



Functional characterization of P2Y₁ and P2X₄ receptors in human neuroblastoma SK-N-MC cells

Title in Spanish: *Caracterización funcional de receptores P2Y₁ y P2X₄ en células SK-N-MC de neuroblastoma humano*

Javier Gualix^{1*}, Miriam León-Otegui¹, María Recuero², María Jesús Bullido², Fernando Valdivieso² and M. Teresa Miras-Portugal¹

¹Departamento de Bioquímica, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain. ²Centro de Biología Molecular “Severo Ochoa” (C.S.I.C.-U.A.M.). Universidad Autónoma de Madrid, 28049, Madrid, Spain. Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain. IdiPAZ - Instituto de Investigación Hospital Universitario La Paz, Madrid, Spain

ABSTRACT: Nucleotides are important signalling molecules in both the peripheral and central nervous system. However, the *in vitro* study of their receptors can be hampered by the heterogeneity of primary neuronal cultures. The use of clonal neuroblastoma cell lines allows to circumvent this difficulty, so these lines are often used as a model to analyze the properties, regulation and physiological role of nucleotide receptors in neural tissues. Expression studies indicated the presence of P2Y₁, P2Y₆, P2Y₁₁, P2Y₁₃, P2X₁, P2X₄, P2X₅, P2X₆ and P2X₇ proteins in SK-N-MC cells. Functional analyses showed transient [Ca²⁺]_i increases upon application of ADP, 2-MeSADP or ADPβS. Responses to these agonists seem to be mediated by a P2Y₁ receptor, as demonstrated by the almost complete blockade exerted by the P2Y₁-selective antagonist MRS2179. ATP was also able to induce [Ca²⁺]_i increases in SK-N-MC cells. Responses to ATP were partially blocked by MRS2179 and the P2X antagonist TNP-ATP, thus suggesting that ATP can interact with two different P2 receptors: a P2Y₁ receptor, inhibited by MRS2179, and a TNP-ATP sensitive P2X receptor. To characterize the P2X receptor responsible for the MRS2179-resistant component of the ATP response, we analyze the effect of several P2X agonists on [Ca²⁺]_i. Cells did not show responses to either α,β-meATP or BzATP, although [Ca²⁺]_i increases could be observed when cells were challenged with CTP. Both the response to CTP and the MRS2179-resistant component of ATP response were potentiated by ivermectin. Such pharmacological profile is consistent with the presence of a functional P2X₄ receptor in SK-N-MC cell line.

RESUMEN: Los nucleótidos son importantes moléculas señalizadoras en el sistema nervioso. El estudio *in vitro* de sus receptores puede verse obstaculizado por la heterogeneidad de los cultivos neuronales. El uso de líneas celulares de neuroblastoma permite eludir esta dificultad y dichas líneas se utilizan frecuentemente como un modelo con el que analizar las propiedades, regulación y función de los receptores de nucleótidos en tejidos neurales. Estudios de expresión indicaron la presencia de proteínas P2Y₁, P2Y₆, P2Y₁₁, P2Y₁₃, P2X₁, P2X₄, P2X₅, P2X₆ y P2X₇ en las células SK-N-MC. Análisis funcionales mostraron incrementos transitorios de [Ca²⁺]_i tras la aplicación de ADP, 2-MeSADP o ADPβS, respuestas que parecen estar mediadas a través un receptor P2Y₁, como se pone de manifiesto por el bloqueo casi total ejercido por el antagonista selectivo P2Y₁, MRS2179. El ATP también indujo incrementos de [Ca²⁺]_i en las células SK-N-MC, siendo su respuesta parcialmente bloqueada por MRS2179 y por el antagonista P2X TNP-ATP, lo que sugiere que el ATP puede interactuar con dos receptores P2 diferentes: un receptor P2Y₁, inhibido por MRS2179, y un receptor P2X sensible a TNP-ATP. Se caracterizó el receptor P2X analizando el efecto de varios agonistas en la [Ca²⁺]_i. Ninguna célula mostró respuestas a α,β-meATP o BzATP, aunque se observaron incrementos de [Ca²⁺]_i cuando las células fueron estimuladas con CTP. Tanto la respuesta a CTP como el componente de la respuesta a ATP resistente a MRS2179, se potenciaron en presencia de ivermectina. Todos estos datos sugieren la presencia de un receptor P2X₄ funcional en las células SK-N-MC.

*Corresponding Author: jgualix@ucm.es

Received: June 22, 2015 Accepted: November 5, 2015

An Real Acad Farm Vol. 81, N° 3 (2015), pp. 247-257

Language of Manuscript: English

1. INTRODUCTION

Extracellular nucleotides have major roles in the activity of neural cells. They act as transmitters or modulators in most nerve types in the peripheral and central nervous system. Furthermore, they also act as important mediators in neuronal-glia or glia-glia communication and are trophic factors participating in cell

differentiation, proliferation, migration and death (1, 2). Nucleotide-mediated signaling mechanisms and specific receptor subtypes have been shown to be involved in various pathological conditions including brain trauma and ischaemia, neurodegenerative diseases involving neuroimmune and neuroinflammatory reactions, as well as in neuropsychiatric diseases (3).

Nucleotide effects are mediated through two subclasses of specific cell surface receptors, ionotropic P2X and metabotropic P2Y receptors (4). Seven distinct P2X subtypes (P2X₁₋₇) have been cloned from mammalian species, showing a topology that includes two transmembrane spanning regions, a large extracellular loop and intracellular N and C termini. These proteins assemble into ATP activated channels either as homomers or heteromers, each functional receptor containing at least three monomers. P2X receptor activation results in Na⁺ and Ca²⁺ influx across the cell membrane, which leads to depolarization. Membrane depolarization subsequently activates voltage-operated calcium channels, thus causing accumulation of Ca²⁺ ions in the cytoplasm. Although the detailed signalling mechanism have not been established for most P2X receptor subtypes, it is well known that cytoplasmic Ca²⁺ elevation triggers a variety of intracellular events, in part, through activation of mitogen-activated protein kinases (MAPKs), protein kinase C (PKC) and calmodulin (5-7).

The P2Y receptors are classical 7-transmembrane domain metabotropic receptors coupled to G proteins. Currently, eight P2Y receptor subtypes (P2Y_{1, 2, 4, 6, 11, 12, 13, 14}) have been cloned. P2Y_{1, 11, 12, 13} receptors are selectively activated by adenine nucleotides, whereas P2Y₄ and P2Y₆ are stimulated by pyrimidines. The P2Y₂ receptor responds equally well to purine and pyrimidine triphosphates (ATP and UTP) and P2Y₁₄ is a sugar-nucleotide (UDP-glucose) responding receptor. Most members of the P2Y family (P2Y_{1, 2, 4, 6, 11}) are functionally coupled to G_{q/11} proteins and stimulate phosphoinositide-specific phospholipase C, resulting in the formation of inositol-(1,4,5)-trisphosphate and diacylglycerol with subsequent mobilization of Ca²⁺ from internal stores. P2Y₁₁ receptor additionally stimulates adenylate cyclase, whereas P2Y₁₂, P2Y₁₃ and P2Y₁₄ promote G_{i/o} inhibition of adenylate cyclase activity. The stimulation of several P2Y receptors is also commonly associated with the activation of MAPKs (in particular extracellular signal-regulated protein kinase 1/2, ERK1/2). Other classes of protein, such as phospholipases A₂ and D, PKC or phosphatidylinositol 3-kinase, can be also activated according to the cell context and the particular P2Y subtype (5, 8, 9).

