tracked using high-performance liquid chromatography (HPLC). Cellular sources of ecto-enzymes were analyzed by immunofluorescence. On the surface of human aortic valves, an active metabolism of NAD+ and NMN was observed. Nicotinamide conversion was not noticed. In the patients with AS, the extracellular NAD+ and NMN hydrolysis enzymes activity of aortic valves were significantly higher than in the control group (0.81 ± 0.07 vs 0.56 ± 0.10 and 1.12 ± 0.10 and 0.71 ± 0.08 nmol/min/cm2, respectively) ($p < 0.05$). Initial experiments with inhibitors have shown that the enzymes - eNPP and CD73, and to a lesser extent CD38, had the largest involvement in the degradation of NAD+. In the case of NMN catabolism, CD73, CD38, and ALP were responsible for these reactions. It has been demonstrated that patients with aortic stenosis have an increased metabolism of NAD+ and NMN on the surface of aortic valves. These changes may be a part of a protective mechanism of the valve. An accurate knowledge of the degradation pathway of NAD+ and NMN could have an impact on the better understanding of the processes occurring in aortic valve pathology. This study was supported by the Polish Ministry of Science and Higher Education.
Keywords: nicotinamide adenine dinucleotide; mononucleotide nicotinamide; ecto-enzymes; aortic valve.

6. Signaling at adenosine A2A receptors (A2aR); crosstalk with Wnt/β-catenin signaling pathway in osteoblasts

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Background: Wnt/β-catenin signaling pathway is a key regulator in bone formation and maintaining bone hemostasis. Wnt signaling upon activation leads to stabilization of the transcriptional regulator β-catenin and its further nuclear localization. Osteoblast differentiation and proliferation are regulated by a number of local and systemic factors, among which adenosine receptors are prominent during bone development. We recently reported cross-talk between Wnt/β-catenin pathway and A2aR in fibroblasts; here we seek to determine whether there is similar cross-talk between Wnt signaling and the purinergic adenosine A2A receptor in osteoblasts. Since nuclear translocation of β-catenin protein is a critical intermediate step in the Wnt signaling pathway, we studied the effect of A2aR signaling on nuclear and cytosolic β-catenin levels in osteoblasts. Methods: We used an osteoblast cell line (MC3T3- E1) as well as primary osteoblasts derived from mesenchymal stem cells of mice. The cells were treated with CGS21680, a selective A2aR agonist, at doses ranging from 0 to 10μM, and for varying incubation periods from 0 to 240 minutes. Using western blot analysis, the levels of total β-catenin and phosphorylated β-catenin at Ser552 (active component) were measured. Additionally, we measured β-catenin levels in the nuclear extracts of the cells, both before and after A2aR activation. We also analyzed nuclear translocation of p-Ser552 β-catenin using immunofluorescence (IF) staining. Results: We observed a significant increase ($p<0.05$), in total β-catenin (169±50% control, n=5) and p-Ser552 β-catenin (253±122%, n=5) levels in MC3T3-E1 cells treated with 1 μM CGS21680 compared to control at 15 minutes following A2aR activation. The immunofluorescence staining results revealed enhanced nuclear accumulation of p-Ser552 β-catenin by approximately 40% among the osteoblastoid cells treated with CGS21680. Similarly, western blot assays showed a significant increase ($p<0.05$) in the nuclear translocation of phosphorylated β-catenin at Ser552 following administration of A2aR agonist in MC3T3-E1 cells (153±37%, n=4), as well as osteoblasts derived from mesenchymal stem cells (148±31%, n=4). Conclusions: These results demonstrate cross-talk between A2aR and Wnt/β-catenin signaling pathway in osteoblasts. Moreover, our results suggest that A2aR activation can bypass blockade of Wnt/Frizzled interactions at the cell surface and thereby maintain bone homeostasis.
Keywords: β-catenin; bone; Osteoblast.

SYMPOSIUM 28 - Electrophysiology and Functions of Purinergic Receptors

1. Interaction of P2X7 receptors with other ion channels

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Objectives/background: P2X7 receptors are cation channels expressed in the cell membrane of immune cells and epithelia. They become activated/opened by extracellular ATP which may be released by cell stretch or under pathological conditions like hypoxia nad necrosis. The opening of the cation channels enables ion fluxes which lead to cell membrane depolarization and increase or decrease of the cytosolic Ca^{2+} or K+ concentration, respectively. Further downstream effects depend on the circumstances of activation and may include stimulation of killing of intracellluar pathogens, secretion of inflammatory mediators, migration, proliferation, apoptosis and necrosis. To accomplish this, interaction with other cellular proteins is necessary, including ion channels. We investigated if P2X7 may interact with other ion chanels of the cell membrane. Methods and results: Votage clamp measurements of cell membrane currents and fluorescence measurements (Förster resonance energy transfer, FRET) were performed in Xenopus oocytes expressing P2X7 and other channels like pannexin, P2X1, P2X4 or Ca^{2+}-dependent anion channels (ANO1, ANO6). P2X7-dependent current kinetics and pharmacological properties were independent of coexpression with P2X1, P2X4, pannexin and ANO6. On the other hand, the P2X7-dependent Ca^{2+} influx may activate ANO1-dependent currents. Experiments testing the association of P2X7 and P2X4 by FRET indicated a physical interaction of P2X7 and P2X4 subunits. Conclusion: P2X7 receptors do not interact functionally with P2X1, P2X4 and pannexin but, via the P2X7-dependent increase of the cytosolic Ca^{2+} concentration, ANO1 channels become activated. P2X7 and P2X4 form heteromers without creation of a new distinct phenotype. The work was funded by the Deutsche Forschungsgemeinschaft (Ma 1581).
Keywords: P2X7; FRET; interaction; P2X4.

2. Cation currents in tumor cells - the Janus face of P2X7 and TRPM7

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Within the ion channel-coupled purine receptor (P2X) family, P2X7 has gained particular interest because of its role in immune responses and in the growth control of several malignancies. Available information is however partly contradictory. For example, it has been shown that blockade of P2X7 by the antagonist brilliant blue G (BBG) can inhibit or promote proliferation in rat C6 glioma brain tumor models (Ryu et al. J. Neuropathol. Exp.Neurol. 70:13, 2011; Fang et al. Int. J. Biochem. Cell Biol 45:1109, 2013). During the characterization of allosteric P2X7 inhibitors, we realized earlier that some modulators abrogated ATP-induced increases in the intracellular Ca^{2+}-concentration but only partially suppressed ATP-induced ionic currents. To resolve this discrepancy, we now tested the possibility that ATP had unexpectedly gated an additional, non-P2X7-associated background conductance. Using complementary electrophysiological and fluorometric methods, as well as Western blotting, we observed then indeed spurious ATP-induced currents in HEK293 cells that neither expressed P2X7 nor displayed ATP-induced Ca^{2+}-influx or Yo-Pro-1 uptake. Although the biophysical properties of these ionic currents resembled those of P2X7 in terms of their reversal potential close to 0 mV, non-rectifying current-voltage relationship, current run-up during repeated ATP application, and augmentation in bath solutions containing low divalent cation (DIC) concentrations, they were poorly inhibited by established non-selective (TNP-ATP) or P2X7-selective P2X-antagonists (AZ-10606120). Because high ATP concentrations would reduce the availability of DICs, these findings prompted us to ask next whether other channel entities may become activated by our experimental regimen. Indeed, a bath solution with no added DICs yielded similar currents, which were inhibited by intracellular Mg^{2+} or extracellular NS-8593 and, hence, conducted via TRPM7 channels (transient receptor potential cation channel subfamily M member 7), a view also corroborated by their observed knockdown in the presence of respective siRNAs. Similar ATP-induced, TRPM7-like currents were additionally found in rat (C6 glioma) and human (GB09/05, LN229, U87) astrocytoma / glioblastoma cell lines. Notably, the currents in the C6 cells, which again did not express P2X7 proteins, were, depending on used the concentration, either augmented (0.1 µM) or inhibited (1.0 µM) by BBG. It is concluded that an atypical P2X7-like conductance may rely on the activation of TRPM7 by ATP, which scavenges free divalent cations and thereby releases these channels from permeation block. Because TRPM7 has a critical role in controlling the intracellular Mg^{2+} homeostasis and in regulating tumor growth, these data imply that the proposed role of P2X7 in glioma proliferation deserves reevaluation. This work was supported by the Deutsche Forschungsgemeinschaft (DFG) within the framework of FOR748 and TRR-SFB 152.
Keywords: P2X7; TRPM7; glioma; proliferation.

3. Modulation of P2X receptor functions by cytoplasmic tails

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P2X receptors (P2XRs) are obligate trimers of identical or homologous membrane-bound subunits. Each subunit comprises two membrane spanning alpha helices, TM1 and TM2, connected by a large ectodomain involved in ATP binding and gating, and flanked by cytoplasmic N- and C-terminal tails of different lengths. The availability of crystal structures proved to be extremely useful in interpreting substituted cysteine-accessibility data to track the conformational changes accompanying ATP-gated opening of the trihelical TM2 pathway of P2XRs including the human (h) P2X7R (Pippel et al 2017). However, important phenotypic characteristics of the P2XRs including desensitizing and non-desensitizing current responses to ATP strongly dependent not only on the TM2 pathway, but also on the exact sequences (and hence structures) of the intracellular tails. For instance, truncation of the C terminal tail of the hP2X7R resulted in greatly reduced diminished current responses to ATP, but co-expression of the tail extension as a discrete protein was capable of fully reconstituting the wild-type hP2X7R function (Becker et al 2008). The structural basis for the function of this split hP2X7R is unknown due to the lack of appropriate high resolution structures. A crucial achievement is this respect is the recent X-ray crystal structure of a slowly desensitizing hP2X3R mutant that in the ATP bound state is kept open by a cytoplasmic cap structure (Mansoor et al 2016). This hP2X3R mutant was identified by converting just three N-terminal residues to those of the non-desensitizing P2XR (Hausmann et al 2014). The implications of these findings for the phenotypic functioning of other P2XR subtypes and mutants will be discussed.

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Keywords: P2X7 receptor; electrophysiology; mutagenesis, Homology modeling.

4. Identification of neurosteroids that are able to interact with allosteric binding sites on purinergic P2X receptors

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Objectives: Family of purinergic P2X receptors (P2XRs) comprises seven subunits (P2X1-7) which can assemble as homo- or hetero-trimers. They are unevenly distributed in the central and peripheral nervous systems, immune system and endocrine system, including pituitary. Each of P2XR subunit consists of a large extracellular domain that contains ATP binding site, two transmembrane domains (TMs) and intracellular N- and C- termini. The activity of the P2XRs does not only depend on the extracellular concentration of ATP, but also on the availability of allosteric modulators. The P2XRs offer multiple binding sites for positive or negative allosteric modulators enhancing or blocking receptor function. Endogenous allosteric modulators of

P2XRs are divalent cations, phospholipids and neurosteroids - neuroactive compounds synthesized in the central nervous system from cholesterol or steroids imported from peripheral sources. For example, sex steroid regulation of peptidergic neurotransmission in the hypothalamic arcuate nucleus plays a critical role in the regulation of pulsatile secretion of gonadotropin-releasing hormone. Endogenous neurosteroids may also modify neuronal activity, and thereby brain function, by acting on ionotrophic receptor channels via a fast, non-genomic action. Methods and results: We tested several testosterone analogues (formiate, propionate, butyrate, for example) for their ability to modulate activity of P2XRs. The effect of drugs was examined electrophysiologically, in HEK293 cells expressing recombinant P2X2R and P2X4R, as well as in pituitary cells endogenously expressing P2XRs. Electrophysiological measurements from HEK293 cells stimulated with 1 μM ATP showed that several testosterone analogues, particularly butyrate and n-pentanoate, potentiated the ATP-induced current of both P2X2R and P2X4R in a concentration dependent manner, threshold concentration was 1 μM. Neurosteroids leftward shifted the ATP concentration-response curve and accelerated recovery of P2X4R from desensitization indicating that these compounds allosterically increased receptor sensitivity to ATP and influenced receptor-channel gating. The comparison of their chemical structures and whole-cell recordings revealed that the interactions of testosterone analogues with the P2XRs depend not only on lipophilicity but also on the length of ester moiety at position C-17 on the D-ring of testosterone. The potentiating effect was also observed on endogenously expressed P2XRs in pituitary gonadotrophs. Conclusion: These results revealed structural requirements of putative steroid site(s) for proper P2XR-mediated interactions that might serve as a guide for synthesis of new molecules. Detailed knowledge about neurosteroid modulatory site is a prerequisite for development of new drugs against P2XR-based disorders. Acknowledgment: Supported by the Czech Science Foundation (grant 18-05413S)
Keywords: ATP-gated receptor-channel; P2X2; P2X4; neurosteroid.

5. Oral Communication: Hits identified by high-throughput screening at the non-desensitizing S15V-rP2X3 receptor

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P2X receptors are ATP-gated cation channels involved in fast signal transduction in many cell types. The P2X3 receptor plays a vital role in sensory neurotransmission and nociception and is thus a probable target of existing and new drugs for the treatment of acute and chronic pain. To overcome problems associated with the fast desensitization of the P2X3 receptor, we generated a 1321N1 astrocytoma cell line expressing a slowly desensitizing receptor mutant, S15V-rP2X3, which we proved to be suited for automated fluorescence-based screening of drug libraries [1]. By using this cell line, we aimed at identifying potent P2X3 receptor ligands in a commercially available library of approximately 2,000 approved drugs and bioactive compounds, called the Spectrum Collection (Discovery Systems, Inc.). For high-throughput screening, we used an automated Fluo-4-based Ca^{2+} imaging system in the 384-well plate format. All compounds of the Spectrum Collection were tested regarding their ability to modulate the responses of the S15V-rP2X3-expressing 1321N1 cells to ATP. We identified 27 compounds that in the 10-20 μM range markedly blocked the ATP-induced Ca^{2+} responses. We retested these primary hits by concentration-response analysis and eventually yielded six compounds that blocked the S15V-P2X3 receptor with IC50 values of < 5 μM. Among these was aurintricarboxylic acid (ATA), which blocked the ATP-induced Ca^{2+} responses with a Ki value of 180 nM in the S15V-rP2X3 astrocytoma cells. By directly recording the ATP-induced inward currents in S15V-rP2X3-expressing oocytes by two-electrode voltage-clamp electrophysiology, we determined an IC50 value of 29 nM for ATA. Selectivity profiling at the P2X subtype receptors revealed that ATA also inhibited the rP2X1 receptor-mediated inward currents with nanomolar potency (Ki = 3 nM). In radiobinding assays, up to 30 μM ATA did not compete with the binding of [3H]-ATP to the S15V-rP2X3 receptor, indicating a non-competitive mechanism of action. Altogether, our results characterize ATA as a potent ATP non-competitive inhibitor selective for the αβ-methylene-ATP-sensitive receptors P2X1 and P2X3.
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Keywords: P2X3; high-throughput screening; P2X1.

SYMPOSIUM 29 - Purinergic Signaling and Inflammation

1. Pro-inflammatory role of the ATP/P2X7 signalling axis in graft-versus-host disease in humanised mice

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Objectives/background: Allogeneic stem cell transplantation is a curative therapy in people with blood cancers, but often results in graft-versus-host disease (GVHD), wherein donor immune cells mediate a destructive inflammatory response against host tissues. Allogeneic mouse models indicate a role for the ATP/P2X7 signalling axis in this process, but similar findings in humans remain to be established. This study aimed to examine the ATP/P2X7 signalling axis in a humanised mouse model of GVHD. Methods and results: NOD scid gamma (NSG) mice were injected i.p. with 10 million human peripheral blood mononuclear cells (PBMCs) or saline. Humanised (engrafted) mice developed GVHD over 4-10 weeks as assessed by weight loss, increased clinical score and reduced survival compared to saline-injected mice. Mice injected with human PBMCs, but not those injected with saline, demonstrated histological and molecular evidence of cutaneous and hepatic GVHD, and early gastrointestinal GVHD. qRT-PCR revealed expression of both murine and human P2X7 in spleens and target tissues of humanised mice. Inhibition of P2X7 by Brilliant Blue G (5 x 50 mg/kg i.p.) reduced serum human interferon-γ, as well as tissue inflammation and damage in humanised mice. In contrast, GVHD development was similar in NSG mice injected with human PBMCs coding for either loss or gain of function P2X7 haplotypes. Collectively this data suggests that host P2X7 plays a greater role than donor P2X7 in GVHD in humanised mice. Furthermore, inhibition of CD39/CD73-mediated hydrolysis of extracellular ATP by αβ-

methylene ADP (6 x 50 mg/kg i.p.) enhanced clinical disease (weight loss) and serum human interleukin-2. In contrast, inhibition of adenosine receptors by caffeine (14 x 10 mg/kg i.p.) had minimal effect on clinical and molecular disease. Collectively this data suggests involvement of extracellular ATP rather than extracellular adenosine in enhancing GVHD in humanised mice. Conclusion: Combined this data indicates that extracellular ATP acts on P2X7 to enhance inflammation during GVHD in humanised mice. Thus, the ATP/P2X7 signalling axis remains a potential therapeutic target to limit GVHD following allogeneic stem cell transplantation in humans.
Keywords: P2X7; inflammation; leukocyte; graft-versus-host disease.

2. Modulating P2X7 – which way do we go?

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P2X7 has emerged as a potential therapeutic target for a plethora of diseases including psychiatric disorders, epilepsy, ischaemic stroke, neurodegenerative diseases, and retinal disease amongst others. The last decade has seen an explosion in the discovery and development of new antagonists acting on P2X7 from a number of pharmaceutical companies including AstraZeneca, Janssen, GSK and Pfizer. In addition, many unrelated chemicals have been documented to have P2X7 antagonist activity including probenecid, paroxetine, berberine, trifluoperazine and a number of kinase inhibitors. It is hoped that this drug discovery effort will pay off in the future with successful clinical trials for neurological and inflammatory disorders. In comparison we know relatively little about activating P2X7 and chemical entities that enhance activation (positive modulators). Activating P2X7 may be a useful therapeutic avenue for driving the killing of intracellular pathogens in macrphages or for killing of cancer cells through apoptosis. Following our discovery of a novel positive allosteric modulator for P2X7 derived from the root extract of the Panax ginseng plant [1], we sought to determine the consequences of positively modulating P2X7 responses in vitro. Using molecular modelling based on crystal structures of zP2X4 and pdP2X7, we have identified a putative binding site for ginsenosides on human P2X7. This positive modulator site is distinct from the orthosteric site and the predicted AZ10606120 antagonist binding site. Site-directed mutagenesis of hP2X7 confirmed residues located in the lower body region beta sheets interact with the ginsenoside chemicals. We also discovered that the lower body region beta sheets were involved in controlling P2X7 sensitivity to ATP and BzATP as mutations enabled P2X7 channel opening at lower agonist concentrations. Downstream consequences of activation with the physiological agonist ATP plus a positive allosteric modulator were investigated in HEK-hP2X7 cells, murine macrophage and microglial cell lines. Ginsenoside CK dramatically increased sustained calcium responses, YOPRO dye uptake and converted a non-lethal concentration of ATP into a lethal signal. These effects only occurred in cells expressing P2X7 and could be blocked with a selective P2X7 antagonist such as AZ10606120. The potential beneficial effects of positively modulating P2X7 will be discussed.
[1] Helliwell RM, ShioukHuey CO, Dhuna K, Molero JC, Ye JM, Xue CC, Stokes L. Selected ginsenosides of the protopanaxadiol series are novel positive allosteric modulators of P2X7 receptors Br. J. Pharmacology 2015 172: 3326-40.
Keywords: P2X7; positive allosteric modulator; ginsenoside.

3. Oral Communication: CD39 Limits P2X7 receptor inflammatory signaling and attenuates sepsis-induced liver injury

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Objectives/background: The severity of sepsis can be linked to excessive inflammatory responses and hepatic injury. Monocyte/macrophages are crucial to the pathogenesis of cellular injury and liver failure. P2X7 receptor (P2X7R) activation by extracellular ATP exacerbates inflammation by augmenting cytokine production while CD39 scavenges extracellular ATP thereby limiting P2X7R activation and contributing to adenosine production. We evaluated whether CD39 and P2X7R functionally interact to control macrophage responses, and whether this interaction dictate outcomes of sepsis and liver injury. Methods and results: For in vitro assays, peritoneal macrophages (MΦ) or bone marrow derived MΦ were primed with 1 μg/mL LPS for 4 h, followed by stimulation with P2X7 agonists (500μM ATP; 100μM Bz-ATP, or 100μM ATPγs) for additional 3 h. Before priming with LPS, cells were also pretreated with select P2X7R antagonists (300μM oxidized ATP, 2 h; or 100nM A740003, 30 min). Sepsis was induced by cecal ligation and puncture (CLP) in wild type (WT) and CD39-/- mice. P2X7R function was inhibited pharmacologically in vivo, using the receptor antagonist brilliant blue G (45.5 mg/kg via i.p. 24 h before CLP). For adenosine A2A receptor activation mice were treated with ATL146e (a specific agonist) at 1 μg/kg immediately after the surgery. CD39 expression in macrophages limits ATP-P2X7 receptor pro-inflammatory signaling. P2X7 receptor paradoxically boosts CD39 activity. Inhibition and/or deletion of P2X7 receptor in LPS-primed macrophages attenuate cytokine production and inflammatory signaling as well as prevent ATP-induced increases in CD39 activity. CD39-/- septic mice exhibit higher levels of inflammatory cytokines and more pronounced liver injury when compared to WT counterparts. P2X7 blockade with a selective antagonist largely prevents tissue damage, cell apoptosis, cytokine production, and the activation of inflammatory signaling pathways (NF-κB and STAT3) in the liver from WT septic mice, but only attenuates these effects in CD39-/- mice. Combination of P2X7 blockade with the administration of ATL146e completely inhibits cytokine production, protects liver injury and decreases the number of apoptotic cells in the liver of CD39-/- septic mice. In addition, BBG+ATL146e treatment improves survival of WT septic mice by 55% (p=0.02). Conclusions: CD39 attenuates sepsis-associated liver injury by both scavenging eATP and ultimately generating adenosine. We propose boosting of CD39 and blocking of P2X7 activation to limit systemic inflammation and restore liver homeostasis during the acute phase of systemic and abdominal sepsis.
Financial support: FAPERJ, CNPq, CAPES, NIH.
Keywords: ATP; ectonucleotidases; macrophages; sepsis.

4. Oral Communication: Stimulation of adenosine A2A receptors regenerates cartilage in osteoarthritis model

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Background: We have previously reported that endogenously produced adenosine, interacting with A2AR, is a critical autocrine factor for maintenance of chondrocyte and cartilage homeostasis and intra-articular injections of liposomal preparations of adenosine inhibit progression of OA in a post-traumatic OA (PTOA) model in rats. We therefore determined whether intra-articular injection of a more selective A2AR agonist could also prevent progression and possibly reverse OA in this model and in the obesity related OA model in mice. Methods: PTOA was induced in SD rats following rupture of the anterior cruciate ligament (ACL) by application of external force to the knee. Starting 4 weeks after injury, when OA has already progressed, knees were injected with 100ul of saline, empty liposomes (LIPO) or liposomes containing CGS21680 (LIPO-CGS) every 10 days (6 injections) before sacrifice. The cartilage volume in OA and normal knees was measured by microCT after staining with hexabrix (40%). Chondrocytes were isolated from neonatal mice and cultured, only first passage chondrocytes were studied. For the obesity-OA model, C57Bl6 mice (5-6/group, 12 weeks old) were fed a 60% fat diet (HFF mice). After 3 months, when OA was present, mice received intrarticular knee injection (10 μl) of LIPO, LIPO-CGS or liposomal adenosine (LIPO-Ado) every 10 days for 4 injections before sacrifice. Results: Injection of LIPO-CGS but not saline or LIPO, significantly reduced swelling of affected rat knees ($p<0.001$). Surprisingly, there was an increase in tibial and femoral cartilage volume in normal knees treated with intra-articular injections of LIPO-CGS but not LIPO or saline (47% increase in tibia and 22% in femur). More importantly, intra-articular injections of LIPO-CGS, but not LIPO or saline, increased tibial and femoral cartilage volume in OA knees, as compared to normal knees and completely abrogated the histologic evidence of OA as well (OARSI score for CGS21680 0.66±0.33 vs 4.55±0.82 in the vehicle group and 3.90±0.89 in the saline group). There was marked chondrocyte proliferation in the deep cartilage of knees of rats treated with LIPO-CGS (Ki67 immunofluorescence). Similarly, LIPO-CGS reversed the OA changes in the obesity related OA model. HFF mice had an OARSI score of 5.17±1.84. Treatments with LIPO-Ado and lipo-CGS decreased OA severity (OARSI score 1.33±0.81 and 1.83±0.98, respectively, $p<0.001$ vs untreated). A2AR stimulation increased TGF-β immunostaining in LIPO-CGS-injected joints and increased TGF-β production by cultured neonatal murine chondrocytes with increased SMAD2/3 phosphorylation and diminished RUNX2 expression. Conclusion: These results demonstrate that intra-articular injection of a long-acting A2AR agonist stimulates chondrocyte and cartilage regeneration, likely by a TGF-β-dependent mechanism. More importantly, these results indicate that treatment with an A2AR agonist can reverse OA in both traumatic and obesity-related OA.
Keywords: Adenosine recepotors; Liposome; Osteoarthritis.

5. Oral Communication: Nucleotide and adenosine converting ecto-enzyme pattern in endothelial inflammation

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Objectives/background: Extracellular nucleotides and adenosine affect vascular pathologies by mediating inflammation and lipid accumulation. Membrane-bound ecto-enzymes effectively control their local concentrations. Our recent studies highlighted that changes in nucleotide and adenosine conversion ecto-enzymes are related to valve dysfunction and atherosclerosis. Current work aimed to investigate cellular sources of specific ecto-enzymes in the vasculature during initial and later phases of vascular pathology and to correlate their activities with markers of vascular activation and inflammation. Methods and results: Human arteries from patients undergone cardiovascular surgery (n=12; M/F:8/4, mean=60ys) were subjected to histological and immunofluorescence to visualize ecto-nucleoside triphosphate phosphohydrolase 1 (eNTPD1), ecto-5'nucleotidase (e5'NT) and adenosine deaminase (ADA). The study was approved by the local ethical committee. At an early stage of the pathological process, eNTPD1 originated from endothelial cells (EC) and vascular smooth muscle cells (VSMC), while e5'NT and eADA derived mainly form endothelial cells. In later stages, eNTPD1 and particularly eADA also originated from immune infiltrate, whereas immune cells were a poor source of e5'NT. Moreover, co-localization of ADA and CD26 was proved that CD26 could bind ADA on the cell surface. The expression of CD26 and adhesion molecules in vessels were analyzed by PCR, while functional activities of ecto-enzymes were estimated by HPLC. eADA activity positively correlated with vascular expression of adhesion molecules (ICAM-1: $r=0.69$, $p<0.05$; VCAM-1: $r=0.65$, $p<0.05$), while the activities of eNTPD1 and e5'NT tended to the negative correlation with these parameters. Ecto-enzyme activities were also analyzed in vitro using LPS-treated primary cultures of human aortic EC (HAEC), VSMC and monocytes/macrophages in the presence of specific ecto-enzyme inhibitors. ADA activity was evaluated in different cell compartments, including intracellular ADA, cell-surface ADA (eADA) and soluble ADA (sADA), which was released by the cells and not anchored on their surface. These studies stressed the role of EC in the origin of e5'NT and hence in the production of protective adenosine. However, LPS-activated EC and immune infiltrate effectively catabolized extracellular adenosine by the increased activity of eADA (endothelial cells) and both eADA and sADA (monocytes/macrophages). Conclusion: This study highlights the importance of extracellular nucleotide and adenosine metabolism in vascular inflammation and endothelial activation. The activities of nucleotide converting ecto-enzymes are adversely modified under these conditions and may be indicated as markers of pathological process within the vessel wall and target for therapy in vascular pathologies. This study was supported by The National Centre for Research and Development of Poland (STRATEGMED1/233226/11/NCBR/2015).
Keywords: adenosine deaminase; inflammation; endothelial activation.

SYMPOSIUM 30 - Purinergic signaling in lung disease

1. Extracellular ATP in sepsis

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ATP is released into the extracellular space during sepsis. Activation of P2X4 and P2X7 receptors suppresses bacterial dissemination, inflammation and mortality in murine sepsis. Macrophages are involved in both the P2X4 and P2X7 receptor-mediated protection against sepsis in vivo. We propose that P2X4 and P2X7 receptors are new targets for the pharmacological management of sepsis.
Keywords: macrophages; killing.

2. Role of adenosine in lung fibrosis caused by silica particles

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Objectives/background: Adenosine is a nucleoside that has been reported to be implicated in fibrosis, being considered as a potential therapeutic target for fibrotic diseases. This study was undertaken to investigate the involvement of adenosine in late phase lung fibrosis in silica-stimulated mice also accessing fibroblast reactivity in vitro. Methods/results: Lung fibroblasts from Swiss-Webster mice were evaluated in vitro, by means of proliferation rate and activation (MCP-1 levels) using BrDU system and ELISA, respectively. Mice instilled with silica particles were analyzed, 28 days after provocation, and the parameters included: lung function as resistance and elastance (invasive whole body plethysmography) and tissue morphology/morphometry (H&E and Picrus sirius staining). Evaluation of lung fibroblasts in vitro revealed that adenosine (100 – 300 μM) and IL-13 (40 ng/mL) stimulation led to increased proliferation and MCP-1 generation, phenomena sensitive to selective A2A receptor (A2AR) antagonists ZM 241385 and SCH58261. This indicated that adenosine might be mediating fibrogenesis caused IL-13, acting through A2AR. In line with this idea, we noted that proliferation of fibroblasts induced by IL-13 was suppressed by inhibitors of CD39 and CD79, enzymes responsible for adenosine generation from ATP. Moreover, fibroblasts, when recovered from lungs of A2AR knockout mice, showed refractory to IL-13 stimulation. In another set of experiments, we observed that silica-provoked mice showed increased expression of the mRNA for CD39 and CD73 enzymes, suggesting elevation of adenosine metabolism. Increased expression of A2AR was noted in the lungs of silicotic mice, which paralleled with decreased of lung function (parameters of resistance and elastance) and tissue fibrosis including granuloma formation. Therapeutical administration of the A2AR antagonist ATL-146e, during 7 consecutive days starting on day 21 post-silica, significantly reversed lung function decrease and tissue fibrosis in silica-challenged mice. Interestingly, mice submitted to deletion of A2AR gene showed similar suppressive profile, of both lung function and tissue alteration also including lower levels of alpha-SMA actin positive cells. Conclusion: Our findings show that adenosine seems to be implicated in fibrogenesis associated with silica stimulation in mice, possibly mediating IL-13 -induced fibrosis, by a mechanism at least partially dependent on A2AR. They also suggest that adenosine A2AR antagonists could provide a useful pharmacological tool for application in fibrotic diseases such as silicosis.
Financial support: CNPq, FAPERJ, CAPES - Brazil.
Keywords: Silica; Lung fibrosis; A2AR.

3. Purinergic-dependent contraction of small intrapulmonary veins: role in a pulmonary arterial hypertension rat model

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Background.- Increasing evidences about the role of pulmonary veins to the total pulmonary vascular resistance has been particularly well supported by studies associated to development of fetal and neonatal pathology. In contrast, the vasoactivity of pulmonary veins in adult mammalians has been more controversial and largely unexplored. Nevertheless, the alterations of the vascular tone of pulmonary veins are believed to play an important role during the development Pulmonary Arterial Hypertension (PAH). In the lung, nucleotides are released from the cytoplasm of many cells including endothelial, smooth muscle and epithelial cells under physiological and pathological conditions. This extracellular ATP and UTP binds to P2Y2/4 and UDP to P2Y6/14 receptors, widely expressed in blood vessels, attributing a pivotal role in the control of vascular tone. However, there are no studies on either the effects of ATP, UTP and UDP on small intrapulmonary vein (SPV) contraction or the mechanisms that couple purinergic signalling to PAH. Here we have used 'living' lung slices and phase-contrast video microscopy to investigate, for the first time, purinergic- dependent dynamic changes in SPV contraction in PAH rats.Methods.- Lung slices (150μm thick), from healthy and Monocrotaline (MCT)-induced PAH Sprague Dawley rats (~200gr), in a vibratome were performed. ATP, UTP and UDP -induced SPV contraction was recorded using phase contrast video microscopy. Statistical differences ($p<0.05$) were performed using non-parametric tests. All experiments were approved by Animal Bioethical Institutional Commite (CICUA). Results.- After 21 days of a single subcutaneous injection of MCT, (60mg/Kg) the rats develop PAH, including right ventricle hypertrophy. ATP-dependent vasoconstriction was strongest in PAH in comparison with healthy rats, but only at lower concentration of ATP. In PAH rats, there was an exacerbated venous constriction in response to UTP versus healthy rats. Similarly, UDP dependent contraction was exacerbated in PAH, but only at higher concentration of UDP. Significant P2Y2 inhibition with ARC118925XX was predominant only in healthy rats, but not PAH rats, suggesting a main role of P2Y4 in PAH. UDP-glucose an specific agonist of P2Y14 receptors contracted SPV only from PAH rats. The presence of P2Y2 and P2Y4 receptors in co-localization with smooth muscle cells was demonstrated by indirect immunofluorescence. Interestingly, P2Y14 receptors was present mainly in the smooth muscle cell from SPV from PAH rats. Conclusions.- These results suggest a novel mechanism involving P2Y4 and P2Y14 receptors in exacerbated vasoconstriction observed in PAH. The study of purinergic therapies to improve survival and quality of life of PAH patients is promising. Supported by FONDECYT N°1140468 and ENL029/2017 to MH
Keywords: P2Y4; P2Y14; SMALL PULMONARY VEINS; PULMONARY ARTERIAL HYPERTENSION.

4. Role of P2 Receptors as Modulators of Rat Eosinophil Recruitment in Allergic Inflammation

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ATP and other nucleotides are released from cells through regulated pathways or following the loss of plasma membrane integrity. Once outside the cell, these compounds can activate P2 receptors: P2X ionotropic receptors and G protein-coupled P2Y receptors. Eosinophils represent major effector cells in the allergic inflammatory response and they are associated with several physiological and pathological processes. Here we investigate the expression of P2 receptors and roles of those receptors in rat eosinophils. In this context, our first step was to investigate the expression and functionality of the P2X receptors by patch clamping, suggesting the presence of P2X1, P2X2 and P2X7receptors. Next, we evaluate by microfluorimetry the expression of P2Y

receptors, our results based in the potency ranking order suggests the presence of P2Y2, P2Y4, P2Y6 and P2Y11. Moreover, we confirmed our findings by immunofluorescence assays. We also assess chemotaxis to verify whether nucleotides could induce migration. After 1 or 2 hours of incubation, ATP increased migration of eosinophils, as well as ATPgS, a less hydrolysable analogue of ATP, while suramin abolished migration. In keeping with this idea, we tested whether these receptors are implicated in the migration of eosinophils to an inflammation site in vivo, using a model of rat allergic pleurisy. In fact, migration of eosinophils has increased when ATP or ATPgS were applied in the pleural cavity, and once more suramin blocked this effect. Selective P2Y2 and P2Y4 agonists failed to promote eosinophils chemotaxis and calcium transients. We have demonstrated that rat eosinophils express P2X and P2Y receptors. In addition, the activation of P2 receptors can increase migration of eosinophils in vitro and in vivo.

SYMPOSIUM 31 - Purinergic Signaling in the Ear

1. Transcriptome profile of purinergic hearing adaptation

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Purinergic hearing adaptation is the term we have applied to the temporary loss of hearing sensitivity when the ear is exposed to sustained elevated sound. Mice lacking expression of the P2X2 receptor (P2X2R) type ATP-gated ion channel (P2X2KO) fail to exhibit this noise-induced hearing loss (evident as a temporary threshold shift (TTS) in the auditory brainstem response (ABR); Housley et al. Proc. Natl. Acad. Sci USA 110(18); 7494, 2013). At high sound levels, the P2X2KO mice are more vulnerable to permanent hearing loss. Thus, purinergic hearing adaptation is oto-protective. The mode of action of the cochlear P2X2R adaptation has not been established, however, given that the adaptation is sustained for many hours after the noise stimulation has ceased, the process likely involves transcriptional and translational responses in the cochlea. We investigated this postulate by analysing the transcriptome response to sustained noise presentation to the mouse ear matching a P2X2R-specific hearing adaptation profile (85 dB SPL, 4 – 32 kHz white noise for 30 minutes). The noise-exposed cochleae and the opposite no-noise control cochleae, were then processed to extract mRNA. The transcript analysis was performed using Affymetrix® mouse gene array 1.1ST, and GenePattern software. Individual transcripts which were identified as having a significant upregulation ($p<0.001$; $q<0.1$; minimum 2-fold change) in response to P2X2R-specific noise were validated by qRT-PCR. These data were then compared with mRNA from P2X2KO mice exposed to this noise level (lacking a TTS). The findings identified sustained activation of signaling cascades for several hours after noise treatment. All experiments were approved by the UNSW Animal Care & Ethics Committee. The work was supported by the National Health & Medical Research Council (grants APP630618; APP1089838).

2. Cochlear rescue from noise-induced hearing loss by inhibition of the molecular complex which regulates adenosine A1 receptor signaling

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Hearing loss is the most prevalent form of sensory impairment, affecting about 360 million people worldwide. Sensorineural hearing loss (SNHL) can result from infection, exposure to excessive noise, ototoxic drugs or progression with age. SNHL is associated with cochlear injury, including loss of sensory hair cells and primary auditory neurons. Hearing aids and cochlear implants can partly restore auditory function, but cannot repair cochlear injury. We and others have previously identified signalling pathways associated with the adenosine A1 receptor (A1R) as important regulators of cellular responses to injury in cochlear tissues. We have shown that the post-exposure treatment with adenosine A1R agonists confers protection against acoustic trauma and other forms of SNHL. Here we describe a novel otoprotective paradigm based on increasing A1R responsiveness to endogenous adenosine. We achieved this by pharmacological targeting of the Regulator of G protein Signalling 4 (RGS4). RGS proteins inhibit signal transduction pathways initiated by G protein-coupled receptors (GPCR) by enhancing GPCR deactivation and receptor desensitisation. RGS4 forms a molecular complex with neurabin, an intracellular scaffolding protein expressed in neural tissues, whose role is to facilitate interactions of RGS4 with the A1R. The neurabin/RGS4 complex is the key negative regulator of A1R activity in the CNS and a promising neuroprotective target. Our studies suggest that this complex may have a similar role in the cochlea. Here, we demonstrate neurabin transcript expression in the rat cochlea and protein distribution in sensorineural tissues which closely mirrors A1R immunolocalisation. We show that intratympanic administration of a small molecule RGS4 inhibitor, CCG-4986, 24 hours after noise exposure attenuates noise-induced permanent auditory threshold shifts by up to 30dB. Significant improvement of auditory thresholds and suprathreshold responses and improved survival of sensorineural tissues (sensory hair cells, primary auditory neurons and their synaptic connections with the inner hair cells) has also been observed when CCG-4986 was administered 48 hours after noise exposure. Our studies thus demonstrate that intratympanic administration of a small molecule inhibitor of RGS4 can rescue cochlear injury and hearing loss induced by acoustic overexposure. This research represents a novel paradigm for the treatment of various forms of SNHL based on regulation of GPCR. The study was supported by the Auckland Medical Research Foundation.
Keywords: Hearing loss; Cochlear rescue; Regulator of G protein signalling 4 (RGS4); Adenosine A1 receptor.

3. A model for P1 and P2 receptor interaction in regulating cell injury and repair in the cochlea

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The cochlea of the inner ear is an exquisite sensory organ, which transduces the mechanical vibrations of sound into impulses in the auditory nerve, coding the physical characteristics of intensity, frequency and time that provide the elements necessary for the perception of sound and speech. There is very good evidence that extracellular purines play a variety of critical roles in cochlear homeostasis, development and adaptation to sound to enhance

hearing in background noise. ATP, acting via P2X and P2Y receptors expressed on cochlear epithelial cells, may also be involved in signalling between the sensory cells and their adjacent supporting cells in response to injury (Lahne and Gale, J Neurosci, 28:4918, 2008). Other studies (Vlajkovic, et. al. BioMed Res Intl, 2014, Article ID 841489) suggest that adenosine may protect sensory cells from injury acting via A1 receptors. Here we explore this further and describe a series of studies which demonstrate a potential interplay between P1 and P2 receptors in the sensory epithelium in regulating cellular injury and repair. Exposure of neonatal organ of Corti explants (postnatal day 3-7) to ATPgS at high concentrations (10μM) causes death of both inner and outer sensory hair cells and erratic and disorganised repair of the sensory epithelium by adjacent supporting cells. The supplementation of adenosine or an A1 receptor agonist CCPA mitigates this effect of ATPgS, on outer hair cells, and where there is cell loss the scar formation by supporting cells is organised to prevent mixing of cochlear fluids. Similarly, addition of ATPgS at a lower concentration (1μM) enhances the extent of cell death in the neonatal organ of Corti following the exposure to ototoxic aminoglycoside antibiotic neomycin. Hair cell survival after with neomycin is substantially increased (average outer hair cell survival from 36% to 80.2%, $p < 0.001$) by the simultaneous addition of the A1 receptor agonist ADAC (1μM). These data suggest an interplay between P1 and P2 receptors localised to the sensory and supporting cells in the sensory epithelium, where ATP is enhancing apoptotic pathways whilst adenosine is enhancing pro-survival pathways and repair of the sensory epithelium by supporting cells. The localised extracellular concentrations of ATP and adenosine are maintained by ectonucleotidases located on the surface of sensory and supporting cells. These ecto-enzymes could play a major role in balancing the two pathways and determining the extent and outcomes of cochlear injury. Supported by the Hearing Research Foundation NZ.
Keywords: Cochlea; Hearing; P2 receptors; P1 Receptors.

4. ATP-activated Ca^{2+} signaling in supporting cells of the organ of Corti in the hearing mouse hemicochlea

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Whilst hair cells are the receptor cells in the hearing organ, supporting cells of the organ of Corti have fundamental role in hearing and its preservation and extracellular ATP-mediated signaling is a major regulator of their functions. Both P2X and P2Y receptors of ATP use Ca2+ as intracellular messenger. We have performed functional Ca2+ imaging in three different supporting cell types in the fura-2/AM bulk loaded hemicochlea preparation of hearing (P>15) CD-1 mice to measure ATP-mediated Ca2+ signaling in pillar, Deiters' and Hensen's cells. ATP evoked reversible, repeatable and dose-dependent Ca2+ transients in all three cell types. Inhibiting the Ca2+ signaling of the ionotropic P2X and metabotropic P2Y receptors by omission of the extracellular Ca2+ and by depleting the intracellular Ca2+ stores using the sarco/endoplasmic reticulum Ca2+-ATPase inhibitor cyclopiazonic acid (CPA), respectively, revealed the involvement of both receptor types. Detection of the mRNAs of P2X2,3,4,6,7 and P2Y1,2,6,12,14 receptors by RT-PCR supported this finding. The extra- and intracellular Ca2+-dependent components of the response showed linear additivity in pillar cells, while the synergistic interaction between the extra- and intracellular Ca2+-dependent signaling pathways in Deiters' and Hensen cells suggested the involvement of calcium-induced calcium release.
In order to achieve a better signal-to-noise in imaging, we have developed the technique of single-cell electroporation in the hearing BALB/c mouse hemicochlea with Oregon Green 488 BAPTA-1, a single wavelength Ca2+ probe. The higher spatial resolution allowed subcellular imaging in Deiters' cells. We noticed that the ATP-induced Ca2+ elevation in the phalangeal process preceded the one in the soma by several seconds. The phenomenon was present in different mouse strains. The lack of effect of both adenosine on the level of intracellular Ca2+ and the adenosine receptor antagonist 8-(p-sulfophenyl)-theophylline on the ATP response suggested that adenosine receptors are not involved in the ATP-evoked Ca2+ transients. The omission of Ca2+ from the extracellular solution and inhibition of L-type voltage-gated Ca2+ channels (L-VGCCs) by nifedipine reduced the time delay between the process and the soma response, while CPA increased it. The amplitudes of the ATP responses were decreased by all three interventions in both compartments. These results confirmed the involvement of both P2X and P2Y receptors in the ATP-evoked Ca2+ transients. The time delay between the transient in the phalangeal process and the soma of Deiters' cells might indicate the different subcellular distribution of the ionotropic and the metabotropic ATP receptors. Differences in ATP-induced Ca2+ signaling between supporting cells and between subcellular compartments in Deiters' cells probably reflect the distinct and highly specialized roles these cells play in cochlear function and pathophysiology.
Supported by TÉT_10-1-2011-0421.

5. Oral Communication: Purinergic hearing adaptation to sustained sound

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P2X2 receptor subunit expression is prominent in the mouse cochlea (Jarlebark et al. Neuroreport 13(15); 1979, 2002). This ion channel is activated in response to sustained sound, which releases ATP from cochlear tissues. We have previously shown that P2rx2 null mice (P2rx2(-/-)) lacking P2X2 receptors maintain their hearing sensitivity after exposure to moderate (85 dB SPL) noise, as measured by auditory brainstem response (ABR) (Housley et al. Proc. Natl. Acad. Sci USA 110(18); 7494, 2013). This is in contrast to wild-type mice which show a reversible threshold shift that recovers over ~ 96 hours. However, at higher sound levels, P2rx2(-/-) mice are more vulnerable to permanent hearing loss. Thus, this purinergic adaptation is oto-protective. In the current study we used wild-type and P2rx2(-/-) mice (C57Bl/6J background; male and female; 9 – 17 weeks old; n = 6 – 11) to further investigate the purinergic adaptation mechanism by measuring the ipsilateral cubic (2f1-f2) distortion product otoacoustic emission (DPOAE). This is a measure of the cochlear outer hair cell gain. We found that there was a significant greater increase in DPOAE thresholds ($P = 0.004$ with 85 dB SPL noise (8 – 32kHz)) in the wild-type mice compared to the P2rx2(-/-) mice measured at 40 minutes. Given that ipsilateral noise can suppress the DPOAE through the efferent medial olivocochlear (MOC) reflex (Froud et al. Nat Commun 6; 7115, 2015), the sustained purinergic hearing adaptation

complements the more rapidly developing MOC efferent reflex. All experiments have been approved by the UNSW Animal Care & Ethics Committee. Our work is supported by the National Health & Medical Research Centre (grant number APP1089838).
Keywords: P2X2 receptor; cochlear hair cells; distortion product otoacoustic emission.

SYMPOSIUM 32 - New Insights on Structure and Functions of Ectonucleotidases

1. Disturbances in purinergic signaling as part of the pathophysiology of hypophosphatasia

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Hypophosphatasia (HPP) is the heritable rare disease that results from ALPL gene mutations leading to deficient activity of the tissue-nonspecific alkaline phosphatase isozyme (TNAP)[1]. HPP features rickets or osteomalacia and early loss of teeth.2 These skeletal and dental manifestations are caused by the accumulation of extracellular inorganic pyrophosphate (PPi), a physiological substrate of TNAP and a potent mineralization inhibitor [1-4]. Additionally, phosphorylated osteopontin (OPN), another potent mineralization inhibitor also accumulates further restricting the degree of extracellular matrix mineralization [3, 4]. Understanding this pathophysiology has provided the rationale for the current therapeutic intervention for HPP using recombinant mineral-targeted TNAP for enzyme replacement [5, 6]. Severely affected HPP patients, as well as Alpl-/- mice, suffer from severe seizures that herald a lethal outcome [1, 2]. The seizures are partly explained by inadequate availability of pyridoxal phosphate, a physiological substrate of TNAP that is a co-factor in the synthesis of neurotransmitters by neuronal cells [1, 2, 4], but aberrant P2X7 signaling in the CNS is also part of the pathophysiology [7]. Other features of HPP are not yet understood, such as what pathophysiological mechanisms lead to the development of craniosynostosis, nephrocalcinosis, muscle weakness, inflammation and pain. During my presentation I will argue that these poorly understood manifestations of HPP are caused at least in part by local changes in the ATP/adenosine ratio as a result of deficient TNAP activity leading to altered purinergic signaling that affects cell behavior and tissue homeostasis.

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Keywords: "alkaline phosphatase"; "mineralization"; "inflammation"; "seizures".

2. CD73 as target for glioblastoma therapy

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Glioblastoma (GBM) is the worst and most common primary brain tumor, characterized by high proliferation and infiltration rates and chemoresistance development. Despite intense efforts, the median survival for patients remains dismal, making necessary the development of new therapeutic strategies. Alterations on purinergic signaling has been involved in cancer progression. CD73 overexpression has been reported in a variety of cancer cells and it is associated to increased tumor malignancy. In addition to generate extracellular adenosine (ADO), CD73 also acts as cell-cell and cell-matrix adhesion protein which favor tumor spread and migration. Here we demonstrate the potential of CD73 as target for GBM treatment. CD73 knockdown by CD73-siRNA strategy decreased in vitro glioma cell proliferation and migration through MMP-2 and vimentin expression modulation. Additionally, CD73 knockdown or enzyme inhibition activity by APCP promoted glioma chemosensitization to temozolomide (TMZ), decreasing the IC50 values for TMZ exposition. Next, therapeutic efficacy of nasal cationic nanoemulsion encapsulating CD73-siRNA (CD73-siRNA-NE) for GBM therapy was evaluated. CD73-siRNA-NE was efficient to knockdown in vitro CD73 in C6 glioma cells and did not promote astrocyte toxicity. Experiments of in vivo CD73-siRNA-NE nasal delivery in Wistar rats showed that the formulation achieved brain tissue and markedly reduced 60% tumor volume when compared to control in a preclinical model of GBM implant. Notably, the reduction of tumor growth was followed by 95% reduction of ADO levels in cerebrospinal fluid, suggesting the in vivo CD73 silencing and indicating the role of extracellular ADO in glioma progression. Of note, treatment did not induce systemic damage or mortality. Taken together, data indicate CD73-siRNA-NE nasal delivery as an interesting strategy for GBM treatment.
Keywords: glioblastoma; CD73; adenosine; nanoemulsion.

3. Identification of new scaffolds as inhibitors of NTPDases and NPPs: Structural, Mechanistic and In-silico Studies

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Ectonucleotidases comprise of four major groups: the ecto-nucleoside triphosphate diphosphohydrolases (NTPDases), ecto-5′-nucleotidase (5′-eNT), ecto-nucleotide pyrophosphatase (NPPs) and alkaline phosphatases (APs). These cell surface located enzymes are responsible for the hydrolysis of extracellular nucleotides to the respective nucleosides. The molecular identification of individual family subtypes have resulted in the significant insights into the structural

and functional role of these ecto-nucleotidases in purinergic signaling. Moreover, the physiological and patho-physiological roles of these cell surface-located members varies considerably between enzyme species. The spatial and temporal expression of ecto-nucleotidases especially; NTPDases and NPPs by various cell types within the vasculature, the nervous tissues and other tissues resulted in several patho-physiological processes. Examples include macrophage activation, contraction of smooth muscle cells, platelet aggregation, differentiation, migration, neurodegeneration, inflammation, apoptosis, cell cytotoxicity, and cell proliferation mechanisms. We are focused on the development of specific inhibitors of NTPDases and NPPs for the treatment of various cancers, neurological disorders and diabetic complications. For this purpose we extend our efforts in investigating the new scaffolds of pyrimidones and pyrazoles analogs at molecular level. We used different approaches for the deep mechanistic studies for example for the preliminary screening; effect on the cell growth was observed by MTT assay, flow cytometry was used for cell cycle analysis, cell morphology was evaluated by nuclear staining and finally, the possible binding interactions of the potent derivatives were determined with mammalian DNA. Binding modes of the identified inhibitors were also explained by molecular docking studies. It can be suggested that the analogues of such effective derivatives can be synthesized and further explored at molecular level to cure the targeted diseases.
Keywords: Purinergic signaling; ecto-nucleoside triphosphate diphosphohydrolases (NTPDases); ecto-nucleotide p.

