REVIEW

P2X3-Containing Receptors as Targets for the Treatment of Chronic Pain

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Abstract

Current therapies for the treatment of chronic pain provide inadequate relief for millions of suffering patients, demonstrating the need for better therapies that will treat pain effectively and improve the quality of patient's lives. Better understanding of the mechanisms that mediate chronic pain is critical for developing drugs with improved clinical outcomes. Adenosine triphosphate (ATP) is a key modulator in nociceptive pathways. Release of ATP from injured tissue or sympathetic efferents has sensitizing effects on sensory neurons in the periphery, and presynaptic vesicular release of ATP from the central terminals can increase glutamate release thereby potentiating downstream central sensitization mechanisms, a condition thought to underlie many chronic pain conditions. The purinergic receptors on sensory nerves primarily responsible for ATP signaling are P2X3 and P2X2/3. Selective knockdown experiments, or inhibition with small molecules, demonstrate P2X3-containing receptors are key targets to modulate nociceptive signals. Preclinical studies have identified that P2X3-containing receptors are critical for sensory transduction for bladder function, and clinical studies have shown promise in treatment for bladder pain and pain associated with osteoarthritis. Further clinical characterization of antagonists to P2X3-containing receptors may lead to improved therapies in the treatment of chronic pain.

Key Words P2X3 · ATP · nociception · sensitization · pain · hyperalgesia

Introduction

Pain is one of the most common reasons for patients to seek medical attention and is a major burden on the quality of life for those who suffer and their caregivers. Chronic pain, defined as pain that lasts or recurs for more than 3 to 6 months [1], is estimated to affect up to 116 million adults in the USA alone with hundreds of millions of dollars in lost productivity annually [2]. While nociceptive pain, also known as "protective pain," is important for survival, many forms of chronic pain linger long after a healed injury and do not appear to serve any useful purpose [3]. Current therapies used to treat chronic pain are woefully inadequate with many dose-limiting side effects. The difficulty in getting effective treatment for

chronic pain is thought to be a key factor in many patients also developing severe psychiatric comorbidities, with 45% suffering from major depressive disorder (MDD) as opposed to 5% in the general population [4]. Increased understanding of the mechanisms that underlie chronic pain is needed to develop new therapies with improved clinical outcomes.

Adenosine triphosphate (ATP) is well known as the principal source of energy for cellular metabolism, but it was first postulated in 1953 that ATP can also serve to modulate neurotransmission. In a rabbit ear preparation, exogenously applied ATP caused antidromic vasodilation that was reduced with the co-application of a cholinesterase inhibitor [5], although at the time the receptors responsible for this effect were not known. The advent of molecular cloning and heterologous expression systems allowed researchers to fully characterize what is now known as the purinergic receptor family. Receptors that activate when exposed to ATP are called P2 receptors and can exist as ligand-gated ion channels (P2X1-P2X7) [6, 7] and G protein–coupled receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11-P2Y14). Adenosine-responding receptors, originally classified as P1 receptors, are now called A1, $A2_A$, $A2_B$, and A3. The nomenclature and pharmacology of

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P2Y and A1–A3 receptors are covered in several excellent reviews [8–13] and therefore will not be a focus here.

P2X receptor subunits form trimeric transmembrane complexes consisting of homomeric and heteromeric ionotropic receptors [7, 14]. Receptors that contain a P2X3 subunit (P2X3 homomers and/or P2X2/3 heteromers) will be referred to as P2X3-containing receptors. Confirmation of the formation of the P2X2/3 heteromer was verified by co-immunoprecipitation experiments and patch-clamp electrophysiology which possessed properties that match ATP-generated currents in neurons [15, 16]. The exact stoichiometry of the P2X2/3 heteromer in native tissue remains to be determined [17]. Experiments with heterologously expressed rP2X2 receptor showed ATP-induced currents exhibited strong cooperativity with a n_H approximately 2, suggesting that more than one ATP molecule must bind before the channel opens [18]. Recent structural data of the human P2X3 receptor reveal the key protein movements that occur upon the binding of ATP to induce a conformational change which leads to the opening of the ion pore [19]. When exposed to ATP, P2X receptors open within milliseconds and are non-selective cation channels, with significant permeability to calcium [20]. Ion channel current properties (e.g., rate of inactivation, tachyphylaxis) and pharmacology can vary dramatically between P2X channels formed by different subunit combinations [6, 7, 20, 21].

P2X3 subunits are primarily expressed in peripheral nerves that innervate a variety of tissues important for nociception [22–26], chemosensation [27], and tissue function such as sensing the stretching of hollow organs (e.g., bladder) [28, 29]. Antagonists selective for P2X3-containing receptors (homomeric or heteromeric) have demonstrated efficacy in a wide range of preclinical models of micturition disorders [30–33], pulmonary hypertension [27], and cough [34, 35], while selective antagonists to P2X3-containing receptors have demonstrated clinical efficacy in chronic refractory cough [36–38]. A common feature of all these symptoms is a key role for extracellular ATP that sensitizes sensory nerve endings thereby driving a hyperexcitable state, primarily acting through P2X3-containing receptors. This review will summarize the importance of ATP as a modulator of nociception, the preclinical evidence for a role of P2X3-containing receptors in pain signaling, and the potential for clinical development of antagonists selective for P2X3-containing receptors as a novel mechanism for treating chronic pain.

