Targeting P2X7 receptors as a means for treating retinal disease

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Age-related macular degeneration and glaucoma are the commonest causes of irreversible vision loss in industrialized countries. The purine ATP is known to regulate a range of cellular functions in the retina via its action on P2 receptors, especially the P2X7 receptor. Although agents that attenuate P2X7 receptor function have been in development for many years, no compound is currently approved for the treatment of eye disease. However, newer compounds that cross the blood–brain barrier could have potential to reduce vision loss. This review will outline recent information relating to the role of P2X7 in age-related macular degeneration and glaucoma and, subsequently, we will discuss recent developments for attenuating P2X7 receptor function.

Introduction

Despite major advances in treatment and management, retinal diseases cause over three-quarters of all cases of irreversible vision loss in the western world. Recent estimates indicate that the leading causes of irreversible severe visual impairment in those >50 years of age are diabetic retinopathy, age-related macular degeneration (AMD) and glaucoma, affecting ~5.4 million people globally [1]. Development of treatments targeting vascular endothelial growth factor (VEGF) over the past 10 years has seen a dramatic reduction in vision loss from retinal diseases involving pathological growth of blood vessels including in one form of advanced AMD and, to a lesser extent, diabetic retinopathy [2]. However, with long-term treatment, many patients with AMD continue to lose vision [3]. Vision loss in those with primary open-angle glaucoma is due to gradual loss of retinal ganglion cells caused by a range of factors including raised intraocular pressure. Despite ongoing treatment that reduces intraocular pressure, vision loss continues to occur in approximately 30% of people. This highlights the need to identify new treatment targets for these and other retinal conditions.

Adenosine triphsophate (ATP) is well known for its intracellular role as an energy metabolite. As an extracellular molecule it can exert a range of effects on cellular function via actions mediated by two families of receptors called P2X and P2Y receptors [4]. There are seven known mammalian isoforms of P2X receptors (P2X1–P2X7), all of which are ligand-gated ion channels. By contrast, P2Y receptors are G-protein-coupled receptors that elicit cellular changes via activation of G protein cascades. The release mechanisms of ATP are atypical for a neurotransmitter. Although they do include standard vesicular release of ATP from cells, which involves the recently characterized vesicular nucleotide transporter (VNUT) [5], conductive mechanisms via hemichannels have also been described [6]. Once in the extracellular space, the cellular effects of extracellular purines are attenuated by a series of ectonucleotidases (NTPDase 1–3) that catalytically convert ATP to adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine. The expression of P2X receptors, P2Y receptors, ectonucleosidases and vesicular nucleotide transporter is widespread and includes cells within the retina, a range of epithelia and also cells of the immune system. Of the purinergic receptors, P2X7, in particular, has been associated with retinal disease, whereas the contribution of other P2X receptors remains poorly understood. Anomalies in P2X7 receptor function potentially contribute to neural loss in glaucoma and inherited retinal degenerations, as well as

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inflammatory changes associated with AMD and diabetic retinopathy [7–9]. The aim of this review is to provide an overview of recent information relating to therapeutic targeting of P2X7 receptors to retinal disease. In particular, we focus on vision loss in AMD and glaucoma and, subsequently, explore the potential role that P2X7 antagonists could have in reducing vision loss.

Structure and function of P2X7 receptors

P2X7 receptors belong to the family of ionotropic receptors that consist of three homomeric subunits [4]. However, unlike other ligand-gated ion channels, P2X7 receptors can mediate distinct functions depending on the concentration and duration of activation by ATP (Fig. 1a–c). In the absence of extracellular ATP, P2X7 receptors expressed on macrophages have been shown to have scavenger activity, initiating binding and phagocytosis of apoptotic cells or microorganisms (Fig. 1a). In the presence of ATP, they require a large concentration of extracellular ATP for activation to cause a depolarizing response, which subsequently allows entry of extracellular sodium and calcium (Fig. 1b). In comparison to other P2X receptors, P2X7 requires a much higher concentration of ATP for activation – the EC_{50} for mammalian P2X7 receptors is in the millimolar range [4]. In addition, following prolonged exposure to ATP, they can form or connect to a large membrane pore which is permeable to organic ions and dyes up to ~900 Da in size [4] (Fig. 1c). Structurally, P2X7 receptors consist of a short intracellular N terminus, two transmembrane domains and a long C terminus. The C-terminal domain is known to be crucial for maximal ion channel function and also pore formation, whereas scavenger activity is thought to be mediated by regions within the extracellular part of the receptor [10]. Notably, when considering the role of P2X7 receptors in retinal disease, the contribution of P2X7 as an ion channel, its role in pore formation or its role in scavenger activity should be considered. In addition, careful consideration of the role of any therapeutic target in modulating ion channel conductance, pore formation and/or scavenger activity of the P2X7 receptor might be warranted.

The crystal structure of P2X7 has recently been described [11]. A single P2X7 subunit resembles a dolphin, where the transmembrane domains form the lower body and tail region, and different regions of the extracellular domain form the head, flippers and dorsal fin. For a functional receptor, three P2X7 subunits are closely associated such that their three 'dolphin noses' form a turret that surrounds a central aperture. ATP binds to key residues located in the lower body region causing a conformational change leading to closure of the turret and channel opening [11]. Although P2X7 antagonists bind in the same pocket between neighboring subunits, no closure of the turret occurs and thus there is no subsequent channel opening [11].

An unusual feature of P2X7 receptors is that exposure to ATP over different time courses is associated with distinct functional features. Notably, ATP binding to P2X7 receptors leads to rapid opening of a cation-permeable channel that occurs within milliseconds. By contrast, exposure to ATP for several seconds, which can occur in the setting of cell death, leads to a gradual continued conformational change leading to increased permeability of organic cations and several dyes. The mechanism(s) leading to the pore formation remains controversial. Permeability of P2X7 receptors to the dye YO-PRO is known to be attenuated when the C terminus is truncated, highlighting the importance of this part of the receptor. However, the contribution of a secondary accessory protein has also been suggested and, although pannexin-1 has received attention, its role in P2X7 pore formation remains a source of debate [12].