4. NTPDase8 regulates integrity and immune function of intestinal epithelial cells

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Extracellular nucleotides are released in large amount during inflammation by various cell types. For example, mounting evidence suggests that when injured, Intestinal epithelial cells (IEC) release nucleotides that activate P2 receptors which affect immune responses and expression of proinflammatory chemokines. P2 receptor agonists are hydrolyzed by ectonucleotidases. Our recent data show that NTPDase8 is the major ectonucleotidase at the apical surface of IEC. We hypothesized that NTPDase8 could regulate the level of nucleotides at the surface of IEC and control their integrity and immune function. Primary IEC were prepared from the colon of WT and Entpd8-/- mice then expression of P2 receptors and ectonucleotidases was evaluated by qPCR. IEC were primed with TNF for 6 hours then stimulated with the TLR4 agonist LPS for 5 or 18 hours for qRT-PCR or ELISA experiments, respectively. The expression of tight junctions were evaluated by qRT-PCR. KC secretion was evaluated by ELISA in IEC supernatants. The integrity and permeability of the IEC monolayer was evaluated by transepithelial electrical resistance (TEER) and the movement of FITC-dextran in Boyden chambers. Neutrophil transmigration and chemotaxis were carried out in a Boyden chambers. While P2Y6 was the dominant P2 receptor expressed in both, WT and Entpd8-/- IEC, NTPDase3, CD73 and P2Y1, P2Y2, P2X4, and A2A expressions were comparable in both IEC. KC secretion was more pronounced in supernatant of Entpd8-/- IEC stimulated with TNF and LPS than that of WT IEC which decreased significantly in presence of P2Y6 antagonist. Accordingly, the supernatant of IEC stimulated with TNF and LPS induced neutrophils chemotaxis and transmigration through IEC monolayer which correlated with the amount of KC secreted in presence or absence of P2Y6 antagonist. Treatment with TNF and LPS also increased mucosal permeability as indicated by enhanced dextran flux, by decreased TEER and by a decreased expression of tight junctions in Entpd8-/- IEC when compared to WT IEC. IEC integrity was restored in presence of P2Y6 antagonist. Our data presented in this study support the view that NTPDase8, by regulating the activation of P2Y6 at IEC surface, contributes to the control of IEC barrier functions and leukocyte recruitment to the intestinal epithelium via KC secretion. This work was supported by grants to J. Sévigny from the Canadian Institutes of Health Research and from the Fondation du CRCHU de Québec – Université Laval.
Keywords: IEC; NTPDase8; P2Y6 receptor; integrity.

5. Oral Communication: Deletion of CD73 leads to the shifts of NAD metabolism

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Objectives/background: Nicotinamide adenine dinucleotide (NAD) is an essential redox carrier, whereas its degradation is a key element of a wide range of signaling pathways. Nicotinamide (NA)- known for its strong anti-inflammatory properties - can be a direct product of many enzymatic reactions, primarily related to NAD catabolism. CD73 – an enzyme of extracellular nucleotide catabolism - degrades NAD to nicotinamide mononucleotide (NMN) and AMP and further to (nicotinamide riboside) NR and adenosine. The aim of the study was to investigate the impact of the CD73 activity absence on the NAD metabolism. Methods and results: 6-month old, male C57BL/6J Wild Type (WT; n=10) and C57BL/6J CD73−/− (CD73−/−; n=10) mice were used for these experiments. Blood and serum were collected and used for the nucleotides and nicotinamide metabolites concentrations as well as enzymes involved in NAD metabolism level. Results are presented as mean ± SEM, unless otherwise indicated. CD73 knock out led to increase in blood NAD+ concentration compared to WT (70.11 ± 3.41 vs. 55.43 ± 5.33 μmol/l; $p<0.05$). Concentration of NA was significantly decreased in CD73-/- mice serum in comparison to WT (0.68 ± 0.04 vs. 0.86 ± 0.06 μmol/l; $p<0.05$). On the other hand, NA metabolites concentrations: N-methylnicotinamide (MetNA), as well as N-Methyl-2-pyridone-5-carboxamide (Met2PY) and N(1)-methyl-4-pyridone-3-carboxamide (Met4PY) were considerably elevated in CD73-/- mice serum as compared to WT. Despite the lack of ecto-5'-nucleotidase activity in CD73-/- mice, concentration of NR was significantly enhanced in CD73-/- serum as compared to WT (0.21 ± 0.02 vs. 0.11 ± 0.02 μmol/l; $p<0.05$). CD73 knock out led to increased poly(ADP-ribose)polymerase 1 (PARP-1), nicotinamide phosphoribosyltransferase (NAMPT) and nicotinamide N-methyltransferase (NMMT) serum levels in comparison to WT. Conclusions: Deletion of CD73 causes substantial changes in the NAD+ and nicotinamide metabolism, which may be involved in the pro-inflammatory phenotype of CD73-/- mice. The increase of NAD+ concentration may be a compensation mechanism aimed at rebuilding the NAD+ pool, after its enhanced degradation. Acknowledgments: This study was supported by National Science Centre of Poland (2015/19/N/NZ1/03435) and The National Centre for Research and Development (STRATEGMED 1/233226/11/NCBR/2015).
Keywords: ecto-5'-nucleotidase; extracellular nucleotides; NAD; nicotinamide metabolism.

SYMPOSIUM 33 - Central Nervous System Regulation by Purinergic Receptors

1. Purinergic regulation of breathing

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The mammalian brain depends on a constant supply of oxygen to meet its energy needs. Failure of this supply for even a few minutes can result in permanent brain damage or death. A host of adaptive responses have evolved to protect brain oxygen. Prominent among these is the biphasic hypoxic ventilatory response in which a fall in oxygen levels (hypoxia) in the blood supplying the brain stimulates peripheral (carotid body) chemoreceptors, triggering an initial, Phase I increase in breathing. If this increase does not restore oxygen levels, breathing is inhibited and falls to a lower steady state Phase II level in what is called the secondary hypoxic respiratory depression. This secondary depression is most severe and life threatening in premature infants with apnea of prematurity (AOP), but the depression and the chronic intermittent hypoxia that can result are also implicated in Sudden Unexplained Death in Epilepsy (SUDEP), sleep disordered breathing including Obstructive Sleep Apnea, congenital chronic hypoventilation syndrome and several neurodegenerative disorders. Dogma holds that the hypoxic ventilatory response results from two main processes, a peripheral chemoreceptor-mediated Phase I excitation followed by a centrally mediated depression to Phase II; i.e., the only contribution of the central nervous system to the hypoxic ventilatory response is inhibition and the secondary hypoxic respiratory depression. Our research, however, challenges this view with evidence that the preBötzinger Complex (preBötC), a brainstem region critical for inspiratory rhythm generation, mounts an excitatory response to hypoxia that attenuates the hypoxic respiratory depression. Specifically, astrocytes in the preBötC appear to sense hypoxia and release ATP (via calcium-dependent, vesicular processes), which in turn acts via P2Y1 receptors to excite inspiratory neurons and increase inspiratory frequency (Angelova et al. J. Neurosci. 35; 10460–73, 2015; Rajani et al. J. Physiol. online, 2017). Discussion will focus on these purinergic and glial signaling mechanisms as well as recent insights into the second messenger cascades and ion channels through which P2Y1 receptor activation excites preBötC neurons and increases ventilation in response to hypoxia. Supported by Canadian Institutes of Health Research, Natural Science and Engineering Research Council, Canadian Foundation for Innovation and the Women and Children's Health Research Institute (University of Alberta).

2. Purinergic regulation of vascular tone in the retrotrapezoid nucleus is specialized to support the drive to breathe

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Cerebral blood flow is highly sensitive to changes in CO2/H+ where an increase in CO2/H+ causes vasodilation and increased blood flow. Tissue CO2/H+ also functions as the main stimulus for breathing by activating chemosensitive neurons that control respiratory output. Considering that CO2/H+-induced vasodilation would accelerate removal of CO2/H+ and potentially counteract the drive to breathe, we hypothesize that chemosensitive brain regions have adapted a means of preventing vascular CO2/H+-reactivity. Here, we show in male Wistar rats that purinergic signaling, possibly through P2Y2/4 receptors, in the retrotrapezoid nucleus (RTN) maintains arteriole tone during high CO2/H+ and disruption of this mechanism decreases the CO2 ventilatory response. Our discovery that CO2/H+-dependent regulation of vascular tone in the RTN is the opposite to the rest of the cerebral vascular tree is novel and fundamentally important for understanding how regulation of vascular tone is tailored to support neural function and behavior, in this case the drive to breathe. Finnancial support: FAPESP (grant 2015/2376-1 and 2016/22069-0) NIH (HL-104101).
Keywords: purinoreceptors; chemoreceptors; cental nervous system; breathing.

3. Purinergic Signaling at Hypothalamus Level: Mechanistic Insights Involved in the Control of Salt-Induced Sympathoexcitation

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Despite large fluctuations in salt and water intake, mammals are able to maintain electrolyte concentrations, and the osmolality of the extracellular fluid, within narrow physiological limits. Two distinct cerebral (osmo)sensory systems, named as as circumventricular organs (CVOs) are constantly monitoring osmolality: the subfornical organ and the organum vasculosum of the lamina terminalis. These CVOs send excitatory projections to the paraventricular nucleus of the hypothalamus (PVN), which is considered to be an important integrative centre for autonomic that modulate sympathetic nerve activity (SNA), and the blood pressure (BP) homeostasis. The parvocellular neurons in the PVN project to premotor sympathoexcitatory neurones located in the rostral ventrolateral medulla (RVLM) in the brainstem, and play a significant role in cardiovascular regulation, particularly in response to homeostatic disturbance including plasma salt loading. Numerous neurotransmitters act within the PVN to regulate SNA, among them the Adenosine-5'-triphosphate (ATP). Immunohistochemical studies have identified the presence of P2X receptors in the PVN neurons that project to the RVLM, suggesting that ATP could act as a neurotransmitter within this hypothalamic nucleus. In this sense, we have shown that purinoceptor activation within the PVN increases SNA in dose-dependent manner, and also that ATP and glutamate can act as co-transmitters to modulate sympathoexcitatory responses via activation of P2 and non-NMDA receptors. Additionally, we were able to evaluate the precise cellular mechanisms, at the synaptic level, underlying the ATP-glutamate interaction in the PVN, and assessed whether this receptor coupling contributed to osmotically-driven sympathetic PVN neuronal activity. We have found that exogenously applied ATP increased firing activity of PVN sympathetic neurons, an effect that was dependent on P2 and glutamatergic receptors. Furthermore, we reported that either the application of ATP or acute hyperosmotic stimulus in PVN slices potentiated AMPA-receptor evoked current in a P2-receptor dependent manner, once the effect was blocked by PPADS (P2 receptor antagonist). Finally, to understand the physiological role to ATP at the PVN in salt-induced sympathoexcitation, we have determined whether alterations in plasma osmolality in awaking animal model would activate P2X2 receptor-expressing PVN neurons. In fact, the increase in plasma osmolality activated PVN neurons (FOS protein expression) that co-expressed the P2X2 receptor subunit. Moreover, the increase in SNA induced by hyperosmotic stimulus involved P2 receptors activation at the PVN level. Collectively, our findings support the idea that the purinergic signalling is involved in the control of salt-Induced sympathoexcitation in the PVN neurons, via activation of P2X receptors. As the perspectives we are seeking to determine the source(s) of ATP that drives osmotically-induced increases in sympathetic outflow.
Keywords: ATP; hypothalamus; hyperosmolality; autonomic nervous system.

4. Astrocyte P2X1 receptors in cortical neurovascular coupling

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The increased energy demand of active neurons is met by a corresponding increase in local blood supply, a phenomenon termed neurovascular coupling. There is considerable debate in the field over which cells mediate neurovascular coupling, and the relative importance of different vascular compartments in this response. We have recently demonstrated that signaling to capillary pericytes and arteriolar smooth muscle cells in the cortex occur via two separate pathways. In cortical slices, neuronal activity evokes both capillary and arteriole dilation. When astrocyte calcium is buffered to arrest calcium increases in astrocyte endfeet, the neuronally-evoked capillary dilation is reduced by 64% while arteriole dilation was unaffected. Capillary dilation depends on calcium entry into astrocytes through the ATP-gated ion channel P2X1 and subsequent synthesis and release of vasoactive prostaglandin E2. In contrast, arteriole dilation does not require astrocyte calcium, but rather depends on NMDA receptor driven generation of nitric oxide, most likely from interneurons. We further demonstrate that both resting vascular diameter and neurovascular coupling of capillaries are reduced following ischemic stroke, which may underlie the long-lasting reduction in blood flow that occurs following stroke. These findings demonstrate a dichotomy in the signaling cascades that regulate cerebral blood flow in different vascular compartments, establish purinergic neuron-astrocyte signaling as an important mediator of capillary neurovascular signaling, and suggest restoration of astrocyte-pericyte signaling as a novel therapeutic approach in stroke treatment.
Keywords: neurovascular coupling; purinergic signaling; astrocytes; ischemic stroke.

5. Oral Communication: Re-evaluation of P2X7 expression in the central and peripheral nervous system using novel mouse models

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The P2X7 receptor channel is activated by high concentrations of ATP as released upon tissue damage. It plays a central role in cytokine release and studies in P2X7 knockout animals have shown its involvement in inflammatory processes and neuronal damage. However, despite its importance as a drug target, its precise cellular localization as well as molecular and physiological functions remain unclear which has been attributed to its complex pharmacology and non-specific antibodies. To resolve this issue, we generated BAC transgenic mouse models in which the murine EGFP-tagged P2X7 is overexpressed under the control of the endogenous P2X7 promoter. The EGFP-tagged P2X7 receptors are efficiently transported to the plasma membrane and functional. Several mouse lines with different expression levels but identical expression patterns were obtained and immunohistochemical analysis show predominant expression in the cerebellum, cortex, hippocampus, and thalamus. Careful immunofluorescence analysis in the CA1 region revealed a dominant P2X7-EGFP protein expression in almost all microglia (57±14% of all GFP-positive cells are Iba1-positive) and oligodendrocytes (47±10% of all P2X7-positive cells are Olig2-positive) and minor expression in S100β-positive astrocytes. These findings were supported by Western blot analysis of brain extracts from microglia and oligodendrocyte-specific P2X7 knockout mice in which protein amounts were reduced by 58 ±4% and 66±3%, respectively. In the peripheral nervous system, P2X7-EGFP was identified in Schwann cells, where it localized to nodes of Ranvier and Schmidt-Lantermann incisures, in perfect agreement with the subcellular distribution pattern of endogenous P2X7. At the neuromuscular junction, however, P2X7-EGFP did neither co-localize with terminal Schwann cells nor with the post- or presynaptyc membrane. Based on the localization and morphology, we suggest its presence on kranocytes, a fibroblast-like cell type at the neuromuscular junction. In the enteric nervous system, P2X7 co-localized with S100β-positive but not with GFAP-positive glia. Taken together, we provide the first quantitative analysis of P2X7 protein expression in the nervous system and will show preliminary data on consequences of its overexpression. This novel mouse model represents an important tool to re-evaluate physiological and pathophysiological roles of the P2X7R.
Supported by the DFG (NI592/7-1)
Keywords: Purinergic P2X7; BAC transgene; conditional knockout.

SYMPOSIUM 34 - Oral communications

1. Dichlorvos exposure at early stages of development alters ecto-5'-nucleotidase and ecto-ADA activities in adult zebrafish (Danio rerio) brain

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Background/objective: Organophosphates are a family of agrochemicals chemically derived from phosphoric acid. Dichlorvos is an insecticide of this family that acts by inhibiting the acetylcholinesterase (AChE) activity, an enzyme that degrades the neurotransmitter acetylcholine (ACh) in cholinergic synapses. ACh is a neuromotransmitter released along with ATP in the synaptic cleft. ATP is the signaling molecule of purinergic system, acting on P2X and P2Y specific receptors. It is inactivated by an enzyme cascade thatconsists of cell surface-located enzymes named Nucleoside triphosphate diphosphohydrolases (NTPDases) and ecto-5'-nucleotidases, which hydrolyzes ATP to adenosine. Adenosine exerts its effects through the activation of specific P1-type purinergic membrane receptors. In the synaptic cleft, adenosine undergoes deamination through adenosine deaminase (ADA), producing inosine. The aim of this study was to evaluate the exposure to dichlorvos at the early stages of development (1 hpf – 7 dpf) on the ectonucleotidase and ADA activities in zebrafish brain at 120 dpf. Methods and Results: Embryos were placed in Petri dishes (30 embryos per dish), and subjected to dichlorvos (PESTANAL®, Sigma-Aldrich, St. Louis, MO) exposure at concentrations of 0 (water, control group), 1, 5 and 10 mg/L for seven days (1 hours post fertilization (hpf) to 7 dpf) (CEUA: 13/00354). After, the animals were placed in 3 L-aquariums with water until 120 dpf when the enzyme and molecular assays were performed. Brain membranes were prepared and NTPDase, ecto-5′-nucleotidase, and ADA activities were determined (J Neurochem 61:1685, 1993; Life Sci 73:2071, 2003; Comp Biochem Physiol B Biochem Mol Biol 139:203, 2004), as well as ecto-5'-nucleotidase and ADA mRNA levels. The results showed that dichlorvos exposure, in the first week of life, was not able to alter the NTPDases activities in brain membranes of adult zebrafish. However, this pesticide promoted an increase in ecto-5'-

nucleotidase activity at all concentrations tested. Moreover, this pesticide decreased the ecto-ADA activity at 5 and 10 mg/L, but did not alter the cytosolic ADA activity. in brain membranes of adult zebrafish submitted to dichlorvos exposure at early stages of development. The RT-qPCR analysis showed no significant changes in gene expression of ecto-5'-nucleotidase and ADA genes. Conclusion: The findings demonstrated the role of nucleotide and nucleoside-metabolizing enzymes on the toxicological effects of dichlorvos and may contribute to a better understanding about the role of purinergic signaling on the actions induced by dichlorvos exposure in the early stages of development.
Keywords: Adenosine deaminase; Dichlorvos; NTPDases; Zebrafish.

2. The effect of changes in the CD73 activity on the aortic valve and endothelium function in mice

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Objectives/background: Aortic stenosis is known to involve inflammation and thrombosis. Changes in activity of ecto-5'-nucleotidase – an enzyme of extracellular nucleotide catabolism - can alter inflammatory and thrombotic responses. The aim of this study was to investigate the impact of the absence of CD73 activity on the function, structure and metabolism of a murine aortic valve and endothelium. Methods and results: Male C57BL/6J Wild Type (WT; n=21) and C57BL/6J CD73−/− (CD73−/−; n=21) mice were used for these experiments. At the age of 9 weeks, the animals were randomly divided into: normal-fat diet WT, normal-fat diet CD73−/−, high-fat diet WT and high-fat diet CD73−/−. Groups were maintained for 15 weeks followed by echocardiographic analysis of aortic valve function, measurement of aortic surface activities of nucleotide catabolism enzymes as well as alkaline phosphatase activity, plasma L-arginine derivatives as well as nicotinamide metabolites concentration, mineral composition and histology of aortic valve leaflets. Results are presented as mean ± SEM. CD73 knock out led to increase in peak aortic flow (1.06±0.26m/s) compared to WT (1.06±0.26m/s vs. 0.79±0.26m/s; $p<0.01$) indicating obstruction. Highest values of peak aortic flow (1.26±0.31 m/s) were observed in high-fat diet CD73-/- mice. Histological analysis showed morphological changes in CD73-/- including thickening and accumulation of dark deposits, proved to be melanin. Concentrations of Ca^{2+}, Mg^{2+} and PO43- in valve leaflets were elevated in CD73-/- mice. Alkaline phosphatase (ALP) activity was enhanced after ATP treatment and reduced after adenosine treatment in aortas incubated in osteogenic medium. AMP hydrolysis in CD73-/- was below 10% of WT. Activity of ecto-adenosine deaminase(eADA), responsible for adenosine deamination, in the CD73-/- was 40% lower when compared to WT. CD73 knock out led to significantly decreased plasma L-Arginine/ N(G), N(G)-dimethyl-L-arginine (ADMA) ratio and decreased N(G),N'(G)-dimethyl-L-arginine (SDMA) level. Conclusions: Deletion of CD73 in mice leads to aortic valve and endothelium dysfuction similar to that induced by high-fat diet. Alterations in CD73 function may contribute to human valve and vascular pathology. Acknowledgments: This study was supported by National Science Centre of Poland (2015/19/N/NZ1/03435).
Keywords: Adenosine; CD73 knock-out mice; Ecto-5′-nucleotidase; Inflammation.

3. Early human sepsis modulates purinergic receptors expression, serum ATP levels and ATPase activity

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Objectives/Background: Sepsis has been recently described as the product of a dysregulated immune response to infection. Although sepsis displays altered innate and acquired immune responses, dysregulation of innate immunity leading to overwhelming inflammation is a more prominent feature in early sepsis. Neutrophil disfunction hallmarks the state of dysregulated inflammation in early sepsis, causing insufficient pathogen clearance and collateral tissue damage, leading to persistent inflammation, organic failure and death. Robust pre-clinical evidences show that purinergic signaling controls innate immunity features in sepsis, including several neutrophil features such as chemotaxis, transendothelial migration, phagocytosis and interleukins release, key processes in initial response to infection. Nevertheless, evidences to support purinergic signaling role in clinical sepsis are scarce. To determine the activity of purinergic signaling in early human sepsis we examined purinergic receptors expression in neutrophils, serum concentration of the purinergic agonist adenosine triphosphate (ATP) and serum activity of enzymes related to purinergic signaling in septic patients and controls. Methods: We enrolled septic patients at the first morning following an intensive care unit admission due to sepsis-led organic failure. The first control group was composed by ward non-septic patients with milder inflammatory conditions such as cancer, diabetes or chronic arterial hypertension but no sepsis or acute (<48 hours) clinical deterioration due to any cause. The second control group was comprised by healthy volunteers with no acute or chronic illness. Neutrophil purinergic receptors expression at mRNA level, serum ATP level and enzymes activity were measured. Results: Approval from human research ethics committee and written informed consent from patients was obtained before enrolling and sampling. Serum ATP was measured in septic (n=18), ward patients (n=18) and healthy controls (n=19). Serum nucleotidase activity was also determined in septic (n=18), ward patients (n=21) and healthy controls (n=20). Receptor expression was measured in septic (n=10), ward patients (n=7) and healthy controls (n=5). As compared to controls, septic patients neutrophils had significantly enhanced expression for P2Y2 ($p < 0.05$ for ward patients and $p < 0.001$ for healthy volunteers) and A2a ($p < 0.01$ for healthy volunteers), but not for P2X7 and P2Y6 receptors. Septic patients also had serum ATP levels ($p < 0.05$ for ward non-septic patients, $p < 0.001$ for healthy volunteers) and ATPase activity ($p < 0.0001$ for both control groups) significantly elevated. Conclusions: Early human sepsis displays a clear phenotype of purinergic signaling activation, suggesting that innate immunity can be regulated through careful modulation of purinergic signaling. ATPergic tonus reduction by removal of excess ATP is a plausible therapy for sepsis. Acknowledgements: CNPq 310846/2014-5, CAPES, FAPERGS
Keywords: SEPSIS; ATP; P2Y2; A2a.

4. The significance of nucleoside diphosphate kinase (NDPK)-dependent transphosphorylation for ADP/ATP carrier (AAC)-mediated mitochondrial proton leak of yeast *Saccharomyces cerevisiae*

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Background: The AAC of S. cerevisiae is considered a main catalyst of the futile (non-phosphorylating) proton leak across the inner mitochondrial membrane (Brand et al. Biochem J 392; 353, 2005). The AAC-mediated proton leak is inhibited by both, carboxyatractyloside (CATR), a classic strong inhibitor of the carrier for nucleotide translocation, and GDP (Echtay et al. EMBO J 22; 4103, 2003). However, we have discovered that the GDP inhibitory effect in mammalian mitochondria is completely suppressed because of the NDPK action (Woyda-Ploszczyca and Jarmuszkiewicz PLoS One 9:e98969, 2014). This enzyme catalyzes the transfer of a γ-phosphate group from NTP to NDP, e.g., ATP + GDP → ADP + GTP. Objectives: The importance of NDPK for AAC-mediated proton leak in S. cerevisiae strains, which are naturally absent of uncoupling protein (UCP), the another major catalyst of the futile proton leak. Methods: Mitochondria were isolated from a wild type yeast strain and its mutant with disrupted gene for NDPK (BY4741 strain, Euroscarf, Germany). Oxygen uptake and mitochondrial membrane electrical potential ($\Delta\Psi$) were measured using a Clark-type oxygen electrode and a tetraphenylphosphonium cation (TPP+)-specific electrode, respectively. Results: In isolated mitochondria of wild type yeast strain, an addition of GDP (1 mM) stimulated the state 4 (non-phosphorylating respiration)-state 3 (phosphorylating respiration) transition revealed as a 32% (± 2 S.E.) increase in respiratory rate (nmol O/min/mg protein) accompanied by a 3% (± 0.3 S.E.) decrease in $\Delta\Psi$ (mV) (n = 5, independent mitochondrial isolations). Because 1 mM ADP led to a similar effect, it means that both, GDP and ADP, are involved in oxidative phosphorylation induction. This conclusion is supported by the fact that CATR totally blunted the ADP and GDP actions. In mutant strain, in the absence of CATR, the ADP action was unimpaired, however the GDP effect was quenched. Although we carried out measurements in the presence of ATP and GDP, the NDPK deficiency resulted in no ADP pool generation responsible for the GDP stimulatory effect. Conclusions: In our opinion, NDPK wins the competition with AAC for GDP. Therefore, GDP cannot be considered a native significant inhibitor of AAC-sustained proton leak. However, to better understand energy transduction in the cell, there is a need to recognize signaling related to physiological inhibition of this energy dissipation pathway. This work was supported by a grant from the National Science Centre, Poland (2015/19/D/NZ3/00087), and partially by the KNOW Poznan RNA Centre (01/KNOW2/2014).
Keywords: nucleoside diphosphate kinase; ADP/ATP carrier; mitochondria; proton leak.

5. P2X7B isoform role in chemoresistance and epithelial-to-mesenchymal transition of human neuroblastoma cells

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Cancer cell resistance to death stimuli is a major challenge in cancer therapy. Even though many alternative therapies have been developed to quench tumor cells, quiescent subpopulations of cancer stem cells lead to chemotherapy endurance, survival and tumor re-establishment. Epithelial-mesenchymal transition (EMT) has been pointed out as a mechanism that mediates those phenomena by switching the phenotype of cells to metastatic and resistant. P2X7 receptor, an ionotropic channel responsive to extracellular ATP, is implicated in many physiological and pathological roles, such as proliferation and stimulation of apoptosis. Paradoxal evidences implicate P2X7 in both pro- and anti-tumoral responses, probably due to expression of alternative splicing isoforms of p2rx7 gene, resulting in a full-length channel, P2X7A, and a truncated version lacking the C-terminal tail that is not capable of pore formation, called P2X7B. METHODS AND RESULTS: Characterization of EMT was performed by relative quantification expression of the EMT marker (vimentin) using RT-qPCR. Data revealed that vimentin expression was downregulated in P2X7A-/B+ cells by increasing concentrations of TGF-β (5-25ng/ml) and EGF (50-100ng/ml), in contrast to the results found for control cells (P2X7A+/B+). Furthermore, aiming to study role of P2X7 isoforms in chemoresistance of human neuroblastoma cells, we performed propidium iodide staining of cells collected from dose-response curve assay. Interestingly, two resistant populations were found, however, just one of them was enriched when vincristine concentration was raised, from 1nM to 1μM. Serum starvation and low glucose supply increased cell death rates independently of vincristine treatment. In order to assess roles of each P2X7R isoforms, we genetically silenced both isoforms (P2X7A-/B-) or isoform A only (P2X7A-/B+), using interference RNAs. P2X7A knockdown increased survival rate during starvation, also ATP stimulation at 1mM lead to an increased survival of cells expressing only isoform B, pointing P2X7B as an important target in chemoresistance. CONCLUSION: Our results has indicated different roles of each P2X7 isoform in cancer, highlighting P2X7B as a main character in EMT and chemoresistance. Financial Support: FAPESP, CNPq
Keywords: neuroblastoma; chemoresistance; emt; p2x7.

6. Cardiac mitochondria function and extracellular vascular nucleotide metabolism in genetic model of hyperlipidemia
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Background: Hyperlipidemia leads to impairment of the mitochondrial function by excessive production of reactive oxygen species and respiratory chain disorders. Moreover, increased level of plasma lipids leads to the development of pathological changes in the blood vessels leading to atherosclerosis and changes in purinergic signaling. The aim of this study was to investigate the effect of experimental hyperlipidemia on the respiratory chain and vascular extracellular nucleotide catabolism pathway. Methods: The analysis of function isolated cardiac mitochondria was performed in 3- and 6-month LDL Receptor knock-out mice (LDLR-/-, n=5 in each age group), following the approval of the local ethics committee. C57Bl/6J wild types (WT, n=5 in each age group) were used as controls. Experimental system examined the transport of electrons in the respiratory chain by adding NAD+-dependent substrates (malate, pyruvate) and FCCP (mitochondrial uncoupler) to mitochondria, followed by rotenone, succinate, antimycin and ascorbate enriched with TMPD (donor of electrons). The analysis was performed using the Seahorse XFp metabolic flux analyzer, by recording the oxygen consumption rate (OCR). The measurement of the rates of ATP and AMP hydrolysis as well as adenosine deamination were evaluated in four segments of aortas

(aortic arch, aortic root, thoracic aorta and abdominal aorta) by analysis conversion of substrate into products using reverse phase high performance liquid chromatography (RP-HPLC). Results: Isolated mitochondria from 6-month LDLR(-/-) mice exhibited lower OCR (179.0±19.04 pmoles/min) than age-matched WT (281.1±22.83 pmoles/min) (mean ± SEM). In turn, no differences were found between mitochondria isolated from 3-month mice. These results negatively correlated with increased activity of vascular ecto-adenosine deaminase (eADA) in 6-month LDLR(-/-) mice in all parts of aorta and for aortic arch eADA was two times higher in LDLR(-/-) vs. WT: 0.19 ± 0.03 vs. 0.08 ± 0.01 nmol/min/mg tissue (mean ± SEM). 3-month mice did not show differences in eADA activity. ATP and AMP hydrolysis were at the same level in both age groups of LDLR(-/-) and WT mice. Conclusions: Lipid abnormalities affected electron flow in the respiratory chain of 6-month-old LDLR (-/-) mice hearts. That correlated with increased activity of vascular eADA that was previously proposed as a maker of endothelial inflammation. Details of the link between adenosine metabolism and mitochondrial function require further studies. Supported by: National Science Centre of Poland NCN/2016/22/M/NZ4/00678
Keywords: mitochondria;hyperlipidemia;adenosine;ATP.

SYMPOSIUM 35 - Purinergic Signalling in Cancer III

1.Effect of P2X7 modulation on the survival and aggressiveness of cancer stem cells from human glioblastoma multiforme

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Obiectives/Background: Glioblastoma multiforme (GBM) is the most common and lethal brain tumor in adult humans. The presence of stem-like cells in GBM (GSCs) with high self-renewal, resistance to radio/chemotherapy and invasiveness/migration potential seems to underlie the unfavorable GBM prognosis. In turn, an important process in tumor genesis, metastasis, and recurrence called epithelial-to-mesenchymal transition (EMT) contributes to GSC aggressiveness. Although still debated in the neuroepithelial context, EMT induces biochemical changes and a mesenchymal phenotype in GSCs, enhancing their migration, invasiveness and resistance to apoptosis. We previously demonstrated that ATP and some related derivatives, mainly acting on the ionotropic P2X7 receptor (P2X7R), restrain GSC growth, also potentiating the cytotoxic activity of temozolomide (TMZ), a drug currently used in GBM therapy (D’Alimonte et al., Purinergic Signal. 11; 331, 2015). Here, using GSCs derived from human surgical GBM specimens, we better investigated anticancer effects of P2X7R agonists/antagonists, such as their activity on EMT-associated genes and GSC migration/invasion. Methods and Results: GSCs were exposed to Transforming Growth Factor β (TGFβ), a known inducer of EMT process, or 2'(3')-O-(4-benzoylbenzoyl)-ATP (Bz-ATP, 50-200μM), a rather selective P2X7R agonist. By qRT-PCR, we observed that 5-10 ng/ml TGFβ significantly increased mRNAs of selected EMT markers (ZEB1, Snail, Slug) in the 24-72 h following its administration to GSCs, whereas BzATP effect (50-200 μM) was shorter, being significant at 12 and 24 h after cell exposure to the drug. As well, western blot (WB) analysis showed that the expression of vimentin and N-cadherin, two other EMT markers, was increased by TGFβ and Bz-ATP in the 24-72 h following their administration. Bz-ATP induced effects were counteracted by the P2X7R antagonist A438079. Moreover, both TGFβ and Bz-ATP, at the used concentrations, enhanced GSC migration without affecting cell viability, as evaluated by scratch and MTS assays, respectively. Finally, we investigated the expression of two P2X7R splicing variants (the full length P2X7RA and the truncated P2X7RB, lacking the carboxylic tail) that may differently affect tumor cell survival. Pivotal results from GSCs of two patients showed a similar mRNA expression of the two P2X7R isoforms, whereas by WB analysis preceded by immune-precipitation we found that the P2X7RB immune-bands were more intense that those for P2X7RA isoform, especially in GSCs which we previously showed to be more resistant to the cytotoxic TMZ action. Conclusions: In human GSCs: i) the activation of P2X7R stimulates the EMT process and cell migration; ii) there may be a prevailing expression of the P2X7RB, that is considered a pro-tumor receptor. Since GBM microenvironment includes elevated levels of ATP required for P2X7R activation, our data support a role of P2X7R in GBM growth and invasiveness.
Keywords: Glioblastoma multiforme; epithelial-to-mesenchymal transition; tumor invasiveness; P2X7 receptors.

2. Purinergic signaling in pancreatic cancer

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Objectives: Pancreatic ductal adenocarcinoma (PDAC) is a fatal disease, which has an overall 5-year survival rate of less than 5% and new therapeutic targets are urgently needed. PDAC is probably of ductal origin, and the tumor is hypovascular and highly fibrotic. The complex tumor microenvironment includes fibrogenic pancreatic stellate cells (PSCs), which are believed to stimulate cancer and metastatic development. Aim of our recent studies is to elucidate how purinergic signaling, which is essential for regulation of pancreatic exocrine secretion in healthy pancreas (1), becomes deregulated and contributes to PDAC development. We focused on the multifunctional P2X7 receptor and its role in PSC and cancer cells.Methods and Results: We found that both human and murine PSCs express the P2X7 receptor, which stimulates cell proliferation and collagen secretion. Furthermore, the P2X7 receptor also regulates secretion of IL-6, which may be an important factor in promoting PDAC cell migration and stimulating cancer development. All processes were inhibited by the inhibitor AZ10606120. The PSCs migration was, however, regulated by the P2Y2 receptor. Studies on human PDAC cell lines show that these express high levels of the P2X7 receptor compared to control duct cells. The receptor regulates cancer cell survival and cell migration/invasion and is sensitive to the inhibitor AZ10606120 (2). Following in vitro studies, we transplanted human PDAC into nude mice, which were then treated with saline or AZ10606120. The inhibitor markedly decreased tumor growth, but it did not eliminate the tumor fully and it did not appear to prevented metastases. However, the inhibitor markedly reduced PSC number and tumor fibrosis (3.) In conclusion: Our studies show that therapeutic targeting of the P2X7 of pancreatic cancer is promising, but other in vivo PDAC models should be explored. More knowledge is needed regarding other factors/cells in the tumor microenvironment, as well as consideration whether fibrosis may prevent cancer from spreading. 1. Novak. Acta Physiol (Oxf) 202; 501, 2011. 2. Giannuzzo et al. Mol. Cancer 14, 203, 2015. 3. Giannuzzo et al. J Cancer 139, 2540, 2016. Acknowledgements:

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Keywords: P2X7; cancer; fibrosis; pancreas.

3. Extracellular nucleotides drive the epithelial or mesenchymal phenotype induction in ovarian carcinoma cells

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Nucleotides and nucleosides are signaling molecules that have a variety of roles mediating paracrine or autocrine activities by acting through specific membrane receptors. Their participation in cancer has been studied but it is still not clear. In this work, we studied the role played by UTP (a nucleotide) and adenosine (a nucleoside) in the migratory capacity of the ovarian carcinoma-derived cells SKOV-3. Stimulation of SKOV-3 cells with UTP (100 μM) increased migration (≈57%), while apyrase (10 U/mL), an ecto-nucleotidase that catalyzes dephosphorylation of purine and pyrimidine nucleotides, decreased basal migration (≈47%).
P2RY2 was found to be the receptor mediating the UTP-induced cell migration since the knock down of this receptor blocked the enhancing effect; this effect was also associated with epithelial to mesenchymal transition (EMT), since it was joined with an increase of snail and twist expression, known EMT inductors, as well as an increase of vimentin expression, a marker for mesenchymal phenotype. By using pharmacological strategies, we determined that the UTP-related effect was dependent on EGFR transactivation. To know with more detail the molecular pathways involved in UTP effects, we performed cDNA microarrays. From this analysis, a set of modified genes related with the Wnt pathway were detected, revealing a crosstalk between purinergic signaling and the Wnt pathway. In turn, the inhibitory effect of apyrase over SKOV-3 cells migration was associated with an enrichment of E-cadherin in the cell contacts, suggesting the establishment of an epithelial phenotype. This observation strongly suggests the possible role of adenosine inhibiting the invasive ability in these cells. To analyze the putative effect of adenosine over cell migration, we studied the effect of adenosine as well as several compounds that modify the adenosine´s activity. By blocking NT5E enzyme with adenosine 5'-(α,β-methylene) diphosphate (APCP) or by degrading adenosine with adenosine deaminase (ADA), migration was unaltered even in the presence of apyrase. However, incubation with dipyridamole (DPR), an inhibitor of the adenosine transporters, induced a reduction of basal migration (≈36%), that was even more accentuated with adenosine (100 μM) (≈64%), strongly suggesting that extracellular adenosine could be acting upon ADORA receptors. In cDNA microarrays of SKOV-3 cells incubated with apyrase or adenosine, it was observed a group of genes whose expression level was modified by both treatments, that included elements related with the signaling of the GTPase Ras and with FGF16. Our results indicate that nucleotides and nucleoside signaling are regulatory switches with the capability to control the cellular phenotype of tumoral cells, opening the way to propose new pharmacological targets. Funded by: PAPIIT-UNAM (IN205114 and IN200815) to F.G.V.-C. and to M.D.-M. We are Grateful with MSc Adriana Gonzalez by Technical Assistance.

4. Regulation of intratumoral purine homeostasis and signaling via concerted action of alkaline phosphatases, CD39 and CD73

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Objectives/background: Extracellular ATP, adenosine and other purines mediate diverse immunomodulatory, angiogenic and other effects via binding to nucleotide- and nucleoside-selective receptors. Given the emerging role of nucleotide-converting ectoenzymes as important regulators governing the magnitude and duration of purinergic signaling cascade, our study aimed to characterize the pattern of nucleotide homeostasis in cancer cells and solid tumors and to further elucidate cellular mechanisms implicated in tumor growth and metastasis.
Methods and results: Pretreatment of metastatic prostate and breast cancer cells with different purinergic agonists and enzyme inhibitors modulated subsequent cell invasion and migration through Matrigel matrix. These effects occurred via two different, receptor-dependent and independent mechanisms, which include cellular uptake of nucleosides, their intracellular interconversion into ADP/ATP and triggering of extra- and intracellular signaling pathways. Our findings also provide evidence for the important tumorigenic role of backward nucleotide-phosphorylating ectoenzymes, adenylate kinase and nucleoside diphosphate kinase (NDPK/NM23) in the maintenance of balanced equilibrium between pro-inflammatory ATP and its counteracting anti-inflammatory metabolite adenosine. Furthermore, enzyme histochemical and immunofluorescence staining analyses of key nucleotidases were performed in snap-frozen biopsies from colorectal cancer patients, by comparing malignant versus surrounding apparently healthy colon tissues (n=30). The results obtained revealed a specific distribution of several ectoenzymes (NTPDase1/CD39, ecto-5'-nucleotidase/CD73, alkaline phosphatase and CD38) in certain tumor areas, including irregular adenocarcinoma glands, reactive connective tissues and lamina propria with tumor-infiltrating lymphocytes.
Conclusion: Collectively, the results confirm the important role of ATP and adenosine in tumorigenesis and further expand current concepts by hypothesizing the co-existence of dynamic ectoenzymatic networks coordinately regulating cellular purine homeostasis and signal transduction pathways in solid tumors.
Acknowledgment: This work was supported by grants from the Academy of Finland, the Sigrid Juselius Foundation and the European Community's Seventh Framework Program (FP7/2007-2013; grant agreement number(602200).
Keywords: Purinergic enzymes; Cancer.

5. Oral Communication: P2X7 suppression as a new innovative treatment option for tumors with lost or mutated p53

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Objectives/Background: The P2X7 receptor is a potent stimulator of inflammation and immunity. An increasing body of evidence describes P2X7R as a promoter of cancer cell growth, and indeed, P2X7R overexpression might be a negative prognostic indicator in several malignancies. These observations make P2X7R an appealing target for anti-cancer therapy.Here we propose a key role for the P2X7 receptor as an oncogene driving the growth-promoting activity due to p53 loss. Methods and results: We found that p53 levels are inversely proportional to P2X7 expression in human cancer and that p53-/- cells display high P2X7 activity. Genetic and pharmacological suppression of P2X7R delays cancer development in p53-/- mice, extending their mean lifespan. Moreover, P2X7R blockers restored sensitivity of p53-/- cancer cells to chemotherapy-mediated apoptosis, thus largely inhibiting the in vitro growth. Conclusion: Taken together our data provide proof of principle that in p53-deregulated cancers and in Li–Fraumeni syndromes P2X7R targeting, might represent an innovative and effective therapeutic strategy.
Keywords: P2X7 RECEPTOR; P53; CANCER.

SYMPOSIUM 36 - Adenosine Receptor Signaling in Neurological and Psychiatric Disorders

1. Adenosine A2A receptors and Parkinson's disease: benefits and challenges

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To develop adenosine A2A receptor (A2AR) antagonists as effective non-dopaminergic drugs for the treatment of Parkinson's disease (PD), it is critical time for us to reassess the potential multiple benefits as well as huge challenges in translating A2AR antagonists for PD treatment. First, based on the concentrated striatal expression of A2ARs, the antagonistic A2AR-dopamine D2 receptor interaction, A2AR antagonists have been preclinically and clinically pursued and emerged as a leading non-dopaminergic treatment for motor deficits in PD. Over the last 8 years, >25 clinical trials were conducted. Six double-blinded, placebo controlled, clinical phase IIb-III trials of istradefylline (KW-6002), preladenant (SCH420814) and tozadenant (SYN115) involving >3500 PD patients were reported, showing a modest but significant motor benefit by reducing the average "OFF" time for ~1.2 hour. However, additional phase III trials for PD with istradefylline, SCH42814, tozadenant and caffeine have not provided the required efficacy data or indicate serious side effects to support the clinical utility of A2AR antagonists in PD. Second, since 2000, several large, long-term (>30 years follow-up) prospective studies have firmly established an inverse relationship between the increased caffeine intake and decreased risk of developing PD (up to five times lower) in men. This epidemiological finding is converged with the animal studies identifying A2AR as the major molecular target of caffeine in the brain and compelling evidence from us and others in support of neuroprotective effect of A2AR antagonists and caffeine against in several neurological disease models, including animals of PD, Alzheimer's disease, stroke and traumatic brain injury. Recent demonstration of the protection against alpha-synuclein-induced neurotoxicity by A2AR inactivation and caffeine, possibly by enhancing autophagy activity, further advance prospective of A2AR antagonists as a novel potential disease modifier. Third, the A2AR in the cortico-striatal pathway is uniquely positioned to integrate incoming information (glutamate signals) and neuronal sensitivity to this incoming information (dopaminergic signals) to control striatal synaptic plasticity, and behavior. Accordingly, a growing body of evidence support that A2AR activation inhibits different cognitive behaviors while inactivation of striatal A2AR is sufficient to enhance working memory (WM), goal-directed behavior and Pavlovian conditioning in normal mice. Furthermore, A2AR inacativation reverses cognitive deficits in animal models of PD, Alzheimer's disease, chronic stress and traumatic brain injury. Our recent demonstration of A2AR antagonist-mediated reversal of working memory impairments in MPTP-induced non-human primate model of PD provide the required pre-clinical data to translate A2AR antagonists in clinical use to improve cognitive deficits in PD.
Keywords: Adenosine A2A receptor; A2A receptor antagonists; Parkinson's disease.

2. The role of adenosine A2A receptor on the tauopathy and cognitive disorder after traumatic brain injury

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Traumatic brain injury (TBI) results in an increase in the level of adenosine and activation of its receptors (including adenosine 2A Receptor, A2AR), and blocking of A2AR significantly alleviates cognitive dysfunction following TBI in animals. Our previous experiments showed in the controlled cortical impact (CCI) model that working memory deficit and excessive accumulation of phosphorylated tau protein (p-tau) presented a short period after TBI, and blocking of A2AR decreased p-tau level through regulating the production, clearing, and accumulation of p-tau in injured or remote regions. On the one hand, we found inactivation of A2AR by the methods of pharmacology and gene knockout reduced the level of p-tau and improved spatial reference memory and working memory in mice. This protection was related to PKA/GSK3 signaling pathways regulated by A2AR. On the other hand, our results indicated that TBI can induce regional disruption of AQP4 polarity in the hippocampal CA1 area, which retarded the clearance of extracellular p-tau and may lead to p-tau be absorbed by neighboring neurons and propagation of p-tau. In addition, we found that A2AR agonist can aggravate the dysfunction of axoplasmic transport, and the overexpression of exogenous aberrant tau protein or A2AR agonist can increase the accumulation of p-tau and cause the spatial memory dysfunction, which may associated with kinesin heavy chain member 2A/translin-associated protein X (KIF2A/TRAX) pathway. These findings provide experimental evidence for clarification of the mechanisms in the rapid progression of cognitive deficits following TBI and for the promising therapeutic strategies targeting A2AR signaling for the progressive cognitive dysfunction after TBI.
Keywords: Traumatic brain injury; Tau protein; cognitive dysfunction.

3. Adenosine A2A and Dopamine D1 Receptors in the Basal Ganglia Regulate Sleep-wake Cycle

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Abstract: The basal ganglia (BG) act as a cohesive functional unit that regulates motor function, habit formation, and reward/addictive behaviors. However, it is still not well understood how the BG regulate sleep-wake cycle to achieve all these fundamental functions until genetically engineered systems developed these years. By using in vivo fiber photometry, optogenetic and chemogenetic approach to manipulate neuron activities, patch-clamp, neuron circuit tracing, immunohistochemistry, and electron microscopy, we recently focused on the adenosine A2A and dopamine D1 Receptors (R) in the BG and obtained following 4 findings: (1) Nucleus accumbens (NAc) dopamine D1R-expressing neurons are essential in controlling wakefulness and are involved in physiological arousal via the lateral hypothalamus and midbrain circuits; (2) The rostromedial tegmental nucleus (RMTg), also called the GABAergic tail of the ventral tegmental area, projects to the midbrain dopaminergic system and other regions. Our findings reveal an essential role of the RMTg in the promotion of non-rapid eye movement (non-REM, NREM) sleep and homeostatic regulation; (3) Opposite to the D1R in the NAc, A2AR made a prominent contribution to sleep control associated with motivation. and (4) Striatal adenosine A2AR neurons control active-period sleep via parvalbumin neurons in external globus pallidus. Taken together, we proposed a plausible model in which the caudate-putamen and NAc integrate behavioral processes with sleep/wakefulness through adenosine and dopamine receptors.
Key words: adeno-associated virus, optogenetics, DREADD, basal ganglia, sleep-wake regulation
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4. Oral Communication: The A2A adenosine receptor mediates a novel regulation of DNA repair machinery implicated in mental disorders

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Adenosine is a neuromodulator that has been implicated in a wide variety of fundamental machineries. There are four adenosine receptors including the A2A adenosine receptor (A2AR), a Gαs- protein-coupled receptor that contains a long C-terminal domain (designated A2AR-C). We have previously reported that A2AR-C interacts with the translin- associated protein X (TRAX), a DNA/RNA binding protein that controls mRNA transport, translation, and DNA repair. Upon genotoxic stresses, TRAX binds with phosphorylated ATM (Ser1981) and contributes to the ATM-mediated DNA repair. Given that stimulation of A2AR by two A2AR-selective agonists markedly ameliorates the double-strand DNA breaks (DSBs) evoked by elevated oxidative stress in human iPSCs-derived neurons, the role of TRAX in mediating the protective role of A2AR is of great importance. Biochemical analyses reveal that TRAX forms a complex with GSK3β and a risk gene for schizophrenia (DISC1). Activation of A2AR leads to dissociation of the TRAX/DISC1/GSK3β complex (TDG complex), and allows the release of TRAX from the TDG complex to enter the nucleus to facilitate DNA repair and the subsequently enhance neuronal survival. Collectively, the TDG complex might serve as a potential therapeutic target for the development of novel treatments for diseases (such as degenerative diseases and mental diseases) with defects in DNA repair.
Keywords: A2AR ; TRAX; DISC1.

SYMPOSIUM 37: Acupuncture

1. Regulation of acupuncture and moxibuxtion on purine receptors in ibs visceral pain

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Objectives / background: Irritable bowel syndrome(IBS)is a common kind of "functional gastrointestinal disorders", characterized by chronic abdominal pain, abdominal distension and alteration of bowel habits. Chronic visceral hypersensitivity (CVH) is the main pathophysiological mechanism which can explain abdominal pain of IBS patients. Glial cells play an important role in the initiation and maintenance of pain and participate in the pathological mechanism of IBS. This research aims to the neurotransmission mechanism of electro-acupuncture (EA) to relieve the visceral hypersensitivity induced by IBS through analyzing the function of P2X3 receptors of the glial cells in the enteric nervous system (ENS) and dorsal root ganglion (DRG). Methods: The visceral hypersensitive IBS model was induced by the colorectal distention (CRD), and assessed by abdominal withdraw reflection scores (AWR) and histopathology. Rats were randomly divided into six groups: Normal group (NG), Model group (MG), electro-acupuncture group (EA), Glial cell inhibitor group (FCA), P2X3 receptor antagonist group (A-317491), P2X3 receptor agonist group (α,β-meATP). NG and MG were only fixed as same as the EA group without any treatment. EA was performed at the Shangjuxu Point (ST37) and Tianshu Point (ST25) with electrical stimulation (dense wave, frequency 2/100Hz, current 1mA, 30 min per time, once a day, continuous treatment for a week). All drugs including FCA, A-317491 and α,β-meATP were intrachecal injected (10-5mmol/μl, 10 μl per time, 3 times a week). AWR scores in the pre-treatment and post-treatment were analyzed. The protein expression of GFAP-P2X3 receptor in colonic myenteric plexus and colon related DRG were analyzed by immunofluorescence (double labeled). The protein and mRNA expression of GFAP and P2X3 receptors in colon were analyzed by Western Blot and real-time PCR. The protein and mRNA expression of GFAP and P2X3 receptors in colon related DRG were analyzed by immunohistochemistry, Western Blot and real-time PCR. Results: 1) After visceral hypersensitive IBS model evaluated, the AWR scores in the model group after CRD stimulation pressure of 20, 40 and 60 mmHg were significantly higher than those of the normal group (P all<0.01). The AWR score in the model group after CRD stimulation pressure of 80 mmHg were also higher than those of the normal group. 2) AWR scores in all EA, FCA and A-317491 group treatment groups after CRD stimulation pressure of 20, 40, 60, and 80 mmHg were significantly lower than those of the model group (P all<0.05). While the AWR scores in α,β-meATP group after CRD

stimulation pressure of 20, 40, 60, and 80 mmHg were higher than those of the normal group.3) The protein and mRNA expression of GFAP in the colon of rats in the model group and α,β-meATP group were significantly higher than those in the normal group (P all<0.01). The protein and mRNA expression of GFAP in the colon of rats in the EA, FCA and A-317491 group were significantly lower than those in the model group (P all<0.01). 4) The protein and mRNA expression of GFAP in colon related DRG of rats in model group and α,β-meATP group were significantly higher than those in the normal group (P all<0.01). The protein and mRNA expression of GFAP in colon related DRG of rats in the EA, FCA and A-317491 group were significantly lower than those in the model group (P all<0.05). 5) The protein and mRNA expression of P2X3 receptor in the colon of rats in the model group were significantly higher than those in the normal group (P all<0.05). The protein and mRNA expression of P2X3 receptor in the colon of rats in the EA and FCA group were significantly lower than those in the model group (P all<0.01). 6) The protein and mRNA expression of P2X3 receptor in colon related DRG of rats in model group were significantly higher than those in the normal group (P all<0.05). The protein and mRNA expression of P2X3 receptor in colon related DRG of rats in the EA and FCA group were significantly lower than those in the model group (P all<0.05). Conclusion: P2X3 receptor may play a closely relationship between the visceral pain of IBS and the enteric glial cells (EGCs), colon related DRG glial cells. EA decreased the protein and mRNA expression of GFAP, P2X3 in the colon and colon related DRG, which may be the mechanism of EA relieving visceral pain in the IBS rats.

