

## Review Article

# The Purinergic System and Glial Cells: Emerging Costars in Nociception

Giulia Magni<sup>1,2</sup> and Stefania Ceruti<sup>1</sup>

<sup>1</sup> Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Via Balzaretti, 9-20133 Milan, Italy

<sup>2</sup> Department of Drug Discovery and Development (D3), Italian Institute of Technology (IIT), Via Morego, 30-16163 Genoa, Italy

Correspondence should be addressed to Stefania Ceruti; [stefania.ceruti@unimi.it](mailto:stefania.ceruti@unimi.it)

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It is now well established that glial cells not only provide mechanical and trophic support to neurons but can directly contribute to neurotransmission, for example, by release and uptake of neurotransmitters and by secreting pro- and anti-inflammatory mediators. This has greatly changed our attitude towards acute and chronic disorders, paving the way for new therapeutic approaches targeting activated glial cells to indirectly modulate and/or restore neuronal functions. A deeper understanding of the molecular mechanisms and signaling pathways involved in neuron-to-glia and glia-to-glia communication that can be pharmacologically targeted is therefore a mandatory step toward the success of this new healing strategy. This holds true also in the field of pain transmission, where the key involvement of astrocytes and microglia in the central nervous system and satellite glial cells in peripheral ganglia has been clearly demonstrated, and literally hundreds of signaling molecules have been identified. Here, we shall focus on one emerging signaling system involved in the cross talk between neurons and glial cells, the purinergic system, consisting of extracellular nucleotides and nucleosides and their membrane receptors. Specifically, we shall summarize existing evidence of novel “druggable” glial purinergic targets, which could help in the development of innovative analgesic approaches to chronic pain states.

## 1. Chronic Pain: Neurons Need Costars to Be Sensitized

Several lines of evidence from basic pain research using diverse animal models indicate that an aberrant excitability of dorsal horn neurons evoked by peripheral sensory inputs is at the basis of both neuropathic and inflammatory pain [1, 2]. While neurons have long been considered the only cell type involved in pain development and transmission, recent studies have shown that pathologically altered neurotransmission requires communication with glial cells [3].

Microglia show a long-term response to a wide range of stimuli controlling physiological homeostasis, including peripheral nerve injury (PNI). In response to PNI, microglia activation in the spinal cord leads to cell hypertrophy, increase in cell number, and altered gene expression [4–6]. By responding to extracellular stimuli, activated glial cells evoke various cellular responses, such as production and release

of bioactive factors including cytokines and neurotrophic factors [7], which in turn lead to the hyperexcitability of dorsal horn neurons and, consequently, to the development of neuropathic pain.

Not only spinal microglia but also astrocytes are involved in neuronal sensitization in the spinal cord. In fact, it has been hypothesized that microglia sense nerve damage or proallogenic inputs from the periphery, and as a consequence they release several mediators that either act directly on dorsal horn neurons or promote reactive astrogliosis [8]. Reactive astrogliosis is a typical double-edged sword phenomenon, showing both neurotoxic and neuroprotective features, depending upon the type of injury and the basal conditions of the affected tissue [9]. This is possibly true also in pain models, where both astrocyte-derived proallogenic and analgesic molecules have been detected [8].

Glial cells are directly involved in nociception not only in the central nervous system (CNS), but also in the periphery. In fact, a peculiar type of glial cells, named satellite glial

cells (SGCs), is located in peripheral sensory ganglia, where they envelop neuronal bodies, thus constituting distinct morphological and functional units [10]. In the past few years, their central role in the development and maintenance of chronic pain has been clearly demonstrated by several authors, reporting an increased expression and release of mediators such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor alpha (TNF $\alpha$ ) [11, 12], as well as their increased gap junction-mediated coupling following nerve injury [13]. In response to chronic pain, SGCs also upregulate the expression of glial fibrillary acidic protein (GFAP) and undergo cell division [14, 15].

The currently established role of glial cells in nociception suggests that the pharmacological manipulation of their reactivity might represent a groundbreaking analgesic strategy, as an alternative to a classical “neuron-centric” approach. It is therefore mandatory to identify the most promising key signaling pathways contributing to glial cell reaction to painful stimulation, which could be exploited pharmacologically. Here, we shall focus on the purinergic system, which in recent years has emerged as one of the most innovative and yet partially unexplored signaling systems controlling glial cell function under various pathological conditions, including pain. Far from being exhaustive in reviewing the exponentially growing number of publications in this field, this review aims at convincing the pain community that extracellular nucleotides and nucleosides are fundamental pro- and antinociceptive signals, mostly through their activity on peripheral and central glial cells.

## 2. Ionic Receptors Activated by Extracellular Nucleotides: The P2X Receptor Family

Extracellular nucleotides, mostly ATP but possibly also UTP, are costored in synaptic vesicles and actively released during physiological or pathological (i.e., excitotoxic) neurotransmission [16]. Glial sources of ATP have been also demonstrated, which contribute to physiological cell-to-cell communication [17]. Additionally, extracellular concentrations of nucleotides increase severalfold at sites of tissue damage, due to leakage of the intracellular nucleotide pool and degradation of nucleic acids [16, 17].

P2X receptors are membrane ion channels that open in response to the binding of extracellular ATP and elicit rapid responses (<10 ms), resulting in selective permeability to Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> cations [18]. In vertebrates, seven genes encode P2X receptor subunits, which are 40–50% identical in their amino acid sequence. Each subunit has two transmembrane domains, separated by an extracellular domain (~280 amino acids). Channels function as trimers of several subunits, and the extracellular domain is the portion containing the binding sites for nucleotides. To date, 7 homomeric (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, and P2X7), and 6 heteromeric P2X receptors (P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, and P2X4/6) have been identified, the latter showing specific pharmacological differences compared to their homomeric counterpart [18]. P2X receptors are differentially distributed among the cell types in different brain areas. Peripheral

neurons mainly express the P2X3 and P2X2/3 receptor subtypes, which are involved in pain and temperature perception (see below) [19]. All P2X receptor subtypes can modulate membrane action potential. They are expressed either at the presynaptic level, regulating neurotransmitter release through the modulation of intracellular levels of Ca<sup>2+</sup> ions, or postsynaptically, where they interact with ionotropic receptors (i.e., nicotinic; gamma-aminobutyric acid, GABA; N-Methyl-D-Aspartate, NMDA) via Ca<sup>2+</sup>-dependent kinases [16]. P2X receptors are involved in a variety of physiological processes, including the modulation of cardiac rhythm and contractility, vascular tone, platelet aggregation, macrophage activation, apoptosis, neuron-glia communication, and modulation of nociception [20].

