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Opposite effects of P2X7 and $P2Y_2$ nucleotide receptors on α-secretase-dependent APP processing in Neuro-2a cells

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ABSTRACT

The amyloid precursor protein (APP) is proteolytically processed by β- and γ-secretases to release amyloid-β peptide (Aβ), the main component found in senile plaques of Alzheimer's disease (AD) patient brains. Alternatively, APP can be cleaved within the Aβ sequence by α-secretase, thus precluding the generation of Aβ. We have demonstrated that activation of the P2X7 receptor leads to a reduction of α-secretase activity in Neuro-2a cells. Moreover, the P2X7 ligand 2′(3′)-*O*-(4-benzoylbenzoyl) ATP (BzATP) can also activate a different P2 receptor in these cells. This receptor, whose pharmacology resembles that of the $P2Y_2$ receptor, has an opposite effect, leading to increases in α-secretase activity. Our study suggests that P2X7R and $P2Y_2$R could be novel therapeutic targets in AD.

1. Introduction

Alzheimer's disease (AD) is a progressive dementia characterized by extracellular deposits of amyloid-β peptide (Aβ) in senile plaques and intracellular neurofibrillary tangles comprising hyperphosphorylated tau assemblies. Aβ is produced when the amyloid precursor protein (APP) is sequentially cleaved by β- and γ-secretases [1]. There is also a non-Aβ-forming pathway in cells involving α-secretase. The α-secretase cleaves APP within the Aβ sequence, thereby precluding the formation of neurotoxic Aβ [2]. Several enzymes capable of mediating non-amyloidogenic α-processing of APP have been identified [3].

P2 nucleotide receptors modulate a wide range of physiological responses in neural tissues. These receptors belong to two major families: a P2X family of ligand-gated ion channels and a P2Y family of G protein-coupled receptors [4]. The G-protein coupled $P2Y_2$ receptor has been shown to stimulate α-secretase-dependent APP cleavage in astrocytoma cells [5]. In neurons, upregulation of $P2Y_2$Rs by interleukin-1β promotes the nucleotide-induced non-amyloidogenic processing of APP [6], suggesting a neuroprotective role for $P2Y_2$ receptors in AD.

Regarding P2X receptors, it is known that the P2X7 subtype becomes upregulated in the brain of patients with AD and in some transgenic mouse models of the disease [7,8]. P2X7 receptor activation induces the generation of superoxide from microglial cells [8] and enhances cytokine secretion elicited by Aβ in human macrophages and microglia [9]. Moreover, neuroinflammatory markers induced by exogenous administration of Aβ are partially prevented by the P2X7R antagonist brilliant blue G (BBG) [10]. These findings suggest that P2X7 receptor could play a role in the inflammatory responses seen in AD. However, little is known about the possible role of P2X7 receptors in Aβ generation. P2X7R is abundantly expressed in neurones where it seems to be targeted to axodendritic fibers and synaptic terminals [11]. Neuronal Aβ release has been linked to the synaptic activity [12]. Thus, any presynaptic receptor able to modulate synaptic transmission has the potential to regulate the generation and release of Aβ from neurons. Indeed, it is well known that activation of the P2X7R leads to changes in the activity of PKC, MAPKs or GSK3 [13], enzymes that are known for their ability to modulate APP processing and Aβ production.

Abbreviations: α-CTF, α-cleaved carboxyterminal fragment of APP (also known as C83); Aβ, amyloid-β peptide; AD, Alzheimer's disease; ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; BACE, β-secretase; BBG, brilliant blue G; BzATP, 2′(3′)-*O*-(4-benzoylbenzoyl) ATP; CTF, C-terminal fragment; GM6001, N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; N2a, Neuro-2a; shRNA, small hairpin RNA; TAPI-1, N-(R)-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-naphthylalanyl-L-alanine 2-aminoethyl amide

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We have analyzed the effect of P2X7 activation on APP C-terminal fragments (CTFs) in Neuro-2a (N2a) cells. P2X7R induced a reduction in the activity of α-secretase, measured by means of α-cleaved carboxyterminal fragment of APP (α-CTF, also known as C83) detection. Alternatively, activation of a $P2Y_2$ receptor stimulates α-secretase-mediated APP processing in the N2a cells. As both P2X7 and $P2Y_2$ receptors are expressed in neural cells, their capacity to modulate the non-amyloidogenic APP processing suggests that they can be novel therapeutic targets in the treatment of AD.

2. Materials and methods

2.1. Cell culture

N2a cells were plated at 8×10^5 cells/well in six-well plates and cultured in DMEM (Sigma) supplemented with Glutamax® (Invitrogen), penicillin/streptomycin (Invitrogen), and 10% heat-inactivated fetal bovine serum (EuroClone). Cells were grown at 37 °C in humidified atmosphere containing 5% CO_2.

2.2. RT-PCR experiments

RT-PCR analysis was performed as previously reported [14]. Briefly, total RNA was extracted from cultured N2a cells and whole adult mice brain using a Rneasy® plus mini kit (Qiagen), following the manufacturer's instructions. After digestion with TURBO DNase (Ambion), total RNA was quantified and reversed transcribed using M-MLV reverse transcriptase (Invitrogen). PCRs were carried out using AmpliTaq Gold® PCR Master Mix and specific commercial oligonucleotide primers for mouse $P2Y_2$ and $P2Y_4$ receptors (Applied Biosystems). Amplified PCR products were electrophoresed on a 1% agarose gel and visualized by SYBR® Safe DNA gel stain (Invitrogen).

