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The 9th International Symposium on VIP, PACAP, and Related Peptides
Program/Abstracts
October 5–8, 2009
Kagoshima, Japan

SCIENTIFIC PROGRAM

Sunday, October 4th, 2009

19:00– **Welcome Reception**
Etoile

Monday, October 5th, 2009

8:30–8:40 **Opening Ceremony**
Kaimon

Session 1: **Central Nervous System**
Session chairs: Akemichi Baba
Katalin Köves

8:40–9:10 PL1 **A role for pituitary adenylate cyclase-activating peptide (PACAP) expression and signaling in the bed nucleus of the stria terminalis (bnst) in stress-induced anxiety-like behavior**
Sayamwong Hammack
University of Vermont, USA

9:10–9:30 O1 **Secretin attenuates the repetitive hyperactive movements in a mouse model**
Katalin Köves
Semmelweis University, Hungary

9:30–9:50 O2 **PAC1-R heterozygous mice have no desire to explore the unknown environment in memory tasks**
Nobuyoshi Hagino
Tulane University, USA

9:50–10:05 O3 **On the cellular and molecular pathways involved in the inhibition of LTP in the CA1 area of the hippocampus**
Diana Cunha-Reis
University of Lisbon, Portugal

10:05–10:20 O4 **Altered emotional and cognitive function in PACAP-deficient mice: a novel animal model for psychiatric disorder**
Norihito Shintani
Osaka University, Japan

10:20–10:30 Coffee break

Session 2: **Neurodevelopment**
Session chairs: David Vaudry
James A. Waschek

10:30–11:00 PL2 **VIP regulates cortical growth through MCPH1/CHK1: relevance to human microcephaly**
Vincent Lelievre
University of Strasbourg and CNRS UPR3212, France

11:00–11:20	O5	**Peripheral administration of vasoactive intestinal peptide ameliorates CNS cytokine induction in mouse model of neonatal diffuse white matter injury** Hiroko Nobuta *University of California, USA*
11:20–11:40	O6	**Roles of the PAC1 receptor in mouse neurogenesis** Sanbing Shen *University of Aberdeen, UK*
11:40–12:00	O7	**PACAP as a neurotrophic signal for astrocyte differentiation** Mario Vallejo *Instituto de Investigaciones Biomedicas, CSIC, Spain*
12:00–13:30		Lunch

Session 3: Neuroprotection (Part A)
Session chairs: William Banks
Ichiro Tatsuno

13:30–14:00	PL3	**PACAP38 protects rat cortical neurons against the neurotoxicity evoked by sodium nitroprusside and thrombin** Paula Grammas *Texas Tech University Health Sciences Center, USA*
14:00–14:20	O8	**Neuroprotective strategy on brain injuries by immunemodulation-lesson from PACAP and hMSCs on stroke?** Hirokazu Ohtaki *Showa University, Japan*
14:20–14:40	O9	**Delivery of pituitary adenylate cyclase activating polypeptide (PACAP) to the brain: targeting with intranasal delivery and cyclodextrins** Naoko Nonaka *Showa University, Japan*
14:40–15:00	O10	**Comparison and possible relationship between PACAP- and enriched environment-induced retinal protection in MSG-treated newborn rats** Peter Kiss *Pecs University, Hungary*
15:00–15:10		Coffee break

Session 3: Neuroprotection (Part B)
Session chairs: Dora Reglodi
Seiji Shioda

15:10–15:40	PL4	**Review of the retinoprotective effects of PACAP** Dora Reglodi *University of Pecs, Hungary*
15:40–16:00	O11	**Protective effects of PACAP against oxidative stress in cochlear cells** Andrea Tamas *Pecs University, Hungary*

16:00–16:20 O12 **Pituitary adenylate cyclase-activating polypeptide (PACAP) receptor expression is altered in the brain of *Podarcis sicula* after nonylphenol administration**
Salvatore Valiante
University of Naples Federico II, Italy

17:00–18:00 Buffet

Session 4: Akira Arimura Memorial Symposium
Chairs: Seiji Shioda
Atsuro Miyata

18:00–18:10 **Opening remark**
Sami I. Said
State University of New York at Stony Brook, USA

18:10–18:30 **Memorial remark**
Sandor Vigh
Ross University, Dominica

Aniko Somogyvari-Vigh
Tulane Cancer Center, USA

18:30–19:10 SL1 **PACAP and the blood-brain barrier**
William A. Banks
Saint Louis University, USA

19:10–19:50 SL2 **Ghrelin: from discovery to translational research**
Kenji Kangawa
National Cardiovascular Center, Japan

19:50–20:30 **Memorial remark**
Hisayuki Matsuo
Miyazaki University, Japan

20:30–20:40 **Closing remark**

Tuesday, October 6th, 2009

8:30–9:30 SL3 **Clinical and pathogenic implications of incretin therapy in patients with type 2 diabetes**
Kun-Ho Yoon
Catholic University, Korea
Chair: Masamitsu Nakazato

Session 5: Endocrine System and Metabolism
Session chairs: Hubert Vaudry
Kinji Inoue

9:30–10:00 PL5 **Secretin: a neurosecretory factor regulating body water homeostasis**
Jessica YS Chu
The University of Hong Kong, China

10:00–10:20	O13	**Central administration of secretin suppresses food intake in mice** Billy KC Chow *The University of Hong Kong, China*
10:20–10:40	O14	**PACAP/VIP inhibits osteoblastic differentiation and stimulates the cytokine production of IL-6 through VPAC2 receptor in MC3T3 cells** Ichiro Tatsuno *Chiba University, Japan*
10:40–11:00	O15	**PACAP stimulates somatolactin release from cultured goldfish pituitary cells** Kouhei Matsuda *University of Toyama, Japan*
11:00–11:10		Coffee break

Session 6: **Integrative Physiology: Gastrointestinal System and Peripheral Nervous System**
Session chairs: Joseph R. Pisegna
Toshihiko Yada

11:10–11:40	PL6	**Paradoxical effects of PACAP and VIP on gastrointestinal physiology** Joseph R. Pisegna *University of California at Los Angeles, USA*
11:40–12:00	O16	**Comparison of intestinal warm ischemic injury on PACAP knock-out and wild-type mice** Andrea Ferencz *University of Pecs, Hungary*
12:00–12:20	O17	**Lipodytrophy and leptin replacement therapy in Japan** Ken Ebihara *Kyoto University, Japan*
12:20–12:40	O18	**Secretin expressing primary sensory neurons in the trigeminal ganglion of rats: in situ hybridization study** Andrea Heinzlmann *Semmelweis University, Hungary*
12:40–14:00		Lunch
14:00–17:00		**Excursion (Heritage Attraction in Kagoshima City)**
18:30–19:00		**Akira Arimura Young Investigator Award Ceremony and Lecture** Kaimon
19:00–21:00		**Poster Session** (cheese-and-wine party) Takakuma

Wednesday, October 7th, 2009

8:30–9:30	SL4	**Physiology and pathophysiology of prostanoids; novel roles revealed by receptor knockout mouse studies** Shuh Narumiya *Kyoto University, Japan* Chair: Kazuwa Nakao

Session 7: **Receptor and Pharmacology**
Session chairs: Mark Laburthe
Eve Lutz

9:30–10:00 PL7 **The human VPAC1 receptor: importance of N-terminal ectodomain in ligand recognition and discrimination between VIP and an antagonist**
Alain Couvineau
INSERM U773/CRB3, France

10:00–10:20 O19 **Analysis of a putative VPAC2 receptor from sturgeon shed light on molecular and functional evolution of VPAC2R in vertebrates**
Leo T.O. Lee
The University of Hong Kong, China

10:20–10:40 O20 **Key pharmacophore elements of the N-terminal domain of PACAP**
David Vaudry
INSERM U413, University of Rouen, France

10:40–11:00 O21 **Alternative splicing of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor contribute to function of PACAP-27**
Mina Ushiyama
Kagoshima University, Japan

11:00–11:10 Coffee break

Session 8: **Immune System and Inflammation**
Session chairs: Mario Delgado
Min Li

11:10–11:40 PL8 **Vasoactive intestinal peptide and its role in inflammatory diseases and self/nonself discrimination**
David Pozo Perez
University of Seville, Spain

11:40–11:55 O22 **Cell-gene therapy as an alternative to deliver VIP for treatment of autoimmune diseases**
Francisco Martin
Andalusian Stem Cell Bank, Spain

11:55–12:10 O23 **Vasoactive intestinal peptide generates human anergic T cells with regulatory functions by inducing cell cycle arrest and CTLA4**
Mario Delgado
CSIC, Spain

12:10–12:25 O24 **Renoprotection with PACAP in cisplatin-induced acute kidney injury**
Min Li
Tulane University, USA

12:25–12:40 O25 **VIP suppresses the inflammatory response to monocrotaline in rat lungs**
Anthony M. Szema
State University of New York at Stony Brook, USA

12:40–13:50 Lunch

Session 9:	**Clinical Application and Therapeutics**	
	Session chairs:	Illana Gozes Akio Inui
14:00–14:30	PL9	**Davunetide (NAP intranasal formulation AL-108) provides cognitive protection in a model of microtubules dysfunction exhibiting schizophrenia-like symptoms** Illana Gozes *Tel Aviv University, Israel*
14:30–14:50	O26	**Effect of PACAP on tear secretion in mouse** Tomoya Nakamachi *Showa University, Japan*
14:50–15:10	O27	**PACAP-38 in human plasma and milk under physiological and pathological conditions: introductory measurements for possible future clinical diagnostic application** Andrea Tamas *Pecs University, Hungary*
15:10–15:30	O28	**Clinical development of nasal GLP-1 compound with a new injector for the treatment of type 2 diabetes** Masanari Mizuta *University of Miyazaki, Japan*
15:30–15:40		Coffee break
Session 10:	**Cardiovascular, Respiratory, and Renal Systems**	
	Session chairs:	Sami I. Said Victor May
15:40–16:10	PL10	**An update on VIP in health and disease** Sami I. Said *State University of New York at Stony Brook, USA*
16:10–16:25	O29	**VIP greatly attenuates monocrotaline-induced pulmonary vasculopathy in rats** Sayyed A. Hamidi *State University of New York at Stony Brook, USA*
16:25–16:40	O30	**Endogenous PACAP attenuated doxorubicin-induced myocardial damage** Hiroyoshi Mori *Showa University, Japan*
16:40–16:55	O31	**Vasoactive intestinal peptide and circadian regulation of the cardiovascular system** Analyne Manzano Schroeder *University of California at Los Angeles, USA*
16:55–17:10	O32	**PACAP protects renal cells against in vitro ischemia and oxidative stress in primary kidney cultures** Gabriella Horvath *University of Pecs, Hungary*
19:00–21:00	**Banquet**	Etoile

Thursday, October 8th, 2009

8:30–9:30 SL5 **The search for novel peptides and for their functions**
Olivier Civelli
University of California, Irvine, USA
Chair: Kazuo Chihara

Session 11: Cell Signaling and Gene Regulation by PACAP and Related Neuropeptides
Session chairs: Lee Eiden
Masayasu Kojima

9:30–10:00 PL11 **PACAP signaling to target genes through cyclic AMP and calcium during the stress response**
Lee Eiden
NIMH, USA

10:00–10:20 O33 **LIF-mediated maintenance of PACAP-induced neurite outgrowths in human SH-SY5Y neuroblastoma cells requires PI3K but not STAT or ERK signaling pathways**
Eve Marie Lutz
Strathclyde University, Scotland

10:20–10:40 O34 **Identification of a novel signaling cascade specifically involved in the light-induced phase advance of circadian rhythm by using PACAP knockout mice**
Michiyoshi Hatanaka
Osaka University, Japan

10:40–11:00 O35 **Involvement of stathmin 1 in the neurotrophic effects of PACAP in PC12 cells**
David Vaudry
INSERM U413, University of Rouen, France

11:00–11:10 Coffee break

Session 12: Regulated Expression of PACAP and Its Receptors
Session chairs: Atsuro Miyata
Masaaki Mori

11:10–11:40 PL12 **Pituitary adenylate cyclase-activating polypeptide (PACAP) induces activity-dependent gene expression in neurons**
Masaaki Tsuda
University of Toyama, Japan

11:40–12:00 O36 **Tissue-type plasminogen activator (tPA) as a PACAP-regulated gene in neuronal cells**
Ludovic Galas
PRIMACEN, University of Rouen, France

12:00–12:20 O37 **Temporal dynamics of gene expression during PACAP-induced PC12 cell differentiation**
Masami Ishido
National Institute for Environmental Studies, Japan

12:20–12:40	O38	**Increased stathmin1 expression in the dentate gyrus causes abnormal axonal arborizations potential relevance to schizophrenia** Kohei Yamada *Osaka University, Japan*

12:40–13:00 Closing Ceremony

A role for pituitary adenylate cyclase-activating peptide (PACAP) expression and signaling in the bed nucleus of the stria terminalis (BNST) in stress-induced anxiety-like behavior

S.E. Hammack
Department of Psychology, University of Vermont, Burlington, VT 05405, USA

The chronic exposure to stressful stimuli has been argued to play an important role in the etiology of anxiety disorders. Consistent with this role, increases in anxiety-like behavior are often observed in rodents repeatedly exposed to environmental stressors. Several brain nuclei have been implicated in coordinating the autonomic, endocrine, and behavioral response to stressor exposure. In particular, the bed nucleus of the stria terminalis (BNST) has been argued to mediate anxiety-like behavioral responding to long-duration anxiogenic stimuli and also coordinate autonomic and endocrine stress responses. Moreover, neuroplasticity in this region is increased following chronic stress; hence, the BNST may be a critical nodal structure whereby chronic stressor exposure produces an anxiogenic behavioral profile. We have shown that chronic stress substantially and selectively increased transcript levels of pituitary adenylate cyclase-activating peptide (PACAP) and its cognate PAC1 receptor, in BNST tissue punches and also increases PACAP immunoreactivity discretely in the oval nucleus of the BNST, a region heavily implicated in mediating anxiety-like behavior. Furthermore, PACAP infusion into the BNST produced an anxiogenic response on baseline acoustic startle responding that persisted for at least 7 days following the initial injection. Current studies using strategies to reduce BNST PACAP signaling during chronic stress treatment also suggest proper BNST PACAP signaling is necessary for enhanced anxiety-like behavior following chronic stress. Based on these data, BNST PACAP signaling appears to mediate the anxiogenic effects of chronic stress, and this mechanism may represent an important target in the treatment of anxiety disorders.

Secretin attenuates the repetitive hyperactive movements in a mouse model

K. Köves[1], G. Kiss[2], M. Mácsai[2], A. Heinzlmann[1], Á. Csáki[1], J.Takács[3], R. Dochnal[2], Z. Boldogkoi[4], G. Szabó[2]
[1*]*Dept. Human Morphology, Semmelweis University*
[2]*Dept. Pathophysiology and* [4]*Dept. Biology, Albert Szentgyörgyi University, Szeged*
[3]*Neurobiological Research Laboratory, Semmelweis University and the Hungarian Academy of Sciences, Budapest, Hungary*

The relation of secretin and autism was suggested 10 years ago: IV secretin improved the mental functions in a few autistic children. The most characteristic morphological features in autistic patients is found in the cerebellum. Secretin influences several functions of the nervous system. In the cerebellum, secretin facilitates the GABA-ergic inhibitory input onto Purkinje cells via a postsynaptic and cAMP-dependent mechanism. Its intracerebroventricular (ICV) administration decreased open-field activity and novel-object approach in white mice. In the present experiment, "Japanese waltzing mice" (JWM) were used to demonstrate the effect of ICV secretin on the repetitive hyperactive movements. Repetitive movements are also characteristic of autistic patients. Secretin very effectively depressed the repetitive movements and normalized the open-field activity in JWM. The number of brush cells of the VI and VII lobes in the cerebellar vermis of JWM was significantly higher than in the individuals of the control strain. The function of these cells is to multiply the incoming signs. It may explain the hyperactive movements of these animals. Because the central amygdala responded to secretin by expressing c-fos, and it is also known that amygdala is involved in the behavior we have looked for connection between the amygdala and the cerebellum using two different tracers in rats. Fluorogold is suitable for demonstrating direct neuronal connections between two structures, and a retrograde virus strain is able to demonstrate neuronal chain between two structures by passing through synapses. Fluorogold, applied iontophoretically into the central amygdala, did not label any structures in the cerebellum; however, we have found labeling in cell bodies of cerebellar nuclei and in the Purkinje cells mainly in the nodular cortex when a retrograde virus was injected into the amygdala. These data indicate that there is a connection between the cerebellum and the amygdala, and this connection is composed of more than one neuron.

PAC1-R heterozygous mice have no desire to explore the unknown environment in memory task

Nobuyoshi Hagino, M.D., Ph.D.
Laboratory of Integrative Medicine, US-Japan Cooperative Biomedical Research Laboratories, Tulane University Herbert Research Center, and Department of Medicine, Tulane University School of Medicine, Belle Chasse, Louisiana, USA

It was observed that the PAC1-R heterozygous (−/+) transgenic mutant mice in the multiple mazes do not respond for the perception of geomagnetic orientation and fear signals, but they respond for the perception of visual orientation. The fear signals also stimulate the perception of olfactory orientation. To confirm that we spray a specific PAC1-R antagonist (PACAP6-38) into the nasal cavity of mouse and we investigate the performance of memory tasks in the multiple mazes. No significant difference in the nasal spray of 50 μl of physiological saline in 18 (−/+) mice and 18 littermates (+/+) are noted.

However, significant increase of the performance time by the nasal spray of 40 μg of PACAP6-38 in 50 μl of physiological saline occur in 18 (+/+) mice, but not in 18 (−/+) mice. What is a desire of (−/+) mouse? To confirm that, we place the mouse into an unknown environment of the multiple mazes and investigate the exploratory-related behaviors. All 18 (+/+) mice explore all four chambers immediately after mouse places in the multiple mazes, however, 18 (−/+) mice explore the first and second chambers after the mouse is placed in the multiple mazes, but they do not explore the third and fourth chambers. This is to show that decline of perception of geomagnetic orientation, olfactory orientation, and fear signals causally mediate the disruption of a desire to explore the unknown environment. The (−/+) mice have an impaired LTP in the mossy fiber in the hippocampus. It is inferred that PAC1-R-mediated signaling of the mossy fiber in the hippocampus is associated with a desire to explore the unknown environment.

On the cellular and molecular pathways involved in the inhibition of LTP in the CA1 area of the hippocampus

Diana Cunha-Reis, Nádia C. Rodrigues, Joaquim A. Ribeiro
Institute of Pharmacology and Neurosciences, Faculty of Medicine, and Neuroscience Unit, Institute of Molecular Medicine, University of Lisbon, Av. Prof. Egas Moniz, 1649-028, Lisbon, Portugal

Vasoactive intestinal peptide (VIP) modulates hippocampal synaptic transmission through several receptor and cellular mechanisms (Cunha-Reis et al., 2004; 2005; 2006) and is fully dependent on GABAergic transmission. VIP containing interneurones are innervated by septal GABAergic and median raphe serotonergic fibers (Papp et al., 1999), suggesting an involvement in theta-related synaptic plasticity. We now evaluated how endogenous VIP influences hippocampal long-term potentiation (LTP) induced by theta-burst stimulation and what the receptor and transduction pathways are involved in this modulation. The role of VIP modulation GABAergic transmission was also investigated.

Extracellular electrophysiological recordings in hippocampal slices were used to access LTP induced by theta-burst stimulation (×5 100 Hz, four stimuli, separated by 200 ms) Selective $VPAC_1$ (PG 97-269) and $VPAC_2$ (PG 99-465) as well a non-selective (Ac-Tyr^1 GRF(1-29) VIP receptor antagonists were used to evaluate the involvement of endogenous VIP in hippocampal synaptic plasticity. The involvement of protein kinases A (PKA) and C (PKC) in these effects was studied using the selective inhibitors H-89 and GF109203×, respectively. How changes in GABAergic transmission contribute to this effect of VIP was tested in the presence of the selective $GABA_A$ antagonist biccuculline.

Theta-burst stimulation caused an enhancement of 28±2.8% (n=15) in fEPSPs slope recorded 50–60 min after stimulation. Ac-Tyr^1 GRF (1–29; 100 nM) increased theta burst-induced LTP to 49±4.1% (n=4). PG 97-269 (100 nM) also increased that theta burst-induced LTP to 40±6.7% (n=6). PG 99-465 (100 nM) did not significantly change theta-burst-induced LTP. Inhibition of PKA with H-89 (1 μM, n=4) did not significantly change the enhancement caused by PG 97-269 on LTP, but that effect was abolished upon inhibition of PKC with GF109203× (1 μM, n=3). Blockade of $GABA_A$ receptors with bicuculline (10 μM) also abolished $VPAC_1$ receptor modulation of LTP induced by theta-burst stimulation (n=5).

These results suggest that endogenous VIP has a restraining effect on hippocampal LTP through tonic activation of $VPAC_1$ receptors and PKC. This effect is also dependent on GABAergic transmission suggesting an indirect effect of VIP on hippocampal glutamatergic synapses, involving modulation of hippocampal GABAergic circuits.

