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Jaanus Suurväli, Pierre Boudinot, Jean Kanellopoulos, Sirje Rüütel Boudinot. P2X4: A fast and sensitive purinergic receptor. *Biomedical Journal*, 2017, 40 (5), pp.245–256. 10.1016/j.bj.2017.06.010 . hal-02391818

**HAL Id: hal-02391818**

**<https://hal.science/hal-02391818>**

Submitted on 26 May 2020

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## Review Article

## P2X4: A fast and sensitive purinergic receptor



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## ARTICLE INFO

## Article history:

Received 23 April 2017

Accepted 30 June 2017

Available online 10 November 2017

## Keywords:

P2X4

Purinergic signaling

Neuropathic pain

ATP

P2X receptor

## ABSTRACT

Extracellular nucleotides have been recognized as important mediators of activation, triggering multiple responses via plasma membrane receptors known as P2 receptors. P2 receptors comprise P2X ionotropic receptors and G protein-coupled P2Y receptors. P2X receptors are expressed in many tissues, where they are involved in a number of functions including synaptic transmission, muscle contraction, platelet aggregation, inflammation, macrophage activation, differentiation and proliferation, neuropathic and inflammatory pain. P2X4 is one of the most sensitive purinergic receptors (at nanomolar ATP concentrations), about one thousand times more than the archetypal P2X7. P2X4 is widely expressed in central and peripheral neurons, in microglia, and also found in various epithelial tissues and endothelial cells. It localizes on the plasma membrane, but also in intracellular compartments. P2X4 is preferentially localized in lysosomes, where it is protected from proteolysis by its glycosylation. High ATP concentration in the lysosomes does not activate P2X4 at low pH; P2X4 gets activated by intra-lysosomal ATP only in its fully dissociated tetra-anionic form, when the pH increases to 7.4. Thus, P2X4 is functioning as a Ca<sup>2+</sup>-channel after the fusion of late endosomes and lysosomes. P2X4 modulates major neurotransmitter systems and regulates alcohol-induced responses in microglia. P2X4 is one of the key receptors mediating neuropathic pain. However, injury-induced upregulation of P2X4 expression is gender dependent and plays a key role in pain difference between males and females. P2X4 is also involved in inflammation. Extracellular ATP being a pro-inflammatory molecule, P2X4 can trigger inflammation in response to high ATP release. It is therefore involved in multiple pathologies, like post-ischemic inflammation, rheumatoid arthritis, airways inflammation in asthma, neurodegenerative diseases and even metabolic syndrome. Although P2X4 remains poorly characterized, more studies are needed as it is likely to be a potential therapeutic target in these multiple pathologies.

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Peer review under responsibility of Chang Gung University.

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<http://dx.doi.org/10.1016/j.bj.2017.06.010>

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The concept of purinergic neurotransmission was first proposed in 1972 [1]. Back then, adenosine 5'-triphosphate (ATP) was mainly recognized as the key molecule responsible for intracellular energy transfer. However, this view had to be adjusted once it became clear that ATP can also act as an extracellular signaling molecule in non-adrenergic, non-cholinergic inhibitory nerve cells from gut smooth muscle [1]. Later works have demonstrated that ATP-mediated neurotransmission is present in most nerves of both peripheral and central nervous system (CNS), and that ATP can be co-released with other neurotransmitters [2]. Even adenosine itself can function as an extracellular signaling molecule.

The receptors for purinergic neurotransmission are divided into three main classes: P1, P2Y, and P2X (to which P2X4 belongs); most of them were cloned in the 1990-ies. It is likely that these receptors originally evolved to bind ATP and/or other purine or pyrimidine nucleotides released from necrotic or apoptotic cells [3]. The neuromodulatory function of ATP is mediated by both P2X (ionotropic nucleotide receptors) and P2Y (metabotropic nucleotide receptors) [1,4–6]. Thus, P2X receptors are ligand-gated ion channels that bind ATP [7], while both P1 and P2Y are G protein-coupled receptors with different binding specificities: P1 receptors bind adenosine, while P2Y bind either ATP or other purine nucleotides, e.g. ADP, UTP, UDP [8].

Both P2X and P2Y receptors play important roles in leukocyte biology and activation: they stimulate a number of signaling pathways, mediating, for example, cell volume regulation, eicosanoid release, phosphatidylserine exposure, hemolysis, impaired ATP release, reactive oxygen species formation, and apoptosis. They also modulate susceptibility or resistance to stress, apoptosis and infection. P2X receptors in particular are distributed widely throughout the body in neural and other tissues, and they mediate a remarkable variety of physiological and pathophysiological reactions especially in microglia [9–14]. P2X receptors are involved in synaptic transmission, muscle contraction, exocrine secretion, platelet aggregation, inflammation, macrophage activation, differentiation and proliferation, neuropathic and inflammatory pain.

P2X receptors are trimeric ATP-gated cation channels [15]. In both human and mouse, trimers consist in combinations of subunits which can be encoded by seven genes (P2X1 to P2X7). To date, six P2X purinergic homotrimeric receptors, and five P2X heterotrimeric receptors have been described [5,16]. Found exclusively in eukaryotes, P2X channels open upon binding of ATP [17], and mediate many of the extracellular actions of this molecule. P2X receptors differ from each other by their ATP binding affinity (nanomolar range for P2X1 to millimolar range for P2X7) [18], and are desensitized with different kinetics. The activity of P2X receptors is affected by many parameters including pH, extracellular concentration of bivalent ions (Zn, Cu, Hg, Ni, Cd), protons, lipids, steroids, ethanol. It is also affected by interactions with other membrane receptors such as rho1/GABA receptors as reviewed in [19]. When expressed alone, all subunits can form trimeric channels that constitute functional receptors [20]. Subunits of P2X hetero- and homotrimers differ by their pharmacological and/or biophysical properties [5,18,20]. Each subunit has two transmembrane-spanning helices, a large extracellular

ligand-binding loop, and intracellular carboxyl and amino termini. The N-terminus contains a consensus site for protein kinase C phosphorylation, whereas the C-terminus is variable.