Several neuroblastoma cell lines, each originating from a single cell that has been isolated from a spontaneously occurring or induced tumor of the nervous system, are now available and can be maintained in culture infinitely. Neuroblastoma derived cell lines have been used as an *in vitro* model for the study of a variety of neurotransmitter receptors including those for nucleotides. Indeed, the P2Y₂ receptor was originally cloned from the mouse neuroblastoma/rat glioma hybrid line NG108-15 (10). Further studies have revealed the presence in these cells of the P2X₇ receptor and metabotropic receptors other than the P2Y₂ subtype (11, 12). Functional P2X₇ receptors can be also detected in different human neuroblastoma lines (13, 14) as well as in the mouse Neuro-2a neuroblastoma cells (6, 15). In addition, a range of cell lines of both

mouse and human neuroblastoma origin have been shown to functionally express pyrimidine selective P2Y receptors (16-18).

SK-N-MC human neuroblastoma are known to constitutively express adrenergic α_{2C} , β_1 and β_3 (19, 20), dopamine D1 (21), neuropeptide Y₁ (22) and muscarinic M₁ receptors (23). The regulation and signalling pathways activated by these receptors have been extensively investigated using SK-N-MC cells as a model (24-28). SK-N-MC cell line also served to analyze the integration or cross-communication of signals upon activation of different types of neurotransmitter receptors (29). More recently, SK-N-MC has been used as a parental line which generate cellular lines that stably overexpress proteins of interest in the study of distinct neurodegenerative disorders, such as the amyloid precursor protein APP (30), carboxyl-terminal fragments of the APP protein (31, 32), or different mutated forms of the Parkin protein (33).

Earlier experiments showed that ATP and other nucleotides are able to induce response (phosphoinositide hydrolysis and formation of inositol phosphates) in the SK-N-MC cells, thus indicating the presence of not fully characterized P2 receptors in this neuroblastoma cell line (23).

In the present study we have analyzed the presence of functional P2 receptors in the human neuroblastoma SK-N-MC cells. If such receptors were present in these cells, SK-N-MC cell line could serve as a useful model to analyze several aspects of the signalling mediated through nucleotide receptors in neural tissues. This includes the regulation and transduction pathways activated by P2 receptors, their interaction with other neurotransmitter receptors or their putative relationship with proteins involved in neurodegenerative processes

2. MATERIALS AND METHODS

2.1. Materials

2-methylthio-adenosine-5'-diphosphate (2-MeSADP), α,β -methylene-adenosine 5'-triphosphate (α,β -meATP), ADP, adenosine-5'-0-(2-thiodiphosphate) (ADP β S), ATP, 2'(3')-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP), CTP, pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP), UDP, UTP and ivermectin were purchased from Sigma (St Louis, Missouri, USA). N⁶-methyl-2'-deoxyadenosine-3',5'-bisphosphate (MRS2179) was purchased from Tocris Cookson (Bristol, UK). Products for cell culture, immunocytochemical and western-blot studies and fluorescent probes are included in the specific methods section. Other analytical grade reagents were purchased from Merck (Darmstadt, Germany).

2.2. Cell culture

The SK-N-MC human neuroblastoma cell line (HTB-10) was purchased from the American Type Culture

Collection (ATCC, Barcelona, Spain). Cells were grown in monolayer in 10-cm plastic culture dishes (BD Falcon, Erembodegem, Belgium) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Culture medium was minimum essential medium (Gibco, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mM glutamine, 1 mM sodium pyruvate, non-essential amino acids (alanine, glutamic acid, aspartic acid, proline and asparagine, each at 0.4 mM), 100 units/mL penicillin, 0.1 mg/ml streptomycin and 0.25 µg/mL amphotericin B (Sigma).

Cells were subcultured about 2 times a week: culture medium was removed and discarded and the cell layer was rinsed two times with 2 mL of a solution containing 0.25% (w/v) trypsin and 0,02% (w/v) EDTA in Hank's Balanced Salt Solution with phenol red (Sigma). Then, culture dishes were placed in an incubator for 1 min at 37° to allow cells to detach. After that, 5 mL of the culture medium were added to the dishes and cells were aspirated by gently pipetting. Cells were counted in a Neubauer chamber using the trypan blue exclusion test and appropriate aliquots of the cell suspension were taken for the immunocytochemical, western blot or calcium imaging assays. Finally, 1 mL of the cell suspension was added to a new culture dish containing 9 mL of the culture medium and cells were maintained at 37°C in 5% CO₂.

2.3. Immunocytochemical assays

Cells were plated at a density of 200,000 cells/mL on glass coverslips precoated with 0.1 mg/mL poly-L-lysine (Biochrom AG, Berlin Germany) and maintained in a humidified incubator at 37 °C in 5% CO₂ for 2-3 days before they were used in the immunocytochemical experiments. Cover slips with the SK-N-MC cells attached were then treated with 4% (w/v) *p*-formaldehyde (Sigma) for 15 min, washed twice with phosphate-buffered saline (PBS) (composition mM: NaCl 137, KCl 2.6, KH₂PO₄ 1.5, Na₂HPO₄ 8.1, pH 7.4) and incubated for 1 h in PBS containing 3% (w/v) bovine serum albumine (BSA), 0.1% (v/v) Triton X-100 and 5% (v/v) normal goat or donkey serum (Sigma). Cells were washed three times with PBS/BSA and incubated with the primary antibody for 1 h at 37° C. Primary antibodies assayed were: rabbit anti-P2Y₁ (serum at 1:100 dilution) (Alomone labs, Jerusalem, Israel), rabbit anti-P2Y₂ (1:200) (Chemicon International, Temecula, CA, USA), rabbit anti-P2Y₄ (1:100) (Chemicon International), rabbit anti-P2Y₆ (1:100) (Alomone labs), rabbit anti-P2Y₁₁ (1:100) (Alomone labs), rabbit anti-P2Y₁₂ (1:100) (Alomone labs), rabbit anti P2Y₁₃ (1:50) (Alomone labs), rabbit anti-P2X₁ (1:100) (Chemicon International), guinea-pig anti-P2X₂ (1:250) (Chemicon International), guinea-pig anti-P2X₃ (1:500) (Chemicon International), rabbit anti-P2X₄ (1:100) (Alomone labs), rabbit anti-P2X₅ (1:200) (Alomone labs), rabbit anti-P2X₆ (1:100) (Alomone labs) or rabbit anti-P2X₇ (intracellular epitope, 1:100 dilution) (Alomone labs). All the antibodies used in this work were polyclonal and most of them were raised against the corresponding human protein. In some

cases, antibodies against the rat protein were also used (if it was the case, cross reactivity with the human protein has been verified by the manufacturer). Next, covers were washed three times with PBS/BSA and incubated for 1 h at 37°C with the secondary antibodies: donkey anti-rabbit IgG coupled to Cy3 (1:400) (Jackson ImmunoResearch, West Grove, PA, USA) or fluorescein conjugated goat anti guinea-pig IgG (1:64) (Sigma). Then, covers were washed three times with PBS and mounted following standard procedures. Control experiments of nonspecific staining were performed without primary antibody or by preadsorption of the primary antibody with the immunizing peptide.

Fluorescent images were taken using a NIKON TE-200 microscope equipped with a S Fluor 40X objective (oil, 1.3 NA) a mercury lamp light source, fluorescein and rhodamine Nikon filter sets and a CCD camera (Kappa ACC 1) controlled by Kappa ImageBase software (Kappa opto-electronics, Gleichen, Germany).