Supported by FCT

Cunha-Reis D et al. (2004) Br J Pharmacol 143:733
Cunha-Reis D et al. (2005) Brain Res 1049:52
Cunha-Reis D et al. (2006) Ann NY Acad Sci 1070: 210
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Altered emotional and cognitive function in PACAP-deficient mice: a novel animal model for psychiatric disorder

Norihito Shintani[1], Hitoshi Hashimoto[1,2,3], Atsuko Hayata[1,2], Katsuya Ogata[1], Ryota Haba[1,2], Akemichi Baba[1]
[1]Laboratory of Molecular Neuropharmacology, Graduate School of Pharmaceutical Sciences, and [2]Center of Child Mental Development, Graduate School of Medicine, Osaka University, [3]United Graduate School of Child Development, Osaka University, Kanazawa University and Hamamatsu University School of Medicine, Japan

Previously, we developed mice lacking PACAP gene (PACAP-KO) which exhibit marked behavioral phenotypes, including hyperactivity and perseverative jumping behavior in an open-field test (OFT) and reduced prepulse inhibition. The phynotypic analysis of PACAP-KO suggests a role for altered PACAP-mediated signaling in certain psychiatric disorders. Here, we examined the impacts of the deletion of PACAP in mice on emotional and cognitive behaviors and their response to antipsychotic drugs and acute intracerebral (ICV) injection of PACAP. PACAP-KO mice showed both emotional and cognitive deficits, including increased immobility in a forced swimming test (FST), and reduced memory retention in a contextual fear-conditioning and novel-object recognition tests (NORT). The atypical antipsychotic risperidone and

the selective serotonin $(5\text{-HT})_2$ antagonist ritanserin normalized the both phenotypic changes in PACAP-KO mice in FST and NORT, whereas hyperkinetic nature in OFT was ameliorated by risperidone but not by ritanserin. The 5-HT_2 agonist (±)-2,5-dimethoxy-4-iodoamphetamine-induced 5-HT syndrome was exaggerated in PACAP-KO mice, which suggests a 5-HT_2-receptor-dependent mechanism in the emotional and cognitive deficits. In contrast, ICV injection of PACAP ameliorated all of the phenotypic changes in PACAP-KO mice in FST, ORT, and OFT. Recent genetic linkage and association studies on human PACAP gene suggested a possible relation between PACAP signaling and psychiatric disorders such as schizophrenia and bipolar disorder. Taken together, the present results suggest that alterations in acute PACAP signaling contribute to the pathogenesis of certain psychiatric disorders amenable to atypical antipsychotic drugs, and PACAP-KO mice is a useful animal model for human psychiatric disorders.

VIP regulates cortical growth through MCPH1/CHK1: relevance to human microcephaly

Sandrine Passemard[1], Pierre Gressens[1], and Vincent Lelievre[2]
[1]*INSERM U676 and IFR37 Paris 7, Hopital Robert Debre, Paris, France*
[2]*CNRS UPR3212 and University of Strasbourg, France*

Human primary microcephaly is a rare genetic disorder that elicits a reduction of cortical outgrowth without severe interference with cortical patterning. Up to date, seven locus candidates (mcph1-7) have been identified, all associated with mutations in genes also known as MCPH1 (BRIT1, encoding for microcephalin), ASPM (MCPH5), CDK5RAP2 (MCPH3), CENPJ (MCPH6), and finally STIL (MCPH7). In brief, these genes encode for proteins involved in either cell cycle control or mitotic spindle formation and centrosomal activities. Anyway, genetic alterations in these key molecules ultimately lead to limited number of mitotic divisions, fewer cortical progenitors and smaller brain size. It has been long proposed that interfering with VIP signaling pathway using pharmacological antagonist of the VIP receptors during murine gestation could generate microcephalic newborn pups (Gressens et al., JCI 1994). In the present work, we revisit this animal model by hypothesizing that VIP antagonist (VA) may interfere with the normal VIP signaling pathway that crosstalks with the identified MCPH-related proteins. Herein, we reported that intraperitoneal injection of VIP antagonist in pregnant females (occurring between E9 and E11) leads to the selective embryonic alteration of MCPH1 expression and function. VA-induced inhibition of MCPH1 expression leads to down-regulation of both BRCA1 and CHK1 expression and CHK1 kinase activity. This decrease turned off neural stem cell proliferation as shown in primary neurosphere cultures. Furthermore, in vitro silencing of MCPH1 in neural stem cells and neural progenitors abolished VA-inhibition of cell proliferation, suggesting that alterations of the VIP/VPAC1/MCPH1/CHK1 signaling could represent an endogenous regulatory pathway crucial for normal cortical development.

Peripheral administration of vasoactive intestinal peptide ameliorates CNS cytokine induction in mouse model of neonatal diffuse white matter injury

Hiroko Nobuta, Cristina A Ghiani, Armine Manukyan, Jean de Vellis, James A Waschek
Interdepartmental Program in Neuroscience, Semel Institute, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, California, USA

Currently, preterm births account for one out of eight births in the US. A subset of afflicted children face serious neurological defects including periventricular white matter injury (PWMI) at high prevalence, which is associated with cognitive/behavioral abnormalities arising from damages to the oligodendrocyte (OL). We have created a new mouse model in which neuroinflammation, a putative causal factor for this disease, is induced by peripheral administration of endotoxin at a time when OL precursor vulnerability is maximum. The phenotype of the model mimicked clinical PWMI, at cellular, molecular, and behavioral levels. Importantly, these phenotypes were preceded by robust central nervous system (CNS) inductions of pro-inflammatory cytokines TNFα, IL1-β, and IL6, which were followed by a significant increase in the activation of microglia. These results prompted us to investigate possible actions of VIP to modulate neuroinflammation and to rescue the damage mediated by abnormal elevation of pro-inflammatory cytokines in the CNS. VIP at dose of 10 pmol per animal was found to be sufficient to significantly reduce TNFα gene expression in the CNS, providing evidence that VIP can potentially control neuroinflammation in this model. Surprisingly, despite the amelioration of cytokine surge, this protocol failed to reverse the microglial activation that preceded white matter damage. Consequently, the loss of OL was not rescued by administration of VIP. These results suggest that (1) peripherally administered VIP may have limited access to the CNS to impose efficient actions on microglia, even in the immature structure of blood-brain barrier in the neonatal animals; (2) higher doses or longer-term treatment with VIP may be necessary to block white matter injury; or (3) white matter damage in this model is not associated with cytokine elevation but rather induced

by other mechanisms. The potential immunomodulatory and protective actions of VIP in the current PWMI model therefore warrant further investigation.

Roles of the PAC1 receptor in mouse neurogenesis

Sanbing Shen, Bing Lang, and Colin D. MiCaig
School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Aberdeen, AB25 2ZD UK

The pituitary adenylate cyclase-activating polypeptide (PACAP) and its high-affinity receptor PAC1 are encoded by genes *ADCYAP1* and *ADCYAP1R1*, respectively. They are highly expressed in the embryonic and adult central nervous system and are recently implicated in the Japanese schizophrenic population. However, it is not known whether expression of the *ADCYAP1* or *ADCYAP1R1* is altered in schizophrenia. We have investigated roles of the PAC1 receptor through the gain-of-function approach, by over-expressing the human PAC1 receptor with a 130 kb transgene in mice. Transgenic mice develop transgene dose-dependent enlargement of the ventricles and reduction of the cerebral cortex and corpus callosum. These neuroanatomical changes are commonly found in hydrocephalus and schizophrenia, and the defects are associated with reduced neural proliferation and increased neuronal apoptosis during embryonic neurogenesis. PAC1 receptor is also expressed in mammalian retina and involved in processing light information. We demonstrate that PACAP signaling plays an important role in the development of retina, particularly in the genesis of GABAergic amacrine cells. Overexpression of the PAC1 receptor leads to an early exit from retinal proliferation, reduced production of GABAergic neurons, and a marked decline in visual function. Possible involvement of PACAP signaling in choroid plexus, CSF flow, hippocampal formation, and adult neurogenesis will also be discussed.

PACAP as a neurotrophic signal for astrocyte differentiation

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During development of the central nervous system the generation of astrocytes is initiated from the same neural precursor cells that generate neurons, but only after neurogenesis has been largely completed. This neurogenic to gliogenic switch is under tight regulation by intrinsic mechanisms that determine the gliogenic competence of neural progenitors. Once this competence is acquired, neural progenitor cells can respond to specific signals that initiate their differentiation into astrocytes. Studies carried out in our laboratory on cortical progenitor cells indicated that pituitary adenylate cyclase-activating polypeptide (PACAP) can trigger their astroglial differentiation by acting on PAC1 receptors and stimulating the cAMP-dependent intracellular signaling pathway. Cyclic-AMP then stimulates the guanine nucleotide exchange factor Epac, leading to the activation of the small GTPase Rap1. In addition, PACAP stimulation of the cAMP-dependent pathway activates *Ras*. Stimulation by PACAP of the expression of the gene encoding glial fibrillary acidic protein (GFAP), a characteristic phenotypic marker of astrocytes, requires the coordinated activation of both Rap1 and *Ras*. Furthermore, PACAP-induced astrocyte differentiation requires the cAMP-dependent entry of extracellular calcium ions into the cells. Both cAMP and calcium stimulate GFAP gene transcription, an effect that requires the integrity of two DNA regulatory elements located in the proximal promoter region that are occupied by the calcium-binding transcription factor DREAM. Mutational studies and transfection of primary cortical progenitor cells revealed that DREAM mediates the transcriptional stimulation of the GFAP gene induced by cAMP and calcium, an effect that requires the integrity of the calcium-binding domains of this transcription factor. Cortical progenitor cells from *dream*-deficient mice fail to differentiate into astrocytes in response to PACAP. In addition, the cerebral cortex of these mice contains a reduced number of astrocytes and an increased number of neurons, indicating the importance of DREAM in the regulation of the neurogenic to gliogenic switch. These studies indicate that the PACAP–cAMP–calcium–DREAM signaling cascade constitutes an important pathway for the regulation of astrogliogenesis during the development of the central nervous system.

PACAP38 protects rat cortical neurons against the neurotoxicity evoked by sodium nitroprusside and thrombin

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Pituitary adenylate cyclase-activating polypeptide (PACAP) 38 is a multifunctional anti-inflammatory and anti-apoptotic neuropeptide widely distributed in the nervous system. The objective of this study is to determine whether PACAP38 is neuroprotective against sodium nitroprusside (SNP) and thrombin, two mechanistically distinct neurotoxic agents. Treatment of primary cortical neuronal cultures with 1 mM SNP for 4 h causes neuronal cell death that is significantly reduced by 100 nM PACAP38. PACAP38 down-regulates

SNP-induced cell cycle protein (cyclin E) expression and up-regulates p57KIP2, a cyclin-dependent kinase inhibitor as well as the anti-apoptotic protein Bcl-2. Similarly, neuronal death induced by 100 nM thrombin or the thrombin receptor activating peptide (TRAP 6) is reduced by PACAP38 treatment. Thrombin-stimulated cell cycle protein (cdk4) expression is decreased by PACAP38 while PACAP38 inhibits thrombin-mediated reduction of p57KIP2. However, the decrease in Bcl-2 evoked by thrombin is not affected by PACAP38. Finally, both SNP and thrombin (or TRAP) increase caspase 3 activity, an effect that is decreased by PACAP38. These data show that PACAP38 supports neuronal survival in vitro suppressing cell cycle progression and enhancing anti-apoptotic proteins. Our results support the possibility that PACAP could be a useful therapeutic agent for reducing neuronal cell death in neurodegenerative diseases.

Neuroprotective strategy on brain injuries by immunemodulation—lesson from PACAP and hMSCs on stroke

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It was considered for a long time that central nervous system is an immune privilege area. Hematopoietic cells are hard to infiltrate into brain parenchyma even if the brain is in pathological conditions because of existing blood-brain barrier. However, latest new evidences drastically changed the hypothesis on development of neural death. After onset of experimental autoimmune encephalomyelitis, after ischemia or on Parkinson's disease, an amount of hematopoietic cells migrate into injury area. In some conditions, they seem to progress neural damage and in other conditions, they work for neuroprotection and neural repair cooperating with resident glial cells.

We have reported proinflammatory cytokine-deficient mice modulate progression of infarct volume or neuronal cell death after ischemia. Moreover, we and other groups pointed out that pituitary adenylate cyclase-activating polypeptide (PACAP) modulates cytokines for suppression of neuronal cell death and of inflammatory responses. In stroke, PACAP increased expression of interleukin-6 (IL-6). Both of PACAP and IL-6 null mice decreased phosphorylation of STAT3 which is downstream of IL-6 receptor. The signaling pathway would increase anti-apoptotic factor bcl-2 and decrease mitochondrial apoptotic signal. PACAP has suppressed IL-1 and TNFa in an appropriate condition of macrophage and microglial culture. We have shown IL-1- or TNFa-gene deficient mice decreased infarct volume after ischemia. Recently, we have reported that human stem progenitor cells from bone marrow (hMSCs also known as mesenchymal stem cells or marrow stromal cells) suppressed dramatically hippocampal neuronal cell death even if it was transplanted into dentate gyrus 1 day later onset ischemia. Gene ontology of microarray survey showed hMSCs decreased ischemia-enhanced immune response 30% or more (21 of 65 non-redundant genes). The detail analyses indicated hMSCs increased activating microglia/macrophages, but they were alternative activating type, were not the classical cytotoxic one. The alternative macrophage worked as antigen-presenting cells and also produced insulin-like growth factor 1.

These lessons indicate that immunomodulation might be a new strategy for brain injuries.

Delivery of pituitary adenylate cyclase-activating polypeptide (PACAP) to the brain: targeting with intranasal delivery and cyclodextrins

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The 38 amino acids from pituitary adenylate cyclase-activating polypeptide (PACAP) has potent neurotrophic and neuroprotective effects when tested in in vitro and in vivo models of ischemia. PACAP has been proposed as a useful treatment of stroke or central nervous system injuries. A major problem with the use of peptides such as PACAP as therapeutics is that their delivery to brain is problematic. Some peptides have been shown to be able to be delivered to the brain by intranasal (IN) administration. Here, we investigated distribution of PACAP radioactively labeled with iodine (I-PACAP) into brain regions, whole brain, and blood by the IN administration and then I-PACAP with cyclodextrin (CD) and I-PACAP with unlabeled PACAP. We compared the abilities of alpha-CD and hydro-β-CD to affect uptake of I-PACAP after IN administration, and we found variations in regional brain uptake between alpha-CD and hydro-β-CD with I-PACAP. Inclusion of unlabeled PACAP in the IN administration significantly increased uptake of I-PACAP by occipital cortex and hypothalamus; olfactory bulb uptake was increased by alpha-CD; uptake by thalamus was increased by hydro-β-CD. Occipital cortex was decreased by alpha-

CD; striatum uptake was decreased by both of alpha-CD and hydro-β-CD, and whole brain uptake was decreased by alpha-CD. Neither alpha-CD nor hydro-β-CD affected blood levels of I-PACAP. These results show that different cyclodextrins have different effects on the distribution in brain of IN I-PACAP. As such, individual cyclodextrins could be used to direct PACAP or other peptides to or away from specific brain regions. In conclusion, we found that IN administration of I-PACAP was absorbed into brain and distributed to all brain regions. Distribution was unique in that occipital cortex and striatum has higher uptakes than the olfactory bulb, especially after unlabeled PACAP probably by competitively inhibiting the brain-to-blood efflux transporter for I-PACAP. Cyclodextrins had variable effects of the uptake of I-PACAP with a general trend towards increasing uptake but actually decreasing uptake into other regions. We conclude that intranasal delivery is a viable route for delivering PACAP to the brain.

Comparison and possible relationship between PACAP- and enriched environment-induced retinal protection in MSG-treated newborn rats

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Pituitary adenylate cyclase-activating polypeptide (PACAP) has retinoprotective effects in different models of retinal degeneration. We have previously shown that PACAP protects against monosodium glutamate (MSG)-induced damage. Furthermore, PACAP induces anti-apoptotic and inhibits pro-apoptotic signaling pathways in the retina. We obtained a similar degree of neuroprotection against MSG toxicity in animals kept in an enriched environment. The present aim was to compare the two neuroprotective strategies alone and together and to find a possible relationship between enriched environment and PACAP. We found that both PACAP and environmental enrichment led to a similar degree of retinal protection, but the two treatments together did not lead to increased protection: their effects were not additive. Enriched environment is known to influence the endogenous levels of trophic factors, although results are very contradictory. In the second part of our experiment, we determined the effects of enriched environment on PACAP levels by RIA. We found that while 1-week-enrichment at adulthood increased PACAP concentration in most examined brain areas, rats kept in enriched environment neonatally had lower PACAP levels in adulthood. Similarly, neonatal rats undergoing MSG treatment had lower PACAP levels at 4 weeks of age than their classical-cage mates, irrespective of environmental enrichment. These results show that the influence of enriched environment on PACAP levels and effects is very complex, similarly to other trophic factors, and it possibly reflects two rather independent trophic influences.

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Review of the retinoprotective effects of PACAP

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In the present study, we give a review on the retinoprotective effects of pituitary adenylate cyclase-activating polypeptide (PACAP). The well-established neuroprotective effects of the peptide can be observed in the retina as well, against different types of toxic agents and pathological conditions. In vitro, it has been shown to protect retinal culture against glutamate toxicity. Subsequently, studies by Seki and colleagues have shown the presence of PACAP and its receptors in the retina, and they provided evidence that it protects against kainic acid-induced injury and optic nerve transection. We have conducted several in vivo experiments that prove that PACAP decreases retinal damage in models of (1) neonatal toxic injury induced by monosodium glutamate (MSG); (2) hypoperfusion injury induced by permanent bilateral carotid artery occlusion; (3) diffuse and focused UV-A-induced retinal degeneration; and (4) streptozotocin-induced diabetic retinopathy. Regarding the molecular mechanism, we have investigated the well-known antiapoptotic protective pathways in the retina, after glutamate-induced toxic injury. We have shown that PACAP treatment conteracts the glutamate-induced increase in caspase-3, JNK, apoptosis-inducing factor, cytochrome c release, and it also reversed the MSG-induced decrease in phospho-PKA, 14-3-3 protein, and phospho-Bad. Furthermore, PACAP increased ERK/CREB activation. Regarding the role of endogenous PACAP, we found that PACAP antagonist administration increases proapoptotic, while decreases antiapoptotic pathway activation and aggravates retinal injury in vivo. Also, PACAP-deficient mice have augmented retinal damage. All these results together show that both endogenous and exogenously given PACAP have retinoprotective effects.

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Protective effects of PACAP against oxidative stress in cochlear cells

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic and multifunctional neuropeptide. Numerous studies prove that PACAP has neuroprotective effects in diverse neuronal systems in vitro and in vivo. The involvement of PACAP in visual and olfactory sensory processing has also been documented, but little is known about its effects in the auditory system. The presence of PACAP and its receptor, the specific PAC1 receptor, has been shown in the cochlea and in brain structures involved in auditory pathways. The aim of the present study was to investigate whether PACAP is protective in cochlear oxidative stress-induced cell death, which is known to play a role in several ototoxic insults. Chicken cochlear cells were exposed to 1 mM H_2O_2, which resulted in a marked reduction of cell viability and a parallel increase of apoptotic and necrotic cells assessed by MTT test and flow cytometry. Co-incubation with 100 nM PACAP increased cell viability and reduced the percentage of apoptotic cells. Furthermore, oxidative stress increased the activation of caspase-3, while simultaneous PACAP treatment reduced it. In summary, our present results demonstrate that PACAP effectively protects cochlear cells against oxidative stress-induced apoptotic cell death.
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Pituitary adenylate cyclase-activating polypeptide (PACAP) receptor expression is altered in the brain of *Podarcis sicula* after nonylphenol administration

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One of the most abundant environmental pollutants, nonylphenol (NP), may interfere with basic functions of central and peripheral organ systems of vertebrates. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic peptide which is involved in a plethora of functions through the binding of three receptors: PAC1, VPAC1, and VPAC2. Here, we report the immunohistochemical distribution of PACAP receptors in the brain of the reptile *Podarcis sicula* after NP administration (3.44 μg/ml/mg of body weight per day) through daily intraperitoneal injections for 7, 14, 28, and 40 days, respectively. In control samples, PAC1 receptor labeling occurs in the cerebellum and adenohypophysis. At 7 days, the labeling is shifted in the optic tectum and telencephalon, decreasing its intensity in the 14- and 28-day specimens. At 40 days, PAC1 labeling also arises from nervous fibers of rhomboencephalon. In control samples, VPAC1 is found in the cerebellum and optic tectum. At 7 days, the optic tectum and the telencephalon are labeled for VPAC1. In 14-, 28-, and 40-day samples, VPAC1 labeling completely disappears. In controls, VPAC2 is found in the optic tectum, cerebellum, and rhomboencephalon. At 7 days, only the optic tectum retains labeling for VPAC2. At 14 days, the optic tectum lacks labeling while cerebellum expresses VPAC2 receptor. At 28 days, VPAC2 labeling occurs in the cerebellum and in telencephalic nervous fibers. At 40 days, no brain structures express VPAC2 labeling. Our data clearly show that: (1) NP administration alters the PACAP receptor distribution pattern in the brain of *Podarcis sicula*; (2) the longer treatment is the deeper are alterations of PACAP receptor expression; and (3) the longest treatment abolishes both VPAC1 and VPAC2 pattern. Hence, it is conceivable that NP may have deep impact on brain functions regulated by PACAP through its receptors.

PACAP and the blood-brain barrier

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The CNS effects of circulating pituitary adenylate cyclase-activating polypeptide (PACAP) likely depend in part on its ability to cross the blood–brain barrier. Previous work has shown that PACAP38 is transported both into and out of the brain by saturable transport systems. PACAP27 enters the brain from the blood by a non-saturable mechanism, but is transported out of the brain by a transport system pharmacokinetically distinguishable from the PACAP 38 efflux system. Thus, a family of transporters likely exists for the PACAPs, and these have been reified as peptide

transport system 6 (PTS-6). The blood-to-brain transport of PACAP38 likely underlies its ability to reverse ischemic damage to the brain after its peripheral administration. The PACAP transporters are expressed throughout the brain and spinal cord and respond differentially to traumatic injury to the spinal cord. In contrast, total body ischemia resulting from cardiac arrest has little effect on PACAP38 transport. More recently, we have isolated the brain-to-blood saturable transporter component for PACAP27. We have identified the protein as ß-F1 ATPase and shown that it colocalizes with PACAP27 on brain endothelial cells. Antisense directed at the mRNA of ß-F1 ATPase selectively inhibits the efflux of PACAP27, but not of PACAP38, iodine, Tyr-MIF-1, or ß-endorphin. Antisenses directed at the mRNA for amyloid precursor protein or preproenkephalin did not affect PACAP27 efflux. Inhibiting the efflux activity of ß-F1 ATPase increased the blood-to-brain uptake of PACAP27 by about 4-fold. This resulted in improved therapeutic effects in animal models of stroke and Alzheimer's disease.

Ghrelin: from discovery to translational research

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A complex network of cell–cell communication system by peptide hormones works for maintaining the mammalian homeostatic balance. To further clarify the intricate mechanisms of the regulation, we have been searching for still-unidentified bioactive peptides. In the course of these studies, we discovered three natriuretic peptides, ANP (1984), BNP (1988), and CNP (1990), in mammalian heart and brain, and adrenomedullin (1993) in human pheochromocytoma. These studies elucidated new regulatory mechanisms of cardiovascular system and also brought their therapeutic potentials on cardiovascular diseases.

