The Purinergic P2X7 Receptor as a Potential Drug Target to Combat Neuroinflammation in Neurodegenerative Diseases

Short running title: P2X7 receptor and neurodegeneration

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Abstract

Neurodegenerative diseases (NDDs) represent a huge social burden, particularly in the Alzheimer's disease (AD) in which all proposed treatments investigated in murine models have failed during clinical trials. Thus, novel therapeutic strategies remain crucial. Neuroinflammation is a common pathogenic feature of all NDDs. As purinergic P2X7 receptors (P2X7Rs) are gatekeepers of inflammation, they could be developed as drug targets for NDDs. Herein, we review this challenging hypothesis, and comment on the numerous studies that have investigated P2X7Rs, emphasizing their molecular structure and functions, as well as their role in inflammation. Then, we elaborate on research undertaken in the field of medicinal chemistry to determine potential P2X7R antagonists. Subsequently, we review the state of neuroinflammation and P2X7R expression in the brain, in animal models and patients suffering from AD, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, multiple sclerosis, and retinal degeneration. Next, we summarize the *in vivo* studies aimed at testing the hypothesis that by mitigating neuroinflammation, P2X7R blockers afford neuroprotection, increasing neuroplasticity and neuronal repair in animal models of NDDs. Finally, we reviewed previous and ongoing clinical trials investigating a plethora of compounds directed toward diverse targets associated with NDDs; we propose that clinical trials with P2X7R antagonists should be initiated. Despite the high expectations for putative P2X7Rs antagonists in various central nervous system diseases, the field is moving forward at a relatively slow pace, presumably due to the complexity of P2X7Rs. A better pharmacological approach to combat NDDs would be a dual strategy, combining P2X7R antagonism with drugs targeting a selective pathway in a given NDD.

KEYWORDS
P2X7 receptors, neurodegenerative diseases, neuroinflammation, P2X7 receptor drugability, P2X7 receptor antagonists

1 INTRODUCTION

Brain diseases affect one billion individuals worldwide, representing an economic burden higher than cancer and cardiovascular diseases combined. In Europe alone, 179 million neuropsychiatric patients represent 35% of the burden associated with all diseases, estimated at an annual cost of 800 billion euros.[1] The increased suffering observed in these patients is frequently associated with serious co-morbidities, multiplying the costs associated with non-medical care *i.e.* nursing homes, as well as indirect costs such as nonattendance at the workplace.[2] As NDDs are age-dependent, increasing longevity will progressively augment their prevalence and hence, the socio-economic burden.

Despite diverse symptomatology and pathophysiology, cumulative evidence suggests that neuroinflammation holds the central stage in NDDs, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), multiple sclerosis (MS), and retinal degeneration. Currently, treatments available for these disorders are merely symptomatic; hence, novel brain-permeable drugs are urgently needed to reduce disease progression. As purinergic P2X7 receptors (P2X7Rs) are considered gatekeepers of neuroinflammation, it is explicable that both academic and pharmaceutical research remain involved in drug development programs focused on the discovery of P2X7R antagonists with therapeutic potential in brain diseases.[3]

Here, we comprehensively review this hot topic, first presenting a concise account of P2X7Rs and purinergic signaling. Then, we highlight the molecular structure and function of P2X7Rs, emphasizing their role in inflammation. Next, we detail the molecular and pharmacokinetic aspects of different groups of P2X7Rs antagonists synthesized to date. Moreover, we comment on the contribution of P2X7Rs and neuroinflammation in association with the pathogenesis of NDDs, in both animal models and patients suffering from NDDs. Finally, we address the proof-of-concept of the therapeutic potential of P2X7R antagonists in animal models of NDDs, concluding with perspectives on clinical trials investigating these compounds.

2 PURINERGIC RECEPTORS AND PURINERGIC SIGNALING

Almost 50 years have passed since Geoffrey Burnstock unveiled “the purinergic nerves” as nerves that could be stimulated by a neurotransmitter distinct from acetylcholine (ACh) and catecholamines, namely adenosine triphosphate (ATP).[4-6] We are now aware that ATP performs its functions by acting on specific receptors expressed in immune cells, blood cells, dendritic cells, vascular smooth muscle cells, secretory cells, neuronal cells, as well as retinal cells. These receptors are divided into two classes depending on their predominant endogenous agonist: P1 receptors are activated by adenosine (ADO), while P2 receptors are activated by purine and pyrimidine nucleotides, mainly ATP and adenosine diphosphate (ADP).

P2 receptors are further classified into two groups: P2Y and P2X receptors. The former are metabotropic receptors and the latter are ligand-gated ion channels. They are homo- or heterotrimers with seven characterized subunits (P2X1-7), composed of two α-helical transmembrane domains (TM), a short intracellular N-terminal, a C-terminal more variable in length, and a large extracellular loop of 269-288 amino acids, mostly folded as β-sheets and loops.[7]. Depending on their distinct activation and desensitization kinetics, P2XRs are classified into three main groups. On one hand, P2X1 and P2X3 homomers are activated in the range of milliseconds and rapidly desensitize; on the other hand, the remaining subtypes are rapidly activated in approximately a second but can undergo slow desensitization or no desensitization. P2X4, P2X4/6, P2X2/3, and P2X2/6 receptors are slowly desensitized, while P2X5, P2X7, and P2X1/5 receptors present a persistent plateau in current during prolonged ATP applications.[8,9]. Except for the P2X5R which is Cl^- permeable, the other P2XRs allow Na^+ and Ca^{2+} influx and K^+ efflux, following their electrochemical gradient through the plasmalemma.

Notably, a high number of purinergic receptors have been identified, along with a rich system of enzymes capable of hydrolyzing endogenous ATP to adenosine, ADP or other active metabolites, thus clarifying the significance of modulating this signaling system in different physiological and pathological processes.[10-12] Furthermore, it has been demonstrated that these receptors are involved in cell homeostasis, degeneration and regeneration of neurons[13] and retinal cells,[14] cell proliferation and tumor growth,[15] immune disorders and inflammation,[16-18] thrombosis,[19] viral infections and replication,[20] epilepsy,[21] fibrosis and gout,[22] renal and cardiovascular inflammation[23], and finally, in

neurodegenerative and neuropsychiatric diseases.[24-28] Table 1 summarizes the subtypes of P2X7Rs, and their endogenous agonists, as well as their synthetic agonists and antagonists.

TABLE 1

3 P2X7 RECEPTORS

Over the past 50 years, researchers have demonstrated the activation of a growing number of cell types in almost all vertebrate tissues mediated by extracellular ATP. Response-eliciting ATP concentrations ($[ATP]_e$) were predominantly in the low μM range; however, in some cells such as macrophages and mast cells, hundreds of μM $[ATP]_e$ caused a distinct pattern of activation, consisting of Ca^{2+} influx and membrane "permeabilization" to fluorescent dyes up to approximately 1 kDa [29-32]. This response was attributed to a specific type of membrane receptors of the purinergic type, initially labeled P2Z [33], and later identified as P2X7 receptors [34]. In the 1990s, P2Z/P2X7 activation was involved in the release of interleukin (IL)-1β, as well as macrophage communication and differentiation [35-37]. F. Di Virgilio integrated the above research with the fact that the receptor was mainly expressed in immune lineage cells, proposing the currently accepted hypothesis that the physiological role of the P2X7 receptor was to mediate chronic inflammatory responses [38,39].

3.1 Molecular structure and function of P2X7 receptors

The P2X7R is a non-selective ligand-gated ion channel permeable to Na^+, Ca^{2+}, and K^+, which assembles as homotrimers. Some studies suggested that it could form heterotrimers with P2X4 subunits;[40,41] however, this hypothesis remains controversial, considering that no physical evidence of P2X4/P2X7 heterotrimers has been obtained (*e.g.* through cross-linking or blue native PAGE studies) in overexpressing systems or native tissue.[42-44]. Using alternative splicing and single-nucleotide polymorphisms (SNPs), various conformational changes of the receptor resulted in enhanced or inhibited activity.[45] This receptor is distinguished from other subtypes by its longer C-terminus of 239 residues,

which also contains a lipopolysaccharide (LPS)-binding domain, modulating LPS-triggered intracellular signal transduction.[46] The recently revealed rat P2X7R structure presented a dinuclear zinc-ion complex and a guanosine nucleotide-binding site in the C-terminal domain. These sites do not influence receptor gating and future studies are needed to determine their involvement in P2X7R signal transduction modulation.[47]. The P2X7R exhibits low affinity for ATP and is permeable to small molecules up to 900 Da in molecular weight.[34] This feature seems to be attributed to distinct characteristics of its ATP binding pocket, which lacks four amino acids with respect to other subtypes and is characterized by more hindered and hydrophobic residues.[48,49] This results in a narrower pocket less likely to be exposed to the solvent, and consequently more inclined to structural fluctuation, decreasing ATP accessibility[47], according to the pocket breathing motion model discussed by Stank *et al*. [50].

Several natural splice variants (P2X7A-J) were discovered in human and rodent tissues; P2X7A is the well-characterized full-length P2X7R. The P2X7B isoform is particularly relevant owing to its sequence similarity with other P2X receptors, a wide tissue distribution, and a lack of the proapoptotic P2X7A actions [51,52]. This suggests that P2X7R responses could be finely tuned by the expression of various P2X7R isoforms. Moreover, this has been elegantly illustrated in a recent study: transfection of P2X7B augmented NFATcl activation, enhanced ATP content, and supported cell growth; however, the transfected cells failed to undergo ATP-triggered apoptosis. Additionally, P2X7B was shown to co-assemble with P2X7A forming a P2X7A+B heterotrimer directly modulating its functions, including pore formation. An interesting hypothesis suggested that P2X7A receptors could be considered the "deadly" molecular evolution of an otherwise harmless P2X7B receptor [53]. Thus, P2X7B is capable of forming functional channels, but not the large pores frequently associated with inflammation and cell death [53].

P2X7K[54] is fully functional in mice, with an 8-fold higher sensitivity to agonists, slower deactivation, and augmented ability to form pores than the human variant. Additionally, nicotinamide adenine dinucleotide (NAD) activated the P2X7K isoform; however, it remained unable to activate P2X7A[55]. In this study, P2X7A was preferentially expressed by macrophages and P2X7K was expressed in T cells. In rodents, this could give rise to differences in P2X7R signaling between different cell types[56,57]. The influence of these and other variants on P2X7R signaling, both *in vitro* and *in vivo*, could present a confounding factor in preclinical studies using knockout or transgenic mice; particularly,

the difference in sensitivity of these variants to P2X7R antagonists being investigated could be relevant, as well as the extrapolation of these outcomes to clinical trials in humans. Moreover, approximately 150 nonsynonymous (or missense) SNPs, with altered human P2X7Rs functions, further complicate an already complex situation [58-60].

To date, no crystal structure of the human receptor (hP2X7R) has been demonstrated by co-crystallization with ATP, neither in the apo-state nor the active state. Nevertheless, receptor activation and inhibition mechanisms were first investigated through homology modeling, by utilizing an already existing hP2X3R and zebrafish P2X4R (zfP2X4R), and more recently through the crystallization of the giant panda (*Ailuropoda melanoleuca*) ortholog (85 % of identity with the human receptor) in its apo-state, or along with several antagonists.[49,61] In these studies, each P2X7 subunit demonstrated the typical conserved dolphin-like structure, composed of the head domain (HD), the upper body (UB), the lower body (LB), the dorsal fin (DF), the left and right flippers (LF and RF, respectively), and the fluke (F). ATP, in its free ion form, binds to the so-called "binding jaws", located in the interface of two subunits, exactly at UB, between HD and DF (Figure 1).

FIGURE 1

ATP adopts a U-shaped conformation upon binding through the interaction of phosphates with the adenine base, similar to the ATP interaction with class II aminoacyl transfer RNA synthetases. However, when ATP binds to other proteins, the preferred conformation is stretched.[62] During activation, the UB subunit shrinks toward the central axis of the receptor, leading the LB to broaden, forming fenestrations allowing the passage of ions through a lateral pathway, away from the central axis. These conformational changes result in a vertical stretch and rotation of the two TM domains, thus opening the channel gate. A structural characteristic of the P2X7R is an extracellular pocket located internally at the top of the UB, which is wider than in other family subtypes. This is the allosteric binding site for several antagonists, as demonstrated by the crystallized panda P2X7R and in subsequent mutagenesis and molecular docking studies.[49,63] This information could help design and develop new selective ligands.

Finally, P2X7Rs sensitivity to ATP differs between species. In detail, ATP has a higher pEC_{50} in rat P2X7R than in mouse and human orthologs (3.4±0.10 against 2.7±0.07 and 2.8±0.06, respectively), demonstrating lower efficacy. Furthermore, these differences are evident when the more potent benzoylbenzoyl-ATP (BzATP) is used as an agonist (pEC_{50}: 5.2±0.09 for rat, <3.5 for mouse, and 4.1±0.06 for human P2X7R) [64] and can be correlated with dissimilarities in current kinetics. In stably transfected HEK293 cells, measured rat P2X7R currents revealed a higher decay time than other orthologs under BzATP stimulation. This depended on agonist association and dissociation rates, determined by protein-ligand interactions, implying that structural differences among species are responsible for these observations[64]. Notably, Young *et al.* demonstrated that mouse P2X7R sensitivity for ATP and BzATP approached that of the rat when mouse aspartate 284 and alanine 127 were converted into the corresponding rat amino acids at these positions, asparagine and lysine (*i.e.* D284N and A127K mutations) [65].