Activation of P2X7 receptors induces a number of cellular signaling pathways [13]. Following opening of the ion channel or pore by ATP, potassium efflux occurs, a change that is crucial for activation of the nucleotide-binding oligomerization domain-like receptor 3 (NRLP3) inflammasome [6,14]. Activation of the NRLP3

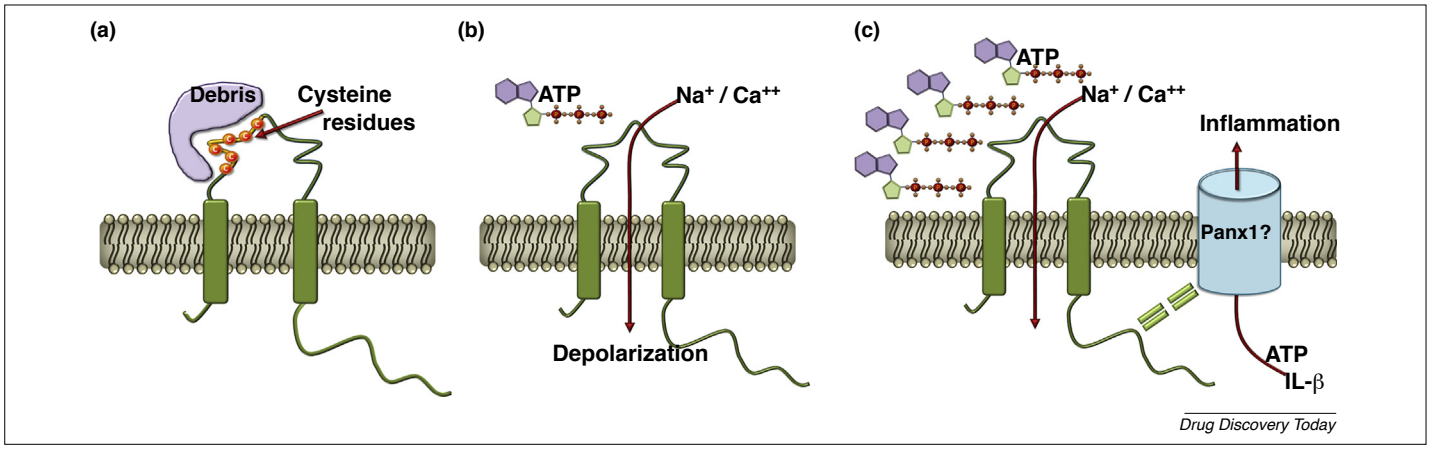

FIGURE 1

Schematic outlining the different functions of the P2X7 receptor. **(a)** In the absence of the P2X7 endogenous ligand ATP, P2X7 receptors expressed on macrophages can act as scavenger receptors, forming an important part of the innate immune response. The presence of ten cysteine residues on the extracellular domain of P2X7 enables it to interact with extracellular waste, pathogens and dying cells, engaging engulfment of the material by the macrophage. **(b)** In the presence of ATP, P2X7 has been shown to act as a traditional ligand-gated ion channel on neurons, mediating entry of Na^+ and Ca^{2+}, and causing depolarization of neurons in a range of tissues such as the hippocampus. **(c)** In the presence of excess levels of ATP, such as in a pathological event where many cells are dying, overstimulation of the receptor causes opening of a large pore in the cell membrane allowing molecules up to 900 kDa to transverse the cell wall. To mediate this pore function, dilatation of P2X7 itself but also modulation of a secondary channel such as pannexin-1 (Panx1) has been suggested. When expressed on immune cells, this pore function of P2X7 plays a part in release of inflammatory mediators such as interleukin (IL)-β, tumor necrosis factor (TNF)α and further ATP.

inflammasome is considered particularly important as a potentiator of inflammation and subsequent cell death during retinal disease [14]. Activation of the NRLP3 inflammasome is associated with cleavage of caspase-1 and release of proinflammatory cytokines including interleukin (IL)-1α, IL-1β and IL-18.

Recently, a novel role for P2X7 in phagocytosis of microorganisms and dead cells has been demonstrated [15,16]. P2X7 receptors form part of a membrane complex with several proteins including structural proteins important for maintaining cellular morphology as well as non-muscle myosin IIA heavy chain [15,16]. Moreover, P2X7 receptors mediate engulfment of apoptotic cells, as well as microorganisms, in a manner that is abolished in the presence of extracellular ATP [17]. Importantly, a region within the extracellular domain of P2X7 that is distinct from the ATP-binding site has been identified as crucial for scavenger activity of P2X7 [10].

In summary, the P2X7 receptor can form an ion channel, elicit cellular changes via the formation of a large conductance pore or have a role in phagocytosis as a scavenger receptor. Development of therapeutic agents that target P2X7 needs to be considered in terms of their effects on one or more of these functions.

Expression and function of P2X7 in the healthy mammalian retina

The P2X7 gene encodes a 595 amino acid receptor. However, there are five known alternatively spliced variants of P2X7, including one full-length variant consisting of translation of 13 exons, one slightly truncated variant that contains most of exon 13 and three variants that are truncated before exon 13 and lack the crucial C-terminal domain which is required for maximal channel function. Expression of three of the known splice variants has been demonstrated in the retina, although their specific cellular localization remains to be determined [8]. It is important to note that one of the P2X7null mice described in the literature (by Solle *et al.* [18]) that has been commonly used to understand the role of P2X7 in the retina lacks only the variant 1 (full-length) splice variant [8].

Expression of P2X7 has been reported on several cell types within the retina including neurons, glia and the retinal pigment epithelium (Fig. 2). Neuronal expression of P2X7 receptors includes retinal ganglion cells (RGC), amacrine cells and photoreceptors [8,19–21]. Retinal function is modulated by application of a P2X7 full agonist: BzATP [21], and attenuated in the P2X7null mouse [8]. As shown in Fig. 2a, high-resolution immunocytochemistry has demonstrated that P2X7 is expressed on processes within rod and cone terminals. A role in photoreceptor function has been further demonstrated by evaluating retinal function following application of the P2X7 agonist BzATP [21] and within the P2X7null mouse [8]. These findings suggest that photoreceptor function can be modulated by ATP and that anomalies in purinergic signaling within the outer retina could influence photoreceptor integrity.

Within the inner retina, immunoelectron microscopy has established that P2X7 receptors are localized to the processes of an amacrine cell type associated with rod bipolar cells and therefore could play an important part in the modulation of the neural circuits important vision at low light levels [20,21]. In addition, several retinal ganglion cell subtypes express P2X7 receptors. As an example, Fig. 2d–h shows an ON-OFF retinal ganglion cell of the mouse retina immunolabeled for P2X7, with P2X7-immunoreactive puncta coincident with the dendrites of a ganglion cell within the ON and OFF sublayer of the inner plexiform layer.

