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Implication of neuronal versus microglial P2X4 receptors in central nervous system disorders

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Abstract

The P2X4 receptor (P2X4) is an ATP-gated cation channel that is highly permeable to calcium and widely expressed in neuronal and glial cell types throughout the CNS. A growing body of evidence indicates that P2X4 plays key roles in numerous central disorders. P2X4 trafficking is highly regulated and consequently in normal situations, P2X4 is present on the plasma membrane at low density and found mostly within intracellular endosomal/lysosomal compartments. An increase in *de novo* expression and/or surface density of P2X4 has been observed in microglia and/or neurons during pathological states. This review aims to summarize knowledge on P2X4 functions in CNS disorders and provide some insights into the relative contributions of neuronal and glial P2X4 in pathological contexts. However, determination of the cell-specific functions of P2X4 along with its intracellular and cell surface roles remain to be elucidated before the potential of P2X4 as a therapeutic target in multiple disorders can be defined.

Introduction

Initially discovered as one of the main sources of energy inside cells, adenosine 5' triphosphate (ATP) is now recognized as a ubiquitous extracellular cell-to-cell signaling molecule in the peripheral (PNS) and central nervous system (CNS), as well as in peripheral organs [1]. Although the release of ATP by sensory nerve cells was observed in the 1950s [2], Geoffrey Burnstock was the first to propose in 1972, the concept of purinergic signaling using ATP as a fast neurotransmitter [3]. ATP was later shown to mediate fast neurotransmission in the PNS and CNS alike, but is nowadays mostly considered as a neuromodulator co-released with other classical neurotransmitters such as GABA or glutamate at inhibitory or excitatory synapses in the CNS [4]. ATP is also a gliotransmitter released by glial cell types such as astrocytes or microglia [5-7]. Neuronal and glial released ATP exerts multiple actions in the neuromodulation of synaptic activity or plasticity, communication in glial networks and directly between glia and neurons via the activation of metabotropic P2Y and ionotropic P2X receptors, or after the conversion of ATP by ectonucleotidases into ADP and adenosine by the activation of P2Y and adenosine P1 receptors [4, 8].

There is also growing evidence for the involvement of purinergic signaling in major CNS disorders including chronic pain, brain trauma, hypoxia/ischemia, epilepsy and neurodegenerative diseases associated with neuro-inflammation (for recent review, see [9]). In these noxious conditions, extracellular levels of ATP increase and serve as a danger signal along with activation of several purinergic receptor subtypes that are widely distributed both in neurons at pre-and post-synaptic sites and in glial cells [4, 6, 8, 10, 11]. Besides P2X7, P2X4, P2Y1 and $A2_AR$ also P2Y6 (microglial

phagocytosis) and P2Y12 (microglial chemotaxis) participate in ATP danger signaling [9], increasing evidence indicates that microglial or neuronal P2X4 receptors are upregulated and play key roles in many CNS disorders. In this review, we focus on the function of neuronal and glial P2X4 function in physiological and pathological conditions.

Properties and function of P2X4 receptors

P2X receptors are trimeric ATP-gated cation channels that are formed by homo or heteromeric associations from 7 different subunits (P2X1 - P2X7) encoded by seven genes in mammals. Functional P2X receptors are non-selective cation channels permeable to sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) and their activation by ATP binding leads to cell depolarization and Ca^{2+} influx. With the exception of P2X6, all homomeric P2X receptors are functional and display different ATP sensitivities, current kinetics and pharmacological properties. Moreover, the variety in expression pattern of the seven P2X subunits and the existence of heteromeric associations between these subunits contribute to the diversity and widespread action of ATP signaling [8]. Among the seven P2X subunits, P2X4 is the most widely distributed in various cell types throughout the body [1, 12]. All P2X subunits are expressed in the CNS, in a heterogeneous manner throughout the different structures, cell types and subcellular compartments [13] , although P2X4 is one of the main subunits found in both neurons and glial cells such as microglia and more controversially in astrocytes, oligodendrocytes and Schwann cells (Table 1and Figure 1) [14]. However, the subunit composition of P2X receptors in most CNS cells is not well defined, and the physiological roles of P2X4 or P2X4-containing receptors is far from being deciphered due to the paucity of selective pharmacological tools. TNP-ATP a weak and non-selective P2X4 antagonist or

5-BDBD a selective allosteric P2X4 antagonist, have been commonly used to show the involvement of P2X4 in rodent CNS tissue [12] *in vitro* and *in vivo*. New potent antagonists of rodent P2X4 (BAY-1797 or NP-1815-PX) were recently identified [15, 16] as well as a highly selective human P2X4 antagonist (BX430) with no effect on rodent P2X4 [17]. Ivermectin, which is an agonist of invertebrate glutamate-gated chloride channels, is used as an antiparasitic drug in human medicine [18]. This compound is also a very potent positive modulator of mammalian P2X4. However, although highly selective for the P2X4 subunit among P2X subtypes, Ivermectin potentiates human P2X7 currents and can modulate other ligand-gated ion channels such as $GABA_A$ or nicotinic receptors [19-22]. Ivermectin is a positive allosteric modulator of P2X4 that stabilizes this subunit in the open state [20], which consequently may also prevent P2X4 internalization [21, 22].

P2X4 trafficking

Indeed, in contrast to other P2X subunits, P2X4 is constitutively and highly internalized, and as a result, is found mainly in endosomal/lysosomal compartments thereby ensuring low surface expression of these receptors in basal states [23]. Recent work showed that intracellular P2X4 in these acidic compartments are functional and may have important functions in lysosome membrane fusion [24]. In various pathological states, such as trauma, ischemia, chronic pain, neurodegenerative processes and several neuropsychiatric disorders, *de novo* expression of P2X4 and/or an increase in cell surface P2X4 density was observed in microglia and/or neurons, thus suggesting possible key and multiple roles of neuronal and microglial P2X4 receptors in the establishment and/or maintenance of these pathologies [25]. Changes in the intracellular expression of P2X4 may also have important consequences in the pathophysiological context.