2.3. Calcium microfluorimetric analysis in single cells

Microfluorimetric studies were performed as previously reported [14]. Briefly, N2a cells cultured on coverslips placed in 35 mm dishes (250 000 cells/well) were washed with Locke's solution (composition in mM: NaCl, 140; KCl, 4.5; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; glucose, 5.5; Hepes, 10; pH 7.4), and loaded with the calcium dye Fura-2 AM (5 μM) for 45 min at 37 °C. After then, cells were placed in a small superfusion chamber and stimulated with a variety of purinergic receptor agonists. Cells were imaged through a Nikon Eclipse TE-2000-E microscope using a Plan Fluor 20×/0.5 lens. The incoming light was set at 340 and 380 nm. Images were acquired with an ORCA-ER C 47 42–98 CCD camera (Hamamatsu) controlled by Metafluor 6.3r6 PC software (Universal Imaging Corp.). Data are represented as the normalized F340/F380 fluorescence ratio, which increases as $[Ca^{2+}]_i$ increases.

2.4. Immunocytochemistry

Immunocytochemical detection of the $P2Y_2$ receptor was carried out as previously described [14]. Rabbit anti-$P2Y_2$R (Alomone Labs) and mouse anti-α-tubulin (Sigma) were used as primary antibodies. Positive immunostaining was revealed using Cy3™-conjugated donkey anti-rabbit (Jackson ImmunoResearch) and Alexa Fluor 488 goat anti-mouse (Invitrogen) IgGs. Images were acquired using a Leica CTR 6500 confocal microscope with a 40× immersion oil objective (NA 1.3).

2.5. Detection of APP CTFs

Cells were grown in six-well plates for 24 h to near confluency and then culture medium was removed and cells treated at 37 °C with a range of APP secretase inhibitors or a variety of P2 receptor agonists and antagonists (as detailed in figure legends). All the compounds added to the cells were dissolved in Mg^{2+}-free Locke's solution, prepared replacing $MgSO_4$ by glucose at a concentration that conserved the solution osmolarity.

Protein extracts were prepared by homogenizing the cells in ice-cold PBS buffer, following by centrifugation at 1200 rpm for 5 min at 4 °C. Supernatant was discarded and pellet was resuspended in 120 μl of Loading Buffer (Tris–HCl 25 mM pH 6.3, glycerol 10%, SDS 10%, β-mercaptoethanol 5%, bromophenol blue 0.01%). Fifty microliters of the protein extracts (containing ~45 μg of protein) were electrophoresed on 16.5% Tris–Tricine–PAGE gels and transferred to nitrocellulose membranes (Whatman). Experiments were performed using anti-APP CTF 8717 (1:1000) and monoclonal anti-α-tubulin (1:10 000) obtained from Sigma. A secondary goat anti-mouse (1:5000) or goat anti-rabbit (1:1000) antibody (Dako Cytomation) was used followed by enhanced chemoluminescence (ECL) detection (Perkin Elmer).

2.6. P2X7 receptor knockdown

P2X7 receptor knockdown was achieved by RNA interference, as previously described [15]. N2a cells were plated at 6×10^6 cells/P-100 dish and transiently transfected with the vector constructs using Lipofectamine™ 2000 (Life Technologies) following the manufacturer's instructions. After 6 h, the medium was removed and cells were further incubated in culture medium. Twenty-four hours after the transfection, cells were plated at 8×10^5 cells/well in six-well plates and maintained for another 24 h before any treatment with the P2 receptor agonists and antagonists was done.

2.7. Western blot quantification of P2X7R

The levels of the P2X7 receptor were quantified in cell lysates by Western blotting. Fifty microliters of the cell lysates (~45 μg of protein) were electrophoresed on 7.5% Tris–glycine–SDS gels and transferred to nitrocellulose membranes (Whatman). Antibodies used were: rabbit anti-P2X7R intracellular epitope (1:250) from Alomone Labs and mouse anti-α-tubulin (1:10 000) from Sigma. Protein bands were detected by using secondary goat anti-mouse (1:5000) or goat anti-rabbit (1:1000) antibodies (Dako Cytomation) followed by ECL detection (Perkin Elmer).

2.8. Cell viability assays

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. This method employs MTT tetrasodium salt, which is reduced to a colored formazan only by metabolically active viable cells. After the treatment with the different P2 agonists or antagonists, MTT tetrasodium salt (Sigma) was added to the cells at a final concentration of 0.5 mg/ml and was maintained for 2 h at 37 °C. Then, an equal volume of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) was added, following incubation for 1 h at room temperature with orbital shaking. The samples were collected and measured spectrophotometrically at 570 nm. Values were normalized with respect to that obtained from untreated cells, considered as 100% survival.

2.9. Statistical analysis

Results were analyzed by unpaired *t*-test using GraphPad Prism 5 (Graph Pad Software Inc.) and expressed as the mean ± standard error of the mean (S.E.M.). Differences were considered to be significant at $P \leqslant 0.05$.

3. Results and discussion

3.1. Characterization of APP CTFs in N2a cells

We reasoned that APP CTFs would provide a sensitive read-out to study secretase dependent regulation of APP processing since they are generated in cells directly after cleavage of APP by α-secretase or β-secretase [1]. First, we characterized the pattern of APP CTFs in N2a cells by immunoblotting using a commercially available C-terminally directed APP antibody. We only observed one band that ran at the correct molecular weight of APP CTFs (Fig. 1A), which range in size from ~8 to 14 kDa. As it has been shown that N2a cells process APP more via the alpha pathway, it is likely that this band correspond with the C83 α-CTF. However, to be certain that the band we were observing is bona fide α-cleaved APP C83, we carried out additional experiments. Treatment of the cells with the broad-spectrum α-secretase inhibitor N-(R)-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-naphthylalanyl-L-alanine 2-aminoethyl amide (TAPI-1) significantly reduced the immunoreactive band detected (Fig. 1A). A similar result was obtained when cells were treated with the non-selective inhibitor of metalloproteinases, N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (GM6001). In contrast, treatment of the cells with the β-secretase (BACE1) inhibitor IV did not induce any reduction in the levels of the observed band but, instead, a marked increase was obtained (Fig. 1A). These data confirmed that the APP-immunoreactive band detected was C83 CTF generated from α-secretase activity.