Additionally, these differences in ligand potency were observed for several allosteric modulators. For instance, AZ11645373 is a potent antagonist of human and dog P2X7Rs at nanomolar concentrations, active only at μM concentrations in mouse orthologs and inactive in rats[66]. These differences were attributed to amino acid 95, which is phenylalanine in human P2X7R and can form a pi-stacking interaction with AZ11645373, differing from leucine 95 in rat orthologs.[66,67]. Another example of species-dependent differences is reportedly the pharmacological activity of GW791343, a potent P2X7 antagonist in humans, but a positive allosteric modulator in rats [68].

3.2 The P2X7 receptor as a gatekeeper of inflammation

A recent review by Francesco Di Virgilio and coworkers addressed the role of P2X7R as the main player in inflammation.[39] This is understandable considering that P2X7Rs are expressed by immune and inflammatory cells and are upregulated in inflammatory processes, including dendritic cells, osteoclasts, and microglia, the most widely studied cells from the monocyte/macrophage axis. Additionally, P2X7Rs are expressed by mast cells, NK cells, and T and B lymphocytes.[69]

Cell components including ATP are released into the extracellular space as a result of stress or tissue damage (danger-associated molecular pattern, DAMP), inducing the activation of protective/regenerative immune responses. It has been extensively

recognized that P2X7Rs are the main sensor for high concentrations of ATP during inflammation, as the concentration required to activate them (EC_{50}=100 μM) is one or two orders in magnitude than for other P2XRs[70], demonstrated under conditions of stress and tissue damage. Additionally, P2X7Rs are the main trigger of the immune response by mediating the maturation and secretion of several interleukins, mainly IL-1β.[71,72] The maturation of IL-1β requires the activation of the NLRP3 inflammasome by ATP-dependent K^+ efflux.[72-75] This intracellular K^+ drop is possibly the best-established mechanism underlying P2X7R-mediated formation of the NLRP3 inflammasome.[76]

Ion fluxes are critical events in NLRP3 inflammasome activation. For example, various inflammasome activators, including ATP, nigericin, and particulate or crystalline molecules, induce K^+ efflux and a concomitant decrease in its intracellular concentration[76,77]. Additionally, Na^+ influx accompanied by water influx diminishes the cytosolic K^+ concentration below the threshold, leading to inflammasome activation[78].

Notably, reactive oxygen species (ROS) induce inflammasome aggregation and IL-1β release, secondary to P2X7R activation.[79,80] Additionally, protein-protein interactions between P2X7Rs and the inflammasome scaffold occur in astrocytes, where they mediate IL-1β maturation and release;[81] this also occurs at restricted subplasmalemmal areas of microglia and macrophages.[82] The synthesis of immature pro-IL-1β occurs through a process requiring the activation of the nuclear factor kappa B (NF-κB) . In turn, maturation of IL-1β requires cleavage by caspase-1 (Casp-1), matured and activated via inflammasome formation.[83,84] This cascade ends with IL-1β release into the extracellular space through non-classical transport mechanisms,[85] finally triggering inflammatory processes (Figure 2).

P2X7Rs have been associated with various neurological disorders exhibiting pathogenic inflammatory features. Indeed, these receptors are expressed by several brain cell types, including astrocytes, microglia, oligodendrocytes, and Schwan cells.[86] However, whether P2X7Rs are additionally expressed in neurons currently remains controversial.[87,88] In Section 5.1 we will further comment on evidence suggesting that P2X7Rs and neuroinflammation may be involved in various NDDs.

FIGURE 2

4 P2X7R ANTAGONISTS: MOLECULAR STRUCTURE AND DRUG-LIKENESS

Initial attempts to synthesize P2X7R ligands were demonstrated in nucleotide analogs with broad-spectrum anti-purinergic receptor activity, including the covalently bound, irreversibly oxidized ATP (oATP) (Figure 3),[89] found to attenuate pro-inflammatory signals via P2 receptor-independent mechanisms.[90] Other frequently used pharmacological tools include the non-selective competitive P2X7R antagonist pyridoxal phosphate derivative PPADS,[91], as well as the non-competitive triphenylmethane dye, Brilliant Blue G (BBG),[63,92] the naphthylsulfonate suramin and its isoquinoline derivative, KN-62,[93] also known for inhibiting CaM kinase II (Figure 3). BBG has been utilized in both *in vitro* and *in vivo* experiments; however, this compound also targets P2X4, P2X5, rat $P2Y_{1,}$ and $P2Y_2$ receptors, as well as voltage-gated sodium channels.[8,94,95] BBG was initially the main compound used for the proof-of-concept of P2X7Rs role in NDDs, such as ALS and AD.[96-98] Its permeability across the blood-brain barrier (BBB) remains dubious as it is a heavy and charged molecule; however, BBG reaches central nervous system (CNS) damaged tissues probably through BBB disruption in animal models of NDDs. [99] During the discovery of novel ligands for P2X7Rs, interest shifted from peripheral disorders such as rheumatoid arthritis[100,101] and Crohn's disease,[102] to pathologies of the CNS; this was reasonable considering the important role that P2X7Rs play in neuroinflammation and subsequently, in NDDs.[16,103]

The search for more potent and selective P2X7R antagonists targeting the CNS with BBB permeable compounds led to the discovery of novel derivatives, including adamantane and *o*-chlorobenzamide moieties (**1** – Figure 3).[104,105] In this context, we will comment on second-generation compounds focused on reproducing P2X7R blockade, thereby blocking IL-1β release, and also on improving their pharmacokinetic properties for targeting CNS diseases. Notably, most work has been performed by *Big Pharma*, underlining the therapeutic relevance of P2X7Rs in implicated diseases. Despite some examples that could be considered "me-too" derivatives, each company has initiated MedChem programs from high-throughput screening (HTS) of their chemical libraries.

FIGURE 3

Kim and coworkers discovered that some analogs of berberine alkaloids possessed hP2X7R blockade capacity. The chemical modifications proposed on hit compounds, in particular the alkyls and benzyl groups, gave rise to a set of quinazolinium derivatives with IC_{50}s around 1 μM, the most potent being the nitro-derivative **2** (IC_{50} = 0.17 nM, 2-fold more potent than KN-62) (Figure 4).[106] Additionally, this compound exhibited the highest potency to block LPS/interferon (IFN)γ-induced IL-1β release from THP-1 cells (IC_{50} = 175 nM). Another screening identified chiral pyrazolodiazepinones with some activity against hP2X7Rs expressed in HEK293 cells. This inhibition and related suppression of IL-1β release were improved following the incorporation of an *N*-methylindole cycle, linked by an ester at the central core;[107] this resulted in ligands with activity similar to KN-62, such as **3** (Figure 4). This second family of compounds was universally less potent than the former, lacking quaternary nitrogens, thereby increasing BBB permeability. More recently, investigators described a series of pyrimidine-2,4-dione related to KN-62, possessing isoquinoline-5-sulfonate and phenylpiperazine moieties in their structure.[108] Substitution at the pending phenyl ring, as well as the presence or absence of a spacer (methyl or carbonyl), conditioned the structure-activity relationship. For instance, benzoyl analogs with different halogen substitutions afforded the best inhibition, as in the case of derivative **4,** which was superior to KN-62 (Figure 4). Furthermore, several researchers demonstrated that the piperazine substructure well accepted other acyl groups of hydrophobic nature (alicyclic or cyclic alkyls, such as adamantyl), offering similar blockade. Compound **4** was selected for investigating some of its pharmacokinetic (PK) parameters, presenting high metabolic stability and low cytotoxicity and cardiotoxicity.

FIGURE 4

Another approach involved a design based on *N*-benzylacetamide with halogen phenyl substitution (Figure 5). Moreover, different aryls and heterocycles were extensively probed. The first hit, bearing an *N*-phenylpyrazole (**5**, R = Ph, X = F, Figure 5), revealed

poor *in vitro* stability. Most attempts to improve blockade and PK profile frequently focused on replacing halogens, the amide spacer, or the pyrazole ring. Removal of the phenyl at the pyrazole (R = H, Figure 5) was essential for enhancing both properties, resulting in ligands **6** (R = H, X = F)[109] and **7** (R = H, X = Cl)[110]. Unfortunately, this additionally inhibited cytochrome P450 3A4 (CYP3A4); hence, researchers selected another hit, providing clues to modify the initial structure by replacing the pyrazole ring, possessing metabolically-labile methyl groups, with cyclic pyroglutamic amides, exemplified by **8** (Figure 5). This change avoided CYP inhibition while maintaining metabolic stability and marginally improving P2X7R blockade, as observed with compound **8**[111] (Figure 5). This compound was evaluated in animal models of pain but in some, its half-life was not as expected. Therefore, to eliminate metabolically-labile positions, pyroglutamic acid was again replaced by other heterocycles, demonstrating satisfactory outcomes with the imidazole moiety, as the IC_{50} of that family was in the nanomolar range. Furthermore, these compounds presented low microsomal clearance. Among these, the unsubstituted imidazolone **9** appeared to stand out[112] (Figure 5).

Based on the work investigating pyroglutamic acid derivatives, Ghinet and coworkers have recently described constrained analogs obtained as unexpected products (**10 –** Figure 5) from the treatment of pyroglutamic acid lactams with the Bredereck's reagent.[113] Despite their synthetic interest, none of the resulting pyrroloimidazolediones offered good P2X7R blocking activity and their intermediates, presenting a pyroglutamide structure and diversely-substituted dimethylaminomethylenes, were markedly agreeable blockers.

FIGURE 5

An *o*-chlorobenzamide derivative, bearing a 1,2,4-triazine heterocycle, showed moderate P2X7R inhibitory activity and extremely high *c*log P (**11**) (Figure. 6). Theoretically, this property is compatible with high BBB permeability, an effect that entails low solubility, a drawback for drug development. Therefore, the program focused on improving the blocking activity by preparing compounds possessing a lower *c*log P.[114] Based on an understanding that a large increase in polarity at the amide compromised inhibitory

activity, the long hydrophobic chain bound to the amide nitrogen was replaced by bulky alkyl, aryl, or heteroaryl groups. In this work, the presence of (1-hydroxycycloheptyl)methyl, as a substituent for the amide (**12**) (Figure 6), lowered *c*log P to 2.9, improving the potency to inhibit IL-1β release by 10-fold with respect compound **11**. Based on this replacement, *c*log P was further lowered by incorporating polar alkyl chains at the 1,2,4-triazine core, as shown in **CE-224,535** (Figure 6). Additionally, the potency to block IL-1β release was 59-fold higher than that observed in **12** and consequently, **CE-224,535** was evaluated in clinical trials for rheumatoid arthritis (see Section 7).

FIGURE 6

Abbott Laboratories disclosed a series of cyanoguanidines with high potency and good pharmacokinetic properties. **A-740003** (Figure 7) was the first to distinguish itself based on its high potency in the nanomolar range.[115] In animal models of neuropathic pain, the compound attenuated tactile allodynia; however, a radiotracer study revealed its poor BBB permeability.[116] Further modifications on this scaffold by retaining the isoquinoline-cyanoguanidine moiety presented cyanoguanidine-piperazine P2X7R antagonists with slight differences in activity with respect A-740003 [117]. Substitution of the piperazine core with a simpler (R)-α-methylbenzylamine resulted in **A-804598** (Figure 7), a ligand with an IC_{50} of approximately 10 nM in rat, mouse, and human P2X7R, as evaluated in calcium flux assays[118]. Furthermore, it proved certain stability in brain tissues, at least after 1 h of oral administration[119]. A-740003 and A-804598 have both been crystallized in the panda P2X7R, demonstrating that they bind in the allosteric binding pocket at the interface of the two subunits' upper bodies [49]. Moreover, they are considered the two P2X7R antagonists presenting the least potent differences between species [120].

Kassiou and coworkers pursued the adamantane cycle to obtain drug-like P2X7R ligands. Thus, they improved the solubility of target ligands by including non-protic, highly polar moieties such as cyanoguanidine.[121] A large set of differentially linked adamantane-cyanoguanidine adducts was evaluated, where the 5-quinolyl derivative **13** (Figure 7) demonstrated the highest potency. However, its *c*log P, close to 5, dissuaded any further

assessment, and was substituted by the more polar compound **14** (Figure 7), with improved PK properties, still far from ideal. In contrast, Kassiou and coworkers proposed derivatives of **1** (Figure 3), defined by either replacing the *o*-chlorobenzamide group by heteroaromatic analogs, such as pyridines or diazines, or replacing some adamantane hydrogens by fluorine atoms[122] (Figure 7); this could be justified by the bioisosteric nature of these chemical modifications, which would presumably reduce lipophilicity and increase metabolic stability. However, although the experimentally obtained log D of the aza-analogs of **1** was slightly reduced, their hP2X7R blockade was potent, particularly for **15** (Figure 7). Regarding the fluorinated derivatives of **1** (Figure 3), the lipophilicity was substantially decreased and hP2X7R blocking activity remained in the same order of magnitude, implying an improvement in the ligand-lipophilicity efficiency. This promising success prompted the evaluation of their PK characteristics, such as stability in liver microsomes, permeability, and CYP inhibition, as well as P-glycoprotein (P-gp)-efflux. The trifluorinated **16** (Figure 7) exhibited the highest metabolic stability, 6-fold higher than **1,** presenting the lowest CYP inhibition rate. Additionally, **16** demonstrated the highest bioavailability, as well as a prolonged brain half-life. All of these data revealed that **16** warranted further preclinical evaluation in models of CNS disorders where P2X7Rs have been implicated.

