The role of adenosine and P2Y receptors expressed by multiple cell types in pain transmission

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Abstract

The role of extracellular nucleotides and nucleosides as signaling molecules in cell-to-cell communication has now been clearly established. This is particularly true in the central and peripheral nervous system, where purines and pyrimidines are involved in both physiological and pathological interactions between neurons and surrounding glial cells. It can be thus foreseen that the purinergic system could represent a new potential target for the development of effective analgesics, also through the normalization of neuronal functions and the inhibition of glial cell activation. Research in the last 15 years has progressively confirmed this hypothesis, but no purinergic-based analgesics have reach the market so far; in the present review we have collected the more recent discoveries on the role of G protein-coupled P2Y nucleotide and of adenosine receptors expressed by both neurons and glial cells under painful conditions, and we have highlighted some of the challenges that must be faced to translate basic and preclinical studies to clinics.

Keywords

Adenosine; extracellular nucleotides; G protein-coupled receptors; glial cells; pain

Highlights

- P2Y and adenosine receptors are expressed by glia and neurons along pain pathways in the brain and periphery
- Each receptor subtype plays peculiar, often opposite, roles in pain transmission
- Interesting pharmacological targets have been identified, but their clinical exploitation is still in its infancy

Abbreviations

2MeSADP: 2 Methylthioadenosine triphosphate; 5-HT: 5-hydroxytryptamine (serotonin); ADP: adenosine diphosphate; AMP: adenosine monophosphate; ATP: adenosine triphosphate; BDNF: brain-derived neurotrophic factor; CB2: cannabinoid receptor type 2; CCL2: C-C Motif Chemokine Ligand 2; CCR5: C-C chemokine receptor type 5; CD: cluster of differentiation; CFA: complete Freund's adjuvant; CGRP: calcitonin gene-related peptide; CNS: central nervous system; COX: cyclooxygenase; CPA: cyclo-pentyl-adenosine; CSF1: colony-stimulating factor 1; CX3CR1: CX3C chemokine receptor 1; CXCL13: C-X-C motif ligand 13; Cx43: connexin 43; ddC: 2',3'-dideoxycytidine; DPCPX: 8-cyclopentyl-1,3-dipropyl-xanthine; DRG: dorsal root ganglia; GFAP: glial fibrillary acidic protein; gp120: glycoprotein 120; HIF-1α: hypoxia-inducible factor 1α; Iba1: ionized calcium-binding adapter molecule 1; IBD: inflammatory bowel disease; IBS: irritable bowel syndrome; IL: interleukin; LPS: lipopolysaccharides; MAPK: mitogen-activated protein kinase; PAP: prostatic acid phosphatase; PNS: peripheral nervous system; SGCs: satellite glial cells; SGK-1: serum/glucocorticoid regulated kinase 1; shRNA: short hairpin RNA; SP: substance P; TG: trigeminal ganglia; TNF: tumor necrosis factor; TRPV1: transient receptor potential vanilloid 1; TSC: Tianshu capsule; TSP4: thrombospondin-4; UTP: uridine-5'-triphosphate

Contents

1. The contribution of different cell types to pain transmission: focus on the purinergic system

In the last decades the purinergic system has clearly emerged as one of the most important signaling system in the whole body and, in particular, in the central and peripheral nervous systems (CNS and the PNS, respectively) under both physiological and pathological conditions, including pain. In fact, both neurons and glial cells can modulate pain transmission by releasing pro- or anti-nociceptive mediators, among which a major role is played by ATP and other nucleotides, which bind to either fast ligand-operated P2X channels or to slowly-acting specific G-protein coupled receptors, the P2Y receptor family widely expressed throughout the body (Magni and Ceruti, 2013).

The extracellular receptor-mediated actions of adenosine have been discovered well before those exerted by extracellular nucleotides, starting from its actions in the cardiovascular system up to every district and cell types of the body (Borea et al., 2018). Nevertheless, its role has been long referred to as simple "neuromodulator" since it is not stored in vesicles as such, but it represents the end product of the hydrolysis of nucleotides. Moreover, its actions are often opposite to those exerted by ATP and other nucleotides; in most cases in the CNS, ATP is excitatory while adenosine is inhibitory on neuronal firing. This has led to the idea that adenosine has the only task to counterbalance and "switch off" nucleotide-mediated actions (Chen et al., 2013). This view has been progressively extended and integrated, with the discovery and cloning of 4 G protein-coupled receptor subtypes selectively activated by adenosine and widely distributed in all tissues and cells of the body (for an extensive review on the state-of-art in adenosine pharmacology please refer to Borea et al., 2018).

Although it is still true that adenosine often plays a compensatory role against nucleotide-mediated activities, also thanks to the delayed raise of its concentrations, it has now emerged that adenosine receptor plays fundamental roles in many physiological and pathological conditions. This is particularly true in pathologies where the local extracellular concentrations of nucleotides rise several folds, such as tissue damage, hypoxia, but also increased neuronal firing as in epilepsy or during chronic pain (Figure 1; Chen et al., 2013).

In the last decade, the hypothesis that non-neuronal cells, including immune cells (macrophages and lymphocytes) and glial cells, play an important role in the generation and maintenance of painful sensation besides neurons has become a matter of fact. Indeed, although several painful conditions are undoubtedly of neuronal origin due to sensitization and increased neuronal firing, the contribution of surrounding glial cells is fundamental to the development and maintenance of the pathologic phenotype, especially concerning the transition from acute to chronic pain. In fact, the classical "neuron-centric" view of pain has progressively moved to a more integrated approach in which glial cells equal neurons in painful networks (Ji et al., 2013), suggesting that the pharmacological modulation of their reactivity could represent an innovative analgesic strategy.

In particular, under pain conditions, neuronal sensitization leads to increased release of neurotransmitters and neuromodulators, which act paracrinally on glial cells. Glial cells, in turn, respond to these stimuli by releasing a variety of signals that could further expand and sustain neuronal sensitization through the so-called "maladaptive plasticity". In fact, one of the main features of the CNS is its ability to be modified by both external and internal stimuli; plasticity of synapses is at the basis of the learning and memory processes and it also involves the contribution of glial cells (Chung et al., 2015). On the other hand, prolonged and pathological changes in the connections within brain areas lead to the disruption of functions which may be considered a disease itself, leading to both functional and structural permanent modifications. In the case of pain transmission, maladaptive plasticity can occur in the periphery (with the development of ectopic spontaneous activity of nociceptors and peripheral sensitization), and at both the spinal and supraspinal levels, with modifications of the balance between nociceptive and anti-nociceptive transmission (May, 2008). Glial cells are at the front row in driving these changes, as demonstrated by their permanent activation not only in animal models of chronic pain, but also in chronic pain patients (Loggia et al., 2015). After nerve injury, the interaction between neurons and activated glial cells induces maladaptive synaptic reorganization and activates intracellular signaling events that permanently contribute to enhance neuropathic pain. (Gwak et al., 2017).

The main non-neuronal actors in the CNS are microglial cells, the resident macrophages of the spinal cord and brain, which become rapidly activated in response to even minor pathological changes (Grace et al., 2014), and astrocytes that, after nerve injury, lose their ability to maintain the homeostatic concentrations of extracellular potassium and glutamate, leading to neuronal hyperexcitability (Ji et al., 2013). The extension of microglial and astrocytic processes into and near the synaptic space accounts for their ability to induce alterations to thousands of synapses, resulting in extensively altered neural networks. Moreover, the interaction between presynaptic and postsynaptic neuronal structures, with the contributions of activated microglia and astrocytes and, likely, of oligodendrocytes, facilitates the transmission of pain-mediating substances produced by activated glial cells. Finally, in the PNS, a fundamental role in controlling neuronal firing is played by satellite glial cells (SGCs) which ensheath the somata of primary sensory neurons within sensory ganglia and by infiltrating macrophages which populate ganglia (see below for details).

Based on literature evidence, the existence of a bidirectional signaling between neurons and glial cells, both in the CNS and in sensory ganglia, which is responsible of the initiation and maintenance of chronic pain conditions has now been ascertained. Its pharmacological modulation could therefore represent a new and more effective approach to chronic painful conditions with respect to currently available drugs which mostly target neurons. The discovery that drugs already

utilized in therapy (such as the antibiotic minocycline or the anti-asthmatic agent Ibudilast; Grace et al., 2014) also act as general glial cell modulators has paved the way towards drug repurposing in the field of pain management (see also Conclusions). In parallel, research is focusing on the comprehensive understanding of the whole network of molecules involved in cell-to-cell communication in pain, with the aim of identifying new potentially "druggable" targets.

Multiple adenosine and P2Y receptors are expressed by all the different cell types involved in pain transmission. Current knowledge is summarized in Figure 2, but the scenario is far from being complete as new pharmacological and biochemical tools become available.