## 3. P2X Receptors and Chronic Pain: Not Only a Matter of Neurons

Since the first publications demonstrating the involvement of ATP in pain transmission [21], the expression levels and functions of P2X receptors have been characterized in neurons from dorsal root ganglia (DRG) and in the trigeminal nerve [22]. All P2X receptor subtypes, with the exception of P2X7, are expressed in sensory neurons; among these, the P2X3 subtype showed the highest expression levels [23]. To date, the most important P2X receptor subtypes involved in pain transmission are (i) the P2X3 and P2X2/3 receptor subtypes, expressed by sensory neurons, (ii) the P2X4 receptor, expressed by CNS microglia, and (iii) the P2X7 receptor, expressed by CNS microglia and astrocytes and by SGCs in sensory ganglia [24].

## 4. Neuronal P2X3 Receptors: Key Players in Nociception

Although this review article is focused on glial purinergic receptors involved in pain transmission, a brief summary of data on neuronal P2X3 subtype, the most studied P2X receptor subtype involved in nociception, cannot be omitted. P2X3 receptors are selectively expressed at high levels in nociceptive primary sensory neurons in trigeminal, nodose, and dorsal root ganglia [25]. The selective expression of P2X3 receptors in these areas involved in pain transmission suggested that they may be crucial in processing pain signals, and several studies have evaluated the relationships between P2X3 receptors and pain sensation.

P2X3 receptors have been initially cloned and functionally characterized from rat DRG [26], showing their unique expression in small and medium size sensory neurons and their absence in large diameter neurons [27, 28]. On the other hand, P2X3 has never been found to be expressed by glial cells, by either immunohistochemical analysis or calcium imaging [29]. P2X3 immunoreactivity has also been observed in other structures indirectly involved in nociceptive transmission, such as the nucleus of the solitary tract, and the spinal trigeminal nucleus [30].

A number of *in vitro* and *in vivo* studies have evaluated the relationships between P2X3 receptors and pain transmission.

Local injection of P2X3 receptor agonists, such as ATP or  $\alpha,\beta$ -methyleneATP, into the hind paws of rats induced nociceptive behaviors and reduced thermal and mechanical thresholds [31, 32]. During inflammation and chronic constriction injuries, ATP concentrations and neuronal expression of P2X3 receptors are upregulated in DRG, trigeminal ganglion (TG), and spinal cord [33, 34]. In animal models of inflammatory and neuropathic pain, P2X3 receptor knockout or its downregulation by antisense oligonucleotides or by short interfering RNA significantly attenuated painful behaviors, including tactile allodynia and mechanical hyperalgesia [35]. In addition, the nonselective P2X3 receptor antagonists TNP-ATP [2',3'-O-(2,4,6 trinitrophenyl) adenosine-5'-triphosphate] and PPADS pyridoxal phosphate-6-azophenyl-2',4' disulfonic acid reduced the excitability of DRG neurons and neuropathic pain [36]. Overall, these findings suggest that neuronal P2X3 receptors play a crucial role in the development and maintenance of chronic pain.

Despite the above-mentioned results, more recent studies have shown a transient decrease in P2X3 expression in a model of partial damage of the mental nerve (belonging to the mandibular branch of the trigeminal nerve), or no significant expression changes following resection of the lingual nerve [37]. It is possible that these differences reflect the type of lesion: after a constriction (i.e., application of a ligature) an increase in the expression levels is observed, while nerve injury or full axotomy leads to reduction or no changes of receptor expression. More importantly, it has been observed that variations in P2X3 expression levels are modulated at different time points after the lesion; a transient decrease in the expression was observed in the first two weeks after nerve injury, followed by an increase during the following weeks, paralleled by the increased expression of glial cell-derived neurotrophic factor (GDNF) and the generation of new neuronal branches (sprouting) in the damaged sensory areas [38]. Therefore, the timing and the type of lesion are critical for the observed results. Finally, it is also worth mentioning that P2X3 receptors are involved in the mechanism of action of known analgesics, as recently shown for the nonsteroidal anti-inflammatory drug naproxen, which inhibits P2X3 activation in the TG [39]. Moreover, it is now emerging that bioactive molecules contained in analgesic preparation from traditional Chinese medicine (such as tetramethylpyrazine, sodium ferulate, and puerarin) can inhibit P2X3-mediated transmission in primary afferent neurons [40].

## 5. Glial P2X4 and P2X7 Receptors

Microglia-neuron communication is bidirectional, and several lines of evidence implicate ATP as a critical signaling molecule [41–43]. Microglia expression of P2X receptors is restricted to the P2X4 and P2X7 subtypes [42, 44], whose activation evokes current responses, increases intracellular  $[Ca^{2+}]_i$ , and causes the release of signaling molecules affecting neuronal functions [45, 46].

The central role of P2X4 receptor subtype in neuropathic pain was first reported by [47]. In this paper, authors showed that intrathecal injection of TNP-ATP, acting as antagonist

at P2X1-4 receptor subtypes, reverted tactile allodynia in nerve-injured rats. On the contrary, treatment with PPADS, antagonist at P2X1-3, 5, and 7 but not at P2X4 receptor, had no effect. Authors therefore concluded that the main P2X receptor subtype involved in central responses to peripheral nerve injury is the P2X4 receptor, and the development and maintenance of tactile allodynia require its activation. To confirm this hypothesis, targeting P2X4 receptors with antisense oligonucleotides [47] or the genetic deletion of the *P2rx4* gene [48] significantly attenuated nerve injury-induced pain phenotypes. Moreover, the development of tactile allodynia correlated with a progressive increase in spinal P2X4 receptor expression, which is generally low in naïve CNS [48].