In 1999, we discovered ghrelin, a novel GH-releasing peptide, from rat stomach. Ghrelin has a marvelous structure modified by fatty acid, *n*-octanoic acid, which is essential to its activity. Ghrelin is primarily produced in distinct endocrine cells, X/A-like cells, in the stomach. Ghrelin-producing neurons are also present in the hypothalamic arcuate nucleus, a region that regulates GH release and food intake. In fact, ghrelin stimulates feeding when administered centrally and peripherally. Ghrelin secretion is up-regulated under conditions of negative energy balance, whereas it is down-regulated under conditions of positive energy balance. Ghrelin is the first neuro-enteric peptide that acts as a starvation-signaling molecule in the periphery. The recent studies indicate that the gastric vagal afferent is the major pathway conveying ghrelin's signals for starvation and GH secretion to the brain. Therefore, beside the stimulatory effect of GH release, ghrelin is also involved in the stimulation of feeding and the regulation of energy metabolism.

Moreover, ghrelin has positive cardiovascular effects. The administration of ghrelin improves cardiac structure and function and attenuates the development of cardiac cachexia in rats with heart failure. In clinical trial, repeated administration of ghrelin improves left ventricular structure and function, exercise capacity, and muscle wasting in heart failure patients. Thus, ghrelin has multifaceted roles in the cardiovascular and metabolic systems and also has therapeutic potentials in various diseases by GH-dependent and GH-independent mechanisms.

Clinical and pathogenic implications of incretin therapy in patients with type 2 diabetes

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The number of people with type 2 diabetes has rapidly and remarkably increased throughout Asia, and the rate of increase shows no sign of slowing. People in Asia tend to develop diabetes with a lesser degree of obesity at younger ages, suffer longer with complications of diabetes, and die sooner than people in other regions. Traditional treatment modalities—even multidrug approaches—for type 2 diabetes are often unsatisfactory at achieving glycemic goals as the disease progresses due to a steady, relentless decline in pancreatic β cell function. To prevent or delay progression of the disease, we have to understand more about the role of incretin to treat the disease and underlying pathogenesis of type 2 diabetes especially focused on islet biology in this region.

GLP-1 receptor agonists not only acutely lower blood glucose resulted by potentiation of insulin secretory response to glucose and suppresses hepatic glucose output by inhibiting glucagon secretion but also engage signaling pathways in the islet β cell that lead to stimulation of β cell replication and inhibition of β cell apoptosis. GLP-1 also decreases β cell workload resulted by reducing peak nutrient absorption and having effects on the central nervous system, resulting in a reduction of food intake. Sitagliptin is an oral, potent, and selective DPP-4 inhibitor. In patients with type 2 diabetes, sitagliptin demonstrated an increase in active GLP-1, GIP, insulin level, and lower glucagon concentration and a reduction in post-glucose load glucose excursion. A once-daily regimen of sitagliptin provides effective improvements in overall glycemic control in monotherapy and combination treatment with metformin, PPARγ agents, and insulin. Comparing with an SU, sitagliptin provides similar

efficacy and superior improvements in measure of β cell function, and less hypoglycemia and no weight gain. Sitagliptin was well tolerated and have showed negligible side effects so far.

In conclusion, sitagliptin has shown efficacy and tolerability in the management of hyperglycemia in type 2 diabetes, without causing weight gain or hypoglycemia. And considerable clinical data support the concept that sitagliptin treatment, alone or in combination with other oral hypoglycemic agents, may potentially reverse the decline in β cell mass that is characteristic of the natural history of type 2 diabetes.

Secretin: a neurosecretory factor regulating body water homeostasis

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Vasopressin (Vp) and oxytocin (Oxt) are generally accepted to be the only two neurosecretory hormones that are released from the posterior pituitary into the systemic circulation. However, recent findings from our group showed that secretin, originally isolated from the upper intestinal mucosal extract, is abundantly expressed in the hypothalamic magnocellular neurons and is also a neurosecretory hormone that is released from the posterior pituitary into the systemic circulation under plasma hyperosmolality conditions. Using secretin-null ($SCT^{-/-}$) and secretin receptor-null ($SCTR^{-/-}$) mice, we found that mutation of either genes could alter the expression and release of Vp under plasma hyperosmolality. Additionally, reduction in the renal expression of water channels including aquaporin-2 and aquaporin-4, as well as altered glomerular and tubular morphology, were also observed in these transgenic littermates. Together with the findings that secretin could (1) act as a dipsogenic agent when injected intracerebroventricularly and (2) directly stimulate the expression and translocation of the aquaporin-2 in the renal medullary tubules, we propose here that the peptide could work at multiple levels in the subfornical organ-hypothalamo-pituitary-kidney axis to regulate body water homeostasis. These findings not only challenge our previous understanding regarding the neuroanatomy of neurohypophysis, but also provide information for at least one of the Vp-independent mechanisms that modulate the process of renal water reabsorption. Future investigations in this direction should be important in developing therapeutic means for treating X-linked nephrogenic diabetes insipidus by therapeutically bypassing the dysfunctional Vp receptors.

Central administration of secretin suppresses food intake in mice

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Secretin is released into the circulation postprandially from the duodenal S cells. The major functions of secretin originated from the gastrointestinal system are to delay gastric emptying, stimulate fluid secretion from pancreas and liver, and hence, optimize the digestion process. In our laboratory, secretin and its receptor have recently been identified in mice hypothalamus where it has long been considered as an important center in the regulation of energy homeostasis. By altering the rate at which nutrients are delivered to compartments of the alimentary canal and being highly expressed in hypothalamic nuclei, secretin could therefore be involved in appetite control. In this study, the functional role of secretin on feeding regulation was investigated. Intracerebroventricular (ICV) administration of secretin (0.15 nmol) into the lateral ventricle was found to suppress food intake in both fasted mice or ad libitum fed wild-type mice but not in fasted mice or ad libitum fed secretin receptor knockout mice (SCTR−/−). In addition, central administration of secretin could induce Fos expression in hypothalamic nuclei, including the paraventricular nucleus (PVN) and the arcuate nucleus (Arc). Consistent with these findings, ICV-secretin could also change expressions of appetite control proteins in various hypothalamic nuclei. It could activate proopiomelanocortin (POMC), reduce agouti-related protein (AgRP) mRNA expression in the Arc, and increase thyrotropin-releasing hormone (TRH) transcripts in the PVN, while these effects of ICV-secretin were not found in SCTR−/−. Altogether, our data suggest that secretin, via its receptor, could inhibit food intake and that this action could be mediated by changing the expressions of POMC and AgRP in the Arc and TRH in the PVN.

PACAP/VIP inhibits osteoblastic differentiation and stimulates the cytokine production of IL-6 through VPAC2 receptor in MC3T3 cells

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Pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the glucagon/vasoactive intestinal peptide (VIP) superfamily, regulates a variety of biological processes such as: neurotropic actions, immune and pituitary function, learning and memory, catecholamine biosynthesis, and regulation of cardiopulmonary function. Both osteoclasts and osteoblasts have been shown to express receptors for PACAP/VIP implicated in their role in bone metabolism. In order o further understand the role of PACAP/VIP family in controlling bone metabolism, we investigated differentiation model of MC3T3-E1 cells, an osteoblastic cell line derived from mouse calvaria. Quantitative RT-PCR analysis demonstrated that MC3T3-E1 cells expressed only VPAC2 receptor, and its expression was up-regulated during osteoblastic differentiation, whereas VPAC1 and PAC1 receptors were not expressed. Consistent with expression of receptor subtype, both PACAP and VIP stimulate cAMP accumulation in a time- and dose-dependent manner with the similar potency in undifferentiated and differentiated cells, while maxadilan, a specific agonist for PAC1-R, did not. Furthermore, down-regulation of VPAC2-R by siRNA completely blocked cAMP response mediated by PACAP and VIP. Importantly, PACAP/VIP as well as forskolin markedly suppressed the induction of alkaline phosphatase mRNA upon differentiation and the pretreatment with 2',5'-dideoxyadenosine, a cAMP inhibitor, restored its inhibitory effect of PACAP. We also found that PACAP and VIP stimulated IL-6 release, a stimulator of bone resorption, and VPAC2-R silencing inhibited IL-6 production. Thus, PACAP/VIP can activate adenylate cyclase response and regulate IL-6 release through VPAC2 receptor with profound functional consequences for the inhibition of osteoblastic differentiation in MC3T3-E1 cells.

PACAP stimulates somatolactin release from cultured goldfish pituitary cells

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Pituitary adenylate cyclase-activating polypeptide (PACAP)-containing neurons directly innervate the adenohypophysis in the goldfish pituitary. In this species, nerve fibers containing PACAP are located in close proximity to somatolactin (SL)-producing cells. However, there is little information available about the effect of PACAP on SL release from pituitary in this species. In order to elucidate this issue, we used the cell immunoblot method. Treatment with PACAP increased the immunoblot area for SL-like immunoreactivity from dispersed pituitary cells. With the use of a pharmacological approach, PACAP-induced SL release was shown to be mediated by PACAP selective receptor (PAC_1R). In addition, the AC/cAMP/PKA- and PLC/inositol 1,4,5-triphosphate/PKC-signaling pathways were shown to be involved in PACAP-induced SL release.
These results suggest that PACAP can potentially function as a hypophysiotropic factor mediating SL release in goldfish pituitary cells.

Paradoxical effects of PACAP and VIP on gastrointestinal physiology

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Previous published studies on pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) effects on gastric acid secretion have shown controversial data in rodents. Our recent working model suggests that PACAP stimulates gastric acid secretion through its actions at PAC1 expressed on the gastric enterochromaffin-like (ECL) cell whereas VIP acting on the VPAC1 receptor expressed on gastric D cells has a net effect of inhibiting gastric acid secretion. To test this hypothesis and to better clarify the role of these neuropeptides in the gastric physiology, a PACAP-specific receptor (PAC1)-deficient and VIP-deficient mouse models were developed and studied. Mice genetically deficient in PAC1 and VIP were generated and genotype analysis performed using specific primers that span the intron–exon junctions. Gastric acid measurements were performed in urethane-anesthetized mice using the pylorus ligation model. Measurements of the basal and stimulated gastric acid secretion and morphological studies on the gastric mucosa were performed in wild-type, PAC1-deficient, and VIP-deficient mice. In addition, using immunohistochemistry, the cellular composition of the gastric corpus was studied. Statistical analysis was performed to determine statistical significance between groups. Compared with the wild-type mice, the PAC1 deficient mice had nearly 3-fold higher basal gastric acid output, increased gastric mucosa thickness and gland height, and proportionally increases in parietal and total cell counts in the gastric mucosa. PAC1-deficient mice also had increased plasma gastrin levels and increased gastrin gene expression in gastric mucosa without cross-responding changes in numbers of ECL cells or

D cells. VIP-deficient mice showed a nearly 2-fold reduction in the basal gastric acid output and decreased gastric mucosa thickness and gland height. The results reported here are the first observations of gastric physiology in both PAC1 and VIP deficient mice. This study indicates that both PACAP and VIP play an important role in the homeostatic mechanisms regulating gastric acid secretion. The paradoxical effect of PACAP and VIP in mice suggests an important role for each peptide to turn on and turn off gastric acid secretion in response to the cephalic phase of gastric acid secretion.

Comparison of intestinal warm ischemic injury on PACAP knock-out and wild-type mice

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is present and plays a central role in the intestinal physiology, mainly in the secretion and motility. The aim of our study was to compare the ischemic injury in PACAP-38 knock-out and wild-type mice following warm mesenteric small bowel ischemia.

Warm ischemia groups were designed with occlusion of superior mesenteric artery in PACAP-38 knock-out (n=20) and wild-type (n=20) mice. Group I: sham-operated, no ischemia; GII: 1 h ischemia; GIII: 3 h ischemia; GIV: 6 h ischemia. Small bowel biopsies were collected after laparotomy (control) and at the end of the ischemia periods. To determine oxidative stress parameters, malondialdehyde, reduced glutathione, and superoxide dismutase were measured. Tissue damage was analyzed by qualitative and quantitative methods on hematoxylin/eosin-stained sections.

In PACAP-38 knock-out animals tissue, MDA increased significantly after 3 and 6 h ischemia (133.97±6,2; 141.86±5,8) compared to sham-operated (100.92±3,6) and compared to wild-type results (112.8±2,1; 118.4± 1.03 umol/g, $p<0.05$). Meanwhile, tissue concentration of GSH and activity of SOD decreased in knock-out mice compared with wild-type in GIII and in GIV (GSH 795.97± 10.4; 665.1±8,8 vs. 893.23±umol/g; SOD 94.4±1.4; 81.2± 3.9 vs. 208.09±3,7 IU/g). Qualitative and quantitative histological results showed destruction of the mucous, submucous and muscular layers, and crypts in knock-out mice compared with wild-type tissues. These processes were time-dependent with the warm ischemia periods.

Our present results propose an important protective effect of endogenous PACAP-38 against intestinal warm ischemia, which provides basis for further investigation to elucidate the mechanism of this protective effect.

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Lipodystrophy and leptin-replacement therapy in Japan

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Lipodystrophies are clinically heterogeneous disorders characterized by a deficiency of adipose tissue. Inherited and acquired lipodystrophies can be generalized or partial. Lipodystrophy is very rare, and no direct estimates are available for the prevalence rate both domestically and internationally. We investigated the number of patients with lipodystrophy in Japan by inquiry survey in members of Japan Endocrine Society and found 31 patients. Of these patients, 21 were generalized, seven were partial, and the rest were unknown. Seipin and AGPAT2 have been identified as causative genes for congenital generalized lipodystrophy (CGL). We examined ten Japanese CGL patients and found eight patients with homozygous mutation of seipin gene. We did not find any AGPAT2 mutations, suggesting that AGPAT2 is a minor causative gene in Japan.

Lack of leptin is implicated in insulin resistance and other metabolic abnormalities in lipodystrophy. We and others demonstrated that the leptin administration or transgenic overexpression of leptin reverses the metabolic abnormalities in lipoatrophic mice. Four-month leptin replacement therapy had been reported to improve glucose and lipid metabolism in lipoatrophic patients in the United States. We also introduced leptin-replacement therapy to ten Japanese patients. The leptin therapy dramatically improved fasting glucose and triglyceride levels within 1 week and reduced insulin resistance. It is important to remember that the efficacy of leptin therapy in patients from Japan, a country in which the prevalence of obesity is relatively low, is excellent. The obese state is thought to be associated with "leptin resistance". Recently, amylin was found to restore leptin responsiveness in obesity. Amylin is a hormone cosecreted with insulin from pancreatic B cells and binds specific receptors in the hindbrain area postrema that activate multiple central nervous system regions to regulate both glucose and energy homeostasis. These results can provide us with novel treatments using leptin for metabolic disorders associated with overweight or obesity.

Secretin-expressing primary sensory neurons in the trigeminal ganglion of rats: in situ hybridization study

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The primary sensory neurons use glutamate as major neurotransmitter. Besides glutamate, several neuropeptides were also demonstrated in these neurons. The neuropeptides modulate the neurotransmission in the primary sensory neurons. In our laboratory, we have demonstrated that secretin is present in primary sensory neurons of several species including human, rat, and cat. In the present experiment utilizing in situ hybridization we have demonstrated for the first time that secretin is not only immunostained but expressed in the primary sensory neurons of the trigeminal ganglion of colchicine-treated male rats. Secretin-expressing cells were observed in all the three subdivisions of the trigeminal ganglion. About 3% of the ganglionic cells express secretin. The secretin synthetizing cells are large and medium sized; however, calcitonin-gene related peptide (CGRP), substance P (SP), and vasoactive intestinal peptide (VIP) immunoreactivities are mainly present in the cells with small size. There number is much higher (CGRP 30%, SP 12%, VIP 10%). These neuropeptides are known to be related to pain sensation; however, secretin is may be involved in proprioception.

Physiology and pathophysiology of prostanoids; novel roles revealed by receptor knockout mouse studies

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Prostanoids including prostaglandin (PG) D2, PGE2, PGF2a, PGI2, and thromboxane (TX) A2 are produced from unsaturated fatty acids such as arachidonic acid by sequential catalysis of cyclooxygenase (COX) and respective synthetases. They are formed in response to various, often noxious, stimuli, released and function in local vicinity of their production. They exert their actions by acting on a family of G-protein-coupled receptors (GPCRs), which include PGD receptor (DP), EP1, EP2, EP3, and EP4 subtypes of PGE receptor, PGF receptor (FP), PGI receptor (IP), and TX receptor (TP). We have generated mice deficient in each of these prostanoid receptors individually, and examined roles each receptor plays in the body under various physiological and pathophysiological conditions. Since prostanoids have been characterized as regulators of smooth muscle contraction and non-steroidal anti-inflammatory drugs (NSAIDs) exert their actions through suppression of prostanoid synthesis by inhibiting COX; prostanoids are generally believed as pro-inflammatory mediators that mediate acute inflammatory responses such as vasodilatation and broncho-spasm as smooth muscle regulators. However, analysis of the receptor–KO mice has revealed that these substances act not only as pro-inflammatory but also anti-inflammatory mediators, and many of these actions are elicited through regulation of gene expression. For example, the PGE2-EP2/EP4 pathway and the PGI2-IP pathway together facilitate elicitation of arthritic response in collagen-induced arthritis, while the PGE2-EP4 pathway exerts anti-inflammatory action in dextran sodium sulfate-induced colitis. In ovalbumin-induced allergic asthma, the PGD2-DP signaling facilitates, and the PGE2-EP3 signaling suppresses allergic reactions. Micro-array analysis has revealed that the prostanoid signaling regulates disease development in these models by modulating expression of disease-associated genes such as chemokines, cytokines, and tissue remodeling factors. Our KO mouse studies have further revealed that prostanoids function not only in processes sensitive to NSAIDs but also in those thus far not known sensitive to NSAIDs. For example, in the brain, while the PGE2-EP3 signaling and the PGE2-EP1 signaling are involved in febrile response and stress-induced ACTH release, two processes sensitive to NSAIDs, the latter signaling unexpectedly regulates also behavioral stress responses. Such an unexpected prostanoid action may be explained by the presence of another prostanoid signaling that opposes that action because we noted various, often-opposing actions of prostanoids in immune response, a process as a whole insensitive to NSAIDs. These results demonstrate that prostanoids collaborate with various bioactive substances including cytokines and regulate many important steps in physiology and pathophysiology. Given the GPCR nature of the prostanoid receptors, the actions we found for prostanoids by our KO mouse studies may be elicited in different contexts by other substances such as VIP/PACAP that also mediate their signals via cognate GPCRs.

The human VPAC1 receptor: importance of N-terminal ectodomain in ligand recognition and discrimination between VIP and an antagonist

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VIP mechanisms of action implicate two subtypes of receptors (VPAC1 and VPAC2) which are members of class B receptors belonging to the super-family of G protein-coupled receptor (GPCR)1. Because the VPAC1 receptor plays regulation of eukaryotic cells and represents an archetype of class B GPCR, we have extensively studied the structure–function relationship of this receptor. Those studies showed the crucial role of the N-terminal ectodomain (N-ted) of VPAC1 in VIP binding. Using different techniques including photoaffinity labeling, NMR, molecular modeling, and molecular dynamic simulation, it has been possible to define how VIP interacts with its receptor 2,3. We have shown that the VIP molecule (sequence 1–28), which is mainly structured in α-helice, tightly binds the N-ted part of the receptor which is itself structured as a «Sushi» domain. Additional data revealed that the PG97-269 analog, a specific antagonist of hVPAC1 receptor, physically interacts also with N-ted but with different region. These differences observed could be related to the presence of N-capping sequence localized in the N-terminal part of all class B receptor ligands4.

In conclusion, these studies define the molecular mechanism involved in ligand recognition of human VPAC1 receptor and should allow the development of new agonist/antagonist molecules.

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Analysis of a putative VPAC2 receptor from sturgeon shed light on molecular and functional evolution of VPAC2R in vertebrates

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Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) are structurally related neuropeptides that exert multiple physiological effects in our body. Their actions are mediated through the activation of three receptors: PAC1R, VPAC1R, and VPAC2R. Despite the importance of these intercellular communicators, there is little information available for PACAP, VIP, and their receptors in extant early vertebrate species. In this study, a full-length VPAC2R cDNA was identified from a sturgeon species, *Acipenser schrenskii*. This cDNA is 1,498 bp in length encoding for a protein with 427 amino acids. Phylogenetic analysis showed that the sturgeon VPAC2R is structurally more similar to tetrapod VPAC2Rs than teleost VPAC2Rs or PHIRs. By real-time PCR, it was found that the sturgeon VPAC2R was widely distributed in various tissues, with the highest expression in gut and with moderate expression in liver and gonad. In functional cAMP assays, the sturgeon VPAC2R could be stimulated by mammalian and fish VIPs but not human and fish PHIs and PACAPs. These data suggested that the ability of VPAC2R to interact with PACAP evolved after the teleost/tetrapod split in the tetrapod lineage. Moreover, the previously isolated fish PHIRs which are structurally related to VPAC2Rs in vertebrates may exist only in teleosts via the teleost-specific 3R genome duplication.

Key pharmacophore elements of the N-terminal domain of PACAP

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Pituitary adenylate cyclase-activating peptide (PACAP) exists in 38 and 27 amino acid forms and participates in the regulation of many physiological processes via the activation of three G protein-coupled receptors (GPCRs) named PAC1, VPAC1, and VPAC2. To better understand the physiological roles of the PAC1 receptor and to validate the therapeutic potential of PACAP as a neuroprotective agent, the development of highly selective PAC1 agonists is a key requirement. The present study describes structure-to-activity relationships (SAR) of the N-terminal domain of PACAP, with an emphasis on the development of selective analogs for PAC1. Hence, using approaches such as Ala-scan, D-scan, *N*-methyl-scan, and single-point mutations, more than 40 analogs of PACAP27 were synthesized. Peptide derivatives were then evaluated for their binding affinity and their ability to induce calcium mobilization using CHO cells stably transfected with human PAC1, VPAC1, or VPAC2 receptors. Our results confirmed the essential role of His^1, Asp^3, and Phe^6 side-chains, as well as their orientation, in the binding affinity and biological activity of PACAP. In fact, their successive substitution with Ala or their D-isomer caused a significant decrease in affinity and activity, compared with the native peptide. Also, we observed that the replacement of Phe^6 with bulky non-natural amino acids modulated the potency and the

binding specificity of PACAP derivatives for PAC1 and VPAC2 receptors, but had no effects on VPAC1 receptor. In addition, the data showed that Gly^4 and Ile^5 are critical for maintaining a specific conformation of the N-terminal domain. Noteworthy, we demonstrated that the orientation of Ser^2 is important for high-affinity receptor interaction and activation. Moreover, some new potent antagonists for PACAP receptors were identified, as well as specific positions that might enhance the specificity for PAC1. These results will facilitate the rational design of PAC1 selective agonists.

