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## Adenosine Triphosphate and Adenosine Receptors and Pain

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The concept of purinergic signaling was proposed in 1972 following early hints of potent extracellular actions of purines in the heart and peripheral ganglia, the demonstration of the release of adenosine 5'-triphosphate (ATP) during antidromic stimulation of sensory nerve collaterals, and evidence that ATP was the transmitter in nonadrenergic, noncholinergic nerves in the gut and bladder [17]. It is now recognized that ATP is a cotransmitter in nerves in both the peripheral and central nervous systems [25] and that receptors for purines and pyrimidines are widely expressed on non-neuronal as well as nerve cells [29]. Two types of purinoceptor, identified as P1 (for adenosine) and P2 (for adenosine triphosphate or diphosphate) were proposed in 1978 [18], but it was not until 1985 that a pharmacological basis was established for distinguishing two types of P2 receptors (P2X and P2Y) [28]. Currently, seven P2X ionotropic receptor subunits and eight P2Y metabotropic receptor subtypes are recognized, and these include receptors that are sensitive to pyrimidines as well as purines [26,128] (see Table I). Four subtypes of P1 receptors have been cloned—A1, A2A, A2B, and A3 (see Table II)—all of which couple to G proteins and have seven transmembrane (TM) domains, with an extracellular NH<sub>2</sub> terminus and a cytoplasmic COOH terminus [50]. P2X<sub>1–7</sub>-receptor subunits show a subunit topology of intracellular NH<sub>2</sub> and COOH termini, two TM-spanning regions (the first gates the ion channel and the second lines the ion pore), and a large extracellular loop. P2X-receptor subunits can combine to form either homomultimers

or heteromultimers [121]. Metabotropic P2Y receptors ( $P2Y_1$ ,  $P2Y_2$ ,  $P2Y_4$ ,  $P2Y_6$ ,  $P2Y_{11}$ ,  $P2Y_{12}$ ,  $P2Y_{13}$ , and  $P2Y_{14}$ ) have an extracellular  $\text{NH}_2$  terminus and intracellular COOH terminus and seven TM-spanning regions [1]. Until recently, apart from vesicular release from nerves, it was usually assumed that the only source of extracellular ATP acting on purinoceptors was damaged or dying cells, but it is now recognized that ATP release from healthy cells is a physiological mechanism. In fact, ATP is released from many non-neuronal cell types during mechanical deformation in response to shear stress, stretch, or osmotic swelling, as well as hypoxia and stimulation by various agents [15]. After release, ATP and other nucleotides undergo rapid enzymatic degradation by ectonucleotidases to result in the formation of adenosine [189].

There were early hints that ATP might be involved in pain, including the demonstration of pain produced by injection of ATP into human skin blisters [13], ATP involvement in migraine [19], and ATP participation in pain pathways in the spinal cord [80]. A significant advance was made in 1995 when the  $P2X_3$  ionotropic ion channel purinergic receptor was cloned and shown to be localized predominantly on small nociceptive sensory neurons in dorsal root ganglia (DRG) together with  $P2X_{2/3}$  heteromultimer receptors [33,101]. A unifying purinergic hypothesis for the initiation of pain was proposed in 1996 [21]; this proposal has been followed by an increasing number of papers expanding on the concept [23,24,45]. A schematic illustrating the initiation of nociception in the periphery and purinergic relay pathways in the spinal cord is shown in Fig. 1a. While  $P2X_3$  and  $P2X_{2/3}$  receptors expressed in sensory neurons were the predominant P2 receptor subtypes recognized to be involved in initiating pain, it has become apparent that P2Y receptors are also present on sensory afferents [137] and that these receptors are also involved in modulating pain transmission [54].

A role for adenosine in nociception first appeared in the 1970s with the use of methylxanthines (first as inhibitors of phosphodiesterase, then as adenosine receptor antagonists) in combination with morphine [70], along with the demonstration of antinociception by systemic administration of an analogue of adenosine [172]. During the 1980s, the spinal cord became a prominent site of investigation following the demonstration that spinal administration of methylxanthines inhibited systemic effects of morphine [85],

**Fig. 1.** (a) Hypothetical schematic of the roles of purine nucleotide and nucleoside receptors in pain pathways. At peripheral sensory nerve terminals,  $P2X_3$  and  $P2X_{2/3}$  receptors are the main P2X purinoceptors;  $P2Y_1$  and possibly  $P2Y_2$  receptors are present on a subpopulation of  $P2X_3$  receptor-immunopositive fibers. Other P2X-receptor subtypes (1–7) are also expressed at low levels in dorsal root ganglia. Adenosine (ADO) also acts on sensory terminals: A1 receptors lead to suppression of pain signaling, while A2A receptors lead to pain facilitation. In the dorsal spinal cord, ATP acts postsynaptically through  $P2X_2$ ,  $P2X_4$ , and/or  $P2X_6$  receptor subtypes; after breakdown to ADO, it acts as a presynaptic inhibitor of transmission through P1 (A1) receptors. ATP and glutamate are cotransmitters in primary afferent central nerve terminals.  $P2X_4$  and  $P2X_7$  receptors on activated microglia have been implicated in neuropathic pain. Yellow dots, molecules of ATP; blue dots, molecules of ADO. (Modified from [30] with permission from Elsevier). (b) Purinergic mechanosensory transduction. Schematic representation of hypothesis for purinergic mechanosensory transduction in tubes (e.g., ureter, vagina, salivary and bile ducts, gut) and sacs (e.g., urinary and gall bladders, lungs). It is proposed that mechanical distension leads to release of ATP from the epithelium lining the tube or sac, which then acts on  $P2X_3$  and/or  $P2X_{2/3}$  receptors on subepithelial sensory nerves to convey sensory/nociceptive information to the CNS. (From [22] with permission from Blackwell.) →

