P2X7 AS COMMON THERAPEUTIC TARGET IN BRAIN DISEASES

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P2X7 AS COMMON THERAPEUTIC TARGET IN BRAIN DISEASES

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Editorial: P2X7 as Common Therapeutic Target in Brain Diseases

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Editorial on the Research Topic

P2X7 as Common Therapeutic Target in Brain Diseases

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Despite differences in disease etiology (e.g., β-amyloid, polyglutamine expansion, or neurodevelopmental abnormalities), several brain diseases (e.g., Alzheimer's disease, epilepsy, or schizophrenia) share common clinical symptoms with overlapping diagnoses including depression, psychotic episodes, cognitive deficits, anxiety, and seizures. This implies the activation of shared pathological pathways in different brain diseases. An emerging concept is that increased hyperexcitability and network changes are universal pathomechanisms in numerous brain diseases (Palop and Mucke, 2010; Cepeda-Prado et al., 2012; Kanner, 2012; Nakahara et al., 2018). As well as neurons, glial cells are involved in network hyperexcitability and the mediation of inflammatory processes by modulating the release of neurotransmitters and pro-inflammatory cytokines (Robel and Sontheimer, 2016).

Purinergic signaling mediated *via* specific purinergic membrane receptors, which are activated by extracellularly released nucleosides (P1 adenosine receptors) and nucleotides [e.g., adenosine triphosphate (ATP)] (P2Y and P2X receptors), were suggested to play an important role in numerous human pathological conditions including diseases of the central nervous system (CNS) (Burnstock, 2020). The P2X7 receptor belongs to the ATP-gated ionotropic P2X receptor family. Among the P2X receptors, it has some unique structural and functional characteristics, that make this receptor a particularly attractive therapeutic target (Sperlagh and Illes, 2014; Jimenez-Mateos et al., 2019; Kopp et al., 2019). In particular, it has a much lower affinity for ATP [activation threshold: 0.3–0.5 mM; however, decreased activation threshold (0.05–0.1 mM) has been reported during inflammation (Di Virgilio et al., 2017)], suggesting that its activation occurs mainly under pathological conditions of high ATP release. Furthermore, it is slowly desensitizing, can induce plasma membrane permeabilization for large molecules, and is a key driver of inflammation (Di Virgilio et al., 2017). Studies have attributed a wide array of pathological processes to P2X7 receptor activation in the brain, most prominently the activation of pro-inflammatory processes and regulation of neurotransmitter release. In addition, P2X7 activation has been linked to other

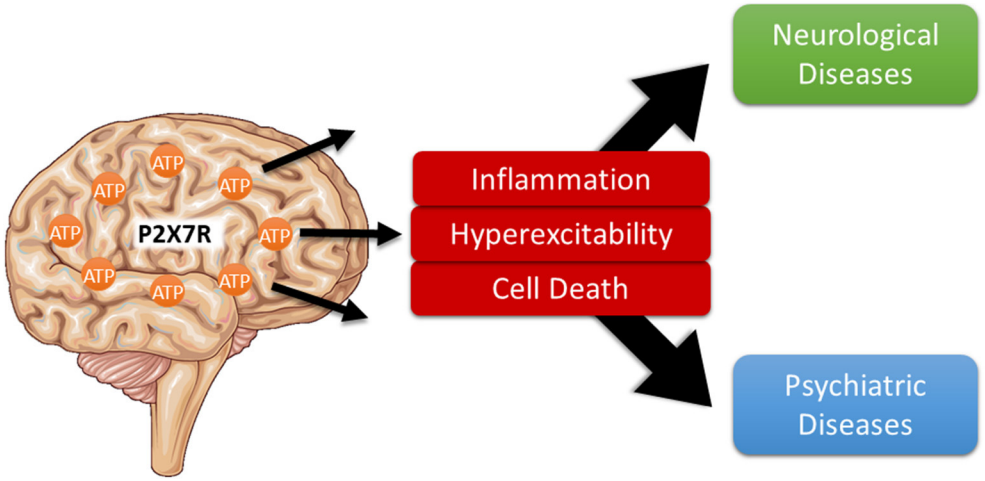

FIGURE 1 | P2X7 receptor activation as shared pathological pathway in brain diseases. Usually found at low extracellular concentrations, ATP levels strongly increase during noxious conditions in the brain. Once released, ATP activates specific purinergic receptors including the ionotropic P2X7 receptor which, in turn, contribute to multiple pathological processes shared among several neurological and psychiatric brain diseases including neuroinflammation, increased hyperexcitability, and neurodegeneration thereby driving disease progression.

damaging processes such as the promotion of cell death, hyperexcitability, and permeabilization of the blood brain barrier (Sperlagh and Illes, 2014). These are shared by the majority of brain diseases and potentially contribute to both primary disease pathology and associated comorbidities (**Figure 1**). In support of this hypothesis, mounting data demonstrate beneficial effects of P2X7 receptor antagonism in numerous brain diseases including neurodegenerative, psychiatric, and neurological diseases (Sperlagh and Illes, 2014).

This Research Topic comprises 11 articles containing five reviews, three original research articles, two brief research reports and one hypothesis/theory article that summarize P2X7 receptor research in different brain diseases and provide up-to date and focused insights into the role of the P2X7 receptor and its potential as a drug target. In the review article written by Francistiová et al. novel findings of the role the P2X7 receptor in Alzheimer's disease are highlighted including data from animal models and humans. Ruiz-Ruiz et al. provided a review about the role of the P2X7 receptor in amyotrophic lateral sclerosis with a particular focus on how the mitigation of neuroinflammation *via* P2X7 receptor blockade may lead to a higher motoneuron survival in patients. Cisneros-Mejorado et al. reviewed the latest findings on the P2X7 receptor in cerebro-vascular disease and Illes et al. contributed an up-to date summary of the P2X7 receptor involvement in major depression and bipolar disorders focussing on possible contributions from both astrocytes and microglia. Finally, Andrejew et al. discusses the molecular mechanisms underlying P2X7 receptor-mediated signaling in neurodegenerative diseases, psychiatric disorders, and brain tumors and highlights the recent advances in the development of P2X7 receptor antagonists. The original research article by Ollà et al. shows for the first time increased P2X7 receptor protein levels in the brain of patients with Huntington's disease supporting the idea that the P2X7 receptor provides a possible therapeutic target in this devastating disease. Interestingly, the observed increases in P2X7 receptor expression in patients are accompanied by disease-specific alterations in the expression of different P2X7 receptor splice variants. Using a mouse model of intraperitoneal phencyclidine (PCP), Calovi et al. support the idea of the P2X7 receptor as a potential therapeutic target in schizophrenia. Using mouse models with either increased or decreased P2X7 receptor expression, the authors show that the P2X7 receptor drives PCP-mediated effects including changes in behavior, basal dopamine concentrations, layer-specific neuronal activation, intrinsic excitability of neurons and the interaction of microglia with hyperactive neurons. In a mouse model of status epilepticus, Conte et al. show for the first time how P2X7 receptor signaling impacts on the expression profile of microRNAs in the brain during normal physiology and following prolonged damaging seizures (i.e., status epilepticus) and suggest a novel pathway of how the P2X7 receptor might contribute to the gene expression landscape during both the maintenance of normal cellular homeostasis and pathological processes. In one of the brief research reports Bibič and Stokes tested the hypothesis of the P2X7 receptor being activated *via* amyloid β peptides. Performing different *in vitro* studies, the authors found, however, no evidence that amyloid β peptides act as agonists of the P2X7 receptor and conclude that amyloid β peptides simply mimic features of P2X7 receptor activation. In another brief research article, Rissiek et al. evaluated the susceptibility of astrocytes and microglia to cell death induced *via* P2X7 receptor activation through its ADP-ribosylation caused by NAD^+. Their data show that treatment of microglia or astrocytes with NAD^+ resulted neither in the activation of the P2X7 receptor nor induction of cell death and explain these results with the finding that astrocytes and microglia preferentially express the ADP-ribosylation-insensitive P2X7a splice variant. Finally, Sanz et al. found a correlation of certain P2X7 receptor single

nucleotide polymorphisms (SNPs) with age and hypothesize that these SNPs may promote an anti-inflammatory phenotype, thereby extending life expectancy among the European and North-American Caucasian population.

In summary, this Research Topic provides a state of-the art description of P2X7 receptor research in the CNS, further supporting the concept of P2X7 activation being a shared pathological pathway among a broad spectrum of brain diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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BRIEF RESEARCH REPORT
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Astrocytes and Microglia Are Resistant to NAD$^+$-Mediated Cell Death Along the ARTC2/P2X7 Axis

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ADP-ribosylation of the P2X7k splice variant on mouse T cells by Ecto-ADP-ribosyltransferase ARTC2.2 in response to its substrate extracellular nicotinamide adenine dinucleotide (NAD$^+$) triggers cell death. Since NAD$^+$ is released as a danger signal during tissue damage, this NAD$^+$-induced cell death (NICD) may impact the survival of other cell populations co-expressing P2X7 and of one of the ARTC2 isoforms (ARTC2.1, ARTC2.2). NICD of brain-resident, non-T cell populations has only been rudimentarily investigated. In this study, we evaluated the susceptibility of two glia cell populations, astrocytes and microglia, towards NICD. We found that astrocytes and microglia strongly upregulate cell surface levels of ARTC2.1 and ADP-ribosylation of cell surface proteins in response to treatment with lipopolysaccharide (LPS) and the mitogen-activated protein kinase kinase (MEK) 1 and 2 inhibitor U0126, but do not respond to extracellular NAD$^+$ with P2X7 activation and induction of cell death. Furthermore, we found that astrocytes and microglia preferentially express the ADP-ribosylation-insensitive P2X7a splice variant, likely accounting for the resistance of these cells to NICD.

Keywords: P2X7, ARTC2, NAD$^+$, ADP-ribosylation, astrocytes, microglia

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INTRODUCTION

P2X receptors are a family of ion channels that are gated by extracellular adenosine triphosphate (ATP). A well-characterized member of this protein family is P2X7, which plays an important role in several immunological processes such as inflammasome formation, release of leaderless pro-inflammatory cytokines, mitochondrial metabolism and cell death (Ferrari et al., 1997; Idzko et al., 2014; Borges da Silva et al., 2018; Linden et al., 2019). In mice, an alternative way of P2X7 activation exists, triggered by a post-translational modification of P2X7 catalyzed by cell surface ADP-ribosyltransferases (ecto-ARTCs). ARTCs use extracellular nicotinamide adenine dinucleotide (NAD$^+$) as a substrate to ADP-ribosylate various cell surface proteins, including P2X7 (Seman et al., 2003). The predominant ecto-ARTC expressed by murine immune cells is ARTC2 with its two isoforms ARTC2.1 and ARTC2.2 (Koch-Nolte et al., 1996). While ARTC2.1 is mainly expressed by cells of the innate immune system such as macrophages and microglia, ARTC2.2 is mainly found on T cells. Of note, the enzymatic activity of ARTC2.1 is enhanced in the presence of reducing agents such as dithiothreitol (DTT), which are thought to break a disulfide bond near the protein surface that is unique to ARTC2.1,

between Cys-80 and Cys-201 (Hara et al., 1999; Hong et al., 2007). Further, it has been demonstrated that both isoforms are able to ADP-ribosylate P2X7 (Hong et al., 2009b). However, the consequence of P2X7 ADP-ribosylation differs among immune cell populations: ADP-ribosylation of P2X7 on T cells induces gating of P2X7, calcium influx, shedding of cell surface proteins, externalization of phosphatidylserine and ultimately cell death (Seman et al., 2003; Rissiek et al., 2015). This can also be induced *via* ATP-mediated P2X7 activation, however, 10-fold lower NAD^+ concentrations (30 μM) are sufficient to induce effects comparable to that of ATP (300 μM). This makes extracellular NAD^+ a potent regulator of T cell death. For macrophages it has been reported that ADP-ribosylation of P2X7 does not induce P2X7 gating, however, it increases the sensitivity of P2X7 towards ATP, thereby lowering the threshold for ATP to induce channel gating (Hong et al., 2009b). Nevertheless, P2X7-mediated induction of cell death can also be achieved in macrophages by prolonged incubation in the presence of ATP. This differential reaction of P2X7 on T cells and macrophages towards ADP-ribosylation has been explained by the expression of two different P2X7 splice variants in macrophages and T cells. While macrophages express the P2X7a variant, T cells express an alternative P2X7 splice variant, termed P2X7k, that differs from the P2X7a in the N-terminal 42 amino acid residues composing the first cytosolic domain and most of the first transmembrane domain (Nicke et al., 2009). Comparative analyses of P2X7a and P2X7k revealed that only the T cell P2X7k variant is gated by ADP-ribosylation, thereby explaining the lack of reactivity of P2X7 on macrophages towards extracellular NAD^+ (Schwarz et al., 2012).

While the role of ARTC2-mediated ADP-ribosylation of P2X7 has been studied extensively in T cell biology and also in the context of macrophages, not much is known about the impact of this post-translational P2X7 modification on other cell populations. Microglia and astrocytes are two glial cell populations in the brain with important functions in e.g., immune surveillance and neuronal nutrition. Our own recent results point towards a potential ADP-ribosylation of P2X7 on microglia (Rissiek et al., 2017). Further, is has been suggested that NAD^+ can also trigger cell death along the ARTC2/P2X7 axis in astrocytes (Wang et al., 2012). The ubiquity of NAD^+ in every metabolically active cell has the consequence that it can be released, analogously to ATP, as danger signal during tissue damage e.g., after ischemic stroke in the brain. Therefore, it is important to know, if the released NAD^+ has an impact on the vitality of microglia and astrocytes in an ARTC2/P2X7-dependent fashion. In the present study, we evaluated this on astrocytes and microglia from mouse mixed glial cultures.

MATERIALS AND METHODS

Mice

C57BL/6 WT, Balb/c WT, Balb/c ARTC2.1ko (Ohlrogge et al., 2002) and NZW WT mice were bred at the animal facility of the University Medical Center (UKE). ICR mice were purchased from Charles River, Sulzfeld, Germany. All experiments involving tissue derived from animals were performed with the approval of the responsible regulatory committee (Hamburger Behörde für Gesundheit und Verbraucherschutz, Veterinärwesen/Lebensmittelsicherheit, ORG-722). All methods were performed in accordance with the relevant guidelines and regulations.

Isolation of Primary Brain Microglia, Peritoneal Macrophages, and Spleen T Cells

For the isolation of brain microglia, mice were sacrificed and single-cell suspensions were prepared by collagenase digestion at 37°C for 30 min. The generated cell suspension was filtered through a 70 μm cell strainer and centrifuged for 5 min at 300 *g*. Microglia were separated from debris by resuspending the pellet in 5 ml 33% percoll solution (GE Healthcare, Chicago, IL, USA). The supernatant was removed and the pellet was resuspended in 1 ml ACK erythrocyte lysis buffer and incubated for 1 min on ice to remove erythrocytes. Cells were washed with 10 ml FACS buffer (PBS + 0.2% BSA/1 mM EDTA) and resuspended in FACS buffer. For the isolation of peritoneal macrophages, mice were sacrificed, 5 ml PBS + 1 mM EDTA were injected into the peritoneal cavity to collect peritoneal macrophages by lavage. For the isolation of spleen T cells, mice were sacrificed, the spleen was collected and minced through a 70 μm cell strainer using a syringe piston. The cell suspension was centrifuged for 5 min at 300 *g*, erythrocytes were removed as described above and the cells were finally resuspended in FACS buffer.

Mixed Glial Cell Cultures and Stimulation With LPS/U0126

Brains from 1 to 2 days old neonatal mice were prepared and transferred into Hanks Balanced Salt Solution (HBSS, Thermo Fisher Scientific, Waltham, MA, USA) containing 10 mM HEPES (Thermo Fisher Scientific, Waltham, MA, USA). After removal of the meninges, brains were minced into smaller pieces, washed and incubated for 25 min in HBSS + 10 mM HEPES with 0.5 mg/ml papain (Sigma-Aldrich, St. Louis, MO, USA) and 10 μg/ml DNAse (Roche Diagnostics, Basel, Switzerland). Cells were then washed in BME medium (Life Technologies, Carlsbad, CA, USA), dissociated and then plated at a density of 3×10^5 cells/ml and cultured in BME media supplemented with 10% FCS and 100U/ml penicillin/100 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Cultures were used for analyses after 14–21 days and contained 20–30% microglia and 60%–70% astrocytes. To induce ecto-ART activity cells were stimulated with LPS (0.1 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) and U0126 (10 μM, Sigma-Aldrich, St. Louis, MO, USA) in culture medium for 24 h at 37°C.

Antibodies and Flow Cytometry

Cells were analyzed using BD FACSCanto II following staining with fluorochrome-conjugated mAbs: anti-ARTC2.1 (clone R18A136#2; UKE), anti-etheno-ADP-ribose (clone 1G4, UKE; Young and Santella, 1988), anti-CD11b (clone M1/70; BioLegend, San Diego, CA, USA), anti-GLAST (clone ACSA-1; Miltenyi), anti-P2X7 (clones Hano43 and Hano44, UKE),

anti-CD45 (30-F11, Biolegend) and anti-CD4 (clone RM4–5; BioLegend, San Diego, CA, USA). Cells were stained and washed in FACS buffer containing PBS + 0.1% BSA + 1 mM EDTA. For flow cytometric analyses microglia were identified as $CD11b^+GLAST^-$ cells and astrocytes as $CD11b^-GLAST^+$.

Calcium Influx Assay

Cells were loaded with 2 μM Fluo-4 (Invitrogen, Waltham, MA, USA) for 20 min at 4°C and 10 min at 37°C, washed once with FACS buffer and resuspended in PBS supplemented with 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$ (Invitrogen, Waltham, MA, USA) and analyzed by flow cytometry (BD FACS-Canto). An infrared lamp was used to maintain a constant sample temperature of 37°C. This was achieved by placing the probe of a digital thermometer in a separate FACS tube filled with PBS in close proximity of the analyzing FACS tube (**Figure 1A**). After baseline measurement for the indicated times, 1 mM ATP, 1 mM NAD^+ or 1 mM NAD^+ + 2 mM dithiothreitol (DTT, Invitrogen, Waltham, MA, USA) was added.

Pore Formation Assay

Cells were resuspended in PBS supplemented with 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$ (Invitrogen, Waltham, MA, USA) and DAPI was added to a final concentration of 1.5 μM. Cells were analyzed by flow cytometry (BD FACS-Canto) using an infrared lamp to maintain a constant sample temperature of 37°C, as described above. After baseline measurement for the indicated times, 1 mM ATP, 1 mM NAD^+ or 1 mM NAD^+ + 2 mM DTT was added.

LDH Assay

LDH release from mixed glial cells was measured after incubation of cells for 24 h by using the Cytotoxicity Detection Kit (Roche, Basel, Switzerland) in order to estimate the frequency of dead cells after NAD/ATP treatment. The assay was used according to manufacturer's instructions.

Etheno-ADP-Ribosylation Assay

Cultured glial cells were incubated for 20 min at 4°C with 100 μM etheno-nicotinamide adenine dinucleotide (etheno-NAD^+, Sigma-Aldrich, St. Louis, MO, USA) in the presence or absence of 2 mM DTT. Etheno-NAD^+ was removed by washing cells twice with FACS buffer. Etheno-ADP-ribose bound to cell surface proteins was detected using fluorochrome-conjugated etheno-adenosine-specific monoclonal antibody 1G4, as described previously (Krebs et al., 2003; Rissiek et al., 2017). Cells were washed twice with FACS buffer and analyzed by flow cytometry. Cells that were not treated with etheno-NAD^+ were stained with 1G4 and used as control.

HEK Cell Transfection

For transfection experiments pCMVSport6.1 plasmids containing mouse P2X7a or P2X7k were used. Expression constructs were transfected into human embryonic kidney (HEK) cells using jetPEI transfection reagent (Polysciences Europe, Hirschberg an der Bergstraße, Germany). Transfected cells were FACS sorted every 3–4 days for high P2X7 expression in order to generate stably transfected HEK cells. These cells were then directly used in experiments or co-transfected with pCMVSport6 encoding for ARTC2.1 in order to evaluate the impact of ADP-ribosylation.

P2X7 Splice Variant Typing

RNA was extracted from FACS sorted murine immune cells (astrocytes and microglia from mixed glial cell cultures, peritoneal macrophages, and spleen CD4 T cells) using RNeasy® Plus Mini Kit (Qiagen, Venlo, Netherlands) followed by cDNA synthesis using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) as recommended by the respective supplier. P2X7 splice variant determination was performed by polymerase chain reaction (PCR) using forward primers specific to exon 1 of P2X7k (5′- gcccgtgagccacttatgc -3′) and P2X7a (5′- cacatgatcgtcttttcctac -3′) and a common reverse primer, which binds to the exon 5 (5′- ccttgtcttgtcatatggaac -3′) of both splice variants. The amplification conditions were 30 cycles of 94°C for 30 s, 55°C for 30 s, followed by 72°C for 30 s, and the final elongation step at 72°C for 6 min. Due to the low expression level of P2X7 on astrocytes, the number of cycles was elevated to 40 to increase the yield of astrocyte-specific P2X7 transcripts. P2X7a (∼380 bp) or P2X7k (∼460 bp) were separated in agarose gel electrophoresis (1.5% agarose).

Software and Statistics

Analysis of flow cytometric data was performed using FlowJo (Treestar). Statistical analyses were performed using Prism 8 software. Two groups were compared by using student's *t*-test and data is presented as mean ± SD.

RESULTS

Astrocytes and Microglia Show No Signs of P2X7 Activation in Response to NAD^+

We recently identified several target proteins of ARTC2.1 on microglia, including P2X7 (Rissiek et al., 2017). Yet, it is unclear whether the ADP-ribosylation of P2X7 on microglia also induces P2X7 activation. Interestingly, a recent study suggests that astrocytes react to NAD^+ in an ARTC2/P2X7-dependent fashion, ultimately resulting in astrocyte cell death (Wang et al., 2012). In order to evaluate the impact of P2X7 ADP-ribosylation on microglia and astrocytes, we set up mixed glial cell cultures from neonatal Balb/c mice consisting mainly of astrocytes and microglia. First, we analyzed the impact of NAD^+ on the immediate effects of P2X7 activation, such as calcium influx and pore formation, using real-time flow cytometry. To distinguish astrocytes and microglia in mixed glial cultures, we used anti-GLAST (astrocytes) and anti-CD11b (microglia) fluorochrome-conjugated antibodies. After 2 min of measuring the baseline signal at 37°C in the absence of an external stimulus, we added either ATP (1 mM) or NAD^+ (1 mM) to the sample and continued measuring for 6–8 min. Treatment with ATP induced a rapid influx of calcium into astrocytes and microglia and pore formation, as evidenced by uptake of the DNA staining dye DAPI. In contrast, treatment with NAD^+ neither induced calcium influx nor pore formation in astrocytes and microglia (**Figure 1B**). Of note, DAPI appeared

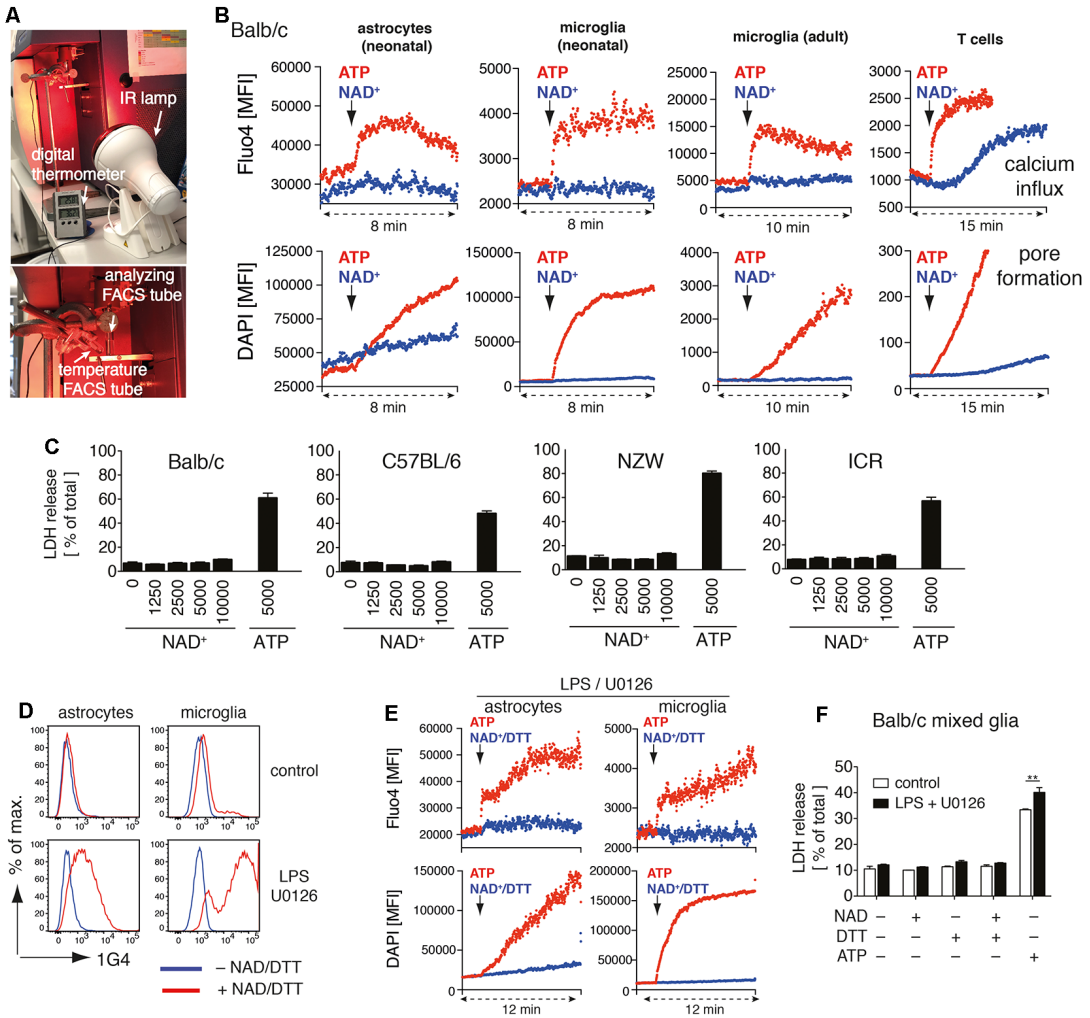

FIGURE 1 | Adenosine triphosphate (ATP) but not nicotinamide adenine dinucleotide (NAD^+) induces activation of P2X7 on astrocytes and microglia. **(A)** Instrumental setup for 37°C real-time flow cytometry. An IR lamp was placed in close distance to the "analyzing FACS tube" to maintain the sample temperature at 37°C. Temperature was monitored using a digital thermometer in a separate "temperature FACS tube" placed next to the "analyzing FACS tube." **(B)** Astrocytes and microglia from Balb/c mixed glial cultures, primary adult brain microglia, and splenic CD4 T cells were loaded with Fluo4 and resuspended in PBS supplemented with calcium and DAPI. Calcium influx and pore formation were measured by real-time flow cytometry in response to the addition of ATP (1 mM) or NAD^+ (1 mM; black arrow) after 2 min of baseline measuring. **(C)** Cell death of mixed glial cells from Balb/c, C57BL/6, NZW and ICR mice in response to NAD^+ or ATP treatment for 24 h was quantified by measuring LDH release. **(D)** Ecto-ART activity of astrocytes ($GLAST^+CD11b^-$) and microglia ($GLAST^-CD11b^+$) from Balb/c mixed glial cultures (stimulated or not with lipopolysaccharide (LPS)/U0126 for 24 h) was analyzed by flow cytometry following incubation of cells with etheno-NAD^+ and detection of incorporated etheno-ADP-ribose with etheno-adenosine-specific mAb 1G4. **(E)** Calcium influx and pore formation by LPS/U0126 stimulated cells were measured by real-time flow cytometry as in **(A)**. **(F)** Cell death of LPS/U0126 treated mixed glial cells from Balb/c mice in response to treatment with NAD^+, NAD^+/DTT or ATP for 24 h was quantified by measuring LDH release. Statistical comparison of two groups was performed by using the student's *t*-test (**$p < 0.01$). Data represent results from two **(B,E,F)** or three **(C,D)** independent experiments.

to diffuse into astrocytes over time. However, the addition of NAD^+ did not enhance DAPI uptake into astrocytes. We repeated our experimental setup with primary microglia from the brain of adult mice. Again, we observed calcium influx and DAPI uptake in response to ATP but not to NAD^+ stimulation. As a positive control we used $CD4^+$ T cells from spleen, which are known to be able to induce P2X7 activation *via* ADP-ribosylation (Adriouch et al., 2008). Here, we detected calcium influx and DAPI uptake in response to ATP or NAD^+ stimulation (**Figure 1B**).

We next compared the capability of NAD^+ and ATP to induce cell death in mixed glial cultures. We incubated mixed glial cells with rising concentrations of NAD^+ (1–10 mM) or ATP (5 mM) for 24 h and measured cell death by the release of lactate dehydrogenase (LDH). Since ARTC2 isoforms ARTC2.1 and ARTC2.2 are differentially expressed among inbred mouse strains (Koch-Nolte et al., 1999) we analyzed mixed glial cultures from Balb/c ($ARTC2.1^+/ARTC2.2^+$), C57BL/6 ($ARTC2.1^-/ARTC2.2^+$), NZW ($ARTC2.1^+/ARTC2.2^-$), and the outbred strain ICR. Interestingly, treatment with up to

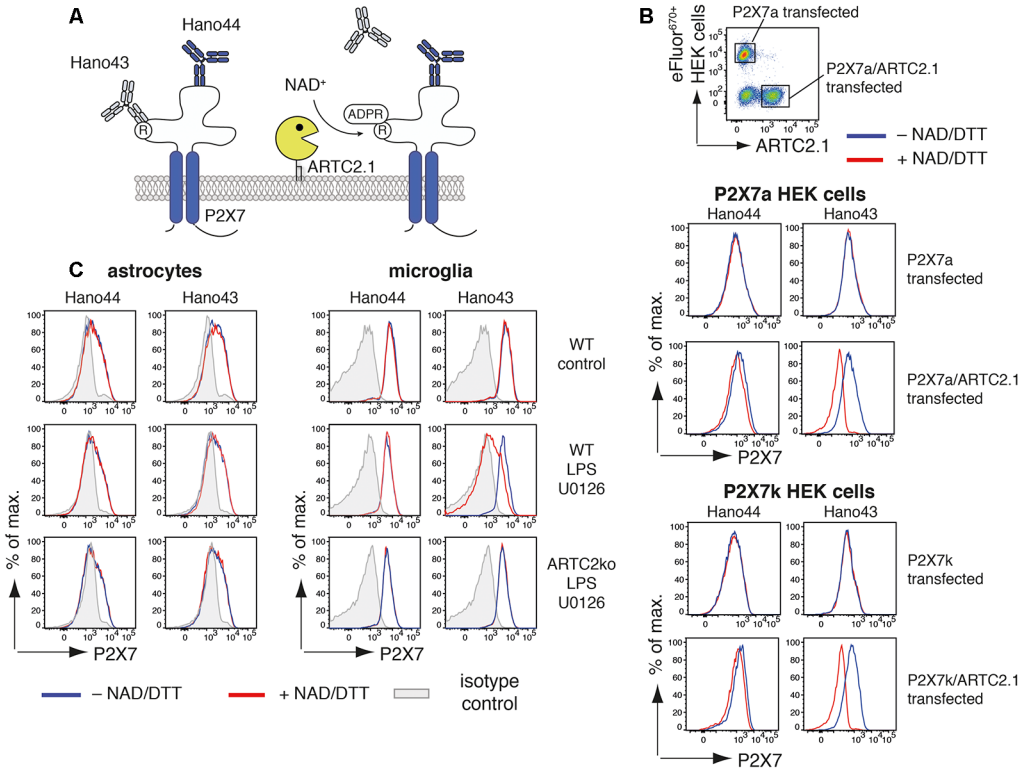

FIGURE 2 | Antibody-aided detection of P2X7-ADP-ribosylation. **(A)** Schematic of the antibody-aided ADP-ribosylation assay. Hano43 and Hano44 are two P2X7-specific mAbs that bind distinct epitopes on P2X7. Binding of Hano43 but not of Hano44 is blocked by ADP-ribosylation of P2X7. **(B)** HEK cells, stably transfected with mouse P2X7a or P2X7k splice variants, were transiently co-transfected with mouse ARTC2.1. HEK cells only expressing P2X7 were labeled with eFluor^{670} and mixed with unlabeled P2X7/ARTC2.1 co-transfected HEK cells. Mixed cells were incubated with or without NAD$^+$/DTT for 20 min at 4°C and binding of Hano43 and Hano44 was measured by flow cytometry. Gating was performed on eFluor$^+$ P2X7-transfected or eFluor$^-$ ARTC2.1/P2X7 co-transfected HEK cells. **(C)** Astrocytes and microglia from mixed glial cells of Balb/c WT or Balb/c ARTC2ko mice were stimulated or not with LPS/U0126 overnight. ADP-ribosylation of P2X7 after incubation with or without NAD$^+$/DTT was measured by binding of Hano43/Hano44 in comparison to isotype control. Gating was performed on CD11b$^+$GLAST$^-$ (microglia) and CD11b$^-$GLAST$^+$ (astrocytes) cells. Data represent results from two independent experiments.

10 mM NAD$^+$ did not induce detectable cell death in mixed glial cultures from all analyzed mouse strains. In contrast, 5 mM ATP induced cell death of 40–70% of mixed glial cells from all analyzed strains (**Figure 1C**).

Since a lack of response to treatment with NAD$^+$ could be a result of low or absent ecto-ART activity, we next stimulated mixed glial cells from Balb/c mice with LPS and U0126 in order to induce ARTC2.1 expression (Hong et al., 2007, 2009a; Rissiek et al., 2017). To measure ecto-ART activity we incubated LPS/U0126 stimulated and unstimulated mixed glial cells with etheno-NAD$^+$ and the reducing agent dithiothreitol (DTT) in order to enhance ARTC2.1 activity (Hara et al., 2000). Incorporation of etheno-ADP-ribose into cell surface proteins was detected with the etheno-ADP-ribose-specific monoclonal antibody 1G4 (Young and Santella, 1988; Krebs et al., 2003). Indeed, treatment with LPS/U0126 markedly increased the ecto-ART activity of both, astrocytes and microglia (**Figure 1D**). We next tested the impact of NAD$^+$/DTT treatment on LPS/U0126 stimulated mixed glial cells using the calcium flux and pore formation assays. Again, we did not detect any notable response to NAD$^+$/DTT treatment, whereas ATP stimulation-induced calcium influx and pore formation, as shown before (**Figure 1E**). Finally, we evaluated the impact of NAD$^+$/DTT on the induction of cell death in LPS/U0126 stimulated mixed glial cultures. Of note, neither NAD$^+$ alone nor NAD$^+$/DTT did induce LDH release from Balb/c mixed glial cultures. In contrast, ATP stimulation induced cell death in a substantial fraction of LPS/U01267 stimulated mixed glial cells, even stronger when compared to unstimulated control (**Figure 1F**). In summary, ATP but not NAD$^+$ induced activation of P2X7 on astrocytes and microglia, and this did not change after LPS/U0126-induced increase of cell surface ART-activity.

ADP-Ribosylation of P2X7 Can Be Detected on Microglia but Not on Astrocytes

Since treatment with NAD$^+$ did not induce any detectable activation of P2X7 we analyzed the ability of ARTC2.1 to ADP-ribosylate P2X7 on astrocytes and microglia. For this, we utilized a pair of P2X7-specific monoclonal antibodies that are differentially affected by ADP-ribosylation of P2X7:

binding of Hano43 to P2X7 is inhibited by ADP-ribosylation of P2X7 whereas Hano44 binds both, ADP-ribosylated and unmodified P2X7 (**Figure 2A**). HEK cells, stably transfected with either P2X7a or P2X7k, were labeled with eFluor^{670} and mixed with HEK cells that were additionally transiently transfected with ARTC2.1 (**Figure 2B**). Treatment of ARTC2.1 co-transfected HEK cells with NAD$^+$/DTT resulted in a strongly reduced binding of Hano43 but not of Hano44. In contrast, staining with Hano43 was not reduced after the treatment of HEK cells that were only transfected with P2X7a or P2X7k with NAD$^+$/DTT. Of note, Hano44 binding was comparable among P2X7-transfected and P2X7/ARTC2.1 co-transfected HEK cells, regardless of NAD$^+$/DTT treatment.

We next applied this tool for measuring ADP-ribosylation of P2X7 in mixed glial cell cultures that had been stimulated for 24 h with or without LPS/U0126 (**Figure 2C**). For microglia but not for astrocytes treatment with NAD$^+$/DTT resulted in a reduced binding of Hano43. Reduced binding of Hano43 was not observed for microglia obtained from ARTC2ko mice, indicating that this effect indeed is mediated by ARTC2.1-catalyzed ADP-ribosylation of P2X7.

Microglia and Astrocytes Express the P2X7a Variant Which Is Insensitive to ADP-Ribosylation

T cells and macrophages express different P2X7 splice variants (Nicke et al., 2009). With our antibody-based ADP-ribosylation assay of P2X7 on HEK cells we could demonstrate that both splice variants can be ADP-ribosylated by ARTC2.1. It has been reported that ADP-ribosylation can trigger the gating of P2X7k but not of P2X7a (Schwarz et al., 2012). Indeed, treatment of P2X7k/ARTC2.1-transfected HEK cells but not of P2X7a/ARTC2.1 transfected HEK with NAD$^+$/DTT induced uptake of DAPI (**Figure 3A**). In contrast, treatment with ATP induced DAPI uptake in both, P2X7k/ARTC2.1- and P2X7a/ARTC2.1-transfected HEK cells.

To determine whether astrocytes or microglia express the P2X7a and/or P2X7k splice variant, we performed a splice variant-specific PCR analysis. As controls we used P2X7a- or P2X7k- expression plasmids and cDNA from peritoneal macrophages, known to express primarily P2X7a, and spleen CD4$^+$ T cells, known to express primarily P2X7k (Schwarz et al., 2012). The results show that both, astrocytes and microglia, predominantly express the ADP-ribosylation-insensitive P2X7a variant (**Figure 3B**). This provides a possible explanation for the apparent resistance of astrocytes and microglia towards NAD-induced cell death along the ARTC2/P2X7 axis.

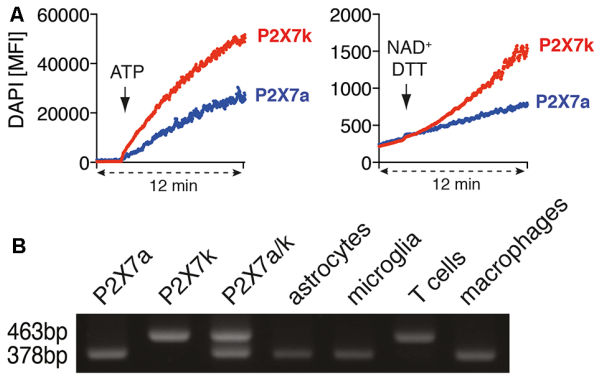

FIGURE 3 | Astrocytes and microglia express the ADP-ribosylation insensitive P2X7a splice variant. **(A)** P2X7a transfected HEK cells were labeled with eFluor670 and mixed with unlabeled P2X7k transfected HEK cells in PBS supplemented with calcium and DAPI. DAPI uptake was measured for 2 min, then ATP (1 mM) or NAD+/DTT was added and measuring continued for 10 min. **(B)** cDNA from astrocytes, microglia, spleen T cells and peritoneal macrophages was used for P2X7 splice variant-specific PCR. Expression plasmids for P2X7a (PCR product: 378bp) and P2X7k (PCR product: 463 bp) or a mixture of P2X7a/k was used as a positive control for the splice variant-specific PCR. The shown data represent results from two independent experiments.

DISCUSSION

In this study, we evaluated astrocytes and microglia for their sensitivity towards NAD$^+$-mediated activation of the ARTC2/P2X7 axis. We found that ATP but not NAD$^+$ induced activation of P2X7 in astrocytes and microglia, even if cell surface ecto-ART activity on both cell types was increased by treatment with LPS/U0126 for 24 h. Consistently, astrocytes and microglia in mixed glial cultures were resistant to NAD$^+$-induced cell death (NICD) but not to ATP-induced cell death. For astrocytes, a possible explanation for NICD resistance is insufficient ADP-ribosylation of P2X7. The capacity of astrocytes to ADP-ribosylate cell surface proteins is lower than that of microglia (see **Figure 1C**). Moreover, the cell surface density of P2X7 is also lower on astrocytes than on microglia (see **Figure 2C**).

Our antibody-based detection system for the ADP-ribosylation of P2X7 is not unique to P2X7. Loss of antibody-binding to ADP-ribosylated proteins has also been described for other ARTC2 target proteins: ADP-ribosylation of CD25 leads to a loss of binging of the clone 7D4 but not of PC61 (Teege et al., 2015). For CD8β, ADP-ribosylation diminishes binding of clones YTS156.7.7 and 53-5.8 but has no influence on the binding of 53-6.7 and H35-17.2 (Lischke et al., 2013). For LFA-1, ADP-ribosylation decreased the binding of mAbs 2D7 and C71/16 but not of mAb M17/4 (Nemoto et al., 1996)). This suggests that pairs of monoclonal antibodies that are affected/unaffected by ADP-ribosylation could be used as a non-radioactive alternative approach to estimate specific target ADP-ribosylation.

Another possible explanation for the lack of detectable P2X7 ADP-ribosylation on astrocytes could be the removal of the ADP-ribose groups by other cell surface enzymes (Zolkiewska and Moss, 1995; Nemoto et al., 1996; Laing et al., 2011). One possible candidate is ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), which can hydrolyze AMP from protein-attached ADP-ribose groups, yielding proteins modified with ribose-5'-phosphate (Palazzo et al., 2016). Whether ENPP1 is expressed by astrocytes or microglia and is capable of partially reversing cell surface

protein ADP-ribosylation on these cells still needs to be investigated.

One important finding of this study is the identification of P2X7a as a predominant splice variant in astrocytes and microglia. Since P2X7a is not gated by ADP-ribosylation (Schwarz et al., 2012), this could account for the resistance of astrocytes and microglia towards NICD. P2X7a and P2X7k differ in their cytosolic N-terminus as well as and in most of the first transmembrane domain (Nicke et al., 2009). Interestingly, by mutating the arginine at position 276 into lysine (R276K), P2X7a can be made sensitive to ADP-ribosylation (Schwarz et al., 2009, 2012). This gives room for speculation that also other modifications e.g., binding of small molecules or other proteins could render P2X7a sensitive to ADP-ribosylation.

Our results are in contrast to those of a previously published study that implicated NAD^+ as an inducer of astrocyte cell death in an ARTC2/P2X7 dependent fashion (Wang et al., 2012). However, Wang et al. neither analyzed whether astrocytes exhibit ecto-ART activity nor whether P2X7 on astrocytes is subject to ADP-ribosylation. Therefore, probably the NAD^+ mediated impact on astrocyte vitality could be triggered by ARTC2/P2X7-independent signaling pathways. Indeed, NAD^+ can also serve as ligand for other receptors, such as the metabotropic P2Y1 receptor (Mutafova-Yambolieva et al., 2007; Hwang et al., 2012). P2Y1 is expressed by astrocytes (Bowser and Khakh, 2004), however, to date it has not been reported that activation of P2Y1 by extracellular NAD^+ or its other ligand ADP can induce cell death in astrocytes. Further, it is conceivable that metabolites of NAD^+ rather than NAD^+ itself trigger astrocyte cell death. Astrocytes reportedly express the NAD^+-hydrolyzing ecto-enzyme CD38 (Yamada et al., 1997) that generates extracellular cyclic ADP-ribose and ADP-ribose. Therefore, future studies will show whether these NAD^+ metabolites influence astrocyte vitality.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Hamburger Behörde für Gesundheit und Verbraucherschutz, Veterinärwesen/Lebensmittelsicherheit.

AUTHOR CONTRIBUTIONS

BR, JS and MC performed the experiments with mixed glial cultures. BR and YD performed the experiments involving transfected HEK cells. SB perform P2X7 splice variant typing. SM, FK-N and TM supervised the experiments and assisted with data interpretation and manuscript preparation. BR assembled the figures and wrote the manuscript, which has been reviewed by all authors.

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Conflict of Interest: FK-N receives royalties from sales of antibodies developed in the lab *via* MediGate GmbH, a 100% subsidiary of the University Medical Center, Hamburg.

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frontiers in Molecular Neuroscience

REVIEW
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Pathological ATPergic Signaling in Major Depression and Bipolar Disorder

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The mood disorders, major depression (MD) and bipolar disorder (BD), have a high lifetime prevalence in the human population and accordingly generate huge costs for health care. Efficient, rapidly acting, and side-effect-free pharmaceuticals are hitherto not available, and therefore, the identification of new therapeutic targets is an imperative task for (pre)clinical research. Such a target may be the purinergic P2X7 receptor (P2X7R), which is localized in the central nervous system (CNS) at microglial and neuroglial cells mediating neuroinflammation. MD and BD are due to neuroinflammation caused in the first line by the release of the pro-inflammatory cytokine interleukin-1β (IL-1β) from the microglia. IL-1β in turn induces the secretion of corticotropin-releasing hormone (CRH) and in consequence the secretion of adrenocorticotropic hormone (ACTH) and cortisol, which together with a plethora of further cytokines/chemokines lead to mood disorders. A number of biochemical/molecular biological measurements including the use of P2X7R- or IL-1β-deficient mice confirmed this chain of events. More recent studies showed that a decrease in the astrocytic release of ATP in the prefrontal cortex and hippocampus is a major cause of mood disorders. It is an attractive hypothesis that compensatory increases in P2X7Rs in these areas of the brain are the immediate actuators of MD and BD. Hence, blood-brain barrier-permeable P2X7R antagonists may be promising therapeutic tools to improve depressive disorders in humans.

Keywords: P2X7 receptor, mood disorders, hippocampus, microglia, astroglia

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INTRODUCTION

The mood disorder major depression (MD) is characterized by extreme sadness, depressed mood, and loss of interest that persist for at least 2 weeks and interferes with the individual's social functioning (Harvey et al., 2007; Deussing and Arzt, 2018; Wei et al., 2018; Ribeiro et al., 2019). During bipolar disorder (BD), the mood state cycles between high (mania) and low (depression) episodes. MD and BD arise from complex interactions between genetic, developmental, and environmental factors (Koenig et al., 2011; Sullivan et al., 2012). The lifetime prevalence estimates for MD vary from 11% to 14% with females having an approximately 2-fold higher disease risk than males (Deussing and Arzt, 2018).

In view of the serious limitations these mood disorders impose upon the life quality of patients and because of their relatively frequent occurrence in the human population, it is of eminent importance to find good curative strategies to combat them. Presently, reuptake inhibitors of monoamines [noradrenaline, dopamine, and 5-hydroxytryptamine (5-HT)] are in the forefront of considerations, although significant drawbacks have to be taken into account: (1) the clinical improvement is achieved only after weeks of treatment; (2) there are multiple side effects; and (3) a substantial group of patients is resistant to therapy (Kulkarni and Dhir, 2009; Deussing and Arzt, 2018). Therefore, intensive search for alternative therapeutic targets and tools is a compelling necessity.

PURINERGIC P2X7 RECEPTOR

Ionotropic P2X7 receptors (P2X7Rs) are members of the P2X purinoceptor family, which were cloned and characterized in 1996 (Surprenant et al., 1996; North, 2002; Burnstock and Knight, 2004). Three properties of the P2X7R are distinguishing characteristics: (1) it is activated by high concentrations of ATP in the millimolar range, clearly surmounting concentrations needed to activate other P2X receptors (P2XRs), which are stimulated by ATP concentrations in the micromolar range; (2) it is a ligand-gated cationic channel, allowing the inward passage of Na^+ and Ca^{2+} and the outward passage of K^+ through the cell membrane. However, its repetitive or longer-lasting activation by ATP results in the opening of membrane pores permeable to large organic cations such as the fluorescent dye YO-PRO, which otherwise do not pass the cell membrane; and (3) the P2X7R consists of three subunits (large extracellular loop, two transmembrane regions, and N- and C-terminal ends) forming a receptor, but each subunit has a much longer C-terminus than that of the other P2XRs.

A particularly intensively discussed issue is the transition of the cationic channel to a large membrane pore, because it appears to be essential for cytokine production and secretion (Illes et al., 2019; Martin et al., 2019). Originally, it was suggested based on equilibrium potential (V_{rev}) measurements with the whole-cell patch-clamp technique that the ion conducting pathway shows progressive dilation (Virginio et al., 1999). However, this suggestion was recently refuted, because the shift in V_{rev} in a medium in which the counterion of intracellular K^+ was $NMDG^+$ instead of Na^+, emerged due to time-dependent alterations in the concentration of intracellular ions rather than channel dilation (Li et al., 2015). Moreover, during long-lasting activation of P2X7Rs, the single-channel current amplitude and the permeation characteristics remained constant (Pippel et al., 2017). Although convincing evidence indicates that pore opening is due to the recruitment of an accessory protein, the pannexin-1 channel (Panx-1; Pelegrin and Surprenant, 2006; Gulbransen et al., 2012; Shoji et al., 2014; Chen et al., 2017), the observation that, for example, the P2X7R pore formation is retained in Panx-$1^{-/-}$ cells supports the opposite notion (Hanley et al., 2012).

The P2X7R C-terminal tail constitutes about 40% of the whole protein, and its deletion or massive truncation prevents effects mediated by receptor activation such as dye uptake and membrane blebbing (generation of exosomes) but also alters channel kinetics (Kopp et al., 2019). In addition, the C-terminus was implicated in regulating signaling pathway activation, protein–protein interactions, and posttranslational modification (Costa-Junior et al., 2011).

P2X7Rs are major drivers of inflammation (Di Virgilio et al., 2017; Burnstock and Knight, 2018; Savio et al., 2018). Secretion of several pro-inflammatory cytokines and chemokines depends on the activation of P2X7Rs by large concentrations of ATP outpouring from damaged central nervous system (CNS) cells. The preferential location of P2X7Rs in the CNS is on the microglia, the resident macrophages of the brain (Bhattacharya and Jones, 2018). Microglia are equipped with a battery of pattern recognition receptors that stereotypically detect pathogen-associated molecules (PAMPs) such as lipopolysaccharide (LPS) from bacterial infection or danger-associated molecular patterns (DAMPs), such as ATP (**Figure 1**; Shao et al., 2015; Young and Górecki, 2018; Illes et al., 2019; Martin et al., 2019). Activation of microglia stimulates the release of interleukin-1β (IL-1β) in a two-step process: the first being the stimulation of toll-like receptor 4 (TLR4) by LPS, leading to accumulation of cytoplasmic pro-IL-1β, and the second being the ATP-dependent stimulation of P2X7Rs, promoting nucleotide-binding, leucine-rich repeat, pyrin domain containing 3 (NLRP3) inflammasome-mediated caspase-1 activation and secretion of IL-1β (Perregaux and Gabel, 1998; Ferrari et al., 2006). Caspase-1 generates IL-1β from pro-IL-1β by enzymatic degradation. It is important to note that the decrease of intracellular K^+ is a major stimulus for P2X7R-dependent NLRP3 inflammasome activation (Muñoz-Planillo et al., 2013; Di Virgilio et al., 2017, 2018).

IL-1β is co-produced/secreted with other pro-inflammatory cytokines such as IL-6 and IL-18 as well as tumor necrosis factor-α (TNF-α). A convincing argument for the idea that P2X7R activation provides the signal that leads to maturation and release of IL-1β and initiation of the cytokine cascade stemmed from experiments showing that P2X7R$^{-/-}$ cells or animals primed with LPS failed to produce IL-1β on the application/injection of ATP (Solle et al., 2001).

The majority of the fully sequenced mammalian genomes include representatives of all vertebrate P2X genes, including P2X4, which in humans is located on chromosome 12 in close proximity to P2X7 (Suurväli et al., 2017). The overlapping expression of P2X4 and P2X7Rs has been documented in macrophages and microglia (Dubyak, 2007; Suurväli et al., 2017). The reason for the co-expression may be the involvement of both receptors in inflammatory processes (de Rivero Vaccari et al., 2012; Hung et al., 2013; Sakaki et al., 2013). Originally, it has been assumed that subunits of P2X4 and P2X7Rs form the heteromeric complex P2X4/P2X7 (Guo et al., 2007), although more recent data lend support to the existence of independent receptors tightly interacting with each other (Nicke, 2008; Antonio et al., 2011). The agonist binding affinities largely differ between P2X7 and P2X4 receptors (P2X4Rs); while the former one is activated by millimolar ATP concentrations, the latter one responds to ATP in the micromolar range (Kaczmarek-Hájek et al., 2012). Hence, non-

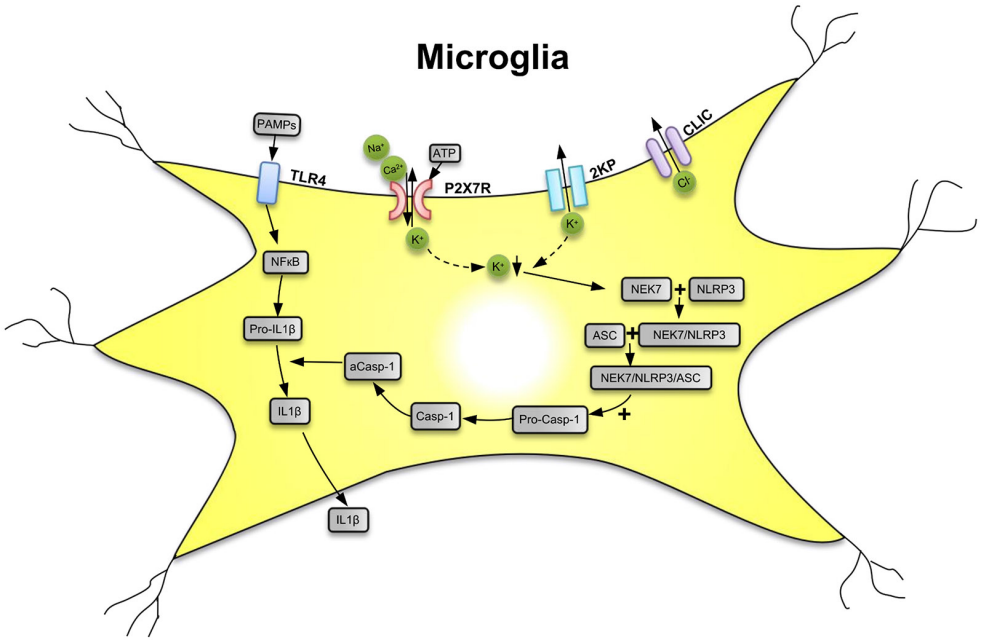

FIGURE 1 | Secretion of interleukin-1β (IL-1β) from microglial cells *via* involvement of the nucleotide-binding, leucine-rich repeat, pyrin domain containing 3 (NLRP3) inflammasome. Pathogen-associated molecular patterns [PAMPs; e.g., bacterial lipopolysaccharide (LPS)] act on toll-like receptor-4 (TLR4) and cause its phosphorylation. In consequence, in the cell nucleus, NF-κB is activated, which promotes the synthesis of the NLRP3 inflammasome and pro-IL-1β, both accumulating in the cytosol in their inactive forms. The activation of NLRP3 is primarily due to a decrease of the intracellular K^+ concentration ($[K^+]_i$), initiated by the stimulation of P2X7Rs by high local concentrations of the molecule ATP, which is considered to be a danger-associated molecular pattern (DAMP). P2X7Rs allow the inward flux of Na^+/Ca^{2+} and in exchange the outward flux of K^+, leading to a fall in $[K^+]_i$. The opening of two-pore domain potassium channels (2KP) may also lead to an impoverishment in cytoplasmic K^+. A further stimulus for NLRP3 activation is the outward flux of Cl^- through chloride intracellular channels (CLICs). TLR4, P2X7Rs, 2KP channels, and CLIC are all located in the cell membrane of the microglia. A sensor for the fall in $[K^+]_i$ is the NEC7 serine/threonine kinase. NEC7 is able to form a complex with NLRP3, which is still inactive, but after constitution of a still larger multimeric complex with apoptosis-associated speck-like protein (ASC) recruits pro-caspase-1 (pro-Casp-1). In consequence, pro-Casp-1 in a complex with NLRP3 and ASC is cleaved to Casp-1, which then by its activated form a-Casp-1 degrades pro-IL-1β to IL-1β. Then, IL-1β leaves the cell by a number of mechanisms to the extracellular space and exerts its effects as a neuroinflammatory cytokine. K^+ ↓, decrease of the K^+ concentration. Artwork by Dr. Hayan Yin.

cell-lytic micromolar ATP release cannot directly stimulate P2X7Rs but easily activates its more sensitive partner, the P2X4R, thereby modifying the function of the P2X4–P2X7R multiprotein complex.

ASSOCIATION OF P2X7 GENE POLYMORPHISM AND MOOD DISORDERS

Linkage studies suggested that variations of the chromosome 12q24,31 containing candidate genes for the P2X7R and calmodulin-dependent protein kinase b (CaMKKb) may be associated with MD and BD. It has been repeatedly reported that the nonsynonymous single-nucleotide polymorphism (NS-SNP) *rs2230912* coding for Gln460Arg-P2X7R is associated with MD (McQuillin et al., 2009; Soronen et al., 2011; Sperlágh and Illes, 2014). However, in the meantime this association has been questioned. Although further studies have supported the possible role of this NS-SNP in mood disorders (Halmai et al., 2013; Vereczkei et al., 2019), other authors failed to detect any association (Green et al., 2009; Grigoroiu-Serbanescu et al., 2009). Two recent meta-analyses also yielded divergent results, one of them confirming (Czamara et al., 2018) and the other one refuting (Feng et al., 2014) the hypothesis on the causal role of the NS-SNP *rs2230912* in MD and BD. Eventually, this led the Psychiatric Genomics Consortium to deny the *P2RX7* gene as a genetic risk factor for mood disorders in large-scale genome-wide association studies (Mühleisen et al., 2014; Wray et al., 2018).

When various *P2RX7* single-nucleotide polymorphism were investigated by electrophysiology/dye uptake studies either in native cells or in HEK293 cells transfected with the respective plasmids, several gain-of-function or loss-of-function allelic mutations were identified (Gu et al., 2001; Roger et al., 2010; Sun et al., 2010). Surprisingly, the ATP-induced inward current was the same through the wild-type (WT) receptor and the Gln460Arg polymorphic receptor (Roger et al., 2010), leading to the suggestion that a haplotype block may explain the lack of the expected decrease of ATP effects (Sluyter et al., 2010). This riddle was dissolved by Aprile-Garcia et al. (2016) who reported that although the variant *per se* is not compromised in its function, co-expression of WT P2X7R with the Gln460Arg-P2X7R results in inhibition of calcium influx, channel current, and intracellular signaling. Moreover, co-immunoprecipitation and FRET studies demonstrated that the Gln460Arg-P2X7R variant physically interacts with the WT

P2X7R. The same group of authors found that humanized mice co-expressing both P2X7R variants showed alterations in their sleep quality resembling signs of a prodromal MD state (Metzger et al., 2017a).

In conclusion, the evidence for an association of the SNP *rs2230912* as an etiologic factor for hereditary mood disorders is far from being equivocal, although its role in P2X7R involvement cannot be excluded either (see above controversial results of epidemiological studies).

THE P2X7R TRIGGERS NEUROINFLAMMATION AND SUBSEQUENT MOOD DISORDERS

Activation of the inflammasome, which precipitates the release of pro-inflammatory cytokines, and activation and migration of microglia and reactive astrogliosis are key regulators of the neuroinflammatory response (Beamer et al., 2016; Liu and Quan, 2018). IL-1β is a master regulator of inflammatory reactions, capable of activating innate immunity by inducing the expression of inflammatory cytokines and chemokines, eliciting leukocyte infiltration into the inflammatory loci, increasing the phagocytic and bactericidal activity of immune cells, enhancing the activity of the complement system, and facilitating the activation of the adaptive immune responses (Dinarello, 2009; Liu and Quan, 2018).

Stress exposure is considered to be the main environmental factor instigating mood disorders in humans, and all animal models of MD are based on exposure to inescapable stress (Ribeiro et al., 2019). Psychological and metabolic stress could induce adrenocorticotropic hormone (ACTH) and glucocorticoid secretion in mice, which were reduced in IL-1 knockouts (KOs) or transgenic animals overexpressing brain IL-1ra, a naturally occurring IL-1 antagonist (Goshen et al., 2003; Liu and Quan, 2018). Intracerebral administration of IL-1 induces corticotropin-releasing hormone (CRH) release in rats (Barbanel et al., 1990), and psychological stress causes brain IL-1 expression (Gadek-Michalska and Bugajski, 2010). Thus, brain IL-1 could mediate physiological responses to stress by stimulating the production of the immunosuppressive glucocorticoid hormone cortisol from the adrenal medulla (Liu and Quan, 2018). In perfect correlation with this idea, IL-1ra suppresses stress-induced depression in animal models (Koo and Duman, 2009; Maes et al., 2012). Consequently, disturbances of the main neuroendocrine stress response system, the hypothalamic–pituitary–adrenal axis including the main initiator CRH and effector glucocorticoids, have been suggested to cause depression (de Kloet et al., 2005; Deussing and Arzt, 2018).

As outlined previously, the primary function of P2X7Rs in the CNS is to initiate (neuro)inflammation. Therefore, it was deduced that the receptor might cause MD and BD, which are reportedly accompanied by neuroimmunological alterations (Bhattacharya and Jones, 2018). The chain of events may be the following: stress causes a massive outflow of ATP in the brain stimulating P2X7Rs, which on their behalf trigger the release of IL-1β. Then, IL-1β induces the secretion of CRH and the consecutive production of ACTH/glucocorticoids, resulting in mood disorders. In fact, acute restraint stress rapidly increases extracellular ATP, the inflammatory cytokine IL-1β, and the active form of the NLRP3 inflammasome in the hippocampus of rodents (Iwata et al., 2016).

Acute and chronic stress may induce in rodent models depressive-like behavior, which can be used to investigate antidepressive pharmaceuticals (**Figure 2**). In contrast to the acute stress models shown in this figure, unpredictable chronic mild stress (UCMS) is delivered for prolonged periods of 8–12 weeks and includes once daily, for example, immobilization, food deprivation, light/dark phase reversal, hot environment, and cage shaking. This procedure leads to depressive-like behavior as measured by reduced sucrose consumption and prolonged immobility in the tail suspension test (TST) and forced swim test (FST) in rodents (Zhang et al., 2015; Su et al., 2017; Wang et al., 2018; Feng et al., 2019). UCMS also resulted in higher protein levels of NLRP3, caspase-1, and IL-1β in the hippocampus of stress-exposed mice (Zhang et al., 2015) and rats (Wang et al., 2018; Feng et al., 2019). Pharmacological blockade of NLRP3 (Zhang et al., 2015) or its genetic deletion (Su et al., 2017) decreased the level of inflammatory mediators and counteracted the symptoms of depressive-like behavior. Microglia has been shown to be essential for these effects, because chronic minocycline treatment known to block the activation of microglia inhibited the following engagement of the NLRP3 inflammasome and the ensuing increased release of inflammatory mediators (Wang et al., 2018). Further, the inflammasome inhibitor Ac-Tyr-Val-Ala-Asp-chloromethyl ketone blocked the behavioral alterations and the production of inflammatory mediators caused by systemic injection of LPS to mice (Zhu et al., 2017). Chronic treatment with the standard antidepressant drug fluoxetine suppressed all symptoms induced by UCMS in rodents (Pan et al., 2014; Du et al., 2016). There is a multitude of review articles available which give further insight to the causal relationship between inflammasome activation and MD (Alcocer-Gómez et al., 2016; Kaufmann et al., 2017; Franklin et al., 2018; Herman and Pasinetti, 2018).

The role of P2X7Rs as essential activators of NLRP3 was also convincingly demonstrated by showing that UCMS elevates hippocampal P2X7R levels (Tan et al., 2017). The selective P2X7R antagonist Brilliant Blue G (BBG) attenuated the increase of immobility time in TST and FST in mice after activation of the inflammasome by LPS (Ma et al., 2014). Similarly, BBG reversed the behavioral deterioration induced by UCMS in mice (Farooq et al., 2018), and clemastine, a nonselective antagonist of P2X7Rs, counteracted the prolonged duration of immobility in TST (Su et al., 2018).

Another piece of evidence for the participation of P2X7Rs in depressive-like behavior was supplied by the use of KO animals. *P2RX7*$^{-/-}$ mice exhibited an antidepressant-like profile in TST and FST; this effect was not accompanied by changes in spontaneous locomotor activity (Basso et al., 2009). In these animals, decreased

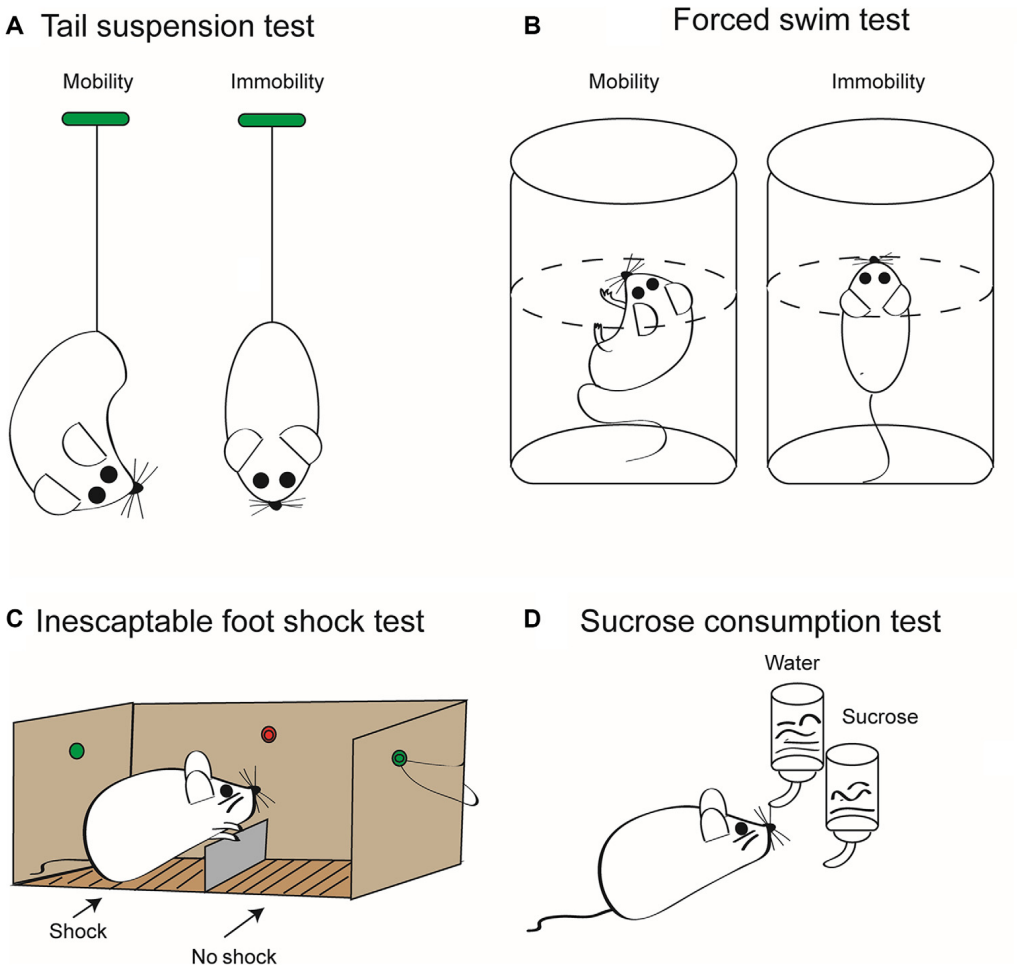

FIGURE 2 | Some relevant tests to measure depressive-like behavior in rodents induced by stressors. These tests are employed to quantify the extent of "learned helplessness" of rats/mice, and thereby, with the necessary precaution, they are supposed to model major depression (MD) in humans. In consequence, they are routinely used to determine the effectiveness of antidepressant pharmacological agents. **(A)** Tail suspension test (TST). Mice are suspended by their tails with tape, in such a position that they cannot escape or hold on to nearby surfaces. Then, the sum of the time periods is measured during which they stop escape reactions; that is, they become immobile. This time period is considered to be a measure of the depressive-like behavior. The duration of the test is maximized usually at 6 min, in order to avoid unnecessary suffering of the animals. Because the weight of rats is much larger than that of mice, rats are not considered to be an appropriate rodent species for this test method. **(B)** Forced swim test (FST). Mice or rats are put into a tank containing water whose temperature is kept at about 23°C. The dimensions of the tank and the depth of water are such that the animals are forced to swim as an escape reaction. Swimming is stopped when the animal notices that it cannot escape and starts to float on the surface of the water. The length of the immobile periods is measured during a maximum of 6 min and is considered to be a measure of the degree of depressive-like behavior. **(C)** Inescapable foot shock test (IFST). The electric foot shock paradigm includes acute or chronic exposures of shocks of varying intensity and duration on an electrified grid floor in a foot shock apparatus. In contrast to the scheme shown, the mice or rat is not able to escape from the chamber where it is subjected to electric shocks to the other chamber where there is no comparable painful stimulation. Animals generally do not habituate to foot shocks in comparison to other stressors, including loud noise, bright light, and hot and cold temperatures. **(D)** Sucrose consumption test (SCT). The two-bottle choice procedure for assessing sucrose preference is a useful test to investigate anhedonia (i.e., inability to feel pleasure) in laboratory rodents. It allows for a comparison between the preference for sucrose solution in drinking water and that for water only. This preference is measured by volume and/or weight of liquid consumed daily, which is then converted to a percent sucrose solution consumed compared to a water only baseline period. As a result of the anhedonia induced by inescapable foot shock, the preference for choosing a sucrose solution decreases in mice or rats. It is important to verify the results of all these tests with separate behavioral tests that measure overall activity such as the open-field test. Moreover, TST, FST, and IFST/SCT should be used in combination to minimize false positivity, and it should be kept in mind that depressive-like behavior in laboratory rodents is not identical with the clinical state of MD in human beings (see above). Artwork by Ms. Lumei Huang.

behavioral despair in FST, reduced immobility in TST, and attenuated amphetamine-induced hyperactivity were detected, indicating an antidepressant phenotype (Sperlágh et al., 2012; Csölle et al., 2013a,b). In addition, several potential mechanisms were identified for these mice such as elevated basal production of brain-derived neurotrophic factor (BDNF), enhanced neurogenesis, and increased 5-HT bioavailability in the hippocampus (Csölle et al.,

2013b). In contrast to these findings, equivalent levels of immobility were observed in *P2RX7*$^{-/-}$ and WT mice on the first exposure to forced swim, but much greater immobility was seen in the WT animals on second and third exposures (Boucher et al., 2011). An explanation for this discrepancy may be that the FST was recently questioned to be an adequate model of despair or helplessness (Molendijk and de Kloet, 2019).

Another factor of insecurity inherent to the P2X7R-deficient mice is that with the two types used routinely for experimentation, some splice variants of the *P2RX7* gene escape inactivation (Bartlett et al., 2014; Sperlágh and Illes, 2014). Experiments with a recently generated conditional humanized P2X7R-deficient mouse, supposed to be devoid of active splice variants of the receptor, could be helpful in this respect (Metzger et al., 2017b).

A further argument for the involvement of P2X7Rs in the etiology of MD is supplied by studies which show that inhibition or genetic abrasion of the P2X7R–Panx-1 pore complex suppresses spreading depolarization and neuroinflammation in mice (Chen et al., 2017). This is in perfect agreement with findings that Cx43- and Panx-1-based channels participate in the induction of neuroinflammation and cerebral neuropathies (Sarrouilhe et al., 2017; for further considerations on the role of connexins/pannexins in MD, see the section "Inhibited Astrocytic ATP Release in the Pre-frontal Cortex").

MICROGLIAL AND ASTROGLIAL FUNCTIONS; CELL DEATH AND PROLIFERATION BY P2X7 RECEPTORS

Microglia are the resident immunocytes of the CNS; unlike other tissue macrophages, they persist for the life of the organism with negligible turnover rates at steady state (Tay et al., 2016; Anderson and Vetter, 2019). Microglia are instrumental in the maintenance of biochemical homeostasis, neuronal circuit maturation during development, and experience-dependent remodeling of neuronal circuits in the adult brain (Szepesi et al., 2018; Anderson and Vetter, 2019; Illes et al., 2019). The cellular processes of quiescent or "resting" microglia are highly mobile (extension and withdrawal) by scanning the environment for disruptions of brain homeostasis (Davalos et al., 2005). When microglia detect danger signals, they rapidly become activated by shortening their processes, eventually being transformed to amoeboid microglia, which produces a number of cytokines, chemokines, and growth factors, as well as developing phagocytotic activity (Kettenmann et al., 2011).

Microglia establish close contact with both neurons (Eyo and Wu, 2013) and astrocytes (Jha et al., 2019), supplementing the "tripartite synapse" (see below) with a microglial component ("quadripartite synapse"; Schafer et al., 2013; Illes et al., 2019). An important regulator of this interaction is ATP/ADP, which is released from neurons and astrocytes/microglia by exocytotic and non-exocytotic mechanisms (Calovi et al., 2019). Microglia possess a range of P2Y receptors (P2YRs). P2Y1 receptors (P2Y1Rs) steer microglial migration (De Simone et al., 2010), P2Y6 receptors (P2Y6Rs) regulate microglial phagocytosis (Koizumi et al., 2007), and P2Y12 receptors (P2Y12Rs) are responsible for chemoattraction of microglial branches to the site of ATP accumulation (Ohsawa et al., 2010).

P2X7, the archetypical macrophage/microglial receptor, mediates two diametrically opposite functions of microglia such as, firstly, proliferation, most likely *via* calcium signaling (Monif et al., 2010, 2016), and, secondly, necrosis/apoptosis *via* the generation of transmembrane pores and activation of the caspase enzymatic cascade (Bartlett et al., 2014; He et al., 2017). Whereas microglial phagocytosis of bacteria and cellular debris is under the regulation of ATP/ADP, P2X7 has been shown to be a scavenger receptor for apoptotic cells even in the absence of its ligand ATP (Gu et al., 2011).

