Mini Review

Role of P2X7 and P2Y2 receptors on α-secretase-dependent APP processing: Control of amyloid plaques formation “in vivo” by P2X7 receptor

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Abstract

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disorder characterized by learning and memory impairments, being the most common form of senile dementia. The main neuropathological characteristics of this disease are the accumulation of intracellular neurofibrillary tangles and extracellular amyloid deposits also known as senile plaques in brain [1]. The amyloid precursor protein (APP) is a single helix transmembrane protein expressed in neural and non-neural tissue, being processed in two different ways by sequential proteases, known as secretases [2,3]. In brain, sequential proteolysis of APP by β- and γ-secretases is at the origin of β-amyloid peptide, Aβ42 peptide, which is the most prominent component of extracellular amyloid deposits. The

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1. Introduction

Alzheimer’s disease (AD) is a progressive and irreversible neurodegenerative disorder characterized by learning and memory impairments,
APP protein can also be processed in a non-amyloidogenic way mediated by α- and γ-secretases. Both types of APP processing occur in the central nervous system (CNS), even in the same cell [4], and many questions arise concerning what are the physiological signals that keep a necessary balance between both APP processing ways to avoid an increase of the amyloidogenic pathway in normal brain [5–7].

Neurofibrillary tangles are the intracellular companion of senile plaques in AD. These inclusions contain the microtubule-associated protein, tau, in its hyperphosphorylated forms. This protein, which is mostly found in neurons, stabilizes microtubules and can be phosphorylated by multiple kinases, among them glycogen synthase kinase 3, GSK-3. When the balance between phosphorylation and dephosphorylation is inaccurate, tau self-assembly occurs. Although most of the effects reported for tau are at the intracellular level [8], it has been recently reported that extracellular tau and its peptide fragments behave as non-desensitizing agonists on muscarinic M1 and M3 receptors, with a sustained cytosolic Ca2+ increase in neural cells [9,10]. On the other hand, phosphorylated tau or its fragments have to be dephosphorylated to be fully active on muscarinic receptors. It is relevant that one of the ecto-phosphatases involved is the tissue nonspecific alkaline phosphatase, TNAP, which is already reported to play a role on the non-amyloidogenic APP processing by diverse signaling cascades has been proved [17].

GSK-3, which plays a role in multiple signaling pathways, is among the enzymes that can influence Aβ production. It has been reported that an increase in GSK-3 activity [18] correlates with the phosphorylation of APP intracellular domain, making it a more suitable substrate for γ-secretase proteolysis, which enhances Aβ42 production [19]. Moreover, as already mentioned, GSK-3 is one of the enzymes responsible for tau phosphorylation [20], thus becoming a link between senile plaques and neurofibrillary tangle formation.

Among neurotransmitters, nucleotides play a relevant role through the activation of ionotropic P2X and metabotropic P2Y receptors. These receptors are widely distributed in CNS, where they regulate calcium homeostasis, neurotransmitter release and a broad diversity of intracellular signalling pathways involved in brain physiology and pathophysiology [21]. The P2X7 receptor, which is abundantly expressed in CNS, being present in diverse cellular subtypes such as microglia, astrocytes or neurons, has emerged as a relevant target among the P2 receptors family [22–25]. Early reports demonstrated the up-regulation of the P2X7 receptor in microglial cells in neuroinflammatory situations, and the beneficial effect of its antagonism [26]. In addition, P2X7 receptor is receiving special attention in neurodegenerative diseases, such as Huntington’s and Parkinson’s disease [27,28]. The role of P2X7 receptor has been more intensively studied in Alzheimer’s disease, where the effects of the signalling cascades coupled to P2X7 activation on APP processing have been reported [29,30]. Other P2 receptors, as it is the case for P2Y2 receptor, have also been involved in APP processing regulation, mainly through activation of the non-amyloidogenic pathway [31–33]. In this review the role of P2X7 and P2Y2 receptors on the APP processing will be discussed, together with the relevance of experimental models and the possibility of use of those receptors as valuable therapeutic targets.
2. Neuro-2a cells as a model for APP processing: Effect of P2Y2 and P2X7 receptors activation

The Neuro-2a cell line, N2a, has been largely employed as a neural model to study signalling pathways, secretory events and neuronal differentiation, thus being a well characterized system [34,35]. These cells have the advantage of constitutively expressing APP, together with functional P2Y2 and P2X7 receptors. P2Y2 receptors are abundant in N2a cells, exhibiting a broad distribution, which can be observed even when neural-like differentiation is induced, as shown in Fig. 1A. The same figure shows the APP distribution along the axon until reaching the axonal growth cone, where a great abundance of the P2X7 receptor can be observed. This distribution agrees with a recent fluorescence-based procedure allowing the study of the axonal transport of APP in cultured hippocampal neurons [36]. The absence of P2Y4 receptor in N2a cells (Fig. 1C), which exhibits a similar agonistic profile as the P2Y2 receptor (Fig. 1B), clearly substantiates a role for P2Y2 receptor activation in APP processing.