ATP as a Modulator of Nociceptive Signaling

The critical role for ATP as a sensory signaling neurotransmitter has been studied for over 50 years [39]. Evidence pointing to a nociceptive role for ATP first came from clinical studies where administration caused a painful reaction when injected into a blister base [40] or when intracutaneously injected into normal skin [41]. Hamilton et al. demonstrated that iontophoretic application of ATP to the skin of human volunteers caused a dose-dependent increase in pain similar to a burning and stinging sensation [42]. Importantly, when applied acutely, ATP induced depolarizing currents in dissociated sensory neurons isolated from dorsal root ganglia (DRG) [24, 43–45] and nodose ganglia [46–48], suggesting purinergic receptors expressed in nociceptors might be mediating the observed clinical effects.

Exogenously applied ATP is readily hydrolyzed *in vivo* into ADP, which is then further metabolized to adenosine, both of which are both bioactive and their downstream activities could confound interpretation of the effects ascribed to ATP [8, 9, 11, 13]. The ATP analog α,β-methylene ATP (α,β-MeATP) is more resistant to hydrolysis making it an ideal tool to study the role of P2X receptors *in vivo*. In rats, intraplantar administration of α,β-MeATP causes nocifensive behavior in a dose-dependent manner [49]. The flinching behavior can be prevented by desensitization via 30-min local pretreatment with α,β-MeATP, whereas pretreatment with α,β-MeATP does not influence nocifensive behavior caused by intraplantar injection of bradykinin (1 nmol) or formalin (0.5%, 0.1 ml). Interestingly, local pretreatment of the TRPV1 agonist capsaicin (100 micrograms) abolished nocifensive behavior caused by α,β-MeATP, bradykinin, and formalin, indicating that receptors for each of these ligands are colocalized with TRPV1 on sensory fibers [49]. Similar findings have been observed in human psychophysical studies, where transcutaneous iontophoresis of ATP produced an increase in pain reporting and prior desensitization of skin with capsaicin abolished the ATP-induced pain responses [42]. Direct evaluation of the effects of ATP analogs on nociceptor subtypes can be done using an ex vivo skin-nerve prep in rodents or microneurography in humans, which allows classification of sensory fiber types based on conduction velocity and responses to thermal and mechanical stimuli. In naïve rodent skin, application of α,β-MeATP to sensory nerve endings generated an increase in activity in about 40% of the nociceptive units, with 75% of those responders identified as C-mechanical and heat sensitive (CMH or polymodal nociceptors), and the remaining being C-mechanonociceptors (CMs) [50]. Microneurography studies in human volunteers demonstrate 64% of CM fibers and 63% C-mechanoinsensitive fibers (CM_i) respond to acute ATP injection. Thermal responses of the CM_i fibers allow further classification as mechanoinsensitive, heat responsive (CH 9/11 units) and mechanoinsensitive, heat insensitive (CM_iH_i 5/11 units) [51]. Collectively, these studies demonstrate that ATP, or ATP analogs, can elicit a painful response through direct excitation of nociceptive fibers.

Further experimentation with local application of α,β-MeATP showed a potentiation of nocifensive behavior under

conditions where the tissue is injured or inflamed. The concentration threshold to detect a behavioral response to intraplantar α,β-MeATP shifted 100-fold lower in skin pretreated with dilute carrageenan solution compared to normal skin [52]. Additionally, intraplantar co-administration of α,β-MeATP with PGE2 generated a nocifensive response greater than that observed with administration of each agent alone, indicating a synergistic relationship exists between peripheral purinergic and prostaglandin nociceptive signaling [52]. Co-administration of α,β-MeATP with a low dose of formalin dose-dependently enhanced the phase 2 response (12–60 min after application) but not phase 1 (0–10 min) implying an enhancement of mechanisms that lead to central sensitization [53]. In skin previously sensitized with carrageenan, activity in C-MH fibers were potentiated with α,β-MeATP at a much lower concentration compared to naïve skin, which temporally correlated with nociceptive behavior in conscious rats highlighting a link between neuronal hyperexcitability and pain-related behavior [50, 52]. A similar increase in α,β-MeATP-induced sensitivity was observed in rat skin pretreated with Complete Freund's Adjuvant (CFA) [54]. In healthy volunteers whose skin was sensitized with capsaicin or UVb irradiation, ATP-induced pain behavior was significantly enhanced compared to naïve skin [42]. Interestingly, ATP alone is not sufficient to sensitize C-nociceptors to thermal or mechanical stimuli [51], suggesting that a co-factor which is released only under sensitized or inflamed conditions may be needed to potentiate the signaling of ATP in sensory fibers. Collectively, these studies demonstrate that ATP, or ATP analogs, can elicit painful responses through direct excitation of nociceptive fibers and potentiate activity in these fibers under inflamed or sensitized conditions, and that many of these findings are translatable to humans.