Further immunohistochemical analysis of wild-type (wt) mice, and studies on transgenic mice selectively expressing the green fluorescent protein in microglial cells revealed that this cell population rather than neurons or astrocytes was expressing the P2X4 receptor subtype [48] and that the increase in P2X4 receptor expression temporally correlated with the development of tactile allodynia. P2X4 receptor upregulation was a consequence, rather than a cause of microglia activation; in fact, preventing the increase in P2X4 receptor did not affect the expression of protein markers of microglia activation, such as complement receptor 3 or the ionized calcium binding adaptor molecule-1 (Iba-1) [47, 48]. Interestingly, activation of microglial P2X4 receptor is sufficient to elicit pain, since the intrathecal injection of P2X4 receptor-stimulated cultured microglia induced mechanical allodynia in naïve animals [41, 47, 49]. Taken together, pharmacological, genetic, and behavioral findings indicate that activation of P2X4 receptor subtype expressed by spinal microglia is both necessary and sufficient to induce tactile allodynia after peripheral nerve injury.

Concerning the bidirectional cross talk between activated microglia and dorsal horn neurons, the chemokine (C-C motif) ligand 21 (CCL21) released from injured neurons has been demonstrated to act as an upstream activator of P2X4 receptor [50], in close collaboration with interferon  $\gamma$ , a cytokine converting resting spinal microglia into its activated state [51], and tryptase, a protease released from mast cells that activates proteinase-activated receptor 2 in microglia [52] (Figure 1). Another critical element for the upregulation of P2X4 receptors expression is the extracellular matrix molecule fibronectin, which modulates the transcriptional and posttranscriptional levels of P2X4 receptor expression in microglia through the activity of Lyn kinase and the downstream activation of intracellular signaling pathways involving phosphatidylinositol 3-kinase- (PI3 K-) Akt and mitogen-activated protein kinase kinase- (MAPK kinase, MEK-) extracellular signal-regulated kinase (ERK) [49, 53, 54]. The implications of this wide modulation network and whether these elements are connected into a common pathway controlling P2X4 receptor expression are still not known.

On the other way around, it has been reported that the activation of microglial P2X4 receptor promoted the synthesis and release of brain-derived neurotrophic factor (BDNF), through the activation of p38 MAPkinase [48, 55].