Astrocytes to a large extent define synaptic connectivity. Indirect effects are exerted by changes in astrocytic functions due to modifications in K^+ uptake and redistribution; Cl^- and water fluxes; Na^+/K^+, Na^+/Ca^{2+}, or Na^+/HCO_3^- exchange; neurotransmitter uptake; etc (Verkhratsky et al., 2017; Mederos et al., 2018; Illes et al., 2019). Astrocytes also directly modify synaptic transmission, because they contact and partially ensheathe synapses with their perisynaptic processes (Allen and Eroglu, 2017). Astrocytes may release "gliotransmitters" [e.g., glutamate, γ-aminobutyric acid (GABA), and ATP] by an exocytotic mechanism modulating neuronal functions; the structural basis for this effect is the "tripartite synapse," which consists of the presynaptic elements, the postsynaptic/dendritic structures, and the astrocytic processes terminating at the synapse (Araque et al., 1999; Halassa and Haydon, 2010; Illes et al., 2019). More recently, the tripartite synapse hypothesis has evolved into the idea of an "astroglial cradle" summarizing all aspects of the synapse function not only those mediated by neurotransmitters (Verkhratsky and Nedergaard, 2018). In addition, astrocytes may also deliver ATP/ADP into the extracellular space by non-exocytotic mechanisms *via*, for example, connexin hemichannels, pannexin channels, maxi-anion channels, and volume-regulated anion channels, contributing to the exocytotic release (Cheung et al., 2014; Dahl, 2015).

For a couple of years, it was doubted whether astrocytes possess the prototypic microglial P2X7R (see, e.g., Jabs et al., 2007); however, more recently convincing functional evidence corroborated this notion (Duan et al., 2003; Oliveira et al., 2011; Illes et al., 2012, 2017). Immunohistochemical investigations in the nucleus accumbens of rats showed that after stab wound injury, P2X7R immunoreactivity was observed in glial fibrillary acidic protein (GFAP)-positive astrocytes (Franke et al., 2001). Similar findings were reported for the cerebral cortex of spontaneously hypertensive rats, where the occlusion of the medial cerebral artery led to the upregulation of P2X7Rs in the penumbra surrounding the necrotic region (Franke et al., 2004). Thus, it was concluded that P2X7Rs induce proliferation of astrocytes upon their stimulation by ATP possibly released from the nearby, massively damaged CNS tissue (Franke et al., 2012; Franke and Illes, 2014; Martin et al., 2019).

INHIBITED ASTROCYTIC ATP RELEASE IN THE PREFRONTAL CORTEX AND HIPPOCAMPUS IS A POSSIBLE LINK TO MOOD DISORDERS

After having discussed the role of P2X7Rs in MD and BD, we turn our attention to a possible role of astrocytes in the pathogenesis of mood disorders by their impeded release of ATP. Numerous lines of evidence support the contention that modification of astrocytic functions or decreased density of astrocytes in the frontolimbic and hippocampal regions is associated with depression (Rajkowska and Stockmeier, 2013; Peng et al., 2015; Rial et al., 2016). Astrocytes are integrated into networks where individual cells communicate with each other *via* gap junctions. Connexins, mainly represented by Cx43, provide the molecular basis for gap junction channels, connecting the cytoplasm of adjacent glial cells (Theis and Giaume, 2012; Verkhratsky and Nedergaard, 2018; Illes et al., 2019). These channels allow direct exchange of a variety of small molecules of less than about 1 kDa, including ions (most importantly Ca^{2+}), energy metabolites, neurotransmitters, and signaling molecules coordinating metabolic and functional activities of connected cells (Pannasch and Rouach, 2013; Cheung et al., 2014). In addition, unopposed connexin hemichannels and Panx-1 channels are conduits for ATP release from astrocytes (Huang et al., 2012; Beckel et al., 2014).

Rats exposed to chronic unpredictable stress exhibited deficits in the sucrose preference test, which signals anhedonic behavior, a core symptom of depression. In the prefrontal cortex of these animals, the diffusion of gap junction channel-permeable dyes as well as the expression of Cx43 is decreased (Sun et al., 2010; Xia et al., 2018). The infusion into the prefrontal cortex of both the gap junction blocker carbenoxolone and the Cx43 mimetic, antagonistic peptide Gap27 caused anhedonia. Similarly, exposure to chronic unpredictable stress of rats also caused a decrease in the expression of prefrontal cortical connexins, while long-lasting treatment with antidepressants with unrelated structure and mode of action invariably increased the expression of connexins (Ren et al., 2018).

In the case of the blockade of connexins, it is unclear whether the gap junction property or the outflow of various neuroactive substances, for example, ATP through (hemi)channels, has been inhibited in the above experiments. However, Panx-1 works only as a channel, and therefore, its blockade in the medial prefrontal cortex by carbenoxolone, ^{40}Panx, and mefloquine appeared to be due to impaired release of an astrocytic signaling molecule (Ni et al., 2018). This molecule may be ATP, because the mefloquine-induced depressive-like behavior was prevented by preconditioning with ATP.

However, opposite results have also been published. Dye uptake experiments in hippocampal slices demonstrated that acute restraint stress, known to instigate depressive-like behavior, induced opening of both Cx43 and Panx-1 channels (Orellana et al., 2015). Moreover, incubation of cultured astrocytes with seven antidepressants inhibited Cx43 channels with different efficacies depending on their therapeutic potencies (Jeanson et al., 2015). An explanation for these divergent results may be that conclusions were drawn based on investigations carried out on different organizational structures (cell culture/brain slice vs. whole animal) and different areas of the brain (hippocampus vs. prefrontal cortex).

When mice susceptible or non-susceptible to social defeat were compared to each other, the brains of the susceptible mice contained lower ATP levels than those of the non-susceptible ones (Cao et al., 2013). Further, FST also caused ATP deficiency in the brain and decreased the ATP content in the microdialysates of their prefrontal cortices. The infusion of ATP into the lateral ventricle of the mouse brain decreased the duration of immobility in the FST. Inositol 1,4,5-trisphosphate (IP3) triggers the release of Ca^{2+} from the endoplasmic reticulum which is a prerequisite for the exocytotic release of ATP. In consequence, IP3 receptor type 2 KO mice exhibited lower ATP release from astrocytes compared with their WT counterparts, as well as a depressive-like phenotype. Comparably, the astrocytic, vesicular release of ATP was blocked, when in mice, a dominant negative domain of vesicular soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) was selectively overexpressed in astrocytes. These transgenic animals also exhibited depressive-like behaviors (Halassa and Haydon, 2010).

Conventional KO and conditioned astrocytic KO of the calcium homeostasis modulator 2 channel (Calhm2) initiated depression-like behaviors in mice (TST and FST), indicating that this channel is the exit pathway for the release of ATP (Jun et al., 2018). In partial disagreement with these findings, the effect of the antidepressant drug fluoxetine has been shown to increase ATP exocytosis (Kinoshita et al., 2018). In consequence, the authors of this latter study concluded that the astrocytic release of ATP involved in depression operates by vesicular exocytosis rather than by Calhm2 opening.

Thus, ample evidence supports the notion that an impaired ATP release from prefronto-cortical astrocytes is the primary reason for depressive-like behavior and probably also MD. However, there is disagreement on whether this damage may be confined to connexin/pannexin hemichannels, the Ca^{2+}-dependent exocytotic machinery, or Calhm2 channels as exit pathways for ATP. In view of the already discussed idea that hyperreactivity of microglial/astrocytic P2X7Rs is causally involved in the pathogenesis of MD/BD, it is quite attractive to hypothesize that the decreased ATP concentration in the prefrontal cortex (and hippocampus) leads to upregulation of P2X7Rs in this area of the brain.

P2X7R ANTAGONISTS AS POSSIBLE THERAPEUTIC AGENTS TO TREAT MOOD DISORDERS

Because P2X7Rs mediate peripheral and central inflammation, a number of pharmaceutical companies developed ligands for this target, and some of them advanced P2X7R antagonistic

compounds even to clinical trials. A major advantage of a P2X7R antagonistic drug would be that the receptor is stimulated only by pathologically high extracellular concentrations of ATP; thus, its blockade will not interfere with effects due to the more physiological release of smaller quantities of ATP (Illes et al., 2019). Although, against expectations, P2X7R antagonists did not produce a beneficial effect on rheumatoid arthritis (Keystone et al., 2012; Stock et al., 2012), they improved symptoms in patients with moderate-to-severe Crohn's disease (Eser et al., 2015). Nonetheless, the development of such compounds for both therapeutic indications was terminated by Pfizer and Astra-Zeneca (Rech et al., 2016; Young and Górecki, 2018), in the case of Crohn's disease probably also because of insufficient safety margins (Bhattacharya and Biber, 2016).

It can be derived from the available literature as discussed in our review that P2X7Rs may be promising targets to treat MD and BD (Bhattacharya, 2018; Wei et al., 2018). However, the following three difficulties are major obstacles in developing new P2X7R antagonists for the treatment of mood disorders: (1) a number of P2X7R antagonists act in rodent receptor orthologs but not in human receptor orthologs when investigated under *in vitro* conditions (Bhattacharya and Biber, 2016); (2) the disease can be modeled by depressive-like states induced by applying acute or chronic inescapable stress to rodents; however, it is most likely that there is no perfect analogy with the human disease (Ribeiro et al., 2019); and (3) P2X7R antagonists have to pass the blood-brain barrier in order to exert effects in the CNS.

The majority of compounds disclosed in the last decade are human-specific P2X7R antagonists with no or weak rodent activity but suffer from lack of robust CNS permeability. However, numerous blood-brain barrier-permeable P2X7R antagonists have been developed by Abbott, Astra-Zeneca, GlaxoSmithKline, and especially Janssen more recently (Bhattacharya, 2018; Wei et al., 2018). The Janssen compounds JNJ-47965567 (Bhattacharya et al., 2013) and JNJ-42253432 (Lord et al., 2014) demonstrated activity in rodent and human P2X7Rs, had good rat pharmacokinetic profiles, and had excellent brain penetration, when dosed subcutaneously.

CONCLUSIONS AND PERSPECTIVES

A tight causal relationship of P2X7Rs with mood disorders is imperatively suggested by their involvement in neuroinflammation and the subsequent modulation/damage of neuronal circuits in mood-relevant areas of the brain. Functional changes in long-term synaptic potentiation (LTP) in the lateral habenula have been observed in rats exposed to inescapable stressors leading to learned helplessness (Li et al., 2011; Park et al., 2017). Similarly, in models of learned helplessness, the expression of synapse-related genes decreased, indicating the loss of synaptic structures. The morphological alterations were manifest as a decrease in spine synapse density in the CA1, CA3, and dentate gyrus of the hippocampus (Hajszan et al., 2009) and were absent in P2X7R-deficient mice (Otrokócsi et al., 2017).

BOX 1 | Microglial cellular effectors modulating neuronal functions

- Resting (ramified) microglia constantly scan their environment for exogenous and endogenous signals indicating a threat to the neuronal homeostasis. They detect PAMPs such as LPS from bacterial infection or DAMPs, such as ATP. DAMPs initiate the transformation of ramified microglia after withdrawal of their cellular processes to microglia with a rounded surface.
- In activated microglia, the assembly/activation of the inflammasome converts pro-caspase-1 to caspase-1, which in turn cleaves the biologically inactive pro-IL-1β to IL-1β. Caspase-1 also activates the apoptotic caspase enzyme cascade to induce programmed cell death (apoptosis). After LPS priming, P2X7Rs largely boost the inflammatory cytokine response executed in the first line by IL-1β, but also by IL-6 and TNF-α.
- Microglial P2X7Rs are termed "suicide receptors" because their activation causes necrosis/cell death.
- Activated microglia release proteases as well as reactive oxygen and nitrogen species into their cellular environment. In addition, they secrete diacylglycerol lipase responsible for endocannabinoid production. These microglia also release ATP and probably also glutamate by vesicular exocytotic mechanisms.
- ATP through activation of microglial P2X7Rs releases extracellular vesicles from the plasma membrane (microvesicles and exosomes), inducing a robust inflammatory reaction in glial cells.
- Activated microglia also acquires phagocytotic properties, thereby eliminating not only cellular debris or pathogenic bacteria but also surplus neurons during development and thereby shaping adult neuronal circuits by phagocytosis or synaptic stripping.

In short, neuroinflammation triggered by inescapable stressors activates microglial cells outpouring cytokines/chemokines, proteases, reactive oxygen, and nitrogen species which damage neurons in the prefrontal cortex and hippocampus (**Box 1**). Microglia also acquires phagocytotic properties, thereby shaping adult neuronal circuits by phagocytosis and synaptic stripping. The classical DAMP ATP initiates the transformation of ramified microglia to microglia with a rounded surface. P2X7Rs have a key role in microglial activation causing *via* multiple signal transduction pathways functional/morphological changes leading to depressive-like reactions in animals and possible also in humans.

In view of good blood-brain barrier-permeable P2X7R antagonists at our disposal and the intensive research activities carried out in academic and pharmaceutical institutions, there is strong hope that newly synthesized and clinically tested drugs of this family will be soon available as potent and side-effect-free antidepressants.

AUTHOR CONTRIBUTIONS

PI drafted the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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frontiers in Molecular Neuroscience

HYPOTHESIS AND THEORY
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Association of Hypomorphic P2X7 Receptor Genotype With Age

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One of the main risk factors for brain diseases is aging. Recent studies have shown that aging is a progressive degenerative process associated with chronic low-level inflammation. The ATP-gated P2X7 receptor (P2X7R) plays an important role in inflammation and has been associated with different brain (e.g., Alzheimer's and Parkinson's) or other age-related (osteoporosis, arthritis, cancer) diseases. Several single nucleotide polymorphisms (SNPs) in the *P2RX7* gene have been identified, including the loss-of-function 1513A>C and 1405A>G SNPs, and the gain-of-function 489C>T and 1068G>A SNPs. We carried out a literature analysis to verify an association between *P2RX7* SNPs' frequency and age. In 34 worldwide eligible studies (11.858 subjects) no correlation between 1513CC genotype frequency and age emerged. On the contrary, analysis of European Caucasian cohorts (7.241 subjects) showed a significant increase in 1513CC frequency with age ($P = 0.027$). In agreement with these findings, analysis of two publicly available datasets, including USA Caucasian cohorts, unveiled an increased frequency of 1513CC and 489CC genotypes with age ($P = 0.0055$ and $P = 0.0019$, respectively). Thus, hypomorphic *P2RX7* genotypes may be positively selected with age in European and North American Caucasian populations. We hypothesize that Caucasian individuals bearing an anti-inflammatory P2X7R phenotype and living in high-income countries may have a longer life expectancy.

Keywords: aging, P2X7, inflammation, polymorphisms, neurodegeneration

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INTRODUCTION

Prevalence of central nervous system (CNS) diseases increases with age, either directly, by a time-dependent accumulation and aggregation of abnormal proteins, e.g., in Alzheimer's, Parkinson's, and Huntington's disease, or indirectly, due to the increase in age-related changes that foster the onset and/or progression of brain diseases. For example, stroke is associated with a high risk of seizures and epilepsy, while type 2 diabetes

and atherosclerosis are a risk factor for Alzheimer's and cerebrovascular disease (Lénárt et al., 2016; Beghi and Giussani, 2018; Hou et al., 2019).

On the other hand, inflammation is a well-recognized pathogenic factor in age-associated disorders, neurological disorders included. A role for chronic, low level, systemic inflammation is hypothesized in psychiatric conditions, epilepsy, cerebrovascular diseases, dementia and neurodegeneration (Vezzani et al., 2011; Najjar et al., 2013; Lénárt et al., 2016; Guzman-Martinez et al., 2019; Ignácio et al., 2019).

The P2X7 receptor (P2X7R) is an ATP-gated cation-selective channel involved in inflammation and host defense. P2X7R activation promotes the release of several pro-inflammatory factors, both in the CNS and in peripheral tissues, and is understood to participate in the pathogenesis of several neurodegenerative diseases such as multiple sclerosis, Alzheimer's, Parkinson's, and Huntington's disease (Savio et al., 2018; Kanellopoulos and Delarasse, 2019). The P2X7R is also involved in the pathogenesis of age-related pathologies such as cancer, osteoporosis, diabetes and arthritis (Tao et al., 2013; Kvist et al., 2014; Sperlágh and Illes, 2014; Novak and Solini, 2018; Adinolfi et al., 2019). The strong pro-inflammatory activity of the P2X7R depends on the ability of this receptor to trigger the generation of reactive oxygen species and release of cytokines and metalloproteases. Some of these responses are mediated through the stimulation of the NLRP3 inflammasome and of caspase-1. P2X7R activation may have opposite effects on cell growth; low level, tonic stimulation promotes cell proliferation, while sustained stimulation triggers cell death by necrosis or apoptosis (Di Virgilio, 2013). P2X7R-dependent cytotoxicity can be exploited for intracellular pathogen killing (Adinolfi et al., 2018).

P2RX7 is a highly polymorphic gene located on chromosome 12q24.31. The most studied *P2RX7* single nucleotide polymorphism (SNP) is the 1513A>C. Homozygous subjects carrying the 1513CC genotype show a non-functional P2X7R pore and a reduced ability to activate inflammation compared to wild-type subjects bearing the AA genotype (Wesselius et al., 2012). The possible association of the 1513CC *P2RX7* genotype with different inflammatory conditions is attracting increasing interest (Di Virgilio et al., 2017). Other important *P2RX7* SNPs are the loss-of-function 1405A>G, and the gain-of-function 489C>T and 1068G>A (Sluyter and Stokes, 2011; Caseley et al., 2014).

In the present study, we tested the hypothesis of an association between age and frequency among polymorphic P2X7 receptor genotypes. To this aim, we carried out a revision of the relevant literature and the analysis of two dbGaP (database Genotype and Phenotype) datasets.

MATERIALS AND METHODS

Publication Search Strategy

A Medline literature search using the keywords rs3751143, 1513A>C, or E496A, which identify the *P2RX7* SNP of interest, allowed a partial retrieval of all pertinent studies. Therefore, the search was extended to the keyword mesh "(P2X7 or P2X7R or P2RX7) and (polymorphism or polymorphisms)." In July 2016, this search produced 178 hits, from which 79 articles analyzing the frequency of 1513A>C *P2RX7* SNP were selected. Forty seven studies were excluded because: (a) two were based on small cohorts (16 and 46, respectively); (b) seven analyzed only diseased subjects, with no cohorts comprising healthy controls; (c) four reported data from already published control cohorts; (d) one article was not found; and (e) the remaining 33 studies did not specify the mean or median age and/or CC frequency of the control cohorts. Thirty-two studies involving a total 34 cohorts (Zhang et al., 2003; Fernando et al., 2007 articles describe two different control cohorts) with 11,858 subjects, were thus identified. With the exception of the study by Sambasivan (Sambasivan et al., 2010), genotype distribution in all control cohorts was in Hardy–Weinberg equilibrium (HWE).

dbGaP Analysis

We received NIH approval to analyze two datasets comprising *P2RX7* SNPs in Caucasian control cohorts which report the age of enrolled subjects:

1. HGVST1 (Human Genoma Variation ST1); Study of prostate cancer; dbGaP Study Accession, phs000207.v1.p1. Dataset Name: CGEMS (The Cancer Genetic Markers of Susceptibility) Prostate_Data; Dataset Accession, pht001105.v1.p1); NIH approval, [#47650-2] [#47650-4]. It is a nested case-control study to identify SNP associated to augmented prostate cancer susceptibility. Control cohort include 1,101 men with European ancestry selected from The Prostate, Lung, Colon and Ovarian (PLCO) Cancer Screening Trial from USA (Yeager et al., 2007).
2. HGVST6; Study of Parkinson's Disease; dbGaP Study Accession, phs000089.v3.p2; Dataset Name cde_ctl. Dataset Accession, pht000177.v3.p2; NIH approval accession: [#47649-3]. This case-control study analyzed genetic variants that may increase risk of Parkinson's disease in the collection of North American Caucasians with Parkinson's disease, as well as neurologically normal controls from the sample population which are banked in the National Institute of Neurological Disorders and Stroke (NINDS Repository) collection (Fung et al., 2006). The control cohort is composed of 802 Caucasian subjects, about 60% are women, and

TABLE 1 | Principal published characteristics of *P2RX7* SNP polymorphisms.

	Base change	Amino acid change	Effect on function	Minor allele frequency
rs2230912	1405A > G	Gln460Arg	Partial loss	0.17
rs3751143	1513A > C	Glu496Ala	Loss	0.175
rs1718119	1068G > A	Ala348Thr	Gain	0.400
rs208294	489C > T	His155Tyr	Gain	0.439

TABLE 2 | Age and 1513CC *P2RX7* genotype frequency in different population cohorts.

Cohort origin	Mean/Median age (years) ± SD (age range)	Genotype 1513CC Freq. (%)	Subjects number	References
China	5.9 ± 4.0 (0.25–16)	11.5	384	Xiao et al. (2009)
Turkey	7.8 ± 4.9	2.6	192	Tekin et al. (2010)
United Kingdom	29 (10–49)	3.0	235	Zhang et al. (2003)
Gambia	30.3 ± 7.5	1.3	297	Li et al. (2002)
Russia	32.2 ± 12.0 (21–71)	2.4	126	Mokrousov et al. (2008)
Peru	32.6 ± 9.4	3.3	513	Taype et al. (2010)
Brazil	32.8 ± 16.5	3.0	263	de Salles et al. (2017)
Oman	35 ± 7	8.2	158	Al-Shukaili et al. (2011)
Tunisia	35 (24–55)	4.0	150	Ben-Selma et al. (2011)
India	35.6 ± 13.3	8.0	100	Sambasivan et al. (2010)
Brazil	35.8 ± 12.0	5	288	Souza de Lima et al. (2016)
Turkey	36.3 ± 19.7 (2–86)	3	120	Somuk et al. (2016)
India	36.4 ± 14.9	2.8	392	Singla et al. (2012)
China	37.2 ± 16.6 (9–80)	6.2	532	Chen et al. (2013)
Australia	37.8 ± 13.0	3.9	102	Fernando et al. (2007)
Turkey	39.3 ± 13.8	13.1	160	Özdemir et al. (2014)
Germany	39.8 ± 11.4	4.6	461	Erhardt et al. (2007)
Korea	40.7	4.0	150	Lee et al. (2007)
Iran	43	1.0	100	Shamsi et al. (2016)
Italy	44.1 ± 12.8	2.0	100	Dardano et al. (2009)
Denmark	44.6 ± 12.2 (21–88)	2.1	808	Hansen et al. (2008)
Australia	46.1 ± 8.9	4.2	167	Fernando et al. (2007)
Italy	46.7 ± 11.1	3.8	131	Ghiadoni et al. (2013)
China	47.0 ± 14.5	10.4	87	Wu et al. (2015)
Denmark	50.7 (45–58)	2.7	1,764	Ohlendorff et al. (2007)
India	55.2 (40–80)	1.7	177	Sharma et al. (2010)
United Kingdom	58 ± 12	4.0	428	Sellick et al. (2004)
United Kingdom	58 (50–90)	3.5	113	Zhang et al. (2003)
Sweden	61 (49–75)	5.0	200	Thunberg et al. (2002)
Germany	62	5.2	97	Nückel et al. (2004)
Sweden	63 ± 6.5	3.2	2,404	Gidlöf et al. (2012)
Denmark	65.3 ± 8.2	3.5	226	Husted et al. (2013)
China	71.8 ± 6.1	2.8	285	Liu et al. (2013)
Italy	73 ± 5.6 (65–93)	7.4	148	Sanz et al. (2014)

Age is specified as found in the original reference: Mean ± SD or Median (age range).

more than 95% of the subjects originate from the USA. Each participant underwent a detailed medical history interview and had no family history of Alzheimer's disease, amyotrophic lateral sclerosis, ataxia, autism, bipolar disorder, brain aneurysm, dementia, dystonia, or Parkinson's disease.

In these control cohorts, we have searched 16 characterized *P2RX7* SNPs (Sluyter and Stokes, 2011) out of more than 300,000 SNPs reported in the databases, but only four polymorphisms were identified. The main published features of these *P2RX7* SNP polymorphisms are shown in **Table 1**.

In the HGVST6 dataset, individual subject age was specified, while in the HGVST1 dataset only decade age was reported, therefore to make data from both datasets homogenous, subjects from the HGVST6 study were re-comprised in the same age decade sub-cohorts as the HGVST1 study, as follows: decade # 3, age range 15–29 (number of subjects, 51); decade # 4, age range 30–39 (number of subjects, 77); decade # 5, age range 40–49 (number of subjects, 99); decade # 6, age range 50–59 (number of subjects, 280); decade # 7, age range 60–69 (number of subjects, 821); decade # 8, age range 70–79 (number of subjects, 507); decade # 9, age range 80–94 (number of subjects, 68). Age decade # 3 was not included in the analysis due to its small number of subjects and because, according to the USA Center for Diseases Control and Prevention (CDC) and the Word Health Association (WHO), the three main causes of death between 15 and 30 years are unintentional injury, suicide and homicide (more than 70% of total deaths), none of which are associated with inflammation[1,2]. All four *P2RX7* SNPs analyzed were in the HWE across all age decades, with the exception of the gain-of-function rs208294 SNP in age decade # 5 ($p = 0.037$).

Statistical Analysis

Data on the rs2230912, rs3751143, rs1718119, rs208294 genotypes, and the age of the subjects enrolled in the two dbGaP datasets, were extracted using SAS 9.4 (SAS Institute, Cary, NC, USA), and analyzed by correlation analysis using the GraphPad InStat 3 software (Graphpad Software, San Diego, CA, USA). The KS normality test (Kolmogorov–Smirnov

[1]https://www.cdc.gov/injury/wisqars/pdf/leading_causesofdeathbyagegroup2015-a.pdf

[2]http://www.who.int/healthinfo/global_burdendisease/estimates/en/index1. html

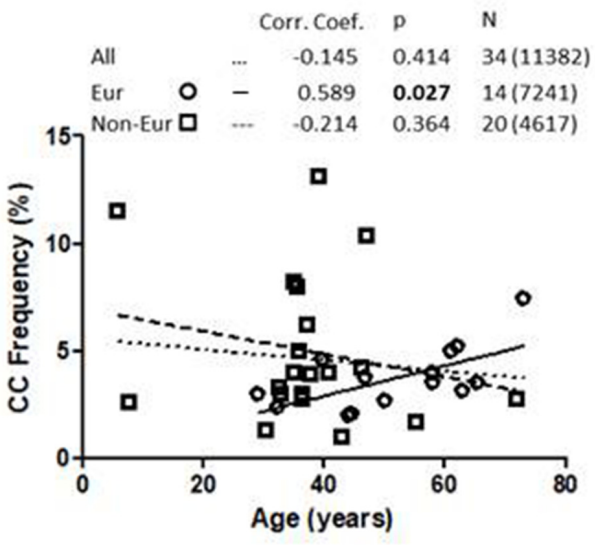

FIGURE 1 | Association of 1513CC P2X7 genotype frequency with the age in All, European and Non-European cohorts. Legend. Corr. Coef., Pearson correlation coefficient. N: cohort number (total number of subjects).

tests with Dallal–Wilkinson–Liliefor *P*-value) was applied, and the Pearson correlation coefficient was calculated. Statistical significance was assumed as $p < 0.05$ for the initial rs375114 studies. When we subsequently analyzed data from the dbGaP and tested three other P2X7 SNPs as a secondary objective (considering eight test hypotheses and according with the Bonferroni correction), statistical significance was reduced to $p < 0.00625$.

RESULTS AND DISCUSSION

The 1513A>C *P2RX7* SNP was initially identified in monocytes from a healthy subject with a nonfunctional P2X7 (Gu et al., 2001). Later it was associated with many different diseases, including tuberculosis, Crohn's disease, rheumatoid arthritis, and psychiatric disorders (Sluyter and Stokes, 2011). Our previous studies showed a higher frequency of the 1513CC *P2RX7* genotype in aged compared with young cohorts, but the sample size was rather small (Cabrini et al., 2005; Dardano et al., 2009; Sanz et al., 2014). Thus, we decided to perform a wide range literature search in PubMed, using the queries "(P2X7 or P2X7R or P2RX7) and (polymorphisms or polymorphism)." Source and data extracted from 34 healthy cohorts specifying mean or median age (29 and 5 cohorts, respectively) and CC % frequency, analyzed across 32 articles, are shown in **Table 2**.

Cohorts were further subdivided into two groups, European (13 articles comprising 14 cohorts) and non-European (19 articles comprising 20 cohorts). Ethnic origin of the cohorts was specified in five European studies (Caucasian origin) and in six non-European studies (non-Caucasian origin). In the remaining cohorts, ethnic origin was not specified, and healthy control subjects were local volunteers. Thus, it was assumed that the majority of the participants belonged to the prevalent ethnicity in the given country.

Linear regression analysis of the association between 1513CC *P2RX7* frequency and age from all cohorts, both European and non-European, is shown in **Figure 1**. Analysis of pooled data from all cohorts showed no correlation between 1513CC *P2RX7* frequency and age. Likewise, no correlation between 1513CC *P2RX7* frequency and age was observed in non-European cohorts. On the contrary, subgroup analysis of European cohorts showed a significant correlation of 1513CC *P2RX7* frequency with age ($p = 0.027$). Separate analysis of European countries with three or more cohorts: Italy (Dardano et al., 2009; Ghiadoni et al., 2013; Sanz et al., 2014), Denmark (Ohlendorff et al., 2007; Hansen et al., 2008; Husted et al., 2013), and the United Kingdom (Starczynski et al., 2003; Zhang et al., 2003; Sellick et al., 2004), numbering three, three, and four cohorts respectively, showed a trend of increase in 1513CC *P2RX7* frequency with age.

To further validate data derived from the literature, we analyzed 1513CC frequency in 1903 Caucasian control subjects included in the HGVST1 and HGVST6 studies. As a secondary objective, three other *P2RX7* SNPs (namely, the 489C>T loss-of-function, the 1068G>A and 1405A>G gain-of-function SNPs) were also included in this analysis (**Table 3**).

Hypomorphic and hypermorphic P2X7R genotype frequency of all SNPs at different age decades was analyzed to verify either a frequency increase in hypomorphic receptor or a frequency decrease of hypermorphic receptor with age (**Table 4**). A statistically-significant association between the increase in hypomorphic 1513CC and 489CC genotype frequency with age was found, and a reduction in the hypermorphic 1068AA genotype frequency with age was observed, but statistical significance was not reached. Instead, 1405A>G SNP frequency was independent of age (**Figure 2**). It is worth mentioning that a decreased frequency of a gain-of-function SNP (308GG) with age has also been

TABLE 3 | *P2XR7* genotype frequency in HGVST1 and HGVST6 dataset.

	Hypomorphic P2X7R	Intermediate P2X7R	Hypermorphic P2X7R	Minor allele frequency	Number of subjects*
1405A > G	GG (%) 44 (2.3%)	AG (%) 540 (28.4%)	AA (%) 1,318 (69.3%)	0.165	1,902
1513A > C	CC (%) 61 (3.3%)	AC (%) 602 (32.5%)	AA (%) 1,192 (64.3%)	0.195	1,855
1068G > A	GG (%) 701 (37%)	AG (%) 932 (49.2%)	AA (%) 263 (13.9%)	0.384	1,896
489C > T	CC (%) 543 (28.5%)	CT (%) 993 (52.2%)	TT (%) 367 (19.3%)	0.454	1,903

** Number subject varies because for some individuals some genotypes are non-specified in the datasets.*

TABLE 4 | Correlation analysis of *P2XR7* genotype frequency with age decade.

SNP	Hypomorphic P2X7R			Hypermorphic P2X7R		
	Genotype	Corr. Coef.	*p*	Genotype	Corr. Coef.	*p*
1405A > G	1405GG	−0.4826	0.3323	1405AA	−0.00817	0.9877
1513A > C	1513CC	0.9390	0.0055[#]	1513AA	−0.4904	0.3234
1068G > A	1068GG	0.3983	0.4342	1068AA	−0.8184	0.0465
489C > T	489CC	0.9359	0.0019[#]	489TT	−0.08178	0.8616

[#]$p < 0.00625$.

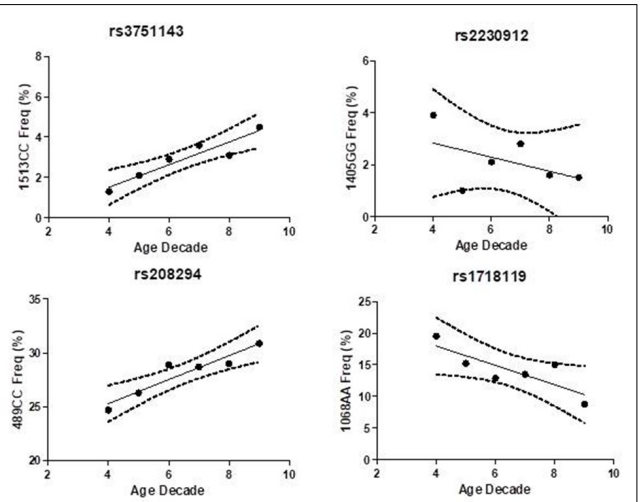

FIGURE 2 | Association of P2X7 genotypes frequency with age decade in Caucasian control cohort.

reported for the potent pro-inflammatory cytokine TNFα (Cardelli et al., 2008).

Differences in 1513CC frequency between European/USA and non-European/non-USA cohorts may be due to ethnic background, as previously reported for immune system-related genes, *P2RX7* included (Lindenau et al., 2013). Also, as suggested by Fuller et al. (2009), environmental factors and prevalent diseases might also cause an allelic selection of *P2RX7* SNPs. Environmental factors, such as hygienic conditions, climate, and food availability, which are extremely variable in different areas of world, have a strong influence on disease prevalence and life expectancy. In low-income countries, nearly 40% of deaths occur in childhood (0 to 15-years age range), while only 20% occur among aged people (70 years and older). In these countries, morbidity and mortality are mainly due to infectious diseases (e.g., lower respiratory tract infections, HIV/AIDS, diarrheal diseases, malaria, and tuberculosis) that collectively account for almost one third of all deaths (World Health Organization data). The P2X7R has been reported to have a protective action against some common infective pathogens, such as Plasmodium, Mycobacterium, and Chlamydia. In high-income countries, however, 70% of deaths occur among people aged 70 years and older, the main causes being chronic diseases where inflammation plays an important and detrimental role, including cardiovascular disease, cancer, dementia, chronic obstructive pulmonary disease, and diabetes (World Health Organization data). Under these conditions, reduced activity of a potent pro-inflammatory receptor such as the P2X7R may turn out to be beneficial. Finally, a limitation of our study is the reduced sample size. Further replication studies are needed to test our hypothesis.

CONCLUSION

Based on these results, we hypothesize that in Caucasian elderly populations from high-income countries, where a hypofunctional P2X7R might afford protection against prevalent chronic inflammatory diseases, hypomorphic *P2RX7* alleles may be positively selected with age. This hypothesis suggests that the P2X7R might be a therapeutic target to alleviate inflammatory brain disorders and others age-related diseases.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in two public available datasets: HGVST1 and HGVST6.

AUTHOR CONTRIBUTIONS

JS: study design, bibliographic research, statistical analysis, writing and discussion of the manuscript. SF: critical reading of the manuscript. MLM: dbGaP statistical analysis and critical reading of the manuscript. GZ and AP: critical reading and discussion of the manuscript. FDV: writing, critical reading and discussion of the manuscript.

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REVIEW
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The Role of P2X7 Receptor in Alzheimer's Disease

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Alzheimer's disease (AD) is the most prevalent neurodegenerative disease characterized by a progressive cognitive decline associated with global brain damage. Initially, intracellular paired helical filaments composed by hyperphosphorylated tau and extracellular deposits of amyloid-β (Aβ) were postulated as the causing factors of the synaptic dysfunction, neuroinflammation, oxidative stress, and neuronal death, detected in AD patients. Therefore, the vast majority of clinical trials were focused on targeting Aβ and tau directly, but no effective treatment has been reported so far. Consequently, only palliative treatments are currently available for AD patients. Over recent years, several studies have suggested the involvement of the purinergic receptor P2X7 (P2X7R), a plasma membrane ionotropic ATP-gated receptor, in the AD brain pathology. In this line, altered expression levels and function of P2X7R were found both in AD patients and AD mouse models. Consequently, genetic depletion or pharmacological inhibition of P2X7R ameliorated the hallmarks and symptoms of different AD mouse models. In this review, we provide an overview of the current knowledge about the role of the P2X7R in AD.

Keywords: amyloidogenic processing, inflammation, oxidative stress, synaptopathy, microglia, induced pluripotent stem cells

HIGHLIGHTS

- Alzheimer's disease (AD) is a multifactorial neurodegenerative disorder.
- P2X7R is upregulated in AD.
- P2X7R is involved in microglial function, synaptopathy, oxidative stress, and amyloidogenic APP processing.
- Induced pluripotent stem cell (iPSC) is a promising new therapeutic approach in AD.

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a devastating neurodegenerative disorder currently affecting more than 47 million people around the world, expecting to reach more than 131 million by 2050. Typical AD onset is after 65 years old, although in less than 5% of cases, onset may be earlier (Alzheimer's-Association, 2019). Approximately between 1 and 3% of AD patients present autosomal dominant form of AD, denominated early familiar AD (eFAD) (Price and Sisodia, 1998). This form is

characterized by mutations in both amyloid-β (Aβ) precursor protein (APP) and enzymes related in its processing, like presenilin-1 and presenilin-2 (PSEN1 and PSEN2) (Price and Sisodia, 1998; Ling et al., 2003).

Symptoms associated to AD follow a progressive course, starting with an impairment in learning and memory, proceeding to later detriments in complex attention, executive functions, language, visuospatial compartment, praxis, gnosis, behavior, and/or social compartment (McKhann et al., 2011). At neuropathological level, postmortem brains from AD patients show atrophy of frontotemporal cortex and hippocampus caused by neuronal loss, neuroinflammation, loss of synapses, and oxidative stress. Typical hallmarks of AD are extracellular senile plaques and intracellular neurofibrillary tangles (NFTs) (Gahtan and Overmier, 1999; Selkoe, 2001; Avila, 2006; Perl, 2010). Senile plaques are formed by β-amyloid Aβ peptides, generated by the sequential proteolysis of APP by β-secretase 1 (BACE1) and γ-secretase (PSEN1 and 2, Nicastrin, and APH-1) (Selkoe, 2001). NFTs are assembled by abnormal accumulation of hyperphosphorylated tau protein [microtubule associated protein tau (MAPT)] (Avila, 2006). Aβ peptide and phosphorylated Tau protein, primary criteria for AD diagnosis, are considered the main toxic species involved in AD (Long and Holtzman, 2019). Indeed, detection of Aβ and tau deposition in cerebrospinal fluid (CSF) or positron emission tomography (PET) imaging presents now an antemortem AD neuropathology diagnosis (Bridel et al., 2019; Lowe et al., 2019).

Current Therapeutic Strategies in AD

There are only four commercial palliative-treatments available for symptomatic AD patients: three acetylcholinesterase inhibitors (donepezil, rivastigmine, galantamine) and memantine, a non-competitive NMDA receptors modulator (Long and Holtzman, 2019). Despite all efforts made, there is no effective treatment available for symptomatic AD patients. Over the last decade, numerous clinical trials have been carried out to avoid the amyloid toxicity associated with AD. One of those was focused on developing specific monoclonal antibodies against Aβ, both soluble and fibrillar forms (Doody et al., 2013; Salloway et al., 2014; Selkoe, 2019) or try to induce an active immunization. Other strategies have attempted to reduce brain Aβ burden designing new potent secretase inhibitors, both against γ-secretase or BACE1 inhibitors (Egan et al., 2018, 2019; Henley et al., 2019; Lopez Lopez et al., 2019). Another trial used anti-inflammatory drugs to avoid Aβ-induced neuroinflammation, focusing on the inhibition of the cyclooxygenase enzyme (Aisen et al., 2000; de Jong et al., 2008). Since some studies have shown increased levels of cholesterol promoting the production of Aβ, other clinical trials used statins likes hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors as cholesterol-lowering agents (Carlsson et al., 2008; Feldman et al., 2010). Regarding Tau-based clinical trials, the followed strategies have been focused on reducing its intracellular phosphorylation rate (Domínguez et al., 2012), avoiding its aggregation (Wischik et al., 1996, 2015), or allowing its removal using immunotherapy approaches. Tau immunotherapy is based on both active and passive immunization approaches. Active immunization must be handled carefully, avoiding the pathogenic activation of the immune system, in particular T-cells, that could lead to aseptic meningo-/encephalitis endangering patient safety. In contrast, passive immunotherapy provides the advantage of control over antibodies' binding properties and their blood concentrations (Vogels et al., 2019). So far, nine ongoing immunotherapy studies are being reported (Alzforum, 2019), two of which are active immunotherapies: AADvac1 (Novak et al., 2017) and ACI-35 (Theunis et al., 2013); and seven passive immunotherapies: R07105705 (Lee et al., 2016), Zagotenemab (LY3303560) (Alam et al., 2017), BIIB076 (Czerkowicz, 2017), ABBV-8E12 (C2N 8E12) (Yanamandra et al., 2015), Gosuranemab (BIIB092) (Boxer et al., 2019), UCB0107 (Alzforum, 2018), and JNJ-63733657 (Alzforum, 2018). While there are several clinical trials ongoing (Long and Holtzman, 2019), complementary approaches targeting alternative pathways still need to be explored.

Over the last two decades, several pieces of evidence suggest that some elements of purinergic signaling, in particular P2X7R, might contribute to AD pathology (Parvathenani et al., 2003; McLarnon et al., 2006; Ryu and McLarnon, 2008; Sanz et al., 2009; Delarasse et al., 2011; Diaz-Hernandez et al., 2012; Sanz et al., 2014; Martin et al., 2019; Martinez-Frailes et al., 2019). The first observation of the possible involvement of P2X7R in AD was based on the upregulation of this receptor in microglial cells surrounding senile plaques both in human AD patients and animal models mimicking AD (Parvathenani et al., 2003; McLarnon et al., 2006; Ryu and McLarnon, 2008). Genetic evidence also provided an association between P2X7R and AD, finding a negative correlation among P2X7R 489C>T polymorphism and AD (Sanz et al., 2014). Later studies supplied additional proofs supporting the involvement of P2X7R in the amyloidogenic APP processing (Delarasse et al., 2011; Diaz-Hernandez et al., 2012), synaptic dysfunction (Lee et al., 2011; Saez-Orellana et al., 2016, 2018; Goncalves et al., 2019), oxidative stress (Parvathenani et al., 2003; Lee et al., 2011; Zhang et al., 2015), and neuroinflammation (Kim et al., 2007; Sanz et al., 2009; Chiozzi et al., 2019; Martin et al., 2019; Martinez-Frailes et al., 2019), associated to AD (**Figure 1**). Supporting the potential therapeutic role of P2X7R, others groups demonstrated that its pharmacological blockade or genetic depletion leads to a significant improvement both symptomatology and neuropathology in AD animal models (Ryu and McLarnon, 2008; Diaz-Hernandez et al., 2012; Chen et al., 2014; Martin et al., 2019). In this review, we are going to examine the different findings supporting a critical role of P2X7R in the regulation of molecular mechanisms underlying AD.

P2X7R

P2X receptors are plasma membrane ligand-gated ion channels, whose activation causes a selective influx of small cations (Na^+, Ca^{2+}) and K^+ efflux from cells (Nicke et al., 1998; Hatorri and Gouaux, 2012; Samways et al., 2014). Similar to other members of P2X family, P2X7 subunit has two transmembrane domains

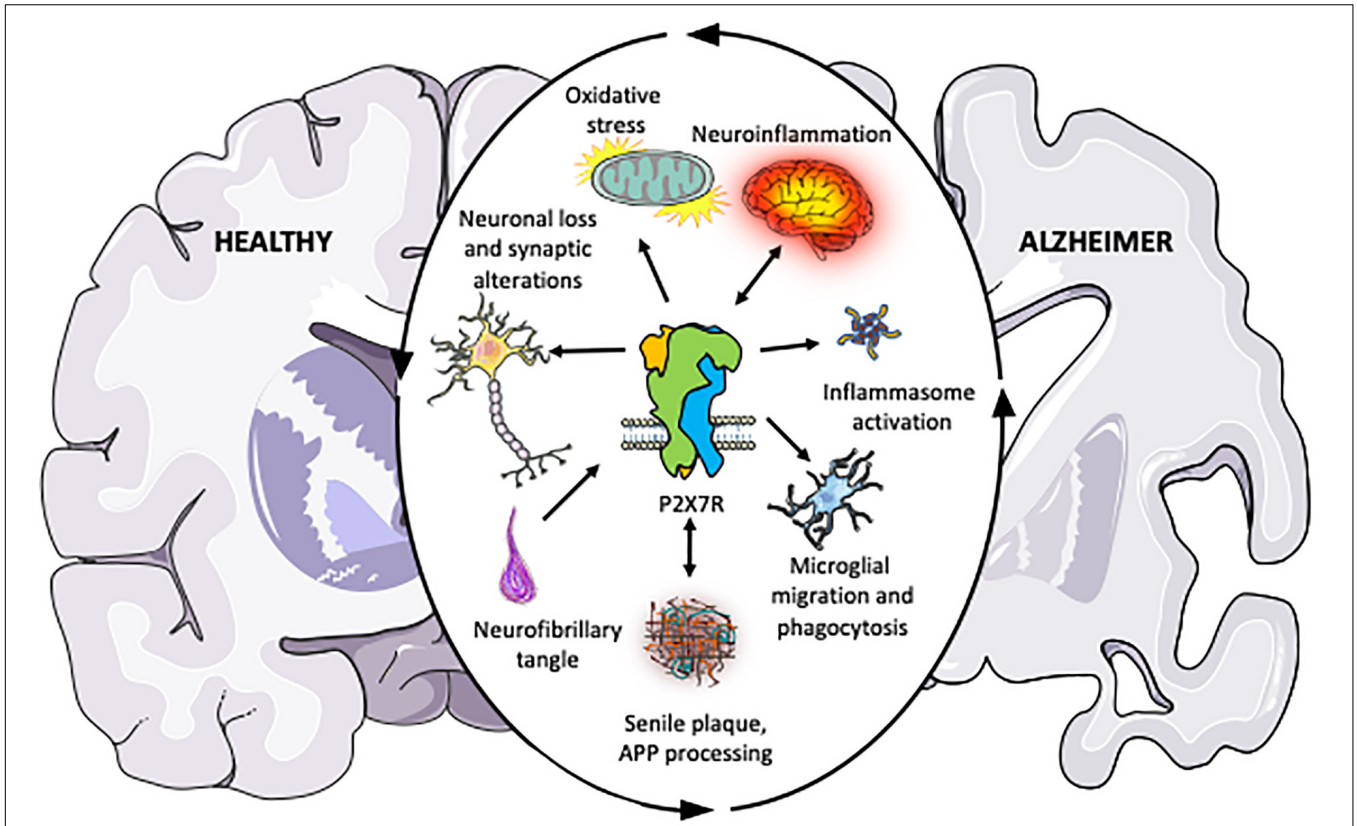

FIGURE 1 | Schematic illustration summarizing the pieces of evidence accumulated over the past years indicating that P2X7R plays a central role in the different physiopathological processes associated with Alzheimer's disease. The outer circular arrows illustrate a chain of interconnected and mutually influenced pathological processes associated with AD. Inner arrows represent the relationships found between P2X7R and these pathological processes, summarizing the studies discussed in the present review. Briefly, P2X7R modulates amyloid APP processing, and it is postulated as a neuroinflammation triggering factor. Upregulated P2X7R expression in AD patients and different AD and neuroinflammation mouse models give it a key role in disease progression. Besides, P2X7R also contributes to neuronal loss and synaptic alterations, oxidative stress; inflammasome assembling; and altered microglial function, being all of them processes contributing to AD progression, as described in the present review.

linked by a large extracellular domain (Nicke et al., 1998). These subunits present a dolphin-shaped structure with the transmembrane helices and the extracellular region similar to the tail and the body, respectively (McCarthy et al., 2019). Among all P2X receptors, P2X7 is the only one found as a homotrimer in physiological models. Human P2X7R protein is a 595 amino acid protein encoded by the *P2RX7* gene located on chromosome 12 (12q24.31 locus) spanning 53,733 bases (NCBI, 2017). Alternative splicing takes place during gene's transcription and may give rise to at least 11 different splice variants of P2X7R, described to date (Rassendren et al., 1997; Cheewatrakoolpong et al., 2005; Skarratt et al., 2005, 2020; Feng et al., 2006; Adinolfi et al., 2010; Sluyter and Stokes, 2011). Specific features of full length P2X7 protein include a large C-terminal domain (Virginio et al., 1999); low sensitivity to its native ligand (ATP) and sensitive to extracellular divalent cations (Surprenant et al., 1996). Different intracellular mediators have been associated with P2X7R activation such as calcium calmodulin kinases II (Diaz-Hernandez et al., 2008), nuclear factor kappa-light-chain-enhancer (NFκB) (Ferrari et al., 1997), ROS/NOS formation (Hewinson and MacKenzie, 2007), glycogen synthase kinase-3 (GSK3) (Diaz-Hernandez et al., 2008), phospholipase D (Humphreys and Dubyak, 1996), inflammasome "NACHT, LRR, and PYD domains-containing protein 3" (NLRP3) (Franceschini et al., 2015). However, sustained activation of P2X7R by high ATP concentrations may induce apoptosis or necrosis in some cellular lineages (Virginio et al., 1999; Delarasse et al., 2009).

Although its specific distribution in the CNS remains debated (Miras-Portugal et al., 2017; Illes et al., 2017), P2X7R expression has been reported in almost all cellular lineages making up the brain tissue, including astrocytes, microglia, oligodendrocytes, and neurons (Matute et al., 2007; Miras-Portugal et al., 2017). Interestingly, P2X7R has also been related to several physiological events including neuronal differentiation (Messemer et al., 2013; Tsao et al., 2013; Glaser et al., 2014; Fumagalli et al., 2017), axonal growth and branching (Diaz-Hernandez et al., 2008), presynaptic regulation and neurotransmitter release (Sperlagh et al., 2002; Miras-Portugal et al., 2003; Leon et al., 2008), microglial activation, migration, and proliferation (Sanz et al., 2009; Rigato et al., 2012; Martinez-Frailes et al., 2019), glial and

microglial phagocytosis (Ni et al., 2013; Gu and Wiley, 2018; Martinez-Frailes et al., 2019).

Upregulation of P2X7R in Alzheimer's Disease

One of the initial pieces of evidence suggesting a possible involvement of P2X7R in AD was the increased P2X7R expression found in microglial cells surrounding amyloid plaques both in AD patients and different AD mouse models (Parvathenani et al., 2003; McLarnon et al., 2006). Later studies using two different mouse models of AD based on the transgenic expression of human APP (APP/PS1 mice and J20 mice, thoroughly described below) confirmed that P2X7R upregulation in activated microglial was parallel with AD progression (Lee et al., 2011; Martinez-Frailes et al., 2019). Another study showed that 9-months-old P301S tau mice, overexpressing mutant human protein tau (MAPT P301S) driven by the mouse prion protein (*Prnp*) promoter (Yoshiyama et al., 2007), show a higher cerebral binding of a radiotracer for P2X7R, [^{123}I]TZ26019, than their corresponding WT control mice. Subsequent analysis revealed that P2X7R was mainly found in hippocampal astrocytes in P301S mice (Jin et al., 2018). However, no robust data about the molecular mechanism causing the glial P2X7R upregulation were provided in these studies. Although astroglial activation is a histopathological mark associated with tau-induced toxicity, including AD, little is know about how this cellular type contributes to tau-induced toxicity (Forrest et al., 2019). Considering the critical role of P2X7R on the astrocytic function (Miras-Portugal et al., 2017), additional studies to determinate whether astroglial P2X7R is contributing to tau-associated pathology should be done.

A recent study reported that PS2 deficient mice are most sensitive to Aβ-induced neuroinflammation due to the upregulation of P2X7R in both glial and neuronal cells in a transcription factor Sp1 (SP1)-dependent manner (Qin et al., 2017). Taking into account that SP1 is a transcription factor promoting P2X7R expression (Garcia-Huerta et al., 2012) and that neuroinflammation causes SP1 upregulation via activation of intracellular kinases cascades (Citron et al., 2008), it is reasonable to think that dysregulation of SP1 may be the factor causing P2X7R upregulation detected in AD. Supporting the involvement of SP1 in the molecular mechanisms underlying Aβ-induced toxicity in AD, a significant increase in both SP1 messenger and protein levels was found in the cortex of AD patients and in two mouse models of AD, APP/PS1 mice and Tg2576 mice (Citron et al., 2008). These mice overexpress human APP containing the Swedish mutation under the control of the hamster prion protein promoter (Hsiao et al., 1996). In this line, it was also reported that SP1 as a transcription factor is able to regulate the expression of both APP and tau proteins (Izumi et al., 1992; Heicklen-Klein and Ginzburg, 2000). However, the potential role of SP1 as a therapeutic target in AD was put in doubt after the observation that its sustained pharmacological inhibition, induced by a selective SP1 inhibitor (mithramycin), caused a significative memory deficit and increased the $A\beta_{1-42}$ and $A\beta_{1-40}$ ratio (Citron et al., 2015). These results indicate that global inhibition of SP1 contributes to neurodegeneration rather than plays a protective role. These side effects could be due to the wide range of different off-target genes regulated by SP1 transcription factor (Bird, 1986; Larsen et al., 1992; Siegfried et al., 1999).

P2X7R in Amyloidogenic APP Processing

Longitudinal studies combining cognitive assessment, PET analysis, and the measurement of pathognostic molecules from the CSF of eFAD and late-onset AD patients showed an early deposition of Aβ in the precuneus and other cortical areas 10–12 years before first AD symptoms appear (Vermunt et al., 2019). Based on these findings, it is currently accepted that Aβ accumulation represents the initial event triggering the disease. Preceding the onset of cognitive impairment, following the initial Aβ accumulation, a sequential tau accumulation can be observed (Morris et al., 2009; Bateman et al., 2012; Fagan et al., 2014; Gordon et al., 2018; Hanseeuw et al., 2019). However, the existence of cases in which senile plaque burden was detected in brains collected from healthy individuals with no dementia (Shankar et al., 2008; Perez-Nievas et al., 2013), put in doubt that Aβ is the only factor triggering AD. As described above, although APP protein may be processed by both amyloidogenic and non-amyloidogenic pathways in the CNS (Hardy and Selkoe, 2002), it is postulated that in healthy brains APP is preferably cleaved via the non-amyloidogenic pathway (Tyler et al., 2002). Nevertheless, deregulation in this balance might favor amyloidogenic processing, leading to Aβ accumulation (Stockley and O'Neill, 2008).

Different studies using both *in vitro* and *in vivo* approaches postulated that P2X7R might be one of the factors controlling APP processing (Delarasse et al., 2011; Leon-Otegui et al., 2011; Darmellah et al., 2012; Diaz-Hernandez et al., 2012). APP protein can be processed in two different ways. The amyloidogenic pathway is mediated by β- and γ-secretase and results in the generation of extracellular sAPPβ, Aβ-peptides, and the intracellular C-terminal fragment C99. On the other hand, the non-amyloidogenic pathway involves the α-and γ-secretases and results in the generation of an intracellular C-terminal fragment, called C88, extracellular peptides sAPP and the P3 peptides (Selkoe, 2001). Preliminary studies, using mouse neuroblastoma cells (N2a) expressing human APP, reported that BzATP-induced P2X7R activation stimulates the release of sAPP in a mitogen-activated protein kinases (MAPK)-dependent manner. This release was inhibited by selective P2X7R knock-down with siRNA and by specific P2X7R antagonists (Delarasse et al., 2011). In a subsequent study, this group reported that Ezrin/Radixin/Moesin (ERM) are required for P2X7R-dependent processing of APP (Darmellah et al., 2012). However, another group, using two different cell lines, found that the inhibition and not the activation of native or overexpressed P2X7R increases α-secretase activity (Leon-Otegui et al., 2011; Diaz-Hernandez et al., 2012). *In vivo* studies confirmed that pharmacological blockade of P2X7R reduces size and number of hippocampal senile-plaques in 8-months-old J20 mice (PDGF-APPSw,Ind). These mice overexpress human APP with the Swedish (APP KM670/671NL) and Indiana (APP V717F) mutations under the

control of platelet-derived growth factor subunit B (PDGFB) promoter (Mucke et al., 2000). This beneficial effect was GSK3-dependent (Diaz-Hernandez et al., 2012). To shed light on these contradictory results, a recent study generated P2X7R deficient APP/PS1 mice. APP/PS1 mice express a chimeric mouse/human APP and human PSN1, with the deletion of exon 9 found in eFAD patients, under the mouse prion protein promoter (APP/PSN1/P2X7$^{-/-}$) (Jankowsky et al., 2004). Results obtained in this study confirmed that genetic depletion of P2X7R leads to a significant reduction in the number of senile plaques in 10-months-old APP/PS1 mice. This decrease was accompanied by a drastic decreasing in Aβ peptides levels and rescue of the cognitive deficit developed by APP/PS1 mice (Martin et al., 2019). All previous data suggest that dysregulation of P2X7R signaling may be one of the factors promoting APP amyloid processing in AD.

P2X7R in Neuroinflammation Associated With AD

It is widely known that accumulation of Aβ in senile plaques initiates the inflammatory process on AD (McGeer et al., 2000) and favors the activation of the microglial cells around them (Selkoe, 2002). In these cells, the P2X7R upregulation suggests its involvement in microglia cells mediated-neuroinflammatory response on AD (Parvathenani et al., 2003; McLarnon et al., 2006). In the following sections, we will describe how the upregulation of P2X7R in microglial cells contributes to neuroinflammation and how this impacts on microglial functionality.

P2X7R in Inflammasome Activation

Microglial cells have dual effects on AD progression. On one side, they promote a decrease in Aβ accumulation by stimulating its phagocytosis, clearance, and degradation. On the other side, chronic microglial activation leads to the release of pro-inflammatory cytokines that can contribute to the neuronal loss (Wang S. et al., 2015; Wang W. Y. et al., 2015). This dual effect may be caused by the activation of microglial cells in two subsets that present different molecular phenotype: the classical (M1) or the selective (M2) activated state (Czeh et al., 2011; Thawkar and Kaur, 2019). M1 state-activated microglia cells promote the release of pro-inflammatory cytokines, playing a pivotal role in the defense against pathogens or tumor cells. M2 state-activated microglia cells secrete anti-inflammatory cytokines promoting tissue repairment (McGeer et al., 2000; Walker and Lue, 2015; Wang W. Y. et al., 2015). However, this is a simplified classification since and microglia cells may acquire other activation states (Ransohoff, 2016). At this regard, a recent genome-wide transcriptome analysis of microglia from models of different neurodegenerative disease has allowed to identify a new disease-associated microglia (DAM) phenotype (Krasemann et al., 2017; Song and Colonna, 2018). DAM, in addition, to express genes characteristic in both classical M1 macrophages and classical M2 macrophages, also express others related to the interferon response, stress response, lysosomal function, and lipid metabolism (Song and Colonna, 2018). One of the first molecules directly linked to activation of DAM was TREM2, a single-immunoglobulin-domain-containing macrophage-specific receptor. Interestingly, recent studies have postulated that this protein plays a central role in the onset and the development of AD because microglia surround and enclose neuritic plaques in a TREM2-dependent manner (Wang Y. et al., 2015; Meilandt et al., 2020).

Data obtained from studies using lipopolysaccharide (LPS)-induced neuroinflammation animal model has contributed clarify the role of microglial cells in neuroinflammation (Boche et al., 2013). LPS activates Toll-like receptor 4 (TLR4) after binding LBP (LPS-binding protein), an intracellular signaling pathway that leads to the activation of nuclear factor NF-kB by a Myeloid differentiation primary response protein MyD88 (MyD88)-dependent mechanism. When activated, NF-kB translocates to the nucleus, binding the DNA, and promoting the transcription of proinflammatory mediators, such as proinflammatory cytokines like pro-interleukin-1 beta (pro-IL1β), pro-interleukin-18 (pro-IL18), and NLRP3 inflammasome (Takeda and Akira, 2004; Venigalla et al., 2016). Activation of this intracellular pathway is a priming event. However, to trigger NLRP3 inflammasome, a cytosolic multiprotein oligomer responsible for the activation of inflammatory responses, a subsequent signal causing a decrease K^+ levels in the cytosolic microenvironment is required to facilitate the oligomerization of NLRP3. Afterward, the inflammasome recruits the apoptosis-associated speck-like protein (ASC) and the procaspase-1 (another apoptosis-related protein), leading to the secretion of IL1β and IL18 (Petrilli et al., 2007; He et al., 2016). Several studies have shown that ATP, found at high extracellular concentrations following insults (Burnstock, 2008, 2016) or released by Aβ peptide (Kim et al., 2007; Sanz et al., 2009; Saez-Orellana et al., 2018; Goncalves et al., 2019), may be one of the signals promoting NLRP-inflammasome assembling and subsequent IL1β processing (Laliberte et al., 1999; Perregaux et al., 2000; Ye et al., 2013). Interestingly, recent studies have reported that NLRP3 activation may also induce tau hyperphosphorylation and aggregation in an IL-1β-dependent manner (Ising et al., 2019). Initially, *in vitro* studies using microglial cells isolated from rat brains showed that fibrillar $A\beta_{1-42}$ peptide-induced ATP-release by P2X7R-dependent mechanism (Kim et al., 2007). Later, using both *in vitro* and *in vivo* approaches, it was suggested that Aβ-induced microglial activation requires the activation of P2X7R via an autocrine/paracrine stimulatory loop (Sanz et al., 2009). Additional research revealed that Aβ causes via a P2X7R-dependent mechanism NF-kB activation and NLRP3 inflammasome expression in microglial cells (Chiozzi et al., 2019). Since pretreatment of cultured microglial cells with potassium chloride (KCl), avoided microglial NRLP3 activation (Gustin et al., 2015), it is reasonable to postulate that efflux of K^+ induced by P2X7R activation is the mechanism by which P2X7R promotes NRLP3 activation in microglial cells. These studies are suggesting that P2X7R/NLRP3/Caspase1 signaling is a crucial pathway in the inflammasome activation once the microglial cell is primed. In accordance with this hypothesis, Martinez-Frailes et al. (2019) found that upregulation of P2X7R in microglial cells takes place in advanced and late stages of AD, but not

in the early stages, when the microglial priming has not yet occurred, and there is a reduced number of senile plaques. In line with this concept, inflammasome activation in APP/PS1 mice occurs in an age- and Aβ deposition-related fashion (Heneka et al., 2013). Recent studies have also proposed the involvement of P2X7R in the initial microglial priming induced by serum amyloid A (SAA) protein via TLR4 activation (Niemi et al., 2011; Facci et al., 2018). SAA is a high-density apolipoprotein generated in the liver and released to the systemic circulation, where it is mainly found associated with HDL, in response to inflammation, reaching different organs including the brain (Gabay and Kushner, 1999). In AD patients, SAA co-localized with cerebral amyloid Aβ-peptide deposits (Kindy et al., 1999) and it is present in high levels in CSF of AD patients (Miida et al., 2006). In cortical microglial cells isolated from rat brain, it was reported that SAA causes microglial priming and inflammasome activation in a P2X7R-dependent manner (Facci et al., 2018).

As, in recent years, it has been suggested that the control or reduction of the chronic neuroinflammation associated with neurodegenerative diseases may be an efficient therapeutic strategy (Beamer et al., 2016; Ribeiro et al., 2019; Thawkar and Kaur, 2019). Therefore, the regulation of neuroinflammation associated with AD by P2X7R is gaining relevance as a possible remedial to fight these disorders. This affirmation is based on the concept that chronic neuroinflammation may contribute to neurodegeneration by promoting the release of proinflammatory cytokines, increasing the permeability of blood–brain barrier (BBB) favoring the recruitment of systemic immune effectors cells and causing a synaptic dysfunction leading to neuronal loss (O'Callaghan et al., 2008; Thawkar and Kaur, 2019). In agreement with this, it has been reported that pharmacological blockade or knocking out the P2X7R in different AD mouse models have positive effects by reducing neuroinflammation (Ryu and McLarnon, 2008; Chen et al., 2018; Martin et al., 2019). Initial studies reported that *in vivo* pharmacological inhibition of P2X7R by Brilliant Blue G (BBG) attenuated inflammatory response and diminished leakiness of BBB induced by intracerebroventricular (i.c.v.) injection of $A\beta_{1-42}$ peptide in rat hippocampus (Ryu and McLarnon, 2008). In accordance, later study revealed that *in vivo* inhibition of P2X7R by i.p. administration of BBG prevented the spatial memory impairment and cognitive deficiency in an AD mouse model (Chen et al., 2014). Another study reported that i.c.v. administration of oxidized ATP (o-ATP), a P2X7R antagonist, attenuated microglial activation and neuronal damage induced by i.c.v. administration of LPS (Choi et al., 2007). On the other hand, a sustained P2X7R inhibition by BBG did not modify either the number or the morphology of astroglia or microglial cells. Although, the specific treatment, did reduce IL1β secretion and promote the non-amyloidogenic APP processing in the hippocampus of young J20 mice (Diaz-Hernandez et al., 2012). Moreover, a recent study has reported that APP/PS1/P2X7R deficient mice present smaller cognitive deficit and better synaptic plasticity than APP/PS1 mice. Furthermore, knocking out P2X7R reduces Aβ-induced chemokines release in glial cells, especially C-C motif chemokine 3 (CCL3), which is related to the pathogenic $CD8^+$ T-cells recruitment (Martin et al., 2019). All these studies suggest that BBB permeable compounds and selective P2X7R antagonists might be considered as good therapeutic drugs to treat chronic neuroinflammation associated with AD.

P2X7R in Microglial Migration

During neuroinflammation, extracellular ATP and other nucleotides seem to act as "find me" and "eat me" signals (Di Virgilio et al., 2009). This hypothesis is based on the fact that extracellular ATP is capable of inducing morphological changes in microglial cells favoring their rapid migration toward local brain injury (Davalos et al., 2005). Initial studies indicated that extracellular purines modulate the microglial migration through their specific metabotropic receptors P2Y12, P2Y1, or P2Y6 (Inoue, 2008; De Simone et al., 2010; Bernier et al., 2013; Langfelder et al., 2015). However, new findings also suggest the involvement of ionotropic purinergic receptors in this phenomenon (Martinez-Frailes et al., 2019). Using *in vitro* and *in vivo* approaches, Martinez-Frailes et al. (2019) have recently confirmed that ATP-induced P2X7R activation promotes microglial migration. These findings might explain why there is an enrichment on P2X7R positive microglial cells around the senile plaques both in AD mouse models and in postmortem brain samples from AD patients (Parvathenani et al., 2003; McLarnon et al., 2006; Lee et al., 2011; Martinez-Frailes et al., 2019). In this line, it reported that P2X7R upregulation in the microglial cell increases in parallel to the incidence of senile plaques (Lee et al., 2011). Moreover, *in vivo* blockade of P2X7R by BBG also caused a reduction of GSK3 activity in P2X7R-expressing microglial cells, by increasing p-GSK3 levels (Diaz-Hernandez et al., 2012). Other *in vitro* studies using BV-2 mouse microglial cells or brain slices also confirmed that GSK3 inhibitors significantly reduced the migratory capacity of microglial cells (Yuskaitis and Jope, 2009). All these findings suggest that P2X7R activation not only promotes NLRP3 inflammasome assembling but also stimulates microglial migration.

P2X7R in Microglial Phagocytosis

Another microglia feature that has been related to purinergic signaling is its phagocytic capacity. First studies suggested that purinergic compounds modulated the phagocytic capacity of microglial cells through the metabotropic P2Y6 receptor (Inoue, 2008). However, recent studies have provided additional data indicating that other purinergic receptors may also regulate this microglial function, as P2X7R. In this line, *in vitro* studies using mouse primary microglial cells isolated from the hippocampus demonstrated that both P2X7R genetic depletion using specific RNAi and its pharmacological inhibition by BBG favors microglial phagocytosis of fibrillar $A\beta_{1-42}$ and decreases IL1β secretion capacity. In this line, the enrichment of the culture medium with IL1β reduced the microglial $A\beta_{1-42}$ phagocytosis (Ni et al., 2013). Additional evidence on P2X7R mediated-regulation of microglial phagocytosis has also been provided by later studies (Janks et al., 2018; Martinez-Frailes et al., 2019). Using both *in vitro* and *in vivo* approaches, it has been found that GSK 1482160A, a selective P2X7R inhibitor, significantly increased the phagocytosis of 2 μm diameter

fluorescence microspheres by microglial cells expressing P2X7R (Martinez-Frailes et al., 2019). Accordingly, other *in vitro* studies using cultured primary human microglial cells confirmed that P2X7R activation induced by 300 μM Bz-ATP decreased the phagocytic capacity of microglial cells. That effect was prevented when they used the selective P2X7R antagonist 50 μM A438079 but not by the selective P2X4R antagonist Bx430 (Janks et al., 2018). In agreement with these findings, ATP induced P2X7R activation causes cytoskeleton changes in microglial, reducing their phagocytic capacity (Fang et al., 2009). In this line, and taking into account the important role that microglial phagocytosis plays in the removing of senile plaques, it is to worth highlighting that, both in the hippocampus of J20 mice and in postmortem cortical samples from human AD patients, the majority of microglial cells in contact with senile plaques did not express P2X7R. Furthermore, the percentage of microglia expressing P2X7R inside extracellular Aβ deposits remains constant along the AD progression, in opposition to the rising of the total number of microglial cells (Martinez-Frailes et al., 2019). Interestingly, in human microglial cells, BzATP-induced P2X7R activation reduced its phagocytic capacity and produced mature caspase-1 by activating the inflammasome, revealing a close relationship between both events (Janks et al., 2018). Supporting this concept, the NLRP3 inflammasome inhibitor, MCC950, stimulates Aβ phagocytosis *in vitro* and reduces the number of senile hippocampal plaques in APP/PS1 mice, causing an improvement in their cognitive function (Dempsey et al., 2017). Indeed, double transgenic mice resulting from the crossbreed of APP/PS1 mice and NLRP3$^{-/-}$ or caspase-1$^{-/-}$ (CASP1) mice exhibited a significant reduction in the loss of spatial memory and an enhanced Aβ clearance, compared with APP/PS1 mice. Furthermore, the NLRP3 inflammasome deficiency promoted the switch of microglial cells to M2 state, resulting in decreased deposition of Aβ in APP/PS1 mice (Heneka et al., 2013). This evidence strongly suggests that therapeutic strategies focusing either on direct inflammasome inhibition or on avoiding its assembling by blocking P2X7R, might be efficient for reducing neuroinflammation and promoting Aβ phagocytosis in AD.

P2X7R in Oxidative Stress Associated With AD

Oxidative stress is a condition where the generation of reactive oxygen species (ROS) exceeds the capacity of antioxidative mechanisms of the cell (Zuo et al., 2015). High levels of ROS are commonly detected in the brain of patients suffering from different neurodegenerative diseases, including AD (Albers and Beal, 2000; Sebastian-Serrano et al., 2019). Although these species cannot trigger the neurodegenerative disease on their own, they might favor its progression by promoting the oxidative damage and interacting with mitochondria (Dias et al., 2013). Thereby, accumulation of ROS might cause mitochondrial alterations, resulting in increased ROS production and consequent mitochondrial dysfunction, favoring the disease progression. Accordingly, the content of ATP in the tissue is reduced in parallel to the disease progression in APP/PS1 mice. This fact is suggesting that this decline is caused by mitochondrial dysfunction provoked by ROS accumulation (Zhang et al., 2015). Moreover, Aβ can lead to ROS production, in particular, hydrogen peroxide (H_2O_2), that causes the damage of proteins, lipids, and nucleic acids (Eckert et al., 2003). Several pieces of evidence point to the fact that P2X7R may be the primary receptor involved in the generation of H_2O_2 by activating microglial cells (Nuttle and Dubyak, 1994). Stimulation of isolated microglial cells from rat brain with ATP or BzATP, induced O_2^- release in NADPH oxidase activation-dependent mechanism. Furthermore, inhibition of phosphatidylinositol 3 kinase, a kinase involved in GSK-3 signaling, attenuated BzATP-induced H_2O_2 release, preventing microglial-induced cortical death (Parvathenani et al., 2003). *In vitro* studies reported that fibrillar $A\beta_{1-42}$ causes ROS production generated via P2X7R activation induced by ATP released from rat microglial cells in an autocrine manner (Kim et al., 2007; Liu et al., 2020). Additional studies revealed that P2X7R positive microglial cells surrounding senile plaques express the catalytic NADPH subunit ($gp91^{phox}$) and produce ROS species in APP/PS1 mice. Hence, P2X7R upregulation in microglial cells may result in excessive ROS production induced by Aβ via P2X7R, which contributes to the synaptic toxicity associated with the early stages of AD (Lee et al., 2011). Recently, a study using P2X7R-deficient microglial cell line (N13R) has demonstrated that Aβ-induced mitochondrial toxicity requires P2X7R in microglial cells (Chiozzi et al., 2019). In agreement with the antioxidative effect of P2X7R antagonists, *in vivo* administration of selective P2X7R antagonist A438073, avoided ROS production and oxidative DNA damage induced by P2X7R activation in spinal cord dorsal horn neurons (Munoz et al., 2017).

P2X7R in Synaptic Dysfunction and Cellular Death in AD

Another major hallmark of AD is the extensive loss of synapses correlating with cognitive impairment (Lansbury, 1999). One of the most robust pieces of evidence indicating that Aβ deposits contribute to synaptic loss is the observation that a degree of synaptic loss is more evident in the proximity of the senile plaques (Lanz et al., 2003). Initial studies postulated that the synaptic loss detected in APP/PS1 mice was due to the dysfunction and collapse of the excitatory synapses. This fact was caused by the interaction of the soluble Aβ peptide oligomers coming from the surrounding plaques with these synaptic contacts (Koffie et al., 2009). However, the molecular mechanism by which Aβ alters synaptic transmission causing a subsequent synaptic loss remains unclear (Brody and Strittmatter, 2018). One hypothesis is that Aβ may interact directly with neuronal synaptic receptors such as metabotropic glutamate receptor 5 mGluR5 (Um et al., 2013), or α7 nicotine acetylcholine receptor α7nAChR (Wang et al., 2000). Others postulate that microglial cells activated by Aβ are responsible for assaulting the synapses (Hong et al., 2016). Nevertheless, new evidence suggests that synaptic dysfunction associated with AD may be due to dysregulation of P2X receptors mediated neurotransmission; dysregulation that may be triggered by increased extracellular ATP concentration induced by Aβ (Chen et al., 2014; Saez-Orellana et al., 2016,

2018; Goncalves et al., 2019). Supporting this hypothesis, higher K^+ depolarization-induced ATP release was found in hippocampal nerve terminals isolated from i.c.v. $A\beta_{1-42}$-treated mice (Goncalves et al., 2019). According to this, pharmacological blockade of P2X7R prevents the increase in the current frequency of the excitatory synapse induced by oligomeric Aβ when binding to excitatory neurons (Saez-Orellana et al., 2016, 2018). Furthermore, pharmacological inhibition of P2X7R prevented Aβ-induced loss of filopodia and spine density in cultured hippocampal neurons (Chen et al., 2018). Other studies have provided additional evidence suggesting that P2X7R-mediated ROS production in Aβ-stimulated microglial is one of the mechanisms explaining oligomeric Aβ-mediated synaptic-toxicity in APP/PS1 mice (Lee et al., 2011). In accordance with this toxic effect, BzATP-induced P2X7R activation caused microglia-induced cortical cell death (Parvathenani et al., 2003). Choi et al. (2014) reported similar results, observing that *in vivo* P2X7R blockade by o-ATP reduces the number of positive caspase-3 neurons in LPS-injected brains (Choi et al., 2007). In concordance with the involvement of P2X7R in the synaptic-toxicity induced by Aβ, P2X7R deficiency rescued the synaptic alterations and LTP deficits detected in APP/PS1 mice (Martin et al., 2019). Interestingly, it was reported that, contrary to what was observed in microglial cells, neuronal P2X7R transcription is reduced in J20 mice both in early and advanced stages. This points to the fact that this phenomenon could be an adaptive physiological response to avoid or at least lessen the neuronal loss associated with AD. However, the loss of this capacity may contribute to the exacerbation of neuronal loss in the late stages of AD (Martinez-Frailes et al., 2019).