In addition to neural expression, P2X7 receptor expression has been reported in glial cells, including retinal Müller cells [22]. Under normal circumstances, Müller cells play a key part in maintaining normal retinal function, by removing neurotransmitters from the synaptic cleft, siphoning extracellular potassium and shuttling energy metabolites between the vasculature to neurons. Retinal glia can also modulate the function of neighboring neurons by releasing 'gliotransmitters' including ATP in response to a range of stimuli [23]. Supporting a functional role, activation of P2X7 receptors on Müller cells has been shown to alter high-affinity glutamate uptake [22].

Purinergic regulation of immune cells, including microglia, the resident immune cells of the central nervous system (CNS), is well described and P2X4 receptors have been localized in primate and rodent retinal microglia (Fig. 2b,c) [24]. Recently, a novel transgenic mouse has been generated where enhanced green fluorescent protein (eGFP) expression was linked with the C terminus of the P2X7 receptor confirming expression of the receptor in microglia [25]. It has been suggested that expression of the full-length variant that allows pore formation in response to extended exposure to high concentrations of ATP is only apparent in microglia and not neurons within the retina [26].

In summary, expression of P2X7 is widespread across different neural and glial populations, as well as being found on the immune cells in the retina. The potential for P2X7 to mediate cation conductance across cellular membranes, form a large conductance pore or exhibit scavenger activity highlights the complexity of functions this receptor can have and also the potential for anomalies in receptor function that underpin disease processes within the retina. In the following sections, we summarize the role that P2X7 receptors can have in the retinal diseases of AMD and glaucoma.

Retinal disease associated with scavenger functions of P2X7 receptors

Phagocytosis of cellular debris requires recognition by 'scavenger receptors' expressed on macrophages, to recognize moieties exposed on the surface of extracellular debris or on dead or dying cells. With relevance to retinal disease, a reduction in scavenger function of P2X7 receptors could be important in the development of AMD. Indeed, early stages of the disease are characterized by the accumulation of small deposits in the posterior eye called drusen. Drusen are known to consist of a range proteins, lipids and other molecules and their size is associated with increasing risk of disease progression [27]. However, the underlying mechanisms leading to the development of drusen in the posterior eye remain to be determined.

Recently, inheritance of single-nucleotide polymorphisms in P2X7 together with P2X4 receptors has been associated with a fourfold increased risk of developing advanced AMD [24]. Notably, phagocytic function of monocytes isolated from patients heterozygous for these single-nucleotide polymorphisms showed a significant reduction in phagocytosis when compared with healthy controls [24]. Further evidence for a role of P2X7 receptors and/or scavenger activity in AMD comes from the observations that aged P2X7null mice show reduced scavenger-receptor activity as well as features of early AMD [7].