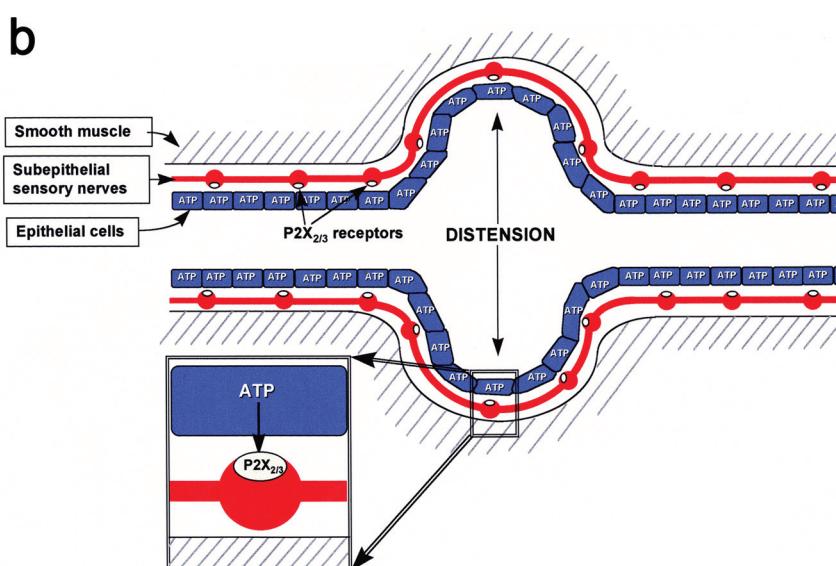
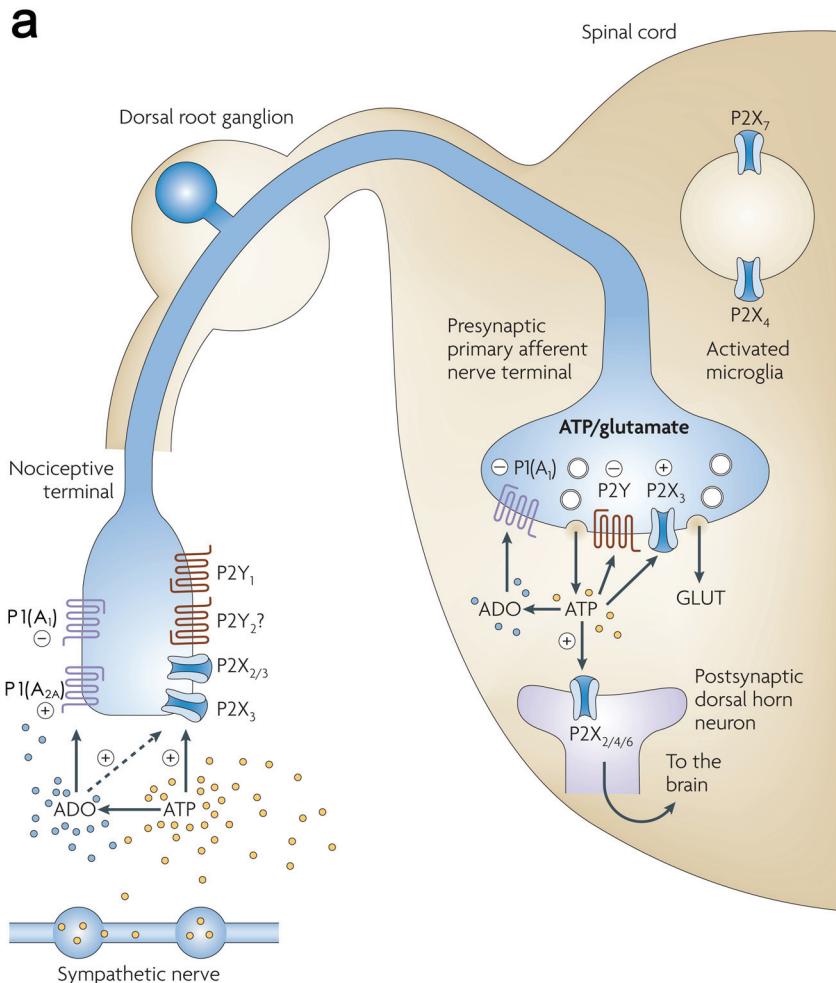


Table I  
Characteristics of P2 receptors

Receptor	Main Distribution	Main Functions
P2X <sub>1</sub>	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurons	Smooth muscle contraction, platelet activation
P2X <sub>2</sub>	Smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia	Sensory transmission and modulation of synaptic function
P2X <sub>3</sub>	Sensory neurons, NTS, some sympathetic neurons	Mediates sensory transmission; facilitates glutamate release in CNS
P2X <sub>4</sub>	CNS, testes, colon	Modulates chronic inflammatory and neuropathic pain
P2X <sub>5</sub>	Proliferating cells in skin, gut, bladder, thymus, spinal cord	Inhibits proliferation and increase differentiation
P2X <sub>6</sub>	CNS, motor neurons in spinal cord	Functions as a heteromeric channel in combination with P2X <sub>2</sub> and P2X <sub>4</sub> subunits
P2X <sub>7</sub>	Apoptotic cells, e.g., immune cells, pancreas, skin	Mediates apoptosis, cell proliferation and proinflammatory cytokine release
P2Y <sub>1</sub>	Epithelial and endothelial cells, platelets, immune cells, osteoclasts, glial cells	Smooth muscle relaxation and mitogenic actions; platelet shape change and aggregation; bone resorption
P2Y <sub>2</sub>	Immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts, astrocytes	Vasodilatation via endothelium and vasoconstriction via smooth muscle; mitogenic actions; mediates surfactant secretion; epithelial cell Cl <sup>-</sup> secretion; bone remodeling
P2Y <sub>4</sub>	Endothelial and epithelial cells, intestine, pituitary, brain (low levels in liver and bone marrow)	Regulates epithelial chloride transport; vasodilatation via endothelium; mitogenic actions
P2Y <sub>6</sub>	Some epithelial cells, placenta, T cells, thymus, spleen, kidney, activated microglia	NaCl secretion in colonic epithelium; role in epithelial proliferation
P2Y <sub>11</sub>	Spleen, intestine, brain, granulocytes	Role in maturation and migration of dendritic cells; granulocytic differentiation
P2Y <sub>12</sub>	Platelets, glial cells, spinal cord	Platelet aggregation; role in dense granule secretion
P2Y <sub>13</sub>	Spleen, brain, lymph nodes, bone marrow, liver, pancreas, heart	Platelet aggregation; role in dense granule secretion; function largely unknown, but present in both immune system and brain
P2Y <sub>14</sub>	Placenta, adipose tissue, stomach, intestine, discrete brain regions, spleen, lung, heart, bone marrow, peripheral immune cells	Chemoattractant receptor in bone marrow hematopoietic stem cells; dendritic cell activation

Source: Reproduced from [27] by courtesy of the Nature Publishing Group.

Abbreviations: CNS, central nervous system; NTS, nucleus tractus solitarius.

while spinal administration of adenosine analogues produced antinociception [127]. Further studies showed that spinal methylxanthines inhibit antinociception by spinal morphine [41,163] and that morphine directly releases adenosine from the spinal cord [163]. Adenosine, with actions mediated by several receptor subtypes, is now known to be involved in pain signaling at peripheral, spinal, and supraspinal sites (see the next section), and several reviews of the actions of adenosine in relation to nociception have been published [43,140,141].

This chapter highlights key functions of ATP and adenosine in relation to pain signaling and considers the potential for pharmaceuticals and approaches based on these mediators to contribute to pain management.

Table II  
Characteristics of P1 (adenosine) receptors

Receptor	Main Distribution	Main Functions
A <sub>1</sub>	Brain, spinal cord, testes, heart, autonomic nerve terminals	Prejunctional neuromodulation of neurotransmitter release; behavioral effects (sedation, anticonvulsive, anxiolytic); cardiac depression
A <sub>2A</sub>	Brain, heart, lungs, spleen	Facilitates neurotransmission; smooth muscle relaxation
A <sub>2B</sub>	Large intestine, bladder	Role in allergic and inflammatory disorders; vasodilatation
A <sub>3</sub>	Lung, liver, brain, testes, heart	Facilitates release of allergic mediators; cardioprotective and cytoprotective

Source: Reproduced from [27] by courtesy of the Nature Publishing Group.

## Role of ATP and Adenosine in Pain Signaling

### ATP and Pain Signaling

#### *The Periphery*

P2X receptors in sensory neurons, including those from dorsal root, trigeminal, nodose, and petrosal ganglia, have been characterized extensively [25]. All P2X-receptor subtypes are found on sensory neurons, although the P2X<sub>3</sub> receptor has the highest level of expression (both mRNA and protein) [91]. P2X<sub>3</sub> and P2X<sub>2/3</sub> receptors are expressed on isolectin B<sub>4</sub> (IB<sub>4</sub>)-binding subpopulations of small nociceptive neurons [16]. P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub> receptor mRNA is also expressed on neurons of DRG, nodose, and trigeminal ganglia, and receptor protein for the P2Y<sub>1</sub> receptor is localized mainly (over 80%) on small neurons; double immunolabeling reveals that ~80% of P2X<sub>3</sub>-receptor-positive neurons also stain for P2Y<sub>1</sub> receptors, while ~30% also stain for P2Y<sub>4</sub> receptors [137].