INDUCED PLURIPOTENT STEM CELLS IN AD RESEARCH

The currently available knowledge regarding AD is mostly based on results acquired from post-mortem patient samples or animal models mimicking the disease. However, because human brain tissue is extremely hard to obtain, especially if there is a need for early-onset materials, a need for a human-derived *in vitro* system arose. Thus, the Nobel Prize awarded induced pluripotent stem cell (iPSC) technology that allows the genetic reprogramming of mature somatic cells into pluripotent stem cells (PSCs) (Takahashi et al., 2007) became widely utilized.

The differentiation of cells from patient-specific iPSCs provides valuable insight into specific molecular phenotypes of neurodegenerative diseases (McKinney, 2017) because the cells possess the complete genetic background of the patient. Moreover, healthy individuals' derived iPSCs can be genetically modified to introduce disease-specific genetic patterns (Ortiz-Virumbrales et al., 2017).

Many neurodegenerative disease models are available to date. For example, AD patient-derived iPSCs are being used by many research groups (Israel et al., 2012; Kondo et al., 2017; Ochalek et al., 2017; Sullivan and Young-Pearse, 2017; Arber et al., 2019; Chang et al., 2019).

Focusing on AD research, the use of iPSC-derived cells has helped to discover new pathological mechanisms underlying AD pathology. For instance, describing for the first time an autophagic dysfunction due to lysosomal depletion and suggesting that modifying the lysosomal biogenesis could present a novel therapeutic intervention (Lee et al., 2014). Besides, iPSC-derived cells responded very differently to drug treatments than APP-overexpressing cell lines and thus demonstrated that it can be a better option for preclinical screening of compounds (Liu et al., 2014). iPSC-models have helped to prove that β-secretase has a higher affinity for neuregulin (NRG1) than for APP, which means that it might be possible to inhibit Aβ production via BACE1 processing without affecting BACE1 interactions with its other substrates (Ben Halima et al., 2016). Importantly, iPSC-based systems are suitable for compound screening. A correlation between CSF profiles from patients and their own Aβ secretome in the differentiated neuronal cultures was found, showing the relevance of iPSC derived systems in AD modeling (Kondo et al., 2017). In AD iPSC-derived neurons, constitutional metabolic changes in ROS production without mitochondrial fission and fusion proteins damage have been described. These findings suggest that increased ROS production might have a more important role in amyloid- and tau-pathology than previously anticipated (Birnbaum et al., 2018). Furthermore, findings in these models shown tau protein species propagation patterns where tau oligomers, but not monomers, induced accumulation of pathological, hyperphosphorylated tau in human neurons (Usenovic et al., 2015). Therefore, it is realistic to expect that this technology will provide valuable insights into the P2X7R research field in the near future.

Application of 3D cell cultures will help to model more reliably the brain tissue cell interactions and microenvironment, including gradients of signaling molecules (Zhang et al., 2014). Particularly for neurodegeneration research, 3D systems promote the formation of specific neuronal cell types with complex interactions and development of AD pathologies, taking into account gradients of signaling molecules such as ATP and the subsequent cellular responses within the tissue (Mungenast et al., 2016).

CONCLUDING REMARKS

Due to the fact that many anti-amyloid clinical trials have failed, Aβ-directed therapies focusing on the reduction of parenchymal Aβ and amyloid deposits in AD brains have been put in doubt (Long and Holtzman, 2019). Many strategies were tested: active and passive immunization, secretase inhibitors or drugs avoiding amyloid aggregation, but none of them was effective in modifying the disease course in symptomatic AD patients (Hara et al., 2019). However, there are still ongoing active Phase III clinical trials based on monoclonal antibodies against Aβ, new anti-inflammatory molecules and to induce an active immunization, whose results could be concluded later (Long and Holtzman, 2019). As results from studies using animal models, mimicking AD pathology are strongly suggesting that Aβ is the triggering disease factor,

perhaps, amyloid-direct therapies would be more useful to treat preclinical cases. Moreover, the vast number of symptomatic AD patients require the urgent development of new therapeutic strategies. In recent years, strategies focused on modulating neuroinflammation, or microglial response have taken strength. So, taking this into account, selective P2X7R antagonists might be considered as potential therapeutic drugs. In addition to reducing the Aβ burden, promoting the non-amyloidogenic APP and processing, P2X7R antagonists have also shown anti-inflammatory, neuroprotective, and antioxidant effects, which might counter the pathological conditions associated with AD. Although human iPSC-based studies have not yet reported on the expression of P2X7R in the in vitro models, either on iPSC-derived neurons or astrocytes, the increasing number of AD patient-derived iPSC disease models will promote the emergence of such investigations toward potential therapeutic targets. Indeed, taking into account the complex pathological state found in AD and other neurodegenerative diseases, nowadays it is postulated that many factors together play a role in facilitating the progressive and detrimental neurodegenerative process. Since major biological systems of the human body are involved—such as the nervous system itself, the immune system, the endocrine system, possibly the digestive system (Jiang et al., 2017; Vogt et al., 2017) and perhaps others, currently unknown features and mechanisms, it is extraordinarily challenging to target only one of the systems or pathological processes in the attempt to cure neurodegenerative diseases. Perhaps the time has come to rethink the therapeutic strategies to treat these diseases, in a way where multiple mechanisms could be pharmacologically targeted at the same time, as long as the individual interventions could add up and lead to the elimination of the progression or even the symptoms of dementia. As we discussed in this review, P2X7R may be an excellent target for this multi-target therapy.

AUTHOR CONTRIBUTIONS

MD-H contributed to conceptualization and writing. MD-H, LF, and CB contributed to original draft preparation. CD, ÁS-S, LD-G, JK, and AD contributed to writing – review and editing. All authors read and approved the final version of the manuscript.

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frontiers in Molecular Neuroscience

REVIEW
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P2X7 Receptor Antagonism as a Potential Therapy in Amyotrophic Lateral Sclerosis

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This review focuses on the purinergic ionotropic receptor P2X7 (P2X7R) as a potential target for developing drugs that delay the onset and/or disease progression in patients with amyotrophic lateral sclerosis (ALS). Description of clinical and genetic ALS features is followed by an analysis of advantages and drawbacks of transgenic mouse models of disease based on mutations in a bunch of proteins, particularly Cu/Zn superoxide dismutase (SOD1), TAR-DNA binding protein-43 (TDP-43), Fused in Sarcoma/Translocated in Sarcoma (FUS), and Chromosome 9 open reading frame 72 (C9orf72). Though of limited value, these models are however critical to study the proof of concept of new compounds, before reaching clinical trials. The authors also provide a description of ALS pathogenesis including protein aggregation, calcium-dependent excitotoxicity, dysfunction of calcium-binding proteins, ultrastructural mitochondrial alterations, disruption of mitochondrial calcium handling, and overproduction of reactive oxygen species (ROS). Understanding disease pathogenic pathways may ease the identification of new drug targets. Subsequently, neuroinflammation linked with P2X7Rs in ALS pathogenesis is described in order to understand the rationale of placing the use of P2X7R antagonists as a new therapeutic pharmacological approach to ALS. This is the basis for the hypothesis that a P2X7R blocker could mitigate the neuroinflammatory state, indirectly leading to neuroprotection and higher motoneuron survival in ALS patients.

Keywords: amyotrophic lateral sclerosis, ALS, neuroinflammation, P2X7, P2X7 receptor antagonists, calcium dyshomeostasis, mitochondrial disruption, motoneuron death

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Abbreviations: $[Ca^{2+}]_c$, Ca^{2+} concentration in the cytosol; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BBB, blood–brain barrier; BBG, Brilliant Blue G; BDNF, brain-derived neurotrophic factor; BzATP, 3'-O-(4-benzoyl)benzoyl-ATP; C9orf72, Chromosome 9 open reading frame 72; CBPs, calcium-binding proteins; CNS, central nervous system; COX-2, cyclooxygenase-2; CSF, cerebrospinal fluid; CT, clinical trial; EAAT2, excitatory amino acid transporter 2; fALS, familial ALS; FDA, U.S. Food and Drug Administration; FUS/TLS, Fused in Sarcoma/Translocated in Sarcoma; GDNF, glia cell-derived neurotrophic factor; H_2O_2, hydrogen peroxide; HIV, human immunodeficiency virus; ip, intraperitoneal; iv, intravenous; MCU, mitochondrial Ca^{2+} uniporter; mNCX, mitochondrial Na^+/Ca^{2+} exchanger; MN, motor neuron; NMDA, N-methyl-D-aspartate; NOX, NADPH oxidase; O_2^-, superoxide radical; OXPHOS, mitochondrial oxidative phosphorylation system; P2X7R, purinergic P2X7 receptor; P90, postnatal day 90; PET, positron emission tomography; PK, pharmacokinetics; R, receptor; RAN, repeat-association non-ATG; ROS, reactive oxygen species; sALS, sporadic ALS; SOD1, Cu/Zn superoxide dismutase; TAR, transactive response; TDP-43, TAR-DNA binding protein-43; VACCs, voltage-activated calcium channels; VEGF, vascular endothelial growth factor; WT, wild type.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease with upper and lower motor neuron (MN) loss in the cerebral cortex, brainstem, and spinal cord. Clinical symptoms start at 55 ± 15 years, with gradual loss of daily motor activities, ambulation, speech and swallowing impairment, compromised respiration, progressive paralysis, and death within 3–5 years after diagnosis. Due to this prompt death, disease prevalence is low; in the UK, the incidence is 1 in 472 women and 1 in 350 men (Alonso et al., 2009), while in the USA, there are about 16,000 affected patients and 5,000 new diagnosed cases every year (The ALS association, 2020).

For the last 25 years, the only pharmacological treatment available has been riluzole, a drug that blocks glutamate release from presynaptic terminals and accelerates glutamate clearance from the synapse. Riluzole also elicits partial blockade of presynaptic sodium channels and inhibits postsynaptic N-methyl-D-aspartate (NMDA) receptors (Doble, 1996). Unfortunately, riluzole is only able to prolong patients' life for approximately 2 months (Miller et al., 2003).

A second drug, edaravone, was approved by the U.S. Food and Drug Administration (FDA) in 2017, with antioxidant effects that presumably preserve MN viability (Cruz, 2018). This approval generated great controversy inasmuch as it was based in a single clinical trial (CT) performed in Japanese patients, with genetic background distinct from that of Caucasian patients. Furthermore, ALS incidence is lower in Asian population, compared with European/American population (Chio et al., 2013). In any case, it is worth mentioning that edaravone has already been approved in Japan (2015), South Korea (2015), USA (2017), Canada (2018), Switzerland (2019), and China (2019).

In light of the scarce therapeutic tools currently available to delay the appearance of symptoms or disease progression, we will analyze here the following aspects of ALS to be able to extract meaningful conclusions for new drug therapy approaches: (i) genetic features; (ii) mouse models of ALS, based on gene mutations found in patients; (iii) pathogenic signaling pathways involved in MN death, and potential drug targets; (iv) neuroinflammation and the role of the purinergic P2X7 receptor (P2X7R) in disease pathogenesis; (v) available ligands for P2X7Rs; (vi) proof of concept of efficacy of some P2X7R antagonists in mouse models of ALS; (vii) past and ongoing CTs; and (viii) conclusions and perspectives.

GENETICS OF ALS

A family history is present in 5–10% of patients of ALS (familial ALS or fALS). The first mutations reported were those in the gene coding for Cu/Zn superoxide dismutase (SOD1) protein, which account for around 15% of all fALS and 1–2% of the sporadic cases (sporadic ALS or sALS) in European population (Rosen et al., 1993; Stephenson and Amor, 2017; Zou et al., 2017). Interestingly, these mutations (nearly 200 different mutations have been reported) do not impair the enzymatic activity of SOD1. Rather, they cause its pathological aggregation in the cytosol or within mitochondria, leading to MN toxicity (Monk and Shaw, 2006). The observation that all cell types express SOD1, yet only MNs degenerate, is puzzling (Pasinelli and Brown, 2006). This could be due to the low capacity of Ca^{2+} buffering exhibited by these neurons (Bezprozvanny, 2009; De Diego et al., 2012).

Around 40 different mutations in the *TARDBP* gene, which encodes for the transactive response (TAR)-DNA binding protein-43 (TDP-43), have been described. Mutations account for the 4–5% of fALS and <1% of sALS in European population (Arai et al., 2006; Neumann et al., 2006; Zou et al., 2017). These mutations cause the aggregation of TDP-43 protein in the cell cytoplasm, which has important functions in RNA processing and regulation. Interestingly, 97% of ALS patients present TDP-43 inclusions that are only absent in patients carrying SOD1 mutations, revealing the high relevance of this protein in ALS pathogenesis.

Mutations in the Fused in Sarcoma/Translocated in Sarcoma (*FUS/TLS*) gene have also been linked to the pathogenesis of ALS (Lagier-Tourenne and Cleveland, 2009). Mutations in FUS are present in 3% of fALS and <1% of sALS European patients (Zou et al., 2017), and they are responsible for the early-onset forms of the disease. This protein has relevant functions in RNA splicing and transport, and it also forms aggregates in the cell cytoplasm of ALS patients with this mutation.

Hexanucleotide repeats (GGGGCC) in intron 1 of chromosome 9 open reading frame 72 (*C9orf72*) gene were identified as the major cause of ALS, being present in 30–40% of fALS and 5% of sALS cases in European population (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Majounie et al., 2012; Zou et al., 2017). Hexanucleotide repeats seem to cause RNA toxicity due to the expansion of sense and antisense RNAs, the aggregation of RNA binding proteins, and the translation of repeat-association non-ATG (RAN) proteins. Patients harboring these mutations also present TDP-43 protein inclusions in the cytoplasm (Liu Y. et al., 2016).

MODELING ALS GENETICS IN MICE

The ALS mutations mentioned above have been identified as leading causes of disease, and based on them, several mouse models have been developed. Ideally, mouse models should present all the hallmarks of the human disease, namely, MN loss, muscle weakness and atrophy, metabolic deficits, TDP-43 inclusions and proteinopathy, gliosis, and changes in innate immunity. Even though available models are far from being perfect, they have been useful to understand ALS pathogenesis, to identify new drug targets with therapeutic potential, as well as to establish the proof of concept of the efficacy and safety of lead compounds (Stephenson and Amor, 2017). This is particularly true for the $SOD1^{G93A}$ mice that is by far the model that has been, and it is being used more frequently.

The discovery of new genes linked to ALS (Chia et al., 2018) and the fast development of gene editing techniques such as CRISPR/Cas9 will surely increase the number of ALS mouse models, giving rise to a drug testing platform that includes the high variability of the human disease. We describe below the models that are more frequently used, emphasizing their

advantages and caveats for drug discovery, previously described in two detailed recent reviews (Stephenson and Amor, 2017; Lutz, 2018).

SOD1 Models

SOD1 overexpressing mouse models were developed in the first place, and they are still the most used when it comes to ALS drug discovery (Rosen et al., 1993; Gurney et al., 1994). In particular, the $SOD1^{G93A}$ mouse model has been the most used and studied of all of them, as it closely resembles the disease progression in humans. These mice develop a progressive MN disease with adult onset and reduced lifespan. Pathogenically, $SOD1^{G93A}$ mice show early onset astrogliosis and microgliosis, glutamate-induced excitotoxicity, deficits in axonal transport, axonal denervation, protein aggregation, aberrant neurofilament processing, and mitochondrial vacuolization. All these processes result in the selective loss of spinal cord MN and the marked wasting, paralysis and atrophy of the forelimbs and hindlimbs. Thus, this model exhibits almost all the hallmarks of ALS (Philips and Rothstein, 2015; Lutz, 2018).

However, some disadvantages are found in this model. As mutations in SOD1 are found in a minority of ALS cases, the model is not representative of the whole patient population. This is of great importance, as SOD1 mutations do not result in TDP-43 inclusions in the $SOD1^{G93A}$ model, with this hallmark of disease pathogenesis being not commonly present in either SOD1 patients or mice (Stephenson and Amor, 2017). However, other SOD1 models such as $SOD1^{G86S}$ do exhibit TDP-43 inclusions and could be used to overcome this pitfall (Jeon et al., 2019). Furthermore, while patients develop the disease with basal quantities of the mutated protein, mice show very high overexpression of the mutated SOD1 protein (Lutz, 2018). In addition, in mice, the disease always commences in lumbar spinal cord MNs, while in humans, ALS onset may be initiated in cortical, bulbar, or spinal cord MNs (Philips and Rothstein, 2015).

TDP-43 Models

Mouse ALS models based in *TARDBP* mutations, encoding for TDP-43 protein, have failed in most cases to mimic the human disease, the overexpressing model $TDP\text{-}43^{A315T}$ being the best one in resembling it. These mice show an adult onset, with progressive and fatal neurodegeneration. Mice present TDP-43 aggregates, activation of astroglia and microglia, as well as MN loss. However, survival is impaired by a severe gastrointestinal pathology that can cause their sudden death previous to neurodegeneration (Wegorzewska et al., 2009; Herdewyn et al., 2014). Further limitations of the model are the overexpression of mutated TDP-43 and a poor loss of spinal cord MNs.

FUS Models

The majority of mouse models based in human *FUS* mutations do not present a complete ALS-like phenotype, neither progression to paralysis or disease end stage. The model that more closely resembles fALS is the wild type (WT) overexpressing mouse or $hFUS^{WT}$. Mice develop paralysis, weight loss, muscle atrophy, and tremor. Moreover, MN loss, protein aggregation, and gliosis are also present. The major caveat of the model is that it does not carry a disease-linked mutation and, hence, the underlying pathology may not be the same as in the human disease (Mitchell et al., 2013).

C9orf72 Models

Again, most C9orf72-based models failed to develop an ALS-like pathology. The most suitable model is the $h(G_4C_2)_{37\text{-}500}$, which shows muscle weakness, paralysis, weight loss, cognitive deficits, and premature death. Moreover, mice show histopathological hallmarks of the disease, such as MN loss, TDP-43 proteinopathy, and gliosis. However, this pathology is only developed by a 30–35% of female subset of mice. Another 40% of females and around 45% of males develop a milder pathology that in some cases progresses to the disease end point (Liu Y. et al., 2016). This heterogeneity seriously limits the use of this model to test the effect of compounds within a drug development program.

ALS PATHOGENESIS

ALS pathogenesis is far from being completely understood. Even though the disease is known to be multifactorial and multisystem, it is still unknown why the selective death of MNs occurs. Our limited knowledge on disease pathogenesis could explain why over 50 CTs with a large variety of compounds targeting different receptors and signaling pathways have provided negative outcomes in ALS patients. We will review here some features of disease pathogenesis such as cell Ca^{2+} dyshomeostasis, mitochondrial Ca^{2+} handling, and production of reactive oxygen species (ROS). Neuroinflammation and the role of P2X7 receptor (P2X7R) will be commented in "Neuroinflammation and P2X7 Receptors in ALS Pathogenesis" section.

Calcium Dyshomeostasis: The Excitotoxic Hypothesis

The excitotoxic hypothesis implies that alterations of some of the multiple receptors, ion channels, and calcium-binding proteins (CBPs), could be the result of some of the ALS mutations above described and the aggregation of pathological proteins (Appel et al., 2001; Van Den Bosch et al., 2006; Bezprozvanny, 2009; Grosskreutz et al., 2010; De Diego et al., 2012). Several factors support the assumption that augmented glutamatergic neurotransmission and Ca^{2+}-dependent excitotoxicity play central relevant roles in MN degeneration (Patai et al., 2017). They are as follows: (i) high levels of excitatory amino acids have been found in the cerebrospinal fluid (CSF) of ALS patients (Plaitakis and Caroscio, 1987; Rothstein et al., 1991; Fiszman et al., 2010); (ii) CSF from ALS patients exerts MN death *in vitro* (Terro et al., 1996; Sen et al., 2005; Anneser et al., 2006; Gunasekaran et al., 2009; Yáñez et al., 2011); (iii) selective loss of astroglial excitatory amino acid transporter 2 (EAAT2) in motor cortex and spinal cord is found in both sporadic and fALS (Rothstein et al., 1995; Fray et al., 1998; Lin et al., 1998; Sasaki et al., 2000) with concomitant impairment of astrocytes' ability to remove glutamate from the synaptic cleft; (iv) a defective glutamate clearance was detected in synaptosomes from brain areas and spinal cord of sALS patients (Rothstein et al., 1992),

but prolonged *in vivo* blockade of astroglial EAAT2 with concomitant extracellular glutamate increase was innocuous for spinal MNs in rats (Tovar et al., 2009); and (v) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) rather than NMDARs seem to contribute more to MN cytotoxicity, firstly because they are expressed at higher density in cultured MNs (Carriedo et al., 1996; Van Den Bosch and Robberecht, 2000) and, secondly, because of their higher Ca^{2+} permeability due to the lack of a GluR2 subunit in the tetrameric receptor complex (Van Damme et al., 2002). Consistent with this are the observations of lower GluR2 mRNA levels in MNs compared with other neurons (Van Damme et al., 2002), and the lower AMPAR mRNA in human spinal MNs (Heath and Shaw, 2002). Furthermore, the lack of GluR2 in $SOD1^{G93A}$ mice accelerated MN loss (Van Damme et al., 2005), and conversely, GluR2 overexpression augmented MN lifespan (Tateno et al., 2004). Of physiological and pharmacological interest are the observations that the GluR2 subunit is regulated by the vascular endothelial growth factor (VEGF; Bogaert et al., 2010) as well as by the brain-derived neurotrophic factor (BDNF) and glia cell-derived neurotrophic factor (GDNF; Brené et al., 2000). Of note is the fact that these growth factors also contribute to maintain MNs in good health. This is the case for VEGF (Wang et al., 2016), BDNF (Lanuza et al., 2019), and GDNF (Thomsen et al., 2018).

Strong or weak capacity to bind free Ca^{2+} ions in the cytosol ($[Ca^{2+}]_c$) by CBPs has immediate consequences for neuron vulnerability during Ca^{2+} loads in ALS-linked MN degeneration. For instance, cortical, lower cranial, and spinal MNs die soon after ALS onset, which correlates with their lack of expression of the CBPs parvalbumin and calbindin-D28k. In contrast, MNs from Onuf's nucleus, oculomotor, trochlear, and abducens nerves that express high levels of parvalbumin and/or calbindin-D28k are resistant to damage or are injured at advanced disease stages (Alexianu et al., 1994). Moreover, in brain slices, the expression level of CBPs by different types of neurons correlate with their specific vulnerability to Ca^{2+} loads (Ho et al., 1996; Beers et al., 2001; Van Den Bosch et al., 2002). In line with these results, another experiment showed that cross breeding SOD1 mutant mice with mice overexpressing parvalbumin in spinal MNs resulted in a delay of disease onset and a longer MN survival (Beers et al., 2001).

Altered Mitochondrial Calcium Handling

Mitochondria are involved in nearly all types of cell death, including necrosis, apoptosis, and necroptosis (Kroemer et al., 1998; Galluzzi and Kroemer, 2008). But notably, the redistribution of mutated TDP-43 from the nucleus to the cytoplasm is being considered a pathological hallmark for most forms of ALS (Kabashi et al., 2008; Sreedharan et al., 2008). A recent study demonstrates that TDP-43 toxicity resides in its mitochondrial location. The aberrant protein specifically impairs the Complex I of the mitochondrial oxidative phosphorylation system (OXPHOS) by preferentially binding to mitochondrial transcribed ND3/6 mRNAs as well as by inhibiting their translation, leading to mitochondrial dysfunction and neurodegeneration. The interest of this study resides in the additional observation that the suppression of WT or mutant TDP-43 in mitochondria restores neuronal viability. This provides a rationale for reducing the aggregated TDP-43 protein inside the mitochondria as a therapeutic approach for ALS (Wang et al., 2016).

Moreover, mitochondria in MNs from $SOD1^{G93A}$ and TDP-43 mice show a swelling shape, have internal vacuoles and disorganized cristae, and aggregate in abnormal clusters. This causes a defect in mitochondrial axonal transport towards the synaptic nerve terminals, leading to neuronal metabolic alterations (Magrane et al., 2014). Mitochondria also show aberrant fission and fusion dynamics that are necessary for damaged mitochondria confinement and repair (Smith et al., 2019).

The Link Between ROS and Mitochondrial Calcium Cycling

ROS are natural metabolic products of cell functions and particularly of oxidative phosphorylation itself. However, impairment of mitochondrial electron transport chain generates an excess of ROS that can damage cell proteins, membrane lipids, and nucleic acids. In fact, several markers of ROS damage, i.e., 3-nitrotyrosine, 8-hydroxy-2′-deoxyguanosine, and 4-hydroxynonenal, have been found in blood, CSF, and urine of ALS patients, as well as in their spinal MNs (Smith et al., 1998; Ihara et al., 2005; Mitsumoto et al., 2008).

Oxidative stress is also caused by a defect in the cellular defense machinery against ROS. Such is the case of SOD1 that catalyzes the conversion of superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2). Physiological reduction of H_2O_2 into oxygen and water through the action of glutathione and catalase offers cell protection from ROS. Interestingly, the mutation of SOD1 in ALS does not result in a loss of its enzymatic function, but rather, it enhances its ability to oxidize cellular antioxidants, reducing molecular oxygen to O_2^- (Liochev and Fridovich, 2003). Nevertheless, the MN toxicity linked to mutated SOD1 is mainly due to the cytoplasmic aggregates created by the aberrant protein (Hardiman et al., 2017).

Large Ca^{2+} loads occurring upon cell stimulation are cleared up by mitochondria through the high-capacity mitochondrial Ca^{2+} uniporter (MCU; Herrington et al., 1996; Xu et al., 1997; Montero et al., 2000). The Ca^{2+} accumulated in the mitochondrial matrix stimulates respiration and ATP synthesis, to couple cell activity and bioenergetics needs (Gunter et al., 1994; Rizzuto et al., 2000). During the excitotoxic process, this accumulation could lead to excessive mitochondrial Ca^{2+} load, oxidative stress, and cell death (Cano-Abad et al., 2001; Orrenius et al., 2003).

Correlation between Ca^{2+} influx with massive mitochondrial Ca^{2+} uptake also occurs in hypoglossal MNs (Ladewig et al., 2003). Such mitochondrial Ca^{2+} uptake is comparatively small in highly Ca^{2+}-buffered ALS-resistant spinal MNs (Carriedo et al., 2000), suggesting that mitochondria could partially compensate for the weak cytosolic Ca^{2+} buffering in vulnerable neurons. In fact, following Ca^{2+} entry through voltage-activated calcium channels (VACCs), substantial mitochondrial Ca^{2+} uptake occurs in weakly buffered neurons (Bergmann and Keller,

2004). Mitochondria may exert an additional control of Ca^{2+} homeostasis through ROS-dependent regulation of excitability. Thus, enhanced ROS production occurs upon inhibition of respiration by sodium cyanide. ROS induces the opening of sodium channels, augmentation of action potential firing and enhanced voltage-dependent Ca^{2+} influx (Bergmann and Keller, 2004). Some studies support the idea that the production of ROS is increased in spinal MN mitochondria as a result of Ca^{2+} overload following excitotoxic stimulation of AMPA/kainate receptors (Carriedo et al., 2000).

Mitochondrial damage elicited by mutant SOD1 aggregates found in the mitochondrial matrix of transgenic mice (Jaarsma et al., 2001; Pasinelli et al., 2004) could decrease the enzymatic activity of the electron transport chain at complexes I, II, and IV (Jung et al., 2002; Mattiazzi et al., 2002). In fact, mutant SOD1 could disrupt the association of complex IV (cytochrome c) with the inner mitochondrial membrane, thereby interfering with respiration (Kirkinezos et al., 2005). This leads to increased ROS production as shown in mutant SOD1-expressing MNs cultures (Kruman et al., 1999) as well as in MNs of brain slices where complex IV was inhibited by sodium cyanide (Bergmann and Keller, 2004).

It seems that ROS produced in MNs can diffuse to neighboring astrocytes to cause oxidative disruption of glutamate transporters (Rao et al., 2003). In turn, this will increase the local extracellular concentration of glutamate, thereby enhancing local excitotoxicity in a vicious circle of MN damage. This model integrates the hypothesis of Ca^{2+}-dependent excitotoxicity and oxidative damage. In this frame, distorted mitochondrial respiration sensitizes MNs to glutamate stimulation and to environmental toxins, thus increasing their vulnerability (Kruman et al., 1999; Andreassen et al., 2001). In this direction, it was observed that in cultured neurons, the chronic mitochondrial inhibition induced by malonate or sodium azide led to selective MN death; free-radical scavengers and AMPAR blockers protected such neurons from death (Kaal et al., 2000).

NEUROINFLAMMATION AND P2X7 RECEPTORS IN ALS PATHOGENESIS

The purinergic P2X7 ionotropic receptor (P2X7R) is considered as one of the main players of inflammation (Di Virgilio, 2015). In fact, this receptor is expressed in immune and inflammatory cells such as dendritic cells, osteoclasts, microglia, mast cells, natural killer cells, or T and B lymphocytes. Additionally, P2X7Rs are upregulated in inflammatory processes (Ferrari et al., 2006).

As a result of cell stress or tissue damage, large amounts of ATP are released into the extracellular space that stimulates the low-affinity target P2X7R, the main sensor for ATP during inflammation. This receptor is also the main trigger of the protective/regenerative immune response, consisting in the maturation and release of several interleukins, mainly IL-1β (Adinolfi et al., 2018).

The maturation of IL-1β requires the activation of the NLRP3 inflammasome, an event that requires Na^+ influx accompanied by water, Ca^{2+} influx, and K^+ efflux. The intracellular K^+ drop is, in fact, the best established mechanism underlying the P2X7R-mediated formation of the NLRP3 inflammasome (Compan et al., 2012; Katsnelson et al., 2015; Jo et al., 2016; Karmakar et al., 2016). This is supported by experiments showing that not only ATP, but also other agents that induce K^+ efflux, such as the ionophore nigericin or crystalline molecules, elicit inflammasome activation too (Compan et al., 2012; Yaron et al., 2015). Noteworthy, ROS, also, induce inflammasome aggregation and IL-1β release due to P2X7R activation (Hung et al., 2013; Minkiewicz et al., 2013).

The role of P2X7Rs in neuroinflammation is supported by the fact that they are expressed in various cells of the central nervous system (CNS), namely, astrocytes, microglia, and oligodendrocytes (Sperlagh et al., 2006; Kaczmarek-Hajek et al., 2018), where they mediate inflammasome signaling (Franceschini et al., 2015). The presence of reactive astrocytes and microglia define the neuroinflammatory process in the CNS. As peripheral immune and endothelial cells, activated microglia and astrocytes produce pro-inflammatory cytokines (IL-1β, IL-16, TNF-α), chemokines (CCL2, CCL9, CCL1), ROS, and secondary messengers (nitric oxide, prostaglandins; DiSabato et al., 2016). However, the activation of P2X7Rs in these cells is known to produce dual and divergent effects depending on the duration of the inflammatory stimulus and the disease stage, presymptomatic or symptomatic.

How neuroinflammation contributes to neurodegeneration has recently been analyzed by Ransohoff (2016). Thus, a chronic mild activation of glial cells *via* P2X7Rs leads, in the long term, to synaptic dysfunction, synapse loss, and neuronal death. The fact that glial cells exhibit a low overall turnover rate make them more susceptible to the neuroinflammatory effects of age, injury, or stress in neurodegenerative diseases (Ajami et al., 2007).

A dual effect of P2X7R-mediated neuroinflammation seems to operate during the time course of ALS. This could be explained in the context of two different phenotypes that microglia develop upon P2X7R activation. The M2 phenotype or anti-inflammatory predominates at earlier disease stages, while the pro-inflammatory M1 phenotype is prevalent at later disease stages in $SOD1^{G93A}$ mice (Liao et al., 2012; Parisi et al., 2016). Consistent with this is an experiment done in $SOD1^{G93A}$ mice with genetic ablation of P2X7Rs; exacerbation of gliosis and enhanced MN death were unexpectedly found in these mice (Apolloni et al., 2013a). Another experiment supporting duality indicated that short P2X7R stimulation augmented autophagy and M2 microglial markers. However, continued P2X7R stimulation impaired autophagy, suggesting a microglial shift to the M1 phenotype. A dual report that implies duality of action at central and peripheral tissues indicated a positive role of P2X7Rs on $SOD1^{G93A}$ muscles (Fabbrizio et al., 2020).

A few preclinical and clinical experiments support the implication of P2X7Rs in ALS pathogenesis. So, the pro-inflammatory action of microglial P2X7Rs was augmented in $SOD1^{G93A}$ mice (D'Ambrosi et al., 2009). Furthermore, spinal cord pathology was ameliorated by P2X7R antagonism, also in these mice (Apolloni et al., 2014). Consistent with this was the observation that P2X7R activation by high ATP concentrations activated NOX2 and the kinase ERK1/2 in the microglia of $SOD1^{G93A}$ mice, thus provoking an increase of ROS (Apolloni

et al., 2013b). Additionally, in co-cultures of astrocytes and MNs from SOD1^{G93A} mice, cell stimulation with P2X7R agonists ATP and 3′-O-(4-Benzoyl)benzoyl ATP (BzATP) elicited a neurotoxic phenotype that was prevented by the P2X7R blocker Brilliant Blue G (BBG; Gandelman et al., 2010). However, unexpected P2X7R down-regulation and Ca^{2+} dyshomeostasis was found in peripheral monocytes of ALS patients (Liu J. et al., 2016). This contrasts with another study showing up-regulation in spinal cord tissue of post-mortem ALS patients (Yiangou et al., 2006).

This cumulative set of data mostly from preclinical *in vitro* and *in vivo* models of ALS, plus a scarce number of clinical studies in ALS patients, support the view in the sense that P2X7Rs mediate the activation of astrocytes and microglia, giving rise to a chronic neuroinflammatory state that is critical in the progression of ALS pathology. However, it should be kept in mind that dependent on disease stage, microglial activation may be anti-inflammatory (early stage, M2 phenotype) or pro-inflammatory (late stage, M1 phenotype).

SOME P2X7R BLOCKERS WITH FAVORABLE PHARMACOKINETICS

Due to its involvement in neuroinflammation, the P2X7R is considered an adequate target for the treatment of several neurodegenerative and neurological diseases, as well as mood disorders (Díaz-Hernández et al., 2009; Hempel et al., 2013; Bhattacharya and Biber, 2016; Jimenez-Pacheco et al., 2016; Volonte et al., 2016; Cieslak and Wojtczak, 2018; Cieslak et al., 2019). Yet, preclinical data in mouse models of ALS leave some uncertainty about it, as previously described. Moreover, P2X7R antagonists have not succeeded in any CT for CNS disorders so far (ClinicalTrials.gov - NIH U.S. National Library of Medicine, 2020). Despite the notable number of P2X7R blockers already existing as pharmacological tools, not all meet the pharmacokinetic (PK) characteristics that are necessary to become a potential drug for the treatment of neurodegenerative diseases, including ALS. This is the case of BBG that has been amply used as a P2X7R blocker only because it is cheap and readily available. However, this compound has a poor selectivity towards P2X7Rs, blocking also P2X4, P2X5, and rat P2Y1 and P2Y2, while potentiating the human P2Y1 at 1–3 μM concentrations (Jacobson et al., 2002, 2006; Jacobson, 2010). Research has led nowadays to very potent antagonists, but unfortunately, this is not enough. Between the main problems of these compounds, there is a lack of favorable PK properties, such as low blood–brain barrier (BBB) permeability and scarce stability in blood and brain tissue, which determine a drop in drug half-life and CNS residence time. Additionally, pronounced differences have been found in potencies and affinities among P2X7R in mouse, rat, and human. Such is the case of AZ11645373 (**Figure 1**) that blocks the human P2X7R at nanomolar concentrations and the mouse P2X7R at micromolar concentrations and has no effect on the rat orthologous (Michel et al., 2009). Another example is compound GW791343, which is a potent allosteric inhibitor of the human receptor, but also a positive allosteric modulator of rat P2X7R (Jacobson, 2010). This complicates the scenario for the development of a drug for clinical use and diminishes the possibility for a new compound of being selected for preclinical and clinical studies.

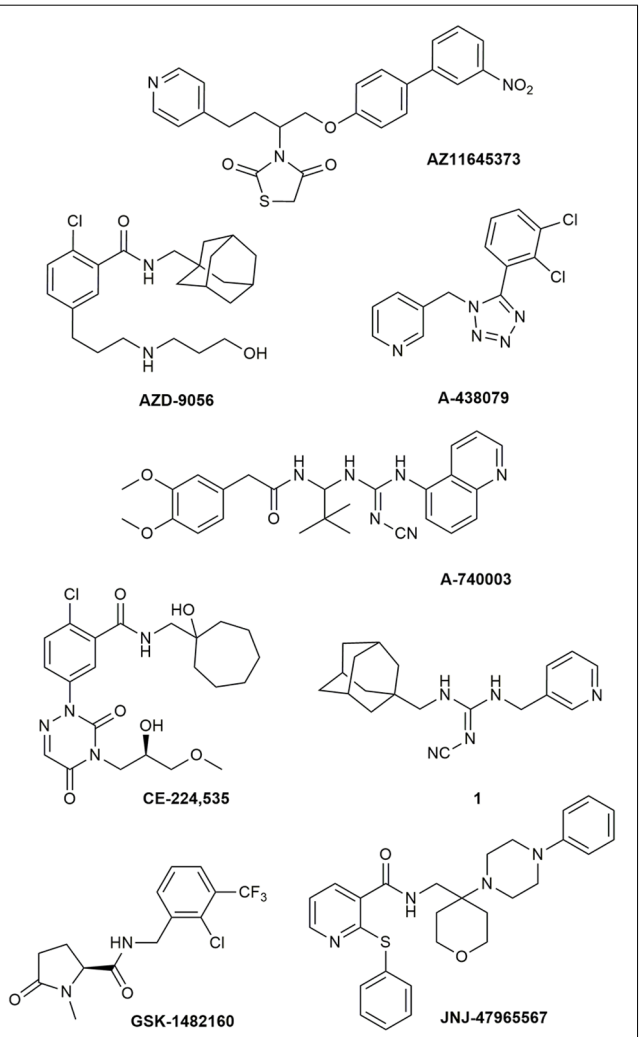

FIGURE 1 | P2X7R antagonists lacking adequate pharmacokinetic properties for the treatment of CNS disorders.

During around the last 10–15 years, the interest in finding new P2X7R clinical candidates has shifted from no-BBB-permeable antagonists for the treatment of peripheral inflammatory diseases (e.g., **AZD-9056** from AstraZeneca, tried in CTs—**Figure 1**) to BBB-permeable blockers, mainly due to the increasing awareness of P2X7R role in CNS disorders (Bhattacharya and Biber, 2016; Bhattacharya, 2018).

However, just increasing lipophilicity is not always highly recommended for the development of BBB-permeable drugs. In fact, this can penalize other important PK properties like solubility, enzyme metabolism, blood protein binding, and half-life. After the development of the high lipophilic *o*-chlorobenzamide P2X7R antagonists by Pfizer, their attempts were focused on the increase of molecular polarity, giving a good clinical candidate that, however, was no longer CNS penetrant (**CE-244,535—Figure 1**; Duplantier et al., 2011).

Abbott Laboratories developed several potent P2X7R antagonists, mainly tetrazoles and cyanoguanidines. Thus, tetrazole derivative **A-438079** (**Figure 1**) was very appealing for its small molecular weight, good potency, and efficacy in reverting allodynia in the Chung model of neuropathic pain. Nevertheless, its half-life of 1 h and low bioavailability after intraperitoneal (ip) administration was discouraging (Guile et al., 2009). Cyanoguanidine **A-740003** (**Figure 1**) was found to have a better half-life after iv administration ($t_{\frac{1}{2}}$ = 4 h) but not a good BBB permeability, as confirmed by its radiotracer [^{11}C]A-740003 (Janssen et al., 2014). Cyanoguanidine **A-804598** (**Figure 2**) has the best balance of pharmacodynamics and PK properties. In fact, it is potent (IC_{50} of around 10 nM for mouse, rat, and human species, vs. the potency of A-740003 ranging from 44 to 150 nM in the different orthologs), selective for the P2X7R, and brain-permeable, persisting in brain tissues at least for 1 h after administration, as shown in *ex vivo* studies of receptor occupancy (Able et al., 2011). More recently, Abbott has developed new potent P2X7R antagonists by adding the adamantine core to cyanoguanidines (compound **1**—**Figure 1**), but without great PK improvements (e.g., $t_{\frac{1}{2}}$ = 0.22 h; O'Brien-Brown et al., 2017).

GlaxoSmithKline developed a series of compounds with excellent physicochemical properties, such as the BBB-permeable **GSK-1482160** in rats (**Figure 1**; Abdi et al., 2010). Unfortunately, deeper studies on its metabolism pathway explained why some parameters, like its half-life, worsened in dog and monkey models, blocking its clinical development. Anyway, new chemical modifications succeeded in overcoming this problem. Among the compounds of this new series, the oxoimidazolidine **2** (**Figure 2**) stands out (Abberley et al., 2010).

It is a fact that Janssen Pharmaceuticals has probably developed the most interesting P2X7R antagonists as clinical candidates for the treatment of CNS diseases. One of the most used ligands in basic and preclinical research is **JNJ-47965567** (**Figure 1**). Unfortunately, its poor oral bioavailability, as well as the lack of deeper details on its residence time in brain, stopped its clinical progress (Bhattacharya et al., 2013). However, new series of dihydro- and tetrahydro-triazolopyridines were discovered and extensively optimized. Among these, **JNJ-54175446** and **JNJ-55308942** (**Figure 2**) have optimal PK properties like solubility, metabolism profile, bioavailability, and half-life, as well as good BBB permeability and tolerability (Letavic et al., 2017; Chrovian et al., 2018). Proof of this is the fact that they were chosen as clinical candidates and that **JNJ-55308942** has completed three phase I CTs, while **JNJ-54175446** is currently in a phase II CT for major depression (ClinicalTrials.gov - NIH U.S. National Library of Medicine, 2020; Recourt et al., 2020).

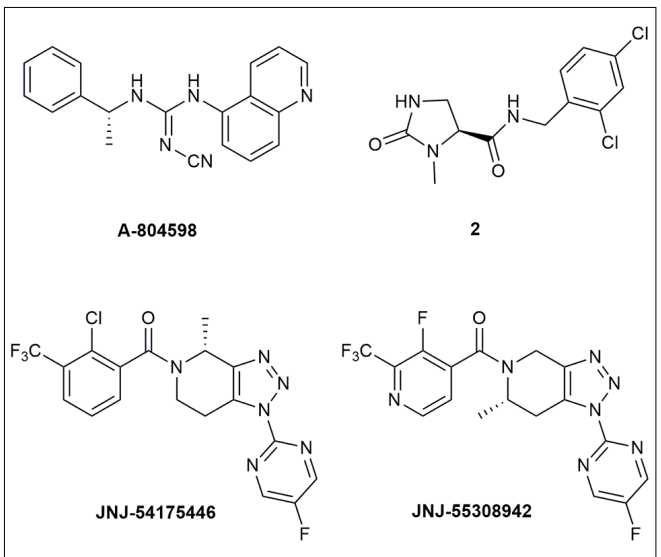

FIGURE 2 | P2X7R antagonists with optimal pharmacokinetic properties for the treatment of CNS disorders.

PROOF OF CONCEPT OF P2X7Rs AS A DRUGGABLE TARGET IN ALS

The proof of concept that a P2X7R antagonist could have promising therapeutic potential in ALS has been tested in the $SOD1^{G93A}$ mouse model of ALS (**Table 1**). In a first study, BBG was administered intraperitoneally at a dose of 45.5 mg/kg every 48 h, starting at postnatal day 90 (P90), a late pre-onset disease stage. The treatment improved deficits in motor performance to a greater extent in males than in females, but no effect on survival was observed (Cervetto et al., 2013).

Taking into account the dual role of P2X7Rs (protection at early stages and promotion of cell death at advanced disease stages), an experiment was designed changing the dose and defining different start points of BBG administration (250 mg/kg, 3 times/week). Starting at late pre-onset stage (P100), higher MN survival and reduced microgliosis were observed in the spinal cord of SOD^{G93A} mice. Moreover, disease onset was delayed and motor performance was improved in both males and females, although survival was not affected. This was not the case when BBG was administered at pre-symptomatic stages, supporting the hypothesis of the dual role of P2X7R in ALS (Apolloni et al., 2014).

In a third experiment, BBG administration at pre-onset stage or P62–P64 (45.5 mg/kg, three times/week) reduced body weight loss in females but not in males, a sign of delayed muscle loss. Survival was also augmented in females but not in males, and motor performance was unaffected in either sex (Bartlett et al., 2017).

Finally, two experiments have used more potent and specific P2X7R antagonists. When the antagonist A-804598 was administered to female mice five times/week at 30 mg/kg, no effects on motor performance, disease onset, or survival were observed (Fabbrizio et al., 2017). Same negative results were obtained with the administration of the antagonist JNJ-47965567 from P100, three times/week at 30 mg/kg (Ly et al., 2020).

These erratic, yet mild positive outcomes suggest that the experimental conditions of future experiments should

TABLE 1 | Proof of concept of P2X7R antagonism on clinical outcomes in SOD1^{G93A} mice.

Reference	Treatment	Treatment starting point	Main outcomes
Cervetto et al. (2013)	BBG, 45.5 mg/kg, every 48 h, ip	P90 (pre-onset) to humane end point	• Improvement in motor performance in both genders, although greater effect in males. • Delayed weight loss in males. • No difference in survival was observed.
Apolloni et al. (2014)	BBG, 50 mg/kg, three times/week, ip	P100 (late pre-onset) and P135 (onset) to humane end point	• Improvement in motor performance in mice treated from onset. • Disease onset and survival not affected.
	BBG, 250 mg/kg, three times/week, ip	P40 (asymptomatic), P70 (pre-onset), P100 (late pre-onset) to humane end point	• Improved behavioral scores and motor performance in mice treated from late pre-onset. • No differences in survival. • Decrease in microgliosis, inflammatory markers and motor neuron loss in late pre-onset treated animals.
Bartlett et al. (2017)	BBG, 45.5 mg/kg, three times/week, ip	P60 (pre-onset) to humane end point	• Reduced body weight loss and prolonged survival in females. • No effect on clinical score or motor performance
Fabbrizio et al. (2017)	A-804598, 30 mg/kg, five times/week, ip	P100 (pre-onset) to humane end point	• No effect on motor performance, behavioral scores or survival observed
Ly et al. (2020)	JNJ-47965567, 30 mg/kg, three times/week, ip	P100 (onset) to humane end point	• No effect on motor performance, ALS score or survival observed. • No altered gene expression in spinal cord. • No altered proportions of lymphoid leukocytes. • No effect on serum cytokines.

be considered as follows: (i) to adjust drug administration starting point; (ii) to define better the dosing and frequency of administration, according to (iii) the PK drug properties, including the BBB permeability and CNS residence time, longer half-life and solubility; and (iv) to include both females and males and, if possible, different ALS mouse models.

FROM BENCH TO CLINICAL TRIALS

Recent reviews (Petrov et al., 2017; Andrews et al., 2019; Chipika et al., 2019) analyze the outcomes of over 50 CTs performed in ALS patients, since riluzole was available. Various druggable potential targets have been addressed: (1) antiglutamatergic compounds (riluzole, memantine, talampanel, ceftriaxone); (2) antioxidant agents (edaravone, coenzyme Q, creatine); (3) anti-inflammatory drugs (valproic acid, NP001, glatiramer acetate, minocycline, pioglitazone, erythropoietin, celecoxib); (4) neurotrophic factors (IGF-1, CNTF, BDNF); (5) lithium; (6) inhibitors of kinase (masitinib, fasudil); and (7) neuroprotective compounds (xaliproden, dexpramipexole, olesoxime, omigapil).

Despite riluzole and edaravone being approved by the FDA after having been tested in CTs, they gave poor clinical outcomes. In the case of riluzole, two out of three late-stage CTs reported negative outcomes. Similarly, in the case of edaravone, two out of three phase III CTs also reported negative results. The rest of the compounds and drugs tested provided negative results in CTs.

Masitinib is emerging as a singular agent to mitigate microgliosis and neuroinflammation. The compound also prolongs survival when administered to SOD1^{G93A} ALS mice at the onset of paralysis (Trias et al., 2016). An add-on riluzole therapy phase III CT reported significant outcomes in ALS patients treated with masitinib [Masitinib in Combination With Riluzole for the Treatment of Patients Suffering From Amyotrophic Lateral Sclerosis (ALS), 2018].

CONCLUSIONS AND PERSPECTIVES

Much is known about clinical features and pathogenic pathways of ALS. Cumulating knowledge, derived from *in vitro* and *in vivo* disease models, as well as from patients and postmortem tissues, outlines the sequence of the pathophysiological events involved in ALS. These could be hypothetically ordered as follows: (1) cytosolic and mitochondrial protein aggregation, affecting mitochondrial ultrastructure and function; (2) distorted mitochondrial calcium handling and circulation; (3) deficits in ATP generation and excess of ROS production; (4) reactive microglia and production of inflammatory mediators; and (5) enhanced MN vulnerability and death.

Regarding disease pathogenesis, a crucial open question remains: are the pathogenic features common for both sporadic and familial ALS? And also, why do only MNs die, if mutated proteins are expressed in all cell types? Given the complexity of ALS pathogenesis, why have CTs been performed using single-target compounds in the last 20 years? In light of the chronic inflammatory background, should we target with

neuroprotective agents only the MNs, or the activated glia, or both? Much further research is required before we can answer these questions.

AUTHOR CONTRIBUTIONS

CR-R and FC contributed equally to this manuscript. They both searched for scientific literature concerning ALS pathogenesis and P2X7R involvement. CR-R reviewed the most considerable ALS mouse models used in research, while FC made a summary of the molecular and pharmacokinetic profiles of the main P2X7 receptor blockers. AG re-elaborated and drafted the final form of the manuscript, reviewing also the subsequent versions.

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frontiers in Molecular Neuroscience

REVIEW
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P2X7 Receptors as a Therapeutic Target in Cerebrovascular Diseases

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Shortage of oxygen and nutrients in the brain induces the release of glutamate and ATP that can cause excitotoxicity and contribute to neuronal and glial damage. Our understanding of the mechanisms of ATP release and toxicity in cerebrovascular diseases is incomplete. This review aims at summarizing current knowledge about the participation of key elements in the ATP-mediated deleterious effects in these pathologies. This includes pannexin-1 hemichannels, calcium homeostasis modulator-1 (CALHM1), purinergic P2X7 receptors, and other intermediaries of CNS injury downstream of ATP release. Available data together with recent pharmacological developments in purinergic signaling may constitute a new opportunity to translate preclinical findings into more effective therapies in cerebrovascular diseases.

Keywords: ATP, pannexin-1, ischemia, neuron, oligodendrocyte

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INTRODUCTION

Cerebrovascular diseases (CVDs) are referred to as a group of conditions that eventually lead to a reduction of blood supply to the brain as a consequence of a blockage (thrombosis or atherosclerosis), malformation (aneurysm), hemorrhage, or transient ischemia. In all instances, a decrease in oxygenation and nutrient supply ultimately leads to brain damage. Cerebrovascular diseases, principally stroke, constitute the second leading cause of death in adults worldwide and are major contributors to disability and reduced quality of life (Truelsen et al., 2000), so it is important to know the mechanisms behind this damage to reduce their consequences with the design and use of therapies against these diseases.

At the cellular level, damaged neurons and glial cells during and after stressful events in CVDs release glutamate into the extracellular space, which finally induces cytosolic Ca^{2+} overload and excitotoxicity (Braun et al., 1998; Jurányi et al., 1999; Melani et al., 2005; Takeuchi et al., 2006; for reviews, see also Rossi et al., 2007; Yenari et al., 2010). More recently, ATP was also defined as a potent excitotoxic signal to oligodendrocytes and neurons (Matute et al., 2007; Domercq et al., 2010; Cisneros-Mejorado et al., 2015b). In addition to ATP being among the molecules that are released by cell damage, recent evidence suggests that ATP acts as a damage-associated molecular pattern (DAMP) to initiate the innate immune response, induce pro-inflammation, and contribute to progressive neurological injury (Braun et al., 2017). Moreover, the massive increase in the

cytosolic concentration of Ca^{2+} is due in part to the overactivation of P2X7 receptors, a nonselective ligand-gated cation channel expressed at the cell surface of various cell types and activated by extracellular ATP. In this review, we summarize the state of the art regarding the P2X7 receptor role in cerebrovascular damage and its possible use as a therapeutic target. In addition, we discuss its relationship with other molecular agents, such as pannexins (Panxs) and calcium-permeable channels, representing all together a pathological orchestrated cluster that can contribute to the onset of tissue damage and its propagation in CVDs.

P2X7 RECEPTORS ARE MAJOR MEDIATORS OF TISSUE DAMAGE IN CEREBROVASCULAR DISEASES

Energy deprivation after stroke causes anoxic and irreversible depolarization. These events subsequently lead to massive release of excitatory neurotransmitters, including glutamate and ATP (Braun et al., 1998; Jurányi et al., 1999; Melani et al., 2005; Rossi et al., 2007), the latter causing neuronal and glial cell death through P2X7 receptor activation (Matute et al., 2007; Domercq et al., 2010; Arbeloa et al., 2012). However, the mechanisms of deleterious ATP release during brain ischemia are only partially known (Dale and Frenguelli, 2009; Cisneros-Mejorado et al., 2015b). Thus, during ischemia, the lack of oxygen causes a reduction in ATP production with an ensuing failure of plasma membrane ion pumps and loss of ion concentration homeostasis, all of which can finally lead to activation of Panx1 and calcium homeostasis modulator-1 (CALHM1) and the subsequent release of ATP (Cisneros-Mejorado et al., 2015b). These events lead to sustained activation of P2X7 receptors and pore formation with ensuing further ATP release that together creates a vicious circle, resulting in enhanced ATP-mediated excitotoxicity (Cisneros-Mejorado et al., 2015b). Indeed, pharmacological blockade or gene ablation of P2X7, Panx1, and CALHM1 results in substantial delayed post-anoxic depolarization following oxygen-glucose deprivation (OGD) and reduced brain tissue damage after transient middle cerebral artery occlusion (MCAO; Cisneros-Mejorado et al., 2015a, 2018; **Figure 1**).

In addition to neuronal and oligodendroglial excitotoxicity, activation of P2X7 receptors triggers the formation of the inflammasome, a multiprotein complex that mediates the release of cytokines, such as IL-1β, IL-18, and IL-33 (Giuliani et al., 2017; Baroja-Mazo et al., 2013), which can expand the initial ischemic damage.

P2X7 receptors are expressed in neurons and glia (see **Table 1**). Neurons express P2X7 receptors (Yu et al., 2008; Díaz-Hernández et al., 2009; Miras-Portugal et al., 2017; but see also Illes et al., 2017), and its blockade prevents ATP excitotoxicity and reduces the damage in models of both *in vivo* and *in vitro* ischemia (Arbeloa et al., 2012; Cisneros-Mejorado et al., 2015a). Likewise, oligodendrocytes, which are the major cellular component of white matter in the CNS, can undergo direct ATP-mediated excitotoxicity *via* activation of P2X7 receptors expressed in their membrane (James and Butt, 2001; Agresti et al., 2005; Matute et al., 2007; Yu et al., 2008; Domercq et al., 2010). Indeed, P2X7 receptors mediate ischemic damage to oligodendrocytes in culture and in optic nerve explants (Domercq et al., 2010). Moreover, oligodendrocyte precursor cells (OPCs) also express P2X7 receptors that contribute to periventricular white matter during perinatal hypoxic-ischemic injury as this condition is attenuated with selective antagonists of these receptors (Wang et al., 2009).

On the other hand, microglia express P2X7 receptors (Ferrari et al., 1996; Collo et al., 1997; Visentin et al., 1999; Sanz and Di Virgilio, 2000; Hide et al., 2000; Verderio and Matteoli, 2001; Chafke et al., 2002) that can promote their activation and proliferation (Bianco et al., 2006; Monif et al., 2009). This can indirectly cause neurotoxicity by stimulating the production of reactive oxygen species (Bartlett et al., 2013) as well as the release of pro-inflammatory cytokines (Suzuki et al., 2004; Shieh et al., 2014). Interestingly, P2X7 receptors in primary adult human microglia kept in culture modulate key components of innate immunity (Janks et al., 2018). Finally, P2X7 receptors are present in astrocytes (Ballerini et al., 1996; Sun et al., 1999; Panenka et al., 2001; Franke et al., 2001; James and Butt, 2001; Kukley et al., 2001), whereby they can raise intracellular Ca^{2+} concentration and ATP release (Suadicani et al., 2006) as well as contribute to non-vesicular glutamate release (Duan et al., 2003). **Table 1** summarizes the evidence supporting the expression of P2X7 receptors in neurons and glia.

Together, these data indicate that activation of P2X7 receptors can activate deleterious signals after ischemia by altering Ca^{2+} homeostasis and promoting the release of pro-inflammatory cytokines as well as causing oxidative stress. Accordingly, P2X7 blockade reduces tissue damage in experimental models of CVDs. Thus, Brilliant Blue G (BBG), a P2X7 selective antagonist, attenuates the extent of brain damage following transient MCAO (Arbeloa et al., 2012; Cisneros-Mejorado et al., 2015a). Similarly, BBG treatment ameliorates neuronal apoptosis in an experimental subarachnoid hemorrhage model (Chen et al., 2013). Moreover, three different P2X7 antagonists (BBG, A0438079, and OxATP) significantly increase survival rates and reduce cognitive deficits and cell death in transient global ischemia-reperfusion injury (Chu et al., 2012). Notably, P2X7 receptor attenuated glial activation and inflammatory cytokine overexpression in the hippocampus (Chu et al., 2012). **Table 2** sums up the protective effects of the inhibition or deletion of P2X7 described above.

ACTIVATION AND BIOPHYSICAL PROPERTIES OF P2X7 RECEPTORS IN NEURONS AND GLIA

P2X7 receptors have two possible states of conductance. First, low concentrations of agonist (ATP or BzATP) induce activation of a nonselective monophasic conductance allowing monovalent (Na^+, K^+) and divalent (Ca^{2+}) cation influx and plasma membrane depolarization (North, 2002). In addition,

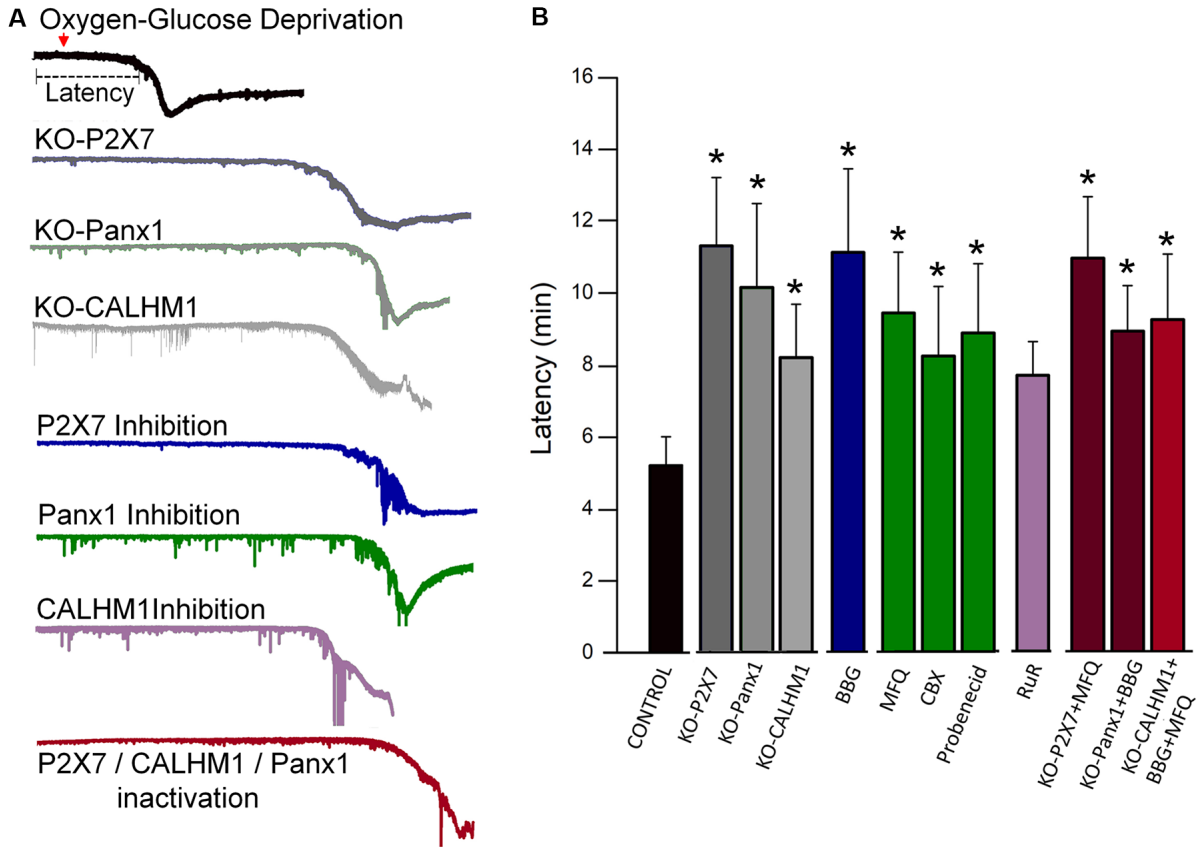

FIGURE 1 | Blockade or genetic ablation of P2X7 receptors, pannexin-1 (Panx1), or calcium homeostasis modulator-1 (CALHM1) channels delays ischemic ionic currents in acute cortical slices. **(A)** Representative electrophysiological recordings in whole-cell configuration (holding potential at −80 mV), of ischemic (oxygen-glucose deprivation, OGD) ionic currents in cortical neurons in acute brain slices from wild-type mice as well as from either P2X7, Panx1, or CALHM1 KO mice in the presence or absence of inhibitors of these channels. **(B)** Histogram showing the latency of the onset of ischemic ionic currents following OGD in the absence (Control) or in the presence of P2X7 receptor antagonist Brilliant Blue G (BBG, 50 nM); Panx1 inhibitors mefloquine (MFQ, 100 nM), carbenoxolone (CBX, 100 μM), and probenecid (1 mM); and CALHM1 inhibitor ruthenium red (RuR, 20 μM). Similar increased latency values were observed in neurons from acute slices obtained from P2X7 receptor, Panx1, and CALHM1 knockout mice (KO-P2X7, KO-Panx1, and KO-CALHM1, respectively). Concomitant blockade of more than one target (red columns) did not result in further delay of the post-anoxic current. All the data included in this graph was published earlier (Cisneros-Mejorado et al., 2015a, 2018; *$p < 0.05$ vs. control).

after prolonged exposure to high agonist concentrations (exposure to high agonist concentrations >1 mM ATP, >30 μM BzATP) P2X7 receptors can form a large nonselective pore allowing the passage of organic cations and molecules of up to 900 Da and the leakage of metabolites, including ATP (North, 2002; Yan et al., 2010), as shown in astrocytes (see above; Suadicani et al., 2006) and microglia (Ferrari et al., 1997). Although it is not clear whether oligodendrocytes leak ATP by this mechanism, there is evidence showing that glucocorticoids increase hemichannel activity in these cells in a P2X7R-dependent manner, suggesting a possible formation of a nonselective pore in these conditions (Maturana et al., 2017). Interestingly, P2X7 receptors in cultured cortical neurons form a large pore only at very high concentrations of BzATP and following a prolonged (10–40 s) exposure to the agonist (Cisneros-Mejorado et al., 2015b) although, in astrocytes *in vitro* or HEK cells expressing P2X7 receptors, the large pore formation occurs more readily (Yan et al., 2010). Macropore formation cannot be due to receptor pore dilation *per se* because the single-channel current amplitude and permeation characteristics remain constant (Riedel et al., 2007; Pippel et al., 2017; Di Virgilio et al., 2018). Thus, it appears that additional mechanisms must underlie the opening of the macropore (see Braun et al., 2017). This suggests that large pore formation in cultured neurons depends not only on prolonged stimulation of the P2X7 receptor. Alternatively, a role in ATP release has also been assigned to membrane channels formed by Panx1, which are large-pore ion channels with broad expression in the CNS (MacVicar and Thompson, 2010). Panx1 is permeable to molecules up to 900 Da and directly mediates ATP release (Locovei et al., 2007; Iglesias et al., 2009). The mechanisms by which P2X7 receptors and Panx1 are involved in pore formation are not well defined. However, the evidence indicates in astrocytes, for example, that Panx1 channels are activated after P2X7 receptor stimulation (Iglesias et al., 2009) or on concomitant membrane depolarization to induce Panx1 opening

TABLE 1 | P2X7 receptor expression in the central nervous system.

Cell type	Preparation	Techniques	References
Neuron	Mouse brain	Ca^{2+} imaging, IHH, qPCR, WB	Díaz-Hernández et al. (2009); Miras-Portugal et al. (2017)
	Rat Brain	*in situ* hybridization	Yu et al. (2008)
Oligodendrocyte lineage	Rat forebrain OPCs *in vitro*	WB, Ca^{2+} imaging	Agresti et al. (2005)
	Rat optic nerve OLs *in situ*	Ca^{2+} imaging	James and Butt (2001)
	Rat brain OLs	qPCR	Yu et al. (2008)
	Rat and human optic nerve OLs	IHH, WB, Ca^{2+} imaging, electrophysiology	Matute et al. (2007); Domercq et al. (2010)
Microglia	Rat and mice brain	WB, IHH, *in situ* hybridization	Collo et al. (1997)
	Rat brain *in vitro*	Ca^{2+} imaging, electrophysiology	Visentin et al. (1999)
	Rat brain *in vitro*	Cytokine reléase, fluorimetry	Hide et al. (2000)
	Rat brain *in vitro*	IHH, fluorimetry	Verderio and Matteoli (2001)
	Mouse brain *in vitro*	Electrophysiology, fluorimetry	Chafke et al. (2002)
Astrocytes	Rat brain *in vitro*	Ca^{2+} imaging	Ballerini et al. (1996)
	Astrocyte cell line	Ca^{2+} imaging	Sun et al. (1999)
	Rat brain *in vitro*	IHH, chemokine signaling	Panenka et al. (2001)
	Rat brain after lesion	IHH	Franke et al. (2001)
	Rat brain	IHH	Kukley et al. (2001)
	Rat optic nerve *in situ*	Ca^{2+} imaging	James and Butt (2001)
	Rat spinal cord	Ca^{2+} imaging, ATP release	Suadicani et al. (2006)

IHC, immunohistochemistry; OL, oligodendrocyte; OPCs, oligodendrocyte progenitor cells; qPCR, quantitative PCR; WB, western blot.

TABLE 2 | Protective effects of blocking of P2X7 receptors.

Antagonist	Model	Effects	References
Brilliant Blue G	OGD, MCAO	Decreases infarct size Protects from neuronal death Relieves neurological symptoms	Arbeloa et al. (2012) Chu et al. (2012) Cisneros-Mejorado et al. (2015a)
	in vitro, EAE	Promotes oligodendrocyte survival, protects myelin, ameliorates neurological symptoms	Matute et al. (2007)
	OGD *in vitro* and *in situ*	Promotes oligodendrocyte survival, protects myelin	Domercq et al. (2010)
	Perinatal hypoxia-ischemia	Reduces white matter injury	Wang et al. (2009)
	experimental subarachnoid hemorrhage	Ameliorates function and reduces neuronal apoptosis	Chen et al. (2013)
A0438079	MCAO	Relieves neurological symptoms increase survival rates Attenuate inflammation	Chu et al. (2012)
	OGD in slices and culture	reduce postanoxic depolarization	Cisneros-Mejorado et al. (2015a)
OxATP	MCAO	Reduces mortality	Chu et al. (2012)
	in vitro, EAE	Promotes oligodendrocyte survival, Protects myelin, ameliorates neurological symptoms	Matute et al. (2007)
Nanobodies	*in vivo* glomerulonephritis	Ameliorates experimental glomerulonephritis in mice	Danquah et al. (2016)

EAE, experimental autoimmune encephalomyelitis; MCAO, transient middle cerebral artery occlusion; OGD, oxygen-glucose deprivation.

as reported earlier (Locovei et al., 2007). Thus, Panx1 can be activated following Ca^{2+} influx *via* P2X7 receptors along or in conjunction with subsequent Ca^{2+}-induced Ca^{2+} mobilization from intracellular stores (North, 2002; Locovei et al., 2006). These findings suggest that P2X7 receptors and Panx1 act synergistically, at least in the CNS, because, in other systems, pore formation after prolonged activation of the P2X7 receptor does not occur through Panx1 channels (Qu et al., 2011; Alberto et al., 2013). One of the best characterized aspects of P2X7 receptor function is its ability to activate indirectly the NACHT, LRR, and PYD domains containing protein 3 (NLRP3) inflammasome in macrophages, thereby initiating caspase-1-mediated IL-1β; processing and release and ultimately inducing macrophage pyroptotic cell death (Chen et al., 2014). In the brain, exogenous ATP induces NLRP3 inflammasome activation in astrocytes and microglia along with an increase in IL-1β production that activates caspase-1 and amplification of the stress response (Murphy et al., 2012). Consistent with this idea, stressed glia increases connexin 43 (Cx43) and Panx1 hemichannel activity in microglia and astrocytes of adult mice (Orellana et al., 2015). Moreover, exposure to high levels of glucocorticoids during gestation induces long-lasting neuroinflammation and activates the inflammasome in hippocampal oligodendrocytes of mouse offspring (Maturana et al., 2017). Thus, oligodendrocytes of control pups showed expression of inflammasome components (NLRP3, ACS, and caspase-1), and their levels were increased by prenatal administration of dexamethasone, a synthetic glucocorticoid. These cells also showed high levels of IL-1β; and TNF-α; in accordance with activation of the inflammasome. Notably, oligodendrocytes showed increased levels of the P2X7 receptors and Panx1, molecules associated with inflammasome activation

(Maturana et al., 2017). However, it is not known whether glucocorticoids also modify the activation properties of the P2X7/Panx1 axis in neurons, a feature that might be relevant in CVDs.

P2X7 RECEPTORS ARE INVOLVED IN ISCHEMIC PRE- AND POST-CONDITIONING EFFECTS

Brief, non-harmful, ischemic preconditioning can confer tolerance and protection from subsequent cerebrovascular damage. Thus, preconditioning attenuates damage in models of cerebral ischemia associated with oxidative stress and glutamate excitotoxicity (Jachova et al., 2019). Although the mechanisms of this phenomenon are unclear, recent data suggest that P2X7 receptors have a role in ischemia preconditioning as this is ineffective in P2X7 receptor knockout mice (Hirayama et al., 2015). In turn, after preconditioning, P2X7 receptors in astrocytes can orchestrate the initiation of neuroprotective cascades including those mediated by HIF-1α; Hirayama et al., 2015). Indeed, P2X7 receptor expression itself is increased in astrocytes and followed by an elevation of hypoxia-inducible factor (HIF)-1α in these cells after preconditioning using an MCAO model in mice (Hirayama et al., 2015). Moreover, inhibiting the astroglial metabolism with fluorocitrate abolished the induction of ischemic tolerance, which strongly suggests that astrocytes play an essential role in its inception (Hirayama et al., 2015).

In addition, hypoxic preconditioning protects cultured neurons against hypoxic stress *via* tumor necrosis factor-α; TNF-α; Jie Liu et al., 2000; Ruscher et al., 1998). Notably, the release of TNF-α appears to be dependent on the P2X7 receptor because microglia treated with BzATP in neuron-microglia cultures leads to significant reduction in glutamate-induced neuronal cell death, and either TNF-α-converting enzyme inhibitor or anti-TNF-α IgG readily suppresses this protective effect (Suzuki et al., 2004). These findings provide evidence that, as in astrocytes, P2X7 receptors in microglia contribute to brain ischemic tolerance though different mechanisms.

The above data argues in favor of a dual role of P2X7 receptors in CVDs with a protective edge during mild ischemia (preconditioning) and a deleterious excitotoxic role in more severe ischemia. This duality adds further complexity to the development of effective therapies to prevent ischemic injury by promoting, for example, preconditioning with P2X7 receptor agonists and/or allosteric modulators while having at hand antagonists as neuroprotectants to limit the extension of the ischemic core into the penumbra once stroke occurs. To define the parameters that limit this double-edged behavior of P2X7 receptors constitutes a major challenge in experimental ischemia as a previous step for an effective translation into CVDs patients.

On the other hand, ischemic post-conditioning may be more amenable and effective for therapeutic use. Thus, repetitive short periods of OGD alternated with reperfusion after prolonged OGD attenuates neuronal apoptosis by increasing Bcl-2 expression while reducing Bax levels and overexpressing heat-shock protein 70 (HSP70; Zhao et al., 2014). Intriguingly, proteomic and functional characterization of the P2X7 receptor signaling complex showed that HSP70 co-immunoprecipitates with P2X7 receptors (Kim et al., 2001). This finding strongly suggests an interaction between HSP70 and P2X7 receptors; however, a direct link between P2X7 activity and post-conditioning neuroprotection is still missing.

THERAPEUTIC POTENTIAL OF P2X7 RECEPTOR IN CEREBROVASCULAR DISEASES

All the above evidence suggests that P2X7 receptor activation or blockade is involved in the onset and propagation of tissue damage as well as in neuroprotection and neuroinflammation in CVDs. Therefore, a thorough understanding of the upstream events leading to P2X7 receptor activation along with subsequent downstream signaling cascades they trigger will eventually allow identifying new cellular and molecular targets amenable for the design of novel drugs to use in clinical studies.