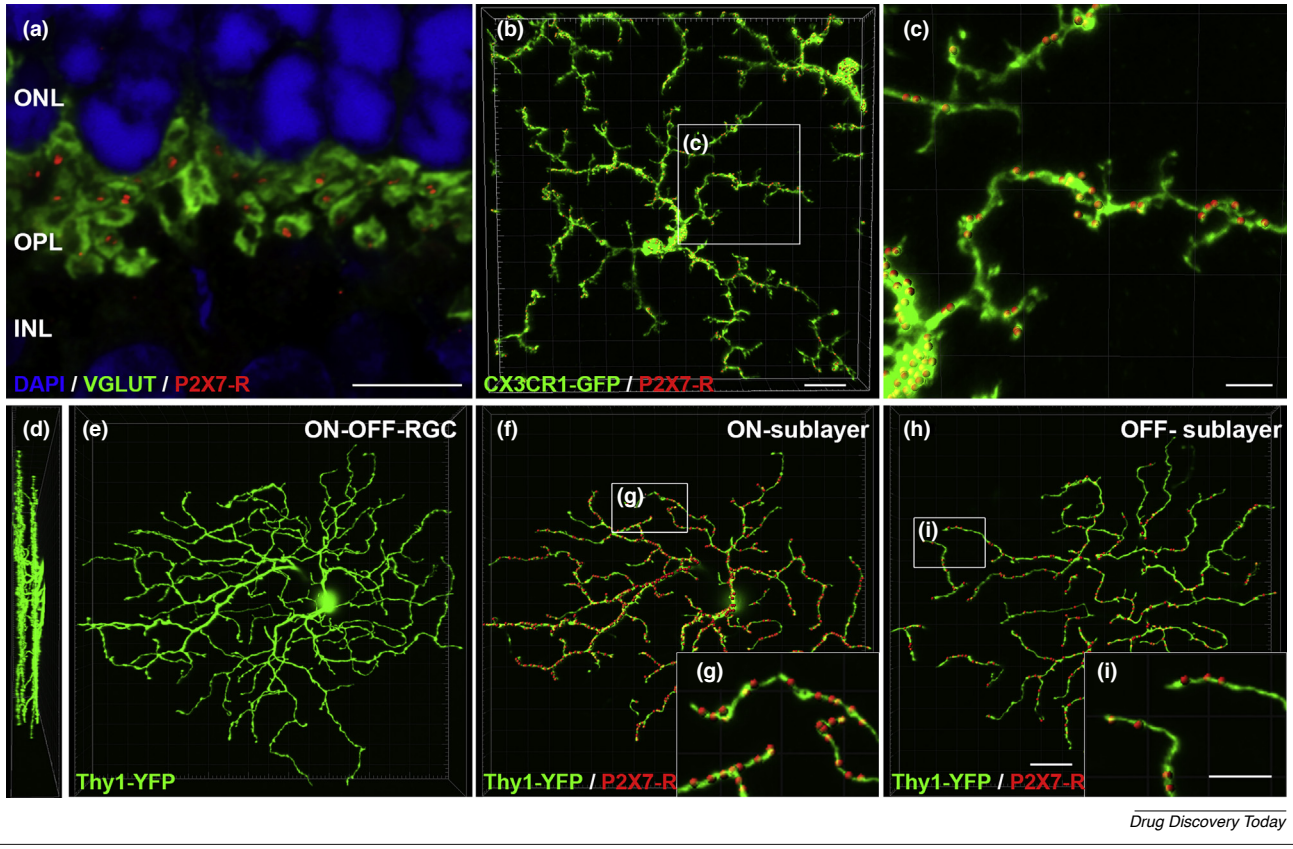

FIGURE 2

Location of the P2X7 receptor in the rodent retina. **(a)** P2X7 is expressed on photoreceptor terminals in the rat retina. Transverse sections of retina from adult dark agouti rats were labeled for P2X7 (red), the vesicular glutamate transporter VGLUT1 (green) and the nuclei label DAPI (blue). Images were collected using Airyscan, super resolution microscopy (LSM880, Zeiss), and showed localization of P2X7-immunoreactivity within VGLUT-positive photoreceptor terminals in the outer plexiform layer (OPL). **(b, c)** P2X7 is expressed on microglia in the mouse retina. Flat-mounted retina from microglia-reporter mice, Cx3cr1$^{GFP/+}$ mice (C 57B6J background), were co-labeled for the P2X7 (red) and for green fluorescent protein (GFP)-positive microglia (green). A confocal Z-stack was collected using the 40x oil objective at high resolution and the 3D image rendered using IMARIS software (v.7.6.5 Bitplane AG, Zürich, Switzerland). P2X7 puncta colocalized with Cx3cr1-positive microglia were defined using the Spot module of IMARIS and are presented as red dots, artificially enlarged for viewing. (c) inset from (b). **(d–i)** P2X7 is expressed on ganglion cells in the mouse retina. Flat-mounted retina from Thy1$^{hYFP/+}$ mice (C57B6J background) were co-labeled for P2X7 (red) and for GFP-positive Thy1 ganglion cells (green). A confocal Z-stack, tile scan was collected using the 40x oil objective at high resolution and the 3D image rendered using IMARIS software. (d, e) Projected view of a Thy1-positive ON-OFF ganglion cell in (d) orthogonal view showing two dendritic plexuses in the inner plexiform layer and (e) as a maximum projection from above. (f) P2X7 puncta colocalized with Thy1-positive ganglion cell dendrites in the ON sublaminae of the inner plexiform layer. (g) Magnified view of inset from (f). (h) P2X7 puncta colocalized with Thy1-positive ganglion cell dendrites in the OFF sublaminae of the inner plexiform layer. (i) Magnified view of inset from (h). P2X7 puncta colocalized with ganglion cell processes were defined using the Spot module of IMARIS and are presented as red dots, artificially enlarged for viewing. Scales: a, 5 μm; b, 15 μm; c, 5 μm; e, f, h, 20 μm; g, i, 10 μm. Abbreviations: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer.

Role of P2X7 in neuronal cell death and disease

High concentrations of ATP are released into the external milieu following cell death and, in some cases, this contributes to bystander cell death. Notably, ATP-induced neural death has been observed in the spinal cord following trauma in a manner that was blocked when animals were treated with P2X7 antagonists including Brilliant Blue [28]. Given the widespread expression of P2X7 across a range of neural subtypes in the retina, it is possible that ATP release from dying cells could exert deleterious effects on neighboring neurons that express P2X7 receptors. Exacerbation of photoreceptor death in inherited retinal degenerations and ganglion cell death in glaucoma are two examples where excessive activation of P2X7 receptors could potentiate neural death.

Photoreceptor death is associated with ~50% of irreversible vision loss in the western world, contributing to vision loss in AMD and inherited retinal degenerations. Photoreceptors are known to express P2X7 receptors, and high levels of extracellular ATP injected into the rodent or feline eye induces rapid and sustained photoreceptor loss (Fig. 3) [29–31]. Moreover, blockade of ATP-induced photoreceptor death was observed when the P2X antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) was co-injected into the eye. This effect was also observed in *rd1* mice, a mouse model of autosomal recessive retinitis pigmentosa, with PPADS, reducing photoreceptor death by ~30% [30]. Subretinal hemorrhage, a feature of one form of advanced AMD, is associated with an increase in ATP concentration within the vitreous humour [32]. In mouse models, subretinal hemorrhage causes photoreceptor loss via mechanisms involving P2X7 receptor activation [32]. Indeed, photoreceptor death induced by hemorrhage was abrogated in P2X7null mice or in animals treated with the P2X7 antagonist Brilliant Blue [32]. These studies highlight the potential for treating photoreceptor death that occurs in

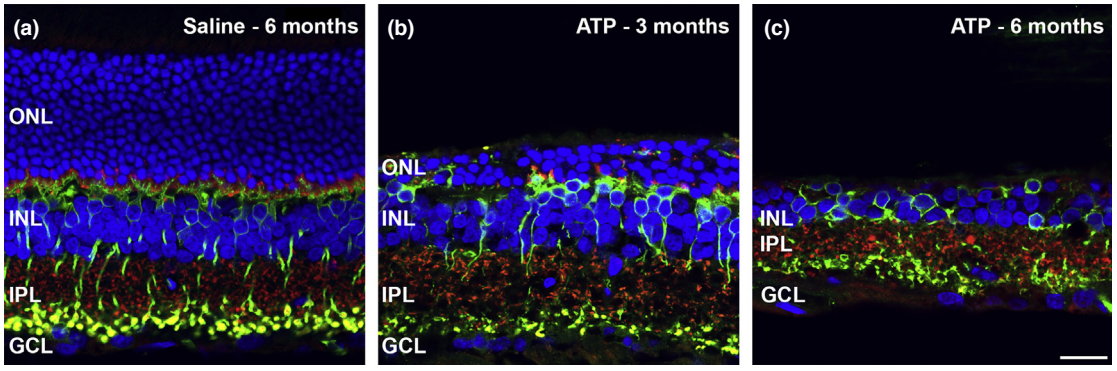

FIGURE 3

Intravitreal administration of high concentrations of ATP induces photoreceptor death and subsequent retinal-degeneration-induced remodeling. The effects of a single intravitreal administration of **(a)** saline versus **(b,c)** 50 mM ATP on the gross structure of the retina were investigated at (b) 3 months and (c) 6 months of age. Transverse sections of retina from adult dark agouti rats were labeled for the vesicular glutamate transporter VGLUT1 (red), rod bipolar cells (PKCa, green) and the nuclei label DAPI (blue). (a) In saline-injected eyes, 6 months after administration, photoreceptor layers and their VGLUT-positive terminals are normal, as is rod bipolar cell morphology. (b) At 3 months after ATP injection, many photoreceptor nuclei are lost and VLGUT synaptic labeling in the outer plexiform layer is irregular; however, bipolar cell morphology is relatively intact. (c) At 6 months after ATP injection, all photoreceptors are lost and secondary remodeling has occurred, including mis-localization of bipolar cell nuclei in the inner nuclear layer. Scale bar: a–c, 20 μm. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

inherited retinal degenerations or in some stages of AMD with agents that attenuate P2X7 receptor function.