2. CD39 and CD73: targets for immune regulation by moxibustion

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Objective: To investigate whether electro-acupuncture and moxibustion can influence Treg cells through CD39/CD73/A2a adenosine metabolic pathway based on Ulcerative Colitis (UC) mice. Methods: Dextran Sulfate Sodium (DSS) induced UC mice were set up and electro-acupuncture and moxibustion were used to treated the mice. The disease activity index (DAI),the length of the colitis and the pathological structure of each group of mice were evaluated and measured. Immunofluorescence (IFC) and Western Blot (WB) were used to detect the expression of CD39, CD73 and A2a proteins in the colon.The levels of CD4+CD39+ T cells, CD4+CD39+Foxp3+ T cells, CD4+CD73+ T cells and CD4+CD73+Foxp3+ T cells in peripheral blood, spleen, and local draining lymph nodes were measured by flow cytometry (FCM). Results: (1) After drinking 2% DSS in mice, the general state gradually deteriorated, bloody stools or pus and blood appeared, and the DAI score was increased. Both electro-acupuncture and moxibustion can improve the general conditions and the DAI scores of the mice. Significantly shorter colons, ulcers and inflammatory cells infiltration were seen in the model group. However, the length of the colon recovered and histopathology improved by the electro-acupuncture group and moxibustion group. (2) Compared with the control group, the fluorescence intensity of CD39, CD73, and A2a, the number of positive cells as well as their protein expression in the colons were decreased in the model group. After treatment, the above detections were significantly improved. In addition,compared with the electro-acupuncture group, the fluorescence intensity of CD39, CD73 and A2a, the number of positive cells, and the expression of the protein in the moxibustion group were higher than that in the electroacupuncture group (P<0.01). (3) The number of CD4+CD39+T cells, CD4+CD73+ T cells in the peripheral blood, lymph nodes and spleen of mice were all decreased in the UC model mice. However, after eletro-acupucture and moxibustion treatment, both of the number of CD4+CD39+T cells and CD4+CD73+ T cells significantly improved. Additionally, the ratio of CD4+ Foxp3+CD39+ T cells to CD4+ CD39+T cells,and the ratio of CD4+ Foxp3+CD73+ T cells to CD4+CD73+T cells in the peripheral blood, lymph nodes and spleen of mice were all increased after treatment (all P values were less than 0.01). (4) Compared with electro-acupunctue group, moxibustion have a greater impact on the ratio of CD4+Foxp3+CD73+T cells in the spleen (P<0.01); but it has a less impact on the ratio of CD4+Foxp3+CD39+ T cells in the peripheral blood and spleen (P<0.01). Conclusion: Both moxibustion and electro-acupuncture have good therapeutic effects on DSS-induced UC mice. Both treatments can increase the expression of CD39, CD73 and A2a protein in colonic tissues. Meanwhile, the ratios of CD4+ Foxp3+CD39+ T cells and the ratio of CD4+ Foxp3+CD73+ T cells in peripheral blood, lymph nodes and spleen were all increased by electro-acupuncture and moxibustion, suggesting that the regulation of Treg cells may be one of the underline mechanisms. Additionally, we also found that the effect of moxibustion treatment on Treg cell-associated CD39/CD73/A2a metabolic signaling pathway was not the same as that of electroacupuncture treatment.
Keywords: Ulcerative colitis, Electro-acupuncture,Moxibustion, Adenosine metabolic pathway, CD39, CD73,A2a

3. P2X3 and acupuncture analgesia

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Objectives: To investigate the analgesic effects of electroacupuncture (EA) at 2 and 100 Hz on type 2 diabetic neuropathic pain (DNP) and on the expressions of the P2X3 receptor and calcitonin gene-related peptide (CGRP) in the dorsal root ganglion (DRG). Methods and results: Rat type 2 DNP was induced by a high calorie and high sugar diet fed for 7 weeks, plus a single intraperitoneal injection of streptozotocin (STZ, 35 mg/kg) after 5 weeks. EA at 2 and 100 Hz were carried out once every day after 7 weeks for 7 consecutive days. Body weight, serum fasting insulin (FINS), fasting blood glucose (FBG), insulin sensitivity index (ISI), and paw withdrawal latency (PWL) were measured. The expressions of L4–L6 DRG P2X3 receptors and CGRP were assessed by immunofluorescence. Data were represented by the mean ± S.E. We found in the model group, 26 of the 44 rats developed type 2 DNP as shown by the increased body weight, FINS, and FBG (≥11.1 mmol/L), as well as the reduced ISI and PWL (≤85% of the base value).EA at both 2 and 100 Hz relieved type 2 DNP (11.94 ± 0.18 for 2 Hz, 10.86 ± 0.1 for 100 Hz, S, N = 9), but the analgesic effect of EA was stronger at 2 Hz. P2X3 receptor expression decreased in L4–L6 DRGs following EA at 2 Hz (26 ± 1 for L4, 19.33 ± 0.89 for L5, 13 ± 1.15 for L6, Positive ratio of P2X3 receptor) and in L5 and L6 DRGs following EA at 100 Hz (23.67 ± 0.88 for L5, 26 ± 0.58 for L6, Positive ratio of P2X3 receptor). EA at both 2 and 100 Hz down-regulated CGRP overexpression in L4–L6 DRGs (For 2 Hz, 29 ± 1.15 for L4, 29.33 ± 0.67 for L5, 24.67 ± 0.88 for L6. For 100 Hz, 29.33 ± 1.2 for L4, 32.67 ± 0.67 for L5, 26.67 ± 0.88 for L6, Positive ratio of CGRP). Conclusion: These findings indicate that EA at 2 Hz is a good option for the management of type 2 DNP. The EA effect may be related to its down-regulation of the overexpression of the DRG P2X3 receptors and CGRP in this condition.
Acknowledgment: the Project was supported by the National Natural Science Foundation of China (No. 81303039), the Specialized Research Fund for the Doctoral Program of Higher Education (No. 20133322120001), the Zhejiang Postdoctoral Science Foundation (No. BSH1302083), the Zhejiang Province Top Key Discipline of Chinese Medicine-Acupuncture & Tuina (No. [2012]80), and the Key Science and Technology Innovation Team of Zhejiang Province (2013TD15), China.

4. P2X7 and acupuncture mechanism

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Objective: To compare the difference of analgesic effects between ipsilateral and contralateral moxibustion and the relation with P2X7 receptor in C57BL/6J mice. Methods:Mice 20±2g used in this experiment were injected with complete Freund's adjuvant (20ul) to the left hind paw to induce inflammatory pain. Four days later, ipsilateral ipsilateral moxibustion, contralateral moxibustion, and with or without P2X7 receptor antagonist A438079 were administrated. Pain thresohold was tested before and after the intervention. Results Compared with the control group, the pain threshold of CFA group markedly decreased after CFA injection($P<0.01$); the pain thresholds of both ipsilateral and contralateral moxibustion groups were significantly higher than that in CFA group,. No statistical significant difference displayed in ipsilateral moxibustion group after intervention at 30min,60min, 90min($P<0.05$) while significant difference was presence in contralateral moxibustion group after intervention at 30min,60min($P<0.05$). Compared with CFA group, there was no statistical significant in ipsilateral moxibustion+ipsilateral A438079 group($P>0.05$); there was statistical significant in ipsilateral moxibustion+contralateral A438079 group at 30min($P<0.05$), but no statistical significant at 60min, 90min,120min and 150min($P>0.05$); Compared with CFA group, there was statistical significant in contralateral moxibustion+contralateral A438079 group($P<0.05$); there was no statistical significant in contralateral moxibustion+ipsilateral A438079 group($P>0.05$). Conclusions:Moxibustion has analgesic effect in inflammatory pain; The analgesic effects were similar between ipsilateral and contralateral moxibustion; Analgesic effects in ipsilateral and contralateral moxibustion were inhibited by ipsilateral A438079 injection. This work was supported by NSFC (NO. 81774437)
Keywords: ipsilateral; contralateral; moxibustion; analgesia; P2X7 receptor

5. Oral Communication: Terahertz-induced analgesia at acupoint and purinergic signaling

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Objectives: To evaluated the analgesic effects of Terahertz(THz) radiation at 'Zusanli' acupoint in mice with experimental inflammation pain model induced by complete Freund's adjuvant(CFA) injection and its role in purinergic signaling. Methods and results: We apply radiation from a continuous-wave optically pumped molecular gas 2.52THz laser ($\lambda = 118.8$ μm) source and the power was determined with a calibrated Scientech laser powermeter. The system includes a temperature controller to conduct temperature-controlled THz exposures. Adult C57/BL/6 mice receiving THz radiation (305.577mW/cm2, 30 min) at 'Zusanli' acupoint of the right hind limb showed significantly increased paw withdrawal latency (ANOVA, $p<0.05$) to a noxious thermal stimulus. This anti-nociceptive effect peaked at 30 minutes post-exposure (7.181±0.428 s), began to eliminate at 90 minutes(4.919 ±0.393s). PCRarray results preliminary showed that P2X receptors may be involved in the process of peripheral analgesia during THz radiation. Conclusion: P2X receptors would be potential target for THz radiation-induced analgesia. Keywords: Terahertz, analgesia, Zusanli acupoint, purinergic signaling. Acknowledgment of financial support: This work was supported by 973 Program of China (NO.2015CB554504), NSFC(NO.81704190) and China Postdoctoral Science Foundation (NO.2016M602662)

SYMPOSIUM 38: Imaging and Electrophysiology for the Study of Purinergic Signaling

1. Neuronal network effects of P2 receptors in schizophrenia

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P2Y and P2X receptors are widely expressed in the hippocampus but their role in network oscillations is still not clear. Gamma oscillations are rhythmic changes of the extracellular field potential at frequencies between 30 and 90 Hz generated by synchronizing fast perisomatic GABAergic inputs onto pyramidal cells. They act as the clockwork of intercellular communication and are associated with higher cognitive functions such as sensory processing, working memory, attention, learning and memory. Pathological gamma oscillations were observed in a line of neuropsychiatric diseases, for example in schizophrenia, autism, and Alzheimer's disease. Here we investigated whether P2Y and P2X receptors are involved in the modulation of hippocampal gamma oscillations in healthy and MK-801-treated rats, an acute first episode model of schizophrenia. We have found that among all investigated P2 receptors, only the P2X4 subtype had a modulatory effect. While in hippocampus slices of healthy control animals activation of P2X4 receptors inhibited gamma oscillations, in hippocampus from MK-801-treated animals a potentiating effect could be observed. None of the other P2X and P2Y receptor subtypes affected gamma oscillations. By means of patch clamp experiments and immunhistochemistry we further investigated the cellular distribution and effects of P2X4 receptors. Our results suggest that P2X4 receptors on the CA3 pyramidal cells are responsible for the network modulatory effects of these receptors. P2X4 receptors on pyramidal cells are able to control hippocampal network oscillations in both healthy and schizophrenic animals.
Keywords: Gamma oscillations; neural network; hippocampus; P2X4 receptor.

2. Role of astroglial purinergic signaling in Alzheimer's disease

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Objectives/background: Astrocytic hyperactivity is a prominent component of network dysfunction in models of Alzheimer's disease (AD). We have previously shown that astrocytic hyperactivity is most pronounced in reactive astrocytes around plaques, and is mediated by purinergic signaling through the P2Y1 receptor. The consequences of long-term treatment with a P2Y1R antagonist for network activity, amyloid metabolism and behavior have remained undetermined.

Methods and results: We aimed to study the consequences of long-term P2Y1R inhibition in an AD mouse model. To this end, we administered P2Y1R antagonists to APPPS1 mice or age-matched littermates intracerebroventricularly using osmotic minipumps. Calcium imaging of neuronal and astroglial activity in the cortex of anesthetized mice using two-photon microscopy revealed that this treatment reduced astrocytic hyperactivity to levels observed in wildtype mice. Moreover, we found that this treatment augmented structural synaptic integrity and preserved hippocampal long-term potentiation. These effects occurred independently from β-amyloid metabolism and degradation or plaque burden, but were associated with a higher morphological complexity of peri-plaque reactive astrocytes as well as reduced dystrophic neurite burden. Importantly, APPPS1 mice chronically treated with P2Y1R antagonists were protected from the decline of spatial learning and memory, as measured using the Barnes Maze paradigm. Conclusion: Our study establishes the restoration of cerebral network homoeostasis by P2Y1R inhibition as a novel treatment target in AD. Acknowledgements: This work was supported by grants from the European Union (EU) Joint Programme – Neurodegenerative Disease Research (JPND) program (EU Horizon 2020 Research and Innovation Program, grant agreement 643417/DACAPO-AD), the Alzheimer's Research Initiative (Alzheimer Forschung Initiative, AFI), and the DZNE.
Keywords: P2Y1 receptors; Alzheimer's disease; Neurodegeneration; Astrocytes.

3. Signaling function of ATP in the central auditory system

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Objectives / background: Understanding how early action potential discharges contribute to the maturation of neuronal networks is one of the major challenges in developmental neurophysiology. Following initial development including neuronal differentiation, migration, axon guidance and synaptogenesis, neuronal activity in maturing circuits is guiding the formation of precise sensory maps. In the auditory system, sensory map formation and sharpening depend on the spontaneous activity before hearing onset and extend to the period of early auditory experience. However, it remains elusive how neuronal activity affects different aspects of maturation in the auditory brainstem circuit that is encoding temporal features of sound to compute sound source location. Methods and results: In the developing ventral cochlear nucleus, the first central station along the auditory pathway that receives inputs from the cochlea through the VIII nerve, paracrine ATP signaling enhances firing in a cell-specific and tonotopically-determined manner. Endogenously released ATP activates the P2X2/3R expressed only in bushy cells, and increases the synaptic efficacy of immature synaptic inputs, i.e. endbulbs of Held, by facilitating postsynaptic AP generation and prolonging APs. Developmental down-regulation of P2X2/3R currents occurs simultaneously with an increase in AMPAR currents from high-to-low frequency area. Conclusion: Our in vivo and slice experiments in P2X2/P2X3Dbl-/- mice demonstrate that the P2X2/3R is required for functional maturation of endbulb of Held synapses during the period of early auditory experience. Acknowledgment: This work was supported by the Deutsche Forschungsgemeinschaft (DFG grant MI 954/3-1) as a part of the priority program 1608 “Ultrafast and temporally precise information processing: Normal and dysfunctional hearing”.
Keywords: "calyceal synapse";"development";"neuronal activity";"P2X2/3 receptor".

4. Dual control of microglial motility by ATP and membrane voltage

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ATP mediates interactions between cells in many tissues, but is particularly important for microglia, the brain’s immune cells. A key feature of these cells is their enormous motility, which can be classified into two modes. In the undisturbed brain, they constantly extend and retract their processes to survey the brain, in order to detect infection, remove dying cells, and prune synapses during brain development. On the other hand, they also promptly send out targeted processes in response to sudden rises of extracellular ATP released by tissue damage, in order to envelop sites of injury and isolate them from undamaged areas. We recently showed that these motility modes differ mechanistically and involve purinergic signaling and K+ channel activity. First, we identified the two-pore domain channel THIK-1 as the main K+ channel expressed in microglia in situ. THIK-1 is tonically active, and its activity is potentiated by P2Y12 receptor activation. Directed motility to an ATP source or laser-induced tissue damage is mediated by P2Y12 receptors but does not require activity of the THIK-1 subunit-containing two-pore domain K+ channels that these receptors gate. In contrast, microglial surveillance of the brain does not require P2Y12 activity (or any signaling that it evokes), but is fundamentally dependent on the tonic activity of THIK-1 channels, which maintain the resting potential of the microglia. We also investigated whether the ceaseless movement of microglia in the undisturbed brain may be driven by constant ATP release from brain cells, since apyrase, an enzyme widely used to manipulate extracellular ATP levels, has been reported to reduce microglial surveillance and ramification. Our experiments revealed that apyrase is highly contaminated with K+ ions, which depolarize microglia, similar to the effect of blockade of THIK-1, and thus inhibit their surveillance and ramification. Dialysis of apyrase to remove K+ retained its ATP-hydrolyzing activity but abolished the microglial depolarization and decrease of surveillance produced by the undialyzed enzyme. Thus, no ATP release and no ambient purinergic signaling are required to maintain microglial surveillance. Finally, we investigated whether the P2Y12-THIK-1 signaling axis is involved in microglial release of immune modulators. We found that block of the ATP-evoked THIK-1 activity inhibited the release of the pro-inflammatory cytokine interleukin-1beta from activated microglia, consistent with K+ loss being needed for inflammasome assembly. Thus, microglial cytokine release requires P2Y12-gated THIK-1 channel activity.
Supported by the ERC and Wellcome Trust
Keywords: ATP; microglia; potassium channel; motility.

5. Oral communication: Cytosolic Ca^{2+} transients evoked by purinergic receptors in hepatocytes: the role of P2Y receptors

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Objectives/background: Extracellular nucleotides are key signaling molecules recognized by hepatocytes and other liver cell types, affecting important hepatic processes. Understanding the role of P2X and P2Y receptors on Ca^{2+} signaling in the hepatic context is of major relevance for liver physiology. Methods and results: Isolated hepatocytes were prepared by collagenase perfusion of rat livers. Freshly isolated hepatocytes were loaded with Fura-2/AM and transferred to a thermostatically regulated microscope chamber for acquisition of fluorescence images of cytosolic Ca^{2+} signals, which typically take the form of periodic Ca^{2+} oscillations. UDP, a P2Y6 receptor-selective agonist, was the only extracellular nucleotide that failed to elicit a Ca^{2+} response in rat hepatocytes. Among the other nucleotides, the transients induced by low doses of each agonist (1-2 μM) showed different spike durations, with distinguishable falling phases. ATP elicited complex Ca^{2+} transient shapes with two main patterns: broader spikes with biphasic decay phase, present in the majority of the cells and narrow spikes with fast decay phase, present in the minority of the cells. Specific activation of P2Y1 receptors by ADP generated a homogeneous short-lasting Ca^{2+} spike pattern with narrow peaks and rapidly declining phase. P2Y2 and P2Y4 receptors, stimulated by UTP, elicited predominantly longer-lasting peaks. In the absence of extracellular Ca^{2+}, the spike widths measured at half-peak height of the first spike were longer than those observed in the presence of extracellular Ca^{2+} at the same agonist dose. The contribution of P2X ligand-gated ion channel receptors in generating cytosolic Ca^{2+} transients was determined with ATP (1-300 μM) in the presence of a Gq protein-specific inhibitor to block P2Y receptor coupling to Ca^{2+}. No Ca^{2+} oscillations occurred under these conditions. At higher ATP doses (400 μM), a slow and constant intracellular Ca^{2+} increase was observed, suggesting Ca^{2+} influx through membrane pore formation by P2X7 receptor activation only at high ATP. Similar results were obtained from treatment with BzATP, a potent P2X7 receptor agonist. Conclusions: In rat hepatocytes, cytosolic Ca^{2+} transients are probably evoked only through IP3 formation by Gq coupled P2Y1, P2Y2 and P2Y4 receptors, but not P2Y6 receptors without contribution of P2X receptors. The differential Ca^{2+} oscillations patterns elicited by activation of these receptors suggests specific and yet undiscovered roles of purinergic signaling in liver physiology.
Acknowledgments: São Paulo Research Foundation, FAPESP; The Brazilian National Council for Scientific and Technological Development, CNPq.
Keywords: Hepatocytes; Calcium imaging; P2Y receptors; cytosolic Ca^{2+} transients.

POSTERS

01 - ADENOSINE AS A KEY FACTOR IN THE LONG-TERM EFFECTS OF EARLY ETHANOL EXPOSURE.

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Fetal Alcohol Syndrome and Alcohol-related neurodevelopmental disease promote morphological and cognitive outcomes depending of the ethanol exposure intensity. Adenosine levels rise in acute ethanol exposure, while A2A adenosine receptors adjust its expression in chronic exposure. We have studied the impact of adenosine modulation in the long-term consequences of early ethanol exposure using zebrafish. Wild-type zebrafish embryos were exposed to 1 or 2% ethanol during Gastrula/Segmentation or pharyngula (developmental phases). Morphological, biochemical and behavioral consequences were evaluated at 7 days (larvae), 3 months (Young-adult) and 1 year (Adult) post-fertilization. At these periods, we accessed: (1) Ecto-5'-nucleotidase and Adenosine deaminase activities and expression; (2) Adenosine levels in brain; (3) Effects of adenosine receptor antagonists (DPCPX and ZM241385) and ecto-5'-nucleotidase (AMPCP) and nucleoside transporter (Dypiridamole) inhibitors on morphological parameters; and (4) effects of AMPCP over locomotor and behavioral/mnemonic aspects. All procedures were approved by CEUA/PUCRS (13/00347 and 15/00468). Our results demonstrated that 2% ethanol at Gastrula/Segmentation phase promotes gross morphological impairment featured by decreased body length, increased distance between eyes and decreased eye's area in zebrafish larvae ($p<0.001$). Dipyridamole (10 and 50 μM) worsened the morphological effects of 2% ethanol exposure, while the pre-treatment with AMPCP did not prevent. A2A receptor antagonism reduced the incidence of pericardial edema (up to 34%) and the reduction on body length ($p<0.001$), while block of A1 receptor had no effect. The ecto-5'-nucleotidase was increased (28%) in zebrafish larvae exposed to 2% ethanol during gastrula/segmentation, with no effect on gene expression. The 1% ethanol exposure did not promote any morphological outcome, and was chosen to evaluate late consequences of early ethanol exposure. Encephalic ecto-5'-nucleotidase activity was elevated (39.2%), and gene expression was normal in young animals exposed to ethanol at gastrula/segmentation phase ($p<0.05$). Early exposure to 1% ethanol affected memory of young animals ($p<0.001$), which was recovered by AMPCP, when gave prior to the training session in the inhibitory avoidance test. Aggressiveness was increased ($p<0.05$), while social interaction was decreased ($p<0.001$) in young animals exposed to ethanol at pharyngula phase, both recovered by AMPCP. Adenosine level increased in the brain of adult animals treated with 1% ethanol at pharyngula stage ($p<0.01$). These results suggest that ethanol effects on adenosine metabolism during early life has long-lasting effects, which could be implicated in behavioral consequences. Ecto-5'-nucleotidase modulation appears to be implicated in adenosine increase, but the nucleoside transporter, another major target of ethanol, must be evaluated regard to these late consequences.
Support: CNPQ and CAPES/Brazil.
Keywords: adenosine, early development; ethanol.

02 - ANTIOXIDANT ACTIVITY OF *SABICEA BRASILIENSIS* AND ITS EFFECTS ON ADENINE NUCLEOTIDES METABOLISM IN A7R5 CELLS.

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Objectives/ background: *Sabicea brasiliensis* is found in Cerrado region of Brazil. It is primarily used as food, but it may also be employed to treat different pathological disorders, including some of the cardiovascular system. Its medicinal use is attributed to the already characterized presence of antioxidant molecules such as scopoletin, ursolic acid, cafeoylquinic acids and triterpenes in *S. brasiliensis* roots (Batista et al. Quím. Nova 37; 4, 2014). Extracellular purines are implicated as important regulators of cardiovascular (patho)physiology. In the vasculature, ATP, ADP and adenosine may influence vasomotor responses, platelet activation, cardiac function, among others. NTPDase1 and ecto-5'-nucleotidase are the major ectoenzymes controlling the availability and effects of nucleotides in vessels surface. Thus, the objective of this study was to evaluate the antioxidant activity of *S. brasiliensis* roots crude extract and fractions and their effects on adenine nucleotide hydrolysis in A7r5 cells. Methods and results: percentage of antioxidant activities of crude extract (CE), ethyl acetate (EA) and hydromethanolic (HM) fractions were determined by the inhibition of DPPH and ABTS radicals comparing to butylated hydroxytoluene (BHT) standard. DPPH showed highest antioxidant activity performance for CE (76%), followed

by EA (46%) and HM (23%) fractions. These results were corroborated by ABTS assay. Total phenolic content was evaluated by Folin-Ciocalteau colorimetric method and showed a significant amount of those molecules in CE (140.6 ± 7.7 mg GAE.g-1), followed by EA (67.1 ± 4.7 mg GAE.g-1) and HM (13.5±8.2 GAE.g-1). A7r5 embryonic rat aorta smooth muscle cells (ATCC® CRL-1444™) were treated with EA at 62.5; 125; 250 and 500 μg·mL−1 for 48 hours. ATP hydrolysis was significantly inhibited at 500 μg·mL−1 (56,65%), evidencing that *S. brasiliensis* can modulate purine levels in vascular tissue. Similarly, EA seemed to diminish ADP hydrolysis. However, new experiments must be performed to confirm these results. Conclusion: CE, EA and HM of *S. brasiliensis* roots present significant antioxidant power and high total phenolic content. Besides, EA alters ATP hydrolysis, modulating the balance of purine levels in A7r5 cells surface. ATP may act as a vasoconstrictor when binding to vascular smooth muscle cells and a as a vasodilator when stimulating endothelial P2 receptors. Thus, other studies must be conducted to better clarify the effect of *S. brasiliensis* in vascular purinergic signaling as well as the impact of this likely interaction in vasomotion processes.
Financial support: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).
Keywords: *Sabicea brasiliensis*; ATP hydrolysis; A7r5; vasomotion.

03 - APPLICATION OF RECOMBINANT NTPDASE-2 FROM *LEISHMANIA INFANTUM CHAGASI* (RLICNTPDASE2) TO DIAGNOSIS OF CANINE VISCERAL LEISHMANIASIS.

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The leishmaniasis are a group of diseases caused by protozoan parasites of Leishmania. These diseases represent major concern on public health and are classified as tropical neglected diseases by the World's Heath Organization (WHO). Canine visceral leishmaniasis (CVL) is caused by *Leishmania infantum* chagasi. CVL has been the aim of several studies given the importance of the dog as a natural reservoir of Leishmania parasite and, as a result, it plays important role on the dissemination of the human leishmaniases. In Brazil, the euthanasia of infected dogs has been used to control the spread of this disease. The diagnosis were done in the past using killed parasites in Indirect Immunofluorescence Assay (RIFI) or using protein extracts of Leishmania by indirect immune assay (ELISA). Currently it is done by new ELISA-DPP® using recombinant antigens from Leishmania. These current assays have known limitations concerning specificity and sensitivity. For this reason, improvements of CVL diagnosis is required. Leishmania have two isoforms of NTPDases, named NTPDase1 and NTPDase2. Previously we showed evidences that recombinant NTPDase-2 from *L. infantum* chagasi (rLicNTPDase) expressed in bacteria system could be good antigen to diagnosis of CVL by ELISA. In the present study, we expand the analyses to a total of 513 sera samples from dogs from different locations of Brazilian southeast. First of all, the rLicNTPDase2-ELISA assay was compared with true positive samples (156 positive sera assayed by parasitological assay) and with the true negative samples (102 negative sera by parasitological and PCR). The agreement of rLicNTPDase2-ELISA and these group of samples was 93.69% (co-sensitivity 89.10% and co-specificity 82.35%). The second step was to compare the rLicNTPDase2-ELISA with other sera groups previously diagnosed as positive: RIFI (concordance=95.45%), RIFI and ELISA protocol (concordance=95.12%) and ELISA-DPP® protocol (concordance=63.53%). This last assay shown the lower level of concordance between the analyzed groups. Our results suggest that ELISA-DPP® could be not the best option to diagnosis and CVL. On the other hand, our data shown the high level of efficacy of rLicNTPase2-ELISA assay to diagnosis of CVL.
Keywords: Leishmaniasis; diagnosis; NTPDase; antigen.

04 - ARE THE TOXIC EFFECTS CAUSED BY NICKEL EXPOSURE RELATED TO PURINERGIC SYSTEM?

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Background/objective: Some studies have shown that the purinergic system can be altered by exposure to metals. Nickel is a heavy metal, naturally present in the earth's crust that, at high concentrations, leads to environmental contamination and causes health problems. The central nervous system has been reported as main target of nickel toxicity, and evidences indicate that the exposure results in a variety of neurological symptoms. In addition, nickel is capable of causing changes in the release of ATP and acts on metalloenzymes. ADA is a metalloenzyme that requires a divalent cation (zinc or cobalt) for its activity. Although this enzyme does not rely on nickel, it is known that interactions between metals occur and, in this sense, nickel may have an effect on this system. Therefore, we evaluated the effects of NiCl2 exposure on behavioral parameters and nucleoside triphosphate diphosphohydrolase, ecto-5'-nucleotidase and ADA activities in brain membranes of adult zebrafish. Methods and Results: Adult zebrafish were exposed to NiCl2 concentrations (2.0, 5.0, and 15.0 mg/L) or water (control) for 96 hours and were tested after the treatment period. Animals were evaluated for exploratory, social and aggressive behavior and memory task. For enzyme evaluation, the brain membranes were prepared and NTPDase, ecto-5′- nucleotidase, and ADA activities were determined (JNeurochem 61:1685, 1993; Life Sci 73:2071, 2003; Comp Biochem Physiol B Biochem Mol Biol 139:203, 2004) (CEUA-PUCRS, permit number 15/00463). The results showed that, exposed zebrafish presented concentration-dependent increases in brain nickel levels compared with controls. The exploratory behavior test showed that nickel exposure induced anxiogenic-like behavior, decrease aggression and impaired memory, whereas no difference was observed in social behavior. There were no significant changes on NTPDase, ecto-5'-nucleotidase, and adenosine deaminase activities after nickel exposure. Conclusion: The exposure to nickel in zebrafish leads to anxiogenic-like effects, impaired memory and decreased aggressive behavior. These alterations may significantly impact the behavioral responses and survival of zebrafish in natural habitats. Since nickel levels were observed in treated animal's brain, the toxic effects of nickel exposure might be related to neurological damages. Our results did not indicate that NiCl2 altered the ATP and adenosine-metabolizing enzymes. However, we postulate that NiCl2 may form a complex with ATP and it is not hydrolyzed, altering the availability of other nucleotides and nucleosides (ADP, AMP and ADO), which may be related to the nickel exposure effects. Further studies are required to investigate the ATP metabolism and its relation to behavioral changes.
Keywords: Nickel; ATP.

05 - BOZEPINIB REDUCES GLIOBLASTOMA GROWTH THROUGH THE MODULATION OF CD39 / CD73 ENZYMES.

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Glioblastoma (GBM) is the most frequent and aggressive primary brain tumor in adults. GBM has a poor prognosis and conventional therapeutic treatments have only a modest effect on the survival of most patients. Bozepinib [[(RS)-2,6-dichloro-9-[1(p-nitrobenzenesulfonyl)-1,2,3,5-tetrahydro-4,1-benzoxazepin-3-yl]-9H-purine] is a potent compound in research for cancer treatment. Recent studies show that Bozepinib induces in vitro cell death by apoptosis in breast and colon cancer as well as in vivo antitumor activity in a breast cancer model. Derived from a purine, Bozepinib is an interesting candidate for purinergic system investigation. The purinergic system consists of nucleosides and nucleotides present in the extracellular milieu modulating a variety of biological actions via the activation of purinergic receptors. In cancer this signaling is controlled manly by the coordinated action of ectonucleotidases, E-NTPDases/CD39 and ecto-5'-nucleotidase/CD73, which progressively hydrolysis ATP to adenosine. The objective of the present study was to explore the antitumor activity of Bozepinib against GBM cell lines and its ability to modulate the purinergic system. In the study was used GBM cell lines (C6 rat and U138 human) and non-tumor cell line (HepG2). The cell viability assay was evaluated by MTS method. The purinergic system evaluation started with a molecular docking study. Then, the expression of CD39 and CD73 enzymes was evaluated by flow cytometry and the enzymatic activity was measured by malachite green method. As preliminary results, Bozepinib induced cell death with low IC50 values, 5.7 ± 0.3 μM and 12.7 ± 1.5 μM in C6 and U138 cell lines, respectively. Bozepinib had safe therapeutic index in vitro (C6 = 9.5; U138 = 4.3). Molecular docking showed Bozepinib interacts energetically with CD73 enzyme to a great extent than quercetin and less than AMPCP, well-known CD73 inhibitors. When CD73 expression was evaluated, Bozepinib did not change the enzyme expression in U138 and C6 cell lines. On the other hand, Bozepinib 5 μM and 10 μM significantly increased the expression of CD39 protein in C6 and U138 cells, respectively (from 23.7 ± 10.0% to 42.4 ± 8.15 and from 4.8 ± 0.3% to 16.3 ± 0.6%, respectively). Regarding enzymatic activity, Bozepinib increased ATP and ADP hydrolysis in U138 cell lines (about 50% in comparison with DMSO), while reduced significantly AMP hydrolysis in both cell lines (C6 = 57%; U138 = 45%). In conclusion, Bozepinib exhibits cytotoxic activity against GBM cell lines leading to an astrocytes-like phenotype, regarding CD39 and CD73 enzymes. Bozepinib was able to upregulate CD39 expression, increasing ATP and ADP hydrolysis while inhibited significantly the AMP hydrolysis without any change in CD73 expression. More studies are necessary to understand the purinergic influence of Bozepinib in GBM cells. Acknowledgements: CNPq/INCT, CAPES.
Keywords: Bozepinib; glioblastoma; CD39/NTPDase1; CD73/ecto-5'-nucleotidase.

06 - CAFFEINE PREVENTS BEHAVIORAL ALTERATIONS AND NEURODEGENERATION IN MICE SUBMITTED TO BILATERAL OLFACTORY BULBECTOMY AS A MODEL OF DEPRESSION.

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Objectives/background: The association between caffeine intake and a diminished risk of depression has been evidenced in longitudinal studies. Similarly, caffeine has also positive effects in experimental models of depression triggered by chronic stress. The Olfactory Bulbectomy (OBX) is an animal model of depression, which presents behavioral and neurochemical alterations similar to those found in depressed patients. This study aimed to investigate the effects of chronic caffeine on neurochemical and behavioral alterations induced by OBX model. Methods and results: CF1 adult mice (42-45g) after receiving caffeine treatment (0.3 or 1g/L in the drinking water) during their active cycle for 2 weeks before OBX. The treatment lasted five weeks after OBX. SHAM animals were submitted to the same procedures, except that the olfactory bulbs were left intact. At the end of the treatment, locomotion was evaluated in the open field, anedhonia in the splash test, recognition memory in the novel object recognition task and spatial memory in the Y-maze task. Besides, immunodetection in the prefrontal cortex, hippocampus and striatum was performed for adenosine A1 and A2A receptors (A1R and A2AR), SNAP-25 (nerve terminals marker) and GFAP (astrocytes marker). Immunohistochemical analysis for GFAP (astrogliosis) and Fluoro-Jade C staining (neurodegeneration) were also performed in brain slices. Caffeine reduced hyperactivity and prevented recognition memory impairment in OBX mice. OBX mice displayed increased A1R and GFAP in the prefrontal cortex (29%, $p<0.05$ and 100%, $p<0.01$, respectively), and decreased SNAP-25 (22%, $p<0.05$) when compared to SHAM mice. While SNAP-25 was also decreased in the striatum of OBX mice (22%, $p<0.05$), A2AR increased in both hippocampus (42%, $p<0.05$) and striatum (38%, $p<0.05$). Although the majority of the proteins analyzed in OBX mice were not modified by caffeine, astrogliosis in the hippocampus and neurodegeneration observed in the striatum and piriform cortex were prevented by caffeine. This study was approved by the ethical committee on use of animals of the Universidade Federal of Rio Grande do Sul (Proc. no. 24477). Conclusion: Caffeine was able to attenuate hyperactivity and cognitive deficits in OBX mice, which might be associated with prevention of hippocampal astrogliosis and neurodegeneration in striatum and piriform cortex. Based on these findings, caffeine confirms its potential as a promising therapeutic tool in the prophylaxis and/or treatment of depression. Financial Support: CAPES, CNPq.
Keywords: Caffeine; Olfactory Bulbectomy; Depression.

07 - CAFFEINE RESTORES PERFORMANCE IN A DECISION-MAKING TASK AND DOPAMINE SIGNALING IN ADHD MODEL IN A SEX DEPENDENT MANNER.

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Objectives / background: Attention deficit and hyperactivity disorder (ADHD) is one of the most commonly diagnosed neurodevelopmental disorder characterized by symptoms of inattention, hyperactivity and impulsivity. Symptomatology differs between sexes, with boys presenting more hyperactivity/impulsivity being more diagnosed than girls. Supporting genetic influence, associations of several genes in ADHD pathophysiology have been reported, including SNAP-25 (Synaptosomal-associated protein of 25 kDa), DAT (dopamine transporter) and DRD4 (dopamine receptor D4). Caffeine is the most consumed psychostimulant worldwide, with beneficial effects on the cognitive functions.

Caffeine has been reported to improve cognitive functions in ADHD animal models, with studies mostly performed in male animals. In this study, we aimed to assess sex differences in the effects of caffeine on a learning and decision making task and on SNAP-25, DAT and D4 receptor immunocontent in brain samples from the most validated ADHD animal model. Methods and results: Male and female spontaneously hypertensive rats (SHR) and control strain Wistar Kyoto 60–70 days old (160 g), received water or caffeine (0.3 g/L) in the drinking water from postnatal day 15 up to 50-55. Ethical committed CEUA/UFRGS (Proc. 29196). Based on scent discrimination paradigm animals were submitted to a learning and decision-making task named Dig Task. Compared to control strain, female SHR rats required more trials the first phase of the tasks, which consist of discrimination phase (n= 5-8; $F(1,27) = 8.35$; $P < 0.05$). For the reversal phase, both male and female SHR rats required more trials in order to complete this phase, showing impaired performance (n=5-8; $F(1,26) = 25.92$; $P < 0.01$). Female SHR rats treated with caffeine showed number of trials similar to control strain females to complete both phases (n=5-8; $F(1,24) = 14.12$; $P < 0.01$) and (n=5-8; $F(1,24) = 5.52$; $P < 0.05$). SHR rats showed reduction of DAT levels (n =6-8; $F(1, 33) = 17.34$;$P < 0.01$), which were counteracted by caffeine in males (n= 7; t= 2.107; $P < 0.05$) and females rats (n= 7; t=2.520; $P< 0.05$). DRD4 receptors were also decreased in both sexes of SHR rats (n=6-8; $F(1, 37) = 7.525$; $P <0.01$), but caffeine was able to restore DRD4 levels only in male SHR rats (n=7; t=2.573; $P < 0.05$). Conclusion: Our findings revealed that female SHR rats displayed full impairment in a task highly dependent of frontal brain functioning, which is usually dysfunctional in ADHD. Caffeine was able to restore the performance in Dig task in both sexes, but dopamine signaling was normalized only in males. Caffeine extends its potential as a promising treatment for cognitive deficits in ADHD with participation of dopaminergic system. Financial support: CNPq and CAPES.
Keywords: Caffeine; ADHD; SHR; sex differences.

08 CARDIAC MITOCHONDRIA FUNCTION AND EXTRACELLULAR VASCULAR NUCLEOTIDE METABOLISM IN GENETIC MODEL OF HYPERLIPIDEMIA.

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Background: Hyperlipidemia leads to impairment of the mitochondrial function by excessive production of reactive oxygen species and respiratory chain disorders. Moreover, increased level of plasma lipids leads to the development of pathological changes in the blood vessels leading to atherosclerosis and changes in purinergic signaling. The aim of this study was to investigate the effect of experimental hyperlipidemia on the respiratory chain and vascular extracellular nucleotide catabolism pathway. Methods: The analysis of function isolated cardiac mitochondria was performed in 3- and 6-month LDL Receptor knock-out mice (LDLR-/-, n=5 in each age group), following the approval of the local ethics committee. C57Bl/6J wild types (WT, n=5 in each age group) were used as controls. Experimental system examined the transport of electrons in the respiratory chain by adding NAD+-dependent substrates (malate, pyruvate) and FCCP (mitochondrial uncoupler) to mitochondria, followed by rotenone, succinate, antimycin and ascorbate enriched with TMPD (donor of electrons). The analysis was performed using the Seahorse XFp metabolic flux analyzer, by recording the oxygen consumption rate (OCR). The measurement of the rates of ATP and AMP hydrolysis as well as adenosine deamination were evaluated in four segments of aortas (aortic arch, aortic root, thoracic aorta and abdominal aorta) by analysis conversion of substrate into products using reverse phase high performance liquid chromatography (RP-HPLC). Results: Isolated mitochondria from 6-month LDLR(-/-) mice exhibited lower OCR (179.0±19.04 pmoles/min) than age-matched WT (281.1±22.83 pmoles/min) (mean ± SEM). In turn, no differences were found between mitochondria isolated from 3-month mice. These results negatively correlated with increased activity of vascular ecto-adenosine deaminase (eADA) in 6-month LDLR(-/-) mice in all parts of aorta and for aortic arch eADA was two times higher in LDLR(-/-) vs. WT: 0.19 ± 0.03 vs. 0.08 ± 0.01 nmol/min/mg tissue (mean ± SEM). 3-month mice did not show differences in eADA activity. ATP and AMP hydrolysis were at the same level in both age groups of LDLR(-/-) and WT mice. Conclusions: Lipid abnormalities affected electron flow in the respiratory chain of 6-month-old LDLR (-/-) mice hearts. That correlated with increased activity of vascular eADA that was previously proposed as a maker of endothelial inflammation. Details of the link between adenosine metabolism and mitochondrial function require further studies. Supported by: National Science Centre of Poland NCN/2016/22/M/NZ4/00678
Keywords: mitochondria; hyperlipidemia; adenosine; ATP.

09 - CD73 DOWNREGULATION INHIBIT IN VITRO AND IN VIVO GLIOBLASTOMA GROWTH THROUGH ADENOSINERGIC PATHWAY.

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Glioblastoma multiform (GBM) is the most devastating primary brain tumor, characterized by high-grade proliferation, invasion and chemoresistance. Alterations in purinergic signaling have been reported in a variety of solid tumors, including GBM. CD73 overexpression in cancer cells may contribute to increasing extracellular adenosine (ADO) in tumor environment, which favor tumor progression, recurrence, chemoresistance, angiogenesis and immunosuppression. Therefore, CD73 has emerging as a drug target to cancer therapy. Here, we investigated the potential of CD73 downregulation for glioma treatment in vitro and in glioma-implanted rats in vivo. To characterize the CD73 role in in vitro and in vivo glioma progression, four experimental groups were applied: (1) Control (vehicle); (2) APCP (pharmacological CD73 inhibitor); (3) GFP-siRNA (scramble); (4) CD73-siRNA. For in vitro experiments, rat C6 and human U87MG glioma cell lines were exposed to treatments above described for 72 h. Glioma cells were analyzed for cell migration capacity by scratch-wound assay, metaloproteinase-2 (MMP-2) and vimentin expression by qPCR; cell proliferation by counting, colony formation assay, propidium iodide

incorporation, cell cycle for flow cytometry and sensitivity to TMZ by MTT assay. For in vivo experiments C6 glioma cells were implanted in rat Wistar male brain (8 weeks old, 250-300g) and animals were divided in 4 groups, as mentioned above. Treatments were administered via intracerebroventricular at 7th, 14th and 21th days following surgery. Rats were euthanized at 23th and the brain was analyzed by HE. Blood samples and cerebrospinal fluid (CSF) were collected for analysis of purine compounds levels by HPLC and treatment safety assessment. Data were expressed as mean ± SD and were subjected to ANOVA followed by Tukey-Kramer post-hoc test. Differences were considered significant for $p<0.05$. In vitro CD73 downregulation/inhibition decreased glioma cell migration in 20%, which was accompanied by complete absence of MMP-2 expression and a 80% decrease in vimentin expression; induced 40% glioma cell death via necrosis and decreased 40% colony formation index. CD73 manipulation resulted in GBM chemosensitization to TMZ, by decreasing IC50 values in 15% and ADO supplementation (1μM) reversed this effect, suggesting the participation of adenosinergic signaling. CD73 also regulates GBM progression in a preclinical model. APCP or CD73-siRNA treatment decreased in vivo glioma growth by ~40 and 45% respectively and reduced in 95% folds ADO levels in CSF. Finally, treatment did not induce systemic damage, no changes were found in ALT, AST, creatinine, urea levels or histopathological changes in the liver, kidney, lung, spleen and heart of the animals. Data indicate the CD73 is an interesting target for the treatment of GBM and its inhibition may provide new opportunities to improve the treatment of brain tumors.
Keywords: Glioma; CD73; Adenosine.

10 - CD73-SIRNA LOADED CATIONIC NANOEMULSION EXHIBITS IN VITRO ANTIGLIOMA ACTIVITY.

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Glioblastoma (GBM) is the most common malignant brain tumor characterized by high invasiveness, poor prognosis and limited therapeutic options. Gene expression knockdown using RNA interference tool (siRNA) has been proposed as new alternative for cancer therapy. siRNA sequences could be delivered complexed to cationic nanoemulsions (NE), which are safe systems that improve the efficiency and specificity of delivery towards tumor tissues. CD73 overexpression is reported in a variety of tumors, including GBM, and it is related to tumor growth, angiogenesis, metastasis and chemoresistance. The aim of this study was to develop a CD73-siRNA loaded NE (CD73-siRNA-NE) and evaluate its in vitro antiglioma activity. Two new rat CD73-siRNA sequences named siRNA-CD73-961 and siRNA-CD73-980 were designed and complexed to NE, which were used as carriers. Selective cytotoxicity of the complexes against cancer cells was determined in primary astrocyte cultures and C6 glioma cells by MTT assay. The CD73-siRNA-NE complex physicochemical characterization was performed by particle size (PS), zeta potential (ZP), polydispersity index (PI) and transmission electron microscopy (TEM) imaging. Silencing efficiency of CD73-siRNA-NE in C6 glioma was determined by the expression and activity of CD73, using immunocytochemistry and malachite green method, respectively. According to the physicochemical characterization, the data +/0.1-charge ratios for siRNA-CD73-GPF (control): PS 348 ± 12.26 nm, ZP -42.1 ± 4.12 mv, PI 0.302 ± 0.074, siRNA-CD73-961: PS 526.52 ± 82.99 nm ZP -43.69 ± 9.30 mv PI 0.67 ± 0.07 and siRNA-CD73-980: PS 414.8 ± 106, 8 nm PZ-41.7 ± 3.38 PI 0.646 ± 0.093, so the complexes were shown within nano parameters reported in the literature. The siRNA sequences also displayed a good ability of complexation, showing through TEM images that they are well adsorbed at the interface of the NE droplets. In addition, NE-siRNA-CD73 presented low toxicity in normal cells and selectivity to tumor cells. The results for siRNA-CD73-961 demonstrated that the CD73 activity and glioma C6 viability was reduced in knockdown cells by 70% and 20%, respectively. The siRNA-CD73-980 decreased the CD73 activity by 63% and the glioma c6 viability by 35%. Finally, our data indicate the potential of siRNA-CD73 loaded nanoemulsion as a therapeutic alternative to GBM treatment.
Keywords: Glioblastoma ; siRNA-CD73 ; cationic nanoemulsion.

11 - CELLULAR MECHANISMS ASSOCIATED TO THE SPONTANEOUS AND THE MECHANICALLY-STIMULATED ATP RELEASE BY MESENTERY ENDOTHELIAL CELLS.

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Objectives/Background: Part of the vascular cell wall communication system mediated by endothelial cells involves the secretion of extracellular ATP. We ascertained whether the cellular mechanisms related to the spontaneous (basal) ATP release by endothelial cells as well as those mediating the mechanically-evoked ATP secretion have common characteristics. If the mechanisms differ, we aimed at investigating the cellular basis that govern each process and looked for endogenous modulators of these processes. Methods and Results: we assessed and compared the mechanisms participating in the spontaneous and mechanically-stimulated secretion using primary cultures of rat mesentery endothelial cells derived from male Sprague Dawley rat (250 g). ATP/metabolites were determined in the cell media of wells maintained without mechanical stimulation (spontaneous) and other wells were subjected to mechanical stimulation by cell media displacement or a picospritzer buffer puff. Mechanical stimulation increased extracellular ATP that peaked within 1 min (basal ATP values was 47 ± 7 (n=24) increased to 271 ± 29 (n=24) pmol/mg protein, $p<0.0001$) and decayed to basal values in 10 min. Interruption of the vesicular transport route by 10 μM monensin elicited a significant 50% reduction in both the spontaneous and the mechanically-induced ATP release, 33 μM nocodazole reduced 65% the spontaneous and 52 % the mechanically-induced ATP release, but 20 μM brefeldin A only inhibited the spontaneous ATP released (50 %). 30 μM 2-APB, a TRPV agonist increased 100% the spontaneous ATP secretion, but reduced 33% the mechanically-induced ATP release. Pannexin1 or connexin blockers and gadolinium, a Piezo1 blocker, reduced the mechanically-induced ATP release without altering spontaneous nucleotide levels. Moreover, thrombin or related agonists increased extracellular ATP secretion elicited by mechanical stimulation, without modifying spontaneous release. Conclusions: Present results allow proposing that the spontaneous extracellular ATP secretion is

essentially mediated by vesicle-stored ATP, while the mechanically-induced released occurs essentially by ATP transport through connexin or pannexin1 hemichannels. The hemichannels-mediated mechanism is fully supported by results from Panx1-/- knock out rodents. Only the latter component is modulated by thrombin and related PAR agonists, highlighting a novel endothelium-smooth muscle signaling role of this anticoagulant molecule.
Acknowledgements: Funded by FONDECYT grant 1170842; additional funds were provided by CEDENNA
Keywords: vesicular ATP; endothelial cell ATP release; extracellular ATP release mechanisms.

12 - CHANGES IN MYOCARDIAL ENERGY AND NUCLEOTIDE METABOLISM BY RESTRICTION DIET IN THE AGING RAT HEARTS.

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Objectives: Heart metabolism is changing with age that may include response to diet manipulation. Our earlier studies identified that food restriction leads to protection of cardiac function during ischemia in the aged rats. We investigated effect of food restriction and re-feeding on cardiac energy and nucleotide metabolism of the aged rat hearts. Methods and results: Hearts of young (6 months) and old (24 months) Wistar male rats were used in this study with ethical approval. Animals were subjected to four different patterns of restriction- re-feeding cycles. Rats were divided into four groups: food restricted (60% daily intake), food restricted with 2 days of re-feeding or food restricted with 4 days of re-feeding and group fed ad libitum (control). For metabolic analyses hearts were frozen in liquid nitrogen immediately after collection. RP-HPLC analyses for the metabolite content were then conducted. Phosphocreatine (PCr) and creatine concentrations were significantly (about 35%) higher in all groups of old animals in comparison to young rats regardless of diet manipulation. ATP, PCr and NAD concentrations were increased in the hearts of dietary restricted old rats when compared to controls. ATP, PCr, NAD concentrations were respectively: 20.7±0.9**; 58.0±2.0* ; 3.7±0.2* mmol/g dry weight in the restrictive diet group and 17.5±0.6; 47.8 ±3.1; 3.1±0.1 mmol/g dry weight in the control group (mean±SE, n=8; p**<0.01; p*<0.05). No such effects were observed in young rats. Conclusion: Cardiac energy and nucleotide metabolism of the old rat is beneficially modified by restrictive diet manipulation in old rats but not in young rats that could explain protective effect in context of cardiac ischemia. However, these effects are lost if interfered with re-feeding episodes.
Acknowledgements: This research was supported by National Science Centre of Poland (2016/22/M/NZ4/00678) and Foundation for Polish Science (TEAM/2011-8/7).
Keywords: heart metabolism; aging; diet restriction.

13 - CHARACTERIZATION OF ADENINE NUCLEOTIDE METABOLISM IN CELLULAR MODEL OF HUNTINGTON'S DISEASE.