A hypothesis has been proposed that purinergic mechanosensory transduction occurs in visceral tubes and sacs (including ureter, bladder, and gut), whereby ATP released from epithelial cells during distension acts on P2X<sub>3</sub> homomeric and P2X<sub>2/3</sub> heteromeric receptors on subepithelial sensory nerves initiating impulses in pain pathways (Fig. 1b) [22]. Evidence supporting this hypothesis is as follows. (a) Mice lacking P2X<sub>3</sub> receptors exhibit reduced inflammatory pain and marked urinary bladder hyporeflexia [35]. (b) ATP is released from urothelial cells during distension; activity in pelvic sensory nerves is mimicked by ATP and α,β-methylene ATP (α,β-meATP) and attenuated by P2X<sub>3</sub> antagonists and in P2X<sub>3</sub> knockout mice; P2X<sub>3</sub> receptors are localized on suburothelial sensory nerve fibers [173]. (c) Botulinum toxin A, which has antinociceptive effects in interstitial cystitis, inhibits distension-mediated urothelial release of ATP in conditions of bladder inflammation [32,107]. (d) Ureteric colic induced by the passage of a kidney stone

causes severe pain. Immunostaining of P2X<sub>3</sub> receptors in sensory nerves in the subepithelial region has been reported [99]; distension of the ureter results in ATP release from the urothelium [92], and distension of the ureter results in increased afferent nerve discharge which is mimicked by intraluminal ATP or α,β-meATP and attenuated by 2',3'-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) [135]. (e) In the gut, purinergic mechanosensory transduction initiates physiological reflex modulation of peristalsis via intrinsic sensory fibers and nociception via extrinsic sensory fibers [23,180]. Distension of the colorectum led to pressure-dependent increase in release of ATP from mucosal epithelial cells and also evoked pelvic nerve excitation, which was mimicked by ATP and α,β-meATP and attenuated by TNP-ATP and pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS). Inflammatory mediators augment P2X<sub>3</sub>-receptor-mediated responses on pelvic afferent fibers in the rat colorectum [179], and P2X<sub>3</sub>-receptor-mediated responses are enhanced in a model of colitis [178]. (f) It is possible that tissue stress or damage in the uterine cervix during late pregnancy and parturition may lead to ATP release and sensory signaling via P2X<sub>3</sub> receptors [125].

ATP and α,β-meATP activate nociceptive sensory nerve terminals in the skin [188], and these responses are increased in inflammatory conditions [65]. Skin cell damage causes action potential firing and inward currents in nociceptive fibers, and this response is eliminated by enzymatic degradation of ATP or blockade of P2X receptors, implicating release of cytosolic ATP [37]. Inflammation upregulates P2X<sub>2</sub> and P2X<sub>3</sub> receptors on DRG neurons [182]. P2Y<sub>2</sub> receptors on the terminals of sensory neurons in mice mediate nociceptive transmission, and P2Y signaling may contribute to mechanotransduction in low-threshold A $\beta$  fibers [160]. A pathogenic role for keratinocyte-derived ATP in irritant dermatitis has been proposed [118]. It has been reported recently that cutaneous sensory neurons expressing a family of G-protein-coupled receptors, named mas-related G-protein-coupled receptors (Mrgprd receptors), sense extracellular ATP and are putative nociceptors [48].

ATP is also implicated in sensory signaling in several other systems. P2X<sub>3</sub> and P2X<sub>2/3</sub> receptors on sensory afferents in tooth pulp appear to mediate nociception [4,132], perhaps from ATP released by mechanical distension or inflammation of odontoblasts. P2X<sub>2</sub>, P2X<sub>3</sub>, and P2Y<sub>1</sub> receptors are abundant on sensory nerve terminals in the tongue [14]. ATP and α,β-meATP were shown to excite trigeminal lingual nerve terminals of rats in an in vitro preparation, mimicking nociceptive responses to noxious mechanical stimulation and high temperature and suggesting the involvement of purinergic mechanosensory transduction [136].

Pain related to the musculoskeletal system (myofascial pain) is common, and ATP has been claimed to excite or sensitize myofascial nociceptors [12]. Nociceptive information arising from the orofacial region via P2X<sub>3</sub> receptors on afferent fibers are carried to trigeminal brainstem sensory nuclei [91]. ATP stimulates group IV receptors in mechanically sensitive muscle afferents [131]. It enhances the muscle pressor response evoked by mechanically sensitive muscle stretch; this response was attenuated

by PPADS [102]. ATP induces sustained facilitation of cranial nociception through P2X receptors on neck muscle nociceptors in mice [109]. ATP acting on P2X<sub>3</sub> receptors in sensory nerve fibers in masseter muscle plays an important role in pressure pain and mechanical hyperalgesia caused by excessive muscular contraction [154]. ATP is a stimulant of articular nociceptors in the knee joint via P2X<sub>3</sub> receptors [47,145]. P2Y<sub>2</sub>-receptor mRNA is expressed in both cultured normal and osteoarthritic chondrocytes taken from human knee joints, and ATP is released by mechanical stimulation [117]. When monoarthritis was induced by injection of complete Freund's adjuvant into the unilateral temporomandibular joint of the rat, the nociception produced was associated with an increase in P2X<sub>3</sub>-receptor-positive small neurons in the trigeminal ganglion [153]. Activation of P2X receptors in rat temporomandibular joint induces nociception, and blockage by PPADS decreases carrageenan-induced inflammatory hyperalgesia [124]. Oxidized ATP inhibits inflammatory pain in arthritic rats by inhibiting the P2X<sub>7</sub> receptor for ATP localized in nerve terminals [42].

### ***The Spinal Cord***

Excitation of dorsal horn neurons by ATP was recognized early [52,80] and described in more detail later (see [25]). ATP-evoked increases in intracellular calcium were demonstrated in both neurons and glia of the dorsal spinal cord [138]. Salter and Hicks later showed that the ATP-evoked release of Ca<sup>2+</sup> from astrocytes was via the phospholipase C-β/inositol triphosphate pathway, suggesting mediation via a P2Y receptor. ATP was shown to inhibit slow depolarization via P2Y receptors in substantia gelatinosa neurons [185]. A recent study has identified P2Y<sub>1</sub>- and P2Y<sub>4</sub>-receptor mRNA in subpopulations of dorsal horn neurons, whereas motor neurons in the ventral horn expressed P2Y<sub>4</sub>- and P2Y<sub>6</sub>-receptor mRNA [93]. This study also showed that astrocytes in the gray matter expressed mRNA for P2Y<sub>1</sub> receptors, and that microglia throughout the spinal cord expressed mRNA for P2Y<sub>12</sub> receptors. Messenger RNA for P2X<sub>2</sub>, P2X<sub>4</sub>, and P2X<sub>6</sub> receptors has been identified within spinal motor nuclei [36].