Excessive activation of P2X7 receptors can also contribute to vision loss in glaucoma. Glaucoma is an optic neuropathy, characterized by death of retinal ganglion cells in response to vascular compromise and/or raised intraocular pressure. Patients with primary angle-closure glaucoma associated with elevated intraocular pressure show up to ninefold increases in ATP levels in the anterior chamber [33]. Moreover, P2X7 receptor expression and ectonucleosidase 1 activity, the primary enzyme important for dephosphorylation of ATP, are altered in rodent models following raised intraocular pressure [34,35]. ATP administration or treatment with the P2X7 agonist BzATP is known to induce death of dissociated retinal ganglion cells *in vitro* and in rat retinas *in vivo* [36,37]. Additionally, following an acute increase in intraocular pressure *in vivo*, or pressure *in vitro*, P2X7 receptor antagonism or ATP breakdown reduced rat retinal ganglion cell loss in a dose-dependent manner [38]. Furthermore, P2X7 receptor blockade, or genetic knockout, protected retinal ganglion cells from an optic nerve crush by delaying cell death and slowing the increase of phagocytic microglia, suggesting a major role of the P2X7 receptors following retinal ganglion cell injury. These findings all point to blockade of P2X7 receptors as providing neuroprotection for retinal ganglion cells.

Whereas the above discussion highlights the role of P2X7 in neuronal cell death, the exact mechanism(s) of neural death in the retina is not fully elucidated. One cause of photoreceptor or ganglion cell death is excessive neural activation by ATP, leading to the sustained influx of Ca^{2+} and cell death via Ca^{2+}-dependent mechanisms such as increased caspase levels [36]. This is supported by findings in rats with laser-induced increases in intraocular pressure, showing a slight, but significant, increase in baseline Ca^{2+} levels compared with control eyes [39].

Alternatively, excessive activation of P2X7 receptors on neurons could lead to their death via actions of associated channels including pannexin-1 channels. Retinal ganglion cells of pannexin-1 knockout mice (*panx1*$^{-/-}$) showed reduced death, reduced Ca^{2+} influx and reduced inflammasome activation following an ischemic injury [40]. Pannexin 1 channels on immune cells and glia could also be implicated. Studies on cultured macrophages found P2X7 receptors and pannexin-1 were necessary to induce a large inward current and open a pore that was permeable to large molecules. This large inward current and permeable pore was induced by the P2X7 receptor agonist BzATP; and blocked by either pannexin-1 blockers or pannexin-1 knockdown using small-interfering (si)RNA. Similarly, pannexin-1 knockout mice showed reduced amplitude of a BzATP-induced inward current in retinal astrocytes, which matched the amplitude from wildtype astrocytes treated with pannexin-1 blockers. Several lines of evidence in differing models strongly support the role of the P2X7 receptor in activating pannexin-1 channels in neurons, glia and immune cells that can contribute to retinal ganglion cell death.

In summary, release of high concentrations of ATP into the extracellular milieu during cell death can lead to bystander death of neurons, including photoreceptors in retinal degenerations or retinal ganglion cells secondary to raised intraocular pressure in glaucoma. Development of inhibitors of P2X7 receptors could therefore have the potential to reduce neural loss in a range of retinal diseases, preserving vision.

P2X7 receptor activation and inflammation

In addition to retinal neurons, P2X7 receptors are also highly expressed in monocytes, macrophages and retinal microglia where they are thought to mediate release of inflammatory cytokines [41]. Following extended exposure to ATP, P2X7 receptors form a nonselective pore that is permeable to molecules up to 900 Da. Efflux of potassium ions also occurs, activating the inflammasome and release of proinflammatory mediators, especially IL-1β and IL-18. Neuroinflammatory mechanisms involving activation of the inflammasome within cells of the innate immune system could

contribute to or even initiate neural death in conditions associated with photoreceptor degenerations and glaucoma [14,41].

Over recent years there has been increasing evidence that accumulation of tissue macrophages within the subretinal space contribute to vision loss in AMD, particularly mediating cone photoreceptor loss in geographic atrophy, a common form of advanced AMD [42]. Inflammatory cytokines, especially IL-1β, are implicated in photoreceptor death [41]. Indeed, photoreceptor death induced by exposure to high levels of light, is associated with increased accumulation of tissue macrophages in the subretinal space, as well as increased IL-1β and increased P2X7 expression in subretinal macrophages [41]. Moreover, blockade of P2X7 receptors with Brilliant Blue G or inflammation with an IL-1-receptor antagonist reduces photoreceptor death [41]. These results, along with a series of *in vitro* studies demonstrating the role of P2X7 in IL-1β release from subretinal macrophages, suggest that high levels of extracellular ATP could induce neural death either directly (as discussed in the section above) or via secondary mechanisms involving neuroinflammation. Indeed, an inflammatory response follows increased intraocular pressure, and is implicated in the potentiation of retinal ganglion cell death. Several studies of patients with glaucoma show increases in proinflammatory cytokines including tumor necrosis factor (TNF)α within the retina as well as a range of cytokines within the aqueous humour including ILs and interferon (IFN)-α and IFN-γ [43,44].

In summary, activation of P2X7 receptors on tissue macrophages and release of inflammatory mediators including IL-1β is implicated in photoreceptor death in AMD and also retinal ganglion cell death. Further work is necessary, however, to establish whether novel compounds targeting P2X7 receptors can abrogate neuroinflammation and reduce neural loss in AMD or glaucoma.

