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Revisión

Physiological role of extracellular nucleotides at the central nervous system: signalling through P2X and P2Y receptors

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ABSTRACT

In the last few years nucleotide receptors, the ionotropic $P2X_{1\text{-}7}$ subunits and the metabotropic $P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$, have acquired an excepcional importance due to their strategic location in organs and tissues, their great variety along with the complexity of the associated signalling pathways and the first evidence of the serious alterations entailed in their dysfunctions. Our group has been pioneer in the characterization of these receptors in the nervous system, where we defined their location and functionality. The abundant presence, at a presynaptic level, of $P2X_3$ and $P2X_7$ should be emphasized, where by means of calcium intake they induce neurotransmitter exocytosis, such as glutamate, GABA, catecholamines and acetylcholine among others, as described in previous works by our group. In addi-

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tion, they induce an extensive remodeling of the terminal's cytoskeleton and exocytotic mechanisms through CaMKII and they can interact widely with other ionotropic and metabotropic receptors co-existing in nearby areas. Neural cells also exhibit the presence of most P2Y receptors signalling through a large variety of intracellular cascades. Recently we have demostrated that P2Y metabotropic receptors of the sub-family activated by ADP, especially $P2Y_{13}$, are connected with the signalling towards GSK3 and β-catenin, opening new ways of understading the nucleotide function in survival and maintenance of neural cells. In addition both P2X and P2Y receptors play a role in early developmental stages and neural maturation where their function has to be fully understanded. Nucleotide receptors are also very abundant in glial cells, and our group has shown that most P2Y receptors are present and fully functional in cultured astrocytes, where, depending on the subtype receptor they activate a large variety of signalling cascades.

Key words: ionotropic P2X receptors, metabotropic P2Y receptors, central nervous system, ATP, synaptic terminals, neurotransmitters release.

RESUMEN

Papel fisiológico de los nucleótidos extracelulares en el sistema nervioso central: señalización vía receptores P2X y P2Y.

En los últimos años los receptores de nucleótidos, receptores ionotrópicos $P2X_{1\text{-}7}$ y metabotrópicos $P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$, han adquirido una importancia excepcional debido a su localización estratégica en órganos y tejidos, a su gran variedad junto con la complejidad de vías de señalización a las que están asociados y a las primeras evidencias de importantes alteraciones debidas a su mal funcionamiento. Nuestro grupo ha sido pionero en la caracterización estos receptores en el sistema nervioso, donde definimos su localización y su funcionalidad. La abundante presencia, a nivel presináptico, de las subunidades $P2X_3$ y $P2X_7$ debe ser resaltada, donde gracias a la entrada de calcio inducen la exocitosis de varios neurotransmisores, como glutamato, GABA, catecolaminas y acetilcolina entre otros, como ha sido descrito por nuestro grupo en trabajos previos. Además, estos receptores inducen una profunda remodelación del citoesqueleto de las terminales nerviosas y de los mecanismos exocitóticos a través de la CaMKII y pueden interactuar con otros receptores ionotrópicos y metabotrópicos co-existentes en sus cercanías. La mayoría de los receptores P2Y también están presentes en las células nerviosas, activando vías de señalización a través de una gran variedad de cascadas intracelulares. Recientemente hemos demostrado que los receptores metabotrópicos P2Y pertenecientes a la sub-familia de receptores activados por ADP, especialmente el $P2Y_{13}$, están conectados con la señalización hacia GSK3 y β-catenina, lo que abre nuevas vías para la comprensión de la función de los nucleótidos en la supervivencia y el mantenimiento de las células nerviosas. Además, tanto los receptores P2X como los P2Y juegan un papel en los estadíos iniciales del desarrollo y en la maduración neuronal donde su función aún ha de ser plenamente comprendida. Los receptores de nucleótidos son también muy abundantes en las células gliales,

y nuestro grupo ha demostrado que la mayoría de los receptores P2Y están presentes y son plenamente funcionales en astrocitos en cultivo, donde, dependiendo del subtipo de receptor, activan una gran variedad de cascadas de señalización.