Expression and Function of P2X3-Containing Receptors in Nociceptive Pathways

Considerable heterogeneity can be observed in ATP-generated currents in sensory neurons, which can consist of rapidly inactivating currents with pronounced tachyphylaxis or those with more sustained current kinetics that do not exhibit tachyphylaxis [24, 29, 44, 45, 56]. The percentage of acutely isolated sensory neurons that generate ATP-sensitive currents range from 40 to 95%, with the differences likely due to the species tested and neuronal culture conditions [24, 43–45]. Messenger-RNA localization and immunohistochemical studies demonstrate that many sensory neurons express both P2X2 and P2X3 subunits [45, 57, 58] in addition to P2X1 [56, 59]; however, see [60]. Heterologous expression of P2X3 [61] and electrophysiology studies in neurons from P2X3$^{-/-}$ animals [28, 29, 62] have identified the P2X3 homomer as responsible for the rapid transient currents in

DRG, while co-expression of P2X2 with P2X3 can produce heteromers [15, 16, 63] and yield currents with significantly slower inactivation kinetics that resemble the sustained currents seen in DRG and nodose neurons [15, 58]. Expression of P2X3 is enriched in small diameter nociceptors that co-express isolectin B4 (IB4) and glial-derived neurotrophic factor (GDNF), indicating these neurons are non-peptidergic, as well as in a subpopulation of Aδ-fibers [24, 45, 64, 65]. In mouse sensory neurons, P2X3 is also co-expressed with MrgprD, which are classified as nociceptors due to the presence of TTX-resistant Na$^+$ currents and Ca^{2+} currents that are inhibited by μ-opioids [66, 67]. P2X3 subunits can also form a cognate receptor with other structurally related Cys-loop receptors. Heterologous co-expression of P2X3 with ASIC3, an important receptor for pain signaling in its own right [68, 69], demonstrates cross-functional regulation of receptor activity in a calcium-dependent manner [70]. P2X3 and ASIC3 can be coimmunoprecipitated from rat DRG membranes demonstrating a close spatial association. *In vivo* studies demonstrate a facilitation of α,β-MeATP-mediated nocifensive behavior with low pH, an activator of ASIC3 receptors, when coadministered intraplantar. Collectively, these data suggest the P2X3/ASIC3 cognate receptor may be important for acidic and purinergic-mediated nociceptive signaling in the periphery [70]. P2X3 is also often co-expressed with the nociceptive marker TRPV1 [22–26]. The axons of TRPV1$^+$/ATP-sensitive neurons project and terminate in lamina II$_{inner}$ in the dorsal horn with P2X3 immunoreactivity concentrated in the central terminals [28, 57, 71–73]. Other neurons that are TRPV1$^-$/ATP-sensitive project deeper into lamina V, indicating distinct purinergic pathways exist with divergent sensory signaling [25, 74]. Peripherally, P2X3 is expressed in sensory nerve terminals found in several tissues, including skin and hollow organs in the viscera such as bladder and endometrium tissue [57, 71, 75, 76]. The proposed mechanism of peripheral and presynaptic P2X3-containing receptors in modulating nociceptive behavior will be a subject of further discussion later in this review (see "Site of Action of P2X3-Containing Receptor Modulation of Nociceptive Signaling").

It is important to note that the expression profile of P2X3 and P2X2/3 in sensory neurons has important differences when comparing preclinical species and primates. While P2X3 homomers and P2X2/3 heteromers are both widely expressed in rodent sensory neurons [24, 29, 44, 45, 56–58], neurons isolated form non-human primate and human DRG show robust P2X3-subunit expression with undetectable P2X2-subunit expression as determined with reverse transcriptase PCR (RT-PCR) and immunohistochemistry (IHC) [77]. This expression profile was confirmed using electrophysiology in non-human primate DRGs where 256/256 neurons have a transient α,β-MeATP-induced current and zero neurons have slowly desensitizing currents consistent with a lack of P2X2/3 receptors [77]. These findings may impact the

translation of preclinical findings to clinical efficacy, particularly when interrogating nociceptive mechanisms driven primarily through P2X2/3 heteromers.

Preclinical Evidence Suggesting Therapeutic Utility of P2X3 Receptor Antagonists for Chronic Pain

Several lines of evidence point to P2X3-containing receptors as an attractive target for the treatment of some types of chronic pain: (a) elevated levels of the P2X agonist ATP are found in injured or inflamed tissue [78–80], (b) P2X3 subunits are expressed in sensory neurons in both preclinical and clinical tissue [57, 72, 77], (c) P2X3 expression in neurons demonstrate plasticity ipsilateral to the site of nerve injury [71, 81, 82] and inflammation [83, 84], and, finally, (d) P2X3-containing receptor agonists directly activate sensory C-fibers and cause nocifensive behavior in both preclinical and clinical studies [42, 50, 51].

Previous efforts to study P2X3-containing receptors and the role they play in sensory transduction *in vivo* were hampered by the lack of selective ligands (see Table 1) and reviewed in [21, 85, 86].