P2X4 is a typical P2X receptor; it is able to form heterotrimers by combining with P2X2, P2X5 and/or P2X6 [16,21]. However, it is also one of the most sensitive purinergic receptors (at nanomolar concentrations), about one thousand times more than P2X7. P2X4 is widely expressed in central and peripheral neurons, in microglia, and also in various glandular tissues (e.g. pancreatic acinar cells and salivary glands) and endothelial cells [22]. Similar to P2X6, another member of the P2X family, it is also expressed in the epithelium of the colon, kidney and lung [23,24]. At the intracellular level, P2X4 tends to localize on the plasma membrane, but also in intracellular compartments such as lysosomes, vesicles, vacuoles and lamellar bodies [25]. Like P2X2, it is involved in neuropathic pain [26], but also in inflammatory pain [27], lung surfactant secretion [28,29], alcohol preference [30,31], cardiac function [32], and neurodevelopmental disorders [33,34]. Pharmacological and genetic strategies that reduce the function of microglial P2X4 alleviate symptoms in neuropathic pain models [25]. In this review, we analyze the multiple roles of P2X4 and show that it is a prominent mediator of pain, alcohol responses and inflammation and thus P2X4 is the subject of a growing interest.

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## P2X4: phylogenetic context and protein structure

### Evolutionary context of P2X4

The first receptors in evolution of life were probably ionic channels sensitive to simple molecules, e.g. protons, nucleotides or amino acids [35]. ATP was one of these, a key biological molecule [36] inducing changes to cell physiology. A recent comprehensive analysis of sequences available in public databases identified ~180 P2X genes across all eukaryotes [37], including animals, fungi, amoebas, and algae [37]. Although P2X genes found in distant organisms share little sequence similarity with mammalian P2X sequences, they possess conserved structural elements and also have high sensitivity to ATP [38].

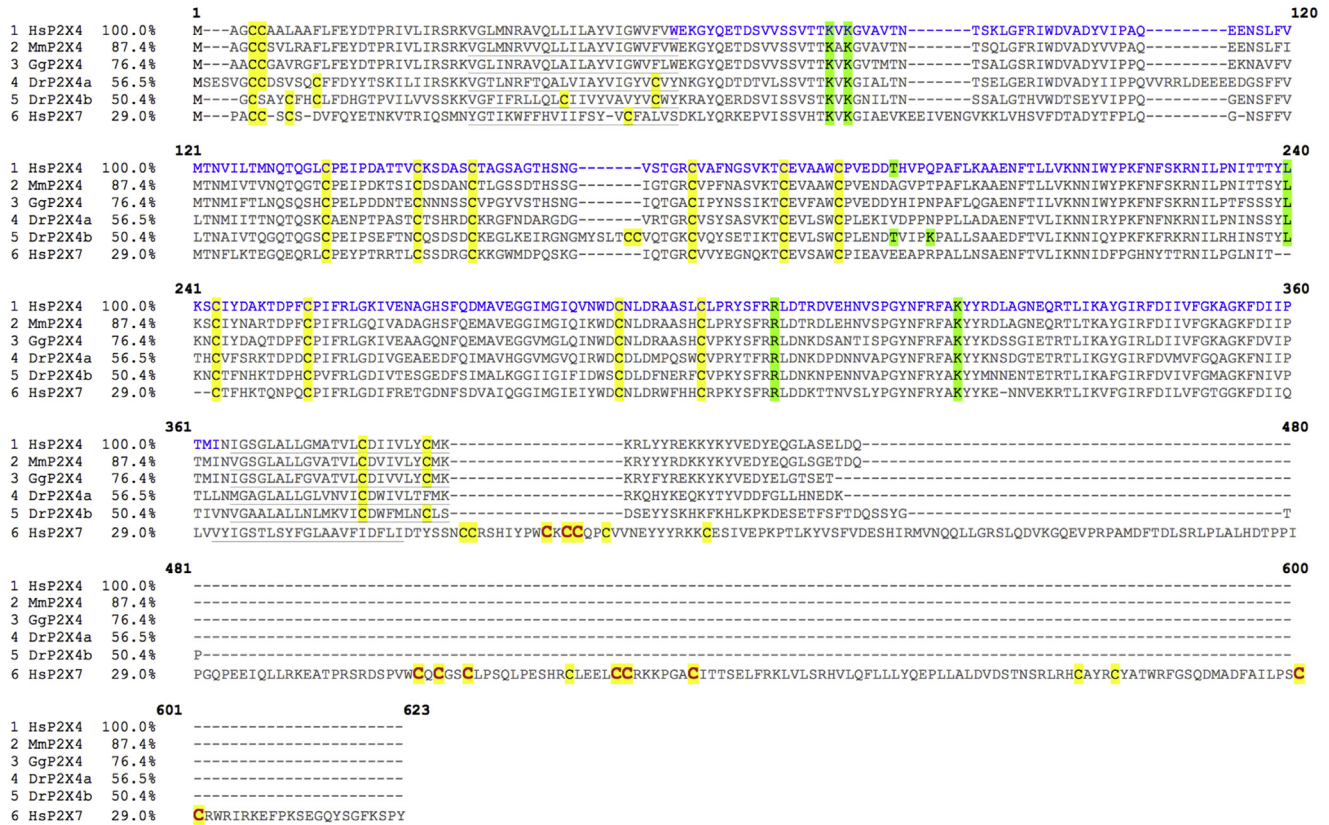
While most groups of animals have 1 or 2 P2X genes, *Drosophila* and *Caenorhabditis elegans*, along with all other known insects and nematodes, lack P2X genes completely [37]. However, other Arthropods such as spiders and crustaceans have a P2X gene, as well as related groups like the enigmatic water bears (Tardigrades) [39]. P2X proteins have also been well characterized in snails and flatworms [40,41], and in amoebas [42]. Lineage-specific expansions and losses have occurred in many groups, most notably in vertebrates, which possess seven distinct classes of P2X.