FIGURE 7

A new series of heterocyclic derivatives were linked to the *o*-chlorobenzamide moiety, and could be considered as a juxtaposition of **JNJ-42253432** (Figure 8)[123] and compound **17**[124] (Figure 8). Position C2 of the quinoline demonstrated diverse alkylamines;[125] their activity on P2X7Rs was scattered and differences between human and mouse orthologs were observed. Compound **18** was considered superior, bearing a 3-(*R*)-hydroxypyrrolidine at C2 (Figure 8), which demonstrated good bioavailability and a significant, though time-dependent, reduction in IL-1ß release in C57BL/6 mice. Moreover, another member of this class of ligands, **JNJ-47965567** (Figure 8), was pharmacologically characterized. In particular, it efficaciously reduced the sensitization of amphetamine-induced locomotion, without exhibiting robust results in models of neuropathic pain, or the forced swim test as a model of depression in rats.[126] JNJ-

47965567 is one of the antagonists crystallized in the panda P2X7R and its binding site was the same allosteric pocket as that for the above-mentioned ligands[49].

FIGURE 8

Simultaneous to the development of these P2X7R ligands, investigators examined the triazolopiperidine scaffold, linked to the *o*-chlorobenzamide moiety (Figure 9). Initially, the influence of aza-aromatic ring substitution at the triazole was examined, as exemplified by **19**[127] (X = H, Figure 9). Aza-aromatics reduced both log P and CYP inhibition. Compound **19** was selected as the lead to be optimized by fixing its pyrimidine heterocycle at *N*1 and replacing the 3-trifluoromethyl-2-chlorophenyl by other halogenated benzenes. However, no significant improvements were obtained in PK values; additionally, in some cases, the potency was diminished, and the cross activity between orthologs deteriorated universally. Preclinical studies demonstrated that the incorporation of a fluorine atom (**20,** X = F, Figure 9) improved PK parameters such as bioavailability and solubility. Further studies aimed to evaluate the effect of an *R*-methylation at the piperidine C4,[128] with the intent of increasing microsomal metabolic stability and potency, like that previously appreciated in the P2X7R antagonist, **JNJ-54166060** (Figure 9); this compound has also demonstrated nanomolar P2X7R antagonist activity, as well as a good PK profile,[129] but was affected by an unavoidable CYP3A interaction. Using an *R*-methylated **19** derivative at C4 as the scaffold, single aza-aromatics were installed at *N*1 of the triazole nucleus, furnishing ligands with nanomolar-ranged potency, highlighting the 4-(*R*)-methyl derivative **20** (**JNJ-54175446** – Figure 9); this compound lacked CYP inhibition and demonstrated elevated metabolic stability, high bioavailability, and low off-target pharmacology. Further pharmacological experiments revealed high tolerability, resulting in compound JNJ-54175446 as a candidate for clinical studies.

By shuffling the methyl group from C4 to C6 using configuration inversion, a new family of triazolopiperidines was revealed[130] (Figure 9), presenting different aromatic *N*-substitutions at the triazole. Their P2X7R activity was as good as observed for previous

families, presenting low solubility and poor stability. To resolve these obstacles, benzamide was replaced with isonicotinamide, as shown in **JNJ-55308942** (Figure 9). Solubility and metabolic stability were improved, but the activity was diminished. The *para*-fluor derivative was again the best of the family, demonstrating an increased plasma free fraction, high tolerability, and cardiovascular safety. Based on these findings, **JNJ-55308942** was selected as a second P2X7R antagonist to enter clinical trials for depression. Phase I ended in March 2018, evaluating the safety of the compound in healthy participants. In 2017, **JNJ-54175446** (Figure 9) was tested in healthy volunteers and patients experiencing major depressive disorders[131]. Currently, it has entered phase II clinical trial for the same indication, as stated in the ClinicalTrials.gov database.

FIGURE 9

Collectively, several P2X7R antagonists have been developed through the years, exploiting different scaffolds and molecular structures. Nevertheless, only a few ligands succeeded in entering clinical trials, and the majority of them in peripheral disorders.[100,102] This is attributed to poor knowledge of the hP2X7R structure, and primarily the lack of its crystal resolution, and additionally the need to discover proper drug-like properties to target the CNS, *i.e.* sufficient lipophilicity, water solubility, and brain tissue half-life. Hence, *BigPharma* pays considerable attention to assess receptor occupancy of the newly developed ligands, as well as their efficacy in the brain, by performing well-established *ex vivo* autoradiography assays or *in vivo* microdialysis in animals. Table 2 summarizes the pharmacodynamics, PK, and drug-likeness of available P2X7R antagonists.

TABLE 2

5 NEUROINFLAMMATION AND P2X7Rs IN NEURODEGENERATIVE DISEASES

5.1 Neuroinflammation, a common pathogenic feature of NDDs

Neuroinflammation is characterized by the presence of reactive astrocytes and microglia in the CNS. These cells, as well as peripheral immune cells and endothelial cells, produce pro-inflammatory cytokines (IL-1β, IL-16, TNF-α), chemokines (CCL2, CCL9, CCL1), ROS, and secondary messengers (NO, prostaglandins).[132,133]

The duality of the neuroinflammatory status remains of interest, as glial cells may exhibit pro- and anti-inflammatory functions. Thus, a low-level of glial inflammation causes corticosteroid release, free radical reduction, phagocytosis, and cellular repair. This may support synaptic plasticity, as well as regulate learning and memory.[134,135] Conversely, a high level of destructive neuroinflammation is observed, with a notable production of pro-inflammatory mediators, infiltration of peripheral immune cells, edema and augmented permeability of the BBB[136-138], as observed in the case of acute CNS infections[139] or stroke.[140]

Between these two extreme cases, lies the concept of low-level glial cell activation, with low to moderate levels of inflammatory mediators present in the parenchyma of the brain or spinal cord in patients with NNDs; in the long term, this low inflammatory status leads to synaptic dysfunction, loss of synapses, and neuronal death.[141] Furthermore, there exists an interesting hypothesis based on the fact that microglia have the same progenitor origin as macrophages.[142] Glial cells exhibit a low overall turnover rate;[143,144] this may explain why glial cells are susceptible to potential pro-inflammatory effects of age, injury, stress or NDDs.[133]

In this context, investigations surrounding the neuroinflammatory status of NDDs have gained momentum during the last few years. Hence, imaging techniques such as positron emission tomography (PET) are being utilized to quantify the inflammatory status of the brain and spinal cord in patients with NDDs. This approach has assisted in identifying pathophysiological features of NDDs, as well as novel targets for the development of drugs that, by acting on neuroinflammatory pathways, could exhibit neuroprotective properties.[145] We will next review the available evidence demonstrating the presence of neuroinflammation and its potential relationship with P2X7Rs, from preclinical *in vitro* and *in vivo* models of NDDs, as well as from the brain of patients suffering from these diseases.

However, results from preclinical studies using P2X7R knockout (KO) mice should be considered with caution. For instance, KO strains from GlaxoSmithKline (GSK)[146] or Pfizer [147] have been extensively used to explore the role of P2X7Rs in inflammatory and neuropathic pain (GSK KO), inflammation, cytokine production, bone formation and various disorders (Pfizer KO)[148]. However, the discovery of P2X7R splice variants suggests that these mice may not be true knockouts.

For instance, a P2X7R variant escaped deletion in the GSK KO and demonstrated its highest expression in the spleen [54]. Furthermore, the P2X7 13B and 13C C-terminal truncated variants escaped deletion in Pfizer KO mice [149]. Moreover, in other studies, anti-P2X7R antibodies detected P2X7R in both mouse strains, although the P2X7A receptor was knocked out in these strains, thus suggesting that antibodies may be detecting splice variants that escaped deletion[150,151]. Therefore, caution is warranted with data interpretation from *in vivo* studies utilizing these KO mice.

5.2 Alzheimer's disease (AD)

The involvement of P2X7R-induced neuroinflammatory responses in AD has been supported by *in vitro* and *in vivo* preclinical studies, as well as postmortem studies in the brain of AD patients. An early study demonstrated that two P2X7R agonists, namely ATP and the more specific BzATP, enhanced $[Ca^{2+}]_c$ and H_2O_2 production in rat microglia. These *in vitro* effects were blocked by three P2X7R antagonists, oATP, BBG, and PPADS. Furthermore, interestingly, ATP and BzATP elicited neuronal death in glia-neuron co-cultures.[152]

In microglia and THP-1 cells, enhanced Ca^{2+} entry triggered by stimulation with BzATP produced an inflammatory response by enhancing p38 MAP kinase and NF-κB, with concomitant release of IL-6, TNF-α, and nitrite ions neurotoxic to SH-SY5Y cells. These effects were ameliorated by Mg^{2+} and the P2X7R antagonists oATP and KN-62. Investigators have suggested that Mg^{2+} could reduce Ca^{2+} induced neuroinflammation in AD.[153]

Microglia activation by Aβ requires P2X7R expression as demonstrated in experiments by Francesco Di Virgilio and coworkers, demonstrating that in microglia from wild type (WT) mice, Aβ induced the release of ATP and IL-1β and augmented plasmalemmal

permeability; these effects were not observed in microglia from P2X7R-deficient mice, suggesting that microglial activation requires the presence of P2X7Rs.[154] Additionally, silencing of P2X7Rs augmented microglial phagocytosis induced by Aβ.[155] A more recent study showed that P2X7R overexpression induced by Aβ oligomers, augmented synaptic failure and neuronal dyshomeostasis in cellular models of AD.[156] Additionally, a paradoxical neuroprotective effect of P2X7R stimulation has been reported.[152] Moreover, P2X7R stimulation causes the activation of the soluble APP ectodomain (sAPPα) from mouse neuroblastoma cells, expressing human APP, as well as from human neuroblastoma cells, mouse astrocytes, or neural progenitor cells. This effect was not observed in P2X7R-deficient mice.[157]

Furthermore, some *in vivo* experiments support a role of P2X7Rs in pathogenic neuroinflammatory features of animal models of AD. For instance, one study showed that P2X7Rs were overexpressed in astrocytes and microglia surrounding the Aβ plaques in the Tg2576 mouse model of AD.[152] A second study in APP/PS1 mice observed that P2X7Rs were mostly located in microglia generating excessive ROS, with ensuing dendritic damage. The investigators correlated these findings with Aβ increase and synaptotoxicity.[158] Furthermore, in the rat hippocampus injected with $A\beta_{1-42}$, a considerable amount of P2X7Rs colocalized with microglia.[159] A similar finding was recently reported in P3015 mice, a model of tauopathy; in this model, P2X7Rs were predominantly expressed by astrocytes.[160]

A study investigating the brain of AD patients showed augmented expression of P2X7Rs in microglia; P2X7Rs were predominantly expressed in association with Aβ plaques and localized in immunoreactive microglia. Additionally, in cultured fetal human microglia, $A\beta_{1-42}$ significantly elevated P2X7R expression. Furthermore, the Ca^{2+} signals evoked by BzATP were increased in these cells and were blocked by *o*ATP. The investigators proposed that P2X7Rs mediated microglial purinergic inflammatory responses in AD brain.[159]

The proof-of-concept of P2X7Rs as a drug target for AD was tested in a study evaluating J20 mice transgenic for human mutant APP. *In vivo* inhibition of P2X7Rs with BBG induced a significant decrease in hippocampal amyloid plaques. This reduction correlated with a decrease in glycogen synthase kinase 3 activity, increasing the proteolytic processing of APP through increased α-secretase activity.[161] Conversely, in rats injected

with $A\beta_{1-42}$, BBG improved cognition and augmented dendritic spines in hippocampal neurons.[96] Furthermore, BBG was neuroprotective and antagonized the neuroinflammatory responses induced by P2X7R agonists in a rat brain injected with Aβ.[162]

5.3 Parkinson's disease

The involvement of P2X7Rs and neuroinflammation in the pathogenesis of PD has extensive experimental support.[23] For instance, *in vitro* experiments revealed that ATP and 6-hydroxydopamine (6-OHDA) induced cytotoxicity in SN4741 dopaminergic neurons via activation of P2X7Rs.[163] In contrast, ATP release induced by stimulation of SH-SY5Y cells was mediated by direct activation of P2X7Rs by α-synuclein.[164] Both postmortem analysis of brain tissue from PD patients and *in vivo* experiments in animal models of PD support the association between P2X7Rs, neuroinflammation, and loss of dopaminergic neurons in PD.