Similar to all seven P2X subunits, P2X4 has a topology with 2 transmembrane regions, linked by a large extracellular domain and two intracellular amino and carboxyl termini [26]. The first X-ray crystal structure of a P2X receptor resolved in a truncated form of the zebrafish P2X4 receptor in

its closed state [27] and then in complex with ATP [28] from crystallized human P2X3 in different states [29] confirmed the subunit topology and trimeric organization of P2X receptors. The structure of head domain of rat P2X4 has also been investigated [30]. These data also revealed the importance of a large extracellular disulfide-rich region packed with N-linked glycosylation moieties and the existence of three inter-subunit binding sites. They also showed that the binding of three molecules of ATP is required to induce conformational changes leading to the opening of the channel pore formed by the three transmembrane regions [28]. P2X subunits share several conserved motifs within the intracellular domains, such as a YXXXK in the C-terminus that regulates surface expression [31] and a TXK/R in the N-terminus which is a putative protein kinase C phosphorylation site regulating current desensitization [32]. However, in terms of sequence and length, the C-terminus domain is the most divergent between P2X subunits [12]. The mammalian P2X4 subunit displays two endocytic tyrosine-based and di-leucine motifs within the C- and N-termini, respectively, which are responsible for its constitutive internalization and main localization in lysosome-related intracellular organelles [33]. This distribution is in contrast to other P2X receptors such as P2X2, which are mainly found at the level of the plasma membrane [34]. Interestingly, the clathrin-dependent constitutive internalization of P2X4 is mediated by an interaction between the non-canonic motif YXXGΦ and the μ2 subunit of the adaptive protein 2 (AP2), whereas the canonic YXXΦ motif is not involved [34, 35]. Mutations of the endocytic motif YXXGL or blockade of clathrin-mediated internalization lead to an increase both in the number of P2X4 at the plasma membrane and associated ATP currents[22, 34-36]. Being highly glycolyzed, the extracellular loop of P2X4 allows it to resist the very acidic intralysosomal environment and proteolysis. Intracellular P2X4 pools can thus remain intracellular or either recycle back to the cell surface and dynamically regulate the number of surface P2X4 receptors [23, 34, 37-39]. Several studies have shown that in macrophages and microglia, the surface trafficking of P2X4 is upregulated in response to stimuli promoting lysosomal exocytosis or altering endocytosis [33, 40, 41]. In numerous

pathophysiological conditions, *de novo* expression of P2X4 and/or increased surface trafficking had been observed [42] suggesting an important role of the dynamic regulation of P2X4 surface trafficking in diseases (Figure 1).

intracellular P2X4 function

Finally, growing evidence suggests that lysosomal P2X4 may also have important physiological functions (for review, see [43]). In lysosomes, the extracellular domain of P2X4 containing the agonist binding sites faces the luminal compartment, which contains high concentrations of ATP [44, 45] transported by the vesicular nucleotide transporter VNUT/SLC17A9 present in the lysosomal membrane. Alkalinization of the intralysosomal pH may lead to P2X4 activation and participate in endolysosomal fusion and vacuolation [43]. Indeed, Ca^{2+} flux through P2X4 activates calmodulin which subsequently associates with the receptor channel and promotes vesicular fusion [46].

Neuronal P2X4

P2X4 is expressed in various tissue cell types of peripheral organs including epithelial cells, kidney, pancreas, lung, liver, cardiovascular system or immune cells (for review, see [12]) in which it has important functions. In the CNS (Table 1), P2X4 along with P2X2 and P2X6, is the most widely expressed P2X subunit in both neurons and glial cells [14, 47]. P2X4 was first identified and cloned from brain tissues in 1996 by two independent groups [14, 48]. Their studies also showed the expression of P2X4 mRNA in the dentate gyrus, CA1/CA3 pyramidal cells of the hippocampus and in cerebellum Purkinje cells [14, 48]. Further immunohistochemical studies with anti-P2X4 antibodies confirmed the presence of P2X4 at the protein level in GABAergic interneurons of the hippocampus, cerebellum and olfactory bulb [13, 49], spiny neurons of the striatum and substantia nigra [49, 50], and in the hypothalamus and hypophysis [51]. Other studies reported P2X4 expression

in somatosensory cortical neurons [52] , sensory nerves or ganglia [53-55] and the retina [56]. Electron microscopy has indicated that neuronal P2X4 is located both pre- and post-synaptically, mainly at the edge of the postsynaptic density in the peri- and extra-synaptic space [13]. This is consistent with the idea that activation of synaptic P2X receptors by ATP coreleased with other neurotransmitters such as glutamate or GABA did not mediate fast synaptic transmission but modulate excitatory or inhibitory synapses in the CNS [6]. The widespread distribution of P2X4 in CNS neurons was confirmed with P2X4 knockout mice expressing ß-galactosidase instead of P2X4 [57] and recently with a transgenic reporter mice expressing tdTomato under the control of a P2X4 promotor and subsequently using a novel internalization-defective P2X4mcherryIN knockin mouse [58, 59].

Neuronal P2X receptors located at pre- and post-synaptic sites act mainly as neuromodulators. However, in most cases the specific contribution of P2X4 has been difficult to ascertain due to the paucity of appropriate pharmacological or genetic tools. In addition, the P2X4 subunit may not only form functional homomeric receptors, but can also assemble in heteromeric association with P2X6 (P2X4/6) or as heterotrimeric P2X2/4/6 [60, 61]. Activation of pre-synaptic P2X4 increases the release of neurotransmitters such as glutamate or GABA in neurons of the arcuate nucleus of the hypothalamus regulating food intake [58]. At the post-synaptic level, ATP released by neurons or glial cells acting on postsynaptic P2X4 receptors modulates activity and plasticity of excitatory and inhibitory synapses in several areas of the brain [10, 62]. P2X4 can modulate excitatory synaptic transmission and plasticity by acting on glutamatergic NMDA and AMPA receptors [63]. The influx of Ca^{2+} *via* the opening of either P2X2 or P2X4 channel pores can trigger the internalization of AMPARs by phosphorylation of the GluA1 subunit [10, 11] *in vitro*, although in hippocampal neurons, this action seems to be mediated by P2X2 rather than P2X4, consistent with the low surface expression of hippocampal neuronal P2X4 in basal conditions [10]. P2X4 receptors can also modulate the expression of NMDA-dependent long-term potentiation (LTP) in CA1 neurons of

the hippocampus. Indeed, the pharmacological blockade of P2X4 facilitates the induction of NMDAR-dependent LTP [64], suggesting that P2X4 have a negative impact on LTP. However, another study has shown that potentiation of P2X4 by ivermectin (IVM), a positive modulator of P2X4, increases LTP in the hippocampal CA1 region [57]. This effect was not observed in P2X4KO mice. The results suggested that the Ca^{2+} influx *via* postsynaptic P2X4, by promoting the incorporation of synaptic NMDARs, would strengthen synaptic activity during LTP [65]. ATP originating from individual excitatory synapses or glial cells, can activate P2X4 in neocortical neurons and in turn reduce the currents induced by NMDAR activation. This effect is not observed in P2X4KO mice in which LTP is increased [62]. Altogether, these results point to the multiple neuromodulator actions of P2X4 at central excitatory synapses.

P2X4 is also capable of modulating GABA inhibitory synapses. The physical interaction between post-synaptic P2X4 and $GABA_A$ receptors in the ventromedial nucleus of the hypothalamus leads to an inhibition of GABA-mediated postsynaptic currents and consequently enhances neuronal excitability [36, 66]. Interestingly, this effect was observed solely after blockade of P2X4 internalization using a competitive peptide, leading to the increase of surface expression of P2X4 [36]. P2X4-mediated attenuation of tonic and phasic GABAergic inhibition was also observed in cortical neurons. In this case the effect is apparently mediated by P2X4-induced Ca^{2+} influx and a PKC-dependent downregulation of $GABA_A$ receptors [6, 67].