P2X<sub>3</sub> immunoreactivity is present on the axon terminals of DRG neurons that extend across the entire mediolateral extent of inner lamina II of the dorsal horn [16,59]. The immunolabeled nerve profiles in lamina II for P2X<sub>3</sub> receptors are located largely on terminals with ultrastructural characteristics of sensory afferent terminals [105]. In contrast, although P2X<sub>2</sub> immunoreactivity is most prominent in lamina II, it is also seen in deeper layers, and only rarely overlaps with P2X<sub>3</sub> immunoreactivity [174]. At central terminals of primary afferent neurons, ATP has been shown to act either presynaptically (facilitating glutamate release) [60] or postsynaptically [9]. P2X receptors are also expressed on glycinergic presynaptic nerve terminals [81]. Distinct subtypes of P2X receptors are functionally expressed at pre- and postsynaptic sites in lamina V neurons in the rat dorsal spinal cord, and it was suggested that purinergic signaling in deep dorsal horn neurons becomes more important during postnatal development [155]. In addition to acting as a fast excitatory synaptic transmitter, ATP facilitates excitatory transmission

by increasing glutamate release and enhances inhibitory neurotransmission mediated by both  $\gamma$ -aminobutyric acid (GABA) and glycine [73,133]. P2X<sub>3</sub> receptors are involved in transient modulation of glutamate release in lamina II of the spinal cord, but a different P2X-receptor subtype (perhaps P2X<sub>1/5</sub> or P2X<sub>4/6</sub>) was involved in long-lasting modulation in lamina V [120].

Activation of P2X receptors in the spinal cord elicits allodynia, and in a seminal study published in *Nature* in 2004, P2X<sub>4</sub> receptors on spinal cord microglia were implicated in neuropathic pain using P2X<sub>4</sub> antisense oligonucleotides [168]. This latter study has led to an explosion of work focused on purinergic signaling in neuropathic pain (see the section below on ATP and neuropathic pain).

## Adenosine and Pain Signaling

### *The Periphery*

Adenosine A1, A2A, A2B, and A3 receptors (ARs) are all present at peripheral sites either directly on sensory afferent nerve terminals (A1R and A2ARs) or on adjacent cell types that can potentially influence nociception under certain conditions (A2AR, A2BR, and A3Rs) (Fig. 1a). A1Rs have been visualized directly in sensory afferent neurons using immunohistochemistry [31,144]. In functional studies, peripheral administration of A1R agonists leads to antinociception against mechanical hyperalgesia [5,164] and thermal hyperalgesia (but not mechanical allodynia) following nerve injury [104]. In vitro studies indicate that A1Rs lead to reduced Ca<sup>2+</sup> entry [64], decreased cyclic AMP (cAMP) generation, and decreased peptide release in sensory neurons [31]. Changes in G proteins, cAMP, and protein kinase A (PKA) activity are implicated in antinociception by A1R agonists [90]. A2ARs are also present in DRG [86]. In functional studies, local peripheral administration of A2AR agonists leads to mechanical hyperalgesia [164] and increased flinching with formalin [44,89]. Hyperalgesia is mediated by increases in cAMP in the sensory nerve terminal, activation of PKA, phosphorylation of Na<sup>+</sup> channels, increased inward currents, and sensory afferent activation [57]. A2ARs also mediate anti-inflammatory and immunosuppressive effects [66,96], and such actions may contribute indirectly to reduced pain signaling in inflammatory states.

Adenosine A2B and A3 receptors have not been identified directly on sensory neurons, but their localization on mast cells and ability to contribute to aspects of inflammation make them potential indirect contributors to inflammatory pain via release of mediators from such cells. Systemic administration of A2BR antagonists produced antinociception in the hotplate test, and this effect was attributed to a peripheral site of action because an analogue that did not cross the blood-brain barrier had the same effect [2]. Furthermore, local administration of a selective A2BR antagonist (PSB-1115) inhibited pain behaviors and paw edema produced by formalin [11], and this finding also supports a pronociceptive and pro-inflammatory role of A2BRs. Adenosine A3Rs are present on several types of immune and inflammatory cells, and they exert complex pro- and anti-inflammatory effects.

Administered acutely to uninjured animals, A3R agonists promote mast cell degranulation and release of mediators that increase nociception [142]. Mice lacking A3Rs exhibit reduced hyperalgesia in response to carrageenan, and this finding is consistent with an involvement of A3Rs in inflammatory pain [177]. In a recent study, local administration of a selective A3R antagonist (PSB-10) inhibited paw edema but had no effect on pain behaviors produced by formalin [11].

### ***The Central Nervous System***

Adenosine receptors, particularly A1Rs, also contribute to pain regulation by actions at central sites. The spinal cord is a prominent site at which A1Rs produce antinociception in models of physiological pain (as revealed by sensory thresholds in the uninjured state), inflammatory pain (in several models of inflammation), and neuropathic pain (produced by various forms of injury to the peripheral nerve) [43,140,141]. A1Rs are concentrated in laminae I and II of the dorsal horn of the spinal cord and are present on intrinsic dorsal horn neurons [144]. Increased K<sup>+</sup> conductance and hyperpolarization of dorsal horn neurons [95,126] are implicated in spinal antinociception by adenosine. While A1Rs have not been directly identified at presynaptic sites on sensory nerve terminals in the dorsal spinal cord, presynaptic actions and inhibition of neuropeptide and glutamate release from such terminals has been demonstrated, suggesting the presence and functional actions of A1Rs contributing to antinociception at such sites [31,103,111,139]. The ability of A1R agonists to inhibit wind-up (a progressive increase in response following repeated stimulation) within the spinal cord has been emphasized, along with their ability to contribute to antinociception in the normal state, following inflammation, and following neuropathy [39,161].

A1Rs also are widely expressed at other sites throughout the CNS, including several areas involved in transmission and regulation of pain. The microinjection of adenosine A1R agonists into the periaqueductal gray (PAG) region produces antinociception (in thermal threshold and formalin tests) and alters ON- and OFF-cell activity in the rostroventral medulla, recognized as sites of supraspinal nociceptive circuitry [108]. Inhibition of both glutamate and GABA transmission within the PAG may contribute to such actions [7]. Supraspinal actions may contribute to antinociception following systemic delivery of A1R agonists that readily access the CNS, and studies in spinalized animals provide direct support for this involvement [130].

There is less information on the role of central A2ARs, A2BRs, and A3Rs in nociception. Microglia are involved in chronic and neuropathic pain, express most adenosine receptor subtypes, including A2ARs, A2BRs, and A3Rs [40], and potentially could contribute to AR-mediated nociception. The spinal administration of selective agonists for A2ARs and A3Rs produces antinociception in the formalin model [183], while spinal administration of selective antagonists for A2ARs, A2BRs, and A3Rs inhibits the action of adenosine in this model [184]. However, it is not known whether these actions reflect effects on neurons or on other types of cells.