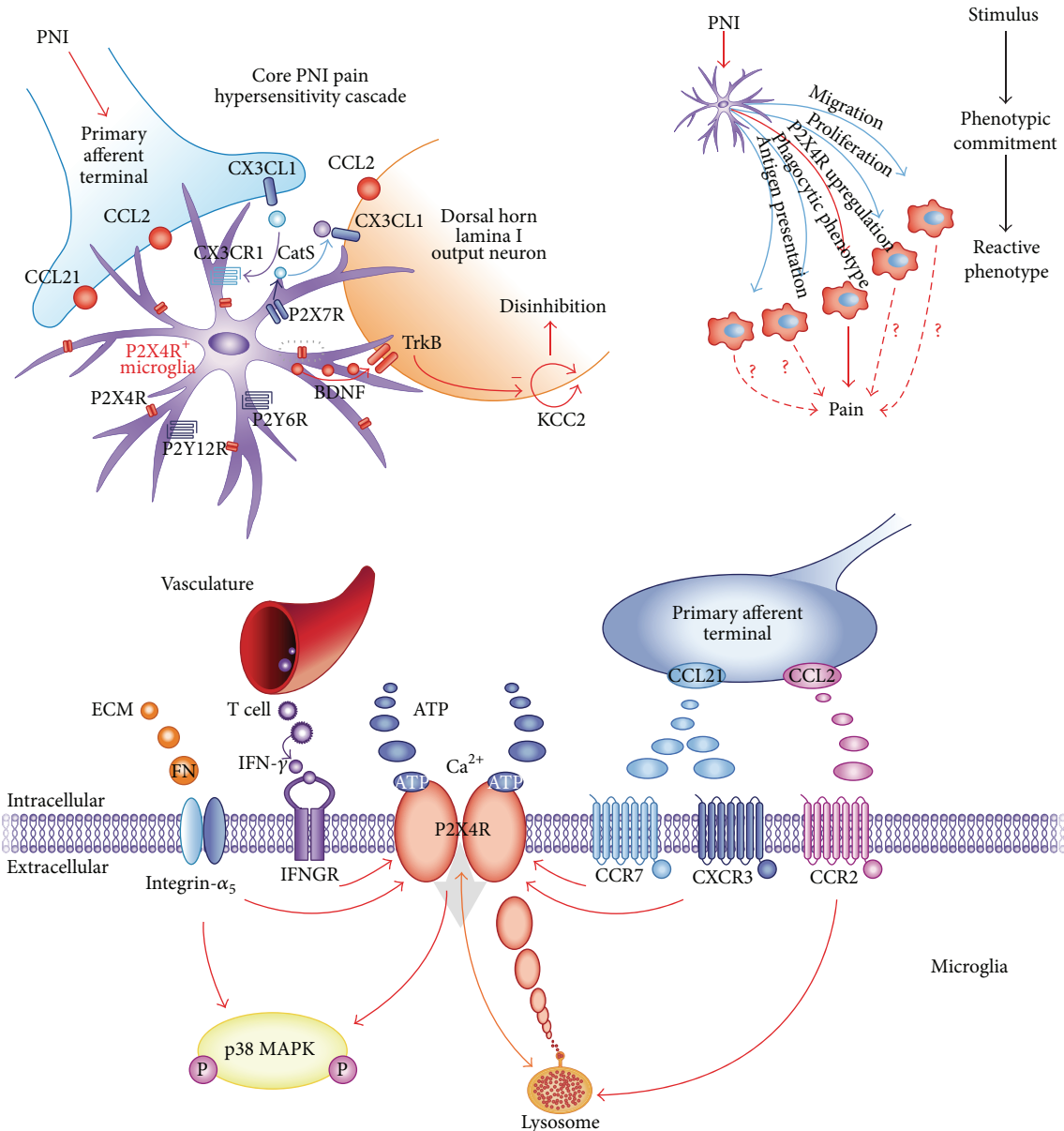


FIGURE 1: Central role of microglial P2X4 receptors in mediating a molecular cascade leading to hypersensitivity after peripheral nerve injury (PNI). The upper left cartoon shows the neuron-to-microglia cross talk triggered by neuronal injury, leading to the appearance of an “activated” microglia phenotype, which expresses higher levels of the P2X4 receptor. The dotted rectangle is expanded in the lower panel, showing the complex molecular network responsible for P2X4 receptor upregulation and involving chemokines, growth factors, and integrins. Activation of the P2X4 receptor ultimately leads to the recruitment and activation of p38 MAP kinase. Reproduced from [56] with permission from Nature Publishing Group.

BDNF in turn altered the transmembrane anion gradient in a subpopulation of dorsal horn lamina I neurons, through the downregulation of the neuronal chloride transporter KCC2. This makes GABA and glycine act as depolarizing rather than hyperpolarizing neurotransmitters, causing an aberrant nociceptive output that contributes to the development of neuropathic pain [41, 56].

In addition to the P2X4 subtype, microglial cells also express functional P2X7 receptors. The P2X7 receptor is a unique member of the P2X receptor family, since it is

only activated by high concentrations of ATP ( $>100 \mu\text{M}$ ) and can open a much larger membrane pore than any other P2X channel [57]. Stimulation of P2X7 receptors is implicated in microglia response to inflammation, release of proinflammatory cytokines, like  $\text{IL-1}\beta$ , and proliferation [58], underlining the involvement of this receptor subtype in inflammatory diseases and pain.

Although P2X7 receptors are expressed on a wide variety of cell types, evidence for involvement of microglial P2X7 receptors in chronic neuropathic pain stems from findings

that its activation causes the p38 MAPkinase-dependent release of IL-1 $\beta$  and cathepsin S, which contribute to the maintenance of mechanical hypersensitivity in the spinal cord [59, 60]. Additional hints on the role of P2X7 receptors in neuropathic pain have been provided by the demonstration of reduced pain sensitivity in P2X7 receptor-deficient mice [61] and by the analgesic effects exerted by P2X7 receptor antagonists [62, 63]. In fact, authors demonstrated the absence of inflammatory and neuropathic hypersensitivity. In fact, in mice lacking the P2X7 receptor, authors demonstrated the absence of inflammatory and neuropathic hypersensitivity after both mechanical and thermal stimuli, accompanied by a reduction of adjuvant-induced increases in IL-1 $\beta$ , IL-6, IL-10, and macrophage chemoattractant protein-1, while normal nociceptive processing is preserved [61]. Moreover, P2X7 receptor was upregulated in human DRG and injured nerves from chronic neuropathic pain patients [61]. Recent studies also showed that P2X7 receptor expression is increased in the ipsilateral spinal cord after sciatic nerve ligation and is highly restricted to microglia; moreover, chronic constriction injury- (CCI-) induced mechanical allodynia and thermal hypersensitivity are reversed by intrathecal administration of the selective P2X7 antagonist Brilliant Blue G [64].

Interestingly, a correlation between single nucleotide polymorphisms (SNPs) within the coding sequence of the *P2rx7* gene and the sensitivity to chronic pain has been provided in both mice and humans. In fact, mice in which P2X7 receptors have impaired pore formation due to the P451L mutation showed less allodynia than mice with the wt pore-forming *P2rx7* allele. Indeed, administration of a peptide, which blocked pore formation but not cation channel activity, selectively reduced nerve injury and inflammatory allodynia only in wt mice. Moreover, in two independent human chronic pain cohorts, a cohort with pain after mastectomy and a cohort with osteoarthritis, authors observed a genetic association between lower pain intensity and the hypofunctional His270 allele of P2X7 receptor, further suggesting that selectively targeting P2X7 pore formation may be a new strategy for individualizing the treatment of chronic pain [65].

Several lines of evidence suggest a functional convergence between the signaling pathways activated by P2X4 and P2X7 receptors in microglia. As previously mentioned, p38 MAPkinase is involved in both the P2X4 receptor-mediated release of BDNF and the P2X7-induced release of IL-1 $\beta$  and cathepsin S (see above). Moreover, a recent paper showed that phosphorylation of p38 MAPK via P2X7 receptor may induce tactile allodynia/hyperalgesia in an orofacial pain model based on the CCI of the infraorbital nerve (CCI-ION), which is most likely mediated by soluble TNF- $\alpha$  released by microglia [66]. Thus, activation of p38 MAPK may be a critical mechanistic step of convergence in the P2X7 and P2X4 receptor signaling pathways in neuropathic pain. P2X7 and P2X4 receptors also interact to form complexes with unique channel properties [44, 67]. This raises the interesting possibility that structural and functional interactions, as well as converging signaling pathways, might be critical cellular mechanisms that underlie the involvement of microglial P2X7 and P2X4 receptors in neuropathic pain. The expression

of P2X4 and P2X7 receptors subtype in a variety of cell types involved in pain transmission represents a promising target for pharmacological intervention. For example, locally administered oxidized ATP, an irreversible inhibitor of P2X7 receptors, had an antihyperalgesic effect on complete Freund's adjuvant-induced mechanical hyperalgesia (paw pressure test) [68].