3.2. Inhibition of α-secretase activity by a P2X7 receptor

We performed experiments to determine whether application of the P2X7 agonist 2′(3′)-*O*-(4-benzoylbenzoyl) ATP (BzATP) modified the levels of C83 α-CTF. BzATP (100 μM) decreased the levels of C83 after 2 and 4 h of incubation, although the reduction was only statistically significant after 4 h of treatment with the nucleotide analog (Fig. 1B). To assess whether our observed BzATP-evoked decrease in C83 levels was due to stimulation of a P2X7-type receptor, we treated N2a cells with BzATP in the absence or in the presence of the P2X7 antagonist BBG. One micromolar BBG completely reverted the inhibitory effect of BzATP (Fig. 1B), thus strongly suggesting that the observed BzATP-induced decrease in C83 levels was mediated trough a P2X7 receptor. Moreover, our measured changes in C83 levels cannot be attributable to any cytotoxic effect caused by the prolonged exposition to the drugs used, as neither BzATP nor BBG modified cell viability, after 4 h of treatment, when assayed by the MTT method (Fig. 1C).

3.3. Biphasic effect of BzATP on α-secretase activity in N2a cells

Next, we challenged N2a cells with different concentrations of BzATP. Based in our previous data of the time course experiments, 4 h were chosen as the incubation time to routinely carry out the experiments. When assayed in a range of different concentrations, BzATP showed a biphasic effect: low concentrations of BzATP, up to 100 μM, induced a reduction in the detected amounts of C83 α-CTF, while higher concentrations of the nucleotide increased α-secretase activity (Fig. 2A). These results suggest that BzATP may be interacting with two different nucleotide receptors to modify α-secretase activity.

As the inhibitory effect of 100 μM BzATP could be reverted by 1 μM BBG, we repeated the dose–response curve for BzATP in the presence of this P2X7 antagonist. BBG completely prevented the inhibitory effect of BzATP on α-secretase activity, whereas the stimulatory component observed at higher BzATP doses was unaffected by the presence of the antagonist (Fig. 2B). These results support our previous assumption that BzATP inhibits α-secretase activity through its interaction with a P2X7 receptor. The stimulatory effects of BzATP on α-secretase activity, however, seem to be mediated through a different receptor, which is resistant to BBG.

P2X7 receptor has been show to be rather insensitive to suramin [16], a non-selective antagonist able to inhibit most of the P2 receptor subtypes [16,17]. When assayed in the presence of 100 μM suramin, BzATP did not show any stimulatory effect on α-secretase activity, but a reduction in the measured levels of C83 α-CTF was observed at any of the concentrations assayed (Fig. 2C).

As a summary, these results clearly demonstrated that BzATP, depending on its concentration, can activate two different nucleotide receptors in N2a cells. These receptors can be pharmacologically differentiated and have opposite effects on α-secretase activity. One of them, that predominates at the lower BzATP concentrations and which activation leads to a reduction in α-secretase activity, is inhibited by BBG, although it has been proved to be resistant to 100 μM suramin. These results clearly point to a P2X7 receptor as the responsible of the BzATP-mediated reduction in α-secretase activity. The other receptor, that seems to be activated at higher BzATP concentrations and which effect is to increase α-secretase activity, shows a completely different pattern of inhibition, being insensitive to BBG and inhibited by suramin.

3.4. P2X7R knockdown prevents the inhibitory effect of BzATP on α-secretase activity

A pSUPER.neo.gfp vector-derived small hairpin RNA (shRNA) strategy was designed to knock down native P2X7 receptor expression. Transfection of N2a cells with a vector carrying the RNA interference for P2X7 (shRNA-P2X7), specifically reduced the expression of the P2X7R protein when compared with control cells, which were transfected with non-specific shRNA-luciferase vector (shRNA-Luc) (Fig. 3A). Next, we analyze the effect of BzATP on C83 α-CTF levels in both shRNA-P2X7 and shRNA-Luc transfected cells (Fig. 3B). Experiments were carried out in the presence of 100 μM suramin to reduce the contribution of the α-secretase activity enhancing receptor. A biphasic curve for BzATP was obtained in control cells (shRNA-Luc), this nucleotide analog being able to reduce α-secretase activity only at its lower concentrations (up to 100 μM). However, it is relevant to note that the inhibitory effect of BzATP on α-secretase activity disappears when P2X7 receptor has been knocked down, the nucleotide analog being unable to decrease C83 α-CTF levels in cells transfected with the shRNA-P2X7 at any of the concentrations assayed (Fig. 3B).

3.5. A $P2Y_2$ receptor mediates the BzATP-induced increase in α-secretase activity in N2a cells

The aforementioned results clearly demonstrated that the inhibitory effects of BzATP on α-secretase activity are mediated through a P2X7 receptor. However, the nature of the receptor that is responsible for the BzATP-induced increase in α-secretase activity needs to be elucidated. Previous reports have shown that α-secretase-dependent APP processing can be enhanced by activation of a $P2Y_2$ receptor [5,6]. Interestingly, $P2Y_2$ receptors can be activated by BzATP [18] and are sensitive to the inhibition with suramin [17], pharmacological properties that are shared by our α-secretase stimulating receptor. In order to analyze whether a $P2Y_2$ receptor could account for the BzATP-induced stimulation of α-secretase activity in N2a cells, we carried out several experimental approaches.