Glial P2X4

Glial cells are themselves capable of releasing ATP, which can play an important role in neuron-glia and glial network signaling such as propagating glial calcium waves [68]. P2X4 is expressed in microglia with P2X7 and both receptors can interact with each other. The existence of P2X4/7 heteromers remains controversial and interaction may also occur by direct or indirect interaction between homomeric P2X4 and P2X7 [69]. A *de novo* expression of P2X4 and/or an increased

surface trafficking of P2X4 observed in activated microglia [42] play key roles in many CNS diseases (see below). The upregulation of microglial P2X4 was revealed following LPS-induced inflammation in the hippocampus and selectively involving activated microglia using *dt*Tomato reporter mice and internalization-defective P2X4mCherryIN knockin mice [58, 59].

The P2X4 receptor has been revealed by immunostaining in S100β-positive astrocytes of the rat hippocampus in the CA1, CA3 regions and the dentate gyrus [70]. P2X4 expression was additionally detected in astrocytes from hippocampal cultures of P2X4mCherryIN mice, as well as in astrocyte end-feet contacting endothelial cells in brain slices of these mice solely by electron microscopy [59]. P2X4 were also detected in GFAP-positive astrocytes of the rat nucleus accumbens [71]. Nevertheless, P2X4 expression in astrocytes remains debated [72], although this discrepancy may merely reflect the heterogeneity of astrocytes across the different regions of the CNS. Indeed, a growing body of evidence suggests that astrocytes as well as microglia and oligodendrocytes display regional and contextual heterogeneities in terms of gene expression pattern, phenotypes and roles in controlling physiological and pathological brain functions [73-75].

Recent studies have shown the presence of P2X4 in satellite glial cells (SGCs) stained by astrocytic markers of dorsal root ganglia. This expression was observed solely during neuropathic pain [76] and diabetes-induced neuropathy [77] suggesting a pathology-induced upregulation of P2X4 in SGCs. The expression of several types of P2 receptors, including P2X4, has also been demonstrated in oligodendroglial precursor cells [78]. However, its function is not yet fully understood, in contrast to P2X7 receptors which contribute to ATP excitotoxicity in oligodendrocytes and the pathogenesis of experimental autoimmune encephalomyelitis[79].

P2X4 has also been observed in Schwann cells, mainly localized intracellularly in lysosomes, under normal physiological conditions. After peripheral nerve injury, an overexpression of P2X4

receptors and their increase on the surface of Schwann cells was shown to promote, through the release of BDNF, motor and sensory functional recovery and ameliorate nerve remyelination [80].

P2X4 in CNS diseases

Neuropathic Pain

Acute pain serves a protective function in promoting reflex withdrawal from painful stimuli and the avoidance of injury or damage. Acute pain is initiated by an activation of nociceptive neurons in peripheral dorsal root ganglia (DRG), with information being processed and integrated within the spinal cord (SC) before transmission to the brain [81]. Whereas acute pain is a normal physiological function of the CNS, chronic pain results from pathological and persistent alterations in the peripheral and central nervous systems [82]. Chronic pain can be induced by nerve damage, so-called neuropathic pain, or by persistent inflammatory pain associated with the healing of damaged tissue[83]. Chronic pain persists even after the expected period of healing and becomes disabling for the individual [84].

Peripheral nerve injury (PNI) was shown to induce an activation of microglia in the spinal cord dorsal horn [85, 86] by several pathways involving interferon-γ (IFN-γ) or platelet-derived growth factor (PDGF) [87, 88]. Activation of spinal microglia causes tactile allodynia, a symptom of neuropathic pain , and several studies following the pioneered work of Tsuda *et al*. [89] showed that a specific microglial response phenotype characterized by the *de novo* expression of P2X4 is critical for the pathogenesis of neuropathic pain [67]. The expression of P2X4 increases exclusively in the reactive microglia after PNI and is confined to the ipsilateral spinal dorsal horn (Figure 2). Moreover, the genetic ablation or downregulation of P2X4 using either P2X4KO mice or RNA

antisense as well as the blockade of P2X4 by the antagonist TNP-ATP dramatically reduces tactile allodynia after PNI [89-91]. Interestingly, an activation of microglia is also observed in P2X4KO mice after PNI [91] indicating that P2X4 is not required for microglial activation but is necessary and sufficient for the development of allodynia by neuro-glial communication. Indeed, activation by ATP of microglial P2X4 reduces GABA or Glycine receptor-mediated inhibitory currents within the spinal cord. This disinhibition results from an increase in the intracellular concentration of chloride that becomes sufficient to block GABA or Glycine mediated-inhibitory responses or to eventually convert inhibition into excitation, thereby inducing the hypersensitivity of spinal neurons and tactile allodynia [92, 93]. Changes in chloride concentration is due to the downregulation of the potassium-chloride co-transporter KCC2, which is mediated by the extracellular release of BDNF and the activation of Trk-B receptors in inhibitory neurons. Several studies have established the key role played by BDNF in this signaling cascade and the resulting disinhibition and pain-related behavior [92, 94]. Also established is the requirement for ATP-mediated activation of P2X4 receptors to elicit BDNF release by microglia [91], although how P2X4 actually triggers the release of BDNF remains to be determined. In the dorsal horn of the spinal cord, ATP can be released by the terminals of primary sensory neurons [95], dorsal horn neurons [96], or glial cells [7].

A recent study showed that PNI increased the expression of the vesicular nucleotide transporter (VNUT) and induced a slight increase in the extracellular concentration of ATP in the spinal cord. By cell-specific deletion of VNUT, the authors elegantly showed that ATP released by spinal cord neurons and astrocytes, but not by sensory neurons, contributes to neuropathic pain[97] very likely by activating microglial P2X4-mediated signaling cascades. However, ATP is co-released with GABA in spinal dorsal horn neurons that can activate simultaneously postsynaptic P2X and $GABA_A$ receptors[96]. A cross-talk between neuronal P2X, including P2X4 and $GABA_A$ receptors,

may downregulate GABA-mediated currents and thus contribute to disinhibition in neuropathic pain [36, 96, 98].