An important role has been also disclosed for the P2X7 receptor subtype in the modulation of reactive astrogliosis. Following trauma, an upregulation of its expression on astrocytes has been demonstrated, and its activation by extracellular ATP or by synthetic agonists led to the release of proinflammatory IL-1 $\beta$ , ATP itself, and glutamate [17]. These molecules can further sustain and propagate astrocytic reaction to injury and can also sensitize nearby neurons in the spinal cord, thus contributing to nociception. More recently, the P2X7-mediated astrocytic release of dynorphins has been demonstrated in the spinal cord [69], thus further corroborating the notion of a direct involvement of astrocytic P2X7 receptor subtype in nociception.

The role of glial P2X7 in nociception is not restricted to the CNS, since recent data established its expression in SGCs, the specialized population of glial cells that envelop neuronal bodies in peripheral sensory ganglia [10]. In sensory ganglia, ATP released from neuronal somata engages P2X7 receptors expressed by surrounding SGCs [12], which in turn inhibits back neuronal algogenic P2X3 receptors [70]. Additional P2X receptor subtypes are upregulated in SGCs upon exposure to proinflammatory conditions [71], but their role in nociception still remains elusive.

## 6. G Protein-Coupled Receptors Activated by Extracellular Nucleotides: The P2Y Receptor Family

The first P2Y receptor (currently named as the P2Y<sub>1</sub> subtype) was cloned in 1993 [76, 77], and since then a total of eight human P2Y receptor subtypes have been identified: the P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub>, and P2Y<sub>14</sub> receptors [78]. The various P2Y receptor subtypes are functionally coupled to different G proteins, and, consequently, downstream signaling pathways, either phospholipase C, with inositol 3 phosphate (IP<sub>3</sub>) formation and intracellular calcium increase, or adenylyl cyclase, leading to modulation of cyclic adenosine monophosphate (cAMP) production [78]. From a pharmacological point of view, P2Y receptors can be broadly subdivided into four groups based on their responsiveness to nucleotides: (i) adenine nucleotide-preferring receptors, mainly responding to ADP and ATP; this group includes human and rodent P2Y<sub>1</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub>, and human P2Y<sub>11</sub>; (ii) uracil nucleotide-preferring receptors, including the human P2Y<sub>4</sub> and P2Y<sub>6</sub> responding to either UTP or UDP; (iii) receptors of mixed selectivity (the human and rodent P2Y<sub>2</sub>, the rodent P2Y<sub>4</sub> and, possibly, P2Y<sub>11</sub>); and (iv) the P2Y<sub>14</sub> receptor, responding to both UDP and sugar nucleotides (mainly UDP-glucose and UDP-galactose) [78, 79].

## 7. Glial P2Y Receptors and Chronic Pain: Still Elusive but Promising Targets for New Analgesic Strategies

Unlike the key and quite well defined role played by P2X receptors in pain transmission, controversial information is currently available concerning P2Y receptor contribution to nociception. Nevertheless, it is well known that these receptors are widely expressed by neurons and glial cells in both the central and the peripheral nervous systems [16], thus leading to hypothesize that their activity is integrated in the complex molecular network associated with the transmission of nociceptive signals. The comprehension of their role in pain pathogenesis is therefore of paramount importance for the discovery of new potential drug targets for pain therapy.

As for P2Y receptors expressed by sensory neurons, the P2Y<sub>1</sub> receptor subtype is often coexpressed with the P2X<sub>3</sub> receptor, acting as its functional antagonist in DRG [80], while the P2Y<sub>12,13</sub> subtypes can inhibit N-type voltage-gated calcium channels [81]. Taken together, these data suggest an overall analgesic profile for ADP-sensitive P2Y receptor activation. Surprisingly, P2Y<sub>1</sub> receptors were also reported to mediate hyperalgesia, possibly through the sensitization of transient receptor potential vanilloid 1 (TRPV<sub>1</sub>) channels [82], thus suggesting that the functional outcome of P2Y<sub>1</sub> receptor activation could depend upon the specific tissue conditions. The same hyperalgesic potentiation of TRPV<sub>1</sub> receptors has been later demonstrated for the UTP-sensitive P2Y<sub>2</sub> receptor subtype in rat lumbar DRGs [83].

Increasing evidence is highlighting a role for glial P2Y receptors in chronic pain. Microglia express a wide range of P2Y receptors (P2Y<sub>1,2,4,6,12</sub>), with the P2Y<sub>6</sub> and P2Y<sub>12</sub> receptor subtypes mediating chemotaxis and migration towards the site of damage [43, 84]. To date, only the P2Y<sub>12</sub> receptor subtype is clearly known to be involved in neuropathic pain onset. In fact, in response to peripheral nerve injury, microglial P2Y<sub>12</sub> receptor expression was upregulated, and its activation was critical for microglial engulfment of myelinated axons in the spinal dorsal horn [43]. Moreover, pharmacological blockade, antisense knockdown of P2Y<sub>12</sub> receptor expression, or genetic deletion of the *P2ry12* gene suppresses both mechanical allodynia and thermal hyperalgesia in nerve-injured rats [85, 86]. Conversely, intrathecal administration of the P2Y<sub>12</sub> receptor agonist 2Me-SADP elicits pain behavior in naïve rats, mimicking what is observed in nerve-injured rats [85]. Moreover, mRNA not only for the P2Y<sub>12</sub>, but also for the P2Y<sub>6,13,14</sub> receptor subtypes was upregulated in microglia in the ipsilateral spinal cord following nerve injury, and a mixture of P2Y<sub>6</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub> selective antagonists showed a longer suppressive effect on pain behavior compared to every single treatment itself [87].

The P2Y<sub>6</sub> receptor can be considered a sensor for microglial cell phagocytosis by sensing diffusible UDP signals in primary cultured microglial cells [84]. Although microglial P2Y<sub>6</sub> receptors expression markedly increases in the spinal cord after peripheral nerve injury [87], its specific role under neuropathic pain conditions still remains unclear.

