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P2X7 receptor: an emerging target in CNS diseases

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Abstract

The ATP-sensitive homomeric P2X7 receptor (P2X7R) has received particular attention as a potential drug target because of its widespread involvement in inflammatory diseases as a key regulatory element of the inflammasome complex. However, it has only recently become evident that P2X7Rs also play a pivotal role in central nervous system (CNS) pathology. There is an explosion of data indicating that genetic deletion and pharmacological blockade of P2X7Rs alter responsiveness in animal models of neurological disorders, such as stroke, neurotrauma, epilepsy, neuropathic pain, multiple sclerosis, amyotrophic lateralsclerosis, Alzheimer's disease, Parkinson's disease, and Huntington's disease. Moreover, recent studies suggest that P2X7Rs regulate the pathophysiology of psychiatric disorders, including mood disorders, implicating P2X7Rs as drug targets in a variety of CNS pathology.

It has been known for almost a decade that P2X7Rs convey important physiopathological functions in the CNS [1]. The aim and scope of the present review are to summarize the latest developments in the description of these functions, to redirect interest to those fields, where there are still significant gaps in our present understanding and to promote further development of those therapeutic areas, in which P2X7R is the most promising as a potential drug target.

The structure and molecular physiology of P2X7Rs

P2X7Rs are ATP-gated, non-selective cation channels belonging to the family of ionotropic P2X receptors. P2X7Rs function in homo-trimeric form and most mammalian P2X7R subunits comprise 595 amino acids [2]. The common structural motifs of P2X7Rs are the two transmembrane domains (TM1, TM2), a large, glycosylated, cysteine-rich extracellular loop, a short intracellular N-terminal domain, and an intracellular C-terminal domain, which is longer than that of other P2X receptor subunits. Within the family of P2X receptors, so far only the crystal structure of zebrafish (zf)P2X4.1R has been solved in the closed [3] and ATP-binding, open state [4]; nevertheless, its considerable homology with mammalian P2X7Rs allowed for the structural modelling of the latter [2]. The molecular architecture of an individual P2X7R subunit is akin to a leaping dolphin, with the extracellular loop forming the body, and the TM domains forming the tail. When co-assembled as a trimeric unit, P2X7R has a chalice-like

structure, overarching the channel pore (Figure 1A). There are three ATP binding sites localized at the interface of the three subunits; occupancy of at least two of the three sites is necessary for the activation of the receptors [5]. The adenine base and the β- and γ-phosphate groups of ATP form hydrogen bonds with the respective amino acid residues of the ATP binding pocket, as suggested for the zfP2X4.1R. However, because a residue corresponding to Leu217, which interacts with the ribose moiety, is missing in the mammalian P2X7R, the affinity of ATP to P2X7Rs is more than a hundredfold lower than to other P2XR-subtypes [2]. On the other hand, non-conserved residues surrounding the ATP binding site might confer differences in agonist sensitivity between mammalian P2XR species, (i.e. rat P2X7Rs display substantially higher sensitivity to ATP and BzATP than their human and mouse counterparts [6]). A distinctive feature of the mouse P2X7R is that it can be activated by extracellular nicotinamide adenine dinucleotide (NAD^+) by ADP-ribosylation with the ADP-ribosyltransferase 2 ectoenzyme [7]. In contrast, less is known about the binding site of antagonists, although potent and selective antagonists of P2X7Rs are now widely available. Earlier data indicated that P2X7R subunits are able to form heterotrimers with P2X4Rs [8], but more recent studies did not confirm this (e.g. [9]).

There are several splice variants of mammalian P2X7Rs, all of which are widely expressed in the nervous system. Hence, a naturally occurring truncated isoform of the human P2X7R (P2X7B) has been found in the CNS [10]; a C-terminally truncated variant of mouse P2X7R has also been identified, which partly retains its functionality, when expressed in tissues of the *P2rx7* gene

89 deficient mice [11]. Another mouse isoform is the P2X7(k) variant, which in
90 contrast to P2X7(a), is sensitive to ADP-ribosylation [12, 13].
91 The gene encoding the human P2X7R (*P2RX7*) is also well known to exhibit a
92 number of non-synonymous single nucleotide polymorphisms (NS-SNPs), which
93 results in a change in amino acid sequence and the expression of different
94 human P2X7 variants, further increasing the structural diversity of P2X7Rs. The
95 functional consequence of several individual NS-SNPs has been determined in
96 native and recombinant systems and their association with various human CNS
97 disease states has been extensively investigated in genetic linkage studies [14].
98 The activation of P2X7Rs results in the opening of the channel pore, allowing
99 the passage of small cations (Na^+, Ca^{2+}, and K^+). In addition, a hallmark feature
100 of the P2X7R is the opening of a non-selective pore in response to repeated or
101 prolonged activation, allowing the permeation of large molecular weight organic
102 cations up to 600-800 Da. The pore forming property of P2X7Rs can be studied
103 by the uptake of high molecular weight cations, such as $NMDG^+$, or dyes, such
104 as Yo-Pro-1 or ethidium bromide; nevertheless, its molecular mechanism has
105 remained a highly debated issue, with two alternative, but non excluding
106 possibilities, both having substantial experimental support (Figure 1 B, C). The
107 first potential mechanism is the progressive dilation of the P2X7R-gated channel
108 itself. A conformational change of the receptor-protein could be the structural
109 basis for channel dilation, as previously confirmed for other P2XRs (P2X2, P2X4)
110 by electrophysiological methods [15]. In agreement with the pore dilation theory,
111 the carboxyl terminal domain [16] and the TM2 region of the P2X7R protein are

essential for pore formation [17]. Moreover, recent studies revealed that the open channel conformation of the P2X7R can allow the passage of negatively charged fluorescent dyes with molecular diameters of up to 1.4 nm [18], and occupation of one or two agonist binding sites favors transition to the desensitized state, whereas occupation of the third binding site favors the transition to the sensitized/dilated state [19].