One of the first non-nucleotide P2X ligands with potent and selective inhibition of P2X3-containing channels was 5-[[[(3-phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl]-1,2,4-benzenetricarboxylic acid (A-317491), a selective P2X3-containing receptor antagonist with >100-fold selectivity over other P2 receptors and non-purinergic receptors [87]. When dosed systemically, A-317491 was efficacious in the CFA model of inflammatory pain (thermal hyperalgesia and allodynia endpoints), as well as in the chronic constriction injury (CCI) model of nerve injury (thermal hyperalgesia and allodynia). The *in vivo* efficacy of A-317491 was likely due to its activity on P2X3-containing receptors since an equivalent dose of the (R)-enantiomer A-317344, which is significantly less active against P2X3-containing receptors *in vitro*, was ineffective in reversing hyperalgesia in these models [87]. Interestingly, A-317491 was inactive in models of acute nociception with

noxious stimuli, including thermal, mechanical, and intraplantar capsaicin. These data support earlier observations of enhanced responsiveness to sensory ATP signaling only under conditions where the tissue is previously sensitized or injured [42, 51, 52]. More recently, structurally distinct antagonists of P2X3-containing receptors have demonstrated efficacy in preclinical models of pain. Gefapixant (AF-219/MK-7264), a selective P2X3-containing receptor antagonist [89], was examined in two models of inflammation: CFA and mono-iodoacetate-induced experimental osteoarthritis model of the knee. Gefapixant showed robust reversal of hypersensitivity when dosed orally at 30 mpk with paw withdrawal threshold (Von Frey) and weight-bearing endpoints with efficacy similar to naproxen in both models. In the spared nerve injury (SNI) model of neuropathic pain, 30 mpk p.o. gefapixant fully reversed mechanical hyperalgesia (Von Frey) and weight-bearing endpoints with efficacy equivalent to that of gabapentin [89]. Finally, two representative compounds from a structurally distinct series of selective P2X3-containing antagonists, MK-3901 [92], and MK-2548 [93], demonstrated efficacy when dosed systemically in preclinical chronic models of pain. Selective P2X3-containing receptor antagonists have also been evaluated in preclinical models of cancer pain. Chronic pain in patients suffering from bone cancer is one of the most commonly reported maladies and is also one of the most difficult to manage [94, 95]. Evidence exists that P2X3 expression is upregulated on nerve fibers in preclinical models of bone cancer pain [96, 97]. In a mouse tumor model, the concentration of extracellular ATP increased from undetectable levels in tumor-free tissue to several hundred micromolar in the tumor interstitium [98], which is high enough to directly activate P2X3-containing receptors [20]. AF-353 (formally RO-4), a potent and selective antagonist of P2X3-containing receptors [99], was tested in a model of bone cancer pain established by the introduction of MRMT-1 mammary gland carcinoma cells onto the tibia of rats, a model that generates an increase in tactile hypersensitivity approximately 10 days after implantation [100]. When dosed prophylactically, AF-353 prevented the development of tactile hypersensitivity and deficits in weight-bearing, although this did not translate to improvements in bone density [101]. In another

Table 1 P2X3-containing receptor antagonists

Compound name	P2X3 IC$_{50}$ (nM)	P2X2/3 IC$_{50}$ (nM)	Reference
A-317491	100	100	[87]
AF-353 (RO-4)	10	80	[88]
AF-792 (compound 28, RO-5)	6	13	[88]
Gefapixant (AF-219/MK-7264)	150	220	[89]
Compound 15 h	280	>2500	[90]
TNP-ATP	1	7	[21]
PPADS	1000	1000	[91]

paradigm, AF-353 significantly reversed tactile hypersensitivity when dosed at day 17 (peak-onset); however, weight-bearing was unaffected. Mechanical stimulation of MRMT-1 cells *in vitro* leads to a 5-fold increase in ATP release; however, this was not affected with the co-incubation of 25 μM AF-353 demonstrating the behavioral effects of AF-353 are not due to changes in ATP release [101]. In a similar model of cancer pain, A-317491 dose-dependently suppressed thermal hyperalgesia produced by the intratibial inoculation of NCTC 2472 fibrosarcoma cells to C3H/HeJ mice [102]. However, when 4T1 mammary carcinoma cells were co-inoculated with NCTC 2472 fibrosarcoma cells, A-317491 did not demonstrate a robust effect, indicating that pain signaling induced only by specific tumor cell types may be sensitive to P2X3-containing receptor antagonists [103]. Large molecule antagonists to P2X3-containing receptors have also been identified. The monoclonal antibody 12D4, directed to the P2X3-subunit, exhibits potent inhibition of P2X3 currents evoked by α,β-MeATP in 1321N1 cells and in acutely dissociated rat DRG neurons [104]. Short-term exposure of cells expressing P2X2/3 show a potentiation of α,β-MeATP currents; however, longer exposures inhibit these currents. Interestingly, 12D4 appears to bind to the desensitized state of P2X3-containing receptors, and prolonged incubations drive membrane internalization leading to a decrease in current density [104]. *In vivo* studies with 12D4 dosed 3 days prior to balloon distention demonstrate efficacy in the TNBS-induced colitis visceral pain model, although 12D4 was inactive in the formalin model and in CFA with a thermal withdrawal endpoint with the same dosing regimen [104]. Post hoc confirmation of successful P2X3-receptor internalization on DRG neurons was not presented in these *in vivo* studies; therefore, a potential explanation for the limited efficacy profile is an insufficient reduction in P2X3 surface expression with systemic dosing.