P2X7 receptor agonists may facilitate ischemia preconditioning by promoting the release of protective factors and signals in neurons and glia that ultimately attenuate major damage (Hirayama et al., 2015). Alternatively, specific P2X7 agonists may enhance the phagocytic capacity of microglia as shown in a model of phagocytic function in fresh human monocytes without promoting pore formation, thus avoiding unwanted side effects, such as excitotoxicity or enhanced neuroinflammation (Ou et al., 2018). This novel role of the P2X7 receptor as a scavenger receptor in microglia/macrophages and possibly in other cells in the CNS creates new pharmacological possibilities as it is not affected by potent selective P2X7 receptor antagonists, and its phagocytic function has features distinct from its pore function (Ou et al., 2018). Therefore, differential drug targeting both on P2X7 pore formation and P2X7-mediated phagocytosis has a great potential as a single or combined treatment in CVDs.

In addition to agonists and antagonists, other P2X7 receptor ligands may have therapeutic value. This includes positive allosteric modulators, such as clemastine, an anti-allergy drug, which binds extracellularly to P2X7 receptors and potentiates their ATP-sensitivity while it increases the release of IL-1β; from lipopolysaccharide-primed macrophages, thus modulating native immune responses (Nörenberg et al., 2011). In turn, clemastine may also favor myelination of damaged myelin and the rescue of behavioral changes that occur after stroke (Liu et al., 2016; Cohen and Tesar, 2017). Likewise, ginsenosides of the protopanaxdiol series potently activate P2X7 receptors, leading to an increase of sustained calcium ion influx in mouse macrophages that may account for their reported immune modulatory actions *in vivo* (Helliwell et al., 2015).

Discovering new uses for approved drugs acting at P2X7 may provide the quickest possible transition from bench to bedside in CVDs. Thus, A-740003 (N-(1-[(cyanoimino)(5-quinolinylamino) methyl] amino-2,2-dimethylpropyl)-2-(3,4-

dimethoxyphenyl)acetamide), a competitive antagonist of P2X7 receptors, produces significant antinociception in animal models of neuropathic and inflammatory pain (Honore et al., 2006) and has been evaluated for neuroinflammation (Janssen et al., 2014). On the other hand, AZD9056, an adamantane amide that was discovered through a program designed to identify potent and selective P2X7 antagonists, provides a significant inhibition of ATP-induced IL-1β release in monocytes ex vivo, suggesting that circulating leucocytes were blocked by P2X7 (Keystone et al., 2012). AZD9056 is well tolerated and induces statistically significant changes in parameters of clinical relevance; however, it failed to ameliorate symptoms in patients with rheumatoid arthritis, an immunologically mediated disease in which cytokines are key regulatory molecules. Similarly, high throughput screening of a compound library provided an attractive lead compound with modest P2X7 receptor antagonist potency and high selectivity against a panel of receptors and channels (Duplantier et al., 2011). Multi-parameter optimization led to a potent P2X7 antagonist, CE-224,535, which was advanced to clinical studies for the treatment of rheumatoid arthritis (Duplantier et al., 2011). This compound is currently under scrutiny for others brain diseases and has therapeutic potential in CVDs.

Another interesting approach in the development of biologics targeting P2X7 receptors are antibodies and nanobodies that antagonize or potentiate gating of P2X7. Their potential advantages over small-molecule drugs include high specificity, lower off-target effects, and tunable *in vivo* half-life (for a recent review see Koch-Nolte et al., 2019). Therapeutic antibodies are commonly injected systemically to maximize delivery; however, they can be also administered as aerosols to treat respiratory tract and lung diseases (Van Heeke et al., 2017). In addition, genetic fusion of P2X7-specific biologics to binding modules may enable targeting of specific cell subsets; besides, directly modulating P2X7 function, antibodies can also initiate specific depletion of P2X7-expressing cells (Koch-Nolte et al., 2019). Furthermore, adeno-associated viral vectors can be used to express P2X7-specific antibodies *in vivo* to achieve long-lasting biological effects and enable modulation of the function of P2X7-expressing immune cells *via* encoded transgenic RNA or proteins (Koch-Nolte et al., 2019). Indeed, functional antibodies and nanobodies have already shown promising therapeutic benefit in animal models of sterile inflammation (Menzel et al., 2018).

CONCLUSIONS

The present review provides an outlook about the role of P2X7 receptors in CVDs and their dual function as a cationic channel and as a precursor of large pore formation. It summarizes a wealth of evidence demonstrating that inhibition of P2X7 receptors promotes neuron and glia protection against brain injury and that they can be essential for the release of cell-supporting factors. At the same time, these receptors are also relevant to preconditioning and post-conditioning and, therefore, emerge as possible targets to attenuate tissue damage in CVDs and modulate neuroinflammation to constrain the expansion of the core lesion into the penumbra.

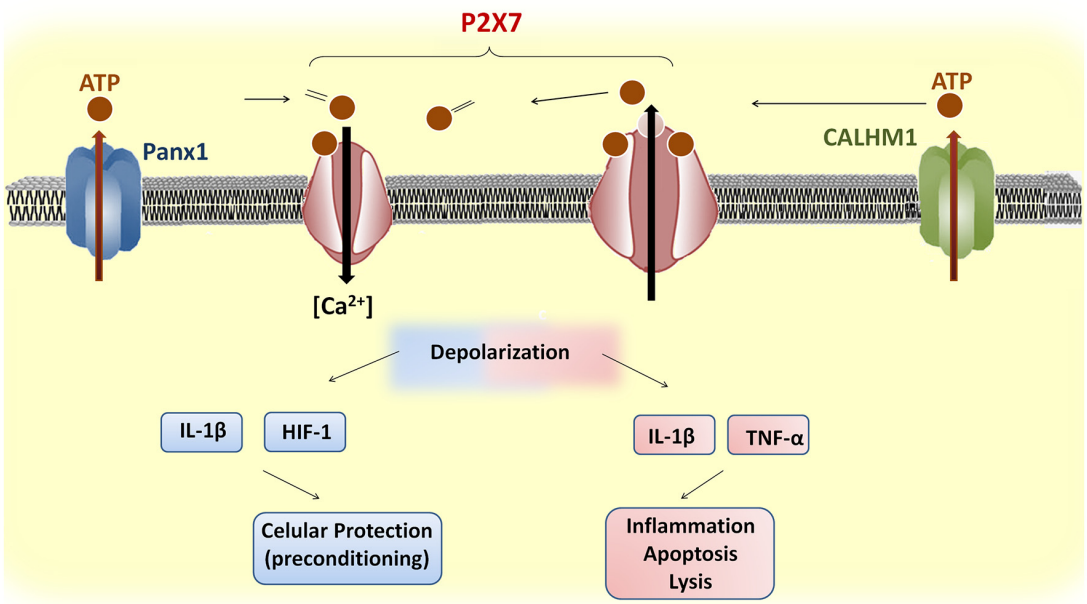

FIGURE 2 | Upstream and downstream events of P2X7 receptor activation in brain cells. ATP release through Panx1 or CALHM1 activates P2X7 receptors, which induces influx of Ca^{2+}. The prolonged stimulation of P2X7 receptors induces pore formation and further ATP release. Brief depolarization during mild cerebrovascular diseases (CVDs) may promote the release of protective factors, such as hypoxia-inducible factor (HIF)-1, that confer cellular protection against subsequent ischemic stimuli. In contrast, severe CVDs trigger a sustained P2X7 receptor depolarization and the release of pro-inflammatory cytokines that cause apoptosis or lysis.

New, groundbreaking research on therapeutic targeting on P2X7 receptors is constantly being made available. A notable example is the relatively recent discovery of a structural basis for subtype-specific inhibition, which provides novel mechanistic insights to facilitate the development of P2X7-specific drugs for treating human diseases (Karasawa and Kawate, 2016). Moreover, new data on the structural and functional properties, in combination with cell-based functional studies, suggest that the P2X7 receptor itself constitutes a lipid-composition-dependent, dye-permeable pore, whose opening is facilitated by palmitoylated cysteines near the pore-lining helix (Karasawa et al., 2017).

In summary, P2X7 receptors contribute to neurotransmission and glia signaling using Ca^{2+} as a key second messenger (**Figure 2**) although the precise mechanisms mediating their effects in neurons and glial cells are still unclear. In mild CVDs, P2X7 receptors are involved directly or indirectly in preconditioning or post-conditioning by conditions by promoting the release of protective factors, such as HIF-1 (Hirayama et al., 2015), resulting in a pro-survival cascade against subsequent harmful events in neurons and possibly in oligodendrocytes. Finally, in severe CVDs, P2X7 receptors promote pore formation, thus allowing the efflux of large molecules including ATP and Ca^{2+} influx causing cytosolic Ca^{2+} overload and generating a detrimental feedback loop that ultimately results in neuronal and oligodendroglial death with the consequent demyelination along with astrocytic and microglial activation as well as pro-inflammatory cytokine release. Further work is warranted to elucidate the exact underlying mechanisms of the P2X7 receptor in the pathophysiology of CVDs and to shed light on therapies that simultaneously target multiple cell types and mechanisms of injury in these diseases.

AUTHOR CONTRIBUTIONS

AC-M and CM conceived and described the initial draft of the manuscript. AC-M prepared graphic material. AP-S, MD, RA, MG, and FK-N contributed numerous comments and suggestions to the final manuscript.

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REVIEW
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The P2X7 Receptor: Central Hub of Brain Diseases

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The P2X7 receptor is a cation channel activated by high concentrations of adenosine triphosphate (ATP). Upon long-term activation, it complexes with membrane proteins forming a wide pore that leads to cell death and increased release of ATP into the extracellular milieu. The P2X7 receptor is widely expressed in the CNS, such as frontal cortex, hippocampus, amygdala and striatum, regions involved in neurodegenerative diseases and psychiatric disorders. Despite P2X7 receptor functions in glial cells have been extensively studied, the existence and roles of this receptor in neurons are still controversially discussed. Regardless, P2X7 receptors mediate several processes observed in neuropsychiatric disorders and brain tumors, such as activation of neuroinflammatory response, stimulation of glutamate release and neuroplasticity impairment. Moreover, P2X7 receptor gene polymorphisms have been associated to depression, and isoforms of P2X7 receptors are implicated in neuropsychiatric diseases. In view of that, the P2X7 receptor has been proposed to be a potential target for therapeutic intervention in brain diseases. This review discusses the molecular mechanisms underlying P2X7 receptor-mediated signaling in neurodegenerative diseases, psychiatric disorders, and brain tumors. In addition, it highlights the recent advances in the development of P2X7 receptor antagonists that are able of penetrating the central nervous system.

Keywords: P2X7 receptor, neurodegenerative diseases, psychiatric disorders, brain tumor, brain diseases, P2X7 receptor antagonists

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INTRODUCTION

The P2X7 Receptor

The investigation of receptors activated by adenosine triphosphate (ATP) has been largely widened since their discovery in 1960s by Geoffrey Burnstock. These receptors are classified into two main types: P1 and P2 receptors. P1 receptors are usually activated by adenosine, have seven transmembrane domains, and are coupled to G proteins. P2 receptors can be divided into two main subtypes, ionotropic P2X receptors and metabotropic P2Y receptors. P2X receptors subunits have just two transmembrane domains and are assembled as homo- or heterotrimers. Such as adenosine-activated P1 receptors, P2Y receptors are coupled to G proteins; however, their ligands are ATP/ADP/ UTP/UDP-glucose (Knight, 2009).

Since the first cloning of the P2X7 receptor from a rat brain cDNA library (Surprenant et al., 1996), it is the most widely investigated purinergic receptor with the largest amount of specific pharmacological tools available (Sluyter and Stokes, 2011).

The *P2RX7* gene is comprised of 13 exons encoding the subunit with 595 amino acids in length that in humans is located at chromosome position 12q24.31 and in mice at chromosome 5. The human *P2RX7* gene is located at the chromosome position also associated with inflammatory and psychiatric disorders (Barden et al., 2006; Lucae et al., 2006). Each one of the three subunits has intracellular amino and carboxyl termini with two hydrophobic transmembrane domains, with a long glycosylated extracellular loop between them, comprising the ATP-binding site. In addition, the P2X7 receptor usually assembles as homotrimer (Sluyter and Stokes, 2011). However, it can also form heteromeric interactions with P2X4 receptor subunits as evidenced in 2007 by Guo et al. (2007) and later confirmed by Schneider et al. (2017).

P2X7 receptor activity is triggered by high concentrations (ranging around 0.05–1 mM) of extracellular adenosine 5′-triphosphate (ATP), mediating the rapid influx of Na^+ and Ca^{2+} and efflux of K^+, and other cations (Burnstock and Kennedy, 2011). Upon long activation, the P2X7 receptor can open pores large enough to allow the passage of organic ions like N-methyl-D-glucamine ($NMDG^+$), $choline^+$ and fluorescent dyes such as $ethidium^+$ and YO-PRO-12^+ (Alves et al., 2014).

Available tools for P2X7 receptor research lack specific agonists. Due to this problem, many literature data need to be carefully analyzed. Studies regarding the activation of P2X7 receptors use agonists, such as ATP and 2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate (Bz-ATP). ATP is a broad agonist for P2X receptors. Bz-ATP is 10–50 times more potent than ATP in activating P2X7 receptors. Besides activating P2X7 receptors, this compound acts as an agonist for P2Y11, P2X1, 2 and 4, and as a weak agonist for P2X5 receptors. Additionally, EC_{50} values for both agonists vary between species. Bz-ATP, for example, activates rat and human P2X7 receptor at 10 times greater concentration than mice P2X7 receptor (Burnstock and Verkhratsky, 2012). As indicated in **Table 1**, some P2X7 receptor antagonists also lack specificity. The widely used Brilliant Blue G (BBG) also antagonizes P2X1, P2X2, P2X3, and P2X4 receptors besides the P2X7 receptor. However, the IC_{50} for the P2X7 receptor is 8–50 times lower compared with other receptors. A-740003, A-438079 and A-804598 are selective for the P2X7 receptor (Burnstock and Verkhratsky, 2012).

Another limitation regarding P2X7 receptors studies is antibody specificity. Available antibodies against the P2X7 receptor are polyclonal, which are prone to cross-reactivity, or monoclonal. Although they did not detect P2X7 receptors in knockout (KO) mice, these monoclonal antibodies failed to consistently and reliably detect and/or block P2X7 receptor signaling pathway in WT mice (Sim et al., 2004; Li et al., 2020). There are at least two P2X7 receptor KO mice commercially available. In the GlaxoSmithKline strain, generated by Chessell et al. (2005), a lacZ transgene and neomycin cassette were inserted into exon 1 (Chessell et al., 2005). These animals express the P2X7K receptor isoform and lack the P2X7A receptor isoform. P2X7K is widely expressed by T lymphocytes, and GlaxoSmithKline P2X7 receptor KO mice possess enhanced P2X7 receptor-mediated responses in T cells. The other available strain from Pfizer, generated by Solle et al. (2001) by inserting a neomycin cassette into exon 13, lacks both P2X7A and K receptor isoforms; however these animals express P2X7 13B and 13C isoforms in the brain and other tissues (Solle et al., 2001; Bartlett et al., 2014). The P2X7 13B isoform was reported to negatively affect P2X7A receptor activity (Masin et al., 2012). Therefore, P2X7 receptor KO mice should be used carefully as a tool to assess P2X7 receptor involvement in brain and inflammation.

Nowadays, P2X7 receptor expression is known to be broadly present throughout diverse tissues and cells, including CNS, such as microglia, oligodendrocytes, Schwann cells, and possibly in astrocytes and neurons. The latter one is still controversial discussed, and various works are still trying to clarify the issue (see Sluyter and Stokes, 2011). Despite several works that demonstrate the presence of P2X7 receptor in neurons (Deuchars et al., 2001; Sperlágh et al., 2002; Wirkner et al., 2005; Yu et al., 2008), its expression and functionality are widely debated (Sim et al., 2004; Anderson and Nedergaard, 2006; Illes et al., 2017; Metzger et al., 2017b). This outlook becomes strengthened when immunoreactivity for this receptor in P2X7 receptor KO strains was detected, evidencing low specificity of anti-P2X7 receptor antibodies (Anderson and Nedergaard, 2006). Recent works with improved methodologies did not find any expression of P2X7 receptors in neurons (Rubini et al., 2014; Kaczmarek-Hajek et al., 2018; Khan et al., 2018). Similarly, the presence of functional P2X7 receptors in astrocytes is also debated c). It is well known, however, that oligodendrocytes and microglia express functional P2X7 receptors (Lord et al., 2015; He et al., 2017; Metzger et al., 2017a; Kaczmarek-Hajek et al., 2018).

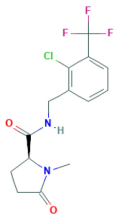
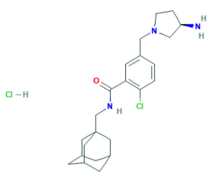
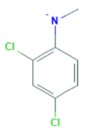
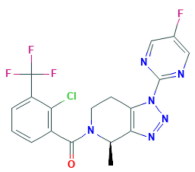
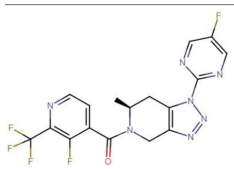
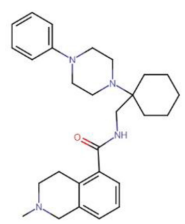
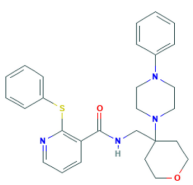
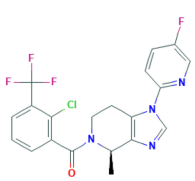
Variants of the P2X7 Receptor

The P2X7 receptor has 10 different alternative splicing isoforms named from P2X7A to P2X7K, the latter has only been identified in rodents (**Figure 1**). The full-length isoform is the P2X7A one. In humans, P2X7B, P2X7H, and P2X7J are the only subunits reported as expressed proteins (Feng et al., 2006; Adinolfi et al., 2010) (**Figure 1**).

The P2X7B isoform is a truncated form, when compared with P2X7A (Cheewatrakoolpong et al., 2005), and assemble as functional channels that cannot form large pores as P2X7A, playing roles in cell proliferation (Adinolfi et al., 2010). The P2X7H is nonfunctional ion channels (Cheewatrakoolpong et al., 2005), whereas the P2X7J can assemble with other splicing variants forming non-functional heterotrimeric receptors (Feng et al., 2006) that are involved in protection against ATP-induced cell death (Feng et al., 2006; Guzman-Aranguez et al., 2017).

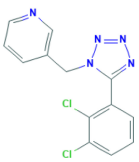
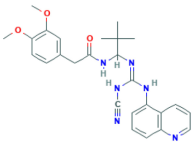
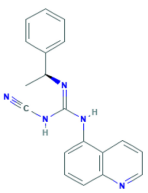
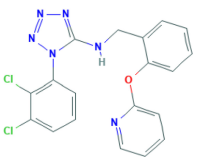
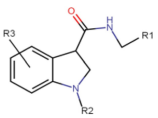
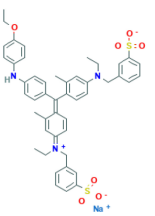
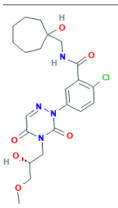
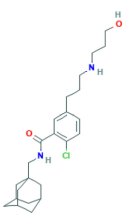
In mice, four splice variants were detected (P2X7B, P2X7C, P2X7D, and P2X7K), besides the canonical P2X7A one. Most of the modifications between isoforms are comprised within the extracellular loop domain. P2X7D and P2X7B can assemble to P2X7A and negatively affect the basal activity of the P2X7 receptor. However, if not assembled to P2X7A, they assemble as receptors forms that show both increased activity and higher sensitivity to agonists (Schwarz et al., 2012; Xu et al., 2012), like the rat P2X7K variant (Nicke et al., 2009). Restricted P2X7 receptor variants present multiple mutations, such as the P2X7 receptor-2 variant that contains H270R and A348T mutations, and the P2X7 receptor-4 variant that has H155Y, H270R, A348T,

TABLE 1 | P2X7 receptor antagonists.

Structure	Compound/IUPAC name	BBB-penetrant	Type	References
	**GSK-1482160** (2S)-N-[[2-chloro-3-(trifluoromethyl)phenyl]methyl]-1-methyl-5-oxopyrrolidine-2-carboxamide	Yes	Preferential P2X7 receptor antagonist	Territo et al., 2017; Kim et al., 2019
	**GSK-314181A** N-(1-adamantylmethyl)-5-[[(3R)-3-aminopyrrolidin-1-yl]methyl]-2-chlorobenzamide;hydrochloride	Yes	Preferential P2X7 receptor antagonist	Broom et al., 2008; Kim et al., 2019
	**Compound 16 (GSK)** (2,4-dichlorophenyl)-methylazanide	Yes	Preferential P2X7 receptor antagonist	Beswick et al., 2010; Kim et al., 2019
	**JNJ-54175446** [2-chloro-3-(trifluoromethyl)phenyl]-[(4R)-1-(5-fluoropyrimidin-2-yl)-4-methyl-6,7-dihydro-4H-triazolo[4,5-c]pyridin-5-yl]methanone	Yes	Preferential P2X7 receptor antagonist	Letavic et al., 2017; Kim et al., 2019
	**JNJ-55308942** (S)-(3-fluoro-2-(trifluoromethyl)pyridin-4-yl)(1-(5-fluoropyrimidin-2-yl)-6-methyl-1,4,6,7-tetrahydro-5H-[1,2,3]triazolo[4,5-c]pyridin-5-yl)methanone	Yes	Non-selective P2X7 receptor antagonist (also binds to P2X1, P2X2, P2X3, P2X2/3, and P2X4 receptors)	Chrovian et al., 2018
	**JNJ-42253432** 2-methyl-N-([1-(4-phenylpiperazin-1-yl)cyclohexyl]methyl)-1,2,3,4-tetrahydroisoquinoline-5-carboxamide	Yes	Preferential P2X7 receptor antagonist	Letavic et al., 2013; Lord et al., 2014
	**JNJ-47965567** N-[[4-(4-phenylpiperazin-1-yl)oxan-4-yl]methyl]-2-phenylsulfanylpyridine-3-carboxamide	Yes	Preferential P2X7 receptor antagonist	Bhattacharya et al., 2013; Letavic et al., 2013; Kim et al., 2019
	**JNJ-54166060** [2-chloro-3-(trifluoromethyl)phenyl]-[(4R)-1-(5-fluoropyridin-2-yl)-4-methyl-6,7-dihydro-4H-imidazo[4,5-c]pyridin-5-yl]methanone	Yes	Preferential P2X7 receptor antagonist	Swanson et al., 2016; Kim et al., 2019

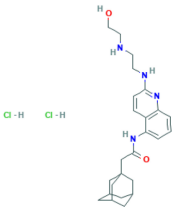
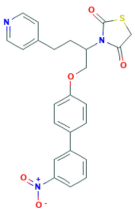
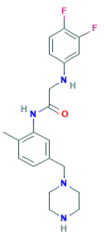
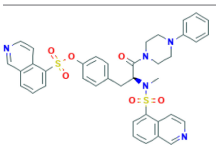
(Continued)

TABLE 1 | Continued

Structure	Compound/IUPAC name	BBB-penetrant	Type	References
	**A-438079** 3-[[5-(2,3-dichlorophenyl)tetrazol-1-yl]methyl]pyridine	Yes	Preferential P2X7 receptor antagonist	Nelson et al., 2006; Kim et al., 2019
	**A-740003** N-[1-[(Z)-[(cyanoamino)-(quinolin-5-ylamino)methylidene]amino]-2,2-dimethylpropyl]-2-(3,4-dimethoxyphenyl)acetamide	Yes	Preferential P2X7 receptor antagonist	Honore et al., 2006; Kim et al., 2019
	**A-804598** 1-cyano-2-[(1S)-1-phenylethyl]-3-quinolin-5-ylguanidine	Yes	Preferential P2X7 receptor antagonist	Donnelly-Roberts et al., 2009; Able et al., 2011; Kim et al., 2019
	**A-839977** 1-(2,3-dichlorophenyl)-N-[(2-pyridin-2-yloxyphenyl)methyl]tetrazol-5-amine	Yes	Preferential P2X7 receptor antagonist	Honore et al., 2009; Kim et al., 2019
	**AFC-5128** indol-3-carboxamide derivative, chemical nomenclature disclosed	Yes	Preferential P2X7 receptor antagonist	Fischer et al., 2016
	**Brilliant blue G (BBG)** 3-[[4-[(E)-[4-(4-ethoxyanilino)phenyl]-[4-[ethyl-[(3-sulfonatophenyl)methyl]azaniumylidene]-2-methylcyclohexa-2,5-dien-1-ylidene]methyl]-N-ethyl-3-methylanilino]methyl]benzenesulfonate	Yes	Non-selective P2X7 receptor antagonist (also binds to P2X1, P2X2, P2X3 and P2X4 receptors)	Savio et al., 2018; Kim et al., 2019
	**CE-224, 535** 2-chloro-N-[(1-hydroxycycloheptyl)methyl]-5-[4-[(2R)-2-hydroxy-3-methoxypropyl]-3,5-dioxo-1,2,4-triazin-2-yl]benzamide	No	High selective P2X7 receptor antagonist (500-fold over P2X1 and P2Y1 receptors)	Savall et al., 2015; Kim et al., 2019
	**AZD9056** N-(1-adamantylmethyl)-2-chloro-5-[3-(3-hydroxypropylamino)propyl]benzamide	No	Preferential P2X7 receptor antagonist	Bhattacharya, 2018; Kim et al., 2019

(Continued)

TABLE 1 | Continued

Structure	Compound/IUPAC name	BBB-penetrant	Type	References
	**AZ-10606120** 2-(1-adamantyl)-N-[2-[2-(2-hydroxyethylamino)ethylamino]quinolin-5-yl]acetamide;dihydrochloride	Not found	Negative allosteric modulator of the human P2X7 receptor.	Kim et al., 2019
	**AZ-11645373** 3-[1-[4-(3-nitrophenyl)phenoxy]-4-pyridin-4-ylbutan-2-yl]-1,3-thiazolidine-2,4-dione	Not found	Preferential P2X7 receptor antagonist (500 times less effective in rat than in human P2X7 receptors)	Kim et al., 2019
	**GW791343** 2-(3,4-difluoroanilino)-N-[2-methyl-5-(piperazin-1-ylmethyl)phenyl]acetamide	Not found	Negative activity modulator of human P2X7 receptors, positive activity modulator of rat P2X7 receptors	Kim et al., 2019
	**KN-62** [4-[(2S)-2-[isoquinolin-5-ylsulfonyl(methyl)amino]-3-oxo-3-(4-phenylpiperazin-1-yl)propyl]phenyl] isoquinoline-5-sulfonate	Not found	Preferential human P2X7 receptor antagonist, however with low affinity to rat P2X7 receptors	Kim et al., 2019

BBB: blood brain barrier; IUPAC: International Union of Pure and Applied Chemistry.

and Q460R mutations (Stokes et al., 2010). These variants in heterologous expression cells also exhibited larger agonist-induced ion currents and dye uptake with a similar agonist sensitivity (Jiang et al., 2013).

Some alternative splicing isoforms of P2X7 receptor show diverse downstream signaling properties. Moreover, P2X7 receptor function varies among human individuals because there are some polymorphisms that can result in loss- or gain-of-function (**Figure 2**). Single nucleotide polymorphisms (SNPs) are widespread in the human P2X7 receptor; some of them are non-synonymous, meaning that there is a change in the amino acid sequence, generating a point mutation. Some of those mutations are related to altered susceptibility to various diseases, shedding new light on the underlying disease mechanisms (Jiang et al., 2013). In this article, we review SNPs involved in Alzheimer's disease (AD) (rs208294, rs3751143), Parkinson's disease (PD) (rs3751143), multiple sclerosis (MS) (rs208294, rs28360457), depressive disorder (rs7958311, rs2230912), anxiety (rs208294, rs2230912), and bipolar disorder (BD) (rs208294, rs1718119, rs2230912, rs3751143) (**Figure 2**).

P2X7 Receptor Function

P2X7 receptor activation induces a number of well-established downstream signaling events in various human cell types. The opening of the channel leads to an increase in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_i$), responsible for a number of P2X7 receptor-induced responses, as AKT activation in astrocytes (Jacques-Silva et al., 2004). Phospholipase (PL) C and A2 (Andrei et al., 2004), src kinase, p38, acid sphingomyelinase (Bianco et al., 2009), caspase 1 (Keller et al., 2008), and gasdermin (Evavold et al., 2018) are involved in P2X7 receptor intracellular signaling.

P2X7 receptor activity mediates cell proliferation and death, rapid and reversible phosphatidylserine exposure, membrane blebbing, release of microparticles and exosomes and multinucleated cell formation, as well as the formation of reactive oxygen and nitrogen species (Sluyter and Stokes, 2011).

P2X7 Receptor in Neuroinflammation

The P2X7 receptor is highly expressed in microglial cells (Lord et al., 2015; He et al., 2017). In healthy tissues, the concentration of extracellular ATP is low at the nanomolar range (Falzoni et al., 2013). Conversely, under stress and cellular damage, the ATP concentration increases considerably, resulting in P2X7 receptor activation. Therefore, it is hypothesized that P2X7 receptor acts as a silent receptor once its activation only occurs in pathological states when there is a rise of extracellular ATP concentrations (Bhattacharya and Biber, 2016).

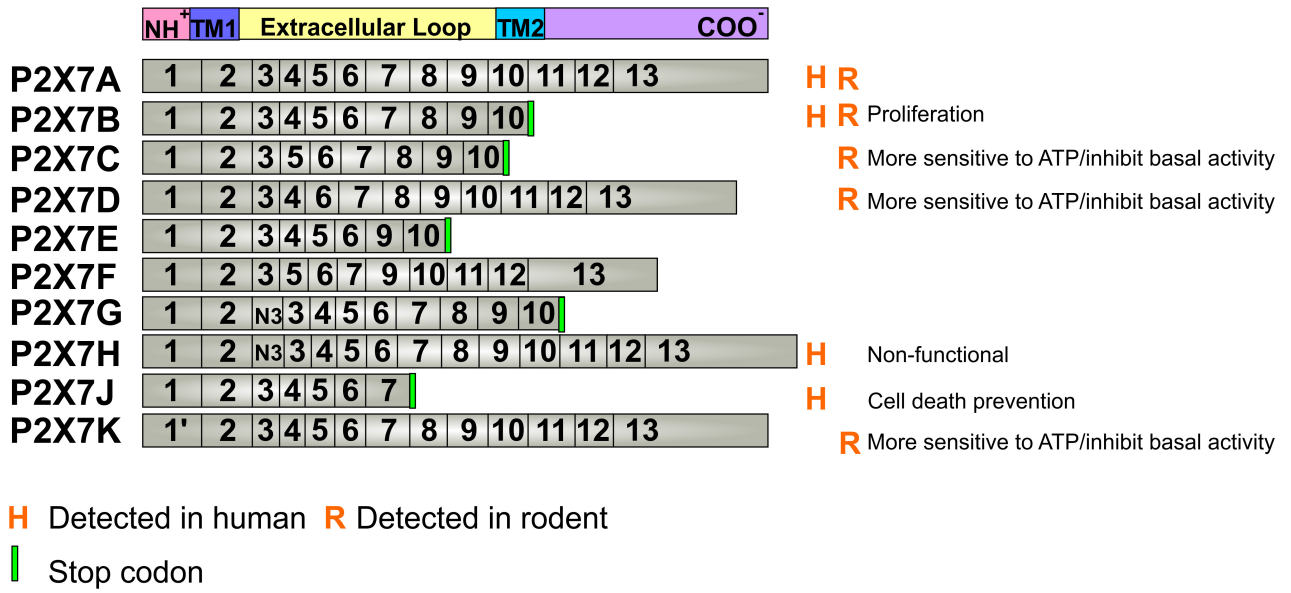


FIGURE 1 | P2X7 receptor splicing variants. The P2X7 receptor has 10 different isoforms derived from alternative splicing and mutations of the 13 exons of the gene. The P2X7A isoform is the native form, expressed in every mammal species. The detected alterative isoforms in humans are P2X7B, H and J, while in rodents, these are P2X7B, C, D and K. The mutations that lead to a stop codon insertion, originate a shortened P2X7 receptor at the carboxy-terminal domain and cannot form pores that induce cell death. P2X7G and H present a copy of exon 3 (N3) near the amino-terminal. Known basic functions for each isoform are described at the right site of the panel. Aminoterminal (NH^+), Carboxyterminal (COO^-), Transmembrane passage 1 (TM1), Transmembrane passage 2 (TM2).

In high concentrations, extracellular ATP can act as a damage-associated molecular patterns (DAMPs) and activate P2X7 receptor (Falzoni et al., 2013). DAMP signal activates the transcription factor NF-κB in the nucleus, which consequently promote the upregulation of proinflammatory cytokines, pro-IL-1β and pro-IL-18, and NLRP3 protein (Jo et al., 2016). Although the precise mechanism is not completely understood, P2X7 receptor it is one of the most potent activators of the NRLP3-associated inflammasome (He et al., 2017). P2X7 receptor activation induces K^+ efflux, which is needed for efficient NLRP3 inflammasome activation (Gustin et al., 2015). NLRP3 inflammasome trigger the activation of caspase-1, which causes the maturation of IL-1β and IL-18 and, consequently, increasing proinflammatory cytokine release (Bernier, 2012; Jo et al., 2016; He et al., 2017; Bhattacharya et al., 2018). This signaling appears to be in functional in microglia and not astrocytes (Gustin et al., 2015). Additionally, the P2X7 receptor may also stimulate the release of TNF, IL-6, CCL2, CCL3, and CXCL2 (Suzuki et al., 2004; Kataoka et al., 2009; Shiratori et al., 2010; Shieh et al., 2014).

It is clear that the P2X7 receptor can modulate the neuroinflammation induced by LPS, once P2X7 receptor blockade may reduce inflammatory mediators release (Bianco et al., 2006; Choi et al., 2007; Monif et al., 2009; He et al., 2017; Yang et al., 2018). Some works showed that LPS enhanced P2X7 receptor expression (Choi et al., 2007; Yang et al., 2018), whereas other studies reported downregulation of P2X7 receptor expression (Bianco et al., 2006; He et al., 2017). Similarly to the LPS-induced effects, P2X7 receptor overexpression was sufficient to trigger microglial activation in primary microglia derived from hippocampus (Monif et al., 2009). Interestingly, a recent study evidenced that the selective P2X7 receptor antagonist, JNJ-55308942, inhibited neuroinflammation development induced in different rodent models by LPS, BCG or chronic stress (Bhattacharya et al., 2018). Recently, efforts were made to detect *in vivo* neuroinflammation. Therefore, radioligands targeting P2X7 receptor were used as a tool to identify brain areas undergoing inflammatory processes. [^{18}F]-JNJ-64413739 and ^{11}C-GSK1482160 were promising in detecting areas of neuroinflammation upon LPS-stimulation of in rodents (Territo et al., 2017; Berdyyeva et al., 2019).

One of the possible pathways for ATP release is from dying cells. Interestingly, diseases that present degeneration of neural cells, as neurodegenerative diseases, psychiatric disorders, and brain tumors, as presented below, may present high local concentrations of extracellular ATP and stimulate pathophysiological P2X7 receptor activity. In view of that, here, we provide evidence that AD, PD, MS, depression, and brain tumors present increased P2X7 receptor expression. P2X7 receptor signal amplification in these diseases is proposed.

P2X7 RECEPTOR ROLES IN NEURODEGENERATIVE DISEASES

Purinergic receptors play a significant role in neurodegenerative diseases (Oliveira-Giacomelli et al., 2018). P2X7 receptors participate in neurodegenerative, neuroinflammatory and neurogenic processes, tightly related to disease development and repair.

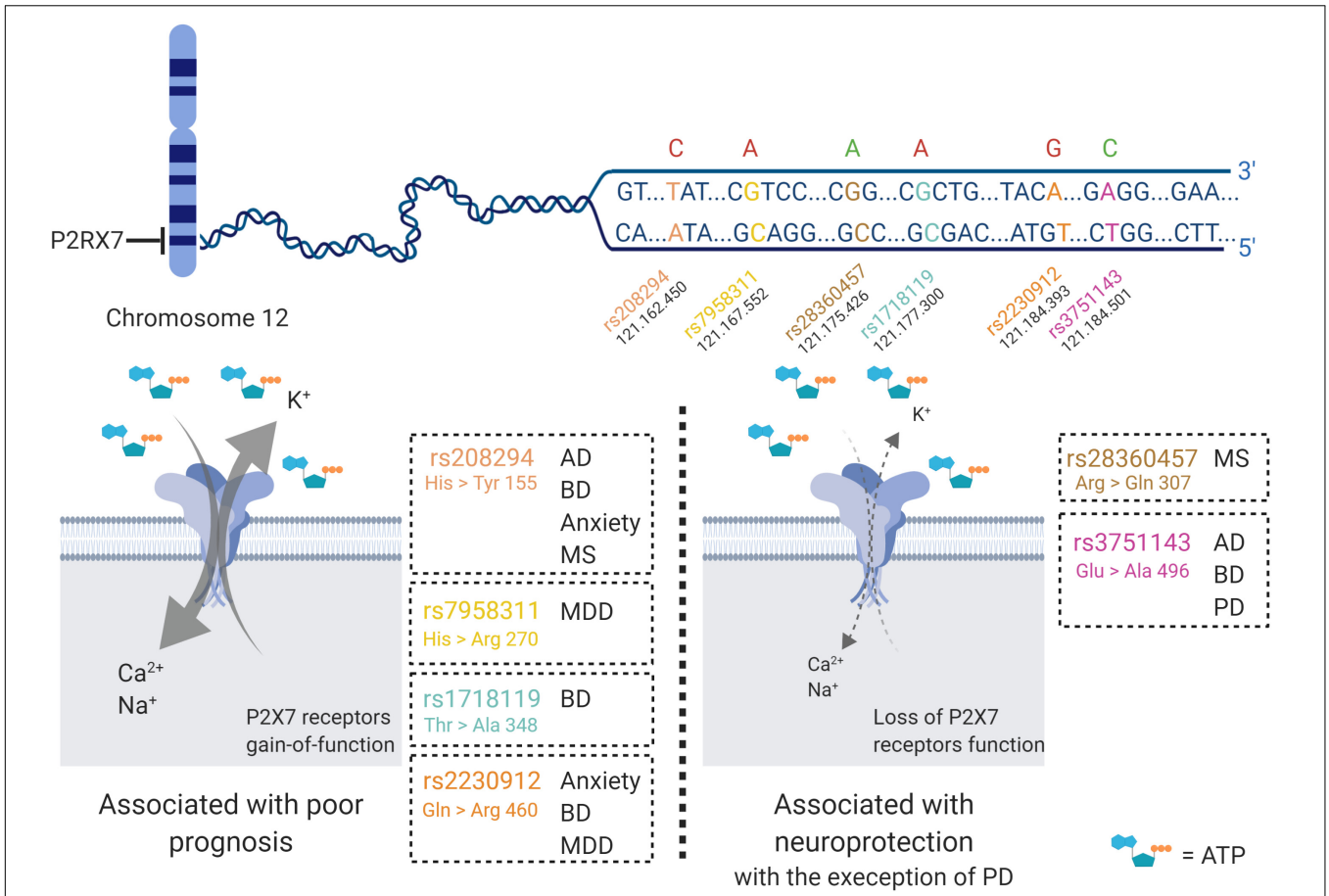


FIGURE 2 | P2X7 receptor single nucleotide polymorphisms (SNP) in brain diseases. Various P2X7 receptor SNPs have been detected and studied in humans. The gene that encodes the P2X7 receptor is located at chromosome 12, and at least seven of the SNPs are related to neurological disorders, such as Alzheimer's disease (AD), bipolar disorder (BD), anxiety, multiple sclerosis (MS), major depressive disorder (MDD) and Parkinson's disease (PD). The red letters represent SNPs that potentialize the response of the P2X7 receptor upon binding to its ligand and generate increased cell death and worsening of diseases. Further, green letters are in line with decreased Ca^{2+} inflow due to loss of function of the P2X7 receptor, and usually lead to neuroprotection. The SNPs are named rs208294, rs7958311, rs1718119, rs2230912, rs28360457, and rs3751143. Created with BioRender.com.

Alzheimer's Disease

Alzheimer's disease is the most common form of dementia in the elderly population (Ballard et al., 2011; Beinart et al., 2012), representing a serious public health problem. Recent estimative indicates that approximately 50 million people have AD worldwide, and this number is expected to reach 132 million by 2050 (Alzheimer's Association, 2015). Processes that trigger AD may start decades before the onset of initial symptoms of dementia (Goedert and Spillantini, 2006; De Felice, 2013), reinforcing the importance of sensitive diagnostic tools for more effective therapeutic interventions.

The main clinical symptom in AD is the cognitive decline, which begins with recent memory lapses, and proceeds with progressively intensified memory loss to total physical dependence. Familial AD (∼5% of all cases) is more severe and initiates earlier than the sporadic form, affecting people from 40 years of age on. Most patients are sporadic cases, presenting AD symptoms from 65 years of life on, and aging is indicated as the leading risk factor for the disease (Evans et al., 1989). The pathophysiologic generation of the neurotoxic β-amyloid oligomers (AβO) by sequential amyloid precursor protein (APP) proteolysis is involved in the development of AD. Familial AD has been directly related to mutations in the genes of APP and presenilin 1 and 2 (Levy-Lahad et al., 1995; Sherrington et al., 1995). The etiology of AD is an association between genetic and environmental factors (Selkoe, 2004; Roberson and Mucke, 2006) which turns disease treatment more difficult. Indeed, the drugs currently available to treat AD have only palliative effects and consist of acetylcholinesterase inhibition to optimize cholinergic activity (Knapp et al., 1994; Rogers and Friedhoff, 1996; Trinh et al., 2003), and the NMDA receptor antagonist memantine (Cosman et al., 2007; Lipton, 2007; Parsons et al., 2007; Xia et al., 2010). Therefore, the development of more effective drugs for AD treatment is needed.

There is evidence that inflammation plays a vital role in AD (Lucin and Wyss-Coray, 2009), as well as in the modulation of neurogenesis (Mishra et al., 2015). Interestingly, there is a significant influence of microglia in both processes (Nunan et al., 2014; De Lucia et al., 2016). AβO also activates

microglia, resulting in secretion of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and IL-1β (Ledo et al., 2013, 2016). Microglial activation may not only compromise their clearance ability (Heneka et al., 2010) but also, surprisingly, contribute to the propagation of AβO in the cerebral parenchyma (Joshi et al., 2014). Interestingly, the P2X7 receptor is involved in these features and in AD as discussed in the following.

Increased P2X7 receptor expression and activation have been involved in the progression of several neurodegenerative diseases, including AD (Savio et al., 2018). Accordingly, P2X7 receptor expression is increased in the brain of AD patients and appears to be concentrated in areas of higher density of amyloid plaques, co-localized with activated microglia (McLarnon et al., 2006). P2X7 receptors expression are also upregulated in the hippocampus of two animal models of AD, such as transgenic mice that express the human APP bearing the Swedish mutation (K670N/M671L) (Parvathenani et al., 2003) and rats injected with amyloid-β peptide (Aβ) 1-42 (1 nmol) into the hippocampus (McLarnon et al., 2006) (**Figure 3**).

Further, the involvement of two P2X7 receptor SNPs were investigated in AD patients and compared to age-matched non-demented elderly, the 1513A > C (rs3751143) and 489C > T (rs208294) (Sanz et al., 2014). This study showed that the presence of the 1513C allele and the absence of the 489C allele (i.e., the presence of both SNPs) decreased the probability of having AD by about four-fold versus the reference subgroup (Sanz et al., 2014). The 1513A > C substitution is associated to the loss of P2X7 receptor function and should confer an "anti-inflammatory" phenotype (Gu et al., 2001). On the other hand, the 489C > T SNP causes a gain of function of this receptor, which may potentiate P2X7 receptor-induced phagocytosis, and subsequent Aβ elimination (Cabrini et al., 2005; Sluyter and Stokes, 2011) (**Figure 2**). Therefore, such mutations may be neuroprotective against AD development (Sanz et al., 2014).

Several studies support the idea that the prolonged activation of P2X7 receptor may result in increased secretion of pro-inflammatory cytokines (such as IL1-β and IL-18) and reduced phagocytic capacity, leading to neuronal damage (Skaper et al., 2006; Sanz et al., 2009; Lee et al., 2011). In accordance with this proposal, injection of fibrillar amyloid-β peptide (fAβ1-42) into the dentate gyrus of the hippocampus enhanced microglial reactivity, astrogliosis and leakiness of the blood-brain barrier (Ryu and McLarnon, 2008a,b). Interestingly, a pronounced increase of P2X7 receptor immunoreactivity was detected in astrocytes and microglia, but not in neurons (McLarnon et al., 2006; Ryu and McLarnon, 2008a). Aβ1-42 treatment also induced ATP release, $[Ca^{2+}]_i$ enhancement and IL-1β secretion in primary microglial cell cultures prepared from wild-type, but not from P2X7 receptor KO mice (Sanz et al., 2009). Intra-hippocampal injection of Aβ1-42 caused a large accumulation of IL-1β in wildtype, but not in P2X7 receptor KO mice (Sanz et al., 2009). Treatment with Bz-ATP increased IL-1β secretion from human microglia cells pre-activated with Aβ1–42, which was completely reversed following pre-incubation with oxidized ATP, P2X7 receptor antagonist (Rampe et al., 2004). This response may be mediated by P2X7 receptors, since the treatment with the selective P2X7 receptor antagonist A-740003 blocked the release of IL-1β induced by ATP treatment of microglial cells from rat cortex incubated with serum amyloid A (**Figure 3**).

Further evidence indicated that P2X7 receptor activation may also induce neuronal damage in AD through the production of reactive oxygen species (ROS). In microglial cultures, Aβ1-42 induced ATP release leading to the production of ROS via P2X7 receptor activation (Soo et al., 2007). A catalytic subunit of NADPH oxidase, which catalyzes the production of ROS, was detected in P2X7 receptor-positive microglial cells in the cerebral cortex of 6-months-old APP/PS1 mice, a double transgenic mice commonly used to study familial AD (Lee et al., 2011). Moreover, postsynaptic density 95-positive dendrites showed significant damage in P2X7 receptor-positive regions in the cerebral cortex of these animals (Lee et al., 2011). Up-regulation of P2X7 receptor expression and ROS production in microglia cells were temporally correlated with Aβ increase and synaptotoxicity in this animal model, since it occurs around the age of 6 months (Lee et al., 2011).

Studies demonstrated that P2X7 receptor activation interferes with processing of APP. APP is proteolytically processed by β- and γ-secretases to release Aβ, the main component of senile plaques found in the brains of AD patients (Zhang et al., 2011). Alternatively, APP can be cleaved by α-secretase, leading to the formation of the nonpathogenic amyloid-α peptide (Aα) (Zhang et al., 2011). In two different cellular lines (HEK293T and neuroblastoma N2a), inhibition of either constitutive expression or overexpression of the P2X7 receptor increased α-secretase activity through inhibition of glycogen synthase kinase 3 (GSK-3) (León-Otegui et al., 2011; Diaz-Hernandez et al., 2012; Miras-Portugal et al., 2015). In addition, systemic administration of P2X7 receptor antagonists in APP_{SweInd} mice, a transgenic animal that expresses the human APP bearing both the Swedish (K670N/M671L) and the Indiana (V717F) mutations, decreased the number of hippocampal amyloid plaques (Diaz-Hernandez et al., 2012; Miras-Portugal et al., 2015). This reduction is correlated with a decrease in GSK-3 activity and consequent increase of α-secretase activity, leading to non-amyloidogenic APP processing (Diaz-Hernandez et al., 2012; Miras-Portugal et al., 2015).

However, results from Delarasse et al. (2011) showed the opposite effect: P2X7 receptor stimulation may enhance α-secretase activity. In this work, four different cell lines (mouse and human neuroblastoma cells, primary murine astrocytes and neural progenitor cells) incubated with ATP or Bz-ATP had activated enzymatic cascades that triggered α-secretase activity, leading to increased levels of Aα, while Aβ was undetectable (Delarasse et al., 2011). Moreover, this study provides evidence to support the idea that ATP- or Bz-ATP-mediated Aα release is mediated by P2X7 receptor activation: (1) three pharmacological inhibitors of P2X7 receptor blocked the release of Aα mediated by Bz-ATP; (2) inhibition of P2X7 receptor synthesis by RNA interference reduced Aα production; and (3) stimulation by Bz-ATP of mouse primary astrocytes and neural progenitor cells from P2X7 receptor-deficient mice did not induce Aα release, while it did in cells derived from wild type animals (Delarasse et al., 2011). Despite such interesting data, it

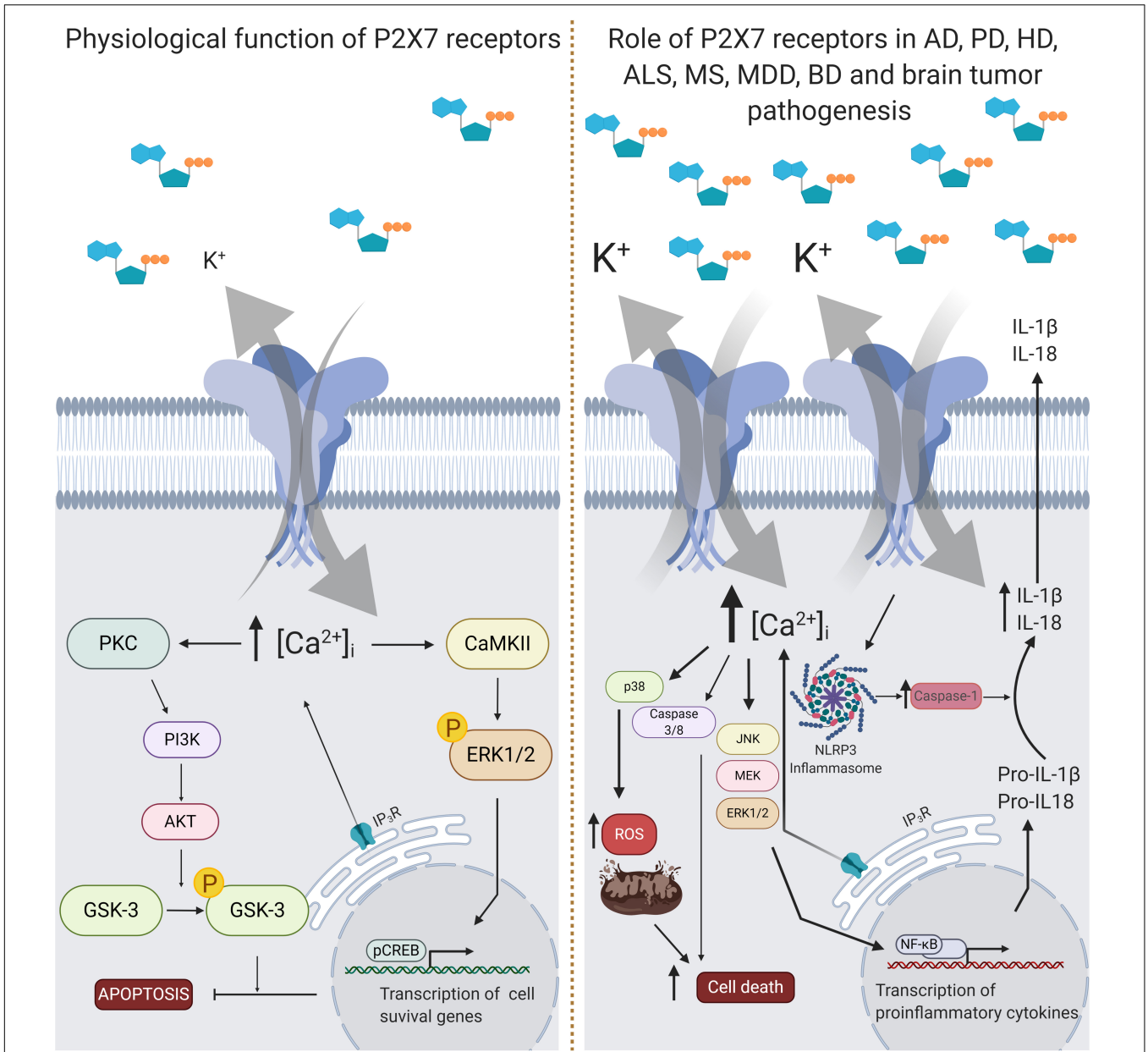


FIGURE 3 | Intracellular signaling pathways triggered by P2X7 receptor activity. The P2X7 receptor is assembled as a homotrimeric protein, and upon ATP binding, receptor subunits change their conformational state and open a pore for the entrance of cations, mainly Ca^{2+}. In physiological conditions (left panel), the increase of intracellular Ca^{2+} concentration levels ($[Ca^{2+}]_i$) leads to the activation of some kinases, like protein kinase C (PKC) and calcium-calmodulin kinase II (CaMKII), which phosphorylates and activates phosphoinositide 3-kinase (PI3K), extracellular signal-regulated kinases 1/2 (ERK1/2), protein kinase B (AKT) and glycogen synthase kinase 3 (GSK3). This signal transduction results in inhibition of apoptosis or increase of the transcription of cell survival related genes. In pathological conditions (right panel), such as in Alzheimer's disease (AD), multiple sclerosis (MS), major depressive disorder (MDD) and Parkinson's disease (PD), P2X7 receptor expression rates are increased. Activation of the P2X7 receptor in AD animal model results in increased release of interleukin 1β (IL-1β) and reactive oxygen species (ROS), and augmented inhibition of GSK3. IL-1β release depends on the formation of the NLRP3 inflammasome together with the activation of the nuclear factor kappa-light-chain-enhancer activated B cells (NF-κB). In ALS, P2X7 receptor activation also induces overproduction of ROS and ERK1/2 signaling. Administration of P2X7 receptor antagonists has been suggested to benefit specific features of AD, PD, MS, MDD, and BD, like improvement of behavior and neuroinflammation. Nevertheless, high concentrations of P2X7 receptor agonists may also enhance *in vitro* cytotoxic effects of temozolomide, a drug of choice for glioblastoma treatment. Created with BioRender.com.

is relevant to emphasize that APP processing depends on the abundance of this protein at the specific cellular model and, in this case, the equilibrium between the different proteolytic pathways could be unbalanced, which could explain the contrast with the results obtained by other authors (León-Otegui et al., 2011; Diaz-Hernandez et al., 2012; Miras-Portugal et al., 2015).

Therefore, the roles of P2X7 receptors in α-secretase activity and APP processing are controversial and should be further investigated. In addition to the aforementioned effects mediated by P2X7 receptors, these receptors have also been involved in memory and cognition impairment, key symptoms of AD frequently attributed to Aβ deposits and neurofibrillary tangles, which spread from the trans-entorhinal and hippocampal regions to the primary areas of the neocortex (Raskin et al., 2015). In accordance with the detrimental role of P2X7 receptor activation in AD, systemic administration of a P2X7 receptor antagonist, BBG, diminished spatial memory impairment and cognitive deficits along with reduced loss of filopodia and spine density induced by the injection of soluble Aβ1-42 into the hippocampal CA1 region of mice, an animal model of AD (Chen et al., 2014). BBG also inhibits, at a lesser extent, P2X4 receptors, which could be responsible for the observed neuroprotective effects. Knockdown of the P2X4 receptor attenuated Aβ1-42-induced neuronal death in neurons primary culture, whereas induction of P2X4 receptor expression in a neuronal cell line that does not express P2 receptors enhanced the toxic effect of Aβ1-42 (Varma et al., 2009).

However, other authors observed that P2X7 receptor inhibition may induce memory deficits. For instance, P2X7 receptor KO mice displayed spatial memory impairment in the Y-maze test, despite their performances in the object recognition task remained unaltered (Labrousse et al., 2009). Additionally, P2X7 receptor KO mice or wild type animals treated with A-438079 presented increased contextual fear recall and impaired acquisition of extinction in mice (Domingos et al., 2018). The treatment with A-740003 elicited dose-dependent impairments in memory acquisition, consolidation and retrieval in rats, whereas P2X7 receptor deletion hampered the aversive memory processes of mice exposed to the contextual fear-conditioning task (Campos et al., 2014). The obtained results indicate that P2X7 receptor inhibition induces memory impairment associated to anxiogenic-like responses. At this point, it is important to highlight that such studies were not conducted in an animal model of AD, but in tests used to evaluate memory and anxiety-related behaviors. The opposite effect observed in an animal model of AD is understandable since experimental conditions were different.

Altogether, literature data indicates that P2X7 receptor inhibition: (1) ameliorates neuronal damage induced by both neuroimmune response activation and ROS production; (2) modulates α-secretase activity and non-amyloidogenic APP processing, in a non-elucidated manner; and (3) attenuated spatial memory impairment and cognitive deficits in an animal model of AD. These results support that P2X7 receptor antagonism may be a possible strategy for AD treatment.

Parkinson's Disease

Parkinson's disease is a neurodegenerative disease that affects more than 1% of the world's elderly population (between 60 and 80 years old) (de Lau and Breteler, 2006). Despite its high incidence, PD etiology is still poorly understood. Dopaminergic neurons of the nigrostriatal pathway undergo neurodegeneration, accompanied by neuroinflammation and oxidative stress. The appearance of protein aggregates formed by α-synuclein aggravating the disease state is also one of the hallmarks of the disease, although it is not the main cause of dopaminergic neuron death (Hornykiewicz, 1966; Hughes et al., 1992; de Lau and Breteler, 2006).

Patients with PD have characteristic symptoms, such as shaking palsy, resting tremor and bradykinesia, as well as non-motor symptoms, including cognitive impairment and mood and sleep disorders (Thenganatt and Jankovic, 2014). Current treatments consisting of remission of symptoms trigger several adverse effects that compromise the quality of life of the individual. There is no known cure for the disease, highlighting the importance of elucidating the mechanisms involved in the disease and possible therapeutic targets (Hornykiewicz, 2002).

In humans, genetic predisposition to PD development was identified in patients carrying P2X7 receptor polymorphisms. In a Han Chinese population, the P2X7 receptor polymorphism rs3751143 (Glu496Ala) was identified as a risk factor for PD (Liu et al., 2013) (**Figure 2**).

Animal models of PD show that the P2X7 receptor is involved in disease development, especially in microglial cell activation. In an animal model of nigrostriatal injury induction by 6-OH dopamine (6-OHDA), a toxic dopamine analog, striatal gene expression of the P2X7 receptor gradually increased over 5 weeks after injury (Oliveira-Giacomelli et al., 2019). Neuroprotective effects of P2X7 receptor antagonism were observed after pretreating animals with A-438079. This treatment prevented the decrease in striatal dopamine stocks triggered by 6-OHDA injection. However, this effect was not accompanied by a reduction of dopaminergic neuron death, indicating that P2X7 receptor inhibition acts on axonal dopamine stores (Marcellino et al., 2010) (**Figure 3**).

Similar results were obtained with BBG treatments. When administered prior to induction of the 6-OHDA injury, intracerebroventricular injection of BBG also protected against decreasing striatal dopamine levels and reduced oxidative stress, mitochondrial dysfunction and apoptosis (Kumar et al., 2017). Treatment with BBG (45 mg/kg) in rats prevented the reduction of striatal and nigral dopamine levels, decreased astrogliosis, striatal microgliosis, and the number of apomorphine-induced rotations (Carmo et al., 2014). Controversially, Hracskó et al., 2011 showed that P2X7 receptor KO animals are equally susceptible to dopaminergic neuron death induction by MPTP (Hracskó et al., 2011). In this study, the Pfizer KO mouse strain was used, known to express P2X7 13C and 13B receptors in the brain (Bartlett et al., 2014).

Additionally, it is suggested that P2X7 receptor inhibition may also promote neuroregeneration of dopaminergic neurons when given 1 week after 6-OHDA-induced injury (Ferrazoli et al., 2017; Oliveira-Giacomelli et al., 2019). Administration of BBG (50 mg/kg) in rats during 7 days, starting 1 week after injury, augmented the number of substantia nigra dopaminergic neurons (Ferrazoli et al., 2017). Likewise, BBG (75 mg/kg) treatment also regenerated striatal dopaminergic fibers. This effect was accompanied by decreased microglial activation in the substantia nigra (Oliveira-Giacomelli et al., 2019) (**Figure 3**).

Treatment of neuronal-differentiated SH-SY5Y cells, an *in vitro* model of dopaminergic neurons, with BBG protected cells from 6-OHDA-induced synaptotoxicity and death (Carmo et al., 2014; Oliveira-Giacomelli et al., 2019). In addition, assays with wild-type and α-synuclein mutants of microglial cells showed that α-synuclein activated microglial P2X7 receptors, inducing NADPH oxidase, modulating the PI3K/AKT signaling pathway and increasing oxidative stress (Jiang et al., 2015). Subsequently, it has been reported that this α-synuclein-promoted effect on microglial cells *in vitro* also involves the stimulation of glutamatergic excitotoxicity (Dos-Santos-Pereira et al., 2018).

Overall, P2X7 receptor inhibition presents neuroprotective and neuroregenerative effects in cellular and animal models of PD. This effect involved anti-inflammatory actions and modulation of the microglial activation state and cytokine release. However, most of these studies used BBG as a tool to assess P2X7 receptor antagonism. Therefore, we cannot discard that P2X4 receptors could be partially responsible for neuroprotective and/or neuroregenerative effects in PD's models (Ase et al., 2015). P2X4 receptor inhibition did not prevent 6-OHDA-induced cell death in SH-SY5Y cell culture (Oliveira-Giacomelli et al., 2019). This result indicates that P2X4 receptor antagonism is not the main mechanism of neuroprotective effect of BBG treatment. On the other hand, there is no reported study of P2X4 receptor antagonism inducing neuroregenerative effects. Thus, P2X4 receptor antagonism could be partially responsible for the regeneration of dopaminergic neurons in the animal model of PD induced by 6-OHDA. In conclusion, P2X7 receptor is an interesting research topic and possible target for PD.

Huntington's Disease

Huntington's disease (HD) is a dominant hereditary disease caused by a mutation in IT15 gene that encodes huntingtin protein (Htt). Abnormal elongation of the (CAG)n repeats localized in 5′ coding sequence results in massive neurodegeneration of the basal ganglia and cortex of patients over the age of 30 (Vonsattel and DiFiglia, 1998; Ross and Tabrizi, 2011; Ross et al., 2014). The role of P2X7 receptor in HD has been still poorly investigated. At the moment, the only study is published by Diaz and collaborators, who by using two distinct mouse models for HD, Tet/HD94 and R6/1 demonstrated that P2X7 receptor expression is increased in HD, and that the receptor channel possesses augmented Ca^{2+} permeability (Díaz-Hernández et al., 2009) (**Figure 3**). The inhibition of the receptor with BBG mitigated motor coordination deficits, cachexia and decreases neuronal loss.

Moreover, *in vitro* analysis revealed that neurons expressing mutant Htt were more sensitive to apoptosis under P2X7 receptor stimulation (Díaz-Hernández et al., 2009). Thus, P2X7 receptors expressed in microglia can promote excitotoxicity in neural cells by inducing glutamate release (Matute, 2012).

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is one of the most prevalent neuromotor diseases in adulthood. The disease is characterized by the death of motoneurons in the motor cortex, brainstem and spinal cord, resulting in muscle impairment and paralysis (Hardiman et al., 2017). Among the mechanisms involved in neuronal death, neuroinflammation is one of the most established factors. ALS patients present alterations in levels of a range of pro-inflammatory cytokines in the cerebrospinal fluid (Mitchell et al., 2009; Moreno-Martinez et al., 2019), as well as increased rates of reactive cerebral microglial cells (Turner et al., 2004). Depending on the stage of the disease, reactive microglia with protective or cytotoxic properties is found, demonstrating the complexity of neuroinflammation in this disorder (Evans et al., 2013). In this sense, studies relating the P2X7 receptor with ALS show a delicate regulation depending on different factors.

Several studies have been conducted with superoxide dismutase 1 transgenic mice harboring the G92A mutation [SOD1 (G93A)], a well-known ALS model. In this model, onset, progression, and animal survival depend on the mouse gender. Cervetto et al. (2013) showed that inhibition of the P2X7 receptor by BBG at a dose of 45 mg/kg slowed down disease progression in males, but not in females (Cervetto et al., 2013).

In addition, Apolloni et al. (2013a) demonstrated that female SOD1 (G93A) mice with the KO of the P2X7 receptor gene showed increased survival but anticipated the onset of the disease and intensified its progression in males and females. Further, increased astrogliosis and microgliosis and augmented motoneuron death were observed, accompanied by increased pro-inflammatory cytokine production (Apolloni et al., 2013a). Authors used Pfizer KO mice, known to express P2X7 13B and 13C receptors in the brain, which present lower membrane migration and channel function when compared to P2X7A receptors (Masin et al., 2012).

The beneficial effects of P2X7 receptor blockade in ALS supposedly did not depend only on the studied gender, but also on the stage of the disease. In the ALS pre-onset phase in SOD1(G93A) mice, Bartlett et al. (2017) used BBG at a dose of 45 mg/kg, three times a week. They reported that this treatment increased female survival without ameliorating motor performance (Bartlett et al., 2017).

Corroborating these results, treatment of late-pre-onset SOD1 (G93A) mice with BBG at 50 mg/kg, three times a week, delayed disease onset and improved motor performance (Apolloni et al., 2014). In addition, this treatment increased motoneurons survival and decreased microgliosis and expression of pro-inflammatory markers. However, when treated in the onset phase, no neuroprotective effect was observed by P2X7 receptor antagonism. On the other hand, P2X7 receptor activation exerted a protective effect on skeletal muscles of SOD1 (G93A) mice (Fabbrizio et al., 2019). Pre-late-onset treatment with Bz-ATP at a dose of 1 mg/kg for 7 days (i.p.) prevented denervation atrophy of the skeletal muscle. The neuroprotective effect of Bz-ATP could be attributed to another purinergic receptor since this compound is not a selective agonist of P2X7 receptors. Despite that, the P2X7 receptor is known to control proliferation, differentiation, and regeneration in healthy skeletal muscle (**Figure 3**).

In vitro, the co-culture of astrocytes and motoneurons from SOD1 (G93A) mice showed P2X7 receptor involvement in astrocyte activity. The addition of Bz-ATP and ATP induced motoneuron death by astrocytic neurotoxicity. When BBG or apyrase (that increases ATP metabolism and decreases

its concentration) was used, inhibition of neuron death was observed (Gandelman et al., 2010). Although BBG treatment also inhibits P2X4 receptors, activation of these receptors appears to protect motor neurons *in vitro* (Cieślak et al., 2019), indicating that the P2X7 receptor subtype is more likely to be activated in the detrimental effect of Bz-ATP. Subsequently, BBG treatment of motoneurons isolated from rat embryonic spinal cord prevented Bz-ATP-induced cell death. In addition, although low concentrations of ATP induced neuronal death, high concentrations of ATP in the cellular media exerted a protective effect, possibly due to its hydrolysis in ADP and the adenosine-induced activation of P1 receptors. ATP and Bz-ATP induced apoptosis by peroxynitrite production, p38 activation and stimulation of the FAS autocrine signaling pathway (Gandelman et al., 2013).

In vitro studies also corroborate microglial participation in disease development in SOD1 (G93A) mice. Using isolated microglia from these animals, Apolloni et al. (2013b) demonstrated that Bz-ATP increased ROS production and activation of the ERK1/2 signaling pathway (**Figure 3**). The pro-inflammatory effects were alleviated following BBG application. Similar results were obtained in SOD1 (G93A) P2X7 receptor KO microglial cells, strengthening the concept of anti-inflammatory effects promoted by P2X7 receptor antagonism (Apolloni et al., 2013a). Besides inducing pro-inflammatory effects, activation of P2X7 receptors in microglia cells isolated from SOD1 (G93A) mice supposedly also modulate autophagy processes. Bz-ATP increased expression of autophagy markers by inhibiting mTOR phosphorylation. This effect was attenuated by treatment with the P2X7 receptor antagonist A-804598 (Fabbrizio et al., 2017).

Finally, peripheral blood mononuclear cells of patients with ALS showed decreased P2X7 receptor expression. Repeated application of ATP to these cells resulted in diminished intracellular calcium transients compared to controls, demonstrating that decreased P2X7 receptor expression induced dysregulation of intracellular calcium homeostasis (Liu et al., 2016).

In conclusion, P2X7 receptor inhibition supposedly promotes dual effects along the course of ALS. Its effects seem to depend on the time window in which the inhibition started. P2X7 receptor ablation before ALS development in mice seems to be detrimental (Apolloni et al., 2013a). In the asymptomatic phase, P2X7 receptor inhibition did not alter disease onset and survival, although it decreases M1 microglial marker expression (Apolloni et al., 2014). In the pre-onset phase, treatment with BBG increased mice's survival but did not alleviate motor symptoms (Bartlett et al., 2017). When administered at the late pre-onset phase, BBG reduced M1 microglial phenotype and increased anti-inflammatory M2 phenotype along with delayed disease onset and decreased motor symptoms (Apolloni et al., 2014). BBG is known to also inhibit P2X4 receptors to a lesser extent, but the role of P2X4 receptors in the ALS development depends on the cell type. While P2X4 receptor inhibition in microglia cells induces the phenotypic change to M1 microglial cells and promotes inflammation, P2X4 receptor activation appears to protect motor neurons against kainate-induced excitotoxicity *in vitro* (Di Virgilio and Sarti, 2018; Cieślak et al., 2019). Since BBG treatment induced a decrease in microglial M1 markers, it is more likely that the neuroprotective effects of BBG treatment involves P2X7 receptor inhibition rather than P2X4 receptor inhibition in ALS.

Multiple Sclerosis

Multiple sclerosis is an autoimmune disease with unknown etiology. It is characterized by chronic inflammation with astrogliosis and microgliosis, death of oligodendrocytes, axonal demyelination and subsequent neuronal transmission impairment. Available drugs alleviate symptoms; however, there is no known cure for this disease (Goldenberg, 2012). Sustained activation of the P2X7 receptor is known to induce oligodendrocyte death and demyelination and neuroinflammatory processes and neurodegeneration, which are characteristic for MS. Thus, studies unraveling functions of this receptor in MS development were conducted.

An animal model of autoimmune encephalomyelitis (EAE) is the gold-standard tool for *in vivo* studies, presenting similar features of MS (Lassmann, 1983). In EAE animals, injection of 10 mM BBG into the optic nerve reduced ATP and Bz-ATP-induced demyelination, suggesting that P2X7 receptor activation induced oligodendrocyte excitotoxicity (Matute et al., 2007). BBG also inhibit P2X4 receptors, but their activation in microglia cells is proposed to trigger remyelination process in EAE mice (Di Virgilio and Sarti, 2018), indicating that P2X7 receptor antagonism could be the responsible for BBG treatment protective effects. P2X7 receptor expression during EAE development in rodents has been demonstrated. In the asymptomatic phase of the disease, overexpression of the receptor in astrocytes was observed. At the peak of the characteristic symptoms of the disease, receptor overexpression occurred not only in astrocytes but also in neuronal terminals (Grygorowicz et al., 2010). Following recovery from the disease, the animals showed P2X7 receptor overexpression in glial cells, whose GFAP labeling was increased in the symptomatic phase without reduction after recovery (Grygorowicz et al., 2011) (**Figure 3**). These results were later confirmed, in which reactive astrocytes in the early phase of the disease expressed P2X7 receptors. Treatment with BBG (50 mg/kg) for 6 days alleviated the appearance of the characteristic symptoms of the EAE rat model, accompanied by reduction in reactive astrocyte labeling (Grygorowicz et al., 2016). Microglial cell analysis also yielded interesting results. In the asymptomatic phase of EAE, microglial cells showed P2X7 expression in active and resting phenotypes, and treatment with 50 mg/kg BBG for 6 days reduced microglial activation and pro-inflammatory cytokine release (Grygorowicz and Strużyńska, 2019).

In the Pfizer P2X7 receptor KO animals, induction of the EAE model resulted in a more severe pathological scenario of the disease. Moreover the authors of this study (Chen and Brosnan, 2006) injected bone marrow cells from P2X7 receptor KO mice into wild-type animals and detected a greater susceptibility to the disease. *In vitro* co-culture of P2X7 receptor KO macrophages and lymphocytes revealed increased lymphocyte proliferation together with decreased apoptotic activity. These results suggest that enhanced disease susceptibility of P2X7 receptor KO

animals may be due to decreased lymphocyte apoptosis rates (Chen and Brosnan, 2006). Controversially, Sharp and colleagues showed that GlaxoSmithKline P2X7 receptor KO mice presented four times less development of the EAE model, with reduced astrocyte activation and axonal damage. On the other hand, they detected an increase in pro-inflammatory cytokine production in splenic T-cells (Sharp et al., 2008), explained by expression of P2X7K receptors in these cells (Bartlett et al., 2014). Although controversial, these results ensure that P2X7 receptors play an important role in the development of the EAE model, both peripherally and in the central nervous system.

Activation of P2X7 receptors is known to induce opening of pannexin-1 associated membrane pores, with increased release of ATP. In this sense, pannexin-1 KO mice showed a decrease in EAE onset rates, accompanied by diminished mortality. In addition, ATP release in the spinal cord was diminished, accompanied by an increase in P2X7 receptor expression. In the long term, these animals developed symptoms as severely as wild-type animals did when submitted to the EAE model. The authors of the work (Lutz et al., 2013) suggested that increased P2X7 receptor expression is a mechanism to counteract the decrease in ATP release due to the absence of pannexin-1, and that this mechanism may be the reason for the similar development of symptoms. When treated with the pannexin-1 inhibitor mefloquine wild type EAE animals showed less severity in EAE development (Lutz et al., 2013).

The P2X7 receptor is associated with reactive microglia, as shown for microglial cells extracted during the autopsy of individuals with MS (Beaino et al., 2017). In addition, P2X7 receptor activation may play a role in the upregulation of IL-1β through nitric oxide synthase expression (Narcisse et al., 2005). P2X7 receptor expression was detected in reactive astrocytes in postmortem brains, showing expression upregulation in the parenchyma of the frontal cortex and in microglial cells from spinal cord and white brain matter (Narcisse et al., 2005; Yiangou et al., 2006; Amadio et al., 2017). P2X7 receptor expression was reduced in peripheral blood mononuclear cells (PBMCs) during acute disease phase, possibly due to autocrine and paracrine mechanisms resulting from inflammatory processes. The obtained results indicate that P2X7 receptor expression downregulation in monocytes and upregulation of expression in astrocytes participate at the inflammatory process of MS (Amadio et al., 2017). In contrast, PBMCs from MS patients had no difference in P2X7 receptor expression when compared to healthy individuals (Caragnano et al., 2012). However, when treated with glatiramer acetate, a compound used for MS treatment, P2X7 receptor and CD39 expression rates were reduced in PBMCs. These data were corroborated by *in vitro* studies of PBMCs, which when treated with glatiramer acetate showed a decrease in P2X7 receptor expression and a tendency to reduced IL-1β and increased CD39 expression (Caragnano et al., 2012).