As already mentioned, ATP-gated P2X<sub>4</sub> receptor channels expressed in spinal microglia actively participate in central sensitization, making their functional regulation a key process in chronic pain pathologies (see above); interestingly, it has been recently reported that P2X<sub>4</sub> receptors function is inhibited by the P2Y<sub>6</sub> receptor subtype, highlighting its possible critical role in regulating neuropathic pain-induced microglial responses [88].

Although not directly associated with pain signaling to date, it is conceivable that also P2Y receptors expressed by astrocytes could significantly contribute to nociception, especially in the spinal cord. P2Y receptors have been directly implicated in the modulation of reactive astrogliosis since the 1990s, also thanks to the contribution of our laboratory (for reviews, see [9, 89]). In particular, activation of the P2Y<sub>1</sub> receptor subtype has been directly correlated with the release of prostaglandins and other proinflammatory mediators [90, 91] and directly or indirectly correlated with the transmission of painful sensations. Targeting astrocytic P2Y receptors could therefore represent an innovative approach to pain, specifically neuropathic pain, through the modulation of astroglial inputs controlling second order spinal neurons firing.

Very few data are currently available on the role of the P2Y receptor family in SGCs, and the vast majority of the available data concerns expression rather than functional studies (for reviews see [75, 92]). Under basal conditions, SGCs in the trigeminal ganglion express functional P2Y<sub>1,2,4,6,13</sub> receptor subtypes [28, 93], whereas glial P2Y expression in DRG cells has been only evaluated at the mRNA level [94], with the exception of P2Y<sub>1</sub> receptors protein which has been detected by immunofluorescence studies [70].

Within the TG, the close interplay between the purinergic system and other classical pain transducing signals has been recently elucidated, starting from the observation that chronic exposure of TG cultures to the algogenic mediator bradykinin induced the upregulation of P2Y-mediated calcium signaling in SGCs [28, 95], thus prompting us to speculate that P2Y receptors expressed by SGCs could represent possible pharmacological targets for the development of innovative therapies against migraine and trigeminal pain in general (for review see [75]). From then on, *in vitro* and *in vivo* studies have revealed that those specific glial P2Y receptors potentiated under proalgogenic conditions are the P2Y<sub>1</sub> and P2Y<sub>2</sub> subtypes (Magni et al., manuscript in preparation). These results have been partially confirmed by a recent paper showing that P2Y<sub>2</sub> receptor inhibition induces analgesia in a rat model of neuropathic pain based on CCI-ION [96], although authors mostly focused on P2Y<sub>2</sub> receptors expressed by trigeminal sensory neurons. Altogether, these data highlight the key role of P2Y purinergic receptors, and especially of the P2Y<sub>2</sub> subtype, as promising targets for the development of new analgesic drugs.

An emerging role for the glial P2Y<sub>12</sub> receptor subtype has been demonstrated in an animal model of trigeminal neuropathic pain [97]. In fact, although not expressed in the TG upon control conditions [14], expression of the P2Y<sub>12</sub> receptor subtype was detected in activated SGCs after unilateral lingual nerve crush. Interestingly, administration

of a P2Y<sub>12</sub>-selective antagonist significantly reverted glial cell activation and alleviated animals' sensitivity to mechanical and heat stimulation of the tongue [97]. These data further sustain the hypothesis that a shift and/or upregulation in purinergic receptor expression can be observed within sensory ganglia, and especially on SGCs, upon proallogenic conditions (Figure 2). This observation opens up the possibility that drugs selectively targeting specific purinergic receptors could exert their analgesic activity at the site of receptor upregulation, thus limiting the occurrence of systemic and unwanted side effects.

## 8. Adenosine Exerts Analgesic Effects through Its Glial Receptors: An Additional Component of Its Neuromodulatory Actions

Adenosine is the major metabolite of the ectonucleotidase-mediated sequential dephosphorylation of ATP. Therefore, it cannot be classified as a true neurotransmitter, but rather as a neuromodulator, through the activation of 4 subtypes of G protein-coupled receptors, that is, the A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> adenosine receptor subtypes, collectively referred to as P1 receptors [98]. The temporal relationship between ATP release and subsequent adenosine generation from its hydrolysis guarantees the generation of a feedback inhibitory loop, which overall counteracts the excitatory effects of ATP. This is particularly true for the A<sub>1</sub> receptor subtype, which is coupled with inhibition of cAMP synthesis and with the activation of potassium conductance, with consequent inhibition of neuronal firing and neurotransmitter release, reduction of heart rate, and other inhibitory effects.

A key role for the A<sub>1</sub> receptor subtype in the modulation of painful sensations has been hypothesized more than 15 years ago. Both spinal and peripheral A<sub>1</sub> receptors have been found directly involved in the analgesic effects exerted by several known drugs, like tramadol, acetaminophen, and amitriptyline, often in close interconnection with 5-HT<sub>7</sub> serotonin receptors [99–101]. Moreover, recent studies also suggest that the analgesic efficacy of some herbal preparations long utilized in traditional Chinese medicine can be at least in part due to a modulation of the adenosine system. In fact, norisoboldine (the major active alkaloid component isolated from the dry roots of *Lindera aggregata* often utilized for chest and abdomen pain, indigestion, cold hernia, and frequent urination) was shown to promote analgesia in both the formalin and the writhing tests in rodents through the activation of A<sub>1</sub> receptor subtype [102].

The cell population expressing the A<sub>1</sub> receptor subtype involved in pain transmission was not clearly identified, until the publication of papers suggesting its microglial identity. An A<sub>1</sub> receptor subtype selective agonist was in fact shown to alleviate neuropathic pain and to reduce spinal microglia activation in the spared nerve injury rat model [103]. Most interestingly, authors later demonstrated that exposure to proallogenic ATP induced a significant increase in A<sub>1</sub> receptor subtype expression in microglia, whose activation in turn led to inhibition of microglial phenotypic changes and to the

reduction of the ability of microglia to promote neuronal firing [104]. Thus, it can be envisaged that microglial A<sub>1</sub> receptors are upregulated and engaged to counteract the allogenic actions induced by massive ATP release at injury or inflammation sites.