Several mechanisms are involved in the increase of P2X4 expression in microglia following PNI. Two transcription factors of the Interferon Regulatory Factor (IRF) family, IRF8 and IRF5, that are expressed in immune cells including microglia [99, 100] are upregulated after PNI in dorsal horn microglia and silencing IRF8 was shown to suppress PNI-induced tactile allodynia[100]. IRF8 promotes several actors involved in chronic pain [100] and IRF5 expression which in turn controls directly P2X4 transcription by binding to the promoter region of p2rx4 gene [100]. Fibronectin also appears to be a key regulator of P2X4 expression through several pathways. The observed increase in extracellular fibronectin after PNI could induce *de novo* P2X4 expression through several fibronectin/integrin signaling pathways [90, 101], as well as promoting P2X4 trafficking from the lysosome to the cell surface through the release of a chemokine CCL2 and subsequent activation of microglia CCR2 receptors [102].

An upregulation of P2X4 in spinal microglia is likely to play a key role in tactile allodynia induced by PNI, although interestingly, recent work has suggested that this mechanism occurs only in males [94, 103]. In contrast to male mice following PNI, no increase in P2X4 expression was observed in dorsal horn microglia of post-PNI female mice and tactile allodynia was not modified by the pharmacological blockade of P2X4 with TNP-ATP [94]. Results suggest that immune cells contribute to pain hypersensitivity in female mice [94, 104], although other studies have indicated that microglial P2X4 and BDNF/TrkB signaling contribute to bone cancer [105] and herpetic pain [16] models in female rodents, thus demonstrating the need for additional studies to clarify this sexual dimorphism.

Besides microglial P2X4 in the spinal cord, P2X4 expression in sensory neurons was recently linked to neuropathic pain. All P2X subunit mRNAs are expressed in sensory DRG neurons [106], but the functional expression and potential involvement in pain processing was only until very recently found to be limited to homomeric P2X3 or heteromeric P2X2/3 receptors expressed in nociceptive neurons [107]. Recent work showed that P2X4 is expressed at mRNA and protein levels in DRG sensory neurons [77, 108-110] and that its expression increases in several neuropathic or inflammatory pain models in rodents [111]. P2X4 receptor expression was also observed in satellite glial cells (SGC) which enwrap the cell bodies of sensory neurons within the ganglia [110, 112]. Although it can be difficult to distinguish P2X4 in the plasma membrane of sensory neurons or SCGs due to the apposition of both elements, several lines of evidence have established that P2X4 expression increases in SCGs under neuropathic pain conditions [77, 111, 113]. After PNI, the coupling between the SGCs themselves and between SGCs and neurons increases via the formation of gap junctions [114, 115] and activated SCGs release inflammatory mediators and other neuromodulators that contribute to neuronal hyperexcitability and pain hypersensitivity [115].

Chronic inflammatory pain

P2X4 receptors expressed in distinct cell types also contribute to inflammatory pain. For example, the presence of P2X4 in skin-resident macrophages contributes to the sensitization of peripheral sensory nerve termini and induces mechanical hypersensitivity due to the production of the inflammatory factor PGE2 [116]. P2X4 also plays a role in mechanical hypersensitivity following chronic inflammation by promoting the hyperexcitability of spinal dorsal horn neurons in chronic inflammation mouse models [116] (Figure 2). On the other hand, such hyperexcitability is downregulated in P2X4-deficient mice [117]. A recent study showed that the expression of P2X4 receptors in small diameter nociceptive neurons of DRGs is increased by twofold during chronic inflammation [109]. In addition, these sensory neurons express BDNF, a key factor in both

neuropathic and inflammatory pain. Indeed, in chronic inflammatory conditions, BDNF contributes to mechanical hypersensitivity using the similar pathway. However, unlike neuropathic pain, during chronic inflammation, BDNF is released in a P2X4-dependent manner by sensory neurons termini in the dorsal horn of the spinal cord, thereby leading *via* the TrkB/KCC2 cascade to spinal disinhibition [118]. P2X4 expressed in SGCs may also contribute to chronic inflammatory pain [112, 119] by releasing pro-inflammatory cytokines such as IL1β and TNFα following activation of the inflammasome NLRP1 [116, 119].

Migraine

Chronic migraine is a neurological disorder characterized by repeated attacks that can be caused by an increase in the excitability of central neurons in the trigeminal nociceptive pathway, principally the trigeminal nucleus caudalis (TNC), leading to central sensitization [120]. Similar to neuropathic pain, microglia surrounding TNC neurons has been suggested to play direct and indirect roles in the establishment of central sensitization [121]. Indeed, microglial activation has been observed when nitroglycerin (NTG) is applied chronically in a model of chronic migraine, with a resultant effect on central sensitization [121]. As observed during neuropathic pain [89, 91], several studies suggested a role for BDNF expressed in the trigeminovascular system in migraine pathophysiology [122] and an increase in P2X4 and BDNF expression has been recently reported in the TNC after chronic intermittent administration of NTG [121, 123]. Moreover, P2X4 pharmacological inhibition has been very recently found to prevent hyperalgesia induced by NTG, associated with an inhibition of p-ERK phosphorylation and calcitonin gene-related peptide (CGRP) release in the TNC [123]. This study also showed that in BV2 microglial cells, ATP triggers BDNF synthesis and release, which is in turn reduced by the P2X4 antagonists 5-BDBD or SB203580 [123]. In addition, a potentiation of P2X4 by ivermectin induced sustained hyperalgesia and significantly increased levels of p-ERK and CGRP release in the TNC [123]. Those results

therefore clearly indicate that microglial P2X4 contributes to TNC neuronal hyperexcitability involved in chronic migraine.

Post-ischemic inflammation

Brain ischemia is associated with post-ischemic inflammation, including glial cell activation along with an increase in extracellular ATP levels. Several studies have reported an upregulation of P2X4 receptors in activated microglial cells after ischemic brain injury [124-127] (Figure 3). During hypoxic conditions, ATP is released in large amounts from cells damaged by ischemia and the activation of both P2Y12 and P2X4 receptors present in a higher density in reactive microglia mediates, *via* the PI3K signaling pathway, chemotaxis of motile microglia towards the lesion site as well as the release of proinflammatory cytokines [125, 128, 129]. More recently, a neuroprotective role of P2X4 during ischemia has also been reported [130]. Specifically, the activation of P2X4 expressed in vascular endothelial cells by ATP release consequently to pressure shocks mediated by vascular ischemia promotes the upregulation of osteopontin, a neuroprotective molecule [130]. These results therefore highlight a new mechanism whereby endothelial P2X4 induces an ischemic tolerance.

Epilepsy

Epilepsy is a chronic neurological condition characterized by recurrent seizures that spread to neighboring cortices, and lead to hippocampal neuron loss and microglia activation (Figure 3). To date, as for P2X7, the implication of P2X4 in epilepsy remains unclear, and whether the latter is up- or down-regulated in the hippocampus following status epilepticus (SE) is still debated. Some studies have reported a lack of change, or even a decrease in P2X4 immunoreactivity, 24 hrs after SE induced either by systemic pilocarpine or by intra-amygdala kainate injection . Kang et al.

reported similar findings in seizure-sensitive compared with seizure-resistant gerbils [131], although the specificity of the antibody anti-P2X4 used in these studies has not been established.