The exon–intron structure and key motifs of vertebrate P2X genes are well conserved [37]. The majority of the fully sequenced mammalian genomes include representatives of all vertebrate P2X genes, including P2X4, which in human is located on chromosome 12 in close proximity to P2X7. This association is conserved, and location as well as phylogenetic analysis show that P2X4 and P2X7 share the same ancestral gene and were likely produced by local gene duplication

(Fig. 1). Furthermore, the two genes have been suggested to be able to complement each other's function, and P2X4 is up-regulated in P2X7 knockout mice [43]. In contrast, two groups found that P2X4 is down-modulated in liver tissue [44], and in whole kidneys from P2X7 KO mice [45].

Interestingly, P2X4 is one of the best conserved P2X across vertebrates [46]. P2X4 has been mostly associated with the nervous system and also with the immune system outside mammals. For example, P2X4 in the Japanese flounder (a species of teleost fish) is involved in the innate immune

**A.**



**B.**

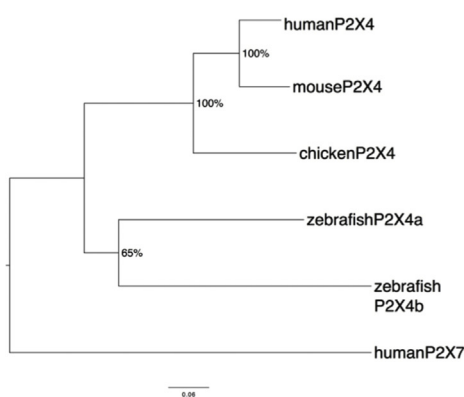


Fig. 1 P2X4 sequences from multiple vertebrates. (A) Multiple alignment of P2X4 from different vertebrate species. Positions of the cysteines are highlighted in yellow, and conserved residues involved in ATP binding are on green background. The two transmembrane regions are underlined, the extracellular (or “out” part of the protein is located between them. Interestingly, the C-terminus region, which in P2X7 contains the palmitoylated cysteines (represented in red on yellow background), is not conserved in P2X4 sequences. (B) NJ distance tree of P2X4 sequences. In zebrafish, P2X4b is located on Chr 8 close to P2X7, while P2X4a is on Chr21; the genomic contexts on these paralogs are different and do not allow to reconstitute a simple duplication history. Accession numbers: Human P2RX4: ENSP00000336607; Mouse P2RX4: ENSMUSP00000031429; Chicken P2RX4: ENSGALP00000006190; Zebrafish P2RX4A: ENSDARP00000108004; Zebrafish P2RX4b: ENSDARP00000089740; Human P2RX7: ENSP00000330696).



response [47], while in chicken P2X4 is involved in the regulation of chondrogenesis [48].

### P2X4 receptor structure

The crystal structure of P2X4 has been resolved in the zebrafish [49,50], and confirmed (at least partially) in rat [51]. Zebrafish P2X4 (zfP2X4) is chalice-shaped and has a trimeric structure; covalent bonds [49] between subunits play an important role in channel gating as well as receptor assembling [49]. The homomeric zfP2X4 has an extracellular domain extending about 70 Å from the membrane and a smaller transmembrane region spreading through the membrane on about 28 Å [49]. The extracellular part of each receptor can be described as large disulfide-rich region [50], packed with protruding N linked glycosylation moieties [49]. The transmembrane region of the receptor complex is formed by six helices, two from each subunit [49]. The helices within a given subunit are antiparallel to one another and are angled 45° from the membrane [49].

For receptor activation, three ATP molecules must bind the extracellular domains, inducing the formation of a non-selective cation channel made of the transmembrane regions [50,52]. In zfP2X4, ATP binding sites are located in deep cavities outside of the trimer [49]. ATP binding is needed for the conformational changes leading to the opening of the channel [5]. Cations entering the cell induce a depolarisation and activate numerous intracellular processes [53,54]. The desensitization of receptor subtypes varies widely; for example homomeric P2X2, P2X4, P2X7 receptors desensitize slowly, while the process is fast for P2X1 and P2X3 [50].

In the absence of extracellular Ca<sup>2+</sup>, a notable increase of the transmembrane pore size can be seen in P2X4 [55]. To clarify the structure of the receptor, as well as conformational changes upon stimulation, atomic force microscopy was used to observe P2X4 behavior at a single channel level. In the presence of Ca<sup>2+</sup>, P2X4 receptor opens just a small cation-permeable channel pore. Alternatively, in the absence of extracellular Ca<sup>2+</sup>, it forms a larger pore that allows larger molecules like propidium iodide, and ethidium bromide to pass [56]. These results demonstrate that extracellular domains of P2X4 are involved in the dynamics of its permeability.

Conformational structure of the P2X4 receptor is critical for its function. For example, Stokes et al showed in 2011 that inheritance of the loss of function mutation “rs 28360472” (Tyr<sub>315</sub> → Cys) in P2X4 is significantly associated with higher pulse pressure [57]. Tyr<sub>315</sub> → Cys mutation introduces an extra cysteine residue in the large ectodomain, which contains 10 highly conserved cysteines, and may induce the formation of an aberrant disulphide bond leading to the disruption in ion channel structure. This loss-of-function mutation impacts large arterial tone because P2X4 activation by ATP leads to NO release by endothelial cells and to arterial smooth muscle relaxation. Hence, Tyr<sub>315</sub> → Cys that disrupts normal P2X4 function, reduces large arterial compliance and accounts for the higher pulse pressure observed in mutant patients [57].