Alternative splicing of the pituitary adenylate cyclase-activating polypetide (PACAP) receptor contributes to function of PACAP-27

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It is reported that pituitary adenylate cyclase-activating peptide (PACAP)-38 represented the majority of PACAP with the much smaller amount of PACAP-27 in peripheral tissues. However, in most regions of the central nervous system, PACAP-27 is more abundant, one third or one fourth of PACAP-38, indicating a possible physiological significance of PACAP-27 distinct from PACAP-38.

The actions of PACAP are mediated through the PACAP-specific receptor, PAC1. It has several variants that result mainly from alternative splicing in the mRNA region encoding the first extracellular (EC1) domain and the third intracellular cytoplasmic (IC3) loop. Previously, we have identified four PAC1 isoforms (N/R, N/HOP1, S/R, and S/HOP1) by the combination of each two variant of EC1 domain (the N or S form) and IC3 loop (the R or HOP1 form) in mouse tissues and characterized receptor property of four murine PAC1 isoforms by assessing the binding properties and the activation of two major second messenger pathways (cAMP production and changes in the $[Ca^{2+}]_i$) with PACAP-38, VIP, and maxadilan. In this study, we focused on PACAP-27 involved in the combinatorial effects of EC1 domain and IC3 loop variants of PAC1 by examining the resulting activation of two major second-messenger pathways. As a result, the potencies of PACAP-27 were increased in the isoforms with S form of EC1 domain but it was decreased in the isoform with HOP form of IC3 loop. Meanwhile, the binding affinities of PACAP-27 to the four PAC1 isoforms were similar. Interestingly, in N/R, S/R, and S/HOP1-expressing cells, PACAP-27 exhibited continuous and biphasic augmentation of calcium mobilization to sub-picomolar concentrations.

These findings are not observed with PACAP-38 and indicate the possibility of specific function of PACAP-27 is medicated by different PAC1 isoforms and help understanding pleiotropic effect of PACAP.

Vasoactive intestinal peptide and its role in inflammatory diseases and self/non-self discrimination

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Since the late 1970s, a number of laboratories have studied the role of vasoactive intestinal peptide (VIP) in the immune system. These studies have pointed out the effects of VIP on immune cell activation and function in key aspects of the immune response from innate to acquire mechanisms of cell networking. During the last years, VIP has emerged as an endogenous anti-inflammatory agent that participates in the regulation of the processes that ensure self-tolerance. By superimposed and redundant mechanisms of action, VIP is an endogenous contributor for shaping the environmental conditions towards the generation of tolerogenic dendritic and Treg cells. This will lead to new opportunities for immune intervention to treat autoimmune/inflammatory diseases and to achieve clinical transplantation tolerance. So far, studies using animal models of disease have indicated that VIP has significant therapeutic and prophylactic potential. New and revisited preclinical data open new venues for VIP-based translational research, while clinical trials in inflammatory-mediated diseases will be also discussed.

Cell–gene therapy as an alternative to deliver VIP for treatment of autoimmune diseases

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The vasoactive intestinal peptide (VIP) is a type-II cytokine with demonstrated therapeutic effect in animal

models of several autoimmune diseases, however, VIP is very unstable, and daily injections of high doses of peptide are required to achieve therapeutic effect. We have studied the possibility of using gene- and stem cell-based strategies to improve efficacy of VIP treatment of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), characterized by degeneration of the central nervous system (CNS) as consequence of inflammatory and autoimmune processes. We aim to compare direct injection of lentiviral vector expressing VIP (LentiVIP) with injection of mesenchymal stem cells (MSCs) transduced with the same vector. A single intraperitoneal injection of LentiVIP reduced disease severity of EAE. Interestingly, treatment is more effective when mice are injected at the peak of disease (chronic stage). We observed a reduction of T cells MOG-proliferative response in LentiVIP-treated mice. The therapeutic activity correlates with increase vector copy number per cells in spleen, liver, and lymph nodes. Alternatively, MSCs were chosen as vehicles to deliver VIP specifically to inflamed central nervous system due to its intrinsic immunomodulatory properties and their ability to migrate to inflammatory sites. We have developed several MSCs cell lines expressing VIP by transducing them with high doses of LentiVIP. In vitro, MSCs transduced with LentiVIP conserved theirs immunosuppresory properties, phenotype, and morphology after different passages, and express high copies of VIP mRNA. We are currently studying potential applicability of this gene-modified MSCs on EAE disease progression and potential therapeutic benefits compared with direct VIP peptide or LentiVIP injection.

Vasoactive intestinal peptide generates human anergic T cells with regulatory functions by inducing cell cycle arrest and CTLA4

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Vasoactive intestinal peptide (VIP) is a potent anti-inflammatory neuropeptide that, by inhibiting Th1-driven responses and inducing the emergence of regulatory T cells (T_{reg}), has been proven successful in the induction of tolerance in various experimental models of autoimmune disorders. T_{reg} have emerged as a unique population of suppressor T cells orchestrating peripheral immune tolerance. Numerous studies have demonstrated the successfully therapeutic use of antigen-specific T_{reg} in various experimental models of autoimmune diseases and allogeneic transplantation, providing long-term tolerance by active and specific regulation of self-antigen and alloantigen-specific T cells. Here, we show that VIP is one of the few reported factors with capacity to generate T_{reg}, a function that is exerted at multiple levels. VIP-treatment in the presence of T cell receptor signaling and CD28 costimulation induces energy in human T cells. VIP blocked G1/S transition of the cell cycle by inhibiting the synthesis of cyclins D3 and E and the activation of the cyclin-dependent kinases cdk2 and cdk4. This inhibition is mediated by the maintenance of the threshold levels of cdk inhibitor $p27^{kip1}$ and the impairment in the PI3K-Akt signaling by VIP plays a major role on all these events. Furthermore, VIP-treated T cells show a regulatory phenotype characterized by high expression of CD25, CTLA4, and FoxP3 and potent suppressive activities against effector T cells. VIP seems to directly program the $CD4^+CD25^-$ cells toward this regulatory phenotype independently of the presence of naturally-occurring T_{reg}. CTLA4 appears critically involved in the generation and suppressive activities of the VIP-induced T_{reg}. Finally, VIP-induced cell-cycle arrest and T_{reg} generation were mainly mediated by elevating cAMP and activating protein kinase A. In summary, we are proposing an alternative strategy for developing T_{reg} immunotherapy by generating human T_{reg} ex vivo from conventional $CD4^+CD25^-FoxP3^-$ T cells, a method that would be more convenient than expanding natural T_{reg} and more feasible for generating antigen/organ-specific T_{reg}. Understanding precisely how VIP controls immunoregulatory mechanisms will help to further its use in the clinic.

Renoprotection with PACAP in cisplatin-induced acute kidney injury

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Acute kidney injury (AKI) remains a significant cause of prolonged hospitalization and increased morbidity and mortality among patients. Virtually no progress has been made toward the development of an effective therapy for AKI. Ischemic and toxic insults to the kidney cause AKI, which is most often manifested as DNA damage, innate immune responses, and apoptosis/necrosis in renal proximal tubule cells. The 38-amino-acid form of pituitary adenylate cyclase-activating polypeptide (PACAP38) has been shown to protect the kidney from ischemic injury and myeloma light chain-induced damage through its well-known modulatory effects on inflammatory responses. In our recent studies, we have demonstrated that PACAP38 can prevent renal tubular injury and protect against kidney dysfunction and histological damage in models of cisplatin-induced AKI. PACAP38 significantly reduced renal tubular injury, inhibited the apoptotic cascade, increased the expression of collagen IV, and decreased the expression of fibronectin to maintain the structural integrity of the tubular basement membrane and prevented tubulointerstitial fibrosis in

vivo and in vitro. The treatment with PACAP38 was more effective than p53 silencing in blocking activation of caspases and DNA single-strand break-activated poly (ADP-ribose) polymerase-1, with the decline in renal tumor necrosis factor-α (TNF-α) preceding the restoration of integrin-mediated cell–extracellular matrix interactions for repairing tubular cell dysfunction. The renoprotection with PACAP38 in cisplatin nephrotoxicity may be associated with the increased expression of the PAC_1 receptor in renal tubule cells and the suppression of p53-mediated up-regulation of apurinic/apyrimidinic endonuclease-1, which decrease apoptosis and TNF-α production after exposure to cisplatin, possibly by both p53-dependent and p53-independent pathways. The administration of PACAP38 before and/or after exposure to cisplatin was renoprotective in vivo and prevented the rise in blood urea nitrogen, serum creatinine, and TNF-α production in mice treated with cisplatin. PACAP38 ameliorated AKI even when given 24 h after the onset of injury and increased tubular regeneration, which contributes to tubular restoration in addition to the anti-apoptotic effects.

VIP suppresses the inflammatory response to monocrotaline in rat lungs

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Background:

Monocrotaline (MCT) induces pulmonary arterial hypertension (PAH), with lung inflammation in rats. The immune cell response in this model has not been fully characterized. We have reported the presence of perivascular and peribronchiolar lymphocytic infiltration in lungs of mice lacking the gene for VIP. Those mice spontaneously develop PAH and airway hyperresponsiveness, both of which are attenuated by VIP replacement therapy. In this current study, we also sought to determine if VIP could suppress inflammatory cell recruitment in MCT-treated rats.

Methods:

We studied three groups of male Sprague–Dawley rats, aged 4–6 weeks. The first group (n=4) received MCT (60 mg/kg, SC). The second group (n=4) also received VIP (150 mg/0.5 ml IP qod for 20 days), beginning simultaneously with MCT injection. A third group of three rats did not receive either drug and served as controls. Three weeks later, under anesthesia with ketamine/xylazine, an endotracheal tube was inserted, bronchoalveolar lavage (BAL) was performed with 3 ml of PBS containing anti-protease. The return-BAL fluid was centrifuged, and the sediment was washed in phosphate-buffered azide and labeled with antibodies to mouse anti-rat TCRa-beta (T cell marker) conjugated to RPE, mouse anti-rat kappa (B cell marker) conjugated to FITC, and mouse anti-rat CD161 (NK cell marker) conjugated to Alexa. Flow cytometric analysis was then conducted on BAL fluid. Data were analyzed by ANOVA and post hoc Tukey's t test.

Results:

MCT-treated rats showed elevated T cell percentages in BAL fluid compared with controls (28.7%±6.4 vs. 8.7%±1.4, P<0.05). Co-treatment with VIP significantly reduced the percentage of T cells in BAL fluid (2.4%±1.9, P<0.001).

MCT treatment significantly increased NK cell percentages (9.3%±3.4 vs. 1.8%±0.8, in control rats P<0.05). Co-treatment with VIP significantly reduced NK percentages in BAL fluid (0.32%±0.14 vs. 9.3%±3.4, P<0.0007) to normal levels.

There were no differences in B cell percentages among all groups.

Conclusions:

These preliminary data show that: (1) MCT induced an inflammatory cell profile in BAL fluid with T and NK cells; (2) VIP treatment suppressed T and NK cell increases down to control levels; (3) B cell numbers were unaffected by MCT or VIP treatment.

Davunetide (NAP intranasal formulation AL-108) provides cognitive protection in a model of microtubules dysfunction exhibiting schizophrenia-like symptoms

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Davunetide (NAPVSIPQ=NAP) is a drug candidate in clinical trials (www.allontherapeutics.com). NAP is a potent neuroprotective peptide derived from activity-dependent neuroprotective protein (ADNP), a protein regulated by the neuropropeptides vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP). In animal models of Alzheimer's disease and frontotemporal dementia, NAP enhanced learning and memory and inhibited tau pathology, suggesting an interaction with the microtubule system. In a proof of concept human clinical trial, the intranasal formulation of NAP, davunetide intranasal (AL-108) improved memory function in patients with amnesic mild cognitive impairment (www.allontherapeutics.com). These studies were recently supported by top-line data

showing positive effects on functional capacity in schizophrenic patients (TURNS/NIMH study in collaboration with Allon Therapeutics Inc.). Here, in a cellular model, NAP significantly affected the tubulin tyrosination cycle (associated with microtubule stability), significantly increasing tyrosinated and detyrosinated alpha tubulin and microtubule dynamics. NAP effects on microtubule dynamics in cells may implicate neuronal plasticity, learning, memory, and neuroprotection. To evaluate the biological background for NAP activity in schizophrenia, we set out to investigate whether a mouse model of schizophrenia that is associated with cytoskeletal deficits exhibited cognitive deficits and whether chronic intranasal NAP treatment was effective in cognitive enhancement in this model. The stable tubule-only polypeptide (STOP) knockout mice have been shown before to provide a reliable model for schizophrenia. Here, heterozygous STOP mice (STOP+/−) showed schizophrenia-like symptoms (hyperactivity in open field and cognitive dysfunction, in a test of object recognition/discrimination) that were ameliorated by chronic treatment with clozapine (a clinically used anti-psychotic drug). Like clozapine, NAP treatment significantly decreased open-field locomotor activity. Furthermore, NAP-treated STOP+/− mice showed significantly improved performance in the object recognition test. Finally, spatial memory was also impaired in the STOP+/− mice and was ameliorated by NAP treatment. These studies provide a mechanistic link between microtubule dysfunction and positive effects on cognitive impairment associated with schizophrenia and support further clinical testing of NAP (davunetide intranasal; AL-108) in this indication.

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Disclosure: Professor Gozes serves as the Chief Scientific Officer of Allon.

Effect of PACAP on tear secretion in mouse

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PACAP null mouse has shown many phenotypes, early postnatal death, reduction of female fertility, and deterioration of ischemic insult. Recently, we fortuitously found a new phenotype on PACAP null mouse that the mice develop dry eye-like symptoms such as keratinization of cornea and reduction of tear volume. To reveal the relations between PACAP and tear secretion, PACAP and PAC1R localization in lacrimal gland were observed by immunohistochemistry. PACAP immunoreactivity was co-localized with NeuN (neuronal marker), and PAC1R immunoreactivity was observed in acinar and duct cells. Physiological function of PACAP on tear secretion was examined by administration of PACAP38 into the vein or on the eye. The eye drops stimulated tear production 15 to 45 min after application, while tear production was also noted when the eyes were checked 4 days after the systemic application of PACAP. This effect was inhibited by pre-treatment of animals with PAC1R antagonist, PACAP6-38, or the adenylate cyclase (AC) inhibitor, SQ22536. Following the application of PACAP eye drops, levels of cAMP and phosphorylated(p)-protein kinase A increased in lacrimal gland. Immunoreactivity to aquaporin 5 (AQP5), a water transporter, in acinar cells was down-regulated in PACAP-null mouse. Eye drops of PACAP increased AQP level in membrane fraction and p-AQP5 level in lacrimal gland via PAC1R/AC pathway. These results suggest that PACAP is a key factor for tear secretion and could be a candidate for the treatment of dry eye disease.

PACAP-38 in human plasma and milk under physiological and pathological conditions: introductory measurements for possible future clinical diagnostic application

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Pituitary adenylate cyclase-activating polypeptide (PACAP) shows widespread occurrence in human tissues. The relationship between PACAP and several pathological conditions have been suggested lately. Although the half-life of the peptide is very short, our aim was to determine the concentration of PACAP38 in human plasma under normal and pathological conditions and also in breastmilk. Considering the important role of PACAP during development and in reproduction, our first set of measurements were conducted in obstetrical, gynecological, and neonatal clinical patients. Here, we describe that PACAP-like immunoreactivity can be reliably measured in human plasma, with concentrations relatively stable (300–320 fmol/ml) in healthy

volunteers and not infuenced by gender, age, and the female hormonal cycle. In gynecological tumor patients, no alteration was found. During pregnancy, a slight elevation was observed, and levels were extremely high in some pathological pregnancy cases, like preecclampsia. PACAP38 concentration in the neonatal peripheral blood was similar to maternal plasma levels at terminus, but about 40–60% lower in the blood obtained from the umbilical artery and vein, respectively. Moreover, 5–15 times higher concentrations of PACAP38 could be measured in the human milk than in the plasma of the same women. Elucidating the functional significance of these results and finding possible correlations between perinatal injuries and maternal PACAP levels would be of great clinical importance. Plasma PACAP38 concentration might be used as a predictive factor influencing possible therapeutic interventions.

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Clinical development of nasal GLP-1 compound with a new injector for the treatment of type 2 diabetes

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Aim: Incretin therapies have been attracting increasing attention as new options treating worldwide expanding type 2 diabetes. We developed nasal GLP-1 compound and a newly designed injector for peptide drug in conjunction with a local company and attempted to clarify the efficacy and safety of the application in the clinical trial.

Methods: The protocol of a double-blind placebo-controlled study was approved by IRB in the hospital. Twenty-six type 2 diabetes patients were enrolled (age 60.5± 1.2 years, BMI 26.36±0.91, HbA1c 7.20±0.12%). Patients using insulin were excluded. Nasal compound containing 1.2 mg of GLP-1 was applied just before every meal using a newly developed injector for 2 weeks. Pharmacokinetics and dynamics were evaluated on the first day of the treatment. Glycoalbumin and 1,5-AG were evaluated. Weight change, amount of food consumption, and adverse events were also evaluated.

Results: A percentage of 99.5% of the compound in the capsule was applied by the injector. Cmax was 82.7 pg/ml, and the half-life was 9.8 min. Early phase of insulin secretion (~15 min) was recovered in GLP-1 group but not in placebo group. Glucagon secretion was suppressed significantly in GLP-1 group during first 30 min. Plasma glucose level was lower in each time point in GLP-1 group but not significantly. Glycoalbumin levels decreased significantly in GLP-1 group after 2-week treatment but not in placebo group (Δglycoalbumin GLP-1 −0.65±0.16; placebo +0.31±0.29%, $P<0.05$). Serum 1,5-AG levels significantly increased after 2-week treatment in GLP-1 group and demonstrated significant difference between two groups. No difference was observed in weight change and amount of food consumption. Three patients out of 18 in GLP-1 group reveled complaints of mild nausea.

Conclusion: Newly developed nasal GLP-1 compound in combination with the injector could be a choice for the treatment of type 2 diabetes. Long-term application of the drug should be evaluated in next trial.

An update on VIP in health and disease

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Almost 40 years after its discovery and isolation, vasoactive intestinal peptide (VIP) is now recognized as a neuropeptide that is widely distributed, has a variety of actions on multiple systems, plays several major physiological roles, and has a promising potential as a therapeutic agent.

VIP exerts major actions on: the cardiovascular, respiratory, endocrine/neuroendocrine, gastrointestinal, nervous, and immune systems, as well as on inflammation and cell survival and proliferation. These actions are mediated via receptors shared with PACAP: VPAC1, VPAC2, and PAC1 and principally the cyclic AMP-PKA pathway.

Functionally, VIP plays a dominant relaxant and anti-proliferative influence on vascular (systemic and pulmonary) and non-vascular smooth muscle, and has important anti-inflammatory and pro-survival effects.

Based on studies in animal models and early clinical trials, VIP appears to have promise as a therapeutic agent in several diseases, including pulmonary arterial hypertension, acute lung injury/acute respiratory distress syndrome, sarcoidosis, auto-immune disorders, and certain malignancies.

VIP greatly attenuates monocrotaline-induced pulmonary vasculopathy in rats

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INTRODUCTION
Targeted deletion of the VIP gene in mice leads to spontaneous expression of pulmonary arterial hypertension (PAH), which is correctable by VIP treatment. We have now investigated the ability of VIP to reverse the pulmonary vascular pathology in monocrotaline (MCT)-induced PAH, a model that is not directly attributable to lack of the VIP gene.
METHODS
PAH was induced in Sprague–Dawley rats, 230–250 g, with a single IP injection of MCT (60 mg/kg). Group1 (n=10) received no additional treatment. Group 2 (n=6) received VIP at 500 mg/kg IP, every other day for 3 weeks, beginning together with MCT. A group of ten rats (controls) received no drugs. All rats were euthanized 3 weeks after MCT for evaluation of: pulmonary vascular thickening by morphometry; RV hypertrophy by RV/(LV+septum) ratio; and intensity and extent of inflammatory infiltrates in lung sections (graded 0–4+).
RESULTS

1. Compared with controls, MCT-treated rats showed pulmonary arterial thickening (medial area/luminal area 3.9±0.51 vs. 0.8±0.1, P<0.05), perivascular inflammation (3.6±0.41 vs. 0.2±0.2., P<0.001), and RV hypertrophy (0.56±0.03 vs. 0.27±0.01, P<0.0001).
2. Co-treatment with VIP significantly reduced vascular remodeling: lower medial area/luminal area (1.5±0.2 vs. 3.9±0.51, P<0.05), minimal inflammatory infiltrates (1.4±0.5 vs. 3.6±0.4, P=0.024), and little or no RV hypertrophy (0.35±0.01 vs. 0.56±0.03, P<0.0001). Values in VIP+MCT rats were closely similar to normal values.

CONCLUSIONS
1. VIP almost totally prevents MCT-induced pulmonary vascular lesions.
2. The results confirm its anti-proliferative and anti-inflammatory efficacy in a PAH model not resulting from its gene deletion.