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Huntington's disease (HD) is a neurodegenerative disorder that is caused by expanded CAG repeats within the exon-1 of the huntingtin (HTT) gene. It has been shown that HTT interacts with the proteins involved in the gene transcription, endocytosis and metabolism, nevertheless the biochemical pathways by which mutant HTT causes a cellular dysfunction remain unclear. Thus, this study aimed to established the role of mutant HTT expansion in energy and nucleotide metabolism deteriorations. This study highlights that the mutant HTT expansion may result in an imbalance in energy and nucleotide metabolism of the cell. We examined HEK 293T cell line transfected with plasmids expressing wild-type or mutant exon 1 of the HTT gene. Analysis of intracellular concentration of ATP and NAD, as well as activity of intra- and extracellular enzymes of nucleotide catabolism, such as AMP deaminase (AMPD), adenosine deaminase (ADA), purine nucleoside phoshorylase (PNP) and ectonucleoside triphosphate diphosphohydrolase (eNTPD), ecto-5'-nuclotidase (e5NT), ecto-adenosine deaminase (eADA) were performed using RP-HPLC. Protein concentration was measured with Bradford method. We found diminished intracellular ATP concentration (22.5±1.6 in HD cellular model, 29.3± 1.4 nmol/mg protein in control cells, **p<0.01), increased ADA activity (27.9±0.9 in HD cells, 21.1±1.6 nmol/mg protein/min in control, **p<0.01) and reduced activities of eNTPD (2.4±0.5 in HD cellular model, 5.8±0.7 nmol/mg protein/min in control cells, **p<0.01), e5NT (0.1±0.01 in HD cells; 0.2±0.01 nmol/mg protein/min in control, *p<0.05) and eADA (0.3±0.03 in HD cellular model, 0.4±0.04 nmol/mg protein/min in control cells, **p<0.01) while NAD concentration, AMPD and PNP activities remained unchanged. This study strongly indicates that the mutant HTT expansion may result in an imbalance in energy and nucleotide metabolism of the cell. One may conclude that the improvement of extracellular nucleotide and energy metabolism of HD affected cell may be considered to be attractive therapeutic targets.
This study was supported by the Polish Ministry of Science and Higher Education (MN – 01-0243/08/256).
Keywords: "Huntington's disease"; "adenine nucleotide metabolism".

14 - CHLOROGENIC ACID PREVENTS ALTERATIONS IN LIVER AFTER STREPTOZOTOCIN INDUCED DAMAGE.

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Background/objective: Streptozotocin (STZ) is a toxic compound to the insulin-producing beta cells of pancreas. STZ causes damage to the DNA after it be transported inside the cell by glucose transporter 2 (GLUT 2) that is also present in liver. In addition, Chlorogenic acid (CGA) is a compound that occurs naturally in coffee. CGA is described as anti-inflammatory, antioxidant, neuroprotector, for this reason, CGA would have protector effect in liver alteration. The aim was to investigate the effects of STZ and/or CGA treatment in parameters of purinergic system in liver of mice after 24h and 6 days of administration. Methods: This project was approved by the Ethics Committee (number: 8240230317). Adult male Swiss mice (45 days old; 25±5g, n=50) were randomly distributed in 4 groups, as follows: Group 1: Control Citrate+Control saline (n=12), Group 2: Control Citrate+CGA 100 mg/kg (n=12), Group 3: STZ 200 mg/kg+Control saline (n=13), Group 4: STZ 200 mg/kg+CGA 100 mg/kg (n=13). The experiments were divided in two

protocols. In the protocol 1, mice received a single dose of STZ (200 mg/kg) after 5h of fasting. One hour after STZ administration, the mice of group 2 and 4 received CGA 100 mg/kg i.p.. After 24h STZ administration, the animals were submitted to euthanasia. The protocol 2, the mice received a single dose of STZ (200 mg/kg) at same condition. One hour after STZ administration, mice of group 2 and 4 received CGA 100 mg/kg. At same time daily by 6 days, the animals received CGA 100 mg/kg i.p.. In the last day, the animals were submitted to euthanasia. The liver was processed to NTPDase and Adenosine Deaminase activities according protocols well-established. Statistical analysis was carried out using two-way ANOVA, using p>0,05 as significant difference in the analysis. Results: We find changes in the ATP hydrolysis. The STZ treatment increase the ATP hydrolysis in liver [F(1,15)=11.13, p=0.0045]. ADA was found decreased by STZ treatment [F(1,20)=5,85, p=0.025]. And there was a significant interaction between factors analyzed (CGA versus STZ) [F(1,20)=11.65, p=0.0028]. The results demonstrated that CGA prevented ADA activity decreased promoted by STZ. At protocol 2, ATP hydrolysis was found increased by STZ [F(1,17)=23.02, p=0.0002] and, CGA [F(1,17)=8.75, p=0.0033] treatments, respectively. ADA activity also was increased by STZ in liver [F(1,20)=19.23, p=0.0003]. And, the interaction between CGA/STZ was significant statistically as demonstrated by F value: [F(1,20)=6.36, p=0.02]. Conclusion: The NTPDase and ADA activities was found altered the liver after 24h and 7 days STZ administration, which no is reversed by CGA administration. However, CGA appears to play a role in adenosine metabolism, since the changes promoted by STZ was reversed by the 24-hour and 7-day CGA treatment.
Keywords: Chlorogenic acid, Streptozotocin, NTPDase; Adenosine Deaminase.

15 - CHLOROGENIC ACID PREVENTS ALTERATIONS IN A1R AND A2AR PANCREATIC RECEPTORS AFTER STREPTOZOTOCIN INDUCED DAMAGE.

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Background/objective: Streptozotocin (STZ) is toxic to insulin-producing beta cells of pancreas. STZ causes damage to the DNA leading β-cell to death inducing a hyperglycemic status. In addition, Chlorogenic Acid (CGA) is a compound that occurs naturally in coffee being associated with beneficial effects in the purinergic system. Our question is could CGA have a protector effect in pancreas alteration promoted by STZ. Then the aim of this study was to investigate the effects of STZ and/or CGA treatment in parameters of purinergic system in the pancreas of mice after 6 days of their administration. Methods: This project was approved by the Ethics Committee (number: 8240230317). Adult male Swiss mice (45 days old; 25±5g, n=50) were randomly distributed in four groups, as follows: Group 1: Control Citrate + Control saline (n=6), Group 2: Control Citrate + CGA 100 mg/kg (n=6), Group 3: STZ 200 mg/kg + Control saline (n=6), Group 4: STZ 200 mg/kg + CGA 100 mg/kg (n=6). Mice received a single dose of STZ (200 mg/kg) after 5h of fasting to induced hyperglycemia. One hour after STZ administration, the mice of group 2 and 4 received CGA 100 mg/kg i.p.. At the same time, daily, by 6 days, the animals received CGA 100 mg/kg i.p. In the last day, the animals were submitted to euthanasia. The pancreas was collected and processed to NTPDase and Adenosine Deaminase (ADA) activities as well as A1 and A2A receptors density. Statistical analysis was carried out using two-way ANOVA, using p>0,05 as significant difference in the analysis. Results: We did not find changes in the ATP hydrolysis and ADA activity. However, we found an increase in the density of A1R promoted by STZ [F(1,12)=5,83, p=0.032] and, the interaction between CGA/STZ was significant statistically as demonstrated by F value: [F(1,12)=40.30, p<0.0001]. Furthermore, STZ [F(1,12)=32.09, p=0.0001] decreased the A2AR density and CGA [F(1,12)=38.45, p<0.0001] increased the A2AR density. Conclusion: The density of A1 and A2A receptors were found increased (77%) and decreased (16%), respectively, after 6 days of STZ administration, which was prevented by CGA 100 mg/kg administration. We have demonstrated that this effect could contribute to the beneficial properties of CGA in illness where adenosine status is altered such as diabetes mellitus. Financial Support: CAPES, CNPq and FAPERGS
Keywords: Streptozotocin; Chlorogenic acid.

16 - COMPARISON OF CAFFEINE EFFICIENCY IN PREVENTING MEMORY IMPAIRMENT AND BDNF CHANGES IN A RAT MODEL OF ATTENTION DEFICIT AND HYPERACTIVITY DISORDER FROM BOTH SEXES.

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Objectives / background: Attention Deficit and Hyperactivity Disorder (ADHD) is a neuropsychiatric disorder with worldwide incidence of about 5% in children. The symptoms include inappropriate inattention, impulsivity and hyperactivity, being more diagnosed in boys than in girls due to hyperactivity/impulsivity symptoms. Some genes involved in the maturation of synapses during brain development such as brain-derived neurotrophic factor (BDNF) may also play a role in the etiology of ADHD. The benefits of caffeine on the cognitive impairment of SHR rats (an experimental model of ADHD) have been reported. However, the studies were designed exploring the effect of caffeine mainly in male rats. In this study, the effect of caffeine treatment administered in childhood and / or adolescence on locomotor activities, exploratory activities and recognition memory were evaluated in male and female SHR rats. Besides, BDNF and its related proteins were assessed in the hippocampus. Methods and results: Males and females SHR rats received caffeine (0.3 g/L) in drinking water from postnatal day 15 (PND) up to 28 (40g) or 50 (160g), which corresponds to infancy and adolescence in humans (n=9 litters). Behavioral tests (open field and object recognition task) were carried out between postnatal day 28-30 and 50-52. Hyperlocomotion, recognition and spatial memory disturbances were observed in SHR rats from both sexes. Moreover, the immunocontent of BDNF and TrkB receptors were evaluated by Western Blot. Ethical committee CEUA/UFRGS (Proc. n° 29196). Our data revealed that females showed lack of habituation ($t = 3.823$; $P < 0.05$) and worsened spatial memory ($t = 3.843$; $P < 0.05$). Caffeine restored recognition memory at both sexes ($t = 6.883$; $P < 0.001$, females; $t = 5.943$; $P < 0.001$, males) and females recovered their spatial memory ($t = 2.795$, $P < 0.05$) but showed exacerbated hyperlocomotion [$F(1,25) = 4.373$; $P < 0.05$]. BDNF and the truncated form of TrkB (TrkB-T) receptors increased the immunocontent in the hippocampus of SHR rats from both sexes and caffeine normalized BDNF [$F(2,19) = 4.451$; $P < 0.05$] in males and TrkB-T [$F(1, 44) = 10.57$; $P < 0.01$] at both sexes. Conclusion: Our results confirm beneficial effects of caffeine in improving recognition memory in male SHR and extended its benefits even better to female SHR rats. In addition, the non-associative learning impairment observed only in female SHR rats was reversed by caffeine treatment. Considering different results in male and female SHR rats and distinct responses to caffeine treatment, our data reinforces the importance of including both sexes in further studies with ADHD models. Thus, sex differences in the response to treatments are also important to be investigated. These evidences strongly highlight the potential of

caffeine as an adjuvant or an alternative treatment for ADHD, and a potential target for development of new drugs to the treatment of ADHD. Financial Support: CNPq and CAPES.
Keywords: ADHD; Caffeine; sex differences.

17 - COMPARISON OF GLIOBLASTOMA STEM LIKE CELLS AND GLIOBLASTOMA TISSUE USING GENE EXPRESSION AND PURINERGIC SIGNALING ANALYSES.

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Objectives: Glioblastoma are the most malignant and aggressive brain tumors. Within the tumor, glioblastoma stem like cells (GSCs) are considered to be responsible for tumor initiating, maintaining, recurrence and chemo- and/or radiotherapy resisting. Purinergic signaling is involved in the progression of glioblastoma and showed influence to the chemoresistance behavior. The aim of the present study were genetic analyses on GSCs and glioblastoma tissue using gene expression and purinergic signaling to get more information of GSCs and improve glioblastoma treatment. Methods: Tumor tissue derived explant cell culture and serum-free culture were established. From the serum-free culture, cell subpopulations were isolated by the two stem cell markers CD15 and CD133 through MACS technique. Tumor tissue, serum-free culture, and the isolated cell subpopulations were analyzed by gene expression and purinergic signaling in a paired design. Raw data of all 47,231 gene-expression probes were extracted by Illumina GenomeStudio. All analyses were done in R (R Core Team 2015). We used the add-on package limma to identify differentially expressed genes. For pathway enrichment, we used hypergeometric tests. As pathway references we used GO, KEGG, Reactome, and DOSE. Genes for global pathway analysis were restricted to FDR 5% level on single-marker analysis and being at least twice times over- and under expressed. Results: The gene expression analyses showed considerable differences between tumor tissue, GSCs, and CD133/CD15 sorted cells. In comparison between tumor tissue and CD133+/CD15+ cells, we detected strong up- and downregulated genes. Whereas 418 genes were upregulated in tumor tissue, 44 genes were downregulated in tumor tissue comparing to CD133+/CD15+ cells. Pathway analyses showed that upregulated genes in CD133+/CD15+ cells in comparison to tumor tissue have mostly influence on regulation of cell cycle processes and tumorigenesis. In contrast, upregulated genes in tumor tissue compared to CD133+/CD15+ cells may influence pathways of immunity and diseases due to immunodeficiency. Furthermore, we analyzed 33 purine metabolism involved genes. Statistical analyses (according to p-values) showed 9 of these genes (ABCB1, ADSL, CDK2, CDKN1B, ENTPD1, GMPS, IMPDH1, KRAS, and PTEN) beeing differentially expressed (globally corrected for multiple testing) when comparing tumor tissue, serum free culture, CD133+CD15+ cells, and CD133-CD15- cells. Applying pathway analyses, these 9 genes showed a higher involvement in general tumorigenesis than the non-differentially expressed purine genes. Conclusions: Generally, we detected some differences when comparing GSCs and tumor tissue using gene expression. Some of the analyzed purine genes showed a significant association comparing these cell populations and were mainly involved in tumor related pathways. However, it still needs more studies to identify novel potential targets for therapy development.
Keywords: "GSC"; "purinergic signaling"; "gene expression".

18 DELETION OF CD73 LEADS TO THE SHIFTS OF NAD METABOLISM.

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Objectives/background: Nicotinamide adenine dinucleotide (NAD) is an essential redox carrier, whereas its degradation is a key element of a wide range of signaling pathways. Nicotinamide (NA)- known for its strong anti-inflammatory properties - can be a direct product of many enzymatic reactions, primarily related to NAD catabolism. CD73 – an enzyme of extracellular nucleotide catabolism - degrades NAD to nicotinamide mononucleotide (NMN) and AMP and further to (nicotinamide riboside) NR and adenosine. The aim of the study was to investigate the impact of the CD73 activity absence on the NAD metabolism. Methods and results: 6-month old, male C57BL/6J Wild Type (WT; n=10) and C57BL/6J CD73−/− (CD73−/−; n=10) mice were used for these experiments. Blood and serum were collected and used for the nucleotides and nicotinamide metabolites concentrations as well as enzymes involved in NAD metabolism level. Results are presented as mean ± SEM, unless otherwise indicated. CD73 knock out led to increase in blood NAD+ concentration compared to WT (70.11 ± 3.41 vs. 55.43 ± 5.33 μmol/l; $p<0.05$). Concentration of NA was significantly decreased in CD73-/- mice serum in comparison to WT (0.68 ± 0.04 vs. 0.86 ± 0.06 μmol/l; $p<0.05$). On the other hand, NA metabolites concentrations: N-methylnicotinamide (MetNA), as well as N-Methyl-2-pyridone-5-carboxamide (Met2PY) and N(1)-methyl-4-pyridone-3-carboxamide (Met4PY) were considerably elevated in CD73-/- mice serum as compared to WT. Despite the lack of ecto-5'-nucleotidase activity in CD73-/- mice, concentration of NR was significantly enhanced in CD73-/- serum as compared to WT (0.21 ± 0.02 vs. 0.11 ± 0.02 μmol/l; $p<0.05$). CD73 knock out led to increased poly(ADP-ribose) polymerase 1 (PARP-1), nicotinamide phosphoribosyltransferase (NAMPT) and nicotinamide N-methyltransferase (NMMT) serum levels in comparison to WT. Conclusions: Deletion of CD73 causes substantial changes in the NAD+ and nicotinamide metabolism, which may be involved in the pro-inflammatory phenotype of CD73-/- mice. The increase of NAD+ concentration may be a compensation mechanism aimed at rebuilding the NAD+ pool, after its enhanced degradation. Acknowledgments: This study was supported by National Science Centre of Poland (2015/19/N/NZ1/03435) and The National Centre for Research and Development (STRATEGMED 1/233226/11/NCBR/2015).
Keywords: ecto-5'-nucleotidase; extracellular nucleotides; NAD; nicotinamide metabolism.

19 - DICHLORVOS EXPOSURE AT EARLY STAGES OF DEVELOPMENT ALTERS ECTO-5'-NUCLEOTIDASE AND ECTO-ADA ACTIVITIES IN ADULT ZEBRAFISH (DANIO RERIO) BRAIN. ALTENHOFEN, S.[1]; NABINGER, D.D.[1]; BITENCOURT, P.E.R.[2]; PEREIRA T.C.B.[2]; BOGO, M.R.[2]; BONAN, C.D.[2]

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Background/objective: Organophosphates are a family of agrochemicals chemically derived from phosphoric acid. Dichlorvos is an insecticide of this family that acts by inhibiting the acetylcholinesterase (AChE) activity, an enzyme that degrades the neurotransmitter acetylcholine (ACh) in cholinergic synapses. ACh is a neuromotransmitter released along with ATP in the synaptic cleft. ATP is the signaling molecule of purinergic system, acting on P2X and P2Y specific receptors. It is inactivated by an enzyme cascade thatconsists of cell surface-located enzymes named Nucleoside triphosphate diphosphohydrolases (NTPDases) and ecto-5'-nucleotidases, which hydrolyzes ATP to adenosine. Adenosine exerts its effects through the activation of specific P1-type purinergic membrane receptors. In the synaptic cleft, adenosine undergoes deamination through adenosine deaminase (ADA), producing inosine. The aim of this study was to evaluate the exposure to dichlorvos at the early stages of development (1 hpf – 7 dpf) on the ectonucleotidase and ADA activities in zebrafish brain at 120 dpf. Methods and Results: Embryos were placed in Petri dishes (30 embryos per dish), and subjected to dichlorvos (PESTANAL®, Sigma-Aldrich, St. Louis, MO) exposure at concentrations of 0 (water, control group), 1, 5 and 10 mg/L for seven days (1 hours post fertilization (hpf) to 7 dpf) (CEUA: 13/00354). After, the animals were placed in 3 L-aquariums with water until 120 dpf when the enzyme and molecular assays were performed. Brain membranes were prepared and NTPDase, ecto-5´-nucleotidase, and ADA activities were determined (J Neurochem 61:1685, 1993; Life Sci 73:2071, 2003; Comp Biochem Physiol B Biochem Mol Biol 139:203, 2004), as well as ecto-5'-nucleotidase and ADA mRNA levels. The results showed that dichlorvos exposure, in the first week of life, was not able to alter the NTPDases activities in brain membranes of adult zebrafish. However, this pesticide promoted an increase in ecto-5'-nucleotidase activity at all concentrations tested. Moreover, this pesticide decreased the ecto-ADA activity at 5 and 10 mg/L, but did not alter the cytosolic ADA activity. in brain membranes of adult zebrafish submitted to dichlorvos exposure at early stages of development. The RT-qPCR analysis showed no significant changes in gene expression of ecto-5'-nucleotidase and ADA genes. Conclusion: The findings demonstrated the role of nucleotide and nucleoside-metabolizing enzymes on the toxicological effects of dichlorvos and may contribute to a better understanding about the role of purinergic signaling on the actions induced by dichlorvos exposure in the early stages of development.
Keywords: Adenosine deaminase; Dichlorvos; NTPDases; Zebrafish.

20 - E-ADA ACTIVITY IN LYMPHOCYTES, NEUTROPHILS AND SERUM OF HIV POSITIVE INDIVIDUALS UNDER ANTIRETROVIRAL THERAPY.

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Infection by human immunodeficiency virus (HIV) is characterized by chronic inflammation and persistent immune activation, even under successful antiretroviral therapy (ARV). Upon infection, ATP is released into the extracellular environment, generating adenosine via E-NTPDase and E-5'-nucleotidase, to restrain inflammation. Adenosine levels are regulated by adenosine deaminase (ADA) that modulates the immune response. ADA has been determined as a marker of T cell activation, inflammation, disease progression and senescence. The aim of this work is to evaluate the activity of E-ADA in lymphocytes and neutrophils as well as serum ADA of HIV infected patients under ARV. Methods and results: 58 HIV positive patients (29 men, 29 women) (mean age 45 years) and 68 healthy individuals (40 men, 28 women) (mean age 37 years) participated in this study. The protocol was approved by the Human Ethics Committee from the Federal University of Santa Maria (Protocol number 2.068.790) and all participants have given written informed consent. All patients were under ARV and on viral suppression for at least 12 months. Serum was separated from SST tubes. Lymphocytes and neutrophils were isolated from EDTA blood and separated by gradient density. ADA was determined as described by Giusti & Galanti (Methods Enzym. Anal 4; 315, 1984) and results expressed in U/mL for serum, and in μM NH3/min/mg of protein for lymphocytes and neutrophils. Data were analyzed by Student's t test for independent samples for serum results and expressed as mean±SEM. Mann-Whitney test was used to analyze the results in cells, shown in median±interquatile range. We found that ADA activity was significantly increased (43%) in serum of HIV patients (n=57) compared to the control group (n=68) ($P<0.001$). In lymphocytes, E-ADA activity was reduced by 25% in HIV patients (n=50) compared to controls (n=54) ($P<0.001$). No differences were observed in E-ADA activity among neutrophils of HIV patients (n=41) and healthy individuals (n=54). Conclusion: Increased activity of serum ADA has been linked to the production of inflammatory mediators and immune cell activation. The rise in the activity of serum ADA observed in this study is consistent with residual inflammation and immune cell activation found in HIV patients under ARV. Conversely, decreased activity of E-ADA in lymphocytes is suggestive of impaired lymphocyte proliferation and function and poor immune recovery. Unlike lymphocytes, E-ADA activity in neutrophils was not altered possibly either because the physiopathology of these cells is not altered in patients under ARV or the adenosinergic signaling is not involved in the process. Acknowledgment of financial support: PROIC/UFSM, CAPES
Keywords: Adenosine deaminase; Human immunodeficiency virus; antiretroviral therapy.

21 - EFFECT OF CURCUMIN AND RUTIN ON THE ACTIVITY OF E-ADA IN LYMPHOCYTES AND NEUTROPHILS OF RATS WITH INDUCED HYPERLIPIDEMIA.

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Objectives/Background: Hyperlipidemia is associated with endothelial dysfunction and inflammatory disorders. Adenine nucleotides and nucleosides modulate immune cells during inflammatory processes, and their levels are controlled by ecto-enzymes. Curcumin and rutin are flavonoids with antioxidant, anti-inflammatory, and cholesterol-lowering effects. The objective of the present study is to evaluate the activity of the E-ADA (ecto-adenosine deaminase) in lymphocytes and neutrophils with induced hyperlipidemia and treated with curcumin and/or rutin. Methods and results: Adult male Wistar rats (n=46) (200-250g), obtained from the UFSM Animal House, were pretreated with curcumin and/or rutin (50mg/kg by gavage for 30 days). Animals were divided into two groups: with and without hyperlipidemia (n=23 each). Each group was divided into 4 subgroups: pretreated with saline (n=5), rutin, curcumin and rutin-curcumin association (n=6 each). Hyperlipidemia was induced by a single intraperitoneal injection of 500 mg/kg of Poloxamer-407. Non-hyperlipidemic rats received the same volume of vehicle (sterile 0.9% NaCl solution). Euthanasia was performed 36 hours after induction. Lymphocytes and neutrophils were isolated from peripheral EDTA blood by density gradient. E-ADA was determined by Giusti & Galanti (Methods Enzym. Anal 4; 315, 1984) method. Data were expressed as mean±SEM. Statistical analysis was performed using two-way ANOVA, followed

by Tukey's test (P<0.05). We found that E-ADA activity increased by 80% in lymphocytes and by 89% in neutrophils of hyperlipidemic rats compared to the control group. Pretreatment with rutin increased the activity of the E-ADA by 24% in lymphocytes compared to the control group but reduced by 30% the activity of this enzyme when compared to the hyperlipidemic group. In neutrophils, rutin reduced E-ADA activity by 112% compared to the control group. Regarding the curcumin pretreatment, E-ADA activity in lymphocytes increased by 10% compared to the control group but is 38% lower than the hyperlipidemic group. In neutrophils, curcumin reduced E-ADA activity by 14% in relation to the control group. The rutin-curcumin association did not have significant effect. Conclusion: The alterations observed in E-ADA activity may be due to the inflammatory process caused by hyperlipidemia and the anti-inflammatory action of these compounds. Both compounds had an anti-inflammatory effect as shown by the lower E-ADA activity in neutrophils and lymphocytes when compared to the control group. In neutrophils, rutin was more effective than curcumin in preventing the damage caused by hyperlipidemia. In lymphocytes, curcumin better prevented the damage caused by hyperlipidemia. Pretreatment with rutin-curcumin association did not affect E-ADA activity, probably due to an antagonistic effect. We can suggest that the pretreatment with rutin or curcumin are promising adjuvants in the treatment of hyperlipidemia. Acknowledgment: FAPERGS and UFSM
Keywords: Adenosine deaminase; Hyperlipidemia; Curcumin; Rutin.

22 -EFFECTS OF BLACKCURRANT ON PURINERGIC SYSTEM ENZYMES IN SCOPOLAMINE-INDUCED AMNESIA IN MICE.

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Background/Objectives: Blackcurrant (Ribes nigrum L.), is rich in biologically active natural molecules as anthocyanins (JIA, N. et al. Meat Sci. 91; 533-9, 2012) that could help in the prevention of neurodegenerative diseases. Thus, this study aimed to investigate the possible neuroprotective properties of Blackcurrant on the purinergic system, in a model of memory loss induced by scopolamine in mice. Methods: Adult male Swiss mice (n=66) were randomly distributed in eight groups: Group 1: Control (Saline); Group 2: Blackcurrant 100 mg/kg; Group 3: Donepezil 5 mg/kg; Group 4: Blackcurrant 100 mg/kg/Donepezil 5 mg/kg; Group 5: Scopolamine 1 mg/kg; Group 6: Blackcurrant 100 mg/kg/Scopolamine 1 mg/kg; Group 7: Donepezil 5 mg/kg/Scopolamine 1 mg/kg; Group 8: Blackcurrant 100 mg/kg/Donepezil 5 mg/kg/Scopolamine 1 mg/kg. Blackcurrant and Donepezil were administered orally once daily at doses of 100 mg/kg and 5 mg/kg, respectively. Scopolamine was administered intraperitoneally once daily and at a dose of 1 mg/kg. Treatment with scopolamine and Donepezil was initiated after the 7th day of the first Blackcurrant administration and lasted 21 days. After 28 days of experimental period, animals were anesthetized and submitted to euthanasia (protocol under no. 8343230616). NTPDase, 5'-nucleotidase and adenosine deaminase (ADA) activities were determined in cerebral cortex synaptosomes, according to well-established methodology. Data were analyzed by two-way ANOVA, Tukey's multiple range test ($p<0.05$) and expressed as mean ± SEM. Results: Scopolamine administration decreased NTPDase activity using ATP (Group 5: 24,97±1,686; Group 6: 21,48±1,79; Group 7: 20,59±2,324) and ADP (Group 5: 14,3±1,995) as substrates, when compared to control group (ATP: 39,55±3,526; ADP: 30,81±3,643). There were no significant alterations in 5'-nucleotidase activity (substrate AMP). In addition, scopolamine caused an increased in ADA activity (Group 5: 4,988±0,521), when compared to control group (0,906±0,568). However, treatment with Blackcurrant and/or Donepezil prevented scopolamine effects on NTPDase activity for ATP (Group 8: 26,53±2,559) and ADP hydrolysis (Group 6: 21,89±2,054; Group 7: 28,32±3,453; Group 8: 31,86±3,838), when compared to scopolamine group (ATP: 24,97±1,686; ADP: 14,3±1,995). Moreover, the increase in ADA activity by scopolamine was also abolished following Blackcurrant and/or Donepezil treatment (Group 6: 4,948±0,503; Group 7: 28,32±0,510; Group 8: 3,339±0,584), when compared to scopolamine group (9.06 ± 0.568). For Blackcurrant 100 mg/kg, Donepezil 5 mg/kg, Blackcurrant 100 mg/kg/Donepezil 5 mg/kg control groups no significant changes on enzymes activities were observed. Conclusion: The results of this study showed that Blackcurrant administration was able to prevent scopolamine effects on purinergic system enzymes. These findings are relevant since the regulation of these enzymes could have important therapeutic potential. Financial support: CAPES, CNPq.
Keywords: Anthocyanins; Scopolamine; NTPDase; 5'-nucleotidase.

23 - EXPRESSION OF ECTO-5'-NUCLEOTIDASE/CD73 AND NTPDASE3 IN THE PROGRESSION OF BLADDER CANCER.

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Objectives/background: Bladder cancer is the seventh most common cancer among men in the world. The current treatments for this malignancy are not efficiently enough to avoid the recurrence and progression. For this reason, researchers continue to look for new therapeutic targets that could result in more effective treatments with less side effects. In this work we investigated the NTPDase3 and ecto-5'-nucleotidase / CD73 expression in bladder cancer progression in an vivo model. Methods: Bladder tumor was induced in 20 male Wistar rats, at the age of 8 weeks, weighing around 500g, by the addition of 0.05% of BBN in the drinking water for 4, 8, 12, 18 and 24 weeks (aproved by CEUA HCPA protocol 13-0296). Following, rats bladders were removed and fixed for enzyme histochemistry, immunohistochemical assays for Ki67, and expression analysis for PCR. Results: After 24 weeks, all rats bladder presented histological alterations correspondent to human transitional cell carcinoma. NTPDase3 expression was decreased in this process while ecto-5'-NT/CD73 showed up and was significantly enhanced in cancerous urothelium The ATP and ADP hydrolysis are higher in the control animal bladders than non-tumoral urothelium of animals, which is in agreement with the expression of NTPDase3. PCR analysis showed that mRNA expression of P2X7R is not present in the control urothelium nor cancerous urothelium, whereas A2AR, A2BR, A3R, P2X5R and P2X6R are expressed. Conclusion: Results present here show ecto-5'-NT/CD73 is involved in cancer progression and malignancy, being a promissory target for pharmacological therapy, although more studies are necessary to better understand the influence of purinergic signaling in cancer development. Acknowledgments: CNPq, CAPES, FAPERGS, FIPE/HCPA

24 - FIBROBLAST GROWTH FACTOR 2 MODULATES EXTRACELLULAR PURINE LEVELS BY ENHANCING ATP RELEASE AND PURINE METABOLIC ENZYME EXPRESSION IN RAT SPINAL ASTROCYTES.

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Objectives/background: Extracellular adenosine (ADO) is an important neuromodulator in the central nervous system (CNS), and mediates neuroprotective effect under pathological conditions. In the CNS, extracellular ADO is mainly produced through metabolism of ATP released from astrocytes. In the previous study, we have shown that astrocytes release ATP via gap junction hemichannels (GJ HCs) by extracellular Ca^{2+} reduction, and the released ATP is metabolized to ADO via ecto-enzymes (Eguchi et al. J Pharmacol Sci 128; 47, 2015). It is well known that astrocytes change their morphology and functions under pathological conditions. However, it is unclear whether the astrocytic changes affect ATP release and purine metabolism. Therefore, in this study, we investigated the change of ATP release and purine metabolism in astrocytes treated with fibroblast growth factor 2 (FGF2) which increases under pathological conditions in the CNS. Methods: Cultured spinal astrocytes isolated from new born rat (Wistar, P0-3) were treated with FGF2 for 2 days. Then, astrocytes were incubated with normal or Ca^{2+}-free artificial cerebrospinal fluid (ACSF). Extracellular purine levels were measured by high-performance liquid chromatography (HPLC) analysis and luciferin/luciferase method. For Enzymatic activity analysis of purine metabolism, ATP, AMP or ADO were added to ACSF and those metabolites were measured with HPLC. Results: In control astrocytes, the Ca^{2+} reduction evoked ATP release, resulting in ADO increase by ATP degradation. FGF2 treatment significantly increased ATP release (control: 257.1 ± 21.5 pmol/mg, FGF2: 385.2 ± 37.9 pmol/mg, n = 9-15, $p < 0.01$), and increased the expression and activity of GJ HCs. FGF2 also enhanced the metabolism of AMP to ADO (control: 72.9 ± 2.6%, FGF2: 94.2 ± 1.1%, n = 9, $p < 0.01$) and ADO to inosine (control: 22.2 ± 2.9 μmol/mg, FGF2: 47.1 ± 6.3 μmol/mg, n = 9, $p < 0.01$). The expression levels of ecto-5'-nucleotidase (NT5E) and adenosine deaminase (ADA), AMP and ADO metabolic enzymes respectively, were increased by FGF2. Especially, extracellular ADA (ecto-ADA) activity was increased in FGF2-treated astrocytes. Conclusion: Our results indicate that FGF2 enhances ATP release and purine metabolism by increasing the expression and activity of GJ HCs, NT5E and ADA. Furthermore, it is suggested that FGF2 induces the ecto-ADA activity, although ADA has been considered to mainly act in cytosol in astrocytes. Astrocytes functionally changed by FGF2 may play an important role in controlling extracellular purine levels under pathological conditions.
Acknowledgment: This work was supported by JSPS KAKENHI JP26450440 to K.O. and JP16J02751 to R.E.
Keywords: astrocyte; adenosine deaminase; ATP; gap junction hemichannels.

25 - GUANOSINE INCREASES GLOBAL SUMOYLATION IN BOTH CULTURED NEURONS AND *in vivo*.

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Objectives: The objective of this work was to determine whether guanosine acts as a global SUMOylation modulator in cultured neurons and in vivo. Background: Guanosine is an endogenous guanine nucleoside that has the potential to act as a trophic agent and neuromodulator. Furthermore, evidence from both animal and cell models suggest guanosine has a number of protective effects. The post-translational protein modification SUMOylation can participate in endogenous protective mechanisms, and defective SUMOylation is observed in a diverse array of neurological disorders. We aimed to test the hypothesis that one of the mechanisms of action of guanosine is to modulate SUMOylation. Methods: Primary cortical neurons were prepared from E18 Wistar rats, and maintained in Neurobasal media for 14 days in vitro until treatment with guanosine (1, 10, 100, 300 or 500 μM) for 30 min or 1 hour. For in vivo experiments, 2 year-old male C57BL/6 mice were treated with guanosine (8 mg/kg, i.p.) every day for 14 days. Behavioural tests were carried out on the 7th day: open field, object relocation test, Y maze, splash test, tail test and MWM. On the last day of treatment, both hippocampi were removed and frozen to analyse global levels of SUMOylation. Data were analysed by Student t-test or one-way ANOVA followed by Neuman-Keuls post hoc test. Results: Global SUMO-2/3-ylation levels increased two-fold in neurons treated for 1h with guanosine 10, 100, 300, and 500 μM (n=6 independent experiments; p=0.0019). At 30 min no significant changes in SUMO-2/3-ylation was observed (n=5 independent experiments; p=0.49). SUMO-1-ylation levels in neurons remained unchanged after guanosine treatment for 30 min (n=4 independent experiments; p=0.99) and 1h (n=7 independent experiments; p=0.48). The effects of guanosine on neuronal survival were assessed by MTT assay. There were no significant differences between the control group and guanosine 500 μM at 30 min and 1h (n=3 independent experiments each time point; p=1.0 and p=0.50, respectively). In aged mice SUMO-1-ylation increased in the hippocampus after chronic administration of guanosine (n=6 mice per group; p=0.03). No differences in SUMO-2/3-ylation levels were observed (n=6 mice per group; p=0.43). No behavioural differences between control and guanosine treated groups were observed in any of the tests performed. Conclusion: We show that guanosine increases SUMOylation in both cultured neurons and in vivo and, to our knowledge, this is the first report showing that guanosine regulates SUMOylation.
We thank CAPES, CNPq and the Royal Society Newton Fund for financial support.
Keywords: SUMOylation; Guanosine.

26 - A3 ADENOSINE RECEPTOR ACTIVATION MECHANISMS PREDICTED USING MOLECULAR DYNAMICS ANALYSIS OF INACTIVE, ACTIVE, AND FULLY ACTIVE STATES.

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Objectives / background: Human (h) A3 adenosine receptor (AR) agonists have potential application in chronic neuropathic pain and as anti-inflammatory, anticancer, and cardioprotective agents. However, the lack of a three-dimensional structure of the hA3AR or of a close homologue G protein-coupled receptor (GPCR) prevented a detailed atomic level investigation of receptor activation mechanisms. Here we model different conformational states of the hA3AR and their behavior using molecular dynamics (MD). Three different A3AR states (R, R* and R*G) were compared with previously identified constitutively active mutant (CAM) A3ARs. Our hypothesis is that the mutations maintain the hA3AR in an active conformation able to couple with the Gi protein. Methods and results: We investigated the activation mechanism of the Gi-coupled A3AR by running 7.2 μs of MD simulations. Homology models were constructed based on GPCR X-ray structures in the apo form for three CAMs and the wild-type (WT) A3AR. The three CAMs

were A229E6.34 (number in superscript refers to Ballesteros-Weinstein notation), R108A3.50, and R108K3.50. Conformational signatures associated with three different receptor states (inactive, active, and bound to Gi protein mimic) were predicted by analyzing and comparing the CAMs with WT receptor and by considering site-directed mutagenesis data available. Persistent salt-bridges involving key residues for activation (including a novel, putative ionic lock), Na+ ion coordination and water molecules were tracked and correlated with the receptor state. Conclusion: Coupling signatures similar to the X-ray structures of the β2 adrenergic receptor-Gs protein and A2AAR-mini-Gs complexes were detected. Our MD analysis suggests that constitutive activation might arise from the destabilization of the D1073.49-R1113.53 ionic lock in R that presumably lowers the energy barrier associated with the transition to the R* state. This study provides new opportunities to understand the activation mechanism of the A3AR and of other related GPCRs. Financial support from the NIDDK Intramural Research Program is acknowledged.
Keywords: drug discovery; GPCR structure; computational modeling; A3 receptor.

27 - ADENOSINE A[1] RECEPTORS INHIBIT EJACULATION AT MULTIPLE SITES.

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Background/Objectives. Adenosine modulates different aspects of male sexual function such as sperm capacitation, penile erection and contractility of male sexual accessory organs. Adenosine A1 receptors (A1R) are widely expressed in brain and spinal cord regions implicated in the modulation of ejaculatory reflex but a regulatory role for the A1R on ejaculation reflex was not investigated before. This study evaluates the role of A1R on ejaculation through a pharmacological analysis of selective A1R ligands effects on different in vitro and in vivo models of ejaculation reflex in rats. Methods. All the experimental procedures were approved by the Institutional Ethics Committee for the Use of Experimental Animals of UNICAMP (process: 4074-1). Adult male (120-180 days old) and female (60-120 days old) Wistar rats were used in the different experiments. In vitro contraction studies. The seminal vesicles (SV), vas deferens (VD) and cauda epididymis (CE) were mounted in 10 ml organ baths to evaluation of isometric contractions and the effects of A1R agonist N6-cyclopentyladenosine (CPA) and antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) on VD, SV and CE contractions induced by electrical field stimulation were evaluated. Additionaly, the effects of in vivo administration of CPA and DPCPX on ejaculation induced by the dopaminergic D3 receptor agonist 7-hydroxy-dipropylaminotetralin (7-OH-DPAT) in urethane-anesthetized rats and on ejaculation in copula were investigated. Data are presented as mean ± sem. Results. In vitro CPA administration inhibited the neurogenic contractions of SV, CE and VD smooth muscle. The CPA inhibitory effects on SV, VD and CE neurogenic contractions were antagonized with high potency by DPCPX (pA2SV: 8.82±0.10, n=4; pA2VD: 8.92±0.30, n=4; pA2CE: 9.16±0.07, n=4) showing A1R-mediated effects of CPA. In vivo administration of CPA (3.0 and 10 ug/kg, iv) reduced the VD contractions, seminal emissions and ejaculations induced by 7-OH-DPAT (100 ug/kg, iv) administration to anesthetized rats and these effects were prevented by DPCPX (30 ug/kg, iv). Administration of CPA (1.0 and 3.0 ug/kg, iv) have no effect on copulatory behavior of male rats but the A1R antagonist DPCPX (30 ug/kg, iv) facilitated the ejaculation decreasing by 53% the ejaculatory latency and by 42% the number of intromissions required for the ejaculation. The DPCPX effects on in copula ejaculation were prevented by the co-administration of CPA (3.0 ug/kg, iv). Conclusions. Altogether, our results show that A1R activation at multiple sites has inhibitory effects on ejaculation highlighting the A1R as a new player in the physiological control of ejaculation reflex. Finantial Support: FAPESP (2015/19677-6).
Keywords: A1R; ejaculation; fertility; smooth muscle.

28 - ADENOSINE AS A POSITIVE REGULATOR OF VASA VASORUM BARRIER FUNCTION IN PULMONARY ARTERIAL HYPERTENSION.

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The vasa vasorum (VV) is a microcirculatory network that provides oxygen and nutrients to the adventitia and media of large blood vessels. Our previous studies demonstrated that neovascularization of the VV network occurs in the pulmonary artery (PA) of chronically hypoxic hypertensive calves. This hypoxia-induced VV neovascularization associates with the impaired endothelial barrier function and perivascular inflammation caused by infiltration of circulating inflammatory cells to the PA wall (1). Adenosine is known for anti-inflammatory and a barrier protective effect (2, 3). We showed that adenosine has a barrier-protective role in VV endothelial cells (VVEC). It involves activation of A1 receptors (A1R), Gαi/PI3K/Akt pathway and cytoskeleton remodeling, however, detailed signaling mechanisms of A1R-mediated barrier enhancement remain not fully elucidated (4). In present study using a small interference RNA (siRNA) technique and Transendothelial Electrical Resistance (TER) assay, we showed that adaptor protein ELMO, regulatory Rac1, and PAK1 proteins can operate downstream of Gαi and mediate adenosine-induced VVEC barrier strengthening. The additional pathway involves Gαi-mediated activation of GAB1 and Shp2, which can lead to PKA activation in cAMP-independent manner, resulting in TER increase. The regulatory cross talk between these pathways may involve Rac1-dependent PKA activation leading to subsequent cytoskeletal changes and barrier protection. Interestingly, we found that actin-interacting GTP-binging protein, Girdin, is not involved in adenosine-mediated VVEC barrier regulation. In a Sprague Dawley rat model of hypoxic PAH, we show that A1R/A2AR agonist, NECA decreased Fulton index (RV/LV+S), right ventricular systolic pressure (RVSP), pulmonary vascular remodeling, and infiltration of inflammatory cells into the lung and PA wall, to a similar extent observed in response to treatment with sildenafil. Together, our data identified adenosine-stimulated signaling pathways in VVEC leading to barrier enhancement via novel mechanism of cAMP-independent PKA/Rac1 activation and proposed that A1R may represent novel pharmacologic target for treatment of PAH and other cardiovascular diseases.
*These authors have an equivalent contribution to this project
Support: P01 HL101902 (A.D. Verin); R01 HL086783 (E.V. Gerasimovskaya)
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Fan Z, Zemskov EA, Alieva IB, Black SM, and Verin AD. Vascular pharmacology. 2010;52(5-6):199-206. Umapathy SN, Kaczmarek E, Fatteh N, Burns N, Lucas R, Stenmark KR, Verin AD, and Gerasimovskaya EV. PLoS One. 2013;8(4):e59733.
Keywords: "Vasa vasorum"; "A1R signaling"; "endothelial barrier"; "PAH".

29 - ADENOSINE MAGNIFIES GAMMA RADIATION TOXICITY IN ZEBRAFISH LARVAE *(Danio rerio)*.

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Background and objectives: Exposure to radiation during radiotherapy can be toxic and often limits its effectiveness (Chargari et al. Presse Med. 42; 342, 2013). Gamma rays damage the DNA, alter cell proliferation and generate reactive oxygen species (Wang et al. Trends Pharmacol Sci. 39; 24, 2018). Zebrafish is a teleost suitable for the studies of human diseases and toxicology, which express the purinergic system components, including adenosine receptors (Cruz et al. Comp Biochem Physiol C Toxicol Pharmacol. 194; 28, 2017). Adenosine, activating P1 receptors can promote tissue protection and repair from excessive inflammation (Jacobson et al. Nat Rev Drug Discov, 5(3); 247, 2006). The aim of this work was to evaluate the effect of adenosine on gamma radiation-induced toxicity. Methods and results: In a 12 well plate, AB wildtype zebrafish with 24 hpf (25 larvae per group, n=3) were treated with 3 different concentrations of adenosine (1, 10 and 100 μM) for 30 min, and were after gamma irradiated at the dose of 15 Gy using a Cobalt Theratron Phoenix equipment (Theratronics Ltd, Ontario, Canada). A survival curve was performed for 7 days, and no significant mortality was observed. At 48 hpf, animals heart rate was counted for 1 min using a stereomicroscope. Gamma radiation increased the number of heart beats per minute (141.3±1.555), and the joint treatment with 1, 10 or 100 μM adenosine potentiated this effect (157.1±15.590; 162.4±1.758; 165.4±1.680, respectively). It was required 10 larvae per group (n=3). In order to evaluate morphological defects, 6 dpf larvae were observed in a stereomicroscope. For measuring the body length and ocular circumference, it was used NIS-Elements D software for Widows 3.2 (Nikon Instruments Inc., Melville, USA). Animals treated with 100 μM adenosine before irradiation presented a significant decrease in body length (2740±24.54) and ocular circumference (33090±487.6). This group also presented an increase of pericardial edema (85.9±2.985%). For those measures we used 10 larvae per group (n=3). Locomotor patterns such as distance, mean velocity and turn angle were evaluated using Ethovision Software®. Adenosine treatments did not alter the effects of radiation in the analyzed parameters. This experiment was made using 20 larvae per group. Statistical comparison was performed by Kaplan-Meier method for the survival curve, and one-way ANOVA followed by Tukey's test for the others experiments. Results are expressed as mean ± S.E., and $P<0.05$ was considered as significant. All protocols were approved by the Institutional Animal Care Committee (SIPESQ/CEUA: 7683, 2017). Conclusion: Our results suggest that adenosine potentiates the toxic effects of gamma radiation treatment in zebrafish larvae. Further experiments will be performed to investigate which adenosine receptors are majorly involved, and the mechanism of this increased toxicity. Financial support: PUCRSINFRA, CAPES, CNPq and FAPERGS.
Keywords: Adenosine receptors; Gamma radiation; Zebrafish.

30 - ADENOSINE REDUCES REACTIVE OXYGEN SPECIES AND INTERLEUKIN-8 PRODUCTION BY *TRICHOMONAS VAGINALIS*-STIMULATED NEUTROPHILS.

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Objectives/background: *Trichomonas vaginalis* is a flagellated protozoan that affects the human urogenital tract causing 276.4 million new infections a year. The parasite elicits a vaginal mucosal infiltration of immune cells, especially neutrophils which are considered to be primarily responsible for cytological change observed at the infection site as well as the major contributor in the inflammatory response against the parasite. Extracellular nucleotides and their nucleosides are signaling compounds involved in several biological processes, including inflammation and immune responses. Once in the extracellular space the nucleotides and nucleosides can directly activate the purinergic receptors. Methods and results: Herein we investigated the involvement of purinergic signaling on the production of reactive oxygen species (ROS) and cytokines by *T. vaginalis*-stimulated neutrophils. Parasites were able to induce an increase in ROS and IL-8 levels while they did not promote IL-6 secretion or neutrophil elastase activity. Adenine and guanine nucleotides or nucleosides were not able to modulate ROS and cytokine production; however, when *T. vaginalis*-stimulated neutrophils were incubated with adenosine and adenosine deaminase inhibitor the levels of ROS and IL-8 were significantly reduced. These immunosuppressive effects were probably a response to the higher bioavailability of adenosine found in the supernatant as result of inhibition of enzyme activity. The involvement of P1 receptors was investigated by immunofluorescence and A1 receptor was the most abundant. Conclusion: Our data show that the influence of purinergic signaling, specifically those effects associated with adenosine accumulation, on the modulation of production of proinflammatory mediators by *T. vaginalis*-stimulated neutrophils contribute to the understanding of immunological aspects of trichomoniasis. Ethical approval: Human blood samples (CEP/UFRGS: CAAE 47423415.5.0000.5347). Reference: Frasson AP, Menezes CB, Goelzer GK, Gnoatto SCB, Garcia SC, Tasca T. Purinergic Signal. 13;569, 2017. Acknowledgments: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil) grant #474930/2012-2. Authors thank Centro de Microscopia e Microanálise, CMM/UFRGS, for technical assistance in the confocal microscopy. T.T. thanks CNPq for researcher fellowship #307447/2014-6.
Keywords: *Trichomonas vaginalis*; reactive oxygen species; interleukin-8; adenosine.

31 - ADENOSINERGIC DRUGS CONTROL STRIATAL METAPLASTICITY IN MICE WITH L-DOPA-INDUCED DYSKINESIA.

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Objectives: The aim of this study was to evaluate the effects of adenosinergic drugs on striatal metaplasticity in dyskinetic mice induced by L-DOPA. Methods and results: C57BL/6 male mice (8-12 weeks) were treated with 6-hydroxydopamine (6-OHDA, 3 μg in 1 μL of 0.02% ascorbic acid diluted in

0.9% NaCl), in two different regions of the right mid-striatum (2 x 2 μL, 0.5 μL/min) to mimic a hemiparkinsonism. After 4 weeks, they were challenged with R(-)-apomorphine (0.6 mg/kg, subcutaneous), to confirm the lesion. During 30 days the animals received a daily intraperitoneal (i.p.) injection with L-DOPA (25 mg/kg) plus benserazide (12.5 mg/kg). The animals were then sacrificed and their brain removed to prepare striatal coronal slices (400 μm) used to measure corticostriatal transmission and synaptic plasticity assessed (WinLTP 2.20b Reanalyzes® software) 30 min after applying a high frequency stimulation protocol (HFS: 100 Hz, 3 times, every 20 seconds). We compared control and 6-OHDA-lesioned striatal slices in the absence and presence of either the selective adenosine A2A receptor antagonist SCH58261 (50 nM) or the analogue of adenosine 2-chloroadenosine (2-CADO, 100 nM) (n=8-12 independent preparations per group). The slices from mice with L-DOPA-induced dyskinesia displayed a shift in striatal plasticity to long-term potentiation (LTP) in control and 6-OHDA side in comparison to both sides in animals who received saline ($F_{3,76}$= 55,11, $p<0.05$). Notably, the incubation of striatal slices from dyskinetic mice with SCH58261 or 2-CADO induced long-term depression (LTD). Conclusion: These findings demonstrate that animals with L-DOPA-induced dyskinesia display alterations in striatal metaplasticity that can be modulated by the blockade and activation, respectively, of adenosine A2A and A1 receptors. Altogether, these results reinforce the potential of adenosinergic drugs in the treatment of L-DOPA-induced dyskinesia. Acknowledgment: Fundação de Amparo à Pesquisa e Inovação do estado de Santa Catarina (FAPESC); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); Santa Casa da Misericórdia; Maratona da Saúde.
Keywords: Adenosinergic drugs; dyskinesia; metaplasticity.

32 - CHRONIC CAFFEINE INTAKE PLUS PHYSICAL EXERCISE IMPROVES EMOTIONAL IMPAIRMENT AND INCREASES SYNAPTIC PROTEIN DENSITY IN THE HIPPOCAMPUS OF SHR RATS, AN ANIMAL MODEL OF ATTENTION DEFICIT HYPERACTIVITY DISORDER (ADHD).