Palabras clave: receptores ionotrópicos P2X, receptores metabotrópicos P2Y, sistema nervioso central, ATP, terminales sinápticas, liberación de neurotransmisores.

INTRODUCTION

The wide distribution and the discovery of new and multiple actions of nucleotides in organs and tissues, through membrane receptors named P2 purinergic receptors, have aroused a lot of interest in investigating their cellular biology, the type of dysfunctions associated with the excess or defect of nucleotide signalling, and the search and design for reliable agonists and antagonists that can be used in pharmacology. In the last two years we have assisted to the growing progress in the number of publications related to nucleotide signalling and its physiopathological implications, which gives evidence of its therapeutic potential in a near future. Between the increasing numbers of revisions, we have selected those written by Professor G. Burnstock, by their historical meaning and seminal contributions (1-3). The prestigious journal Trends in Pharmacological Sciences, commemorated the 75th anniversary of the British Pharmacological Society foundation with the publication of a special volume, in which the revision of Burnstock entitled «ATP as a neurotransmitter» (4), is included as one of the 6 most relevant and historical revisions. 36 years ago, Pharmacological Reviews published an article entitled «Purinergic Nerves» (5) that first laid the foundations of purinergic neurotransmission, but that finding was scarcely attractive to the scientific community at that time.

The extracellular role of ATP, or any other nucleotide, requires the presence of this compound outside the cell. In this way, the presence of ATP and other nucleotides and dinucleotides in a large variety of storage and secretory vesicles, such as the cholinergic and aminergic ones, from neural or neuro-endocrine tissues, or the ones from platelets and mastocytes, suggested an extracellular role, as co-transmitter, or extracellular functions for nucleotides (6). The

vesicular storage and exocytotic release of nucleotides with other neurotransmitters is now well established, and the kinetic properties of the granular and vesicular nucleotide transporter, exhibiting a characteristic mnemonic regulation, have been studied by our group (7, 8). However, the extracellular signalling mediated by ATP and nucleotides is so broadly extended, that other mechanisms for nucleotide release should exist. For a non-quantal, or non Ca^{2+} dependent exocytotic release, numerous membrane proteins have been postulated such as some members of the ABC transporter family, members of the ecto-ATPase family known as CD-39, or connexins hemichannels, and constitutes a fertile area of debate and research (9).

The physiological effects of extracellular nucleotides conclude by the action of ectonucleotidases, rendering phosphate and the nucleosides. The broad variety and abundance of these enzymes in all studied cells have made difficult the study of nucleotide receptors responses (10).

NUCLEOTIDE RECEPTOR FAMILIES

The nucleotide receptors are grouped in two big families, the ionotropic, known as P2X, and metabotropic, called P2Y.

Metabotropic P2Y family

The metabotropic P2Y family contains 8 members, $P2Y_{1,2,4,6,11,12,13,14}$ receptors. Due to their variety they are trying to divide them in three families, essentially depending on the G protein with which they couple preferentially, and the nature of the nucleotide agonist. This way we have the P2YA group coupled to Gq and Gs and which prefer adenine nucleotides ($P2Y_1$ and $P2Y_{11}$). The next group is P2YB, coupled to Gq and Gi/o, which can recognize both uridine and adenosine nucleotides ($P2Y_2$, $P2Y_4$, $P2Y_6$). The P2YC group, couple essentially to Gi and recognize with great efficiency ADP ($P2Y_{12}$, $P2Y_{13}$), while $P2Y_{14}$ is activated by UDP-glucose. Attempts to assign other members to the P2Y family have been rejected (11). The effects