2.4. Western blot

Cells were seeded on 10-cm plastic Petri dishes (BD Falcon) at a density of 400,000 cells/mL and maintained at 37 °C in 5% CO₂ for 2-3 days before any treatment. Western blot analysis were performed using protein extracted from the SK-N-MC cells by homogenization in a buffer containing 100 nM HEPES (pH 7.4), 0.5 mM EDTA, 50 µg/ml phenylmethylsulfonyl fluoride, 0.5 µg/ml leupeptine, 0.5 µg/ml pepstatine, 0.2 M NaCl and 1% (v/v) Triton X-100. The samples were homogenized at 4°C and protein content determined by Bradford assay. Total protein (40 µg) was electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Whatman, Maidestone, UK). The membranes were blocked with 5% nonfat dried milk for 1 h at room temperature and further incubated overnight at 4°C with the different P2 receptor antibodies at a dilution of 1:500. Blots were then washed and incubated for 1 h at room temperature with the corresponding secondary antibody coupled to horseradish peroxidase (DakoCytomation, Glostrup, Denmark). Protein bands were detected with enhanced chemoluminescence detection (Perkin Elmer, Boston, MA, USA). Incubation with the control peptide was made following the manufacturer instructions.

2.5. Calcium imaging

Cells were plated at a density of 200,000 cells/mL on glass coverslips precoated with 0.1 mg/mL poly-L-lysine (Biochrom) and maintained in a humidified incubator at 37 °C in 5% CO₂ for 2-3 days before they were used in the calcium imaging experiments. Cells attached to coverslips were then washed with Locke's solution (composition in mM: NaCl, 140; KCl, 4.7; MgSO₄, 1.2; KH₂PO₄, 1.2; CaCl₂, 2.5; glucose, 5.5; HEPES, 10; pH 7.4) supplemented with 1 mg/mL BSA and loaded with 5 µM Fura-2 AM (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C. Next, the coverslips were washed with Locke's medium and mounted in a small superfusion

chamber on the stage of a Nikon TE-200 microscope. Cells were superfused with Locke's solution, and different agonists of the P2 receptors were applied in 30s pulses. When the experiments were performed using P2 receptor antagonists (MRS2179, TNP-ATP) or potentiators (ivermectin), these compounds were superfused for 2 min before the application of the corresponding agonist.

Cells were alternately excited at 340 and 380 nm, these wavelengths corresponding to the fluorescence peaks of Ca^{2+} -saturated and Ca^{2+} -free Fura-2 solutions. The wavelength of the incoming light was selected with the aid of a Lambda 10-2 optical filter changer (Sutter Instrument, Novato, CA, USA) and emitted light was isolated with a dichroic mirror (430 nm) and a 510 nm bandpass filter (Omega Optical). Cells were imaged through a NIKON 40X lens (S Fluor 1.3 oil iris) and 12-bit images were acquired using an ORCA-ER C 47 42-80 camera from Hamamatsu (Hamamatsu City, Japan) controlled by MetaFluor 6.2r6 PC software (Universal Imaging Corp., Cambridge, UK). Time course data represent the average light intensity in a small elliptical region inside each cell. Background and autofluorescence components were subtracted at each wavelength and the 340/380 ratio was calculated. The data are represented as the normalized F340/F380 fluorescence ratio, which increases as $[\text{Ca}^{2+}]_i$ increases.

3. RESULTS

3.1. Immunological characterization of P2X subunits in SK-N-MC cells

In order to investigate the presence of native purinergic receptors in the SK-N-MC cells, the expression of P2X subunits was analyzed by using commercially available subunit-specific antibodies.

Western blot experiments demonstrate that most of the P2X subunits are expressed in SK-N-MC cells, as bands corresponding to monomeric P2X₁, P2X₄, P2X₅, P2X₆ and P2X₇ proteins can be immunodetected (results not shown).

As expected, immunocytochemical detection of P2X subunits correlated well with the results obtained by western blot. All P2X subunit antibodies, except that for the P2X₂ and P2X₃ subtypes, labeled the SK-N-MC cells. Immunostaining was specific as it disappeared when antibodies were pre-adsorbed with the corresponding antigen peptide (Figure 1).

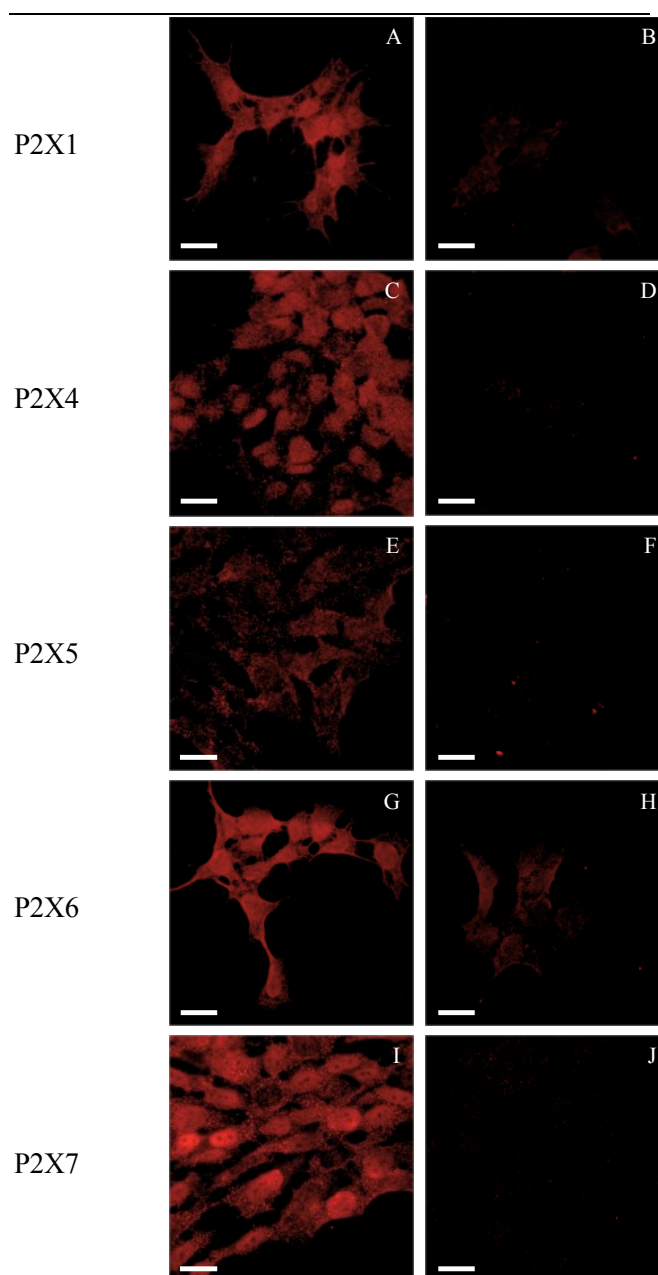


Figure 1. Immunological detection of P2X receptor subunits in SK-N-MC cells. Fluorescence image of SK-N-MC cells labeled with anti-P2X₁ (A), anti-P2X₄ (C), anti-P2X₅ (E), anti-P2X₆ (G) and anti-P2X₇ (I) antibodies. To confirm a specific immunoreaction, primary antibodies used in A, C, E, G and I were pre-absorbed with the corresponding control peptide and immunostaining are shown in B, D, F, H and J, respectively. Scale bar= 20 μm in all micrographs.