P2X7 receptor immunolabelling shows a distribution similar to that observed for APP, being present not only in the neuronal cell body, but also in the axon-like extension [37,38] (Fig. 2A). The presence of a functional P2X7 receptor has been demonstrated by calcium imaging fluorescence techniques, challenging neural cells with the selective P2X7 receptor agonist benzoyl ATP (BzATP) in the presence or absence of Mg$^{2+}$ ions (Fig. 2B), and also by electrophysiological techniques in which the current elicited by stimulation with BzATP and the inhibition exerted by the specific reversible antagonist A438079 were measured (Fig. 2C).

APP processing in N2a cells can be followed by detection of the specific proteolytic fragments present in the plasma membranes. The presence of the carboxy-terminal C83 fragment, α-CTF, indicates that membrane protein APP has been processed by α-secretase, which results in the simultaneous release of the extracellular protein moiety, APPα. The C83 fragment is further hydrolysed by γ-secretase that cleaves the carboxy-terminal fragment in the middle of APP transmembrane helix, which results in the release of the extracellular peptide, P3, and an intracellular C-terminal fragment, AICD. The AICD fragment is identical in both the amyloidogenic and non-amyloidogenic APP processing, and a role on the control of gene expression has been postulated for this fragment [39].

P2Y2 receptor agonists are able to significantly increase the α-secretase-mediated APP processing in N2a cells, this stimulatory effect being consequently blocked by the broad spectrum P2 antagonist, suramin, as shown in Fig. 3A. These results agree with those obtained by other groups (as Gary Weisman’s group), supporting the role of P2Y2 receptor in neuroprotection, an effect that is mediated at least in part via the activation of the APP non-amyloidogenic pathway through α-secretase processing [31-33,40].

The activation of P2X7 receptor in N2a cells decreases the levels of C83 fragment, which is the indicator of the α-secretase non-
amyloidogenic hydrolytic pathway of APP processing (Fig. 3A). This effect can be overturned by using P2X7 receptor antagonists; both the reversible and more specific A438079 and the wider spectrum less specific Brilliant blue G (BBG) were able to increase the C83 fragment product of \( \alpha \)-secretase (Fig. 3A). These results contrast with those obtained by other authors, but it is relevant to emphasize that APP processing depends on the abundance of this protein at the specific cellular model and in addition, when it is overexpressed, the equilibrium between the different proteolytic pathways could be unbalanced, making it more difficult to understand the process [29,41].

The effect of P2X7 receptor-activated signaling cascades on GSK-3 activity has been already reported by several authors. Activation of P2X7 receptor in granule cells from cerebellum results in GSK-3 phosphorylation through the PI3K/AKT cascade, which in turn promotes neuroprotection [23,42]. However, P2X7 receptor activation induces a reduction of GSK-3 phosphorylation and stops axonal elongation in embryonic hippocampal neurons during differentiation, both effects being reverted by P2X7 receptor antagonists or by the reduction of the extracellular levels of ATP ligand by the use of alkaline phosphatase [38]. N2a cells, behave in a similar way to cultured hippocampal neurons, as stimulation with P2X7 receptor agonist results in a decrease of GSK-3 phosphorylation in serine 9/21 as shown in Fig. 3B, which correlates with a reduction in the \( \alpha \)-secretase-generated C83 APP fragment (Fig. 3A). Consequently, the P2X7 receptor antagonists, A438079 and BBG, significantly increased GSK-3 phosphorylation and production of C83 fragment (Fig. 3A and B). A similar effect was obtained with the GSK-3 inhibitor SB216763 (Fig. 3A), thus corroborating the pharmacological relevance of the inhibitors of this enzyme in AD.

### 3. P2X7 receptor and its role in animal models of Familial Alzheimer’s Disease

There are many models of genetically modified mice that develop cerebral amyloid deposits. The transgenic mice known as J20 hAPP have been chosen because they develop the characteristic amyloid peptide deposits by 6–8 months of age. These transgenic mice, labelled as B6.Cg-Tg(PDGFB-APPSwInd)20Lms/2J strain, express a mutant form of the human amyloid protein precursor bearing both the Swedish (K670N/M671L) and the Indiana (V717F) mutations [APPswInd], [43]. All procedures were carried out in accordance with European and Spanish regulations (86/609/CEE; RD1201/2005) when working with these animals in our laboratory.

The main question was to understand the balance between both APP proteolytic pathways in vivo situations and whether it was possible to...
change the dynamics of amyloid deposits by impacting P2Y2 and P2X7 receptors.

Concerning P2Y2 receptor, there are not selective agonists or antagonists with good pharmacokinetic parameters for in vivo administration to date. However, its relevance has been confirmed in the TgCRND8 mouse model of Alzheimer’s disease, where loss of P2Y2 nucleotide receptors enhances the β-amyloid (Aβ) deposit and also the soluble Aβ1–42 levels in the cerebral cortex and hippocampus [31].

The availability of P2X7 receptor ligands for in vivo studies is slightly better, as the antagonist BBG is able to infiltrate the brain parenchyma. The efficacy of this antagonist in mice has been already reported in the beneficial effects on Huntington’s disease symptomatic and the seizure suppression and neuroprotection in status epilepticus [27,44]. Recently, BBG has proved to improve cognition in an animal model of Alzheimer’s disease [45]. In addition to BBG, there are many other P2X7 receptor antagonists able to reach the brain, as it is the case of A438079 [46].