Notably, P2X7Rs are upregulated in the brain of PD patients.[165] This is in line with the supposition that excessive microglial activation and chronic neuroinflammation contribute to dopaminergic neuron degeneration in the substantia nigra and striatum of PD patients. Furthermore, the accumulation of oligomerized α-synuclein is postulated to mediate neurotoxicity by activating microglia, followed by the production of ROS and pro-inflammatory mediators.[166] Importantly, α-synuclein binds to P2X7Rs in microglia and stimulates P2X7R transcription,[167] thus explaining the upregulation of P2X7Rs in PD brain.[165]

In the brain of PD patients, notable microgliosis has been observed; this can be mimicked in a rat model of PD by unilateral intranigral injection of 6-OHDA, inducing DA depletion, nigrostriatal lesions, and microgliosis. P2X7R antagonists such as BBG or A-438079 attenuate microgliosis and mitochondrial dysfunction, as well as motor and memory impairment, reinforcing that P2X7Rs take center stage in neuroinflammation and microglia activation in PD. [168-170]

In PD, the *in vivo* proof-of-concept that P2X7R blockers can protect DA neurons from degeneration has been investigated in several studies. For instance, using tyrosine hydroxylase (TH) as a marker, it was observed that BBG prevented the loss of DA

neurons in rats injected with 6-OHDA.[168,171] Similar neuroprotective effects of BBG have been demonstrated in an intranigral LPS animal model of PD, presenting marked reduction of activated microglia and protection of DA neurons.[172]

Additionally, some reports were unable to establish the neuroprotective effects of P2X7R antagonists. For instance, an intranigral injection of the P2X7R antagonist, A-438079, attenuated DA store depletion; however, it failed to reduce the loss of DA neurons in the 6-OHDA model of PD.[170] Similarly, either deletion of P2X7Rs or their pharmacological inhibition failed to afford neuroprotection in the MPTP mouse model of PD.[173] Despite these findings, overall evidence supports the hypothesis that P2X7R-elicited microglial activation is linked to PD pathogenesis. However, doubts persist regarding whether the neuroprotection conferred by P2X7R antagonists results solely from microgliosis inhibition, or whether derived from a direct effect on neuronal P2X7Rs. Two types of approaches emphasize microglia as targets for P2X7R antagonists. First, the immunocytochemical observations discussed above showed P2X7R expression colocalizing with nigrostriatal microglia in animal models of PD. Second, a recent brain analysis of conditional humanized P2X7R mice confirmed the expression of P2X7Rs in all major non-neuronal lineages throughout the brain, including microglia, astrocytes or oligodendrocytes; P2X7Rs were further observed in pyramidal neurons of the hippocampus, questioning their presence in DA neurons.[174]

5.4 Amyotrophic lateral sclerosis

The challenges in establishing new ALS treatments are partly attributed to the unknown disease pathophysiology. However, there is evidence implicating neuroinflammation and P2X7Rs in disease pathogenesis. The initial evidence appeared when protein levels of P2X7Rs were found to be upregulated in the postmortem spinal cord tissue of ALS patients, along with the enzyme cyclooxygenase 2 (COX-2). Expression of this enzyme is increased upon injury or inflammation, stimulated by the release of the pro-inflammatory cytokines IL-1β and TNF-α, through the activation of P2X7Rs.[175] In ALS, the relationship between P2X7R activation and motoneuron death was further established in co-cultures of astrocytes and motoneurons from $SOD1^{G93A}$ and WT mice. Repeated stimulation of P2X7Rs with ATP or BzATP triggered a neurotoxic phenotype in control

and SOD1^{G93A} astrocytes, inducing motoneuron death prevented by treatment with BBG.[176]

Despite these results, which suggest negative effects of P2X7Rs in ALS, genetic ablation of the receptor in SOD1^{G93A} mice accelerated and worsened disease progression, inducing earlier disease onset[177] and suggesting a dual role of the receptor in the disease, with protective activity at some disease stages. This dual role could be explained by the two different phenotypes presented by microglia upon P2X7R activation: M1 or pro-inflammatory and M2 or anti-inflammatory. Reportedly, M2 is found to be predominant in the early disease stages, while M1 appears more prevalent at later stages, as observed in aged SOD1^{G93A} mice.[178,179]

The rationale why microglia evolve toward an M1 phenotype at a certain stage of the disease remains elusive. However, ALS genetics could partly explain this through the dysregulation of miRNA production owing to the above-mentioned mutations in *FUS* and *TDP-43* genes. Several miRNAs were found to be upregulated in SOD1^{G93A} microglia, with miR-125b being the most relevant.[180] The involvement of miRNA-125b in microglia activation toward M1 phenotype and motoneuron death was established in microglial primary cultures from SOD1^{G93A} mice. Reportedly, miRNA-125b activated the NF-κB signaling pathway, leading to transcription of the pro-inflammatory genes *TNF-α*, *Ncf-2*, and *NOX2*. Upregulation of miRNA-125b correlated with an increase in the transcription and activation of P2X7Rs, which upon ATP stimulation resulted in the release of the pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-10, and further miRNA-125b transcription. Under normal conditions, this vicious circle of neuroinflammation would be controlled by the anti-inflammatory protein A20, which blocks the NF-κB signaling pathway. However, in ALS, microglia A20 was downregulated upon P2X7Rs stimulation with BzATP, contributing to the chronic neuroinflammatory environment present in SOD1^{G93A} mice.[179]

Furthermore, other signaling pathways are altered and implicated in the development of ALS, such as the NADPH oxidase (NOX) pathway, a major source of extra and intracellular ROS in microglia. In SOD1^{G93A} mice, stimulation of microglial P2X7Rs with ATP promoted the stimulation of NOX2 and the kinase ERK1/2, which together provoked an increased generation of ROS.[177] Additionally, P2X7Rs have been found to modulate autophagy in SOD1^{G93A} microglia, resulting in microglial activation. A short

stimulation of P2X7Rs augmented autophagy markers and M2 microglial phenotype markers. However, persistent stimulation of the receptor resulted in impairment of the autophagic flux, which could correspond with the change to M1 phenotype.[181]

Although these studies presented the general idea that P2X7Rs and their signaling pathways are upregulated in ALS patients and SOD1^{G93A} mice, one study reported downregulation of the receptor and intracellular calcium dysregulation in peripheral blood mononuclear cells from ALS patients;[182] the reason for this discrepancy has not been explored. In short, the P2X7R seems to display a clear involvement in the development and pathogenesis of ALS; hence, its role as a potential therapeutic target for the treatment of ALS deserves further elucidation and characterization. Furthermore, the proof-of-concept supporting the hypothesis that P2X7R antagonists have therapeutic potential in ALS patients has been explored in a few studies.

The use of P2X7R antagonists for ALS treatment has been evaluated in the SOD1^{G93A} mouse model of the disease. Reportedly, BBG is the most explored antagonist, tested at different concentrations, dosing frequencies, and initiated at various stages of the disease. In a first study, BBG was administered intraperitoneally at a concentration of 45 mg/kg every 48 h, starting at the late pre-onset stage of the disease, at postnatal 90 days (P90). BBG treatment ameliorated motor performance deficit in treated mice, which was greater in males than in females; however, no effect on survival was observed.[183]

As previously mentioned, P2X7Rs appeared to possess a dual role in the course of ALS, protective during the early stages and promoting cell death at late stages. Hence, it is crucial to adjust the time at which P2X7R antagonist therapy is initiated in mice. In a comparison between different administration start points, BBG (250 mg/kg, 3 times/week) at late pre-onset (P100) enhanced motor neuron survival and reduced microgliosis in the spinal cord of SOD1^{G93A} mice; this was not observed when treatment was initiated at earlier stages. In both males and females, disease onset was delayed, as well and motor performance was improved, although survival was not affected.[98] Bartlett and colleagues started BBG administration (45.5 mg/kg, 3 times/week) at pre-onset or P62-P64, reducing body weight loss in females but not in males, as a sign of delayed muscle loss. Similarly, it prolonged survival in females but not males; however, motor performance was not affected in either sex.[97] Lastly, the administration of PX7R antagonists, A-804598 and JNJ-47965567, which are both more potent and specific than

BBG, failed to improve motor performance, disease onset, or survival in SOD1^{G93A} mice (30 mg/kg, 5 times/week for A-804598 and 30 mg/kg, 3 times/week for JNJ-47965567).[181,184]

Collectively, P2X7R antagonists seem to exert beneficial effects in the SOD1^{G93A} mouse model of ALS. However, to reveal these beneficial effects, it appears crucial that the starting point of treatment is adjusted, as well as the dosing and frequency of P2X7R antagonist administration. Moreover, chronic administration of P2X7R antagonists to SOD1^{G93A} mice presented an intriguing gender difference that warrants further examination. Future experiments in mouse models of ALS should carefully consider the pharmacokinetics of brain-permeable P2X7R antagonists, focusing on a longer half-life and higher solubility, to allow their continuous administration either with food, drinking water, or sustained-release minipumps.

5.5 Huntington's disease

The role of P2X7Rs in HD has not been extensively investigated, with only a few facts known about this possible relationship. In mouse and cellular models of HD, an increase in P2X7R levels was observed in HD neurons, as well as a dysregulation in calcium permeability mediated by the receptor. Moreover, mutant huntingtin-expressing neurons were prone to undergo apoptosis upon P2X7Rs stimulation.[24] Although these results suggest an involvement of P2X7Rs in HD, further investigations are needed to better define the potential role of these receptors in disease pathogenesis.

The proof-of-concept for the involvement of P2X7Rs in the development of HD has been examined only in one *in vivo* study performed in the R6/1 mouse model of HD. BBG treatment (45.5 mg/kg, every 48 h during 4 weeks) in 8-month old mice prevented body weight loss and improved motor coordination. However, treatment with the antagonist **A-438079** (34.2 mg/kg, every 24 h) failed to present any difference when compared with controls, possibly due to the extremely short half-life and limited bioavailability of this compound.[24]

5.6 Multiple sclerosis

The involvement of P2X7Rs in the pathogenesis of MS is based on postmortem observations, with P2X7Rs demonstrating high expression in activated microglia and astrocytes in the spinal cord and brains of patients.[175,185] In a rat model of MS, experimental autoimmune encephalomyelitis (EAE),[186] upregulation of P2X7Rs was observed in astroglia at an early asymptomatic stage[187] and in neurons and oligodendrocytes at a later symptomatic stage.[188] P2X7R deficiency suppressed the development of EAE.[189]

Interestingly, P2X7Rs remained elevated in EAE rats even 20 days after immunization, correlating with sustained astrocytosis in advanced disease stages in both EAE and MS patients.[190,191] These data are reinforced by the recent observation of increased P2X7Rs in frontal cortex astrocytes from patients presenting progressive MS.[192] These augmented P2X7Rs lead to increased ATP signaling, with concomitant oligodendrocyte excitotoxicity and death. Moreover, a proof-of-concept of P2X7R involvement in MS pathogenesis is the finding that, in EAE rats, the administration of P2X7R antagonists (BBG or oATP) decreased astrogliosis and demyelination, thereby improving neurological symptoms.[188,193] This supports the view that P2X7R blockers could have a role in preventing or improving MS symptoms.[23]

5.7 Retinal degeneration

The mammalian retina expresses most of the purinergic receptor subtypes, as well as the vesicular transporters ATP, VNUT, and the molecular machinery required for ATP degradation.[194-196] P2X7Rs are widely expressed in several types of retinal cells (Figure 10), including retinal pigmented epithelium (RPE), pericyte-containing retinal microvessels, and glial cell types such as astrocytes, microglia, and human Müller cells.[196-201]. Concerning neuronal cells, immunohistochemical studies demonstrated the expression of P2X7Rs in the outer plexiform layer (synaptic sites in rods and cones), inner nuclear layer (in horizontal and amacrine cells), inner plexiform layer, and retinal ganglion cells.[196,200,202] A large body of studies, both in isolated cells and in retinal tissue samples of different animal species, support the expression of P2X7Rs in neurons, as well as their role in the modulation of neurotransmitter release and regulation of synaptic function; however, the expression of P2X7Rs in neurons remains controversial.[87,88,203]

In the eye, the purinergic system has predominant roles in most physiological and pathological processes. Purine signaling through P2X7Rs is prominent in physiological processes in the lacrimal gland, cornea, trabecular meshwork, lens, RPE, and retina.[200] Purines have a modulatory role in the inner and outer retina. Functional electrophysiological studies have evidenced that P2X7R activation induces changes in a- and b-waves of the electroretinogram (ERG), as well as in ERG oscillatory potentials.[195,200,204-206]

FIGURE 10

Purinergic signaling plays a central role in the induction of retinal gliosis, as well as in mediating degeneration of the injured and diseased retina. Notably, stressed cells release large quantities of ATP. P2X7R overstimulation mediates the formation of plasma membrane pores contributing to cytotoxic calcium overload and cytolysis. Furthermore, the P2X7R pore is associated with the activation of the inflammasome, as well as the inflammasome-dependent cell death pathway.[14] Under pathological conditions, not only sustained stimulation but additionally, P2X7Rs upregulation may predispose retinal neurons to damage. Through P2X7Rs, ATP released by neurons or glial cells can induce the activation and proliferation of microglia, resulting in the release of pro-inflammatory cytokines such as TNF-α and IL-1β, which can further promote microglial activation and the propagation of retinal gliosis from a focal injury into the surrounding non-injured tissue; this could induce secondary cell death in the retina[207,208], worsening the neurodegenerative process.[14,209]

It is well accepted that increased extracellular ATP and P2X7R activation play a relevant role in retinal degenerative diseases.[210,211] As stated previously, P2X7Rs are involved in oxidative stress,[212,213] inflammatory processes[23,39], and cell death.[16] In the retina, P2X7R activation has been related to inflammation, as well as the oxidative processes.[214,215] Hence, pharmacological modulation of purinergic receptors may have a clinical therapeutic impact on retinal neurodegenerative diseases.[14,209,212] We next discuss how P2X7Rs are associated with the pathogenesis of several retinal neurodegenerative diseases.