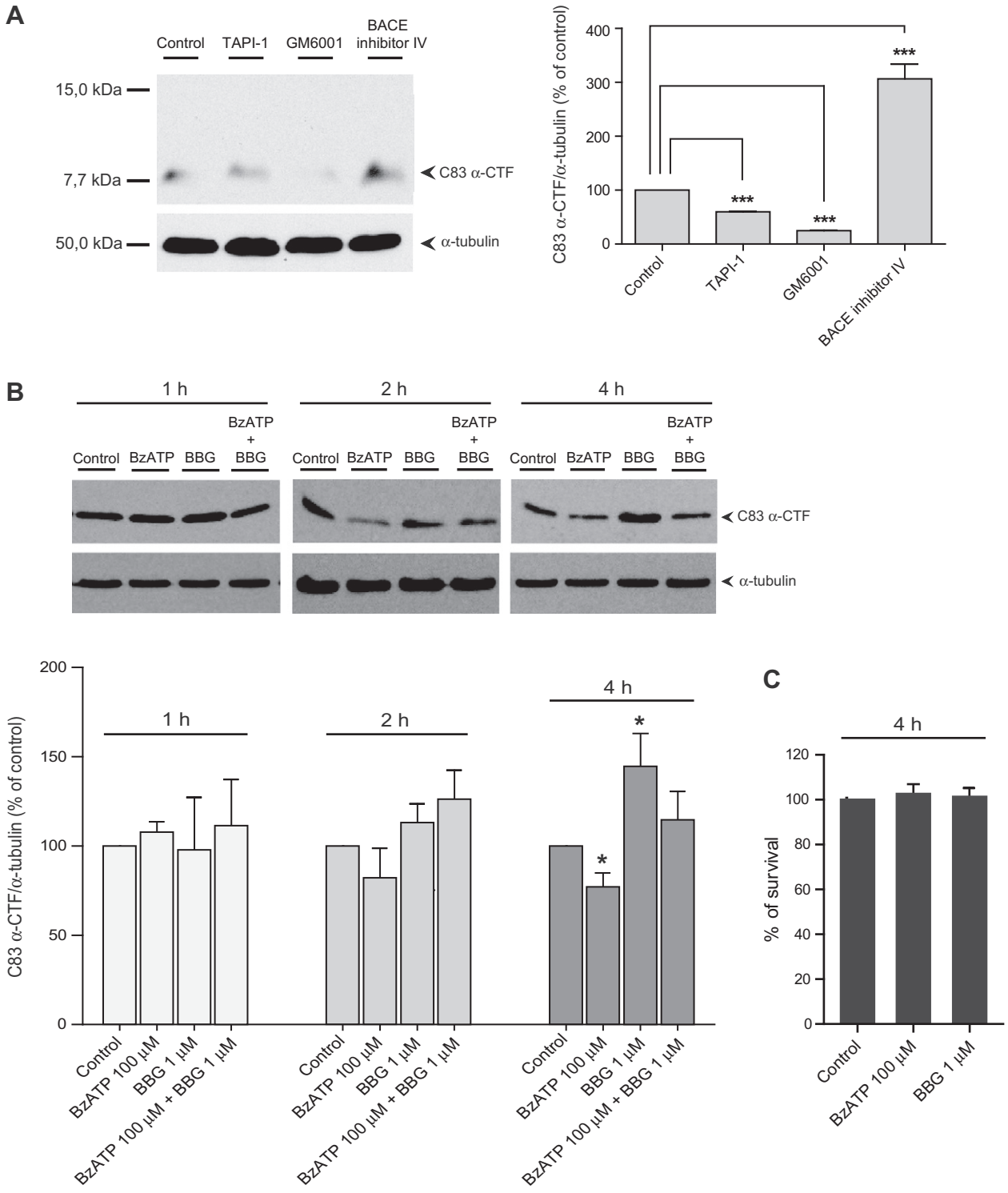

Fig. 1. C83 α-CTF detection and BzATP-induced α-secretase inhibition in N2a cells. (A) Representative immunoblot of C83 α-CTF detection in N2a cells treated with TAPI-1 (10 μM), GM6001 (100 μM) or BACE inhibitor IV (10 μM). α-Tubulin was used as a loading control and the ratio C83 α-CTF/α-tubulin was calculated. Ratio values are represented as a percentage of the ratio in untreated cells (control) that was set as a 100%. Data are the mean ± S.E.M. of four independent experiments. $^{***}P < 0.001$. (B) Cells were treated with BBG (1 μM), BzATP (100 μM) or BzATP (100 μM) in the presence of BBG (1 μM). C83 α-CTF and α-tubulin were quantified by Western blotting after different incubation times. Representative immunoblots of C83 α-CTF and α-tubulin detection at the different treatment times are shown. Histogram represents mean ± S.E.M. of C83 α-CTF/α-tubulin ratios normalized with respect to the control ($n = 4$). $^{*}P < 0.05$. (C) N2a cells were incubated with BzATP (100 μM) or BBG (1 μM) and cell viability was analyzed, after 4 h of treatment, by the MTT assay. Data are the mean ± S.E.M. of three experiments performed in triplicate.

First, we analyzed the calcium responses when cells were stimulated with different nucleotide agonists that are known to activate $P2Y_2$ receptors. As it can be observed in Fig. 4A, equal (100 μM) concentrations of UTP and ATP evoked similar $[Ca^{2+}]_i$ increases in N2a cells, UDP giving rise to responses of much less amplitude. N2a cells also showed transient $[Ca^{2+}]_i$ increases upon application of 100 μM Up_4U, this pattern of responses being compatible with the presence of a functional $P2Y_2$ receptor