The role of additional adenosine receptor subtypes in nociception is much less clear. Conflicting results have been obtained by administering either A<sub>2A</sub> receptor subtype-selective agonists or antagonists in animal pain models, showing both analgesia and hyperalgesia [105]. In fact, it has been demonstrated that A<sub>2A</sub> receptor knock-out mice have a reduced sensitivity to pronociceptive stimuli, which seems to be mediated by the loss of pronociceptive A<sub>2A</sub> receptors on DRG primary sensory neurons [106]. It should be mentioned, however, that microglia express A<sub>2A</sub> receptors, which are directly involved in the modulation of the release of proallogenic stimuli, like nerve growth factor (NGF), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and nitric oxide (NO) [107]. Based on the above-mentioned data, it can therefore be envisaged that selective A<sub>2A</sub> receptor antagonists could counteract the proinflammatory and proallogenic features of microglial cells and ultimately prove to be useful as antiallogenic agents, at least in inflammatory pain conditions. Conversely, the intrathecal injection of a selective A<sub>2A</sub> receptor agonist produced a significant and long-lasting reversal of mechanical allodynia and thermal hyperalgesia consequent to the CCI of the sciatic nerve [108]. A reduction of glial cell activation and a significant upregulation in the production of anti-inflammatory IL-10 in the spinal cord were also observed, thus suggesting that activating rather than inhibiting the A<sub>2A</sub> receptor subtype could represent a better option for neuropathic pain states [108].

Finally, the availability of selective agonist ligands has allowed demonstrating an antiallogenic role for the A<sub>3</sub> receptor subtype in neuropathic pain induced either by the CCI of the sciatic nerve or by chemotherapeutic agents in rodents [109]. The cellular localization of the target receptors involved in nociception has not been directly identified, although it has been demonstrated that the A<sub>3</sub> receptor subtype is involved in ADP-mediated microglia chemotaxis and process extension [110].

Although their direct role in pain transmission has not been proven yet, it is worth mentioning that all the four adenosine receptor subtypes are expressed by spinal cord and brain astrocytes and significantly contribute to the modulation of reactive astrogliosis (for review see [111]). It is therefore conceivable that they can participate to astrocytic modulation of neuronal functions, especially under chronic pain conditions, when high amounts of adenosine are produced as a consequence of the hydrolysis of extracellular nucleotides and of the leakage of nucleic acids from damaged cells [111]. To date the role of adenosine receptors in peripheral ganglia has not been studied in detail.

A major limitation to the systemic use of drugs acting on adenosine receptors is represented by their wide distribution, especially in the CNS and in the cardiovascular system, which could account for significant and harmful side effects. Specifically, activation of the A<sub>1</sub> adenosine receptors in the CNS could lead to inhibition of locomotor activity

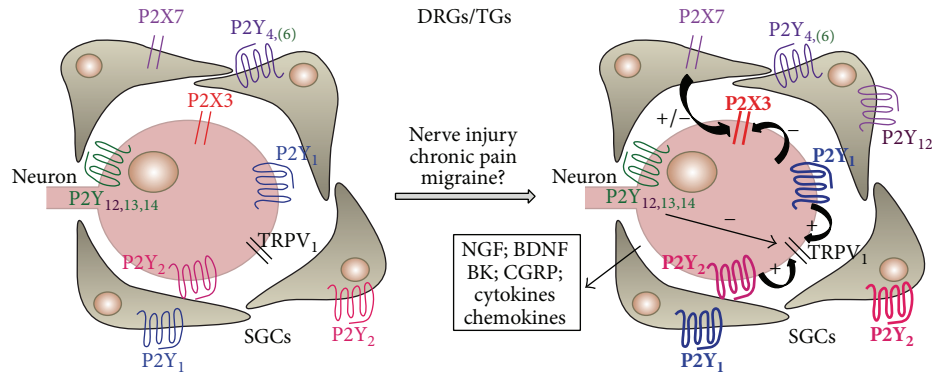


FIGURE 2: Plasticity of purinergic signaling within sensory ganglia upon proinflammatory conditions. The cartoon schematically summarizes currently available data on the modulation of P2 receptor expression by proallogenic and proinflammatory conditions in sensory ganglia. An increased line weight indicates receptor upregulation, which has been observed in both sensory neurons and surrounding satellite glial cells. Some receptor subtypes (i.e., glial P2Y<sub>12</sub> receptor subtype) are only detected upon allogenic conditions. Activation of P2 receptors by extracellular nucleotides leads to the modulation of other systems involved in nociception, like TRPV<sub>1</sub> receptors, and in the release of additional cytokines, growth factors, and other substances (see box), further contributing to the development and maintenance of a proallogenic milieu. Reproduced from [75] with permission from Elsevier.

and catalepsy, whereas in the heart severe bradycardia and atrioventricular block may occur [112]. Acting on the A<sub>2A</sub> subtype with selective agonists may lead to a significant drop in blood pressure, accompanied by tachycardia [112]. Conversely, despite the significant increase of circulating histamine that was observed in mice, clinical studies in cancer patients have shown no significant side effects after administration of selective A<sub>3</sub> receptor agonists [112].

## 9. When Will an Analgesic Drug Targeting Glial Purinergic Receptors Be Finally Available?

The incomplete and still growing list of scientific studies reviewed in this paper clearly demonstrates that targeting glial purinergic receptors might significantly improve the currently available armamentarium of analgesic drugs to be utilized in chronic pain state, still lacking a satisfactory pharmacological control. Nevertheless, to date no new purinergic drug entities have reached the market. This is mostly due to (i) the widespread expression of all the different receptor subtypes, which renders the discrimination between beneficial and side effects extremely complicated, (ii) the lack of good subtype-selective agents, and (iii) when selective ligands are available, the lack of an acceptable route of administration to humans.