3.2. Immunological characterization of P2Y receptors in SK-N-MC cells

Immunological characterization of P2Y receptors was carried out using commercially available antibodies for the P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂ and P2Y₁₃ subtypes.

Several P2Y receptors are simultaneously expressed in SK-N-MC cells, as specific bands corresponding to P2Y₁,

P2Y₆, P2Y₁₁ and P2Y₁₃ proteins can be detected in the western blot assays (results not shown). Again, immunocytochemical experiments correlated with the results obtained in the western blot experiments: SK-N-MC cells showed specific immunostaining with antibodies against P2Y₁, P2Y₆, P2Y₁₁ and P2Y₁₃ receptor subtypes (Figure 2). No immunostaining were observed with the antibodies for the P2Y₂, P2Y₄ and P2Y₁₂ subtypes (results not shown).

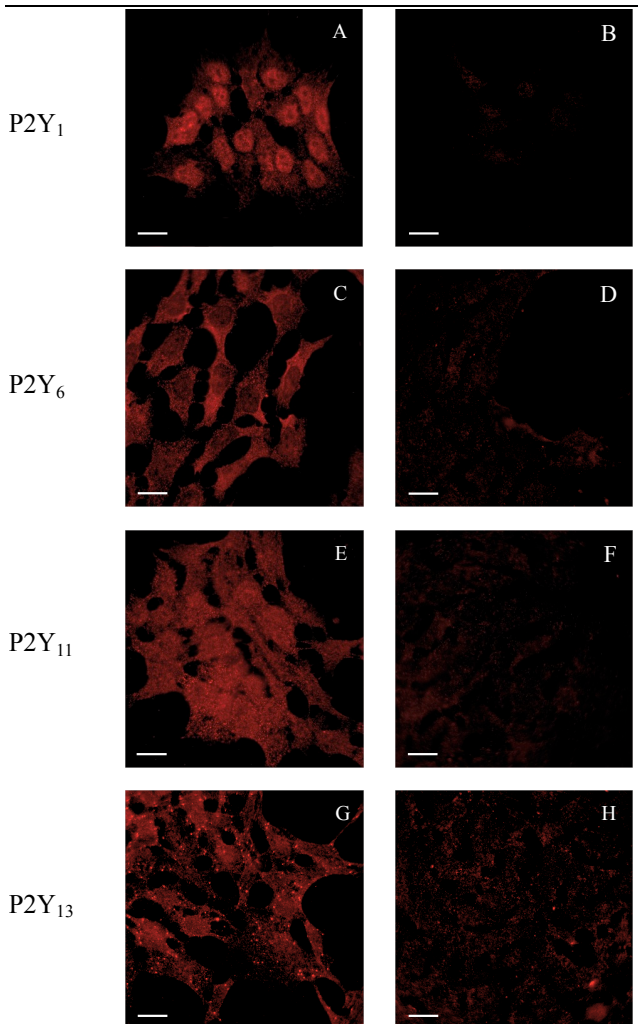


Figure 2. P2Y receptors immunodetection in SK-N-MC cells. Fluorescence image of SK-N-MC cells labeled with anti-P2Y₆ (C), anti-P2Y₁₁ (E) and anti-P2Y₁₃ (G) antibodies. To confirm a specific immunoreaction, the primary antibodies used in A, C, E and G were pre-absorbed with their corresponding control peptide and immunostaining are shown in B, D, F and H, respectively. Scale bar= 20 μm in all micrographs.

3.3. Characterization of functional P2 receptors by calcium imaging in SK-N-MC cells

The immunodetection of different P2X subunits or P2Y receptors indicates the presence of the protein but does not give information concerning their functionality. To approach this specific issue, microfluorimetric techniques were used.

We analyzed the response of SK-N-MC cells to the

most commonly utilized P2 agonists by single-cell imaging after loading cells with the fluorescent calcium dye Fura-2 AM. A significant percentage of the cells ($91.5 \pm 1.5\%$ of total cells, $n = 240$) showed transient $[Ca^{2+}]_i$ increases upon application of 10 μM ADP. Response to ADP is likely mediated by a P2Y₁ receptor, as demonstrated by the almost complete blockade exerted by the P2Y₁-selective antagonist MRS2179 (10 μM, Fig. 3A). Other P2Y₁ agonists such as 2-MeSADP (10 μM) or ADPβS (10 μM) were also able to elicit $[Ca^{2+}]_i$ increases in the SK-N-MC cells, these responses being almost completely blocked by 10 μM MRS2179 (Fig. 3B,C). Neither UDP (100 μM) nor UTP (100 μM) elicited calcium responses in the SK-N-MC cells (results not shown), thus precluding the presence of functional pyrimidine receptors.

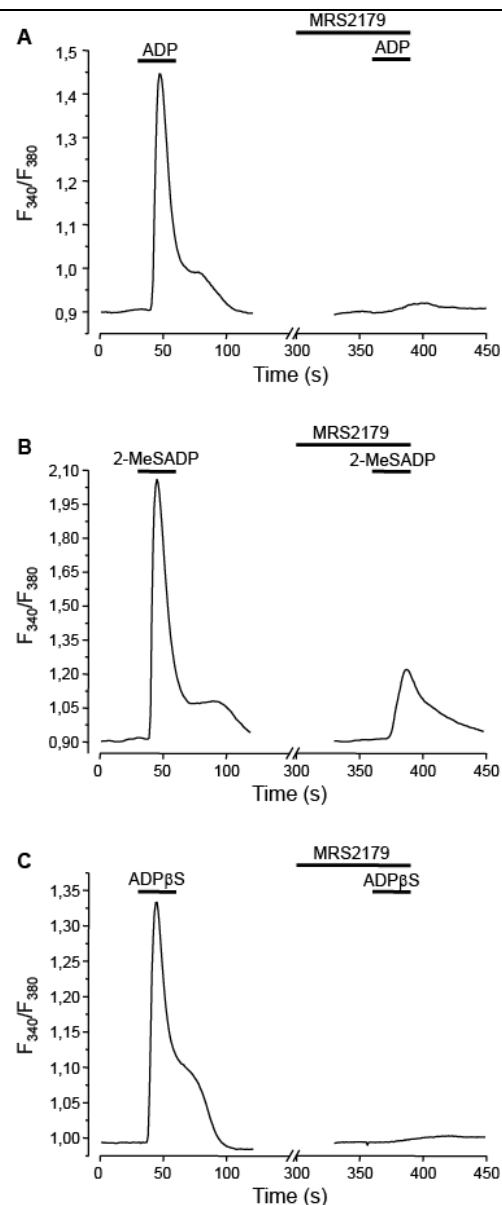


Figure 3. Stimulation of the SK-N-MC cells with ADP, 2-MeSADP or ADPβS induces intracellular transients that are inhibited by the P2Y₁ antagonist MRS2179. Changes in the

intracellular Ca^{2+} concentration were measured when cells were challenged with 10 μ M ADP (A), 10 μ M 2-MeSADP (B) OR 10 μ M Ad β S (C), both in the absence and in the presence of the selective P2Y₁ antagonist, MRS 2179 (10 μ M). Traces represent the mean of the responses measured in 156 (A), 140 (B) or 144 (C) individual cells. Horizontal bars above traces indicate duration of drug applications.