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Objectives / background: Attention-deficit/hyperactivity disorder (ADHD) is a persistent neurodevelopmental disorder that affects 5% of children and adolescents and 2.5% of adults worldwide. In addition to classic symptoms (inattention and/or hyperactivity–impulsivity) many psychiatric comorbidities are associated to ADHD, such as depression and anxiety. Nowadays, the pharmacological treatments for ADHD are palliative and focused in the improvement of behavioral deficits, with low efficacy on emotional impairments. The current study was conducted to evaluated the effects of the association of caffeine (a non-selective adenosine receptor antagonist) plus physical exercise as therapeutic strategy to improve behavioral and neurochemical impairments observed in an animal model of the ADHD, the spontaneously hypertensive rats (SHR). Methods and results: Adolescent male inbred SHR (30 days old) were assigned randomly to a sedentary control group or a voluntary exercise group (equipped with a running wheel). Both groups received water or caffeine (0.3 mg/ml in drinking water) during six weeks (n=10/group). After chronic caffeine intake plus voluntary running, a battery of behavioral tests was conducted. After behavioral studies, the animals were euthanized for biochemical assays by Western blotting analysis. In the forced swimming test, chronic caffeine intake ($F_{1, 36}$=27,785, $p<0.05$) and physical exercise ($F_{1, 36}$=53,703, $p<0.05$) reduced the immobility time. Post-hoc Dunnett comparisons revealed a significant reduction in the immobility time in the animals from the caffeine, exercise and caffeine plus exercise groups in comparison to control group (vehicle-treated), indicative of an antidepressant effect. Chronic caffeine intake plus physical exercise did not alter anhedonic- and anxiety-like behaviors. Concerning the synaptic proteins density in the hippocampus, two-way ANOVA showed a significant caffeine x exercise interaction effect ($F_{1, 19}$=6,9040, $p<0.05$) on SNAP-25 immunoreactivity. There was a significant main effect of chronic caffeine intake ($F_{1, 20}$=5,8690, $p<0.05$) on syntaxin immunoreactivity. However, there was no interaction effect ($F_{1, 20}$=3,3517, $p=0.08208$) in this parameter. There was no significant effect of caffeine ($F_{1, 20}$=2,6961, $p=0.11622$) and exercise ($F_{1, 20}$=,12135, $p=0.73121$) on synaptophysin immunoreactivity. Conclusion: This study provides the first evidence of beneficial effects of chronic caffeine intake plus physical exercise on emotional impairment observed in an animal model of the ADHD. In addition, it was associated to synaptic modifications in the hippocampus, namely increases in the SNAP-25 and syntaxin density. Ethics committee on the use of animals (Protocol: Ceua-Ufsc PP0830). Acknowledgments: Ufsc, Capes, Cnpq, Fct.
Keywords: Adhd; caffeine, shr, exercise.

33 - EPITHELIAL MESENCHYMAL TRANSITION AND MIGRATORY ABILITY IN OVARIAN CARCINOMA CELLS ARE REGULATED BY A2B ADENOSINE RECEPTOR.

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Extracellular nucleotides are signaling elements present in the tumor microenvironment; in previous studies, we described that hydrolysis of extracellular ATP (exATP) by apyrase (Apy) inhibits migration and favors an epithelial phenotype in SKOV-3 ovarian cancer cells. A pharmacological characterization of this effect suggested that the reduced migration was consequence of extracellular adenosine (ADO) accumulation (Martínez Ramírez et al. J Cell Biochem 2017,118(12):4468). The aim of the present study was to define the ADORA receptor mediating ADO actions and the cellular mechanisms involved. Thus, we did cellular migration analysis applying pharmacological tools in SKOV-3 cells and ADORA2B receptor was over-expressed. To have a better idea on the ADO´s mechanism of action, micro arrays were done in stimulated cells with ADO, and the results were analyzed with a gene ontology strategy. By scratch assay, we found that ADORA2B activated with the selective agonist BAY-606583, diminished cell migration (1μM 69.8 ± 5.3, n=3; 10μM 62.4 ± 2.6, n=3; control 100 ± 0, n=3). We also over-expressed ADORA2B using the plasmid pEYF-N1-A2BR. The results indicated that ADORA2B over-expression in SKOV-3 cells reduce migration (A2BR 41.2 ± 6, n=14; control 100 ± 3.6, n=15). To evaluate cell migration capacity in a physiological context, we inoculated SKOV-3 cells over-expressing ADORA2B in chick embryo chorioallantoic membrane (CAM); preliminary results showed that ADORA2B overexpression decreased SKOV-3 cell migration in this essay. In addition, SKOV-3 cells incubation with NECA, a non-selective ADORA agonist, induced relocation of E-cadherin suggesting the promotion of an epithelial phenotype. Al together, these results highlight ADORA2B as a receptor involved in the modulation of cell migration. To further explore the mechanism mediating ADO effects on SKOV-3 cells we performed a microarray with a library of cDNAs of 35K from the whole human genome in cells stimulated with 100 μM ADO by 12 h. The treatment with ADO importantly reduced the expression of

WNT2, 6 and 10B and FGF18, whose signaling pathways are involved in EMT in ovarian cancer. In contrast, ADO treatment increased the expression of ARPC4 and RAPGEF1 transcripts, which are associated with cytoskeleton rearrangement. In parallel, a microarray was made in SKOV-3 cells incubated with 10 U/mL Apy, to remove ex-ATP and promote the generation of ADO. This treatment decreased the expression of FGF4, WNT3 and RHOF while enhanced PTK2 and CFL2 expression. Interestingly, both treatments reduced RAC1, a small GTPase linked to migration in ovarian carcinoma cells while augmented ARHGAP4, a gene that codifies for FilGAP, a GAP protein that inhibits Rac1. Overall, our results indicate new elements of ADORA2B-nediated adenosinergic pathways that mediate cellular migration in cancerous cells. Funding by PAPIIT-UNAM IN201017. We are grateful with Adriana González by technical assistance.
Keywords: adenosine A2B receptor; cellular migration; ovarian carcinoma.

34 - ABSENCE OF PURINERGIC P2X7 RECEPTOR ALTERS THE PATTERN OF ILEAL CONTRACTION IN MICE.

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Introduction: In intestinal smooth muscle, acetylcholine (Ach) produces contraction by activating muscarinic receptors. The muscarinic contraction generally is mediated via M3 subtype receptor and depends on Ca^{2+} entry via Ca^{2+} channels opened inducing membrane depolarization. The purinergic system influences intestinal motility in mammals being mediated by purine nucleotides and nucleosides via purinergic receptors. In particular, the ionotropic P2X7 receptors are found on different types of cells within the gut, as well as in neurons of the enteric nervous system which regulates the intestinal motility. The present work aimed to investigate if there is any change in the contraction in response to Ach of the ileum strip in P2X7-/- mice. Methods: Animals were anesthetized. Segments of terminal ileum (1 cm long) were removed from C57BL/6 (WT) and P2X7-/- mice (2-3 months old) of either sex. The longitudinal muscle strips were vertically mounted in a 5-ml organ bath filled with Krebs-Ringer solution (in mM; NaCl 118.3; KCl 4.7; CaCl2 2.5; MgSO2 1.2; KH2PO4 1.2; NaHCO3 25; glucose 11.1, pH 7,4) which was bubbled with carbogen (5% CO2 and 95% O2) at 37 oC. The tissues were equilibrated under a tension of 1 g (~ 10 mN) for 30 min until baseline tension was stable. At the end of the equilibration period, to test tissue viability the ileum was contracted by adding KCl (100 mM) for ~5 min and 10 uM carbachol. Then the tissue was washed twice with the warm aerated Krebs solution in an interval of 15 min. Dose-response curves to Ach (0.01 – 30 μM) were constructed. The contractile responses were measured using an isometric force transducer and expressed as mN. Data were analyzed by nonlinear regression to estimate potency (the agonist concentration that produces 50% of the maximal effect, EC50) and efficacy (Emax). For concentration-response curves, statistical analysis was performed using two-tailed unpaired t-test ($P < 0.05$). Results are presented as mean ± SEM (n=4). Results: It was found that Ach produced a dose-dependent increase in contraction in both WT and P2X7-/- mice ileum. However, there was a significant difference in the Emax values for Ach-induced contraction between the WT (1.98 ± 0.01 mN, n=3) and P2X7-/- (4.20 ± 0.48, n=4, p = 0.01) groups, showing a higher contraction effect on the ileum of the P2X7-/- animals than on the WT. Otherwise, there was no significant difference in the EC50 values for Ach-induced contraction between the groups, being 1 uM and 1.3 uM for WT and P2X7-/- mice, respectively (P = 0.66). According Antonioli et al. (2014), under normal conditions, P2X7 receptors acting at neuronal level has an effect in a tonic inhibitory control on excitatory cholinergic motility which could explain to a greater contraction induced by Ach observed on the ileum of the P2X7-/- animals. Conclusion: These initial studies suggest that P2X7 receptor plays an important role in modulating cholinergic-induced ileal contraction in mice.
Keywords: isolated ileum; intestinal motility; purinergic signaling.

35 - ATP AND CALCIUM OSCILLATIONS IN HUNTINGTON'S DISEASE: TARGETING NEURAL STEM CELLS.

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Background: Purinergic receptors have been attributed with developmental functions including gastrulation and neural differentiation. Upon activation, P2 purinergic receptors trigger intracellular calcium transients controlling cellular processes. Huntington's disease (HD) is a genetic neurodegenerative disease caused by the loss of GABAergic neurons (GABANs) from the basal ganglia and the symptoms onset start around the third decade of life spam. Thus recent studies propose that the disease actually starts much earlier, and HD is a neurodevelopmental disease. For clarifying this correlation, we induced GABAN differentiation of neural stem cells derived from a HD patient and recorded the intracellular calcium oscillations during the process of differentiation and compared to ATP-promoted response and cell death rates. Methods and results: First of all, P2Y2 receptor stimulation along differentiation of mouse embryonic stem cells increases the efficacy of GABANs differentiation (27±5 vs 42±2.2, n=3) by boosting the frequency of spike-like calcium oscillations. Using a technique that couples the imaging of alterations in cytosolic calcium concentration by Fluo4-AM (calcium imaging) and Ascl-1 or Neurogenin 2 by luciferase activity (stable transfected cells with Ascl-1 or Ngn2 promoter-protein fusion to luciferase reporter construct), we observed the effectiveness improvement was due to prolonged expression of Ascl-1. In view of that, we investigated HD-NPC and control NPC differentiation patterns. HD-NPCs differentiated from patient iPS cells did not reveal any spike-like oscillations, while they showed increased cell death / apoptosis rates when compared to the healthy donor (30±8.3 vs 8.6±4.1, n=3), despite of caspase3/7 activation. Moreover, HD NPCs challenged with ATP showed higher amplitude of cytosolic calcium transients if compared to the healthy donor (4.2±0.4 n=51 vs 2.4±0.06 n=35). Blockade of intracellular calcium mobilization by P2Y2 receptors using thapsigargin could not prevent cell death. Thus, NPC differentiation status was changed, by decreasing the pool of undifferentiated NPCs when calcium oscillations were blocked, indicated by nestin expression detected by flow cytometry (90.7±1.1vs 48.4±10.4, n=3). Conclusion: Altogether these data suggest that P2Y2 receptor activation or inhibition modulates spontaneous calcium oscillations during neural differentiation and consequently changes the expression pattern of Ascl-1, thus controlling the cell fate decision to GABAergic neurons. This process is altered in HD patients' cells, compromising the pool of NPCs during early nervous system development. Acknowledgment: This research is financially supported by FAPESP and CNPQ.
Keywords: P2Y2; spike-like oscillations.

36 - BOTH P2X7-KO AND CD73-KO MICE DISPLAY NORMAL HEMATOPOIESIS IN NON-STRESS MEDIATED STEADY STATE CONDITIONS.

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Background/Objectives: We recently reported that mice that are deficient for P2X7 purinergic receptor and CD73 ectonucleotidase mobilize hematopoietic stem progenitor cells differently in response to administration of granulocyte colony stimulating factor (G-CSF). We found that while P2X7-KO mice are poor mobilizers, in contrast mobilization is significantly enhanced in CD73-KO animals. Based on these findings we become interested in steady state conditions status of hematopoiesis in these mutant animals. Methods/Results: Pathogen-free, 4–6-week-old C57BL/6J wild-type (WT), B6.129P2-P2rx7tm1Gab/J (P2X7–/–), and B6.129S1-Nt5etm1Lft/J (CD73–/–) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). We measured in these animals i) values of peripheral blood counts (HB, leucocytes, monocytes, lymphocytes, platelets), ii) number of circulating in peripheral blood and residing in bone marrow hematopoietic stem cells by employing FACS (Sca-1+Kit+Lin- and Sca-1+CD45+Lin- cells), and iii) number of clonogeneic progenitors (CFU-Mix, CFU-GM, BFU-E, CFU-E) in bone marrow by employing in vitro colony assays. We noticed that in steady state conditions both mutant mice display normal hematological parameters as compared to wild type normal littermates. Conclusion: Despite a fact that purinergic signaling plays an important role in development of hematopoietic cells as well as in response to stress situations mice that do not express P2X7 receptor as well animals that do not express cell surface ectonucleotidase CD73 have normal hematopoietic parameters. Hematopoietic defects visible in defective release of HSPCs from bone marrow into peripheral blood occur first after induction of mobilization by G-CSF. Currently we are testing if sublethal irradiation of these animals (650 cGy) will reveal delay in recovery of hematopoietic parameters. This work was supported by NIH grants 2R01 DK074720 and R01HL112788.
Keywords: "P2X7-KO mice"; "CD73-KO mice".

37 - BRADYKININ EFFECTS ON P2X7 ISOFORMS TO REGULATE STEMNESS AND CELL INVASION IN NEUROBLASTOMA.

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P2X7 receptor has been shown to induce tumor proliferation as well apoptosis in cancer cells, depending on which of its isoforms are activated. The isoform A (P2X7A) has a C-terminal important for opening membrane large pores that leads to apoptosis whereas the truncated isoform B (P2X7B) has a reduced C-terminal, inducing proliferation of tumor cells. Recently, our group showed that neuroblastoma (NB) cells treated with bradykinin (BK) presented higher expression of both isoforms, favoring the upregulation of P2X7B and the metastatic behavior of NB cells. Therefore, we hypothesized that P2X7 overexpression retains cells in the NB mass with a stem cell phenotype (cancer stem cells – CSC), increasing tumor aggressiveness, chemo- and radiotherapy resistance, and metastatic potential. Aiming to investigate the contribution of isoforms A and B in the maintenance of CSC in NB, we used human ACN NB cells with knocked down P2X7A (P2X7A-/B+) and P2X7A plus B (P2X7A-/B-). First of all, we observed by flow cytometry that P2X7A+/B+ tumorspheres express pluripotency markers (Sox-2 and CD133). Afterwards we accessed the best medium supplementation for enrichment of CSCs in tumorspheres. Interestingly, cells supplemented with EGF/bFGF/N2 resulted in increased tumorsphere formation and more cells positively stained for CD133 and Sox-2. Thereafter, adherent cells (low CSC rate) showed a decreased invasion which was less pronounced for P2X7A-/B+ cells. Despite P2X7A-/B+ cells, all BK primed cells showed enhanced invasion by chemotaxis assay. In contrary to BK priming in tumorsphere-derived cells. Our findings suggest that CSC have to go through a differentiation process firstly to acquire an invasive phenotype. Also, P2X7B plays an important role in the invasion of adherent ACN cells and BK plays a prime effect on the truncated isoform B invasion process induction. Furthermore, our results have indicated that the absence of the P2X7R showed to be important to revert the invasive capability. Financial Support: FAPESP
Keywords: P2X7 isoforms; cancer stem cell; neuroblastoma; invasion.

38 CANNABINOIDS INDUCE CELL DEATH AND MODULATES P2X7 RECEPTOR-MEDIATED CALCIUM RESPONSES IN DEVELOPING RETINAL CELL CULTURES.

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P2Y1 and 13 receptors are required to late retinal progenitors proliferation (Jacques et al. Cell Signal. 35:95-106, 2017). P2X7 receptors are involved with retinal new born neurons cell death (Anccasi et al. Purinergic Signal. 9(1):15-29, 2013). CB1 and CB2 receptors are important for a wide range of physiological phenomena in the brain, between them calcium currents, neurotransmission, neuroplasticity and neuroprotection (Schwitzer et al, Neural Plast. 2016; 2016). This work aims to investigate the actions of cannabinoids on the proliferation, death and calcium signal on chick retinal cells in culture treated with ATP and ADP. Cultures of retinal cells were obtained from the White-Leghorn chicken embryos at E7 (Approval in ethics committee: CEUA-UNIRIO 2016.02). Cells were seeded on culture dishes (3100 cells/mm2) and cultured for 24 hours at 37°C in a humidified atmosphere of 95% air / 5% CO2. Immunofluorescence microscopy revels that both CB1 and CB2 receptors are expressed on nestine, β-tubulin III and 2M6 positive cells. In order to verify cell proliferation, incorporation of [3H]-thymidine assay was realized. Treatment with 0.5 μM WIN 55,212-2, a non selective CB1 and CB2 agonist, for 24 hours inhibited ~ 84.14% (n=5) of ATP-induced cell proliferation. In the same way, 50 μM URB 602, a MAGL inhibitor, an enzyme that hydrolyze 2-arachidonoylglicerol, inhibited ~ 75% (n=03) of 100 μM ADP-induced cell proliferation. To evaluate cell viability, cultures in E7C1 were treated with increasing concentrations of WIN 55.212-2 (0.5; 1.0 and 5.0 μM) for 24 hours, and submitted to the cell viability assay (MTT). WIN 55,212-2 reduced cell viability (% of effect vs. control ± S.E. Control = 100 ± 2.3; 0.5 μM; 0.5 μM WIN = 98 ± 7.0; 1 μM WIN = 64 ± 2.3; 5.0 μM WIN

= 40 ± 2.6. n = 4; p<0.001). Cell death induced by WIN 55,212-2 was completely reverted by 1 μM AM251 and 1μM AM630, CB1 and CB2 antagonist receptor, respectively. Using 5 mM fura-2 as calcium probe, the addition of 50 mM KCl induced ~77% of calcium increase only in neurons, while 1 mM ATP has no effect in E7C1 retinal cultures. However, KCl was not able to increase calcium signal in cultures treated with 0.5 μM WIN 55,212-2 for 24 hours, while 1 mM ATP induced ~37.5% of calcium increase only in Müller glial cells. At least 2600 cells were analyzed (n = 3). Finally, cultures in E7C1 were treated with 100 nM A438079, a selective antagonist of P2X7 receptors, plus 1 μM WIN 55,212-2 for 24 hours, and submitted to the cell viability assay (MTT). A438079 completely inhibited the cell death induced by WIN 55,212-2 (% of effect vs. control ± S.E. Control = 100 ± 2.1; WIN 1.0 μM = 68.4 ± 1.6; A438079 100nM = 103.1 ± 3.6; A438079 100 nM + WIN 1.0 μM = 106.7 ± 5.9. n = 3). These data together suggest that cannabinoids, through CB1 and CB2 receptors, inhibits ATP/ADP-induced cell proliferation, induce Müller glial cell response to ATP and promotes P2X7 dependent cell death.
Keywords: Retina; Cell Death; P2X7 receptor; Cannabinoid.

39 - CONTRIBUTION OF P2X7 RECEPTOR DURING *Toxoplasma gondii* INDUCED GUT INFLAMMATION.

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Background and Objectives: Toxoplasmosis, an important disease affecting 30% of the world's population, is caused by the protozoan parasite Toxoplasma gondii (Pappas et al. Int J Parasitol. 2009). The infection starts in the gut, which induces an inflammatory response that is critical for initiating a Th1 mediated immune response (Chia-Hsin Ju et al., J Immunol., 2012). The pro-inflammatory response to T. gondii is important to protect the host and keep the disease under control (Chia-Hsin Ju et al., J Immunol., 2012). However, a stronger inflammatory event against T. gondii leads to an IBD-like phenotype in C57BL/6 mice. The P2X7 receptor is reported to be a key component in the T. gondii control in several contexts, promoting a pro-inflammatory scenario (Moreira-Souza, ACA et al., Immunobiol, 2017). Interestingly, the absence of P2X7 receptor protects the gut during experimental IBD (Figliuolo VR, et al., Purinergic Signal. 2017). In this context, we propose to analyze the role of P2X7 receptor during intestinal inflammation (Ileitis) induced by T. gondii. Methods and results: C57BL/6 - WT or P2X7-/- mice from 6-8 weeks old were orally infected with 10 cysts of Toxoplasma gondii, strain Me-49. After a follow-up of 8 days, animals were euthanized and ileal samples collected for histopathological and immunohistochemical analysis. The gut's weight and size were measured, while ileal and blood cytokines levels were detected by ELISA and CBA kits, respectively. We found no difference between ileal size, but the gut from P2X7-/- mice showed an increased weight when compared with C57BL/6 mice($p<0.005$). Histopathological analysis revealed an intense inflammation both in C57BL/6-WT and P2X7-/- animals. However, we found a more extensive ileal lesion in P2X7-/- mice with an increased number of T. gondii parasites when compared with the ileum from C57BL/6 mice ($p<0.05$). The immunohistochemical analysis confirmed the P2X7 receptor overexpression in the gut of C57BL/6 mice post-T. gondii infection. The production of the pro-inflammatory cytokine IL-1β in the ileal section from the P2X7-/- mice was lower compared with C57BL/6-WT mice($p<0.05$). In the peripheral blood, collected by cardiac puncture, we found lower levels of proinflammatory cytokines such IL-6 and IFN-γ in the serum of P2X7-/- animals compared with C57BL6-WT mice($p<0.005$). Conclusion: We conclude that P2X7 receptor has a critical protective function during gut inflammation induced by T. gondii infection, promoting systemic proinflammatory cytokine secretion and parasite control. Finnancial support: Faperj, Capes, CNPq
Keywords: P2X7R; Ileitis; Toxoplasma gondii; gut inflammation.

40 – CYTOSOLIC CA^{2+} TRANSIENTS EVOKED BY PURINERGIC RECEPTORS IN HEPATOCYTES: THE ROLE OF P2Y RECEPTORS.

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Objectives/background: Extracellular nucleotides are key signaling molecules recognized by hepatocytes and other liver cell types, affecting important hepatic processes. Understanding the role of P2X and P2Y receptors on Ca^{2+} signaling in the hepatic context is of major relevance for liver physiology. Methods and results: Isolated hepatocytes were prepared by collagenase perfusion of rat livers. Freshly isolated hepatocytes were loaded with Fura-2/AM and transferred to a thermostatically regulated microscope chamber for acquisition of fluorescence images of cytosolic Ca^{2+} signals, which typically take the form of periodic Ca^{2+} oscillations. UDP, a P2Y6 receptor-selective agonist, was the only extracellular nucleotide that failed to elicit a Ca^{2+} response in rat hepatocytes. Among the other nucleotides, the transients induced by low doses of each agonist (1-2 μM) showed different spike durations, with distinguishable falling phases. ATP elicited complex Ca^{2+} transient shapes with two main patterns: broader spikes with biphasic decay phase, present in the majority of the cells and narrow spikes with fast decay phase, present in the minority of the cells. Specific activation of P2Y1 receptors by ADP generated a homogeneous short-lasting Ca^{2+} spike pattern with narrow peaks and rapidly declining phase. P2Y2 and P2Y4 receptors, stimulated by UTP, elicited predominantly longer-lasting peaks. In the absence of extracellular Ca^{2+}, the spike widths measured at half-peak height of the first spike were longer than those observed in the presence of extracellular Ca^{2+} at the same agonist dose. The contribution of P2X ligand-gated ion channel receptors in generating cytosolic Ca^{2+} transients was determined with ATP (1-300 μM) in the presence of a Gq protein-specific inhibitor to block P2Y receptor coupling to Ca^{2+}. No Ca^{2+} oscillations occurred under these conditions. At higher ATP doses (400 μM), a slow and constant intracellular Ca^{2+} increase was observed, suggesting Ca^{2+} influx through membrane pore formation by P2X7 receptor activation only at high ATP. Similar results were obtained from treatment with BzATP, a potent P2X7 receptor agonist. Conclusions: In rat hepatocytes, cytosolic Ca^{2+} transients are probably evoked only through IP3 formation by Gq coupled P2Y1, P2Y2 and P2Y4 receptors, but not P2Y6 receptors without contribution of P2X receptors. The differential Ca^{2+} oscillations patterns elicited by activation of these receptors suggests specific and yet undiscovered roles of purinergic signaling in liver physiology. Acknowledgments: São Paulo Research Foundation, FAPESP; The Brazilian National Council for Scientific and Technological Development, CNPq.
Keywords: Hepatocytes; Calcium imaging; P2Y receptors; cytosolic Ca^{2+} transients.

41 - DEVELOPMENT OF AVERMECTINS AS AN ADJUNCTIVE THERAPY FOR PARKINSON DISEASE.

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Avermectins are a group of compounds derived by fermentation of soil micro-organism, Streptomyces avermitilis and have been frequently used for treatment of parasitic infections. Ivermectin (IVM), the first in class molecule, proved to be an effective medication on basis of its unique pharmacological properties. IVM was reported to be a positive modulator of purinergic P2X4 receptors (P2X4Rs) which are cation permeable ion channels activated by adenosine-5'-triphosphate (ATP) expressed in the brain (Priel at al. J. Gen. Physiol 123, 2004). P2X4Rs have been reported to modulate dopamine (DA)-dependent behaviors including sensorimotor gating, drug reward behavior. Interestingly, IVM was reported to induce deficits in sensorimotor gating and increase thigmotactic behavior in open field paradigm indicating that IVMp maybe potentiating DA function via P2X4Rs to cause these behavioral abnormalities (Bortolato et al. Intl.J. Neuropsychopharmacol 16;5, 2013). Furthermore, IVM increased dopamine and cyclic-AMP regulated phosphoprotein of 32 kDa (DARRP-32) in the striatum, which is characteristic of DA receptor agonists (Khoja et al. J. Neurochem 139;1, 2016). These findings indicate there is interaction between P2X4Rs and DA systems. This led us to hypothesize that avermectins can be used for treatment of disorders characterized by DA hypofunction such as Parkinson's disease. We addressed this hypothesis by using the 6-hydroxydopamine (6-OHDA) model of DA depletion. We stereotaxically injected the left medial forebrain bundle of 8-10 month old adult male C57BL/6J with 6-OHDA to induce ablation of DA neurons on left side of striatum, leading to striatal DA imbalance and causing the mice to rotate to one side only. Mice were injected with levodopa (L-DOPA; 5mg/kg s.c.), a commonly used therapy for Parkinson's disease, which causes mice to rotate away from lesioned side of striatum (contralateral rotations). IVM (5 mg/kg i.p.) alone had no effect on rotational behavior, but in combination with L-DOPA, significantly potentiated L-DOPA's effects on rotational behavior. Furthermore, these effects of IVM were significantly attenuated in male P2X4R knockout (KO) mice, indicating that the IVM-mediated increase in rotational behavior is P2X4R dependent. In addition to IVM, we also tested the effects of another avermectin; moxidectin (MOX) on L-DOPA induced motor behavior. When MOX was administered (2.5 mg/kg i.p.) on its own and in combination with L-DOPA (5mg/kg s.c.) no significant alteration in rotation behavior was observed. These contrasting findings indicate that structural differences in these compounds can influence their mechanism of action, thus affecting their activity on motor behavior. Overall, our findings indicate that avermectins can be repurposed as adjuvant therapies for Parkinson's disease. Support: AA022448 (DLD) and USC School of Pharmacy.
Keywords: Avermectins; Parkinson', P2X4 receptors; therapy.

42 - DISCOVERY OF A POTENTIAL ANTAGONIST FOR P2Y2 AND P2Y4 RECEPTORS FROM BRAZILIAN FLORA.

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Objectives/ background: P2Y2 and P2Y4 are UTP-activated receptors that have many physiological functions, including intracellular calcium mobilization. These receptors are associated with diseases such as inflammation and cancer, which make them important therapeutic targets. However, the scarcity of selective antagonists for these receptors limits their application in clinical therapy, encouraging the search for new molecules with antagonistic activity. Thus, we performed the screening of approximately 100 extracts from Brazilian biomes and found that JA2 extract significantly inhibited calcium responses mediated by P2Y2/4 receptors. Methods and results: JA2 extract was acquired from an institutional natural library under a confidentiality agreement. It was obtained by the chemical methanolic extraction process. The experiments were performed using J774.G8 macrophage cell line plated in 96-well plates ($2x10^5$ cells/well). The intracellular calcium measurements were performed on a FlexStation III microplate reader using cells loaded with Fluo-4AM [2 μM] in Dulbecco's PBS containing probenecid [2.5 mM]. These cells were pretreated with JA2 extract [50 μg/mL], PPADS [300 μM] or Reactive Blue-2 [20 μM] during 30 minutes and then, were stimulated with different P2 agonists. JA2 extract significantly inhibited calcium mobilization induced by UTP [10 μM] (133.6 ± 18 vs 67.6 ± 24) ($p< 0.05$) but did not inhibit this response in DPBS solution without extracellular calcium (18.5 ± 6.1 vs 27.7 ± 9.6). JA2 also significantly inhibited intracellular calcium responses induced by selective P2Y2 and P2Y4 agonists: 2-thio-UTP [10 μM] (112.8 ± 11.2 vs 34.9 ± 14.2) and MRS4062 [10 μM] (38.5 ± 11.3 vs 17.1 ± 6.3), respectively ($p< 0.05$). However, JA2 did not inhibit calcium mobilization induced by ATP [100 μM] (120.9 ± 35 vs 94.5 ± 47.6), BzATP [100 μM] (118 ± 50.7 vs 95.3 ± 50.5), ADP [10 μM] (45.9 ± 22.8 vs 44.5 ± 27.1) and UDP [100 μM] (102.9 ± 36.6 vs 73.1 ± 23.6) ($p> 0.05$). This inhibition effect was not due to quenching of calcium indicator by molecules of the extract since JA2 did not inhibit calcium mobilization induced by Ionomycin [1 μM] (199.1 ± 35.2 vs 188.2 ± 31.8) ($p> 0.05$). J774.G8 cells pretreated with JA2 [50 μg/mL] were stimulated with ATP [5 mM] during 15 minutes and subsequently, received YO-PRO-1 [1 μM] to perform dye uptake assay. As result, JA2 did not inhibit the uptake of YO-PRO-1 (21.7 ± 6.5 vs 24.1 ± 6.6) ($p> 0.05$). MTT assay was also performed to rule out a possible cytotoxicity of JA2 [50 μg/mL] treatment during 1, 6 and 24h. The cell viability means were 99.8 ± 16.6, 80.8 ± 14.7 and 109 ± 31.3, for 1, 6 and 24h, respectively, and these results were similar to controls (cells without treatment): 95.2 ± 6.9, 94.5 ± 6.7 and 92.5 ± 15.7 ($p> 0.05$). Conclusion: JA2 extract showed the ability to inhibit intracellular calcium mobilization possibly acting on P2Y2 and P2Y4 receptors, and without cytotoxicity effects. Acknowledgments: IOC and CNPq.
Keywords: P2Y2; P2Y4; UTP; antagonist.

43 - DYSREGULATED P2Y6 RECEPTOR SIGNALING INCREASES A RISK FOR PATHOGENESIS OF GLAUCOMA.

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Glaucoma is a progressive optic neuropathy and the second leading cause of blindness worldwide. The blindness in glaucoma is mediated by damages of retinal ganglion cells death (RGCs). Although the highest risk factor for glaucoma is elevated intraocular pressure (IOP), detailed mechanisms of IOP regulation are poorly understood. Here, we report that dysregulation in P2Y6 receptor signaling causes age-dependent hypertensive glaucoma-like phenotypes. We found that instillation of uridine diphosphate (UDP), an endogenous agonist for P2Y6 receptors, showed transient reduction in IOP. Its maximum effect was obtained about 10% IOP reduction. P2ry6 deficient (P2Y6KO) mice showed no hypotensive effect by UDP. Immunohistochemistry and in situ hybridization revealed that P2Y6 receptors were expressed in non-pigmented epithelial cells of ciliary body which are essential for producing aqueous humor. Because IOP is balanced by production/draining of aqueous humor, we then analyzed dynamics of aqueous humor using fluorophotometry. We found that fluorescein inflow to the anterior chamber was significantly suppressed by UDP and timolol but not by latanoprost whereas fluorescein outflow from the anterior chamber was enhanced by latanoprost but not by UDP or timolol, indicating that the effect of UDP was likely to reduce production of aqueous humor. P2Y6KO mice showed elevated IOP regardless of their ages. Young-adult P2Y6KO mice (3 months old) showed elevated IOP but no histological or functional abnormalities in the eye. Middle-aged P2Y6KO mice (6–12 months old) showed significant structural and functional abnormalities including optic nerve atrophy, thinning of GCL/IPL, degeneration of RGCs and disordered visual functions. These changes were attenuated by an IOP lowering agent. Taken together, our data shows that dysfunctional P2Y6R signaling causes IOP elevation, resulting in glaucomatous optic neuropathy.
Keywords: P2Y6; intraocular pressure; retina; glaucoma.

44 - EARLY HUMAN SEPSIS MODULATES PURINERGIC RECEPTORS EXPRESSION, SERUM ATP LEVELS AND ATPASE ACTIVITY. RAFAEL OLIVÉ LEITE1;

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Objectives/Background: Sepsis has been recently described as the product of a dysregulated immune response to infection. Although sepsis displays altered innate and acquired immune responses, dysregulation of innate immunity leading to overwhelming inflammation is a more prominent feature in early sepsis. Neutrophil disfunction hallmarks the state of dysregulated inflammation in early sepsis, causing insufficient pathogen clearance and collateral tissue damage, leading to persistent inflammation, organic failure and death. Robust pre-clinical evidences show that purinergic signaling controls innate immunity features in sepsis, including several neutrophil features such as chemotaxis, transendothelial migration, phagocytosis and interleukins release, key processes in initial response to infection. Nevertheless, evidences to support purinergic signaling role in clinical sepsis are scarce. To determine the activity of purinergic signaling in early human sepsis we examined purinergic receptors expression in neutrophils, serum concentration of the purinergic agonist adenosine triphosphate (ATP) and serum activity of enzymes related to purinergic signaling in septic patients and controls. Methods: We enrolled septic patients at the first morning following an intensive care unit admission due to sepsis-led organic failure. The first control group was composed by ward non-septic patients with milder inflammatory conditions such as cancer, diabetes or chronic arterial hypertension but no sepsis or acute (<48 hours) clinical deterioration due to any cause. The second control group was comprised by healthy volunteers with no acute or chronic illness. Neutrophil purinergic receptors expression at mRNA level, serum ATP level and enzymes activity were measured. Results: Approval from human research ethics committee and written informed consent from patients was obtained before enrolling and sampling. Serum ATP was measured in septic (n=18), ward patients (n=18) and healthy controls (n=19). Serum nucleotidase activity was also determined in septic (n=18), ward patients (n=21) and healthy controls (n=20). Receptor expression was measured in septic (n=10), ward patients (n=7) and healthy controls (n=5). As compared to controls, septic patients neutrophils had significantly enhanced expression for P2Y2 ($p < 0.05$ for ward patients and $p < 0.001$ for healthy volunteers) and A2a ($p < 0.01$ for healthy volunteers), but not for P2X7 and P2Y6 receptors. Septic patients also had serum ATP levels ($p < 0.05$ for ward non-septic patients, $p < 0.001$ for healthy volunteers) and ATPase activity ($p < 0.0001$ for both control groups) significantly elevated. Conclusions: Early human sepsis displays a clear phenotype of purinergic signaling activation, suggesting that innate immunity can be regulated through careful modulation of purinergic signaling. ATPergic tonus reduction by removal of excess ATP is a plausible therapy for sepsis. Acknowledgements: CNPq 310846/2014-5 CAPES FAPERGS
Keywords: SEPSIS; ATP; P2Y2; A2a.

45 - EFFECT OF P2Y6 RECEPTOR ANTAGONISM IN AN ANIMAL MODEL OF PARKINSON'S DISEASE.

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Objectives / background: Parkinson's Disease (PD) is a neurodegenerative disease characterized by diminished dopamine bioavailability in substantia nigra and striatum. Taking into account that ATP is intensely released in the 6-hydroxydopamine (6-OHDA) animal model of PD, a screening of purinergic receptors gene expression was performed and receptor activity was modulated in order to investigate the effect on preventing or reversing hemiparkinsonian behavior and dopaminergic deficit in this animal model. As expected, P2X7 receptor expression was altered in the PD model. P2X7 receptor antagonism was demonstrated to reverse dopaminergic deficit in the PD's animal model of 6-OHDA (Ferrazoli et al. Cell Transplant 26; 669, 2017). P2Y6R gene expression was altered as well, and its antagonist was used to investigate beneficial effects in this animal model. Methods and results: All animal procedures were approved by the Ethics' Committee of the Institute of Chemistry-USP. Male Sprague-Dawley rats were injected with 6-OHDA (2 μl, 7μg/μl) in the medial forebrain bundle of the right brain hemisphere, and the apomorphine rotational test was performed after 1, 3 or 5 weeks. At each time point, samples were collected and qRT-PCR for purinergic receptor expression was performed. P2X7 (0.743286+-0,068438, $p<0.001$) and P2Y6 (3.861810+-0,159544, $p<0.001$) receptor expression levels were increased in comparison to control hemisphere (0.361330+-0.015946, 2.670790+-0.437817, respectively). Animals were injected prior or 5 weeks after lesion with the P2Y6 antagonist MRS2578 (100μM, 2μl, striatum). MRS2578 injection prior to 6-OHDA lesion partially prevented dopaminergic deficit in the substantia nigra as analyzed by tyrosine-hydroxylase immunostaining

(0,457216+-0,040663, p<0.001) in comparison to the lesioned group (0,24543+-0,009561). Statistical analysis were performed with one or two-way analysis of variance (ANOVA) followed by the post hoc test. A (p) value of 0.05 was assumed significant. Conclusion: The present work showed that striatal gene expression of P2X7 and P2Y6 receptors are altered in the progression of the lesion during 5 weeks. Taking into account that these purinergic receptors subtypes are involved in inflammatory responses, the preventive effect in dopaminergic deficit demonstrated by P2Y6 receptor antagonism might be due to anti-inflammatory action. Altogether, these data bring evidence that subtype-specific purinergic receptor activity modulation is a promising tool for PD treatment. Financial support: Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (Projects No. 141979/2014-3; 306429/2013-6). Fundação de Amparo a Pesquisa do estado de São Paulo- FAPESP (Project No. 2012/50880-4).
Keywords: Parkinson's Disease; P2Y6 receptor; purinergic receptors; 6-OHDA model.

46 - EFFECTS OF BRILLIANT BLUE G ON MEMORY DEFICITS AND OXIDATIVE STRESS IN A ALZHEIMER'S DISEASE MODEL INDUCED BY STREPTOZOTOCIN IN MICE.

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Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by a progressive decline in cognitive functions. Intracerebroventricular (icv) injection of streptozotocin (STZ), in a sub-diabetogenic dose has been likened to sporadic dementia of Alzheimer's type (Liu et al., 2016, Brain Res. 1631: 137-146). Since accumulating evidence demonstrates the neuroprotective effect of Brilliant Blue G (BBG) in different models of neurodegenerative diseases, we now tested the effect of this P2X7R antagonist on memory deficits and oxidative stress induced by STZ. Male Swiss mice received bilateral icv injections of STZ (3 mg/kg) dissolved in artificial cerebrospinal fluid. Two days after the first STZ administration, injections were repeated and the animals were treated with BBG (50 mg/kg) 1 h after surgery and once a day until the last day of behavioral evaluation (18th after the first STZ injection). The animals subjected to STZ administration showed significant recognition memory deficits evaluated by the object recognition task, and early and late memory deficits in the passive avoidance test. No differences of locomotor activity in the open field test were observed between groups. STZ also increased malonaldehyde and nitrite contents in the prefrontal cortex and hippocampus. BBG treatment was able to prevent the early memory deficits (sham: 155.2±28.2 s; STZ: 69.9 ±14.9 s; STZ+BBG: 173.8±55.0 s, p<0.05) and the increase of nitrite production in the hippocampus (sham: 15.6±2.0 μM; STZ: 28.0±3.6 μM; STZ+BBG: 24.2±1.7 μM, p<0.05). These data highlight the therapeutic potential of P2X7R antagonists in AD, however the role of P2X7R on STZ-induced AD model requires more investigation. Supported by CNPq, CAPES and Funcap.
Keywords: Streptozotocin; Brilliant Blue G; Oxidative stress; Memory.

47 - EFFECTS OF PHARMACOLOGICAL MODULATION OF P2Y12 RECEPTOR IN GLIOMA CELLS PROLIFERATION.

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Objectives/background: Glioblastoma multiform (GBM) is considered the most lethal intracranial tumor, and patients affected by this tumor present median survival time of approximately 14 months. Another important characteristic of this type of glioma is its high proliferative rate. Purinergic signaling has been implicated in the development and proliferation of tumors, including gliomas. ADP acts as P2Y12 ligand, and the expression of this receptor has been documented in different kinds of cancer. Nevertheless, few is known about the role of this receptor on glioma proliferation. The aim of this study was to evaluate the effect of P2Y12 receptor (P2Y12R) activation, via ADP, in the proliferation of glioma cell lines. Methods and results: Human U251-MG and rat C6 glioma cell lines were cultured in DMEM medium supplemented with fetal bovine serum and kept under ideal conditions of cultive. PCR assay was performed in order to investigate the expression of the P2Y12R on glioma cell lines. GAPDH was used as the reference gene. PCR analysis showed that both glioma cell lines studied presented the P2Y12R significantly expressed when compared to the reference gene. To evaluate cell proliferation, the cells were exposed to 100 μM of ADP and/or to different concentrations of the P2Y12R antagonist, clopidogrel (150 and 300 μM) for the C6 cell line, and clopidogrel (150, 300 and 500 μM) for U251-MG cell line. The cell number was determined by cell counting through the exclusion of dead cells by Trypan blue labeling. The clonogenic assay was performed to evaluate colonies formation after 10 days of treatment. Our results showed that treatment with ADP promoted an increase in the number of both glioma cells studied. After 48 h of treatment, the specific antagonist clopidogrel was able to reduce the number of human glioma cells in all concentrations used. Similar results were observed for C6 lineage after 24 h of treatment. In addition, after 24 h of treatment with the P2Y12R antagonist, clopidogrel 300 μM, both cell lines presented a significant reduction in the ability to form colonies when compared to the respective controls. As expected, treatment with the nucleotide ADP induced cell proliferation. Conclusion: Glioma cell lines tested expressed P2Y12R. In addition, the modulation of this receptor with its agonist, ADP, promoted glioma cells proliferation and the treatment with the antagonist reduced this effect. In conclusion, we can suggest that the activation of P2Y12R is involved with glioma cell growth.
Keywords: glioma; proliferation; purinergic system; P2Y12R.

48 - EVALUATION OF P2X7R EXPRESSION AND SNP 1513A>C AND 489C>T POLYMORPHISMS IN GLIOBLASTOMA AS POTENTIAL NEW BIOMARKERS FOR PROGNOSIS.

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Objectives / background: Gliomas are the most frequent malignant tumors of central nervous system, corresponding to about 80% of the neoplasms of this organ. Despite intense efforts to develop new pharmacological therapies and new diagnostic methods, effective agents are not yet available. The purinergic system is strongly involved in the progression of different tumor types and P2X7R is one of the most studied purinergic receptors in cancer. The effects mediated by this receptor are very variable, depending on the cell type, the form of activation and the concentration of the agonist, and can, in a dual way, trigger effects of increasing or decreasing cell proliferation. In gliomas, the expression of this receptor was analyzed only in cell lines and is related to both tumor growth and cell death. In addition, P2X7R has two single nucleotide polymorphisms (SNP), SNP 1513A>C (leads to loss of receptor function), and SNP 489C>T (leads to gain in receptor function), which are considered markers of malignancy in some types of cancers. However, in gliomas these polymorphisms have not yet been analyzed. Therefore, the aim of this study was to investigate whether the expression of the P2X7 receptor and its SNP (1513A>C and 489C>T) is related to the malignancy of this type of cancer. Methods and results: A meta-analysis of gene expression and total genome data from a cohort of patients with glioblastomas (GBM) (n = 339) provided by The Cancer Genome Atlas (TCGA) was performed and the presence of SNP 1513A>C and 489C>T was evaluated by real-time PCR in different human GBM lineages. The expression of P2RX7 showed prognostic value only for younger patients, below 50 years of age, (low tumor gene expression is related to a twofold higher chance of death; HR = 0.5; CI = 0.27-0.91). Only 5% of the cohort biopsies had mutations in P2RX7, however SNP 1513A>C and 489C>T were not detected. SNP 1513A>C was identified only in heterozygosity in M059J, T98G and U87 lineages and SNP 489C>T was also found in heterozygosity only in T98G and U138 GBM cell lines. Conclusion: The gene expression of P2X7R and the presence of mutations in its gene did not present value as a global marker of malignancy in GBM. Because its activation is associated with a complex of modulatory actions on tumor growth, the study of the gene expression of proteins involved in these modulatory pathways may have relevance to predict the outcome of GBM patients. However, further studies are needed to elucidate this hypothesis. Acknowledgment: This work is supported by CNPq/PDJ and INCT/Doenças cerebrais, excitotoxicidade e neuroproteção.
Keywords: P2X7R; glioblastoma; SNP.

49 - EXTRACELLULAR ATP DAMPENS PLANT DISEASE BY BOOSTING IMMUNITY.

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Extracellular ATP, a damage-associated molecular pattern (DAMP) released upon exposure to cellular stresses, acts as an important signal for cellular responses to abiotic and biotic stresses. In plants, extracellular ATP is perceived by a purinoceptor, P2 receptor kinase 1 (P2K1), that causes downstream signaling for defense responses. Our recent study (Tripathi et al., Plant Physiology, 176: 511-523, 2018) revealed a synergistic interaction between extracellular ATP and other plant stress hormones, especially jasmonates (JAs), for the activation of plant defense responses. This synergistic response provided strong resistance against a necrotrophic fungus, Botrytis cinerea, which has a broad host range and considerable economic impact. Interestingly, extracellular ATP directly changes the formation of the JA receptor complex, thereby enhancing JA signaling. This signaling crosstalk was increased in a P2K1 overexpression line, whereas there was no crosstalk observed in a P2K1 knockout mutant, suggesting that the functional P2K1 receptor is required for maximizing plant defense responses. The signaling crosstalk requires the formation of the secondary messengers, i.e., cytosolic calcium, reactive oxygen species, and nitric oxide. This finding has given a new direction to understanding defense signaling pathways activated by DAMPs in plants. We discussed possible insights into how extracellular ATP signaling interacts with other hormonal signaling pathways for plant defense responses. This work was supported by NSF (IOS-1557813).
Keywords: DAMP; plant defense responses; P2 receptor kinase; jasmonates

50 - FUNCTIONAL EXPRESSION OF P2Y RECEPTORS IN HEPATOCYTES DURING HEPATIC FIBROSIS.

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Hepatic fibrosis is an accumulation of extracellular matrix. Throughout chronic cellular damage, liver cells release pro-inflammatory molecules, resulting in a continuous state of inflammation and aberrant scarring that drives the liver into a fibrotic state (Forbes et al. Best Pract Res Clin Gastroenterol 25(2):207-17, 2011). One of the signaling pathways involved in the regulation of inflammatory responses to damage is the activation of purinergic receptors by ATP and its metabolites (Burnstock et al. Purinergic Signal 10(1):51-70, 2014). However, the role of purinergic communication on fibrotic processes in the hepatocyte is not well understood. Our objective was to investigate if ATP could act as a promoting-damage mediator in the onset of this pathological state. We administrated CCl4 during 4 weeks as model of hepatic fibrosis in male C57BL6 mice of 6 weeks of age. After demonstrating establishment of fibrosis, we proceeded to identify purinergic receptors by qPCR. We observed differential expression of the receptors mRNAs between groups; P2YR2 and P2YR6 increased their relative expression in treated animals (7.55±2.06 fold of control n=6 p=0.0067 and 7.18±1.76 fold of control n=6 p=0.0015, respectively) whereas P2YR13 and P2YR14 significantly reduced their expression (0.18±0.04 fold of control n=5 p=0.0066 and 0.47±0.09 fold of control n=5 p=0.0003, respectively), for statistical analysis ANOVA test was used. By immunofluorescence it was determined the distribution of P2YR2 receptor within liver structure and observed that the control group P2YR2 exhibited central zonation, which was obliterated by the increase of its expression observed in the CCl4-treated livers. Primary cultures of hepatocytes were obtained from both groups and were stimulated with UTP, a selective agonist for P2Y2 receptor. Then, we analyzed activation of extracellular regulated kinase (ERK) by Western blot and proliferation by MTS assay. Induction of phosphorylation of ERK (5 min, 100 μM) was almost 3 times greater after stimulation with UTP in the fibrotic group compared with the control group (385.7±108 fold of basal n=7 p=0.0203). MTS proliferation assays showed a tendency to increase proliferation in a UTP concentration-dependent manner, although not significant. We conclude that purinergic receptors express differentially after chronic liver damage. Furthermore, increased expression of P2YR2 eliminated the zonated expression found in controls. Changes in expression is also related to greater receptor-mediated responses in the fibrotic condition. Results suggest the activation of these receptors could have an important role in the onset of this pathological state, driving processes such as uncontrolled cellular proliferation. However, these possibilities should be further investigated. Funded by PAPIIT-UNAM, number IN201017
Keywords: "P2YR2"; "hepatic fibrosis".

51 - IMPACT OF P2X7 RECEPTOR, CASPASE-1 AND CASPASE-11 ON IL-1&BETA; EXPRESSION AND BACTERIAL LOAD CONTROL IN GINGIVAL TISSUE OF MICE CO-INFECTED WITH BACTERIA PORPHYROMONAS GINGIVALIS AND FUSOBACTERIUM NUCLEATUM.

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Introduction: Periodontal disease (PD) is a chronic inflammatory disease of the oral cavity. Porphyromonas gingivalis (Pg) and Fusobacterium nucleatum (Fn) have virulence factors that induce DP. Purinergic signaling may play a key role in PD, involving the purinergic P2X7 receptor. Activation of caspase-1 leads the cleavage of pro-IL-1β in IL-1β which is central in the host inflammatory response in PD. The caspase-11 is important against bacterial pathogens due to induction of cell death and IL-1 release. The aim the study was to verify the impact of the absence of the P2X7 receptor and caspase-1 (Casp-1) and caspase-11 (Casp-11) on IL-1β expression and control of bacterial load on the gingival tissue of mice co-infected by bacteria Pg and Fn. Methods: The animals C57BL/6 (WT), P2X7-/-, Casp11-/- and Casp1/11-/- (2 months old) were treated with sulfamethoxazole (0.87 mg/mL) and trimethoprim (0.17 mg/mL) in drinking water ad libitum for 10 days, followed by 3 days without antibiotics. Then the mice were anesthetized and infected in the oral cavity with 50 μL of 2% carboxymethyl-cellulose containing 109 CFU (colony forming units) of each bacterium (Pg applied four hours after Fn). Three inoculations were applied at 2-day intervals (total period of 7 days). After the experimental period, were evaluated in gingival tissue: IL-1β relative expression and relative amount of Pg. ANOVA and Tukey test were used for parametric variables, and Kruskal-Wallis test and Dunn test for non-parametric variables. Up to 5% significance was adopted. Data represent means ± SEM. Results: The IL-1β expression was increased in Pg/Fn infected WT animals (2.5 ± 0.6, n = 5, $p < 0.05$) and P2X7-/- Pg/Fn (0.8 ± 0.1, n = 4, $p = 0.05$) in relation to WT (0.9 ± 0.2, n = 4, $p < 0.05$) and P2X7-/- (0.26 ± 0.04, n = 5, $p = 0.05$), respectively. The same set of data demonstrated that the presence of the P2X7 receptor contributes to the expression of IL-1β in coinfection, since mRNA levels for this cytokine were decreased in the P2X7-/- Pg/Fn animals when compared to the WT Pg/Fn animals. Similar results were obtained in the other knockout groups, with increased IL-1β mRNA levels of the WT Pg/Fn (3.0 ± 0.5, n = 4, $p < 0.01$) and CASP 11-/- Pg/Fn (2.4 ± 0.2, n = 4, $p < 0.01$) relative to their respective controls, WT (1.0 ± 0.2, n = 4, $p < 0.05$) and CASP 11-/- (0.8 ± 0.1, n = 4, $p < 0.05$). Similarly what was found to P2X7-/- the absence of caspase-11, IL-1β cytokine expression was decreased in co-infection. About bacterial load, it was observed that the co-infection caused the increase of the amount of Pg in relation to the healthy mice, and that in the absence of the P2X7 receptor, Casp-1 and Casp-11, the control of the bacterial load was reduced, demonstrating the importance of these routes to fight the bacteria. Conclusion: These initial studies suggest that P2X7 receptor-signaling pathways, Casp-1 and Casp-11 are involved in host response against coinfection of Pg and Fn via IL-1β production.
Keywords: Periodontal disease; dysbiosis; purinergic signaling.