Therapeutic agents targeting P2X7 receptors and their potential use in retinal diseases

There has been well over a decade of development in antagonists that target P2X7 receptor function. To date, no P2X7 receptor antagonists for treatment of retinal disease are commercially available nor are there any undergoing investigations in Phase I, II or III clinical trials for treatment of eye disease. Two P2X7 antagonists have been the subject of clinical trials for non-eye-related diseases including two Phase II trials in rheumatoid arthritis (dose escalation 50–400 mg AZ9056 administered once per day [45]; 500 mg CE224535 administered twice per day [46]) and one in Crohn's disease (200 mg AZ9056 administered orally once per day) [47]. Those clinical trials evaluating safety and efficacy for treatment of rheumatoid arthritis failed to demonstrate efficacy either with serum markers including C-reactive protein or objective clinical criteria [45,46]. A recent evaluation of AZD9056 for Crohn's disease also failed to show a reduction in C-reactive protein, although improvements in abdominal pain were observed [47]. Progress in developing suitable P2X7 antagonists for treating retinal disease or other diseases of the central nervous system (CNS) has been hampered by the species specificity of available agents (Table 1), lack of penetration into the brain (and therefore the retina) and the potential to induce non-P2X7-mediated effects.

TABLE 1

Summary of P2X7 antagonists and their actions on ion channel, pore formation and scavenger activity

Compound name	Species specificity (rat or human)	Ion channel function ($[Ca^{2+}]$ IC_{50}[a]	Pore formation (ethidium bromide; IL-1β release)	Scavenger activity	Brain: plasma ratio	Human trials	Refs
KN62	Human>>rat	4.88	7.12	na		No	[52]
PPADS	Human, rat	5.45	5.92	na		No	[53]
Brilliant Blue	Rat	<4	6.22	No affect		No	[53]
A740003	Human, rat	7.36	7.00		0.1	No	[11,53]
A438079	Human, rat	6.90	6.40	No affect	2.0	No	[50,53]
AF27139	Human		Blocks	No affect		No	[50]
A804598	Human, rat, mouse	10.9	8.5	na	0.2	No	[11,54]
AZ10606120	Human, rat, mouse	8.9	Blocks	No affect		No	[11,50]
AZ11645373	Human>>rat	8.15	7.68	na		No	[53,55]
GW791343	Human, rat	6.9	na	na		No	[11]
JNJ47965567	Human, rat, mouse	8.3	6.7	na	1	No	[11]
JNJ55308942	Human, rat	7.97	7.21	na	0.1	No	[48]
JNJ42253432	Human, rat, mouse	7.7	67.4	na	1	No	[56]
Nanobodies (13A7, Dano1)	Mouse, human	12	Blocks	na	na	No	[51]
Glatiramer acetate				Blocks	na	Multiple sclerosis	[57]
GSK1482160	Human, rat	8.5	3	na	0.5	Phase I dose-escalation study in healthy individuals	[58,59]
JNJ5417446	Human, rat	8.46	7.7	na	0.8	Phase I healthy individuals	[49,60]
AZD9056	Human	10	na	na	0	Phase IIa Rheumatoid arthritis Crohn's disease	[45,47]
CE224,535	Human, rat	na	1.4	na	0	Phase IIa rheumatoid arthritis	[46]

Abbreviation: na, not available.

[a] IC_{50} values are provided for human P2X7 where relevant.

One of the major effects of P2X7 receptor activation is release of IL-1β in response to NRLP3-inflammasome activation. Recently, several P2X7 antagonists were developed that attenuate IL-1β and also cross the blood–brain barrier, making them of potential value in treating diseases of the CNS [48]. JNJ55308942 reduces calcium conductance through P2X7 receptors, IL-1β release from blood monocytes and cultured microglia [48]. Moreover, following oral administration, evidence for increasing occupancy of P2X7 receptors with increasing dose was observed suggesting that this compound crossed the blood–brain barrier. Consistent with its crossing the blood–brain barrier, treatment with JNJ55308942 reduced inflammation and clinically relevant signs in rodent models of anhedonia and chronic stress [48]. Importantly, similar compounds (JNJ55471300, JNJ55308942) that lacked P2X7 occupancy within the brain were found to have reduced efficacy in the same model of chronic stress [48]. Moreover, evaluation of a similar brain-permeable orally available P2X7 antagonist: JNJ5417446, in healthy individuals confirmed penetration into the brain and effects on IL-1β release from peripheral blood monocytes [49]. In view of the similarities in structure of the blood–retinal and blood–brain barriers, it is likely that the brain-permeant antagonists including JNJ-55308942 or JNJ-5417446 couldJNJ55308942 and JNJ5417446 could be effective in treating P2X7-associated retinal disease. Further studies are needed, however, to evaluate the potential use of these and other CNS-permeable P2X7 antagonists in retinal disease.

As noted above, P2X7 receptors can act as ion channels, induce cellular effects via pore formation and also have a role as a scavenger receptor. Future development of P2X7 antagonists might need to consider the effect any compound has on one or more of these functions. Ou *et al.* evaluated the potential for three P2X7 antagonists to block ion channel pore formation and scavenger activity. P2X7 antagonists of three different classes all blocked pore formation with IC_{50} values in the nanomolar range but had no effect on scavenger activity [50]. A range of small peptides based on the structure of the P2X7 receptor have been developed that modulate P2X7 receptor scavenger activity [10]. These peptides were found to enhance phagocytosis of apoptotic cells and bacteria; however, more work is needed to confirm the site of interaction with the receptor and the effects of these compounds on P2X7 receptor cation conductance and pore formation.

Nanobodies are a recently identified alternative to using small-molecule inhibitors for attenuating P2X7 receptor function [51]. Nanobodies are small proteins, one-tenth the size of conventional antibodies, that are based on the single-antigen domain of camelid or shark antibodies. Like antibodies, they have high specificity, low toxicity and an extended half-life that underpin their potential. Recently, nanobodies have been developed that target P2X7 receptors [51]. Dimeric nanobodies have been developed that can reduce (called 13A7) or enhance (called 14D5) P2X7 receptor current conductance, respectively [51]. Moreover, pore formation and IL-1β release were reduced following treatment of macrophages and T cells with the P2X7 blocking nanobody, 13A7. Consistent with a reduction in inflammation, *in vivo* application of this nanobody reduced inflammation associated with allergic contact dermatitis and experimental glomerulonephritis [51].

Concluding remarks

P2X7 receptors are highly expressed in a range of cell types of the retina including neurons, glia and microglia. P2X7 receptors have been implicated in AMD, inherited retinal degenerations and ganglion cell death via mechanisms involving excessive activation of P2X7 receptors, pore formation leading to release of inflammatory cytokines or through the role of the P2X7 receptor as a scavenger. To date, there are no small-molecule inhibitors of P2X7 available commercially to treat retinal disease, and nor are there any currently undergoing clinical trial investigation. The recent development of brain-permeant P2X7 antagonists that attenuate the release of P2X7-induced IL-1β could have potential in the treatment of retinal disease. In addition, molecules that enhance P2X7-mediated scavenger activity could have a role in reducing AMD. However, more work is needed, to understand how best to target retinal disease with small-molecule inhibitors or molecules that modulate scavenger activity.