Further evidence supporting the role of P2X3-containing receptors in nociceptive signaling comes from selective knockdown of P2X3 and P2X2 subunits. Cockayne et al. confirmed selective knockdown of P2X3 by the absence of all transient ATP and α,β-MeATP-induced currents in both DRG and nodose ganglia and the elimination of P2X3 immunoreactivity in DRG and the central terminals of the spinal cord [28, 29]. These P2X3$^{-/-}$ mice had a reduced response to intraplantar flinching in response to α,β-MeATP [28], and in both acute and persistent phases of the formalin model [28, 62]. Consistent with what has been observed with pharmacological tools, P2X3$^{-/-}$ mice had normal responses to acute noxious stimuli, including hot plate, paw flick, and tail pressure when compared to wild type [62]. Interestingly, anesthetized P2X3$^{-/-}$ mice demonstrated a deficit in the coding for innocuous warmth as measured using *in vivo* spinal cord recordings, although mechanical coding remained intact [62]. However, conscious P2X3$^{-/-}$ animals exhibited an enhanced thermal avoidance behavior, indicating compensatory

changes may contribute to the P2X3$^{-/-}$ phenotype [105]. Selective knockdown of P2X3 using intrathecally delivered antisense confirmed many of these findings, showing significant blockade of acute behavior associated with intraplantar injections of α,β-MeATP and formalin, as well as in models of chronic pain [106, 107]. Furthermore, acute thermal hyperalgesia post-carrageenan with P2X3 antisense was not different than control which is consistent with findings in experiments with P2X3$^{-/-}$ mice [62]. P2X3 antisense knockdown also reversed mechanical allodynia in the partial sciatic ligation (PSL) [106] and SNL neuropathic injury models [107]. These findings are particularly interesting, as previous studies demonstrated a significant decrease in P2X3 expression in small diameter neurons in L5-L6 DRG ipsilateral to the site of injury, but an increase in P2X3 expression in large diameter neurons [108], consistent with findings in sciatic nerve axotomy [71, 82]. Taken together, these data suggest that increased P2X3-receptor activity on large diameter neurons may play a significant role in the transmission of mechanical allodynia associated with nerve injury and neuropathic pain. P2X2$^{-/-}$ mice, where P2X3 homomers are intact and P2X2/3 heteromeric receptors are absent, demonstrate a phenotype distinct from that of the P2X3$^{-/-}$ counterparts. For example, intraplantar injection of α,β-MeATP generated similar nocifensive responses in P2X2$^{-/-}$ mice compared to wild type, indicating P2X3 homomers are likely responsible for the acute behavior seen with α,β-MeATP [29]. Of particular interest, P2X2$^{-/-}$ mice exhibited acute flinching behavior similar to that of wild type after formalin injection while the persistent phase was unaffected [28, 29, 62]. These data contrast with P2X3$^{-/-}$ animals, with both P2X3 homomers and P2X2/3 heteromers absent, which showed a deficit in both the acute and persistent phases. The use of selective knockdown of P2X3 and P2X2 subunits suggests that P2X3 homomers have a key role in acute peripheral sensory transmission while P2X2/3 receptors may contribute to the mechanisms that underlie central sensitization. This topic will be explored further in more detail in the next section.

Site of Action of P2X3-Containing Receptor Modulation of Nociceptive Signaling

Sensory nerves are pseudounipolar, with axons extending from the soma that receive signals from the periphery and relay this information to the central nervous system via central terminals innervating the spinal cord [109]. Immunohistochemical evidence of sensory nerves demonstrate that P2X3-subunits are concentrated in both peripheral and central terminals as well as the cell bodies of the primary sensory afferents [57, 72, 75, 110, 111], which place P2X3-containing receptors in key locations to initiate and modulate nociceptive activity.