## Purines and Neuropathic Pain

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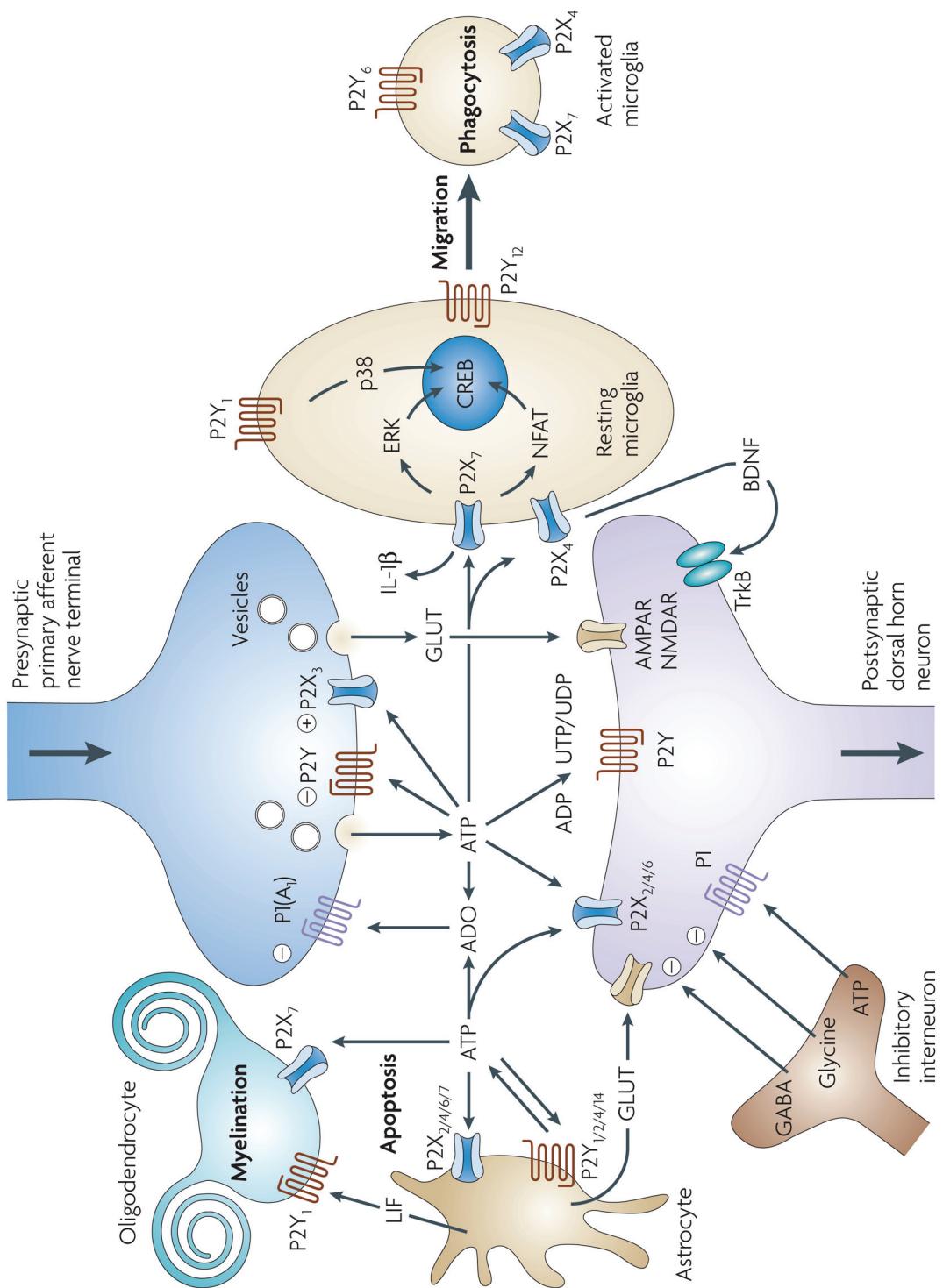
### ATP and Neuropathic Pain

Neuropathic pain, which results from damage to peripheral nerves or injury to the CNS, is difficult to treat clinically. Thus, while several classes of drugs are currently in clinical use, they are limited by partial efficacy and adverse effects. Neuropathic pain involves neurons, immune cells, and glia [143]. In addition to P2X<sub>3</sub> receptors on neurons, P2X<sub>4</sub>, P2X<sub>7</sub>, and P2Y<sub>12</sub> receptors on microglia in the spinal cord are implicated in neuropathic pain; the location of such receptors in relation to pain signaling is depicted schematically in Fig. 2.

### *P2X<sub>3</sub> and P2X<sub>2/3</sub> Receptors*

There is considerable evidence that P2X<sub>3</sub> receptors are involved in neuropathic pain; the literature includes evidence of peripheral receptors located on sensory afferent nerves, as well as central receptors on primary afferent nerve terminals in the spinal cord [176] and in the trigeminal brainstem sensory nuclei [91]. Nerve injury can lead to complex effects on P2X<sub>3</sub>-receptor expression in DRG neurons, as there are reports of both increases [122] and decreases [16,87] in receptors following various forms of nerve injury. Some of this difference may reflect the state of the nerve, because when the investigators distinguished between injured and uninjured neurons (using the transcription factor ATF3), P2X<sub>3</sub> receptors were reduced in injured DRG neurons but increased in uninjured neurons [171]. Functional studies reveal enhanced pain signaling in response to local application of ATP and/or  $\alpha,\beta$ -meATP into the hindpaw following nerve injury [115]. Some of the increased functional response may reflect increased translocation of the P2X<sub>3</sub> receptor to the cell membrane [110]. In the spinal cord, P2X<sub>3</sub> receptors on

**Fig. 2.** Purinergic signaling in the spinal cord. Presynaptic primary afferent nerve terminals in the dorsal horn → of the spinal cord are depicted releasing both glutamate (GLUT) and adenosine triphosphate (ATP) as co-transmitters by exocytosis. ATP acts postsynaptically on P2X<sub>2</sub>, P2X<sub>4</sub>, and P2X<sub>6</sub> receptors and on various P2Y-receptor subtypes. Glutamate acts postsynaptically on  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) and N-methyl-D-aspartate receptors (NMDARs). ATP is broken down by ectonucleotidase to adenosine (ADO), which acts as a presynaptic inhibitory modulator via P1 (A1) receptors, but ATP itself also can act presynaptically to inhibit the release of transmitter through P2Y receptors or to enhance release of glutamate through P2X<sub>3</sub> receptors. ATP is also released from astrocytes (and probably also microglia) together with glutamate to participate in glial-neuron interactions. Both P2X- and P2Y-receptor subtypes are expressed by astrocytes. Leukemia-inhibiting factor (LIF) released by astrocytes in response to ATP promotes myelination in oligodendrocytes and remyelination through P2Y<sub>1</sub> receptors. P2X<sub>7</sub> receptors on oligodendrocytes mediate apoptosis. Resting microglia express P2X<sub>4</sub> and P2X<sub>7</sub> receptors involved in neuropathic pain. ATP, through P2X<sub>7</sub> receptors, promotes interleukin 1 $\beta$  (IL-1 $\beta$ ) release. Activation of P2X<sub>4</sub> receptors leads to release of brain-derived neurotrophic factor (BDNF), which acts on tropomyosin-related kinase B (TrkB) receptors on neurons in the pain pathway. Activation of P2X<sub>7</sub> receptors (via extracellular regulated kinase [ERK] and/or nuclear factor of activated T cells [NFAT]) activates transcription factor cyclic AMP response element-binding protein (CREB), whereas P2Y<sub>1</sub> receptors activate CREB through p38 signaling. P2Y<sub>12</sub> receptors on resting microglia mediate cell migration after injury, whereas P2Y<sub>6</sub> receptors on activated ameboid microglia mediate phagocytosis of debris at the site of damage. Inhibitory interneurons co-release  $\gamma$ -aminobutyric acid (GABA), glycine, and ATP and also modulate nociception. (From [27] with permission of Nature Publishing Group.)



terminals of primary afferent nerves in the inner lamina of the dorsal spinal cord mediate facilitation of glutamate release [60]. Furthermore, ATP co-released with GABA in spinal interneurons is probably involved in modulation of nociceptive pathways [84]. Curiously, supraspinal P2X<sub>3</sub> and P2X<sub>2/3</sub> receptors may play an inhibitory role in pain transmission [51].