The alternative mechanism involves the recruitment of an additional pore-forming protein, most likely the pannexin-1 hemichannel (Panx1). Evidence derived from studies using genetic knockdown of Panx1 indicate that this protein is indispensable for the pore formation (e.g. [20]) and can be selectively affected pharmacologically by colhicine [21]. However, other data conflict with the involvement of Panx1 in the formation of the membrane pore (e.g. [22]). Therefore, it appears that although recruitment of pannexin hemichannels is a downstream signaling event closely linked to P2X7R activation, it is not an absolute requirement [23]. A potential dissolution of conflicting results is that different P2X7R splice variants display distinct pore forming properties [12, 23].

The opening of the large pore might eventually result in membrane blebbing and cell death; however, this is not an obligatory consequence of P2X7R activation. Pore formation might gain significance in the pathological sensitization underlying chronic pain as highlighted by a recent study [24]. This paper reported that mutations of the gene encoding the P2X7R, which result in hypofunctional pore formation, affect chronic pain sensitivity in both mice and humans. Moreover treatment with a peptide corresponding to the P2X7R C-terminal domain, which

blocks pore formation, but not cation channel activity, selectively reduced allodynia only in mice with the pore-forming P2rx7 allele. These findings illustrate that the pore formation associated with P2X7R, by itself could be a potential target of personalized therapy to combat chronic pain disorders.

Tissue and cell type specific distribution of P2X7Rs

P2X7Rs are expressed by many cell types, including cells of hematopoietic origin (lymphocytes, monocyte-macrophages, microglia) and intrinsic cells of the nervous system (neurons, astrocytes, oligodendrocytes, Schwann cells). P2X7R binding sites have been explored in autoradiographic studies using the radioligand [^{3}H]-A-804598, and a dense P2X7R binding was found throughout the brain and spinal cord [25], including hypothalamic nuclei, thalamic nuclei, hippocampus, spinal trigeminal nucleus and tract, cortical regions, cerebellum and caudate putamen [25]. Nevertheless, the cell-type specific localization of the P2X7Rs in the CNS has been the subject of a long-standing debate, which has not reached general consensus even after a decade: immunohistochemical findings are inhomogeneous and contradict findings obtained by physiological and neurochemical methods. Whereas early studies found a prominent expression of P2X7R immunoreactivity (IR) on excitatory nerve terminals [26], and later studies confirmed these findings throughout the CNS [27, 28]; other groups questioned these findings, revealing P2X7R-immunoreactivity in brain sections obtained from P2X7R deficient animals [29]. Subsequently however,

functional splice variants of rodent P2X7R [11, 12] were identified which are likely to be responsible for P2X7-pseudo-immunoreactivities, found in the brain of $P2X7R^{-/-}$ mice. These variants represent either gain- or loss-of function P2X7Rs, and may explain the high variability of responses induced by P2X7R stimulation. Other studies reported an activity-dependent expression pattern of P2X7Rs, induced or upregulated following an insult such as a seizure [30], ischemia [31], sleep deprivation [32], undernourishment [33], or morphine tolerance [34]. A recent study utilizing single particle tracking photoactivated localization microscopy (sptPALM) revealed that Dendra2 tagged P2X7Rs transfected to hippocampal neurons formed two dynamic populations within the extrasynaptic membrane of proximal dendrites: one was composed of rapidly diffusing receptors and another stabilized within nanoclusters, both being rarely appositioned to synaptic sites [35].

In contrast to immunohistochemistry, the available evidence on functional P2X7Rs on different cell types of the CNS is convincing. Functional studies, verifying P2X7Rs on neurons, astrocytes and microglia are presented in Table 1. The most parsimonious explanation for the contradictory findings is that the expression of P2X7Rs dynamically changes in response to experimental variables such as age or different levels of stressful stimuli prior to sample collection (freshly prepared *vs.* fixed sections). Moreover, under *in vivo* conditions even mild stimuli, such as saline injection, may cause a dramatic change in the expression level of P2X7Rs.

Physiopathology of P2X7 receptors

P2X7R function can be studied with a selection of pharmacological and genetic tools (Box 1). The activation of P2X7Rs is followed by Ca^{2+} influx and a variety of cellular responses depending on the cell type investigated (Figure 2). Outside the nervous system, the most prominent role of P2X7R is in the regulation of cytokine response to inflammatory challenge. In fact, P2X7R is a key regulatory element of the inflammasome molecular complex, providing the external stimulus necessary for the posttranslational modification and subsequent release of the pro-inflammatory cytokine IL-1β. The role of P2X7Rs has been confirmed in the regulation of central cytokine response after LPS priming [36]. This effect could be involved in physiological and pathological actions controlled by P2X7Rs, such as memory formation [37]; sleep [32], fever [38], hyperalgesia [39] and depression [40, 41].