The expression and traffic of the P2X4 receptor have been studied by several groups. Qureshi et al. [58] have shown, in primary cells such as microglia, vascular endothelial cells and macrophages, that P2X4 is preferentially localized in

lysosomes. In addition, they identified motifs in the N- and C-termini which are required to address the P2X4 to lysosomes. Mutations of the following tyrosine-motifs (Y<sub>372</sub> xx V and Y<sub>378</sub> xx GL) in the C-terminus of P2X4 decrease the levels of P2X4 in the lysosomal compartment. In addition, it was established that the Y<sub>378</sub> xx GL motif is required for the endocytosis of P2X4 by a clathrin-dependent pathway because this motif binds the μ2 subunit of the clathrin adaptor complex [59]. The L22 and I23 mutations to alanine, at the N-terminus, increased the amounts of P2X4 at the plasma membrane. When mutations at the N- and C-terminus were combined, a major increase of P2X4 at the plasma membrane was observed [58]. Qureshi et al. [58] also showed that in the acidic and proteolytic lysosomal environment, P2X4 was not cleaved because it is protected from proteolysis by about 6 N-linked complex oligosaccharides. Interestingly, the lysosomal localization of P2X4 coincides with the presence of ATP in lysosomes of astrocytes [60], microglia [61] and hepatocytes [44]. Huang et al. [62] have shown that the highest concentrations of ATP were found in lysosomes and mitochondria. In addition, using whole-lysosome patch clamp, these authors demonstrated that the lysosomal P2X4 receptor is activated by luminal ATP provided that the pH was raised to 7.4 [62]. These results explained that, in physiological conditions, the high levels of ATP and P2X4 receptor in lysosomes do not lead to P2X4 activation because the lysosomal pH is comprised between 3.5 and 5.0. It is well established that P2X4 is activated by ATP in its fully dissociated tetra-anionic form, ATP<sup>4-</sup>. This finding is in agreement with the ATP-P2X4 receptor crystallographic structures showing that the α, β and γ negatively charged phosphate groups interact with highly conserved basic and polar amino-acids of two subunits of P2X4 [50]. The presence of high levels of ATP in lysosomes is due to the vesicular nucleotide transporter (VNUT)/SLC17A9 that is present in lysosomal membrane and transports ATP across it [63]. Silencing of VNUT/SLC17A9 in a mouse cell line induced a dramatic decrease of ATP concentrations in lysosomes, and the accumulation of lipofuscin in it, leading to cell death [63,64]. A synthetic view of P2X4 intracellular distribution is shown in Fig. 2.

An original strategy was recently developed to follow the cellular distribution of the P2X4 receptor among various compartments [25]. Using a construct of P2X4 fused with pH-sensitive fluorescent protein (pHluorin), the authors demonstrated that P2X4 preferentially localizes to acidic cellular compartments [25]. Triggering of P2X4 by intra-lysosomal ATP is pH-dependent [62,63], and the triggered P2X4 is involved in the fusion of late endosomes and lysosomes. More precisely, P2X4-stimulated Ca<sup>2+</sup> release activates calmodulin, which then associates with P2X4 itself and promotes vesicle fusion and vacuolation [65].

## Functional roles of P2X4 in the nervous system

### P2X4 modulates major neurotransmitter systems

Growing evidence suggests a central role for P2X4 in the homeostasis of major neurotransmission pathways. P2X4 knockout mice exhibit deficits in sensorimotor gating, social interactions, and ethanol drinking behavior [34,66], all of