In developed countries, age-related macular degeneration (AMD) is the leading cause of severe and irreversible vision loss in the elderly.[216] In this pathology, alteration of RPE cells, followed by photoreceptor degeneration and vascular angiogenesis is the most critical cause of visual impairment.[209] A high concentration of ATP was observed in the vitreous humor of patients with AMD and vitreous hemorrhage.[217] Genetic variations of P2X7Rs and P2X4Rs increase the risk of AMD.[218] Results from *in vitro* and *in vivo* experiments using models mimicking dry and wet AMD demonstrated that P2X7Rs mediate AMD-like defects.[219] In mice, the loss of function of P2X7Rs induced retinal changes similar to those observed in early AMD, associated with reduced phagocytosis; hence, this could be an important determinant in the development of AMD and/or its progression.[220,221] P2X7Rs activation contributed to calcium signaling and apoptosis in RPE.[222] In geographic atrophy, the late-stage of the dry form of AMD, reduced levels of the microRNA processing enzyme DICER1 were observed in RPE, which increased Alu RNA transcripts (noncoding transcripts belonging to the Alu family of retrotransposons) that induce cell death. In a mouse model of geographic atrophy, a relationship of increased Alu levels was observed following activation of P2X7Rs, and the formation of the inflammasome was established.[223] In this study, nucleoside reverse transcriptase inhibitors were able to suppress the laser-induced choroidal neovascularization[224] and prevented P2X7Rs-mediated NLRP3 inflammasome activation induced by Alu RNA; therefore, these inhibitors have been suggested for drug repurposing in P2X7R-driven diseases.[225]

Diabetic retinopathy (DR) is the most common complication of diabetes and a leading cause of vision loss worldwide, induced by changes in retinal vascular cells.[226] In diabetic patients, fibroblasts demonstrate a high level of ATP release or increased P2X7R reactivity.[227] Furthermore, diabetic mice upregulate P2X7Rs in the retina and retinal endothelial cells,[228] consistent with the fact that P2X7Rs are involved in the microvascular complications of DR.[229] Diabetes increases the susceptibility of retinal microvessels to P2X7R activation, which can reduce retinal blood flow and disrupt vascular function in DR.[230,231] Moreover, P2X7Rs are involved in the inflammatory state in diabetes, as Müller cells can induce the P2X7R-dependent expression of cytokines by microglial cells, contributing to the development of early experimental DR.[232]

Glaucoma is a leading cause of vision loss, characterized by retinal ganglion cell (RGC) death and optic nerve damage, with increased intraocular pressure as one of the principal

risk factors. In animal models of glaucoma, increased intraocular pressure can induce the release of excessive extracellular ATP in the retina and cause damage in ganglion cells by acting on P2X7Rs.[233] Additionally, the expression of the nucleotide transporter VNUT is significantly increased during glaucoma progression.[234] In an experimental model of glaucoma, degrading extracellular ATP or blocking P2X7Rs prevented acute pressure-induced damage in ganglion cells.[235] In a rat chronic ocular hypertension model, P2X7R antagonists protected RGC by inhibiting microglial activation.[207] Alterations in purinergic signaling have been associated with inherited NDDs such as retinitis pigmentosa (RP); inhibition of P2X7R signaling delayed photoreceptor degeneration in rd1 mice, a model of human recessive retinitis pigmentosa[211]

Both *in vitro* and *in vivo* models are being used to establish the relationship between P2X7Rs and the different retinal degenerative diseases. For instance, primary cultures of neuronal retinal cells have been used to demonstrate that P2X7R overstimulation induces a sustained increase in $[Ca^{2+}]_c$ and cell death.[236-238] Primary cultures allow examining the protective role of P2X7R antagonists.[214,236,238,239] Primary cultures of human RPE cells from donors have been used to demonstrate that activation of P2X7Rs induces apoptosis through P2X7R-mediated calcium influx.[222] Cultured human retinal pericytes exposed to high glucose levels have been used as an *in vitro* model of early DR. Using this model, it was shown that P2X7R inhibition reverts the cytotoxic effects of high glucose.[240]

To date, retinal cell lines have not been widely used to investigate purinergic signaling. However, several immortalized retinal cell lines have been described, such as those derived from ganglion cells,[241] photoreceptors,[242,243] Müller glia cells,[244-250] astrocytes,[251] endothelial cells,[252,253] perycites,[254] and RPE.[255] The photoreceptor-derived 661W cell line is a mouse photoreceptor-derived cell line, obtained from a retinal tumor formed in a transgenic mouse expressing SV40 large T-antigen under the control of the human IRBP (interphotoreceptor retinoid-binding protein) promoter.[242,256] This cell line has been used to study cytotoxicity induced by ATP and the protective effect of substances such as saffron.[257] Additionally, the human RPE cell line, ARPE-19, has been widely used, mostly as an *in vitro* model of AMD. Using this model, a pivotal role of P2X7R-pannexin-1 in oxysterol toxicity has been demonstrated in retinal cells.[258] The mouse ganglion cell line RGC-5 has been used to investigate the involvement of miR-187/P2X7R signaling in retinal cell apoptosis and its relationship with oxidative stress.[259]

In experiments with human retinal explants, RGC death was elicited following P2X7R activation.[260,261] Moreover, retinal organotypic cultures can offer a reliable system to study neurodegeneration and neuroprotection, as long as the *in vitro* architecture and cellular connections are maintained within the tissue[262,263]. This model presents an option in between cell line studies and *in vivo* models, and has been used to demonstrate that P2X7R activation mediates RGC death in human retinas.[260]

In different animal models of retinal diseases, several studies have demonstrated that through P2X7Rs, ATP is associated with cell death. To develop pharmacological therapies to restore visual function, animal models of retinal degeneration and blindness are an essential tool for proof-of-concept trials. A good model of retinal neurodegeneration should mimic the human degenerative process, affecting the same neuronal cell types involved in human pathology. Additionally, P2X7R pharmacology varies in different animal species, although selective and competitive P2X7R antagonists demonstrate fewer species differences than earlier non-selective antagonists.[120]

P2X7R-deficient mice have helped demonstrate the involvement of P2X7Rs in the regulation of inner retinal responses of rod and cone pathways.[196] Furthermore, in these mice, the lack of P2X7Rs induced a delayed loss of retinal ganglion cells and decreased phagocytic microglia after optic nerve axotomy.[264] To evaluate whether P2X7Rs play a causative role in oxidative stress-induced AMD, P2X7R deficient mice have been cross-bred with Sod1-deficient mice, a model of AMD. This P2X7R/Sod1 double-knockout demonstrated that P2X7Rs are involved in microparticle accumulation within RPE/choroid tissues, and play a critical role in inducing the accumulation of microglia/macrophages in the subretinal space.[212] Furthermore, other animal models for AMD have been used to study the relationship of P2X7Rs with the initiation of disease progression in AMD, including an intravitreal injection with toxic Alu RNA transcripts used as a model of geographic atrophy,[223] as well as laser injury to induce choroidal neovascularization as a model of the wet form of AMD.[224] Both models have been used to analyze the effect of nucleoside reverse transcriptase inhibitors on P2X7R-related inflammation and neovascularization processes, as well as their therapeutic potential in AMD. In a mouse model of subretinal hemorrhage induced by an injection of autologous blood into the subretinal space, extracellular ATP induced an acceleration of apoptosis via activation of P2X7Rs.[217]

To study inherited retinal degenerative diseases, both naturally occurring and genetically modified mutants have been found or developed.[265,266] Several mouse models carrying genetic mutations in the rhodopsin gene (rd), mimicking human RP hereditary disease, have been described.[267,268] To date, these mouse models have not been extensively used to investigate purinergic pharmacology or its relationship with degenerative diseases. In BALBCrds mice, an early upregulation of neuronal P2X7Rs has been associated with injury and retinal damage.[269] Additionally, the rd1 mouse model was used to demonstrate the protective effect of the purinergic antagonist PPADS on photoreceptor survival during the degenerative process.[211]

Notably, other experimental models mimic retinal degeneration by causing toxicity and cell death. Intravitreal injections of ATP or Bz-ATP have been validated as models of degeneration; an ATP injection causes cell death and reorganization of the outer retina, gliosis, and progressive retinal degeneration and remodeling,[211,238,270-272] which is common in all retinal degenerative processes.[209] This effect was evident in control mice, but not in P2X7R deficient mice.[273] The phototoxicity observed in ATP or BzATP injected eyes was possibly attributed to the activation of P2X7Rs on photoreceptors and other retinal cells, and this model allows the testing of potential therapeutic compounds in a relatively short period.[270,273] As in glaucoma, injury to RGCs is mimicked using an injection of N-methyl-D-aspartate (NMDA) with hypertension models, as well as with injuries elicited by axotomy or optic nerve crush. P2X7R antagonists protected ganglion cells after the NMDA injection.[274] In a rat model of chronic ocular hypertension by cauterizing episcleral veins, P2X7R antagonists protected RGCs by inhibiting microglial activation.[207] Furthermore, P2X7R antagonists effectively protected RGCs after several insults, i.e. optic nerve injury[214] or optic nerve crush.[214]

As multiple cell death pathways exist, inhibition of P2X7Rs may be insufficient to prevent cell death *in vivo*, and the simultaneous inhibition of more than one death pathway could be necessary for efficacious neuroprotection.[14] Hence, primary cultures and cell lines offer a useful model to investigate cell death and neuroprotective mechanisms. Despite extensive limitations, the well-regulated conditions afforded by these models allows the evaluation of more specific cellular functions.

6 STATE-OF-THE-ART CLINICAL TRIALS IN NEURODEGENERATIVE DISEASES

Before attempting to explain the delay in access to clinical trials (CTs) investigating P2X7R antagonists in NDDs, we need to briefly establish previous and ongoing CTs in these diseases. This will clarify why P2X7R antagonists are considerably neglected in their logical and eventual pathway from bench to CTs.

For instance, in AD the frame remains highly disappointing, as exemplified in two recent reviews. One identified 2,072 CTs in the database ClinicalTrials.gov: only 229 were fully published.[275] The other review included 744 CTs at different phases; 80% involved drug investigations.[276] Despite extensive efforts and enormous investment, only two classes of drugs, presenting mild symptomatic effects, have been introduced in clinical practice nearly two decades ago: cholinesterase blockers (rivastigmine, donepezil, galantamine) and one antagonist of glutamate NMDA receptors (*i.e.* memantine). A drug capable of modifying the natural trajectory of AD, thereby reducing its progression, is still lacking.

In terms of AD CTs, reasons for possible failure are diverse in nature. One possible rationale is the mechanism of action, which has focused on at least 30 different targets[277], indicating that the origin and pathogenesis of the disease are possibly being overlooked. Most drugs tested (small molecules or monoclonal antibodies) focused on the inhibition of Aβ synthesis or aggregation and inhibition of GSK3β, based on the fact that Aβ and phospho-tau (*P*-tau) have been considered as the pathogenic hallmarks of AD for over 30 years. As acetylcholine is tightly linked to learning and memory, and inhibitors of cholinesterase were successful, several agonists for nicotinic and muscarinic receptors were additionally explored. As AD progression affects synapses other than the cholinergic, different neurotransmitter systems were considered targets for drug development in AD. Growth factors, calcium channel blockers, and agents acting on oxidative stress and mitochondrial bioenergetics, as well as non-steroidal anti-inflammatory drugs, have also been investigated.

As the pathogenesis of PD implicates the degeneration of dopaminergic neurons of the substantia nigra pars compacta, the deposition of dopamine remains the gold standard, with the introduction of L-dopa nearly half-century ago. This is a symptomatic therapeutic strategy and as in the case of AD, several compounds designed for disease modification failed to meet primary endpoints in several CTs undertaken over the past

several decades in patients with PD. A targeted approach for new therapies includes α-synuclein aggregation, mitochondrial dysfunction, neuronal vulnerability, iron deposition, neuronal network alterations, neurotransmitter systems other than dopaminergic, and neuroinflammation [278]. Some small molecules are in the pipeline in CTs of different pharma companies. For instance, nilotinib, a tyrosine kinase inhibitor approved for chronic myelogenous leukemia and behaving as a protector of dopaminergic neurons in MTPP models of PD, is in phase II CTs. Notably, α-synuclein remains the most interesting target for disease modification, although limitations have been recorded owing to a lack of biomarkers for target engagement, tolerability, and safety.

Inhibition of LRRK2 by small molecules such as DNL201 is another disease-modifying strategy in the initial phases of CTs. Calcium dyshomeostasis renders dopaminergic neurons more vulnerable; thus a 5-year phase III disease-modifying CT with the calcium channel blocker isradipine is currently ongoing in patients with early PD. Another CT is exploring the neuroprotective effect of the iron chelator deferiprone. Compounds impacting the mitochondria, as well as oxidative stress, such as pioglitazone or urate are under investigation. Finally, neuroinflammation targeting compounds, including the irreversible inhibitor of myeloperoxidase, AZD3241, or the glucagon-like peptide 1, are also being tested.[278]

In the case of ALS, a recent review has summarized the results of more than 50 CTs conducted in the last 20 years since riluzole was approved.[279] Antiglutamatergic compounds (ceftriaxone, memantine, talampanel, riluzole), antioxidants (coenzyme Q10, creatine, edaravone), neuroprotective compounds (dexpramipexole, olesoxime, TCH346, xaliproden), neurotrophic factors (brain-derived neutrophil factor (BDNF), ciliary neurotrophic factor (CNTF), insulin-like growth factor-1 (IGF-1)), anti-inflammatory compounds (celecoxib, erythropoietin, glatiramer acetate, minocycline, NP001, pioglitazone, valproic acid) and lithium, have been explored in CTs in patients with ALS. Except for riluzole and edaravone (the only two compounds approved), the clinical outcomes for all compounds were negative. Notably, in the case of riluzole, two out of three late-stage trials reported negative results; for edaravone, two out of three Phase III CTs also reported negative results.[279] Masitinib, a tyrosine kinase inhibitor, is emerging as a unique compound to combat microgliosis and neuroinflammation, and prolonged survival when administered to $SOD1^{G93A}$ ALS mice at the onset of paralysis.[280] An add-

on riluzole therapy in Phase III CT reported significant outcomes in patients treated with masitinib.