52 - IN VIVO AND IN SITU ANALYSIS OF THE INTERACTION BETWEEN P2X4 AND P2X7 RECEPTORS. IS THERE A PHYSIOLOGICAL RELEVANCE?

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P2X receptors are trimeric non-selective cation channels gated by extracellular ATP. Among the seven mammalian P2X receptor subunits, P2X7 and P2X4 share the highest sequence similarity with 48% identity of the murine proteins and their encoding genes are adjoining on the same chromosome. Together with the fact that both receptors are coexpressed in epithelial and immune cells where they might serve a common function, this raised the question if both subtypes can physically interact or even assemble as heterotrimers. Thus, a number of studies arguing for and against an interaction of P2X7 and P2X4 were published in recent years. Although there is evidence that homomeric P2X4 and P2X7 receptors may physically interact, it is still an open question if this interaction takes place under physiological conditions in native tissue. Here, we use a novel P2X7R-EGFP BAC transgenic mouse model and subtype-specific knockout mice to investigate the distribution as well as the physical interaction and mutual interrelation of the P2X7 and P2X4 subtypes in native mouse tissue. Quantitative PCR and Western blot analysis of different tissue revealed that neither mRNA nor protein expression of each P2X subunit is significantly altered by P2X7R overexpression or genetic ablation of the respective other P2X receptor. Immunofluorescence staining of lung sections from BAC transgenic mice show that both subtypes are co-expressed in immune cells, but display distinctly different expression patterns within the respiratory epithelium: P2X7R seems to be equality distributed in all epithelia cells, whereas P2X4R is expressed in single cells at crossing-points of the epithelia, which most likely represent the secretory alveolar epithelial type II cells, in agreement with previous findings. A more detailed view of the protein expression of both subtypes in the same cell further demonstrates a clear difference in their subcellular localization. While P2X7R is mainly localized at the cell membrane, the majority of the P2X4R signal is detected in the cytosol, showing overlapping signal with the lysosome marker protein CD68, similar to previous observations. Using the GFP tagged P2X7R as a bait in biochemical pull-down experiments, we could show that the P2X4 subunit can be co-purified upon heterologous expression of both subunits in Xenopus oocytes, while there was no evidence for an interaction in protein extracts from P2X7-EGFP BAC transgenic mouse tissue. Furthermore, use of the P2X7R-EGFP BAC transgenic mouse model to identify novel interaction partners of P2X7 by protein cross-linking coupled with mass spectrometry (XL-MS) failed to identify P2X4 receptors as interaction partners. In conclusion, our data argue against a significant interaction between P2X4 and P2X7 receptors and suggest that if such an interactions exists at all, it might be of minor physiological relevance. This study was funded by the DFG (NI 592/7-1).
Keywords: P2X4; P2X7; Protein-Protein Interaction; BAC transgenic mouse model.

53 - INHIBITION OF P2X4 RECEPTOR SIGNALING IMPEDES HIV REPLICATION IN PRIMARY CD4 T CELLS.

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Background/Objective: T cell receptor engagement results in the release of ATP by panx-1 channels. Subsequent to the release of ATP, P2X1 and P2X4 receptors translocate to the immunologic synapse and promote T cell activation through enhancement of downstream Ca^{2+}

signaling and IL-2 expression. Despite the importance of P2 receptor mediated signaling in T cell activation, few studies have examined the role of purinergic receptor signaling in HIV infection, yielding contradictory results. Here we explored the impact of P2X mediated signaling on HIV replication. Methods: Memory CD4 T cells of healthy donors (n=5) were infected with the replication competent subtype B HIV-1 strain AD8. The P2 receptor antagonist 5-BDBD was added to infected cultures at concentrations between 0.5-10 μM, with or without Indinavir, a virus protease inhibitor. At 48 hours post infection, intracellular staining of HIV Gag p24 was performed to determine the frequency of infected cells. In parallel, the number of integrated HIV DNA copies was measured by qPCR, and the number of spliced (Rev) and unspliced (gag) HIV mRNA copies were quantified in the cultures by qPCR (n=3). Data were analyzed by 2-way ANOVA. Results: Compared to untreated controls, 5-BDBD treatment of memory CD4 T cells after HIV inoculation led to a significant decrease in the frequency of infected cells (>10-fold, $p<0.0001$). This effect was also observed at the DNA and RNA level: both the number of integrated HIV DNA copies (5-fold decrease, $p=0.0001$), as well as the number of spliced Rev RNA copies (5-fold decrease, $p=0.0001$) and unspliced Gag RNA copies (5.6-fold decrease, $p=0.0001$) were significantly decreased in 5-BDBD treated cells compared to untreated controls. In contrast, in the samples treated with 5-BDBD and Indinavir, the frequency of p24 expressing cells, as well as the number of integrated HIV DNA copies, spliced Rev and unspliced Gag mRNA copies was not statistically different between 5-BDBD treated cells and controls. Conclusions: These results indicate that inhibition of P2X4 signaling affects HIV replication at a step downstream of integration and transcription. Possible points of interference are virus assembly or virus budding (entry was ruled out experimentally). Another possibility is that the effect stems from an overall altered cell metabolic state. Currently, experiments are being carried out to test these hypotheses.
Keywords: "P2X4"; "HIV replication".

54 - INOSINE INCREASES THE INCORPORATION OF [3H]-THYMIDINE IN RETINAL CULTURES THROUGH A P2Y RECEPTOR-DEPENDENT MANNER.

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Aims: In the present study, we investigated the involvement of adenosine and inosine in the proliferation of glial progenitor cells in chick embryo retina cultures. Methods and results: [3H]-thymidine incorporation was performed in retinal cultures obtained from 7-day-old chick embryos cultivated for 2 days. When retinal cell cultures were treated with 100 μM ATP during 24 h, a significant increase of 83.5% in the [3H]-thymidine incorporation was noticed. Addition of 100 μM ARL67156, an inhibitor of ectonucleotidase activity, inhibited proliferation in the cultures (in cpm/culture: control = 4334 ± 698; ATP = 7952 ± 887; ARL = 2227 ± 409; ATP + ARL = 4113 ± 353; $n \leq 4$), suggesting the involvement of ADP and/or adenosine in the proliferation stimulated by purinergic signaling. While addition of 10 μM of the adenosine A2b receptor antagonist PSB 1115 to the cultures did not change significantly the incorporation of [3H]-thymidine induced by ADP, their incubation with ADP in the presence of 500 nM of the A2a adenosine antagonist ZM-241385 decreased proliferation by 63.9%, suggesting that activation of A2a, but not A2b, adenosine receptors is required for ADP-induced glial progenitor proliferation. Moreover, treatment of the cultures with 100 μM ADO induced an increase in [3H]-thymidine incorporation, an effect that was inhibited by the co-incubation of the cultures with 10 μM EHNA, an inhibitor of adenosine deaminase (ADA) (in cpm/culture: control = 1932 ± 407.1; 100 μM ADP = 8025 ± 1773; ADO = 4117 ± 911; EHNA + ADP = 9309 ± 1973; EHNA + ADO = 3093 ± 1093, n = 2), suggesting the participation of inosine in cell proliferation. Incubation of the cultures with 1 U/mL ADA promoted a significant increase [3H]-thymidine incorporation (in cpm/culture: control = 881.5 ± 158; 0,1 U/mL ADA = 1348 ± 151; 0,5 U/mL ADA = 2409 ± 480.4; 1 U/mL ADA = 3216 ± 536.6; n = 3). An increase in cell proliferation also was observed, when retinal cultures were incubated with 100 or 300 μM inosine, (in cpm/culture: control = 861.8 ± 74.61; 100 μM Ino = 3595 ± 482; 300 μM Ino = 3295 ± 335; n = 3), corroborating that inosine can induce the proliferation of retinal glial progenitors in culture. Conclusion: These results suggest that ADP, adenosine and/or inosine are required for the proliferation of retinal glial progenitors in culture. Financial support: CAPES, CNPq, PROPPI-UFF, FAPERJ.
Keywords: Retina; Proliferation; Glial progenitors; Inosine.

55 - MAPPING "MISSING" CONFORMATIONS OF ATP-GATED P2X RECEPTOR ION CHANNELS.

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Objectives/Background: Crystallographic work has provided snapshots of P2X receptors in apo, agonist-bound open and desensitised states as well as antagonist bound forms. As protein structure is dynamic, it is likely there is variation and movement around these states. Our previous biochemical studies (Roberts et al. Proc. Natl. Acad. Sci USA 109; 4663-67, 2012) have shown the accessibility of the upper vestibule that was not predicted from the zfP2X4 receptor based homology model. This suggests there is an additional distinct "relaxed" apo state with increased access to the upper vestibule. The aim of this study was to use biochemical and bioinformatics methods to provide validated models of the new state of the receptor. Methods and results: Cysteine accessibility was used to estimate the dimensions of the upper vestibule with residues chosen lining the upper vestibule (e.g. A94C) and control residues on the surface of the receptor (e.g. K190C). Accessibility data was obtained for MTSEA-biotin (6.7x2.9 Å, 381.52 kDa), MTS-TAMRA (9.4x3.0 Å, 567 kDa) and MTS-TPAE (6.9x4.2 Å, 446.55 kDa). Interestingly relative access was size-dependent (MTS-TPAE< MTS-TAMRA). These data show that large MTS-reagents can bind in the upper vestibule and that there must be more space available than predicted based on the apo structure. Manual docking using Pymol was done that showed the MTS-reagents do not fit in the current apo-based homology model. Molecular dynamics simulation showed a trend towards increased diameter of the upper vestibule i.e. relaxation. Work is ongoing to further characterise these movements. Conclusions: Data about the size of the upper vestibule in the "relaxed" apo state could point towards a looser association of subunits at the apex. Further molecular dynamics simulation could provide information for the molecular movements leading to the different states. This work was funded by the BBSRC.
Keywords: modelling; mts-reagents; molecular movements; accessibility.

56 - HYDROLYSIS OF ATP, ADP AND AMP IS INCREASED IN PLASMA BLOOD OF PROSTATE CANCER PATIENTS.

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Prostate cancer (PC) is the second most diagnosed neoplasm in men, with the exception of non-melanocytic skin tumors. Its global incidence is 1.1 million cases per year and its mortality rate is around 307,000 deaths. Around the world, there are 3,850,000 CP survivors, since their survival in five years reaches values exceeding 80%. Adequate stratification in the diagnosis and follow-up of these patients is so relevant to ensure successful of the treatment. In this context it is necessary to discover new biomarkers that help in this process. Adenine nucleotides are important signaling molecules that mediate innumerous biological functions in pathophysiological conditions, including cancer. CD39 and CD73 promote the sequential hydrolysis of ATP producing adenosine in the extracellular medium. This metabolism is evidenced as an important mechanism that corroborate to cancer progression. In addition, these enzymes can be found in cell membrane surface or soluble coupled to exossomas surface in the blood stream and thus breaking the antitumor immune answer. Thus, the present study aimed to evaluate the hydrolysis of adenine nucleotides (ATP, ADP and AMP) in the plasma blood of patients with prostate cancer. Peripheral blood samples of twenty-nine patients were collected and questionnaires were filed based on the clinical data of the medical records. Seventeen healthy individual were voluntaries and their blood were also collected after consentient. The blood samples are processed and plasma was extracted to analysis of nucleotide hydrolysis. Malachite green method was used to verify ATP, ADP and AMP degradation. As results, PC patients presented an elevated hydrolysis of all nucleotides evaluated when compared to healthy individuals. The correlation of ATP, ADP and AMP hydrolysis with clinicopathological data showed that patients with lower clinical stage (CS-IIA) presented an elevated ATP hydrolysis when compared to more advanced clinical stages (CS-IIB and CS-III). All clinical stages presented elevated AMPase activity. In conclusion, we can suggest that the hydrolysis of ATP, ADP and AMP can produce adenosine in the blood stream, and thus could be associated to prostate cancer progression, favoring the suppression of immune tumor. Therefore, the correlation of this hydrolysis profile with clinical stage of patients, suggest this biochemical nucleotide analysis as a promissory blood biomarker to PC. (Ethical Committee of Approval - CAAE: 62424416.0.0000.5336/PUCRS, Porto Alegre-RS and 2017- 001/CACON, Cruz Alta-RS).
Keywords: Prostate cancer; ATP; adenosine; ectonucleotidases.

57 - INFLUENCE OF TREATMENT WITH IMATINIB ON PURINERGIC SIGNALING IN CHRONIC MYELOID LEUKEMIA CELLS.

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Objectives/background: Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm characterized by the occurrence of the t (9;22) (q34;q11.2) translocation. This rearrangement is known as the Philadelphia chromosome (Ph+) and its molecular consequence is the generation of a BCR-ABL1 fusion oncogene, which is translated into BCR-ABL oncoprotein. Imatinib is the first line therapy in CML's treatment and acts as a selective inhibitor of BCR-ABL protein, trough competition for ATP's binding site. The adenine nucleotide signaling is modulated by the ectonucleotidases that actuate in sequence, composing an enzymatic cascade. Considering the relation of the purinergic signaling and cancer, the objective of this study was to characterize enzymatic activity, and expression of NTPDases and ecto-5'-nucleotidase in a chronic myelogenous leukemia human cell line (K-562) after treatment with imatinib. Methods and results: NTPDases and ecto-5'-nucleotidase activities were determined using ATP, ADP and AMP as substrate, by the measure of the amount of inorganic phosphate released, in a colorimetric assay after 24 and 48 hours treatment. These enzymes expression was determined by RT-PCR and Real Time PCR. The pattern of extracellular ATP metabolism was evaluated by HPLC analysis. The results demonstrate that ATP, ADP and AMP hydrolysis activities are linear up to 90 min of incubation. An interesting difference in ATP and ADP degradation rate was observed between 24 and 48 hours treatment. K-562 cell line expresses Entpd 1–3, 5, 6 and ecto-5'nucleotidase (CD73), responsible for modulating extracellular nucleotides level. Furthermore, after treatment with imatinib there was an increase in Entpd 3 and Entpd 5 expressions, respectively 3,32 ± 0,96 and 2,45 ± 0,94 fold when compared to the control cells and increased hydrolysis activity which could be caused by these enzymes activation in response to ATP's imatinib-induced accumulation, mainly after 24h treatment. Conclusion: Considering that these enzymes have an important catalytic activity and control purinergic nucleotide concentrations in cancer, the ectonucleotidases presence in CML cells can be important to regulate the levels of extracellular adenine nucleotides. Acknowldgements: Financial support from Capes/Brazil and PROPESq/UFRGS.
Keywords: purinergic signaling; chronic myeloid leukemia; imatinib.

58 - INOSINE, AN ENDOGENOUS PURINE NUCLEOSIDE, IMPROVE VASODILATORY RESPONSE AND EXERTS ANTI-INFLAMMATORY EFFECTS THROUGH ENOS ACTIVATION IN A HYPERCHOLESTEROLEMIC MODEL IN RATS.

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Introduction: Atherosclerosis is characterized as a chronic process closely related to inflammatory process and proliferative responses of the endothelium after injury (Ross RN, Engl J Med., 340(2): 115-26, 1999). Inosine, an analog of adenosine, results of adenosine deamination by adenosine deaminase (Nishikura, Annu Rev Bioquem., 79: 321-349, 2010). Adenosine and its analogs can change a variety of inflammatory diseases mediated by the immune system and has shown important effects at different models. The present study aims to evaluate the pharmacological properties of inosine, administered sub chronically in a hypercholesterolemic model in rats. Methods: The animal protocols were approved by the Ethics Committee for Experimental Research of the Federal Fluminense University (CEUA/UFF 858/2016). Adult male Wistar rats (200-250g) were randomly divided into three groups (n= 8, for each group): control group (C) fed standard chow diet, hypercholesterolemic diet group (HC) and hypercholesterolemic diet group + inosine (HC+ INO). At 31º diet day, was performed the sub chronic treatment with inosine (10mg/kg/orally) once daily, totalizing 15 days of treatment. The animals were euthanized by cervical dislocation under ketamine and xylazine anesthesia. Blood samples were collected for ELISA and biochemical analysis. Thoracic aortas were excised for vascular reactivity and western blot assays. Data were analyzed using one-way ANOVA followed by a post-

hoc Bonferroni Multiple Comparison Test, P<0.05. Results: The hypercholesterolemic diet increased serum lipid profile and aorta malondialdehyde levels in the HC group. Inosine reduced these levels (p<0.05), demonstrating its lipid lowering and antioxidant role. Furthermore, the HC diet increased IL-6 (C: 41,32 ± 1,25 pg/ml x HC: 110,7 ±5,44 pg/ml) and TNF-alpha; (C: 30.93 ±2.93 pg/ml x HC: 42.68 ±; 2.34 pg/ml) in serum, decreased the maximum relaxation induced by acetylcholine (83.02 ±4.07%) when compared to control group (89.17 ±1.36%); The treatment with Inosine reduced IL-6 (HC+INO: 47,96 ±4,17 pg/ml) and TNF-alpha; (HC+INO: 27.55 ±4.90 pg/ml) levels, iNOS, VCAM-1 and NF-κB protein expression in aortas of hypercholesterolemic rats (p<;0.05). Inosine increased the maximum relaxation promoted by aceylcholine (98.23 ± 2.21%), eNOS phosphorylation, PKA and PKG protein expression (p<0.05). Conclusion: This study demonstrated the ability of the hipercholesterolemic diet to promote vascular damages through increased of pro-inflammatory cytokines and proteins. On the other hand, we also showed that the treatment with inosine was capable to improve vascular function, probably by increase of PKA and PKG protein expression and the activation of eNOS, culminating in decrease of the inflammatory process through NF-κB and iNOS inhibition. This study provides results that indicate inosine as a potential drug for the treatment of cardiovascular disorders such as atherosclerosis. Financial support - CNPq, CAPES, PROPPI-UFF, FAPERJ
Keywords: Inosine; Inflammation; Atherosclerosis; Purine.

59 - LOCALIZATION OF ENTPDASES FROM *LEISHMANIA INFANTUM CHAGASI* REVEAL THAT LICNTPDASE1 COULD BE SECRETED AND HIGHER EXPRESSED IN PROMASTIGOTES WHILE LICNTPDASE2 WAS NOT SECRETED AND HIGHER EXPRESSED IN AMASTIGOTES.

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Objectives/background: *Leishmania infantum* chagasi has two known isoforms of ectonucleotidases from E-NTPDase family: NTPDase-1 (~70 kDa) and NTPDase-2 (~40kDa). These enzymes are known as possible virulence factors and there are evidences of their participation in important processes for the establishment of infection like adhesion and infection of host cells and the influence on host purinergic signaling. Those actions are dependent of ecto-localization of those enzymes as ecto-membrane or secreted proteins. Until now only evidences of ecto-membrane localization are shown and the secretion was not evaluated. In this context, the aim of this work was to evaluate the possible secretion of those enzymes which is important to direct the research in the field. Methods: Immunolocalization- *L. infantum* chagasi promastigotes in logarithmical growth were fixed and ultrathin sections were cut and incubated with an anti-recombinant LicNTPDase-2- antibody (1:400) except for control sections. Samples were photographed in transmission electron microscope (Zeiss EM 109). Secretion assay- Promastigotes were washed in secretion buffer and transferred to 1 mL of this same buffer supplemented with protease inhibitors. Samples were collected after 26° for 0min, 15min, 30min, 1h and 2h. Supernatants containing the secreted proteins were evaluated by WB using anti-recombinant LicNTPDase-2- antibody. LicNTPDases (1 and 2) mRNA expression in four different conditions were evaluated by RT-qPCR. LicNTPDases presence in total extract of parasites by promastigotes and axenic amastigotes forms were evaluated by western blot using the same antibody described above. Results: EM analysis revealed the presence of the LicNTPDases reactive gold immune staining on the out and inner cell surface and other at the extracellular medium, showing labeling in punctuate regions near the cell that highlight the possible secretion of LicNTPDases. The results from western blots (secretion assay) show a unique protein band with apparent MW of ~70kDa secreted after 1h and 2h. In addition, the LicNTPDase1 mRNA and protein was higher expressed by promastigotes in logarithmic stage used to do the secretion assay. On the other hand, LicNTPDase2 showed higher mRNA and protein levels in amastigote form. Conclusion: We shown for the first time for *L. infantum* chagasi promastigotes the possible secretion of LicNTPDases. Taken together, the presence of a signal peptide and a cleavage site in LicNTPDase1 gene, the visualization of EM images suggestive of secretion and the higher expression of LicNTPDase1 mRNA and protein in promastigote forms used in the secretion assay lead us to believe that LicNTPDase-1 could be secreted by promastigotes. LicNTPDase-2 seems to be higher expressed in amastigote form and was not secreted by promastigotes. Those results could be related with different roles of LicNTPDases during Leishmania infection. Acknowledges: CAPES, CNPq and FAPEMIG for finnancial support
Keywords: Visceral Leishmaniasis; Ectonucleotidases; Neglected Tropical Diseases.

60 - LYCORINE INHIBITS *TRICHOMONAS VAGINALIS* NUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASE AND ECTO-5'-NUCLEOTIDASE ACTIVITIES.

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Background: Trichomoniasis, the curable sexually transmitted infection (STI) caused by *Trichomonas vaginalis*, represents a profound impact on sexual and reproductive health worldwide3. There is an increase on metronidazole resistant isolates1 and drug discovery from plants plays an important role in the pharmaceutical therapy field, revealing the alkaloid lycorine a potential candidate. The ectonucleotidases nucleoside triphosphate diphosphohydrolase (NTPDase) and ecto-5′-nucleotidase (E5'-NT) have been characterized in *T. vaginalis*. The aim of this study was to evaluate the effect of lycorine on *T. vaginalis* viability and on ectonucleotidases activities. In addition, the alkaloid toxicity was also investigated. Methods and results: The ATCC 30236 *T. vaginalis* isolate was used in this study and the IC50 value was determinate. Hemolysis, in vivo/in vitro toxicity, the effect of lycorine on ROS production and on *T. vaginalis* NTPDase and E5'-N activities were evaluated. IC50 value was 32 μM. After 1 and 24 hours of incubation at 37 °C, lycorine did not cause significant hemolysis. The CC50 values for lycorine were 3.5 μM (HMVII cells) and 10 μM (fibroblasts), with selectivity indexes (SI) of 0.11 and 0.31, respectively. Lycorine was not toxic against G. mellonella larvae. Lycorine induced ROS production by neutrophils incubated with treated parasites. Conversely, trichomonads treated with lycorine did not present ROS accumulation. Ectonucleotidases activities were inhibited by lycorine (250 μM) on 24 h-treated parasites. Conclusion: Considering the cytotoxic and pro-inflammatory roles of ATP, the regulation of extracellular nucleotide levels could be relevant in increasing susceptibility of *T. vaginalis* to host immune response in the presence of lycorine. The effect on ROS liberation observed in neutrophils incubated with lycorine-treated trichomonads could be another adjuvant on

trichomoniasis treatment. On the other hand, lycorine did not increase ROS production on trichomonads, suggesting low resistance mechanism. Importantly, lycorine did not demonstrate toxicity in the in vivo model revealing a promising molecule for the design of new drugs.
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Ethical approval: Human blood samples (CEP/UFRGS: CAAE 47423415.5.0000.5347). Acknowledgements: B.P.S. acknowledges the fellowship from PIBIC/CNPq/UFRGS. This work was supported by CNPq (Brazil) grant # 305.173/2013-8 awarded to J.A.S.Z.
Keywords: Lycorine; *Trichomonas vaginalis*; nucleoside triphosphate diphosphohydrolase; ecto-5′-nucleotidase.

61 - PHYSICAL EXERCISE PREVENTS ATP AND ADP INCREASE IN SYNAPTOSOMES OF SEPSIS-INDUCED RATS.

ASSMANN, C.E.[1]; MIRON, V.V.[1]; COSTA, P.[1]; PALMA, T.V.[1]; LOPES, T.F.[1]; CASAGRANDE, A.G.[1]; MOSTARDEIRO, V.B.[1]; DRUZIAN, L.T.[1]; CARDOSO, A.M.[2]; SCHETINGER, M.R.C[2]; MORSCH, V.M.M.[2]
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Objectives/Background: Physical exercise has been well-known to positively influence in cases of inflammation-related diseases as sepsis, which alters many enzymes activity. Therefore, the aim of this study was to investigate whether physical exercise could have a protective effect on purinergic system enzymes activity in a lipopolysaccharide(LPS)-induced sepsis model. Methods: Adult male Wistar rats (30-60 days, 220-300g) were used in this experiment. All animal procedures were approved by the Animal Ethics Committee of the UFSM (protocol number: 5975191216). Rats were divided in control, LPS, exercise, and LPS+exercise. Animals of exercise groups were submitted to weathered exercise in a ladder for 12 weeks (Scheffer et al. App.Phys.Nutr.Met. 37;1239-1246, 2012) 3 times/w; in animals of LPS groups, sepsis was induced by intraperitoneal injection of LPS (2,5 mg/kg) (Schneiders et al. Brain.Beh.Imm. 48;147-164, 2015) 48 hours after the last session of exercise. These animals were euthanized 24h after induction of sepsis (Vuaden et al. Life Sci. 80;1784–1791, 2007). Synaptosomes were prepared according to Nagy & Escueta (J.Neurochem. 43,1114-23, 1984). Ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) and 5'-nucleotidase activities were determined as described by Balz et al. (Int.J.Dev.Neur.21,75-82, 2003). The released inorganic phosphate(Pi)was assayed by the method of Chan et al. (Analy.bioch.157;375-80,1986). Adenosine Deaminase (ADA) activity was determined according to Giusti and Gakis (Enzyme 12,417-425, 1971). Data were analyzed using 2-way ANOVA followed by Tukey's multiple range test, considering $p<0.05$. Results: ATP and ADP hydrolysis were increased in synaptosomes of LPS sedentary rats compared to control group [$F(1, 26) = 16,79$, $p = 0,0004$; $F(1, 18) = 7,077$, $p = 0,0159$] and exercise was able to prevent this increase [$F(1, 26) = 12,44$, $p = 0,0016$; $F(1, 18) = 10,48$, $p = 0,0046$]. AMP and adenosine hydrolysis did not present significant changes compared to control group although exercise had a tendency do decrease AMP hydrolysis. Conclusion: Sepsis triggered an increase in ATP and ADP levels of synaptosomes and this increase may be one of the mechanisms which explain the proinflammatory condition verified in septic animals. Physical exercise was able to prevent the increase of ATP and ADP in synaptosomes of animals with sepsis suggesting a new mechanism by which exercise may exert its anti-inflammatory effects. Financial support: CAPES, CNPq.
Keywords: Purinergic system; Nucleotides; Inflammatory diseases; Physical exercise.

62 - PHYSICAL EXERCISE PREVENTS ATP INCREASE IN SERUM OF ANIMALS WITH SEPSIS.

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Objective/background: Physical exercise has been used as a preventive tool in cases of diseases related to inflammation. Thus, the aim of this study was to verify the protective effect of physical exercise on purinergic system enzymes activity and ATP levels in a model of sepsis induced through the administration of lipopolysaccharide(LPS). Methods: Adult male Wistar rats (30–60 days; 220–300g) were used in this experiment. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol under number: 5975191216). Rats were divided in: control (CTL), exercise (EX), LPS and LPS+exercise (LPS+EX). The animals of exercise groups were submitted to weathered exercise in the ladder for 12 weeks (Scheffer et al. Applied Physiology, Nutrition, and Metabolism 37; 1239-1246, 2012) 3 times/w and in the animals of LPS groups the sepsis was induced by intraperitoneal injection of LPS (2,5 mg/kg) (Schneiders et al. Brain, Behav. Immunity 48; 147-164, 2015)48 hours after last session of exercise. These animals were euthanized 24h after the sepsis induced (Vuaden et al. Life Sci. 80;1784–1791,2007). Blood was collected by cardiac puncture and serum and lymphocytes were isolated for analysis. The quantitative adenosine triphosphate(ATP)determination was developed using commercial kit by bioluminescence assay (Karamohamed et al. Biotechniques 31; 420-425, 2001). Ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) activity was determined as described by Leal et al. (Biochimica Biophysica Acta 18, 1721; 9-15, 2005). The released inorganic phosphate(Pi)was assayed by the method of Chan et al. (Analyt. Bioch. 157; 375-80, 1986). Adenosine Deaminase (ADA) activity was determined according to Giusti and Gakis (Enzyme 12, 417-425, 1971). Data were analyzed using 2-way ANOVA followed by Tukey's multiple range test, considering $p<0.05$. Results: The levels ATP in serum was increased in animals of LPS group when compared to CTL group (0,5±0,03 vs. 0,3±0,04 in CTL) and exercise was able to prevent this increased (0,5±0,03 vs. 0,4±0,06 in LPS+EX). ATP hydrolysis decreased in sedentary animals after LPS administration when compared to CTL group and exercise was able to partially prevent this decrease. ADP hydrolysis increased in animals of LPS+EX group when compared to EX group. Adenosine hydrolysis was increased in animals of LPS group and exercise was able to partially prevent this increase. Conclusion: Sepsis triggers an increase in extracellular ATP levels as well as a decrease in NTPDase activity in lymphocytes, which may be one of the mechanisms that explains the proinflammatory condition verified in septic animals. The exercise

was able to prevent the increase of ATP concentration in serum of animals with sepsis as well as prevent or partially prevent the ectonucleotidases alterations, suggesting a new mechanism in which exercise exerts its anti-inflammatory properties in sepsis. Financial support: CAPES, CNPq.
Keywords: Physical exercise; Sepsis; Purinergic System.

63 - PRELIMINARY RESULTS IN GUANOSINE NANOASSEMBLIES.

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Objectives / background: Guanosine (GUO) is a natural compound with important role in many central nervous system (CNS) process, however peripheric and extracellular metabolism reduce its concentration and effect (Alonso-Andrés et al. Brain Pathol. doi:10.1111/bpa.12592; 2018). Besides, GUO shares signaling mediated-neuroprotection with adenosine through existing receptors, i.e. adenosine A1 and A2a receptors (Ciruela. J. Neurochem. 126; 425, 2013). Bioactive delivery remains the main challenge of CNS drug development, as have been observed for GUO (Di Liberto et al. Front Pharmacol. 7; 2016). Nanotechnology is one of the most promising technologies to develop new strategies for controlled protection and release of the bioactive compounds (Zhang and Ma. Nano Today, 5, 337; 2010). In the present study, the aim is performing preliminary production of nanoassemblies conjugating GUO to natural and biocompatible lipid. Methods and results: The polyunsaturated fatty acid (PUFA; C30:6n-omega2) was covalently linked onto the amino group of the GUO to form the prodrug PUFA-GUO. Nanoassemblies were prepared by nanoprecipitation method and characterized by size, polydispersity index (PDI), Zeta potential and pH determination. The stability of the nanoparticles formulation was evaluated at different temperatures (± 4 °C, ± 25 °C and ± 40 °C) during 15 days. It was observed that the PUFA-GUO nanoparticles showed approximately particle diameter of 160 nm, polydispersity index of 0.257, zeta potential values of -16.9 mV and pH 7.0. Stability study indicated no significant alterations for nanoassemblies at 25 °C after 7 days, but important increasing in all parameters after 15 days for all exposition temperatures. Conclusion: The conjugation of GUO to the lipid and the subsequent formation of nanoassemblies indicated the efficient spontaneous formation of the PUFA-GUO nanoparticles, which were stable for up to 7 days at 25 °C. However, there is a need for further investigations improving the nanoassemblies formulation, increasing physical stability during storage. Also, applying nanotechnology to these agents may be delivering strategy to sites of action within the CNS and useful to evaluate its neuroprotective effect.
Keywords: nano-conjugation; drug delivery; guanosine.

64 - PURINERGIC SIGNALING DIFFERENTIALLY MODULATES TISSUE IMMUNE RESPONSE FATE IN EXPERIMENTAL CHAGAS DISEASE.

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Background and Objective: Chagas cardiomyopathy, caused by the intracellular parasite *Trypanosoma cruzi*, constitutes a major public health problem in Latin America due to its prevalence and mortality. Despite the development of life-long immunity, the immune system fails to completely clear the parasites that persist within host tissues. One potential immunosuppressive system that could regulate anti-parasitic immune functions is CD73 ectoenzyme/adenosine (ADO) pathway. We previously reported that transient pharmacological inhibition of CD73 during the early acute phase of murine *T. cruzi* infection induced microbicidal mechanisms, a reduction in cardiac parasite load and the consequent improvement in chronic cardiomyopathy outcome (Ponce et al. J Immunol, 2016, 197:3). The aim of this study was to characterize the effect of the genetic abrogation of CD73 on the microbicidal immune response in different *T. cruzi*-target tissues. Methods and Results: To this aim, immune system modulation and its implications in *T. cruzi* infection response were comparatively studied in heart, liver and visceral adipose tissue (VAT) from CD73 knockout (KO) and C57BL/6 (WT) infected mice (Tulahuen strain trypomastigotes). The kinetics of cardiac macrophage (Ma) subsets showed a predominant inflammatory/M1 (CD86+ CD206-) ($p<0.001$) subset throughout the acute infection with higher frequencies of IL-1^{2+} and iNOS+ M1 Ma ($p<0.05$) and augmented cardiac nitric oxide levels ($p<0.001$) in KO compared to WT mice. Moreover, KO cardiac tissue exhibited increased IFN-γ+ and CD107a+ CD8 T cells frequency ($p<0.05$) and consequent lower parasite load (9.26± 0.45 AU) compared to WT (453.2±23.1 AU) ($n=6$; $p<0.05$) at 21 days post-infection (dpi). Strikingly, KO mice exhibited higher parasitemia compared to WT mice ($n=6$; $p<0.05$). Moreover, VAT parasite load was augmented at 21 and 28 dpi (7.83±1.62; 9.11±2.14 AU) in KO compared to WT (0.077±0.013; 0.051±0.006 AU) ($n=8$; $p<0.01$), likely due to an increased basal VAT/body weight ratio (0.97±0.07%) compared to WT mice (0.64±0.05%) ($n=12$; $p<0.001$), thus generating an important reservoir for parasite growth. Furthermore, no differences were observed in M1 Ma kinetics in VAT. As for liver, parasite burden was augmented in KO (327.5±54.3 AU) compared to WT mice (122.5±10.0 AU) ($n=4$; $p<0.001$) at 21 dpi. These findings could be explained by the purinergic signaling differential impact on *T. cruzi*-target tissues evidenced by the significantly augmented ATP/ADO ratio in KO heart (15.4±4.12) compared to WT (2.83±1.46) ($n=5$; $p<0.01$). Likewise, cardiac ATP/ADO ratio was increased compared with liver (3.23±1.11) and VAT (5.63±0.92) in KO mice ($n=5$; $p<0.05$). Conclusion: ATP catabolic machinery inhibition generates a unique tissue purinergic milieu that contributed to the modulation of host immune response which modifies parasite-host interaction and, consequently, *T. cruzi* persistence. Fundings: SECyT-UNC; ANPCyT-FONCyT; CONICET.
Keywords: CHRONIC CHAGAS CARDIOMYOPATHY; MACROPHAGE; ATP; ADENOSINE.

65 - PURINERGIC SIGNALING PROFILE IN B-PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA.

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B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a neoplasm characterized by an anomalous clonal proliferation of B lymphoid precursor cells which deregulates physiological and immunological processes. The ectoenzymes E-NTPDase (CD39), E-5'-NT (CD73), and ecto-adenosine deaminase (E-ADA) regulate the extracellular concentrations of ATP, ADP, AMP and adenosine. Objectives: This study aimed to evaluate, in peripheral lymphocytes, the activities of E-NTPDase and E-ADA, as well as the expression of CD39 and CD73. Besides, we evaluated the activities of NTPDase, ADA and xanthine oxidase (XO), and also the levels of purines and cytokines in serum of BCP-ALL patients. Methods and results: Newly diagnosed pediatric patients (mean age 8.4 years) were evaluated in two timepoints: at diagnosis (D0) (n=32) and 15 days after treatment (D15) (n=14) and compared to a control group (C) (34 healthy individuals, mean age 8.7 years). The study was approved by the UFSM Human Ethics Committee, protocol number 293.865. E-NTPDase activity was determined following Leal et al. (Acta Biochim Biop, 1721, 2005) in isolated peripheral lymphocytes, and Oses et al., (Life Sci, 74; 3275, 2004) in serum, and expressed in Pi/min/mg of protein and Pi/min, respectively. E- ADA in lymphocytes and ADA in serum were determined by the method described by Giusti & Galanti (Methods Enzym. Anal 4; 315, 1984), and expressed in U/mg protein and U/L, respectively. Cytokines levels in pg/mL were assessed by flow cytometry. Serum purine levels in nmol/mL were evaluated by HPLC. One-way ANOVA and Mann-Whitney tests were used for statistical analysis. Results were expressed as mean±SEM ($P<0.05$). E-NTPDase activity (ADP hydrolysis) was reduced on D15 (51%) while E-ADA activity was increased on D0 (135%) and on D15 (82%) in relation to the C group. On D0, in serum, NTPDase activity was decreased by 45% for ATP and by 29% for ADP hydrolysis and XO was 46% lower, whereas ADA activity was increased by 126%. CD39 and CD73 expression in lymphocytes were distinct according to the cell maturation degree. In lymphoblasts, D0 showed lower CD39 (54%) and higher CD73 (70%) expression in relation to lymphocytes within the same group. On D0, serum levels of adenosine (155%), inosine (317%) and xanthine (119%) were significantly increased, whereas of hypoxanthine (53%) were decreased compared to the control group. IL-6 (22.15±3.57), IL-17 (50.65±8.51) and IL-10 (8.99±2.47) levels were significantly increased on D0 when compared to C group. Conclusion: The results showed an inflammatory status of BCP-ALL at diagnosis and a possible modulation of cytokines and purinergic signaling profile on neoplasic lymphocytes, which may influence in the immunosuppression status and the changes in the immune response, enabling neoplasic cell proliferation. Acknowledgment of financial support: PROIC/UFSM, FAPERGS, CNPq, CAPES
Keywords: BCP-ALL; E-NTPDase; E-ADA.

66 - SEARCH FOR RECOMBINANT *TRYPANOSOMA CRUZI* NTPDASE-1 INHIBITORS REVEALS ENZYME INHIBITORY ACTION AND LEISHMANICIDAL ACTIVITY OF QUERCETIN DERIVATIVE.

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Chagas disease caused by *Trypanosoma cruzi* is one of 13 major neglected diseases. It is estimated that there are about 6 to 7 million people infected in the world. *T. cruzi* has on its surface an ectonucleotidase called NTPDase-1. This enzyme is capable of hydrolyzing tri- and diphosphate nucleotides. Extracellular nucleotides play important roles in cell signaling, acting on several cellular processes, such as the modulation of the immune response of mammalian hosts. Our research group has been studying *T. cruzi* and Leishmania NTPDases and their relation with parasite-host interaction. Previously we demonstrated that TcNTPDase-1 plays important role in the infectivity, virulence and adhesion of the parasite to the host cell. This allow us to believe that TcNTPDase-1 is a good target for new drugs for Chagas disease chemotherapy. Therefore, this work aimed to search for inhibitors of recombinant TcNTPDase-1. For this, the nucleotidase activity of the recombinant enzyme was measured using the Malachite Green method. All the assays were done in triplicate and the statistical analyzes used ANOVA in software GraphPad Prisma versão 5.01. Interestingly, from chemical screening two plant extracts and one purified compound shown total inhibitory activity were identified: the extracts BCFE and BCFEA, and the compound isolated from BCFE CIII-L, a quercetin derivative. The CIII-L compound was chemically synthesized. CIII-L and several synthetic intermediates were also tested as TcNTPDase-1 inhibitory activity. The intermediates of the synthesis IL-05 and IL-09 shown the highest inhibitory activity (47% and 94% respectively). Next, the toxicity assay of IL-05, IL-09 and IL-18 in Leishmania braziliensis promastigotes and in Raw 264.7 macrophages were evaluated. We used the compounds at concentrations of 1 to 100 μM for 48 hours and the effects were measured by the Resazurin Method. In macrophage IL-05, IL-09 and IL18 showed low toxicity, with IC50 close to 100 μM to all compared to control. Moreover, leishmanicidal activity IC50 40,1 μM; 10 μM and 31.62 μM respectively compared to control. The results allow us to conclude that it was possible to select extracts and compounds with inhibitory capacity on the recombinant TcNTPDase-1 enzyme. In addition, the compounds IL-05, IL-09 and IL-18 demonstrated low toxicity in macrophage and significant leishmanicidal activity.
Financial support: CNPq, INBEQMeDI, A-ParaDDisE, e FAPEMIG. Keywords: NTPDase-1 inhibitors; quercetin derivative; *Trypanosoma cruzi*; leishmanicidal activity.

67 - SELECTION OF APTAMERS TARGETING CD73 BY USING CAPILLARY ELECTROPHORESIS-BASED PROTOCOLS.

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BACKGROUND: The metabolism of ATP into its metabolites ADP, AMP, and adenosine, and consequently, the regulation of purinergic signaling, is a tightly regulated process by a family of cell surface-located ecto-nucleotidases, including nucleoside triphosphate diphosphohydrolases (NTPDases) and ecto-5'-nucleotidase (CD73). AMP is generated by the stepwise catabolism of ATP via the intermediate ADP in two reactions predominantly carried out by CD39 (NTPDase-1), whereas CD73 is required for the conversion of AMP to adenosine. Adenosine, in turn, is able to activate receptors expressed in membranes of immune system cells, playing a profound immunosuppressive effect. This draws attention to CD73 as a potential therapeutic target against cancer, in which immune system suppression is crucial for the initiation of malignant neoplasms and the progression of established tumors. Indeed, recent evidence suggests that CD73 inhibition reduces tumorigenesis and metastasis, as well as enhances the efficacy of conventional therapies. However, still

only available very few CD73 inhibitors are available for pre-clinical trials. OBJECTIVES: Here we propose to develop capillary electrophoresis methods for the selection of aptamers targeting CD73. These oligonucleotides developed by an in vitro selection protocol show affinity and specificity comparable to those obtained with monoclonal antibodies, are easily produced and optimized and lack of immunogenicity, representing a promising alternative for the use of small molecules and antibodies. METHODS AND RESULTS: Here we combined molecular biology and capillary electrophoresis techniques for the development of selection protocols of aptamers against CD73. Tris and borate buffers were tested as run buffers at different concentrations. Although increasing Tris concentrations have had the positive effect of reducing the protein absorption in the capillary walls, they also resulted in more time-consuming separations. The same was observed when we used ethyleneglycol to decrease the electroosmotic flow. The best resolution between the DNA and target peaks was obtained with 50 mM and 100 mM Tris-acetate when the protein and a catalytic site-corresponding peptide were used as targets. After determining the aptamer collection window and performing one selection cycle against 5 nM target, PCRs were carried out in order to determine the best number of cycles, which was found to be 20. CONCLUSIONS: In summary, intermediate run buffer concentrations resulted in better shaped peaks, higher resolution and non-time-consuming capillary electrophoresis protocols for the separation of aptamers targeting CD73. The first selection cycle has been already performed and this method will be further applied in order to obtain aptamers with higher specificity and affinity. ACKNOWLEDGMENTS: This work was financial supported by FAPESP.
Keywords: CD73; aptamers; capillary electrophoresis.

68 - SELECTION OF POTENTIAL HUMAN ECTO-5'-NUCLEOTIDASE (CD73) INHIBITORS BY VIRTUAL SCREENING.

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Objectives/background: Human ecto-5'-nucleotidase (ecto-5'NT, CD73) is a GPI membrane-anchored protein, which plays a pivotal role in purinergic signaling pathways (1). Ecto-5'NT hydrolyzes AMP to adenosine, acting as a major control point for the extracellular provision of this signal molecule (1,2). Recent studies have shown that ecto-5'NT is upregulated in tumor cells from several types of cancer (2). Indeed, CD73-generated adenosine has been shown to accumulate in the tumor microenvironment, triggering immunosuppressive responses that favors neoplastic progression (3). Accumulated adenosine is also known to regulate tumor angiogenesis, as well as proliferation, differentiation and apoptosis of cancerous cells (2,3). Despite its relevance as a potential target for cancer and even for many other disorders, so far only few ecto-5'NT inhibitors have been reported, and most of them are not suitable as drug candidates. Here, to search for novel potential ecto-5'NT inhibitors, virtual screening (VS) models have been proposed (4,5). Methods and results*: Two VS models (VS-1 and VS-2) were generated, applying a sequence of pharmacophore, drug-like, docking and visual inspection filters to the ZINC database (~23x106 compounds). Model VS-1 was based on ecto-5'NT open conformation crystal structure, complexed with a peptidonucleoside inhibitor (PSB11552). Model VS-2 was based on ecto-5'NT closed conformation crystal structure, complexed with AMPCP inhibitor. From each of these crystal structures, a pharmacophore model, generated using LigandScout 3.01, was applied to the ZINC database as a first filter. Subsequently, compounds which violate one or more Lipinski's Rule of 5 parameters limits were eliminated. In a third step, the remaining compounds were docked into PSB11552 binding site (Model VS-1) or into AMPCP binding site (Model VS-2), using GOLD 5.2. Finally, docked compounds were subjected to visual inspection. Model VS-1 selected 12 compounds from the ZINC database (~99% reduction), from which 6 were purchased and submitted to enzymatic assays for VS experimental validation, using the malachite green method. Model VS-2 selected 13 compounds from the ZINC database (~99% reduction), from which 2 were purchased and submitted to enzymatic assays, using the malachite green method. So far, four tested compounds showed moderated inhibitory activity against ecto-5'NT. Conclusion: The generated VS models, applied to the ZINC database, selected 4 structurally diverse compounds with moderated inhibitory activity against ecto-5'NT.
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Keywords: Ecto-5'-nucleotidase; virtual screening; inhibitors.

69 - STABILITY AND FUNCTION OF GLIOBLASTOMA-DERIVED EXTRACELLULAR VESICLES.

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Background/Objectives: Glioblastoma (GBM) is the most malignant and has the poorest survival rate of the glial tumors. The tumoral tissue is composed by many proliferating neoplastic cells, fibroblasts and cells of immune system. Tumor proliferation depends on complex network factors, such as cytokines, adenosine and extracellular vesicles. It has been suggested these vesicles may activate the immune system against tumor growth. In this context, we seek to understand the role of glioblastoma-derived extracellular vesicles (GEVs) in peripheral lymphocytes modulation and GBM progression. Methods and Results: GEVs were isolated by differential centrifugation of C6 cell line supernatant. GEVs stability was analyzed by Zetasizer equipment on days 1, 4 and 18. For better characterization of these vesicles, extracellular ATP metabolism was analyzed by HPLC. GEVs (8 μg) were incubated with ATP 50 μM for 30 minutes and treated with inhibitors of CD39 and CD73 enzymes (ARL-67156 and APCP, respectively), as well as with an ADO uptake inhibitor (dipyridamole). Further, C6 glioma cells were treated with different concentrations of GEVs during 96 h (n=3) and cell viability was assessed by MTS assay. Then, GEVs were incubated (8 μg) with mesenteric lymphocytes isolated from adult Wistar rats (300-400 g). After 48 h of incubation, the expression of CD39 and CD73 enzymes was evaluated by flow cytometry. The in vivo GBM models were performed by co-

injecting GEVs with C6 GBM cells into the striatum by stereotactic surgery of adult Wistar rats. After 14 days of tumor growth, the rats were decapitated and the entire brain was removed for tumor size quantification (Protocol #33505). GEVs presented uniform size (175,2±6,14nm) and the stability, at 4°C, remained constant during the 18 days tested (186,8±6,64nm). Inhibitors of CD39 and CD73 enzymes reduced ADO formation (52,1% and 57,8%, respectively) while ADO transporter inhibitor did not alter extravesicles amount of this nucleoside. The percentage of viable cells was significantly reduced after treatment with 16 and 32 μg/mL of GEVs (from 120±2,12% to 82,52±5% and 92,1±7,9%, respectively). The incubation of T-lymphocytes with GEVs did not alter the expression of CD39 and CD73 protein in any tested subset of T-lymphocytes. Moreover, the co-injection of GEVs reduces the GBM size from 192,8±38,1 mm^3 to 99,6±47,7 mm^3 in comparison to GBM group. Conclusion: According these preliminary results, it is possible to assume the size of GEVs remain stable when stored at 4°C and these vesicles express CD39 and CD73 enzymes, but not the nucleoside transporters. After the treatment with GEVs, we observed the capacity of these vesicles in reduce viability of C6 GBM cells, but they didn't affect peripheral T-lymphocytes. After these results, we aim to investigate, as our main perspective, if vesicles are able to modulate lymphocytes in the tumor microenvironment. Financial Support: CAPES, CNPq and FAPERGS
Keywords: Glioblastoma; Extracelular Vesicles; Adenosine.

70- THE ANTI-*TRICHOMONAS VAGINALIS* PHLOROGLUCINOL DERIVATIVE ISOAUSTROBRASILOL B MODULATES EXTRACELLULAR NUCLEOTIDE HYDROLYSIS.

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Objectives/background: *Trichomonas vaginalis* causes trichomoniasis, a neglected non-viral sexually transmitted infection with 276.4 million new cases annually worldwide1,2. Treatment relies on 5-nitroimidazole drugs however; adverse effects and 10% of resistance already reported highlight the need for new alternatives3. Phloroglucinols detected in Hypericum perforatum species, presents biological activities and antiparasitic properties against *T. vaginalis* and L. amazonensis. Biochemical pathways provide excellent targets in parasites, as the control in nucleoside and nucleotide levels by ectonucleotidases, directly modulating inflammation4. The study aimed the anti-*T. vaginalis* activity of phloroglucinols derivatives in NTPDase and ecto-5'-nucleotidase activities and modulation of the immune system. Methods and results: H. perforatum phloroglucinol derivatives were obtained through chromatography. Screening was performed using ATCC-30236 *T. vaginalis* isolate and those derivatives with better activities were further used. Determination of the half maximal inhibitory concentration (IC50) and in vivo/in vitro toxicity assays were performed. The effect of the most active compound, isoaustrobrasilol-B, was tested against erythrocytes, determination of reactive oxygen specimen and proinflammatory cytokines produced by neutrophils, and modulation of NTPDase and ecto-5′-nucleotidase enzyme activities. Isoaustrobrasilol B (IC50 38 μm) showed better activity and, despite of some cytotoxicity against mammalian cell lineage, in vivo model of Galleria mellonella and erythrocytes showed no toxicity. Interestingly, enzymes were significantly inhibited and leading to accumulation of pro-inflammatory nucleotides. The immune response was also favored by the increase in the production of IL8. Conclusion: The associative mechanism of trophozoites death and ectonucleotidases modulation by isoaustrobrasilol B may increase the susceptibility of *T. vaginalis* to host innate immune cells consequently, contributing to parasite clearance. References: World Health Organization, Geneva 2012. Secor et al. Am. J. Trop. Med. Hyg. 90; 800, 2014. Schwebke et al. Antimicrob. Agents Chemother. 50;4209, 2006 Bours et al. Pharmacol. Ther.112; 358 2006. Ethical approval: Human blood samples (CEP/UFRGS: CAAE 47423415.5.0000.5347). Acknowledgment: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil), Marine Biotechnology Program and Universal Program supported this study.
Keywords: *Trichomonas vaginalis*; isoaustrobrasilol B; NTPDase; ecto-5'-nucleotidase.

71 - THE EFFECT OF CHANGES IN THE CD73 ACTIVITY ON THE AORTIC VALVE AND ENDOTHELIUM FUNCTION IN MICE.