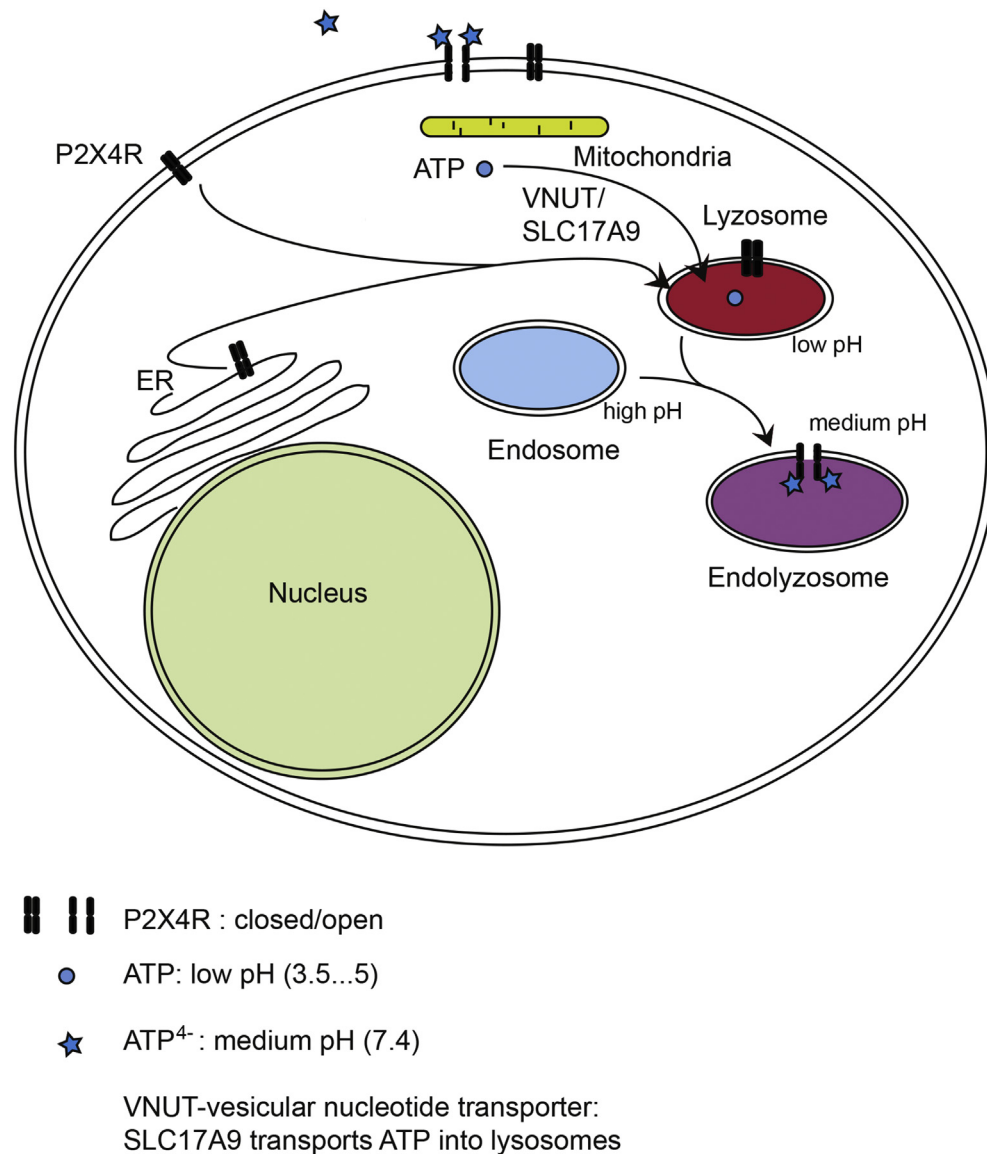


Fig. 2 **P2X4 intracellular distribution.** P2X4 is located mainly in lysosomes and is involved in the fusion of late endosomes with lysosomes as a  $\text{Ca}^{2+}$ -channel. Arrows represent transfer of P2X4 between different compartments. P2X4 is represented in open or closed configuration, depending on the availability of  $\text{ATP}^{4-}$  (represented as a blue star).

which have been previously linked to disruption of glutamatergic and/or GABAergic functions [67–69]. Indeed, knockout mice also exhibit altered subunit expression of multiple glutamatergic and GABA<sub>A</sub> receptors across multiple regions of the brain [34,66]. These mice can be rescued from the sensorimotor gating-perturbed phenotype by treatment with dopamine agonists [70].

#### **P2X4 regulates alcohol-induced responses in microglia**

Alcohol is known to cause structural and functional abnormalities to the brain, including perturbations in microglial cells. More specifically, the processes of phagocytosis, cell proliferation, migration, and expression of brain-derived neurotrophic factor (BDNF) are all altered after exposure of

microglial cells to ethanol [71]. It is also known that in more drastic cases, alcohol can even induce apoptosis in microglia [72]. Many of these effects can be attributed to the alcohol-induced expression of P2X4 in microglial cells, and involve the inhibition of the AKT and ERK cascades [73]. This increase in P2X4 is observed at both mRNA and protein levels [71]. When alcohol is available, P2X4 knockouts drink more than wild type mice, correlating with the reduction of detrimental effects [66].

Other studies have shown that alcohol can also allosterically inhibit the function of already expressed P2X4, likely through interactions with its transmembrane domain [74]. Native P2X4 channels are sensitive to ethanol at intoxicating concentrations [75,76]; a point mutation in the ectodomain-transmembrane interface eliminates the inhibitory effects of

ethanol on P2X4 [77]. More precisely, tryptophan 46 is a critical site for the action of both ethanol and ivermectin (IVM), an antiparasitic drug that potentializes P2X4 (but not the other P2X) [78]. Accordingly, IVM antagonizes ethanol-mediated effects on P2X4 both *in vitro* and *in vivo*, and significantly reduces ethanol intake and preference in murine models of ethanol self-administration [66,74,79]. Thus, tryptophan 46 represents a potential therapeutic target that could be used for medication development against alcohol use disorders.

Taken together, alcohol and P2X4 are in a complex interplay, in which alcohol both increases the expression of P2X4 but inhibits its function once expressed. In any case, it is clear that P2X4 is a key player in alcohol-mediated effects on microglial cells.

### ***P2X4 is one of the key receptors mediating neuropathic pain***

The role of microglia in pain pathophysiology is considered more and more important [80]. P2X4 upregulation in spinal microglia and CCL2-dependent relocalisation from lysosomes to cell surface are critical for pain hypersensitivity (allodynia) after peripheral nerve injury [58,81]. P2X4 knockout mice do not show pain behavior after nerve injury [82]. In wild type mice, blocking P2X4 function by its antagonist NP-1815-PX has been shown to inhibit the chronic pain induced either by mechanical damage to the 4th lumbar spinal nerve or by herpesvirus HSV-1 infection of the dorsal root ganglion neurons [83]. In the herpesvirus model, activation of spinal microglia correlated with an increase in P2X4R mRNA expression, which reached a peak at 7 days post-HSV-1 infection [83].

Brain-derived neurotrophic factor (BDNF) is clearly involved in the P2X4-mediated pain response. During pain response, microglial cells activated by ATP (via P2X4) start secreting BDNF. Interestingly, BDNF signaling is blocked in P2X4 knockout mice [82]. Furthermore, direct injection of BDNF to Sprague–Dawley rats leads to sustained pain hypersensitivity [84], and BDNF pathway inhibitor Trk-Fc (synthetic decoy receptor for BDNF) inhibits allodynia in several chronic pain models, including in the HSV-1-induced allodynia. The spinal dorsal horn neurons were recently identified as being involved in neuropathic pain, via the exocytic ATP release after peripheral nerve injury, a process dependent on the vesicular nucleotide transporter VNUT [85]. Thus, P2X4 is a key factor of a core pathway leading to pain: activation by ATP at the cell surface leads to calcium influx, phosphorylation of p38, then synthesis and release of BDNF causing disinhibition of nociceptive projection neurons [81,86].

As BDNF is also known to regulate synaptic plasticity [84], suggesting that P2X4 might have a role in this phenomenon. To determine whether purines released by microglia are able to modulate synaptic transmission and plasticity, George et al. [87] added lipopolysaccharide (LPS)-stimulated microglia to mouse hippocampal slices. These results show convincingly that ATP released by LPS-triggered microglia and adenosine produced by dephosphorylation of extracellular ATP trigger pre-synaptic P2X4 and Adenosine A1 receptors respectively to modulate synaptic plasticity.