Subsequently, in an epileptic mouse model using intraperitoneal injection of kainate, hippocampal P2X4 transcript and protein levels were found to increase 24 and 48 hrs after the SE, and particularly in activated microglia in the stratum radiatum [132]. In P2X4KO mice, a decrease in microglial activation as well as subsequent cellular death were observed after the induction of the SE [132]. Even though epileptic seizures induced by kainate are not strongly inhibited in P2X4KO mice, there is still a significant reduction of cell death in the hippocampus as well as a decrease in the amplitude of hyperpolarizing outward current activated by microglia [132]. As for neuropathic pain, the increase in *de novo* P2X4 expression in activated microglia after the induction of epilepsy could facilitate BDNF release and the induction of hyperexcitability, thereby promoting epileptogenesis.

Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder and is characterized mainly by the accumulation of insoluble aggregates of beta-amyloid fragments ($A\beta_{1\text{-}42}$) that in turn lead to synaptic dysfunction and neuronal death causing a progressive loss of memory [133, 134]. Like other neurodegenerative diseases, the increase of extracellular nucleotides concomitantly with exacerbated neuroinflammatory events play a crucial role in AD pathogenesis [135].

It has been proposed that ATP, acting through P2X receptors, could participate directly in some of the toxic effects of Aβ peptide observed in neurons, with a particularly important role of P2X4 receptors [136, 137]. Indeed, an accumulation of P2X4 has been observed in hippocampal neurons exposed to the Aβ peptide [138]. This expression increase was found to be restricted to the cell body after 6 hrs of treatment, before also reaching the neurites after 12 hrs [138]. In parallel, after exposure to an Aβ fragment, the authors revealed an accumulation of a smaller P2X4 fragment, which could

be prevented by exposure to a caspase inhibitor, thus suggesting occurrence of a proteolytic cleavage of P2X4 receptors (Figure 3). They concluded that the cleavage by caspase-3 slows down the closing time of the receptor's channel pore and prevents receptor agonist-dependent internalization. This leads to an increase in membranal surface trafficking of the neurons that can in turn induce neuron toxicity due to increased calcium influx [138]. In this study, moreover, the overexpression of neuronal P2X4 was found to promote the toxic effect of the Aβ fragment while inhibition of receptor expression has a significant positive effect on neuronal death after Aβ exposure. Consequently, these findings led to the conclusion that neuronal P2X4 receptors are directly implicated in neuronal death induced by Aβ [138]. Consistent with the implication of neuronal P2X4 in AD, an increased surface density of neuronal P2X4 was recently shown to induce synaptic deficits and alterations in learning and memory functions of P2X4 internalization-defective knock-in mice. Surprisingly, however, the authors also reported a decrease in P2X4 protein levels in the middle frontal and temporal gyri of AD patients with severe cognitive impairment [138].

P2X7 has been shown to play a key role in neuroinflammatory processes of AD and other neurodegenerative diseases, including Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and Huntington disease (HD), in promoting proinflammatory cytokine production by microglia and astrocytes [139-141]. Like P2X7, P2X4 could also be implicated in neuroinflammation associated with AD [142] and the resultant potentiation of Aβ-mediated neuronal death, since similarities exist between these two receptor subtypes as well as a neighboring chromosomal localization [69, 143], and because PX4 has been correlated with several other inflammatory mechanisms [144, 145] and microglial activation processes [89].

Parkinson's disease

PD is characterized by the progressive loss of dopaminergic nigrostriatal neurons specifically, causing impaired dopamine (DA) homeostasis and associated behaviors leading to motor troubles. A hallmark of PD is the presence of abnormal intracytoplasmic aggregates of α-synuclein, called Lewy bodies (LB), within neuronal cell bodies. It is well known that P2X4 modulates major neurotransmitter systems including glutamatergic and GABAergic functions [10, 11, 36, 57, 62, 64-67, 146-150]. Several early studies suggested that P2X4 receptors are indirectly involved in dopamine (DA) neurotransmission [151, 152] and behavioral deficits were recovered in P2X4 KO mice that may also express DA dysfunction [153, 154]. A recent study has confirmed the receptor's direct role in regulating DA homeostasis that is impaired in PD [155]. Specifically, using P2X4 KO mice, the authors showed that a *p2rx4* deficit affects DA synthesis and transport, and increases DA receptor levels that could in turn alter DA neuron function and neurotransmission, with a resulting impact on DA associated behaviors such as motor control and sensorimotor gating [155]. These findings further indicate that P2X4 could be implicated in multiple neurological disorders such as PD and psychiatric diseases that involve DA homeostasis impairment.

Neuroinflammation is also a main hallmark of PD [156-159] and microglia seems to be central in this neurodegenerative disease [160, 161]. As previously discussed, microglial P2X4 could be involved in those neuroinflammatory mechanisms that are common to neurodegenerative diseases, and for which, in the case of PD, P2X7 is known to play a role [162, 163]. Nevertheless, the implication of microglia in the physiopathology of PD is still unclear and it remains unknown whether microglial alterations are causal to, or a consequence of, DA neuron degeneration [164].

Amyotrophic lateral sclerosis (ALS)

ALS is a fatal neurodegenerative disease characterized by the progressive and selective loss of spinal motoneurons (MN) as well as cortical motor neurons (Figure 2). The causes of ALS are multifactorial and the mechanisms underlying the selective degeneration of MN are still unknown.

A link has been established between ALS and P2X4 [165]. For example, a strong increase in P2X4 immunoreactivity in ventral horn MN immediately preceding their death has been reported in the SOD1-G93A ALS rodent model, suggesting an important role of neuronal P2X4 receptors in the pathogenesis of the disease [166]. P2X4 immunoreactivity has also been associated with a degeneration of other neuronal populations including noradrenergic neurons in the *locus coeruleus*, cerebellar Purkinje cells and serotoninergic neurons of raphe nucleus [166]. Surprisingly, the same authors subsequently showed, by using anti-P2X4 antibodies directed against the intracellular C-tail of P2X4 subunits, that SOD1-G93A misfolded proteins were detectable in MN but not in microglia [167]. This finding led to the suggestion that neuronal conformers of SOD1-G93A proteins that are reactive to anti-P2X4 antibodies could play a pathogenic role and induce neuroinflammation activating microglia and astrocytes when injected intracerebrally in normal animals [167].

An increase in P2X4 expression, both at mRNA and protein levels, was also reported in microglia of SOD1-G93A mice [168]. More recently, Volonté and colleagues demonstrated for the first time an increase in P2X4 and P2X7 expression in the peripheral nervous system of SOD1-G93A mice, particularly in the sciatic nerves [165].

