

Review

ATP receptors in pain sensation: Involvement of spinal microglia and P2X₄ receptors

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Abstract

There is abundant evidence that extracellular ATP and other nucleotides have an important role in pain signaling at both the periphery and in the CNS. At first, it was thought that ATP was simply involved in acute pain, since ATP is released from damaged cells and excites directly primary sensory neurons by activating their receptors. However, neither blocking P2X/Y receptors pharmacologically nor suppressing the expression of P2X/Y receptors molecularly in sensory neurons or in the spinal cord had an effect on acute physiological pain. The focus of attention now is on the possibility that endogenous ATP and its receptor system might be activated in pathological pain states, particularly in neuropathic pain. Neuropathic pain is often a consequence of nerve injury through surgery, bone compression, diabetes or infection. This type of pain can be so severe that even light touching can be intensely painful; unfortunately, this state is generally resistant to currently available treatments. An important advance in our understanding of the mechanisms involved in neuropathic pain has been made by a recent work demonstrating the crucial role of ATP receptors (i.e., P2X₃ and P2X₄ receptors). In this review, we summarize the role of ATP receptors, particularly the P2X₄ receptor, in neuropathic pain. The expression of P2X₄ receptors in the spinal cord is enhanced in spinal microglia after peripheral nerve injury, and blocking pharmacologically and suppressing molecularly P2X₄ receptors produce a reduction of the neuropathic pain behaviour. Understanding the key roles of ATP receptors including P2X₄ receptors may lead to new strategies for the management of neuropathic pain.

Abbreviations: IL-1 β – interleukin-1 β ; IL-6 – interleukin-6; MAPK – mitogen-activated protein kinase; PPADS – pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid; TNF- α – tumor necrosis factor- α ; TNP-ATP – 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate

Introduction

Although the possibility of extracellular ATP being able to evoke pain in the periphery was raised about 30 years ago [1, 2], significant advances in our understanding of the molecular mechanisms by which ATP causes pain have been made quite recently by the discovery of cell-surface receptors for detecting extracellular ATP, and other nucleotides, via P2 receptors on sensory neurons [3–7]. In a subset of primary afferent sensory neurons, ATP or its analogues produce electrophysiological and biological responses via ligand-gated ion-channel receptors, namely P2X receptors (P2XRs) [8–13], and G protein-coupled receptors, namely

P2Y receptors (P2YRs) [14–19]. The P2XR and P2YR subclasses have been further divided into seven (P2X_{1–7}Rs) and eight (P2Y_{1,2,4,6,11–14}Rs) subtypes, respectively [6, 20]. mRNA and protein for both P2XRs and P2YRs are expressed in sensory neurons, but accumulating evidence using gene deletion methods, antisense technologies, and selective receptor antagonists now suggests that the molecular targets of ATP on primary sensory neurons could be P2X₃Rs, P2X_{2/3}Rs (heteromultimeric P2X₂ and P2X₃ receptors), P2Y₁Rs and P2Y₂Rs [11, 17, 19, 21–26]. ATP responses evoked via these receptors in primary sensory neurons could code for the sensation of pain *in vivo* in laboratory animals [5–7, 11, 12, 23, 24, 26–35]. All cells contain millimolar levels of ATP and release ATP into the extracellular milieu when tissues are damaged [36] or stimulated mechanically [37]. The released ATP activates neighboring sensory nerve endings via P2X and P2YRs [36, 37]. These make ATP an interesting candidate for a tissue-damage signal at the periphery that may activate

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nociceptive sensory neurons [5–7, 12, 23, 24, 38]. In the dorsal horn of the spinal cord, ATP, presumably released from primary afferent central terminals [39], modulates excitatory (glutamatergic) and inhibitory (GABAergic and glycinergic) neurotransmission in dorsal horn neurons [40–47]. Activation of P2XRs in the spinal cord enhances pain behaviour [29, 48, 49], suggesting that ATP has a role in pain processing in the spinal cord as well. However, blocking P2XRs or P2YRs pharmacologically or suppressing their expression molecularly in sensory neurons or in the spinal cord had little effect on acute physiological pain evoked by heat or mechanical pressure in normal animals [23, 24, 26, 30, 31, 33]. It thus seems likely that endogenous ATP and its receptors system may be activated rather in pathological pain states, particularly in neuropathic pain, than in normal conditions [26, 30, 31, 50].