However, a major caveat in our understanding of the physiopathology of P2X7R function is how the endogenous activation of P2X7Rs is achieved, given the low affinity of the endogenous agonist ATP. ATP is present in the synaptic vesicles and is co-released as a co-transmitter with various other transmitters in the autonomic nervous system under physiological conditions [42]. This holds also true to a certain extent for central synapses and the increase in extracellular ATP in response to normal neuronal activity might transiently reach the high micromolar concentration required for the activation of P2X7R, at least in the synaptic cleft. However, a more widespread activation of P2X7Rs is expected

under pathological conditions, when tissue damage, trauma or other pathological signals provide an ATP-rich extracellular milieu, which might lead to the activation of extrasynaptic and extraneuronal P2X7Rs. In addition, the possibility of constitutive activity without the presence of the endogenous agonist cannot be excluded either and should be further investigated. In the CNS, the best characterized consequence of P2X7R activation is the release of neurotransmitters, in particular of glutamate to the extracellular space [43]. This effect could be evoked both from synaptosomes [44] and from astrocytes [45]. In nerve terminals and cell lines expressing recombinant P2X7Rs, the P2X7R mediated glutamate release appears to be both exocytotic and non-exocytotic, [46, 47]. P2X7R mediated excitatory amino acid efflux can be detected in acutely prepared brain slices by neurochemical (e.g. [48, 49]) and electrophysiological techniques [50]. In rat hippocampal (hilar neurons; [51] CA1 neurons [52]), and midbrain slices (locus coeruleus; [50]), stimulation of P2X7Rs by BzATP elicited an increase of the frequency but not amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) and miniature (m)EPSCs. Occasionally [49, 50] the P2X7R-mediated glutamate release was sensitive to blockade by fluorocitric acid, a glia-selective metabolic poison, and to antagonists of glutamate receptors. These findings imply that glutamate release induced by P2X7R stimulation from neurons could also be indirect, mediated by glutamate release from astrocytes, acting subsequently on glutamatergic nerve terminals.

To add further complexity to neuron-glia and glia-neuron P2X7R signaling, P2X7R stimulation elicits or reinforces the release of ATP, thereby providing an

auto-stimulatory loop. This effect was observed in retinal ganglion cells [53] hippocampal brain slices [49] and cultured spinal cord astrocytes [54]. The mechanism of P2X7R-driven ATP release could be exocytotic, as observed by total internal reflection microscopy in neuroblastoma cells [55], whereas in other studies it appears to involve connexin and/or pannexin hemichannels [49, 54].

A further interesting function of P2X7Rs is to regulate differentiation and cell-fate during development. P2X7Rs are expressed by both embryonic [56] and adult neural progenitor cells (NPCs) in the subventricular zone of the lateral ventricle [57]. Whereas stimulation of P2X7Rs induces neuronal differentiation in embryonic NPCs [56], other studies indicated that P2X7Rs stimulate gliogenesis [58]. In contrast, the activation of P2X7Rs on adult, cultured NPCs decrease cell proliferation and induce necrotic/apoptotic cell death [57].

Of note, a very recent study showed that P2X7Rs regulate ion channel density and protein composition/function of the axon initial segment, a key structural element of neuronal excitability and in consequence action potential initiation in cultured hippocampal neurons and brain slices [59].

It has been known for a long time that P2X7R activation might lead to cell death through pore formation as it has been described for peripheral immune cells. However, a more recently emerging view is that P2X7Rs also convey trophic function against cell-death promoting physiological or pathological stimuli: for example the microglial “suicide” P2X7R promotes cell cycle progression and proliferation [60, 61], and this receptor might act as a scavenger for the removal of apoptotic cells in the absence of its ATP ligand [62, 63].

P2X7R as a potential target in neurological diseases

ATP is released in large quantities following any kind of cell injury, and the ensuing stimulation of the low affinity P2X7R results in necrosis/apoptosis or proliferation as the two opposing end-points of neuroinflammation. P2X7R antagonists are potential therapeutics of traumatic brain injury, stroke, epilepsy, neuropathic pain, and neurodegenerative illnesses, because in these cases secondary cell damaging conditions accompany the primary pathological condition.

Middle cerebral artery occlusion, the most widely used animal model of cerebral ischemia, results in cell death in the core of the affected neuronal tissue, while around it, in the so called penumbra, the cellular damage is reversible. Both infarct size and neurological deficits were reduced by P2X7R antagonists [64, 65]. In combination with the sequential up-regulation of P2X7R-IR in microglia and then in astrocytes and neurons, this receptor-type was considered to be a primary target of the considerable amounts of ATP released. Similar results were reported for subarachnoid hemorrhage [66], traumatic brain [67, 68] or spinal cord injury [69] and ischemic retina degeneration [70]. However, a later study failed to reconfirm the protective action of P2X7R in spinal cord injury [71]. Reperfusion after transient global cerebral ischemia exacerbates the consequences of oxygen/glucose deprivation (OGD) due to microglial and astroglial activation [72]. The ensuing neuroinflammatory reaction is also

alleviated by P2X7R antagonists [73, 74]. BBG partially reversed the OGD-induced anoxic depolarization and cell damage in cultured oligodendrocyte cells [75]. Accordingly, left common carotid artery occlusion decreased P2X7R-immunoreactivity at oligodendrocyte precursor cells in cerebral cortex, subcortical white matter and hippocampus [76].