Other work has shown that a potentiation of P2X4 function by ivermectin has a dual effect on MN survival depending on ATP concentrations, with a beneficial action at low ATP levels, but becoming deleterious at higher levels [146]. The authors deduced that ivermectin potentiates a neuroprotective effect of P2X4 on MN at low ATP levels, probably by increasing P2X4 function and/or the receptor's cell surface density [20, 22]. Moreover, the oral administration of ivermectin via the animal's drinking water from a presymptomatic stage (P50), caused a 10% increase in the lifespan of SOD1-G93A mice [146].

As for other neuroinflammatory diseases, P2X4 receptor is also likely to be involved in neuroinflammation processes associated with ALS and known to be implicated in MN degeneration mechanisms [169]. Several studies have highlighted a role of P2X7 in this disease [170], notably via a modulation of autophagy [171] as well as of microglia-mediated neuroinflammation processes that may be both beneficial or deleterious according to the stage of the disease, [172, 173]. An interaction between P2X4 and P2X7 receptors signaling pathways could also be involved in microglial inflammatory actions, with one pathway activating signaling of the other. Such a cross-talk is already known for macrophages, where P2X4 receptors regulate the inflammatory functions of P2X7 receptors [174]. Although the implication of P2X4 in the pathogenesis of ALS remains to be clearly established, this receptor subtype appears to be a potential therapeutic target for confronting this disease.

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic T-cell mediated auto-immune disease characterized by a massive infiltration of immune cells, demyelination and axonal loss. This neurodegenerative disease is also characterized by an early inflammation that is beneficial and initially delays the onset of neurodegeneration, which then evolves in a complex manner as the disease progresses [175]. The initial regenerative phase is due to the repairing actions of anti-inflammatory infiltrating macrophages and microglia.

Interestingly, P2X4 has been shown to be overexpressed in microglia and macrophages in a rat model of MS and experimental autoimmune encephalomyelitis (EAE) [127] (Figure 3). But in contrast to neuropathic pain, where P2X4 blockers have been proposed to be potential therapeutic drugs [16], P2X4 antagonists exacerbate neurological symptoms in MS animal models, whereas P2X4 potentiation by ivermectin or P2X4 expression upregulation are both beneficial in EAE [176]. Moreover, microglial P2X4 activation is known to induce BDNF release [92] and it has been shown

that BDNF release from microglia as a result of P2X4 potentiation or overexpression in Schwann cells promotes remyelination [80, 176].

In contrast, P2X4 potentiation by ivermectin increases myelin engulfment and degradation [176] and P2X4 activation in microglia induces endolysosomal membrane fusion and lysosome pH acidification, both of which are essential to phagocytic pathways [46, 176] that could control myelin phagocytosis. Lysosomes are also involved in secretion and myelin biogenesis [177, 178] and P2X4 has been shown to be implicated in lysosome exocytosis contributing to surfactant secretion in lung tissue [179]. Altogether these findings therefore suggest a potential role for lysosomal P2X4 receptors in secretion and phagocytosis in myelin disorders.

Alcohol-related disorders

Several studies have pointed to a role of P2X4 in alcohol-induced behavior [180-183]. P2X4 receptors are expressed in brain regions involved in the reinforcing properties of alcohol and other drugs [184]. Furthermore, a recent study suggested the involvement of P2X4 in regulating striatal dopamine homeostasis [155], supporting a role of P2X4 in the mesolimbic dopamine system implicated in the reward system of the brain (Figure 3).

An increase in ethanol intake is observed in P2X4 KO mice, suggesting that P2X4 could also be implicated in alcohol intake and/or preference [182, 185]. Other studies have demonstrated a lower P2X4 expression in rats that prefer alcohol, confirming the correlation between P2X4 expression and alcohol preference [183, 184]. Moreover, a direct link between P2X4 and the regulation of dopamine neurotransmission in the mesolimbic system has been established, further indicating that through its presence in dopaminergic neurons of the mesolimbic system, P2X4 plays a role in mediating alcohol drinking behavior [183].

Interestingly, low ethanol concentrations are able to inhibit P2X4 *in vitro*, probably by blocking opening of the channel without affecting its deactivation [186, 187]. Conversely, ivermectin is able to

antagonize the inhibition of P2X4 by ethanol, indicating that this positive modulator interferes with the linkage site of ethanol on the P2X4 receptor [188]. Altogether, these findings suggest that ethanol can reinforce alcohol intake *via* P2X4 inhibition and that pharmacological potentiation of P2X4 may reduce alcohol intake and preference.

Alcohol abuse is also known to promote neuroinflammation [189] and microglial P2X4 has been recently reported to play a role in this mechanism [190, 191]. Gofman and collaborators demonstrated that an alcohol-induced increase of P2X4 expression in microglia affects microglial migration and phagocytosis, and can be reversed by a P2X4 selective antagonist [190]. Later, the same group found that *via* P2X4, alcohol decreases the phosphorylation of key regulatory proteins and increases CREB transcriptional activity [191]. Cerebral ischemic events have also been reported in alcohol abuse, involving an upregulation of P2X4 in microglia following ischemia and hypoxia as described above [128, 192].

Neuropsychiatric disorders

Depression, bipolar disorder, schizophrenia, attention deficit hyperactivity and anxiety are neuropsychiatric disorders where DA homeostasis is impaired. As mentioned above, P2X4 has been shown to be implicated in the regulation of DA homeostasis and sensory motor gating [155] and there is increasing evidence that P2X4 plays a critical role in psychiatric disorders [59, 126, 155, 193, 194].

Consistent with an involvement of P2X4 receptors, ivermectin has been found to produce anxiolytic-like and depressive-like behavior in mice [193]. Very interestingly and in line with these findings, data from a new conditional P2X4 internalization-defective knock-in mouse, namely P2X4mCherryIN, that display an increase in number of P2X4 receptors at the surface of targeted cells, further supported the link between neuronal P2X4 and anxiety-like behavior as well as in memory [59]. The anxiolytic effects of a selective increase in surface P2X4 density in mouse

excitatory forebrain neurons further underlined the role of neuronal P2X4 in anxiety [59]. Another study using P2X4KO mice on the role of P2X4 in ischemic stroke showed that P2X4 deletion predisposes animals to chronic depression-like behavior after stroke [126]. Significantly in this context, a recent work demonstrated that the potentiation of P2X4 by ivermectin can lead to DA hyperactivity and disruption of information processing because of a potential perturbation of the interaction between P2X4 and DA receptors [194]. Together these results suggest that P2X4 antagonists could serve as novel anti-psychotic treatments for psychiatric disorders arising from sensorimotor gating impairments linked to the disruption of DA homeostasis [194].