Neuropathic pain, which often develops when nerves are damaged through surgery, bone compression, diabetes or infection, is a type of pathological pain that does not resolve even when the overt tissue damage has healed [51–53]. Neuropathic pain can be so severe that even light contact with clothing can be intensely painful (tactile allodynia – an abnormal hypersensitivity to innocuous stimuli) and is relatively resistant to most current treatments. Accumulating evidence concerning how peripheral nerve injury creates neuropathic pain has suggested that molecular and cellular alterations in primary sensory neurons and in the spinal dorsal horn after nerve injury have important role in the pathogenesis of neuropathic pain [51–53]. While there is an increasing body of evidence suggesting that P2X₃R in primary sensory neurons have a role in neuropathic pain [26, 30, 31, 54, 55], other P2XR and P2YR subtypes are also beginning to be investigated in terms of their changes in expression using cDNA microarray [56–58]. Recently, we revealed that the P2X₄R subtype in the spinal cord is required for the expression of neuropathic pain [59]. Importantly, the expression of P2X₄R in the spinal cord is enhanced, and this is highly restricted to microglia that are activated after nerve injury. Until recently, glial cells were generally considered to serve primarily housekeeping roles in the nervous system, but that study directly implicated activated glia, particularly microglia, in the pathogenesis of neuropathic pain [59]. Here we review the progress in the current understanding of how the ATP receptor in spinal microglia participate in the pathophysiology of neuropathic pain.

Microglia and nerve injury-induced pain

Glial cells make up over 70% of the total cell population in the CNS and are classified into astrocytes, oligodendrocytes and microglia. In the adult, microglia are ubiquitously distributed throughout the CNS and represent a morphologically unique type of cell; under normal conditions, microglia have a small soma bearing thin and branched processes [60, 61]. Once activated by neuronal injury, trauma, ischemia, infection, or neurological diseases, microglia show a stereotypic program of changes in morphology, gene expression, function and number

[60–62]. Activated microglia change their morphology from a resting, ramified shape into an active, amoeboid shape. The changes in expression of cell-surface molecules (i.e., complement receptor 3, which is recognized by the antibody OX42) and in morphology are widely used as the key diagnostic markers of activated microglia [60, 61].

Clinical evidence that neuropathic pain results from damage to peripheral nerves in humans led to the development of a variety of models for studying neuropathic pain in laboratory animals. In most animal models of neuropathic pain which have been intensively studied so far peripheral nerves are directly damaged [63–67]. Evidence from studies using such models has revealed that peripheral nerve injury leads to a dramatic change in microglia within the spinal dorsal horn [68–72]. Within the first 24 h after peripheral nerve injury, spinal microglia become hypertrophic in their short and thick processes [68]. This is followed by a burst proliferation of microglia with a peak at around 2–3 days after the nerve injury [73]. Activated microglia exhibit upregulated OX42 labeling in the dorsal horn [68, 70–72, 74], which starts to increase as early as 1 day after nerve injury and peaks at around 14 days [70]. The temporal pattern of OX42 upregulation in the dorsal horn correlated with that of the development of tactile allodynia [70], suggesting the role of microglia in neuropathic pain. While there have been many studies showing that activation of microglia in the dorsal horn is correlated with the development of pain hypersensitivity in a wide variety of nerve injury models [68, 70–72, 74, 75], until recently it remained an open question whether spinal microglia play a causal role in neuropathic pain behaviour.

P2X₄R in spinal microglia are necessary and sufficient for neuropathic pain

A clue to identifying P2X₄R in the spinal cord as being required for neuropathic pain first came from pharmacological investigations of pain behaviour after nerve injury using the P2XR antagonists TNP-ATP and PPADS [59]. We found that the marked tactile allodynia that develops following injury of a spinal nerve was reversed by acutely administering TNP-ATP intrathecally but was unaffected by administering PPADS. TNP-ATP had no effect on acute pain behaviour in the uninjured state nor on motor behaviour. From the pharmacological profiles of TNP-ATP and PPADS, it was inferred that tactile allodynia depends upon P2X₄R in the spinal cord. The expression of P2X₄R protein, normally low in the naïve spinal cord, progressively increased in the days following nerve injury with a time-course parallel to that of the development of tactile allodynia. Immunohistochemical analysis demonstrated that many small cells in the dorsal horn on the side of the nerve injury were intensely positive for P2X₄R protein. These cells were identified as microglia rather than neurons or astrocytes by double immunolabelling using cell-specific markers. The cells expressing P2X₄R in the nerve-injured side of the dorsal horn were more numerous than under