Besides rare mutations in the P2X7 receptor gene found in familial MS (Sadovnick et al., 2017; Zrzavy et al., 2019), patients with mutations of Arg307Gln (rs28360457), which cause a substantial loss in membrane pore formation, are up to twice less frequent in MS patients, indicating a protective effect of this mutation (Gu et al., 2015). The opposite occurs when the mutation involves a P2X7 receptor gain-of-function that increases receptor channel permeability for Ca^{2+} such as the Ala76Val polymorphism, which is more common in MS patients (Oyanguren-Desez et al., 2011) (**Figure 2**).

Altogether, *in vivo* and *in vitro* evidence in animal models and patient samples indicates that the P2X7 receptor is closely related to MS pathology. Its expression is increased in microglia and reactive astrocytes resulting from inflammatory processes, and interventions that downregulate expression or activity of this receptor have neuroprotective effects. Moreover, although several studies used BBG as antagonist for P2X7 receptors, and this compound also inhibits P2X4 receptors, activation of the latter is known to induce microglial changes towards the M2 phenotype exerting remyelination effects in EAE mice (Di Virgilio and Sarti, 2018). Additionally, outcomes of P2X7 receptor ablation before EAE development are not clear, since different P2X7 receptor KO mice present different outcomes.

P2X7 RECEPTOR ROLES IN PSYCHIATRIC DISORDERS

As reviewed by Cheffer et al. (2018), a range of purinergic receptors are involved in psychiatric disorders. As discussed below, the P2X7 receptor also seems to influence development, vulnerability and severity of these disorders.

Depressive Disorders

Major depressive disorder (MDD) is estimated to affect about 322 million people worldwide, which represents 4.4% of the global population (World Health Organization, 2017). Prevalence rates vary by sex (5.1% of females and 3.6% of males) and by age (peaking in the older adulthood, between 55 and 74 years old) (World Health Organization, 2017). As described by several studies, MDD has a high social and economic impact (Wang et al., 2003; Greenberg et al., 2015), which could be attenuated by more appropriated treatments (Chisholm et al., 2016). However, about 65% of patients with MDD fail to achieve remission and about 33% do not respond to the treatment initially prescribed (Schatzberg, 1999; Trivedi et al., 2008). A possible explanation for the ineffectiveness of antidepressants in some patients is that most of them acts through facilitation of monoaminergic neurotransmission and studies from the last decade show that depression etiology involves more than this system (Kendler et al., 2006; Dean and Keshavan, 2017).

Depressive disorders result from a combination of environmental influence, personality traits, genetic and epigenetic factors leading to neuroendocrine dysfunction (hypothalamic–pituitary–adrenal axis imbalance), neurochemical alterations (impaired monoaminergic neurotransmission, increased glutamate levels and enhanced neuroimmune response) and decreased neuroplasticity (Kendler et al., 2006; Dean and Keshavan, 2017). As recently reviewed by Ribeiro and co-workers the P2X7 receptor is a core regulator of such neurochemical and neuroplastic mechanisms (Ribeiro et al., 2019a). Based on that, it is not surprising that several

studies indicate P2X7 receptor involvement in mood disorders as discussed in the following.

A pioneering work showed an association between the presence of the SNP rs2230912 in the gene coding for P2X7 receptor with MDD development (Lucae et al., 2006). Accordingly, the SNP rs2230912 was also associated with mood disorders, longer depressive episodes (Soronen et al., 2011) and increased severity of the depressive symptoms (Hejjas et al., 2009). However, the case-control study performed by Hejjas et al. (2009) found no differences in the presence of these polymorphisms between patients suffering from MDD and controls. Moreover, opposite results were found by two meta-analysis studies: Feng et al. (2014) reported that there was no association between rs2230912 polymorphism and MDD development; however, Czamara et al. (2018) showed a positive correlation (Feng et al., 2014; Czamara et al., 2018) (**Figure 2**). It is noteworthy that the latter work included more validated studies, which could explain the different results.

In addition, mice expressing either normal human P2X7 receptors (hP2X7 receptor – wild type) or receptors expressed by an altered gene (hP2X7 receptor – rs2230912), did not present any behavioral changes (Metzger et al., 2017b). However, hP2X7 receptor – rs2230912 mice showed increased vulnerability to chronic social defeat stress. These results indicate that heterozygotic individuals may be more susceptible to development of MDD through interactions between genetic predisposition and stress exposure (Metzger et al., 2017b). In accordance with this idea, the gene polymorphism rs7958311 in P2X7 receptor was correlated with MDD development in individuals with previous history of stress exposure (Gonda et al., 2018) (**Figure 2**).

Beyond the evidence provided by human studies, *in vitro* and *in vivo* experiments may also help to understand the role of the P2X7 receptor in depression and in the mechanisms underlying therapeutic and/or side effects induced by antidepressants. For this purpose, the effects of antidepressant treatment on the expression/function of the P2X7 receptor has been investigated. In a whole-cell patch-clamp study, paroxetine, but not fluoxetine nor desipramine administration, reduced the inward currents evoked by Bz-ATP on cloned rat P2X7 receptors expressed in HEK293 cells (Wang et al., 2016). In another study, paroxetine inhibited, while fluoxetine and clomipramine potentiated ATP-induced dye uptake in HEK-293 cells expressing recombinant human P2X7 receptors (Dao-Ung et al., 2015). *In vivo*, antidepressant-like effect induced by clemasine (Su et al., 2018), ketamine (Tan et al., 2017) and imipramine (Ribeiro et al., 2019b) were associated with diminished P2X7 receptor levels in the hippocampus of stressed animals. These results suggest that P2X7 receptor activity/expression can be modulated by different antidepressants, revealing a potential mechanism by which these drugs may induce their therapeutic effects. Accordingly, mice exposed to chronic unpredictable mild stress (CUMS) (Su et al., 2018) or chronic restraint stress (Tan et al., 2017) presented enhanced P2X7 receptor expression in the hippocampus. However, there are also animal studies showing no alterations (Yue et al., 2017) or even a reduction (Kongsui et al., 2014) in hippocampal P2X7 receptor levels induced by stress exposure. The discrepant data may be explained by different techniques used to determine P2X7 receptor levels (Western blotting versus immunohistochemistry), different stress protocols, or it may indicate a more complex role of P2X7 receptor in stress induced consequences (**Figure 3**).

Aiming to better understand P2X7 receptor involvement in stress response, the effects of P2X7 receptor inhibition has been studied. P2X7 receptor KO mice presented antidepressant-related behavior in both forced swim test (FST) and tail suspension test (TST), two experimental approaches to predict antidepressant effects of drugs (Basso et al., 2009; Csölle et al., 2013a,b). In addition, P2X7 receptor KO mice demonstrated improved responses to a sub-effective dose of imipramine in the FST (Basso et al., 2009). Despite these results, Boucher and co-workers observed a decrease in the immobility time of P2X7 receptor KO mice only after repeated exposure to the FST (Boucher et al., 2011). Altogether, data from P2X7 receptor KO mice indicate that P2X7 receptor absence results in increased resilience to stress, and a phenotype showing antidepressant-related behaviors.

Pharmacological studies in rodents using antagonists with different affinities for P2X7 receptor further support this hypothesis. Pereira and co-workers observed that acute treatment with PPADS (12.5 mg/kg), a pan antagonist for P2 receptors, or iso-PPADS (12.5 or 25 mg/kg), an antagonist of P2X receptors, decreased the immobility time in the FST (Pereira et al., 2013). Csölle et al. (2013b) observed that systemic administration of BBG at dose of 50 mg/kg/day during 4 days, increased sucrose consumption and decreased the immobility time in the TST of mice pretreated with LPS. In another study from the same research group subchronic (7 days) but not acute treatment with BBG (50 mg/kg/day) decreased the immobility time of mice exposed to TST (Csölle et al., 2013a). Mice systemically treated with BBG (50 mg/kg/day) during 8 weeks (Farooq et al., 2018) or rats treated with A-804598, at a dose of 5 mg/kg twice daily for 4 weeks (Iwata et al., 2016), reversed behavioral alterations induced by CUMS exposure. In accordance with these data, 7 days of treatment with BBG (50 mg/kg/day) decreased the number of escape failures induced by inescapable foot shocks application (Ribeiro et al., 2019b). Additionally, 7 days of treatment with A-804598 (30 mg/kg/day) induced antidepressant-like effects in the flinders sensitive line rats, an animal model of depression based on selective breeding (Ribeiro et al., 2019c). Intracerebral administration of P2X7 receptor antagonists have been also carried out in order to investigate the role of these receptors in specific brain regions. Interestingly, microinjection of P2X7 receptor antagonists (BBG or A-438079) into the rat hippocampus during 3 weeks prevented the development of depression-related behaviors induced by CUMS exposure, while the administration of P2 receptors agonists (ATP or Bz-ATP) for the same period caused depressive-like behaviors similar to those observed after stress exposure (Yue et al., 2017).

Altogether, pharmacological and genetic findings indicate that P2X7 receptor inhibition induces antidepressant-related effects in animals. This response may be mainly associated with the blockade of P2X7 receptors expressed in the hippocampus,

although the involvement of other brain structures needs to be further investigated. Regardless the region responsible for the effects induced by systemic administration of P2X7 receptor antagonists, the behavioral response points this receptor as a possible target for depression therapy.

Bipolar Disorder

Bipolar disorder is an incapacitating, chronic and severe mental disorder that occurs in a cyclic course. Patients with bipolar I disorder (BDI) present an exacerbated mood elevation, mania episodes and usually experience major depression. Bipolar II patients (BDII) exhibit an elevation of mood, named hypomania, and a history of major depression without mania episodes. The whole spectrum of BD is prevalent in approximately 2.4% of population, whereas the prevalence of BDI and BDII are 0.6 and 0.4%, respectively (Merikangas et al., 2011). There is several evidence that BD may progress and present neurodegenerative components, once patients exhibit symptoms worsening, gradual cognitive impairment and brain atrophy (Rao et al., 2010).

The neurobiological processes of BD remain poorly understood. The pathways most associated hitherto include monoaminergic neurotransmission, such as dopaminergic, serotonergic, and noradrenergic systems (Grande et al., 2016), redox imbalance (Versace et al., 2014) and neuroinflammation. Some contradictory results exist regarding the neuroinflammation state in BD. BD is a highly heterogeneous disorder and the classification, cycling phase, number of episodes, and medication can vary widely among patients, which can implicate different inflammatory cytokine patterns present in BD patients. Using a meta-analytic approach, serum or plasma samples evidenced highly concentrated soluble IL-2 receptor, TNF-a, soluble TNF receptor type 1, soluble IL-6, and IL-4 in bipolar patients. Overall, there were not any differences between other analyzed anti-inflammatory and pro-inflammatory cytokines (Munkholm et al., 2013).

Bipolar disorder is extremely difficult to model in rodents since the mechanism behind the maniac and depressive cycle is not well established. Thus, animal models are employed that mimic the state of mania. A mouse strain that naturally presents a mania-like phenotype showed downregulation of P2X7 receptor expression (Saul et al., 2012). In contrast, genetic deletion of P2X7 receptor protected the abnormal locomotor activity by acute amphetamine administration (Csölle et al., 2013b; Gubert et al., 2016). In the mania animal model induced by chronic administration of amphetamine, pharmacological antagonism with A-438079 and genetic deletion of P2X7 receptor completely reverted increased locomotor activity induced by amphetamine (Gubert et al., 2016). Additionally, A-438079 abolished the release of pro-inflammatory cytokines IL-1β and TNF-α and lipid peroxidation in hippocampus (Gubert et al., 2016). Using the same animal model, BBG treatment prevented hyperlocomotion, DOPAC augmentation in the hippocampus, increased NTPDase3 expression and astrogliosis induced by amphetamine (Gubert et al., 2019b) (**Figure 3**). Although in the last work only the non-specific antagonist BBG was used, Gubert et al. (2016) found similar results when BBG or the specific antagonist A-438079 were administrated. These studies evidence a reproducibility in P2X7 receptor antagonism in the mania model induced by amphetamine, strengthening the possible role of P2X7 receptor in mania-like state in BD.

There are several studies of genetic associations between P2X7 receptor polymorphisms and BD development. However, inconsistent findings made the identification of any association impossible. The rs2230912 is a SNP in the P2X7 receptor gene that promotes gain of function and was previously associated with increased risk of BD development in patients from the United Kingdom and Ireland (McQuillin et al., 2009) and Canada (Barden et al., 2006). Further, BD patients that presented rs2230912 and rs208294 polymorphisms spent more time in the symptomatic stage than patients without these alleles (Soronen et al., 2011). Nevertheless, this finding was not appropriately replicated in other populations studies. A multi-centric analysis conducted in individuals from Germany, Poland, Romania, and Russia evidenced no allelic or genotypic association between rs2230912 and BDI (Grigoroiu-Serbanescu et al., 2009). Studies in Swedish BD patients revealed an association between rs1718119 and rs1621388 polymorphisms and cognitive features of mania – distractibility, thought disorder, and talkativeness. Still, the rs2230912 polymorphism presented no association with BD (Backlund et al., 2011). A study that analyzed nine variants of P2X7 receptor polymorphisms, such as rs591874, rs208293, rs1186055, rs208298, rs503720, rs1718133, rs1718119, rs2230912, and rs1621388, in United Kingdom individuals found that these polymorphisms did not have any effects on BDI susceptibility (Green et al., 2009). A recent study conducted in Brazilian patients evidenced a decrease in 1513C allele frequency and a potential increase in 1513A A/AC genotype frequency of rs3751143 polymorphism in BD patients (Gubert et al., 2019a) (**Figure 2**). All these polymorphisms in the P2X7 receptor gene represent a gain of function, which could indicate potential influence of the P2X7 receptor behind the genetic predisposal of BD development.

Schizophrenia

Schizophrenia (SCZ) is a complex, multifactorial, heterogeneous, and severe psychiatric disorder. SCZ symptomatology is classified by three major categories: (1) positive symptoms, in which the patient may present disturbance of thinking, delusions and hallucinations, named psychotic symptoms; (2) negative symptoms that are characterized by impaired motivation, decrease in spontaneous speech, and social withdrawal; and (3) cognitive symptoms, which the core features may present impairments in working memory, attention, problem-solving, and executive functioning (van Os and Kapur, 2009). Many efforts have been placed to understand the molecular mechanisms that cause SCZ, however, the full complexity of this disorder remains unknown. SCZ is a highly polygenic (Owen et al., 2016) and many environmental factors have been already associated (Byrne et al., 2004; Allardyce and Boydell, 2006; Varese et al., 2012; Cantor-Graae and Pedersen, 2013; Moustafa et al., 2017). Besides, it is already known that SCZ is a neurodevelopmental disorder and maternal complications may be risk factors (Khashan et al., 2008; Brown, 2011, 2012; Khandaker et al., 2013). There are multiple lines

of evidence supporting the impaired function in dopamine, glutamate and GABA neurotransmission (Schwartz et al., 2012). Similarly, several neurochemical dysfunctions are stated in the kynurenine pathway (Kindler et al., 2019), redox dysregulation (Do et al., 2015), and neuroinflammation (Na et al., 2014; Marques et al., 2019).

Few clinical data are available regarding P2X7 receptor participation in SCZ pathophysiology. Two antipsychotics drugs, prochlorperazine and trifluoperazine, may inhibit human P2X7 receptor function (Hempel et al., 2013). Further, prochlorperazine, a drug with strong antipsychotic action, could act as a negative allosteric modulator of P2X7 receptor activity (Hempel et al., 2013). A populational study conducted with SCZ patients from Denmark analyzed nine SNPs of the P2X7 receptor – rs28360447, rs208294, rs28360457, rs1718119, rs2230911, rs2230912, rs3751143, rs1653624, and rs35933842 – and did not observe any associations between SCZ and these polymorphisms of P2X7 receptor (Hansen et al., 2008) (**Figure 2**).

It is a tremendous challenge to mimic SCZ using animal models due to its high complexity, multifactorial component, and difficulty to distinguish and analyze positive symptoms of these disorders. Phencyclidine (PCP) is a compound largely used as an inductor for animal models of SCZ once the rodents present some similar features in their behavior. In the acute PCP mouse model, the pharmacological blockade with JNJ-47965567 and genetic deletion of the P2X7 receptor alleviated some behavioral parameters and also alteration of gene expression of GABA receptor subunits and neuregulin 1 in the prefrontal cortex (Koványi et al., 2016). Overall, there is lack of evidence supporting the role of the P2X7 receptor in the neurobiology of SCZ. It is a poorly explored field and more studies are needed to indicate whether or not there is association.

Anxiety

Anxiety disorders belong to the most prevalent and disabling psychiatric disorders, substantially impacting life quality. It is estimated that 25% of the population will suffer at least one episode of this disease in adulthood. Types of anxiety disorders include separation anxiety disorder, specific phobia, social anxiety disorder, panic disorder, agoraphobia, generalized anxiety disorder, and drug-induced anxiety disorder. Symptoms include anxiety, excessive fear and other mood disturbances (Kessler et al., 2005). Anxiety disorders are often accompanied by other psychiatric disorders, such as MDD and BD (Schaffer et al., 2012).

Current treatments include serotonin and norepinephrine reuptake inhibitors, benzodiazepines and antidepressant drugs. However, these are partially efficient according to patient histories and the type of anxiety disorder (Murrough et al., 2015). Thus, the identification of specific targets for novel therapeutic approaches is urgent.

In PBMCs from patients with anxiety and depression, an increase in P2X7 receptor expression was found after ATP stimulation. In the same cells, patients with comorbidity of anxiety and Sjogren's syndrome have higher P2X7 receptor expression when compared to control healthy individuals (Xie et al., 2014).

Several studies show the relationship between the chromosome 12q2431, in which the P2X7 receptor gene is inserted, and the development of mood disorders. Thus, polymorphisms of this receptor are widely studied in mood disorders. The SNP rs1718119 with the Thr348Ala mutation was not related to anxiety onset in patients (Erhardt et al., 2007). Although the P2X7 receptor rs2230912 Gln460Arg polymorphism did not present any relation to mood disorders in case-control analysis, this receptor induces higher symptomatic severity scale scores of patients with G-allele (Nagy et al., 2008; Hejjas et al., 2009). In a cohort study, this same SNP was associated with a higher risk of developing mood disorders and alcoholism, including anxiety (Soronen et al., 2011). This study also identified the rs208294 His155Tyr polymorphism as a possible risk factor for disease development (Soronen et al., 2011). In addition, the P2X7 receptor variant rs208294 has been associated with neuroticism-mediated outcomes of mood disorder, a personality trait that indicates vulnerability to the onset of anxiety in stressful situations (Mantere et al., 2012) (**Figure 2**).

P2X7 receptor KO mice show controversial results regarding anxiety-like behavior. Despite showing decreased depressive behavior, Pfizer P2X7 receptor KO animals showed no anxiolytic effect in the elevated plus maze test (Basso et al., 2009). In contrast, Boucher et al. (2011) found anxiety-like behavior in this same test, but not in the light dark emergence test (Boucher et al., 2011). P2X7 receptor KO mice also exhibited anxiety-like behavior in the elevated plus maze test when subjected to contextual fear condition (Domingos et al., 2018).

The P2X7 receptor also presented discrepant results regarding its involvement in inducing anxiety-like behavior in different animal models. Inhibition of the P2X7 receptor with A-438079 (10 mg/kg) augmented anxiety-like behavior of mice subjected to the contextual fear condition model (Domingos et al., 2018). Antagonism using intraperitoneal injections of A804598 for 25 days decreased this behavior in mice subjected to high fat diet (Dutheil et al., 2016), possibly by blocking the formation of inflammasomes. However, this same compound had also an anxiolytic effect in an unpredictable chronic stress model, blocking the release of IL-1β, TNF-α and inflammasome formation (Iwata et al., 2016).

Overall, effects of P2X7 receptor activity modulation on animal anxiety parameters has yet to be elucidated. Ablation of P2X7 receptor expression did not prevent the onset of symptoms, and receptor antagonism induce pro- and anti-anxiety effects in different animal models.

BRAIN TUMORS

Brain tumors are intracranial neoplasms that account for 2% of all cancers (Gould, 2018), while being the second most common cancer among 0 to 14-year-old children. Surpassing even leukemia, brain cancers are the leading cause of oncologic death in this age group (American Brain Tumor Association, 2019). Importantly, the brain is a very fertile soil for metastatic seeding, so that brain metastases incidence is estimated to be at

least 10 times higher than that of primary brain tumors (Vargo, 2017). In fact, 30% of all people with cancers in other body parts will present brain metastases (Gould, 2018). Among primary malignant brain tumors, 80% of all cases are gliomas, malignant tumors raising from glial cells (Gould, 2018).

Although prognosis greatly varies, the incidence of near- and long-term disabilities is notably high (Mukand et al., 2001). Both the tumor itself and the frequently associated perilesional edema, which can reach a several-fold greater volume than the tumor itself, account for the functional neurological consequences (Tran et al., 2019). Indeed, brain tumors cause severe economic impacts not only due to direct treatment and rehabilitation costs, but also due to productivity loss (Su and Abdullah, 2016).

Among candidate molecular targets for anti-cancer drug development, the P2X7 receptor has received great attention. In fact, high ATP levels are a common feature in the tumor microenvironment, reaching concentrations of up to hundreds of micromolar (Pellegatti et al., 2008), a range of concentration capable of activating P2X7 receptors (North and Barnard, 1997). Thus, it is not surprising that P2X7 receptors emerge as central players of purinergic signaling in the tumor microenvironment. In agreement, P2X7 receptor expression is upregulated in several tumor types (Adinolfi et al., 2002; Slater et al., 2004; Solini et al., 2008; Ryu et al., 2011; Arnaud-Sampaio et al., 2019). Glioma cell lines of human (U-138MG, U-251MG, M059J) (Gehring et al., 2012), rat (C6) (Wei et al., 2008), and mouse (GL261) (Tamajusuku et al., 2010) origin express P2X7 receptors as well. Importantly, glioma cells have decreased sensitivity to the cytotoxic effects of extracellular ATP in comparison to healthy tissue cells (Morrone et al., 2005), and glioma cells show less ATP hydrolysis (Wink et al., 2003), favoring the maintenance of high extracellular ATP concentrations. Furthermore, stimulation by extracellular ATP drives the release of glutamate by GL261 glioma cells, an effect partially reversed by P2X7 receptor antagonism (Strong et al., 2018). Elevated levels of both ATP and glutamate mediate cytotoxic effects on the boundaries of the tumor, favoring its expansion and growth (de Groot and Sontheimer, 2011; Strong et al., 2018).

Brain tumor microenvironment is composed by tumor and stromal cells, as reactive astrocytes, fibroblasts and myeloid-derived cells, including microglia (Volak et al., 2018). Therefore, P2X7 receptor expression in the tumor mass may occur in different cell types, leading to particular downstream responses, which may be pro- or anti-tumoral depending on the context. The analysis of human glioma samples revealed that microglial cells confined within the tumor had increased P2X7 receptor expression, and pharmacological inhibition of the receptor significantly decreased the number of glioma cells (Monif et al., 2014).

In a brain tumor model established by intrastriatal injection of C6 glioma cells in rats, pharmacological inhibition of P2X7 receptor by BBG decreased tumor growth. *In vitro*, BBG treatment decreased the receptor expression and prevented chemotaxis induced by Bz-ATP (Ryu et al., 2011), pointing to a pro-tumoral intrinsic activity of P2X7 receptor in this model. In agreement, stimulation of human glioma cells with Bz-ATP increased cell proliferation and migration, an effect counteracted by an inhibitor of the MEK/ERK pathway, implicating this pathway in P2X7 receptor-mediated proliferative effects (Ji et al., 2018) (**Figure 3**). Consistently, overexpression of the P2X7 receptor in a naturally low-expressing human glioma cell line conferred modest *in vitro* growth advantages, but largely accelerated tumor growth *in vivo* (Bergamin et al., 2019), reinforcing a trophic role for this receptor. Also, in a mouse model of neuroblastoma, a rare intracranial tumor that affects immature or developing cells of the nervous system, chronic blockade of the P2X7 receptor in tumor-bearing mice diminished progression and metastasis (Ulrich et al., 2018).

In contrast, another study found that P2X7 receptor blockade by BBG increased C6 glioma cell proliferation, an effect corroborated by enhanced tumor growth observed in rats that received intracranial transplantation of C6 glioma cells either due to *p2rx7* gene knockdown or pharmacological P2X7 receptor blockade (Fang et al., 2013). Conflicting findings were attributed by the authors to different periods and doses of BBG treatment, which would lead to distinct microglial responses.

When expressed both in glioma cells and in glioma-infiltrating microglia, the P2X7 receptor mediates the release of pro-inflammatory factors, as monocyte inflammatory protein 1α (MIP-1α) (Fang et al., 2011), monocyte chemoattractant protein 1 (MCP-1) (Wei et al., 2008; Fang et al., 2011; Braganhol et al., 2015), IL-8 (Wei et al., 2008; Braganhol et al., 2015) and VEGF (Wei et al., 2008). In fact, P2X7 receptor expression in tumor bearing-hosts is essential for mounting an effective anti-tumoral immune response, so that genetic deletion or pharmacological blockade of the receptor increased the incidence of tumors in a murine colitis-associated cancer model (Hofman et al., 2015). Furthermore, P2X7 receptor-deficient tumor-bearing mice undergo a shift toward an immunosuppressive response (De Marchi et al., 2019) and show accelerated tumor progression (Adinolfi et al., 2015).

A comparison between human glioma cell lines showed that those with upregulated P2X7 receptor expression exhibited higher sensitivity to irradiation (Gehring et al., 2012). Further studies corroborated that the P2X7 receptor acts synergistically with radiotherapy promoting cytotoxicity, and the level of P2X7 receptor expression is a good prognosis predictor for radiotherapy response in gliomas (Gehring et al., 2015). Treatments with high ATP and Bz-ATP concentrations also potentialized *in vitro* cytotoxic effects of temozolomide, a drug of choice for glioblastoma treatment, in human glioblastoma cells (D'Alimonte et al., 2015). In agreement, the P2X7 receptor is implicated in the ATP-induced necrotic death of glioblastoma murine cells, supporting its role in killing tumoral cells (Tamajusuku et al., 2010), despite the evidence of glioma resistance to ATP-induced cytotoxicity (Morrone et al., 2005).

In summary, responses triggered by P2X7 receptor highly depend on the expression levels of the receptor, on the stimulation tonus and on the cell type, and the context of tumor microenvironment seems crucial for determining whether P2X7 receptor activation will end up being pro- or anti-tumorigenic. Ultimately, translating existing evidence into therapeutically useful approaches demands a fine

resolution between the distinct phenomena mediated by P2X7 receptors. Adopting optimized experimental designs is crucial to move forward, highlighting how and when P2X7 receptor actions are relevant for tumoral pathophysiology. Experimental design should take into account the complexity of the tumor microenvironment, the different stages of tumor development and the numerous existing splicing variants of the P2X7 receptor gene. Furthermore, findings should combine multiple strategies and rely on both gene expression modulation tools and specific agonists and antagonists, so that conclusions are reproducible and robust. In fact, a considerable part of the available evidence relies on pharmacological modulators that could target other purinergic receptors, as previously mentioned. IC_{50} values for inhibition of other purinergic receptors by BBG fall in the micromolar range, and experimental concentrations for P2X7 receptor inhibition are traditionally limited to hundreds of nanomolar. However, especially in human cells, in which IC_{50} values for P2X7 receptor and P2X4 receptor inhibition differ by only approximately an order of magnitude, much closer than those observed, i.e., in rats (Jiang et al., 2000), overlapping inhibition of both receptors may occur. In spite of that, evidence implicating P2X4 receptor functions in tumor biology is scarce, and mostly related to its inflammatory roles (Guo et al., 2004). In fact, gliomas poorly express P2X4 receptors, and its presence has no prognostic value (The Human Protein Atlas, 2020).

BRAIN-PENETRANT P2X7 RECEPTOR ANTAGONISTS

As discussed so far, P2X7 receptor blockade may be a viable approach for treating brain diseases. Although a range of P2X7 receptor antagonists were developed, some of them are not capable of passing the blood-brain barrier (**Table 1**).

Compounds produced by GlaxoSmithKline (GSK-1482160) and Janssen (JNJ-54175446 and JNJ-55308942) were the first to present both effects in rodents and CNS permeability (Letavic et al., 2017; Territo et al., 2017; Chrovian et al., 2018). The observed *in vivo* activity stimulated the use of target engagement assays to drive development of new drugs, as well as allowed pharmacological tests in rodent models of diseases (Bhattacharya, 2018). In this way, GSK and Janssen advanced in developing other P2X7 receptor antagonists capable of penetrating the blood-brain barrier: GSK compound 16 (Beswick et al., 2010), JNJ-42253432 (Letavic et al., 2013; Lord et al., 2014), JNJ-47965567 (Bhattacharya et al., 2013; Letavic et al., 2013), and JNJ-54166060 (Swanson et al., 2016). In addition, Abbott Laboratories synthetized brain-penetrant P2X7 receptor antagonists, namely: A-438079 (Nelson et al., 2006), A-740003 (Honore et al., 2006), A-804598 (Donnelly-Roberts et al., 2009; Able et al., 2011), and A-839977 (Honore et al., 2009).

Despite the development of several compounds [for detailed reviews see Rech et al. (2016), Pevarello et al. (2017)], the only CNS-permeable P2X7 receptor antagonist that advanced to clinical trials was GSK-1482160. Besides promising initial data, the GSK-1482160 did not present the safety margins to achieve such sustained inhibition, and consequently its development was terminated (Ali et al., 2013).

Currently, Affectis Pharmaceuticals disclosed the use of the brain-penetrant P2X7 antagonist AFC-5128 for neuropathic pain and MS treatment, as stated at the company's website[1]. Moreover, Alzheimer's Drug Discovery Foundation has been supporting Axxam to identify selective P2X7 receptor antagonists for AD treatment.

CONCLUDING REMARKS

The P2X7 receptor has become a very popular target in the purinergic signaling research. This review collected evidence for P2X7 receptor role in CNS diseases, although further studies are needed for a better understanding of this involvement. The neuroinflammation process is largely prominent in CNS diseases, mainly those covered in this review. It is robustly established that P2X7 receptor activation promotes proinflammatory cytokines release, whereas P2X7 receptor blockade efficiently inhibit the neuroinflammatory process. Additionally, blockade of P2X7 receptor signaling may reduce hippocampal amyloid plaques in AD; regenerate dopaminergic neurons of nigrostriatal pathway in PD; delay the ALS onset, progression, and motor performance; decrease MS-related symptoms and microglial activation in this condition; exhibit anti-depressant properties; reduce features related to mania; and decrease tumor growth. Degeneration of neural cells as presented in these conditions may increase the extracellular ATP levels, leading to overactivation of P2X7 receptors. Furthermore, AD, PD, MS, MDD, and brain tumors present increased P2X7 receptor expression. In view of that, we propose a signal amplification of P2X7 receptors in these diseases.

Pharmacological and genetic studies also contributed to elucidate the neurobiology of these conditions. However, here we provide evidence of the lack specificity of some antagonists and antibodies related to the P2X7 receptor. BBG, for example, is still widely used in the literature due to the low cost and blood-brain barrier permeability despite its non-specificity. Therefore, critical analysis regarding P2X7 receptor studies is extremely necessary.

The most studied SNPs of the P2X7 receptor result in loss or gain-of-function, and several studies associate these SNPs with disease development, symptomatology or disease worsening concerning AD, BD, MS, MDD, PD, and anxiety. Regarding SCZ and anxiety, the role of P2X7 receptor should be further explored to clarify its involvement in the pathogenesis of these disorders. Altogether, the studies presented here show the involvement of the P2X7 receptor in pathologies and the therapeutic potential of inhibiting this receptor in the treatment of brain diseases. Herewith, we suggest that these effects are due to the resolution of neuroinflammation components of the aforementioned diseases.

[1] http://www.affectis.com/afc5128.html

AUTHOR CONTRIBUTIONS

RA wrote the Schizophrenia, Bipolar Disorder, and Conclusion sections and prepared the **Figures 2**, **3** and **Table 1**. ÁO-G wrote the Parkinson's Disease, Amyotrophic Lateral Sclerosis, Multiple Sclerosis, Anxiety, and Conclusion sections and prepared the **Table 1**. DR wrote the Alzheimer's Disease, Depressive Disorders, and Brain Penetrant Drugs sections and prepared the **Table 1**. TG wrote the Introduction and Huntington's Disease sections and prepared the **Figure 1**. VA-S wrote the Brain Tumor section. HU and CL conceptualized, supervised manuscript elaboration, edited, revised, and critically overviewed the manuscript. All authors contributed to the article and approved the submitted version.

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P2X7 Receptor-Dependent microRNA Expression Profile in the Brain Following Status Epilepticus in Mice

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The ionotropic ATP-gated P2X7 receptor is an important contributor to inflammatory signaling cascades *via* the release of Interleukin-1β, as well as having roles in cell death, neuronal plasticity and the release of neurotransmitters. Accordingly, there is interest in targeting the P2X7 receptor for the treatment of epilepsy. However, the signaling pathways downstream of P2X7 receptor activation remain incompletely understood. Notably, recent studies showed that P2X7 receptor expression is controlled, in part, by microRNAs (miRNAs). Here, we explored P2X7 receptor-dependent microRNA expression by comparing microRNA expression profiles of wild-type (wt) and P2X7 receptor knockout mice before and after status epilepticus. Genome-wide microRNA profiling was performed using hippocampi from wt and P2X7 receptor knockout mice following status epilepticus induced by intra-amygdala kainic acid. This revealed that the genetic deletion of the P2X7 receptor results in distinct patterns of microRNA expression. Specifically, we found that in vehicle-injected control mice, the lack of the P2X7 receptor resulted in the up-regulation of 50 microRNAs and down-regulation of 35 microRNAs. Post-status epilepticus, P2X7 receptor deficiency led to the up-regulation of 44 microRNAs while 13 microRNAs were down-regulated. Moreover, there was only limited overlap among identified P2X7 receptor-dependent microRNAs between control conditions and post-status epilepticus, suggesting that the P2X7 receptor regulates the expression of different microRNAs during normal physiology and pathology. Bioinformatic analysis revealed that genes targeted by P2X7 receptor-dependent microRNAs were particularly overrepresented in pathways involved in intracellular signaling, inflammation, and cell death; processes that have been repeatedly associated with P2X7 receptor activation. Moreover, whereas genes involved in signaling pathways and inflammation were common among up- and down-regulated P2X7 receptor-dependent microRNAs during physiological and pathological conditions, genes associated with cell death seemed to be restricted to up-regulated microRNAs

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during both physiological conditions and post-status epilepticus. Taken together, our results demonstrate that the P2X7 receptor impacts on the expression profile of microRNAs in the brain, thereby possibly contributing to both the maintenance of normal cellular homeostasis and pathological processes.

Keywords: purinergic signaling, P2X7 receptor, status epilepticus, hippocampus, microRNA

INTRODUCTION

Purinergic signaling is increasingly recognized to play an important role in diseases of the central nervous system (CNS), including epilepsy (Engel et al., 2016; Burnstock, 2020). Epilepsy, characterized by the occurrence of unprovoked seizures, is one of the most common chronic brain diseases affecting approximately 65 million people worldwide (Moshé et al., 2015). Prolonged seizures (status epilepticus) are harmful to the brain and can trigger lasting changes in brain excitability through cell death, changes in neuronal plasticity, and inflammation (Klein et al., 2018). Although much effort has been invested in identifying what drives these pathological changes, the molecular mechanisms remain incompletely understood.

ATP-gated P2 receptors comprise the ionotropic P2X receptors and metabotropic P2Y receptors. Both classes are increasingly linked to the control of brain excitability in health and disease, including processes that influence seizure generation and the development of epilepsy (Engel et al., 2016; Rassendren and Audinat, 2016; Alves et al., 2018; Burnstock, 2020). Among the P2X receptor family, the P2X7 receptor subtype has attracted the most attention (Henshall and Engel, 2015; Beamer et al., 2017). The P2X7 receptor has the lowest affinity for extracellular ATP suggesting that its activation mainly occurs under pathological conditions where high amounts of ATP are released (Surprenant et al., 1996). The P2X7 receptor is an important driver of inflammation *via* induction of the NLRP3 inflammasome and release of Interleukin-1β (IL-1β) but is also known to affect cellular survival, influence neurotransmitter release and control aberrant synaptic plasticity (Sperlágh et al., 2002; Adinolfi et al., 2005; Di Virgilio et al., 2017; Miras-Portugal et al., 2019). Expression of the P2X7 receptor is found to be elevated in the hippocampus and cortex of rodents subjected to status epilepticus and in the brains of patients with drug-resistant epilepsy (Engel et al., 2012; Jimenez-Pacheco et al., 2013, 2016). While some studies have shown this upregulation to occur primarily on microglia (Rappold et al., 2006; Kaczmarek-Hajek et al., 2018), others have suggested that P2X7 receptor expression is also increased in neurons (Doná et al., 2009; Engel et al., 2012; Jimenez-Pacheco et al., 2016). There is also evidence that P2X7 receptor antagonism can be anticonvulsive and neuroprotective following acute seizures (Engel et al., 2012; Jimenez-Pacheco et al., 2013; Mesuret et al., 2014; Huang et al., 2017; Rodriguez-Alvarez et al., 2017). However, others have observed limited or no protection by P2X7 receptor antagonism (Fischer et al., 2016; Nieoczym et al., 2017), and in some studies P2X7 receptor antagonism was reported to promote seizures (Kim and Kang, 2011; Rozmer et al., 2017). Finally, P2X7 receptor antagonists have also been shown to reduce the duration (Amhaoul et al., 2016) and number (Jimenez-Pacheco et al., 2016) of spontaneous seizures in epileptic rodents. The mechanism(s) of these effects remain, however, poorly understood.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at a post-transcriptional level (O'Carroll and Schaefer, 2013). To function, miRNAs are uploaded to the RNA-induced silencing complex (RISC) where Argonaute proteins facilitate complementary base-pairing to target mRNAs resulting in translational repression or degradation of transcripts (Czech and Hannon, 2011). A single miRNA can have numerous targets, either in the same or different pathways. Altered expression of miRNAs has been extensively documented in experimental and human epilepsy (Henshall et al., 2016). Importantly, the targeting of specific miRNAs in animal models has provided compelling evidence that miRNAs influence pathophysiological outcomes after status epilepticus and in chronic epilepsy (Jimenez-Mateos et al., 2012, 2015; Henshall et al., 2016; Tiwari et al., 2018). Notably, the P2X7 receptor was recently identified as a target of miRNAs in the brain (Jimenez-Mateos et al., 2015; Engel et al., 2017; Reigada et al., 2019). How miRNA expression becomes dysregulated following seizures remains, however, incomplete understood.

In the present study, we investigated how genetic deletion of the P2X7 receptor affects miRNA expression in the brain. By using a mouse model of unilateral status epilepticus and P2X7 receptor knockout (*P2rx7*$^{-/-}$) mice, we demonstrate that the loss of the P2X7 receptor alters the expression of several miRNAs under normal physiological conditions and following status epilepticus. Our study demonstrates that P2X7 receptor-controlled downstream signaling pathways include the regulation of an extensive class of miRNAs and thus extends the range of mechanisms by which this receptor influences brain function in health and disease.

MATERIALS AND METHODS

Mouse Models

All animal experiments were performed following the principles of the European Communities Council Directive (2010/63/EU). All procedures carried out in the present manuscript were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland (RCSI; REC 1322) and Health Products Regulatory Authority (HPRA; AE19127/P038; AE19127/P001). Procedures were undertaken as described previously (Torres-Peraza et al., 2013) using 8–12 weeks old male C57Bl/6 wild-type (wt) and *P2rx7*$^{-/-}$ mice [6NTac;B6N-P2rx7tm1d(EUCOMM)Wtsi/Ieg] which lack exon 2 of the *P2rx7* gene. Mice were bred at the Biomedical Research Facility (BRF)

at RCSI and housed in a controlled facility on a 12-h light/dark cycle at 22 ± 1°C and humidity of 40–60% with food and water provided *ad libitum*. During stereotaxic procedures, mice were anesthetized using isoflurane (5% induction, 1–2% maintenance) and maintained normothermic using a feedback-controlled heat blanket (Harvard Apparatus Limited, Kent, UK). Once fully anesthetized, mice were placed in a stereotaxic frame and a midline scalp incision was performed to expose the skull. A guide cannula (coordinates from Bregma; AP = −0.94 mm, *L* = −2.85 mm) was fixed in place with dental cement. Status epilepticus was induced by microinjection of 0.3 μg KA [in 0.2 μl phosphate-buffered saline (PBS); Sigma-Aldrich, Dublin, Ireland] into the right basolateral amygdala 3.75 mm below the dura. Vehicle-injected control animals received 0.2 μl of PBS. The anticonvulsant lorazepam (6 mg/kg; Wyetch, Taplow, UK) was delivered intraperitoneal (i.p.) 40 min following intra-amygdala KA or vehicle to curtail seizures and to reduce morbidity and mortality. In a subset of mice, which were not included in the miRNA array analysis, the electroencephalogram (EEG) was recorded from cortical implanted electrodes, one on top of each hippocampus with the reference electrode on top of the frontal cortex. EEG was recorded using an Xltek recording system (Optima Medical Limited, Guildford, UK) starting 10 min before the administration of intra-amygdala KA. Hippocampal tissue was obtained from either vehicle-injected control mice or 8 h after intra-amygdala KA injection.

Electroencephalogram (EEG) Analysis

To analyze seizure onset and EEG frequency and amplitude signal (power spectral density and EEG spectrogram of the EEG data), EEG data were uploaded into Labchart7 software (AD Instruments Limited, Oxford, UK) and analyzed as before (Engel et al., 2018). EEG total power (μV^2) is a function of EEG amplitude over time and was analyzed by integrating frequency bands from 0 to 100 Hz and the amplitude domain filtered from 0 to 50 mV. The duration of high-frequency (>5 Hz) and high-amplitude (>2 times baseline) polyspike discharges of ≥5 s duration, synonymous with injury-causing electrographic activity (Araki et al., 2002), was counted manually by a reviewer unaware of treatment (Alves et al., 2019).

RNA Extraction and OpenArray Analysis

Total RNA was extracted from the ipsilateral hippocampus from wt and *P2rx7*$^{-/-}$ mice 8 h post-intra-amygdala vehicle or KA using the Trizol method (Engel et al., 2013). RNA quantity was measured using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only samples with an absorbance ratio at 260/280 between 1.8–2.2 were considered acceptable. RNA degradation was not assessed. RNA dilutions were made up in nuclease-free water.

MiRNA profiling was performed using the OpenArray platform (Thermo Fisher Scientific, Waltham, MA, USA; Jimenez-Mateos et al., 2015). OpenArray reverse transcription reaction was performed according to the manufacturer's protocol using 1 μg of total RNA from each sample (each sample was a pool of two hippocampi from different mice). Before loading samples onto the OpenArray, cDNA was pre-amplified following the manufacturer's recommendation. The pre-amplified product was then diluted with 0.1× TE (1/40). Subsequently, 22.5 μl of the diluted pre-amplified product was added to the same volume of 2× Taqman OpenArray Real-time PCR Master Mix (Cat No. 4462164, AB). Finally, the mix of Pre-Amp product and Master-Mix was loaded onto a 384-well OpenArray plate. OpenArray panels were automatically loaded by the OpenArray AccuFill System (Thermo Fisher Scientific, Waltham, MA, USA) and run on a QuantStudio 12 K Flex Real-Time PCR system. 754 murine miRNAs were amplified from each sample together with 16 replicates of four internal controls [ath-miR159a (negative control), RNU48, RNU44, and U6 rRNA]. OpenArray Ct values were normalized to the global mean (GMN). Heat maps were generated using heatmap.2 [RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA, USA[1]] and hierarchical cluster (Cytoscape 3.7.1; Shannon et al., 2003). Only miRNAs were detectable in all samples with a Ct value <28 were used for the analysis (**Supplementary Table S1**). To detect differences between genotypes and treatment groups two different analyses were performed. To determine differences between all groups (treatment and genotype) data were normalized to the average of the control group (vehicle-injected control wt mice). To identify differences between wt and *P2rx7*$^{-/-}$ mice post-status epilepticus, data from *P2rx7*$^{-/-}$ mice post-status epilepticus were normalized to data from wt mice post-status epilepticus. Because of our relative low *n* numbers on the OpenArray (*n* = 3 per group), instead of using false discovery rate corrections, miRNAs showing a fold change higher than 1.5 were considered as up-regulated, and miRNAs showing a fold change lower than 0.6 were considered as down-regulated (**Supplementary Table S2**).

Individual RT-qPCR (miRNAs)

OpenArray results were validated using small-scale real-time quantitative polymerase chain reaction (RT-qPCR; Mitchell et al., 2008). Extracted total RNA was reverse transcribed using the Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) and Taqman primers (mmu-miR-155, ID: 002571; miR-134, ID: 001186) and quantified by RT-qPCR. U6 snRNA (ID: 001973) was selected as an endogenous control. Ct values were normalized to U6 snRNA using the $2^{-\Delta\Delta Ct}$, where ΔΔCt = ΔCt miRNA sample X − ΔCt miRNA reference sample and ΔCt = Ct miRNA (mmu-miR-X) − Ct U6 snRNA.

Pathway Analysis

For miRNA target identification and Gene Ontology (GO) enrichment analysis, experimentally validated targets were retrieved from miRTarBase Release 7.0 (Chou et al., 2018) and TarBase v.8 (Xiao et al., 2009; Karagkouni et al., 2018) while predicted targets were retrieved from TargetScan Release 7.2 (Agarwal et al., 2015) and miRDB Version 6.0 (Liu and Wang, 2019) and processed as described previously (Raoof et al., 2018) with some modifications. Briefly, prediction scores of TargetScan targets were rescaled between 0 and 1 while those of miRDB targets were rescaled between 0.5 and 1 (since the original miRDB

[1]http://www.rstudio.com/

database excluded all targets with scores <50 while the max score is 100). Targets with rescaled prediction scores <0.5 were removed from further analysis. Enrichment analysis of GO terms was only performed on genes that have been targeted by at least two up- or down-regulated miRNAs in each condition (control or status epilepticus) using ReactomePA R/Bioconductor package (Yu and He, 2016). GO terms with adjusted enrichment p-values < 0.05 were considered significant.

Individual RT-qPCR (mRNAs)

Complementary DNA (cDNA) was produced by reverse transcription using SuperScript III reverse transcriptase enzyme (Invitrogen, CA, USA) primed with 50 pmol of random hexamers (Sigma, Dublin, Ireland) using 500 ng of total RNA. qPCR was performed using the QuantiTech SYBR Green kit (Qiagen Limited, Hilden, Germany) and the LightCycler 1.5 (Roche Diagnostics, GmbH, Mannheim, Germany). Each reaction tube contained 2 μl cDNA sample, 10 μl SYBR Green Quantitect Reagent (Qiagen Limited, Hilden, Germany), 1.25 μM primer pair (Sigma, Dublin, Ireland) and RNAse free water (Invitrogen, CA, USA) to a final volume of 20 μl. Using LightCycler 1.5 software, data were analyzed and normalized to the expression of β-*actin*. Primers used (Sigma, Dublin, Ireland): *P2ry1* forward: GTAGGTAGTACGCCAGGG TC, reverse: AAGTAGTTCGGCTGTTCCCA; *c-Fos* forward: GGAATTAACCTGGTGCTGGA, reverse: CATTCAGACCAC CTCGACAA; *P2rx2* forward: ATGGGATTCGAATTGACGTT, reverse: GATGGTGGGAATGAGACTGAA; *P2rx4* forward: TA TGTGGTCCCAGCTCAGGA, reverse: TCACAGACGCGTTG AATGGA and β-*actin* forward: GGGTGTGATGGTGGGAAT GG, reverse: GGTTGGCCTTAGGGTTCAGG.

Western Blotting

Western blot analysis was performed as described previously (Alves et al., 2017). Lysis buffer (100 mM NaCl, 50 M NaF, 1% Tx-100, 5 mM EDTA pH 8.0, 20 mM HEPES pH 7.4) containing a cocktail of phosphatase and protease inhibitors was used to homogenize hippocampal brain tissue and to extract proteins, which was quantified using a Tecan plate reader at 560 nm. Thirty microgram of protein per sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted using the following primary antibodies: P2X7 receptor (1:200, Cat no: APR-004, Alomone Labs, Jerusalem, Israel), Iba1 (1:400; Cat no: 019-19741; Wako, Neuss, Germany) and β-Actin (1:2,000, Cat no: A5441. Sigma–Aldrich, Dublin, Ireland). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Plymouth, PA, USA) and bands visualized using Supersignal West Pico Chemiluminescence Substrate (Pierce, Rockford, IL, USA). Images were captured using a Fuji-Film LAS-3000 (Fuji, Sheffield, UK).

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5.00 and STATVIEW software (5.0.1.0). Data were presented as means ± standard error of the mean (SEM). Analysis of variance (ANOVA) with *post hoc* Fisher's protected least significant difference test was used to analyze three or more group data. For two-group comparison, Student's t-test was used to determine statistical differences between groups analyzed *via* OpenArray. Significance was accepted at $^*p < 0.05$.

RESULTS

Similar Seizure Severity of P2X7 Receptor Knockout Mice and Wild-Type Mice During Status Epilepticus

To establish whether the P2X7 receptor impacts on the miRNA expression profile in the brain, ipsilateral hippocampi from wt and *P2rx7*$^{-/-}$ mice were analyzed under physiological conditions (vehicle-injected control mice) and post-status epilepticus using a genome-wide high-throughput qPCR-based miRNA platform (OpenArray; n = 3 per group, each sample was a pool of two hippocampi from different mice). The OpenArray is a high-density qPCR assay plate that enables the analysis of hundreds of miRNAs with high reproducibility (Farr et al., 2015). P2X7 receptor-dependent miRNA expression was analyzed 8 h post-status epilepticus, the time-point at which a peak in hippocampal P2X7 receptor expression in the intra-amygdala KA mouse model of status epilepticus has been described (Engel et al., 2012). Status epilepticus was triggered *via* unilateral microinjection of KA into the basolateral amygdala in mice (Mouri et al., 2008; **Figure 1A**). In this model, status epilepticus leads to neurodegeneration in the brain which is mainly restricted to the ipsilateral brain hemisphere including the cortex and the CA3 subfield of the hippocampus. Hippocampal neurodegeneration is absent in vehicle-injected control mice (Mouri et al., 2008). Western blotting confirmed the absence of the P2X7 receptor expression (∼72 kDa) in the ipsilateral hippocampus of *P2rx7*$^{-/-}$ mice subjected to status epilepticus (**Figure 1B**). We first wanted to establish whether the absence of the P2X7 receptor has an impact on the expression of other P2 receptors for which a role during status epilepticus had been suggested previously. This included the P2X family members P2X2 and P2X4 and the $P2Y_1$ receptor, which belongs to the P2Y receptor family. Among the P2X receptor family, the P2X4 receptor is the receptor sharing most similarities with the P2X7 receptor (Craigie et al., 2013). Moreover, while expressional changes have been reported for both the P2X2 and P2X4 receptors (Avignone et al., 2008; Engel et al., 2012), P2X4 receptor deficiency has also been shown to aggravate seizure-induced neurodegeneration (Ulmann et al., 2013). In contrast, the $P2Y_1$ receptor is involved in both seizure generation and seizure-induced cell death (Simoes et al., 2018; Alves et al., 2019). Interestingly, $P2Y_1$ receptor expression has been observed to be particularly increased on microglia post-status epilepticus (Alves et al., 2019), the main cell type expressing the P2X7 receptor (Kaczmarek-Hajek et al., 2018). qPCR revealed that *P2rx7*$^{-/-}$ mice showed normal transcript levels of P2X2 and P2X4 receptors in the hippocampus (**Supplementary Figure S1A**). *P2rx7*$^{-/-}$ mice showed also similar transcript levels of the $P2Y_1$ receptor. Furthermore, *P2rx7*$^{-/-}$ mice displayed a reduction in protein levels of the microglial marker Iba1 in the hippocampus

(**Supplementary Figure S1B**), in line with the known role for P2X7 receptor driving microglia activation (Monif et al., 2009). Finally, hippocampal mRNA levels of the neuronal activity-regulated gene *c-Fos* and baseline EEG recordings were similar between wt and *P2rx7*$^{-/-}$ mice suggesting that a loss of the P2X7 receptor does not noticeably alter normal brain function (**Supplementary Figures S1C,D**).

When subjected to intra-amygdala KA-induced status epilepticus, *P2rx7*$^{-/-}$ mice experienced a similar seizure phenotype during the 40 min between injection of KA and administration of lorazepam when compared to wt mice [Total power: wt (39,670 ± 6,227 μV^2) vs. *P2rx7*$^{-/-}$(31,040 ± 9,174 μV^2), p = 0.4659; Amplitude: wt (411.8 ± 38.70 μV) vs. *P2rx7*$^{-/-}$(412.5 ± 76.45 μV), p = 0.9931; high-frequency high amplitude (HFHA) polyspiking: wt (682.5 ± 214.2 s) vs. *P2rx7*$^{-/-}$(613.8 ± 252.9 s), p = 0.8425; **Figures 1C–E** and **Supplementary Figures S1E,F**]. Reinforcing that *P2rx7*$^{-/-}$and wt mice experience a similar seizure severity during status epilepticus, levels of the neuronal activity-regulated miRNA-134 (Jimenez-Mateos et al., 2012) were comparable between genotypes 8 h post-status epilepticus (**Supplementary Figure S1G**). Since there were no significant differences in seizure severity during status epilepticus, this indicates that differences in miRNA profiles result directly from changes in signaling rather than being secondary to an effect of altered seizure severity.

Altered miRNA Expression Profile in P2X7 Receptor Knockout Mice Under Physiological Conditions and Following Status Epilepticus

Next, we profiled miRNA expression within the hippocampus of wt and *P2rx7*$^{-/-}$ mice (**Figure 2A**). MiRNAs were included if detected in all samples of all four groups (vehicle- and KA-injected wt and *P2rx7*$^{-/-}$ mice). This resulted in 335 miRNAs (**Figures 2A,B**, and **Supplementary Table S1**). Demonstrating OpenArray results to reproduce earlier findings, our analysis identified several miRNAs shown to alter their expression following seizures and/or during epilepsy in previous studies (e.g., miR-134, miR-21, and miR-27a* were found to be up-regulated and miR-18a down-regulated post-status epilepticus; Roncon et al., 2015; Cava et al., 2018; **Supplementary Figures S1G, S2**). We then compared miRNA profiles between vehicle-injected control wt and *P2rx7*$^{-/-}$ mice. This revealed that 50 miRNAs are up-regulated and 35 miRNAs are down-regulated in *P2rx7*$^{-/-}$ mice (**Figure 2C**). Then, we investigated to what extent the lack of the P2X7 receptor had an effect on the miRNA expression profile after status epilepticus. When both genotypes subjected to intra-amygdala KA were compared to vehicle-injected control wt mice, 58 miRNAs were up-regulated and 26 miRNAs were down-regulated in wt mice after status epilepticus and 53 miRNAs were up-regulated and 28 miRNAs down-regulated in *P2rx7*$^{-/-}$ mice after status epilepticus (**Figure 2C**). Average fold changes in miRNA expression were similar between conditions and genotypes (**Figure 2D** and **Supplementary Figure S3**). Further analysis revealed 33 miRNAs were commonly regulated after status epilepticus between genotypes whereas 22 miRNAs were unique to wt and 20 miRNAs to *P2rx7*$^{-/-}$ mice among the up-regulated miRNAs post-status epilepticus. Among the down-regulated miRNA pool, eight were common between both genotypes, 17 miRNAs were unique to *P2rx7*$^{-/-}$and 18 unique to wt mice. Only three miRNAs were up-regulated in wt and down-regulated in *P2rx7*$^{-/-}$ mice and one single miRNA was at the same time up- in *P2rx7*$^{-/-}$ mice and down-regulated in wt mice (**Figure 2E**). Interestingly, miRNA expression differences between wt and *P2rx7*$^{-/-}$ mice are more pronounced during physiological conditions when compared to post-status epilepticus (85 in control conditions and 59 post-status epilepticus) with more miRNAs being up-regulated under both conditions 50 up- (e.g., miR-671-3p, fold change (FC) = 6.66; miR-129, FC = 4.69) and 35 down-regulated (e.g., miR-431*, FC = 0.27; miR-20b, FC = 0.28) in control conditions and 44 up- (e.g., miR-770-3p, FC = 34.1; miR-409-5p, FC = 14.92) and 13 down-regulated (e.g., miR-721, FC = 0.41; miR-490, FC = 0.46) post-status epilepticus; **Figure 2F** and **Supplementary Table S2**). Again, fold changes in miRNA expression were similar between conditions (**Figure 2G**). Notably, minimal overlap in altered miRNAs was observed between vehicle-injected control *P2rx7*$^{-/-}$ mice when compared to *P2rx7*$^{-/-}$ mice subjected to status epilepticus with only 11 miRNAs out of 64 common among up-regulated miRNAs and 3 out of 37 among down-regulated miRNAs (**Figure 2H**). This suggests P2X7 receptor-driven changes in the miRNA profile are dependent on physiological context.

In summary, P2X7 receptor deficiency leads to a distinct miRNA signature in the hippocampus during normal physiology and following status epilepticus with increased miRNA expression being the predominant response under both conditions.

Pathways Targeted *via* P2X7 Receptor-Dependent miRNAs

We then explored what genes and pathways are potentially regulated *via* P2X7 receptor-dependent miRNAs during physiological conditions and following status epilepticus. To ensure meaningful results, only experimentally validated and reliably predicted target genes (with comparable prediction scores across databases) were taken into account. Our analysis predicted 9,927 genes to be targeted *via* up-regulated miRNAs and 6,144 genes targeted *via* down-regulated miRNAs during physiological conditions. Post-status epilepticus, 8,254 genes were identified to be targeted *via* up-regulated miRNAs and only 235 genes were predicted to be targeted *via* down-regulated miRNAs (**Supplementary Table S3**).

Enrichment analysis of GO terms of putative target genes of P2X7 receptor-dependent miRNAs shows that genes involved in signaling pathways (e.g., "Signaling by VEGF," "Signaling by Receptor Tyrosine Kinases," "Intracellular signaling by second messengers," "MAPK family signaling cascades," "MAPK1/MAPK3 signaling," "PIP3 activates AKT signaling,"

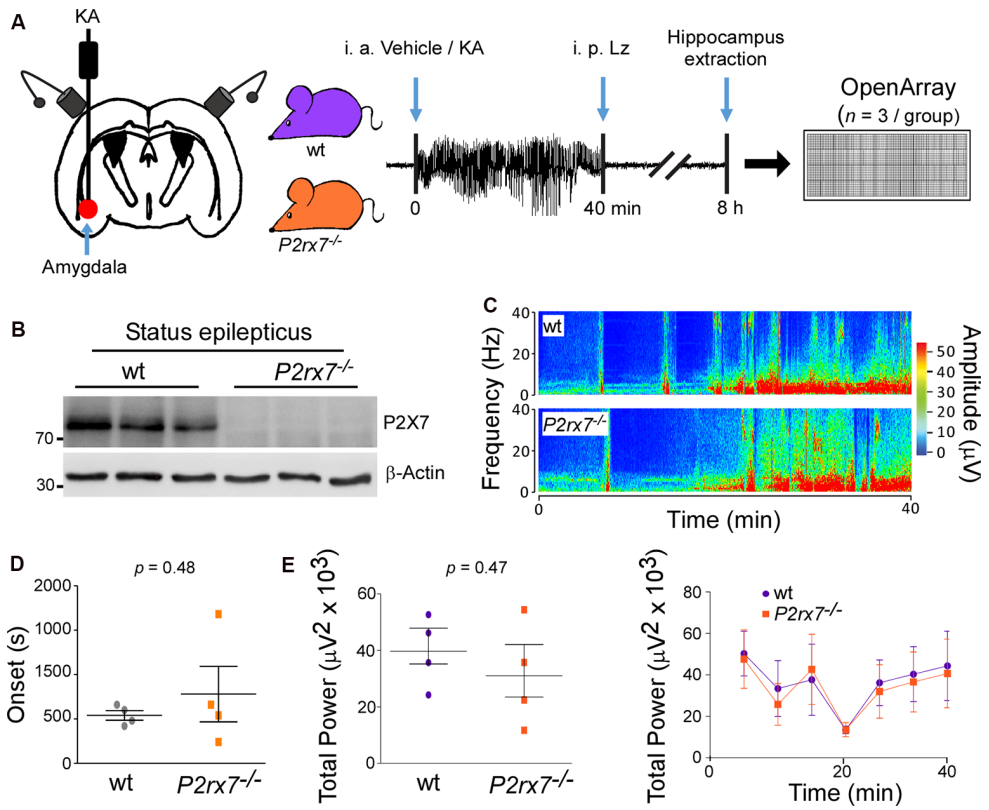

FIGURE 1 | P2X7$^{-/-}$ mice experience similar seizure severity during intra-amygdala Kainic acid when compared to wild-type mice. **(A)** Experimental design: Kainic acid (KA) was injected intra-amygdala to trigger status epilepticus in mice, which was interrupted after 40 min through the injection of the anticonvulsant lorazepam (Lz). Ipsilateral hippocampi were extracted 8 h post status epilepticus from wt and *P2rx7*$^{-/-}$ mice, the peak of the P2X7 receptor expression post-status epilepticus in the intra-amygdala KA model. MiRNAs were profiled *via* OpenArray. **(B)** Western blot (n = 1 per line) showing the absence of the P2X7 receptor signal (∼72 kDa) in the hippocampus of *P2rx7*$^{-/-}$ mice 8 h post-status epilepticus. **(C)** Representative electroencephalogram (EEG) recordings presented as heat maps of frequency and amplitude data showing no difference between wt and *P2rx7*$^{-/-}$ mice during intra-amygdala KA-induced status epilepticus. **(D)** Graph showing no difference in the onset of seizures between wt mice when compared to *P2rx7*$^{-/-}$ mice (n = 4 per group). **(E)** Graphs showing no difference in EEG total power between wt and *P2rx7*$^{-/-}$ mice during 40 min of status epilepticus starting at the time of intra-amygdala KA injection until the administration of the anticonvulsant lorazepam (n = 4 per group).

"Signaling by TGF-beta family members") were overrepresented under both vehicle-injected control conditions (up- and down-regulated miRNAs) and post-status epilepticus (up-regulated miRNAs) with signaling cascades associated with the serine/threonine-specific protein kinase AKT [also known as Protein kinase B (PKB)] particularly abundant. Of note, pathways associated with the mitogen-activated protein kinase (MAPK) were only present among the up-regulated miRNAs in vehicle-injected control mice. Genes involved in the regulation of the immune system (e.g., "Antigen processing: Ubiquitination and Proteasome degradation," "Class I MHC mediated antigen processing and presentation," "Cytokine Signalling in Immune system") are overrepresented in P2X7 receptor-dependent up- and down-regulated miRNAs in vehicle-injected control mice and post-status epilepticus, in line with the P2X7 receptor being a major contributor to inflammatory signaling cascades (Di Virgilio et al., 2017). In contrast, pathways regulating cell death seem to be mainly restricted to the up-regulated miRNA pool (control conditions and post-status epilepticus; e.g., "Apoptosis," "Programmed cell death," "Death receptor signaling"; **Figures 3**, **4** and **Supplementary Figures S4, S5**). Other pathways include: (a) "Axon guidance" (targeted by both up- and down-regulated miRNAs in control conditions and up-regulated miRNAs after status epilepticus); (b) "EPH-Ephrin signaling," involved in neuronal migration and targeted by up-regulated miRNAs post-status epilepticus); (c) "Clathrin-mediated endocytosis" and "Regulation of TP53 activity" (targeted by down-regulated miRNAs in control conditions); (d) Phagocytosis "Fcgamma receptor (FCGR) dependent phagocytosis" and "Protein ubiquitination" (targeted by up-regulated miRNAs post-status epilepticus); and (e) "Neddylation" and "Signaling in Hippo" (targeted by down-regulated miRNAs post-status epilepticus; **Figures 3**, **4** and **Supplementary Figures S4, S5**). Thus, P2X7 receptor-regulated miRNAs seem to impact on numerous cellular pathways during both physiological and pathological conditions with a strong overrepresentation of genes involved in intracellular signaling pathways, cellular survival, and inflammation processes repeatedly linked to the P2X7 receptor (Kopp et al., 2019).

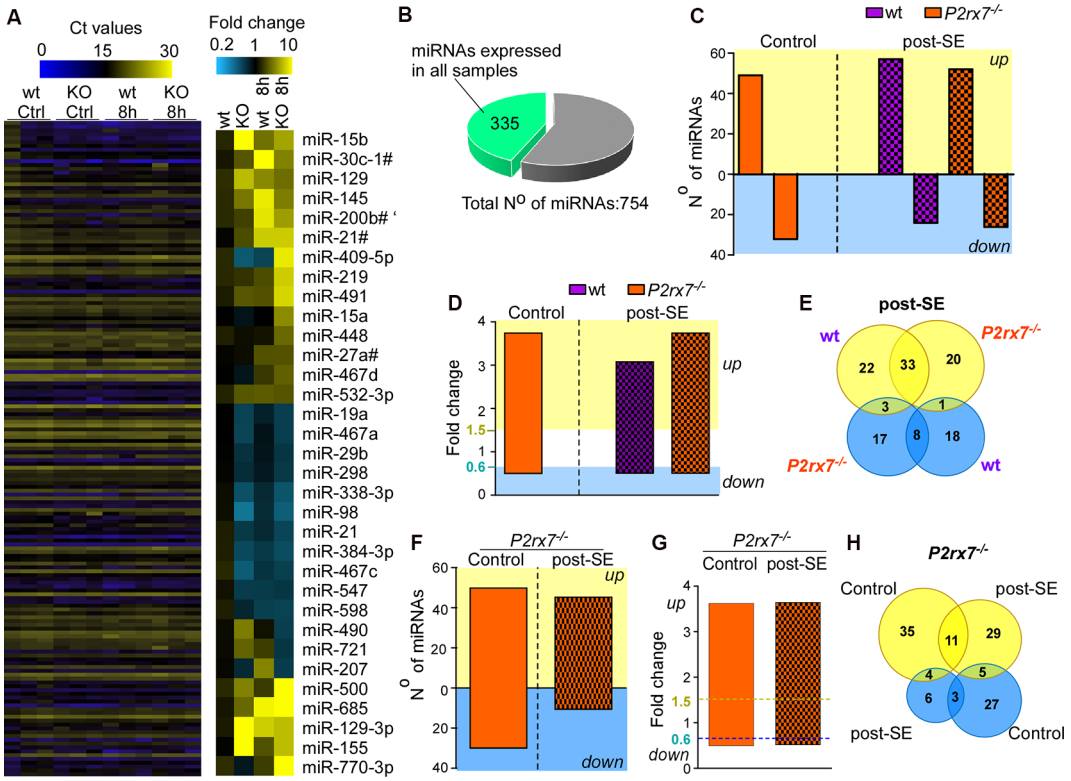

FIGURE 2 | P2X7 receptor-dependent changes in hippocampal miRNA levels. **(A)** Illustrative heat map showing hippocampal miRNA levels in the different groups including vehicle-injected control mice and mice subjected to status epilepticus [wild-type (wt) and *P2rx7*$^{-/-}$(KO)]. Colors on the left indicate Ct values for each miRNA. While blue represents a higher Ct value which corresponds to lower miRNA levels, yellow represents a lower Ct value corresponding to higher miRNA levels. The panel on the right shows miRNAs with the highest fold changes (blue, lower fold-change (FC); yellow, higher FC). Vehicle-injected wt control mice were used as a normalizing control. **(B)** Pie chart indicating the number of miRNAs used for the analysis. The OpenArray analysis included 754 murine miRNAs. Three-hundred and thirty five miRNAs were detected in all hippocampal samples analyzed with Ct values <28. **(C)** Bar chart showing differentially regulated miRNAs in vehicle-injected control mice (*P2rx7*$^{-/-}$) and mice subjected to status epilepticus (wt and *P2rx7*$^{-/-}$). MiRNA expression was normalized to vehicle-injected wt control mice. **(D)** Bar chart showing similar fold changes (FC) of differentially expressed miRNAs among different treatment groups [control and post-status epilepticus (post-SE)] and genotypes (wt and *P2rx7*$^{-/-}$). **(E)** Venn diagram showing the number of up- and down-regulated miRNAs unique and common to wt and *P2rx7*$^{-/-}$ mice post-status epilepticus (post-SE). **(F)** Bar chart showing differentially regulated miRNAs in *P2rx7*$^{-/-}$ mice [control and post-status epilepticus (post-SE)] when compared to wt mice. **(G)** Bar chart showing similar fold changes (FC) of differentially expressed miRNAs in *P2rx7*$^{-/-}$animals (control and post-status epilepticus). The blue dashed line indicates the cut-off of down-regulated miRNAs, the yellow dashed line indicates the cut-off of up-regulated miRNAs. **(H)** Venn diagram showing the number of up-regulated and down-regulated miRNAs in *P2rx7*$^{-/-}$ mice during control conditions and post-status epilepticus (post-SE).

Finally, the P2X7 receptor has been described as an important contributor to inflammatory processes (Adinolfi et al., 2018). We, therefore, analyzed specifically expression changes of P2X7 receptor-dependent miRNAs with a known role during inflammation (**Figure 5A**). While 8 out of 14 anti-inflammatory miRNAs were either up- or down-regulated in *P2rx7*$^{-/-}$ mice, only two out of six pro-inflammatory miRNAs showed altered expression in *P2rx7*$^{-/-}$ mice (**Figure 5A**). In line with more miRNAs undergoing expression changes in vehicle-injected control *P2rx7*$^{-/-}$ mice, more inflammation-associated miRNAs seem to be dysregulated during physiological conditions when compared to post-status epilepticus (**Figure 5A**). Inflammatory miRNAs differentially expressed in vehicle-injected *P2rx7*$^{-/-}$ mice include: (a) down-regulated anti-inflammatory miRNAs let-7e, miR-21, and miR-181c; (b) up-regulated anti-inflammatory miRNAs let-7d*, let-7a, let-7f; and (c) up-regulated pro-inflammatory miRNA-155. Inflammatory miRNAs differentially expressed in *P2rx7*$^{-/-}$ mice post-status epilepticus include: (a) down-regulated anti-inflammatory miRNAs let-7e* and miR-181c; (b) up-regulated anti-inflammatory miRNA miR-223; and (c) up-regulated pro-inflammatory miRNAs miR-27b* and miRNA-155 (Barnett et al., 2016; Gaudet et al., 2018; Gui et al., 2018; Song et al., 2019; Sun et al., 2019; Zhang J. et al., 2019). Among these, miR-155 was detected as one of the most consistently dysregulated miRNA in *P2rx7*$^{-/-}$ mice *via* our OpenArray analysis showing increased levels in vehicle-injected *P2rx7*$^{-/-}$ control mice and *P2rx7*$^{-/-}$ mice subjected to status epilepticus (**Figure 5A**). Moreover, miR-155 has a well-established role during inflammation (Mahesh and Biswas, 2019) and has repeatedly been associated with epilepsy (Huang et al., 2018; Fu et al., 2019; Zhang W. et al., 2019). While no expression changes could be observed during control conditions, individual qPCR

FIGURE 3 | Predicted pathways targeted *via* P2X7 receptor-dependent miRNAs in the hippocampus during control conditions. Bar charts showing the top 20 pathways predicted to be targeted *via* P2X7 receptor-dependent **(A)** up-regulated and **(B)** down-regulated miRNAs in the hippocampus of vehicle-injected control *P2rx7*$^{-/-}$ mice.

confirmed miR-155 to be upregulated in *P2rx7*$^{-/-}$ mice following status epilepticus (**Figure 5B**). Therefore, in line with the P2X7 receptor being an important contributor to inflammatory processes, miRNAs previously associated with inflammation undergo widespread expression changes in *P2rx7*$^{-/-}$ mice.

DISCUSSION

In the present study, we show that the ATP-gated P2X7 receptor impacts on the miRNA expression profile in the hippocampus during normal physiology and following status epilepticus. While the lack of the P2X7 receptor leads to a predominant up-regulation of miRNAs during physiological and pathological conditions, we found very little overlap in identified miRNAs between both conditions. Our results, therefore, suggest a context-specific contribution of the P2X7 receptor to gene expression in the brain *via* the regulation of miRNAs during both normal physiology and pathology.