of P2Y receptors in different signalling pathways are interconnected and it requires to be analyzed from an integrated point of view (12). It should be emphasized the great contributions by the group of K. Harden, E. Barnard, J.M. Boeynaems, J.T. Neary, M.P. Abbracchio and more recently J.P. Gachet among others (13-16). Recent evidences of homo- and heterodimerization of nucleotide receptors have been reported. This fact provides new possibilities of signalling and new pharmacological approaches (17). The family of metabotropic P2Y receptors exhibits a very abundant presence in all neural and glial cells, where more than one subtype co-exists. The physiological nucleotide agonists of P2Y receptors are very diverse, some of them such as $P2Y_{1,12,13}$ are specific for ADP, $P2Y_{11}$ is the only one specific for ATP, others as $P2Y_2$, $P2Y_4$, and $P2Y_6$ are activated mainly by the pyrimidine nucleotides UTP and UDP. These metabotropic receptors are coupled to G proteins and activate a large variety of downstream cascades, such as PI3K, AKT, MAP kinases, and recently it has been reported the glycogen synthase kinase-3, GSK3, regulation. Glial cells, exhibit a broad diversity of functional P2Y receptors, coupled to Gq or Gi proteins and signalling to intracellular cascades related to survival and proliferation. Figure 1 shows the main signalling cascades described for the P2Y subtypes and Table 1 summarises the agonists, antagonists and G proteins coupling of the P2Y receptors subtypes.

As described for other seven helix G protein coupled receptors, the possibility of P2Y metabotropic receptors forming homodimers or heterodimers increases the signalling and modulation posibilities and should be considered in the future (18).

Ionotropic P2X family

P2X ionotropic receptor family contains a total of seven subunits, $P2X_{1,2,3,4,5,6,7}$. They present simple structures, with only two transmembrane helixes, internal amine and carboxy terminals and a large extracellular domain where the nucleotide binding site is located. It is widely accepted, although not completely demonstrated, that a minimum of three subunits are required to obtain a functional ionotropic receptor. As in most of the studied tissues more than one

subunit is expressed, it is probably that the majority of P2X receptors are heteromeric in nature (18). Unlike P2Y, these P2X receptors only recognize adenine nucleotides, in concentrations able to be reached in physiological conditions.