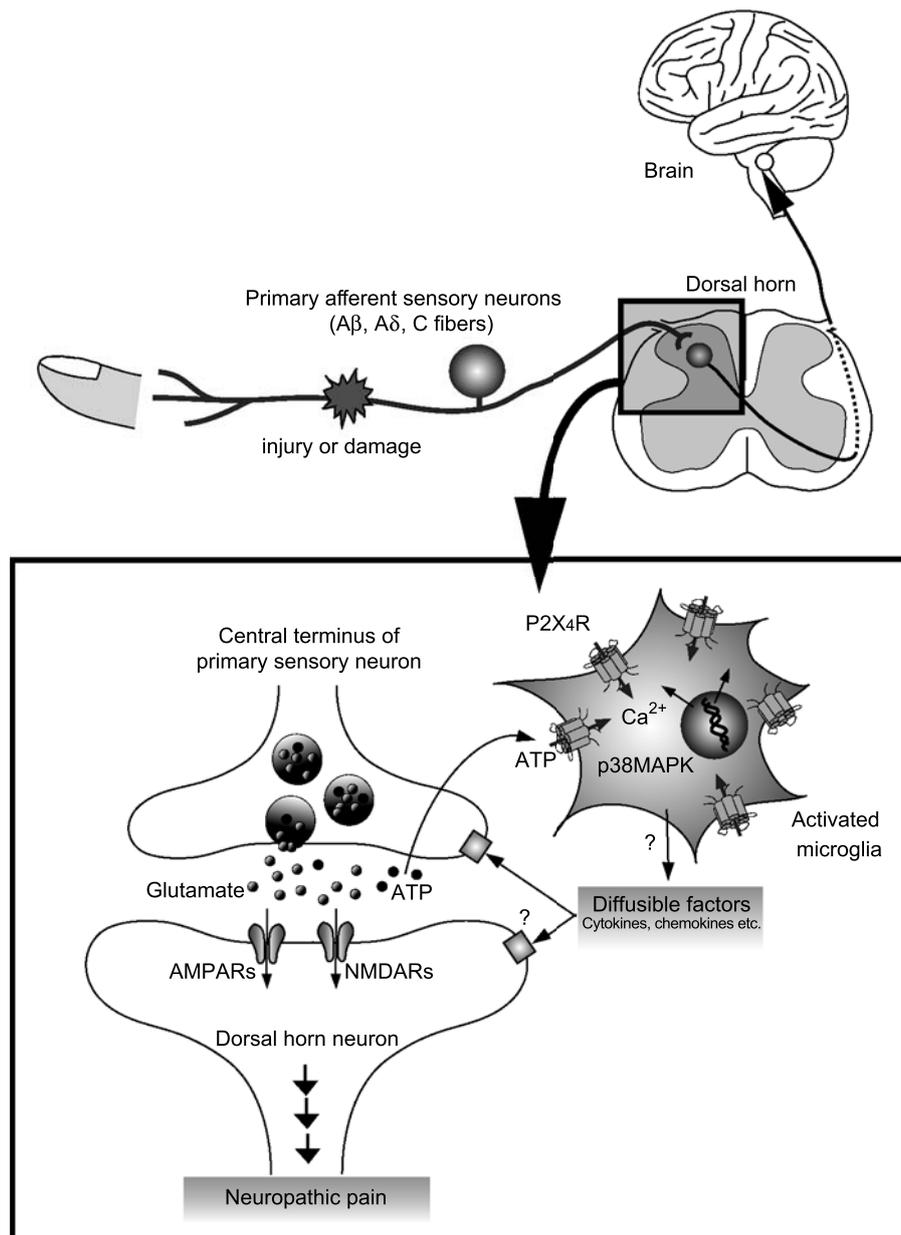


Figure 1. Schematic illustration of possible mechanisms by which P2X₄R in activated microglia modulate pain signaling in the dorsal horn. Peripheral nerve injury leads to molecular and cellular alterations in the spinal dorsal horn after nerve injury. Activated microglia after nerve injury increase the expression of P2X₄R. The P2X₄R are activated by ATP which is presumably released from primary sensory neurons (or astrocytes), and in turn cause a rise in the intracellular calcium and activate p38MAPK. These signals lead to the release of bioactive diffusible factors such as cytokines and chemokines. The diffusible messengers released from microglia may then interact with excitatory and inhibitory synapses of neighboring dorsal horn neurons and enhance the excitability in dorsal horn neurons, which in turn leads to increased transmission in pain signaling neurons. The enhanced transmission in the dorsal horn pain network might be due to the facilitation of glutamatergic synaptic transmission or the suppression of GABA/glycinergic inhibition.

control conditions and showed high levels of OX42 labeling and morphological hypertrophy, all of which are characteristic markers of activated microglia. Moreover, it was found that reducing the upregulation of P2X₄R protein in spinal microglia by means of intrathecally administered antisense oligodeoxynucleotide targeting P2X₄R prevented the development of the nerve injury-induced tactile allodynia. Collectively, this evidence implies that P2X₄R activation is necessary for pain hypersensitivity following nerve injury, and that microglia are required for this hypersensitivity since the expression of these receptors in the dorsal horn is restricted to this type of cell.