Status epilepticus (SE)-like seizures, modelled in rodents by pilocarpine or kainate, up-regulate P2X7R-immunoreactivity in microglial cells [77] astrocytes and neurons [78]; quantification by western-blotting confirmed these results [79, 80]. Utilizing the intra-amygdala application of kainate as an epileptic stimulus [79, 80], it was shown that (1) Bz-ATP facilitated and prolonged the EEG activity caused by seizures, and (2) P2X7R antagonists had a neuroprotective effect after epilepsy due to suppression of IL-β production and microglial response. More recent findings suggest that the effect of P2X7Rs during SE depends on the nature of the chemical stimulus used. A-438079 decreased pilocarpin-induced seizure susceptibility in mice by interrupting a direct facilitatory interaction between P2X7- and muscarinic receptors [81] or blockade of the release of the protective TNF-α [82]. P2X7R activation also influenced leukocyte infiltration [83] and reactive astrogliosis following SE [84].

The involvement of P2X7Rs in different models of inflammatory and neuropathic pain and the potential therapeutic effect of P2X7R antagonists are well documented [85]. Down regulation of P2X7Rs with siRNA or BBG prevented the induction of spinal long-term potentiation *in vitro* and at the same time alleviated mechanical allodynia in naive rats *in vivo* [39]. Central sensitization of

296 nociceptive neurons could be produced by intrathecal superfusion of Bz-ATP and
297 was depressed by P2X7R antagonists [86]. Additional studies extended these
298 findings to mechanisms participating in the development of neuropathic or
299 orofacial pain [87-89], bone cancer pain [90] and migraine [91]. Recent studies
300 highlighted the association between human P2X7R variants with chronic pain
301 sensitivity [24].

302 Multiple sclerosis (MS) is a chronic degenerative disease of the CNS that is
303 characterized by focal lesions with inflammation, infiltration of immune cells,
304 demyelination, oligodendroglial death and axonal damage [92]. A putative
305 association of the *P2X7R* gene with this illness was indicated by the most
306 frequent expression of the gain-of-function T allele of rs17525809 polymorphism
307 of the receptor, which yields an Ala-76 to Val change in its extracellular domain
308 [93]. The overexpression of P2X7Rs was detected in experimental autoimmune
309 encephalomyelitis (EAE), an animal model of SM [94], whereas the amelioration
310 of EAE was found in P2X7R deficient animals [95, 96], but see [97]. Further,
311 pannexin-1 knockout mice with restricted ability to mediate pore development/dye
312 uptake after P2X7R stimulation, also displayed a delayed onset of clinical signs
313 of EAE and decreased mortality when compared with wild-type mice [98].

314 Amyotrophic lateral sclerosis (ALS) is characterized by the progressive
315 degeneration of motor neurons in the spinal cord, brainstem and motor cortex,
316 leading to respiratory failure and death of the affected patients within a few years
317 of diagnosis [99]. Microglia and astrocytes are major contributors to motor neuron
318 dysfunction in ALS through the maintenance of a chronic inflammatory response.

Transgenic mice expressing a mutant protein Cu^{+}/Zn^{+} superoxide dismutase SOD1-G93A, which directly enhances the activity of the main reactive oxygen species producing enzyme in microglia (NADPH oxidase 2; NOX2) is used widely as a model of ALS [100]. P2X7R activation by BzATP induced the death of motor neurons in mixed astrocytic/neuronal cultures prepared from wild-type mice [101]. Further, apyrase, an enzyme degrading ATP or BzATP, decreased neuronal death observed in cultures prepared from SOD-G93A spinal cord. Bz-ATP also increased the activity of NOX2, leading to motor neuron damage, an effect which did not occur in primary microglia cultures of SOD-G93A mice lacking P2X7Rs [102].

A neuropathological hallmark of Alzheimer's disease (AD) is the appearance of plaques consisting of extracellular β-amyloid peptide (Aβ) surrounded by reactive microglial cells [103]. Aβ triggered increases in intracellular Ca^{2+}, ATP release, IL-1β secretion and plasma membrane permeabilization in microglia from wild-type but not $P2X7R^{-/-}$ mice [104]. These findings and the neuroprotective effects of BBG against intrahippocampally injected Aβ suggest that Aβ activates a purinergic autocrine/paracrine stimulatory loop of which the P2X7R is an obligatory component. In fact, *in vivo* inhibition of the P2X7R in mice transgenic for mutant human APP indicated a significant decrease of the number of hippocampal amyloid plaques [105].

Parkinson's disease (PD) is a motor disease affecting the striatal dopaminergic system, by damaging dopaminergic neurons in the substantia nigra. In the disease model induced by unilateral intrastriatal injection of 6-

hydroxydopamine, BBG and A-438079 prevented the ensuing synaptotoxicity, gliosis and neurotoxicity [106]. In another study, A-438079 prevented the depletion of striatal dopamine stores by 6-hydroxydopamine treatment, but this was not associated with a reduction of dopaminergic cell loss [107]. Similarly, the effects of P2X7R antagonists appeared to depend on the neurotoxin used, because in MPTP- or rotenone-induced Parkinson models, the genetic deletion of the P2X7R did not increase survival rates of mice compared to wild-type counterparts [108].

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a triplet repeat expansion coding for a polyglutamine sequence in the N-terminal region of the huntingtin protein. A higher P2X7R level was documented by western-blot analysis in the striatum of transgenic mice models of this disease [109]. In addition, P2X7R antagonists prevented neuronal apoptosis and attenuated body weight loss and motor-coordination deficits.