Functional studies using receptor antagonists and antisense oligonucleotides have revealed a prominent role of P2X<sub>3</sub> receptors in pain regulation in nerve injury states. Thus, selective receptor antagonists produce antinociceptive actions in behavioral [113] and electrophysiological paradigms [149] in several nerve injury models. Downregulation of P2X<sub>3</sub> receptors using antisense oligonucleotides revealed a significant role for these receptors in neuropathic pain [71]. A recent study indicates that P2X<sub>3</sub> receptors mediate thermal hyperalgesia in a rat model of trigeminal neuropathic pain [152].

One particular condition in which ATP may contribute prominently to neuropathic pain is in sympathetically maintained pain states. Normally, the sympathetic nervous system does not interact directly with sensory nerves; however, after nerve injury, a sympathetic-afferent interaction can occur whereby sympathetic activity can enhance pain [82]. This interaction results from sprouting of sympathetic nerves in response to growth factors and cytokines and has been demonstrated directly in DRG. Different nerve injury models lead to different time courses and patterns of sympathetic-sensory coupling, which can reflect different origins and mechanisms of sprouting at DRG vs. peripheral sites [98]. Several growth factors cause upregulation of P2X<sub>3</sub> receptors in DRG neurons [16,129]. ATP is co-released with norepinephrine and neuropeptide Y from sympathetic nerves [20]. Norepinephrine potentiates pain behaviors induced by local administration of α,β-meATP into the hindpaw, as well as excitatory responses of ATP/α,β-meATP on DRG neurons *in vitro* [175]; this potentiation is particularly prominent following nerve injury [110,115]. Adrenergic regulation of P2X<sub>3</sub> receptors involves α<sub>1</sub>-adrenergic receptors and intracellular signaling via PKC [110,116]. Adrenergic-purinergic interactions on sensory nerves are depicted schematically in Fig. 3. While neuropeptide Y is co-released with norepinephrine and ATP from sympathetic nerves and can augment vascular effects of norepinephrine and ATP [75], its role in regulating peripheral sympathetic influences on pain has not been elaborated.

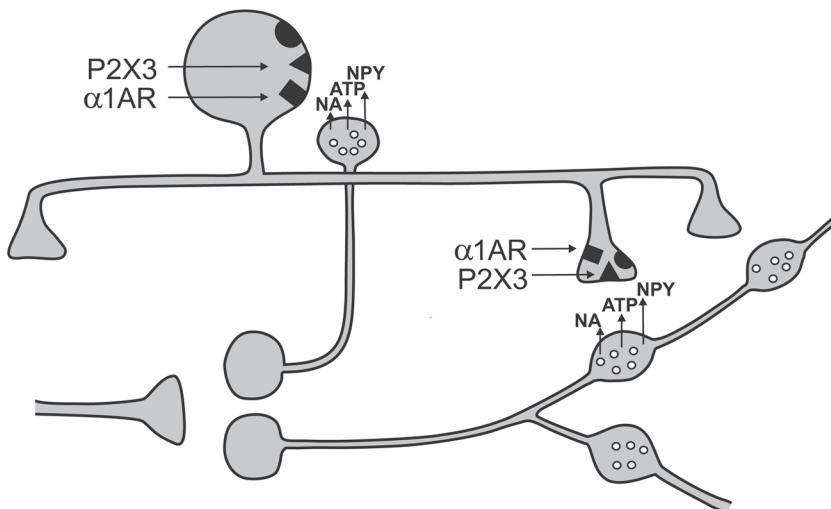
### **P2X<sub>4</sub> Receptors**

A number of papers have described the role of P2X<sub>4</sub> receptors on spinal microglia in neuropathic pain following the initial discovery by Tsuda and colleagues in 2003 (see [77]). Microglial P2X<sub>4</sub> receptors were upregulated and microglial reactivity was produced during allodynia in rats with experimental autoimmune neuritis [187]. Brain-derived neurotrophic factor is released from microglia by the stimulation of P2X<sub>4</sub> receptors [38]. The involvement of increased levels of spinal fibronectin/integrin following peripheral nerve injury in upregulation of microglial P2X<sub>4</sub> receptors and neuropathic pain has been considered [169]. Ligands for toll-like receptors and nucleotide-binding

oligomerization domain 2 receptors stimulate microglial P2X<sub>4</sub>-receptor upregulation, suggesting that microglia sense the presence of inflammatory stimulation using multiple recognition systems [62]. A recent study showed that Lyn tyrosine kinase plays an important role in the pathogenesis of neuropathic pain and is required to mediate nerve-injury-induced upregulation of P2X<sub>4</sub> receptors [170]. In rats displaying allodynia, the level of p38 was increased in microglia. Intraspinal administration of the p38 inhibitor, SB203580, suppressed allodynia, suggesting that neuropathic pain hypersensitivity depends on the activation of the p38 signaling pathway in microglia. Platelet activating factor, which is released from activated microglia, is a potent inducer of tactile allodynia and thermal hyperalgesia after intrathecal injection into the spinal cord, and this response is mediated by ATP [119]. The possible mechanisms that underlie the role of P2X<sub>4</sub> receptors in neuropathic pain and the involvement of inflammatory cytokines have been discussed [76,167].

### **P2X<sub>7</sub> Receptors**

P2X<sub>7</sub> receptors were shown to be expressed in a mouse microglial cell line in 1997 (see [29]) and subsequently on microglia in rat brain [186]. The P2X<sub>7</sub>-receptor antagonist oxidized ATP relieves inflammation-induced mechanical hyperalgesia in rats [42]. Both



**Fig. 3.** Schematic depicting sympathetic innervation of dorsal root ganglion (DRG) cell bodies and peripheral aspects of sensory afferent nerves by sympathetic nerves. Following nerve injury, growth factors and cytokines cause sympathetic nerve sprouting, leading to abnormal sympathetic terminal arborizations around some DRG neurons. Different nerve injury models lead to different time courses and patterns of sympathetic-sensory coupling at the two sites, which can reflect actions in distinct populations of sympathetic nerves (releasing NA, ATP, and neuropeptide Y [NPY] as cotransmitters) and different mechanisms of sprouting. Norepinephrine (noradrenaline, NA) augments ATP and/or  $\alpha_1$ -meATP responses at P2X<sub>3</sub> receptors at peripheral sites (following local drug application to the rat hindpaw) and on DRG cell bodies (following drug application to isolated DRG neurons). Alpha-1 adrenergic receptors ( $\alpha_1$ -ARs) are implicated in adrenergic augmentation of P2X<sub>3</sub>-receptor-mediated responses at both sites, and intracellular signaling involves protein kinase C.

inflammatory pain and neuropathic pain were abolished in P2X<sub>7</sub> knockout mice [34]. P2X<sub>4</sub> and P2X<sub>7</sub> receptor knockout mice share a common pain phenotype, but this phenotype appears to be conferred via different mechanisms [74].