Several studies have demonstrated that P2X7 receptor expression in the brain is partly regulated *via* miRNAs including miR-22 and miR-135a (Jimenez-Mateos et al., 2015; Reigada et al., 2019). In line with the P2X7 receptor driving seizures and epilepsy development, suppression of miR-22 in the intra-amygdala KA mouse model led to increased P2X7 receptor expression, an increase in inflammation, and the formation of a secondary epileptic focus (Jimenez-Mateos et al., 2015). Blocking of miR-135a on the other hand protects

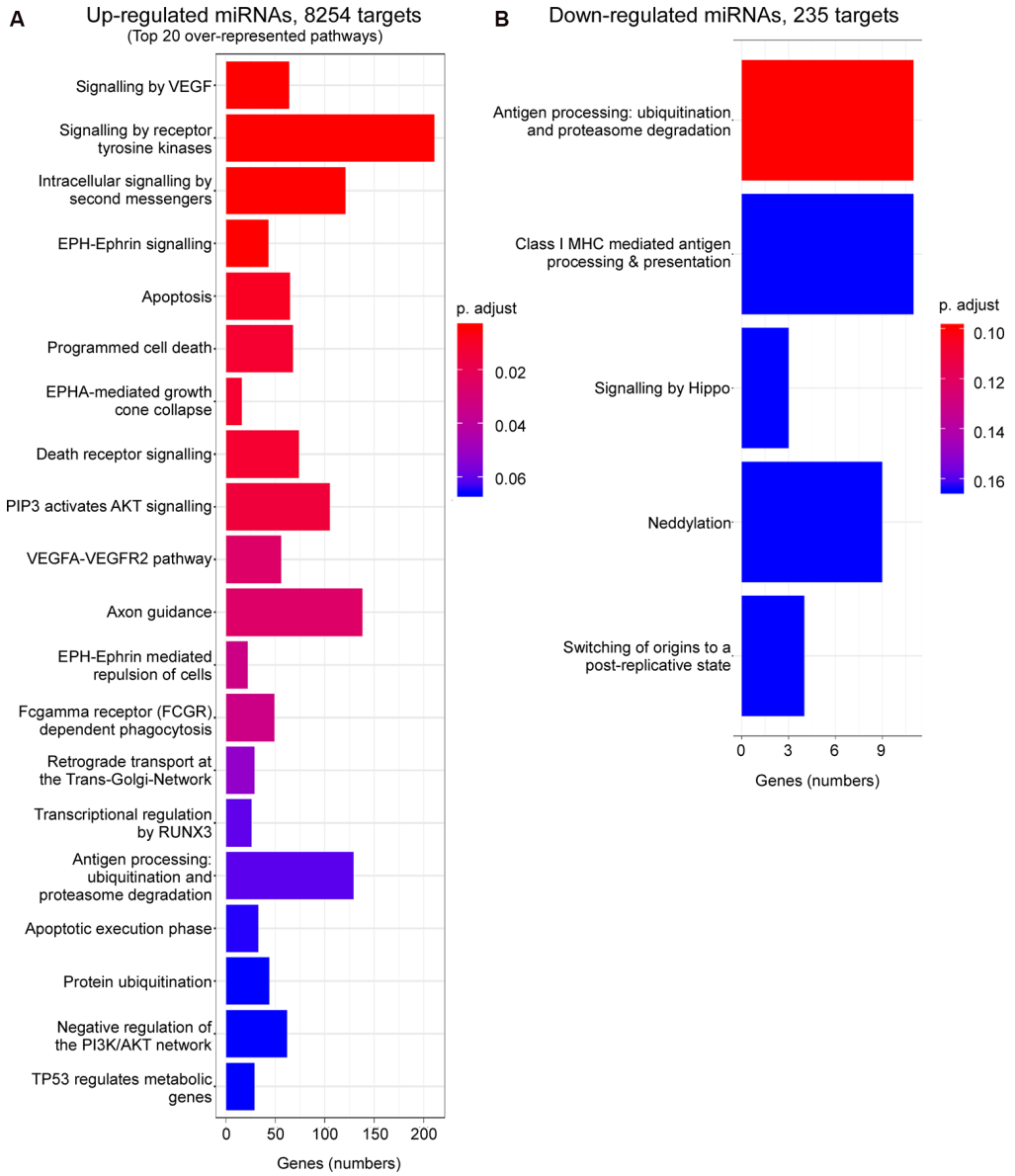

FIGURE 4 | Predicted pathways targeted *via* P2X7 receptor-dependent miRNAs in the hippocampus post-status epilepticus. Bar charts showing pathways predicted to be targeted *via* P2X7 receptor-dependent **(A)** up-regulated and **(B)** down-regulated miRNAs in the ipsilateral hippocampus of *P2rx7*$^{-/-}$ mice post-status epilepticus.

against excitotoxicity in a model of traumatic spinal cord injury (Reigada et al., 2019). Whether the P2X7 receptor impacts on the miRNA expression profile in the brain has, however, not been investigated to date.

A first unexpected finding of our study was that the absence of the P2X7 receptor leads to a higher number of dysregulated miRNAs under physiological conditions than post-status epilepticus. This is even more remarkable, as previous studies using *P2rx7*$^{-/-}$ mice have provided little evidence of altered P2X7 receptor down-stream signaling during physiological conditions with the regulation of cytokines representing one of the few studies reported (Solle et al., 2001; He et al., 2017). Moreover, the affinity of the P2X7 receptor to extracellular ATP is much lower (activation threshold: 0.3–0.5 mM) than that of other P2X receptor subtypes, and extracellular ATP concentrations are only thought to activate the receptor during pathological conditions (Idzko et al., 2014). It is, however, important to keep in mind that our studies used a constitutive *P2rx*$^{-/-}$ mouse; therefore, molecular changes may have developed over a prolonged period leading to more pronounced changes than during the 8 h following status epilepticus. An alternative explanation is a possible ceiling effect of dysregulated miRNAs post-status epilepticus masking possible differences between wt and *P2rx7*$^{-/-}$ mice.

A

MicroRNAs	*P2rx7*$^{-/-}$	
Anti-inflammatory miRNAs	Control	post-SE
mmu-let-7a*	1.40	1.20
hsa-let-7e	▽ 0.54	0.79
hsa-let-7c	0.77	0.79
hsa-miR-21	▽ 0.52	0.74
mmu-let-7d*	△ 1.74	0.90
mmu-let-7c-1*	0.83	1.04
hsa-let-7a	△ 3.86	0.98
hsa-let-7i*	1.35	1.09
hsa-let-7f	△ 1.61	0.97
hsa-let-7e*	1.49	▽ 0.59
mmu-let-7g*	1.17	1.06
hsa-let-7g	0.91	1.45
hsa-miR-223	1.23	△ 2.04
hsa-miR-181c	▽ 0.50	▽ 0.56
Pro-inflammatory miRNAs		
hsa-miR-27b*	0.85	△ 1.76
mmu-miR-155	△ 13.36	△ 2.91
hsa-miR-125b-1*	0.67	1.21
hsa-miR-206	1.05	1.23
hsa-miR-26a	0.70	0.65
mmu-miR-93	0.70	0.91

B

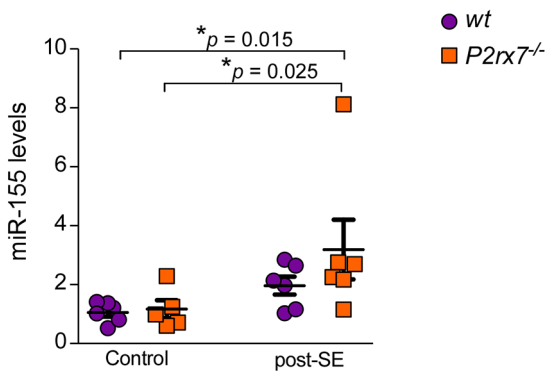

FIGURE 5 | Inflammatory miRNAs regulated *via* the P2X7 receptor. **(A)** Table showing the average fold changes (FC) of anti-inflammatory and pro-inflammatory miRNAs in the ipsilateral hippocampus of *P2rx7*$^{-/-}$ mice during control conditions (Control) and post-status epilepticus (post-SE). **(B)** Graph showing hippocampal miR-155 levels in the different treatment groups (n = 6 per group; analysis of variance (ANOVA) with Fisher's *post hoc* test: 1.057 ± 0.1418 (Control, wt) vs. 3.191 ± 1.013 (post-SE, *P2rx7*$^{-/-}$), F = 2.987, df = 3, p = 0.015; 1.167 ± 0.3006 (Control, *P2rx7*$^{-/-}$) vs. 3.191 ± 1.013 (post-SE, *P2rx7*$^{-/-}$), F = 2.987, df = 3, p = 0.025).

Another surprising finding is the almost complete lack of overlap in identified P2X7 receptor-regulated miRNAs between physiological and pathological conditions. Again, the reason for this remains elusive. The P2X7 receptor, however, may activate different pathways depending on its activation status and the availability of extracellular ATP, which may increase during status epilepticus (Beamer et al., 2019). The P2X7 receptor may also be expressed in different cell populations according to physiological context, thereby differently affecting their miRNA expression profiles. While the P2X7 receptor has been described to be mainly expressed on microglia and oligodendrocytes during physiological conditions (Collo et al., 1997; Matute et al., 2007; Kaczmarek-Hajek et al., 2018), the P2X7 receptor has also been found on neurons following status epilepticus and during epilepsy (Doná et al., 2009; Engel et al., 2012; Jimenez-Pacheco et al., 2016). To establish the exact cell-specific P2X7 receptor-dependent miRNA expression profile will, however, require the use of cell-specific *P2rx7*$^{-/-}$ mice.

What are the molecular mechanisms by which the P2X7 receptor impacts on the expression profile of miRNAs? The P2X7 receptor has been shown to regulate the activity of a variety of different transcription factors [e.g., Nuclear Factor-κB (Nf-κB), Activator protein 1 (AP-1), Glycogen synthase kinase-3β (Barth et al., 2016; Kopp et al., 2019)] and signaling pathways [e.g., MAPK/ERK pathway (Chen et al., 2013)] which may influence the expression of miRNAs. MiRNAs are transcribed as long primary-miRNAs (pri-miRNAs) in the nucleus. Once transported into the cytoplasm, miRNAs are further processed *via* an enzyme called DICER into their mature form. This mature form is then incorporated into the RISC complex for complementary base-pairing with their corresponding target mRNAs (Czech and Hannon, 2011). The activity of DICER is regulated *via* cleavage by proteases, including the calcium-activated protease calpain, and by caspases (Lugli et al., 2005) which are both shown to be activated by the P2X7 receptor (Kong et al., 2005; Jimenez-Mateos et al., 2019). Interestingly, patients with temporal lobe epilepsy presented, despite showing no changes in pri-miRNA levels, reduced levels of mature miRNAs and full-length DICER (McKiernan et al., 2012). Thus, we cannot exclude that the observed P2X7 receptor-dependent alterations of the miRNA expression profiles are due to changes in the processing of pri-miRNAs rather than increased transcription rates of miRNAs. P2X7 receptor-dependent miRNA expression may also be altered *via* other P2X7 receptor-regulated processes including inflammation or, in the case of changes occurring during status epilepticus, differences in the severity of seizures and seizure-induced neurodegeneration between genotypes. However, the latter is unlikely since *P2rx7*$^{-/-}$ mice and their wt littermates showed a similar seizure phenotype during status epilepticus. This is an unexpected finding, as a previous study has shown that P2X7 receptor deficiency leads to seizure suppression during status epilepticus in the intra-amygdala KA mouse model (Engel et al., 2012; Jimenez-Pacheco et al., 2013). However, a different *P2rx7*$^{-/-}$mouse model which expresses a P2X7 receptor splice variant in the CNS (Masin et al., 2012) had been used in the previous study. Whether P2X7 receptor-mediated alterations in miRNA levels result in altered functions of miRNAs has not been analyzed. MiRNA expression has however been shown to correlate with miRNA binding to Ago-2 which is the main component of the RISC complex (Martinez and Gregory, 2013). Finally, our results show that the absence of

the P2X7 receptor leads to more miRNAs being up-regulated than down-regulated under both physiological and pathological conditions. Again, we do not know the reason behind this. P2X7 receptor deficiency-mediated up-regulation of miRNAs, however, suggests P2X7 receptor deficiency having an overall negative impact on protein expression possibly promoting thereby a protective phenotype (Jimenez-Mateos and Henshall, 2009). Another possibility is that the loss of the *P2rx7* gene "de-represses" miRNAs normally bound to the *P2rx7* mRNA, thereby increasing their abundance.

The P2X7 receptor has been involved in numerous molecular processes regulating vital cellular functions with a particular emphasis on pathological conditions, which in part can probably be attributed to its low affinity to extracellular ATP (Surprenant et al., 1996; Jimenez-Mateos et al., 2019). The P2X7 receptor has been described as a gatekeeper of inflammation regulating the activation of the NLRP3 inflammasome and the release of various cytokines (Di Virgilio et al., 2017). In addition to inflammation, the P2X7 receptor has, however, also been implicated in numerous other pathological processes pertinent to epileptogenesis including disruption of the blood-brain barrier, neurogenesis, regulation of neurotransmitter release and cellular survival (Sperlágh and Illes, 2014; Barros-Barbosa et al., 2016; Miras-Portugal et al., 2019). Most notably, pathway analysis of genes targeted *via* P2X7 receptor-dependent miRNAs revealed that most identified pathways have previously been associated with the P2X7 receptor including intracellular signaling (e.g., VEGF or AKT), inflammation and cell death (Amoroso et al., 2015; Di Virgilio et al., 2017; Miras-Portugal et al., 2019). Why genes involved in signaling pathways are strongly overrepresented among up-regulated miRNA targets and are not present among down-regulated miRNAs in *P2rx7*$^{-/-}$ mice post-status epilepticus remains to be determined; however, decreasing the activation of these pathways may serve a neuroprotective strategy reducing cellular energy depletion (Jimenez-Mateos and Henshall, 2009). Interestingly, inflammatory pathways are particularly enriched among P2X7 receptor-dependent miRNA targets, suggesting altered immune responses in *P2rx7*$^{-/-}$ mice, which is in line with previous studies showing reduced cytokine release in these mice (Solle et al., 2001). Regulation of the inflammatory process by the P2X7 receptor is further supported by the fact that P2X7 receptor deficiency leads to altered levels of pro- and anti-inflammatory miRNAs. Evidence for the P2X7 receptor regulating inflammatory signaling during seizures and epilepsy stems from data showing that blocking of the P2X7 receptor during status epilepticus leads to a decrease in the release of the proconvulsant cytokines IL-1β and Tumor necrosis factor-α (TNF-α) and a reduction in Nf-κB-mediated inflammation in the hippocampus (Kim et al., 2011; Engel et al., 2012; Huang et al., 2017). Moreover, P2X7 receptor antagonist-treated epileptic mice show a strong decrease in both astrogliosis and microgliosis (Jimenez-Pacheco et al., 2016). In contrast, genes involved in apoptotic pathways are predominantly overrepresented among up-regulated miRNAs, suggesting that *P2rx7*$^{-/-}$ mice could be protected from cell death, which is in good agreement with a pro-apoptotic function of the P2X7 receptor and the observed neuroprotection *via* P2X7 receptor antagonism from seizure-induced cell death (Adinolfi et al., 2005; Engel et al., 2012; Miras-Portugal et al., 2019). Other pathways linked to the P2X7 receptor include a role in "Axon guidance" in line with previous studies showing that blocking of the P2X7 receptor promotes axon growth and axon branching (Díaz-Hernandez et al., 2008). Of note, genes of the "Signaling by Hippo" pathway are overrepresented within the down-regulated miRNA pool post-status epilepticus. This is in line with a recent study showing the P2X7 receptor driving proliferation following seizures (Rozmer et al., 2017). Importantly, processes predicted to be targeted *via* P2X7 receptor-regulated miRNAs have all previously been associated with status epilepticus. This includes particularly pathways involved in inflammation (Vezzani et al., 2019), but also pathways linked to intracellular signalings such as the MAPK pathway (Hansen et al., 2014), cell death (Engel et al., 2010) and aberrant neurogenesis (Cho et al., 2015). Interestingly, the P2X7 receptor itself has been shown to regulate neurogenic processes following status epilepticus (Rozmer et al., 2017) as has also miR-22 (Beamer et al., 2018), which targets the P2X7 receptor during status epilepticus (Engel et al., 2017). Thus, the P2X7 receptor may impact on the epileptic phenotype directly and indirectly *via* the activation or suppression of selected miRNAs.

Possible limitations of our study include the fact that we have not established whether P2X7 receptor-regulated miRNAs contribute to pathological changes following status epilepticus previously attributed to the P2X7 receptor. Because P2X7 receptor-dependent miRNAs seem to impact mainly on pathways/networks linked to the P2X7 receptor, it is, however, tempting to speculate that these miRNAs act as an additional mechanism to fine-tune and/or amplify P2X7 receptor signaling. It is also important to keep in mind that our approach used a constitutive knockout of the P2X7 receptor. While *P2X7*$^{-/-}$ mice showed similar brain levels of different P2 receptors in the brain, we cannot exclude that the observed changes in miRNA expression are in part due to either developmental alterations or possible compensation mechanisms. Thus, our results should be validated in future studies using either conditional P2X7 receptor knockout mice or P2X7 receptor antagonists. While we acknowledge the low n number for our OpenArray analysis, our study aimed to provide the proof-of-principle data that P2X7 receptor signaling impacts on the miRNA expression profile in the brain extending thereby the potential mechanisms by which the P2X7 receptor impacts on brain function in health and during pathological processes. Also, our analysis has focused on the hippocampus which is one of the main brain structures affected within the intra-amygdala mouse model and patients with temporal lobe epilepsy (Chang and Lowenstein, 2003; Mouri et al., 2008). However, seizures may also affect extra-hippocampal brain areas, such as the cortex, which also show increased expression of the P2X7 receptor (Jimenez-Pacheco et al., 2013). Lastly, rather than decreasing, P2X7 receptor expression levels increase during pathology (Beamer et al., 2017; Miras-Portugal et al., 2019). While outside of

the scope of the present manuscript, the recent development of the P2X7 receptor overexpressing mice (Kaczmarek-Hajek et al., 2018) may allow the analysis of the effects of increased P2X7 receptor expression/function on miRNA profiles in future studies.

In summary, our data demonstrate that P2X7 receptor signaling affects the expression profile of miRNAs in the brain thereby possibly contributing to the gene expression landscape during normal physiology and pathology which should be taken into consideration when analyzing P2X7 receptor-driven molecular pathomechanisms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found in the Gene Expression Omnibus: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153204, accession number GSE153204.

ETHICS STATEMENT

The animal study was reviewed and approved by Research Ethics Committee of the Royal College of Surgeons in Ireland.

AUTHOR CONTRIBUTIONS

GC carried out qPCRs and Western blotting, analyzed the OpenArray, and wrote parts of the manuscript. NN carried out pathway analysis. LD-G carried out Western blotting. MA analyzed EEG, performed qPCR and carried out Western blotting. AK generated heat maps. AN bred and provided P2X7 knockout mice and edited the manuscript. DH edited manuscript. EJ-M performed experiments with OpenArray and edited manuscript. TE supervised the study, carried out *in vivo* work and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2020.00127/full#supplementary-material.

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frontiers in Molecular Neuroscience

BRIEF RESEARCH REPORT
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Revisiting the Idea That Amyloid-β Peptide Acts as an Agonist for P2X7

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The P2X7 receptor (P2X7) is a cell surface ligand-gated ion channel, activated by its physiological nucleotide agonist ATP and a synthetic analog (BzATP). However, it has also been suggested that there may be structurally unrelated, non-nucleotide agonists such as the amyloidogenic β peptide. Here we aimed to reassess the effect of amyloid β peptides in various *in vitro* cell models, namely HEK293 overexpressing human P2X7, the microglial BV-2 cell line, and BV-2 cells lacking P2X7. We measured YO-PRO-1 dye uptake in response to full-length amyloid β peptide (1–42) or the shorter amyloid β peptide (25–35) and there was a concentration-dependent increase in YO-PRO-1 dye uptake in HEK-hP2X7 cells. However, these amyloid β peptide-induced increases in YO-PRO-1 dye uptake were also identical in non-transfected HEK-293 cells. We could observe small transient increases in $[Ca^{2+}]_i$ induced by amyloid β peptides in BV-2 cells, however these were identical in BV-2 cells lacking P2X7. Furthermore, our metabolic viability and LDH release experiments suggest no significant change in viability or cell membrane damage in HEK-hP2X7 cells. In the BV-2 cells we found that high concentrations of amyloid β peptides (1–42) and (25–35) could reduce cell viability by up to 35% but this was also seen in BV-2 cells lacking P2X7. We found no evidence of LDH release by amyloid β peptides. In summary, we found no evidence that amyloid β peptides act as agonists of P2X7 in our *in vitro* models. Our study raises the possibility that amyloid β peptides simply mimic features of P2X7 activation.

Keywords: Alzheimer disease, P2X7 receptor, microglia, reproducibility, amyloid beta peptide

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INTRODUCTION

The most prevalent cause of dementia is Alzheimer's disease, a fatal neurodegenerative disorder that is characterized by a progressive cognitive and functional impairment and memory loss (Heppner et al., 2015). In the field of Alzheimer's disease research, the amyloid cascade hypothesis has been the major hallmark of pathogenesis. This states that the generation of amyloid plaques, primarily composed of the amyloid-β peptide (Aβ), represents the initial event triggering neurobiological dysfunction (Hardy and Selkoe, 2002). Over two decades of research (Hardy and Higgins, 1992) have recently revealed many layers of complexity (Lee et al., 2018), however, the bulk of data still supports the role of the Aβ peptide as the primary initiator of Alzheimer's disease pathogenesis (Musiek and Holtzman, 2015; Cheignon et al., 2018).

Previous data have indicated that the immune system may have a role in Alzheimer's disease and that activated microglia have been observed in patients (Sarlus and Heneka, 2017). Microglia, the resident macrophages of the CNS, alter their morphology and phenotype to adopt

a so-called activated state in response to neurophysiological brain insults (Heneka et al., 2015; Sarlus and Heneka, 2017). Morphologically activated microglia are believed to contribute to the progression of Alzheimer's disease via receptors such as the scavenger receptor CD36 (El Khoury et al., 1996), α6β1 integrins (Koenigsknecht and Landreth, 2004), the formyl peptide receptor-like protein (Le et al., 2001), TLR2 (Chen et al., 2006), TLR4 (Michaud et al., 2013) and the TLR-interacting molecule CD14 (Fassbender et al., 2004). As a result, this leads to inflammatory mediator secretion and microglial responsiveness to injury as comprehensively reviewed by Sarlus and Heneka (2017). However, another receptor that is also highly expressed by microglial cells is P2X7, an ATP-gated ion channel (Chessell et al., 1997; Ferrari et al., 1997; Bartlett et al., 2013). In recent years, several studies suggested the participation of P2X7 in Aβ-mediated brain damage (Haughey and Mattson, 2003; Parvathenani et al., 2003; Rampe et al., 2004; McLarnon et al., 2006). Furthermore, Sanz et al. (2009) suggested that P2X7 may participate in microglia activation by Aβ peptides and it has since been proposed that ATP might not be the only endogenous agonist for P2X7 receptors (Di Virgilio et al., 2018). This may, in turn, open new avenues for the development of novel therapies for Alzheimer's disease. Intrigued by this proposal of alternative agonists, we set out to determine if Aβ could act as a P2X7 agonist and investigate the effect of positive allosteric modulators of P2X7 that we have previously characterized (Helliwell et al., 2015). However, we have been unable to validate the results that suggest that Aβ-induced responses require the expression of P2X7. Based on our data, we conclude that Aβ peptides may not directly cause P2X7-dependent signaling in microglial cells.

MATERIALS AND METHODS

Cell Culture

Microglial BV-2 and BV-2 P2X7-deficient cells were maintained in DMEM/F12 with L-glutamine (Gibco 11320-074, Fisher Scientific, United Kingdom), containing 10% (v/v) FBS (Gibco, US origin), penicillin and streptomycin (Fisher Scientific, United Kingdom). HEK-293 cells were maintained under the same media conditions. HEK-293 cells stably expressing human P2X7 were generated previously (Bhaskaracharya et al., 2014) and maintained under similar conditions with the addition of 400 μg/ml geneticin (Fisher Scientific, United Kingdom). All cells were maintained at 37°C with 5% CO_2 in a humidified incubator.

Materials

Aβ peptides corresponding to human Aβ amino acids $A\beta_{25-35}$, $A\beta_{35-25}$ (inactive scrambled peptide) and $A\beta_{1-42}$ were purchased from GenScript, United States and prepared as 10 mM stock solutions in either water or DMSO. Apyrase (an ATP-hydrolyzing enzyme), ATP and BzATP were purchased from Sigma Aldrich, United Kingdom. Stock solutions of P2X7 antagonists AZ10606120 and JNJ47965567 (Tocris Bioscience, Bio-Techne, United Kingdom) were prepared in DMSO (10 mM) and stocks were kept frozen at −20°C.

Measurements of Intracellular Ca^{2+}

The cells were seeded at 2×10^5 cells/well (100 μl) and plated on poly-D-Lysine coated 96-well plates (Nunc 167008, Fisher Scientific, United Kingdom) and used for experiments 24 h after plating. Cells were loaded with the indicator dye Fura-2-AM using a concentration of 2 μM (HelloBio, United Kingdom) in HBSS for 45 min at 37°C. Loading buffer was removed and replaced with 180 μl of assay buffer containing 147 mM NaCl, 2 mM KCl, 0.1 mM $CaCl_2$, 13 mM Glucose, 10 mM HEPES; pH 7.35. The plate was warmed in the FlexStation 3 (Molecular Devices, United Kingdom) for 10 min before recording using excitation wavelengths 340 and 380 nm and emission at 520 nm (Bibic et al., 2019).

Agonists (10x concentration) were automatically injected at 30 s using the Flex function. Data was converted to Fura-2 ratio (340/380) and normalized using a zero baseline correction. Area under the curve was calculated and plotted. All data was collected in triplicate.

Measurements of YO-PRO-1 Dye Uptake

Impermeant dye uptake was measured with the extracellular fluorescent tracer YO-PRO-1, a probe that enters the cells through P2X7 activation-induced pores and emits fluorescence when it binds DNA (Idziorek et al., 1995). A solution of 2 μM YO-PRO-1 in assay buffer (see above) was added to wells, and the 96-well plate was placed at 37°C for 10 min. The fluorescence signal in response to drug injection was then measured using a Flexstation 3 (Molecular Devices) as described previously (Bidula et al., 2019; Dhuna et al., 2019). Excitation wavelength was 490 nm and emission was measured at 520 nm. Machine settings include bottom read fluorescence, PMT medium, 6 reads/well with a sample interval of 3.5 s. RFU data was normalized using a zero baseline correction and area under the curve was calculated and plotted. All data was collected from triplicate wells in each independent experiment.

Cell Viability Measurements

Cells were plated at 2×10^4/well (in a volume of 100 μl) and plated on non-coated 96-well plates (Fisher Scientific, United Kingdom) in culture medium containing 1% FBS 24 h before stimulation. Stimuli included concentrations of Aβ peptides (10–60 μM), ATP at 500 μM and 3 mM concentrations, as well as staurosporine (5 μM), and the vehicle control (DMSO). Following incubation with different stimuli, resazurin (0.1 mg/ml in PBS, Sigma Aldrich) was added to cells for final 2 h at 37°C. The plate was then read on a Flexstation 3 plate reader using an excitation wavelength of 570 nm and emission wavelength of 600 nm (Bidula et al., 2019).

LDH Release Assay

Lactate dehydrogenase (LDH) release into cell culture supernatants was measured using an LDH assay kit (Pierce, Fisher Scientific, United Kingdom) following the manufacturer's instructions. Control cells were lysed with the lysis buffer provided to harvest total intracellular LDH. For measuring LDH release, cells were cultured in 96-well plates, stimuli applied for

24 h, and supernatants were collected. Absorbance of duplicate 50 μl aliquots of supernatants were measured on a Flexstation 3 plate reader at 490 nm.

Data Analysis and Statistics

All results are expressed as mean ± SD using data from collated experiments. All experimental data was collected from triplicate wells. All data for cell viability and cytotoxicity were obtained as relative fluorescence units (RFU) and are expressed as a percentage of the negative control (culture medium). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison *post-hoc* test (GraphPad Prism v8). Statistically significant differences from controls are indicated by * using $p < 0.05$ as a threshold.

RESULTS

A study using N13 microglial cells indicated that Aβ peptide, both the full-length 1–42 and the shorter 25–35, may induce responses similar to the activation of P2X7 (Sanz et al., 2009). We show that stimulation of BV-2 microglial cells with ATP and BzATP, both known agonists for P2X7, led to $[Ca^{2+}]_i$ increases (**Figures 1A,C**). Using a P2X7-deficient BV-2 cell line generated by CRISPR/Cas9 gene editing (Dhuna et al., 2019), the $[Ca^{2+}]_i$ responses were different to the parental BV-2 line, with the response to BzATP almost completely abolished (**Figures 1B,D**). We first tested $A\beta_{25-35}$ and found that this peptide did induce a transient increase in $[Ca^{2+}]_i$ increase in BV-2 cells (**Figures 1A,E**) that displayed a concentration-dependent effect. We observed a similar $A\beta_{25-35}$ -induced $[Ca^{2+}]_i$ increase in the P2X7-deficient BV-2 cells (**Figures 1B,F**). Furthermore, we show that the $A\beta_{25-35}$ induced $[Ca^{2+}]_i$ increases were not affected by the P2X7-selective antagonist AZ10606120 whereas the ATP-induced $[Ca^{2+}]_i$ increase was reduced 30% by AZ10606120 (**Figure 1A**) in BV-2 cells. As expected, there was no significant effect of AZ10606120 in P2X7-deficient BV-2 cells (**Figure 1B**). The inactive scrambled $A\beta_{35-25}$ peptide did not increase $[Ca^{2+}]_i$ to the same level as $A\beta_{25-35}$ in BV-2 microglial cells, whether expressing P2X7 (**Figure 1G**) or not (**Figure 1H**). Some evidence suggests that the solvent, such as DMSO, acetonitrile, and water, may influence the self-assembly and thus the biological activity of Aβ peptides (Busciglio et al., 1992; Shen and Murphy, 1995; Zagorski et al., 1999). In our experiments $A\beta_{25-35}$ was prepared in either DMSO or water (**Figure 1**) and peptide dissolved in DMSO displayed higher $A\beta_{25-35}$ -induced $[Ca^{2+}]_i$ increases (**Figures 1E,F** and **Supplementary Figure S1**).

We next tested whether $A\beta_{25-35}$ would induce responses in a stable HEK-293 cell line over-expressing human P2X7 (HEK-hP2X7). We used a standard YO-PRO-1 dye uptake assay to assess the P2X7 large pore formation (Bibic et al., 2019). We observed that $A\beta_{25-35}$ (**Figures 2A,B**), but not the inactive scrambled $A\beta_{35-25}$ (**Figure 2C**), induced a significant YO-PRO-1 uptake over the concentration range 30–100 μM. Notably, this $A\beta_{25-35}$-induced dye uptake was not abrogated by the P2X7-specific antagonist AZ10606120 despite complete blockade of ATP- and BzATP-induced dye uptake in this cell line (**Figure 2A**). Furthermore, $A\beta_{25-35}$-induced dye uptake was not affected by the ATP-degrading enzyme apyrase (**Supplementary Figure S2**). In addition, we observed that $A\beta_{25-35}$ induced YO-PRO-1 dye uptake into non-transfected HEK-293 cells which fail to display ATP-induced dye uptake (**Figure 2D**) supporting the hypothesis that the observed effects of $A\beta_{25-35}$ are not dependent on the expression of P2X7. The inactive scrambled $A\beta_{35-25}$-peptide did not induce YO-PRO-1 dye uptake responses (**Figures 2B,C**). The $A\beta_{25-35}$-induced dye uptake was affected by the solvent used with the DMSO-peptide having a greater effect than the water-solubilized peptide (**Figure 2B** and **Supplementary Figure S2**).

We investigated the full-length Aβ peptide, 1–42, which was also reported by Sanz et al. (2009) to have an effect on microglial IL-1β secretion. We did not observe $[Ca^{2+}]_i$ increases in BV-2 (**Figure 3A**) or YO-PRO-1 dye uptake in HEK-hP2X7 (**Figure 3B**) in response to $A\beta_{1-42}$ peptide at concentrations of 10, 60 or 100 μM. Both ATP and BzATP induced robust responses in both cell lines and the P2X7-selective antagonists AZ10606120 and JNJ47965567 could abolish these responses (**Figure 3**). Even at the highest concentration of $A\beta_{1-42}$, this peptide did not cause a significant increase in $[Ca^{2+}]_i$ release relative to the buffer control.

Once activated, P2X7 is endowed with the ability to kill microglia, either by necrosis or apoptosis, as well as to trigger many responses such as inflammation and oxidative stress (Surprenant et al., 1996; Ferrari et al., 1997; Brough et al., 2002; Volonte et al., 2012). Thus, we examined whether $A\beta_{25-35}$ or $A\beta_{1-42}$ could trigger cell death in BV-2, P2X7-deficient BV-2 cells, or HEK-hP2X7 cells at either 10, 30 or 60 μM, using a cell viability assay (**Figure 4**). In BV-2 (**Figure 4A**) and P2X7-deficient BV-2 cells (**Figure 4B**), $A\beta_{1-42}$ or $A\beta_{25-35}$ (60 μM) reduced cell viability similarly up to 35 and 30% of control, respectively, suggesting that this effect was not P2X7 dependent. Lower concentrations of $A\beta_{1-42}$ or $A\beta_{25-35}$ (10, 30 μM) had lesser effects and no significant decrease in cell viability was found with $A\beta_{1-42}$ or $A\beta_{25-35}$ on HEK-hP2X7 cells (**Figure 4C**). The inactive scrambled $A\beta_{35-25}$ peptide did not have any effect on the cell viability (**Figures 4A–C**). Cell supernatants were also measured for the presence of LDH, which is released upon cell lysis. **Figures 4D–F** shows LDH levels from BV-2 cells, P2X7-deficient BV-2 cells, and HEK-hP2X7 cells. None of the Aβ-treated HEK-hP2X7 cell supernatants contained significant levels of released LDH as compared with control samples of cells stimulated with staurosporine or 3 mM ATP. In the BV-2 cells and P2X7-deficient BV-2 cells, we found a relatively high spontaneous LDH release and none of the Aβ peptide treatments were higher than this (**Figures 4D,E**).

DISCUSSION

ATP is a crucial extracellular messenger serving as the physiological agonist of both P2Y and P2X receptors (Wang et al., 2004; Davalos et al., 2005; Haynes et al., 2006; Koizumi et al., 2007). The identification of alternative agonists for P2X receptors is of particular interest when ascribing physiological roles to individual receptors in different cells and tissues. There

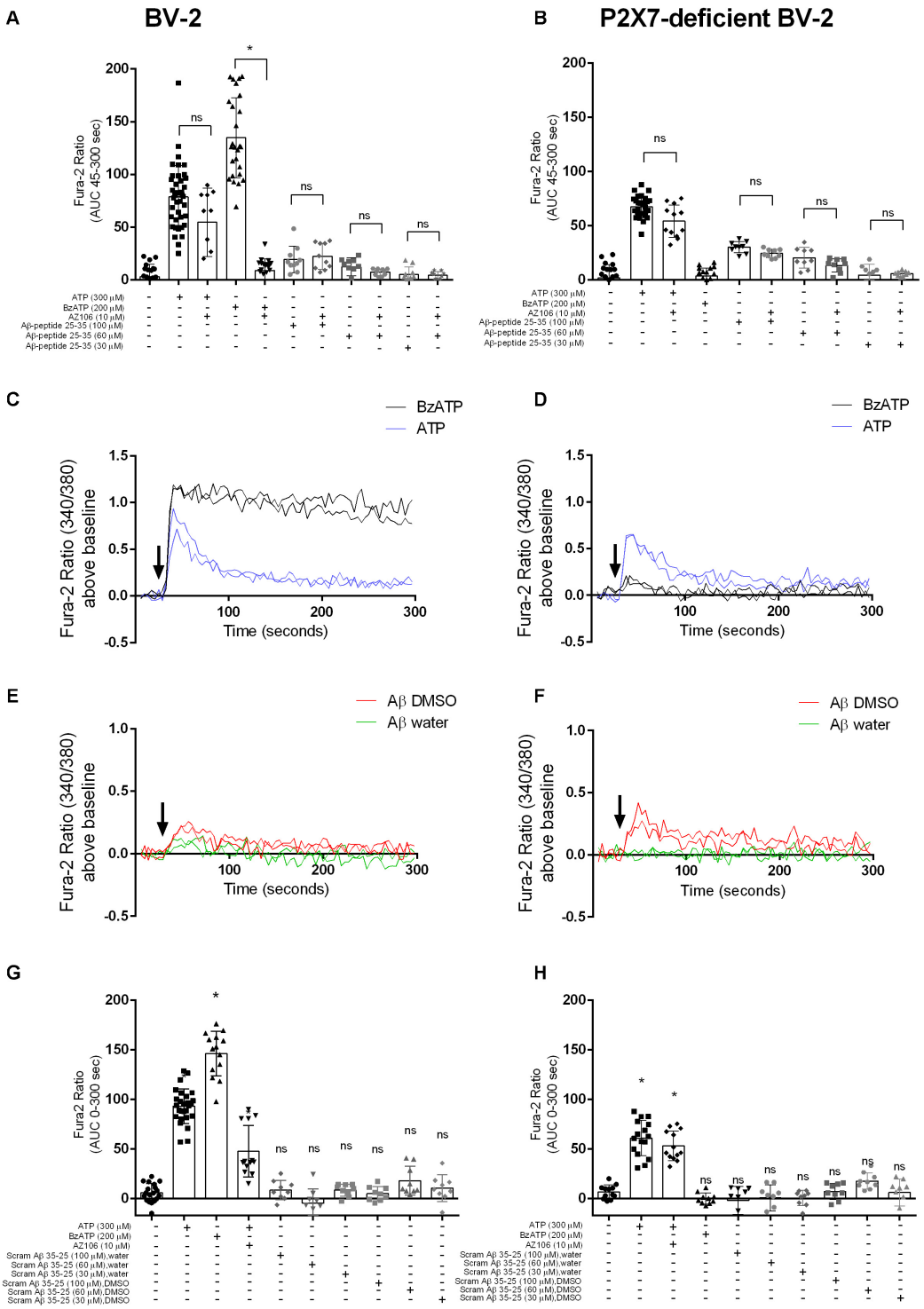

FIGURE 1 | $A\beta_{25-35}$ induced similar $[Ca^{2+}]i$ responses in microglial BV-2 cells in the presence/absence of P2X7. BV-2 cells were loaded with Fura-2AM (2 μM) and challenged with the $A\beta_{25-35}$ peptides (DMSO) in the concentration range 30–100 μM in either BV-2 **(A)** or P2X7-deficient BV-2 **(B)**. One-way ANOVA was performed with Tukey's multiple comparison test where * indicates a significant difference to the paired control (with AZ1060610) and ns denotes no significant difference. Each symbol represents a single well and data has been collated from all independent experiments. Kinetics of the $[Ca^{2+}]i$ response in either BV-2 **(C,E)** or P2X7-deficient BV-2 **(D,F)** are plotted from a representative experiment. Similarly, BV-2 cells were loaded with Fura-2AM (2 μM) and challenged with the inactive scrambled $A\beta_{35-25}$ peptide in the concentration range 30 μM -100 μM in either BV-2 **(G)** or P2X7-deficient BV-2 **(H)**. AZ10606120 is a selective antagonist of P2X7, and BzATP is a synthetic agonist for P2X7 receptors. Experiments were repeated five times with triplicates on each plate, and the results are presented as area under curve (mean ± SD). One-way ANOVA was performed with Tukey's multiple comparison test where * indicates a significant difference ($P < 0.05$) to the control (buffer alone) and ns denotes no significant difference.

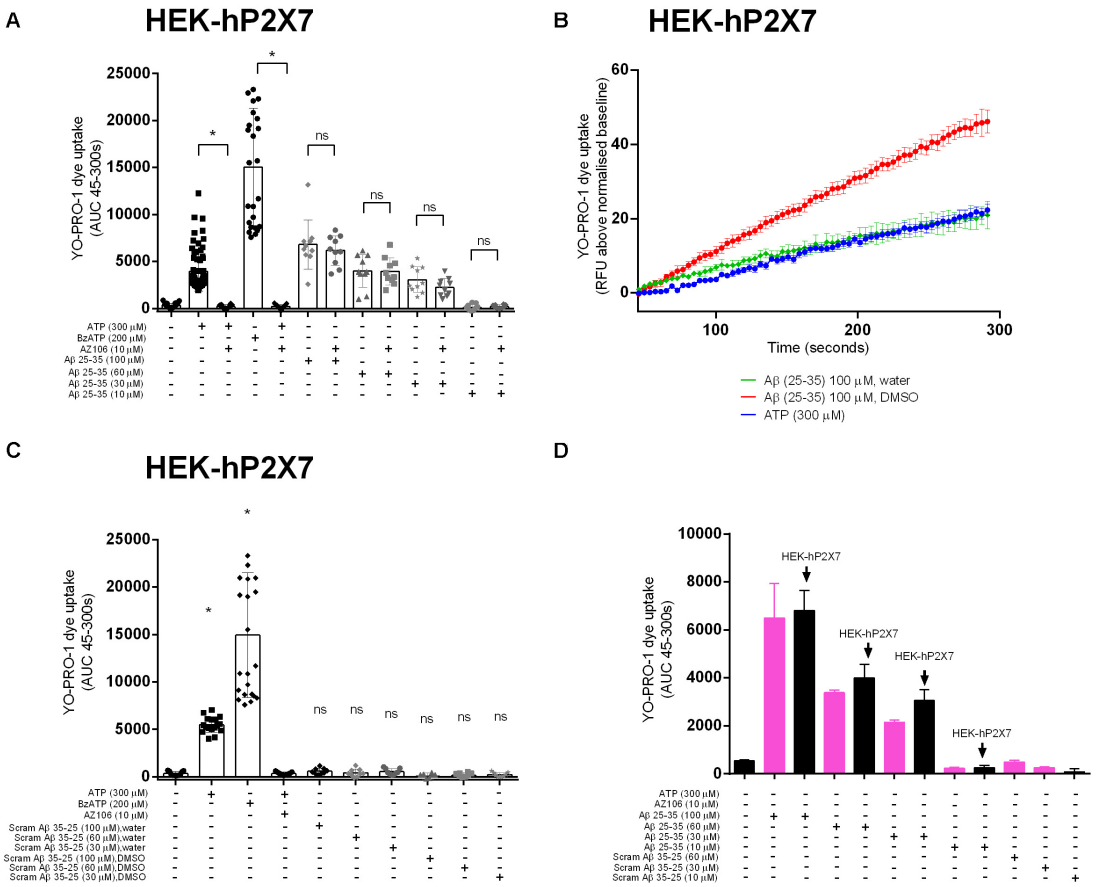

FIGURE 2 | Aβ peptides triggered a non-specific YO-PRO-1 uptake in HEK-hP2X7 and HEK-293 cells. HEK-hP2X7 and plain HEK-293 cells were incubated with YO-PRO-1 dye in low-divalent assay buffer (2 μM) and challenged with either $A\beta_{25-35}$ **(A,B)** or the inactive scrambled version $A\beta_{35-25}$ **(C)**. Kinetics of the $A\beta_{25-35}$ dye uptake response in HEK-hP2X7 are plotted together with the appropriate controls **(B)**. AZ10606120 is a selective antagonist of hP2X7, and BzATP is a synthetic agonist for hP2X7 receptors. **(D)** shows summary data from HEK-293 cells comparing responses to HEK-hP2X7 cells. Experiments were repeated five times with triplicates on each plate, and the results are presented as mean ± SD. One-way ANOVA was performed with Tukey's multiple comparison test where * indicates a significant difference ($P < 0.05$) to the paired control [with AZ1060610 in **(A)** or control in **(C)**] and ns denotes no significant difference.

has been a suggestion that ATP may not be the only agonist at P2X7 receptors (Di Virgilio et al., 2018). It is proposed that agents such as amyloid–β (Sanz et al., 2009), serum amyloid (Niemi et al., 2011) and the cathelicidin LL-37 peptide (Elssner et al., 2004), may function as non-nucleotide agonists at P2X7. In the current study, we revisited the role of the amyloid-β peptide as an agonist for the P2X7 receptor using a microglial cell line and HEK-293 cells over-expressing human P2X7. We have previously used the microglial BV-2 cells and a clonal P2X7-deficient BV-2 cell line, generated using Cas9 gene editing, in order to assess Ca^{2+} influx upon P2X7 activation with ATP and the effect of positive modulators (Dhuna et al., 2019).

The biological effect of synthetic amyloid-β peptides, including $A\beta_{25-35}$, may vary due to differences in aggregation states (Pike et al., 1993; Wei and Shea, 2006), therefore we performed the experiments using two common solvents, DMSO (Mattson et al., 1993) and water (Whitson et al., 1994). Our data shows that $A\beta_{25-35}$ directly induced intracellular Ca^{2+} responses in BV-2 microglial cells regardless of the solvent (**Figure 1**) although responses were noted to be higher for the DMSO-dissolved peptide. We saw no increase in $[Ca^{2+}]_i$ to the full length human $A\beta_{1-42}$ peptide. Furthermore, when these two amyloid-β peptides ($A\beta_{25-35}$ and $A\beta_{1-42}$) were studied in a HEK-hP2X7 stable cell line using a dye uptake assay (Helliwell et al., 2015; Bibic et al., 2019; Bidula et al., 2019; Dhuna et al., 2019), we observed some YO-PRO-1 dye uptake in response to $A\beta_{25-35}$. However, this also occurred in non-transfected HEK-293 cells that do not express P2X7 receptors (**Figure 2**). We saw no increase in YO-PRO-1 dye uptake to the full length human $A\beta_{1-42}$ peptide. Collectively this data suggests that amyloid-β peptides may act on other receptors that can induce similar responses to P2X7 or the peptide may act by insertion into the lipid bilayer forming similar pores. It is known that other agents such as maitotoxin and ionomycin can induce dye uptake responses to P2X7 stimulation (Schilling et al., 1999; Verhoef et al., 2004) likely through insertion into the membrane and subsequent pore formation. There is evidence that amyloid-β peptides

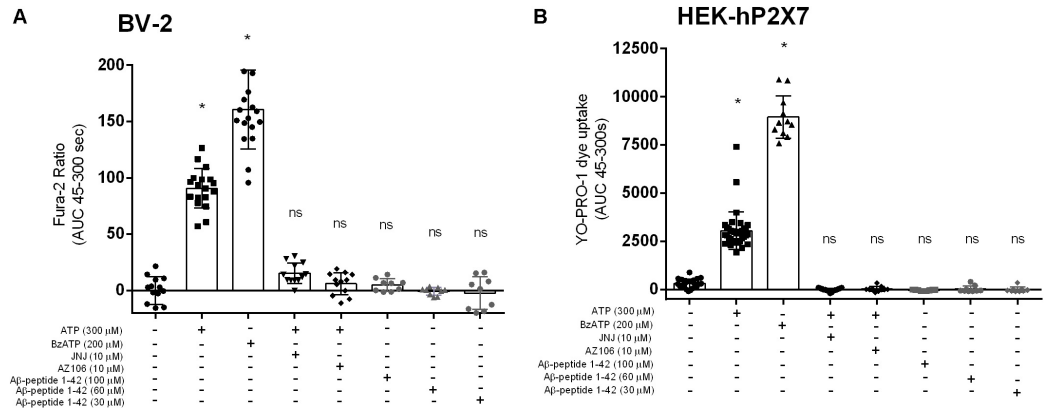

FIGURE 3 | $A\beta_{1-42}$ do not trigger responses in BV-2 cells or HEK-hP2X7 cells. BV-2 cells were loaded with Fura-2AM (2 μM) and challenged with the $A\beta_{-1-42}$ **(A)** peptides in the concentration range 30–100 μM. HEK-hP2X7 cells were exposed to YO-PRO-1 dye in low-divalent assay buffer (2 μM) and challenged similarly as BV-2 cells **(B)**. AZ10606120 is a commercially available antagonist of hP2X7. Experiments were repeated five times with triplicates on each plates, and the results are presented as mean ± SD. One-way ANOVA was performed with Tukey's multiple comparison test where * indicates a significant difference ($P < 0.05$) to the control (buffer alone) and ns denotes no significant difference.

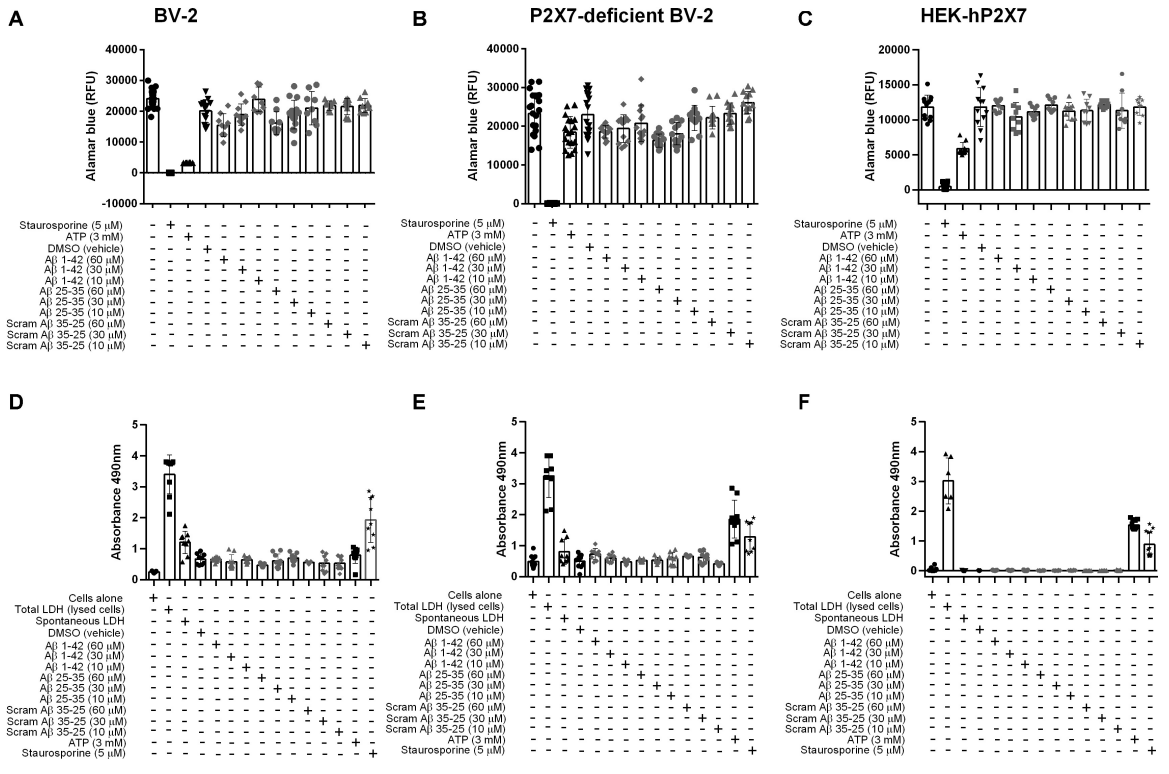

FIGURE 4 | Aβ peptides do not trigger P2X7-specific cell death nor compromise the cell membrane in microglial BV-2 cells or HEK-hP2X7 cells. **(A–C)** An AlamarBlue assay and **(D–F)** an LDH assay were performed to assess cell viability and cell death respectively after 24 h administration of Aβ peptides. At indicated times (see "Materials and Methods"), the extracellular medium was collected and assayed for lactate dehydrogenase (LDH) activity. Control cells were lysed with the lysis buffer to induce maximal LDH release for normalization of the LDH release (% of max). Staurosporine and 3 mM ATP were used as a positive control. Data points represent the mean ± SD of 5 replicated experiments with triplicates on each plate. One-way ANOVA was performed with Dunnett's multiple comparison test where * indicates a significant difference ($P < 0.05$) to the control (media alone).

may act via other routes such as on pannexin-1 (Orellana et al., 2011) to cause ATP release from cells rather than acting to directly activate P2X7. This was also suggested to be the likely (indirect) effect on microglial cells in the work by Sanz et al. (2009). We did not directly measure ATP release in our study but we hypothesized that any ATP

released by the amyloid-β peptides would elicit responses at P2X receptors and therefore we would have seen an effect of the P2X7-selective antagonists, apyrase, or in the BV-2 cells with P2X7 deficiency.

Human and rodent (rat/mouse) full length amyloid-β peptide (1–42) are highly similar and differ only in three amino acid substitutions at the N-terminus. It is not clear which isoform of the $A\beta_{1-42}$ was used in the study by Sanz et al. (2009) however, we believe this minor sequence difference is unlikely to contribute to our lack of effect at P2X7. Indeed, should there be a species difference, we would expect to see responses at human P2X7 (which we did not). The amino acid sequence for $A\beta_{25-35}$ peptide is identical between human and rodent. In our study we focused on addressing whether the amyloid-β peptides could act as agonists at P2X7. We did not extend our experiments to investigate the effect of amyloid-β peptides on IL-1β secretion from microglial cells. Gustin et al. demonstrated that $A\beta_{25-35}$ could induce IL-1β secretion from LPS-primed mouse microglia but did not detect any ATP secretion in response to $A\beta_{25-35}$. Furthermore, this team of researchers used primary microglia from the $P2X7^{-/-}$ mouse and observed a similar IL- 1β secretion in response to $A\beta_{25-35}$ (Gustin et al., 2015). This notion led them to conclude that $A\beta_{25-35}$-induced IL-1β secretion was P2X7-independent (Gustin et al., 2015). This contradicts the earlier work from Sanz et al. who demonstrated that amyloid peptides could induce IL-1β release from primary microglia but only when P2X7 was present (Sanz et al., 2009). Furthermore, *in vivo* evidence showed that amyloid β-induced IL-1β secretion in the hippocampus was reduced in P2X7 knockout mice (Sanz et al., 2009). Other studies have shown that activation of microglia with $A\beta_{1-42}$, followed by exposure to BzATP, may result in enhanced secretion of IL-1β (Rampe et al., 2004). This suggests that amyloid-β peptides may be working as positive allosteric modulators rather than as agonists. Furthermore, McLarnon et al. demonstrated that Ca^{2+} responses in adult microglial cells from Alzheimer's disease patients were significantly increased following $A\beta_{1-42}$ pre-treatment when activated by a P2X7 selective agonist BzATP (McLarnon et al., 2006). We have tested the idea that amyloid-β peptides could act as positive allosteric modulators since this was our entry point into this set of experiments. However, we saw no enhancement of ATP-induced $[Ca^{2+}]_i$ responses by $A\beta_{1-42}$ peptide and no potentiation of ATP-induced YO-PRO-1 dye uptake in HEK-hP2X7 cells (**Supplementary Figure S3**).

Microglial cell death (measured as a reduction in cellular viability) can be observed when cells were stimulated by 3 mM ATP (Nishida et al., 2012; Dhuna et al., 2019). When the BV-2 cells were stimulated by either $A\beta_{1-42}$, $A\beta_{25-35}$ or the inactive scrambled $A\beta_{35-25}$ peptide (60 μM), microglial cell viability was reduced by 35% compared to control. There was no significant increase in LDH release above vehicle treatment or spontaneous LDH release induced by water. Our data indicate that Aβ peptides may not act as non-nucleotide agonists of the P2X7 receptor and that Aβ peptides are unable to induce cytotoxicity or decrease cell viability directly via P2X7. We are not discarding P2X7 as an emerging therapeutic target for Alzheimer's disease. Others have shown that P2X7 is involved in amyloid protein precursor (APP) processing (Delarasse et al., 2011) and affects phagocytosis of Aβ peptides (Ni et al., 2013). More recently, the P2X7 knockout mouse was investigated in the APPPS1 mouse model of Alzheimer's disease (Martin et al., 2019). $P2X7^{-/-}$mice had a reduced amyloid-β load and were protected from cognitive defects. In this model the chemokines CCL3, CCL4, and CCL5 were elevated only when P2X7 was present and this affected $CD8^+$ T cell recruitment to the choroid plexus and hippocampus (Martin et al., 2019). It appears that there is still more to be discovered about how P2X7 can influence the development of neurodegenerative disorders.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

LB conceived and designed the study together with LS. LB performed the experiments and analyzed the data. LS and LB wrote the manuscript and edited the final version. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2020.00166/full#supplementary-material

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P2X7 Receptor Upregulation in Huntington's Disease Brains

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Huntington's disease (HD) is a fatal degenerative disorder affecting the nervous system. It is characterized by motor, cognitive, and psychiatric dysfunctions, with a late onset and an autosomal dominant pattern of inheritance. HD-causing mutation consists in an expansion of repeated CAG triplets in the huntingtin gene (*HTT*), encoding for an expanded polyglutamine (polyQ) stretch in the huntingtin protein (htt). The mutation causes neuronal dysfunction and loss through multiple mechanisms, affecting both the nucleus and cytoplasm. P2X7 receptor (P2X7R) emerged as a major player in neuroinflammation, since ATP – its endogenous ligand – is massively released under this condition. Indeed, P2X7R stimulation in the central nervous system (CNS) is known to enhance the release of pro-inflammatory cytokines from microglia and of neurotransmitters from neuronal presynaptic terminals, as well as to promote apoptosis. Previous experiments performed with neurons expressing the mutant huntingtin and exploiting HD mouse models demonstrated a role of P2X7R in HD. On the basis of those results, here, we explore for the first time the status of P2X7R in HD patients' brain. We report that in HD postmortem striatum, as earlier observed in HD mice, the protein levels of the full-length form of P2X7R, also named P2X7R-A, are upregulated. In addition, the exclusively human naturally occurring variant lacking the C-terminus region, P2X7R-B, is upregulated as well. As we show here, this augmented protein levels can be explained by elevated mRNA levels. Furthermore, in HD patients' striatum, P2X7R shows not only an augmented total transcript level but also an alteration of its splicing. Remarkably, P2X7R introns 10 and 11 are more retained in HD patients when compared with controls. Taken together, our data confirm that P2X7R is altered in brains of HD subjects and strengthen the notion that P2X7R may represent a potential therapeutic target for HD.

Keywords: P2X7, purines, ATP, Huntington's disease, neuroinflammation, neurodegenerative disease

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INTRODUCTION

Huntington's disease (HD) is a neurodegenerative disorder characterized by motor dysfunction, cognitive decline, and psychiatric symptoms (McColgan and Tabrizi, 2018). The HD-causing mutation lies in the huntingtin (*HTT*) gene. In normal population, *HTT* exon 1 includes from 6 to 35 repeats of the CAG triplet, while HD patients have 40 or more repeats (MacDonald et al., 1993). This mutation has an autosomal dominant inheritance, is highly penetrant, and initiates the disease through various mechanisms (Duyao et al., 1993). On one hand, the expanded CAG repeats of

the *HTT* mRNA are able to trap several RNA-binding proteins (Mykowska et al., 2011), thus likely provoking their loss of function. Interestingly, splicing factors and spliceosome components are among the sequestered proteins (Schilling et al., 2019). As a consequence, at least two pathogenic mis-splicing events, affecting *HTT* and *MAPT*, have been reported (Fernández-Nogales et al., 2014; Neueder et al., 2018). On the other hand, the expanded CAG triplets encode for an abnormally long polyglutamine (polyQ) trait in the N-terminus of the huntingtin protein (htt), which accounts for a toxic gain of function, as well (MacDonald et al., 1993). Both exon 1 and exon 1-like fragments contain the expanded polyQ trait and have been reported to trigger HD through toxic protein–protein interactions. Besides, htt fragments are prone to aggregate into microaggregates, fibrils, and inclusion bodies, which in turn may sequester other proteins, both in the nucleus and in the cytoplasm (Bates et al., 2015). All this leads to global neuronal impairment and death. Medium spiny neurons of the striatum are the most vulnerable cell type to mutant htt, which triggers striatal atrophy, the main hallmark in HD patients (McColgan and Tabrizi, 2018). Nevertheless, mutant htt is expressed throughout the whole brain, provoking degeneration in many other regions of the CNS.

P2X7 receptor (P2X7R) is a cation channel modulated by endogenous ATP. A single P2X7R subunit comprises a short intracellular N-terminus, two transmembrane motifs (TM1 and TM2) separated by an extracellular loop, and an intracellular C-terminus tail. P2X7R subunits coassemble in a trimeric chalice-like architecture provided of three ATP binding pockets (McCarthy et al., 2019; Rump et al., 2020). When ATP binds to P2X7R, the channel is open, allowing the transit of small cations, e.g., Na^+, Ca^{2+}, and K^+ (Surprenant et al., 1996). However, when the ATP activation is prolonged or repeated, P2X7R acts as a non-selective pore, which allows transit of large-molecular-weight molecules (Surprenant et al., 1996). It has been shown that both the TM2 domain (Sun et al., 2013) and the C-terminus (Alloisio et al., 2010) play a pivotal role in the pore formation. P2X7R is expressed in many cell types, including neurons and brain glial cells (microglia, astrocytes, and Muller cells) (Armstrong et al., 2002; Hervás et al., 2003; Miras-Portugal et al., 2003). ATP is released as cotransmitter via synaptic vesicles, and thus, a transient and localized increase in extracellular ATP can follow neuronal activity. Accordingly, a function of P2X7R in neuron–neuron communication and plasticity has been established (Sperlágh et al., 2006). However, wider ATP release is observed in response to adverse events, including ischemia, hypoxia, mechanical stimuli, bacteria, or toxin exposure (Csölle and Sperlágh, 2010). This kind of high levels of extracellular ATP leads to P2X7R-mediated neuron–glia crosstalk and glial activation, which in turn causes P2X7R upregulation. In general, P2X7R stimulation leads to a Ca^{2+} influx, which has different consequences depending on the cell type where it occurs. P2X7R provokes glutamate release from presynaptic nerve terminals and from astrocytes, responsible for an excitotoxic effect (Sperlágh et al., 2002), while astrocytes and microglia are accountable for IL-1β, IL-6, and TNF-α release, triggering neuroinflammation (Solle et al., 2001). ROS production, combined with BDNF downregulation, is a further mechanism by which neuronal damage and reactive gliosis are achieved via P2X7R (Ficker et al., 2014). Under prolonged stimulation, P2X7R pore opens, prompting plasma membrane blebbing and lastly cell death (Sorge et al., 2012). All this makes P2X7R a key player in neuroinflammation.

Notably, different P2X7R transcript variants deriving from alternative splicing exist in both human and mouse. In particular, 10 isoforms (from *A* to *J*) have been described for the human receptor (Cheewatrakoolpong et al., 2005). *P2 × 7R-A* comprises 13 exons and corresponds to the canonical transcript (Buell et al., 1998). *P2 × 7R-B* differs from it for the retention of the 84-nucleotide-long intronic region between exon 10 and exon 11, while variants *P2 × 7R-C/D/E/F/J* lack either exon 4, 5, 7, 8, or 7 and 8 together. *P2 × 7R-G* and *P2 × 7R-H* have an extra exon named N3 between exon 2 and exon 3. *P2 × 7R-I* lacks both exon 2 and N3. Transcripts *E*, *G*, and *I* also present the intron 10–11 retention. Of such transcripts, four have been extensively studied since they originate proteins. Therefore, not a unique P2X7R exists. Rather, four P2X7Rs have been described based on alternative splicing: P2X7R-A, P2X7R-B, P2X7R-H, and P2X7R-J (Cheewatrakoolpong et al., 2005; Feng et al., 2006).

P2 × 7R-A encodes the well-characterized full-length P2X7R-A. It includes 595 aa constituting the N-terminus, TM1 and TM2 separated by an extracellular loop, and the intracellular C-terminus of the protein. The N-terminus can form intracellular complexes with many substrates including heat shock proteins, β2-integrin, α-actin, and several protein kinases and phosphatases (Kim et al., 2001). The extracellular loop owns the ligand-binding sites and a number of N-glycosylation sites (Wang et al., 2005). The C-terminus of P2X7R-A, due to multiple protein–protein and protein–lipid interaction motifs (Denlinger et al., 2001), contributes to its communication with cytoskeletal and intracellular proteins (Kim et al., 2001) and is required for the formation of a pore, hence eliciting many functions of the receptor. *P2 × 7R-B* is the transcript for P2X7R-B and lacks the C-terminus as a consequence of the premature stop codon introduced by the intron 10–11 retention. Accordingly, this protein comprises 364 aa, where the last 18 aa are different from those of P2X7R-A. Interestingly, *P2 × 7R-B* seems to be the predominant P2X7R transcript in multiple human tissues, including the brain (Cheewatrakoolpong et al., 2005; Adinolfi et al., 2010). Experiments in HEK293 cells expressing P2X7R-B demonstrate its ability to form homotrimers and maintain all the ATP-stimulated channel functions, despite being unable to form a non-selective pore and trigger apoptosis. Thus, P2X7R-B is free of the cytotoxic activity linked to the C-terminal tail and is generally considered a less "dangerous" form of P2X7R. However, when coexpressed, P2X7R-A and P2X7R-B can heterotrimerize efficiently. In this case, P2X7R-B potentiates P2X7R-A functions, including the formation of a pore and proapoptotic activity. Therefore, cells could modulate ATP responses by P2X7R-A and P2X7R-B expression ratio and combination in trimers (Adinolfi et al., 2010). A pathophysiological role of P2X7R-B has been described in multiple conditions, including bone cancer (Giuliani et al., 2014) and bone differentiation (Carluccio et al., 2019). It has also been described in neural progenitors (Glaser et al.,

2014), neuroblastoma cells (Ulrich et al., 2018), and glioblastoma cells (Ziberi et al., 2019). P2X7R-H contains 505 aa and is also known as P2X7R-ΔTM1, since the TM1 is absent. Indeed, *P2 × 7R-H* contains the N3 exon, which creates a new start codon responsible for the absence of the first part of the protein. However, when transfected in HEK293 cells, P2X7R-H is an inactive receptor (Cheewatrakoolpong et al., 2005). P2X7R-J includes only 258 aa and lacks the C-terminus, the TM2, and part of the extracellular loop. Still, P2X7R-J can form heterotrimers with P2X7R-A. It emerged to act as a dominant negative, since it antagonizes the function of P2X7R-A in cervical cancer cells (Feng et al., 2006). To date, the implication of such variety around P2X7Rs expression in both physiological and pathological backgrounds has never been explored in the nervous system.

Cell injury is a common feature of neuroinflammatory and neurodegenerative disorders, including traumatic brain injury, stroke, epilepsy, neuropathic pain, amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer's disease, Parkinson's disease, and HD. When damaged, cells release a great amount of ATP, which acts on P2X7R. Therefore, P2X7R has been proposed as a pivotal player in all these conditions and a potential common target for their treatment (Díaz-Hernández et al., 2009; Kim et al., 2009; Sanz et al., 2009; Amadio et al., 2011; Lämmer et al., 2011; Kimbler et al., 2012; North and Jarvis, 2013; Carmo et al., 2014). Besides, and regarding HD, P2X7R upregulation has been described in the striatum of R6/1 and Tet/HD94 mouse models of HD (Díaz-Hernández et al., 2009). Moreover, cultures of primary neurons from such mice showed that P2X7R exhibits an altered permeability to calcium, suggesting a different functional state of the receptor and higher vulnerability to P2X7R-mediated apoptosis (Díaz-Hernández et al., 2009), thus indicating a direct link between P2X7R and HD. Remarkably, the report of a lower rate of neuronal apoptosis and motor impairment recovery in HD mice following the administration of Brilliant Blue-G (BBG), a P2X7R antagonist, further suggested a contribution of P2X7R to this pathology (Díaz-Hernández et al., 2009), pinpointing P2X7R blockade as a possible therapeutic approach for HD. As, to date, P2X7R has not been explored in brains of HD subjects, here, we aim to further validate the possible role of P2X7 in HD pathogenesis.

MATERIALS AND METHODS

Human Brain Tissue Samples

Striatal tissues, including the caudate and putamen, from control and HD individuals were provided by Banco de Tejidos Fundación Cien (BT-CIEN, Madrid, Spain; CTRL n = 5, HD n = 7), the Netherlands Brain Bank (Amsterdam, The Netherlands; CTRL n = 4, HD n = 5), and Banc de Teixits Neurològics (Barcelona, Spain; CTRL n = 6, HD n = 6). Controls and HD subjects are matched by age (CTRL = 56 ± 3.8; HD = 63 ± 3.5) and sex (of the total, CTRL: M = 70%, F = 30%; in HD: M = 60%, F = 40%). Postmortem interval (PMI) is lower than 24 h in all cases. HD subjects' mean of CAG repetitions is 45.7 ± 1.4. Written informed consent for brain removal after death for diagnostic and research purpose was obtained from brain donors and/or next of kin.

Antibodies

In order to explore P2X7Rs at the protein level in brain samples, different antibodies have been employed:

Host	Brand	Reference	Directed to	Epitope location	Epitope sequence	Detects
Rabbit	Alomone Lab	APR-004	C-terminal	rat epitope 576–595	KIRKEFPKTQ GQYSGFKYPY	P2X7R-A
						P2X7R-H
Goat	NovusBio	NBP1-37775	N-terminus	human epitope 13–26	YETNKV TRIQSMNY	P2X7R-B
						P2X7R-J
Rabbit	Alomone Lab	APR-008	extracellular	mouse epitope 136–152	KKGWMDP QSKGIQTGRC	all P2X7Rs

Western Blot Analysis

Different cohorts of samples from human brains were stored at −80°C and ground with a mortar in a frozen environment with liquid nitrogen to prevent thawing of the samples, resulting in tissue powder. Protein extracts were prepared by homogenizing tissue powder in ice-cold extraction buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 20 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 1 μM okadaic acid, 5 mM sodium pyrophosphate, 30 mM β-glycerophosphate, 5 mM EDTA, protease inhibitors (Complete, Roche, cat. no. 11697498001). Homogenates were centrifuged at 15,000 rpm for 15 min at 4°C. The resulting supernatant was collected, and protein content determined by Quick Start Bradford Protein Assay (Bio-Rad, 500-0203). Ten micrograms of total protein was electrophoresed on 10% SDS-polyacrylamide gel, transferred to a nitrocellulose blotting membrane (Amersham Protran 0.45 μm, GE Healthcare Life Sciences, 10600002), and blocked in TBS-T (150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 0.1% Tween 20) supplemented with 5% non-fat dry milk. Membranes were incubated overnight at 4°C with different anti-P2X7R antibodies directed to C-terminus (rabbit, 1:1,000, Alomone Lab, APR-004), N-terminus (goat, 1:500, NovusBio, NBP1-37775), or extracellular domain (rabbit, 1:200, Alomone Lab, APR-008) in TBS-T supplemented with 5% non-fat dry milk, washed with TBS-T, and next incubated with HRP-conjugated anti-rabbit IgG (1:2,000, DAKO, P0448) or anti-goat IgG (1:5,000, Bethyl, A50-101P) and developed using the ECL detection kit (PerkinElmer, NEL105001EA). As a loading control, β-actin (1:50,000, Sigma, A2066), α-tubulin (1:20,000, Sigma, T9026), and vinculin (1:20,000, Abcam, ab129002) were used. Densitometric analysis was carried out by using a densitometer (Bio-Rad GS800). Quantification was performed by using Image Lab 5.2 software (Bio-Rad). In all cases, the average intensity value of the pixels in a background-selected region was calculated and was subtracted from each pixel in the samples. The densitometry values obtained in the linear range of detection with the antibody was normalized with

respect to loading controls to correct for any deviation in loaded amounts of protein.

Immunohistochemistry

Formalin-fixed (4%, 24 h), paraffin-embedded tissues from the striatum were used (CTRL $n = 3$, HD $n = 3$). Sections (5 μm thick) were mounted on SuperFrost Plus tissue slides (Menzel Gläser) and deparaffinized. Brain sections were immersed in 0.3% H_2O_2 in methanol for 45 min to quench endogenous peroxidase activity. Sections were then immersed for 1 h in blocking solution (PBS containing 0.5% fetal bovine serum, 0.3% Triton X-100, and 1% BSA) and incubated overnight at 4°C with C-terminal-directed anti-P2X7R (rabbit, 1:1,000, Alomone Lab, APR-004) or N-terminal-directed anti-P2X7R (goat, 1:500, NovusBio, NBP1-37775), diluted in blocking solution. After washing, brain sections were incubated first with biotinylated anti-rabbit or anti-goat secondary antibody and then with avidin–biotin complex using the Elite VECTASTAIN kit (Vector Laboratories, PK-6101 and PK-6105). Chromogen reactions were performed with diaminobenzidine (SIGMAFAST DAB, Sigma, D4293) for 10 min. Sections where first dehydrated and then mounted with DePeX (SERVA). Images were captured using an Olympus BX41 microscope with an Olympus camera DP-70 (Olympus Denmark A/S).

RNA Extraction and cDNA Synthesis

Total tissue RNA was extracted from the striatum of CTRL and HD patients using the Maxwell® 16 LEV simplyRNA Tissue Kit (Promega, AS1280). Quantification and quality of RNA were done on a NanoDrop ND-1000 spectrophotometer and NanoDrop 1000 v.3.7.1 (Thermo Scientific). Retrotranscription (RT) reactions were performed using the iScript cDNA synthesis kit (Bio-Rad, PN170-8891) following manufacturer's instructions. Briefly, 1 μg of total RNA from each sample and 4 × Master Mix (which includes all necessary reagents, a mixture of random primers, and oligo-dT for priming) were brought to a final volume of 40 μl with DNase/RNase-free distilled water (Gibco, PN10977). Thermal conditions consisted of 5 min at 25°C, 30 min at 46°C, and 5 min at 95°C.

P2X7R Quantitative and Semi-Quantitative PCR

Quantitative RT-PCR was performed using gene-specific primers and TaqMan MGB probes for human P2X7R (forward, 5′-GTGAACCAGCAGCTACTAGGGAG-3′; reverse, 5′-TGAAGTCCATCGCAGGTCTTG-3′), β-actin, and GAPDH. Fast thermal cycling was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems) as follows: denaturation, one cycle of 95°C for 20 s, followed by 40 cycles each of 95°C for 1 s and 60°C for 20 s. The results were normalized as indicated by parallel amplification of the endogenous controls β-actin and GAPDH. Semi-quantitative RT-PCR was performed by designing specific primers in P2X7R exon 10 and exon 11 (forward, 5′-CATCGGCTCAACCCTCTCCTA-3′; reverse, 5′-TTTGGCTCCACAATGGACTCG-3′) to amplify the intron 10–11 retaining isoforms in human brain cDNA. PCR amplification protocol used 5 min 94°C + 30 cycles (30 s at 94°C + 30 s at 58°C + 2 min at 72°C) + 7 min at 72°C. The PCR product was resolved on 1.5% agarose/GelGreen (Biotium, 41004) gels run at 120 V for 1.5 h. Images were taken in a UviDoc transilluminator (UviTec) and then scanned with densitometer (Bio-Rad, GS-900) and quantified with Image Lab 5.2 (Bio-Rad).

P2X7R Transcript Level and Splicing Alteration in HD by RNA-Seq

P2X7R expression levels in HD and CTRL postmortem striatum samples were evaluated from our RNA-seq data (CTRL = 3 vs. HD = 3) (Elorza et al., 2020). For total mRNA transcript levels, reads were aligned against the *Homo sapiens* genome (GRCh38.p2 version) using the TopHat2 aligner (Trapnell et al., 2009), and differentially expressed genes were obtained with the Cuffdiff software (Trapnell et al., 2013). For the splicing analysis, percent spliced in (PSI) values were obtained by running vast-tools (Vertebrate Alternative Splicing and Transcription Tools) (Irimia et al., 2014). Alternatively, TopHat-aligned reads were run with the rMATS software for the detection of altered spliced events (Shen et al., 2014).

Statistics

All experiments were repeated at least three times, and the results are presented as the mean ± sem. Statistical analysis was performed with SPSS 21.0 (SPSS Statistic IBM). Where indicated, Student's *t*-test was applied, and false discovery rate (FDR) was calculated in the case of RNA-seq data.