In the periphery, there are several sources of ATP that could initiate P2X3-mediated signaling. It is estimated that the cytosolic concentration of ATP is approximately 1–10 mM [112]; therefore, large amounts of ATP can be released upon tissue injury. Indeed, in a human incision model, levels of extracellular ATP increased from 0.18 to 14 μM 3–5 min post injury to the skin [78], concentrations high enough to directly activate P2X3-containing receptors [7, 20]. Utilizing an *in vitro* model of tissue injury, lysis of skin cells adjacent to cultured DRG neurons induced depolarizing currents that were abolished with enzymatic degradation of ATP, prior desensitization with ATP, or pharmacologic blockade of P2X receptors [113]. Peripherally, ATP can be released from inflammatory cells via active vesicular release as an "injury" signal [114] that can directly act on P2X3-containing receptors on peripheral nerves thereby initiating a nociceptive response [75, 113]. Synaptic release of ATP has also been studied in peripheral sympathetic nerves. Pioneering work by Geoffrey Burnstock demonstrated that ATP is a cotransmitter with noradrenaline (NA) in sympathetic nerves that innervate guinea pig vas deferens which control vascular tone [115]. Experiments in healthy volunteers demonstrated purinergic antagonists delivered via microdialysis significantly inhibited vasoconstriction induced by whole-body cooling [116]. Primary afferent sensitization can also be subject to modulation via sympathetic efferent activity through P2X3-mediated mechanisms. Recording from single fibers, intraplantar capsaicin enhanced the response of Aδ- and C-fibers to mechanical stimuli only in animals with intact sympathetic postganglionic efferents [117]. Further experimentation showed that the P2X3-containing receptor antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS [21]) could prevent the capsaicin-enhanced mechanical sensitivity in these fibers in sham sympathectomized animals and pretreatment with α,β-MeATP could restore this sensitivity in sympathectomized rats [118]. In naïve animals, capsaicin-induced sensitization of primary afferents also led to an increase of TRPV1 and calcitonin gene-related peptide (CGRP) expression in sensory neurons, which was not observed in sympathectomized rats. In these sympathectomized rats, the addition of α,β-MeATP facilitated the capsaicin-induced neuronal upregulation of TRPV1 and CGRP in sensory neurons. Application of uridine triphosphate (UTP), a P2Y-preferring partial agonist, was not effective thereby highlighting the importance of P2X3-containing receptors in this mechanism of plasticity [119].

In addition to peripheral mechanisms, synaptic release of ATP from the central terminals of primary afferents has a significant role in modulating nociceptive signals in the spinal cord. Evidence supporting presynaptic release of ATP comes from experiments using spinal cord synaptosomes which show elevated levels of ATP upon evoked release [120]. Furthermore, evoked excitatory postsynaptic potentials (EPSCs) in a transverse spinal cord slice generate a current in lamina II sensitive to suramin, a P2X antagonist [121]. Exogenously applied ATP has been demonstrated to influence glutamate signaling in the spinal cord. When recording from the superficial dorsal horn (substantia gelatinosa), bath-applied ATP elicits fast inward glutamate currents that were inhibited by P2X antagonists [122, 123]. Gu and MacDermott showed that in a DRG-dorsal horn coculture, ATP can increase the frequency of miniature evoked postsynaptic currents (mEPSCs) that were inhibited by NMDA antagonists, indicating an increase in presynaptic glutamate release [124]. Direct application of ATP to isolated dorsal horn neurons did not potentiate glutamate currents, evidence that ATP is likely exerting its effects presynaptically. In a spinal cord slice, application of α,β-MeATP increased glutamatergic transient activity in lamina II and long-lasting activity in lamina V [73]. Selective ablation of IB4-containing neurons eliminated both the presynaptic P2X3 immunoreactivity in lamina II and the transient α,β-MeATP-induced signaling while leaving the long-lasting modulation in lamina V intact, evidence that P2X3-containing receptors are primarily responsible for the modulatory effects of ATP in lamina II [73]. In total, these studies indicate that release of ATP from primary afferent central terminals can act presynaptically on P2X3-containing receptors facilitating glutamate release which could influence nociceptive signaling. Data supporting this hypothesis comes from studies with intrathecal delivery of α,β-MeATP which caused a dose-dependent increase in thermal hyperalgesia that was blocked by co-administration of PPADS and 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP), a P2X3-containing receptor antagonist [21, 125]. This effect was not seen with the P2X1 preferring agonist β, γ-methylene-L-ATP demonstrating the specific contribution of P2X3-containing receptors to this effect.

The site of action where P2X3-containing receptors play a key role in behavioral responses has been evaluated pharmacologically using local administration of antagonists. In the CFA model of inflammatory pain, intraplantar TNP-ATP caused a partial reversal of mechanical hyperalgesia [126]. Spinal, but not intraplantar, administration of A-317491 produced robust reversal of mechanical allodynia in CCI and L5-L6 spinal nerve ligation nerve injury models [127]. Both routes of administration of A-317491 demonstrated efficacy in the acute and persistent phases of the formalin model; however, the reversal of flinching behavior was more robust when dosed intrathecally. In models of inflammation, intraplantar A-317491 elicited a partial dose-dependent reversal of thermal hyperalgesia in the CFA model but was ineffective in acute carrageenan with the same endpoint consistent with earlier reports using antisense P2X3 and in P2X3$^{-/-}$ mice [62, 107]. Data supporting peripheral P2X3-containing receptors playing a key role in