Recent data unveiled a sexual dimorphism of mechanical allodynia, suggesting that distinct cell types mediate pain in

male and female mice. Mechanical allodynia in mice of both sexes was induced using spared nerve injury (SNI), a procedure producing persistent neuropathic pain. While the levels of pain hypersensitivity in both sexes were comparable, P2X4 was not upregulated in the microglia of female mice, and microglial depletion had no apparent effect on the female pain response [88]. Instead, the mechanism appeared to be mediated by lymphocytes (involving, for example, either GABA(A) or P2 receptors other than P2X4). This may be one of the factors contributing to higher numbers of resident T-lymphocytes in the periphery of female mice (about twofold more than in males) [89]. However, in the absence of adaptive immune cells, females still resort to the use of microglia [88]. In any case, injury-induced upregulation of P2X4 expression is gender dependent and plays a key role in pain difference between male and female mice. Such a sexual dimorphism suggests that male mice cannot be used as proxies for females in pain research and distinct strategies targeting neuro-immune signaling might be required for the treatment of chronic pain in men and women [88].

Therefore, P2X4 appears as a potentially important drug target to treat pain states that are resistant to available therapies [90,91]. Morphine treatment is the gold standard for both post-operative and chronic pain. However, it may lead to a pain hypersensitization called morphine-induced hyperalgesia [92], via a P2X4-BDNF signaling pathway between microglia and neurons of the spinal dorsal horn. The expression of P2X4 in microglia and the subsequent release of BDNF are both required for hyperalgesia, which can be reversed by blocking BDNF signaling. Importantly, the increase in P2X4 expression is morphine receptor dependent while BDNF release is not, suggesting P2X4-BDNF pathway as a therapeutic target to prevent hyperalgesia without affecting the morphine analgesia itself [93].

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## **P2X4 and inflammation**

### ***ATP as a pro-inflammatory molecule***

The occurrence of pathological events associated with cellular destruction promotes a massive accumulation of ATP, which serves as a key danger signal, triggering an inflammatory cascade. In chronically inflamed tissues, both extracellular ATP and adenosine may be elevated for extended periods [94], which suggests a key role for purinergic signaling.

### ***P2X4 in brain post-ischemic inflammation***

Post-ischemic inflammation is a good example of such a situation, in which inflammation is correlated with a high ATP release. Whereas energy deficit and glutamate excitotoxicity cause neuronal death immediately after ischemia, activation of brain inflammatory cells causes lingering damage that evolves during the following hours. A number of studies report an increase of P2X4 expression in microglial cells following spinal cord injury, brain ischemia and brain trauma [95]. These cells quickly get activated and acquire motile amoeboid form due to activation of P2X4 by ATP released from cells damaged by ischemia. The peak of P2X4 upregulation

occurs two days later [96], suggesting that leukocyte activation may induce damages long after the ischemia. Indeed, inflamed lesions in the brain can persist for days or even weeks after an ischemic stroke [97].

P2X4 has also been reported to have a role in neuroprotection [98]: when stimulated in the wall of blood vessels by pressure shocks, it promotes the induction of osteopontin, a neuroprotective molecule. Thus, P2X4 is involved in a mechanism whereby vascular endothelial cells induce an ischemic tolerance.

#### **P2X4 in rheumatoid arthritis**

P2X4 promotes joint inflammation in rheumatoid arthritis (RA). In both human patients and in a mouse model of RA, targeting P2X4 by antisense RNA suppressed the production of pro-inflammatory cytokines [99]. Furthermore, P2X4 inhibition negatively regulates NLRP1 inflammasome activation, improving joint inflammation and reducing joint destruction in RA. In fact, P2X4-induced  $\text{Ca}^{2+}$  influx is required for effective production of IL-1 $\beta$  and IL-6, via activation of P2X7 receptor [100]. Thus, P2X4 blockade could be regarded as a potential strategy in the treatment of RA [99].

#### **P2X4 also contributes to microglial cell death in neuroinflammatory and degenerative diseases**

It is probable that the mechanisms that cause P2X4 receptor up-regulation in microglia during neuropathic pain are also engaged in brain disorders that are accompanied by microglia activation and neuroinflammation [101,102]. If so, P2X4 receptors may constitute therapeutic targets for inflammatory and neurodegenerative pathologies (e.g. Parkinson's Disease, Alzheimer's Disease and Multiple Sclerosis) [103]. Neuroinflammation provoked by *in vivo* LPS injection in mice induces a rapid microglial loss in the spinal cord. This loss can be either mitigated or increased by P2X4 receptor blockade or facilitation, respectively [104]. In a mouse model for epilepsy, P2X4 deficiency similarly leads to partial protection from microglia activation and subsequent cell death. While kainate-induced epileptic seizures are not visibly inhibited in P2X4 deficient mice, there is a notable reduction in hippocampal cell death, as well as in the amplitude of outward currents elicited by depolarization [105].

What could be the mechanism underlying these observations? Signaling pathways coupled to P2 receptors in the CNS include the MAPK/ERK pathway, NGF expression, and calcium mobilization [71]. Expression of P2X4 itself is up-regulated in inflammatory foci and in activated microglia in the spinal cord of rats with experimental autoimmune encephalomyelitis (EAE) as well as in the optic nerve of Multiple Sclerosis patients [104]. The expression of P2X4 in microglia is also increased during nerve injury and drives both the release of prostaglandin E2 and stimulation of the neurotrophin BDNF [27,82].

P2X4 thus mediates early microglial cell death during neuroinflammation and provides new avenues to control the fate of activated microglia, and possibly to manipulate microgliosis towards a beneficial phenotype in CNS injury and disease.

