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

Miriam León-Otegui, Rosa Gómez-Villafuertes, Juan Ignacio Díaz-Hernández, Miguel Díaz-Hernández, Maria Teresa Miras-Portugal, Javier Gualix *

*Departamento de Bioquímica, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain*

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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

* Corresponding author. Address: Departamento de Bioquímica y Biología Molecular IV, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain. Fax: +34 91 3943909.
*E-mail address:* jgualix@vet.ucm.es (J. Gualix).

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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 $8 \times 10^5$ 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 $6 \times 10^6$ 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 $8 \times 10^5$ 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 $P \leqslant 0.05$.