mechanical allodynia in chronic inflammation is supported in rat skin-nerve preparations where A-317491 completely blocked mechanical sensitization of C-fibers caused by α,β-MeATP in CFA-treated skin [54]. *In vivo* studies using selective antagonists to P2X3 homomers have also shed light on the specific role of these receptors in nociceptive signaling. Cantin et al. reported that systemically dosed compound 15 h, reportedly > 10-fold selective for P2X3 homomers over P2X2/3 heteromers, dose-dependently reverses mechanical hyperalgesia 96 h post-CFA using a mechanical endpoint with free plasma exposures approximately equal to the rat P2X3 IC_{50} at the highest dose tested [90]. In contrast to A-317491, local spinal delivery of compound 15 h was ineffective in CFA while both compounds were effective when dosed intraplantar [90, 127]. Several notable differences between the studies using compound 15 h and A-317491 may account for the discrepancies, such as duration post-CFA when compounds were tested (96 h vs. 48 h), measured endpoints (mechanical allodynia vs. thermal hyperalgesia), and P2X3/P2X2/3 pharmacology (> 10-fold selective for P2X3 homomers vs. non-selective). Assuming local delivery of compound 15 h achieved concentrations that enabled selective inhibition P2X3 homomers over P2X2/3 heteromers, which was 5 mM at the top dose, peripheral P2X3 receptors appear to play a primary role over P2X2/3 in mechanical inflammatory pain and efficacy is possible with peripherally restricted compounds. On the other hand, these data also suggest that P2X3 homomers expressed in the central terminals may play a minor role compared to P2X2/3 heteromers in modulating nociceptive signaling due to mechanical inflammatory pain. However, interpreting *in vivo* results with local delivery of compounds (e.g., intraplantar and intrathecal) is hampered by poor control of compound exposures, therefore potentially engaging other targets important for nociception which can confound interpretations. Experiments with systemically delivered compounds with well understood pharmacokinetics and target pharmacology will be needed to understand if engaging P2X3-containing receptors on central terminals provides added efficacy compared to peripherally restricted compounds. It is also not yet known whether selective inhibition of P2X3 homomers will demonstrate efficacy in preclinical neuropathic pain models as is observed with the non-selective P2X3/P2X2/3 antagonists, or if efficacy with selective inhibition of P2X3 homomers is limited to inflammatory conditions. Given the low expression of P2X2/3 receptors in primate sensory neurons [77], this is an area where additional investigation is needed as both selective P2X3 homomer antagonists and non-selective P2X3/P2X2/3 antagonists are in clinical development.

Clinical Programs Investigating P2X3-Containing Receptor Antagonists in Chronic Pain

Sensory afferents that innervate the urinary bladder originate from the pelvic nerves and primarily consist of Aδ- and C-fibers, along with sympathetic efferents from the thoracolumbar spinal cord [31], and respond in a graded manner to distension of the bladder [128]. Hydrostatic extension of rabbit bladder induced the release of ATP which occurred in the presence of intraluminal TTX and required an intact epithelial layer, indicating that a non-vesicular mechanism is involved [129, 130]. The physiologic role of P2X3-containing receptors in this signaling pathway was investigated using P2X3$^{-/-}$ and P2X2$^{-/-}$ mice. Immunohistochemical staining in bladder tissue identified P2X3 expression in the subepithelial plexus which was absent in P2X3$^{-/-}$ mice while the overall organization of the tissue remained unchanged [28, 131]. Intraluminal ATP release in response to bladder distension of P2X3$^{-/-}$ mice was indistinguishable from wild type [131]; however, pelvic afferents showed an attenuated response to bladder distension indicating P2X3-containing receptors are important for downstream purinergic signaling and not ATP release [29, 131]. Cystometry studies can measure the repetitive urinary muscle contractions of the micturition reflex in response to slow intravesical infusion of saline. Compared to wild-type animals, both P2X3$^{-/-}$ and P2X2$^{-/-}$ mice show an increase in the volume of saline required to initiate the reflex, and a decrease in the number of evoked contractions [28, 29]. Local inhibition of P2X3-containing receptors via desensitization with intravesical infusion of α,β-MeATP [132], or inhibition with the antagonists TNP-ATP or PPADS [131], suppressed bladder afferent activity in response to distension. Spinal administration of the selective P2X3-containing receptor antagonist AF-792 (formally RO-5) dramatically suppressed bladder contractions to a slow intravesical infusion of saline [91]. Therefore, as in somatosensory nociception, P2X3-containing receptors play a key role in bladder signaling pathways at both a peripheral and spinal level. Evidence supporting a role for P2X3-containing receptors in human bladder pathology comes from studies using tissue isolated from patients. *In vitro* investigation of bladder urothelial cells (BUCs) from healthy volunteers and patients suffering from interstitial cystitis (IC) demonstrates greater ATP release in response to stretching in IC BUCs [133] which correlated with a significant increase in P2X3 immunoreactivity [134]. Additionally, tissue biopsies taken from patients suffering from intractable detrusor overactivity (DO) demonstrated a significant decrease in PGP9.5(+) P2X3-immunoreactivity after intravesical treatment with botulinum neurotoxin type A (BoNT/A), which correlated with reduction of urgency episodes at 4 and 16 weeks [135]. Although BoNT/A has known effects on presynaptic neurotransmitter release [136], the