## **Other roles of P2X4**

### **P2X4 and secretion in epithelial cells**

One of the main functions of epithelial cells is the polarized transport of ions and fluids. This process is regulated by ATP (and other nucleotides) and involves purinergic P2 receptors of both P2X and P2Y subtypes [106]. The evolutionarily related P2X4 and P2X7 receptors are both mainly associated with secretory epithelia, allowing for direct transmembrane transport of ions such as  $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$  and  $\text{K}^{+}$  [24]. In particular, both of these receptors are well studied in pancreatic and salivary secretory cells – they are ATP-responsive and the increase in intracellular  $\text{Ca}^{2+}$  levels mediated by these receptors is a key part of the mechanism leading to exocytosis [24,106,107].

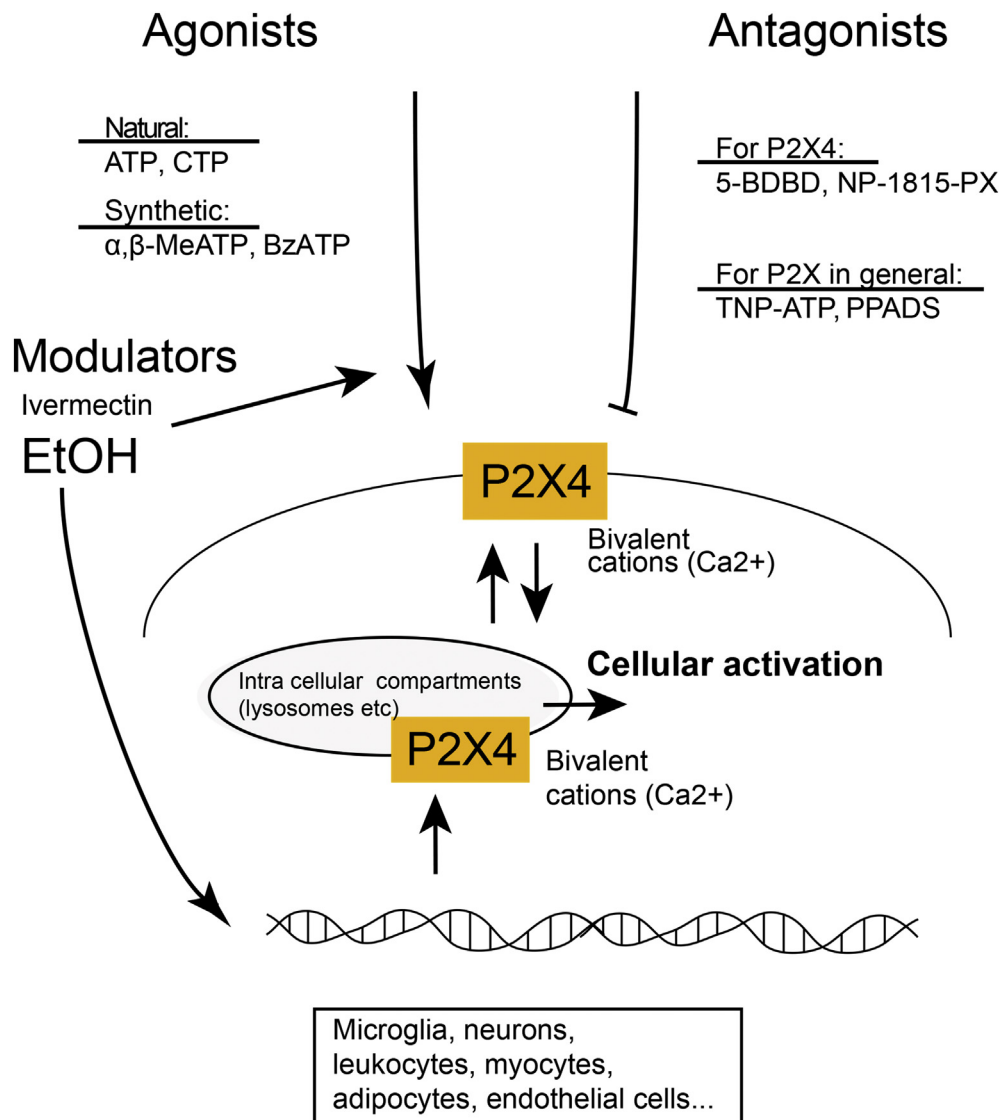
In lung epithelial cells, P2X4 is associated with both exocytosis of lung surfactants and alveolar fluid transport [28], making it a key player in airway homeostasis. It is elevated in asthmatic patients and in a mouse model of asthmatic disease [108]. In the mouse model of asthma, P2X4 antagonists can be used to efficiently alleviate many of the symptoms: eosinophilia, inflammation, mucus production, and cellular infiltration in the airway [108,109]. P2X4-deficient mice are also less prone to Th2 responses, the same can be observed in mice which received an adoptive transfer of P2X4-deficient bone marrow cells [108]. Taken together, these data suggest that P2X4 in lungs may be a novel potential therapeutic target for the treatment of asthma and other airway diseases.

Whether P2X4 and P2X7 directly interact with each other is a controversial topic. Even in the airway epithelia, both seem to be required for carrying out some of the necessary functions – in a study on the effects of industrial chemicals on lungs, the release of asthma-associated enzymes was found to depend on the activity of both P2X4 and P2X7 [110]. It has been suggested that under some conditions, P2X4 and P2X7 might be able to form heteromers, particularly in the mouse salivary glands [111,112]. However, a more recent study of these two receptors in parotid acinar cells demonstrated clearly distinct patterns for the two receptors: P2X7 is mainly localized in the sub-luminal regions; activation of P2X7 leads to a fast luminal-to-basal  $\text{Ca}^{2+}$  spike (~18  $\mu\text{m/s}$ ). In contrast, P2X4 is mostly found in the basal areas; the  $\text{Ca}^{2+}$  spike evoked by selective P2X4 activation is in reverse direction (basal-to-luminal) and several times slower (~4  $\mu\text{m/s}$ ). Regardless of differences on the mechanistic level, activation of either (or both) of these receptors leads to spatially overlapping exocytosis [107].