Concluding remarks

P2X4 is the most widespread P2X subunit expressed in several neuronal and glial cell types throughout the CNS. Homomeric or heteromeric P2X4-containing receptors are ATP-gated channels that are highly permeable to calcium and in basal conditions, are localized in the plasma membrane at low density and mostly in intracellular endosomal/lysosomal compartments. Growing evidence suggest that endo/lysosomal P2X4 receptors are functional and play important intracellular signaling roles. Intracellular P2X4 can also be rapidly mobilized to the cell surface and contribute, together with *de novo* expressed receptors, to the upregulation observed in specific cell types in many central disorders such as pain, neurodegenerative disorders and neuropsychiatric diseases. However, establishing the function of upregulated P2X4 expressed in glial *versus* neuronal cells remain elusive. We have recently developed conditional internalization-defective P2X4mCherryIN knockin-mice with increasing numbers of P2X4 at the surface of targeted cells and have demonstrated the role of surface neuronal P2X4 in synaptic plasticity and memory[59]. The development of such a transgenic knockin mouse, along with the possibility of generating a conditional P2X4KO line that targets specific cell populations, represent major tools

to gaining in the near future a better understanding of the role of surface P2X4 in various physiological and pathological conditions and to evaluate the potential of P2X4 as a therapeutic target in these various disorders. Intracellular P2X4 can also play important roles and extensive studies are now needed to evaluate the respective functions of intracellular *versus* surface P2X4 receptors, which may differ between the various cell types expressing P2X4. It will also be important to identify mechanisms and regulation of P2X4 trafficking leading to the cell surface increase in pathological conditions, as well as determining the functional significance of the equilibrium between intracellular and surface P2X4 receptors.

Conflict of Interest

All authors declare that they have no competing interests

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Figure Legends

Figure 1: P2X4 expression in CNS diseases

Left, P2X4 in normal states is mostly intracellular with low surface expression at the surface of neurons and glia. P2X4 expression in astrocytes and oligodendrocytes precursors remain controversial (indicated by a question mark). Activation of P2X4 by extracellular ATP released by neurons or glial cells exerts mostly neuromodulatory actions in physiological state. Right, in pathological states, higher extracellular ATP concentration and *de novo* expression of P2X4 as well as increased surface trafficking of intracellular pools of P2X4 lead to the increase of the number of surface P2X4 in distinct cell types in numerous central disorders and exacerbation of purinergic signaling.

Figure 2: Implication of P2X4 in spinal cord disorders

de novo P2X4 expression and/or an increased surface trafficking of intracellular pools occur in glia or neurons of the spinal cord (inset) in various chronic pain conditions and amyotrophic lateral sclerosis (ALS). The contribution of P2X4 in spinal cord (as well as in sensory ganglia for pain) is indicated. P2X4 potentiation was induced by ivermectin.

Figure 3: Implication of P2X4 in brain disorders

de novo P2X4 expression and/or an increased surface trafficking of intracellular pools occur in various brain disorders in either microglia, neurons or in both cell types. The relative contributions of neuronal and glial P2X4 in brain disorders is indicated (MS, multiple sclerosis; AD, Alzheimer's disease; PD, Parkinson disease). P2X4 potentiation was induced by ivermectin. Increased P2X4 correspond to observation from P2X4mCherryIN knockin mice.

Table 1. Expression of P2X4 in the CNS

CNS regions	P2X4 mRNA[a]	P2X4 protein	P2rx4 reporter mice [82, 83]	P2X4 mCherryIN mice [84]	Cell types specificity	References
Amygdala	++	NA	NA	NA	NA	
Basal ganglia	++	+	NA	NA	Neurons, Astrocytes	[48, 49, 71]
Cerebellum	++	++	++	+	Neurons	[48, 49, 57, 58, 59,]
Cerebral cortex	+++	+	++	++	Neurons Astrocytes[b]	52, 58] [59]
Hindbrain	+	++	++	NA	Neurons	[58]
Hippocampus	++	++	++	++	Neurons, Microglia, Astrocytes[b]	[13, 14, 57, 58, 59]
Hypothalamus	+	+	++	NA	Neurons	[51, 58]
Midbrain	+	+	++	NA	Neurons	[58]
Olfactory region	++	+++	+++	++	Neurons, Epithelial cells	[49, 57, 58, 59]
Spinal Cord	++	++	++	**NA**	Motoneurons, Microglia	[58, 89, 109]
Thalamus	+	+	+	NA	Neurons	[58]

Expression levels are indicated on a semi-quantitative scale where (+) indicates the presence and relative abundance of P2X4 expression. (NA) no specific data set. (a) data are from the Allen brain atlas obtained by in situ hybridization and available at http://mouse.brain-map.org/experiment/show/75551474. (b) P2X4 expression was detected by electronic microscopy in astrocytes of P2X4mCherryIN mice using anti-RFP antibodies.