We also tested the ability of ATP (100 μ M) to induce $[Ca^{2+}]_i$ increases in the SK-N-MC cells. Almost all SK-N-MC cells ($96.8 \pm 0.4\%$ of total cells, $n = 882$) showed responses when challenged with this nucleotide. Thus, ATP was used in some of our assays as an indicator of cell functionality (see Figures 5A and B). Responses to 100 μ M ATP were partially blocked by MRS2179 (10 μ M), the residual response observed in the presence of the antagonist being a 13.3 % of the original one (Fig 4A). The P2X antagonist TNP-ATP (100 μ M) also acted as a partial inhibitor of ATP responses, reducing by 50.2 % the amplitude of the calcium transients elicited by the agonist (Fig 4B). When SK-N-MC cells were treated with both antagonists, MRS2179 (10 μ M) and TNP-ATP (100 μ M), responses to 100 μ M ATP were completely blocked (results not shown). This is indicating that ATP is exerting its effects through the interaction with two different P2 receptors: a P2Y₁ receptor, sensitive to MRS2179, and a P2X receptor which is blocked by TNP-ATP.

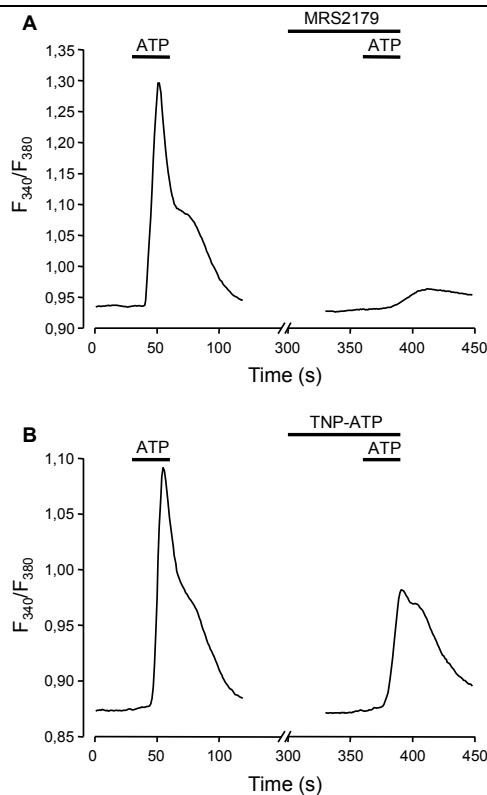


Figure 4. ATP induces calcium responses in the SK-N-MC cells that are inhibited by MRS2179 and TNP-ATP. Changes in the intracellular Ca^{2+} concentration were measured when cells were stimulated with 100 μ M ATP applied alone or in the presence of the selective P2Y₁ antagonist, MRS 2179 (10 μ M),

or the P2X antagonist, TNP-ATP (100 μ M, B). Traces represent the mean of the responses obtained in 128 (A) or 94 (B) individual cells. Horizontal bars above traces indicate duration of drug applications.

To further characterize the P2X receptor that is responsible for the MRS2179 resistant component of the ATP response, we analyze the effect of several P2X agonists on the SK-N-MC cells. No cells showed responses to either 100 μ M α,β -meATP or 100 μ M BzATP (Fig 5A). However, responses to CTP (300 μ M) were present in a $67.6 \pm 10.1\%$ of the analysed cells ($n = 396$, Fig 5B). Moreover, both the response to CTP (300 μ M) and the MRS2179-resistant component of the ATP (100 μ M) response were potentiated in the presence of 5 μ M ivermectin (Fig 5B and C), a positive allosteric modulator of the P2X4 receptor (34, 35).

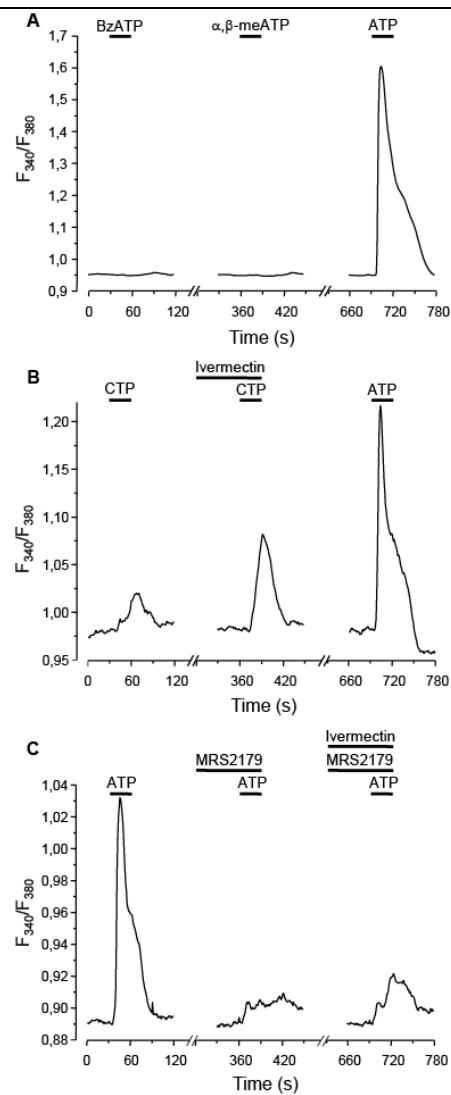


Figure 5. Intracellular calcium increments evoked by different purinergic agonists and effect of the P2X4 potentiator ivermectin in the SK-N-MC cells. (A) Effect of 100 μ M BzATP or 100 μ M α,β -meATP on the intracellular Ca^{2+} concentration in the SK-N-MC cells. At the end of the experiments, cells were challenged with 100 μ M ATP to test their

functionality. Traces represent the mean of the responses obtained in 108 individual cells. **(B)** SK-N-MC cells were stimulated with 300 μ M CTP both in the absence and in the presence of the P2X₄ positive modulator ivermectin (5 μ M). At the end of the experiments, cells were challenged with 100 μ M ATP to test their functionality. Traces represent the mean of the responses obtained in 135 individual cells that showed responses to CTP. **(C)** SK-N-MC cells were stimulated with 100 μ M ATP, both in the absence and in the presence of the P2Y₁ antagonist MRS2179 (10 μ M), to selectively analyze the MRS2179-resistant component of the ATP response. Afterwards, cells were challenged with 100 μ M ATP in the presence of MRS2179 (10 μ M) and ivermectin (5 μ M), in order to analyze the effect of the positive modulator of the P2X₄ receptor on the MRS2179-resistant component of the ATP response. Traces are the mean of the responses measured in 140 individual cells. Horizontal bars above traces indicate duration of drug applications in all cases.

4. DISCUSSION

The limited biological material and the cellular heterogeneity of primary neuronal cultures frequently represent a disadvantage for the *in vitro* study of the nucleotide mediated signalling in the nervous system. The use of homogeneous cultured neuroblastoma cell lines that constitutively express P2 receptors allows avoiding these inconveniences, so these lines could constitute a reliable and convenient model with which analyze the signal transduction pathways and intracellular events associated with the activation of nucleotide receptors in neural tissues.

In the present study we have investigated the presence of functional P2 receptors in human neuroblastoma SK-N-MC cells by a combination of immunological and calcium measurement experiments. Immunological assays showed the expression of P2Y₁, P2Y₆, P2Y₁₁ and P2Y₁₃ receptors in the SK-N-MC cell line. P2X₁, P2X₄, P2X₅, P2X₆ and P2X₇ subunits can also be detected in the SK-N-MC cells by means of immunocytochemical and western blot assays.