A recent review has addressed the state-of-the-art CTs in HD.[281]. As observed with other NDDs, failure rates remain high.[282] Only two symptomatic drugs are currently approved to treat HD chorea, including tetrabenazine and deutetrabenazine. Potential effective therapies include restoring synaptic function, reducing cellular stress, promoting support of trophic factors, and reducing and/or modulating neuroinflammation. During 2015-2018, CTs investigating small molecules addressed the improved mitochondrial function and reduced oxidative stress (creatine, coenzyme Q10, BN82451B, ethyl eicosapentaenoic acid), inhibition of phosphodiesterase 10A to increase cyclic nucleotide signaling (PF-02545920), reduction of metal-induced aggregation of mHTT (PBT2), reduction of oxidative stress and increased BDNF levels (pridopidine), inhibition of noradrenaline and dopamine uptake (bupropion), modulation or reduction of neuroinflammation (laquinimod, cannabinoid mixture). Most compounds tested in patients with HD were well-tolerated, but showed no efficacy; only pridopidine, an activator of sigma-1 receptors, elicited a possible slowing of clinical progression, with laquinimod demonstrating a significant reduction in caudate and whole-brain atrophy, as well as in white matter loss of volume.[281]

Although investigations involving disease-modifying therapies in NDDs have encountered continuous setbacks, simultaneous research in MS has been encouraging. As with other NDDs, MS is a heterogeneous disease with marked phenotypic clinical variability, as well as radiological, pathological, and genetic phenotypes. Despite these differences, in some forms of MS, drugs that can alter the natural disease course have been reported. Therapeutic agents in clinical use focus on inhibiting inflammation and blunting the immune system; small molecules include glatiramer acetate, fingolimod, teriflunomide, and dimethyl fumarate, as well as interferon and monoclonal antibodies. Limitations of currently available medications include serious adverse effects, drug affordability, and routes of administration. Presently, second-generation SP1-receptor modulators, siponimod, ozanimod, and ponesimod, are in late-stage development. Fingolimod, the first approved drug in this class, has presented important adverse effects such as bradycardia, reduction in atrioventricular conduction, lymphopenia, and leukopenia with potentially serious infections; as the new agents are more selective, acting on SP-1 receptor subtypes, a lower incidence of serious adverse effects is

anticipated. Near 100 CTs with agents for MS are ongoing; however, most are investigating approved molecules or new monoclonal antibodies.[283]

7 FROM THE LAB BENCH TO CLINICAL TRIALS WITH P2X7R ANTAGONISTS

In light of the preclinical research reviewed, the question emerges whether a P2X7R antagonist can enter CTs with a reasonable probability of positive outcomes in patients presenting a given NDD. This decision needs to consider several factors, including the polymorphic nature of P2X7Rs, the drug-likeness of newly synthesized compounds, the proof-of-concept with P2X7R antagonists in animal models of disease, and the state-of-the-art of past and present CTs being conducted for the different NDDs reviewed. Additionally, the concept of multi-target compounds fits well in this analysis.

Regarding drug-likeness, some compounds have been found to display an adequate PK profile. Additionally, concerns on safety have been clarified both preclinically and clinically as some CTs, although with negative outcomes, have been performed in patients with rheumatoid arthritis either with the AstraZeneca compound AZD9056[**100**] or with the Pfizer compound CE-224,535[**101**], showing a safe profile. A further argument in favor of safety is derived from the fact that P2X7Rs are activated only under inflammatory or tissue damage conditions in the presence of extremely high ATP concentrations; this poses P2X7Rs an ideal candidate target with few expected side effects,[**26,28,284,285**] elaborating the case for the more drug-like Janssen compounds, JNJ-54166060 and JNJ-55308942.

However, a note of caution has emerged from the above-mentioned CTs evaluating AZD9056 and CE-224,535 in patients with rheumatoid arthritis. It is puzzling that in these trials, while effective P2X7R antagonism was achieved, assessed by IL-1β release from circulating monocytes, clinical endpoints were not attained. Additional challenges could arise in the targeting of neuroinflammation in NDDs, as compounds need to readily cross the BBB and possess a prolonged residence time in brain tissues. Furthermore, as mentioned in Section 6, some P2X7R antagonists evaluated in rodent models of NDDs may play key roles only during specific time points of disease progression.

In addition to drug-likeness and safety, the transition from bench to patient should be based on the proof-of-concept of efficacy in animal models of NDDs. This has been detailed in Section 5, but for the present analysis, it would be practical to reexamine this concept. For instance, BBG afforded some positive neuroprotective outcomes in the J20 mouse model of AD[161], as well as in rats subjected to an intracerebral injection of Aβ.[96,162] Additionally, neuroprotection by BBG has been proven in three studies using animal models of PD;[168,171,172] however, some other studies with A-438079 and other P2X7R antagonists have reported negative results.[170,173] Two studies in the SOD1^{G93A} mouse model of ALS have reported interesting positive outcomes with BBG;[97,98] however, a negative result has also been reported with compound A-804598.[181] Conversely, positive data with BBG were determined in the R6/1 mouse model of HD; however, this was not observed for compound A-438079.[24] However, in a rat model of MS, positive outcomes were observed with P2X7R antagonists (BBG and oATP).[188,193] Finally, in some *in vivo* models of retinal degeneration elicited by several insults, P2X7R antagonists protected ganglion cells.[207,214,274] In addition to these *in vivo* experiments with P2X7R antagonists, data on the ablation of genes coding for P2X7Rs support their leading role in neuroinflammatory processes underlying the pathogenesis of various NDDs. Finally, numerous *in vitro* tissue and cell models have confirmed the protagonist role of P2X7Rs in neuroinflammation.

Additionally, it is crucial to highlight that most studies in murine disease models to test the *in vivo* potential benefit of P2X7R antagonism have been performed with agents such as BBG, *o*ATP, PPADS, suramin, or KN-62 (see Section 4). This over-reliance on compounds with poor PK and underappreciated off-target effects has been largely attributed to their ready availability and low costs. Thus, it is necessary to perform further studies with compounds selective for P2X7Rs, with a good PK profile, and higher BBB permeability and residence time in the brain (see Section 4). Unfortunately, these agents are expensive for *in vivo* chronic administration to mouse disease models, or are available only from Pharmaceutical companies.

Classically, the "one-target one-compound" paradigm has been extraordinarily successful across several diseases, enabling the discovery of new small molecules with therapeutic effects. Biologists define a key target and medicinal chemists design and synthesize a molecule that selectively interacts with these targets. Recently, genomic and proteomic studies have given rise to a plethora of new potential targets in the complex pathogenic

signaling pathways of several diseases, including NDDs. However, during the last two decades, this approach has revealed limited success; this could be attributed to the fact that these complex cellular networks are highly buffered to prevent major changes in the physiological parameters under their control.[286-288] Thus, in complex disorders such as cardiac heart failure, cancer, or NDDs, which result owing to multimolecular alterations, target-directed medicine is unlikely to capable of modifying disease progression.

For a prolonged time, combination therapies have been increasingly used with reported success, including in AIDS treatment, secondary prevention of heart infarction, or cancer treatment. During the last two decades, therapeutic success in several cancer types has been attributed to drug combinations tested in CTs based on complementary mechanisms of action. This strategy has been proven efficacy in neuroprotection, in both *in vitro* and in *in vivo* models of neuronal damage. For instance, the combination of sub-effective concentrations of drugs with antioxidant properties causes synergistic protection in SH-SY5Y neuroblastoma cells.[289] An alternative approach is the design of molecules possessing an affinity for two or more targets; this "multitarget-directed ligand" approach has been extensively investigated during the last decade but restricted to preclinical laboratory experiments.[290-292]

Table 3 summarizes the specific and common pathogenic targets in NDDs. We propose the hypothesis of a dual drug approach, combining a specific disease target with a neuroinflammation target. In choosing the compound directed to a specific target, the more promising small molecule investigated so far should be selected. For instance, a blocker/modulator of glutamatergic receptors would be appropriate in patients with AD; as memantine has shown some therapeutic value at mild-moderate disease stages, this agent could be selected. As P2X7Rs behave as gatekeepers of neuroinflammation, a drug-like antagonist should be chosen. Utilizing this drug combination, we seek to combat neuroinflammation while affording neuroprotection, by restoring calcium homeostasis. In other NDDs, a similar rationale could be applied.

TABLE 3

8 CONCLUSIONS AND PERSPECTIVES

P2X7Rs are involved in multiple organismic physiological and pathological conditions in different body tissues. Their increased expression and prolonged activation owing to elevated ATP concentrations released during cell death or tissue injury contribute to the pathogenesis of NDDs. Therefore, under these conditions, ATP/P2X7R signaling stimulates the production of neuroinflammatory mediators, promoting a state of chronic inflammation, cell death, and disease progression.

Considering the immense literature reviewed here, a new therapeutic concept seems to be emerging: neuroinflammation in NDDs could be combated utilizing drug-like, brain-permeable P2X7R antagonists. Following the assertion of the hypothesis, several points emerge:

1. The presence of reactive glia and augmented inflammatory mediators is a common feature of all NDDs.
2. This implies that combating neuroinflammation using a P2X7R antagonist could exert a therapeutic effect against all diseases.
3. However, depending on the disease stage and type of disease, neuroinflammation could have beneficial or deleterious effects.
4. Observational studies have concluded that chronically administered NSAIDs could be neuroprotective in some NDDs. However, CTs with NSAIDs have provided negative outcomes in AD and PD.
5. The proof-of-concept in animal models of familial or sporadic NDDs is far from consistent: the chronic administration of a P2X7R antagonist is yet to provide a clear picture of efficacy.
6. Drug-likeness of available P2X7R antagonists may vary considerably.
7. P2X7R antagonists present variable kinetic binding characteristics, and the receptors themselves show great variability among mammalian species.

This complex picture could help clarify why some CTs with P2X7R antagonists are yet to be been initiated in NDDs. Although there are numerous ongoing CTs with investigating distinct targets in various NDDs (particularly in AD), it remains perplexing that despite their complexities, a considerable number of available P2X7R antagonists fail to enter into clinical development. At this juncture, we need to question the consistency and strength of available preclinical data supporting the transition of compounds associated with different NDDs targets into clinical trials. However, the

inherent pathogenic complexity of these diseases should be best approached with drug combinations, presenting complementary mechanisms of action. Based on the thorough analysis performed in this review, we postulate that these combinations should involve a P2X7R antagonist to combat background neuroinflammation, along with a more selective compound acting on a specific pathogenic pathway relevant to the specific NDD.

Table 1 Summary of purinergic P2X receptor subtypes, their functional characteristics, and their ligands, agonists, and antagonists.

Purinergic receptors							
Family		Subtype	Receptor type	Endogenous agonist	Function	Most potent agonists	Antagonists
P2	P2X	P2X1	Ionotropic	ATP	Permeable to Na^+, Ca^{2+}, K^+	BzATP, ATP, 2-MeSATP	NF-449, IP_5I
		P2X2				ATP, 2-MeSATP	RB2, iso PPADS
		P2X3				ATP, 2-MeSATP	TNP-ATP
		P2X4				ATP	5-BDBD
		P2X5			Permeable to Cl^-	ATPγS, ATP	BBG
		P2X6			Permeable to Na^+, Ca^{2+}, K^+	-	-
		P2X7				BzATP	JNJ-54175446, JNJ- 47965567, A-74003, KN-62

Table 2 Principal pharmacodynamic (PD) and pharmacokinetic (PK) characteristics of the P2X7R antagonists summarized in this review.

Under the red line is a list of all antagonists selective for P2X7R, while under the green line is a list of blood-brain barrier (BBB)-permeable ones, unless otherwise indicated.

Antagonist name	PD activity	PK properties	Clinical trials
oATP	Irreversible	Only pharmacological tool	-
PPADS	P2X selective	Non-BBB permeable	-
BBG	P2X selective	BBB permeable	-
Suramin	No P2 selective Antitrypanosomal drug $GABA_A$ antagonist [293]	Non -BBB, but circumventricular organs permeable Long half-life (around 50 days)	-
KN-62	mP2X7R selective CaM kinase II inhibitor	Only pharmacological tool	-
Quinazolinium derivative (**2**)	Subnanomolar range activity on P2X7R	-	-
N-methylindole pyrazolodiazepinone (**3**)	Less potent than **2**	Better properties than **2**	-
Pyrimidine-2,4-diones (**4**)	IC_{50} = 27 nM	High metabolic stability Low cytotoxicity and cardiac toxicity	-
N-phenylpyrazoles (**6** – **7**)	IC_{50} = 10 – 20 nM	CYP3A4 inhibition	-
Pyroglutamic derivative (**8**)	IC_{50} = 3.2 nM	Metabolic stability Short half-life (1.5 h)	-
Imidazolone derivative (**9**)	IC_{50} = 10 nM	Slow microsomal clearance	-
1,2,4-triazine (**11**)	IC_{50} = 1.1 μM	*c*log P > 6	-
CE-224,535	IC_{50} = 4 nM	*c*log P = 2.9	Phase 2 – Osteoarthritis Phase 3 – Rheumatoid arthritis
Adamantane-cyanoguanidine (**14**)	IC_{50} = 69 nM	High *c*log P	-
Trifluoroadamantane-*o*-chlorobenzamide (**16**)	IC_{50} = 33.9 nM	High metabolic stability High bioavailability	-

		Short brain half-life (75.6 m)	
Quinoline derivative (**18**)	IC_{50} = 35 nM	Good bioavailability	-
JNJ-47965567	hP2X7R IC_{50} = 5.3 nM	Low brain half-life (4 - 6 h in mice)	-
JNJ-54166060	hP2X7R IC_{50} = 4 nM	High microsomal stability CYP3A4 inhibition	-
JNJ-54175446	hP2X7R IC_{50} = 3 nM	High metabolic stability High bioavailability No CYP3A4 inhibition Low off-target effects	Phase 1 – Major depressive disorder
JNJ-55308942	hP2X7R IC_{50} = 10 nM	High water solubility High brain half-life (20 h in dogs)	Phase 1 – Healthy participants

Table 3 Specific and common pathways involved in the pathogenesis of neurodegenerative diseases (Adapted from [281])

SPECIFIC PATHWAYS			
Disease	**Causes**	**Misfolded protein**	**Predominant cell type affected**
AD	Mutations of APP, PSN1, PSN2	Aβ plaques; *p*-tau neurofibrillary tangles	Hippocampal and cortical neurons
PD	Mutations of LRRK2, PARK7, PINK1, PRKN, SNCA	Lewy bodies	Substantia nigra
ALS	Mutations of SOD1, TDP43, e9orf72, FUS	Aggregates of TDP43 and SOD1	Motor neurons
HD	Mutation of HTT	Huntingtin inclusion bodies	Striatal and cortical neurons
MS	Autoreactive T lymphocytes	Not applicable	White matter and oligodendrocytes
COMMON PATHWAYS			
Reduced BDNF			
Post-translational protein alterations			
Mitochondrial dysfunction			
Excitotoxicity and/or Ca^{2+} dysregulation			
Axonal and/or neuronal damage			
Neuroinflammation and glia activation			

AD, Alzheimer's disease; PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis; HD, Huntington's disease; MS, multiple sclerosis; BDNF, brain-derived neurotrophic factor; SOD1, superoxide dismutase 1; TDP43, TAR DNA-binding protein 43.