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Objectives/background: Aortic stenosis is known to involve inflammation and thrombosis. Changes in activity of ecto-5'-nucleotidase – an enzyme of extracellular nucleotide catabolism - can alter inflammatory and thrombotic responses. The aim of this study was to investigate the impact of the absence of CD73 activity on the function, structure and metabolism of a murine aortic valve and endothelium. Methods and results: Male C57BL/6J Wild Type (WT; n=21) and C57BL/6J CD73−/− (CD73−/−; n=21) mice were used for these experiments. At the age of 9 weeks, the animals were randomly divided into: normal-fat diet WT, normal-fat diet CD73−/−, high-fat diet WT and high-fat diet CD73−/−. Groups were maintained for 15 weeks followed by echocardiographic analysis of aortic valve function, measurement of aortic surface activities of nucleotide catabolism enzymes as well as alkaline phosphatase activity, plasma L-arginine derivatives as well as nicotinamide metabolites concentration, mineral composition and histology of aortic valve leaflets. Results are presented as mean ± SEM. CD73 knock out led to increase in peak aortic flow (1.06±0.26m/s) compared to WT (1.06±0.26m/s vs. 0.79±0.26m/s; $p<0.01$) indicating obstruction. Highest values of peak aortic flow (1.26±0.31 m/s) were observed in high-fat diet CD73-/- mice. Histological analysis showed morphological changes in CD73-/- including thickening and accumulation of dark deposits, proved to be melanin. Concentrations of $Ca^{2+,}$ Mg^{2+} and PO43- in valve leaflets were elevated in CD73-/- mice. Alkaline phosphatase (ALP) activity was enhanced after ATP treatment and reduced after adenosine treatment in aortas incubated in osteogenic medium. AMP hydrolysis in CD73-/- was below 10% of WT. Activity of ecto-adenosine deaminase(eADA), responsible for adenosine deamination, in the CD73-/- was 40% lower when compared to WT. CD73 knock out led to significantly decreased plasma L-Arginine/ N(G), N(G)-dimethyl-L-arginine (ADMA) ratio and decreased N(G),N'(G)-dimethyl-L-arginine (SDMA) level. Conclusions: Deletion of CD73 in mice leads to aortic valve and endothelium dysfuction similar to that induced by high-fat diet. Alterations in CD73 function may contribute to human valve and vascular pathology. Acknowledgments: This study was supported by National Science Centre of Poland (2015/19/N/NZ1/03435).
Keywords: Adenosine; CD73 knock-out mice; Ecto-5′-nucleotidase; Inflammation.

72 - THE EXTRACELLULAR NAD+ AND NMN METABOLISM ON THE SURFACE OF HUMAN AORTIC VALVES.

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Nicotinamide adenine dinucleotide (NAD+) plays a crucial role in the energy metabolism as a redox carrier and an important coenzyme. Furthermore, NAD+ is a substrate for enzymes responsible for intra- and extracellular signaling pathways. Previous studies have pointed to an important role of extracellular enzymes in controlling adenine nucleotide levels in pathological conditions, for instance, aortic stenosis (AS). The aim of this study was to investigate the catabolic pathways of NAD+, mononucleotide nicotinamide (NMN), and nicotinamide (Nam) on the surface of human aortic valves in patients with AS. Stenotic aortic valves have been obtained from patients undergoing aortic valve replacement (AVR, study group, n=50), while non-stenotic valves after Bentall surgery (control group, n=10) followed by the approval of the local Bioethical Committee. The fragments of aortic valves were immediately placed in a specially designed 24-well plate, which allowed for the exposure of the well-known surface. A buffer and the corresponding substrate: NAD+, NMN or Nam, respectively, were then added to wells and 2 hours incubation was carried out. Additionally, an experiment with inhibitors for CD73 (ecto-5'nucleotidase), NPP (nucleotide pyrophosphatase/phosphodiesterase), ALP (alkaline phosphatase), and CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase) was performed to demonstrate, which of the enzymes could be responsible for the catabolism of NAD+ and its derivatives. The analysis of the products concentrations was tracked using high-performance liquid chromatography (HPLC). Cellular sources of ecto-enzymes were analyzed by immunofluorescence. On the surface of human aortic valves, an active metabolism of NAD+ and NMN was observed. Nicotinamide conversion was not noticed. In the patients with AS, the extracellular NAD+ and NMN hydrolysis enzymes activity of aortic valves were significantly higher than in the control group (0.81 ± 0.07 vs 0.56 ± 0.10 and 1.12 ± 0.10 and 0.71 ± 0.08 nmol/min/cm2, respectively) ($p < 0.05$). Initial experiments with inhibitors have shown that the enzymes - eNPP and CD73, and to a lesser extent CD38, had the largest involvement in the degradation of NAD+. In the case of NMN catabolism, CD73, CD38, and ALP were responsible for these reactions. It has been demonstrated that patients with aortic stenosis have an increased metabolism of NAD+ and NMN on the surface of aortic valves. These changes may be a part of a protective mechanism of the valve. An accurate knowledge of the degradation pathway of NAD+ and NMN could have an impact on the better understanding of the processes occurring in aortic valve pathology. This study was supported by the Polish Ministry of Science and Higher Education.
Keywords: nicotinamide adenine dinucleotide; mononucleotide nicotinamide; ecto-enzymes; aortic valve.

73 - THE POTENTIAL ROLE OF THE PURINERGIC SYSTEM IN AN ANIMAL MODEL OF SCHIZOPHRENIA

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Background: Schizophrenia is an incapacitating, chronic and debilitating psychiatric disorder. It is known that genetic susceptibility and environmental insults during early development can disrupt brain maturation that can be a trigger for the disease onset. However, the whole complexity of this multifactorial disorder is widely unknown. In this sense, the goal of this work is to investigate the role of the purinergic signalling in the central nervous system in social isolation model. Methods: Male Wistar rats were allocated at post-natal day 21 in two groups: animals that were reared in social isolation (SI) (1 animal/cage) or group housed (GH) condition (3-4 animals/cage) for 8 weeks (CEUA-UFRGS 33550). After, rats were submitted to behavioral tests: open field (OFT), spontaneous alternation, social interaction and prepulse inhibition (PPI). Level of purines (ATP, ADP, AMP, adenosine, guanosine, inosine, hypoxanthine, xanthine and uric acid) was measured in cerebral spinal fluid (CSF) by High Performance Liquid Chromatography (HPLC). Bioinformatics analysis for differential expression for all genes of the purinergic system was performed in postmortem brains of controls and patients with schizophrenia. Expression datasets were obtained from GEO. For microarray studies were used the limma package. RNASeq studies were computed using DESeq2 package. These analyses were implemented in R environment. Data analysis was expressed as mean±s.d. Results: SI rats showed a sensorimotor gating deficit at 77 and 85 dB (GH: 13,68± 3,39; SI: 3,51± 2,11, p=0,0151; GH: 24,06± 2,82; SI: 9,60± 2,86, p=0,0004, respectively; two-way ANOVA with Bonferroni post hoc test) prepulses intensities when compared with GH group in the PPI test (n=10 and 9, respectively) with no differences at 71 dB prepulse (p>0,05). There were no differences in travelled distance in the OFT and percentage of spontaneous alternation (p>0,05, t test) (n=10/group). In social interaction test, the time of interaction with the stranger animal was increase in SI rats (GH: 121,8±21,04 s, n=10; SI: 224,6±22,6 s, n=10; p<0,0001, t test). HPLC analysis of CSF showed an increase in ADP levels in SI group (GH: 1,38±0,047 μM, n=8; SI: 2,71±1,46 μM, n=7; p=0,0224, t test). All others purines analyzed have no differences between groups (p>0,05). Finally, bioinformatics analysis showed a decrease of ADORA3 in prefrontal cortex; P2RX2, P2RX5, P2RY11 and SLC29A1 in dorsolateral prefrontal cortex (DLPFC); P2RY13 in striatum; P2RY13 and ENTPD3 in hippocampus (HPC) of patients. Also, it was seen an increase of P2RY14, NT5E, P2RY2 in DLPFC; ADORA2B and ENTP2 in HPC of patients. These results will be evaluated in the present model. Conclusions: So far, our data showed that purinergic system might potentially be involved in pathophysiology in the social isolation model. As perspective, we planned to perform hippocampal and striatum slices to evaluate the nucleotide hydrolysis pattern. Support: CNPq and INCT 2014.
Keywords: Schizophrenia; Social Isolation model; Purinergic signalling.

74 - THE PURINERGIC SYSTEM AND ITS RELATION WITH THE IMMUNE SYSTEM IN DIABETIC RATS INFECTED OR NOT WITH *CANDIDA ALBICANS*.

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Objectives/background: The involvement of ectonucleotidases during infections has been described, but little information is available on how these enzymes would contribute to the understanding of the pathophysiology of fungal infections in patients with diabetes mellitus (DM). The objective of this study was to evaluate changes in purinergic and inflammatory system in DM rats infected or not by *Candida albicans*. Methods and results: Male Wistar rats were divided into four groups (n=6): G1, control; G2, *Candida albicans* (CA); G3, diabetic (DM); G4, DM + CA. DM was induced by a single intraperitoneal (IP) injection of streptozotocin (60 mg/kg). *C. albicans* yeasts (105 UFC/mL) were inoculated (IP) in the respective groups after 15 days of DM induction (Fisher et al. Antimicrob Agents Chemother, 33: 1042-1045, 1989). After 21 days ADA (U/L), 5′NT and NTPDase (ATP, ADP; nmol Pi/min/protein) activities (Schmatz R et al. Life Sci, 84: 345-350, 2009) and IL 1β, IL10, TNF-α and INF-γ were measured in serum. Data were expressed as mean ± S.E. Ethic Committee number 074/2014. An signicant increase in serum ADA, 5'-NT and NTPDase (ATP and ADP) activities was observed in CA (7.21±0.44; 3.21±0.19; 2.10±0.11; 1.08 ±0.09), DM (8.56±0.3; 3.46±0.19; 2.30±0.08; 1.10±0.10) and DM+CA (7.71±0.52; 3.87±0.25; 2.15±0.08; 1.14±0.06) when compared to control (3.08±0.26; 2.33±0.06; 0.69±0.08; 0.27±0.02). DM promoted an increase in 5'-NT and NTPDase activities in serum as a response to metabolic insult (Lunkes G et. Thromb Res,109: 189-194, 2003). The animals inoculated with *C. albicans* also showed an increase in ATP, ADP and AMP hydrolysis in serum, contributing to an increase in adenosine production. Levels of the pro-inflammatory cytokines were increased in DM, CA and DM+CA when compared to control ($p<0.01$). Furthermore, we observed a decrease in IL10 levels in DM, CA and DM+CA when compared to control ($p<0.01$). Hyperglycemia is known to increase the production of free radicals and to induce inflammation, which can lead to a permanent stimulation of immune cells (Kim JS et al. Biol Sport, 31:73-79, 2014), promoting the extremely strong inflammatory response observed. Conclusions: Our findings indicate that the upregulation of ectoenzymes in serum, which reflects the immune system status, may play an important role in controlling cellular responses induced by diabetes complications and attenuating systemic changes caused by the fungal infection. Acknowledgment: CNPq; CAPES
Keywords: Diabetes; animal model; *Candida albicans*; purinergic system.

75 - THE SIGNIFICANCE OF NUCLEOSIDE DIPHOSPHATE KINASE (NDPK)-DEPENDENT TRANSPHOSPHORYLATION FOR ADP/ATP CARRIER (AAC)-MEDIATED MITOCHONDRIAL PROTON LEAK OF YEAST Saccharomyces cerevisiae.

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Background: The AAC of S. cerevisiae is considered a main catalyst of the futile (non-phosphorylating) proton leak across the inner mitochondrial membrane (Brand et al. Biochem J 392; 353, 2005). The AAC-mediated proton leak is inhibited by both, carboxyatractyloside (CATR), a classic strong inhibitor of the carrier for nucleotide translocation, and GDP (Echtay et al. EMBO J 22; 4103, 2003). However, we have discovered that the GDP inhibitory effect in mammalian mitochondria is completely suppressed because of the NDPK action (Woyda-Ploszczyca and Jarmuszkiewicz PLoS One 9:e98969, 2014). This enzyme catalyzes the transfer of a γ-phosphate group from NTP to NDP, e.g., ATP + GDP → ADP + GTP. Objectives: The importance of NDPK for AAC-mediated proton leak in S. cerevisiae strains, which are naturally absent of uncoupling protein (UCP), the another major catalyst of the futile proton leak. Methods: Mitochondria were isolated from a wild type yeast strain and its mutant with disrupted gene for NDPK (BY4741 strain, Euroscarf, Germany). Oxygen uptake and mitochondrial membrane electrical potential ($\Delta\Psi$) were measured using a Clark-type oxygen electrode and a tetraphenylphosphonium cation (TPP+)-specific electrode, respectively. Results: In isolated mitochondria of wild type yeast strain, an addition of GDP (1 mM) stimulated the state 4 (non-phosphorylating respiration)-state 3 (phosphorylating respiration) transition revealed as a 32% (± 2 S.E.) increase in respiratory rate (nmol O/min/mg protein) accompanied by a 3% (± 0.3 S.E.) decrease in $\Delta\Psi$ (mV) (n = 5, independent mitochondrial isolations). Because 1 mM ADP led to a similar effect, it means that both, GDP and ADP, are involved in oxidative phosphorylation induction. This conclusion is supported by the fact that CATR totally blunted the ADP and GDP actions. In mutant strain, in the absence of CATR, the ADP action was unimpaired, however the GDP effect was quenched. Although we carried out measurements in the presence of ATP and GDP, the NDPK deficiency resulted in no ADP pool generation responsible for the GDP stimulatory effect. Conclusions: In our opinion, NDPK wins the competition with AAC for GDP. Therefore, GDP cannot be considered a native significant inhibitor of AAC-sustained proton leak. However, to better understand energy transduction in the cell, there is a need to recognize signaling related to physiological inhibition of this energy dissipation pathway. This work was supported by a grant from the National Science Centre, Poland (2015/19/D/NZ3/00087), and partially by the KNOW Poznan RNA Centre (01/KNOW2/2014).
Keywords: nucleoside diphosphate kinase; ADP/ATP carrier; mitochondria; proton leak.

76 - TRANSPOSON PIGGYBAC: AN EFFICIENT METHOD FOR STABLE GENE TRANSFER TO STUDY PURINERGIC SIGNALING IN IN VITRO AND IN VIVO GLIOMAS.

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Background: The search for new therapeutic strategies for the treatment of glioblastoma (GBM), a highly invasive and malignant tumor of the central nervous system, has been one of the great challenges in recent years. Previous work from our group has demonstrated that alterations in the purinergic signaling in glioma cell lines are linked to increase in proliferation and invasion of this tumor type. In these studies, we observed that the enzymes involved in the degradation of extracellular ATP and ADP, such as NTPDase 1 and 2, are low expressed, leading to an accumulation of nucleotides in the tumor microenvironment. In agreement, injections of potato apyrase (that degrade ATP and ADP) in a rat glioma model lead to a reduction in tumor size. Thus, the NTPDases are good tools to modulate the nucleotide levels in the tumor microenvironment. Different tools for genetic modification have

already been described to overexpress or silence targets to study cancer biology. However, the majority are time consuming and expansive. Transposon-based gene transfer is a non-viral method capable of inducing stable expression, being a simpler and straight tool. Therefore, the objective of this work was to develop, with the Transposon PiggyBac, a glioma cell line stably expressing the soluble NTPDase1 gene (ENTPD1) and analyze in vitro the cellular characteristics, enzyme functionality and potential for in vivo studies. Methods and results: after transduction of C6 cells, we confirmed its efficiency through gene expression by PCR, which showed that only the cells transduced expressed the sequence of ENTPD1 and iRFP fluorescent protein (Fig. 1B). The iRFP was also confirmed by flow cytometry and immunofluorescence, as demonstrated by representative figures (Fig 1C and 1D, respectively). In addition, all cells presented similar cell proliferation (Fig.2A, n=3, p=0,315), migration (Fig.2B, n=4, p=0,462) and adhesion (Fig.2C, n=3, p=0,338). The assay of the enzymatic activity from the cell supernatant reveals that ENTPD1 cells secreted an active soluble enzyme with ATP/ADPase activities (4.5±0.8 nmol Pi/min/mL and 2.5±0.3 nmol nmol Pi/min/mL to ATP and ADP, respectively, n=5, p=0,0002) (Fig. 3A), which were confirmed by HPLC (Fig. 3B, n=3). Next, we analyzed the functionality of the enzyme in vivo. The transduced cells were injected in the right striate of Wistar rats and after 23 days the animals were analyzed. The images obtained from the ex vivo imaging system (IVIS) demonstrate that the cells maintained the tumor formation potential (Fig. 4A) and cerebrospinal fluid (CSF) samples confirmed the presence of the functional soluble NTPDase1 (Fig. 4B). Conclusion: This methodology allowed the generation of cells stably expressing soluble NTPDase1 enzyme in vitro and in vivo. Therefore, these cells can be used as aconstant "scavenger" of extracellular ATP to better understand the role of this nucleotide in the tumor biology. This work was financed by CNPq, CAPES and FAPERGS.
Keywords: Glioma; NTPDase1; Transposon; PiggyBac.

77 - *TRYPANOSOMA CRUZI* ALTERS SERUM NUCLEOTIDES AND NUCLEOSIDES LEVELS OF INFECTED MICE.

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Objectives/background: Approximately seven million people are infected by *T. cruzi* worldwide in endemic regions, and present risk of developing the chronic form of disease (World Health Organization, Wkly Epidemiol. Rec. 87:519–522, 2012). The immunoregulatory mechanisms are an important form to control of infection (Dutra et al. Parasite Immunol. 36:377–87, 2014), and the purinergic signaling system have an important role in immunomodulation of inflammatory and immune responses by extracellular purines (Yegutkin, G.G. Biochim. et Bioph. Acta. 1783:673-694, 2008). The aim of this study was to evaluate the consequences of *T. cruzi* infection on seric levels of purine and their contributions to host immunomodulation. Methods and results: The blood trypomastigotes of *T. cruzi*, Colombian strain (Federici et al. Am. J. Trop. Med. Hyg. 13:272–280, 1964) were used for this study. Animals were obtained from the Central Animal House of the UFSM, six female (Swiss) mice (45 days, 20–30g) constituted the infected group inoculated by intraperitoneal route with 0.2 mL of blood trypomastigotes. The control group (n=6) received intraperitoneal route of saline. Animals were maintained at a constant temperature (23 ± 1°C) on a 12h light/dark cycle with free access to food and water. The number of blood trypomastigotes of *T. cruzi* was recorded every 2 days up to day 20 post-infection (PI), the number of trypanosomes was expressed as parasites per mL (Brener Z. Rev. Inst. Med. Trop. São Paulo 4:386–396,1962). Samples were collected on day 20 PI, animals were anesthetized with isoflurane, and blood was collected by cardiac puncture. Serum samples were obtained for purine analyses by HPLC (Voelter W et.al. J Chromatogr 199:345–354, 1980). The quantification of purine levels and metabolic residues were measured by absorption at 254 nm and expressed as ηmoles/mL. The statistical analysis was performed by analysis of variance (ANOVA) followed by the Student t test and were considered statistically significant when p values < 0.05. Animals does not show clinical signs of the disease, but trypomastigote forms of *T. cruzi* were observed in the bloodstream at 6 days PI and the parasitemia peak occurred on day 10 PI. Seric levels of ATP and ADP significantly increased ($p < 0.05$) in infected animals (ATP: 4.71 ± 0.9; ADP: 10.28 ± 2.9) compared with the control group (ATP: 1.59 ± 0.5; ADP: 4.5 ± 1.0). On the other hand, AMP levels were lower ($p < 0.05$) in the infected group (33.8 ± 2.3) compared with control group (44.2 ± 5.0). Adenosine levels were higher (1.71 ± 0.15) in the infected group ($P < 0.05$) compared with control group (1.009 ± 0.21). Conclusion: *T. cruzi* infection alters seric nucleotides and adenine nucleosides levels, which affects the host immune system generating a pro- and anti-inflammatory response, which may lead to an immunomodulation. Financial support: CAPES, CNPq
Keywords: Nucleotides; parasite; Chagas disease.

78 - TUCUMÃ (*ASTROCARYUM ACULEATUM)* PREVENTS CHANGES IN PURINERGIC ENZYMES IN PLATELETS OF RATS WITH POLOXAMER-407-INDUCED HYPERLIPIDEMIA.

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Objectives/background: Cholesterol and triglycerides are the most relevant lipids in human metabolism. Their excessive levels characterize hyperlipidemia and may lead to chronic inflammation as cardiovascular diseases. Adenine nucleotides and nucleosides trigger a cascade of reactions involved in the onset and maintenance of immune responses and are thereby regulated by ectonucleotidases. Tucumã (*Astrocaryum aculeatum*) is rich in carotenoids and flavonoids, with notable antioxidant and anti-inflammatory activities. We investigated the possible preventive effect of tucumã and its role in modulating purinergic enzymes in platelets of rats submitted to hyperlipidemia. Methods and results: Adult male Wistar rats (350–450g) from UFSM Central Animal House were pretreated with tucumã extract (250mg/kg), rutin (3.2mg/kg) and β-carotene (6.5mg/kg) by gavage for 30 days. Rutin and beta-carotene were used as a comparative standard. The animals were divided into 8 groups (n=7): control (C); control+tucumã (C+T); control+rutin (C+R); control+β-carotene (C+B); hyperlipidemic (H); hyperlipidemic+tucumã (H+T); hyperlipidemic+rutin (H+R); hyperlipidemic+β-carotene (H+B). Hyperlipidemia was induced by a single intraperitoneal injection of P-407 (500mg/kg). Non-hyperlipidemic rats received the same volume of vehicle (sterile 0.9% NaCl solution). Rats were anesthetized with isoflurane and submitted to euthanasia 36h post-induction. Blood was drawn in citrate by cardiac puncture. E-NTPDase and 5'-nucleotidase activities were determined as described by Lunkes et al. Thromb. Res. 109; 189, 2003 and results expressed in nmol Pi released/min/mg of protein. E-ADA activity followed Giusti & Galanti. Methods Enzym. Anal. 4; 315, 1984, with results expressed in μM NH3/min/mg of protein. Data were analyzed by two-way ANOVA followed by Tukey's test and expressed as mean ± SEM. ATP hydrolysis was

significantly higher (51%) in H when compared with C (P<0.05). ADP hydrolysis in H was twice as high as C (P<0.001) and 75% higher in H+B than in C (P<0.05). Conversely, H+T and H+R showed an ADP hydrolysis decreased by 36% (P<0.05) and 53% (P<0.01), respectively, when compared to H. 5'-nucleotidase activity was higher in H (53%, P<0.05) and H+B (97% P<0.001) than in C. E-ADA activity showed a significant decrease in H (69%, P<0.05) and H+T (60%, P<0.05) when compared to C. However, adenosine deamination was two times higher in H+B (P<0.05) than H. Conclusion: In conclusion, we demonstrate that pretreatment with tucumã partially modulated the activities of ectonucleotidases in P407-induced hyperlipidemia. Tucumã and rutin prevented hyperlipidemia-induced increase in ADP hydrolysis, while beta-carotene prevented the hyperlipidemia-induced decrease in E-ADA activity. Although requiring further study, tucumã and its compounds may be a promising complementary therapy to reduce the impact of hyperlipidemia on chronic inflammatory diseases. Acknowledgment: UFSM, CAPES
Keywords: Hyperlipidemia; tucumã; ectonucleotidases; inflammation.

79 - 1α,25-dihydroxyvitamin D3 alters ectonucleotidase expression and activity in human cutaneous melanoma cells.

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Objectives/background: We hypothesize that vitamin D decreases rates of adenosine formation in human cutaneous melanoma cells through the inhibition of extracellular ATP breakdown, thereby affecting tumor cell viability. Thus, the objective of this study was to verify the mechanisms of action of 1,25(OH)2D3 on the activity and expression of ectonucleotidases in cutaneous melanoma cells. Methods: Human melanoma cell line, SK-Mel-28, were cultured and treated with 1-50 nM concentrations of the active vitamin D metabolite (1,25(OH)2D3) during 24h followed by determination of NTPDase/CD39 and ecto-5'-nucleotidase/CD73 activity and as well as of expression rates of the purinergic system-related NTPASE1, NT5E and adenosine deaminase and vitamin D receptor. MTT was utilized to evaluate the cellular viability. Results: The results show that 1,25(OH)2D3 was able to decrease AMP hydrolysis by ecto-5'-nucleotidase/CD73 and expression of CD73, while do not change NTPDase/CD39 activity but increases the CD39 expression. It was also observed an increase of the cell viability in the concentration 1nM, but this viability decreased as the concentrations of vitamin D active metabolite was increased to 50nM. No differences were observed in genes expression. Conclusion: We show for the first time a mechanism of control in adenosine production through the modulation of the purinergic system in cutaneous melanoma cells treated with the active metabolite of vitamin D. This study provides original information on mechanisms, by which vitamin D plays a key role in preventing tumor progression in human melanoma cells. Acknowledgments: The authors would like to thank the Laboratory of Biotechnology and Animal Reproduction – BioRep and the financial support of CAPES and CNPq (MDB proj. No. 449485/2014-5), Brazil. HU acknowledges grant support from the São Paulo. Research Foundation (FAPESP proj. No. 2012/50880-4), Brazil.
Keywords: Vitamin D; Enzymes; Cutaneous melanoma.

80 - 4-PIRYDONE-3-CARBOXAMIDE-1β;-D-RIBONUCLEOSIDE (4PYR) SIGNIFICANTLY SUPPRESSES INVASIVE POTENTIAL OF 4T1 BREAST CANCER CELLS BUT HAS A TENDENCY TO INCREASE METASTASIS FORMATION.

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Background: During metastasis tumor cells acquire increased motility and invasiveness and actively modulate their attachment to vascular endothelium and its permeability. However, metastasis is a complex, multistep process and it can be influences by tumor-associated endothelium activation, hypoxia and associated platelet activity as well many other factors [Blazejczyk et al. Pharmacol Rep. 67; 711, 2015]. One of such factors could be 4PYR (4-pirydone-3-carboxamide-1β-D-ribonucleoside), a nicotinamide metabolite, whose elevated levels have been reported in cancer [Schram, Mass Spectrom. Rev. 17; 131, 1998]. 4PYR is converted into its derivatives by erythrocytes [Slominska et al. J. Biol. Chem. 281; 32057, 2006] as well by cancer cells leading to their decreased energy metabolism through decreased glycolytic rate. 4PYR can also regulate intracellular adenine nucleotide pool, e.g., through inhibition of adenine deaminase activity [Pelikant-Malecka et al., Int. J. Biochem. Cell Biol. 88; 31, 2017]. This could suggest, that 4PYR, as an endogenous metabolite, may influence tumor metastatic potential. Methods and results: Effects of 4PYR (end conc. 100 μM) on invasive potential of 4T1 triple-negative breast cancer cell line were analysed both in vitro and in vivo. Direct cell migration was inhibited in wound healing assay by 24% (± 1%, p<0.001). In Transwell assays both chemotactic migratory response and ECM invasion were decreased by 32% (± 12%, p<0.01) and 49% (± 10%, p<0.001), respectively. 4PYR have also inhibited 4T1 cells attachment to murine EC line H5V in cell attachment assay by 43% (± 9%, p<0.001) and their transmigration through EC layer by 44% (± 14%, p<0.001) in transmigration assay. However, 4PYR treatment after intravenous injection of 4T1 cells into female BALB/c mice (6–8-week old) have resulted in a tendency to higher metastases number and increased in vitro permeability of mouse lung EC layer by 14% (± 4%, p<0.01). Furthermore, we have observed interrelation between 4PYR and adenosine metabolism, e.g., 4PYR effect on 4T1 cells invasiveness was reversed by decreased extracellular adenosine levels (p<0.001). Additionally, just interaction between 4T1 and H5V cells reduced extracellular ATP hydrolysis by 86% (± 9%, p<0.001) in Transwell chamber co-culture assay. Conclusion: 4PYR metabolite is able to inhibit invasive potential of breast cancer cells, however, the changes in endothelium induced by tumor cells and 4PYR interrelation with an extracellular adenosine metabolism could lead to its pro-metastatic activity.
This work was supported by grant STRATEGMED1/233226/11/ NCBR/2015 strategic programmes "Prevention practices and treatment of civilization" - STRATEGMED: "Prostacyclin, nitric oxide and carbon monoxide - based pharmacotherapy of endothelial dysfunction and platelet activation - a novel strategy to inhibit cancer metastasis" (METENDOPHA).
Keywords: 4T1 breast cancer; 4-pirydone-3-carboxamide-1β-D-ribonucleoside (4PYR); cancer metastasis.

81 - FURTHER STUDIES ON THE CARDIAC RELEVANCE OF HUMAN A2A ADENOSINE RECEPTORS.

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We have generated mice with cardiac specific expression of A2A adosine receptors. The isolated perfused hearts of these mice are less prone to hypoxia reperfusion damage than wild type litter mates (Boknik et al. Frontiers in Pharmacol. doi.org/10.3389/fphar.2018.00013). In isolated perfused hearts at cycle lengths of 100 ms, the A2A receptor agonist CGS 21680 reduced the action potential duration at 75% repolarization (APD 75) in WT. In TG, due to arrhythmias, no stable APDs were recordable. These arrhythmias manifested as non-sustained atrial fibrillation or atrial tachycardia. In telemetric ECG measurement, one of three freely moving TG exhibited atrial extrasystoles (21/hour) whereas none was noted in WT. In telemetric measurements, basal spontaneous heart rates were higher in TG than WT but after injection of isoproterenol the same heart rate was obtained in WT and TG. Under sedation however, basal heart rates were not different between TG and WT. CGS 21680 injection increased heart rate (under telemetric conditions) in TG but not in WT. In 38 weeks old animals, a higher incidence of extrasystoles was noted in TG than in WT, which increased after stimulation with CGS 21680. In isolated left atrial preparations (electrically stimulated), we noted positive inotropic effects using 10 μM CGS 21680 as agonist in presence of adenosine deaminase (1 μg/m) to degrade endogenous adenosine and DPCPX (10 μM) to block A1 adenosine receptors. Hence, it was of interest whether these effects are also present in human cardiac preparations. To our surprise we noted under the above conditions only in right atrial strips (electrically stimulated) from two patients (males, 47 and 73 years of age) a positive inotropic effect was apparent while no inotropic effect was detectable in isolated strips from additional ten patients. In summary, cardiac A2A adenosine receptors might contribute to arrhythmias but their role in humans seem to vary broadly for reasons that require further studies.
Keywords: cardiac function;A2a.

82 - IMPACT OF GENETIC VARIATIONS IN ADORA2A GENE AND SYMPTOMS OF DEPRESSION: A CROSS-SECTIONAL POPULATION BASED STUDY.

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Variants in genes involved in adenosine metabolism and adenosine receptors were associated with increased risk for psychiatric disorders, including anxiety, depression and schizophrenia. In this study, we examined associations between a single nucleotide polymorphism (SNP) in A2A receptor gene (ADORA2A, rs2298383), current depressive episode and symptoms profile. In a cross-sectional population-based study, 1,253 individuals were analyzed by the Mini International Neuropsychiatric Interview 5.0. Our data showed that the TT genotype of ADORA2A rs2298383 SNP was associated with reduced risk for major depression when compared to the CC/CT genotypes (p=0.020). This association remained significant after adjusting for confounding variables as smoking, gender, socioeconomic class and ethnicity [OR=0.631 (95% CI 0.425-0.937); p=0.022]. Regarding the symptoms associated with major depression, we evaluated the impact of the ADORA2A SNP in the occurrence of sad/ discouraged mood, anhedonia, appetite changes, sleep disturbances, motion changes, loss of energy, feelings of worthless or guilty, difficulty in concentrating and presence of bad thoughts. Notably, the TT genotype was independently associated with reduced sleep disturbances [OR=0.438 (95% CI 0.258-0.743); p=0.002] and less difficulty in concentrating [OR=0.534 (95% CI 0.316-0.901; p=0.019]. Hence, our data support an important role for ADORA2A variants in clinical heterogeneity associated with major depression. The presence of at TT genotype was associated with decrease risk for major depression and protection against disturbances in sleep and attention, two of the most common symptoms associated with this disorder.
Keywords: depression; A2A receptors; polymorphism.

83 - PHARMACOLOGICAL ACTIVATION OR BLOCKADE OF A2A ADENOSINE RECEPTORS MODULATES CONTEXTUAL FEAR MEMORY CONSOLIDATION AND GENERALIZED FEAR EXPRESSION IN MALE WISTAR RATS.

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Adenosine acts as a neuromodulator and several of their biological effects are mediated by A2A adenosine receptors (A2AR), including synaptic plasticity, learning and memory processes. However, its role in fear memories and psychiatric disorders such as posttraumatic stress disorder (PTSD) remains poorly explored. An inability to restrict fear response to the appropriate context, the generalized fear expression (GFE), is a common PTSD feature. The objective of the present study was to investigate the role of A2AR on contextual fear memory (CFM) consolidation and GFE. Male Wistar rats (n= 8-10/group, 3 to 4 months old) were subjected to a contextual fear conditioning (CFC) protocol, re-exposed to the paired Context A (Test A) 1 day later and exposed to a novel and unpaired Context B (Test B) 2 days after CFC (Ethics Committee number UFSC/CEUA PP00830). In some experiments, Test A and Test B were repeated 14 and 15 days after CFC, respectively. In Experiment 1, animals received an intraperitoneal (i.p.) injection of vehicle (VE) or the selective A2AR antagonist SCH 58261 (0.05, 0.1 or 0.2 mg/kg) immediately after a CFC with 3 shocks of 0.7 mA. In Experiment 2, animals were treated i.p. with VE or SCH 58261 (0.1 mg/kg) immediately after a CFC with 1 shock of 0.5 mA. In Experiment 3, animals received an i.p. injection of VE or the selective A2AR agonist CGS 21680 (0.05, 0.1 or 0.2 mg/kg) immediately after a CFC with 3 shocks of 0.7 mA. VE or Caffeine (20 or 40 mg/kg), a non-selective A2AR antagonist, were injected i.p. immediately after a CFC with 3 shocks of 0.7 mA in Experiment 4. Freezing behavior in each session was measured as an index of CFM and analyzed by one-way ANOVA and Newman-Keuls post-hoc test. In Experiment 1, all groups presented similar freezing time when exposed to Test A (F3.32= 2.75, P= 0.059). The treatment with SCH 58261 (0.1 mg/kg) induced GFE, observed by the increase in freezing time on Test B at 2 days (F3.32= 4.71, P= 0.008; P= 0.003) and 15 days (F3.32= 4.30, P= 0.012; P=

0.015) after CFC. One-way ANOVA revealed no significant differences in freezing time evaluated in Test A (F1.18= 0.05, P= 0.828) or Test B (F1.18= 0.01, P= 0.912) in Experiment 2. In Experiment 3, the treatment with CGS 21680 (0.1 and 0.2 mg/kg) impaired CFM consolidation, observed by the reduction in the freezing time on Test A 1 day after CFC (F3.33= 5.01, P= 0.006; P= 0.018 and 0.03, respectively) and similar finding was observed at 14 days after CFC with the higher tested CGS 21680 dose (0.2 mg/kg) (F3.33= 3.59, P= 0.024; P= 0.0459). Finally, in Experiment 4, statistical analysis revealed no significant difference in Test A (F2.25= 0.79, P= 0.46) or Test B (F2.25= 0.91, P= 0.41) performed 1 and 2 days after CFC, respectively. In summary, the present results suggest that the pharmacological activation or blockade of adenosine A2AR immediately after CFC affect CFM consolidation and GFE in rats. Acknowledgments: CNPq, CAPES, PPGFMC.
Keywords: generalized fear expression; memory consolidation; A2A adenosine receptor.

84 - REAL-TIME ALLOSTERIC MODULATION AT HUMAN AND RAT ADENOSINE A1 RECEPTOR.

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Background: Allosteric binding sites are less conserved across receptor subtypes or species, therefore allosteric modulators are promising tools to increase target specificity. In the case of Adenosine receptors (A1AR, A2AAR, A2BAR, A3AR) highly distributed throughout the human body, this principle will reduce off-target side effects in a treatment with subtype-unspecific agonists. In this study we investigated the adenosine receptor subtype A1AR with a subtype-specific positive allosteric modulator (PAM) PD 81,723. Our approach allows us to monitor receptor conformational changes caused by allosteric modulators in living cells. Methods and results: We used our recently developed fluorescence resonance energy transfer (FRET) approach and designed human and rat A1 receptor based FRET-sensors. These sensors are modified with a cyan fluorescent protein at the C-terminus and a six amino acid FlAsH-binding motif within the third intracellular loop as previously described (1). Using a recently developed Gi-protein based FRET sensor (2) we measured allosteric modulation by PD 81,723 at the G-protein level for the human A1 receptor. All described sensors were well expressed at the cell surface and retained their functional properties. The affinity of PD 81,723 in combination with adenosine was found in the lower micro molar range. Co application of 10 μM PD 81,723 caused a threefold left shift of the concentration response curve for adenosine in living cells. Furthermore, we demonstrated probe dependency of PD 81,723 using the non-selective receptor agonist NECA. To investigate the role of the second extracellular loop as a putative allosteric binding site we introduced three point mutations S161A, E172A and I175A (3, 4) in our rat and human A1 adenosine receptor sensors and tested for altered allosteric behavior. Adenosine response curves were left-shifted one to twofold (I175A<S161A<E172A) for the human A1 mutants treated with 10μM PD 81,723. Conclusions: In summary, this study demonstrated that our designed human and rat A1 FRET-sensors are highly suitable to record allosteric modulation at receptor and G-protein level in real-time. With this we provide kinetic and mechanistic insights into allosteric modulation in combination with the endogenous ligand adenosine. This work was performed within the framework of the TR166 ReceptorLight granted by the German Research Foundation (DFG).
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Keywords: allosteric modulation; adenosine A1 receptor.

85 - SWIMMING PREVENTS MEMORY IMPAIRMENT IN THE L-NAME RAT MODEL OF HYPERTENSION WHICH CORRELATE WITH FLUCTUATION IN P1 AND P2 RECEPTOR EXPRESSION.

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Objective/background: Evidences for the involvement of purinergic signaling in regulating Central Nervous System function has been emerging as a new approach to understand hypertension-mediated memory dysfunction and chronic exercise is able to modulate purinergic system. Herein, we investigated the effect of chronic swimming training on memory and on purinergic system receptors expression in cortex and hippocampus of L-NAME-induced hypertensive rats. Methods and results: Male Wistar rats were divided in four groups: Control, Exercise, L-NAME and Exercise L-NAME. Inhibitory avoidance test was the tool used to access memory status. P2 receptors expression were measured by qRT-PCR. Data were analyzed using 2-way ANOVA test, considering $p<0.05$. Our results showed that L-NAME-treated group exhibited a low latency (~50 s) when compared to control group (~200 s, $P<0.05$). This result shows that L-NAME group has an impairment in memory. Hypertensive animals (L-NAME group) submitted to exercise exhibited an increase in latency (~150 s) when compared to a sedentary hypertensive group. This result indicates that this exercise protocol reverted the impairment of memory induced by L-NAME ($P<0.05$). A2A and A2B receptors were upregulated in L-NAME treated rats in cortex and hippocampus. Exercise was able to prevent A2A increased expression in cortex and hippocampus to go back to the levels of the control groups not treated with L-NAME. P2X2 and P2Y6 expression was increased in exercise group in hippocampus and cortex, respectively. P2X6 was upregulated in exercise and L-NAME treated rats; however, hypertensive animals submitted to exercise exhibited a normalized expression (to control values) of P2X6 and P2X2 in hippocampus. Conclusion: These changes may suggest that hypertension increases adenosine generation which may act via A2A as a possible mechanism of memory impairment in hypertension and exercise was able to prevent these effects. These data may indicate a possible mechanism by which exercise may prevent memory impairment induced by L-NAME.
Keywords: Swimming; hypertension model; memory; purinergic receptors.

86 - THE A2A ADENOSINE RECEPTOR MEDIATES A NOVEL REGULATION OF DNA REPAIR MACHINERY IMPLICATED IN MENTAL DISORDERS.

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Adenosine is a neuromodulator that has been implicated in a wide variety of fundamental machineries. There are four adenosine receptors including the A2A adenosine receptor (A2AR), a Gαs- protein-coupled receptor that contains a long C-terminal domain (designated A2AR-C). We have previously reported that A2AR-C interacts with the translin- associated protein X (TRAX), a DNA/RNA binding protein that controls mRNA transport, translation, and DNA repair. Upon genotoxic stresses, TRAX binds with phosphorylated ATM (Ser1981) and contributes to the ATM-mediated DNA repair. Given that stimulation of A2AR by two A2AR-selective agonists markedly ameliorates the double-strand DNA breaks (DSBs) evoked by elevated oxidative stress in human iPSCs-derived neurons, the role of TRAX in mediating the protective role of A2AR is of great importance. Biochemical analyses reveal that TRAX forms a complex with GSK3β and a risk gene for schizophrenia (DISC1). Activation of A2AR leads to dissociation of the TRAX/DISC1/GSK3β complex (TDG complex), and allows the release of TRAX from the TDG complex to enter the nucleus to facilitate DNA repair and the subsequently enhance neuronal survival. Collectively, the TDG complex might serve as a potential therapeutic target for the development of novel treatments for diseases (such as degenerative diseases and mental diseases) with defects in DNA repair.
Keywords: A2AR ; TRAX; DISC1.

87 - THE ERGOGENIC EFFECTS OF CAFFEINE DEPEND ON A2A RECEPTORS.

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Objectives/background: Ergogenic aid is a substance or method used for enhancing exercise and sports performance. The efficacy of many of these techniques is controversial. Caffeine is the most used ergogenic aid for amateur and professional athletes; the ergogenic effects of caffeine are clear and very well documented. However, the mechanisms of action have not yet been clarified, currently limited to three hypotheses. The increased (1) intracellular Ca+2 mobilization and (2) cAMP activity were only demonstrated at toxic mM concentrations. At non-toxic concentrations (uM), caffeine acts as an antagonist of adenosine receptors. First, we evaluated the ergogenic and thermogenic effect of caffeine (a non-selective antagonist of A2AR) and SCH-58261 (a selective antagonist of A2AR) on female wildtype mice. Then we confirm the role of A2AR in knockout mice. Methods and results: 42 adult female mice (19±0.6 g body weight, 10-12 weeks old) from a global A2AR knockout colony (FMUC, University of Coimbra) were used. The animals underwent a period of familiarization on the treadmill (3 days x treadmill 10 min, 15 cm/s, inclination 5°, saline i.p) for ergospirometry evaluation (running power and respiratory gases – O2 and CO2) on the 5th day. Caffeine (15 mg/kg, i.p., -15 min) and SCH 58261 (1 mg/kg, i.p., -15 min) were administered on the 4th day of open field behavioral task (15 min) and on the 5th day of ergospirometry test. The animals ran at increasing speeds (incremental test) until they reached exhaustion. The temperature at rest and after exercise was evaluated by infrared thermography. The estrous cycle of the females was determined by vaginal lavage and microscopy evaluation. The animals were perfused for immunohistochemistry of prefrontal cortex. Caffeine was psychostimulant (distance and average speed) for wildtype animals in the open field, but not for SCH-58261-treated or A2AR-KO animals. Caffeine and SCH-58261 were ergogenic for wildtype mice, that is, they increased 68±9% and 82±19% the running performance (vertical power) on the incremental treadmill test. In addition, caffeine and SCH-58261 also increased 36±11% and 47±8% maximal O2 consumption (V̇O2max) in wildtype mice, respectively. CO2 production (V̇CO2) had similar kinetics. But caffeine unchanged running power, V̇O2max and V̇CO2 of A2AR-KO animals. Acute physical activity increased cFos density in the prefrontal cortex of the animals. The different genotypes and treatments did not modify the body and tail temperature at rest and exercise-induced hyperthermia. The estrous cycle of the females did not influence the ergogenic effects of caffeine. Conclusion: Our results suggest that the ergogenic effects of caffeine are mediated by A2AR, possibly in the central nervous system. Financial support: Maratona da Saúde, CAPES-FCT, CNPq
Keywords: A2AR; caffeine; exercise; fatigue.

88 - THE IMPACT OF PHARMACOLOGICAL MANIPULATION OF ADENOSINE A1 AND A2A RECEPTORS IN CORTICAL NEURON CULTURES FROM RAT MODEL OF ATTENTION DEFICIT AND HYPERACTIVITY DISORDER.

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Objectives / background: Attention deficit and hyperactivity disorder (ADHD) is the most prevalent psychiatric disorder in children and adolescent, characterized by a triad of symptoms that include hyperactivity, inattention and impulsivity. Caffeine is the most widely consumed psychostimulant, which antagonizes adenosine actions at adenosine A1 and A2A receptors. Caffeine has shown benefits against cognitive impairments observed in ADHD model. Adenosine is a neuromodulator and its influence over neuronal growth has been postulated. In this study, we explored the action of adenosine receptors namely A1 and A2A receptors (A1) upon neurite outgrowth of cultured cortical neurons from spontaneously hypertensive rats (SHR), the most validated ADHD animal model. Methods and results: Frontal cortical neuron cultures were prepared from E17 embryos of SHR and Kyoto (control strain) rats. Cells were plated in 24-multiwell dishes at 0.25 x106 cells/mL density. Seven to ten independent cultures (50 neurons per culture) were analyzed per condition. After 1 day in vitro (DIV) or 4, neurons were incubated during 24 hours with 100 nM CPA or 50 nM CGS 21680 (A1 and A2A receptors agonists, respectively), as well as 50 nM DPCPX or 50 nM SCH 58261 (antagonists, respectively) and caffeine (30 μM). Neurons were immunostained for Tau and microtubule-associated protein 2 (MAP-2). SHR neurons presented a reduction of 28.47% (P = 0.012) in the number of neurite branches at 2 DIV when compared to Kyoto. Furthermore, SHR neurons showed a reduction of 47.99% (P = 0.0018) for Tau immunoreactivity at DIV 5. Only selective blockade of adenosine A2A receptors

counteracted Tau decrease in SHR neurons. SHR neurons treated with caffeine showed increase of 34.54% (P = 0.0042), in the total length of neurites and 26.54% (P = 0.0196) in maximal length of neurites at DIV 2. The levels of adenosine A1 and A2A receptors at 5 DIV assessed by immunoblotting was not different in neurons from both strains. Conclusion: This is the first time that the morphology of neurons from prefrontal cortex of the most validated ADHD model was analyzed. Reductions in the number of branches and Tau protein from axons found here may be related to behavioral alterations previously reported for this ADHD model. We also highlighted that the blockade of neuronal A2A receptors may play a role in the morphological alterations observed in the ADHD model.
Acknowledgment: CAPES. Approved by the ethics committee on the use of animals of Federal University of Rio Grande do Sul (Protocol number 29196).
Keywords: ADHD; Neurons; Adenosine; Caffeine.

89 - MODELING AND DYNAMICS OF P2X7 CHANNEL IN AN OPEN CONFIGURATION.

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Structure-function relationship studies of membrane proteins remain a challenge for scientists, since their current unsuitability to conventional structure determination methods like NMR and X-ray crystallography. Ion channels are membrane proteins that play major role in many physiological functions in prokaryotes and eukaryotes. Understanding mechanisms of ion channel activity as ligand binding, gating and permeability are crucial to develop new strategies that could be used in variety of pathologies. In this context, comparative models appear to be a strategy to study ion channels. Comparative models are made from a template of a similar (often homolog) protein with known structure. This tool is crucial for P2X receptors, which has three members crystallized: P2X3, P2X4 and P2X7 receptors. P2X receptors are trimeric ion channels expressed in many tissues regulating many physiological and pathological functions. There are seven subunits cloned until now in mammals. When it is activated by the physiological ligand, ATP, it opens a cation channel that lead to membrane depolarization and calcium influx according to electrochemical gradient. Within P2X receptors, zebrafish P2X4, human P2X3 and, recently, the chicken P2X7 and panda P2X7 were resolved by x-ray crystallography. Those structures provide unique data about binding, gating, and permeability of these receptors. Moreover, the resolved structures serve as model to in silicon experiments. The P2X7 receptor has some unique features in relation to others members of the family, as high agonist doses for activation and a progressive membrane permeabilization to large molecules (up to 900 Da). The P2X7 receptor is also vital in inflammation processes, as multiple sclerosis, asthma and pain. It is believed that occurs through releasing of IL-1β and activation of inflammasome complex. Thus, many molecules have been generated to treat inflammatory diseases based on P2X7 antagonism. Through the structural point of view, the P2X7 receptor is poorly understood. Therefore, the knowledge of P2X7 structure could help to understand the enigmatic function of this channel that shifts from a low to high conductance channel and to make a rational design of new P2X7 antagonists. So, we addressed whether we could simulate how P2X7 works based in the P2X4 zebra fish x-ray structure and validate our data with panda P2X7. In this way, we generate a model of a water-filled environment separated by a membrane in silico. Our membrane model resembled some physical-chemical proprieties of a biological membrane and, thus, used to insert a comparative model of human P2X7 receptor based on a zebrafish P2X4 structure. In addition, we refined the structure and used it to observe cation selectivity of the channel model. Our results show that our P2X7 comparative model (available on PDB) could be used to gain insights on the structure to aim experimental design, mainly in docking and mutation approaches.
Keywords: P2X7; in silico; compartive model; ion conducion.

90 - MOLECULAR RECOGNITION OF AGONISTS AND ANTAGONISTS BY G PROTEIN-COUPLED P2Y2 AND P2Y4 RECEPTORS.

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The nucleotide-activated G protein-coupled P2Y2 (ATP- and UTP-activated) and P2Y4 (UTP-activated) receptors (R) are promising drug targets, e.g., for the treatment of neurodegenerative and inflammatory diseases [1,2]. However, there is still a lack of potent, selective and bioavailable ligands that would be suitable for target validation studies. We constructed homology models of the human P2Y2 and the P2Y4R based on the recently published X-ray structures of the human P2Y1R [3,4]. Docking studies were performed with agonists selective for the P2Y2 or the P2Y4R (ATP, UTP, AP4A and MRS4062) as well as antagonists (AR-C118925, Reactive Blue 2 (RB-2) and several anthraquinone derivatives). Receptor mutants were created to probe the identified binding interactions. An ionic lock between an aspartic acid in extracellular loop 2 (ECL2) and an arginine in transmembrane (TM) region VII, which is probably involved in agonist-induced receptor activation, was observed for both, the P2Y2 and the P2Y4R [5]. Distant from the orthosteric binding site, Arg190 (TMV) likely forms a second ionic lock in the P2Y4R with aspartic acids. The interaction profile of the anthraquinone derivatives with the P2Y2R differed between the large RB-2 and smaller compounds. We expect that anthraquinone derivatives can bind to the orthosteric or an allosteric binding site depending on their structure and the receptor subtype. Our results suggest that the ECL2 plays a major role in agonist and antagonist recognition and interactions.
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Keywords: P2Y2; P2Y4; Homology Modeling; Mutagenesis.

91 - MONOSODIUM URATE (MSU) CRYSTALS INDUCES STERILE SECRETION OF IL-1βBETA; BY P2X7 RECEPTOR DEPENDENT OF HIGH MOBILITY GROUP BOX 1(HMGB1).

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Introduction: The classic theory of NLRP3 inflammasome activation by MSU involving two signals is controversial (Riteau et al, Cell Death Dis 2012, 3, e403), and the ability of MSU to induce inflammation in the absence of the first signal has already been described (Hoffman et al, Arthritis Rheum, 2010, 2170-2179). HMGB1 is a important DAMP and during sepsis-induced by CLP the inhibition of pannexin-1 channel contributes to decrease of HMGB1 release reduzing the inflammatory process. Once in the extracellular environment, this protein can be recognized by TLR-4 receptors. Objective: Knowing that HMGB1 and ATP are danger signals that can be actively released when cells are subjected to injury process, we investigated the involvement of P2X7 receptor in the inflammatory response induced by MSU without LPS, and the participation of ATP and HMGB1 in this phenomenon. Methods and Results: In vitro: Macrophages obtained from C57/BL6 (WT)and C57/BL6 P2X7 knockout mice (P2X7 KO) (both sexes, 8 weeks old) by peritoneal wash were stimulated with 500 ug/ml MSU for 18h. The supernatants were collected to measure ATP, LDH and cytokines by ELISA. Peritoneal macrophages released less IL-1β and ATP when stimulated by MSU crystals in the presence of 25nM A740003 and 300μM oATP (P2X7 receptor antagonists). Macrophages from P2X7 KO did not respond in vitro to MSU treatment considering the release of IL-1β. Moreover, murine macrophages from (WT and P2X7 KO) obtained by the above description were exposed to 5 mM ATP (15 minutes) or MSU 500 ug/ml (60 minutes) in the presence of ethidium bromide (BE) at 37°C to measure fluorescence dye uptake. Using flow cytometry to quantify the fluorescence dye uptake, we noticed that MSU crystals induced cell membrane permeabilization only in macrophages from WT animals. THP-1 cells (ATCC TIB 202 ™) were differentiated into human macrophages with 10 ng/ml PMA for 48 h. After the differentiation, the cells were stimulated with 500 μg/ml MSU for 18h and the supernatants collected for measure HMGB1 and IL-1β by western blotting and ELISA respectively. The data showed that THP-1 cells released less HMGB1 and IL-1β in the present of 25nM or 100nM A740003, 6u apyrase or 300μM oATP. Using fluorescence dye uptake assay we noticed that the permeabilization induced by MSU was depend of P2X7 receptor in THP-1 cells. In vivo: It was injected subcutaneously 20μl MSU crystals (20 mg / mL) or PBS alone into the right hind footpad of WT and P2X7 KO mice. We observed a reduction of edema formation in hind footpad from P2X7 KO mice. Conclusion: The data show that IL-1β secretion induced by MSU through P2X7 receptor activation is dependent of HMGB1 secretion. Financial support: CAPES, FAPERJ, CNPq
Keywords: sterile inflammation; MSU; P2X7.