Despite the abundant expression of P2 receptors in the SK-N-MC cells only a few range of P2X or P2Y agonists are able to elicit calcium responses. A similar feature can be also observed in other neuroblastoma cell types. For instance, murine N2a cells express a wide range of P2X subunits, including P2X₁, P2X₃, P2X₄ and P2X₇. However, of these, the P2X₇ receptor was the only functional receptor (6).

The finding that ADP, 2-MeSADP and ADP β S induce calcium increases that are inhibited by the subtype-selective P2Y₁-antagonist MRS2179 (36, 37), strongly support the presence of a functional P2Y₁ receptor in the SK-N-MC cell line. ADP and its derivatives also activate the P2Y₁₃ receptor (36, 38, 39), which can be immunologically detected in SK-N-MC cells. However if the calcium transient induced by these compounds were, at least in part, due to their interaction with a P2Y₁₃ receptor, a residual response should be observed in the presence of MRS2179 that, at concentrations up to 100 μ M, had no significant effect on the P2Y₁₃ receptor (36, 38). This is not the case: responses to ADP and ADP derivatives are almost completely blocked by MRS2179, thus indicating

that these agonists increase the intracellular calcium concentration due to their exclusive interaction with a P2Y₁ receptor in the SK-N-MC cells. On the other hand, the lack of effect of UDP, a selective agonist at the pyrimidineric P2Y₆ receptor (36, 37), precludes the presence of functional P2Y₆ receptors in the SK-N-MC cells, in spite of our data showing their robust expression.

Regarding ATP, this nucleotide was also able to induce calcium transients in the SK-N-MC cells. The partial inhibition that both the specific P2Y₁ antagonists MRS2179 and the P2X antagonist TNP-ATP (40) exert on the ATP responses indicates that this nucleotide is activating at least two different receptors in the SK-N-MC cells: the previously mentioned P2Y₁ receptor, which is blocked by MRS2179, and a P2X receptor, inhibited by TNP-ATP. P2Y₁₁ receptor, which is sensitive to ATP (36, 37), can be also detected at the protein level in the SK-N-MC cells. However, if ATP were activating also this receptor, a residual response to the agonist should be observed in the combined presence of MRS2179 and TNP-ATP. This is not the case: ATP responses in SK-N-MC cells are completely blocked by means of a combination of MRS2179 and TNP-ATP. Additionally, the lack of effect of BzATP and UTP, both potent agonists at the P2Y₁₁ receptor (36, 41), also precludes the presence of functional P2Y₁₁ receptors in the SK-N-MC cell line.

We tried to establish the identity of the P2X receptor that is responsible for the MRS2179-resistant component of the ATP response. SK-N-MC cells did not respond to α,β -meATP or BzATP but increased their $[Ca^{2+}]_i$ when challenged with CTP. Although P2X₁ and P2X₆ subunits can be immunodetected in the SK-N-MC cells, the presence of functional P2X₁ or P2X₆ channels can be ruled out due the lack of action of α,β -meATP, a potent agonist of both receptors (42-44). α,β -meATP is a weak or inactive agonist at the P2X₅ and P2X₇ receptors but, if these channels were functional in the SK-N-MC cells, responses to BzATP should be obtained, as both receptors are activated by this nucleotide analog (42, 45, 46). The pattern of agonist activity observed in the SK-N-MC cells is, however, consistent with the presence of a functional P2X₄ receptor. P2X₄ channels have been proved to be nearly insensitive to methylene-substituted ATP analogs, whereas they can be activated by CTP, this compound being a less potent agonist than ATP (42, 47). An unusual property of the P2X₄ receptor that differentiates it from all other homomeric P2X channels is its potentiation by ivermectin. This antiparasitic agent is a positive allosteric modulator that specifically augments the response of P2X₄ receptor to its agonists (34, 35). SK-N-MC cells showed responses to CTP that increased in the presence of ivermectin, this being an additional evidence favouring the presence of a functional P2X₄ receptor in the SK-N-MC cell line. Moreover, the residual responses to ATP in the presence of MRS2179 were also potentiated by ivermectin, which could indicate that the P2X₄ receptor is responsible for the MRS2179-resistant component of the ATP response in the SK-N-MC cells.

As SK-N-MC cells express different P2X subunits, the possible formation of heteromeric assemblies, in which P2X4 subunits were present, should be taken into consideration. Three different P2X4-containing heteromeric channels have been described so far: P2X1/4, P2X4/6 and P2X4/7 receptors (48-50). P2X4/7 heteromeric receptor shares some of the pharmacological properties of the P2X receptor in the SK-N-MC cells, as it is potentiated by ivermectin (48). However there is a clear difference between both receptors: heteromeric P2X4/7 receptor is activated by BzATP (48) whereas the P2X receptor in SK-N-MC cells has been proved to be insensitive to this nucleotide analog. Regarding P2X1/4 and P2X4/6 receptors, the phenotype of such heteromers differs in some aspects from that of P2X4. For example, heteromeric P2X1/4 and P2X4/6 receptors are activated by low concentrations of α,β -meATP, whereas homomeric P2X4 receptors are relatively insensitive to this substance (49, 50). As P2X receptors in SK-N-MC cells appear to be unaffected by α,β -me-ATP, it seems more probable that they were formed by a homomeric combination of P2X4 subunits rather than a heteromeric combination of P2X4 with the P2X1 or the P2X6 subunits. However, as SK-N-MC cells also express some additional P2X subunit, such as P2X5, a heteromeric association between this subunit and the P2X4 one can not be ruled out.

Taken together, these pharmacological data likely indicate the presence of two functional P2 receptors in the human neuroblastoma SK-M-MC cell line: one metabotropic, P2Y₁, and the other ionotropic, P2X4 (or a heteromeric channel including the P2X4 subunit). Responses to ADP and its derivatives will be mediated through the activation of the P2Y₁ receptor, although responses to ATP will be due to the interaction of such nucleotide with both the P2Y₁ and the P2X4 receptors. CTP, on their hand, will only activate the ionotropic P2X4 receptor.

P2X4 receptor, which is expressed in several regions of the brain and spinal cord (51, 52), has received considerable recent attention due to its emerging role in the modulation of chronic inflammatory and neuropathic pain (53-55). There are also evidences suggesting that P2X4 receptors could be involved in the mechanism sustaining neuronal cell death caused by oxygen/glucose deprivation (56, 57). More recently, a role of the P2X4 receptor in the long term potentiation in the hippocampus has been described (58).

Regarding P2Y₁, immunohistochemical studies showed an abundant and widespread distribution of this receptor throughout the human brain, suggesting its involvement in a number of important functions (59). In post-mortem brain sections from persons with Alzheimer's disease, the P2Y₁-like immunoreactivity in the hippocampus and entorhinal cortex was localized to neurofibrillary tangles, neuritic plaques and neuropil threads, which are characteristic Alzheimer's structures (60). On the other hand, it is now recognized that interactions between G protein-coupled receptors can take place through the

formation of oligomers, a process commonly referred to as receptor dimerization. There is evidence that P2Y₁ receptors can form both homodimers and heteromeric complexes with the P2Y₁₁ or A1 receptors (61, 62). Such kinds of interaction will contribute to add complexity to the purinergic signalling

5. CONCLUSIONS

In summary, P2Y₁ and P2X4 receptors are widespread in the CNS where they can participate in a number of different and important functions. As these receptors are constitutively expressed and functional in SK-N-MC cells, such cells can constitute a useful model with which analyze several aspects of the physiology of both nucleotide receptors. These include their regulation, associated intracellular signalling pathways, oligomerization, cross-talk with other neurotransmitter systems and putative implication in neurological or neurodegenerative disorders.