Figures

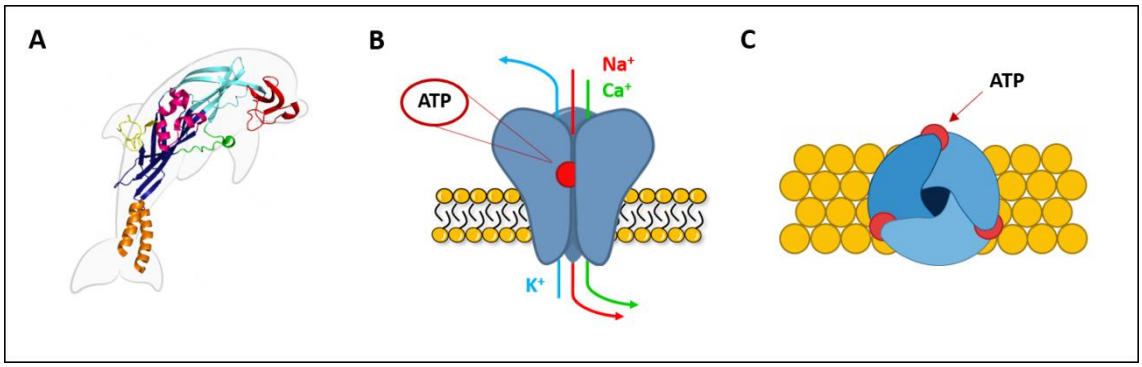

FIGURE 1 Diagrammatic representation of the molecular structure of P2X7R and its subunits. A) Crystal structure of the single subunit of the *Ailuropoda melanoleuca* P2X7R, as reported in the RCSB database (www.rscb.org).[49] The different structural parts are depicted in different colors: head domain (HD) in red, upper body (UB) in cyan, lower body (LB) in blue, dorsal fin (DF) in yellow, left flipper (LF) in green, right flipper (RF) in magenta and the fluke (F) in orange. B) Schematic representation of the activation of the P2X7R by ATP, and the concomitant ion fluxes through the pore. C) Schematic representation of the top view of the P2X7R. The three ATP binding sites are in the UB, at the interface of two subunits.

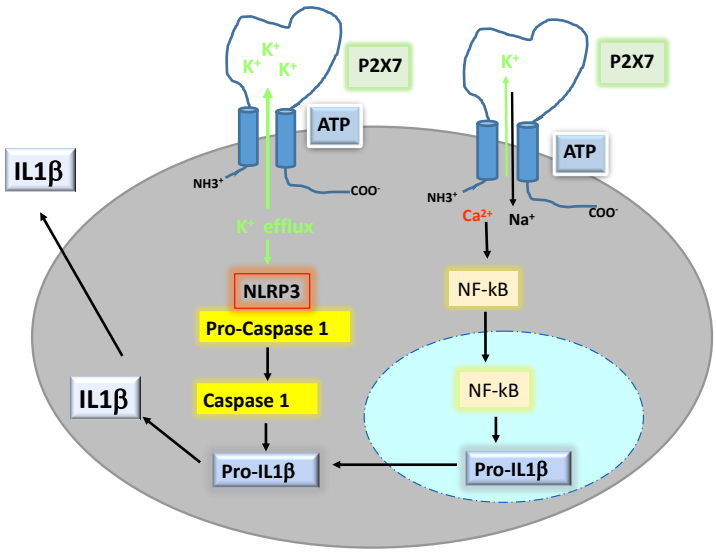

FIGURE 2 Activation of P2X7Rs induces interleukin (IL)-1β release by (i) K^+ efflux: the macropore formed by P2X7R releasing K^+, thereby causing the formation of the inflammasome complex that cleaves pro-caspase 1 into caspase-1; this results in the maturation of pro-IL-1β into IL-1β, subsequently released into the extracellular space. (ii) P2X7R activation promotes intracellular Ca^{2+} increase, which in turn activates NF-κB, which is then translocated into the nucleus eliciting the activation of immature pro-

IL-1β transcription; next, caspase 1 catalyzes the conversion to mature IL-1β, which can be subsequently released to the extracellular space (Adapted from [39]).

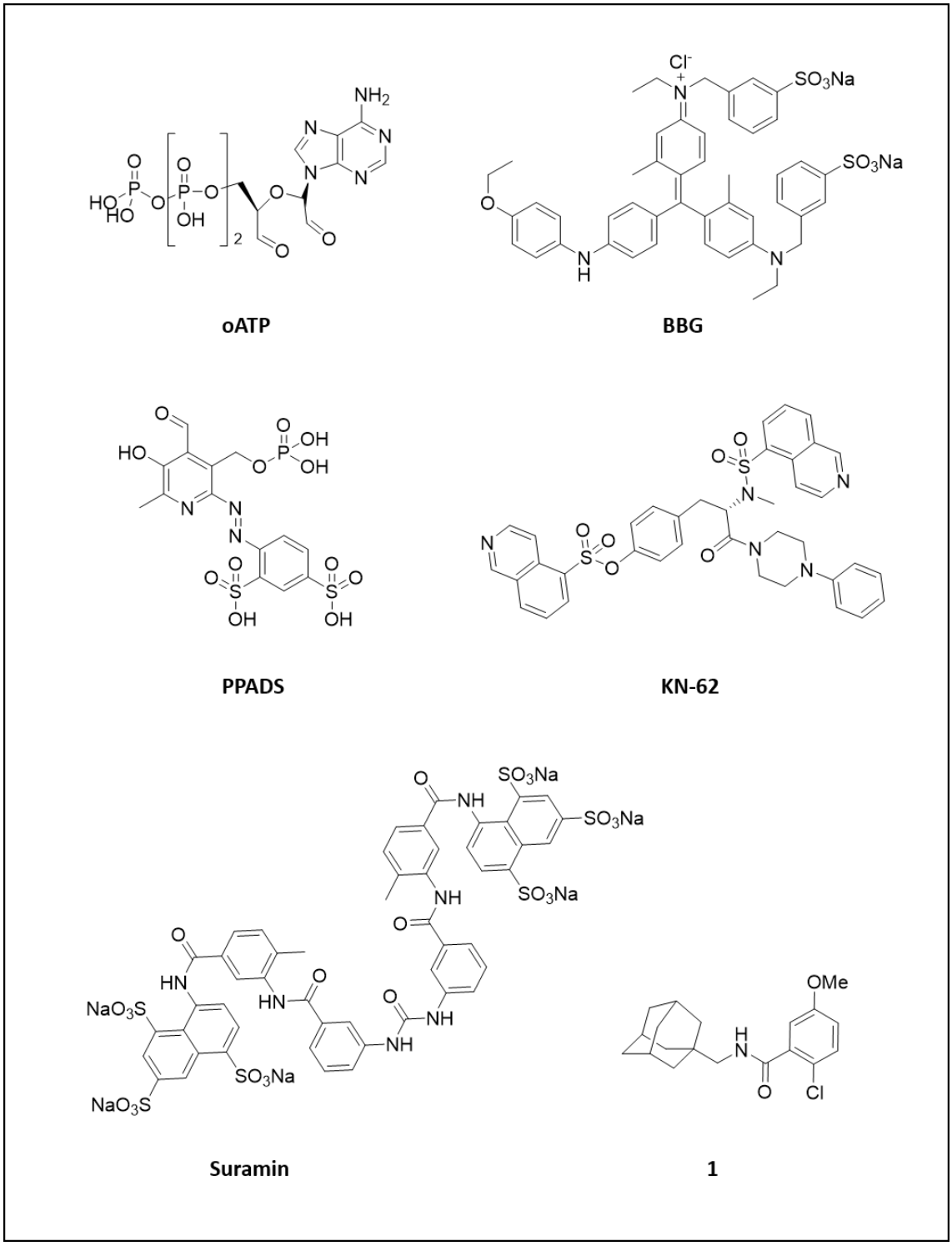

FIGURE 3 Chemical structure of first-generation P2X7R antagonists: oATP, BBG, PPADS, KN-62, suramin, and one of the paradigmatic adamantane-*o*-chlorobenzamide adducts of Astra-Zeneca (**1**).

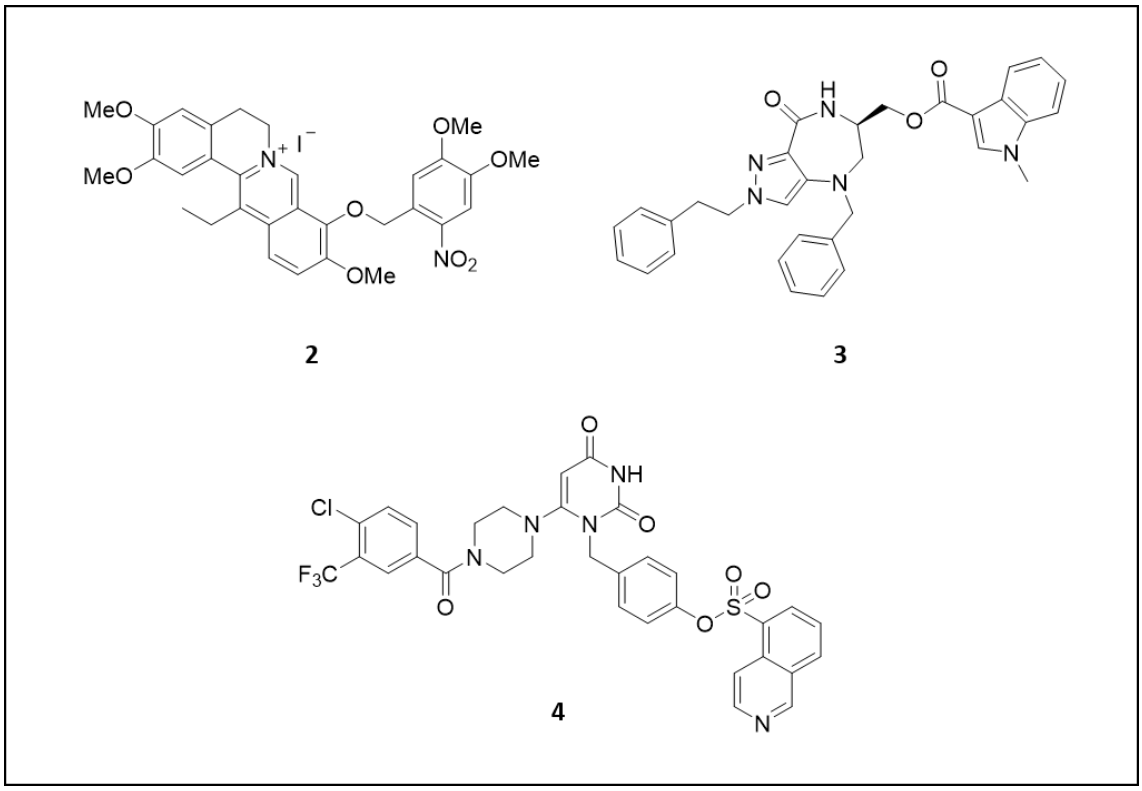

FIGURE 4 Selected examples of P2X7R antagonists reported by Kim and coworkers.[106-108]

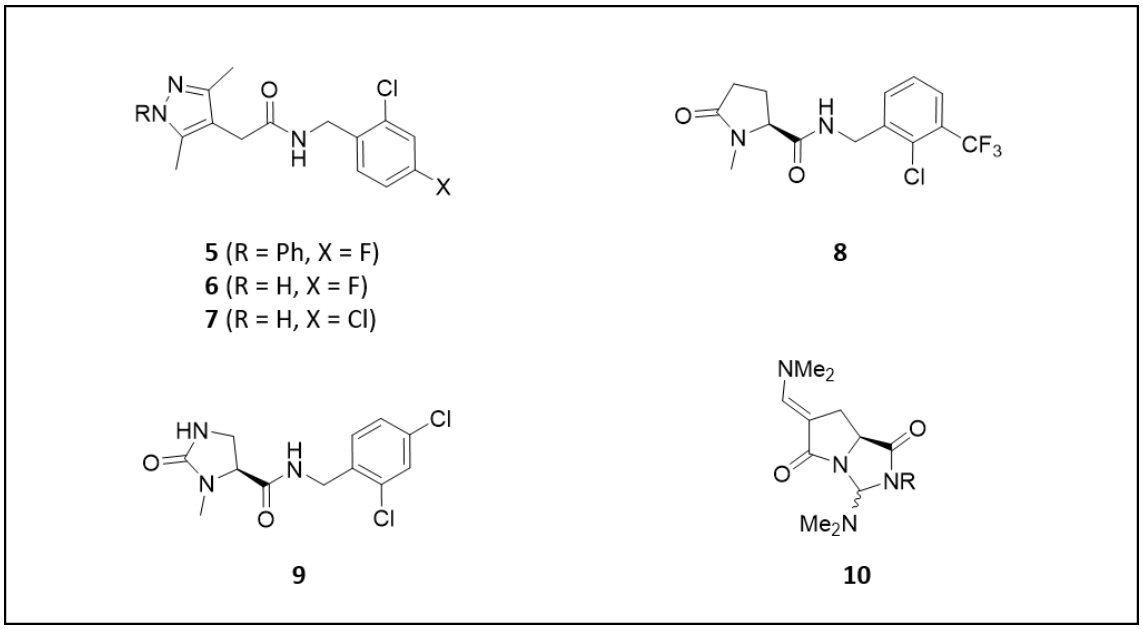

FIGURE 5 Benzylacetamides (**5 – 9**) from GSK and their constrained analogs (**10**) from Inserm (see text for references).