Endogenous PACAP attenuated doxorubicin-induced myocardial damage

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is known as a neuroprotective polypeptide. PACAP and its receptors are expressed in mouse heart, but it is unclear whether PACAP exerts its protective effect on the cardiac tissue in vivo. The aim of the present study was to investigate whether endogenous PACAP has a cardioprotective effect on doxorubicin-induced cardiomyopathy.
Wild-type (WT) and PACAP heterozygous knockout (PACAP+/−) mice were studied at 10 to 12 weeks of age. A single intraperitoneal injection of doxorubicin hydrochloride at a dose of 20 mg/kg was performed to induce cardiomyopathy. Survival rate for 15 days was evaluated. Blood samples were collected at 5 days after the injection. Echocardiography and histological examination were performed at day 10.
PACAP+/− mice died from day 6, and the survival rate of PACAP+/− mice (51.7%) was significantly less than that of WT mice (81.1%) at day 15. Cardiac function measured by echocardiography was significantly lower in PACAP+/− mice than in WT mice. Morphological examination of cardiac tissue stained with hematoxylin–eosin and sirius red showed degenerative change and relatively increased fibrosis in PACAP+/− mice. Electron micrographs of cardiomyocytes showed mitochondrial degeneration and swelling and focal myofilament disarray in PACAP+/− mice compared with WT mice. Reactive oxygen metabolites (oxidative stress marker) in serum were significantly higher and 8-hydroxy-deoxyguanosine-positive nuclei identified by immunostaining significantly increased in PACAP+/− mice. Besides, TUNEL-positive nuclei in cardiac tissue increased in PACAP+/− mice.
These results suggest that endogenous PACAP attenuated doxorubicin-induced myocardial damage, and its mechanism could associate with the reduction of oxidative stress.

Vasoactive intestinal peptide and circadian regulation of the cardiovascular system

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A variety of recent evidence indicates that the neuropeptide vasoactive intestinal peptide (VIP) is critical for normal functioning of the circadian system. SCN neurons containing VIP are part of the circuit through which the circadian system regulates autonomic function. Therefore, we sought to examine the impact of the loss of VIP on circadian rhythms focusing on physiological parameters highly dependent upon autonomic regulation. First, we characterized the circadian rhythms of heart rate (HR) and body temperature (T_b) in freely behaving mice using telemetry measurements. As expected, wild-type (WT) mice dis-

played diurnal changes with peaks in T_b and HR during the night. These rhythms continued when the WT mice were held in constant conditions with a period of approximately 23.6 h. In contrast, the VIP-deficient mice did not exhibit significant day/night variation in T_b or HR. In light dark (LD) conditions, the physiological outputs of the mutant mice were either arrhythmic or expressed low amplitude rhythms, in which the onset began during the first half of the day rather than in anticipation of the night. In dark dark (DD) conditions, some of the VIP-deficient mice exhibited low amplitude rhythms in T_b with a shortened free-running period of approximately 22.5 h. None of the VIP-deficient mice exhibited rhythms in HR under these same conditions. In contrast, the loss of VIP did not appear to influence the acute autonomic regulation of HR. Rhythms of clock genes (*per1*, *per2*, *and bmal1)* are disrupted in VIP-deficient mice in LD and constant conditions. In an attempt to rescue circadian rhythms of HR in VIP-deficient mice, we find that the addition of a running wheel strengthened HR, and T_b rhythms. Overall, our data demonstrate that VIP is essential for the circadian regulation of cardiovascular function and body temperature.

PACAP protects renal cells against in vitro ischemia and oxidative stress in primary kidney cultures

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Neuroprotective effects of pituitary adenylate cyclase-activating polypeptide (PACAP) have been described in different models of neuronal damage. PACAP has also been shown to have cytoprotective effect in non-neuronal cells such as endothelial cells, cardiomyocytes, lymphocytes, prostate cells, and ovarian follicular cells. PACAP has protective effects against myeloma kidney injury and renal ischemia. The aim of our study was to investigate the protective effect of PACAP under in vitro circumstances using primary rat kidney cell cultures, by which methods further insight regarding the nephroprotective effects of PACAP could be gained in the future. The effects of PACAP against oxidative stress and in vitro hypoxia were investigated using renal cells treated with 1, 1.5, 3, or 6 mM H_2O_2 for 2 and 4 h or with 100 and 300 uM $CoCl_2$ for 48 h, respectively. Cell viability of rat renal cells exposed to H_2O_2 and $CoCl_2$ was examined by colorimetric MTT assay. PACAP treatment alone did not influence the survival rate. The applied $CoCl_2$ and H_2O_2 treatment significantly decreased renal cell viability. Co-incubation with PACAP significantly increased the survival of cells exposed to 300 uM $CoCl_2$ PACAP treatment of each cell group exposed to different concentrations of H_2O_2 led to a significant increase in cell viability compared with cells treated with the different concentrations of H_2O_2 alone. In summary, PACAP protected rat renal cells against H_2O_2-induced oxidative stress and $CoCl_2$-evoked in vitro hypoxia. (Support: OTKA F67830, K72592, CNK78480, ETT, PTE AOK Research Grant 2009, Bolyai Scholarship, Richter Foundation)

The search for novel peptides and for their functions

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Orphan G protein-coupled receptors (GPCRs) are receptors lacking endogenous ligands. Found by molecular biological analyses, they became the roots of reverse pharmacology, in which receptors are attempted to be matched to potential transmitters. The deorphanization of GPCRs has been an ongoing effort since the first GPCRs were cloned by homology approaches. More recently, orphan GPCRs have been used as targets to identify novel neuropeptides. In this presentation, we will discuss one such "deorphanized" system, the melanin-concentrating hormone (MCH) system and discuss the search for its functions. We will show that while originally found to regulate skin pigmentation in fish, the MCH system carries different functions in mammals, some of which are unexpected. In particular, we will focus on the role of the MCH system in modulating dopamine-related responses and show that the MCH receptor is coexpressed with the dopamine receptors in the neurons known to modulate reward that MCH can potentiate dopamine-induced responses and that blockade of the MCH system decreases cocaine-motivated behaviors. Together, our data point at the MCH system as a potential target for therapeutic interventions aimed at modulating the dopamine tone.

PACAP signaling to target genes through cyclic AMP and calcium during the stress response

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Pituitary adenylate cyclase-activating polypeptide (PACAP) appears to be a general stress-transducing transmitter (emergency response peptide) involved in both detection of and allostatic responses to metabolic and psychogenic stressors. It is unclear whether mimicry, to enhance the allostatic response, or antagonism, to mitigate allostatic

overload, is the most appropriate pharmacological model for PACAP-based approaches to treating stress-associated disease. PAC1 receptor activation controls the transcription of multiple genes through calcium mobilization and influx, cAMP elevation, and combinatorial calcium and cAMP signaling. Using microarray, PACAP-responsive gene regulation in cultured cells and the signaling pathways required for gene expression can be correlated with PACAP-dependent stress responses in vivo. For example, PACAP sustains adrenomedullary catecholamine biosynthesis during metabolic stress in part through cAMP/PKA-dependent regulation of tyrosine hydroxylase enzymatic activity, while cellular plasticity changes at the transcriptome level appear to require a PKA-independent signaling pathway initiated by PACAP that involves both elevation of cAMP and activation of ERK. Maximal induction of neuroprotective proteins such as stanniocalcin (Stc1) involves both cAMP elevation and calcium influx in combination. PACAP-dependent control of expression of Ier-3/Iex-1, which promotes cell death or survival in a cell-type-dependent fashion, requires cAMP elevation and activation of calcineurin via calcium mobilization, but not the calcium influx needed for PACAP-dependent stimulation of exocytosis from secretory cells. Remarkably, PACAP is also involved in neuronal activation in the central nervous system after both systemic and psychogenic stress. Novel PACAP-responsive genes activated following PACAP-dependent immediate-early gene induction are now being identified using microarray hybridization: these may represent potential drug targets in the context of neuroresilience upon activation of CNS stress circuits. As PACAP-dependent transcripts are identified in pathophysiological paradigms in vivo, and their signaling pathways elucidated in cell culture models, it will be important to identify a pharmacology specific for PACAP mediation of primary organismal stress responses versus PACAP signaling that may be neuroprotective at the cellular level, during either systemic or psychogenic stress.

LIF-mediated maintenance of PACAP-induced neurite outgrowths in human SH-SY5Y neuroblastoma cells require PI3K but not STAT or ERK signaling pathways

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Previously, we have shown that pituitary adenylate cyclase-activating polypeptide (PACAP) induces neuritogenesis in human SH-SY5Y cells through a cAMP-dependent but protein kinase A-independent mechanism involving activation of the extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK) signaling pathways. However, the morphological changes induced by PACAP are transient, with the increase in number of neurite-bearing cells peaking at day 4 before returning to control cell levels over an 8-day time course. Control cells maintain an undifferentiated neuroblast morphology during the 8-day experiment. On the other hand, neurite extensions are maintained by cells co-treated with PACAP and the neuropoietic cytokine leukemia inhibitory factor (LIF), with similar levels in the number of neurite-bearing cells observed at day 8 to that at day 4. Interestingly, the induction of neurite outgrowths in SH-SY5Y cells requires a minimal 2-h treatment with PACAP, with 4–6 h of treatment inducing similar peak levels of neurite-bearing cells observed at day 4 to that of continual PACAP treatment. LIF is not required until day 4 after which continual treatment with LIF is necessary for neurite maintenance in PACAP-treated cells. The signaling pathways activated by LIF were investigated also. LIF elicits signal transducer and activator of transcription 3 (STAT3) but not STAT1 tyrosine phosphorylation along with a transient activation of the ERK and prolonged activation of phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB/Akt) in PACAP-treated and control SH-SY5Y cells. Phosphorylation of serine 727 on STAT3 was detected in PACAP-treated but not control cells, and this was prevented by the MEK-1 inhibitor PD98059. Activation of p38 MAPK or c-jun N-terminal kinase (JNK) were not detected, however, an elevated basal level of phosphorylated p38 MAP kinase was observed in PACAP-treated cells. Neither PD98059 nor siRNA knockdown of STAT3 prevented LIF-mediated maintenance of PACAP-induced neurite extensions. However, the PI3K inhibitor wortmannin was found to effectively block LIF maintenance of neurite outgrowths in PACAP-treated cells. Thus, the ability of LIF to stabilize and maintain PACAP-induced neurite outgrowths requires PI3K but not the STAT3 or ERK signaling pathways in SH-SY5Y cells.

Identification of a novel signaling cascade specifically involved in the light-induced phase advance of circadian rhythm by using PACAP knockout mice

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is one of the neurotransmitters that transfer light signals from the retina to the suprachiasmatic nucleus (SCN), where the master clock of circadian rhythm locates. We previously reported that the phase advance of the circadian rhythm, but not the phase delay, induced by light exposure (20 l×, 30 min) was attenuated in PACAP knockout (KO) mice. To reveal the responsible molecular mechanism underlying the asymmetrical phenotype in photoentrainment, here, we performed the SCN-specific gene chip analysis and the following functional analyses on the candidate molecules. Out of about 22,000 genes, 53 genes including lipocalin-type prostaglandin D_2 synthase (L-PGDS) were induced by phase advance-inductive light in wild-type mice, but not in PACAP-KO mice. L-PGDS mRNA was induced by phase advance-inductive light, but not by phase delay-inductive light in the SCN of wild-types. Since L-PGDS and PGD2 receptor (DP1 and CRTH2) mRNAs are expressed in the SCN; we next examined which PGD2 receptors being concerned in light-induced phase advance. The light-induced phase advance was significantly attenuated in CRTH2-KO mice, but not DP1-KO. In addition, the light-induced phase advance in wild-type mice was significantly attenuated by intracerebral injection of CRTH2 antagonist (CAY10471, 1.5 mg/kg). These results suggest for the first time that *L*-PGDS-PGD_2-CRTH2 signaling is specifically involved in the light-induced phase advance of circadian rhythm.

Involvement of stathmin 1 in the neurotrophic effects of PACAP in PC12 cells

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The rat pheochromocytoma PC12 cell line is a well-established model to investigate the signaling mechanisms involved in the neurotrophic activities of PACAP. In particular, PACAP has been shown, to promote differentiation and to inhibit apoptosis of PC12 cells. In order to investigate the mechanisms involved in these effects, we have been looking for proteins that get phosphorylated after PACAP treatment. Using high-performance liquid chromatography (HPLC) and 2D gel electrophoresis analysis, coupled with mass spectrometry (MS), we found stathmin 1 to be strongly phosphorylated within only 5 min of exposure to PACAP. PACAP-induced phosphorylation was observed on two sites of this protein, i.e., Ser^{25} and Ser^{38}. Western blot experiments confirmed the remarkable phosphorylation of stathmin 1 by PACAP in a transient manner with a maximum of induction observed after 1 h of treatment (15-fold higher than control cells). However, neither stathmin 1 gene expression nor total amount of the protein were affected by PACAP. Investigation of the signaling pathways known to be activated by PACAP in PC12 cells showed a reduction of PACAP-induced stathmin 1 phosphorylation in cells preincubated with the selective protein kinase A (PKA) inhibitor, H89; the protein kinase C (PKC) inhibitor, Gö6983; the PKA and PKC inhibitor, H7; the mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor, U0126 and the p38 MAP kinase inhibitor, SB203580. Total blockage of stathmin 1 phosphorylation was observed in cells co-treated with H89 and U0126, which suggests that phosphorylation of stathmin 1 involves PKA and mitogen-activated protein kinase (MAPK) signaling pathways. Blocking stathmin 1 expression with small interfering RNA (siRNA), did not affect growth arrest, cell size increase, or neurite outgrowth induced by PACAP. However, inhibition of stathmin 1 expression suppressed the ability of PACAP to block caspase-3 activity and significantly decreased its neuroprotective action.

Pituitary adenylate cyclase-activating polypeptide (PACAP) induces activity-dependent gene expression in neurons

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Pituitary adenylate cyclase-activating polypeptide (PACAP) and its specific receptor PAC1 receptor (PAC1-R) are widely distributed in the brain and implicated in versatile neurological functions including synaptic plasticity. Recently, it is well accepted that the gene expression is required for the long-term synaptic plasticity like memory consolidation. Therefore, it is important to understand the molecular mechanisms controlling gene expression of not only the PACAP gene itself but also the genes regulated by PACAP. In our previous study, we demonstrated that the PACAP gene transcription is activated by the calcium (Ca2+) signals evoked via membrane depolarization, in which the CRE located about –200 bp upstream of transcription initiation site is involved. The same Ca2+ signals

prolonged the half-life of PACAP mRNA. On the other hand, several groups recently demonstrated that PACAP potentiates *N*-methyl-D-aspartate receptor (NMDA-R) through activation of PAC1-R. Therefore, we next analyzed the gene expression induced by PACAP, particularly in terms of neuronal activity-dependency. Using a microarray analysis, we found that potentiation of NMDA-R mediated by stimulation of PAC1-R with PACAP-38 immediately induced a limited number of genes encoding factors involved in neuronal functions, such as brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated protein (Arc), the Ca2+ signal dependency of which varied. After Ca2+ entry, the BDNF-promoter IV that promotes the BDNF exon IV–IX mRNA expression was activated by the Ca2+ signaling pathway including CREB. PACAP also increased the Arc mRNA expression activity-dependently but much less than BDNF did because of different mechanisms for promoter activation. In contrast, PACAP mRNA expression was not increased, due to its mRNA instability accelerated by PKA but attenuated by Ca2+ signals. Thus, a cascade of gene expression is induced by not only the PKA and PKC pathways but also the Ca2+ signaling ones, which should strengthen the structure and function of synapses through the expression of BDNF and Arc, possibly resulting in a development of PACAP-ergic system.

Tissue-type plasminogen activator (tPA) as a PACAP-regulated gene in neuronal cells

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On PC12 cells and immature cerebellar granule neurons, pituitary adenylate cyclase-activating polypeptide (PACAP) induces neuritogenesis, promotes cell survival, and inhibits motility through activation of a number of target genes. In the present study, we have shown that PACAP (10^{-7} M) strongly stimulates in PC12 cells the expression of tissue-type plasminogen activator (tPA), a serine protease which can affect cell survival and/or migration, through activation of the PKA-dependent signaling pathway. Similarly, exposure of rat granule cells to PACAP (10^{-7} M) promotes the synthesis of tPA by 2.4-fold after 3 h of treatment, and this effect was blocked with PACAP6-38. In contrast, PACAP had no effect on the expression of any component of the proteolytic cascade linked to activation of tPA including plasminogen (a substrate of tPA) and endogenous tPA inhibitors named plasminogen activator inhibitor 1 (PAI-1), neuroserpine or protease nexin-1. Immunocytochemical labeling showed the presence of tPA in the cytoplasm and processes of cultured granule neurons. Long-term treatment with PACAP (10 h) also induced a 3-fold increase of tPA release in the culture medium as revealed by zymography experiments. On cerebellar tissue microexplants, application of PACAP (10^{-7} M) reduced the speed of granule cell motility by 40%. In contrast, application of tPA, plasminogen or PAI-1 (10^{-7} M) did not modify granule cell motility. Studies will now be conducted to determine the possible implication of tPA in the neuroprotective effect of PACAP. Altogether, these data indicate that tPA is a target gene regulated by PACAP in PC12 and cerebellar granule cells but the functional relevance of this effect still remains to be identified.

Temporal dynamics of gene expression during PACAP-induced PC12 cell differentiation

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a 38-amino-acid neuropeptide. Addition of PACAP into the cultured PC12 cells promoted neurite outgrowth of the cells, indicating cell differentiation. A complete description of the cellular pathway by PACAP to promote neuronal differentiation is lacking. In this study, we first examined the kinetics of levels of gene expression which were altered by PACAP during PACAP-promoted neurite outgrowth of PC12 cells. RNA samples were harvested before and at a time of 6 h treatment with 1 nM PACAP, when neurite outgrowth was remarkably observed under the condition used. The level of gene expression of synaptotagmin IV and tissue-type plasminogen activator was time-dependently increased by PACAP about 7- and 5-fold for 6-h treatment, both of which might be involved in vehicle trafficking and neuritogenesis. Messages of ornithine decarboxylase, and nuclear tyrosine phosphatase were steady expressed 2.5~4-fold through neurite outgrowth. It was noteworthy that PACAP increased gene expression of STAT3, at least up to 24 h, being maximum 9.5-fold with 3-h treatment of the neuropeptide. Furthermore, NGF-responsible genes such as NGF-induced protein A and VGF8a were also increased by PACAP, suggesting the cross regulation between these two signal pathways. Secretogranin, which has sequence similarities to VGF8a, was increased less than the ancient.

Among these, we further investigated the kinetics of STAT3 molecule. PACAP increased the amount of STAT3 proteins and phospho-STAT3 Tyr 705 in the nuclei; the protein levels were steadily lasting at least up to 24 h.
Thus, our data indicate that PACAP altered the expression of various genes and possibly their consequent proteins with different dynamics to achieve the molecular networks necessary for cell differentiation.

Increased stathmin1 expression in the dentate gyrus causes abnormal axonal arborizations potential relevance to schizophrenia

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is involved in multiple brain functions. To clarify the cause of schizophrenic abnormal behavior in PACAP-deficient mice, we tried the identification of the gene altered by PACAP deficiency in the dentate gyrus of the mice using the differential display method. Expression of stathmin1 was up-regulated in the dentate gyrus at both the mRNA and protein level. PACAP stimulation inhibited stathmin1 expression in PC12 cells, while increased stathmin1expression in neurons of the subgranular zone and primary cultured hippocampal neurons induced abnormal arborization of axons. Next, we investigated the pathways involved. Ascl1 binds to the E10 box of stathmin1 and increases its expression. Inhibitory bHLH proteins (Hes1 and Id3) were up-regulated rapidly by PACAP stimulation, and Hes1 could suppress Ascl1 expression, and Id3 could inhibit Ascl1 signaling. We also detected an increase of stathmin1 expression in the brains of schizophrenic patients. These results suggest that up-regulation of stathmin1 in the dentate gyrus secondary to PACAP deficiency may create abnormal neuronal circuits that cause schizophrenic abnormal behavior.

Poster Session

The activation of PACAP receptor (PAC1 receptor) differentially targets NR2A containing NMDA receptors and favors LTP induction

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NMDAR activity at CA3-CA1 hippocampal synapses is regulated by cell signaling activated by various GPCRs. We have shown that a variety of GPCRs including mGluR5 and LPA receptors enhance NMDAR-mediated currents via PKC/Pyk2/Src signaling pathway. Recently, we found that PACAP acts via the PAC-1 receptor to enhance NMDA-evoked currents in CA1 hippocampal neurons, and it does so by stimulating a sequential Gαq/PKC/Pyk2/Src signal transduction cascade. In the present study, we showed that the activation of PACAP receptor (PAC1 receptor) differentially targets NR2A containing NMDARs and favors LTP induction.
The application of the NR2B antagonist R0-25-6981 at a concentration that entirely blocks the NR2B failed to prevent the potentiation of peak NMDAR currents by stimulation of the PAC1R. In contrast, a concentration of NVP that depressed NR2A currents by about 80% eliminated the potentiation. It suggested that the activation of PAC1R enhances NR2A and not NR2B receptors on CA1 pyramidal neurons. We further confirmed this result in two different ways. Zinc shows a much greater selectivity for NR2A versus NR2B-containing receptors. We found that 300 nM Zn^{2+} blocked the potentiation evoked by PACAP application. Finally, we examined NMDA responses of CA1 neurons acutely isolated from N2A knockout and matched wild-type mice. In wild-type cells, PACAP enhanced the currents as anticipated but these effects were absent in cells from NR2A knockout mice. In addition, we found that the application of PACAP38 increased tyrosine phosphorylation of NR2A but not that of NR2B.
Using field recordings, we employed a fixed intensity of stimulation to induce synaptic plasticity using a range of stimulation frequencies. PACAP shifted the BMC relationship to the left, essentially reducing LTD at low frequencies and shifting the threshold for LTP to lower frequencies. Furthermore, in the presence of PAC1 receptor antagonist

MD65, high-frequency stimulation can not induce LTP anymore.