### **P2Y Receptors**

The contributions of the metabotropic P2Y receptors to normal and pathological pain have been less well examined compared to P2X receptors [54]. However, the expression of P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub> mRNA in DRG neurons suggests that these receptors may be involved in peripheral somatosensory transmission [25]. Activation of uridine 5'-triphosphate (UTP)-sensitive P2Y<sub>2</sub> and/or P2Y<sub>4</sub> receptors and the uridine diphosphate (UDP)-sensitive P2Y<sub>6</sub> receptor, in contrast to P2X receptors, inhibits spinal pain transmission [123]. P2Y<sub>1</sub> and P2Y<sub>4</sub> receptors were identified on predominantly small-diameter sensory neurons in a subpopulation that were colocalized with P2X<sub>3</sub> receptors [137]. Activation of P2Y<sub>1</sub> receptors on DRG neurons modulates currents generated through *N*-type calcium channels and P2X<sub>3</sub> receptors [54]. Indeed, activation of *N*-type calcium channels in cultured DRG neurons was inhibited by ATP and even more potently by the P2Y<sub>1,12,13</sub>-receptor agonist ADP. The effects of ATP were blocked by the selective P2Y<sub>1</sub>-receptor antagonist MRS 2179. The outcome of these P2Y<sub>1</sub>-related inhibitions is likely to result in a decreased release of nociceptive transmitters into the spinal cord [25]. P2Y<sub>1</sub> receptor mRNA is upregulated in the lumbar DRG after peripheral axotomy [181]. In the isolated skin-nerve preparation, 54% of cutaneous C fibers and 12% of A-mechanoreceptors responded to UTP (approximately 70–80% were capsaicin-sensitive) [160]. However, an additional 22–26% of large-diameter A $\beta$  fibers responded to UTP, suggesting that P2Y<sub>2</sub> receptors also may be directly involved in the transmission of low-threshold mechanical inputs to the spinal cord. It is also possible that activation of the hetero-oligomeric P2Y<sub>2</sub>/A1 receptor complex [162] may also negatively modulate the antinociceptive effects of A1-receptor agonists [45]. The rostral ventromedial medulla serves as a critical link in bulbospinal nociceptive modulation, and it has been suggested that while ON-cells preferentially express P2X receptors, OFF-cells express P2Y receptors in this region [146]. Activation of P2Y receptors inhibits P2X<sub>3</sub>-receptor channels via G-protein-dependent facilitation of their desensitization [55]. It has been reported recently that P2Y<sub>12</sub> receptors expressed on microglia [68] are required for neuropathic pain after peripheral nerve injury [166].

### **Adenosine and Neuropathic Pain**

Interest in the role of adenosine in treating neuropathic pain developed following a clinical report that spinal delivery, by intrathecal injection, of an A1R agonist produced long-lasting relief of neuropathic pain [88], as well as a preclinical report that intrathecal adenosine analogues, particularly A1R agonists, were very effective in relieving hypersensitivity responses (mechanical allodynia) after spinal nerve ligation in rats [100]. Subsequent pre-clinical studies confirmed the efficacy of spinal A1R agonists (or adenosine itself) in the spinal nerve ligation model [97], the chronic constriction model [157], and a model of

central pain [158]. Following safety evaluation, intrathecal adenosine was shown to reduce neuropathic pain in humans [10,49].

Several mechanisms have been implicated in spinal analgesia by A1Rs following nerve injury. These include: (a) inhibition of release of glutamate from sensory afferent nerves [103], (b) interactions with spinal noradrenergic systems [8,58], and (c) inhibition of spinal wind-up processes [39,161]. Other effects implicated in nociceptive mechanisms in general, such as inhibition of neuropeptide release from sensory afferents and increased K<sup>+</sup> conductance and postsynaptic inhibition, have not specifically been examined following nerve injury but are still presumed to potentially contribute to spinal analgesia.

Several clinical studies have also examined effects of intravenous (i.v.) infusions of adenosine for neuropathic pain, and the authors reported pain reductions following treatment [106,156]. However, Eisenach et al. [49] reported reduced allodynia in neuropathic pain following intrathecal, but not i.v., administration of adenosine. Clinical reports and studies of adenosine in neuropathic pain, in several experimental pain models in humans, and in other clinical situations have been reviewed recently [67]. The analgesic action of i.v. adenosine is not fully understood due to the short half-life of adenosine in the blood, but it has been presumed to reflect a spinal site of action for A1Rs; contributions from supraspinal and peripheral actions at adenosine A1Rs are also possible.

## Potential Clinical Applications

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### Clinical Applications of ATP Agents

There is increasing interest in developing P2 receptor antagonists for the treatment of chronic and neuropathic pain [24,27,45,56,134]. Towards this end, an active search is ongoing for selective P2X<sub>3</sub>, P2X<sub>2/3</sub>, P2X<sub>4</sub>, P2X<sub>7</sub>, and P2Y<sub>12</sub> receptor antagonists that are orally bioavailable, can cross the blood-brain barrier, and are metabolically stable as potential novel therapeutics for the treatment of pain.

#### *P2X<sub>3</sub>-Receptor Antagonists*

TNP-ATP is a potent P2X<sub>3</sub>-receptor antagonist; it has a low nanomolar affinity for P2X<sub>3</sub> receptors but also a high affinity for P2X<sub>1</sub> receptors [79]. This compound is rapidly degraded in situ [121], and in vivo studies have been limited to intrathecal administration [83] or direct administration into a site of peripheral tissue damage [83]. Several non-nucleotide small-molecule P2X<sub>3</sub> antagonists have been developed. A-317491 has nanomolar affinity for blocking both P2X<sub>3</sub> and P2X<sub>2/3</sub> receptors [113]; while it is generally stable and shows high systemic bioavailability following subcutaneous administration, it lacks oral bioavailability. A recent paper reported that intraplantar injection of A-317491 improved experimental cystitis in rats [78]. Systemic administration of A-317491 reduced nociception in inflammatory and neuropathic pain models [113]. A-317491 also blocked persistent pain in formalin and acetic acid abdominal constriction tests, but it was inactive in models of acute noxious stimulation [113]. RO-3 is another antagonist that potently blocks P2X<sub>3</sub>

receptors; it has lower protein binding compared with A-317491 (48% versus 99%) and good CNS penetration [56]. RO-3 reduced nociceptive sensitivity in several animal pain models [56].

Antisense oligonucleotides also have been used to downregulate P2X<sub>3</sub> receptors. In models of neuropathic and inflammatory pain, this approach has been shown to relieve hypersensitivity responses [71]. P2X<sub>3</sub> antisense oligonucleotides (and antagonists) appear to be less effective for treating diskogenic pain (of the lumbar intervertebral disks) than for cutaneous tissue pain [6]. Combined antisense and RNA interference-mediated treatment for specific inhibition of the recombinant rat P2X<sub>3</sub> receptor appears to be promising for pain therapy [69]. P2X<sub>3</sub> double-stranded short interfering RNA relieves chronic neuropathic pain and opens up new avenues for therapeutic pain strategies in humans [46].

### **P2X<sub>4</sub>-Receptor Antagonists**

The lack of selective P2X<sub>4</sub>-receptor antagonists has hindered the pharmacological validation of the role for P2X<sub>4</sub> receptors in pain. However, 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro-[3,2-e]-1,4-diazepin-2-one was recently shown to block P2X<sub>4</sub>-receptor-mediated currents expressed in Chinese hamster ovary cells [45], and may prove to be useful in this regard. A series of benzofuro-1,4-diazepin-2-ones have been reported to be effective P2X<sub>4</sub> antagonists in a Bayer Health Care, AG patent [151]. Antidepressants have been shown to be effective in relieving neuropathic pain [114] and a recent communication [77] suggests that paroxetine is a P2X<sub>4</sub>-receptor antagonist, but this needs to be confirmed and tested clinically.