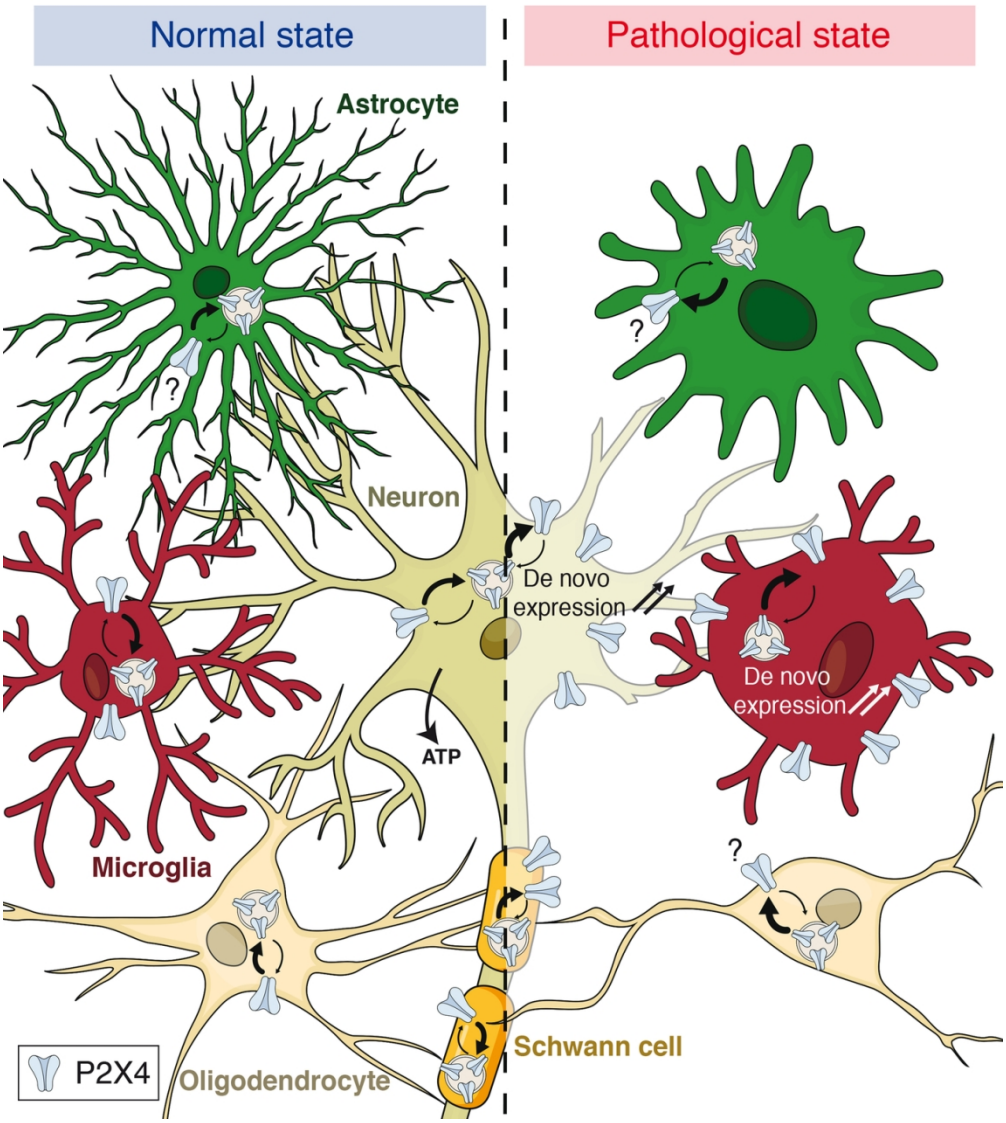
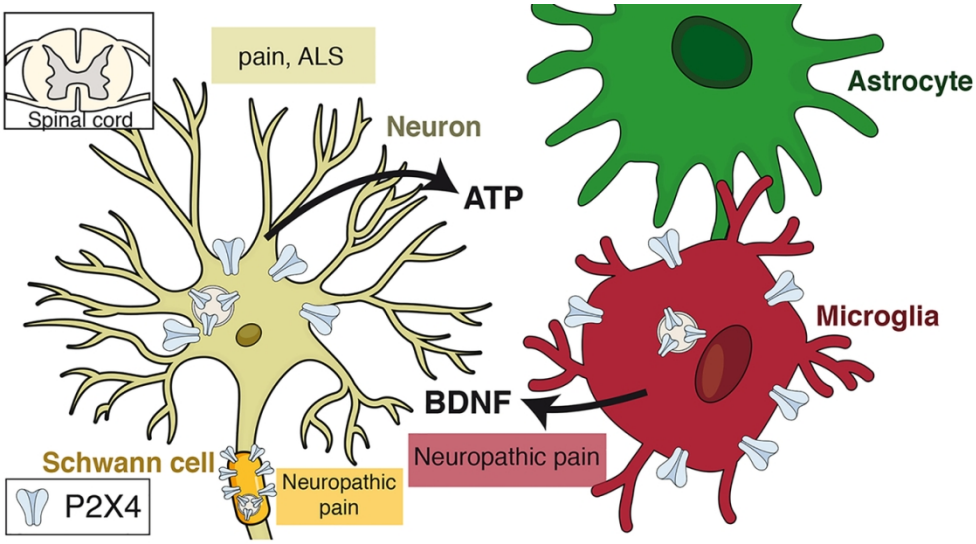


Figure 1: P2X4 expression in CNS diseases

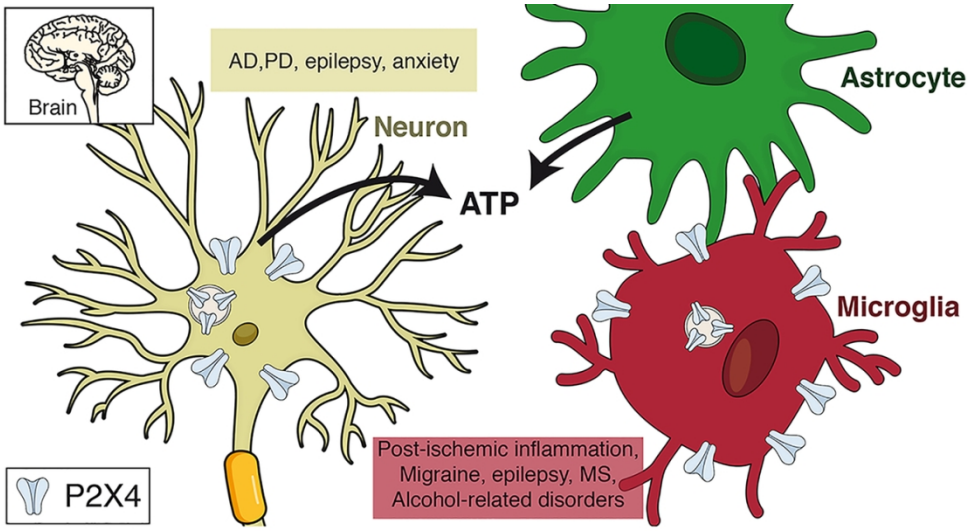
119x134mm (300 x 300 DPI)



	Increase of P2X4	Function of P2X4
Neuropathic pain	spinal microglia, sensory neurons, Schwann cells, SGC	release microglial BDNF and activate BDNF-TrkB-KCC2 pathway, spinal neuron hyperexcitability, tactile allodynia
Inflammatory pain	macrophages, sensory neurons, SGC	release of cytokines, release of BDNF by sensory neurons termini
ALS	spinal motoneurons, microglia, sciatic nerves	P2X4 potentiation: MN degeneration (high ATP) or neuroprotective effect (low ATP)

Figure 2: Implication of P2X4 in spinal cord disorders

118x96mm (300 x 300 DPI)



	Increase of P2X4	Function of P2X4
Post-ischemic inflammation	brain microglia, vascular endothelial cells	chemotaxis of microglia, release of cytokines upregulation of osteopontin (neuroprotective)
AD	hippocampal neurons	promote the toxic effect of beta-amyloid fragments increased P2X4: induced alteration of synaptic plasticity and memory deficits
Migraine	trigeminal nucleus caudalis microglia	microglial release of BDNF P2X4 potentiation: MN degeneration (high ATP), Increase p-ERK and CGRP release
Epilepsy	hippocampal neurons, stratum radiatum microglia	release of BDNF, neuronal hyperexcitability, cell death
MS	microglia, macrophages	release of microglial BDNF; Increase of myelin phagocytosis P2X4 potentiation: promotes remyelination
Alcohol-related disorders	dopaminergic neurons, microglia	ethanol induced P2X4 inhibition, reinforce alcohol intake/preference
PD	dopaminergic neurons	regulate DA homeostasis
Anxiety	forebrain neurons	increased P2X4: induced anxiolytic effects

Figure 3: Implication of P2X4 in brain disorders

114x123mm (300 x 300 DPI)