Conflicts of interest

The authors declare no conflicts of interest.

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References

1 Flaxman, S.R. *et al.* (2017) Global causes of blindness and distance vision impairment 1990–2020: a systematic review and meta-analysis. *Lancet Glob. Health* 5, e1221–1234
2 Rosenfeld, P.J. *et al.* (2006) Ranibizumab for neovascular age-related macular degeneration. *N. Engl. J. Med.* 355, 1419–1431
3 Rofagha, S. *et al.* (2013) Seven-year outcomes in ranibizumab-treated patients in ANCHOR, MARINA, and HORIZON: a multicenter cohort study (SEVEN-UP). *Ophthalmology* 120, 2292–2299
4 Coddou, C. *et al.* (2011) Activation and regulation of purinergic P2X receptor channels. *Pharmacol. Rev.* 63, 641–683
5 Ho, T. *et al.* (2015) Vesicular expression and release of ATP from dopaminergic neurons of the mouse retina and midbrain. *Front. Cell. Neurosci.* 9, 389
6 Mugisho, O.O. *et al.* (1862) The inflammasome pathway is amplified and perpetuated in an autocrine manner through connexin43 hemichannel mediated ATP release. *Biochim. Biophys. Acta Gen. Subj.* 3, 385–393
7 Vessey, K.A. *et al.* (2017) Loss of function of P2X7 receptor scavenger activity produces an early age related macular degeneration phenotype in mice. *Am. J. Pathol.* 187, 1670–1685
8 Vessey, K.A. and Fletcher, E.L. (2012) Rod and cone pathway signalling is altered in the P2X7 receptor knock out mouse. *PLoS One* 7, e29990
9 Mitchell, C.H. *et al.* (2009) The P2X(7) receptor in retinal ganglion cells: a neuronal model of pressure-induced damage and protection by a shifting purinergic balance. *Purinergic Signal.* 5, 241–249
10 Gu, B.J. *et al.* (2011) P2X(7) is a scavenger receptor for apoptotic cells in the absence of its ligand, extracellular ATP. *J. Immunol.* 187, 2365–2375
11 Karasawa, A. and Kawate, T. (2016) Structural basis for subtype-specific inhibition of the P2X7 receptor. *Elife* 5, e22153
12 Di Virgilio, F. *et al.* (2018) The elusive P2X7 macropore. *Trends Cell Biol.* 28, 392–404
13 Bartlett, R. *et al.* (2014) The P2X7 receptor channel: recent developments and the use of P2X7 antagonists in models of disease. *Pharmacol. Rev.* 66, 638–675