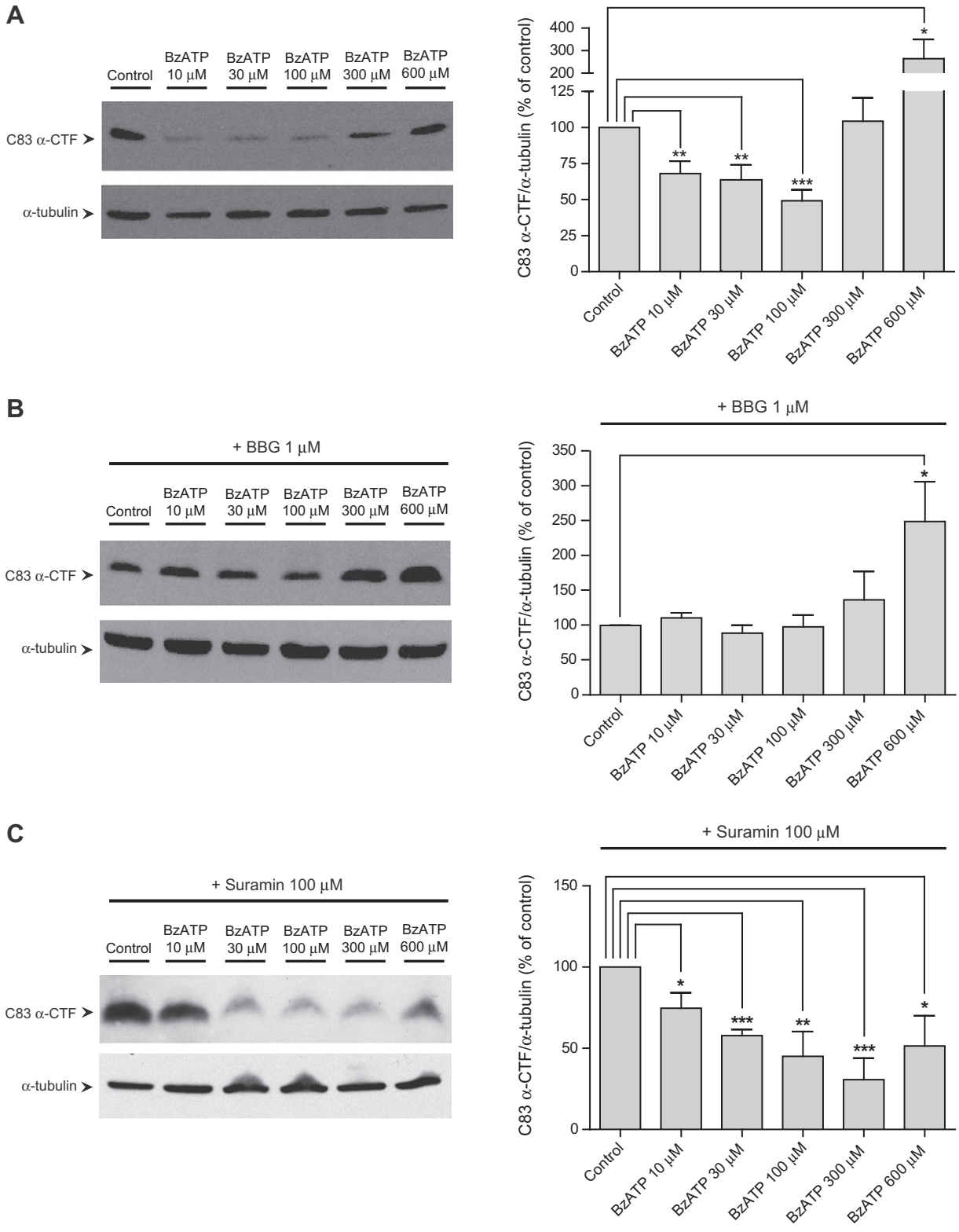

Fig. 2. BzATP activates two different receptors with opposite effects on α-secretase activity. (A) N2a cells were stimulated with several BzATP concentrations, ranging from 10 to 600 μM. (B, C) Cells were treated as in (A) but in the presence of 1 μM BBG (B) or 100 μM suramin (C). When BBG or suramin were present, cells were preincubated for 30 min with the antagonist, prior to the addition of BzATP. C83 α-CTF and α-tubulin levels were quantified by Western blotting after 4 h of treatment with the agonist. Representative immunoblots of C83 α-CTF and α-tubulin detection are shown. Histograms represent the mean ± S.E.M. of C83 α-CTF/α-tubulin ratios, normalized with respect to the control, in 12 (A) or 6 (B, C) independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

[17,19]. The involvement of other pyrimidine-activated P2 receptors, such as the $P2Y_6$, can be ruled out, as $P2Y_6$ is a nucleoside diphosphate preferring receptor with UDP being a much more potent agonist than UTP [17]. However, on the basis of this