Pituitary adenylate cyclase-activating peptide (PACAP) expression and signaling in the bed nucleus of the stria terminalis (BNST) mediate increased anxiety-like behavior following chronic variate stress

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Chronic exposure to stressors has been argued to play an important role in the etiology of anxiety disorders. Consistent with this role, increases in anxiety-like behavior are often observed in rodents repeatedly exposed to environmental stressors. Moreover, these behavioral changes have been associated with changes in neuroplasticity in specific brain nuclei that mediate emotional behavior. The bed nucleus of the stria terminalis (BNST), in particular, has been argued to mediate anxiety-like behavioral responding to long-duration anxiogenic stimuli, and neuroplasticity in this region is increased following chronic stress. We have shown that chronic variate stress substantially and selectively increased transcript levels of PACAP and its cognate PAC1 receptor, in anterolateral BNST tissue punches. Furthermore, PACAP infusion into the BNST produced an anxiogenic response on baseline acoustic startle responding that persisted for at least 7 days following the initial injection. Based on these data, we have argued that BNST PACAP increases mediate the effects of chronic stress on subsequent increases in anxiety-like behavior. We now show that chronic variate stress increases PACAP immunoreactivity discretely in the oval nucleus of the BNST, in patterns distinct from those for corticotrophin-releasing hormone immunoreactivity. To demonstrate that stress-induced changes in PACAP signaling mediate increases in anxiety-like behavior, the PACAP receptor antagonist PACAP(6-38), was infused into the lateral ventricles during 1 week of chronic variate stress. Initial results suggest that PACAP (6–38) infusion attenuated the anxiogenic effects of chronic stress and also attenuated stress-induced reductions in weight gain. In good agreement, PACAP knockout mice also demonstrated reduced chronic stress-induced anxiety behavior compared with their wild-type controls. These results suggest that central PACAP receptor activation mediates some of the behavioral and physiological consequences of chronic stress.

Memory, cAMP, and PACAP—a phylogenetically conserved function? Studies in *Lymnea stagnalis*

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a highly conserved polypeptide, which, along with its receptors (PAC1-R, VPAC1, and VPAC2), is expressed in both vertebrate and invertebrate nervous systems. In the first part of the present study, we showed the presence of PACAP in *Lymnea stagnalis* by mass spectrometry and investigated whether PACAP elevates cAMP in cerebral ganglia. VIP, PACAP, and maxadilan increased cAMP levels by ~47, 79, and 82% of control levels, respectively, and this effect could be blocked by the co-application of the antagonist PACAP6-38 and maxadilan antagonist.

Long-term memory (LTM) forming after single-trial classical reward conditioning in *Lymnaea* requires cAMP-activated protein kinase. In the second part of our study, we tested whether PACAP plays a role in the formation of LTM in this model. PACAP6-38 injected 60 min before training blocked LTM. Another classical conditioning paradigm is repeated pairing of a tactile stimulus to the lips with a food reward. This "weak" paradigm leads to reliable memory formation only after >10 trials. In the third part of our study, we tested whether PACAP accelerates this slow memory formation process. We found that pre-treatment of intact animals with PACAP accelerated learning: a significant response to the stimulus was found after as few as three to five training trials. Our new observations strongly support the notion that a PACAP (or PACAP-like peptide) is present, active, and plays a key role in learning-induced activation of AC in *Lymnaea*.

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PACAP plays a crucial role in the stress-induced activation of HPA-axis

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Distribution of pituitary adenylate cyclase-activating polypeptide (PACAP) is closely related to signal transduction in stress-induced physiological responses. It is known that acute restraint stress can induce neuronal activation in various brain regions, including the hypothalamic paraventricular nucleus (PVN), followed by the activation of the hypothalamic–pituitary–adrenal (HPA) axis. In this study, we examined endogenous roles of PACAP in the stress-induced HPA-axis response by using of PACAP-deficient (KO) mice. Mice were subjected to immobilization (tube-restraint) stress by enclosing the mice in a polypropylene tube. Plasma corticosterone levels were determined by radioimmunoassay. The expression of c-Fos as a marker of neuronal activation was analyzed by immunohistochemistry. In wild-type mice, a 2-h immobilization stress induced a marked increase in plasma corticosterone levels and c-Fos expression in the PVN. In contrast, both of these responses were significantly reduced in PACAP-KO mice, whereas peripheral administrations of corticotropin releasing factor or adrenocorticotropic hormone caused a similar magnitude of increase in plasma corticosterone levels in PACAP-KO mice and their wild-type littermates. Furthermore, we found that stress-induced c-Fos expression in medial amygdala (MeA) but not in medial prefrontal cortex, was significantly reduced in PACAP-KO mice. As MeA is known to play a prominent role in promoting stress responses to psychogenic stressors, impairment of stress-induced PVN/HPA-axis response in these mice may be partially resulted from the reduced neural activation in MeA. Taken together, these results suggest that central PACAP plays a crucial role in the MeA–PVN pathway to regulate the stress-induced activation of HPA axis.

15-Deoxy-Δ-prostaglandin J_2 enhances NGF-induced neurite outgrowth in PC12 cells via a CRTH2 receptor-p38 MAP kinase pathway

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Nerve growth factor (NGF)-induced neurite outgrowth in pheochromocytoma cells is an appropriate experimental model of neuronal differentiation. We previously showed in PC12h cells that simultaneous treatment with pituitary adenylate cyclase-activating polypeptide (PACAP) and NGF synergistically increased both neurite outgrowth and PACAP mRNA expression. The former was specifically governed by ERK whereas the latter was preferentially regulated by p38 MAPK activations. In a series of experiments on the relation between neurite outgrowth and MAPK signals, we found that 15-deoxy-Δ-prostaglandin J_2 (15d-PGJ_2), an endogenous metabolite of prostaglandin D_2 (PGD_2), promoted NGF-induced neurite outgrowth in PC12 cells. PGD_2 (0.1–3.0 μM) and 15-deoxy-PGJ_2 (0.1–1.0 μM), which are ligands to classical D-prostanoid receptor (DP) and recently identified chemoattractant receptor-homologous molecule expressed on T helper type-2 cells (CRTH2), enhanced the neurite outgrowth induced by NGF (50 ng/ml) in a dose-dependent manner. CRTH2 receptor antagonist CAY10471, but not DP receptor antagonist BWA868C, suppressed the promoting effect of 1.0 μM 15d-PGJ_2 on NGF-induced neurite outgrowth. NGF increased the phosphorylation of Akt, ERK, and p38 MAP kinase with a peak at 5 min, whereas 15d-PGJ_2 increased the phosphorylation of ERK and p38 MAP kinase, but not Akt, with a peak at 1 h after the treatment. The effects of 15d-PGJ_2 and NGF on the MAP kinase activation were sustained for 24 h. Co-treatment with 15d-PGJ_2 and NGF induced ERK and p38 MAP kinase phosphorylation additively. Pretreatment of CAY10471 suppressed the 15d-PGJ_2-induced phosphorylation of p38 MAP kinase, but not that of ERK. These data suggested that 15d-PGJ_2 enhances the NGF-induced neurite outgrowth in PC12 cells via a CRTH2 receptor-p38 MAP kinase pathway. We will also discuss the possible signal cross-talk between PACAP and 15d-PGJ_2 in the neurotrophic effects of NGF.

Effects of environmental factors during development on abnormal phenotypes in PACAP knock-out mice

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon family of peptides. We have previously found that mice lacking PACAP (PACAP-KO mice) display behavioral abnormalities, such as hyperactivity and jumping behavior, depression-like behavior, memory impairment, and prepulse inhibition (PPI) deficits. In addition, single-nucleotide polymorphism association studies show the relation of PACAP genes to schizophrenia. On the other hand, clinical research shows that not only genetic factors

but also environmental factors play an important role in etiology of many psychiatric disorders. In the present study, we examined how environmental factors could alter the behavioral changes in PACAP-KO mice. Locomotor activity and jumping behavior were measured for 60 min using an infrared photocell beam detection system. Depression-like behavior was evaluated by the immobility time in a 6-min forced swim test. For PPI testing, pulses were randomly presented and acoustic startle responses were measured. Memory performance was examined in novel object recognition test. Isolation rearing (for 2 weeks from 4-week-old) induced hyperlocomotion and aggressive behaviors in PACAP-KO mice without affecting behavioral performance of wild-type controls. Rearing under enriched conditions (for 4 weeks from 4-week-old) attenuated the hyperactivity and jumping behavior, shortened the immobility time, and ameliorated memory performance in PACAP-KO mice, but did not affect PPI deficits. In contrast, rearing under enriched conditions (for 4 weeks from 8-week-old) had no effect on the abnormal behaviors in PACAP-KO mice. Exposure to enriched environment increased hippocampal protein levels of brain-derived neurotrophic factor in both groups, while there was no significant difference in the levels between PACAP-KO and wild-type mice. These results indicate that some abnormal phenotypes in PACAP-KO mice depend on environmental factors during development.

Pituitary adenylate cyclase activating polypeptide (PACAP) enhances blood-brain barrier (BBB) functions of rat brain microvascular endothelial cells in vitro

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is an endogenous ligand to stimulate cAMP formation and widely distributed within the nervous system and also in vascular endothelial cells. There have been no reports on the influence of PACAP for the tight junctions (TJs) of the blood–brain barrier (BBB). We found first that the in vitro effect of PACAP in the cultured rat brain microvascular endothelial cells (RBEC), a BBB-cell. RBEC were cultured on Transwell^{TM}. Barrier functions were estimated by measuring the transendothelial electrical resistance (TEER) and the permeability of sodium fluorescein (Na-F, 376 Da), and Evans' blue albumin (EBA, 67 kDa). The expression of the TJs proteins (claudin-5, occludin, ZO-1) were observed by using an immunostaining-method for PACAP. In the RBEC, PACAP dose-dependently increased TEER and decreased permeability of Na-F. This effect was enhanced by inhibitor of type 4 phosphodiesterase (RO- 20-1724). PACAP-induced barrier function enhancements were blocked by anti-PAC1 receptor antibody. The expression of the TJs proteins changed from zipper-like to linear type. In the RBEC, the PAC1 receptor abundantly expresses to luminal side (blood side) compared with the abluminal side (brain side). Thus, PACAP enhanced the barrier function at the RBEC through the PAC1 receptor. Our results suggest that circulating PACAP regulates the BBB function.

Presence of galarin-like immunoreactivity at different stages of murine embryonic development

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The galanin family of neuropeptides consists of three members: galanin, galanin-like peptide (GALP), and a splice variant of GALP called alarin. Recently, galanin was identified in undifferentiated mouse embryonic stem cells as one of the most abundant transcripts [Anisimov SV et. al.:Genomics 79: 69–176 (2002)], and galanin-like immunoreactivity was observed in mesenchyme, heart, bone, and neural crest derivates at embryonic day E10 to E15 [Jones M et. al.:Anat Rec 292:481–487 (2009)].

The aim of the present study was to determine the distribution of alarin during the most important developmental stages in murine embryogenesis. Immunohistochemistry was performed on paraffin-embedded tissue of NMRI mice at stages E14.5 and E17 using an affinity-purified polyclonal anti-alarin antibody. Strong alarin-like staining at both developmental stages was detected in the intermediate zone of the cortex, choroid plexus, epidermis, and cartilage of the developing bones. Further staining was observed in the eye lens at stage E14.5 and in the leptomeninges, as well as in neurons of the spinal cord at E17.

These data indicate a function of alarin in morphogenesis and a developmental role of this peptide in ectodermal and neural-crest origin tissues in the mouse embryo. Whether

alarin has a growth and/or differentiative role, remains to be demonstrated.

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Localization of pituitary adenylate cyclase-activating polypeptide (PACAP) receptors in peripheral tissues during mouse perinatal development

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic peptide which is involved in a number of functions during development. PACAP have affinity for a family of three receptors: PAC1, VPAC1, and VPAC2. Here, we report the immunohistochemical distribution of PACAP receptors during perinatal stages of mouse development at 17dpc (pre-natal), 19dpc (newborn), 21dpc (post-natal), respectively. Interestingly, many tissues show PACAP receptor labeling, and a differential expression of each receptor subtype occurs. At stage 17dpc, during late embryonic life, PAC1 is expressed within the liver, adipose tissue, and kidney while VPAC1 and VPAC2 are expressed in the adipose tissue exclusively. At 19dpc, in newborn stage, all receptors are expressed in the adrenal gland and submaxillary gland. VPAC1 is found in the smooth muscle layer of the intestine while adipose tissue VPAC2 labeling disappears. At this stage, germ cells and gonadal interstitial cells are faintly labeled for VPAC1 and VPAC2. At stage 21dpc, during early post-natal development, lungs become labeled for PAC1 and VPAC2; adrenal gland and gonads retain labeling for VPAC1 and VPAC2 exclusively while adipose tissue expresses again all three receptors. Our data show that the expression of PACAP receptors during perinatal stages of mouse development is precisely time-modulated in several tissues suggesting that the effect of PACAP on tissue differentiation is regulated by PACAP receptor differential expression.

Neuroprotective effects of PACAP against alcohol toxicity in the developing rat cerebellum

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The brain is vulnerable to alcohol exposure throughout development, and most structures of the central nervous system can be affected. In particular, it has been established that exposure of 8-day-old rats to ethanol exacerbates cerebellar granule cell death. The deleterious effect of alcohol on cultured granule neurons can be blocked by PACAP, but no in vivo data were available yet. The aim of this project was thus to establish the effects of PACAP against alcohol toxicity in vivo and to determine some of the mechanisms involved. Real-time PCR revealed that, 4 h after treatment, *fos* gene levels were significantly reduced by ethanol, and this inhibition was partially blocked by PACAP, whereas bcl-2 expression was only regulated by PACAP. Moreover, the expression of the proapoptotic genes *jun* and *bax* was significantly increased by alcohol administration, and those effects were reduced by PACAP. Some consequences on the animal motor functions were analyzed using the negative geotaxis test. Two days after injection, sham animals turn 90° within less than 20 s, whereas ethanol-treated animals required 103 s. Interestingly, treatment with PACAP significantly reduced the deleterious effect of alcohol (67 s). Quantification of the thickness of the cerebellar cortical layers revealed that, 3 days after treatment, ethanol induced a 35% reduction of the thickness of the internal granule cell layer (IGL) which could be totally blocked by PACAP. Treatment of rats with inhibitors of caspase-3 and JNK mimicked the effect of PACAP on ethanol-decreased IGL thickness, confirming that these pathways are involved in the toxic effect of alcohol on cerebellar granule cells and explaining how PACAP counteracts alcohol toxicity. All these results suggest that PACAP is a putative candidate to block, in vivo as in vitro, the deleterious effects of ethanol during brain development.

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Extent of retinal damage in carotid artery occlusion-induced hypoperfusion model in PACAP-deficient mice

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuroprotective peptide exerting protective effects in neuronal injuries. We have provided evidence that PACAP is neuroprotective in several models of retinal degeneration in vivo. Our previous studies showed that PACAP treatment ameliorated the damaging effects of chronic hypoperfusion modeled by permanent bilateral carotid artery occlusion. We have also demonstrated in earlier studies that treatment with PACAP antagonists further aggravates retinal lesions. It has also been shown that PACAP-deficient mice have larger infarct size in cerebral ischemia. The aim of the present study was to compare the degree of retinal damage in wild-type and PACAP-deficient mice in ischemic retinal insult. Mice underwent 10 min of bilateral carotid artery occlusion followed by 2-week reperfusion period. Retinas were then processed for histological analysis. It was found that PACAP-deficient mice had significantly greater retinal damage, as shown by the thickness of the whole retina and the morphometric analysis of the individual retinal layers, such as inner and outer plexiform and inner and outer nuclear layers. These results clearly show that endogenous PACAP reacts as a stress-response peptide that is necessary for endogenous protection against different neuronal insults.
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Effects of PACAP in streptozotocin-induced rat model of diabetic retinopathy

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Pituitary adenylate cyclase-activating polypeptide (PACAP) has been shown to exert protective effects in models of neurodegenerative diseases, cerebral ischemia, and retinal degeneration. Diabetic retinopathy is a leading cause of adult blindness. In the early stages, the amacrine cells characteristically undergo degeneration. The aim of the present study was to investigate whether PACAP is effective in a model of diabetic retinopathy induced by 70 mg/kg streptozotocin in Wistar rats. We administered intravitreal PACAP (100 pmol) injection into the right eye, saline in the other, and retinas were evaluated with histologial, immuncytochemical (vertical sections and whole mount preparations), and molecular biological (Western blot, RT-PCR) methods. In streprozotocin-induced diabetic rats, dopaminergic amacrine cells appeared to be degenerated, as revealed by thyrosine hydroxylase (TH) immunopositivity. Severe degeneration of dopaminergic amacrine cells was seen in the inner nuclear layer, shown by the shape of their soma and their connection. Morphometrical analysis showed significant reduction in the number of cells. Neuroprotective effects of PACAP were observed in streptozotocin-induced retinal degeneration. Intraocular PACAP treatment led to a nearly intact appearance of the soma, connections, and cell number. According to RT-PCR and Western blot analyses using TH primary antibody, intensity of immunostaining was increased by PACAP treatment compared with diabetic retinas. In summary, intravitreal administration of PACAP protected dopaminergic amacrine cells, demonstrating its therapeutic potential in streptozotocin-induced diabetic retinopathy.
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Signaling of neuroprotective peptides through the microtubule/tubulin tyrosination cycle

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Microtubules are key cytoskeletal elements participating in cell division, cell motility, cellular transport, and maintenance of cell shape. Post-translational modifications of the alpha–beta heterodimer tubulin subunits of microtubules add to the dynamic nature of these essential cellular components. The tubulin tyrosination cycle includes the removal of the C-terminal tyrosine of microtubule alpha tubulin, resulting in Glu-tubulin and the re-addition of tyrosine (Tyr) to soluble alpha-tubulin. Tyr-tubulin, a marker for dynamic microtubules, is generally found in

the neuronal growth cones and soma, while Glu-tubulin, characterizing stable microtubules, is commonly found along the axons. Here, a cell-based assay for tyrosination that allows the assessment of the relative degree of stable microtubule versus dynamic microtubules using confocal microscopy and quantitative in cell immunodetection, termed in-cell westerns was implemented for the evaluation of the neuroprotective drug candidate davunetide [NAPVSIPQ, also termed NAP (www.allontherapeutics.com)]. NAP is a potent neuroprotective peptide derived from activity-dependent neuroprotective protein (ADNP). Early studies have shown that ADNP synthesis and cellular secretion is regulated in part by the neuropeptides vasoactive intestinal peptide and pituitary adenylate cyclase-activating poplypeptide. Both peptides provide neuroprotection, and it is hypothesized that this neuroprotective activity is provided in part by ADNP, with NAP constituting the neuroprotective active site of ADNP. In animal models of Alzheimer's disease and frontotemporal dementia, NAP enhanced learning and memory and inhibited tau pathology, suggesting an interaction with the microtubule system. In a proof of concept human clinical trial, the intranasal formulation of NAP, AL-108 (davunetide) improved memory function in patients with amnesic mild cognitive impairment (www.allontherapeutics.com). Here, changes in the tubulin tyrosination cycle were used to study the effects of NAP on microtubule dynamics. Rat PC12 cells were subjected to a 2-h treatment by NAP, and control compounds followed by cell permeabilization to wash out free tubulin. As expected, colchicine significantly reduced microtubule content, and paclitaxel significantly increased detyrosinated tubulin. NAP treatment significantly affected the tyrosination cycle, increasing tyrosinated and detyrosinated alpha tubulin in a dose-dependent manner. NAP effects on microtubule dynamics in cells that can be differentiated into neurons may implicate neuronal plasticity, learning, memory, and neuroprotection.

Molecular cloning and characterization of a VPAC receptor in the inshore hagfish, *Eptatretus burgeri*

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Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) are pleiotropic polypeptides with diverse tissue distribution. They belong to the same VIP/PACAP/secretin peptide superfamily and have the ability to interact with the class II G protein-coupled receptors. Three receptor subtypes namely $VPAC_1$, $VPAC_2$, and PAC_1 are responsible for interaction with VIP and PACAP, each differing in ligand-binding affinities and signal-transduction mechanisms. However, characterization of these ligand–receptor pairs has not yet been well studied in living primitive fish species such as lampreys and hagfish, which represent the earliest vertebrates to exist. In this study, a VPAC receptor (hfVPAC) sequence was identified in the inshore hagfish, *Eptatretus burgeri*. By real-time PCR, it was found predominately in the brain, while trace amounts were also detected in the muscles. This contrasts the diverse tissue distribution pattern of VIP/PACAP receptors in other vertebrates, suggesting the first function of the ligand–receptor pair to be restricted to the brain and that functions elsewhere evolved only later. Functionally, both VIP and PACAP ligands from fish and mammals were able to stimulate cAMP responses in the hfVPAC-transfected cell line, further suggesting it's identity as a VPAC receptor. This is also verified by phylogenetic analyses showing the hfVPAC to be clustered together with other vertebrate VIP and PACAP receptors. As the hfVPAC does not belong exclusively to $VPAC_1$, $VPAC_2$, or PAC_1 receptor subtype groups, this may suggest a more ancient evolutionary origin and a closer resemblance to the ancient VIP- and PACAP-receptor genes. Through the discovery of this receptor in the hagfish, we hope to provide clues to better understand the early events in the molecular evolution of VIP and PACAP receptors in vertebrates.

Pituitary adenylate cyclase-activating polypeptide and its receptors in diet-induced obese rat adrenal glands

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic peptide first isolated from ovine hypothalamus which is involved in a number of central and peripheral functions such as energy homeostasis and regulation of lipid metabolism. PACAP have affinity for a family of three receptors: PAC1, VPAC1, and VPAC2. Here, we report the immunohistochemical distribution of PACAP and its receptors in the adrenal gland of rats fed for 7 weeks with hyperlipidic diet (HF) compared with low fat controls (LF). PACAP is expressed in the medullary chromaffin cells of both HF and LF adrenals, although HF adrenals show a marked increase of PACAP content of chromaffin cells. Furthermore, PACAP receptors have

different but specific distribution among the adrenal zones. In LF, VPAC1 shows a specific cortical distribution in the zona glomerulosa (ZG) and reticularis (ZR); PAC1 and VPAC2 show, to a minor extent, the same distribution pattern, labeling ZG, ZR, and, in addition, medullary cells. Interestingly, in HF adrenals, VPAC1 and PAC1 expression is completely abolished while VPAC2 retains a faint labeling pattern. Our results clearly demonstrate that, in the adrenal gland of HF rats, PACAP and its receptors can be up- and down-regulated, respectively, thus suggesting that, at least in the adrenal glands, hyperlipidic diet can modulate the PACAP-based regulatory pathways of energy homeostasis.