In the last five years we and others have published many extensive reviews on the role of the purinegic system in controlling pain signaling (see for example Magni and Ceruti, 2013; 2014; Sawynok, 2016; Burnstock, 2017; Magni et al., 2018). Thus, in the next sections we shall focus on the more recent advances on the role of G protein-coupled P2Y nucleotide receptors and of P1 adenosine receptors expressed by various cell types in modulating pain transmission with the aim of identifying the most relevant pharmacological targets to be further exploited in clinics. Updates on the contribution of ionotropic P2X nucleotide receptors in pain are provided elsewhere in this issue.

2. Adenosine and P2Y receptors in neuropathic pain: modulating glial cell reaction to nerve injury.

2.1 Microglia purinergic equipment driving neuropathic pain

Spinal cord microglia have been strongly implicated in the pathogenesis of neuropathic pain. In particular, after peripheral nerve injury a marked proliferation and activation of microglia has been observed, in parallel with the up-regulation of the typical markers Iba1 and CD11b (Suter, 2016). Different mediators participate in microglia-neuron crosstalk during pain conditions, including ATP, colony-stimulating factor-1 (CSF1), proteases (e.g. cathepsin S), caspase-6 and chemokines (e.g. CCL2 and fractalkine), which derive from the central terminals of dorsal root ganglia (DRG) primary sensory neurons (Zhang et al., 2017; Figure 3).

In parallel, the expression of microglial purinergic receptors for nucleotides (in particular P2X4, P2X7 and $P2Y_{12}$) and fractalkine receptor CX3CR1 is increased (Beggs et al., 2012; Clark & Malcangio, 2014). In turn, their activation results in an intracellular signaling cascade involving the phosphorylation of p38 MAP kinase, which drives the synthesis and release of TNF-α, IL-1β, IL-18, and BDNF, and in the increased expression of cyclooxygenase (COX) and subsequent synthesis of prostaglandin E2 (Ji et al., 2016). All these neuromodulators regulate the balance between excitatory and inhibitory synapses within the spinal cord, triggering pain signal transmission to the brain.

Interestingly, it has been recently demonstrated that, although nerve injury results in spinal microglia activation in both sexes, pharmacological or genetic interference with microglial cell functions reduces allodynia in male mice only (Sorge et al., 2015), and purinergic P2X4 receptors seem to be mainly involved in this sexually dimorphic pain transmission (Mapplebeck et al., 2018; see also "P2X receptors in pain" in this issue).

In a similar way to inflammatory pain, an overall anti-algogenic role for adenosine A_1 receptor subtype in neuropathic pain emerges from literature data (for review, see Magni et al., 2018). Apart from its known inhibitory role on neuronal transmission, thanks to the coupling to potassium channels in neurons, which was long considered as the main mechanism to explain the analgesic activity of A_1 receptor agonists (Varani et al., 2017), a clear-cut demonstration of the involvement of this receptor subtype in pain transmission came from the discovery of its expression on activated microglia cells following induction of neuropathic pain (Luongo et al., 2014). Moreover, the administration of a new A_1-selective agonist, named 5'-chloro-5'-deoxy-(±)-ENBA, led to reduced microglia activation and inhibited its ability to sensitize sensory neurons (Luongo et al., 2012). This suggests that A_1 receptor upregulation on microglia represents an attempt to reduce the development of pain following injury and adds this receptor subtype to the growing number of microglia purinergic receptors which represent possible targets for the development of centrally-active analgesics.

In this respect, the leading role as purinergic receptors expressed by central microglia involved in pain transmission is entrusted to the $P2Y_{12}$ and $P2Y_6$ subtypes.

$P2Y_{12}$ receptors on microglia are a primary site at which nucleotides act to induce chemotaxis of microglial processes during the initial phases of pain transmission (Haynes et al., 2006), leading to increased neuronal sensitization and persistent pain. $P2Y_{12}$ is known to deeply influence microglial dynamics *in vivo:* the receptor is highly and preferentially expressed in ramified resting microglia, and undergoes rapid downregulation following the initiation of inflammation, indicating that it primarily acts during rapid responses to perturbations of brain homeostasis (Sipe et al., 2016). Moreover, mice lacking $P2Y_{12}$ receptors show reduced spinal microglial proliferation after spinal nerve injury and reduced neuropathic pain compared to *wild type* mice (Gu et al., 2016).

Recently, it has been shown that dorsal horn spinal cord microglia contribute to cannabinoid CB2 receptor-mediated analgesia, and activation of CB2 receptor reduces the expression of $P2Y_{12}$ and $P2Y_{13}$ receptors in rats subjected to neuropathic pain (Niu et al., 2017). The latter nucleotide receptor subtype, which shares many structural and pharmacological properties with $P2Y_{12}$ (for example they are both activated by ADP; Alexander et al., 2017) has been long neglected in the evaluation of purinergic contribution to neuropathic pain. Nevertheless, $P2Y_{13}$ receptors have been found upregulated in spinal microglia following nerve injury (Kobayashi et al., 2012), via

RhoA/ROCK protein pathway (Tatsumi et al., 2015), and in diabetes-related neuropathic pain leading to mechanical allodynia with receptor activation linked to increased expression of IL-1β and IL-6 (Zhou et al., 2018).

It is also worth mentioning that the intrathecal administration of non-selective $P2Y_{1,12,13}$ receptor agonists, namely ADPβS and 2meSADP, was able to induce neuropathic pain and increased thermal and mechanical sensitivity in naïve rats (Tatsumi et al., 2015; Niu et al., 2017; Sugawara et al., 2017). This suggests that the upregulation of spinal cord P2Y receptor expression which is detected after nerve injury is not necessary to trigger pain and that basally expressed receptors are involved as well.

Together with the $P2Y_{12}$ receptor subtype, $P2Y_6$ receptors are mainly known for their role in the regulation of CNS microglial cell functions, namely phagocytosis (Xu et al., 2016). Concerning the role of this receptor in pain transmission, authors have described both pro-algogenic and analgesic actions (reviewed in Magni et al., 2018). A very recent paper seems to point to pro-algogenic effects for this receptor, since the intrathecal administration of a selective $P2Y_6$ antagonist reduced both thermal hyperalgesia and mechanical allodynia in rats exposed to chronic constriction injury of the sciatic nerve, with a parallel decrease of microglia activation (Huang et al., 2018).

Recently, the involvement of $P2Y_2$ receptor subtype on microglia in neuropathic pain has also been demonstrated. Authors evaluated the role of intrathecal administration of ulinastatin, a serine protease inhibitor with anti-inflammatory and neuroprotective effects which is already on the market in Far East countries (i.e., Japan, India, South Korea and China), in a rat model of sciatic nerve ligation. Results show that increased expression of $P2Y_2$ receptors is involved in microglia activation after nerve injury. Administration of ulinastatin: i) prevented the development of mechanical allodynia and thermal hypersensitivity; ii) reduced the level of extracellular ATP; iii) down-regulated $P2Y_2$ receptors, and iv) inhibited microglia activation in the dorsal horn of the spinal cord (Shi et al., 2017). These data confirm the crucial role played by $P2Y_2$ receptors in modulating glial cell activation both in the CNS and PNS and pave the way for the use of ulinastatin in the management of neuropathic pain.

2.2 Contribution of spinal astrocytes to neuropathic pain: a role for the purinergic system?

At variance with microglial cells, astrocyte activation in chronic pain conditions is more persistent, indicating their major contribution to the chronicization of pain (Ji et al., 2013). Astrocytes activation following nerve injury is characterized by changes in morphology, increased expression of glial fibrillary acidic protein (GFAP), proliferation and secretion of pro-inflammatory molecules and growth factors (Jensen et al., 2013). The released factors exert both autocrine and paracrine actions,

thus further sustaining astrocytic reactivity and increase neuronal sensitization (Skaper et al., 2016). In particular, astrocytes can directly communicate with neurons via gap junctions, and nerve injury induces the up-regulation of connexin-43 (Cx43) in astrocytes, followed by a switch of Cx43 function from gap junction communication to paracrine modulation. The latter sustains late-phase neuropathic pain through the release of astrocytic chemokines, together with increased release of glutamate and ATP, which potentiate excitatory synaptic transmission (Chen et al., 2014). These mediators contribute to facilitate neuropathic pain induction and maintenance via bidirectional neuron-astrocyte interactions. For example, a recent study showed that the chemokine CXCL13 is up-regulated in spinal cord neurons after nerve injury, and can in turn activate astrocytes via CCR5 receptor, contributing to maintain chronic neuropathic pain (Jiang et al., 2016). Nerve injury also induces astrocytes to up-regulate thrombospondin-4 (TSP4) protein, which contributes to sustain neuropathic pain through the formation of new synapses (Kim et al., 2012).