6. ACKNOWLEDGMENTS

This work was supported by research grants from Ministerio de Ciencia e Innovación (BFU2014-53654-P) and Comunidad de Madrid (S2013/ICE-2958 BRADE-CM).

7. ABBREVIATIONS LIST

2-MeSADP, 2-methylthio-adenosine-5'-diphosphate; α,β -meATP, α,β -methylene-adenosine 5'-triphosphate; ADP β S, adenosine-5'-0-(2-thiodiphosphate); APP, amyloid precursor protein; BSA, bovine serum albumin; BzATP, 2'(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate; ERK1/2, extracellular signal-regulated protein kinase 1/2; Fura-2 AM, fura-2 acetoxymethyl ester; MAPKs, mitogen-activated protein kinases; MRS2179, N⁶-methyl-2'-deoxyadenosine-3',5'-bisphosphate; PBS, phosphate-buffered saline; PKC, protein kinase C; PPADS, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid; TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate.

8. REFERENCES

1. Franke H, Illes P. Involvement of P2 receptors in the growth and survival of neurons in the CNS. *Pharmacol Ther* 2006; 109: 297-324.
2. Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* 2007; 87: 659-797.
3. Burnstock G, Krügel U, Abbracchio MP, Illes P. Purinergic signalling: From normal behaviour to pathological brain function. *Prog Neurobiol* 2011; 95: 229-74.
4. Burnstock G. Purine and pyrimidine receptors. *Cell Mol Life Sci* 2007; 64: 1471-83.
5. Erb L, Liao Z, Seye CI, Weisman GA. P2 receptors: intracellular signaling. *Pflugers Arch* 2006; 452: 552-62.
6. Gomez-Villafuertes R, del Puerto A, Diaz-Hernandez

- M, *et al.* Ca²⁺/calmodulin-dependent kinase II signalling cascade mediates P2X₇ receptor-dependent inhibition of neuritogenesis in neuroblastoma cells. *FEBS J* 2009; 276: 5307-25.
7. Ortega F, Pérez-Sen R, Morente V, Delicado EG, Miras-Portugal MT. P2X₇, NMDA and BDNF receptors converge on GSK3 phosphorylation and cooperate to promote survival in cerebellar granule neurons. *Cell Mol Life Sci* 2010; 67: 1723-33.
 8. Morente V, Pérez-Sen R, Ortega F, Huerta-Cepas J, Delicado EG, Miras-Portugal MT. Neuroprotection elicited by P2Y₁₃ receptors against genotoxic stress by inducing DUSP2 expression and MAPK signaling recovery. *Biochim Biophys Acta* 2014; 1843: 1886-98.
 9. Ortega F, Pérez-Sen R, Miras-Portugal MT. Gi-coupled P2Y-ADP receptor mediates GSK-3 phosphorylation and β -catenin nuclear translocation in granule neurons. *J Neurochem* 2008; 104: 62-73.
 10. Lustig KD, Shiau AK, Brake AJ, Julius D. Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc Natl Acad Sci USA* 1993; 90: 5113-17.
 11. Kaiho H, Matsuoka I, Kimura J, Nakanishi H. Identification of P2X₇ (P2Z) receptor in N18TG-2 cells and NG108-15 cells. *J Neurochem* 1998; 70: 951-57.
 12. Sak K, Samuel K, Kelve M, Webb TE. Pharmacological characterisation of pyrimidinoceptor responses in NG108-15 cells. *Eur J Pharmacol* 2001; 415: 127-33.
 13. Larsson KP, Hansen AJ, Dissing S. The human SH-SY5Y neuroblastoma cell-line expresses a functional P2X₇ purinoceptor that modulates voltage-dependent Ca²⁺ channel function. *J Neurochem* 2002; 83: 285-98.
 14. Raffaghello L, Chiozzi P, Falzoni S, Di Virgilio F, Pistoia V. The P2X₇ receptor sustains the growth of human neuroblastoma cells through a substance P-dependent mechanism. *Cancer Res* 2006; 66: 907-14.
 15. Wu PY, Lin YC, Chang CL, *et al.* Functional decreases in P2X₇ receptors are associated with retinoic acid-induced neuronal differentiation of Neuro-2a neuroblastoma cells. *Cell Signal* 2009; 21: 881-91.
 16. Cavaliere F, Nestola V, Amadio S, *et al.* The metabotropic P2Y₄ receptor participates in the commitment to differentiation and cell death of human neuroblastoma SH-SY5Y cells. *Neurobiol Dis* 2005; 18: 100-9.
 17. Sak K, Webb TE, Samuel K, Kelve M, Jarv J. Only pyrimidinoceptors are functionally expressed in mouse neuroblastoma cell lines. *Mol Cell Biol Res Commun* 1999; 1: 203-8.
 18. Lee H, Choi BH, Suh BC, Lee SK, Kim KT. Attenuation of signal flow from P2Y₆ receptor by protein kinase C- α in SK-N-BE(2)C human neuroblastoma cells. *J Neurochem* 2003; 85: 1043-53.
 19. Esbenshade TA, Han C, Theroux TL, Granneman JG, Minneman KP. Coexisting beta 1- and atypical beta-adrenergic receptors cause redundant increases in cyclic AMP in human neuroblastoma cells. *Mol Pharmacol* 1992; 42: 753-9.
 20. Schaak S, Cayla C, Blaise R, Quinchon F, Paris H. HepG2 and SK-N-MC: two human models to study alpha-2 adrenergic receptors of the alpha-2C subtype. *J Pharmacol Exp Ther* 1997; 281: 983-91.
 21. Sidhu A., Fishman PH. Identification and characterization of functional D1 dopamine receptors in a human neuroblastoma cell line. *Biochem Biophys Res Commun* 1990; 166: 574-9.
 22. Gordon EA, Kohout TA, Fishman PH. Characterization of functional neuropeptide Y receptors in a human neuroblastoma cell line. *J Neurochem* 1990; 55: 506-13.
 23. Fisher SK, Landon RE. Identification of multiple phosphoinositide-linked receptors on human SK-N-MC neuroepithelioma cells. *J Neurochem* 1991; 57: 1599-608.
 24. Sidhu A, Olde B, Humblot N, Kimura K, Gardner N. Regulation of human D1 dopamine receptor function and gene expression in SK-N-MC neuroblastoma cells. *Neuroscience* 1999; 91: 537-47.
 25. Chan AS, Ng LW, Poon LS, Chan WW, Wong YH. Dopaminergic and adrenergic toxicities on SK-N-MC human neuroblastoma cells are mediated through G protein signaling and oxidative stress. *Apoptosis* 2007; 12: 167-79.
 26. Chen J, Rusnak M, Luedtke RR, Sidhu A. D1 dopamine receptor mediates dopamine-induced cytotoxicity via the ERK signal cascade. *J Biol Chem* 2004; 279: 39317-30.
 27. Sheriff S, Qureshy AF, Chance WT, Kasckow JW, Balasubramaniam A. Predominant role by CaM kinase in NPY Y₁ receptor signaling: involvement of CREB. *Peptides* 2002; 23: 87-96.
 28. Bahouth SW, Sowinski KM, Lima JJ. Regulation of human β 1-adrenergic receptors and their mRNA in neuroepithelioma SK-N-MC cells: effects of agonist, forskolin, and protein kinase A. *Biochem Pharmacol* 2001; 62: 1211-20.
 29. Chan AS, Yeung WW, Wong YH. Integration of G protein signals by extracellular signal-regulated protein kinases in SK-N-MC neuroepithelioma cells. *J Neurochem* 2005; 94: 1457-70.
 30. Recuero M, Serrano E, Bullido MJ, Valdivieso F. A β production as consequence of cellular death of a human neuroblastoma overexpressing APP. *FEBS Lett* 2004; 570: 114-8.
 31. Sebastia J, Pertusa M, Vilchez D, *et al.* Carboxyl-terminal fragment of amyloid precursor protein and hydrogen peroxide induce neuronal cell death through different pathways. *J Neural Transm* 2006; 113: 1837-45.
 32. Sopher BL, Fukuchi K, Smith AC, Leppig KA, Furlong CE, Martin GM. Cytotoxicity mediated by