92 - New pharmacological effects of approved drugs targeting P2X7 receptors against the release of IL-1β from microglial cells and neuropathic pain after peripheral nerve injury.

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P2X7 receptors (P2X7R) are a family of ATP-gated non-selective cation channels. In microglial cells, one of the harmful functions of P2X7R is to release of pro-inflammatory cytokines such as interleukin-1β (IL-1β), which is also a critical mediator in the pathogenesis of neuropathic pain. Therefore, P2X7R is a potential therapeutic target for treating neuropathic pain. In the present study, we screened a chemical library of clinically approved drugs (1,979 compounds) by high throughput screening (HTS) and showed that three compounds succeed to inhibit the function of both calcium responses and the pore-forming on rodent and human P2X7R. In primary cultured rat microglial cells, these compounds also inhibited not only P2X7R mediated calcium responses but also the ATP-induced release of IL-1β. Moreover, in a rat model of neuropathic pain, intrathecal administration of a potent compound among them to reduce the release of IL-1β produced a reversal of nerve injury-induced mechanical allodynia, a cardinal symptom of neuropathic pain. These results suggest that three compounds discovered by HTS are able to inhibit P2X7R function and the release of P2X7R-induced IL-1β from microglial cells. In addition, a potent compound to reduce the release of P2X7R-induced IL-1β shows promising an antiallodynic effect in a model of neuropathic pain.
Keywords: P2X7 receptor; microglia; approved drugs; neuropathic pain

93 - NLGN4X/Y AND PURINERGIC RECEPTORS ASSOCIATION IN A CELLULAR EMBRYONIC NEURODEVELOPMENT MODEL.

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Objectives / background: The presynaptic neurexins, postsynaptic neuroligins and SHANK3 (NRXN/NLGN/SHANK3 complexes) are implicated with long-term potentiation (LTP) and long-term depression (LTD) in hippocampus, which affects synapses' plasticity and hence learning and memory processes (Verpelli et al., 2011, J Biol Chem, 286(40):34839–50). In the NLGN family, mutations of the human genes NLGN3 and NLGN4 were related to autism. These two members of NLGN are broadly expressed in the brain, particularly in postsynaptic glutamatergic neurons in humans (Bemben et al., 2015, Nat Neurosci, 17(1):56-64). Alterations in purinergic signaling could cause convergent molecular abnormalities in autism. For a preliminary evaluation of the influence of NLGN4 mutations on purinergic signaling, we analyzed expression levels of NLGN4 and P2X1-7, P2Y1, P2Y12, and of the P1 receptors (R) in glutamatergic neurons obtained from human induced pluripotent stem cells (hiPSC) differentiation, from neuroprogenitor cell (NPC) stage to mature neurons. Methods and results: The hiPSCs differentiation on glutamatergic neurons follow Shi et al (2012) Nat prot, 7(10):1836 protocol. The expression of NLGN4 and other neuronal markers, and the purinergic receptors were evaluated by real-time PCR (A1, A2A, A2B, A3, P2X2-3, P2X5-7, P2Y1, P2Y12), flow cytometry (A2A, A2B, A3, P2X1-3, P2X5, P2X7) and immunocytochemistry (P2X1-3). NLGN4, βIII tubulin, EEAT1, GluR1, NMDAR and P2Y1R, P2X3/5R, A2A/2B/3R had increased expression levels since day seven, VGLUT1 (glutamatergic neuron marker) showed expression

since day 21, and P2X1-3R showed increased expression level on day 30. We did not find P2X7R expression since day seven of differentiation. Calcium signaling by microfluorimetry showed response to 100 μM ATP, inhibited by 30 μM EGTA, and was maximal with 10μM ionomycin, on day 30 of differentiation. Conclusions: P2Y1R and P2X3R have early expression in neurodevelopment and are essential for embryonic neurogenesis (Cheung et al., 2003, Dev Dyn 228:254–266). P2X5R had been investigated in epilepsy and it is closely related to glutamatergic system. That could explain their high expression in glutamatergic neurons (Henshall et al., 2013, Front cel neurosc 7:237). During neurodevelopment, P2X7R has decreased expression related to neuronal differentiation (Glaser et al., 2014, PLoS One, 9(5):e96281). This is an ongoing study and further investigations are going to include NLGN4/purinergicR structural and functional interaction also on neurons with NLGN4 loss of function. Acknowledgments: Grant and fellowship support by CNPq and FAPESP, Brazil.
Keywords: NLGN4; purinergic receptors; neurogenesis.

94 - P2X7 RECEPTOR AND METABOLISM OF CD4+ T CELL DURING EXPERIMENTAL MALARIA.

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Introduction: The molecular pathways involved in activation and regulation of the immune response are important targets for studies aiming to produce vaccines and to develop new therapeutic approaches. The cells of the immune system recognize not only pathogen-associated molecular patterns (PAMPs), but also intracellular molecules named DAMPs (damage-associated molecular patterns), such as ATP, that are released in the extracellular milieu during cellular damage or stress. The detection of extracellular ATP (eATP) by purinergic receptors (P2X1-7) alerts immunological cells that trigger the inflammatory response. Some studies show that recognition of ATP by P2X7 receptors on CD4+ T lymphocytes is important for cell activation and death. Furthermore, enzymes that cleave eATP participate in the control of tissue damage and inflammation. In malaria, an intense activation of CD4+ T cells is observed, which contributes to IFNγ production (Th1 cells), B cell activation (Tfh cells) and regulation (Treg and Tr1 cells). Methods and Results: To evaluate whether the P2X7 receptor contributes to protection against blood stages of Plasmodium chabaudi AS (PcAS), the disease progression was analyzed in C57BL/6 (B6) and P2rx7-/- mice. Our research group found that the P2X7 receptor promotes Th1 cell differentiation and controls the Tfh cell population during PcAS infection. Additional experiments are being performed to understand the mechanisms involved in P2X7 receptor-dependent differentiation of CD4+ T cells during experimental malaria. A proteomic analysis of CD4+ T cells showed glycolytic pathways activated during the peak of parasitemia with an increase of Th1 cells. On day 4 post infection, we observed an increase in the expression of Tbet (transcriptional factor), IFNγ and glucose transporter-1 (Glut-1) mRNA in CD4+ cells. Conclusion: The engagement of specific metabolic pathways profoundly affects cell differentiation and function. In addition, P2X7 receptors can modulate energy metabolism and T cell growth. Altogether, the main objective of this study is to provide new insight into purinergic signaling and immune system by showing the importance of P2X7 receptor in CD4+ T cells during experimental malaria. Financial support: FAPESP and CNPq.
Keywords: P2X7 receptor; CD4 T cell; metabolism; malaria.

95 - P2X7 RECEPTOR CONTRIBUTES TO LONG-TERM BRAIN ALTERATIONS IN SEPSIS-SURVIVING MICE.

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Objectives / background: Sepsis is a severe clinical condition characterized by an uncontrolled, excessive, and systemic inflammation that impacts multiple organs, including the brain. As a result, septic patients may present neurological dysfunctions termed sepsis-associated encephalopathy (SAE). SAE have been detected in up to 70% of patients with severe systemic infection and a substantial cognitive deficit has been reported in the survivors. Extracellular nucleotides, pro-inflammatory cytokines and oxidative stress have been associated to the pathophysiology of SAE. We have already shown that the ATP-gated P2X7 receptor contributes to sepsis associated acute brain dysfunction by inducing IL-1β release and stimulating the production of oxygen reactive species. Therefore, here we sought to investigate the contribution of P2X7 receptor to long-term brain alterations and cognitive dysfunction verified in mice that survived from sepsis. Methods and results: Sepsis was induced by cecal ligation and puncture (CLP) in wild type (WT) and P2X7 deficient mice (P2X7-/-). Biological samples were collected 24 hours or 13 days post-surgery. Acetylcholinesterase (AChE) activity was measured by determining the rate of hydrolysis of acetylthiocholine iodide (0.8 mM) in 300 μL assay solution with 30 mM phosphate buffer, pH 7.5, and 1.0 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) at 25 °C. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). All samples were run in triplicate. Our results showed a significant increase in AChE activity in cerebral cortex and hippocampus from both septic WT and P2X7-/- mice 24 hours after surgery when compared with their respective sham groups ($p<0.05$). However, when we assessed the AChE activity in mice that survived from sepsis (13 days after surgery) we observed and increase in both brain structures analyzed only in WT septic mice ($p<0.05$). P2X7-/- sepsis-surviving mice did not show changes in AChE activity ($p>0.05$), suggesting that the absence of this receptor might reduce the brain sequels in sepsis survivors. Behavioral tests will be performed soon to evaluate the contribution of the P2X7 receptor to the cognitive impairment verified in sepsis survivors. Conclusions: Our results suggest that P2X7 receptor might contributes to brain alterations detected in mice that survived from sepsis. Therefore, P2X7 receptor might be a suitable therapeutic target to limit brain sequels in sepsis survivors. Financial support: FAPERJ, CNPq, CAPES.
Keywords: Neuroinflammation; P2X7; Sepsis; Brain.

96 - P2X7 RECEPTORS IN VENTRAL HIPPOCAMPUS ARE INVOLVED IN STRESS RESPONSE AND ANTIDEPRESSANT EFFECT.

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Introduction: P2X7 receptors (P2X7R) play a central role in stress-related processes such as activation of neuroimmune response, glutamate release, and reactive oxygen species formation. P2X7R polymorphisms have been associated to the severity of depressive symptoms. Moreover, P2X7R inhibition prevents the stress-induced consequences in forced swimming test (FST) and tail suspension test. Based on that, the involvement of P2X7R in the neurobiology of depression and in stress response has been suggested. However, the effects of stress and antidepressant treatment on P2X7R expression was unknown. Additionally, the effect of P2X7R blockade and the mechanisms underlining this response in the Flinders Sensitive Line (FSL) rats, an animal model of depression based on the selective breeding, had not been studied until now. Aims: 1. To investigate the effects of stress and antidepressant treatment on P2X7R levels on frontal cortex (FC) and hippocampus (HIP) of rats submitted to the learned helplessness (LH). 2. To determine the effect of P2X7R blockade on the behaviour and brain-derived neurotrophic factor (BDNF) signalling in FC and HIP of FSL rats. Methods: 1. Male Wistar rats were submitted to pretest session of LH (40 inescapable foot shocks, 0.8mA, 10s, 60s ± 30s interval) or habituation (40 min in the same context without shocks). Animals of each group were treated with antidepressants (desipramine 25mg/Kg/day or imipramine 15 mg/Kg/day) or vehicle for one (acute) or seven (repeated) days. One hour after the last injection, animals were submitted to the test session of LH (30 escapable foot shocks, 0.8mA, 10s, 60s ± 30s interval) or had FC and HIP dissected for posterior evaluation of the P2X7R levels by western blotting (WB). 2. FSL and its control counter partner flinders resistant line (FRL) rats were treated with vehicle or P2X7 receptor antagonist (A-804598 3, 10 or 30 mg/Kg/day) for one or seven days. One hour after the last injection, animals were exposed to FST. Following, FC and HIP were dissected for evaluation of BDNF, Akt, Erk, mTor and p70 S6 kinase levels by WB. Results: 1. Repeated but not acute antidepressant treatment reverted the stress-induced consequences in the LH model. Stress increased while repeated treatment with antidepressants decreased P2X7R levels on ventral HIP. 2. Repeated but not acute treatment with A-804598 (30 mg/Kg/day) induced antidepressant-like effect on FSL rats. FSL rats presented decreased BDNF and p70S6 kinase levels on vHIP while repeated treatment with A-804598 (30 mg/Kg/day) attenuated this feature. P2X7R blockade increased Akt activation in ventral HIP. Conclusion: Antidepressant effect may involve attenuation of stress-induced P2X7R expression in ventral HIP. P2X7R blockade induces antidepressant-like effects in FSL rats, which is associated with BDNF signalling activation in this structure. Altogether, our data suggest that P2X7R in ventral HIP is involved in stress response and antidepressant effect.
Keywords: P2X7 receptor; stress; antidepressant.

97 - P2X7B ISOFORM ROLE IN CHEMORESISTANCE AND EPITHELIAL-TO-MESENCHYMAL TRANSITION OF HUMAN NEUROBLASTOMA CELLS.

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Cancer cell resistance to death stimuli is a major challenge in cancer therapy. Even though many alternative therapies have been developed to quench tumor cells, quiescent subpopulations of cancer stem cells lead to chemotherapy endurance, survival and tumor re-establishment. Epithelial-mesenchymal transition (EMT) has been pointed out as a mechanism that mediates those phenomena by switching the phenotype of cells to metastatic and resistant. P2X7 receptor, an ionotropic channel responsive to extracellular ATP, is implicated in many physiological and pathological roles, such as proliferation and stimulation of apoptosis. Paradoxal evidences implicate P2X7 in both pro- and anti-tumoral responses, probably due to expression of alternative splicing isoforms of p2rx7 gene, resulting in a full-length channel, P2X7A, and a truncated version lacking the C-terminal tail that is not capable of pore formation, called P2X7B. METHODS AND RESULTS: Characterization of EMT was performed by relative quantification expression of the EMT marker (vimentin) using RT-qPCR. Data revealed that vimentin expression was downregulated in P2X7A-/B+ cells by increasing concentrations of TGF-β (5-25ng/ml) and EGF (50-100ng/ml), in contrast to the results found for control cells (P2X7A+/B+). Furthermore, aiming to study role of P2X7 isoforms in chemoresistance of human neuroblastoma cells, we performed propidium iodide staining of cells collected from dose-response curve assay. Interestingly, two resistant populations were found, however, just one of them was enriched when vincristine concentration was raised, from 1nM to 1μM. Serum starvation and low glucose supply increased cell death rates independently of vincristine treatment. In order to assess roles of each P2X7R isoforms, we genetically silenced both isoforms (P2X7A-/B-) or isoform A only (P2X7A-/B+), using interference RNAs. P2X7A knockdown increased survival rate during starvation, also ATP stimulation at 1mM lead to an increased survival of cells expressing only isoform B, pointing P2X7B as an important target in chemoresistance. CONCLUSION: Our results has indicated different roles of each P2X7 isoform in cancer, highlighting P2X7B as a main character in EMT and chemoresistance. Financial Support: FAPESP, CNPq
Keywords: neuroblastoma; chemoresistance; emt; p2x7.

98 - P2Y1 RECEPTORS BLOCKADE REDUCES PROLIFERATION IN RETINOBLASTOMA CELLS IN VITRO.

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Adenine nucleotides are present in both healthy and tumoral microenvironment and several studies are considering the understanding of purinergic signaling as a target for novel therapy development. In addition, P2Y1 purinergic receptor has already been described as participant in distinct tumoral tissues as a proliferation modulator. Retinoblastoma is a malignant eye tumor of early childhood mostly related to RB1 gene deletion in neural stem cell at the retina. In this present study, we aimed to evaluate the effects of purinergic signaling modulation on the proliferating of retinoblastoma cells. Y79 retinoblastoma strain cells were cultivated in 25cm^2 culture flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin, in an atmosphere with 5% CO2 at 37°C. Cells were treated with MRS2179, a P2Y1 receptor antagonist, at the concentrations of 1μM, 10μM, 50μM and 100μM, ADP or with ATP at 100μM for 24, 48 or 72 hours. After the treatment, cells were counted in the Neubauer chamber by trypan blue exclusion assay and MTT assay was performed to measure cell viability. Our previous results indicate that P2Y1 receptor blockade for 24h reduced the number of retinoblastoma cells in a dose-dependent manner. Moreover, stimulation with ADP, but not ATP, also reduced cell number but without altered number of cells labeled for Ki-67, a proliferation marker. In the other hand, MTT assays showed no great reduction in cell viability in 24h of treatment (Control = 100%; MRS 1μM =105.4 ± 7.1; 10μM = 100 ± 1.0; 50μM = 108 ± 5.4; 100μM = 87.5 ± 6.4; ADP = 89.8 ± 4.9; ATP = 90.5 ± 5.9; n=4). Preliminary results suggest that P2Y1 blockade and ADP treatment for 48h might reduce cell number: (CTR = 137500 ± 17500; MRS 1μM = 70000 ± 25000; 10μM = 70000 ± 20000; 50μM = 90000 ± 5000; ADP = 73500 ± 21250; ATP = 95000 ± 2500; n=2). Meanwhile in 72h of treatment the

reduction effect seems to belong only to ATP: (Control = 100000 ± 7217; MRS 1μM = 108333 ± 17638; 10μM = 77500 ± 28976; 50μM = 116667 ± 11024), (ADP = 128333 ± 4410; ATP = 65833 ± 6667; n=3). These findings suggest that P2Y1 receptors are not inducing cell death in retinoblastoma cells but rather playing a role in proliferation itself even though previous data showed no alteration in Ki-67 expression. This mechanism might be probably dependent upon activation of other P2Y receptors such as P2Y12 since ADP stimulus had a similar effect. Financial support: CAPES, CNPQ e Proppi-UFF.
Keywords: P2 receptor; Proliferation; Retinoblastoma.

99 - P2Y12 RECEPTOR BLOCKAGE TRIGGERS AUTOPHAGY IN GLIOMA CELLS.

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Background/objectives: Glioblastoma multiform is considered the most aggressive tumor of central nervous system, being associated with low survival prognosis and chemotherapy resistance. P2Y12 is a purinergic receptor that has affinity to adenosine diphosphate (ADP), and its expression has been documented in some cancer cells, such as C6 rat glioma, renal and colon carcinoma lineages. However, its role in tumor progression and in the resistance mechanism of chemotherapy is not well elucidated. Clopidogrel bisulfate is a potent antithrombotic drug that inhibits ADP-induced platelet aggregation, acting as an irreversible inhibitor of P2Y12 receptor. Preview studies showed that glioma cells can express P2Y12 and its activation can induce proliferation. The aim of this study was to investigate alterations of cell cycle progression and to elucidate cell death mechanisms induced by clopidogrel in two glioma cell lines, human U251-MG and rat C6. Methods and results: Cell lines were cultured in DMEM medium supplemented with 5-10% of fetal bovine serum and kept under ideal conditions of cultivation. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT assay) using a vast range of antagonist concentrations (1 to 500 μM) as treatment, during 24 and 48 h. Based on MTT results, different concentrations of clopidogrel were used to cell cycle and cell death analysis: 150, 300 and 500 μM (U251-MG) and 150 and 300 μM (C6), for 24 h. Cell cycle investigation was performed by flow cytometry, using propidium iodide. Cell death pathway was determinated by flow cytometry, using annexin V-FITC–propidium iodide double staining and acridine orange staining. All data were analyzed using FlowJo Software (Tree Star Inc, Ashland, OR, USA). Our results showed that treatment with clopidogrel (above 150 μM) lead to a significant viability reduction in C6 cells, but the same result was not observed in U251-MG cell line, even at higher concentration (500 μM) after 24 h of treatment. U251-MG cells were sensitive to P2Y12 receptor antagonist (200 μM) only when exposed for a longer period (48 h). Additionally, clopidogrel (500 μM) was able to alter the cell cycle progression by arrest the cells in the G2 phase (from 9.26 to 19.47 ± 1.93, % cell cycle ± SD) with consequent decreasing in the S phase (from 27.43 to 16.84 ± 1.12) in U251-MG cells. Similar results were not observed in C6 lineage. Treatment with P2Y12 receptor antagonist did not induce cell death by apoptosis or necrosis in tested concentrations for both lineages. Interestingly, clopidogrel triggered autophagy in U251-MG human (150 μM and 500 μM) and C6 rat glioma (150 μM and 300 μM) cells. Conclusion: These data show the importance of P2Y12 inhibition, by a specific antagonist, in order to control glioma growth. Acknowledgments: CAPES, CNPq, LACOG and FINEP.
Keywords: glioblastoma multiform; P2Y12; purinergic receptor; clopidogrel.

100 - P2Y2-DEFICIENCY AFFECTS ADULT NEUROGENESIS.

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Background: Purinergic signalling in the brain has been implicated in integrity of neuronal circuits and is essential for neuroprotection and adult neurogenesis [1]. We showed earlier that adult neurogenesis is affected in mice with disrupted molecular clockwork [2] and P2 receptors, including P2Y2, are rhythmically expressed in the circadian rhythm generator [3]. Interestingly, purinergic signalling regulates the proliferation of embryonic neural stem cells (NSCs) [4]. Adult neural progenitor cells (NPCs) express functional P2Y receptors in vitro [5]. Moreover, P2Y receptors are abundantly expressed in neurogenic niches in the subventricular zone (SVZ) and in the hippocampus [6]. However, little is known about the role of P2Y2 in adult neurogenesis in vivo. Methods: In mice with a targeted deletion of the P2Y2 receptors (P2Y2-/- mice) we studied adult neurogenesis in the two neurogenic niches namely the subgranular zone of the hippocampus (SGZ) and the SVZ. Proliferation of NPCs cells was analysed by BrdU assay. BrdU+ cells in the hippocampus were further colabeled with NeuN to investigate their differentiation. Migration of NPCs along the rostral migratory stream into the olfactory bulb (OB) was also studied. All experiments were approved by North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection, Germany (AZ:84-02.04.2015.A273). Results: We found significantly less BrdU+ cells in the SGZ and SVZ in P2Y2-/- mice. However, commitment into neuronal lineages was not affected. Alteration in the histological architecture of the OB was observed in P2Y2-/- mice. Conclusion: Our results indicate that deletion of P2Y2 receptors negatively impacts the proliferation of NPCs in SGZ of the hippocampus and SVZ; however, without affecting their neuronal fate decision.
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Keywords: P2Y2; BrdU; hippocampus; SVZ.

101 - PURINERGIC SIGNALLING OF PRIMARY CULTURES FROM GASTRIC CANCER BIOPSIES.

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Gastric Cancer (GC) is the one of the most prevalent cancer and one of the leading causes of cancer-induced deaths. Previously, we have found that cells lines derived from gastric adenocarcinomas (AGS, MKN-45, MKN-74) expresses functional P2 receptors and that the purinergic signaling of these cells differs from that one found at a cell line derived from healthy gastric mucosa (GES-1). In this work we explore the expression and functional responses of purinergic receptors in biopsies and primary cultures derived from human gastric tumors and compare to normal cells from adjacent healthy mucosa. We found that similar to cell lines, some P2 receptors are over-expressed, specially the P2Y2 receptor, whereas the expression of other subtypes, such as the P2X4 receptor, is dramatically decreased. Proliferation studies and the use of purinergic agonists and antagonists demonstrated that similar to cells lines, the activation of P2Y2 increases whereas the activation of P2X4 decreases cell proliferation. These results demonstrate the involvement of different purinergic receptors and signaling in GC; the change in the expression pattern of purinergic receptors and the increase of intracellular ATP in tumoral cells, probably directs ATP and nucleotide signaling from an anti-proliferative effect in healthy cells to a proliferative effect in tumoral cells. Thus, purinergic signaling could constitute an interesting target for the develop of anticancer therapies.
Keywords: Gastric Cancer; P2Y2; P2X4.

102 - ROLE OF P2X7 RECEPTOR AND ADENOSINE TRIPHOSPHATE IN THE MODULATION OF THE IMMUNE RESPONSE AFTER IMMUNIZATION WITH OVALBUMIN.

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Introduction: The activation and regulation of the immune system are important targets of studies for the vaccine development and therapeutic approaches. The release of intracellular molecules, such as DAMPs (damage-associated molecular patterns), in particular adenosine triphosphate (ATP) when exposed in the extracellular environment, is recognized by purinergic receptors, such as the P2X7 receptor. Recently, P2X7 receptor has been shown to be important in the activation of Th1 response and T helper cell (Tfh) control in malaria-infected mice. In order to broaden the understanding of the immune system, we must evaluate the role of P2X7 receptor and ATP, as an adjuvant, in modulating the immune response in CD4 T cells following immunization with the ovabulmin protein. For this, the use of a transgenic OT II animal is required in studies of antigen-specific CD4 T lymphocytes. Its ability to express αβ-TCR, which recognizes the ovabulmin protein, allows a greater induction of response associated with the P2X7 receptor and ATP. Methods and results: The generation of knockout animals for the signaling genes P2rx7, FOXP3 GFP+ and OTII, which has αβ-TCR is being performed in the laboratory of the immunology department of the University of São Paulo, the first lineage of heterozygous animals have already been obtained, polymerase chain reactions (PCR) are being performed in order to verify their genes for the promotion of a posterior cross. A first immunization with ovalbumin (OVA - 10μg or 50μg) with or without adenosine triphosphate (ATP - 300 and 500μM) is being performed subcutaneously in Foxp3GFP+OTII or Foxp3GFP+OTII P2rx7+ mice After 3 days, the immune response in the spleen and lymph nodes will be evaluated. Conclusions: The crossing of animals to obtain transgenic mice is being carried out, but the studies do not yet present conclusive results. Furthermore one of the objectives of this study is to evaluate the P2X7-ATP axis in differentiation of CD4 T cells and their possible role as an adjuvant in immunization with OVA.
Keywords: adenosine triphosphate (ATP); P2X7 receptor; transgenic OT II animal; differentiation of CD4 T cells.

103 - ROLE OF P2Y2 PURINERGIC RECEPTOR IN PROLIFERATION AND ADHESION IN HUMAN ESOPHAGEAL CANCER CELLS.

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Background: Esophageal cancer (EC), an aggressive illness, ranks as the 8th most common cancer in the world and 6th in mortality. There are two main subtypes of EC: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC is the most incident type of esophageal cancer, and cases of EAC have increased over the years. Different P2YR subtypes are widely distributed in the body, and play important roles in physiological and pathological processes. Activation of P2Y2R by ATP leads to apoptosis and cell cycle arrest in ESCC cell line (Kyse-140). P2Y2R has also been related with tumor growth, invasion and metastasis in several types of cancer. Aims: To investigate if the pharmacological modulation of P2Y2R with agonists and antagonist (AR-C118925XX) can affect cell proliferation and adhesion in human esophageal cancer cell lines of ESCC (Kyse-30 and Kyse-450) and EAC (OE-33). Methods: Cell lines were maintained under standard culture conditions. To verify P2Y2R involvement in cell proliferation, we performed cell counting. Cells were seeded (2 x 104 per well) in 24-well plates and treated as follows: (1) control group: RPMI medium 0.5% SFB; (2) ATP 100μM; (3) UTP 100μM; (4) AR-C 118925XX 20μM; (5) AR-C118925XX 20μM plus ATP or UTP 100μM. After 24h of treatment, we used an automatized cell counter. We also analyzed if the adhesion capacity of cells after treatment. The cells were seeded in 96-well plates (5 x 104 per well) and treated as described: (1) Control group: RPMI medium 10% SFB; (2) ATP 50 and 100 μM; (3) UTP 50 and 100 μM; (4) AR-C118925XX 20 μM; (5) AR-C118925XX 20 μM plus ATP 50 and 100 μM; (6) AR-C118925XX 20 μM plus UTP 50 and 100 μM. After 2h of incubation, adherent cells were fixed and stained with crystal violet, and cell adhesion was evaluated by measuring optical density at 570nm in a plate reader. Results: ATP and UTP (100 μM) lead to a significant increase of cell proliferation in OE-33 and Kyse-450 cells ($p<0.05$) in comparison to the control. In contrast, when we treated with the P2Y2 antagonist AR-C118925XX the three cell lines showed a significant decrease ($p<0.05$) in cell number, which was not reversed by the subsequent addition of agonist nucleotides ATP and UTP (100 μM). Regarding cell adhesion, Kyse-30 cell line treated with AR-C118925XX showed a significant decrease when compared to control ($p<0.05$), and the treatment with UTP (100 μM) lead to an increase of cell adhesion when compared to AR-C118925XX treated cells, AR-C118925XX plus UTP 50, or 100 μM ($p<0.05$). The tested treatments did not alter Kyse-450 cell adhesion. In addition, OE-33 lineage showed a significant

decrease in cell adhesion when treated with the antagonist AR-C 118925XX, AR-C 118925XX plus ATP, or UTP in both concentrations used were compared to control group ($p<0.05$). Conclusion: the pharmacological modulation of P2Y2R was able to alter proliferation and cell adhesion events. Financial support: CAPES and LACOG.
Keywords: Adenocarcinoma; esophageal cancer; nucleotides; purinergic receptors.

104 - SINGLE MODERATE DOSE OF CAFFEINE AMELIORATES ACUTE ISCHEMIC CELL DEATH IN AVIAN DEVELOPING RETINA.

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Objectives/background: Ischemia is a debilitating condition in which the tissue is deprived of blood supply. In the retina, such event is part of several eye pathologies such as glaucoma, and retinopathy of prematurity. Adenosine is a nucleoside that acts through metabotropic receptors, A1 and A3, A2A and A2B. Caffeine is a psychoactive compound that, acts through inhibiting adenosine A1 and A2A receptors. It has been demonstrated that caffeine exerts beneficial effects to cognitive function in adults, although, developing tissue seem to be affected oppositely when exposed to high concentration of the xanthine. Here we sought to investigate the effect of moderate doses of caffeine on the avian developing retina submitted to oxygen and glucose deprivation (OGD). Methods and Results: at sixteen embryonic days (E16), the retinas previously injected to caffeine (30 mg/kg of egg at E14) through a single in ovo injection were processed to different approaches. Caffeine-treated retinas showed lower levels of extracellular LDH (CTR=100±28.38%, n=6; OGD= 309.2±43.98%, n=8; CAF=97.37±32.68, n=6; CAF+OGD=194.1±43.86%, n=6) after 50 minutes of OGD (Ringer solution 95%N2/5%O2, without glucose), indicating diminished cell death. Western blot analyzes showed increased levels of signaling proteins including, pERK (CTR=100±25.49%, n=6; OGD=15.56±2.14%, n=5; CAF=369.6±40.5%, n=4; CAF+OGD=28.72±10.10%, n=4), pCREB (CTR=100±16.3%, n=6; OGD=21.70±1.45%, n=5; CAF=191.5 ±55.97%, n=4; CAF+OGD=32.03±11.02%, n=5), and BDNF (CTR=100±13%, n=5; CAF=170.4±23.9%, n=5) after caffeine exposure. Functional [3H]-MK-801 binding studies revealed that caffeine treatment was increasing NMDA receptor activity (CTR basal = 4.90±1.84 n=3; CAF basal = 18.11±1.58 n=3; CTR stimulated = 15.17±1.10 n=3; CAF stimulated = 19.74±4.72 n=3), raising the possibility that this could be the trigger to BDNF production, involving ERK and CREB. Furthermore, we observed that the levels of the K-Cl-cotransporter 2 (KCC2), the main responsible for the maintenance of the chloride inward electrochemical gradient, was decreased (CTR=100±17%, n=8; CAF=54.41±11.03, n=6), probably contributing for increased excitability, and possibly NMDA activity. Finally, when we co-injected an antagonist of the TrkB receptor (K252A) with caffeine, the protective effect of caffeine exposure disappear (CTR=100±33.3%, n=10; OGD=491.4±61.6% n=8; CAF=173.6±25.7, n=9; CAF+OGD=361±35.1%, n=8; K252A=174.9±40.7%, n=6; K252A+OGD=450.6±81.5%, n=6; CAF+K252A=80.54±17.95%, n=6; CAF+K252A+OGD=481.9±96%, n=7) indicating that BDNF binding to its receptor is essential for caffeine effect. Conclusion: We conclude so far that caffeine at moderate doses seem to exert beneficial effects upon developing retina under ischemic conditions. This effect seems to involve the regulation of chloride transporters, increase in excitability and activation of NMDA receptors as well as BDNF production. Financial support: CAPES, CNPq, Faperj.
Keywords: caffeine; adenosine, retina, ischemia.

105 - THE INFLUENCE OF THE PURINERGIC SYSTEM ON TUMOR MORPHOLOGY AND METASTASIS.

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Introduction: Neuroblastoma is an important cause of mortality in pediatric oncology and is the most common tumor among infants. Cells isolated from the neuroblastoma usually display high rates of invasion, proliferation and metastasis. Cancer Stem Cells (CSC) are described as a population of cells in the neuroblastoma tumor mass with features of normal stem cells, which are responsible by tumor aggressiveness and metastatic potential. Based on that, the two main strategies that are currently being explored are therapies that specifically direct CSCs to cell death or promote differentiation of CSCs by depleting them from the tumor reservoir. Therefore, we explored in this study the possible roles of purinergic signaling in an in vitro model of CSC called tumorspheres, focusing on the maintenance and the metastasis processes. Methods and Results: Firstly, our data showed that ATP-treated tumorspheres culture at increasing concentrations lead to formation of spheroids with different morphologies and increased sizes when compared to controls. In addition, by immunofluorescence assay and tissue cytometry analysis, differences in the intensity of pluripotency markers were observed in ATP-treated tumors. Analysis of pluripotency markers by confocal microscopy allowed the identification of CSCs inside the tumorspheres. Furthermore, xenotransplanted animals receiving 50mg/kg of BBG (P2X7 antagonist) intraperitoneally showed decreased spread of neuroblastoma cells to the metastatic niches. Conclusion: Based on our findings, we suggest that the purinergic system may be involved in the maintenance of CSCs in the neuroblastoma tumor mass. Thus, the balance of ATP in the tumor environment would maintain a higher percentage of cells in a stemness state in neuroblastoma tumor, which would elevate its aggressiveness.
Keywords: Neuroblastoma; Cancer Stem Cells (CSC); tumorspheres and ATP.

106 - THE METABOTROPIC P2Y1 RECEPTOR AS NOVEL DRUG TARGET IN EPILEPSY THE METABOTROPIC P2Y1 RECEPTOR AS NOVEL DRUG TARGET IN EPILEPSY.

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Objectives/Background: The treatment of epilepsy remains symptomatic, with 30% of patients not responding to currently available anti-epileptic dugs (AEDs). The mechanism of action of the majority of AEDs is based on blocking Na+ and/or Ca^{2+} channels, promotion of GABA or inhibition of glutamate

signaling. There is therefore an urgent need to identify new drug targets with a different mechanism of action. Brain inflammation, in particular, has attracted much attention over recent years. Emerging evidence demonstrates a causal role for brain inflammation in lowering seizure thresholds and driving epileptogenesis. Consistent with this, intervening in pro-inflammatory cascades has shown promise in animal models of epilepsy, with clinical trials of anti-inflammatory agents already underway. ATP is released into the extracellular space during pathologic processes including increased neuronal firing and neuroinflammation resulting in changes to glial function and neuronal network excitability. Whereas the fast-acting ATP-gated ionotropic P2X receptor family has attracted most interest in the search for new AEDs, recent evidence also suggests involvement of the P2Y receptor family in the pathogenesis of epilepsy1. Among the P2Y receptor family, the P2Y1 subtype represents one of the most promising targets with P2Y1 promoting astrocyte calcium waves and P2Y1 expression being increased in animal models of epilepsy and in patients1. Methods and results: To determine the role of P2Y1 signalling during acute seizures, epileptogenesis and chronic epilepsy, two different animal models were used including the intra-amygdala kainic acid and intraperitoneal pilocarpine-induced status epilepticus mouse model. Both models develop epilepsy following a short seizure-free latent period. While P2Y1 antagonists, when administered shortly following the induction of status epilepticus, potently decreased seizure severity and the resulting neurodegeneration, P2Y1 agonists increased seizure severity and seizure-induced cell death. Treatment of mice once status epilepticus was terminated with P2Y1 antagonists delayed the onset of spontaneous seizures and treatment with P2Y1 antagonists during chronic epilepsy reduced daily seizure rate by ~50%. Conclusion: In summary, our results show potent anticonvulsive and neuroprotective potential for P2Y1 antagonism during acute seizures and chronic epilepsy suggesting P2Y1 antagonism represents a new treatment target. Acknowledgments: This work was supported with grants from Science Foundation Ireland (13/SIRG/2098) and the Health Research Board Ireland (HRA-POR-2015-1243).

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Keywords: Status epilepticus; Epilepsy; P2Y1 receptor; Anticonvulsant.

107 - THE P2X7 RECEPTOR CONTRIBUTES TO SEPSIS-INDUCED OXIDATIVE DAMAGE IN MICE LIVER.

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Objectives/background: Sepsis is a severe illness characterized by a systemic inflammation and consequent organ dysfunction generated in response to an infection. This life-threatening condition is the leading cause of death in intensive care units worldwide and represents a major public health issue since there is no effective treatment for this disease. The liver has crucial roles in sepsis promoting the bacterial clearance and the production of acute-phase protein and cytokines. The development of liver failure in sepsis is recognized as a major complication, which contributes to the disease severity. Plasma ATP levels increase during sepsis, suggesting a possible role for this pro-inflammatory molecule in the development of excessive systemic inflammation. ATP acting via P2X7 receptor induces the maturation and release of pro-inflammatory cytokines, such as IL-1β and IL-18, and the production of reactive nitrogen and oxygen species. Excessive reactive species lead to tissue oxidative damage. Therefore, the oxidative stress has been considered one of the pathological mechanism that contributes to initiation and progression of liver injury. Here, we sought to investigate the role of ATP-gated P2X7 receptor in sepsis-induced oxidative damage and liver injury. Methods and results: Sepsis was induced by cecal ligation and puncture (CLP) in wild type (WT) and P2X7-/- mice. The oxidative stress in liver of septic mice was assessed by 2',7'-dichlorofluorescein oxidation reaction (DCF), thiobarbituric acid-reactive substances (TBARS), nitrite levels (NO) (quantified by the Griess reaction), and by the reduction of sulfhydryl groups (quantified by the reduction of 5,5-dithio-bis (2-nitrobenzoic acid- DTNB). The status of endogenous defense system was evaluated through catalase (CAT) and superoxide dismutase (SOD) activities. All the results are expressed as mean± SEM with 4-6 animals per group. We observed an increased in the reactive species in the liver of septic WT animals (79.82 ± 32.77 nmol/mg protein, * $p < 0,05$) and an increase in lipid peroxidation (2.652 ± 0.6929 nmol MDA/mg protein **$p< 0,05$) compared to sham animals. Septic P2X7-/- mice did not show increased levels of reactive species and in lipid peroxidation. The nitrite and sulfhydryl concentrations were not altered after CLP in both WT or P2X7-/- mice. Finally, we found an imbalance between SOD and CAT activities expressed as an increase in SOD/CAT ratio (0.6058 ± 0.1326, * $p < 0,05$) in the liver of WT septic animals. This imbalance in the enzyme activities was not observed in P2X7-/- septic. Conclusions: In summary, we showed that sepsis increases reactive oxygen species generation and lipid peroxidation, as well as, decreases antioxidant defenses in liver tissue in a P2X7-dependent manner. Therefore, P2X7 receptor might be a therapeutic target to limit oxidative stress damage and liver injury during sepsis. Funding Information: This work was supported by CAPES, CNPq, FAPERJ.

Keywords: P2X7; sepsis; oxidative stress; liver.

108 - THE P2X7 RECEPTOR IS OVEREXPRESSED IN CRONH'S DISEASE ILEITIS.

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Introduction and aims: Crohn's disease (CD), one of the two major forms of inflammatory bowel disease (IBD) is a chronic inflammatory disorder of unknown etiology and unsatisfactory therapeutic outcomes. The P2X7 receptor expression is increased in colonic samples from patients with CD, and is critical to the development of experimental colitis. Oral infection with Toxoplasma gondii cysts triggers a CD-like enteritis in mice. The aim of this study was to investigate whether P2X7 receptor is involved in CD-associated ileitis. Methods and Results: Surgical ileal samples were obtained from patients with CD (n=10) and controls (n=10), with ages ranging from 30–50 years old. Ileal mucosal samples were used to analyze histological parameters, while P2X7 receptor expression by real-time PCR and immunohistochemistry. C57BL/6 male mice with ages between 6-8 weeks were orally infected with 10 cysts of T. gondii ME49 strain, to the induction of

experimental ileitis (n=7-11 per group). After a follow-up of 8 days, animals were euthanized and ileal samples collected for histopathological and immunohistochemical analysis, and P2X7 receptor expression by qPCR. Study protocols were approved by the Human and Animal Ethical Committee of the Federal University of Rio de Janeiro. Hematoxylin-eosin staining showed that all mucosal samples from the control group were histologically normal. In respect of histological grading of inflammation, inflamed ileum of CD patients was classified as severe and moderate comparing to mucosal samples from the control group (p=0.001). The assessment of collagen fibers and goblet cells were performed through the analysis of paraffin sections stained with phosphomolibidic acid-picrosirius red dye and periodic acid-Schiff, respectively. A significant increase of collagen density (p=0.001), and a decrease in the number of goblet cells was observed in slides from patients with CD compared with normal controls (p=0.001). In experimental ileitis, T. gondii-infected animals lost more weight, and ileal samples had higher inflammatory scores (p<0.001) using histological parameters such as ulceration, hyperplasia, and the presence of inflammatory infiltrate (severity ranging from 0 to 4). A significant increase of collagen density (p=0.008), and a decrease in the number of goblet cells was detected in ileal samples of T. gondii-infected mice compared with controls (p=0.001). The immunohistochemical labeling demonstrated that P2X7 receptor is highly expressed throughout the epithelium and lamina propria mononuclear cells of the inflamed ileum compared with controls, in both human and mice samples (p=0.001). Analysis by qPCR revealed that the P2X7 receptor gene expression is increased in human and mice inflamed ileum, compared with controls (p=0.05). Conclusion: The results of this study suggest that P2X7 receptor might be involved in the development and progression of human CD ileitis.
Keywords: Ileitis; Toxoplasma gondii; P2X7 receptors; Crohn's disease.

109 - THE ROLE OF THE P2X7 RECEPTOR IN MICROGLIA IN RELATION TO GLAUCOMA.

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Objectives / Background. In glaucoma, loss of vision is caused by degeneration of retinal ganglion cells (RGCs) whose function is to carry information from the retina to the brain via the optic nerve. Much evidence implicates the P2X7 receptor in neurodegeneration in glaucoma and our research has indicated that the death of RGCs and release of the interleukin-1β (IL-1β) can be caused by activation of the P2X7Rs in the human retina. Retinal microglia may be involved these processes, therefore we have used a microglial cell line (BV2) to investigate the role of the P2X7R in microglial survival and IL-1β expression. Methods: A P2X7R knockout (KO) BV2 mouse microglial cell line was developed by CRISPR/Cas9 gene editing. Absence of the P2X7R was assessed by Western blot, flow cytometry and P2X7-dependent calcium signalling. Cell viability and death were quantified by MTS and LDH assays respectively. Induction of IL-1β mRNA was evaluated using RT-PCR. Results: Western blots and flow cytometry confirmed the knockout of P2X7R in BV2 (n=3). ATP and BzATP (50μM-1mM & 5μM-100μM respectively) significantly increased intracellular Ca^{2+} in BV2 cells (n=3;p<0.05). ATP caused a biphasic response, showing an initial peak followed by a lower sustained phase of raised intracellular Ca^{2+}. BzATP caused only the sustained phase. The P2X7R antagonist AZ10606120 (10μM) inhibited the sustained phase of the Ca^{2+} response. In P2X7 KO BV2 cells, no sustained phase was seen with ATP and BzATP caused no response (n=3). BV2 cells exhibited dose-dependent decreases in cell viability and increases in cell death in response to ATP (50μM–3mM) (n=4;p<0.05) and BzATP (5μM–300μM) (n=4;p<0.05). AZ10606120 (10μM) significantly protected against ATP- and BzATP-induced cell death (n=4;p<0.05). In P2X7R KO BV2 cells, no significant decrease in cell viability was observed at any ATP concentration tested (10μM-5mM) (n=4). ATP (300μM) increased IL-1β mRNA expression in BV2 cells by 5-fold compared with control at 24h (n=4;p<0.05). A similar effect was demonstrated in P2X7R KO BV2 cells, (n=4). BzATP (30μM) caused no significant changes in IL-1β expression in either cell line (n=4). Conclusion: We successfully developed a stable P2X7 receptor knockout BV2 cell line and demonstrated that P2X7 has an important role in cell viability/death of these cells. ATP-mediated IL-1β mRNA induction was not mediated by the P2X7R. Microglia play an important role in retinal homeostasis and cytokine production; understanding the role of the P2X7R in microglial activation and neuroinflammation may provide insight into the role of ATP signalling in neurodegeneration in glaucoma.
Keywords: P2X7; eye; microglia; neurodegeneration.

110 - PURINERGIC SIGNALING IN BIPOLAR DISORDER.

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Background and aims: The physiopathology of Bipolar Disorder (BD) shows the involvement of different neurotransmission systems, including the purinergic signaling pathway as participant of depressive and manic behavior. An important feature of BD pathophysiology is a neuroinflammatory status, which has been associated to both onset and progression of the disorder. In this work, we characterized the inflammatory status in an animal model of mania induced by GBR12909 during different time points and the expression of purinergic receptors associated to neuroinflammation responses. Methods: Young adult C57Bl/6 mice (female and male) were subjected to a single (n = 7) or multiple (n = 7) intraperitoneal injections of 12,5mg/kg GBR 12909 (Sigma Aldrich) or vehicle (NaCl 0,9%; n = 10). Open field test was performed 1 hour after last injection, and animals were euthanized 1 hour after behavioral test. Brain structures were collected and 1. immediately frozen in dry ice and stored in -80°C until the further gene expression experiments (n=4); or 2. immediately digested in trypsin and fixed in paraformaldehyde 4% for further flow cytometry analysis. Results: Open field test analysis shows higher locomotor response from mice that received multiple injections than those that received a single injection. Gene expression data shows increased expression of IL-1b in both striatum and hippocampus of mice chronically induced in comparison to control group, while no significant changes were observed between control and single-injection groups. No alterations were observed in NF-kB gene expression. Among purinergic targets, A2B receptor expression was significantly decreased in both brain regions, and this condition is cell-type dependent, as confirmed by flow cytometry assay. Conclusions: GBR 12909 is a selective dopamine reuptake inhibitor that induces mania-like phenotype by increasing locomotor activity. Our results indicate a better response when injected chronically in comparison to single injections. Chronic administration induces neuroinflammation, which can be modulated by A2b adenosine receptor in both striatum and hippocampus of mice showing manic-like phenotype.
Keywords: inflammation; a2b; mania.

111 - AN ALTERED NUCLEOTIDE METABOLISM AS A NOVEL MECHANISM LEADING TO HUNTINGTON DISEASE RELATED CARDIOMYOPATHY.

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Huntington's disease (HD) is a neurodegenerative disorder with a significant peripheral component to the disease pathology. This includes an HD-related cardiomyopathy, with an unknown pathological mechanism. In this study, we characterized changes in cardiac nucleotides metabolism in the HD mouse models. Moreover, we aimed to assess the concentrations of adenine nucleotides catabolites in plasma of HD patients. We examined R6/2 mice (n=5, male,12 weeks of age),HdhQ150 mice (n=5,male,22 months of age) and their WT littermates. Plasma samples from HD and control patients (n=5 per group) were obtained from the European Huntington's Disease Network. To investigate changes in the nucleotides metabolism, the concentration of adenine and guanine nucleotides, creatine, NAD and NADH were measured. Activity of eNTPD, AMPD, e5'NT,ADA and PNP as well as serum concentration of nucleotides catabolites were measured with HPLC. We evaluated cardiac substrate preferences using 13C glucose and LC-MS method. Analysis of genes transcripts were performed using RT- qPCR. Moreover, level of AMPK phosphorylation was measured with ELISA KIT. We observed a notable energy metabolism deterioration in hearts of HD mice (ATP/ADP ratio=6.39±0.46 in R6/2 and 3.57±0.57 in WT, *$p<0.05$;8.56 ±1.84 in HdhQ150 and 5.98±0.55 in WT, *$p<0.05$). We demonstrated AMPK over-activation in hearts of HD mice that was accompanied by shift in a cardiac substrate preference from glucose to fatty acids. We found a reduced activity of AMPD and e5'NT, while the activity of ADA was increased. Moreover, we found a significant down-regulation of genes involved in purine de novo biosynthesis and up-regulation of transcripts of genes involved in adenosine degradation. This was accompanied by an increases in concentration of nucleotide catabolites in HD mouse model serum, in comparison to their wild type littermates. Interestingly, we observed prominently increased levels of hypoxanthine and uridine also in HD patients plasma, in comparison to their healthy controls. Moreover, hypoxanthine and uridine levels strongly correlated with HD disease progression parameters. This study highlights a profound deregulation in cardiac energy and nucleotides metabolism in HD mouse models. We suggest that mutant huntingtin disrupts coupling of cardiac energy metabolism with its regulatory pathways that despite its activation is unable to ensure recovery. Consequently, hearts and possibly other organs remains energy depleted that translate into elevated nucleotide catabolites concentration and suppression of nucleotide synthetic pathways. Furthermore, for the first time, our study identified biomarkers that might be linked to HD progression both in pre-clinical and clinical settings. Restoration of energy equilibrium in HD hearts may be important therapeutic target in HD. This work was supported by the National Science Center of Poland (2015/17/N/NZ4/028410) and Polish Ministry of Science and Higher Education (MN – 01-0243/08/256).
Keywords: "nucleotide metabolism"; "Huntington disease", "heart".

112 - INHIBITION OF AMP DEAMINASE IS CARDIOPROTECTIVE IN ACUTE OXYGEN DEPRIVATION.

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Background. Clinical analysis of the effect of genetic polymorphisms known to affect cardiac AMP deaminase (AMPD) activity in cardiovascular disease demonstrated diverse results with uncertain mechanisms indicating need for experimental studies. This study evaluated effects of a genetic alteration of AMPD activity in mice hearts subjected to oxygen deprivation. Methods and Results. Double knock-outs for Apolipoprotein E (ApoE) and Low Density Lipoprotein Receptor (LDLR) mice were crossed with AMPD-/- CRE+ strain. Target genetic pattern was confirmed by genotyping for ApoE, LDLR, AMPD, and CRE. Functional confirmation of the genetic alterations was performed by analysis of the heart homogenates of ApoE-/-LDLR-/-AMPD-/-CRE+ (3KO), ApoE-/-LDLR-/- (2KO), AMPD-/-CRE+ (DKO) and wild type (WT) male mice strains (n=7). Activities of AMPD, adenosine deaminase (ADA), ecto-5'nucleotidase (e5NT) and purine nucleoside phosphorylase (PNP) in the mouse hearts were measured by monitoring the conversion of substrates into products by HPLC as described previously. AMPD activity decreased to 25% in 3KO when compared to 2KO strain. Activities of PNP, ADA and e5NT were similar in all groups of animals. Analysis of mRNA expression revealed absence of AMPD1 mRNA in all group, detectable mRNA for AMPD2 and depressed expression of the AMPD3 in KO and 3KO mice as compared to 2KO. When subjected to hypoxia (breathing 7% O2 for 7.5 min), inhibition of AMPD in 3KO male mice resulted in attenuation of ECG STU area changes and in decrease of troponin T concentration in the serum 6h after hypoxia when compared to 2KO male mice- from 188.5±17.2 pg/ml in 2KO to 147.7±8.4 pg/ml in 3KO, n=7, $p<0.001$. In addition, we found that phosphorylation status of AMP regulated protein kinase was significantly 30% elevated in the 3KO mice hearts. Conclusion. This study shows that reduced AMPD activity is protective in acute oxygen deprivation and that activation of AMPK cascade is likely to be the mechanism. This study is consistent with potential use of AMPD inhibitors in cardiac acute oxygen deprivation. Acknowledgements. This research was supported by National Science Centre of Poland (2016/22/M/NZ4/00678) and Foundation for Polish Science (TEAM/2011-8/7).
Keywords: AMP deaminase; purine metabolism; hypoxia; AMP-activated protein kinase.