Astrocytes have been the first brain cell population where expression and function of A_3 adenosine receptors has been demonstrated (Abbracchio et al., 1997). In the last five years, the notion that this receptor subtype is an innovative target for the control of pain has emerged, also thanks to the availability of selective pharmacological entities, namely the old IB-MECA and chloro-IB-MECA agonists and new more selective and potent compounds (Janes et al., 2016; for review, see Magni et al., 2018). This receptor subtype is expressed not only by astrocytes, but also by neurons and immune cells, which all contributes to the overall analgesic effect of agonists, thus highlighting the complex modulation of the cell-to-cell cross-talk driving chronic pain (Janes et al., 2016). Interestingly, dysregulation of A_3 adenosine receptor recruitment is at the basis of chemotherapy-induced pain. In fact, oxaliplatin administration induces the expression of adenosine kinase in astrocytes, which contributes to the reduction of extracellular adenosine concentrations by promoting its phosphorylation to AMP. This in turn limits the activation of analgesic A_3 receptors and promotes the generation of a pro-inflammatory and pro-algogenic milieu, which is reversed by the intrathecal administration of selective A_3 agonists (Wahlman et al., 2018). These recent data strongly support the further development of selective A_3 receptor agonists for different types of chronic neuropathic pain syndromes, which could be accelerated by already ongoing clinical development for different indications (see below).

A pathological and bidirectional astrocyte/microglia cross-talk has been also demonstrated in several brain pathologies. Activated microglia can promote the appearance of a neurotoxic subpopulation of astrocytes, the so-called A1 astrocytes (Liddelow et al., 2017), with the generation of a highly pro-inflammatory and detrimental milieu. Reactive astrocytes in turn release a vast variety of pro-inflammatory mediators which further contribute to the activation of microglia cells and to its

polarization towards a so-called pro-inflammatory M1 phenotype (Zhang et al., 2017; Figure 3). ATP and its derivatives play a crucial role as signals in this pathological communication. The $P2Y_{14}$ receptor subtype, the only officially-recognized P2Y purinergic receptor activated by uridine sugars (i.e. UDP-glucose and UDP-galactose) which is expressed by leukocytes and promotes neutrophil chemotaxis (Lazarowski et al., 2015), has been found upregulated in spinal microglia after peripheral nerve injury (Kobayashi et al., 2012). The demonstration that its activation on microglia cells reduced the proliferation of astrocytoma cells in *in vitro* cocultures (Curet & Watters, 2018) suggests that it may represent an additional member of the purinergic network controlling astrocyte-to-microglia communication in the brain in pathological conditions, possibly including pain.

The cross-talk between these two glial cell types is also responsible for the diurnal exacerbation of mechanical allodynia, a highly invalidating condition that affects patients suffering from several types of pain and the $P2Y_{12}$ receptor has a major role in it. It is in fact well known that pain comes in waves during the day, and day-night changes in symptoms have been observed in patients with cancer, diabetic neuropathy, fibromyalgia and multiple sclerosis (Koyanagi et al., 2016). In the search of the underlying mechanisms, researchers have demonstrated in a rat model of neuropathic pain (partial sciatic nerve ligation) that diurnal exacerbation of pain is directly connected to the circadian variations in glucocorticoid release from the adrenal gland. Glucocorticoids activate the serum- and glucocorticoid-inducible kinase-1 (SGK-1) in spinal astrocytes, which in turn promotes the release of ATP through pannexin hemichannels. The hydrolysis of ATP leads to increased ADP concentrations which activate $P2Y_{12}$ receptors on surrounding microglia leading to exacerbation of mechanical allodynia (Koyanagi et al., 2016). Targeting $P2Y_{12}$ receptors could therefore prove useful in limiting the worsening of symptoms in pain patients.

Interestingly, $P2Y_1$-mediated proliferation of astrocytes is inhibited by the concomitant presence of microglia expressing the $P2Y_{13}$ receptor subtype in co-cultures *in vitro* (Quintas et al., 2018). Based on the emerging role of astrocytic $P2Y_1$ receptors in driving their transition towards an activated, detrimental phenotype (Shinozaki et al., 2017), data suggest that microglia could contribute to the modulation of astrocytic reactivity also through the $P2Y_{13}$ receptor subtype.

2.3 The neuronal side of purinergic signaling in neuropathic pain

Several adenosine and P2Y receptors are expressed and functional on sensory neurons; adenosine A_1 receptors are generally coupled to K^+ channels, and can therefore limit neuronal firing (Borea et al., 2018), whereas generation of Ca^{++} waves after activation of various P2Y nucleotide receptors has been demonstrated (Ceruti et al., 2008). Nevertheless, the overall pro- or anti-algogenic role exerted by each receptor subtype has not been clearly established, often due to the difficulties in

discriminating between a neuronal and/or a glial site of action *in vivo*. For example, in a rat model of neuropathic pain associated to spinal cord injury, named the spinal neuropathic avulsion pain (which allows the unilateral generation of allodynia without overt motor deficits, paralysis and other health problems; Kwilasz et al., 2018), one single intrathecal injection of the A_{2A} agonist 2-p-(2-carboxyethyl-phenethylamino-5′-N-ethylcarboxamido-adenosine (CGS21680) was able to abolish mechanical allodynia for up to six weeks. This was paralleled by a reduction of signs of inflammation which represent the driving force for deficits and problems arising from the second chronic phase of tissue damage following spinal cord injury (Kwilasz et al., 2018). Since inhibition rather than activation of A_{2A} receptors has proved beneficial in migraine pain (see below), the different sites of action and/or the different types of pain syndrome could represent a possible explanation for the opposite effects exerted by this receptor subtype, which add further complexity to an already quite complicated scenario when developing new analgesic strategies.

Expression of the $P2Y_{11}$ receptor subtype has been described in sensory neurons in rodents, but its participation in pain is still scarcely understood. $P2Y_{11}$ receptors inhibition reduces tactile allodynia in both neuropathic (Barragán-Iglesias et al., 2014) and formalin-induced pain models (Barragán-Iglesias et al., 2015), but the existence of a rodent ortholog of human $P2Y_{11}$ receptors is still a matter of debate, since no cloned receptor but only functional data are currently available.

Not only receptors, but also enzymes controlling the complex bidirectional interconversion between nucleotides and nucleosides are involved in pain transmission. As mentioned before, adenosine is not released from nerve terminals per se, but it is likely generated extracellularly through the action of various enzymes, mostly ectonucleoside triphosphate diphosphohydrolase (CD39) and ecto-5-nucleotidase (CD73; Borea et al., 2018). Prostatic acid phosphatase (PAP) is one of the less studied nucleotide-hydrolyzing enzymes; it is expressed by small diameter nociceptors and hydrolyzes AMP to Ado. Interestingly, $PAP^{-/-}$ animals show a higher nerve injury-associated pain, similarly to A_1 adenosine receptor KO mice which show increased nociceptor response (Wu et al., 2005). In a mouse model of neuropathic pain, where small-diameter sensory nerves are selectively depleted by resiniferatoxin (a capsaicin analogue that acts on TRPV1 receptors) animals showed downregulation of PAP expression leading to increased AMP and reduced adenosine generation (Kan et al., 2018). The exogenous administration of either PAP or adenosine reversed mechanical allodynia, and this effect was inhibited by the A_1-receptor antagonist dipropyl-cyclopentylxanthine (DPCPX; Kan et al., 2018). Taken together, these results demonstrate that an imbalance in adenosine-mediated signaling, which can act autocrinally on sensory neurons and/or paracrinally on surrounding cells contributes to the development of neuropathic pain.

2.4 Do oligodendrocytes contribute to neuropathic pain?

Although no direct studies have been performed up to now, a possible role for oligodendrocytes in pain transmission can be speculated based on recent evidence demonstrating that myelination of axons is dynamically modulated by both vescicular and non-vescicular axo-glial communication. Increased neuronal firing leads to increased release of neurotransmitters at the so-called axo-glial signaling complex, which in turn promotes the synthesis of myelin proteins (Fields, 2015). Since it is known that alterations in myelin structure are at the basis of peripheral neuropathies and neuropathic pain (Veronica et al., 2016), it is tempting to speculate that the cross-talk between firing axons and oligodendrocytes could represent an interesting target to be further exploited to manage neuropathic pain. Glutamate has been directly involved in this effect, but it cannot be excluded that other neuro-and co-transmitters (such as ATP) can contribute.