14 Yerramothu, P. *et al.* (2018) Inflammasomes, the eye and anti-inflammasome therapy. *Eye (Lond.)* 32, 491–505
15 Kim, M. *et al.* (2001) Proteomic and functional evidence for a P2X7 receptor signalling complex. *EMBO J.* 20, 6347–6358
16 Gu, B.J. *et al.* (2009) Extracellular ATP dissociates nonmuscle myosin from P2X(7) complex: this dissociation regulates P2X(7) pore formation. *Am. J. Physiol. Cell Physiol.* 297, C430–439
17 Gu, B.J. *et al.* (2010) The P2X7-nonmuscle myosin membrane complex regulates phagocytosis of nonopsonized particles and bacteria by a pathway attenuated by extracellular ATP. *Blood* 115, 1621–1631
18 Solle, M. *et al.* (2001) Altered cytokine production in mice lacking P2X(7) receptors. *J. Biol. Chem.* 276, 125–132
19 Brandle, U. *et al.* (1998) Expression of the P2X7-receptor subunit in neurons of the rat retina. *Brain Res. Mol. Brain Res.* 62, 106–109
20 Puthussery, T. and Fletcher, E.L. (2004) Synaptic localization of P2X7 receptors in the rat retina. *J. Comp. Neurol.* 472, 13–23
21 Puthussery, T. *et al.* (2006) Evidence for the involvement of purinergic P2X receptors in outer retinal processing. *Eur. J. Neurosci.* 24, 7–19
22 Pannicke, T. *et al.* (2000) P2X7 receptors in Muller glial cells from the human retina. *J. Neurosci.* 20, 5965–5972
23 Newman, E.A. (2001) Calcium signaling in retinal glial cells and its effect on neuronal activity. *Prog. Brain Res.* 132, 241–254
24 Gu, B.J. *et al.* (2013) A rare functional haplotype of the P2RX4 and P2RX7 genes leads to loss of innate phagocytosis and confers increased risk of age-related macular degeneration. *FASEB J.* 27, 1479–1487
25 Kaczmarek-Hajek, K. *et al.* (2018) Re-evaluation of neuronal P2X7 expression using novel mouse models and a P2X7-specific nanobody. *Elife* 7, 36217
26 Innocenti, B. *et al.* (2004) ATP-induced non-neuronal cell permeabilization in the rat inner retina. *J. Neurosci.* 24, 8577–8583
27 Ferris, F.L., 3rd *et al.* (2013) Clinical classification of age-related macular degeneration. *Ophthalmology* 120, 844–851
28 Wang, X. *et al.* (2004) P2X7 receptor inhibition improves recovery after spinal cord injury. *Nat. Med.* 10, 821–827
29 Aplin, F.P. *et al.* (2014) ATP-induced photoreceptor death in a feline model of retinal degeneration. *Invest. Ophthalmol. Vis. Sci.* 55, 8319–8329
30 Puthussery, T. and Fletcher, E. (2009) Extracellular ATP induces retinal photoreceptor apoptosis through activation of purinoceptors in rodents. *J. Comp. Neurol.* 513, 430–440
31 Vessey, K.A. *et al.* (2014) Adenosine triphosphate-induced photoreceptor death and retinal remodeling in rats. *J. Comp. Neurol.* 522, 2928–2950
32 Notomi, S. *et al.* (2013) Dynamic increase in extracellular ATP accelerates photoreceptor cell apoptosis via ligation of P2RX7 in subretinal hemorrhage. *PLoS One* 8, e53338
33 Li, A. *et al.* (2011) Sustained elevation of extracellular ATP in aqueous humor from humans with primary chronic angle-closure glaucoma. *Exp. Eye Res.* 93, 528–533
34 Lu, W. *et al.* (2015) Rat, mouse, and primate models of chronic glaucoma show sustained elevation of extracellular ATP and altered purinergic signaling in the posterior eye. *Invest. Ophthalmol. Vis. Sci.* 56, 3075–3083
35 Zhao, D. *et al.* (2017) Characterization of the circumlimbal suture model of chronic IOP elevation in mice and assessment of changes in gene expression of stretch sensitive channels. *Front. Neurosci.* 11, 41
36 Zhang, X. *et al.* (2005) Stimulation of P2X7 receptors elevates Ca2+ and kills retinal ganglion cells. *Invest. Ophthalmol. Vis. Sci.* 46, 2183–2191
37 Hu, H. *et al.* (2010) Stimulation of the P2X7 receptor kills rat retinal ganglion cells *in vivo*. *Exp. Eye Res.* 91, 425–432
38 Resta, V. *et al.* (2007) Acute retinal ganglion cell injury caused by intraocular pressure spikes is mediated by endogenous extracellular ATP. *Eur. J. Neurosci.* 25, 2741–2754
39 Niittykoski, M. *et al.* (2010) Altered calcium signaling in an experimental model of glaucoma. *Invest. Ophthalmol. Vis. Sci.* 51, 6387–6393
40 Dvoriantchikova, G. *et al.* (2012) Genetic ablation of Pannexin1 protects retinal neurons from ischemic injury. *PLoS One* 7, e31991
41 Hu, S.J. *et al.* (2015) Upregulation of P2RX7 in Cx3cr1-deficient mononuclear phagocytes leads to increased interleukin-1beta secretion and photoreceptor neurodegeneration. *J. Neurosci.* 35, 6987–6996
42 Guillonneau, X. *et al.* (2017) On phagocytes and macular degeneration. *Prog. Retin. Eye Res.* 61, 98–128
43 Chua, J. *et al.* (2012) Expression profile of inflammatory cytokines in aqueous from glaucomatous eyes. *Mol. Vis.* 18, 431–438
44 Takai, Y. *et al.* (2012) Multiplex cytokine analysis of aqueous humor in eyes with primary open-angle glaucoma, exfoliation glaucoma, and cataract. *Invest. Ophthalmol. Vis. Sci.* 53, 241–247
45 Keystone, E.C. *et al.* (2012) Clinical evaluation of the efficacy of the P2X7 purinergic receptor antagonist AZD9056 on the signs and symptoms of rheumatoid arthritis in patients with active disease despite treatment with methotrexate or sulphasalazine. *Ann. Rheum. Dis.* 71, 1630–1635
46 Stock, T.C. *et al.* (2012) Efficacy and safety of CE-224,535, an antagonist of P2X7 receptor, in treatment of patients with rheumatoid arthritis inadequately controlled by methotrexate. *J. Rheumatol.* 39, 720–727
47 Eser, A. *et al.* (2015) Safety and efficacy of an oral inhibitor of the purinergic receptor P2X7 in adult patients with moderately to severely active Crohn's disease: a randomized placebo-controlled, double-blind, Phase IIa study. *Inflamm. Bowel Dis.* 21, 2247–2253
48 Bhattacharya, A. *et al.* (2018) Neuropsychopharmacology of JNJ-55308942: evaluation of a clinical candidate targeting P2X7 ion channels in animal models of neuroinflammation and anhedonia. *Neuropsychopharmacology* 43, 2586–2596
49 Timmers, M. *et al.* (2018) Clinical pharmacokinetics, pharmacodynamics, safety, and tolerability of JNJ-54175446, a brain permeable P2X7 antagonist, in a randomised single-ascending dose study in healthy participants. *J. Psychopharmacol.* 32, 1341–1350
50 Ou, A. *et al.* (2018) The scavenger activity of the human P2X7 receptor differs from P2X7 pore function by insensitivity to antagonists, genetic variation and sodium concentration: relevance to inflammatory brain diseases. *Biochim. Biophys. Acta Mol. Basis Dis.* 1864, 1051–1059
51 Danquah, W. *et al.* (2016) Nanobodies that block gating of the P2X7 ion channel ameliorate inflammation. *Sci. Transl. Med.* 8, 366ra162
52 Gargett, C.E. and Wiley, J.S. (1997) The isoquinoline derivative KN-62 a potent antagonist of the P2Z-receptor of human lymphocytes. *Br. J. Pharmacol.* 120, 1483–1490
53 Donnelly-Roberts, D.L. and Jarvis, M.F. (2007) Discovery of P2X7 receptor-selective antagonists offers new insights into P2X7 receptor function and indicates a role in chronic pain states. *Br. J. Pharmacol.* 151, 571–579
54 Donnelly-Roberts, D.L. *et al.* (2009) [3H]A-804598 ([3H]2-cyano-1-[(1S)-1-phenylethyl]-3-quinolin-5-ylguanidine) is a novel, potent, and selective antagonist radioligand for P2X7 receptors. *Neuropharmacology* 56, 223–229
55 Stokes, L. *et al.* (2006) Characterization of a selective and potent antagonist of human P2X(7) receptors, AZ11645373. *Br. J. Pharmacol.* 149, 880–887
56 Lord, B. *et al.* (2014) Pharmacology of a novel central nervous system-penetrant P2X7 antagonist JNJ-42253432. *J. Pharmacol. Exp. Ther.* 351, 628–641
57 Gu, B.J. *et al.* (2016) Innate phagocytosis by peripheral blood monocytes is altered in Alzheimer's disease. *Acta Neuropathol.* 132, 377–389
58 Abdi, M.H. *et al.* (2010) Discovery and structure-activity relationships of a series of pyroglutamic acid amide antagonists of the P2X7 receptor. *Bioorg. Med. Chem. Lett.* 20, 5080–5084
59 Ali, Z. *et al.* (2013) Pharmacokinetic and pharmacodynamic profiling of a P2X7 receptor allosteric modulator GSK1482160 in healthy human subjects. *Br. J. Clin. Pharmacol.* 75, 197–207
60 Letavic, M.A. *et al.* (2017) 4-Methyl-6,7-dihydro-4H-triazolo[4,5-c]pyridine-based P2X7 receptor antagonists: optimization of pharmacokinetic properties leading to the identification of a clinical candidate. *J. Med. Chem.* 60, 4559–4572