P2X7R as a potential target in psychiatric disorders

Mood disorders arise from complex interactions between genetic, developmental and environmental factors [110, 111]. Linkage studies suggested that variations of the chromosome 12q24.31 containing candidate genes for P2X7R, P2X4R and calmodulin-dependent protein kinase b (CaMKKb) may be associated with major depressive, bipolar and anxiety disorders. It has repeatedly been reported that the NS-SNP rs2230912 coding for the P2X7R-Glu460Arg is associated with

major depressive disorder [112, 113]. Further, relevant SNP mutations identified by linkage studies were introduced into the human recombinant P2X7R and were expressed in human embryonic kidney cells [114]. The measurement of their functional properties by the patch-clamp technique indicated that some of them, including Glu460Arg, exhibited a strong impairment of the current response to ATP, while other mutants demonstrated significant increases in sensitivity. In contrast, other studies failed to confirm the allelic or genotypic association of rs2230912 or other SNPs of P2X7R with mood disorders [115, 116]. The reasons for this discrepancy are presently unknown. Eventually, variations in the *P2X7R* gene were described to be associated with cognitive manic symptoms in bipolar disorders [117], but not in schizophrenia [118].

Production of TNF-α and IL-6 is initiated by the activation of Toll-like receptors (TLRs) by e.g. bacterial lipopolysaccharide. The formation of IL-1β also requires TLR4 induction of gene transcription but requires an additional step, the processing of pro-IL-1β to the mature form of IL-1β, which is then released via NLRP3 referred to as the “inflammasome” [110, 119]. P2X7Rs are indispensable activators of NLRP3. Inflammatory cytokines have been suggested to play key roles in the development of depressive behavior. Their levels are elevated in depressed patients [110, 120] and rodents exposed to stressful stimuli [111]. These cytokines are potent activators of the hypothalamic-pituitary-adrenal axis through which the secretion of hypothalamic corticotropin releasing hormone (CRH), pituitary adrenocorticotropic hormone (ACTH) and corticosterone are stimulated. In this respect it is interesting to note that P2X7R stimulation also

directly leads to increased ACTH secretion from the terminals of hypothalamic magnocellular neurons [121].

The interrelationship between inflammatory cytokines, P2X7Rs and mood related behavior has been intensively studied in animal models. The genetic deletion of P2X7Rs resulted in antidepressive-like behavior in the forced swim and tail suspension tests and alleviated amphetamine induced hyperactivity [40, 41]. Although P2X7Rs are present at peripheral/central immunocytes, glial cells and neurons, it was shown that macrophages and microglia are not responsible for the detected changes in mood measured by tail suspension test and amphetamine-induced hyperlocomotion in $P2X7R^{-/-}$ mice [41]. On a larger scale, several potential mechanisms were identified for the antidepressant phenotype of $P2X7R^{-/-}$ mice, such as the absence of P2X7R-mediated glutamate release, elevated basal brain-derived neurotrophic factor (BDNF) production, enhanced neurogenesis and increased serotonin bioavailability in the hippocampus [48]. It has also been observed that P2X7Rs are downregulated in the hippocampus in response to chronic stress [122] and $P2X7R^{-/-}$ mice exhibited impaired adaptive coping responses to repeated stress [123], which enlighten the potential role of P2X7Rs as a protective adaptive mechanism in the process leading to mood disorders.

The above data illustrate that P2X7R seems to be activated in a number of different pathological conditions raising the possibility that the receptor is one common avenue of cellular stress signaling pathways (Figure 2). However, one should keep in mind that the pathophysiology of CNS diseases is very complex

involving a multiplicity of mediators and signaling pathways and the P2X7R is only one among the multiple signaling pathways activated. Moreover, the significance of this avenue is probably not uniform in all CNS pathologies and could be more prominent in certain disease conditions (e.g. chronic pain, status epilepticus) than in other ones (e.g. Parkinson's disease), depending on the expression of P2X7Rs in the brain area afflicted. Finally, important physiological functions mediated by P2X7Rs should not be neglected. For instance, taking into account that the purportedly necrotic/apoptotic P2X7Rs also convey trophic and adaptive changes, their role might vary or even reverse during the course of the same disease, because neuroinflammation, regulated by P2X7Rs has also a double-faced role. In fact, inflammation initially is a protective reaction and becomes detrimental only, when it progresses to an excessive or chronic phase. These aspects serve as explanations to conflicting results with P2X7R inhibition on the disease outcome (e.g. [95-97]) and should also be addressed when P2X7R is considered as a potential human drug-target.

Current development of P2X7R ligands

Although end-products of the pioneering developments of P2X7R antagonists, such as CE-224,535 [124] and AZD 9056 [125] have not proved efficacious in Phase II trials in rheumatoid arthritis patients, clinical studies revealed an acceptable safety and tolerability profile of such antagonists as a whole [124-

126], opening up the possibility of developing P2X7R-targeting compounds in new areas, such as CNS disorders.

In recent years, a number of different classes of small molecular weight, drug-like P2X7R ligands have been developed (Table 2), and P2X7Rs have been qualified as the most “druggable” target within the P2X receptor family [85, 127]. More recently, the development of centrally penetrating potent P2X7R antagonists has also been reported (Table 2). In addition, systematic search through compound libraries resulted in the further discovery of novel P2X7R antagonists and allosteric modulators utilizable either for basic research or drug development. Analyses of natural compounds have also resulted in several valuable P2X7R ligands (Table 2).

Concluding remarks

In conclusion, P2X7R mediated pathways appears to be a common avenue of many CNS disorders of different aetiology and P2X7R antagonists are potential drugs to treat them. Their immense advantage may lie in the absence or low density of P2X7Rs in healthy tissue and therefore in the limited systemic side effects of these compounds. However, major caveats in our understanding of the physiopathological functions of central P2X7Rs should be further elucidated (Box 2). Though the majority of known antagonists fail to pass the blood-brain barrier, BBG and some new and high affinity P2X7R antagonists readily enter the CNS [128]. Further, recently identified negative allosteric modulators of P2X7Rs (e.g.