2.5 Glial cells in sensory ganglia as direct modulators of neuronal firing also through purinergic signaling

Concerning the PNS, research in the last two decades has demonstrated that sensory ganglia (i.e. DRGs, and trigeminal ganglia, TG) are important sites in processing pain information. Sensory neurons express different receptors for neurotransmitters and hormones and can also release a variety of mediators, including glutamate, ATP, substance P (SP), and CGRP (Figure 4; Hanani, 2015). Under painful conditions, satellite glial cells (SGCs), the peculiar glia population in sensory ganglia, become activated before central glia and contribute to the release of additional inflammatory mediators, thus sensitizing neuronal bodies. In particular, following nerve injury, SGCs undergo an increased expression and release of pro-algogenic mediators including IL-1β and TNF-α, as well as an increased gap junction-mediated coupling (Magni and Ceruti, 2014; Figure 4).

Moreover, during pain conditions, SGCs upregulate the expression of GFAP and undergo cell division (Magni et al., 2015; Donegan et al., 2013), Intriguingly, a subpopulation of SGCs acts as neuronal progenitors giving birth to new functionally active CGRP-positive neurons which contribute to pain development and maintenance (Zhang et al., 2019).

In parallel with the CNS, extracellular nucleotides and nucleosides play a fundamental role in delivering information back and forth from neurons to glial cells within sensory ganglia as well, with the involvement of specific membrane receptors. Expression of $P2Y_{12}$ mRNA and protein in SGCs in the DRGs was increased in a rat model of type 2 diabetes mellitus. Targeting the receptor by short hairpin RNA (shRNA) counteracted the upregulated expression of $P2Y_{12}$ mRNA and protein, reduced its co-expression with GFAP, as well as signs of SGC activation, including overexpression of GFAP, IL-1β, phospho-P38 MAPK and TNF receptor 1, and inhibited mechanical and thermal hyperalgesia

(Wang et al., 2018). Interestingly, administration of nanoparticle-encapsulated curcumin to rats suffering from diabetic neuropathy decreased thermal hyperalgesia, as well as the mRNA and protein expression of $P2Y_{12}$ receptors in the DRG. *In silico* molecular docking of curcumin on rat $P2Y_{12}$ protein showed that curcumin has a high affinity to interact with residues in the $P2Y_{12}$ receptor agonist-binding pocket (Jia et al., 2018), thus raising the intriguing hypothesis that it could act by directly binding to the receptor.

As a further confirmation of the role of $P2Y_{12}$ receptor expressed by SGCs, another study focused on neuropathic pain induced by HIV envelope glycoprotein 120 (gp120) combined with 2′,3′-dideoxycytidine (ddC). Exposure of peripheral nerves to HIV-gp120+ddC induces mechanical and thermal hyperalgesia and increases $P2Y_{12}$ receptor mRNA and protein expression in DRG SGCs. Moreover, primary cultures of SGCs treated with gp120+ddC displayed significantly increased levels of intracellular calcium after application of the $P2Y_{12}$ receptor agonist 2-methylthio-adenosine 5′-diphosphate (2-MeSADP), and this effect was counteracted by $P2Y_{12}$ receptor shRNA treatment (Yi et al., 2018). Intrathecal administration of $P2Y_{12}$ shRNA reduces both the release of pro-inflammatory cytokines and the phosphorylation of p38 MAPK in the DRG of gp120+ddC-treated rats, as well as the levels of mechanical and thermal hyperalgesia (Shi et al., 2018), thus confirming *in vitro* data on a key role played by $P2Y_{12}$ receptors in HIV-induced neuropathic pain. Furthermore, the interaction between activated SGCs and CGRP-immunoreactive neurons via $P2Y_{12}$ receptors seems to contribute to neuropathic pain in the tongue after lingual nerve injury (Sugawara et al., 2017), confirming the receptors role also in this pain model, as previously postulated (Katagiri et al., 2012).

Recent data on the role of adenosine and P2Y receptors in preclinical models of neuropathic pain are summarized in Table 1.

3. Adenosine and P2Y receptors in inflammatory pain and in migraine

High adenosine concentrations are generated at inflammatory sites, due to the rapid hydrolysis of ATP in the inflammatory hypoxic milieu. Adenosine-mediated signaling plays a pivotal role in controlling inflammation in any body district, thanks to the massive expression of its receptors in immune cells, fibroblasts, endothelial cells and platelets (for review, see Magni et al., 2018). When analyzing their role in pre-clinical models of inflammatory pain it is therefore quite difficult to discriminate between the contribution of neuronal/glial receptors and receptors expressed by other cell types. For example, activation of A_{2A} receptors expressed by circulating cells has an overall anti-inflammatory outcome, but their role in pain is still controversial. In fact, both anti- and pro-algogenic actions have been described for receptors expressed by both neurons and glial cells in the CNS (for review, see Magni et al., 2018). It is worth mentioning that A_{2A} receptors are directly involved in the induction of reactive astrogliosis promoted by basic fibroblast growth factor (bFGF; Brambilla et al.,

2003). Additionally, their activation on microglia cells leads to the modulation of the transcription factor hypoxia-inducible factor alpha (HIF-1α), which is upregulated under hypoxic and/or inflammatory conditions (Merighi et al., 2015; Figure 1). These data suggest that the role played by these receptors in the modulation of glial cell reactivity in pain should be further explored.

A pro-inflammatory role has been instead postulated for the A_{2B} receptor subtype which, similarly to the A_{2A} receptor subtype, is highly expressed in inflammatory cells (Feoktistov & Biaggioni, 2011). Interestingly, this receptor subtype has the peculiar feature to be activated by high micromolar adenosine concentrations which are only reached in pathological situations (Chen et al., 2013; Borea et al., 2018), thus suggesting that its selective targeting could be devoid of significant side effects in healthy tissues.

Few studies have been recently published which directly investigate the role of specific adenosine and P2Y receptors in inflammatory conditions, including migraine (Table 2). Inhibition of the $P2Y_1$, $P2Y_6$, $P2Y_{11}$ receptor subtypes prevented the flinching behavior in rats injected with formalin in the hind paw (Barragán-Iglesias et al., 2015). Conversely, the $P2Y_2$ receptor subtype, expressed along with the $P2Y_1$ receptor subtype by SGCs in the TG, is responsible for the development of orofacial mechanical allodynia in a rat model of sub-chronic inflammatory trigeminal pain, as demonstrated by the potent anti-allodynic action exerted by a selective antagonist (Magni et al., 2015).

Of interest due to the clinical availability of several selective antagonists (see below), a recent paper demonstrated that $P2Y_{12}$ receptors expressed by platelets contribute to hyperalgesia and local inflammation in a chronic Complete Freund's Adjuvant (CFA)-induced inflammatory pain model in mice (Bekő et al., 2017). Authors demonstrated that $P2Y_{12}$-/- mice developed milder signs of CFA-induced pain with respect to wild-type animals, in parallel with reduced signs of inflammation of the hind paw. Platelet depletion decreased hyperalgesia and attenuated the proinflammatory cytokine response in wild-type but not in $P2Y_{12}$-/- mice, thus suggesting for the first time the contribution to pain development of $P2Y_{12}$ receptors expressed on platelets.

A role for adenosine receptors expressed along the spinal-trigeminal pathway have been postulated in migraine. In fact, electrical stimulation of the TG, taken as a surrogate model of migraine due to the development of plasma extravasation and neurogenic inflammation, led to increased expression of CGRP and A_{2A} adenosine receptors in both the TG and the trigeminal nucleus caudalis and to a reduction of A_1 receptors both at the mRNA and at the protein level. These changes were reverted by the pretreatment with a Chinese drug named Tianshu capsule (TSC) which is widely utilized in China as acute and prophylactic treatment for migraine with no known mechanisms of action (Lu et al, 2016). Despite the lack of the cellular localization of receptors and of direct functional

correlates, making it impossible to discriminate whether the observed changes simply represent an epiphenomenon, results are suggestive of a pro-algogenic and an anti-algogenic role for trigeminal A_{2A} and A_1 receptors, respectively.

A_{2A} receptors could also play a fundamental role in migraine thanks to their ability to promote dilation of small diameter vessels, through the intracellular increase of cAMP concentrations. In fact, adenosine application to the rodent isolated middle meningeal artery exerts vasodilation, which is considered one of the triggering events for a migraine attack (Haanes & Edvinsson, 2014). This effect is observed also in living animals thanks to *in vivo* videomicroscopy and is blocked by selective A_{2A} receptor antagonists and by caffeine, a non-selective adenosine receptor antagonist, which is often included in over-the-counter drugs for acute migraine attacks (Haanes et al. 2018). Thus, the modulation of cerebral vessel diameter through A_{2A} receptors can be one of the mechanisms through which caffeine contribute to reduce migraine and headache attacks.

4. Adenosine and P2Y receptors in visceral pain

Visceral hypersensitivity is a hallmark of several gut pathologies associated with pain, including Irritable Bowel Syndrome (IBS). Many different events contribute to its development, including the loss of endothelial barrier integrity (leading to the so-called “leaky gut”), which favors the contact of the intestinal content with surrounding tissues. This activates resident immune cells and increases the production of pro-inflammatory and pro-algogenic molecules, including histamine and serotonin. Overall, these events sensitize enteric nociceptors and primary afferent nerves, which in turn contribute to trigger central sensitization in the spinal cord and, subsequently, the shift towards chronic pain (Asano & Takenaga, 2017).