RESULTS

P2X7R Protein Level Is Increased in the Striatum of HD Patients

To further explore the potential role of P2X7R in HD pathogenesis, we first analyzed by western blot the protein level of this receptor in the striatum of HD subjects, the most affected brain region in patients. As previously mentioned, human P2X7R mRNA can undergo differential splicing, thus originating multiple P2X7R variants. Of these, four are protein encoding and generate the P2X7R-A, P2X7R-B, P2X7R-H, and P2X7R-J isoforms, with expected molecular weights of 68.6, 41.8, 58.2, and 29.3 kDa, respectively. In order to detect P2X7R-A and P2X7R-H, which are provided with in the canonical C-terminus, we used a C-terminus-directed antibody. With this antibody, we observed a unique band of about 70 kDa, most likely corresponding to P2X7R-A, which was increased (2.8-fold, $p = 0.03$) in brain striatal extracts of HD patients (**Figure 1A**). This P2X7R upregulation in HD specimens is in accordance with the one previously obtained in the same brain region in two mouse models of HD (Díaz-Hernández et al., 2009). P2X7R-H, which indeed presents the same C-terminus sequence of P2X7R-A, has not been detected with this antibody, even after a prolonged exposure, neither in normal nor in pathological conditions. Regarding the C-terminus truncated isoforms, P2X7R-B and P2X7R-J, we opted for an N-terminus-directed antibody, which should

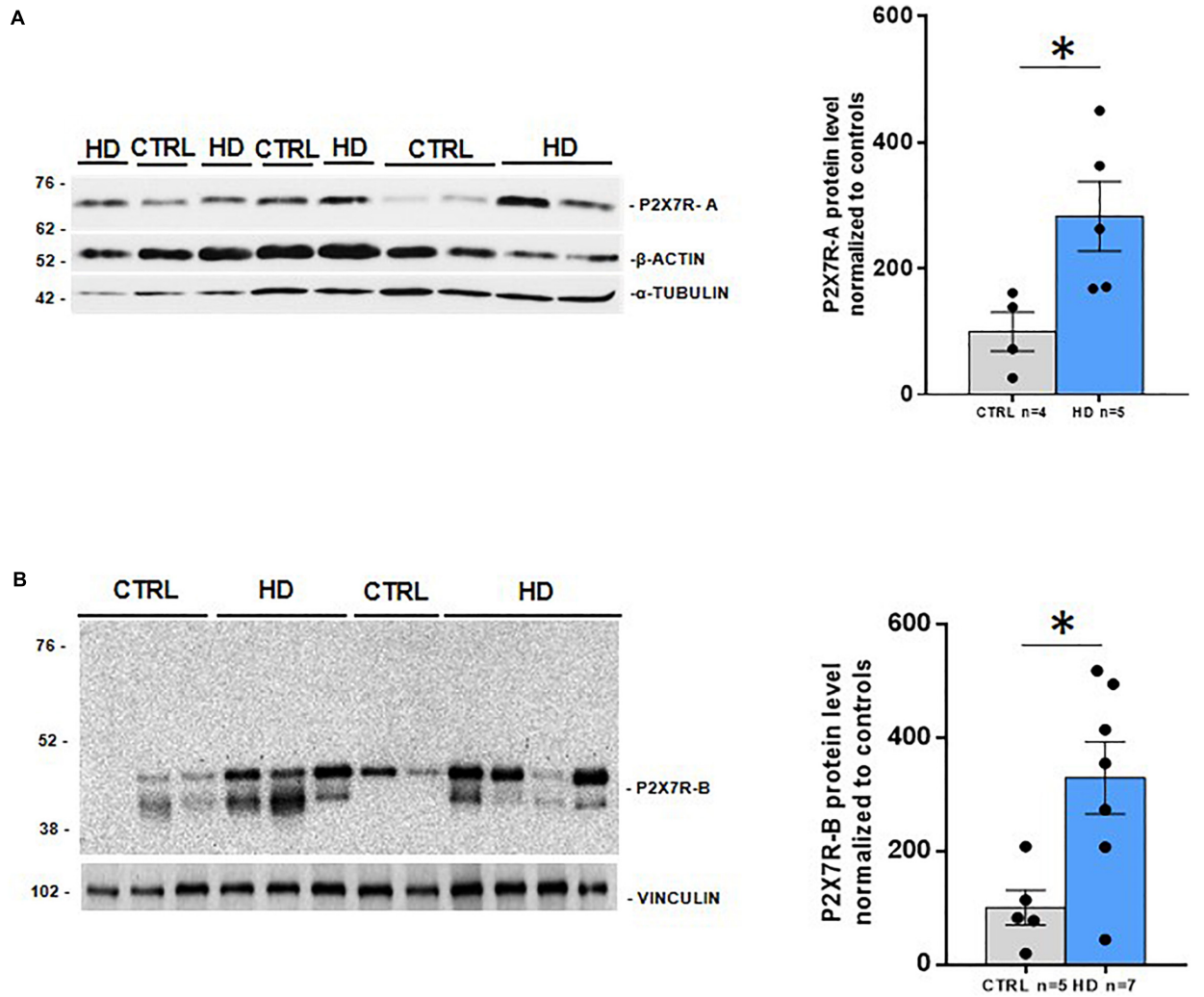

FIGURE 1 | Increased P2X7R protein levels in striatal post-mortem samples from HD patients. Western blot analysis of **(A)** P2X7R-A (CTRL $n = 4$, HD $n = 5$) with a C-terminus oriented antibody, and **(B)** P2X7R-B (CTRL $n = 5$, HD $n = 7$) with a N-terminus directed antibody, with their loading controls (below) and the corresponding quantifications (right). Graphs show mean ± SEM. Dots represent individual values. Student's *t*-test, $^*p < 0.05$.

also recognize the full-length form. First, after short exposure, we observed a doublet at 42–45 kDa, possibly corresponding to P2X7R-B. Such doublet results strongly increased (3.3-fold, $p = 0.01$) in the striatum of HD patients (**Figure 1B**). Second, following long exposure, a band can be observed in the range of 30–38 kDa, possibly corresponding to P2X7R-J (**Supplementary Figure S1**). In HD, such band shows a non-statistically significant increase due to the great dispersion of data within the HD group ($p = 0.2$). This antibody also detects a doublet at approximately 60 kDa. Such a molecular weight could correspond to a homodimerization of P2X7R-J; however, no change occurs between control and HD specimens ($p = 0.5$). Finally, a shade at approximately 70 kDa, possibly corresponding to P2X7R-A, can be detected. It seems, therefore, that this antibody is somehow less efficient than the C-terminus-directed one in detecting the full-length protein (**Supplementary Figure S1**). Since the extracellular loop represents a shared feature of all P2X7R isoforms, an extracellular loop-directed antibody has been used for the simultaneous detection of P2X7R-A and P2X7R-B (Carluccio et al., 2019; Ziberi et al., 2019). Unfortunately, this antibody was not sensitive enough to detect P2X7R in human postmortem brain samples (data not shown). Taken together, these results indicate that P2X7R is upregulated at the protein level in HD, with an augmented level of both full-length (P2X7R-A) and C-terminus lacking (P2X7R-B) forms of the receptor.

P2X7R Immunoreactivity Is Augmented in HD Brains

We further investigated P2X7R status in HD brains through immunohistochemistry, with the same two antibodies that were used for the western blot analysis. Detection of P2X7R immunoreactivity with the C-terminal antibody in the striatum

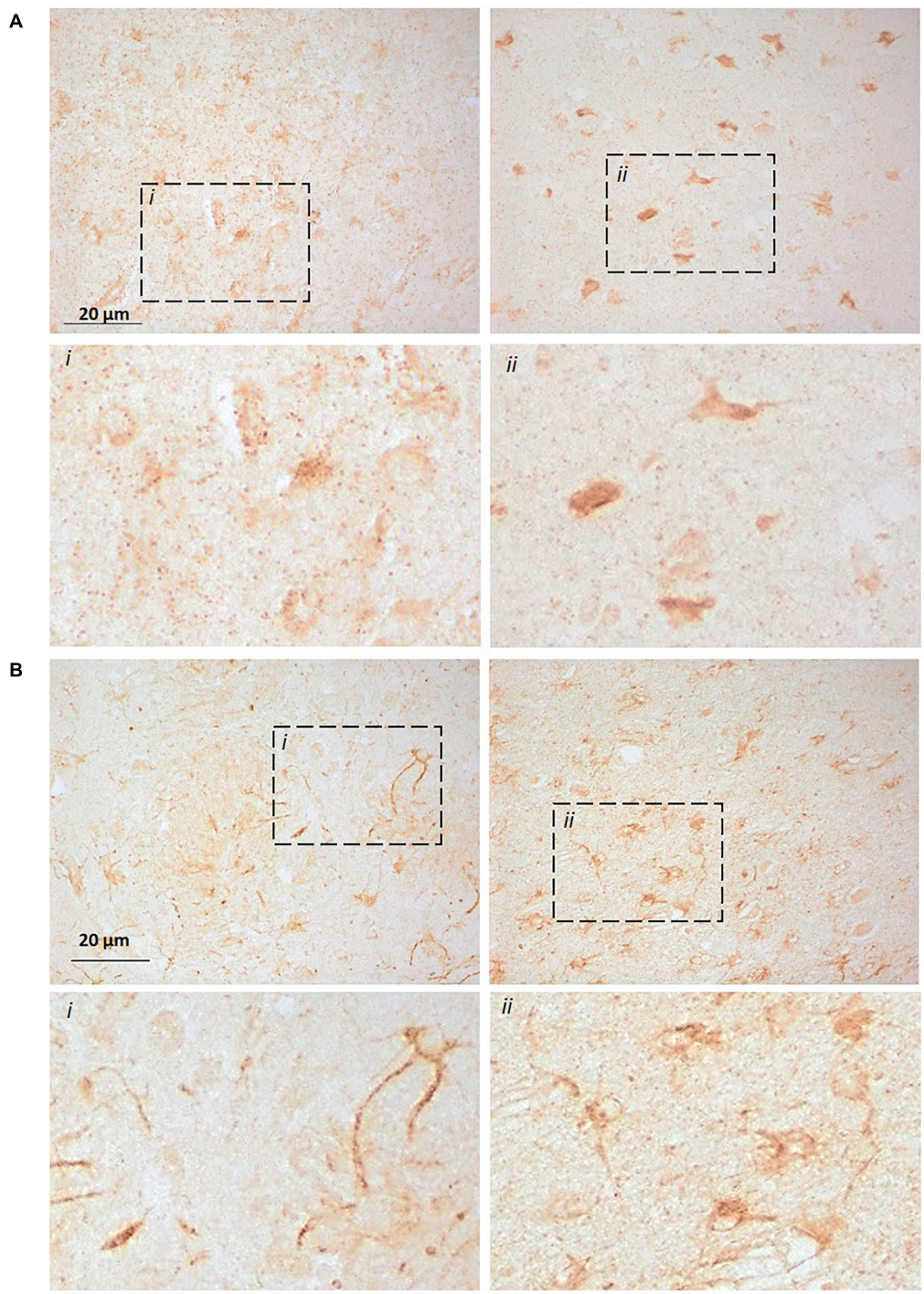

FIGURE 2 | Increased P2X7R-associated immunoreactivity in striatal sections from HD patients (CTRL *n* = 3, HD *n* = 3). Immunohistochemistry assay was carried out with **(A)** the C-terminus directed antibody or **(B)** the N-terminus directed antibody. In both cases, an augmented signal was observed in HD sections when compared to controls. Dash line boxes show the zoomed areas (i;ii) in both cases.

of control subjects shows a weak punctate and cytoplasmic pattern mainly in neurons and neuropil; in striatal sections from HD patients, we observe a more diffuse and intense reactivity, which in neurons appears to extend to their processes, and a higher number of clearly immunoreactive cells was seen (**Figure 2A**). Since no P2X7R-H-associated band has been

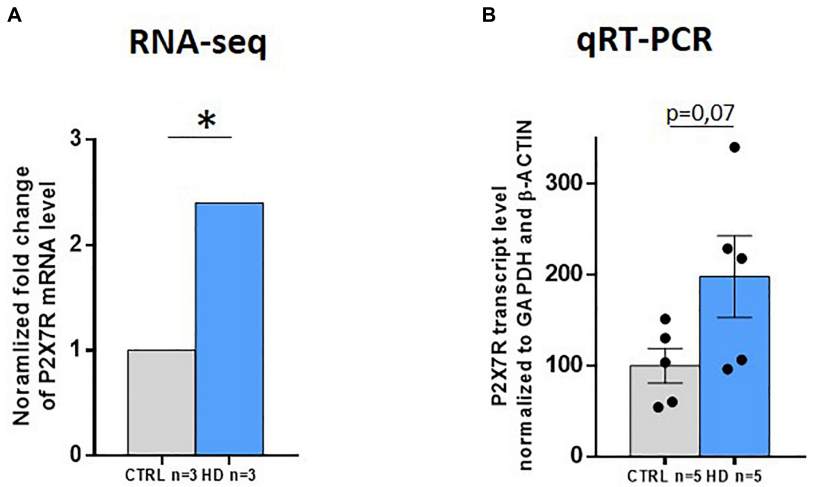

FIGURE 3 | P2X7R total transcript levels strongly increase in HD striatum. **(A)** P2X7R transcript level analyzed by RNA-seq (CTRL $n = 3$, HD $n = 3$). Fold-change with respect to controls. q = adjusted p-value. *$q < 0.05$. **(B)** P2X7R total transcript levels by qRT-PCR (CTRL $n = 6$, HD $n = 6$). GAPDH and β-actin were used as housekeeping genes for normalization. Student's t-test. Graphs show mean ± SEM. Dots represent individual values.

detected by western blot in this brain region, we can speculate that the signal detected here essentially corresponds to P2X7R-A.

Regarding the N-terminal antibody, the pattern in the striatum of controls was also cytoplasmic and punctate mainly in neurons, but neuronal processes also get clearly stained. This pattern seems to be maintained in HD striatal sections, but with more abundant immunoreactive somata (**Figure 2B**). Since by western blot this antibody identified mainly P2X7-B, while other bands emerged only after a prolonged exposure, we can speculate that such signal could essentially be related to P2X7R-B. This result, together with the increased protein level detected by western blot, highlights an alteration of P2X7R-A and P2X7R-B in HD brains.

P2X7R Transcript Alteration in HD Brains

Since P2X7R protein levels are augmented in HD striatum, we investigated whether this increase is associated with a higher amount of total P2X7R transcript in this brain area. To achieve such aim, we analyzed an RNA-seq study of the striatum of HD patients and controls that we have recently performed (Elorza et al., 2020). We found a strong increase in total P2X7R transcript level (2.4-fold, $q = 0.005$) in HD subjects (**Figure 3A**). We further analyzed P2X7R mRNA level in the striatum by quantitative-PCR (Q-PCR) in an independent cohort of patients. Coherently, we detected a tendency of having a higher amount of P2X7R transcripts (2.0-fold, $p = 0.07$) in this region when comparing HD subjects with controls (**Figure 3B**). Taken together, these results suggest that the striatal increase in P2X7R protein levels detected in HD is likely associated with augmented P2X7R total transcript levels in the same brain region. This is consistent with the previously reported trend of an increase in P2X7R mRNA in the brain of R6/1 and Tet/HD94 mouse models of HD (Díaz-Hernández et al., 2009).

On one hand, previous data have shown the existence of naturally occurring isoforms of P2X7R generated by differential splicing, which could play a role in physiological and/or pathological conditions (Cheewatrakoolpong et al., 2005; Feng et al., 2006; Adinolfi et al., 2010). On the other hand, splicing alterations have been reported to contribute to HD pathogenesis (Fernández-Nogales et al., 2014; Neueder et al., 2018). Thus, we wondered whether P2X7R suffers from splicing alterations in HD, as well. We therefore analyzed the RNA-seq study performed in the striatum with two different bioinformatic tools. By using vast-tools, we found that the inclusion level of the 84-nucleotide-long intronic region located between exon 10 and exon 11 is increased by 39% in the HD striatum ($p = 0.007$, **Figure 4A**). We also found that exon 4 inclusion was decreased about 12% in patients ($p = 0.01$, **Supplementary Figure S2A**). When we performed the equivalent analysis by using rMATS, we confirmed that intron 10–11 retention is increased by 41% (FDR = 0.002) (**Figure 4B**) and that exon 4 inclusion is about 8% lower (FDR = 0.04) in HD patients (**Supplementary Figure S2B**).

While no function has been annotated regarding exon 4, there is literature about intron 10–11 retention. As mentioned, it is involved in the production of the P2X7R-B protein (Cheewatrakoolpong et al., 2005), which has been described to play a role *in vitro* (Adinolfi et al., 2010). Therefore, we validated in an independent set of samples the increased retention of intron 10–11 by semi-quantitative RT-PCR with primers hybridizing in the flanking exons (10 and 11). This confirmed that, indeed, intron 10–11 are more retained in HD postmortem brains, since the proportion of transcripts including this region with respect to the non-intron-containing ones is more abundant in our HD sample set (3.5-fold, $p = 0.008$) (**Figure 4C**). Taken together, these results demonstrate that, in the striatum of HD subjects, P2X7R undergoes mis-splicing with slightly decreased inclusion of exon 4 and markedly increased retention of intron 10–11, the latter explaining the increase in the P2X7R-B isoform observed by western blot.

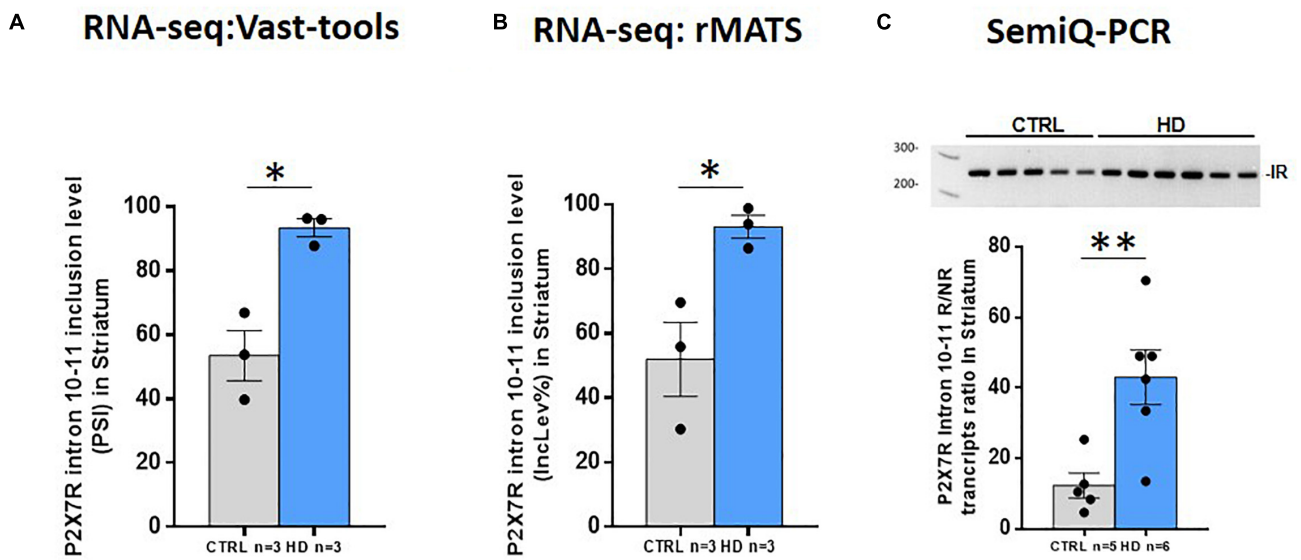

FIGURE 4 | P2X7R altered intron retention in HD striatum. **(A)** Percentage of spliced in (PSI) values for P2X7R intron 10–11 retention in control and HD according to Vast-tools analysis (ΔPSI = 39%). Student's *t*-test. *$p < 0.05$. **(B)** P2X7R intron 10–11 inclusion level percentage (IncLev%) for control and HD according to rMATS analysis (IncLevDiff% = 41%). *FDR < 0.05. **(C)** Semi-qRT-PCR directed to P2X7R intron 10–11, represented as the ratio between intron retaining/non-retaining transcripts, confirms increased retention in independent HD cases (CTRL $n = 5$, HD $n = 6$). Student's *t*-test. **$p < 0.01$. Graphs show mean ± sem. Dots represent individual values.

DISCUSSION

Here, we perform, to our knowledge, the first analysis of P2X7R status in brains of HD subjects. By analyzing P2X7R proteins through western blot and immunohistochemistry and P2X7R transcript isoforms through RNA-seq and subsequent validation by RT-PCR, here, we report a clear increase in the total levels of P2X7R. Both the full-length isoform P2X7R-A and the lower-molecular-weight isoform P2X7R-B show increased levels that can be explained by the changes observed at the transcript level, the latter affecting both the total transcript levels and the increased retention of intron 10–11 which originate the P2X7R-B isoform.

To date, no disease-modifying treatment is available for HD subjects, who only receive palliative care. Although a number of clinical trials have been completed during the past years, no compelling results emerged. Thus, the research of new therapeutic approaches, possibly acting on the multiple features of the disease, remains open. Previous data demonstrated that P2X7R participates in the modulation of neurotransmitter release (Deuchars et al., 2001) and also in microglial (Ferrari et al., 1997) and astroglial activation (Verderio and Matteoli, 2001), thus highlighting a role of this receptor in neuroinflammation. Since neuroinflammation is a shared feature of many CNS diseases, including HD, P2X7R has been proposed to be a potential therapeutic target for their treatment. More compelling evidence of P2X7R as a likely therapeutic target specifically for HD arose from experiments performed in various HD cellular and mouse models, where the receptor was found to be upregulated and functionally altered (Díaz-Hernández et al., 2009), thus suggesting a role of P2X7R in the pathophysiology of HD. Interestingly, antagonizing P2X7R through the administration of BBG provokes less body weight loss and improves motor coordination in HD mice (Díaz-Hernández et al., 2009). Those results strongly supported the hypothesis of P2X7R as a target for the treatment of HD. However, evidence of P2X7R being in fact altered in the brains of HD subjects was missing. In order to address the status of P2X7R in HD brains, here, we focused on the striatum, the most affected brain region in the disease.

In the present study, we investigated the protein level in control and HD striatal samples first by western blot with an antibody raised against a C-terminal epitope. This antibody is expected to recognize the full-length P2X7R-A isoform. In good agreement with the expected weight of 68.6 kDa, we detected a band at around 70 kDa, thus fitting P2X7R-A. Interestingly, experiments performed on a cervix cancer cell line with the same antibody showed that P2X7R-A can reach the weight of 85 kDa (Wang et al., 2005) due to the N-glycosylation at five different sites (Asn-187, Asn-202, Asn-213, Asn-241, and Asn-284) (Surprenant et al., 1996). Since a unique band was identified in our study, it seems that the N-glycosylated P2X7R is not detectable in striatal samples. Moreover, although the antibody could potentially detect P2X7R-H as well, we did not observe any signal at its expected molecular weight. We therefore believe that the P2X7R-H isoform is not expressed or that it is expressed at a very low amount in the human striatum. In summary, the western blot experiments with the C-terminal antibody allowed us to conclude that P2X7R-A is expressed in the human striatum and is upregulated in HD patients (**Figure 1A**). This result is in accordance with the previous one obtained in R6/1 mice in the same brain region (Díaz-Hernández et al., 2009). We have also explored at protein level the P2X7R isoforms lacking the

C-terminus, which are generated following differential splicing: P2X7R-B and P2X7R-J (Cheewatrakoolpong et al., 2005). We wondered whether these variants were expressed in the striatum and whether changes occur in the pathological context of HD. To address these questions, we used an antibody that binds an N-terminal sequence. We observed a doublet at 42/45 kDa possibly corresponding to P2X7R-B, which is upregulated in HD (**Figure 1B**). Regarding P2X7R-J, we observed a band between 30 and 38 kDa, which could correspond to such form, since the predicted molecular weight based on its sequence (UniProtKB–Q15G98) is 29.3 kDa. There is a non-significant tendency to an increase in HD regarding such band ($p = 0.2$) (**Supplementary Figure S1**). It must be considered that P2X7R-J has been discovered in cervical cancer cells (Feng et al., 2006). There, the group describes a 42/45 kDa doublet as P2X7R-J, as this isoform maintains four out of the five N-glycosylation sites of the full-length form that, after posttranslational modification, would increase its weight till 41.8 kDa. Since we did not obtain the 85-kDa band equivalent to the N-glycosylated form of P2X7R-A in our samples, we believe that the forms we are observing are the unglycosylated ones. In such condition and taking into consideration that the molecular weights of 42/45 kDa are closer to the one P2X7R-B is expected to show, 41.8 kDa, we consider that such doublet could correspond to P2X7R-B rather than to P2X7R-J.

The immunohistochemical analysis of P2X7R in striatal sections shows increased staining mainly in neurons, opposite to Alzheimer's disease tissue and mouse models which show increased P2X7R staining in glial cells (Martin et al., 2019), possibly indicating a major role of increased P2X7R in altered neurotransmission rather than in neuroinflammation in HD. The immunohistochemical analysis has been carried out by exploiting the same antibodies used for the western blot analysis. Since the C-terminus-directed antibody substantially recognizes P2X7R-A while the N-terminus-directed one recognizes mostly the P2X7R-B-associated bands, we can assume that these are the proteins accountable for the observed immunoreactivities. However, we cannot exclude that the signal observed in this brain sections could be associated with other P2X7Rs not clearly detectable by western blot. Interestingly, the fact that the N-terminal antibody stains neuronal processes that are not detected with the C-terminus-directed antibody in control tissue suggests that P2X7R-A and P2X7R-B isoforms may have different spatial distributions in physiological conditions. Regardless of this, the increased number of somata with clear immunostaining detected with both antibodies is in accordance with the increased protein levels detected by western blot in HD. To date, we do not know the meaning of the increased protein level of both P2X7R-A and P2X7R-B in the neurons of HD patients. In this regard, it has been reported that P2X7R-A and P2X7R-B play a role in undifferentiated and neural-differentiated embryonic stem cells (ESC) (Glaser et al., 2014). The authors there demonstrated that while before differentiation both isoforms are well expressed by ESC, under neural differentiation, the ratio between P2X7R-A and P2X7R-B favors the full-length isoform (Glaser et al., 2014). They speculate that the good expression of P2X7R-B in the undifferentiated condition eases cell proliferation and differentiation, avoiding cellular death (Glaser et al., 2014). Indeed, P2X7R-B trophic activity had been previously described in HEK293 cells as well as in a human osteosarcoma cell line (Adinolfi et al., 2010; Giuliani et al., 2014). However, neurons in the striatum are post-mitotic cells, which do not enter cell cycle or differentiate. On one hand, it is possible to speculate that P2X7R-A and P2X7R-B increase in HD could represent a consequence of the inflammatory mechanisms related to HD. On the other hand, it is possible that such increase, and especially of P2X7R-B, could be an adaptive mechanism to favor neuronal survival in HD. Further experiments are required to establish P2X7R-associated pathophysiological mechanisms in neurons.

At the mRNA level, we observed increased total P2X7R mRNA levels in the HD striatum, which in turn may explain the increase in total protein levels, but we do not know the precise underlying mechanisms. These might take place at the transcriptional level, as there are multiple transcription factors altered in HD and global transcriptomic alteration is well-documented (Valor, 2015). Another possibility is that it is due to a posttranscriptional mechanism, for instance, involving microRNAs, given their ability to control stability and translation of their target transcripts. In this regard, it is known that the P2X7R transcript can be regulated by microRNA-22 (mir-22) (Jimenez-Mateos et al., 2015). More precisely, experiments performed in a mouse model of status epilepticus demonstrated that the P2X7R transcript is a target of mir-22, which normally inhibits its translation (Jimenez-Mateos et al., 2015). On the other hand, it has been reported that many microRNAs are downregulated in HD mouse models, including mir-22 (Lee et al., 2011). Indeed, the overexpression of mir-22 inhibited neurodegeneration in rat primary striatal cultures exposed to a mutated human huntingtin fragment (Htt171-82Q) (Jovicic et al., 2013). Thus, P2X7R increase in HD could be, at least in part, explained via mir-22 diminution in this pathology, but further investigation regarding the status of mir-22 in samples from HD patients would be necessary to ascertain this. Finally, and specifically regarding the increase in P2X7R-B isoform at both mRNA and protein levels, it can be explained by the increased retention of intron 10–11 in the P2X7R transcript in HD reported here. The likely underlying mechanism could be the already-reported alteration of many splicing factors in HD brain tissue and cell and mouse models such as MBLN1 (Mykowska et al., 2011) or SRSF6 (Fernández-Nogales et al., 2014; Cabrera and Lucas, 2017). Thus, we here provide for the first time a connection between HD dysregulated splicing and P2X7R-mediated mechanisms in HD. Nevertheless, further research is needed to identify the specific splicing factors binding near or at P2X7R intron 10–11.

In conclusion, here, we report an upregulation of P2X7R in HD brains that, together with the analogous increase in HD mouse models and the preclinical studies – also in the mouse models – showing efficacy of P2X7R manipulation, further provides evidence of a role of P2X7R in this pathology and strengthens its potential as a drug target.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JL directed the study, designed experiments, performed the manuscript revision, and read and approved the submitted version. AE and IO performed the RNA sequencing analysis. IO performed q-RT-PCR and semi-q-RT-PCR analyses. MS-G carried out western blotting and the immunostaining. IO analyzed data and wrote the draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2020.567430/full#supplementary-material

FIGURE S1 | P2X7R protein levels detected with an N-terminus directed antibody in control and HD striatum. Blot image (left) is able to highlight multiple bands after prolonged exposure. The band at around 30 kDa could correspond to P2X7R-J. As shown by its quantification (right), such band does not significantly vary between HD and controls. Doublets can be observed at around 60 kDa, which could correspond to P2X7R-J dimers. However, no significant change occurs in HD samples. Only a light shade is observed in the range of P2X7R-A. Graphs show mean ± SEM. Dots represent individual values. Student's *t*-test, ns: non-significant.

FIGURE S2 | P2X7R exon 4 inclusion is decreased in HD. RNA-seq analysis of control and HD striatum by **(A)** Vast-tools result (Student's *t*-test, $*p < 0.05$) was confirmed by **(B)** rMATS (*FDR < 0.05). Graphs show mean ± SEM. Dots represent individual values.

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frontiers in Molecular Neuroscience

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P2X7 Receptor-Dependent Layer-Specific Changes in Neuron-Microglia Reactivity in the Prefrontal Cortex of a Phencyclidine Induced Mouse Model of Schizophrenia

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Background: It has been consistently reported that the deficiency of the adenosine triphosphate (ATP) sensitive purinergic receptor P2X7 (P2X7R) ameliorates symptoms in animal models of brain diseases.

Objective: This study aimed to investigate the role of P2X7R in rodent models of acute and subchronic schizophrenia based on phencyclidine (PCP) delivery in animals lacking or overexpressing P2X7R, and to identify the underlying mechanisms involved.

Methods: The psychotomimetic effects of acute i.p. PCP administration in C57Bl/6J wild-type, P2X7R knockout (P2rx7$^{-/-}$) and overexpressing (P2X7-EGFP) young adult mice were quantified. The medial prefrontal cortex (mPFC) of P2rx7$^{-/-}$ and heterozygous P2X7-EGFP acutely treated animals was characterized through immunohistochemical staining. The prefrontal cortices of young adult P2rx7$^{-/-}$ and P2rx7$^{tg/+}$ mice were examined with tritiated dopamine release experiments and the functional properties of the mPFC pyramidal neurons in layer V from P2rx7$^{-/-}$ mice were assessed by patch-clamp recordings. P2rx7$^{-/-}$ animals were subjected to a 7 days subchronic systemic PCP treatment. The animals working memory performance and PFC cytokine levels were assessed.

Results: Our data strengthen the hypothesis that P2X7R modulates schizophrenia-like positive and cognitive symptoms in NMDA receptor antagonist models in a receptor expression level-dependent manner. P2X7R expression leads to higher medial PFC susceptibility to PCP-induced circuit hyperactivity. The mPFC of P2X7R knockout animals displayed distinct alterations in the neuronal activation pattern, microglial organization, specifically around hyperactive neurons, and were associated with lower intrinsic excitability of mPFC neurons.

Conclusions: P2X7R expression exacerbated PCP-related effects in C57Bl/6J mice. Our findings suggest a pleiotropic role of P2X7R in the mPFC, consistent with the observed behavioral phenotype, regulating basal dopamine concentration, layer-specific neuronal activation, intrinsic excitability of neurons in the mPFC, and the interaction of microglia with hyperactive neurons. Direct measurements of P2X7R activity concerning microglial ramifications and dynamics could help to further elucidate the molecular mechanisms involved.

Keywords: P2X7 receptor, schizophrenia, microglia, phencyclidine, prefrontal cortex

INTRODUCTION

Schizophrenia (SCZ), which has a prevalence of 0.7% in adults, is a severe psychiatric disorder characterized by abnormalities in thought and cognition (Powchik et al., 1998). The pathology of SCZ is typically recognized by multiple psychotic episodes, along with a progressive worsening of social and cognitive abilities and multifactorial tissue deterioration of key brain areas, such as the prefrontal cortex (PFC; Lesh et al., 2011).

Studies of the molecular events related to SCZ point to a basal dysregulation of the glutamatergic system, which consequently damages the dopaminergic and local circuits, affecting distinct, defined areas of the central nervous system (McCutcheon et al., 2020).

However, the effects and mechanism of SCZ, as well as the consequential detrimental cascade, remain unclear. There is a lack of SCZ treatments that adequately treat cognitive symptoms (Tripathi et al., 2018).

The P2X7 receptor (P2X7R) is an ionotropic purinergic nucleotide receptor that is activated by high extracellular adenosine triphosphate (ATP) concentration and triggers an influx of extracellular calcium (Janks et al., 2018). While it is known that P2X7R is expressed in the brain, the exact cell-type specific localization of the receptors has been the subject of extensive debate (Illes et al., 2017; Teresa Miras-Portugal et al., 2017). Novel findings using the enhanced green fluorescent protein P2X7-EGFP reporter mouse line, which overexpresses the EGFP C-terminally tagged receptor protein, indicates that P2X7R, at least in the mouse brain, is preferentially expressed by microglia and oligodendroglial cells (Kaczmarek-Hajek et al., 2018). In addition to its recognized function as a ligand-dependent ion channel receptor, more than 20 proteins involved in immune functions have been reported to interact with the long intracellular C-terminal tail (Kim et al., 2001; Kopp et al., 2019) of the P2X7R protein.

P2X7R has received considerable interest in recent years due to its widespread involvement in a variety of central nervous system pathologies (Sperlágh and Illes, 2014), including mental health disorders (Bhattacharya, 2018). The first observation that suggested the involvement of P2X7R in psychotic episodes was that receptor deficiency blocked amphetamine-induced hyperactivity in P2X7R deficient mice (Csölle et al., 2013), which observation was further supported by others (Bhattacharya et al., 2013; Lord et al., 2014; Gubert et al., 2016).

More recently, we showed that P2X7R genetic deletion or pharmacological blockade alleviates the acute psychotomimetic effects of phencyclidine (PCP, 2, and 5 mg/kg; Koványi et al., 2016), which is a validated model for SCZ symptomatology (Paasonen et al., 2017). These changes were accompanied by alterations in SCZ-related genes in the PFC (Koványi et al., 2016). However, it is unclear how changes in the activity of P2X7R leads to behavioral alterations.

Extracellular purines, including ATP, adenosine diphosphate (ADP), and adenosine, act as signaling molecules, guiding numerous types of rapid microglial dynamics and interactions in the brain, and modulate long-term physiological cellular functions (Calovi et al., 2019). Among their receptors, P2X7Rs promote microglial proliferation and activation (Bianco et al., 2006; Monif et al., 2010) and the release of proinflammatory cytokines such as interleukin-1β (IL-1β; He et al., 2017).

Activation of P2X7R in the central nervous system as a trigger of microglia-associated neuroinflammation in the prefrontal and hippocampal regions has been recently hypothesized to play an important role in psychiatric disorders (Illes et al., 2020).

PCP is a potent non-competitive N-methyl-D-aspartate receptor (NMDA-R) antagonist that can result in symptoms such as hallucinations, delusions, and disorganized speech, thereby mimicking the episodic phase of psychosis in humans (Bubeníková-Valešová et al., 2008). The class of arylcyclohexylamine anesthetics, such as ketamine and PCP, exerts a complex array of behavioral and neurochemical effects in the brain, which are considered part of the basis of the NMDA-R hypofunction hypothesis of SCZ (Javitt and Zukin, 1991; Castañé et al., 2015). Systemic injection of PCP in rodents results in a psychotomimetic effect, characterized by hyper locomotor activity and stereotypical rotational behavior (Ishmael et al., 1998). Moreover, it is established that acute PCP administration alters the activity of several cortical and subcortical areas, which can be observed by the increased number of c-Fos expressing neurons (Celada et al., 2013; Hervig et al., 2016). In contrast, acute PCP administration in brain slices (Bourne et al., 1983; Wang and Liang, 1998) or local drug delivery in freely moving rats (Suzuki et al., 2002) inhibits most multi-synaptic excitatory neuronal activity.

Interestingly, one of the target areas of PCP-induced enhancement of neuronal activity in rodents is the medial PFC (mPFC; Jodo et al., 2005). The mPFC is intimately involved in cognitive functions, hereunder working and episodic memory, which is disrupted in the PCP model (Arime and Akiyama, 2017).

There are different hypotheses, supported by evidence and not mutually exclusive, to explain the paradoxical effect of systemic vs. local PCP on neuronal circuits. First, the direct preferential inhibition of GABAergic interneurons, predominantly the parvalbumin (PV) axo-axonic cell population, which, by disinhibition of the pyramidal cells, leads to a loss of activity synchronization (Lewis and Gonzalez-Burgos, 2006). Second, indirect activation occurs *via* excitatory firing from the thalamocortical projections of midline nuclei-relay neurons. In this sense, it is supposed that the main site of the PCP inhibitory action is in the reticular nucleus of the thalamus (Celada et al., 2013). The study of thalamocortical projections requires experiments to be conducted *in vivo*. It is also known that acute PCP deeply affects the midbrain dopaminergic system, increasing extracellular levels of dopamine in the frontostriatal region concomitantly with the behavioral psychotomimetic effect (French, 1994; Moghaddam and Adams, 1998). More recently, it has been confirmed that glutamate, noradrenaline, and serotonin rapidly increase in the mPFC upon acute PCP (Kehr et al., 2018).

As mentioned above, PCP-induced neuropathological mechanisms unleash desynchronization and ungoverned activation in most SCZ-related CNS regions (Kargieman et al., 2007), resulting in lower inter-area functional connectivity (Paasonen et al., 2017).

Rodents that undergo repeated PCP treatments experience a complex cumulative effect, which persists during drug withdrawal, leading to a well-documented mimicry of SCZ-negative symptomatology (Jentsch et al., 1998; Castañé et al., 2015; Cadinu et al., 2018). The nature of this long-lasting detrimental effect is still unclear, yet physiological changes are consistently observed in rodent models of NMDA-R antagonists. Deterioration of PV interneurons (Toriumi et al., 2016), network-level abnormalities (Seshadri et al., 2018), and task-related recruitment of neurons (Arime and Akiyama, 2017) are similar to the schizophrenic pathological events in the mPFC of rodents, which corresponds to the dorsolateral frontal cortex in humans. The PFC is not the only area involved in the pathology of PCP models (Uramura et al., 2014).

Interestingly, mild brain inflammation is well documented in SCZ (Müller, 2018), and hampering the microglia proinflammatory shift is suggested to be beneficial in SCZ models (Mattei et al., 2014). Whether neuroinflammation also occurs in the mPFC in PCP SCZ models remains unknown (Zhu et al., 2014).

Therefore, we have aimed to investigate the development of a chronic, unceasing proinflammatory state in the PFC during PCP subchronic treatment, since neuroinflammation might be an interesting, novel pathway through which P2X7R may contribute to the symptomatology of SCZ. However, we have not found evidence that suggested neuroinflammation at any point in the project. Therefore we rejected the potential involvement of chronically activated microglia and neuroinflammation in the P2X7R mediated symptomatology during SCZ.

The present study was designed to study the role of mPFC P2X7 receptors in an acute PCP model for positive symptoms. We have also extended our previous observations to a P2rx7$^{-/-}$ subchronic PCP model, which better reflects the disease phenomenon in humans. Moreover, we tested the heterozygous animals overexpressing P2X7-EGFP (Kaczmarek-Hajek et al., 2018) using the acute treatment model of either a regular or a low dosage of PCP, which better depicts an array of negative symptoms (Paasonen et al., 2017).

MATERIALS AND METHODS

Animals

The behavior, histology, microscopy, cytokine quantification, and ^{3}H-Dopamine release experiments were conducted following the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee of the Institute of Experimental Medicine (Budapest, Hungary, ref. No. PEI/001/778-6/2015). The electrophysiology experiments were approved and performed in compliance with National and European regulations (RD1201/2005; 86/609/CEE) following the guidelines of the International Council for the Laboratory Animal Science at the University of the Basque Country UPV/EHU (CEEA 290/2015).

Sixty to ninety days old adult male, drug and test naïve, wild-type (P2rx7$^{+/+}$), and P2X7R knockout (P2rx7$^{-/-}$) mice were housed in a light- (12 h on, 12 h off) and a temperature-controlled room with food and water available *ad libitum*.

Homozygous P2X7R P2rx7$^{+/+}$ mice were bred on the C57Bl/6J background. The P2rx7$^{-/-}$ mice were kindly supplied by Christopher Gabel from Pfizer, Inc. (Groton, CT, USA) or purchased (for electrophysiology experiments, Charles River). The animals contained the DNA construct previously shown to delete the P2X7R (Solle et al., 2001). Offspring of this mouse line was cross-bred with P2rx7$^{+/+}$ mice, and the resulting heterozygotes were used as breeding stock for the F1 generation offspring employed in the behavioral studies. Genomic DNA was isolated from the tails of P2rx7$^{+/+}$ and P2rx7$^{-/-}$ animals, and the genotypes were confirmed by polymerase chain reaction (PCR) analysis.

The overexpressing P2X7-EGFP C57Bl/6J mouse (line 17 in C57Bl6N) was obtained from Annette Nicke (Kaczmarek-Hajek et al., 2018) and bred heterozygously. Mice were raised in grouped cages until the 2 month of life. Male animals were tested using the experiments detailed below.

Treatments

All treated animals were handled and habituated to the behavioral unit at least 24 h before the first injection. Injections were performed in a treatment room, and behavioral protocols were recorded in a separate experimental room.

Acute PCP treatment consisted of a single i.p. injection (volume of 10 ml/kg) of the vehicle (0.9% NaCl sterile) or PCP at two dosages, referred to as PCP and low-dose PCP (PCP-HCl 10 mg/kg and low dose 2 mg/kg, Sigma–Aldrich Kft, Budapest, Hungary) freshly dissolved in the vehicle. Ten animals per genotype (P2rx7$^{-/-}$ and P2rx7$^{+/+}$) and 12 animals per genotype (P2rx7$^{tg/+}$ and P2rx7$^{+/+}$) were deeply anesthetized (Nembutal 100 mg/kg, Sigma–Aldrich Kft, Budapest, 10 ml/kg

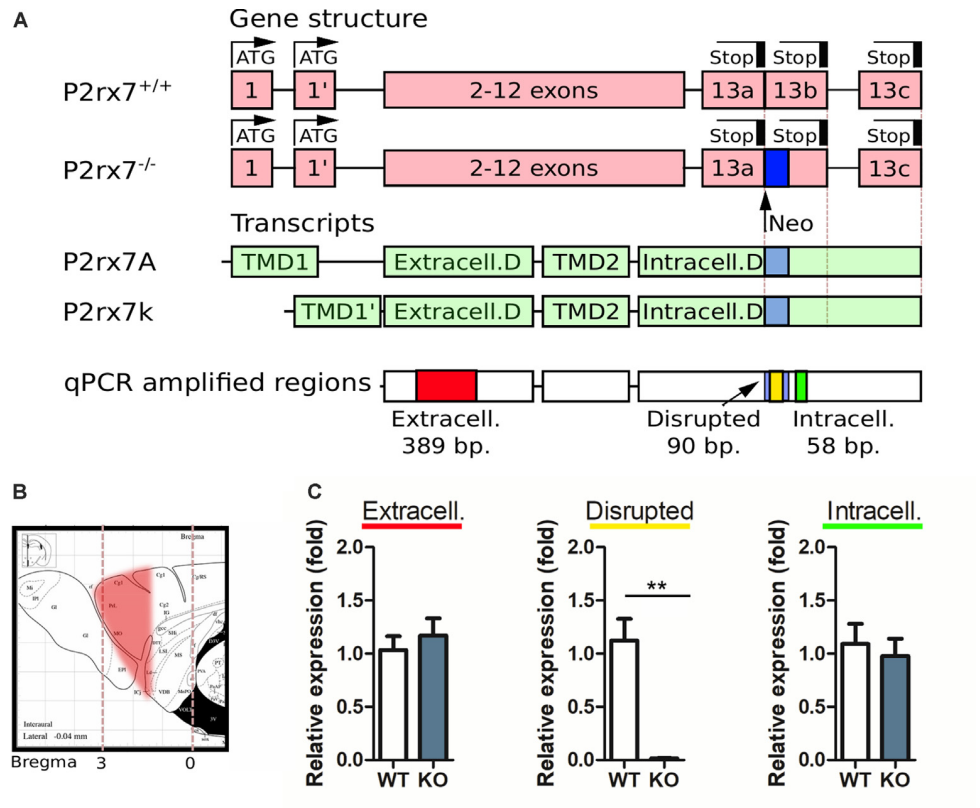

FIGURE 1 | Purinergic receptor P2X7 (P2X7R) expression analysis in the prefrontal cortex with Real-time quantitative polymerase chain reaction (RT-qPCR). **(A)** Schematic representation of the P2rx7 gene in P2rx7$^{+/+}$ and P2rx7$^{-/-}$ mouse lines, the principal splicing variants found in C57bl/6 line, and the amplified regions in the qPCR. **(B)** Schematic representation of the prefrontal cortex (PFC) cut, subsequently analyzed. Paxinos ATLAS, sagittal. **(C)** Purinergic receptor P2X7 (P2X7R) expression analysis in P2rx7$^{+/+}$ and P2rx7$^{-/-}$ mouse (N = 5, both groups) prefrontal cortex by real-time qPCR. Relative expressions were given as fold changes normalized to GAPDH. Shown is the mean ± SEM. Statistical analysis was performed with unpaired Student's t-test, p-value **< 0.01 **(C)**.

injection volume) 180 min after receiving PCP treatment, and transcardially perfused 10 or 20 min afterward (**Figures 2A**, **9A**). For the low-dose PCP experiment, 18 animals homozygous for P2X7R and heterozygous for P2X7-EGFP protein overexpression (Kaczmarek-Hajek et al., 2018), referred to as P2rx7$^{tg/+}$, were randomized into a vehicle and low-dose PCP groups. Animals were habituated 30 min before the experimental environment, received the treatment, and were placed back in the house cage for 45 min. Then, they were tested in pairs for 10 min in a circular open field (**Figure 8A**). Subsequently, they were deeply anesthetized (Nembutal) and transcardially perfused.

Subchronic treatment consisted of the daily vehicle or PCP injections for seven consecutive days (Zain et al., 2018). A cohort of 26 P2rx7$^{+/+}$ and 30 P2rx7$^{-/-}$ animals was subchronically treated, followed by a withdrawal period of 7 days before sacrifice (**Figure 10A**). Four independent treatments with a maximum of eight animals per genotype were conducted. The animals were decapitated after deep anesthesia (Nembutal).

Real-Time qPCR

To measure the expression of the P2X7 receptor in the prefrontal cortex (PFC), five wild type and knockout mice were sacrificed. The PFC (cut at Bregma 1.20) was snap-frozen in liquid nitrogen and kept at −80°C.

Total RNA isolation was performed using TRI Reagent® (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. In brief, chloroform was used for phase separation, the supernatants were aspirated and mixed with propane-2-ol (Sigma–Aldrich, St. Louis, MO, USA) for RNA precipitation. RNA pellets were washed with 70% ethyl alcohol and dissolved in RNase-free water. RNA concentrations were measured using Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The RNA integrity was verified by electrophoretic separation on 1% agarose gel. Reverse transcription from 1 μg RNA into cDNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's protocol. Based on the previously published primer sequences (Sánchez-Nogueiro et al., 2005), the following three regions of the P2X7 receptor were measured by Real-time quantitative PCR (RT-qPCR): (1) the extracellular region; (2) the intracellular region; and (3) the absent region in the knockout mice (disrupted). Primer sequences used to amplify the disrupted region was 5′-TGCATCACCACCTCCAAGCTCTTC CAT-3′ (forward primer) and 5′-CACCAGCAAGGGATCCTG

GTAAAGC-3′ (reverse primer); to amplify the extracellular part 5′-GCACGAATTATGGCACCGTC-3′ (forward primer) and 5'-ACACCTGCCAGTCTGGATTCCT-3′ (reverse primer); and to amplify the intracellular part 5′-AGGATCCGGAAGGAGTT-3′ (forward primer) and 5′-TAGGGATACTTGAAGCCACT-3′ (reverse primer) was used. The housekeeping gene, GAPDH, was used for normalization to account for intra-well variability [5′-TTCACCACCATGGAGAGGGC-3′ (forward primer) and 5′-GGCATGGACTGTGGTCATGA-3′ (reverse primer)]. The RT-qPCR reactions were performed using the SensiFast SYBR Green No-Rox kit (Bioline Reagents Limited, London, UK) according to the manufacturer's protocol in 10 μl total volume. The RT-qPCR products of the P2X7 receptor were visualized by electrophoretic separation on 1% agarose gel (data not shown).

Behavioral Studies

Acute Treatments

$P2rx7^{+/+}$, $P2rx7^{-/-}$ and $P2rx7^{tg/+}$ post-natal day 71–81 male mice were housed in individual cages for 3 days in the behavioral unit, and randomly assigned to the two treatment groups: six received acute PCP treatment and four received vehicle treatment for the wild type vs. knockout experiment, while nine received acute PCP treatment and three received vehicle treatment for the wild type vs. P2X7R overexpressing line experiment. The experiments were repeated independently two and three times for genotype tested, with a maximum of 10 animals tested at a time. In the wild type vs. knockout experiments, the mice within their home cages were placed in the experimental room at dim light for 1 h before each behavioral protocol was performed (**Figure 2A**). Following this habituation period, the animals were individually transported to the treatment room and injected according to their weight. Once treated, each animal was returned to the experimental room in a clean home-cage positioned over an infrared backlight platform (IR, 850 nm). Each animal left the experimental room to receive treatment for a maximum of 3 min. A fixed camera (Basler aca1300-60gc GigE camera—computer H3Z4512 CS-IR 4.5–12.5 mm F1.2 lens) equipped with an IR filter (Heliopan 35.5 RG850) was mounted on the ceiling of the experimental room. This IR system-generated high-contrast movies that were devoid of reflected-light artifacts, which are common with plexiglass arenas under dim light conditions. In the wild type vs. P2X7R overexpressing line experiments, the protocol was performed in normal light conditions. The animals were recorded continuously for 90 min after the injections. After the recording, the animals were placed back in their home cages for additional 90 min.

For the low-dose PCP study, $P2rx7^{+/+}$ and $P2rx7^{tg/+}$ animals were housed in individual cages in the behavioral unit 24 h before receiving the treatment. For each genotype, eight and six animals were treated with vehicle, and 10 and 12 were subjected to low-dose PCP, respectively. The experiment was independently repeated three times. Animals were habituated to the experimental room for 30 min and were transferred for injection in pairs to the treatment room. Forty-five minutes later, mice were subjected to the social withdrawal test (**Figure 8A**). The test was performed in dim light, a circular open field, and recorded for 10 min, following a protocol previously described (Koványi et al., 2016). Briefly, two unfamiliar mice receiving the same pharmacological treatment were placed in a circular open field. Mice were placed on opposite sides of the open field. The following behavioral variables were recorded: distance traveled, social interaction, ataxia, and stationary stereotyped behavior. Line crossings and social interactions were recorded for the entire duration of the test using a computer-based event recorder. Social investigations were defined as sniffing and nosing when the nose of the scored mouse touched (or was very close to) the body of the partner and followed toward the partner. PCP-induced stereotyped behavior and ataxia were scored manually according to the protocol described by Nabeshima et al. (1987) and Sams-Dodd (1995), respectively.

Subchronic Treatment

$P2rx7^{+/+}$ and $P2rx7^{-/-}$ 2-month-old male mice were housed in individual cages in the behavioral unit and randomly assigned to PCP or vehicle subchronic treatment. On day 3 of the treatment, 90 min after the injection, four randomly selected animals per group were placed into a glass cylinder (10 cm diameter) and recorded for 5 min in dim light. The tests were repeated the day after the fourth injection. After 3 days of treatment washout (72 h from the last injection), the animals were tested in a Y-maze (custom-built: arm length 30 cm; width 7 cm; walls height 20 cm; angle 120° equal) for 10 min at dim light and recorded.

One $P2rx7^{-/-}$ animal acutely treated with PCP was excluded from the analysis as it escaped the cage during the experiment. Subchronically treated animals that did not reach 20 alternations in the Y-maze were excluded. Alternations were considered when an animal changed from visiting one arm to visiting a second arm, with the central body point over 20% of the arm length. Repeated passages from the center of the maze to the same arm were considered a single-arm exploration. Behavior was quantified and analyzed offline using Noldus EthoVision XT®.

Histology

Acute Treatment

To study the acute effect of PCP on *in vivo* neuronal activity during the psychotomimetic phase (40–90 min after the injection), animals were sacrificed after reaching the peak of c-Fos IEG protein expression, 90 min after the neuronal activating event (Chaudhuri et al., 2000; Arime and Akiyama, 2017). Therefore, 180 min after the treatment, each animal received 100 mg/kg pentobarbital i.p. dissolved in saline (Nembutal, Sigma–Aldrich Kft, Budapest, 10 ml/kg injection volume in 0.9% NaCl sterile). After 10–20 min, the animal was transcardially perfused for 3 min with saline (flow rate 5 ml/min), followed by 15 min perfusion with 4% paraformaldehyde (PFA, Merck-Sigma) in 0.1 M phosphate buffer (PB, $Na_2HPO_4 \cdot 2H_2O$; NaH_2PO_4, Merck-Sigma) solution. To obtain comparable c-Fos staining, the time window was strictly respected. Following fixation, the brain was rapidly removed from the skull and post-fixed in 4% PFA in PB at 4°C overnight. The following day, the collected brains were extensively washed in PB. The prefrontal cortices were coronally sliced (40 μm thickness, Vibratome Leica VT 1200 Wetzlar, Germany) and rinsed

in PB before staining. Coronal slices of the PFC (Bregma +1.70 − +2.10) were selected for immunohistochemistry (Franklin and Paxinos, 1997).

C-Fos DAB

Two slices from three saline- and four PCP-treated animals per genotype ($P2rx7^{+/+}$ and $P2rx7^{-/-}$) were incubated for 10 min in 3% H_2O_2 in PB, rinsed three times for 10 min in PB, and three times for 10 min in PB. The pre-made blocking buffer (ImmPRESS UNIVERSAL REAGENT, Vector Laboratories, MP-7500) was supplemented with 0.5% Triton X-100 (Tx, Merck-Sigma), and 7.5% of normal donkey serum (NDS, Jackson Immunoresearch, Europe). After 1 h of blocking, slices were incubated with the primary rabbit anti-c-FOS antibody (1:1,000, Santa Cruz Biotechnology sc-52, Dallas, TX, USA) in PB containing 0.05% sodium azide (Merck-Sigma) for 24 h at room temperature (RT) and 72 h at 4°C. Eventually, the slices were rinsed in PB and incubated with the premade secondary anti-mouse/rabbit HRP-conjugated ImmPRESS UNIVERSAL REAGENT (Vector Laboratories, MP-7500) for 1 h. DAB was developed using the commercially available DAB-REACTION KIT (Vector Laboratories, SK-4105) according to the manufacturer's instructions.

Fluorescence Immunohistochemistry

A similar protocol to the above DAB staining was performed for single fluorescence immunostaining for c-Fos staining ($P2rx7^{+/+}$ and $P2rx7^{tg/+}$, four and five PCP-treated animals respectively) and double fluorescence immunostaining. Slices were rinsed in PB three times for 10 min and blocked for 1 h (10% NDS; 0.5% Tx in PB). Antibody against murine c-Fos (1:1000, 226.004 Synaptic System, Göttingen, Germany) was dissolved in PB-0.05% sodium azide, 0.1% NDS, and 0.2% Tx. Slices were incubated with the c-Fos primary solution for 1 day at RT and 2 days at 4°C. On the 3 day, the primary antibodies against murine Parvalbumin (PV, 1:500, PVG-213, Swant, Marly, Switzerland), against murine P2Y12R (1:400, AS-55043, AnaSpec, San Jos, CA, USA), NeuN (1:400, MAB377, Merck Millipore, Darmstadt, Germany), and anti-tyrosine hydroxylase (TH, 1:400, EP1532Y, Abcam, Cambridge, UK) were added to the wells for an additional 24 h. Eventually, the slices were rinsed with PB (three times 10 min) and incubated for 1–2 h at RT with the corresponding secondary antibodies (1:500, 706-605-148 Alexa Fluor 647 Donkey anti-guinea pig for c-FOS; 711-605-152 Alexa Fluor 647 Donkey anti-rabbit for TH;705-585-003 Alexa Fluor 594 Donkey anti-goat for PV; 711-545-152 Alexa Fluor 488 Donkey anti-rabbit for P2Y12R, Jackson Immunoresearch, Europe). For the demonstrative co-localization of P2X7R and microglia, PFC slices from P2X7-EGFP mice were rinsed in PB three times for 10 min and blocked for 2 h (5% NDS; 0.3% Tx in PB). The primary antibodies against murine P2Y12R (1:400, AS-55043, AnaSpec, San Jose, CA, USA) and chicken anti-GFP (1:500, GFP-1020, Aves Labs) were dissolved in PB, 5% NDS, 0.3% Tx. Slices were incubated with the primary antibody solution overnight at 4°C. On the 2 day, the slices were rinsed with PB three times for 10 min and incubated for 2 h at RT with the corresponding secondary antibodies (1:500, 711-545-152 Alexa Fluor 488 Donkey anti-rabbit and A-11039 Alexa Fluor 488 goat anti-chicken).

After DAB-revelation or secondary antibody incubation, slices were rinsed in PB three times for 20 min before being mounted on microscopy slides and coverslipped (Thermo-Fisher Scientific) with ProLong™ Gold Antifade Mountant (P36934, Thermo-Fisher Scientific) and kept at 4°C.

Microscopy

Microscopy was carried out at the Nikon Microscopy Center in the Institute of Experimental Medicine at the Hungarian Academy of Science. Pictures were taken with a confocal Nikon C2 microscope (20× and 60× -oil immersion objectives). Fluorescent picture z-stacks: 11/20 steps z-steps 0.5–1 μm (c-Fos, c-Fos/PV; c-Fos/TH; c-Fos/P2Y12R/NeuN); 30–38 steps z-steps 1 μm (c-Fos/P2Y12R). Bright field c-Fos DAB pictures were taken with a color camera (DS-Fi3), 11 steps per z-steps 2.5 μm. Fluorescent z-stack microscopy images were analyzed using the software FIJI ImageJ (Schindelin et al., 2012). c-Fos counting: "Sum of the slices" (DAB) or "Maximal projection" (fluorescence) images were divided into regions of interest, and the individual layers were analyzed (layers width: *I* = 120–140 μm; II/III = 80 μm; *V* = 320–350 μm; VI = minimum 280 μm, **Figure 3A** right). The c-Fos positive nuclei mean gray values from the original images were measured over the auto-threshold-counted objects with automated macro processing in FIJI ImageJ. The 2D/3D Sholl analysis was performed using the FIJI ImageJ application (Ferreira et al., 2014).

[^{3}H]-Dopamine ([^{3}H]-DA) Release Experiment

The [^{3}H]-DA release experiments were conducted using the method described in our previous articles (Csölle et al., 2013; Koványi et al., 2016). Briefly, 2–3-month-old male mice ($n = 32$ in $P2rx7^{+/+}$, $n = 11$ in $P2rx7^{-/-}$and $n = 4$ in $P2rx7^{tg/+}$), were anesthetized under light CO_2 inhalation, decapitated, and the brain was extracted from the skull. The PFC dissected in ice-cold Krebs' solution saturated with 95% O_2 and 5% CO_2. Coronal 400 μm-thick PFC slices were chopped (McIlwain tissue chopper) and incubated for 45 min at 37°C in 1 ml Krebs solution with 5 μCi/ml [^{3}H]-DA (specific activity 60 Ci/mmol; ARC, St. Louis, MO, USA) bubbled with 95% O_2 and 5% CO_2. Once loaded with [^{3}H]-DA, the slices were continuously superfused with 95% O_2, and 5% CO_2-saturated modified Krebs solution (flow rate: 0.7 ml/min). After a 90 min washout period, perfusate samples were collected over 3 min periods and assayed for tritium content. The temperature was maintained at 37°C. At the 20th min, after starting to collect samples, the slices were subjected to 3 min of 20 μM veratridine (Sigma Chemical Co., St. Louis, MO, USA) perfusion. The radioactivity of the samples was measured using a Packard 1900 Tricarb liquid scintillation spectrometer, using an Ultima Gold Scintillation cocktail. The release of tritium was expressed as a percentage of the amount of radioactivity in the tissue at the sample collection time (fractional release). The tritium uptake in the tissue was determined as the sum of release + the tissue content after the experiment and expressed in Bq/g.

For the evaluation of the basal tritium outflow, the fractional release measured in two consecutive 3 min samples under drug-free conditions was considered. The veratridine-induced [^{3}H]-DA efflux was calculated as the net release in response to the respective stimulus by subtracting the release before the stimulation from the values measured after stimulation.

Electrophysiology

Eight P2rx7$^{+/+}$ and six P2rx7$^{-/-}$ male mice 60–75 days old were anesthetized with isoflurane (IsoVetR 469860, Braun) and decapitated. The brain was extracted, put on ice and immediately immersed in ice-cold *N*-methyl D-glucamine based cutting solution (NMDG 92 mM, $NaHCO_3$ 30 mM, NaH_2PO_4 1.25 mM, HEPES 20 mM, glucose 25 mM, Na-ascorbate 5 mM, Na-pyruvate 3 mM, Thiourea 2 mM, KCl 2.5 mM, $MgSO_4$ 10 mM, $CaCl_2$ 0.5 mM; all from Merck-Sigma; pH 7.3) for 2 min, before being embedded in 2% agarose gel (Merck-Sigma) and sliced at 300 μm thickness on a Compresstome (VF-300-0F Precisionary). Coronal PFC slices (Bregma +1.70 − +2.50) recovered for 30 min in an NMDG-based cutting solution bubbled with 95% O_2 and 5% CO_2 at 37°C. Slices were transferred for an additional hour in aCSF (300/306 mOsm, pH 7.3, NaCl 119 mM, KCl, 2.5 mM, $MgCl_2$ 1.6 mM $NaHCO_3$ 26 mM, NaH_2PO_4 1 mM, HEPES 5 mM, D-glucose 10 mM, $CaCl_2$ 2.5 mM; Merck-Sigma) at RT. Patch-clamp recordings of neurons in the prelimbic mPFC cortical layer V were made using infrared differential interference contrast video microscopy (CleverExlore, MCI Neuroscience). For whole-cell patch-clamp recordings, a pipette solution containing K-gluconate 125 mM, KCl 5 mM, HEPES 10 mM, EGTA 1 mM, 4 mM Na_2GTP, 0.3 mM, NaP-creatine 10 mM, ascorbic acid 3 mM (280–295 mOsm, pH 7.3; all from Merck-Sigma) was used, yielding a tip resistance of 4–5 MΩ. Recordings were obtained 200 s after the entire patch-clamp configuration to allow steady-state conditions between the pipette solution and the cytosol. The resting membrane potential (Vm) was recorded in current-clamp mode at 0 pA for 10 s. Current/Voltage (I/V) relationship and action potential firing were obtained with 0.4 s square pulse current step injections from −100 pA to +280 pA at 20 pA increments (I step, 1 per second). At 100 ms before Istep, a 1 ms current pulse at −75 pA was injected to normalize the Vm after depolarization. The membrane potential at 0 pA before and after Istep, were taken from the average voltage of the 30 ms before Istep (Vm pre) and of the last 0.1 s after 0.3 s from I step (Vm post). Ramp injection of 0–500 pA current over 1 s was used to determine the current required to elicit an action potential, that is, rheobase. All currents and voltages were registered and controlled using a HEKA EPC10 amplifier. Data were analyzed using the Fitmaster software (HEKA electronic).

Cytokine Quantification

Cytokine quantification from the PFC of subchronically treated animals was performed as described in our previous article (Horváth et al., 2019). Briefly, on day 14, after the first PCP or vehicle injection, brain samples were collected after light CO_2 anesthesia. Tissue was homogenized and centrifuged, as described previously (Dénes et al., 2010), and supernatants were collected to measure the levels of the following inflammatory mediators: IL-1α, IL-1β, IL-10, TNF-α, and CXCL1 (KC) using BD Cytometric Bead Array Flex Sets (BB Biosciences). Measurements were performed on a BD FACSVerse flow cytometer, and data were analyzed using FCAP Array version 5 (Soft Flow). Cytokine concentrations in the brain tissue were normalized to total protein levels measured by photometry using a BCA Protein Assay Kit (Thermo Fisher Scientific, Pierce). Absorbance was measured at 560 nm using a Victor 3V 1420 Multilabel Counter (PerkinElmer). Plasma cytokine levels are expressed as picograms per milligram.

Statistics

Statistical analyses were performed using STATISTICA version 64 (StatSoft Inc., Tulsa, OK, USA) and Graph Prism version 5 (GraphPad Software, San Diego, CA, USA). The RT-qPCR data were analyzed using the − delta-delta Ct ($2^{-\Delta\Delta Ct}$) calculation method, for comparison was used unpaired nonparametric Mann–Whitney *U* test. Differences between two groups were analyzed using the Student's *t*-test, between multiple groups one-way analysis of variance (ANOVA) followed by a *post hoc* Kruskal–Wallis or Dunn's comparison test or two-way ANOVA followed by Bonferroni's *post hoc* test or Mann–Whitney *U*-test, as appropriate for multiple comparisons was used. The specific tests used are reported in the captions of the figures. All data are expressed as a mean ± standard error of the mean (SEM; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). A *p*-value of lower than 0.05 was considered to be statistically significant.

RESULTS

Acute PCP Treatment-Induced Hyperactivity and Layer-Specific Neuronal Activation in the mPFC Was Alleviated in P2rx7$^{-/-}$ Mice

To test the validity of the P2X7R knockout model, real-time qPCR (RT-qPCR) reaction detecting P2rx7 gene transcripts was performed (**Figure 1A**). The RNA has been extracted from the whole frontal cortex (**Figure 1B**). The RT-qPCR measurement revealed that the disrupted sequence in P2rx7$^{-/-}$ mice was markedly reduced compared to the P2rx7$^{+/+}$ mice, while no difference was detected regarding the extra- and intracellular sequences between the knockout and WT mice (**Figure 1C**). The visualization of RT-qPCR products by electrophoretic separation confirmed that the band (90 bp), corresponding to the disrupted sequence in P2rx7$^{-/-}$ mice, appeared only in the P2rx7$^{+/+}$ (data not shown), while the bands corresponding to the extra-, and intracellular regions of P2X7 receptor (at 389 bp and 58 bp, respectively) were detected in both the WT and in the KO mice (data not shown). Implications of the presence of P2rx7 transcripts will be further elaborated in the "Discussion" section. For sake of clarity, in the current text, this model is referred to as the genetically deficient mouse strain for the P2X7 receptor (P2rx7$^{-/-}$).

To evaluate the involvement of P2X7R in the PCP-induced hyperactivity of the mPFC circuit, locomotor activity in an

open-field arena was evaluated, and neuronal activation was monitored using c-Fos immunohistochemistry. The proto-oncogene c-Fos is a transcription factor responsible for the formation of Activator-Protein-1 (AP-1) together with c-Jun. It is widely accepted that c-Fos activation and neuronal activity directly correlate (Day et al., 2008). Every animal followed a strict schedule from the beginning of the behavioral protocol until brain extraction (**Figure 2A**).

P2rx7$^{+/+}$ and P2rx7$^{-/-}$ mice were treated with 10 mg/kg PCP i.p. and immediately placed in a new cage. PCP increased locomotion and elicited stereotypic behavior. Hyperlocomotion and rotational stereotypic behavior peaked between 40 and 80 min after injection (**Figures 2B,C**).

The effects of PCP in P2rx7$^{+/+}$ animals were more pronounced in terms of stereotypy and longer-lasting hyperlocomotion than P2rx7$^{-/-}$ mice (**Figure 2C**). Along the mPFC of treated animals, PCP induced a clear band of c-Fos immuno-positive neuronal somata, primarily at the level of layer V (**Figure 3A**). The ventrodorsal gradient of active neuron concentration revealed a robust PCP-driven engagement of the infralimbic (IL) and prelimbic (PL) areas (**Figure 3B**). P2rx7$^{-/-}$ mice displayed reduced PCP-induced hyperlocomotion and stereotyped behavior (**Figure 2C**). This was accompanied by a lower number of c-Fos-positive nuclei in the ventral region of Layer II/III, but not Layer V (**Figure 3B**). Since the number of c-Fos-positive cells and the level of c-Fos protein expression was established as a neuronal activity marker (Chung, 2015), a deeper inspection of the IL and PL areas in PCP-treated mice was performed. Two different anti-c-Fos antibodies with different protocols were employed to enhance reliability (**Figures 3A,C**). Plotting the number of c-Fos-positive neurons against the intensity of the signal revealed different distribution between genotypes (**Figure 3D**). Pragmatic discrimination between activated (c-Fos^{+}) and strongly activated (c-Fos^{++}) neurons, setting the dimension and intensity threshold to 200 μm^2 and 400 mean gray value (ImageJ standardized units), respectively, between c-Fos^{+} and c-Fos^{++}, revealed a significant reduction in the concentration of c-Fos^{++} neurons in the P2rx7$^{-/-}$ IL and PL areas, specific to layer V (**Figure 3E**). These results suggest that the genetic deletion of P2X7R in part buffers the psychotomimetic effects of PCP and the consequent layer-specific neuronal activation in the mPFC.

P2X7R Deficiency Does Not Affect PCP-Related Changes in PV Interneurons in the PFC and Dopamine Release From the Striatum

To determine the underlying mechanism involved in the differential effect of acute PCP in P2rx7$^{+/+}$ and P2rx7$^{-/-}$ animals, double immunohistochemistry for c-Fos, and PV were performed, and microscopic images were manually analyzed

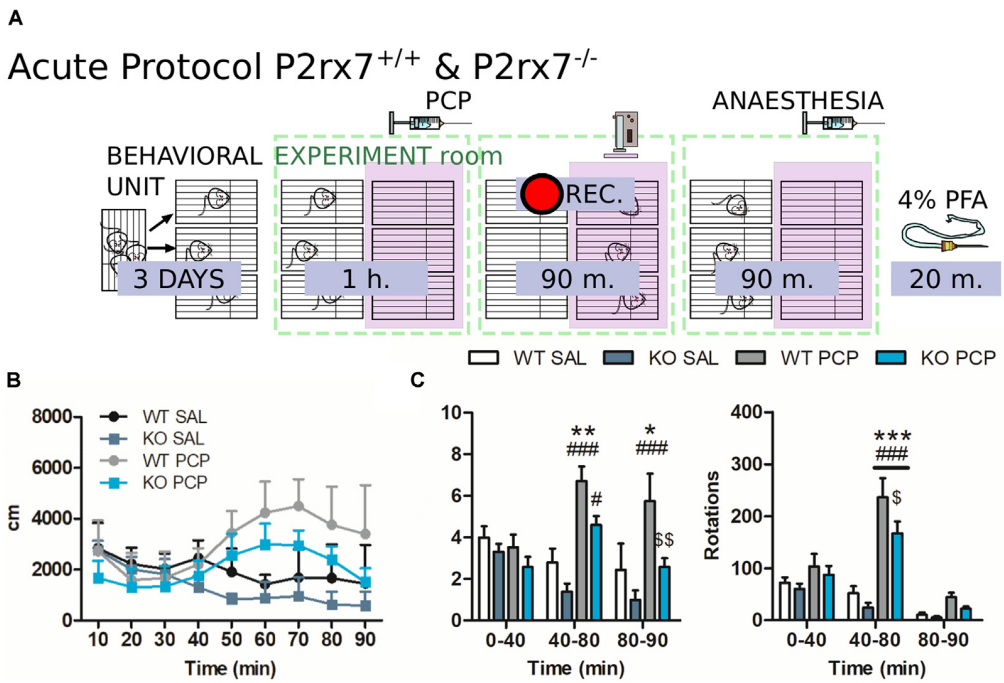

FIGURE 2 | P2rx7$^{-/-}$ mice are less susceptible to the acute phencyclidine (PCP) psychotomimetic effect. **(A)** Schematic representation of the behavioral protocol for acute treatment. The vehicle or PCP 10 mg/kg i.p. injection is represented by the PCP syringe, the pink panel represents the IR backlight, and the pink rectangle in front of the camera represents the infrared (IR) filter. **(B)** Distance moved by the animals after the injection, in 10 min time bins. **(C)** Average velocity (left) and numbers of circle rotations (right) averaged for the first and second 40 min, plus the remaining recorded time. The PCP psychotomimetic effect was evident 40 min after the PCP injection. *N* = 4 (WT Sal), *N* = 5 (KO PCP), and *N* = 6 (WT PCP). Shown is mean ± SEM. Statistical analysis was performed with two-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. Scoring: +; ++; +++; mean *p*-values < 0.05; 0.01; 0.001. Symbols: * vs. WT SAL; # vs. KO SAL; $ vs. WT PCP.