457 certain phenothiazine-type antipsychotic drugs), already registered for human
458 use [129], may become important therapeutic tools.
459 The future development of new P2X7R antagonists has to take into
460 consideration that P2X7R isoforms may exhibit large variability between different
461 species in their agonist/antagonist sensitivities. Therefore, the classic search for
462 new pharmacologically active compounds based on the use of laboratory
463 animals, may lead to spurious negative or positive results. A further complicating
464 factor is the presence of numerous splice variants and SNPs widely distributed in
465 the animal and human organism; their sensitivities to pharmacological blockade
466 is often different from that of the wild-type receptor. Hence, the development of
467 new and therapeutically valuable P2X7R antagonists is a tedious task but the
468 reward may be enormous.
469

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Boxes

Box 1. Tools to study P2X7 receptors

The continuously evolving interest in this receptor resulted in the generation of various tools to study its function. P2X7Rs could be identified based on the following distinctive pharmacological features:

- The affinity of the endogenous agonist ATP is low, in the high micromolar-millimolar range.
- BzATP is a more potent agonist than ATP itself. It has been frequently used mistakenly as a selective agonist of P2X7R. This is, however, not valid, because BzATP also binds to other P2X receptors with high affinity.
- The effect of ATP and BzATP are potentiated by a low Ca^{2+}/no Mg^{2+}-containing external medium.
- There are several potent antagonists available, such as A-438079, A-740003, the negative allosteric modulator AZ-10606120 and Brilliant blue G (BBG); among them BBG is selective in concentrations below 1 μM. This antagonist is also a useful tool in *in vivo* experiments. The penetration of BBG through the blood-brain barrier has already been determined and using doses not higher than 50 mg/kg, the resultant brain concentration remains below 1 μM [105]. It should be noted, however, that many P2X7R antagonists, including BBG also inhibit Panx1 channels. Therefore, BBG alone is inadequate to prove the involvement

of P2X7Rs [130]. In this respect, a valuable compound could be Brilliant blue FCF, which inhibits Panx1, but not P2X7R [131].

- Novel radioligands, i.e. [^{3}H]A-804598 are also available to characterize the affinity of newly developed compounds to rodent P2X7Rs [25].

In addition to pharmacological approaches,

- genetic knock-down by siRNA has been increasingly used to silence P2X7Rs in the past years in both *in vitro* and *in vivo* studies (e.g. [34, 39]).
- Mouse lines, genetically deficient in P2X7Rs, initially generated by the companies Glaxo (LacZ gene and neomycin cassette insertion into exon 1; [132]) and Pfizer (Neo insertion in exon 13, close to the carboxyl terminal; [133]), have also been widely used. However, none of these mouse lines could be regarded as fully deficient in P2X7Rs, as individual splice variants evaded inactivation [11, 12].
- For studies of P2X7R function in morphologically identified neurons, astrocytes or microglia, the GFP-P2X7 reporter mouse seems to be a crucial tool [134].

945 **Box 2. Outstanding Questions**

946

947 Despite the large interest in P2X7Rs and the correspondingly high number of
948 publications dealing with this receptor, many questions still remain unresolved.

949 • The C-terminus of the P2X7R has been implicated in regulating receptor
950 function including signaling pathway activation, cellular localization, protein-
951 protein interactions, and post-translational modification [135]. It would be
952 important to learn the three-dimensional structure of the P2X7R C-terminal
953 tail, which is yet to be determined [4].
954 • Although repetitive or long-lasting stimulation of P2X7Rs by ATP allows the
955 passage of 600-800 Da organic molecules through the cell membrane, the
956 mechanism of pore opening is still a matter of debate. There are good
957 arguments favouring an accessory protein, with Panx1-hemichannels
958 probably involved in this effect, but the cationic channel-dilation theory is also
959 an attractive alternative.
960 • Original work based on co-immunoprecipitation with epitope tagged subunits
961 demonstrated that overexpressed recombinant P2X1-6 subunits could form
962 hetero-oligomeric complexes, while P2X7 was able to form only homomeric
963 receptor channels [136]. However, it remains to be established whether true
964 functional P2X4/7 heteromers are formed in native systems, which might have
965 great significance for CNS immune functions e.g. in microglia.
966 • A lot of controversy has arisen on the issue of whether P2X7Rs are located
967 exclusively at microglia and astroglia in the CNS or also at neurons (see the

discussion on “Tissue and cell type specific distribution of P2X7Rs”). The solution of this enigma might be that under normal conditions P2X7Rs are dormant but after various types of damaging conditions (mechanical trauma, ischemia, inflammation, etc.) they become unmasked, mostly at central immunocytes but probably also at neurons. Already the tissue damage afflicted to cells during the culturing procedure or the preparation of brain slices may be sufficient to induce the expression of previously absent P2X7Rs.

- Although endogenous activation of P2X7Rs under disease conditions has repeatedly been proven, its exact mechanism is not fully understood, given the low affinity of ATP. The possibility of constitutive activity of this receptor as well as its potential endogenous ligands other than ATP should be explored.
- Whereas available gene deficient mouse models are not fully deficient in P2X7Rs, more advanced mouse models, such as cell-type specific and/or inducible knockouts, optogenetic constructs, as well as humanized mouse models reproducing human gene polymorphisms in rodents are yet to be generated for probing P2X7R function.