In this scenario, the contribution of adenosine and P2Y receptors has now emerged, as summarized in Table 3.

Pain arising from colonic distension can be inhibited by the intracisternal injection of orexin, a neuropeptide that localizes in neurons in the lateral hypothalamus. The analgesic effect of orexin is mediated by adenosine through the A_1 receptor subtype; in fact, centrally administered cyclo-pentyl-adenosine (CPA, a selective A_1 receptor agonist; Borea et al., 2018) demonstrated an analgesic effect which was blocked by DPCPX. Interestingly, DPCPX also blocked the effects of orexin, thus demonstrating that modulation of adenosine signaling is involved in the analgesic actions of other systems (Okumura et al. 2016).

Interesting hints on the role of adenosine receptors in visceral pain also came from the demonstration of an abundant expression of A_{2B} receptors in the gastrointestinal tract, with a role in the regulation of intestinal fluid secretion by epithelial cells, of enteric motor functions and of resident

immune cells (Asano & Takenaga, 2017). Excessive activation of A_{2B} receptors could cause diarrhea, and an additional role in the development of visceral hypersensitivity can be also foreseen. In parallel with the observation that blocking A_{2B} receptor-mediated signaling can resolve several types of inflammatory painful conditions (for review see Magni et al., 2018), it has been recently demonstrated that aminophylline, acting as A_{2B} receptor antagonist, reduces visceral pain and colonic propulsion in a rat model of stress-induced IBS (i.e., following maternal separation or wrap-restraint stress; Asano et al., 2017), thus highlighting this adenosine receptor subtype as a novel potential target to treat pain and colonic dysfunctions in IBS. Whether the effects of A_{2B} receptors are also linked to the modulation of the cross-talk between enteric neurons and glial cells has not been addressed yet. It is nevertheless worth mentioning that a complex role for this receptor subtype has been demonstrated in LPS-activated microglia *in vitro*, with cooperation with the A_{2A} subtype in promoting the induction of HIF-1α but a specific anti-inflammatory effect demonstrated by reduction of TNF-α production (Merighi et al., 2015) and upregulation of pro/anti-inflammatory IL-6 (Merighi et al., 2017). These data suggest that opposite effects for a specific receptor subtype can be observed depending upon the cell types and tissue, especially concerning central and peripheral sites of action. This must be taken into careful consideration when designing new therapeutic approaches, Nevertheless, the A_{2B} receptor subtype has now been included into the novel and promising pharmacological targets for the management of pain and other symptoms in IBS, which should be further exploited to counteract the so-called “opioid crisis” due to significant opioid abuse and overuse to get relief from visceral pain (Camilleri, 2018).

Several P2Y receptor subtypes are also involved in the modulation of visceral pain. $P2Y_1$-selective antagonists have potential therapeutic value in treating abdominal pain as demonstrated in experimental IBS (Wu et al., 2017). Another research group showed that $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors are expressed in thoracolumbar gut-projecting sensory neurons, and that their activation led to neuronal sensitization (Hockley et al., 2016). Moreover, the voltage-gated Nav1.9 sodium channel, a major regulator of visceral afferent excitability to noxious mechanical and inflammation stimuli, is expressed by 100% of $P2Y_2$-positive colonic neurons and a reduced afferent fiber response to UTP has been observed in Nav1.9$^{-/-}$ mice (Hockley et al., 2016).

Interestingly, UTP elicits calcium oscillations in a model of human enterochromaffin cells to stimulate 5-HT release primarily through a $P2Y_4$ receptor-mediated mechanism (Liñán-Rico et al., 2017). Although the activation of the $P2Y_4$ subtype seems to play a major role, UTP also activates $P2Y_6$ receptors in enterochromaffin cell, likely following its hydrolysis to UDP, thus increasing intracellular free Ca^{2+} levels and leading to 5-HT release (Liñán-Rico et al., 2017).

No data are currently available on the possible expression of any P2Y receptor subtypes on enteric glia. However, based on data on P2Y receptors expression on many different glial cell subtypes, it is quite conceivable that they could contribute to control glial cell function in the enteric nervous system as well, with a possible contribution to visceral pain.

5. When will a purinergic-based drug reach the market as innovative painkiller?

As emerging from historical and more recent data, the purinergic system represents a potential bottomless pit of new targets to be exploited pharmacologically for the management of virtually all types of pain, including pain syndromes with unsatisfactory control by currently available drugs. Despite clear basic and preclinical evidence, unfortunately no purinergic-based analgesic has been made available to patients so far.

Three main issues can be identified which have slowed down the development of analgesics (and drugs in general) targeting the purinergic system so far: i) the ubiquitous distribution of purinergic receptors with the possible development of serious side effects (see for example the role of $P2Y_{12}$ receptors in platelet aggregation or the role of A_1 adenosine receptors in controlling cardiac functions); ii) the high number of receptor subtypes, and ii) the lack of truly subtype-selective agonists and antagonists to be dispensed through clinically relevant routes of administration. To further complicate this already intricated scenario, several purinergic receptors couple to different second messengers depending upon the tissue and the pathological or physiological conditions. Additionally, they undergo tolerance upon prolonged activation, and they can also homo- or etero-dimerize (and dimers often present a different pharmacological profile when compared to homomers; for review see Chen et al., 2013; Nishimura et al., 2017).

In the field of adenosine-mediated signaling, some of the above-mentioned problems have now started to be solved, thanks to the invaluable efforts of medicinal chemists. New selective and potent A_3 adenosine receptor agonists are now available (Jacobson et al., 2018), new A_1 receptor agonists devoid of any cardiac actions have been designed and tested as analgesic agents (Petrelli et al., 2017; 2018), and new selective A_{2A} receptor antagonists acting as vasodilators in migraine are currently under development (Haanes et al., 2018). When dealing with adenosine agonists, an important issue to be considered is represented by the massive (ab)use of the methylxanthine caffeine, which acts as antagonist at various adenosine receptor subtype and could therefore alter the results of clinical trials and, prospectively, the beneficial effects of any agonist which will reach the market (Chen et al., 2013).

It is also worth mentioning that the opposite roles sometimes played by one receptor subtypes in pain modulation, possibly depending on the type of pain and/or the site of action (see above), makes

quite difficult to address the development towards agonist or antagonist molecules. The generation of inverse agonists, as for the A_{2A} receptor subtype (Varano et al., 2016), could represent an alternative helpful strategy. In fact, inverse agonists stabilize the receptor in its inactive conformation and inhibit both the action of the agonists, like classical antagonists, and the constitutive activity of the receptor which can sometimes be involved in pain maintenance.

One possible interesting opportunity is represented by the repurposing of drugs acting on the purinergic system which are already marketed or are currently undergoing clinical trials for different indications. For example, aminophylline has been long utilized as anti-asthmatic drug as generic adenosine receptor antagonist and phosphodiesterase inhibitor, with a very favorable clinical profile. The recent discovery of its action on the A_{2B} receptor subtype which is involved in the development of visceral pain (see above) has paved the way for its clinical exploitation in IBD patients (Asano et al., 2017). A_3-selective agonists are currently in clinical trials for rheumatoid arthritis (Phase III; Piclidenoson, CanFite BioPharma) and liver diseases (Phase II; Namodenoson, CanFite BioPharma). Data could be further utilized in view of a possible application of these drugs to neuropathic pain syndromes (see above).

In the field of P2Y receptors, where the high number of receptor subtypes and endogenous agonists adds further difficulties to the development of really selective agents, a key example is represented by $P2Y_{12}$ antagonists. The most studied effect of $P2Y_{12}$ receptors is their crucial role in platelet aggregation, so that selective antagonists are blockbuster drugs in the management of thrombosis and in the secondary prevention of ictus and cardiac ischemia (Kupka & Sibbing, 2018). Although the concomitant activity on platelets could pose some safety problems, the evaluation of these drugs in different types of pain syndrome could therefore be accelerated by the availability of pharmacokinetics and pharmacodynamics data despite that the appropriate route of administration is identified to reach the CNS target on microglia cells. Interestingly in view of preclinical data showing a possible role for platelet $P2Y_{12}$ receptors in the modulation of pain (see above; Bekő et al., 2017), genetic variability in the *P2Y12* gene seems to contribute to individual differences in pain and opioid sensitivity. A recent study genotyped 31 single nucleotide polymorphisms (SNPs) throughout the *P2Y12* gene and compared the different genotypes with pain severity in 90 cancer patients, and with a cohort of patients suffering from post-operative pain. Authors have identified five *P2Y12* gene SNPs that are positively associated with increased pain severity in both cohorts of patients (Sumitani et al., 2018). Since two out of five SNPs are associated to higher platelet activation, it is conceivable that the increased $P2Y_{12}$ receptor signaling contributes to higher pain perception in patients, thus adding further interest to this receptor subtype as new target for pain.