### **P2X4 in metabolic syndrome**

The metabolic syndrome is typically associated with high levels of blood glucose [113], circulating free fatty acids [114], and with low-grade chronic inflammation. In a context of vascular disease progression, endothelial cell damage and lysis at the site of inflammation increase ATP levels in the extracellular space, which can activate cells via P2X4 and P2X7, leading to an increase in endothelial cell permeability [113]. P2X4 thus mediates at least in part the metabolic stress-induced endothelial dysfunction and could be a therapeutic target.





**Fig. 3 P2X4 regulation pathways.** Binding of ATP activates P2X4R and initiates signalling: agonist binding is closing the cleft in the intersubunit binding site – that is opening the ion channel. The open state of P2x4R is accessible for binding of allosteric modulators for example ivermectin. Note that  $\alpha,\beta$ -methylene ATP ( $\alpha,\beta$ -MeATP) is a partial agonist when compared to ATP because the currents triggered by  $\alpha,\beta$ -MeATP are lower than those elicited by ATP. P2X4 is in an open conformation when bound to ATP in reconstituted high-density lipoproteins as shown in zebrafish [123]. In contrast, when P2X4 was bound to  $\alpha,\beta$ -MeATP, the transmembrane domain and the lower body of P2X4 are in equilibrium between closed and open conformations. The small proportion of P2X4 in the open conformation may thus spark partial activation of the receptors. 5-BDBD: 5-(3-Bromophenyl)-1,3-dihydro-2H-Benzofuro[3,2-e]-1,4-diazepin-2-one; TNP-ATP: 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate – antagonist of P2X1-4R; PPADs: pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid = an antagonist of P2X1-3,5,7R but not P2X4R; NP-1815-PX: (5-[3-(5-thioxo-4H-[1,2,4]oxadiazol-3-yl)phenyl]-1H-naphtho[1,2-b][1,4]diazepine-2,4(3H,5H)-dione); BzATP: 2',3'-O-(4-benzoyl-benzoyl)-ATP.

#### P2X4 in liver regeneration

After partial hepatectomy in the rat, extracellular ATP rises in blood and bile and contributes to liver regeneration [115]. Partial liver surgery showed P2X4, which is expressed in hepatocytes and Kupfer cells, to be a key target for this ATP. Delayed regeneration, hepatocyte necrosis, and cholestasis were observed in P2X4 knockout mice. In these mice, biliary adaptation was impaired with a smaller increase in bile flow and altered biliary composition, with reduced ATP and

lysosomal enzyme release [44]. Thus, during postsurgical liver regeneration, P2X4 contributes to the complex control of biliary homeostasis through mechanisms involving pericanalicular lysosomes.

#### P2X4 and infection

While the role of P2X7 in inhibition of infection by *Mycobacterium tuberculosis* [116,117] *Bacillus Calmette-Guerin* [118] and *Chlamydia trachomatis* [119] is well documented, the potential

role of P2X4 on infected host cells has been much less studied. HeLa cells infected by the intra-cellular bacteria *C. trachomatis* release ATP, which inhibits bacterial growth through the stimulation of P2X4 [120]. In gingival epithelial cells, P2X4 potentiates ROS production and NLRP3 inflammasome activation that is otherwise mediated by P2X7 triggering [121].

## Conclusions

P2X4 is one of the most sensitive and widely expressed purinergic receptors. It appears to be preferentially located in lysosomes, where it is inactive at low pH. It gets activated by high ATP concentration in this compartment but only when pH increases to 7.4, converting ATP into ATP<sup>4-</sup>, for example after fusion with endosomes. Although a few agonists (in addition to ATP) and antagonists (5-BDBD, NP-1815-PX) are known (Fig. 3), P2X4 mechanisms of activation, intracellular transport, and regulation remain surprisingly elusive.

P2X4 is expressed by multiple cell types, which explains its involvement in many functions and pathologies. The production of conditional P2X4 KO mice targeting well-defined cell types will certainly be of major importance to characterize P2X4 functions, in physiological or pathological conditions. This strategy is now feasible because several P2X4 KO mice or P2X4-ES cells with conditional potential are available (cf. [http://www.mousephenotype.org/data/alleles/MGI:1338859/tm1a\(EUCOMM\)Wtsi](http://www.mousephenotype.org/data/alleles/MGI:1338859/tm1a(EUCOMM)Wtsi)). In the P2X4 KO mouse currently available [122], the absence of P2X4 in all cells that naturally express it precludes a comprehensive analysis of its function in well defined sub-populations of cells. Furthermore, the neomycin cassette inserted in the P2X4 locus may down-modulate the expression of closely linked genes such as the P2X7R gene, and lead to misleading conclusions.

P2X4 is a mediator of pain, inflammation, a modulator of lung and cardiac functions; it has been involved in neurodevelopmental and metabolic disorders, in the inflammatory components of auto-immune diseases, in neuroinflammation and several other pathologies. Importantly, it plays a key role in pain and also in response to alcohol, which underscores its potential importance as a therapeutic target. Considering its multiple functions, it is striking that P2X4 remains overall poorly characterized. In comparison, mechanisms by which P2X7 modulate immunity have been dissected in detail. Extensive studies will be necessary to evaluate the potential of P2X4 as a therapeutic target in the multiple pathologies in which it is implicated.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgements

This work was financially supported by institutional grants from Tallinn University of Technology and Estonian Research

Council grant PUT685 and by Campus France travel grant No 885510B (French embassy support for scientific exchange) (SRB). JS is a fellow of the German Research Foundation grant DFG-SPP1819. JK was supported by grants from Institut national du cancer (INCa) project P2X7R 2015-137 and Agence Nationale de la Recherche (P2SX7RFAS (ANR-13-ISV6-0003)). PB was supported by institutional grants from INRA. We warmly thank Drs David Ojcius and Colette Kanellopoulos-Langevin for their critical review of our manuscript.

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