Autocrine regulation of osteoblastic differentiation by pancreatic polypeptide in osteoblastic MC3T3-E1 cells

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Although the neuropeptide Y (NPY) family has been demonstrated to control bone metabolism, the role of pancreatic polypeptide (PP), which has structural homology with NPY and peptide YY (PYY) to share the NPY family receptors, has remained unknown in peripheral bone tissues. In the present study, the regulatory roles of PP and its Y receptors were studied using MC3T3-E1 cells, a murine-transformed osteoblastic cell line, as a model for osteoblastic differentiation. We found that (1) PP mRNA was detected and increased during cell-contact-induced differentiation in MC3T3-E1 cells; (2) the immunoreactivity of PP was detected by radioimmunoassay and increased in culture medium during differentiation; (3) all the types of NPY family receptor mRNAs (Y1, Y2, Y4, Y5, and Y6) were found to increase during differentiation; (4) PP stimulated differentiation in MC3T3-E1 cells in terms of ALP mRNA and BMP-2 mRNA. These findings suggested that MC3T3-E1 cells produce and secrete PP, which may in turn stimulate the differentiation of MC3T3-E1 through its specific receptors in an autocrine manner.

Intra-islet PACAP protects pancreatic beta-cells against glucotoxicity and lipotoxicity

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AIMS: Pituitary adenylate cyclase-activating peptide (PACAP), a potent insulinotropic peptide, is localized in pancreatic islets. In this study, we examined whether endogenous PACAP protects islet β-cells against toxicities induced by hyperglycemia and hyperlipidemia.
METHODS: Pancreatic islets were prepared from both wild-type and PACAP null mice. Islets were cultured in medium containing either 5.6 (low glucose) or 25 mM glucose (high glucose) in the presence or absence of 0.4 mM palmitate for 48 h. Subsequently, the abilities of islet β-cells to respond to glucose stimulation with increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and insulin secretion were examined.
RESULTS: A rise in the superfusate glucose concentration from 2.8 to 8.3 mmol/l induced the first phase increases in $[Ca^{2+}]_i$, which were often followed by oscillations of $[Ca^{2+}]_i$ in both wild-type and PACAP null mice islets cultured in medium containing 5.6 mM glucose for 2 days. The glucose-induced $[Ca^{2+}]_i$ increases were observed in islets of wild-type mice following culture with high glucose or palmitate, whereas they were severely impaired in islets of PACAP null mice. Furthermore, treatment with high glucose or palmitate also impaired glucose-induced insulin secretion in islets of PACAP null mice but not wild-type mice.
CONCLUSIONS: Intra-islet PACAP attenuates glucotoxicity and lipotoxicity. It is suggested that endogenous PACAP plays a role in maintaining β-cell survival and functions including insulin secretion in vivo.

Expression patterns of incretin-producing cells in mouse intestine during embryonic development

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To clarify the development of incretin-producing cells, expression patterns of incretin-producing cells were examined in mouse intestine from embryonic day 15 (E15) to

E17 by immunocytochemistry. Triple immunofluorescent labeled cells were observed using a confocal laser scanning microscope. The localizations of glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 and F-actin were analyzed. We first determined that incretin-producing cells in adult mouse intestine are located singly among epithelial cells, which is consistent with previous findings that enteroendocrine cells including incretin-producing cells are observed singly, and are regarded as terminally differentiated cells derived directly from intestinal epithelial stem cells, which are localized at the crypt in adult animals. It is generally thought that only such intestinal epithelial stem cells can divide. However, we frequently observed in mouse embryo that the two incretin-producing cells were located adjacently and usually symmetrically in contact with each other in the intestinal epithelium. These findings strongly suggest that these pairs of incretin-producing cells are produced by cell division of pre-existing incretin-producing cells or intermediate precursor cells derived from intestinal epithelial stem cells. Thus, incretin-producing cells or their putative precursor cells may exhibit significant cell proliferative activity in embryonic stages that is almost lost in adults.

Regulation of somatolactin release from cultured goldfish pituitary cells by PACAP and MCH

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Pituitary adenylate cyclase-activating polypeptide (PACAP)- and melanin-concentrating hormone (MCH)-containing neurons directly innervate the adenohypophysis in the goldfish pituitary. In this species, nerve fibers containing PACAP and MCH are located in close proximity to somatolactin (SL)-producing cells. However, there is little information available about the effect of PACAP and MCH on SL release from pituitary in this species. In order to elucidate this issue, we used the cell immunoblot method. Treatment with PACAP increased the immunoblot area for SL-like immunoreactivity from dispersed pituitary cells. With the use of a pharmacological approach, PACAP-induced SL release was shown to be mediated by PACAP selective receptor. In addition, the AC/cAMP/PKA- and PLC/inositol 1,4,5-triphosphate/PKC-signaling pathways were shown to be involved in PACAP-induced SL release. On the other hand, treatment of dispersed pituitary cells with MCH decreased the area of SL-like immunoreactivity on immunoblots. MCH-induced reduction in SL release was shown to be mediate by MCH receptor and subsequent inhibition the AC/cAMP/PKA-signaling pathway via Gi protein.

These results suggest that PACAP can potentially function as a hypophysiotropic factor mediating SL release and that MCH can potentially function as a hypothalamic factor suppressing SL release in goldfish pituitary cells. We propose that MCH and PACAP exert novel neuroendcrine control over SL release from pituitary in goldfish.

The role of the circulatory system in the transportation of PACAP-like compounds in earthworms

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Some hormones that regulate the gametogenesis, regeneration, and homeostasis in earthworms have been identified earlier. Earthworms have no pituitary or any endocrine organ, and all known hormones are mainly synthesized by the neurosecretory cells of the central nervous system (CNS). Certain endocrine cells are located in the body wall and alimentary canal epithelium. Recentlym, we have shown the occurrence of PACAP-like compounds in various body parts (e.g., CNS, body wall, alimentary canal, seminal vesicles) of the earthworm, *Eisenia fetida*, but the site of synthesis remained unknown. The main question was whether all of the peripheral tissues had PACAP synthesis capacity or it was synthesized by neurosecretory cells and transported to various body parts with the closed circulatory system of the earthworms. Applying tissue fractionation methods and radioimmunoassay, the PACAP concentration of various parts of the CNS, blood, and the capillary-rich part of the body wall (prostomium) in earthworms was determined. All parts of the CNS contained high amounts of PACAP; however, a decreasing gradient from the supraesophageal ganglion (so-called brain) to ventral nerve cord ganglia was found. PACAP-concentration of the medial part of the brain (that contains a number of neurosecretory cells) was significantly higher than in its lateral parts. Extremely high PACAP concentration was found in the blood, and the capillary-rich body

part namely in prostomium. Our present results suggest that certain neurosecretory cells of the earthworm CNS synthesize most of the PACAP (or PACAP-like compounds), and it is transported to various tissues by the closed circulatory system of earthworms. This finding supports the neurohormone characteristics of PACAP in an invertebrate species.
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Impaired nocifensive behaviors and mechanical hyperalgesia, but enhanced thermal hyperalgesia in PACAP knockout mice

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Pituitary adenylate cyclase-activating polypeptide-38 (PACAP-38) and its receptors are present in the spinal cord, dorsal root ganglia, and capsaicin-sensitive sensory neurons. Since its role in nociception is contraversial, our aim was to investigate acute somatic and visceral nocifensive behaviors, sciatic nerve ligation-evoked neuropathic as well as resiniferatoxin-induced inflammatory mechanical and thermal hyperalgesia in PACAP-deficient ($PACAP^{-/-}$) mice to elucidate its overall function in pain transmission.
The number of paw lickings in the early (0–5 min) and late (20–45 min) phases of the formalin test reflecting somatic chemonociception and inflammatory nociception, respectively, was markedly diminished in $PACAP^{-/-}$ mice. Intraperitoneal acetic acid-evoked abdominal contractions referring to visceral chemonociception was also significantly attenuated, and neuropathic mechanical hyperalgesia was absent in PACAP knockouts. Intraplantarly injected resiniferatoxin-evoked mechanical hyperalgesia of the paw, which involves both peripheral and central mechanisms was decreased, but thermal hyperalgesia mediated by only peripheral mechanisms was increased in $PACAP^{-/-}$ mice.
The overall role of PACAP in pain transmission originating from both exteroceptive and interoceptive areas is excitatory; it is involved in central sensitization. In contrast, it is an inhibitory mediator at the level of the peripheral sensory nerve endings and decreases sensitization of the capsaicin-sensitive terminals to heat. This result, which is in agreement with our previous data showing that PACAP inhibits sensory neuropeptide release from sensory nerve endings, suggests the existence of a potential, presently unknown, inhibitory mechanism in the peripheral nerve terminals.
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Expression and regulation of PACAP/VIP and receptors in micturition reflex pathways of nerve growth factor (NGF) overexpressing (OE) mice

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Previous studies have demonstrated expression and regulation of PACAP, PAC1, VPAC1, VPAC2 transcripts in urinary bladder, and lumbosacral (LS) dorsal root ganglia (DRG) with cyclophosphamide (CYP)-induced cystitis. Enhanced target-derived nerve growth factor (NGF) availability increases PACAP expression in small nociceptive DRG cells. NGF may play a role in urinary bladder dysfunction by mediating inflammation and functional changes in sensory and sympathetic neurons innervating the urinary bladder. To explore the role of NGF in bladder sensory function and neurochemical plasticity of PACAP/receptors, we used a transgenic mouse model of chronic NGF overexpression in the bladder using the urothelial-specific uroplakin II promoter. NGF was over-expressed at the mRNA and protein level in the bladders of transgenic compared with wild-type (WT) littermate control mice. Transgenic mice had a reduced bladder capacity in conscious open-voiding cystometry studies and an increased referred pelvic somatic hypersensitivity. Quantitative PCR was used to determine PACAP/VIP and receptor expression in bladder reflex pathways in NGF overexpressing (OE) and WT mice. In NGF, OE mice, PACAP mRNA was significantly ($p \leq 0.05$) increased in the LS spinal cord and DRG compared with WT control mice whereas VIP mRNA was not affected. PACAP/VIP receptors showed differential responses in LS DRG in NGF OE compared with WT mice. PAC1 and VPAC2 receptor mRNA was decreased in LS DRG in NGF OE mice whereas VPAC1 receptor mRNA was increased in LS DRG compared with WT mice. Substance P and galanin mRNA in LS DRG and spinal cord in NGF OE mice were also examined, but changes were not as dramatic as those observed for PACAP mRNA. We are currently determining if changes in PACAP/receptor mRNA expression are associated with immunoreactivity changes. These studies are consistent with NGF regulation of PACAP/receptors in bladder reflex pathways suggesting that target-derived NGF affects neurochemical plasticity and reflex function.
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Effects of PACAP on mitochondrial antiapoptotic pathways and cytokine expression in rats subjected to renal ischemia/reperfusion

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The neuroprotective effects of pituitary adenylate cyclase-activating polypeptide (PACAP) are well-known, but the neuropeptide is able to exert similar actions in non-neuronal cells. Recently, we have shown that PACAP prolongs ischemic time, decreases mortality, and attenuates tubular degeneration in a rat model of renal ischemia/reperfusion, but the mechanism of renoprotection is not known. The aim of this study was to obtain further insight into the renoprotective effects by examining the direct effects of PACAP on mitochondrial permeability transition in vitro and on the expression of the antiapoptotic bcl-2 in kidney tissues following 45- and 60-min renal ischemia/reperfusion in vivo. Furthermore, we also investigated the effects of PACAP on cytokine/chemokine expression in the same in vivo model using a cytokine array kit. We found that PACAP did not affect mitochondrial permeability transition and limited effects were seen in cytokine/chemokine expression. While PACAP counteracted some of the changes caused by ischemia/reperfusion, such as RANTES, CNTF, TIMP-1, and thymus–chemokine expression, most interleukins and other mediators remained unchanged. Dramatic effects were seen in bcl-2 expression in rats subjected to renal ischemia. PACAP counteracted the ischemia/reperfusion-induced decrease in the antiapoptotic bcl-2, both after 45- and 60-min ischemia. Taken together, the present results indicate that the antiapoptotic effects of PACAP involving the mitochondrial bcl-2 pathway may be a major factor playing a role in attenuation of kidney injury by PACAP.

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The role of endogenous PACAP in protection against hypoxia and oxidative stress: in vitro studies in PACAP knockout mice

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One of the well-known effects of pituitary adenylate cyclase-activating polypeptide (PACAP) is its neuro- and cytoprotective actions, including renoprotective effects. PACAP-deficient mice display several behavioral, metabolic, and developmental alterations. Furthermore, it has been shown that PACAP-deficient mice have larger infarct volume in a model of cerebral ischemia, delayed axonal regeneration, and increased cell death in cerebellar oxidative stress. These results show that endogenous PACAP plays a protective role against different stressors. The aim of the present study was to investigate whether endogenous PACAP has protective effect in the kidney against oxidative stress and in vitro hypoxia. Kidney cell cultures were isolated from wild-type and PACAP-deficient mice, and cell viability was assessed following oxidative stress induced by 0.5, 1.5, and 3 mM H_2O_2. In vitro hypoxia was induced by $CoCl_2$. We found that the sensitivity of cells from PACAP-deficient mice was greatly increased to oxidative stress: both after 2 or 4 h of exposure, cell viability was significantly reduced compared with control wild-type mice. These results show that endogenous PACAP protects against oxidative stress in the kidney and that PACAP may act as a stress sensor in renal cells.

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Presence of VIP, PACAP, and their receptors in control and explant cultured mouse major pelvic ganglia

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The major pelvic ganglion (MPG) innervates the urogenital organs and lower bowel. It is a unique autonomic ganglion because it contains sympathetic and parasympathetic postganglionic neurons. During surgical procedures such as prostatectomy or lower bowel resection, MPG neurons can be injured or transected. However, the change in

chemical phenotype occurring in response to injury is not established. Some cholinergic MPG neurons express vasoactive intestinal polypeptide (VIP). However, no information has been reported concerning the presence of pituitary adenylate cyclase-activating polypeptide (PACAP) or which type of VIP-sensitive receptor may be present in the MPG neurons. We have analyzed using quantitative PCR, whether VIP and PACAP transcripts and VPAC and PAC1 receptor transcripts are present in extracts of the male mouse MPG and using immunocytochemistry, whether PACAP as well as VIP is expressed by the MPG neurons. In control MPG, VIP transcript expression is much greater than PACAP transcript expression. Low levels of transcripts for VPAC1, VPAC2, and PAC1 receptors are present in extracts from control MPG. In freshly isolated or 4-h explant cultured MPG, there are numerous VIP-IR cells and fibers, whereas no PACAP-IR cells and only a few PACAP-IR fibers are noted. We also tested whether the phenotype of the MPG neurons changes during explant culture, an in vitro injury model. In 72-h explants, PACAP transcript expression increased 18-fold, and there are numerous PACAP-IR cells and fibers in the MPG. There is no marked change in VIP transcript expression and many VIP-IR cells and fibers remain. Also, there is no change in transcript levels for PAC1 and VPAC1, but the level of VPAC2 receptor transcript increases (5-fold) in 72-h explants. These results indicate that PACAP is up-regulated following injury. With a variety of functional effects for PACAP reported in smooth muscle of pelvic viscera, PACAP likely contributes to pelvic organ dysfunction after neural injury.

The roles of pancreatic PACAP in cerulein-induced pancreatitis

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Pancreatic PACAP stimulates hormone secretion from pancreatic endocrine and exocrine glands. Using mice over-expressing PACAP in pancreatic β-cells (PACAP-βTg), we previously showed that pancreatic PACAP inhibits islet hyperplasia under type 2 diabetes. Here, we examined the effect of multiple injections of cerulein in PACAP-βTg and mice with ubiquitous deficiency in PACAP (PACAP-KO) to reveal the roles of pancreatic PACAP in acute pancreatitis. Acute pancreatitis was induced by 50 μg/kg cerulein administered seven times IP at hourly intervals. Acute pancreatitis was monitored by serum amylase and lipase activities and histological analysis of pancreas. Cerulein-induced pancreatitis was characterized by a time-dependent rise in serum amylase and lipase activities with the maximal rises at 12 h accompanied by severe pancreatic tissue damages. In PACAP-βTg, the cerulein-induced enzyme release and accompanied pancreatitis were enhanced. In PACAP-KO, cerulein-induced rise in serum enzyme activities and pancreatic damages were ameliorated compared with wild-type mice, though their responses were highly variable. Accordingly, PACAP-KO injected cerulein were segregated into two groups in group (A) where cerulein caused pancreatitis with similar magnitudes observed in wild-type mice and in group (B) where cerulein caused no pancreatitis. After cerulein injection, group (A) did not die by 48 h, but group (B) began to die by 24 h, and most of them died within 48 h. Upon cerulein injection, the body temperature was not altered in group (A), but it was dramatically decreased to about 25°C in group (B). As this time, it is not clear the reason of why PACAP-KO were segregated for two groups on the cerulein response. All together, the present study shows that over-expression of pancreatic PACAP aggravates acute pancreatitis induced by cerulein.

Comparison of intestinal cold preservation injury on PACAP knock-out and wild-type mice

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Tissue injury caused by cold preservation remains an unsolved problem during small intestinal transplantation. Pituitary adenylate cyclase-activating polypeptide (PACAP) is present and plays a central role in the intestinal physiology. The aim of our study was to compare the cold ischemic injury on PACAP-38 knock-out and wild-type mice after small bowel cold storage.

Cold ischemia was produced with small bowel preservation in University of Wisconsin solution at 4°C in PACAP-38 knock-out (n=20) and wild-type (n=20) mice. In group I: sham operated, no-ischemia; GII: 1-h ischemia; GIII: 3-h ischemia; and GIV: 6-h ischemia. Small bowel biopsies were collected after laparotomy (control) and at the end of the ischemia periods. To determine oxidative stress parameters, malondialdehyde, reduced glutathione, and superoxide dismutase were measured. Tissue damage was analyzed

by qualitative and quantitative methods on hematoxylin/eosin stained sections.
In PACAP-38, knock-out animal tissue lipid peroxidation was elevated. These changes were significant after 6 h (153.04±7.2) compared with sham-operated (110.44±5.5) and compared with wild-type results (120.0±1.1 umol/g, $p<0.05$). Meanwhile, the capacity and activity of endogenous antioxidant system decreased significantly after 3 and 6 h preservation (GSH 808.7±5.2; 720.4±8.7 vs. 910.4± umol/g; SOD 125.1±1.4; 103.3±1.9 vs. 212.11±5.8 IU/g). Qualitative and quantitative histological results showed destruction of the mucous, submucous, and muscular layers and crypts in knock-out mice compared with wild-type tissues. These processes were dependent on the time of the cold preservation periods.
Our present study showed that PACAP-38 has a key role in the protection against intestinal cold preservation injury.
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The role of the MCH system in regulating metabolism through peripheral mechanisms

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Melanin-concentrating hormone (MCH) is a cyclic, 19 amino-acid neuropeptide which was originally discovered in fish to lighten skin color by affecting melanosome aggregation. MCH is conserved in various organisms from fish to mammals and predominantly expressed in the hypothalamus. In rodents, MCH gene is overexpressed upon fasting, and central MCH injection increases food intake. Interestingly, the receptor for MCH, MCH1R, is widely expressed in both central and peripheral tissues suggesting that the MCH system might regulate various peripheral functions in addition to central functions.
We developed a specific MCH1R antagonist which does not cross the blood-brain barrier and therefore, is useful to study the peripheral MCH system function. When this MCH1R antagonist is injected peripherally to mice, it affects food intake and fat metabolism. We confirmed this finding by injecting MCH peripherally to see an opposite effect. These suggested that peripheral MCH system can regulate energy homeostasis.
To identify peripheral target tissues, distribution pattern of MCH1R in rat tissues was examined by using quantitative real-time PCR. MCH receptors are highly expressed in several peripheral tissues, in particular, pituitary. In particular, we found that MCH1Rs are localized in thyroid-stimulating hormone (TSH)-expressing cells of the pituitary suggesting that the MCH system can modulate TSH release. Next, we used an MCH1R antagonist as well as an agonist, MCH to examine how peripheral activation and inactivation of the MCH system can regulate metabolism by modulating TSH release. We also used MCH1R knockout mice to study whether genetic inactivation of the MCH system affects TSH and related hormones and how it modulates animals' metabolism.
In conclusion, our findings indicate that peripheral MCH system can regulate energy homeostasis, and this might be due to the fact that MCH receptors are highly expressed in the pituitary, in particular, TSH expressing cells. This is a novel finding that the peripheral MCH system can play an important role in regulating energy homeostasis by modulating pituitary function.

Involvement of pituitary adenylate cyclase-activating polypeptide (PACAP) in diabetic neuropathy of streptozotocin (STZ) treated mice

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide which was originally isolated from the ovine hypothalamus. There are many evidences that PACAP functions as a hypothalamic hormone, neurotrophic factor, neurotransmitter, or neuromodulator. Furthermore, it has been reported that PACAP might be associated with regulation of insulin secretion. Regarding pain transmission, we reported that the intrathecal (IT) administration of PACAP exhibits nociceptive effect. However, we also reported that the intracerebroventricular (ICV) injection of PACAP in mice reduced hot plate response, tail pinch response, and formalin-induced response, suggesting that ICV injection of PACAP exhibits analgesic effect. Moreover, it was reported that PACAP might be associated with the allodynia (Tamaki, 2004). These findings prompted us to evaluate the involvement of PACAP in neuropathy associated with diabetes mellitus using streptozotocin (STZ)-induced diabetic mice. STZ-induced diabetic mice (8–9 weeks of age; blood glucose above 300 mg/dl) were used for experiments 4 weeks after the injection. In the assessment of allodynia by von Frey test,

the maximum decrease in pain threshold was observed at 4 weeks after STZ injection compared with control mice. RT-PCR analysis then demonstrated that the expression level of PACAP mRNA was not changed in brain and spinal cord tissues of control and diabetic mice and that the expression level of PAC1, one of receptors which PACAP, have high affinity for, mRNA in the brain of diabetic mice was higher than that of control mice, although that was not changed in the spinal cord. In addition, von Frey test demonstrated that the IT injection of PACAP elicited mechanical allodynia in control mice and reduced significantly the withdrawal threshold in diabetic mice, suggesting that PACAP–PAC1 signaling might be involved in diabetic neuropathy. Now, we are examining whether IT injection of PAC1-selective antagonist remedy the diabetic neuropathy.