Tables

Table 1. Examples from recent studies verifying functional P2X7Rs on different cell types of the rodent central nervous system.

Cell type/Brain area, preparation	Technique	Refs
Neurons		
Cerebral cortex, purified synaptosomes	neurochemistry, Ca^{2+} fluorimetry	[44]
Midbrain, synaptic terminals	Ca^{2+} microfluorimetry	[137]
Neurohypophysis, nerve terminals	patch clamp electrophysiology	[138]
Caudal brainstem, nerve terminals	neurochemistry	[139]
Hippocampus, isolated hilar neurons	patch clamp electrophysiology	[51]
Retina, isolated ganglion cells	patch clamp electrophysiology	[53]
Suprachiasmatic nucleus, isolated neurons	Ca^{2+} imaging	[140]
Embryonic spinal cord, cultured neurons	neurochemistry	[141]
Cortex, cultured neurons	neurochemistry	[142]
Astrocytes		

Cortex, *in situ*	patch clamp electrophysiology	[143]
Cortex, cultured	patch clamp electrophysiology	[144]
Cerebellum, cultured	neurochemistry	[145]
Human, cultured	Ca^{2+} fluorimetry	[146]
Bergmann glia		
Cerebellum, *in situ*	patch clamp electrophysiology, Ca^{2+} imaging	[147]
Satellite glia		
Immature dorsal root ganglion, isolated	electrophysiology	[148]
Microglia		
Cortex, *in situ*	patch clamp electrophysiology	[149]
N9 microglia, cultured	neurochemistry	[150]

990

991

992 Table 2. Non-comprehensive list of different classes of P2X7 receptor

993 antagonists and allosteric modulators. For more information see [151]

994

Class/Compound	Function	Refs
Novel, small molecule		
(1H-pyrazol-4-yl) acetamides	antagonist	[152, 153]
benzamides	antagonist	[154, 155]
tetrasubstituted-imidazoles	antagonist	[156]
2-oxo-N-(phenymethyl)-4-imidazolinecarboxamides	antagonist	[157]
Novel, small molecule, CNS active		
JNJ-47965567	antagonist	[128]
polycyclic carboranes	antagonist	[158]
Identified by screening compound libraries		
clemastine	Positive allosteric modulator	[159]
perazine-type antipsychotic drugs	Negative allosteric modulator	[129]
ivermectine	Negative allosteric modulator	[160]

Natural compounds		
teniposide	antagonist	[161]

995

996

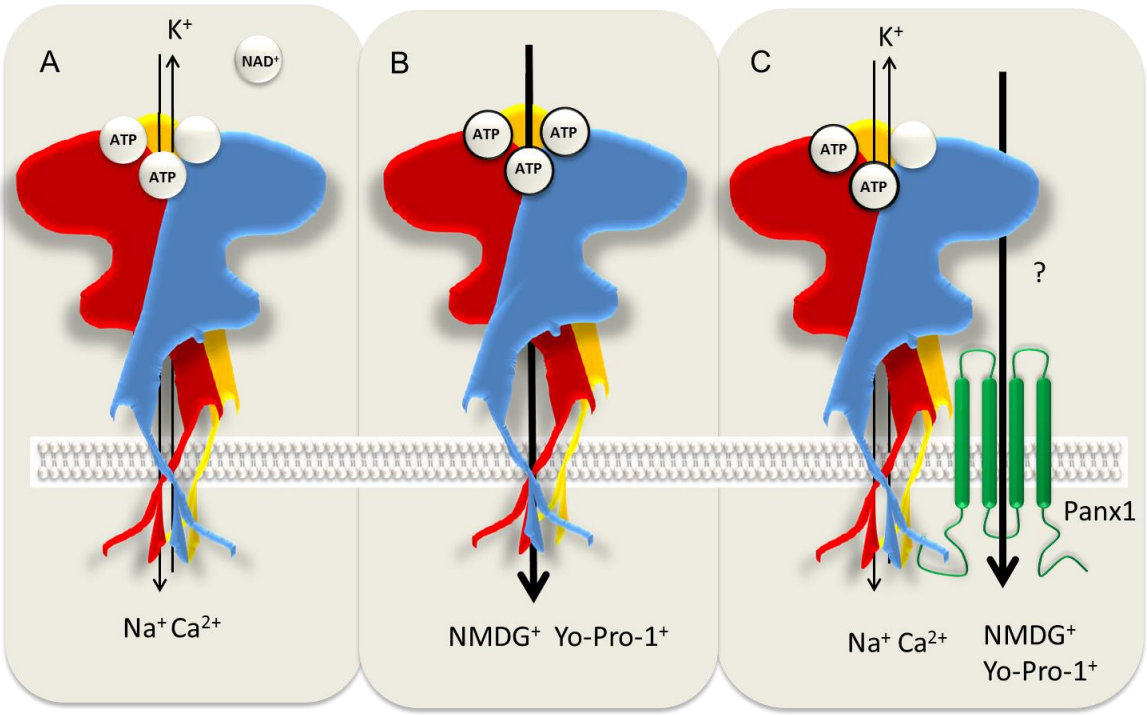
Figure Legends

Figure 1. The simplified schematic structure of the P2X7R in open state (A) and during pore formation (B and C). The P2X7R functions as a homo-trimer, forming a chalice-like structure, while the individual P2X7R subunit is akin to a leaping dolphin. The agonist binding sites are located at the subunit interfaces and the occupation of two out of three binding sites is necessary for opening of the channel. In addition to ATP, which is the presumed endogenous agonist, the mouse P2X7R receptor could also be activated by NAD^+ through ADP-ribosylation. The activation of the receptor-ion channel leads to the inward flux of cationic current. Prolonged and /or repeated activation of P2X7R and occupation of the third agonist binding site renders the membrane permeable for high molecular weight organic cations and dyes such as $NMDG^+$ and Yo-Pro-1^+ (B and C). B. One potential mechanism of the pore formation is the dilation of the P2X7R-mediated channel pore itself. C. Alternatively, but not exclusively, additional pore forming proteins, such as pannexin (Panx1) might be recruited, which seem to be indispensable for pore formation under certain circumstances.