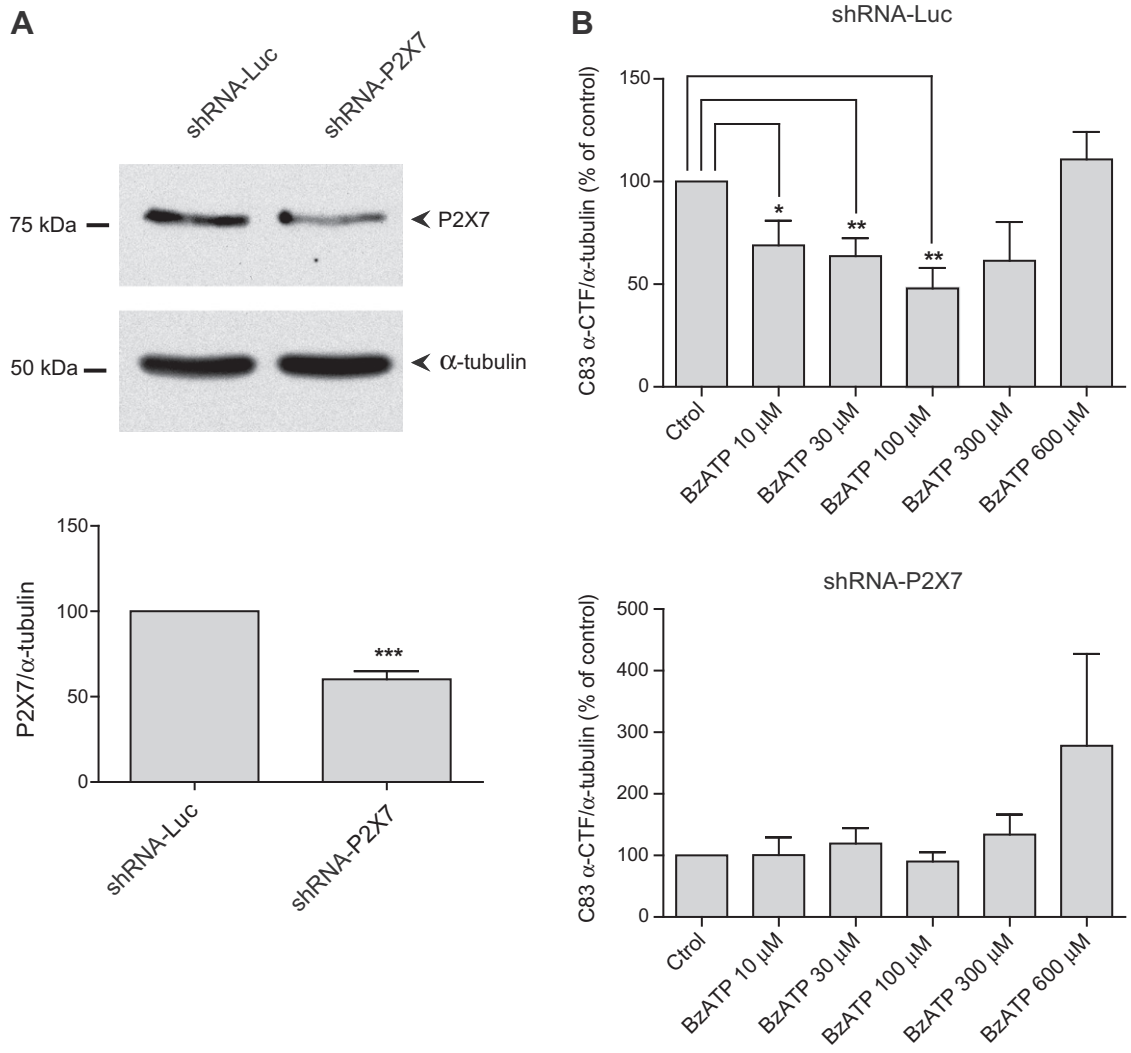

Fig. 3. BzATP induces α-secretase inhibition via a P2X7R. (A) Western blotting of N2a cells transfected with either the RNA interference for P2X7 (shRNA-P2X7) or a non-specific shRNA-luciferase vector (shRNA-Luc). P2X7/α-tubulin ratio was used to estimate the efficiency of P2X7R knockdown. $^{***}P < 0.001$. (B) BzATP was applied at different concentrations, in the presence of 100 μM suramin, to both shRNA-P2X7 and shRNA-Luc transfected cells. Suramin was preincubated for 30 min prior to the addition of the agonist. C83 α-CTF and α-tubulin levels were quantified after 4 h of treatment with BzATP. Histograms represent the mean ± S.E.M. of C83 α-CTF/α-tubulin ratios normalized with respect to the control ($n = 5$). $^{*}P < 0.05$; $^{**}P < 0.01$.

pharmacological profile we cannot discard the participation of a $P2Y_4$ receptor, as this receptor has proved to be sensitive to Up_4U [19], its mouse ortholog being equipotently activated by ATP and UTP [17].

In order to elucidate whether $P2Y_2$ and/or $P2Y_4$ receptors are present in N2a cells, we analyzed the expression of both nucleotide receptors at the transcriptional level. RT-PCR analysis demonstrated that only $P2Y_2$ receptors are expressed at a measurable level in N2a cells (Fig. 4B). Immunocytochemical assays confirmed the presence of $P2Y_2$ receptors in these cells (Fig. 4C).

Once we have established the presence of a functional $P2Y_2$ receptor in N2a cells we wonder whether the activation of such receptor could lead to increases in α-secretase activity. Stimulation of the cells with the $P2Y_2$ agonist Up_4U increased the levels of C83 α-CTF in a dose dependent manner (pEC_{50}, 6.96 ± 0.20; n_H, 0.96 ± 0.38), reaching saturation at ~1.0 μM of added ligand (Fig. 4D). Indeed, Up_4U responses were inhibited by suramin (Fig. 4E), which is also a pharmacological characteristic of the $P2Y_2$ receptor. Treatment of N2a cells with Up_4U (even at the higher concentration used, 100 μM) or suramin (100 μM) did not modified cell viability, which was assessed by the MTT method (Fig. 4F).

From these data it seem reasonable to conclude that the increases in α-secretase activity observed at high (>100 μM) BzATP concentrations are mediated through the $P2Y_2$ receptor, as BzATP has been previously described as an activator of this type of receptor [18] and these BzATP responses were also reduced in the presence of suramin (Fig. 2 A and C).

4. Concluding remarks

Identifying receptors capable of modulating the processing of APP is an important goal, which could open new perspectives for the treatment of AD. Up to now, inhibitors of β- and γ-secretases have been the focus of intense research. However, the side effects associated to the decrease in BACE1 and γ-secretase activities have restricted this therapy. Modulation of α-secretase activity could represent an alternative approach for the therapeutical treatment of AD. It has been previously shown that activation