- conditional expression of a carboxyl-terminal derivative of the β -amyloid precursor protein. *Brain Res Mol Brain Res* 1994; 26: 207-17.
33. Hyun DH, Lee M, Hattori N, *et al.* Effect of wild-type or mutant Parkin on oxidative damage, nitric oxide, antioxidant defenses, and the proteasome. *J Biol Chem* 2002; 277: 28572-77.
 34. Khakh BS, Proctor WR, Dunwiddie TV, Labarca C, Lester HA. Allosteric control of gating and kinetics at P2X₄ receptor channels. *J Neurosci* 1999; 19: 7289-99.
 35. Priel A, Silberberg SD. Mechanism of ivermectin facilitation of human P2X₄ receptor channels. *J Gen Physiol* 2004; 123: 281-93.
 36. von Kugelgen I. Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. *Pharmacol Ther* 2006; 110: 415-32.
 37. Abbracchio MP, Burnstock G, Boeynaems JM, *et al.* International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* 2006; 58: 281-341.
 38. Marteau F, Le Poul E, Communi D, *et al.* Pharmacological characterization of the human P2Y₁₃ receptor. *Mol Pharmacol* 2003; 64: 104-12.
 39. Communi D, Gonzalez NS, Detheux M, *et al.* Identification of a novel human ADP receptor coupled to G_i. *J Biol Chem* 2001; 276: 41479-85.
 40. Virginio C, Robertson G, Surprenant A, North RA. Trinitrophenyl-substituted nucleotides are potent antagonists selective for P2X₁, P2X₃, and heteromeric P2X_{2/3} receptors. *Mol Pharmacol* 1998; 53: 969-73.
 41. White PJ, Webb TE, Boarder MR. Characterization of a Ca²⁺ response to both UTP and ATP at human P2Y₁₁ receptors: evidence for agonist-specific signaling. *Mol Pharmacol* 2003; 63: 1356-63.
 42. Gever JR, Cockayne DA, Dillon MP, Burnstock G, Ford AP. Pharmacology of P2X channels. *Pflugers Arch* 2006; 452: 513-37.
 43. Jones CA, Vial C, Sellers LA, Humphrey PP, Evans RJ, Chessell IP. Functional regulation of P2X₆ receptors by N-linked glycosylation: identification of a novel α,β -methylene ATP-sensitive phenotype. *Mol Pharmacol* 2004; 65: 979-85.
 44. Evans RJ, Lewis C, Buell G, Valera S, North RA, Surprenant A. Pharmacological characterization of heterologously expressed ATP-gated cation channels (P2x purinoceptors). *Mol Pharmacol* 1995; 48: 178-83.
 45. Bo X, Jiang LH, Wilson HL, *et al.* Pharmacological and biophysical properties of the human P2X₅ receptor. *Mol Pharmacol* 2003; 63: 1407-16.
 46. Rassendren F, Buell GN, Virginio C, Collo G, North RA, Surprenant A. The permeabilizing ATP receptor, P2X₇. Cloning and expression of a human cDNA. *J Biol Chem* 1997; 272: 5482-86.
 47. Garcia-Guzman M, Soto F, Gomez-Hernandez JM, Lund PE, Stuhmer W. Characterization of recombinant human P2X₄ receptor reveals pharmacological differences to the rat homologue. *Mol Pharmacol* 1997; 51: 109-18.
 48. Guo C, Masin M, Qureshi OS, Murrell-Lagnado RD. Evidence for functional P2X₄/P2X₇ heteromeric receptors. *Mol Pharmacol* 2007; 72: 1447-56.
 49. Le KT, Babinski K, Seguela P. Central P2X₄ and P2X₆ channel subunits coassemble into a novel heteromeric ATP receptor. *J Neurosci* 1998; 18: 7152-59.
 50. Nicke A, Kerschensteiner D, Soto F. Biochemical and functional evidence for heteromeric assembly of P2X₁ and P2X₄ subunits. *J Neurochem* 2005; 92: 925-33.
 51. Soto F, Garcia-Guzman M, Gomez-Hernandez JM, Hollmann M, Karschin C, Stuhmer W. P2X₄: an ATP-activated ionotropic receptor cloned from rat brain. *Proc Natl Acad Sci USA* 1996; 93: 3684-8.
 52. Bo X, Zhang Y, Nassar M, Burnstock G, Schoepfer R. A P2X purinoceptor cDNA conferring a novel pharmacological profile. *FEBS Lett* 1995; 375: 129-33.
 53. Inoue K, Tsuda M, Koizumi S. ATP- and adenosine-mediated signaling in the central nervous system: chronic pain and microglia: involvement of the ATP receptor P2X₄. *J Pharmacol Sci* 2004; 94: 112-4.
 54. Tsuda M, Shigemoto-Mogami Y, Koizumi S, *et al.* P2X₄ receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* 2003; 424: 778-83.
 55. Guo LH, Trautmann K, Schluesener HJ. Expression of P2X₄ receptor by lesional activated microglia during formalin-induced inflammatory pain. *J Neuroimmunol* 2005; 163: 120-7.
 56. Cavaliere F, Dinkel K, Reymann K. Microglia response and P2 receptor participation in oxygen/glucose deprivation-induced cortical damage. *Neuroscience* 2005; 136: 615-23.
 57. Cavaliere F, Florenzano F, Amadio S, *et al.* Up-regulation of P2X₂, P2X₄ receptor and ischemic cell death: prevention by P2 antagonists. *Neuroscience* 2003; 120: 85-98.
 58. Sim JA, Chaumont S, Jo J, *et al.* Altered hippocampal synaptic potentiation in P2X₄ knock-out mice. *J Neurosci* 2006; 26: 9006-9.
 59. Moore D, Chambers J, Waldvogel H, Faull R, Emson P. Regional and cellular distribution of the P2Y₁ purinergic receptor in the human brain: striking neuronal localisation. *J Comp Neurol* 2000; 421: 374-84.
 60. Moore D, Iritani S, Chambers J, Emson P. Immunohistochemical localization of the P2Y₁ purinergic receptor in Alzheimer's disease. *Neuroreport* 2000; 11: 3799-803.
 61. Ecke D, Hanck T, Tulapurkar ME, *et al.* Hetero-oligomerization of the P2Y₁₁ receptor with the P2Y₁

Functional characterization of P2Y₁ and P2X₄ receptors in human neuroblastoma SK-N-MC cells

receptor controls the internalization and ligand selectivity of the P2Y₁₁ receptor. *Biochem J* 2008; 409: 107-16.

62. Yoshioka K, Nakata H. ATP- and adenosine-mediated signaling in the central nervous system: purinergic receptor complex: generating adenine nucleotide-sensitive adenosine receptors. *J Pharmacol Sci* 2004; 94: 88-94.