Suppression of oxidative stress by PACAP

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Oxidative stress is a major mediator of tissue and cell injuries. Oxidative stress increases after brain injuries such as ischemia and Alzheimer's disease of which in vivo models were implicated by PACAP treatment. PACAP has been reported to suppress an apoptotic cell death by H_2O_2 in cerebellar granullar cells, cardiomyocytes, and endothelial cells. PACAP suppressed cell death induced by glutamate, C2-ceramide, lipopolysaccharide, aconitase, and ethanol as well. All of these in vitro and in vivo stimulants increase an oxidative stress. A few studies have reported a decrease of an oxidative stress by PACAP. However, no direct evidences have shown PACAP regulates oxidative stress.

Plasma in PACAP +/+, +/−, and −/− mice was collected under anesthesia in an age-dependent manner (day 40–day 240). Plasma in young adult wild-type mice was collected in a time-dependent manner for 24 h after bolus intravenous injection with PACAP (0.5–500 ng/kg), VIP (500 ng/kg), or PACAP (500 ng/kg) with PACAP 6–38 (500 ng/kg). Then, an oxidative metabolite and anti-oxidative potential was determined by free radical evaluator. Plasma in PACAP −/− mice was gained greater oxidative metabolite level than the aged wild-type one, but not in the younger one. The anti-oxidative potential was not significantly different among the mice in any age, but PACAP −/− had relatively smaller level than the others. Plasma obtained from PACAP-injected mice showed significantly decrease of oxidative metabolite and increase of anti-oxidative potential at a highest doses. The effect was not seen by injection of VIP and was canceled by PACAP co-treatment of PAC1R antagonist, PACAP6-38. Moreover, hydroethidium signals which are an indicator for O_2^- in aged PACAP −/− mice were greater than the wild-type one in brain. These results suggested PACAP might be involved in the regulation of oxidative in aged.

VPAC1 receptor: identification of different binding sites of VIP and an antagonist by photolabeling and modeling

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VIP is a widespread neuropeptide which exerts many biological functions through interaction with the VPAC1 receptor, a class B G protein-coupled receptor. Photoaffinity labeling studies associated to three-dimensional molecular modeling demonstrated that the central and C-terminal parts of VIP (segment 6-28) interact with the N-terminal ectodomain (N-Ted) of VPAC1 receptor structured as a sushi domain. However, the domain of the VPAC1 receptor interacting with the N-terminus of VIP (1-5) is still unknown. In order to more delineate the interaction model of VIP with VPAC1 receptor, we determined: (1) the interaction site of VIP N-terminus; and (2) differences of binding sites between VIP and a specific VPAC1 antagonist, PG97-269. The identification of these differences by photolabeling and peptidic mapping would first better position N-terminal parts of VIP and PG97-269 with the receptor and also determine domains implied in agonist or antagonist effects of both peptides. Use of a photoreactive probe Bpa^0-VIP revealed, after photolabeling, receptor cleavage and NuPAGE electrophoresis, that position 0 of VIP interacts with sequence 130-137 of VPAC1 receptor N-Ted, and more precisely, with the Gln 135. Photolabeling studies of the central part (6-28) of PG97-269 showed that this portion of the antagonist interacts with the same region than the central part of VIP. In contrast, photolabeling studies of Bpa^0-PG97-269 identify the 60-66 fragment of N-Ted and more precisely, with residue Gly 62. These results indicate that (1) major part (sequence 6-28) of both peptides binds in the same manner; (2) N-terminal parts of VIP and antagonist physically interact with N-Ted of VPAC1 receptor. This study clearly demonstrates that the N-terminal parts of VIP and PG97-269 bind differently to VPAC1 receptor.

Upregulation of galanin and substance P RNA in organs of septic mice

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Substance P (SP) has been shown to be an important mediator in lung injury during polymicrobial sepsis. SP exhibits its proinflammatory effects via increased capillary permeability and neutrophil extravasation. For the neuropeptide galanin, so far, no data concerning septic conditions are available. However, in murine skin, galanin has been shown to inhibit plasma extravasation induced by SP and calcitonin-gene related peptide (CGRP). This suggests galanin as an opponent of SP and CGRP in neurogenic inflammation. To study a possible role of galanin also during systemic inflammation, we investigated the expression of galanin and SP mRNA in two different animal models of sepsis [bacterial lipopolysaccharide (LPS)-induced sepsis and the colon ascendens stent peritonitis (CASP)]. Sepsis was induced in NMRI mice by implantation of a stent in the colon ascendens (CASP) or intraperitoneal injection of LPS. Quantitative real-time PCR was performed to determine the relative expression of galanin and SP mRNA. We were able to detect upregulation of the expression of galanin (34-fold) and SP (23-fold) in lungs of CASP mice compared with healthy animals. The increased galanin expression was not found in the LPS model. A significant increased expression of both peptides was also observed in liver, spleen, and kidney of CASP mice. Our data indicate differences of the two mouse models of sepsis, although, we were not able to compare the severity of the septic conditions directly. However, CASP has been reported to resemble more closely human septic conditions than the LPS model. Our results implicate that galanin plays a role in septic conditions and may have an anti-inflammatory activity in systemic inflammation similar to its functions in the skin.

Keratinocyte-derived galanin message-associated peptide inhibits growth of *C. albicans*

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Several epithelial surfaces are continuously exposed to potential pathogens but rarely become infected because specific secretions protect them. Antimicrobial peptides participate in this innate immune response by providing a rapid first-line defense against infection. Recently, we could show that galanin message-associated peptide (GMAP) belongs to this group of peptides. It inhibits growth and the yeast-to-hyphal transition of *Candida albicans*. GMAP is derived from the precursor ppGAL encoded by the galanin gene, which is processed into the 29 amino acid peptide galanin and the 59 amino acid peptide GMAP. No data upon processing of this peptide in human skin and activities of endogenously derived GMAP have been reported.

Using a keratinocyte cell line (HaCaT), we generated stable transfectants in which ppGAL expression is under the control of a tetracycline-regulated expression system (T-REx^{TM} System). Stably transfected clones were tested for doxycyclin-inducible overexpression using qRT-PCR. Functional processing and secretion of galanin peptide into the cell culture supernatant was analyzed by radioimmunoassay. The effect of crude purified cell culture supernatants of ppGAL overexpressing cells on growth of *C. albicans* was examined utilizing a microbial viability assay.

Using qRT-PCR, we could show that ppGAL mRNA expression of HaCaT/ppGAL is approximately 100-fold upregulated upon 24 h induction with 100 ng/ml doxycyclin. Radioimmunoassay showed a 350-fold higher concentration of galanin peptide in cell culture supernatants of induced HaCaT/ppGAL (indHaCaT/ppGAL) compared with untransfected HaCaT. In microbial cell viability assays, conditioned supernatants of indHaCaT/ppGAL significantly inhibited growth of *C. albicans*.

Endogenously produced GMAP strongly inhibits fungal growth, demonstrating the physiological relevance of the effect already observed with a synthetic peptide. These studies establish GMAP as a new component of the innate immune system, which has implications for prophylactic and therapeutic strategies of *Candida* infections.

PACAP protects mice with dextran sodium sulfate-induced acute colitis

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Inflammatory bowel disease results from chronic dysregulation of the mucosal immune system involving aberrant activation of innate and adaptive immune responses. Pituitary adenylate cyclase-activating polypeptide (PACAP) plays a crucial role in immunity and inflammation. Our aim was to obtain insight in the role of PACAP in experimental colitis in mice and thus its possible role in inflammatory bowel disease. Here, using gene-targeting, we have identified a novel role for PACAP as a regulator in colonic inflammation. We initially investigated the susceptibility of PACAP-deficient mice to the development of dextran sodium sulfate (DSS)-induced acute colitis by analyzing mortality, the disease activity index (DAI), and histology of the distal colon. PACAP-deficient mice showed severe mortality on administration of 5% DSS in drinking water for 7 days. In accordance with the observed difference in survival, PACAP-deficient mice showed much more severe weight loss compared with wild-type (WT) control mice. PACAP-deficient mice had a significantly higher DAI score compared with WT control mice on day 4 to day 7 after DSS administration. We next show that distal colon of PACAP-deficient mice produced extremely high levels of IFN-gamma, IL-1beta, IL-6, IL-12, and KC on day 4 after DSS administration, indicating inflammatory cytokines and chemokine production are controlled by PACAP in vivo. Our findings indicate that PACAP has previously undocumented roles in the protection of mucosal epithelial cells and the elimination of acute inflammation in the colon.

Signaling pathways involved in PACAP and cytokine interactions regulating adrenomedullary neuropeptide biosynthesis

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Along with catecholamines, several neuropeptides are stored in and secreted from chromaffin cells of the adrenal medulla. Two of these, galanin and VIP, affect steroid production by adrenocortical cells and thus may have a role in homeostatic regulation during inflammation. To test the hypothesis that PACAP, a co-transmitter with acetylcholine at the adrenomedullary synapse, affects neuropeptide biosynthesis under inflammatory conditions, we examined neuropeptide expression in adrenal medulla after treatment with LPS, a model for septic shock. Messenger RNAs encoding VIP and galanin are up-regulated at 24 h (8- and 2-fold, respectively). Up-regulation of VIP and galanin by LPS is abrogated in PACAP-deficient mice, suggesting an interaction between LPS or LPS-induced cytokines and PACAP released in adrenal medulla from the splanchnic nerve (Ait-Ali et al., Neuropharmacology, In press, 2009). Microarray analysis of PACAP and TNF-alpha gene induction in bovine chromaffin cells indicates that TNF signaling through the TNFR2 receptor induces a cohort of genes with NF-kB together with one or more additional *cis*-active elements in their promoters, while PACAP signaling activates a distinct cohort of transcripts whose promoters contain a more variegated combination of *cis*-active elements. However, treatment of cultured chromaffin cells with 100 nM PACAP and 10 nM TNF-alpha, a cytokine whose production is elevated by LPS, results in long-term synergistic up-regulation of VIP and galanin mRNA. PACAP blocks induction by TNF-alpha of mRNA encoding IkB, normally a negative autoregulator of TNF-alpha signaling through NF-kB, without affecting the induction of TNFAIP3, another NF-kB-dependent gene induced by TNF-alpha in chromaffin cells. Thus, PACAP acts downstream of NF-kB to inhibit IkB gene induction by TNF-alpha, attenuating IkB-dependent negative autoregulation of NF-kB signaling and prolonging TNF-alpha-dependent neuropeptide induction in chromaffin cells. This mechanism may underlie PACAP-dependent neuropeptide gene induction by LPS in vivo, during which elevation of serum TNF-alpha concentrations is large and sustained.

Engineering of silver-protected VIP nanoparticles: chemical synthesis and functional characterization

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The primary aim of the current study was to provide a method for silver nanoparticle conjugation to VIP that would be also useful for tailor-made applications based on nanoparticle multifunctional capabilities. VIP was conjugated to tiopronin-capped silver nanoparticles of a narrow size distribution, by means of proper linkers, to obtain VIP functionalized silver nanoparticles with two different VIP orientations (Ag/tiopronin/PEG/succinic/[His]VIP and Ag/

tiopronin/PEG/VIP[His]). VIP intermediate nanoparticles were characterized by TEM, FTIR, Raman, ^{1}H-NMR, and TOCSY. Two different types of VIP functionalized silver nanoparticles were obtained; both expose the C-terminal part of the neuropeptide, but in the first type, VIP is attached to silver nanoparticle trough its free amine terminus Ag/tiopronin/PEG/succinic/[His]VIP, while in the second type, VIP N-terminus remains free Ag/tiopronin/PEG/VIP[His]. VIP functionalized silver nanoparticles did not compromise cellular viability and inhibited microglia-induced stimulation under inflammatory conditions. We have exploited the potential of nanoparticle functionalization as an alternative approach to improve the therapeutic prospect of the endogenous cytokine-like peptide VIP. Our results showed the proof-of-concept for its use, as the chemical synthesis procedure developed to obtain VIP functionalized silver nanoparticles rendered functional products, in terms of biological activity, without any observed cytotoxic effects. The present work provides functional data that demonstrates that VIP can be conjugated to tiopronin-capped silver nanoparticles in two alternative orientations, involving or not the VIP N-terminus, without loss of biological activity. This information is especially valuable for other studies aiming at including VIP in formulations where the possibility of chemical synthesis constraints exists depending on the nanosurface to be functionalized. Our study provides, for the first time, a proof-of-principle to enhance the therapeutic potential of VIP with the valuable properties of metal nanoparticles for imaging, targeting, and drug delivery.

Pituitary adenylate cyclase-activating polypeptide (PACAP) analogs increase the therapeutic index of anticancer agents for blood cancers

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Chemotherapy is the preferred method of treatment for disseminated cancers, including blood cancers and metastatic tumors. The maximal tolerable dose of the most commonly used anticancer agents is limited by their toxic effects on one or more major organs. One strategy to increase the therapeutic index of anticancer agents is to preferentially protect normal tissues against their cytotoxic side effects. We have recently synthesized a series of PACAP analogs that are resistant to proteolysis and/or filtration by the kidney. Exposure of human renal proximal tubule epithelial cells in vitro to cisplatin or doxorubicin caused extensive apoptosis. PACAP analogs protected the proximal tubule epithelial cells against the cytotoxic effects of both cisplatin and doxorubicin in a dose-dependent manner. Exposure of human lung epithelial cells in vitro to bleomycin caused extensive apoptosis. PACAP analogs also protected the human lung epithelial cells against the cytotoxic effects of bleomycin in a dose-dependent manner. The administration of cisplatin to mice increased serum creatinine, blood urea nitrogen, and kidney levels of tumor necrosis factor-α, and the daily administration of PACAP analogs for 3 days reversed these three effects. We are currently testing PACAP analogs in a preclinical mouse model of doxorubicin-induced congestive heart failure. However, PACAP analogs also protected breast cancer and pheochromocytoma cells in vitro against doxorubicin- and cisplatin-induced apoptosis, respectively. In contrast, PACAP analogs enhanced the killing of hematopoietic tumor cells in vitro by the commonly used anticancer agents carmustine, vincristine, and thalidomide in a dose-dependent manner. PACAP analogs also inhibited the proliferation of hematopoietic tumor cells, and this inhibitory effect was reduced by the MEK inhibitor PD98059 in a dose-dependent manner but was unaffected by various doses of the phosphodiesterase inhibitor isobutylmethylxanthine. These experiments suggest that PACAP analogs can be used effectively as adjunctive agents with commonly used chemotherapeutics for blood cancers.

The in vivo role of endogenous PACAP kidney ischemia/reperfusion: studies with knockout mice and radioimmunoassay

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The protective potential of PACAP is established in models of ischemia/reperfusion, myeloma kidney injury, and against gentamicin- and cisplatin-induced tubular degeneration. Our aim was to investigate the extent of

renal damage in PACAP-deficient ($PACAP^{-/-}$) mice and to measure the concentration of endogenous PACAP before and after ischemia/reperfusion kidney injury in rats. Mice underwent renal artery ligation for 45 or 60 min, 2 weeks reperfusion, then histological analysis. We found no histological differences between wild-type and $PACAP^{-/-}$ mice with no ischemia. However, ischemia/reperfusion induced a more severe tubular degeneration and resulted in a thinner cortex in $PACAP^{-/-}$ mice. Radioimmunoassay measurements were done in rats before and after ischemia/reperfusion kidney injury. PACAP38 concentration was significantly higher then PACAP27, and the cortex had higher concentrations of both forms of the peptide than the medulla. PACAP concentrations showed a marked decrease 1 and 6 h after ischemia/reperfusion, while a significant increase was found after 24 h. These results show that PACAP is endogenously present in the kidney in high concentrations, and it has a protective role in the kidney, since PACAP-deficient mice had a greater extent of tubular damage.

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The cell-specific promoter in upstream region of human PACAP testis-specific exon

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is significantly localized also in testis. Based on the report of rat PACAP testis-specific exon (PB Daniel et al., 2000), we identified mRNA of human PACAP testis-specific exon (hTE). Although there are some neuropeptides that also have testis-specific mRNA, the detailed regulatory mechanism of gene expression in testis remains unknown. Therefore, in order to clarify the mechanism of gene expression in testis, we attempted to characterize the upstream region of PACAP testis-specific exon. In 1.2 kb of the 5'-flanking region of hTE, we previously identified 80 bp as the region to demonstrate the potent promoter activity in a cell-specific manner in F9 mouse testicular teratoma cell. In the 80 bp, there are potential transcription factor-binding elements for AML-1, GATA1, and 2 (Tominaga A, 2007).

In the present study, we try to characterize the binding proteins to the 80 bp. Using electrophoresis mobility shift assay, the probe of the 80 bp generated several complexes with proteins of F9 nuclear extract, which were partially completed by two short probes of four equipartition of the 80 bp. Furthermore, some complexes were dissociated by high salt concentration of monovalent cation (0.4 M NaCl solution) although other complexes remained to retain. Because the dissociation of the complex under at low salt concentration means non-specific binding, we performed affinity purification using beads coupled to the 80 bp under the condition of high salt concentration. After washing the beads with 0.4 M NaCl solution, the bound proteins were eluted with 1 M NaCl solution and analyzed on SDS-PAGE. In result, the four bands corresponding to 105, 85, 45, and 35 kDa were observed. The detailed analyses of these proteins are in progress now.

The alternative regulation of pituitary adenylate cyclase-activating polypeptide (PACAP) gene expression by neural-restrictive silencer

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PACAP is a pleiotropic neuropeptide that belongs to the secretin/glucagon/VIP family. Previously, we undertook the detailed analysis of 5' upstream region of mouse PACAP gene and identified the neural restrictive silencer (NRS)-like elements (NRSLE1 and 2) in approximately 1.7–1.9 kbp upstream from the translation start site. Recently, we identified the third element (NRSLE3) which located in 200 bp downstream of the former sites. NRSLE3 is highly homologous to the consensus NRS sequence (Schoenherr et al., 1996) and conserved among mouse, rat, and human PACAP gene. In Swiss-3 T3 cells (non-neuronal cell line), SVL4 vector in which 5'-flanking region of mouse PACAP gene containing NRSLE1, 2, and 3 was inserted in the upstream of SV40 promoter showed about 40% of the activity of SV40 promoter (pGL3-promoter vector). However, in PC12 cells treated with NGF (neuronal cell line), SVL4 vector showed almost equi-potent activity with pGL3-promoter vector. The site-directed mutation in NRSLE3 only significantly attenuated the repression activity to the almost equi-potent activity with pGL3-promoter vector in Swiss-3 T3 cells. On the other hand, the repression of SV40 promoter activity was not changed by the site-directed mutation in NRSLE1 and/or NRSLE2. In the electrophoretic mobility shift assay with nuclear extracts of Swiss-

3 T3 cells and oligonucleotide probe of NRSLEs, a specific complex was observed to have the same migration as compared with the NRS probe of rat type II sodium channel gene (Mori et al., 1992), respectively. NRS binding factor (NRSF) has been shown to repress the expression of neuron-specific genes in non-neuronal cells. In the RT-PCR analysis, trichostatin A, a histone deacetylase inhibitor which indirectly inhibits NRSF-mediated gene silencing, increased PACAP mRNA level in PC12 cells. These data suggest that each NRSLE can associate with NRSF and NRSLE3 function as a repressor element in Swiss-3 T3 cells.

Regulatory mechanism of PAC1 gene expression by nerve growth factor (NGF) in PC12 cells

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Pituitary adenylate cyclase-activating polypeptide (PACAP) functions as a pleiotrophic neuropeptide like hypophysiotropic hormone, neurotransmitter, and neuromodulator or neurotrophic factor. PACAP exerts a variety of physiological functions through three types of G protein-coupled receptors, PAC1, VPAC1, and VAPC2. PAC1 binds to PACAP with higher affinity than VIP, and VPAC1 and VPAC2 bind to both PACAP and VIP with equally high affinity. PAC1 has several variants, by alternative splicing which of the mRNA may contribute to the functional pleiotropism of PACAP. Recently, we reported that nerve growth factor (NGF) augments neuroprotective effect of PACAP in PC12 cells, and that NGF increases PAC1 gene expression. In the present study, we attempted to clarify the regulatory mechanism of PAC1 gene expression by NGF. At first, by PCR, we cloned the 5' flanking region of human PAC1 gene of -2160~+268 bp including exon 1 and several transcription factor binding sites, such as genetic Sp1,GATA, and Zac1. In the next, we prepared a series of deleted mutants of promoter region and evaluated their promoter activity by a luciferase reporter assay. As a result, it was demonstrated that augmentation of promoter activity by NGF was attenuated by deleting −372~−252 bp containing two SP1 sites. The treatment with U0126 (MEK inhibitor) or mithramycin A (Sp1 DNA inhibitor) significantly attenuated its promoter activity, which was augmented by NGF. These indicate that activation of SP1 by *Ras*/MAPK pathway might participate in neuron-specific expression mechanism of PAC1 gene.

Pituitary adenylate cyclase-activating polypeptide (PACAP) induces activity-dependent gene expression through the potentiation of *N*-methyl-D-aspartate receptor (NMDA-R) in neurons

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of vasoactive intestinal peptide (VIP)/secretin/glucagon family and contributes to expressing a variety of neuronal functions including neuronal survival and plasticity. Recent studies suggest that PACAP is also related to psychiatric disorders, such as schizophrenia. In order to understand how PACAP is involved in expressing neuronal functions and causing disorders, in this study, we particularly focused on PACAP-induced gene expression in primary cultured rat cortical neurons. Using microarray analysis, we found that a limited number of genes, which encode factors involved in neuronal function, such as brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated protein (Arc), were up-regulated by the treatment with 100 nM PACAP38. The effect of PACAP-induced gene expression was dose-dependent, and greater than that of VIP. Interestingly, more than 50% of PACAP-inducible genes were up-regulated by PACAP, at least in part, through *N*-methyl-D-aspartate receptor (NMDA-R). Moreover, NMDA-induced gene expression was potentiated by PACAP, suggesting that PACAP modulates NMDA-R function but not affect glutamate release. To further elucidate the mechanisms for PACAP-induced gene expression, we performed promoter assay using luciferase reporter analysis. BDNF gene promoter IV, which is one of the alternative BDNF gene promoters and activated by neuronal activity, was entirely controlled by the calcium signals evoked via NMDA-R after PACAP-treatment. The promoter activation was dependent on cAMP-response element (CRE) and its binding transcription factor, CRE-binding protein (CREB), suggesting that PACAP induces NMDA-R-derived CRE/CREB-dependent transcription. These results indicate that PACAP can induce activity-dependent gene expression through the potentiation of NMDA-R function and may contribute to neuronal development.