The hippocampi of the J20 mice showed abundant amyloid plaques at the age of 6–8 months. These deposits were clearly identified with anti-Aβ antibodies and the Thioflavin-T dye that is able to intercalate between the β-sheet structures of amyloid deposits. To study the role of P2X7 receptor on β-amyloid deposits in vivo, J20 mice were treated before the appearance of the first hippocampal plaques, at the age of 4 months, with BBG (intraperitoneally injected every 48 hours at 45.5 mg/Kg), or vehicle solution, PBS, for 4 months. BBG concentration in brain was around 200 nM at the dose used. It is relevant to emphasize that the concentration reached in vivo is in the range of the IC50 of BBG to antagonize P2X7 receptor (10–200 nM) [47].

After BBG treatment the number and size of amyloid plaques at the hippocampal structures of J20 mice were significantly reduced compared to their littermates treated with vehicle, as shown in Fig. 4A and B. In addition, the treatment with BBG did not significantly modify either the P2X7 receptor or murine APP and human APP mRNA expression. The levels of these proteins and their distribution patterns in the hippocampus were also not modified by the treatment with BBG, as demonstrated by western blot and immunohistochemical studies. However, a dramatic change was observed concerning the pattern of the C83 and C99 peptides, which correspond to the carboxyterminal fragments generated by APP cleavage by α-secretase and β-secretase, respectively. C99 fragment was under the limits of detection in wild type mouse brain, but was very abundant in the brain of J20 mice, which exhibit a much lower concentration of the α-secretase generated C83 fragment. However, BBG reversed the situation and a significant increase in the C83 fragment was achieved in the brain of BBG-treated J20 mice [48]. This situation mimics the APP processing pattern observed in N2a cells treated with P2X7 receptor inhibitors. On the other hand, an increase in the phosphorylated form of GSK-3 was observed in the hippocampus of BBG-treated J20 mice, when compared with vehicle-treated animals. Thus, BBG was able to reduce hippocampal GSK-3 activity in FAD animal models in the same way as in N2a cells.

J20 animals at the end of their lives, about 20 months-old, exhibit a profusion of hippocampal senile plaques that were surrounded by microglial cells. However, at the first stages of the FAD neurodegenerative disease a significant presence of microglial positive cells cannot be observed when considering the total hippocampal structures (Fig. 4C and D). Only some few cells surrounding the senile plaques expressed microglial markers, together with P2X7 receptor and hypophosphorylated GSK-3. This fact is relevant because to date, most of the effects of P2X7, and also P2Y2, receptors have been explained by microglia activation around the senile plaques due to the amyloid Aβ1–42 peptide or the release of extracellular nucleotides and cytokines, thus helping in the extracellular clearance of the amyloid deposits [26,29,31,49].

4. Summary and Outlook

In spite of the complexity of Alzheimer’s disease physiopathology, it is however relevant to tackle one of the main characteristics of the

![Fig. 4. Brilliant Blue-G (BBG) treatment reduces the number of amyloid plaques and microglia in the J20 mouse hippocampus. (A) Immunostaining of V92 in hippocampal slices from 6 to 8 months-old J20 mice injected intraperitoneal with vehicle or BBG. Scale bar, 500 μm. (B) Histogram represents the mean ± SEM of Aβ1–42 amyloid plaques per slice in the hippocampus of J20 mice treated with vehicle or BBG, being 16 slices per mouse (n = 7 mice per condition). ***p < 0.005, unpaired Student’s t test. (C) Immunostaining of microglial marker Iba-1 in hippocampal slices from 6 to 8 months-old J20 mice treated with vehicle or BBG. Scale bar, 500 μm. (D) Quantification of microglial cells in the hippocampus of J20 mice treated with vehicle or BBG. Histograms represent the mean ± SEM of microglial cells per hippocampal area of 0.1 mm² being 16 slices per mouse (n = 7 mice per treatment). For methods see Ref. [49].]
disease, the formation of amyloid plaques, using an in vivo mouse model of FAD. In this model, it was demonstrated for the first time that the in vivo inhibition of P2X7 receptors significantly reduces the amyloid plaques formation in brain hippocampal structures. The molecular mechanisms underneath this relevant effect, reported here, were the phosphorylation and consequent reduction of GSK-3 activity, which correlates with an increase in α-secretase activity. Apparently, the prolonged BBG treatment is efficient and non-toxic, thus providing a suitable therapeutic approach to prevent amyloid deposition on FAD. However, remarkable differences exist when studying the effects of P2X7 receptor agonists and antagonists in various neuronal cell models. N2a cells and primary cultures of embryonic hippocampal neurons, behave as the adult hippocampus neurons regarding APP processing. By contrast, P2X7 receptor activation in cultured cerebellar granule neurons result in GSK-3 inhibition and neuroprotection. Consequently, although significant progress has been made over the past decade understanding AD and β-amyloid deposition, it is relevant to emphasize that the brain is a complex entity where many different structures coexist.

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