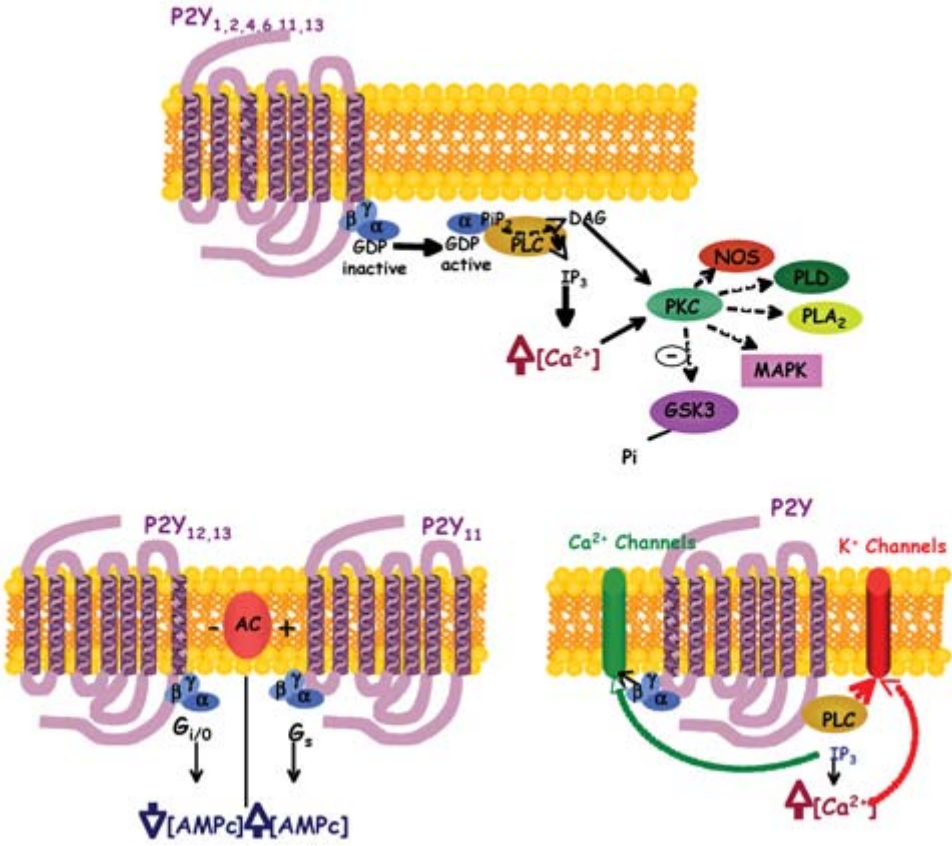

FIGURE 1. ***Signal transduction mechanisms of P2Y receptors.*** *In general, P2Y receptors are coupled, through G protein, to PLC, and induce intracellular calcium mobilization, and finally, PKC activation. In addition, P2Y activation can induce other signalling mechanisms such as PLD, PLA2, MAPK and NOS activation. Moreover, P2Y receptors can be coupled to AC through Gi proteins and to potassium and calcium channels.*

Table 1. *Characteristics of P2Y purinoceptors*

Receptor	Agonist	Antagonist	Signal transduction mechanisms	Major distribution
$P2Y_1$	2MeSADP>ADPβS >MeSATP=ADP>ATP UTP, UDP inactives AP_3A>Ap_2A	MRS 2279>MRS 2179> Suramin>PPADS	$G_q/_{11}$ PLCβ/IP_3/Ca^{2+}	Platelets, brain, placenta, prostate, endothelial cells, heart, skeletal muscle, glial cells, digestive tract, osteoclasts
$P2Y_2$	ATP≈UTP>Ap_4A ADP, UDP y 2MeSATP inactives	Suramin> RB 2, ARC126313	G_q/G_{11};G_i? PLCβ/IP_3/Ca^{2+}	Skeletal muscle, respiratory epithelium, bone, lung, pituitary gland, spleen, lymphocytes, osteoblasts, kidney
$P2Y_4$	UTP>UTPγS >>ATP, UDP (partial agonists) UTP=ATP=Ap_4A (rat)	PPADS RB 2 (rat)>Suramin	G_q/G_{11};G_i? PLCβ/IP_3/Ca^{2+}	Gut, brain, pituitary gland, placenta, heart, lung, vascular smooth muscle, endothelial cells
$P2Y_6$	UDPβS>UDP>UTP>ADP >2MeSATP	RB 2>PPADS>Suramin, MRS2578	G_q/G_{11} PLCβ/IP_3/Ca^{2+}	Placenta, spleen, kidney, heart, aorta, gut, brain, timus, lung.
$P2Y_{11}$	ATPγS≈BzATP>ATP >2MeSATP	Suramin>RB 2, NF157, 5´-AMPs	G_q/G_{11} y G_s PLCβ/IP_3/Ca^{2+} AC Activation	Brain, placenta, pituitary gland, gut, immune system, granulocytes

Table 1. ***Characteristics of P2Y purinoceptors*** *(cont.)*

Receptor	Agonist	Antagonist	Signal transduction mechanisms	Major distribution
$P2Y_{12}$	2MeSADP=2MeSATP >>ADP>>ATP UTP, UDP inactives	AR-C69931,AR-C66096, Clopidogrel, Tioclopidine, RB 2, Suramin, 2MeSAMP, CT50547, AZD6140, INS49266, PSB0413, PPADS inactive, BzATP (rat)	Gi AC Inhibition	Human platelets, brain
$P2Y_{13}$	2MeSADP≥2MeSATP>ADP >>ATP UTP, UDP inactives	AR-C69931 >PPADS>Suramin 2MeSAMP MRS2211	G_q/G_{11}/Gi $PLC\beta/IP_3/Ca^{2+}$ AC Inhibition	Spleen, brain, glial cells, spinal cord, linfatic ganglia endothelial cells
$P2Y_{14}$	UDP-glucose>UDP-galactose>UDP-Glucuronic >UDP-N-acetilglucosamine ATP, ADP, UTP, UDP inactives	¿?	G_q/G_{11}/Gi	Placenta, stomach, gut, brain, spleen, heart, lung