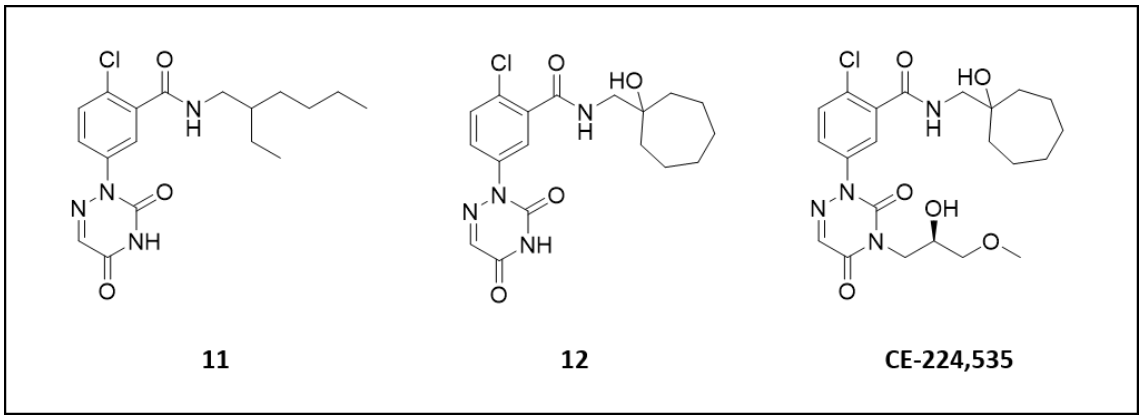

FIGURE 6 1,2,4-triazines with P2X7R blocking properties[114]

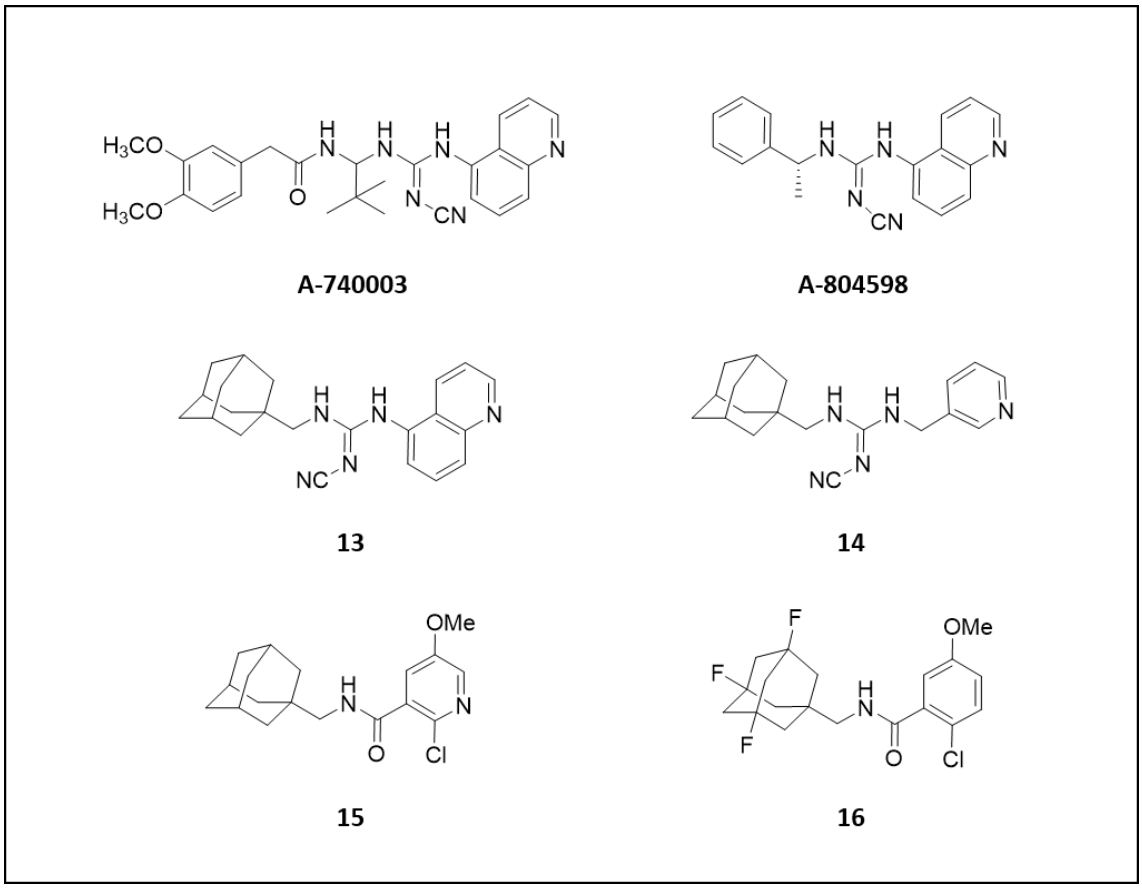

FIGURE 7 Cyanoguanidines from Abbott Laboratories (A-740003 and A-804598). Adamantane-cyanoguanidines and adamantane-*o*-chlorobenzamide adducts from Kassiou and coworkers.[121]

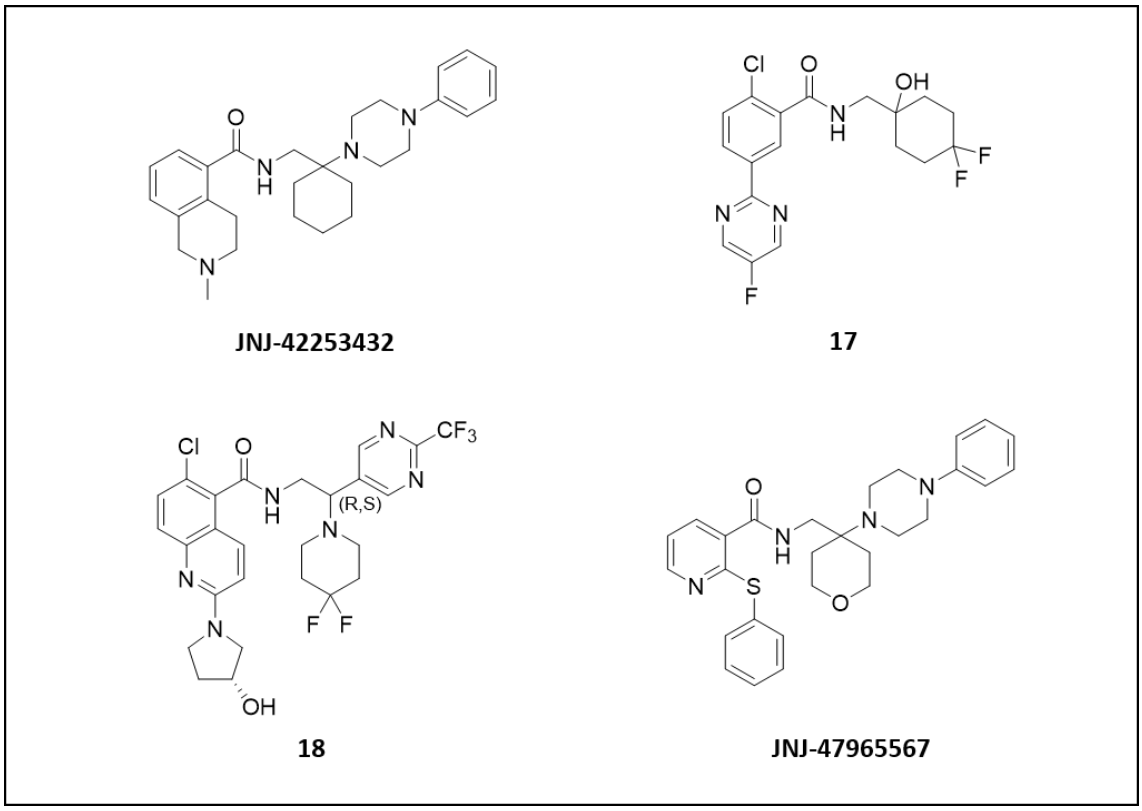

FIGURE 8 First P2X7R antagonists developed by Janssen.

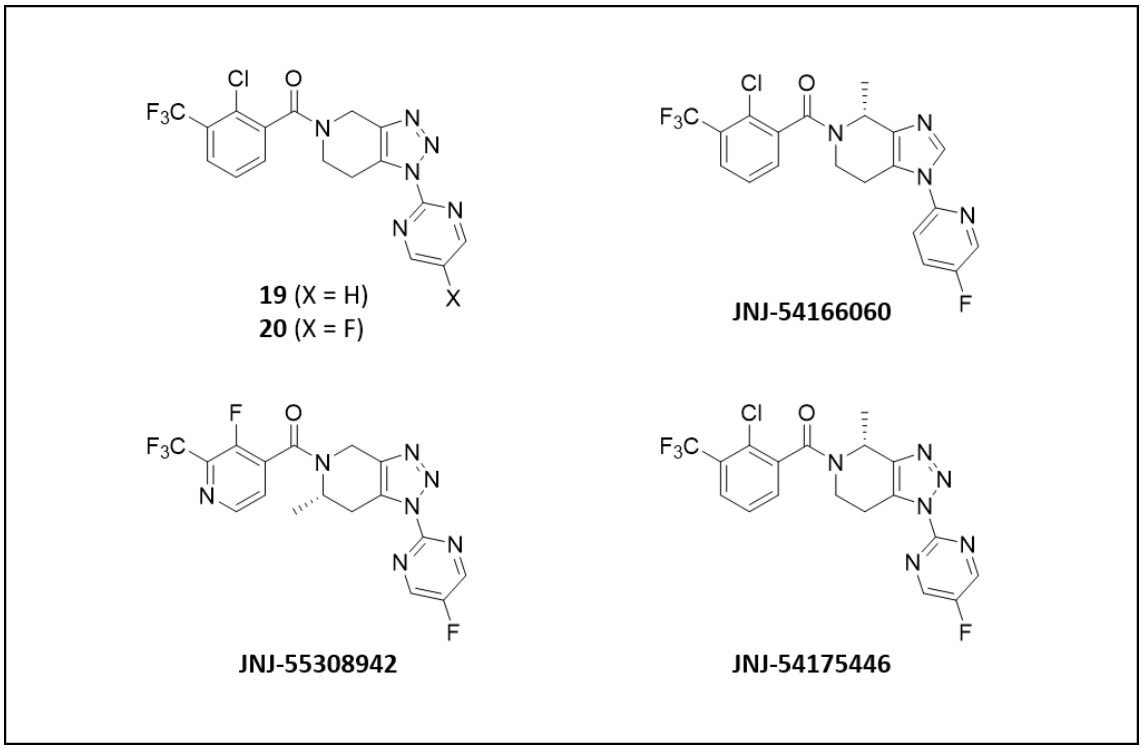

FIGURE 9 Piperidine-fused heterocycles with improved pharmacokinetics (PK) and P2X7R blocking properties (see text for references).

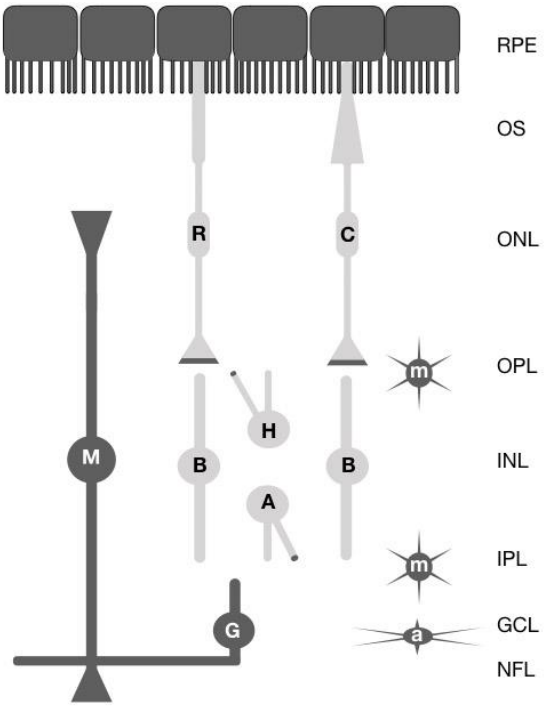

FIGURE 10 Scheme of cells expressing P2X7Rs in the mammal retina (in dark gray). RPE, retinal pigmented epithelium; OS, outer segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer; R: rods; C: cones; M: Müller cells; B: bipolar cells; H: horizontal cells; A: amacrine cells, G: ganglion cells; m: microglial cells; a: astrocytes.