Finally, $P2Y_2$ receptor-selective agonists, diquafosol and denufosol, have been recently discontinued from clinical trials in cystic fibrosis despite initial highly encouraging results. The former has been later marketed in Japan and Korea as eye drops for dry eye syndrome (Burnstock, 2017); based on interesting preclinical data on the role of this receptor subtype in trigeminal pain, a possible future analysis of its analgesic potential can be foreseen.

In conclusion, recent basic and preclinical research fully confirm the crucial role played by the purinergic system in the development of chronic pain conditions, and in conjunction with new developments in medicinal chemistry, results are fostering our hope to welcome the marketing of the first purinergic-based analgesic in a limited period of time.

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Figure legends

Figure 1. Increased extracellular adenosine concentrations under pathological conditions.

At variance with physiological conditions (upper panel), under pathological situations extracellular adenosine concentrations are increased from low nanomolar to high micromolar range, thanks to multiple mechanisms, including increased ATP release and catabolism. Adenosine receptors are upregulated as well, which contribute to increase the role of this signaling pathway in pathology. These events take place not only at brain synapses, but wherever vescicular or non-vescicular ATP release can be observed.

AK: adenosine kinase; ENT: equilibrative nucleoside transporter; ENTPD1: ectonucleoside triphosphate diphosphohydrolase 1, also known as CD39; HIF-1α: hypoxia-inducible factor-1α; NT5E: ecto-5′-nucleotidase, also known as CD73; SAH: S-adenosyl-homocysteine. Reproduced with permission (RightsLink licence #4403531025645) from Chen et al., 2013.

Figure 2: P2Y and adenosine receptor expression in sensory neurons and glial cells.

Expression of both A_1 and A_{2A} receptor subtypes has been detected in the trigeminal ganglion, but no cell-specific localization has been provided (Lu et al., 2016).

Figure 3: Neuron-glia crosstalk in the spinal cord.

In painful conditions, the activation of peripheral nociceptors with cell bodies in sensory ganglia (i.e., DRGs or TGs) induces the release of several mediators from their central terminals (blue), which in turn activate microglial cells and astrocytes in the spinal cord dorsal horn. Activated microglia (yellow) and astrocytes (red) further release neuromodulators which influence second order neurons (green), act back on nociceptor terminals (blue), and contribute to the generation of a vicious circle of glial cell activation, which sustains neuronal sensitization and the transition from acute to chronic pain.

ATP: Adenosine triphosphate; BDNF: brain-derived neurotrophic factor; CCL2: C-C motif ligand 2; CSF1: macrophage colony-stimulating factor 1; CXCL13: C-X-C motif chemokine ligand 13; Glu: glutamate; ILs: interleukins; TNF-α: tumor necrosis factor alpha.

Figure 4: Neuron-glia crosstalk in sensory ganglia.

Bidirectional signaling between sensory neurons and satellite glial cells (SGCs) in peripheral ganglia involves the release of different mediators, which act both autocrinally and paracrinally thus contributing to neuronal sensitization. In fact, neuronal pro-algogenic signals, mostly CGRP and SP but also ATP and glutamate, stimulate IL-1β release by SGCs (blue), enhance glial COX activity with

the subsequent production of pro-inflammatory PGE_2 (green), and promote P2Y receptors expression/activation via phospho-ERK1/2 signaling pathway (orange). A long-lasting and sustained stimulation of SGCs further potentiate the network, by promoting the coupling of SGCs through GAP junctions and their release of additional pro-inflammatory molecules, which in turn drive the transition from acute to chronic pain.

ATP: adenosine triphosphate; CGRP: calcitonin gene-related peptide; COX: cyclooxygenase; Cx43: connexin 43; Glu: glutamate; IL-1β: interleukin 1 beta; pERK1/2: phosphorylated Extracellular signal-regulated kinases 1/2; PGE_2: prostaglandin E_2; SP: substance P; TNF-α: tumor necrosis factor alpha.

Experimental model	Species	Pharmacological agents tested	Receptor subtype(s) involved	Functional outcome(s)	References
Spinal nerve ligation	Rat	MRS2578 ($P2Y_6$ antagonist) NF340 ($P2Y_{11}$ antagonist)	$P2Y_6$, $P2Y_{11}$	Inhibition of tactile allodynia	Barragán-Iglesias et al., 2014
		MRS2500 ($P2Y_1$ antagonist)	$P2Y_1$		Barragán-Iglesias et al., 2016
		MRS5698 (A_3 agonist)	A_3		Little et al., 2015
Sciatic nerve ligation	Rat	Ulinastatin (serine protease inhibitor)	$P2Y_2$	Inhibition of mechanical allodynia and thermal hyperalgesia with downregulation of $P2Y_2$ receptors	Shi et al., 2017
Chronic constriction injury of the sciatic nerve	Rat	MRS2500 ($P2Y_1$ antagonist)	$P2Y_1$	Inhibition of tactile allodynia	Barragán-Iglesias et al., 2016
		MRS2578 ($P2Y_6$ antagonist)	$P2Y_6$	Inhibition of mechanical allodynia and thermal hyperalgesia	Huang et al., 2018
		AM1241 (CB2 antagonist)	$P2Y_{12}$, $P2Y_{13}$	Inhibition of thermal hyperalgesia with downregulation of purinergic receptors	Niu et al., 2017
	Mouse	ABT-702 (adenosine kinase inhibitor) MRS5698 (A_3 agonist)	A_3	Inhibition of mechanical allodynia	Little et al., 2015
Spared nerve injury	Rat	MRS2500 ($P2Y_1$ antagonist)	$P2Y_1$	Inhibition of tactile allodynia	Barragán-Iglesias et al., 2016
	Mouse	MRS2578 ($P2Y_6$ antagonist)	$P2Y_6$	No effect on neuropathic pain behavior	Syhr et al., 2014
Lingual nerve crush	Rat	MRS2395 ($P2Y_{12}$ antagonist)	$P2Y_{12}$	Attenuation of tongue hypersensitivity to mechanical and heat stimulation	Sugawara et al., 2017
Diabetic peripheral neuropathy	Rat	Nanoparticle-encapsulated curcumin	$P2Y_{12}$	Inhibition of mechanical and thermal hyperalgesia	Jia et al., 2018
		$P2Y_{12}$ short hairpin RNA (shRNA)	$P2Y_{12}$	Inhibition of mechanical and thermal hyperalgesia	Wang et al., 2018
		MRS2211 ($P2Y_{13}$ antagonist)	$P2Y_{13}$	Inhibition of mechanical allodynia	Zhou et al., 2018
	Mouse	IB-MECA (A_3 agonist)	A_3	Inhibition of mechanical and thermal hyperalgesia	Yan et al., 2016
HIV gp120-induced	Rat	$P2Y_{12}$ short hairpin RNA (shRNA)	$P2Y_{12}$	Inhibition of mechanical and thermal hyperalgesia	Shi et al., 2018 Yi et al., 2018

neuropathic pain					
Small-fiber neuropathy (resinifera-toxin)	Mouse	Prostatic Acid Phosphatase (PAP)	Adenosine receptors (A_1)	Reduction of mechanical allodynia	Kan et al., 2018
Spinal neuropathic avulsion pain (SNAP)	Rat	CGS21680, ATL313 (A_{2A} agonists)	A_{2A}	Reduction of mechanical allodynia	Kwilasz et al., 2018
Chemotherapy -induced neuropathic pain	Rat	ABT-702 (adenosine kinase inhibitor) MRS5698 (A_3 agonist)	A_3	Inhibition of mechanical allodynia and hyperalgesia	Little et al., 2015 Wahlman et al., 2018