Figure 2. Common disease mechanism by P2X7R mediated pathways in CNS disorders of different etiology. P2X7 receptors are expressed on nerve terminals, astrocytes and microglia and they are upregulated upon various disease conditions. Stress signals, such as hypoxia/ischemia (metabolic limitations),

mechanical injury, and bacterial or chemical toxins elicit the endogenous activation of P2X7R and leads to a self-amplifying ATP release and to further activation of P2X7 receptors on neighbouring cells. Following the influx of Ca^{2+} through the receptor ion channel complex, P2X7 receptor activation (a) releases glutamate from nerve terminals and astrocytes by both exocytotic and non-exocytotic mechanisms, which may give rise excitotoxicity; (b) leads to the posttranslational processing of pro-IL-1β to the leaderless, mature IL-β and to its further release by the NLRP3 inflammasome and that of other cytokines, which contribute to neuroinflammation; (c) enhance ROS production and thereby aggravate protein misfolding and neuronal damage; (d) leads directly or indirectly to cell death and the following reactive astrogliosis (e) directly or indirectly downregulates the production of BDNF and the following neuroplasticity. These key mechanisms could be manifested and contribute to disease pathology in Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), status epilepticus (SE), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), stroke, pain and mood disorders in different forms and proportion, depending on the etiology. GLU, glutamate, ROS, reactive oxygen species.

1042



1043 Figure 1

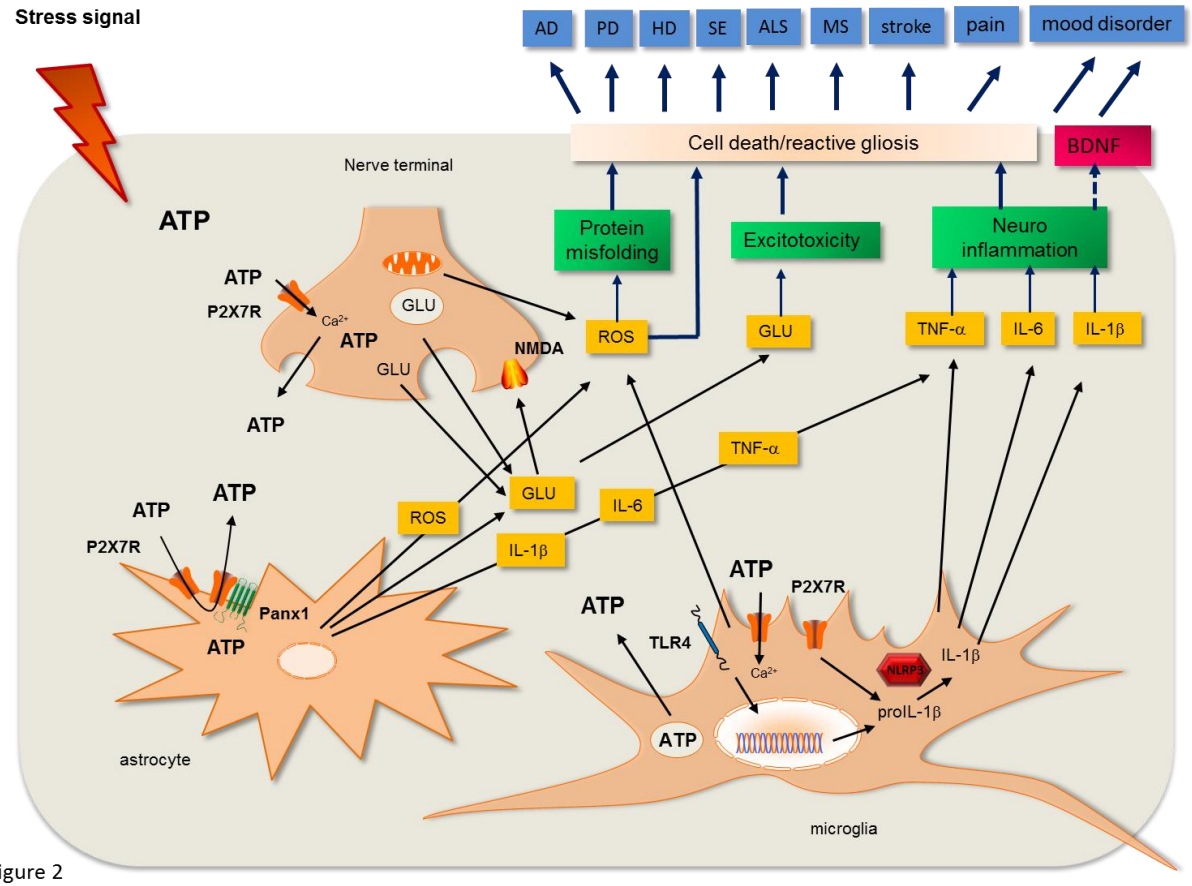


Figure 2

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