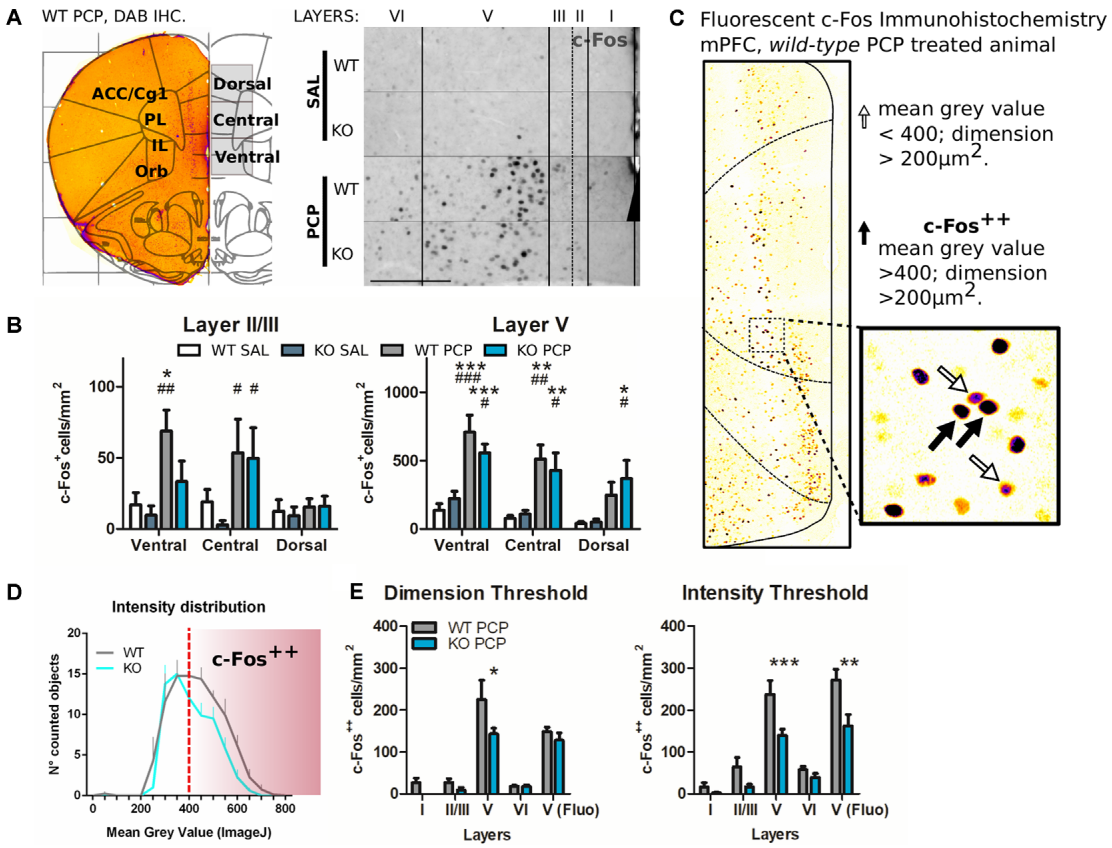

FIGURE 3 | The reduced PCP related behavioral outcome in P2rx7$^{-/-}$ is correlated with lower PCP-driven hyperactivation of the medial PFC mPFC neurons. **(A)** Representative c-Fos/DAB immunostaining of the coronal PFC from a P2rx7$^{+/+}$ PCP treated mouse overlapped with the Paxinos ATLAS (Bregma +2.00; Franklin and Paxinos, 1997). The left hemisphere represents the field of view of the pictures quantified (left). Representative immunostaining from projected images used for automated c-Fos nuclei counting. Layer width ranges: *I* = 120–140 µm; II/III = 80 µm; *V* = 320–350 µm; VI = minimum 280 µm. Scale bar: 200 µm (right). **(B)** Quantification of c-Fos positive nuclei in layers II/III and V of the mPFC. **(C)** Representative picture of the coronal PFC immunostaining (WT PCP) with a different c-Fos antibody (SYSY Cfos-226 004 guinea pig/fluorescent Jackson IR secondary). In the V-centered insert layer, the white arrows indicate c-Fos positive nuclei (mean gray value <400, dimension >200 µm^2), while the black arrows indicate strongly immunostained nuclei (mean gray value >400; dimension >200 µm^2), referred to as c-Fos^{++}. The length of the insert square side is 165 µm. **(D)** Averaged histogram of the mean gray value for the automatically detected c-Fos positive nuclei in layer V. PCP-treated P2rx7$^{-/-}$ and P2rx7$^{+/+}$ prelimbic and infralimbic areas were quantified as the central and dorsal portions of the ventral mPFC pictures. The red dashed line represents the threshold (400 units of mean gray value, FIJI ImageJ), which were considered strongly activated neurons c-Fos^{++}. **(E)** Quantification of prelimbic and infralimbic content of strongly activated c-Fos^{++} nuclei, by dimension (left) and intensity (right) threshold. Automatic counting with FIJI ImageJ in each medial PFC layer for DAB immunostaining, and in the isolated layer V for fluorescent immunostaining. *N* = 3 (Sal), *N* = 4 (PCP). Shown is mean ± SEM. Statistical analysis was performed with two-way ANOVA followed by Bonferroni's *posthoc* test. Scoring: +; ++; +++; mean *p*-values < 0.05; 0.01; 0.001. Symbols: **(B)** * vs. WT SAL; # vs. KO SAL; $ vs. WT PCP; **(E)** * vs. WT PCP.

by an investigator blinded to the treatments and genotype (**Figure 4A**). No difference was detected between the genotypes in either the total number of PV positive interneurons (**Figure 4B**) or in the percentage of those activated by systemic PCP (**Figure 4C**) in the layers II/III and V of the mPFC. Next, we explored the influence of P2X7R deficiency on the density of dopaminergic fibers. We did not observe major genotype-related anatomical differences by analyzing the double immunostaining for the c-Fos increase and tyrosine hydroxylase (TH, dopamine- and noradrenaline-synthesizing axon terminals marker) of the mPFC (**Figure 5A**). In this preliminary study, longitudinal quantification of signal intensity confirmed an increase in c-Fos expression (**Figure 5B**, upper panel) in wild-type PCP-treated animals within layer V (200/220 µm lateral to the midline). At the same time, there was no change in TH positive fiber density (**Figure 5B**, lower panel, and **Figure 5C**). Next, we have studied the release of dopamine with tritiated dopamine from the local dopaminergic afferents in the PFC to clarify the involvement of P2rx7$^{-/-}$ or P2rx7$^{tg/+}$. Analysis of 20 µM veratridine-induced (Fekete et al., 2009), Na^+ channel-mediated tritiated dopamine release (perfusion for 3 min leads to a transient Na^+ channel activation, **Figure 5D**) did not differ between genotypes in both sets of experiments. Confirming our previously published results (Koványi et al., 2016), in the PFC of P2rx7$^{-/-}$ animals, the basal dopamine release was lower (**Figure 5E**, left panel). On the other hand, dopamine uptake of P2rx7$^{tg/+}$ PFC was lower in comparison to the corresponding wild type controls (**Figure 5F**, right panel).

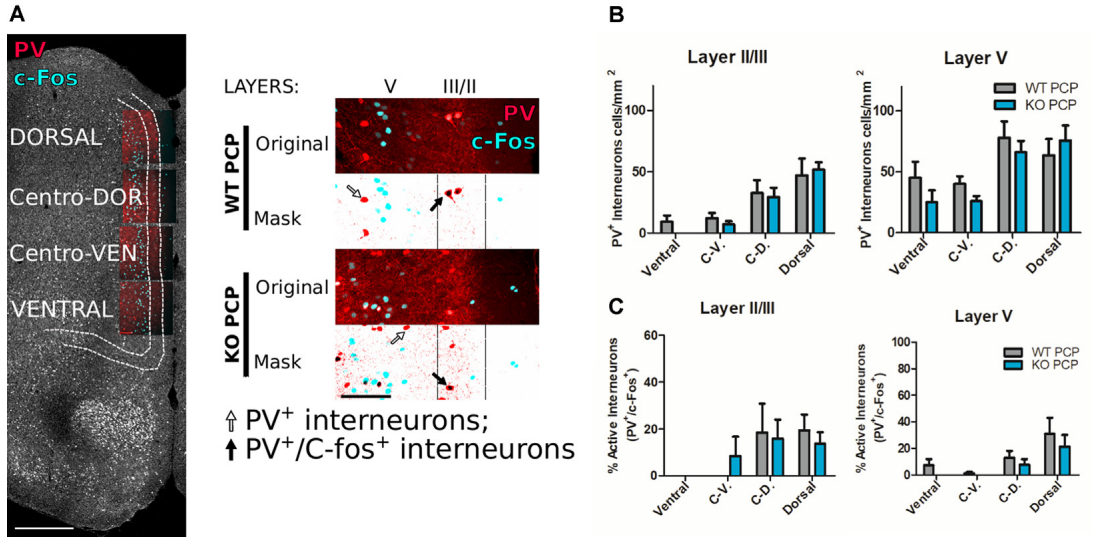

FIGURE 4 | Medial prefrontal parvalbumin (PV) interneurons do not differ in terms of numbers and levels of activity in PCP-treated P2rx7$^{-/-}$ and P2rx7$^{+/+}$ animals. **(A)** Examples of double immunostaining for c-Fos and PV. Reconstructed mPFC from a P2rx7$^{+/+}$ phencyclidine (PCP) treated (WT PCP) mouse. Scale bar, 500 μm (left). Representative images of dorsal mPFC double immunostaining (right). The original pictures are the stacks maximal projections while masks are the correspondent threshold images, combined to facilitate the manual counting of PV$^+$ interneurons (black arrow) and double PV$^+$ and c-Fos$^+$ (white arrow) interneurons in the II/III (80 μm) and V (320–350 μm) layers. Scale bar 100 μm. **(B)** Results of manual counting of PV$^+$ interneurons concentrations in mPFC of PCP-treated animals. **(C)** Results of the manual counting of double-positive PV$^+$ and c-Fos$^+$ interneurons expressed as the percentage of the counted PV$^+$ interneurons in the mPFC. $N = 4$ (WT PCP), $N = 3$ (KO PCP). Shown is mean ± SEM. Statistical analysis was performed with two-way ANOVA followed by Bonferroni's *posthoc* test. Scoring: no significant differences, p value > 0.05.

P2X7R Gene Deficiency may Play a Role in Microglial Contacts With Hyperactive Neuronal Soma

Next, we addressed the role of the P2X7R expressed by microglial cells in the context of PCP-driven neuronal activation. To label microglia, P2Y12 receptor (P2Y12R) immunohistochemistry was used, which is widely used as a marker of resting, but not activated microglia (Haynes et al., 2006; Cserép et al., 2020), and is a part of the exclusive microglia signature (Butovsky et al., 2014; Calovi et al., 2019).

Coronal IL and PL cortical layer V immunohistochemical staining against P2Y12R (**Figures 6A,C**) or P2Y12R and c-Fos (**Figures 6G,I**) from P2rx7$^{-/-}$ and P2rx7$^{+/+}$ animals treated with saline or PCP were performed. Moreover, additional triple immunohistochemistry, with the inclusion of the NeuN (neuronal nuclear marker) of slices from PCP-treated animals (**Figure 6E**), was performed. Manual and automated analyses showed no difference in microglial density and general ramification by treatment or genotype, while a slightly smaller P2Y12R immunoreactivity was noted in P2rx7$^{-/-}$ concerning P2rx7$^{+/+}$ after acute PCP (**Figure 6B**). Maximal projections (10 μm thick stacks) of IL and PL areas stained for P2Y12R allowed us to perform a soma-centered 2D-Sholl analysis over non-isolated microglial cells (**Figure 6C**, left). Thus, this analysis does not present the characteristic "crossing branches count" curve from single-cell ramifications. Microglia ramification in the cortex shapes an intricate network of processes, in which single cells marked by a unique morphology can extend up to tens of microns, rendering complex reconstruction and isolation of cellular microglia in a 3D space. This novel analysis returned in a mixed Sholl profile where, instead of a bell-shaped curve, the number of crossing branches increased linearly with the extension of the Sholl radius (**Figure 6D**). It is reasonable to consider that the area included by small Sholl circles (0–20 μm radius, red bar **Figures 6C,D**) usually represents the ramification of a single cell since microglial nuclei are evenly distributed in the cortex. Bigger Sholl circles (20–40 μm radius, green bar **Figures 6C,D**) cover the territory patrolled by several cells, therefore representing an inter-cellular territory (**Figure 6C**, right panel). Adopting as the y Cartesian axis graph the graph identity function ($f_{(x)} = x$, red-dashed line **Figure 6D**) allows better visualization of differences in the microglial ramification profiles between the groups (**Figure 6D″**). This illustrates that the difference between groups was not related to single-cell ramifications (**Figure 6C**; red bar), while P2rx7$^{-/-}$ microglial processes were significantly denser in the intercellular territory (**Figure 6C**, left panel; green bar, **C**, right panel; the green-colored area, **D"**; green bar). Genotype-related differences faded with acute PCP treatment (**Figure 6D″**).

To better understand the relationship between neurons and microglia in the context of PCP-driven hyperactivation, mPFC slices were triple-immunostained for P2Y12R, NeuN, and c-Fos (**Figure 6E**). Neurons whose nuclei were NeuN$^+$ and c-Fos$^-$ were considered to be not recruited by the PCP-driven effect and are herein referred to as inactive neurons. We examined whether the

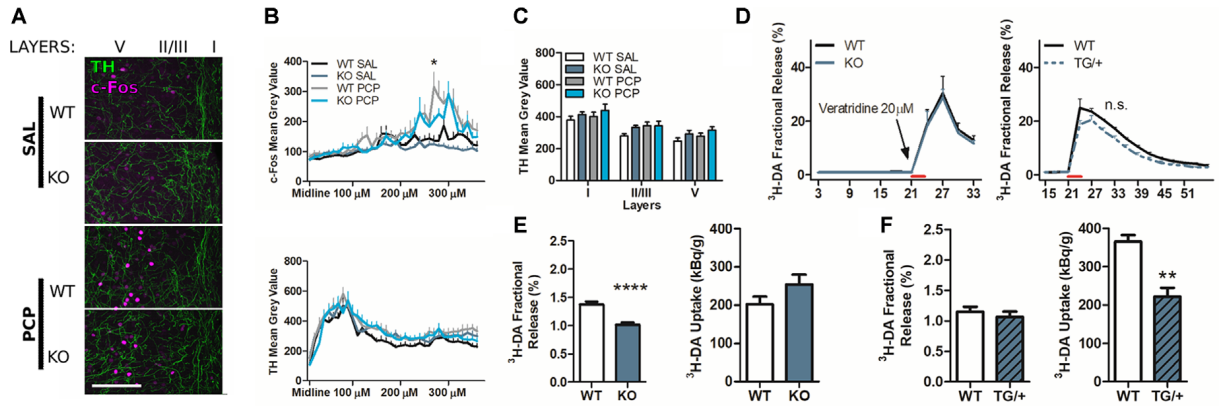

FIGURE 5 | The P2rx7$^{-/-}$ prefrontal cortex displays a lower dopamine release, while P2rx7 overexpression leads to lower dopamine uptake compared to the respective wild-type (WT) prefrontal cortex. **(A)** Example of P2rx7$^{-/-}$ and P2rx7$^{+/+}$ mPFC double immunostaining for c-Fos and the dopamine- and noradrenaline-synthesizing fiber marker tyrosine hydroxylase (TH). Z-stack maximal projections. Scale bar 100 µm. **(B)** Longitudinal quantification, with FIJI ImageJ, of the c-Fos (up) and TH (down) immunoreactivity. The profiles of the average signal along the mPFC medial-distal axis show that layer I, where the peak expression of TH fibers is detected, does not correspond with the peak of c-Fos activated neurons (layer V). **(C)** Quantification of TH expression. The profile intensities were grouped by layers. *N* = 2; 6 pictures per animal. Shown is mean ± SEM. Statistical analysis was performed with two-way ANOVA followed by Bonferroni's *posthoc* test. Scoring: +; means *p*-value < 0.05. Symbols: * WT PCP vs. KO PCP at 270 µm and 280 µm. **(D)** Graph displays stimulated fractional release from PFC slices induced by 20 µM veratridine perfusion. *N* = 28 (WT); *N* = 11 (KO), left; *N* = 4 (WT); *N* = 4 (TG/+), right. Shown is mean ± SEM. Statistical analysis was performed with two-way ANOVA followed by Bonferroni's *posthoc* test. Scoring: no significant differences, *p*-value > 0.05. **(E)** Results of the P2rx7$^{-/-}$ [^{3}H]-DA release experiments. The quantification of dopamine release and uptake confirm the previously published data (Koványi et al., 2016). *N* = 14 (WT); *N* = 8 (KO). **(F)** Results of the P2rx7$^{tg/+}$ [^{3}H]-DA release experiments. The quantification of dopamine release and uptake suggests that the newly tested P2X7R-EGFP line display lower DA uptake concerning the wild type cortex; *N* = 4 (WT) *N* = 4 (TG/+). Shown is mean ± SEM. Statistical analysis was performed with unpaired Student's *t*-test with Welch corrections. Scoring: ++; ++++; means *p*-values < 0.01; 0.0001. Symbols: **(E,F)** * vs. WT.

guidance of microglial branches toward active nuclei would be detectable by applying the aforementioned 2D Sholl analysis, this time centered in active or inactive neurons of the mPFC layer V (**Figure 6E**). We confirmed that c-Fos$^+$ neuronal nuclei, both in the analyzed P2rx7$^{-/-}$ and P2rx7$^{+/+}$ PCP treated animals, are surrounded by a higher number of microglial branches compared to inactive neurons in the same field of view (**Figure 6F**). While revealing a significant treatment effect, the 2D Sholl analysis did not detect differences between genotypes concerning microglial branches surrounding the nearby parenchyma of both inactive and activated neurons (**Figure 6F″**).

As for microglia organization concerning c-Fos positive IL and PL neurons in PCP-treated animals (**Figure 6G**), we noticed that in P2rx7$^{-/-}$ mice, the strongly activated neurons (c-Fos^{++}) tended to be more often in contact with microglial somata. However, this was not statistically significant (Student's *t*-test $p = 0.0967$, **Figure 6H**). To obtain a precise quantification of the microglia branches in the proximity of hyperactive neurons, larger z-stacks over 30 µm thick were taken. A 3D Sholl analysis of microglial staining, centered on c-Fos$^+$ nuclei, was performed (**Figure 6I**). The 3D Sholl analysis, performed over a radius of 15 µm, revealed a greater number of microglial branches recruited by c-Fos$^+$ neurons in the P2rx7$^{-/-}$ prefrontal cortical layer V (**Figure 6J**).

Intrinsic Properties of P2rx7$^{-/-}$ mPFC Layer V Pyramidal Neurons

To investigate whether the difference in PCP-driven c-Fos immunoreactivity could be related to congenital/developmental intrinsic hypoexcitability of neurons in P2rx7$^{-/-}$ animals, patch-clamp recordings of mPFC layer V neurons were performed (**Figure 7A**). To reduce developmental variability, neurons from mice older than 59 days were recorded.

Thirteen neurons from eight P2rx7$^{+/+}$ animals and 21 neurons from six P2rx7$^{-/-}$ animals were patch-clamped and analyzed. No difference in series resistance (35.7 ± 3.2 MΩ P2rx7$^{+/+}$; 33.4 ± 2.4 MΩ P2rx7$^{-/-}$) and membrane resistance (375.6 ± 38.7 MΩ; 433.5 ± 56 MΩ) were found between the genotypes. Similarly, no difference in resting membrane potential recorded at 0 pA current-clamp was observed (**Figure 7B**). As measures of excitability, rheobase and IV relationships were recorded in current-clamp mode using the ramp and step protocols, respectively. We found no difference in these values between P2rx7$^{-/-}$ and P2rx7$^{+/+}$ control animals (**Figures 7C,D**). Following action potential-induced depolarization, P2rx7$^{-/-}$ PFC neurons displayed faster repolarization of the membrane potential than wild-type (red segment **Figures 7A,E**). Moreover, during depolarizing current injections, P2rx7$^{+/+}$ neurons fired more action potentials than P2rx7$^{-/-}$ neurons at the same current pulse, indicating a difference in spike accommodation (**Figure 7F**).

Our data confirm that in mPFC layer V of P2rx7$^{-/-}$ and P2rx7$^{+/+}$, pyramidal neurons of young adult C57Bl/6J animals present similar input-related response characteristics, and no difference in the current amount necessary to reach the action potential threshold (rheobase). We found that P2rx7$^{+/+}$ neurons respond more robustly to membrane depolarization

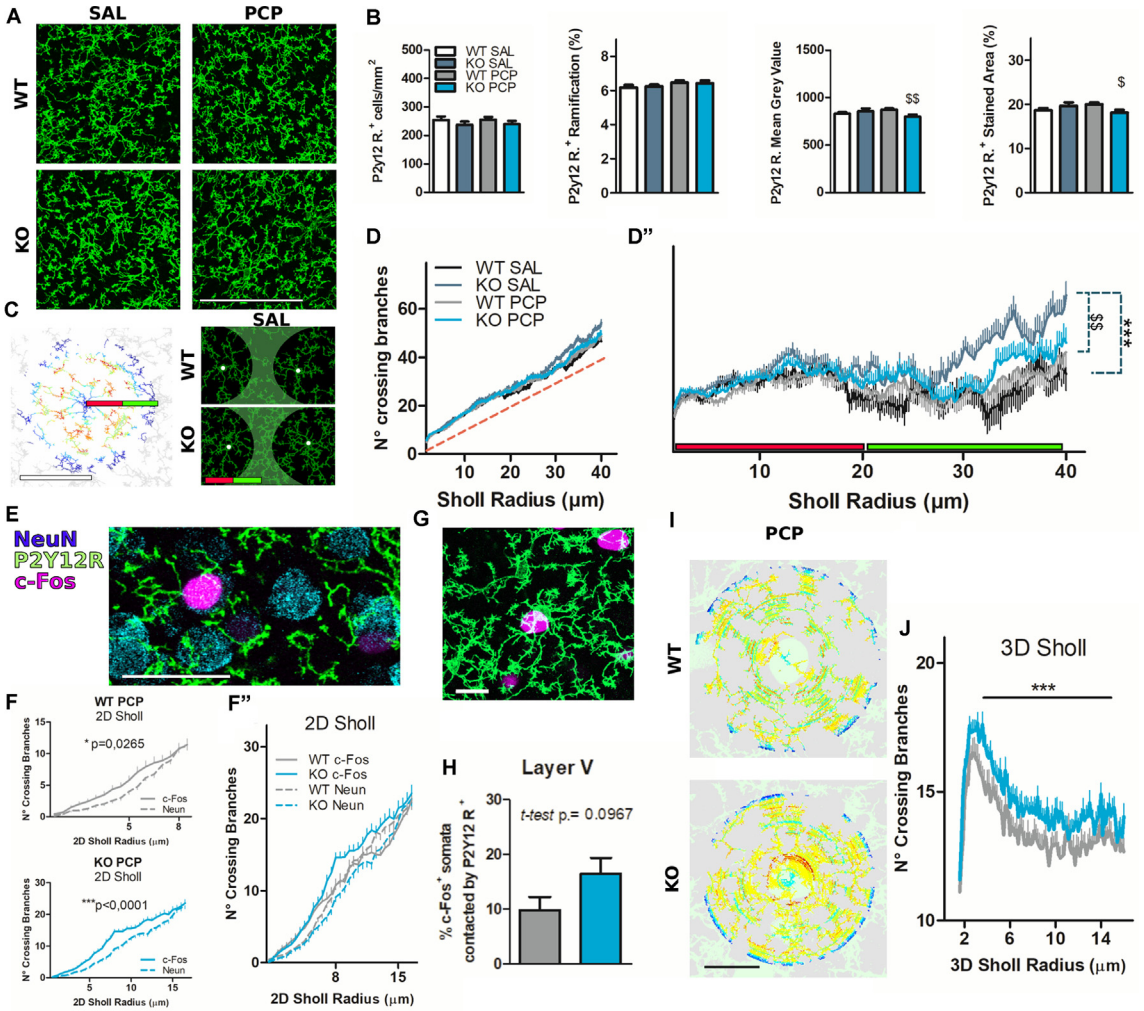

FIGURE 6 | Microglial cells organize their processes around hyperactivated neurons, and their ramification is partly dependent on P2X7 receptor (P2X7R) expression. **(A)** Representative images of mPFC layer V immunostaining for the microglial membrane marker P2Y12R. Scale Bar, 100 µm. **(B)** Bar diagrams quantify P2Y12R positive cell properties characterization. Cell count was manually performed (left). The general ramification was quantified with FIJI ImageJ as the percentage of the stained area from the skeleton stacks (left center). The signal intensity was assessed with FIJI ImageJ as the mean gray value (center-right) and percentage of threshold stained area (right). *N* = 3 (Saline, Sal), *N* = 4 (phencyclidine, PCP). **(C)** Example of 2D Sholl analysis of the mPFC layer V non-isolated microglia (WT SAL), after processing the skeleton of the maximal stack projection, all performed in FIJI ImageJ. The first 20 µm of the Sholl radius is likely to be occupied by a single-cell harborization (green line); from 20 µm to 40 µm of Sholl radius (inter-cellular territory) are present ramifications of several cells (red line). Scale bar, 40 µm (left). Representative images of the difference in inter-cellular territory ramification from vehicle-treated $P2rx7^{+/+}$ and $P2rx7^{-/-}$ microglia. The green-colored areas were approximately 20 µm from the microglia nuclei signed with white spots (right). **(D)** Quantification of the 2D Sholl analysis is represented as the number of crossing branches per radius value of non-isolated microglial cells. The red dashed line is the identity function $y = x$. **(D")** The same graph presented in **(D)**, but the y-axis has changed with the identity function $y = x$ for better visualization. *N* = 3 (Sal), *N* = 4 (PCP); 2D cell-center numbers analyzed: *n* = 38 (WT SAL), *n* = 48 (KO SAL), *n* = 60 (WT PCP), *n* = 59 (KO PCP). Shown is mean ± SEM. Statistical analysis was performed Mann–Whitney *U*-test over the curves between 0 and 20 µm (not significant) and 20–40 µm. **(E)** Example of triple immunostaining of the mPFC layer V for microglia (P2Y12R), neuronal nuclei (NeuN), and activated neuronal nuclei. Scale Bar 30 µm. **(F)** Quantification of the 2D Sholl profiles of microglial branches surrounding PCP-activated (continuous line) or inactive (dashed line) neuronal nuclei. Wild-type PCP-treated animals, in prelimbic and infralimbic layer V areas, displayed preferential microglial contact in the 8 µm radius, still targeting c-Fos$^+$ activated neurons after 180 min from the PCP-induced psychotomimetic effect plus 20 min after Nembutal anesthesia. The preferential microglial contact towards activated neurons was even larger, in terms of the radius (15 µm) in $P2rx7^{-/-}$ animals. *N* = 4; 2D cell-center numbers analyzed: *n* = 52 (WT NeuN), *n* = 51 (KO NeuN), *n* = 34 (WT c-Fos), *n* = 36 (KO c-Fos). Shown is mean ± SEM. Statistical analysis was performed with two-way ANOVA followed by Bonferroni's *posthoc* test, exact *p*-values are displayed on graphs. **(F")** Values presented in *F* grouped in a single chart. In the 8 µm Sholl radius, c-Fos vs. NeuN in two-way ANOVA Bonferroni post hoc test, for both genotypes, return *p*-values > 0.05. **(G)** Example of mPFC layer V double immunostaining representing P2Y12R positive microglia in which the cell body is in physical contact with an activated c-Fos$^+$ neuronal nucleus. Scale bar 15 µm. **(H)** Quantification of the manually counted cell-body contacts between the microglia and activated neurons expressed as a percentage of counted c-Fos$^+$ nuclei. *N* = 4. Shown is mean ± SEM. Statistical analysis was performed with unpaired Student's *t*-test, the exact *p*-value shown on the graph, not significant. **(I)** Example of c-Fos$^+$ nuclei centered 3D Sholl analysis of P2Y12R$^+$ microglial cell membrane in the mPFC layer V from PCP-reacted $P2rx7^{+/+}$ and $P2rx7^{-/-}$ mice. Scale bar 15 µm. **(J)** Results from the quantification of the 3D Sholl analysis. *N* = 3; 3D cell-center numbers: *n* = 52 (WT PCP); *n* = 45 (KO PCP). Shown is mean ± SEM. Statistical analysis was performed with Mann–Whitney *U*-test. Scoring: +; ++; +++; means *p*-values < 0.05; 0.01; 0.001. Symbols: **(B)** $^{\$}$ vs. WT PCP; **(D")** $^{\#}$ vs. KO SAL; $^{\$}$ vs. WT PCP; **(F)** * c-Fos vs. NeuN; **(J)** * KO PCP vs. WT PCP.

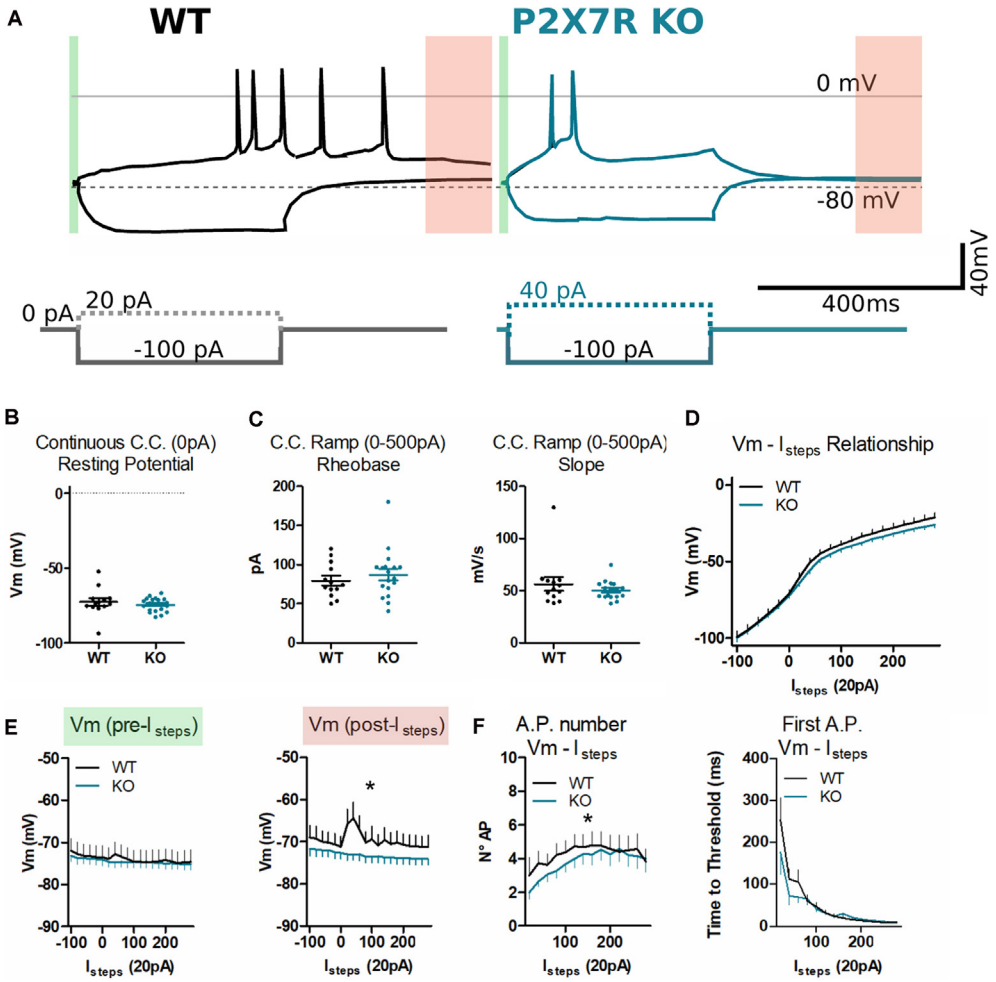

FIGURE 7 | P2rx7$^{-/-}$ mPFC layer V neurons display similar depolarization but faster re-polarization after firing action potentials. **(A)** Example of traces from P2rx7$^{+/+}$ and P2rx7$^{-/-}$ mPFC layer V pyramidal neurons during the whole-cell patch-clamp current step injection (Istep) protocol. Recording of the 400 ms hyperpolarizing step (−100 pA) and the first step triggering action potential firing (+20 pA for P2rx7$^{+/+}$, left; 40 pA for P2rx7$^{-/-}$, right) have been reported. **(B)** The voltage of membrane in the current-clamp configuration. Holding current at 0 pA for 10 s. **(C)** Analysis of the ramp current injection (0–500 pA) over 1 s. Column analysis **(B,C)**, each point represents an individual neuron. *N* = 8 P2rx7$^{+/+}$ (13 neurons); *N* = 6 P2rx7$^{-/-}$ (21 neurons). Shown is mean ± SEM. Statistical analysis was performed with unpaired Student's *t*-test. Scoring: no significant differences, *p*-value > 0.05. **(D)** Current clamp step protocol (Istep) IV relationship. Istep minimum of −100 pA, Istep maximum of +280 pA, Istep size of 20 pA, Istep time of 400 ms, and sweep frequency of 1 Hz. Averages ± SEM voltage over 400 ms Istep. **(E)** Membrane voltage before and after Istep in the current clamp at 0 mV. Average voltage over 30 ms before Istep [A green, Vm (pre-Istep), left], and over 100 ms [A red, 300–400 ms after Istep, Vm (post-Istep), right]. **(F)** Count of action potentials fired during the 400 ms Istep (left) and plot of the time to the threshold of the first action potential. *N* = 8 P2rx7$^{+/+}$ (12 neurons); *N* = 6 P2rx7$^{-/-}$ (19 neurons). Shown is mean ± SEM. Statistical analysis was performed with Mann–Whitney *U*-test. Scoring: *indicates *p*-value < 0.05.

than P2rx7$^{-/-}$. Wild-type neurons present a shorter refractory period, therefore firing a higher number of action potentials, and has longer-lasting depolarization of the membrane after action potential firing. This indicates that P2rx7$^{-/-}$ could be less responsive to strong neuronal activation.

P2X7R-EGFP Overexpressing Reporter Mice Displayed Higher Sensitivity to Acute Low-Dose PCP Treatment

Our work conducted on P2rx7$^{-/-}$ and P2rx7$^{+/+}$ suggests that P2X7R deficiency ameliorates acutely induced PCP psychotomimetic hyperactivity in the mPFC, likely involving lower basal dopamine release, increased microglia-hyperactive neuron interaction, and faster neuronal repolarization after action potential firing. These findings collectively result in lower activation of a subgroup of layer V mPFC neurons.

To corroborate these findings in P2X7R knockout animals, it is crucial to test an alternative animal model to evaluate possible predominant collateral artifacts, such as developmental abnormalities.

Therefore, we carried out a behavioral study on a recently developed mouse model, that is, the heterozygous

C57Bl/6J mouse line overexpressing the P2X7-EGFP protein (Kaczmarek-Hajek et al., 2018), referred to as P2rx7$^{tg/+}$. To assess acute PCP-susceptibility, P2rx7$^{tg/+}$ and P2rx7$^{+/+}$ mice were subjected to a low-dose of PCP (2 mg/kg i.p.) followed by a modified protocol of the open field and social withdrawal tests, previously validated for PCP treatment evaluations. Their behavior was recorded over a 10 min trial period (45 min after the PCP or vehicle, **Figure 8A**) and analyzed, as previously described (Koványi et al., 2016).

Basal levels of locomotion, social behavior, stereotypical behavior, and ataxia were not different between the genotypes, while PCP treatment affected several behavioral parameters (**Figure 8B**). In P2rx7$^{+/+}$, acute PCP treatment strongly diminished social interaction and promoted stereotypical behavior (**Figure 8B**, left-center panel, and right-center panel). However, this lower dose of PCP did not significantly alter the locomotion of P2rx7$^{+/+}$ animals and failed to trigger ataxia (**Figure 8B**, left and right panels). In contrast, the acute PCP effect involved all tested behavioral parameters in P2rx7$^{tg/+}$ mice, which exhibited hyperlocomotion and features related to ataxia already appearing in some mice (**Figure 8B**, left and right panels).

To visualize the P2X7R protein expression in the mPFC, we have performed immunostaining against P2X7R-EGFP and the purinergic receptor P2Y12R, using the P2rx7$^{tg/+}$ reporter mice. **Figure 8C** shows the overall P2X7R expression pattern (violet), by immunostaining against the EGFP part of the P2X7R and the P2Y12R (green). The presence of the receptor is conspicuous in the microglia of this mice model, yet interestingly we observed EGFP positive/P2Y12R negative fibers as well, implying that the receptor is also expressed by other neural cell types (**Figure 8C**).

Acute PCP Treatment-Induced Hyperactivity and Layer-Specific Neuronal Activation in the mPFC Was Exacerbated in P2rx7$^{tg/+}$ Mice

To corroborate the involvement of P2X7R in the PCP-induced hyperactivity of the mPFC circuit, the acute PCP experiment was repeated similarly (normal light), this time testing the P2rx7$^{tg/+}$ mouse line (**Figure 9A**).

PCP increased locomotion and elicited stereotypic behavior. Hyperlocomotion and rotational stereotypic behavior peaked between 30 and 70 min after injection (**Figures 9B,C**).

The effect of PCP in P2rx7$^{tg/+}$ animals were strongly pronounced in terms of stereotypy in respect to the P2rx7$^{+/+}$ mice (**Figure 9C**). Fluorescent c-Fos immunostaining of treated animals in both analyzed bregmas showed the typical band of c-Fos immuno-positive neuronal somata corresponding to the layer V of the IL and prelimbic PL areas (**Figure 9D**).

A similar analysis to the one performed in **Figure 3** was performed on 5 μm thick stacks maximal projections images, focusing on the layer V of the IL and PL areas of PCP-treated mice. The exacerbated stereotypy of P2rx7$^{tg/+}$ mice was accompanied by a higher number of strongly activated (c-Fos^{++}) positive nuclei, specific to the Bregma 1.70 but not to the Bregma 2.10, in the IL and PL region of the Layer V (**Figure 9E**).

In this case, the dimension and intensity threshold to count c-Fos^{++} neurons were set to 100 μm^2 and 1,100 mean gray value (ImageJ standardized units), respectively, selected according to the visual comparison with the previously selected nuclei. These results suggest that the overexpression of P2X7R has opposite effects concerning the receptor-deficient model, and exacerbated the psychotomimetic effects of PCP and the consequent layer-specific neuronal activation in the mPFC.

Subchronic PCP Failed to Induce Working Memory Deficits in P2X7R Deficient Animals

We decided to evaluate the possible impact of P2X7R deficiency on the cognitive symptoms induced by repeated PCP administration (daily 10 mg/kg i.p. per 7 days, Zain et al., 2018) with a simple battery of behavioral tests (**Figure 10A**).

Before the first treatment, P2rx7$^{-/-}$ animals had a higher body mass compared to the P2rx7$^{+/+}$ animals (Giacovazzo et al., 2018), which normalized after the 1 day (**Figure 10E**).

To confirm whether repeated PCP injections did not induce long-lasting alterations in basal locomotor activity, four animals per group were tested in a confined environment, a glass cylinder, 90 min, and 20 h after receiving the third PCP or vehicle treatment. After drug clearance, no difference in locomotor behavior was observed (**Figure 10B**).

At the end of the subchronic PCP or vehicle treatment, animals experienced 72 h of withdrawal before being tested in a Y-maze. We were particularly interested in assessing the status of the working memory. The working memory is strictly dependent on the mPFC microcircuit integrity, and at the same time, is a major cognitive symptom of SCZ that is still largely untreated by available treatments (Elsworth et al., 2014).

Both PCP and vehicle-treated P2rx7$^{-/-}$ animals covered a significantly shorter distance during the trial, yet the number of total alternations was not affected by either genotype or treatment (**Figure 10C**, left and center panels).

P2rx7$^{+/+}$ PCP-treated mice tested for spontaneous alternations displayed fewer alternations concerning the P2rx7$^{+/+}$ and P2rx7$^{-/-}$ PCP-treated animals. The scores of P2rx7$^{-/-}$ animals were unaffected by subchronic PCP, indicating that subchronic PCP failed to induce working memory deficits in P2X7R deficient animals (**Figure 10C**, right panel).

After two additional days of washout, an open field test was performed for 10 min on each animal, which did not show any influence by either the genotype or treatment in locomotor activity (**Figure 10D**).

Finally, we quantified cytokine levels in the brain, considering that neuroinflammatory events are accompanied by changes in cytokine concentrations, and are reportedly depend on P2X7R-mediated mechanisms (Di Virgilio et al., 2017; He et al., 2017).

A significant constitutive increase in the fractalkine ligand (CX3CL1) level was found in the P2rx7$^{-/-}$ PFC, after both PCP and vehicle subchronic treatments, whereas the levels of other measured cytokines were not affected by either the treatment or genotype (**Figure 10F**). The effect of subchronic

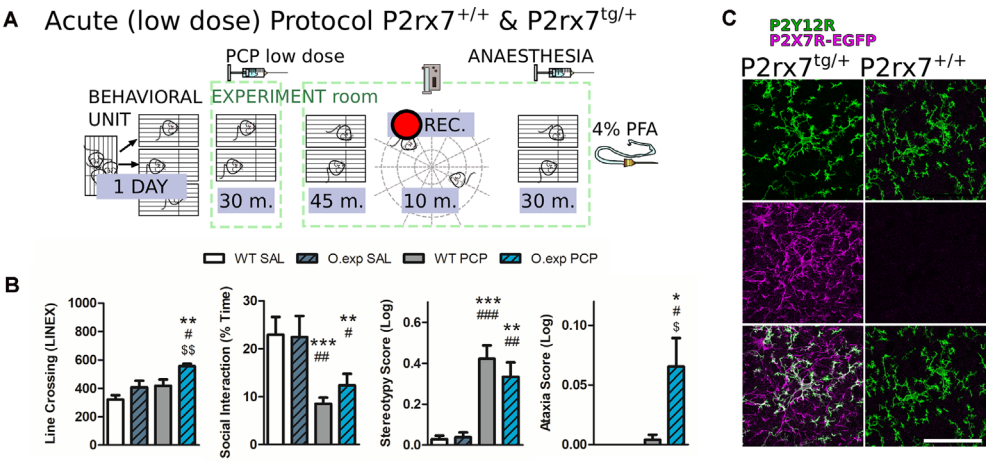

FIGURE 8 | P2rx7$^{tg/+}$ mice are more susceptible to the acute low dose of phencyclidine (PCP) psychotomimetic effect. **(A)** Schematic representation of the behavioral protocol for acute treatment. The vehicle or low dose PCP 2 mg/kg i.p. injection is represented by the PCP syringe. Animals were tested 45 min after receiving the treatment. **(B)** Quantification of different aspects of the PCP-induced behavior in the coupled open-field test. Locomotor activity (left), time spent performing social behavior (left-center), and scores for stereotypical behavior (center right) and ataxia (right) were quantified and analyzed. *N* = 10 (WT SAL), *N* = 12 (O.exp SAL), *N* = 6 (WT PCP), *N* = 12 (O.exp PCP). Shown is mean ± SEM. Statistical analysis was performed with one-way ANOVA followed by Dunn's comparison *posthoc* test (left and left-center); and Kruskal-Wallis test (center-right and right). Scoring: +; ++; +++; mean *p*-values < 0.05; 0.01; 0.001. Symbols: * vs. WT SAL; # vs. O.exp SAL; $ vs. WT PCP. **(C)** Example of the P2rx7$^{tg/+}$ layer V mPFC double immunostaining for P2Y12R (green) and GFP, labeling the P2X7R-GFP protein (violet). Scale bar 50 μm.

PCP on IL-1β was not significantly different between the two genotypes or when expressed as a percentage of the control (**Figure 10F″**).

Our subchronic PCP experiments, which suggest a beneficial role of P2X7R deficiency in the context of the PCP-induced loss of working memory in rodents, do not point to a major change in the consequent neuroinflammatory phenomenon in the prefrontal region.

DISCUSSION

In this study, we found that the effect of PCP in mice is modulated by P2X7R in terms of positive and cognitive symptoms. Genetically deficient animals for the receptor displayed a lower susceptibility to the psychotomimetic effects of the dissociative anesthetic, while the overexpression of the purinergic receptor produced a typically positive symptom response within a sub-optimal PCP dose for psychosis-like activity (Paasonen et al., 2017) and exacerbated stereotypy with an acute psychotomimetic 10 mg/kg PCP dose. Most importantly, after a subchronic PCP treatment, P2rx7$^{-/-}$ mice did not display a reduction in working memory, typical of the model's cognitive symptomatology. Before discussing the results obtained with P2rx7$^{-/-}$ mice, it is appropriate to review the current genetic model used (Solle et al., 2001), since it is a source of some controversy. The gene of the P2rx7 receptor protein is located in the mouse chromosome Chr.5, 62.50 cM. Persistent stimulation of P2X7R triggers the ATP-dependent opening of an aqueous plasma membrane pore. Most of the pathophysiological functions of P2X7R are thought to be dependent on this macropore formation, suggested to be an intrinsic property of the channel itself (Di Virgilio et al., 2018). While nine splicing variants are reported in humans, in the mouse four alternative variants that generate four P2X7R subunits have been identified (Sluyter, 2017; Adinolfi et al., 2010). The full-length variant, named P2X7A in human and mouse, is generally found co-expressed with the mouse naturally occurring P2X7k variant, which seems to escape inactivation, representing a gain of function isoform (Kaczmarek-Hájek et al., 2012). Regarding receptor knockout animal models, at least two strains of mice are currently commercially available: a P2X7R knock out strain with reporter function produced by GlaxoSmithKline, in which exon 1 has been replaced with a lacZ gene and neomycin cassette (Neo); and a strain from Pfizer (commercially available from The Jackson Laboratory, the strain used in the current study), in which a portion of the exon 13, encoding Cys506 to Pro532, has been deleted and replaced with a Neo, truncating the long C–terminal cytoplasmic tail (Sikora et al., 1999; Solle et al., 2001). Nonetheless, antibody detection and Ca^{2+} responses indicative of P2X7R functional expression in cerebellar neurons and midbrain synaptosomes from the Pfizer knockout mice line have been reported, yet the pharmacological assays suggest a P2rx7 loss of function in the knockout line (Sánchez-Nogueiro et al., 2005; Marín-García et al., 2008). In addition, whether two C-terminally truncated splice variants of the P2X7R escape the Pfizer Knock out inactivation strategy cannot be excluded; however, currents mediated *via* these C-terminally truncated P2X7 splice variants are of much lower amplitude compared to the P2X7A mediated receptor currents (Adinolfi et al., 2010; Masin et al., 2012). As a result of the presence of the truncated and probably partly functional variant of P2X7 (Sánchez-Nogueiro et al., 2005), it might contribute to the relatively modest amelioration

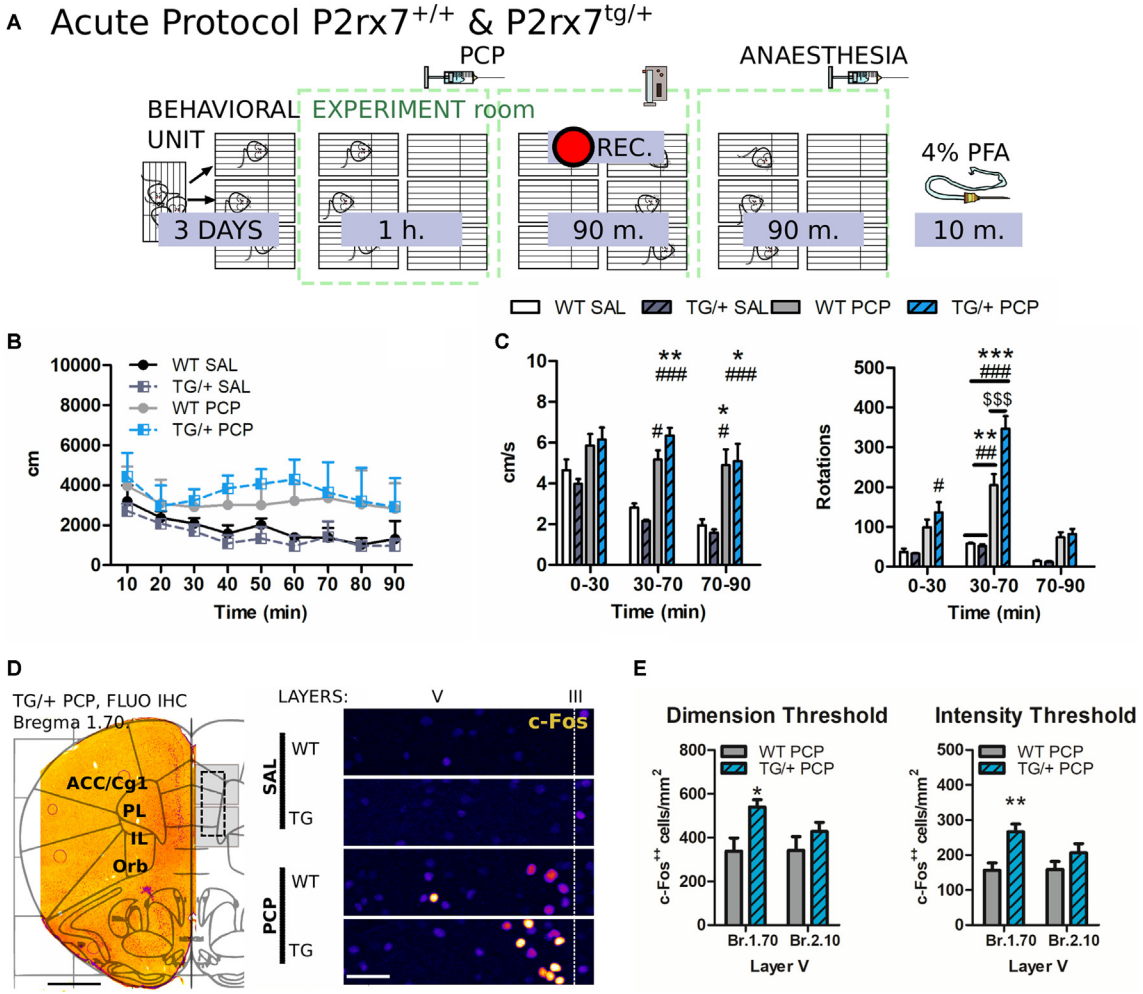

FIGURE 9 | P2rx7$^{tg/+}$ mice display exacerbated acute phencyclidine (PCP) psychotomimetic effect correlated with a higher PCP-driven hyperactivation of the mPFC neurons. **(A)** Schematic representation of the behavioral protocol for acute treatment. The vehicle or PCP 10 mg/kg i.p. injection is represented by the PCP syringe. **(B)** Distance moved by the animals after the injection, in 10 min time bins. **(C)** Average velocity (left) and numbers of circle rotations (right) averaged for the first 30 and second 40 min, plus the remaining recorded time. The PCP psychotomimetic effect was evident 30 min after the PCP injection. *N* = 3 (WT and TG/+ Sal), *N* = 9 (TG/+ PCP), and *N* = 9 (WT PCP). Shown is mean ± SEM. Statistical analysis was performed with two-way ANOVA followed by Bonferroni's *post hoc* test. **(D)** Representative c-Fos fluorescent immunostaining of the coronal prefrontal cortex (PFC) from a P2rx7$^{tg/+}$ PCP treated mouse overlapped with the Paxinos ATLAS (Bregma +2.00). The left hemisphere represents the field of view of the pictures quantified. Scale bar: 1 mm (left). Representative immunostaining from 5 μm projected images used for automated c-Fos nuclei counting. Layer V width range *V* = 280–350 μm. Scale bar: 50 μm (right). **(E)** Quantification of prelimbic and infralimbic content of strongly activated c-Fos^{++} nuclei, by dimension (left) and intensity (right) threshold. Automatic counting with FIJI ImageJ in the isolated layer V for fluorescent immunostaining. Shown is mean ± SEM. Statistical analysis was performed with unpaired Student's *t*-test. Scoring: +; ++; +++; mean *p*-values < 0.05; 0.01; 0.001. Symbols: **(C)** * vs. WT SAL; # vs. TG/+ SAL; $ vs. WT PCP; **(E)** * WT PCP vs. TG/+ PCP.

of symptoms in the P2X7-KO mice. Additionally, the Pfizer produced P2X7R knockout mouse line is bred over a C57bl/6, which constitutively co-express the P2X7A and P2X7k splicing variants of the receptor in all the systems. Our RT-qPCR results confirm the presence of P2rx7 RNA in the prefrontal cortex of the Pfizer strain, yet the disrupted portion is found only in wild type tissue suggesting that a COOH truncated isoform with a lowered functionality is expressed by the tested animals.

Our current results confirm and extend our previous observations (Koványi et al., 2016) and others (Lord et al., 2014; Gubert et al., 2016) obtained with genetic deletion and pharmacological inhibition of P2X7R. A significant novel observation of the present study is the alleviation of neuronal activation in a restricted area of the mPFC (layer V of prelimbic and infralimbic areas) in response to PCP treatment in mice genetically deficient for P2rx7, while the opposite effect is obtained with the receptor overexpression. This finding identifies a potential primary site of action, whereby P2rx7 might affect PCP-induced behavior.

The rodent mPFC primarily involved in the PCP psychotomimetic effect is of particular interest since this

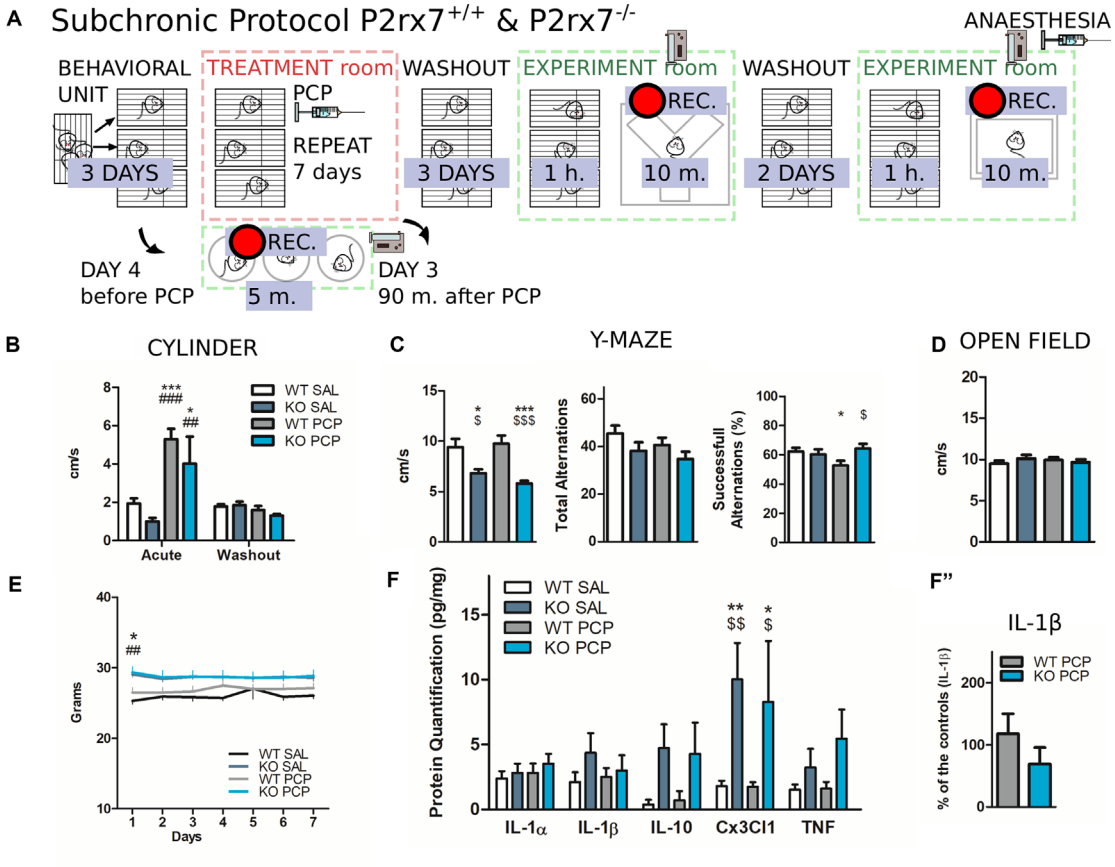

FIGURE 10 | P2rx7 gene-deficient animals show reduced deficit in working memory after PCP subchronic treatment independently of prefrontal neuroinflammation. **(A)** Schematic representation of the behavioral protocol for the subchronic treatment. The vehicle or PCP 10 mg/kg i.p. injections were repeated for seven consecutive days in a treatment room (red-dashed square). Animals were tested during and after the treatment washout a separated experimental room (green-dashed square). **(B)** Velocity inside a glass cylinder during a 5-min-long test. "Acute" refers to the test performed 90 min after the third PCP/Saline injection; (third day); "Washout" refers to the test performed 20 h after the third PCP/Saline injection. *N* = 4. Shown is mean ± SEM. Statistical analysis was performed with two-way ANOVA followed by Bonferroni's *post hoc* test. **(C)** Results of the Y-maze spontaneous alternation test performed the third day after withdrawal from the subchronic treatment. The animals were placed in the arena and recorded for 10 min. Semi-automated analysis with EthoVision® XT. *N* = 8, Shown is mean ± SEM. Statistical analysis was performed with two-way ANOVA followed by Bonferroni's *posthoc* test (left, center); unpaired Student's *t*-test (right, successful alternations). **(D)** Average velocity over 10 min open field test. **(E)** Weight of the animals during the PCP subchronic treatment. *N* = 8, Shown is mean ± SEM. Statistical analysis was performed with two-way ANOVA followed by Bonferroni's *post hoc* test. **(F)** Interleukins quantification with bead-array FACS analysis from prefrontal cortices of P2rx7$^{-/-}$ and P2rx7$^{+/+}$ vehicle or PCP subchronically treated mice. Brain extraction 15 days after the first injection. **(F")** Effect of PCP subchronic treatment on the level of IL-1β protein expressed as a percentage of the control. *N* = 8 (Sal), *N* = 12 (PCP), Shown is mean ± SEM. Statistical analysis was performed with two-way ANOVA followed by Bonferroni's *posthoc* test. Scoring: +; ++; +++; mean *p*-values < 0.05; 0.01; 0.001. Symbols: * vs. WT SAL; # vs. KO SAL; $ vs. WT PCP.

area is affected by SCZ pathology (dorsolateral frontal cortex for humans) and SCZ models of both positive, negative, and cognitive symptoms are a crucial node for cognitive abilities (Celada et al., 2013). We confirmed that PCP treatment produced a lower number of heavily stained c-Fos neuronal nuclei in the P2rx7$^{-/-}$ genotype, which is an indirect measurement of *in vivo* neuronal activity (Chung, 2015). In the same area, heterozygous P2X7R-EGFP mice displayed an increased concentration of strongly stained c-Fos neurons compared to wild type controls, therefore showing an opposite readout concerning the knockout animals. Our data imply that the difference in P2X7R expression potentially modulates the PCP effect concerning the prefrontal activity in mice. However, we cannot exclude that the P2rx7 genetic manipulations could lead to artifacts, such as altered protein degradation dynamics.

To explore possible protective mechanisms happening in the P2rx7$^{-/-}$, we examined different aspects of the fifth layer of the infralimbic and prelimbic cortices. Our observation from the double and triple immunostaining, [^{3}H]-DA release experiments, and electrophysiology were consistent with a possible pleiotropic role of P2rx7 gene deletion as a negative modulator of the PCP psychotomimetic effect.

Differences in PV interneuron activation were excluded from playing a role linked to the lower c-Fos immunoreactivity detected in PCP-treated P2rx7$^{-/-}$ mice. It is worth mentioning that a previous c-Fos study reported an increase in PV interneuron activity in areas involved in acute PCP-induced

hyperactivity, but different from the mPFC (Celada et al., 2013; Hervig et al., 2016). The detrimental effect of subchronic PCP models on prefrontal GABAergic interneurons is well documented (Piyabhan et al., 2019).

To rule out developmental abnormalities in our model strain we investigated and found no gross anatomical deviation of the dopaminergic and noradrenergic fibers. Furthermore, $P2rx7^{-/-}$ and $P2rx7^{tg/+}$ displayed no difference in the chemically stimulated local release of tritiated dopamine. We confirmed a small but significant effect on basal dopamine release from the local dopaminergic fibers in the $P2rx7^{-/-}$ PFC (Koványi et al., 2016). Interestingly, in the $P2rx7^{tg/+}$ PFC slices, we could detect a lower tritiated dopamine uptake. These direct measurements may imply a functional role of P2rx7 in regulating basal dopamine local concentration *via* modulating receptor expression. Additionally, dysfunctional P2rx7 expression hints to a deregulated prefrontal dopaminergic tone, which leads to a complex scenario: it is predicted that basal dopamine release and the related compensatory mechanisms in the PFC have an inverted "U" relationship with PFC performance and was shown to be relevant to SCZ symptomatology (Rolls et al., 2008).

We also observed marked change in the microglia morphology at the level of PL and IL layer V, raising the possibility that P2X7 regulates the microglia-neuron interaction, and thereby affects the behavior of the animals.

The number of microglial cells is not altered upon PCP treatment, which is not surprising, considering that migration of microglia cell bodies works on the scale of hours to days (Eyo et al., 2018). Microglia pro-inflammatory activation is also a phenomenon hardly considered to take place in PCP models, except for the Olney's lesion in the retrosplenial cortex (Olney et al., 1989; Zhu et al., 2014).

We detected a small decrease in P2Y12R immunoreactivity in $P2rx7^{-/-}$ PCP-treated animals compared to the PCP treated wild-type, which could be interpreted as a sign of an initial shift toward a pro-inflammatory profile (Haynes et al., 2006). Nevertheless, no difference was found between the vehicle and PCP-treated groups, indicating that acute neuroinflammatory reactions, if any, are not detected by tissue immunostaining. Indeed, the most convincing information from P2Y12R immunostaining lies in the precise morphological outline of the microglial membrane (Cserép et al., 2020). With unbiased image analysis, using the software FIJI ImageJ, it is possible to obtain an accurate 1-pixel skeleton of the microglia reticule, which renders a Sholl analysis of the skeleton largely independent of immunoreactivity signal oscillations (Schindelin et al., 2012; Ferreira et al., 2014). From the 40 μm thick slices, image stacks of 10–12 μm thickness, starting 5 μm down/up from the slice surfaces, resulted in the best images with the adopted antibodies and protocols. Therefore, the material to analyze was undersized in the z dimension to obtain any single microglial cell ramification fully reconstructed in three dimensions. Indeed, the resulting 3D isolated cells displayed cut branches, and therefore the whole-cell dimension depended on the orientation of its branches with respect to the coronal cut (data not shown). Considering the limitations of the isolated cell analysis, we applied the Sholl analysis on the whole microglia skeleton in the tissue slice. The general microglia ramification (IL and PL cortices, layer V) displayed similar stained areas, suggesting that PCP (10 mg/kg) and P2rx7 gene deletion did not give rise to a plain hyper- or hypo-ramified microglia phenotype.

It was recently reported by Liu et al. (2019), that noradrenaline is the master regulator of microglia general ramification. Two-photon *in vivo* imaging of rodents transitioning from the awake state to the anesthetized state, *via* lowered noradrenergic tone, revealed the expansion of microglial surveillance territory in minutes (Liu et al., 2019). In the current study, we could not detect any PCP-induced anesthesia-like effect on microglial ramification. This observation is in line with the observed acute long-lasting increase, but not decrease, of noradrenergic tone at the level of the rodent mPFC within the PCP psychotomimetic dose range (Kehr et al., 2018). We could not track possible differential effects induced by anesthesia (Nembutal, 100 mg/kg), which probably affected the prefrontal noradrenergic tone in the 20 min following the injection (Pan and Lai, 1995). Since all animals underwent the same procedure, we acknowledge the systematic error.

Microglia cell-centered analysis revealed that microglia of saline-treated $P2rx7^{-/-}$ mice displayed a higher number of branches after the first 20 μm of Sholl radius compared to the saline-treated $P2rx7^{+/+}$ ones. Additionally, PCP treatment dissolves the genotype-related difference in ramification.

It has been reported that P2X7R may retain specific migration and phagocytic properties, as it is essential for CX3CL1 chemoattraction (Fernandes et al., 2016), promote migration toward amyloid senile plaques upon activation while inhibiting microglial phagocytic capacity (Martínez-Frailes et al., 2019), and appears to have a permissive role for NF-κB activation, NLRP3 inflammasome formation and mitochondria toxicity in the microglia (Chiozzi et al., 2019). Regarding the possible role of the $P2rx7^{-/-}$ lower dopaminergic tone, microglia ramification was reported to increase in the presence of a dopamine-3 receptor antagonist (Elgueta et al., 2017), where extracellular dopamine has been reported to exert an anti-inflammatory effect by modulating the inflammasome cascade (Yan et al., 2015). However, we could not find specific information in the literature related to P2X7R expression and microglial ramification.

To match the specific area and location of PCP-driven hyper-activated neurons with microglial branches, we performed double and triple immunohistochemical staining of the mPFC of the treated animals.

Microglial branches are in continuous movement and dynamically contact and explore the surrounding parenchyma concerning external stimuli, as in the case of purine-driven chemotaxis (Calovi et al., 2019). However, recent evidence demonstrates that around neuronal bodies, microglial contacts are a rather specialized communication route that is stable for a few tens of minutes (Cserép et al., 2020). They have been proposed to function as a restraint of neuronal hyper-activation (Sharma et al., 2020). From the PCP-related event that drives mPFC neuron hyperactivity until brain fixation (maximal cumulative c-Fos expression), it took 110 min,

which should outdistance the neuronal hyper-activity-related recruitment of microglial branches around neuron somata. The neuron-centered 2D Sholl analysis revealed that microglial branches were more abundant in the proximity of c-Fos positive neurons relative to the negative ones in both genotypes. We observed that the contacts between microglial branches and hyperactive neurons *in vivo* might be stable within a few hours, rather than tens of minutes, in specific pathological contexts. A series of c-Fos and P2Y12R immunostained images with a minimum 30 μm z-stack thickness were taken to perform a 15 μm Sholl radius 3D analysis centered in c-Fos^{+} nuclei located in the central 15 μm along the z-axis. Within the 15 μm radius sphere, in PCP-treated P2rx7$^{-/-}$ animals layer V mPFC hyper-activated neuronal somata were contacted by one to two more microglial branches (approximately 9–16%) than corresponding neurons in wild-type animals. This difference may reflect an increased tendency of P2rx7$^{-/-}$ c-Fos^{+} neuron somata to be in direct contact with microglia somata.

Microglia-neuron contacts are an intriguing subject that recently started to be molecularly characterized. However, it is still too early to conclude any relationship between P2rx7 expression and microglial behavior. Moreover, it is important to verify whether intrinsic neuronal properties could explain the differences observed between the genotypes since neurons can directly attract microglial branches. Patch-clamp recordings of mPFC layer V neurons identified no functional differences between genotypes concerning resting membrane potential or rheobase. However, in the current clamp pulse step protocol, we found a difference in spike accommodation and re-polarization, with an increased firing rate and slower re-polarization to resting membrane potential in wild-type animals. This identifies the subtle excitatory effect of P2rx7 on these neurons.

The role of P2X7R during development seems to be commonly related to necrosis and apoptosis (Kanellopoulos and Delarasse, 2019), yet some *in vitro* studies have pointed out different possible regulatory effects in axonal growth and branching (Díaz-Hernandez et al., 2008). The functionality of these receptors in early and neuronal development remains unknown, as current studies are mainly focused on adults and aging. To our knowledge, this is the first report of patch-clamp analysis on young adult P2rx7$^{-/-}$ prefrontal IL and PL layer V neurons. The electrophysiological profile of P2rx7$^{-/-}$ neurons is in line with our c-Fos results, in that P2rx7$^{+/+}$ mPFC layer V neurons were prone to fire a higher amount of action potential for the same amount of current compared to P2rx7$^{-/-}$ neurons. We cannot exclude that this difference is general to more areas of the mouse brain. If the diminished P2rx7$^{-/-}$ neuronal excitability corresponds to a lower PCP-driven mPFC neuronal hyperactivity (c-Fos signal), it is surprising to observe that a higher number of microglial branches were attracted to the P2rx7$^{-/-}$ strongly activated neuronal bodies. Indeed, microglia/neuron interaction is supposed to be driven by ATP/ADP neuronal release, which has been observed as dependent on neuronal activity (Cserép et al., 2020; Sharma et al., 2020). In contrast, the higher number of attracted microglial branches could also be a compensatory phenomenon. A better characterization of *in vivo* or *ex vivo* microglial dynamics could shed further light on the significance of this observation.

We observed that P2rx7$^{tg/+}$ displayed exacerbated schizophrenic-like behavior from the low-dose PCP treatment, which may explain the milder effects of PCP on sociability scores. Mice often followed and circled close to each other, while sniffing each other briefly but more continuously than WT mice. Beyond susceptibility, the P2rx7$^{tg/+}$ mice experience a strongly accentuated stereotypical behavior which correlates with values of concentration of strongly stained c-Fos neurons in the same area where were reduced in P2rx7$^{-/-}$, the layer V of IL and PL areas. The higher PCP sensitivity in terms of behavior and medial prefrontal neuronal hyperactivation adds credibility and relevance to the model, as patients with schizophrenia have an increased response to arylcyclohexylamines anesthetics relative to dopamine-related excitant drugs (Lahti et al., 2001). Interestingly, the lower dopamine uptake of P2rx7$^{tg/+}$ animals suggests that the overexpressing strain has prefrontal hyperdopaminergia, which might also account for the exacerbated PCP effect. The P2X7-EGFP, expressed by heterozygous P2rx7$^{tg/+}$ mice, was localized in the layer V mPFC microglia but was expressed by other cells throughout the mPFC.

To summarize our findings, P2rx7$^{-/-}$ animals might display small cumulative effects pointing to a hypo-excitability mPFC circuit and tempered psychotomimetic PCP effect, namely:

- The lower dopaminergic tone in the PFC.
- PL and IL layer V enhanced microglial branches/hyperactive neuron interactions.
- PL and IL layer V neurons with faster post-action potential firing polarization and lower initial action potential frequency.

While symptoms related to psychotic schizophrenic episodes, which are mimicked by the acute PCP effect, can be alleviated by the available antipsychotic drugs, there is an unmet clinical need concerning cognitive deficits (Tripathi et al., 2018). We examined the working memory of P2rx7$^{-/-}$ mice using an established subchronic PCP treatment that compromises memory performance (Zain et al., 2018). Along with the treatment, PCP tended to induce a smaller acute hyperlocomotion in knock-out animals, without compromising the behavior in the short-term washout. After the subchronic treatment withdrawal period of 3 days, the animals were tested for the spontaneous alterations test in a Y-maze. The lower locomotion displayed by P2rx7$^{-/-}$ animals in the Y-maze was contextual to this test, as we could not rule out genotype locomotion-related differences in tests before and after the spontaneous alternation test. As mentioned, we did not take into consideration the repeated entries in the same arm as "valid alternations". Therefore, a different velocity between genotypes may reflect different behavioral explorative strategies. However, these did not influence the total number of different arm explorations, and thus alternation, during the test. Subchronic PCP treatment significantly impaired the working memory

performance in wild-type mice concerning the P2rx7$^{+/+}$ vehicle group and the P2rx7$^{-/-}$ PCP treated group. The following analysis of the prefrontal cytokine levels did not reveal any PCP-related effects on the brain inflammatory profile. These data confirm that the noncompetitive antagonism of NMDA receptors directly modulates neuronal activity and does not trigger a robust neuroinflammatory phenomenon. Interestingly, the prefrontal level of fractalkine, or CX3CL1, was strongly upregulated in both genotypes subjected to PCP treatment. Fractalkine is a pivotal trophic factor of the brain and is strongly involved in microglial physiology, with many implications (Arnoux and Audinat, 2015). Since P2X7R also has a trophic effect on microglia (Bianco et al., 2006; Monif et al., 2016), we cannot exclude the possibility that fractalkine upregulation could play a compensatory role in P2rx7$^{-/-}$ animals.

In addition to the mechanistic explanations detailed above, we cannot exclude different pharmacokinetics of PCP in the different genotypes. In rats, PCP has a fast brain uptake and hours-timescale washout (Kalinichev et al., 2008), and subchronic PCP is cumulated in plasma and tissues (Balla et al., 2003). Arime and Akiyama reported that when rodents were involved in working memory tasks, there was increased recruitment of neurons at the level of the prelimbic layer II/III, which correlated with poorer performance (Arime and Akiyama, 2017).

Acute PCP treatment is also known to trigger a stress response, affecting the hypothalamus-pituitary-adrenal axis by stimulating pituitary ACTH release and consequently increasing plasma corticosterone levels (Pechnick et al., 1990, 2006). After PCP subchronic treatment, induced stress triggers greater stress responses in terms of behavior and plasma levels of ACTH (Tejedor-Real et al., 2007). Interestingly, our group previously published evidence that P2rx7$^{-/-}$ animals exhibit an anti-depressed profile, since they respond better to a broad spectrum of stressors (physical restraint, amphetamine, and lipopolysaccharide treatments) by reducing centrally dependent HPA axis activation (Csölle et al., 2013). This, along with other evidence, points to a hypothetical cascade of neuroinflammatory-related events that would explain the protective role of P2X7R against repetitive stressful events (Illes et al., 2020). Also, our previous study (Koványi et al., 2016) pointed to a set of subtle expression changes in several genes implicated in SCZ in a small dose acute PCP model, which was partially reversed in P2rx7$^{-/-}$ animals.

Collectively, our findings support the notion that P2X7R exerts multiple roles, depending on the context and the cell type. It seems reasonable to assume that short-term activation of P2X7R could impact the functionality of neuronal networks in the long term, such as gene expression regulation, behavior, and memory formation. The P2X7-driven action identified by the current study, such as increased layer-specific neuronal activation and intrinsic excitability of neurons in the mPFC and preferential interaction of microglia with hyperactive neurons, might be responsible for the alterations detected in PCP-induced behavior. However, the mechanism by which P2X7R is endogenously activated under these conditions needs to be further examined.

CONCLUSION

These results confirm the role of P2X7R in SCZ-like behaviors in an animal model, which we propose as a potential therapeutic target. A decrease of neuronal activation in a restricted area of the mPFC in response to PCP treatment in mice genetically deficient for P2rx7 was observed. Specifically, P2X7R gene deletion elicited a lower number of strongly activated neurons in the mPFC specific to the V layer of the prelimbic and infralimbic areas. Opposite results were found testing a P2X7R overexpressing line, which is more susceptible to PCP in terms of behavior and prefrontal specific circuits involvement. This finding identifies a potential primary site of action, whereby P2rx7 might affect PCP-induced behavior. Moreover, we observed a change in microglial morphology in the same area regarding the contact sites of microglia over the neuronal somata. This raises the possibility that P2X7 regulates microglia-neuron interaction and thereby affects animal behavior.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Local Animal Care Committee of the Institute of Experimental Medicine (Budapest, Hungary, ref. No. PEI/001/778-6/2015). International Council for the Laboratory Animal Science at the University of the Basque Country UPV/EHU (CEEA 290/2015).

AUTHOR CONTRIBUTIONS

SC, BS, ESV, and JT designed the research. SC, AI, PT, and PM-A performed the research and analyzed the data. AN and SM provided transgenic mouse models. BS, ESV, and JT supervised the study. SC wrote the article with input from all authors. All authors contributed to the article and approved the submitted version.

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