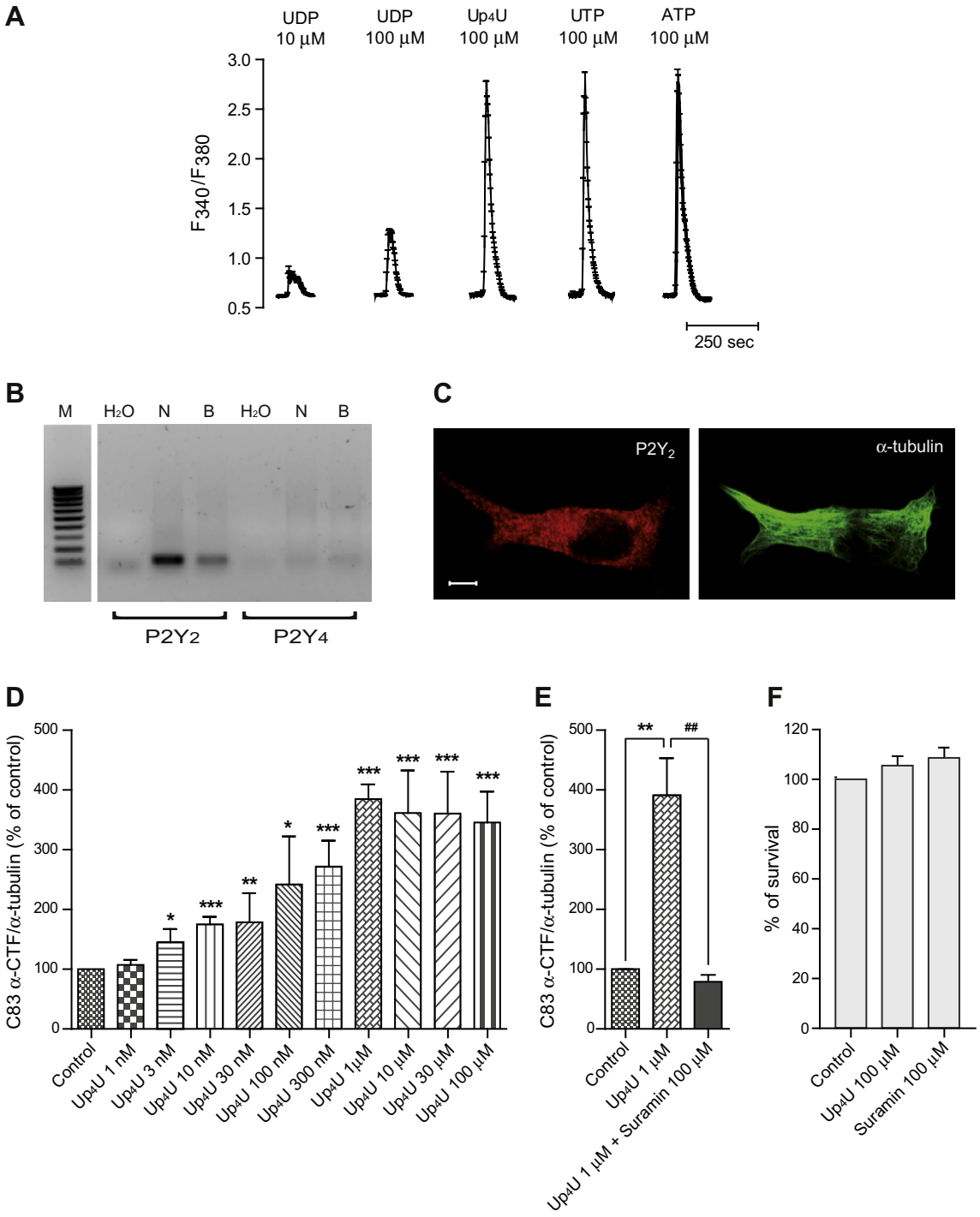

Fig. 4. $P2Y_2$ receptor increases α-secretase activity in N2a cells. (A) Intracellular calcium increments evoked by 30 s stimulation with different nucleotide agonists in N2a cells. Traces represent mean ± S.E.M. from 156 individual cells. (B) Expression of $P2Y_2$ and $P2Y_4$ receptors was analyzed by RT-PCR in both N2a cells, N, and adult whole mouse brain mRNA extracts, B, H_2O, control carried out without template. M, DNA ladder. (C) Immunocytochemical detection of $P2Y_2$ receptors in N2a cells (red). Cell morphology was defined using anti-α-tubulin antibodies (green). Scale bar, 5 μm. (D) N2a cells were stimulated with several Up_4U concentrations, ranging from 1 nM to 100 μM. C83 α-CTF and α-tubulin levels were quantified after 4 h of treatment with the $P2Y_2$ agonist. Histogram represents the mean ± S.E.M. of C83 α-CTF/α-tubulin ratios normalized with respect to the control ($n = 5$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (E) Cells were preincubated for 30 min with 100 μM suramin and then stimulated with the same Up_4U concentrations used in (D), but in the presence of the antagonist. Results obtained at 1 μM Up_4U are shown. Histogram represents the mean ± S.E.M. of C83 α-CTF/α-tubulin ratios normalized with respect to the control ($n = 4$). $^{**}P < 0.01$, control vs 1 μM Up_4U; $^{\#\#}P < 0.01$, 1 μM Up_4U vs 1 μM Up_4U + 100 μM suramin. (F) N2a cells were incubated with Up_4U (100 μM) or suramin (100 μM) and cell viability was analyzed, after 4 h of treatment, by the MTT assay. Data are the mean ± S.E.M. of three experiments performed in triplicate.

of the $P2Y_2$ nucleotide receptor enhances α-secretase-dependent APP processing in astrocytoma cells and primary cortical neurons [5,6]. Our results confirm the stimulatory effect of $P2Y_2$ receptors on α-secretase activity and support a putative role for these receptors in AD.

More interestingly, we have shown that BzATP stimulation of the P2X7R leads to a reduction of α-secretase activity in N2a cells. Several lines of evidence demonstrate that BzATP-mediated α-secretase inhibition is specifically dependent on P2X7R: both the pharmacological blockade of the P2X7 receptor and the inhibition

of P2X7R synthesis by RNA interference, completely prevent the inhibitory effect of BzATP on α-secretase activity.