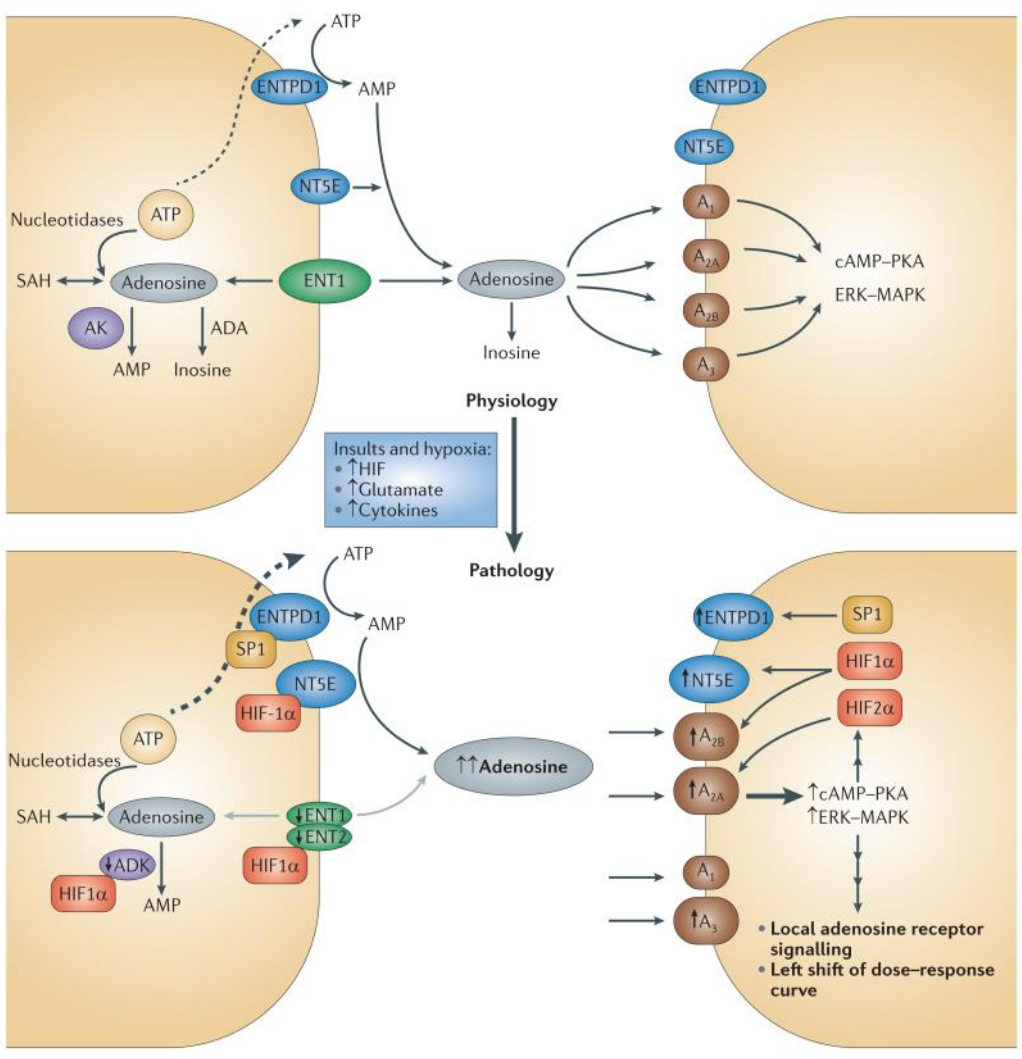
Table 1. Recent in vivo preclinical studies evaluating the role of adenosine and P2Y receptors in neuropathic pain

Experimental model	Species	Pharmacological agents tested	Receptor subtype(s) involved	Functional outcome(s)	References
Injection of Complete Freund's Adjuvant (CFA) – hind paw	Mouse	Cangrelor, PSB-0739 ($P2Y_{12}$ antagonists)	$P2Y_{12}$	Inhibition of the local inflammatory response, of CFA-induced hyperalgesia and of chronic inflammation (involvement of platelet $P2Y_{12}$ receptors)	Bekő et al., 2017
Injection of Complete Freund's Adjuvant (CFA) – temporomandibular joint	Rat	ARC118925 ($P2Y_2$ antagonist)	$P2Y_2$	Inhibition of orofacial mechanical allodynia	Magni et al., 2015
Formalin injection – hind paw	Rat	MRS2500 ($P2Y_1$ antagonist) MRS2578 ($P2Y_6$ antagonist) NF340 ($P2Y_{11}$ antagonist)	$P2Y_1$, $P2Y_6$, $P2Y_{11}$	Inhibition of the flinching behavior	Barragán-Iglesias et al., 2015
Closed cranial window model	Rat	Various JNJ compounds (A_{2A} antagonists with different receptor selectivity)	A_{2A}	Inhibition of CGRP- or CGS21680-induced meningeal vasodilation	Haanes et al., 2018

Table 2. Recent in vivo preclinical studies evaluating the role of adenosine and P2Y receptors in inflammatory pain and migraine

Experimental model	Species	Pharmacological agents tested	Receptor subtype(s) involved	Functional outcome(s)	References
Intracolonic administration of acetic acid	Rat	MRS2179 ($P2Y_1$ antagonists) Aminophylline (A_{2A}, A_{2B} antagonists) MRS2174, PSB1115 (A_{2B} antagonists)	$P2Y_1$ A_{2B} (partially A_{2A})	Reduction of abdominal sensitivity (abdominal withdrawal reflex and electromyography)	Wu et al., 2017 Asano et al., 2017
Intraperitoneal injection of acetic acid	Mouse	Several inverse agonists (A_{2A} receptor)	A_{2A}	Significant reduction of writhing behavior	Varano et al., 2016
Colonic distension by colorectal balloon	Rat	CPA (A_1 agonist)	A_1 (centrally expressed)	Reduction of the abdominal withdrawal reflex	Okumura et al., 2016

Table 3. Recent in vivo preclinical studies evaluating the role of adenosine and P2Y receptors in visceral pain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AMP
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ENTPD1
NT5E
A1
A2A
A2B
A3
cAMP–PKA
ERK–MAPK
Physiology
Insults and hypoxia:
• ↑HIF
• ↑Glutamate
• ↑Cytokines
Pathology
ATP
ENTPD1
SP1
NT5E
HIF-1α
AMP
ATP
Nucleotidases
SAH
Adenosine
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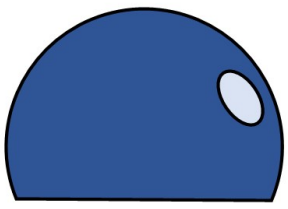
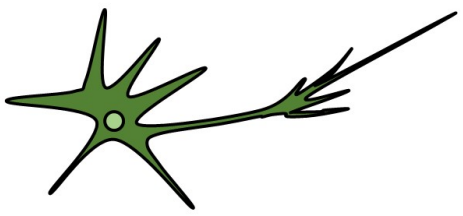
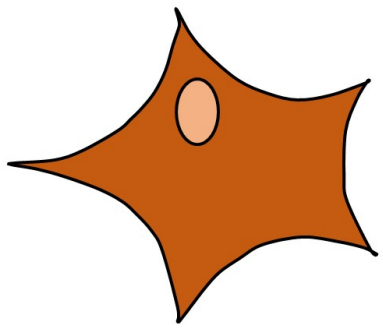
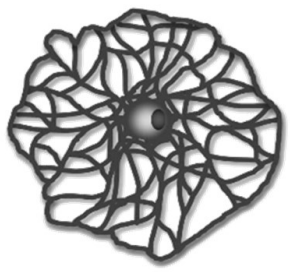
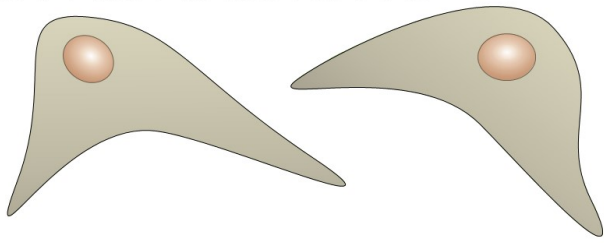
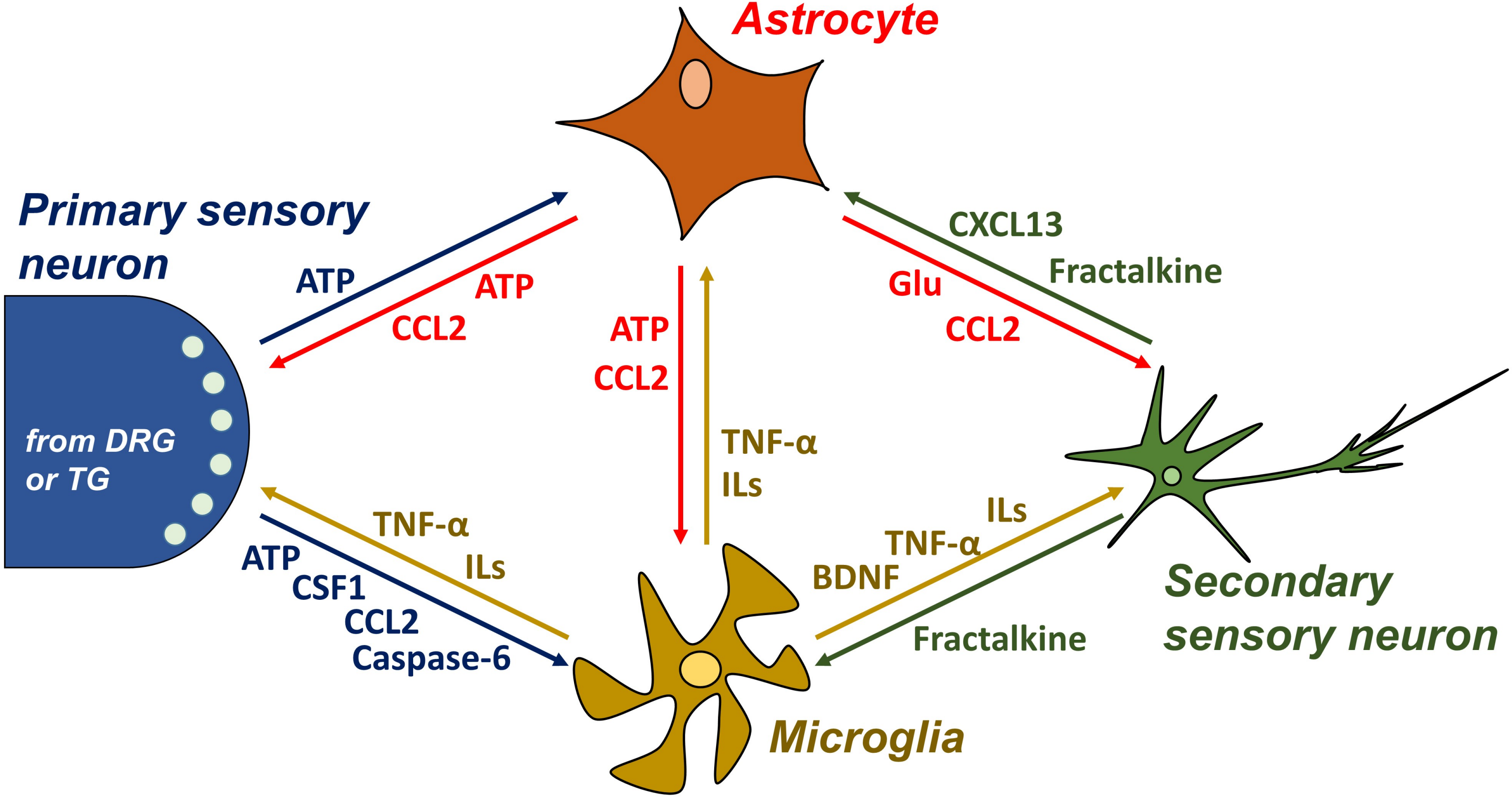
↓ENT2
↑↑Adenosine
↓ADK
HIF1α
HIF1α
AMP
↑ENTPD1
SP1
HIF1α
↑NT5E
HIF2α
↑A2B
↑A2A
↑cAMP–PKA
↑ERK–MAPK
A1
↑A3
• Local adenosine receptor signalling
• Left shift of dose–response curve