Concerning the most studied purinergic receptor in nociception, the neuronal P2X<sub>3</sub> subtype, no clinical studies were available so far, despite the massive preclinical *in vivo* and *in vitro* efforts. This was mainly due to the lack of selective antagonists with a chemical structure suitable for administration to humans [37]. Very recently, however, two clinical trials employing the new orally active P2X<sub>3</sub>-selective antagonist AF-219 [113] were concluded (see Table 1), although results have not been disclosed yet. Recently, the extensive use of high-throughput screening followed by a hit-to-lead program

has prompted a number of pharmaceutical companies to identify highly selective, potent, and metabolically stable small-molecule inhibitors for both P2X<sub>4</sub> and P2X<sub>7</sub> receptor, which provide a novel therapeutic approach to the treatment of pain and inflammatory disorders [114, 115]. Nevertheless, no significant results have emerged from the first clinical trials performed with different P2X<sub>7</sub>-selective antagonists (Table 1), thus suggesting that the role of this receptor subtype should be reconsidered, at least in rheumatoid arthritis-related pain.

As for P2Y receptors, although important progress in exploring structure-activity relationships has been recently achieved with the publication of the first crystal structure of a member of the family [116], most of the P2Y receptor subtypes are still lacking potent and selective synthetic agonists and antagonists [78], which has greatly hampered both research in this field, and the translation of *in vitro* data to *in vivo* and clinical settings. Additionally, the only P2Y-selective antagonists available so far, acting on the P2Y<sub>12</sub> receptor subtype, are widely utilized for their strong antiplatelet activity [116], which poses serious concerns about possible side effects in case of their administration for painful conditions.

Finally, in the case of the A<sub>1</sub> adenosine receptor subtype, its widespread distribution has greatly limited the preclinical and clinical use of the available A<sub>1</sub>-selective agonists, due to significant systemic side effects (see above). Recently, it has been demonstrated that a novel and potent A<sub>1</sub> receptor allosteric modulator, named TRR469, exhibits antinociceptive activity in the formalin and writhing tests in mice, without showing the usual side effects of A<sub>1</sub>-selective agonists (see above) [117].

It is also worth reaffirming that expression of purinergic receptor on glial cells is highly plastic and can therefore significantly change during the course of chronic pain conditions (Figure 2). For example, it has been hypothesized that the purinergic system is fundamental for the so-called “modal shift” of microglia, namely, their acquisition of different



TABLE 1: Summary of past and current clinical trials evaluating purinergic ligands in pain.

Name of ligand	Company	Clinical trial number	Type of pain	Main outcomes
AF219 (P2X3 antagonist)	Afferent Pharmaceuticals	NCT01554579	Osteoarthritis of the knee	No published results yet
		NCT01569438	Interstitial cystitis/bladder pain syndrome	No published results yet
CE-224,535 (P2X7 antagonist)	Pfizer	NCT00628095	Rheumatoid arthritis	CE-224,535 was not efficacious, compared with placebo, for the treatment of RA in patients with an inadequate response to metotrexate [72]
		NCT00418782	Osteoarthritis of the knee	CE-224,535 failed to demonstrate efficacy in a 2-week study of knee pain in osteoarthritis patients
AZD9056	AstraZeneca	NCT00520572	Rheumatoid arthritis	AZD9056 does not have significant efficacy in the treatment of RA [73]
GSK1482160 (P2X7 antagonist)	GlaxoSmithKline	NCT00849134	“First-in-human” study to assess the pharmacokinetic profile of the drug	No major safety or tolerability concerns were identified in this study. Nevertheless, monitoring the release of IL-1 $\beta$ in subjects led to the decision to discontinue drug development for inflammatory pain conditions [74]
Adenosine (e.v.)	Wake Forest School of Medicine	NCT00349921	Neuropathic pain (comparison with clonidine)	No published results yet
	Xsira Pharmaceuticals	NCT00298636	Perioperative pain (hysterectomy/myomectomy)	No published results yet
GW493838	GlaxoSmithKline	NCT00376454	Neuropathic pain as a result of postherpetic neuralgia or nerve injury	No published results yet

phenotypes over time, based on the time-dependent modification of the expression profile of P1 and P2 receptors [118]. This additional fascinating, but complicating, issue should be taken into consideration when deciding which is the best target option for the development of an antihyperalgesic rather than an antiallodynic purinergic-based agent.

Therefore, based on these promises and pitfalls, a closer collaboration among pharmacology, biochemistry, molecular biology, and pharmaceutical chemistry is highly recommended to clearly identify and target the most promising “druggable” receptors, which could be successfully utilized in preclinical and clinical studies to accelerate the process towards new therapeutic options for chronic pain patients.

### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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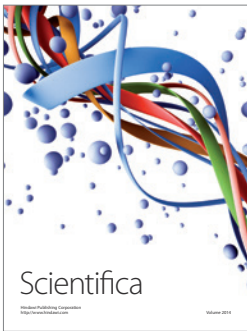
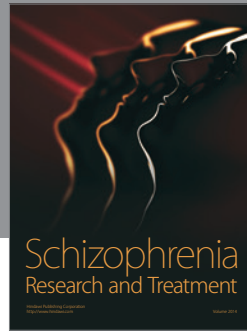
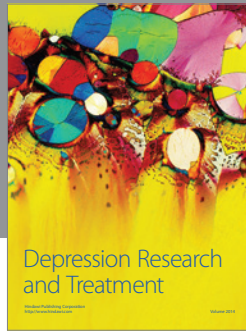
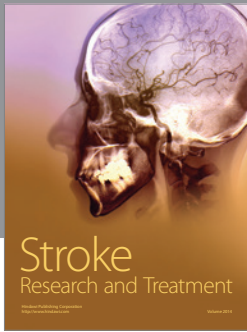
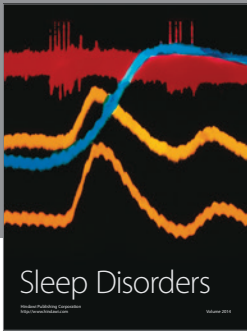
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