### **P2X<sub>7</sub>-Receptor Antagonists**

Several P2X<sub>7</sub>-receptor antagonists have been developed recently including oxidized ATP, Brilliant Blue G, the tyrosine derivatives KN-62 and KN-04, cyclic imides, adamantane and benzamide derivatives [61], compound 4g [134], chelerythrine and other benzophenanthridine alkaloids [150], U73122 and U73343 [165] and compounds such as cyanoguanidines and aminotetrazoles [151]. Direct support for a role of P2X<sub>7</sub> receptors in pain modulation has been provided by studies using such selective antagonists. Thus, systemic administration of the P2X<sub>7</sub>-receptor-selective antagonists, A-438079 and A-740003, produced dose-dependent antinociceptive effects in models of neuropathic and inflammatory pain [45].

### **P2Y-Receptor Antagonists**

2-Methylthio-ADP is a potent agonist of mammalian P2Y<sub>1</sub> receptors and N<sup>6</sup>-methyl-2'-deoxyadenosine 3',5'-bisphosphate (MRS2179) and MRS2500 have been identified as selective antagonists [72]. At P2Y<sub>2</sub> and P2Y<sub>4</sub> receptors in the rat, ATP and UTP are equipotent, but the two receptors can be distinguished with antagonists. MRS2578 appears to be a selective antagonist at P2Y<sub>6</sub> receptors and there are a number of selective antagonists to the P2Y<sub>12</sub> receptor, including ARL-66096, INS-4266, and AZ-6140 [26]. Intrathecal administration of a P2Y<sub>12</sub>-receptor antagonist, AR-C69931MX, has been shown to prevent the development of tactile allodynia [166].

### ***Other Approaches***

Other therapeutic approaches to pain management using ATP systems are being considered, including development of agents that control expression of ATP receptors and those that inhibit ATP breakdown by selective inhibition of ectonucleotidases. In the meantime, while it is now clear that many different cell types release ATP physiologically in response to mechanical distortion, hypoxia, and various agents, we still await clear understanding of the mechanisms that underlie ATP transport. When this functioning becomes clearer, agents can be developed that can enhance or inhibit ATP release, another potential way forward as a therapeutic strategy. At this time, there are no publications to date describing clinical evaluations of P2-receptor antagonists and related purinergic compounds for the relief of pain. However, clinical trials for some compounds are in progress [56].

### **Clinical Applications of Adenosine Agents**

The preclinical profile of adenosine A1R agonists in nociception, whereby selective agonists have inhibited pain in several inflammatory and neuropathic pain conditions, appeared promising. Based on these findings, several novel selective A1R agonists have been developed. However, the orally active A1R agonist SDZ WAG 994 did not produce analgesia in postoperative dental pain [147]. Similarly, GR79236X, which exhibited antinociceptive actions in a preclinical model and was well tolerated, also lacked analgesic effects following third molar dental extractions [159]. It remains to be seen if these agents exhibit analgesic properties in other pain conditions.

In addition to direct effects on neurons, adenosine A2A, A2B, and A3 receptors can have widespread effects on immune and inflammatory cells, and potentially they could contribute to antinociception via such indirect actions. Recent reviews describing progress in the development of adenosine receptor ligands as anti-inflammatory drugs are available [3,96].

An additional line of exploration of potential adenosine-based agents for pain has been in developing inhibitors of adenosine metabolism via adenosine kinase [94,112]. Several selective compounds have emerged (ABT-702, A134974); these agents exhibit a spectrum of antinociceptive actions in inflammatory and neuropathic pain models following systemic spinal and local administration; they also exhibit a wider separation between antinociceptive actions and (adverse) hypomotor and cardiovascular effects than do A1R agonists [112]. However, despite these promising preclinical observations, these agents have not undergone clinical development.

One area of clinical application of adenosine for pain management that has been explored has been potential use for postoperative pain; this line of research was based on early reports of anesthetic and analgesic sparing effects of i.v. adenosine [67]. A recent systematic review of such studies reported on eight randomized, double-blind, placebo-controlled parallel-group studies between 1996 and 2006 [53]. In three trials, i.v. infusions of adenosine during surgery led to anesthetic sparing and some analgesic sparing

effects, and in two trials, i.v. adenosine was superior to remifentanil for pain relief and reduced opioid analgesia requirements following surgery. Two trials reported no beneficial effects of i.v. adenosine in the perioperative setting, while one trial reported no analgesia from intrathecal adenosine prior to anesthesia. While such studies suggest that adenosine is worthy of further investigation, some caution is necessary. (1) Methodological issues were identified in trials that showed anesthetic and analgesic sparing effects [53]. (2) The strongest support for analgesic properties of i.v. adenosine was from trials in which this treatment was compared to remifentanil. However, remifentanil can increase postoperative pain and increase analgesic requirements due to acute opioid tolerance [63], and this hyperalgesic action may account for the observed differences between groups. (3) A recent study administered adequate doses of adenosine intrathecally—both early and late, to test for possible preemptive as well as analgesic effects—and found no analgesic effects of adenosine [148]. Further studies will need to resolve these issues.

## Conclusions and Future Directions

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Multiple P2 receptor subtypes are involved in pain pathways, where they serve both as initiators and modulators of pain transmission. Peripheral homomeric P2X<sub>3</sub> receptors on sensory afferent neurons probably contribute to acute nociception and to some aspects of acute inflammatory pain [25,45], whereas heteromeric P2X<sub>2/3</sub> receptors appear to modulate longer-lasting nociceptive sensitivity associated with nerve injury or chronic inflammation [83]. Furthermore, under conditions of persistent nociceptive input activation, P2X<sub>4</sub>, P2X<sub>7</sub>, and P2Y<sub>12</sub> receptors on microglia may serve to maintain nociceptive sensitivity through complex neural-glial cell interactions or via sensitization (via P2Y<sub>2</sub>) of other nociceptive receptors such as TRPV1 channels. There is still an urgent need to understand mechanisms underlying the successful application of P2X<sub>3</sub>, P2X<sub>4</sub>, P2X<sub>7</sub>, and P2Y<sub>12</sub> receptor antagonists for the treatment of neuropathic pain.

With respect to adenosine systems, the preclinical literature demonstrates widespread antinociceptive actions of A1R agonists and inhibitors of adenosine metabolism (particularly via adenosine kinase) in models of inflammatory and neuropathic pain, potentially reflecting spinal, supraspinal, and peripheral actions. However, there are no convincing reports of analgesia in clinical trials using A1R agonists or adenosine kinase inhibitors. The largest body of clinical evidence relates to perioperative i.v. adenosine for pain management, and while studies of adenosine in comparison with remifentanil appeared promising, the reported differences in pain outcomes could reflect an acute opioid tolerance with remifentanil rather than an analgesic action of adenosine. Intrathecal adenosine does not produce either pre-emptive or postoperative analgesia in clinical trials. The most promising area for investigation of adenosine and its analogs is for neuropathic pain, where there are several reports of pain relief following both i.v. and intrathecal adenosine, and this area merits further exploration.

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