effect on decreasing the number of P2X3-containing sensory fibers and associated downstream purinergic signaling in the bladder may also contribute to the clinical benefit [137, 138]. Based on the evidence supporting a role for P2X3-containing receptors in bladder pathophysiology, a phase 2a study with gefapixant was initiated in patients suffering from interstitial cystitis/bladder pain syndrome (IC/BPS) [139, 140]. Women aged 18–80 years of age who were diagnosed with IC/BPS for more than 6 months and moderate to severe pain measured by the mean daily Numeric Pain Rating Scale (NPRS) were entered into the study. The trial design was a 4-week double-blind treatment with 74 female patients (38 placebo and 36 gefapixant). Patients taking gefapixant were dose titrated starting from 50 mg BID and increased 50 mg daily to a maximum dose of 300 mg BID. After the 4-week treatment, gefapixant-treated patients had a decrease in the NPRS score from 6.2 at baseline to 3.3 compared to 6.5 to 4.5 in placebo-treated patients ($p = 0.019$). Patients in the treatment arm also had significant improvements in urge to urinate and in both Patient's and Clinician's Global Impression of Change compared to placebo ($p = 0.038$). The most frequent reported adverse event in treated patients was dysgeusia/hypogeusia [139].

Several studies have demonstrated a link between increased levels of ATP in synovial fluid (SF) and joint pain associated with arthritis. For example, SF analyzed from patients diagnosed with osteoarthritis (OA) with knee pain showed elevated levels of ATP compared to SF from patients that had recovered from knee pain [79]. After 5 weeks of treatment with intraarticular injections of the viscosupplement high molecular weight hyaluronic acid (HA), the OA patient group had a significant reduction in SF ATP levels that strongly correlated with a reduction in the Visual Analog Scale (VAS) score [79]. In preclinical studies, elevated SF ATP concentrations were also observed in the stifle joints of dogs with urate-induced synovitis and also in dogs with naturally occurring OA compared to normal joints [80]. In the rat, a subpopulation of C- and Aδ-fibers that innervate the knee joint demonstrates increased excitation upon intraarticular injection of α,β-MeATP and ATP which is prevented with co-application of PPADS [55]. Also in rats, intraarticular co-administration of A-317491 with carrageenan dose-dependently prevents the development of hyperalgesia which temporally correlates with lower joint inflammatory cytokine levels and neutrophil infiltration, evidence supporting a role for P2X3-containing receptors in joint pain associated with inflammation [141]. In light of the preclinical evidence and human biology, gefapixant was evaluated in a phase 2 clinical study in patients suffering from pain associated with OA [142, 143]. Patient inclusion criteria included having OA of ≥ 6-month duration and required baseline average daily pain between 5 and 9 on the Numeric Pain Rating Scale (NPRS). The primary endpoint was change from baseline (week 4) of weekly average daily NPRS. A total of 134 patients completed 4 weeks treatment (gefapixant $n = 56$, placebo $n = 78$). Patients in the gefapixant treatment arm demonstrated a reduction in NRPS over placebo at all time points, with the greatest effects seen at week 2 ($p = 0.04$). Placebo patients took significantly more rescue medication over all 4 weeks [142]. No serious adverse events were noted, and most common adverse finding in the gefapixant treatment arm was dysgeusia/hypogeusia.

P2X3 subunit expression is found in vagal sensory neurons that innervate the viscera [144–146] which can transmit mechanosensory signals and nociception [147]. A major source for visceral pain is associated with endometriosis, defined as the presence of functional endometrium outside the uterine cavity which can result in dysmenorrhea, dyspareunia, infertility, and severe pelvic pain [148, 149]. Although the mechanism behind the pain is not clear, biopsies from patients suffering from endometriosis-related pain (ERP) show an increase in the density of sensory nerve fibers innervating the endometriotic lesions [150–152], including those expressing the nociceptive marker CGRP [153]. In endometriotic biopsies, the expression level of P2X3 was greater in patients compared to age-matched controls and positively correlated with pain severity [76]. Consistent with the human biology data, systemic delivery of an enhanced formulation of A-317491 effectively reversed both mechanical and thermal hyperalgesia in a preclinical rodent model of ERP [154]. According to publicly available information, several companies have active clinical programs to test antagonists to P2X3-containing receptors in patients suffering from ERP [155, 156].

It is important to note that structurally diverse compounds in clinical development with varying degrees of P2X3/P2X2/3 receptor selectivity have reported alterations in taste that, in some cases, overlap with clinical efficacy [36, 37, 139, 142]. Care must be taken to proceed with clinical exposures that minimize the risk for potentially unblinding the patient to treatment, which could inadvertently affect clinical outcomes that use a subjective endpoint like patient-reported outcomes commonly used in pain trials.

Conclusion

ATP is a key nociceptive modulator, which can act peripherally to heighten the activity of sensitized sensory nerves and centrally to enhance presynaptic glutamate release, thereby potentiating the processes that underlie chronic hypersensitivity. Peripheral and central expressing P2X3-containing receptors play a significant role in these mechanisms, and further clinical exploration may lead to better therapies for the treatment of chronic pain.

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