PRIMARY SENSORY NEURON 	$P2Y_1$ $P2Y_2$ $P2Y_4$	Magni et al., 2015 Molliver et al., 2002 Ruan and Burnstock, 2003
SECONDARY NEURON 	$P2Y_1$ $P2Y_4$ A_1 A_{2B}	Kobayashi et al., 2006 Kobayashi et al., 2006 Borea et al., 2018 Borea et al., 2018
MICROGLIA 	$P2Y_1$ $P2Y_2$ $P2Y_6$ $P2Y_{12}$ $P2Y_{13}$ $P2Y_{14}$ A_1 A_{2A} A_{2B} A_3	Bianco et al., 2005 Shi et al., 2017 Xu et al., 2016 Koyanagi et al., 2016 Kobayashi et al., 2012 Kobayashi et al., 2012 Luongo et al., 2014 Duarte et al., 2019 Borea et al., 2018 Borea et al., 2018
ASTROCYTES 	$P2Y_1$ $P2Y_2$ $P2Y_4$ $P2Y_6$ $P2Y_{11}$ $P2Y_{14}$ A_{2A} A_{2B} A_3	Shinozaki et al., 2017 Tran, 2011 Tran, 2011 Kim et al., 2011 Barragán-Iglesias et al., 2014 Kinoshita et al., 2013 Borea et al., 2018 Borea et al., 2018 Borea et al., 2018
OLIGODENDROCYTES 	$P2Y_1$ $P2Y_{12}$ A_{2A}	Agresti et al., 2005 Amadio et al., 2014 Borea et al., 2018
SATELLITE GLIAL CELLS 	$P2Y_1$ $P2Y_2$ $P2Y_4$ $P2Y_6$ $P2Y_{12}$ $P2Y_{14}$	Magni et al., 2015 Magni et al., 2015 Villa et al., 2010 Ceruti et al., 2008 Wang et al., 2018 Kobayashi et al., 2006


Astrocyte
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CXCL13
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TNF-α
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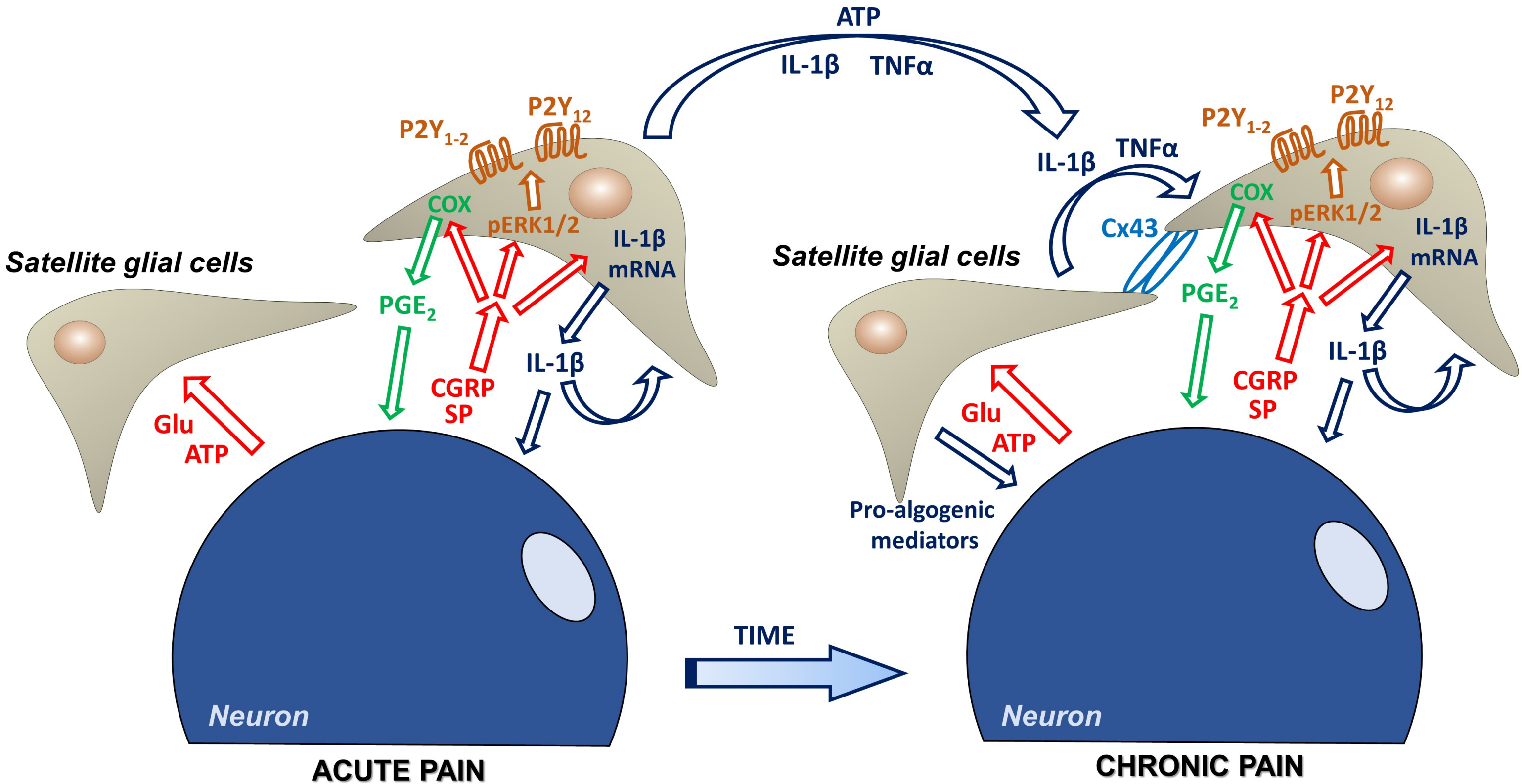
Caspase-6
BDNF
TNF-α
ILs
Fractalkine
Microglia
Secondary sensory neuron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CGRP
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IL-1β
Glu
ATP
Satellite glial cells
Neuron
ACUTE PAIN
TIME
IL-1β
TNFα
Cx43
Pro-algogenic mediators
CHRONIC PAIN