While this manuscript was in preparation, a work by Delarasse et al. [20] appeared describing an opposite effect of P2X7 on α-secretase activity. However, it is relevant to note that our data support a neuroprotective role of P2X7 antagonism in AD, which has been previously described in the literature [10]. P2X7 induces the release of proinflammatory mediators from macrophages and microglia [8,9] and the neuroprotective effect of P2X7 blockade has been linked with a reduction in the levels of such inflammatory molecules. Our data suggest that blocking of the P2X7 receptor could be also beneficial by enhancing the non-amyloidogenic processing of APP, thus leading to a reduction in the generation of the neurotoxic Aβ.

In summary, this study provides the first evidence that non-amyloidogenic processing of APP can be regulated by two different nucleotide receptors with opposite effects on α-secretase activity. Because both P2X7 and $P2Y_2$ receptors are widely expressed in the brain, these findings could open new challenging perspectives on the therapeutic treatment of AD. I.e. the use of activators of the $P2Y_2$ receptor in combination with P2X7R blockers could have beneficial effects by reducing the amyloid plaques.

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References

[1] Selkoe, D.J. (2001) Alzheimer's disease: genes, proteins, and therapy. Physiol. Rev. 81, 741–766.
[2] Esch, F.S., Keim, P.S., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D. and Ward, P.J. (1990) Cleavage of amyloid beta peptide during constitutive processing of its precursor. Science 248, 1122–1124.
[3] Kuhn, P.H. et al. (2010) ADAM10 is the physiologically relevant, constitutive α-secretase of the amyloid precursor protein in primary neurons. EMBO J. 29, 3020–3032.
[4] Burnstock, G. (2007) Purine and pyrimidine receptors. Cell. Mol. Life Sci. 64, 1471–1483.
[5] Camden, J.M., Schrader, A.M., Camden, R.E., Gonzalez, F.A., Erb, L., Seye, C.I. and Weisman, G.A. (2005) $P2Y_2$ nucleotide receptors enhance α-secretase-dependent amyloid precursor protein processing. J. Biol. Chem. 280, 18696–18702.
[6] Kong, Q. et al. (2009) IL-1β enhances nucleotide-induced and α-secretase-dependent APP processing in rat primary cortical neurons via up-regulation of the $P2Y_2$ receptor. J. Neurochem. 109, 1300–1310.
[7] McLarnon, J.G., Ryu, J.K., Walker, D.G. and Choi, H.B. (2006) Upregulated expression of purinergic $P2X_7$ receptor in Alzheimer disease and amyloid-β peptide-treated microglia and in peptide-injected rat hippocampus. J. Neuropathol. Exp. Neurol. 65, 1090–1097.
[8] Parvathenani, L.K., Tertyshnikova, S., Greco, C.R., Roberts, S.B., Robertson, B. and Posmantur, R. (2003) $P2X_7$ mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's disease. J. Biol. Chem. 278, 13309–13317.
[9] Rampe, D., Wang, L. and Ringheim, G.E. (2004) P2X7 receptor modulation of β-amyloid- and LPS-induced cytokine secretion from human macrophages and microglia. J. Neuroimmunol. 147, 56–61.
[10] Ryu, J.K. and McLarnon, J.G. (2008) Block of purinergic $P2X_7$ receptor is neuroprotective in an animal model of Alzheimer's disease. NeuroReport 19, 1715–1719.
[11] Miras-Portugal, M.T., Diaz-Hernandez, M., Giraldez, L., Hervas, C., Gomez-Villafuertes, R., Sen, R.P., Gualix, J. and Pintor, J. (2003) $P2X_7$ receptors in rat brain: presence in synaptic terminals and granule cells. Neurochem. Res. 28, 1597–1605.
[12] Cirrito, J.R. et al. (2008) Endocytosis is required for synaptic activity-dependent release of amyloid-β in vivo. Neuron 58, 42–51.
[13] Ortega, F., Perez-Sen, R., Morente, V., Delicado, E.G. and Miras-Portugal, M.T. (2010) P2X7, NMDA and BDNF receptors converge on GSK3 phosphorylation and cooperate to promote survival in cerebellar granule neurons. Cell. Mol. Life Sci. 67, 1723–1733.
[14] Gomez-Villafuertes, R. et al. (2009) Ca^{2+}/calmodulin-dependent kinase II signalling cascade mediates P2X7 receptor-dependent inhibition of neuritogenesis in neuroblastoma cells. FEBS J. 276, 5307–5325.
[15] Diaz-Hernandez, M., del Puerto, A., Diaz-Hernandez, J.I., Diez-Zaera, M., Lucas, J.J., Garrido, J.J. and Miras-Portugal, M.T. (2008) Inhibition of the ATP-gated P2X7 receptor promotes axonal growth and branching in cultured hippocampal neurons. J. Cell Sci. 121, 3717–3728.
[16] Gever, J.R., Cockayne, D.A., Dillon, M.P., Burnstock, G. and Ford, A.P. (2006) Pharmacology of P2X channels. Pflugers Arch. 452, 513–537.
[17] Abbracchio, M.P. et al. (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol. Rev. 58, 281–341.
[18] Wildman, S.S., Unwin, R.J. and King, B.F. (2003) Extended pharmacological profiles of rat $P2Y_2$ and rat $P2Y_4$ receptors and their sensitivity to extracellular H^+ and Zn^{2+} ions. Br. J. Pharmacol. 140, 1177–1186.
[19] Pendergast, W., Yerxa, B.R., Douglass 3rd, J.G., Shaver, S.R., Dougherty, R.W., Redick, C.C., Sims, I.F. and Rideout, J.L. (2001) Synthesis and P2Y receptor activity of a series of uridine dinucleoside 5′-polyphosphates. Bioorg. Med. Chem. Lett. 11, 157–160.
[20] Delarasse, C., Auger, R., Gonnord, P., Fontaine, B. and Kanellopoulos, J.M. (2011) The purinergic receptor P2X7 triggers α-secretase-dependent processing of the amyloid precursor protein. J. Biol. Chem. 286, 2596–2606.