The sufficiency of P2X₄R activation in microglia for the development of allodynia was demonstrated by intrathecal administration of activated, cultured microglia in which these receptors had been stimulated *in vitro* by ATP [59]. In otherwise naïve animals, intrathecal administration of cultured microglia that were preincubated with ATP to activate P2X₄R on microglia produced tactile allodynia progressively over the 3–5 h following the administration. In contrast, intrathecal administration of unstimulated microglia did not cause allodynia nor did administering vehicle or ATP alone. Microglia also express another subtype of P2XR, P2X₇R, but this receptor subtype appears

not to be involved because activation of P2X₇Rs typically requires a higher concentration of ATP than used [76, 77] and because TNP-ATP, which does not affect P2X₇Rs [78], prevents ATP from stimulating microglia to produce allodynia. Moreover, in rats in which tactile allodynia was caused by the ATP-stimulated microglia this allodynia was reversed by administering TNP-ATP [59]. Thus, stimulation of P2X₄Rs is required in the tactile allodynia caused by ATP-stimulated microglia and this tactile allodynia therefore resembles that caused by nerve injury. Collectively, these findings indicate that P2X₄R stimulation of microglia is not only necessary for tactile allodynia, but is also sufficient to cause the allodynia. Furthermore, this finding makes a strong case that microglia activation is not simply correlated with neuropathic pain behaviour. Rather, microglia within the dorsal horn play an active and ongoing role in the tactile allodynia produced by injury to peripheral nerves.

Possible mechanisms underlying microglial modulation of dorsal horn pain signalling

The variety of biological effects produced by ATP in microglia in *in vitro* studies using purified microglia in culture may provide hints towards clarifying the mechanisms by which microglia produce altered processing of information in the spinal cord dorsal horn. We have previously shown that ATP stimulates the release of plasminogen in a concentration-dependent manner from 10 to 100 μ M with a peak response at 5–10 min after the stimulation [79]. ATP also potently stimulates tumor necrosis factor- α (TNF- α) release following an increase in the TNF- α mRNA expression by activating mitogen-activated protein kinases (MAPKs) [80, 81]. A *de novo* synthesis and release of interleukin-6 is also induced by ATP through pathways mediated by both Ca²⁺-dependent protein kinase C and p38 MAPK [82], a member of the MAPK family [83]. Interestingly, we and others have found that nerve injury leads to persistent activation of p38 MAPK, and, importantly, that the activation of p38 MAPK in the spinal cord is entirely restricted to microglia [84, 85]. Furthermore, an inhibitor of this enzyme, SB203580, administered intrathecally, reverses mechanical allodynia following spinal nerve ligation [84, 85]. Also, continuous infusion of SB203580 starting before the nerve injury prevents the development of tactile allodynia [84]. These indicate that p38MAPK could be an important intracellular event in the development of nerve injury-induced pain. Moreover, several cytokines such as IL-1 β , IL-6 and TNF- α in the dorsal horn are increased after nerve lesion [86–88] and have been implicated in contributing to nerve-injury pain [75, 86–90].

The ability of ATP to release cytokines raises the possibility that microglia might release one, or more, diffusible factor(s) that act either directly or indirectly on neurons (Figure 1). Several cytokines have been reported to alter synaptic transmission in the CNS including the spinal cord [91–93]. For example, the exogenous application of IL-1 β enhances NMDA receptor-mediated Ca²⁺ responses

via activating tyrosine protein kinase Src [94], which is known to enhance NMDA receptor activity in dorsal horn neurons [52, 95]. IL-1 β also decreases GABA_A receptor-mediated currents [96]. More recently, Moriguchi et al. [97] have shown in acute cortical slices that the application of a microglial-conditioned medium potentiates NMDA receptor-mediated postsynaptic responses, but not when the medium is boiled or incubated with proteinase K. Furthermore, they fractionated the medium into six sharp peaks by anion-exchange chromatography and found that the fraction contained a relatively strong protein band with a molecular mass of approximately 70 kDa that showed the most potent enhancing activity on the NMDA receptor-mediated responses. Although they have not identified the molecule(s) yet, they suggested that both heat- and protease-labile molecules released from microglia regulate NMDA receptor-mediated excitatory synaptic transmission in the CNS. Thus, diffusible factors released from activated microglia by activating P2X₄Rs may also have modulatory effects on neurons in the pain processing network within the dorsal horn (Figure 1).

Conclusion

Damage or injury to peripheral nerve leads to changes in the spinal cord that cause the activation of microglia (Figure 1). These activated microglia express P2X₄R; endogenous ATP can then activate these receptors and lead to neuropathic pain. Almost all currently known drugs for neuropathic pain were developed to target neurons, and these drugs do not exhibit adequate therapeutic effects in patients with neuropathic pain [98]. We expect that efforts to elucidate how P2X₄R signaling in microglia causes neuropathic pain will provide us both with exciting insights into pain mechanisms and with clues to developing new therapeutic agents which may fundamentally change the management of intractable pain. Such strategies could not have been anticipated based on the prevailing neuron-centric view of pain.

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