The properties and functional characterization of homomeric and heteromeric receptors built with the P2X subunits was carried out by a broad number of scientific groups, and will still require extensive experimental work (19-25). Recently the $P2X_7$ receptor has deserved a special attention, not only due to the existence of polymorphisms and the pronostic of chronic myeloid leukaemia (CML) in humans and immune system functions, but also due to recent data of a possible role in other aspects of neuronal functioning (26-28). For this reason, $P2X_7$ has become a preferential target for pharmaceutical enterprises (29).

From an electrophysiological point of view, all P2X receptors are cation selective ligand gated ion channels, with permeability similar for Na^+ and K^+, but very high for Ca^{2+}, depending on the implicated subunits. It is probable that an excess intake of calcium through these receptors could contribute in vivo to pathologies associated with P2X receptors (25, 30-36). P2X subunits are formed by two transmembrane domains with intracellular N and C termini and a large extracellular ligand binding loop connecting them (Figure 2). Functional receptors require the assembling of at least three subunits to form homomeric or heteromeric receptors. It was generally accepted that $P2X_7$ exclusively formed homomeric receptors, although recent data points to a coasembling almost with PX_4 (37). The main physiological agonist of functional P2X receptors is ATP, and for the moment there is a limited battery of nucleotide synthetic agonists to pharmacologically identify each one of the homomeric or heteromeric P2X combinations. The same situation occurs with the antagonist's disponibility, as it can be seen in Table 2. In spite of this situation, combination of diverse nucleotide agonists and antagonists, together with the kinetic behaviour exhibiting or not desensitization allows the identification of different P2X functional receptors. Molecular biology and immunohistochemical techniques are relevant tools to confirm the tissular and cellular location of the P2X receptor subunits.

TABLE 2. *Characteristics of P2X homomeric purinoceptors*

Receptor	Agonists	Antagonists	Ion modulation	Desensitization	Major distribution
$P2X_1$	ATP=2MeSATP≥Ap_6A ≥α, β-meATP >BzATP>ADP	NF449>TNP-ATP>Ip_5I >MRS2257>PPADS (nM) Suramin>RB 2 (μM)	$H^+\downarrow$ $Zn^{2+}\downarrow$	Fast	Brain, spinal cord, smooth muscle, platelets, sympathetic ganglia
$P2X_2$	ATP≥ATPγS≥MeATP >Ap_4A α, β-meATP y ADP inactives	RB 2=NF279, NF770 >PPADS=TNP-ATP =BBG≥Suramin (μM)	$H^+\uparrow$ $Zn^{2+}\uparrow$ $Cu^{2+}\uparrow$ $Ca^{2+}\uparrow$	Slow	Brain, spinal cord, smooth muscle, sympathetic ganglia, chromafin cells, retina
$P2X_3$	2MeSATP≥ATP= α,β-meATP>Ap_5A >ADP	TNP-ATP>MRS2257= A317491>MRS2159= PPADS (nM)NF279= NF449>Ip_5I≥Suramin> NF023>RB 2 (μM) A317491, NF110, phenol red	$Zn^{2+}\uparrow$ $H^+\downarrow$ $Ca^{2+}\uparrow$	Fast	Brain, spinal cord, smooth muscle, sympathetic ganglia, sensory neurons
$P2X_4$	ATP>2MeSATP α, β-meATP y ADP inactives Potentiation by Ivermectin, Propofol and Cibacron blue	TNP-ATP>BBG (μM) phenolphthalein	$H^+\downarrow$ $Zn^{2+}\uparrow$ $Cd^{2+}\uparrow$	Slow	Brain, spinal cord, smooth muscle, sympathetic ganglia, testis, colon

Table 2. ***Characteristics of P2X homomeric purinoceptors****(cont.)*

Receptor	Agonists	Antagonists	Desensitization	Major distribution
$P2X_5$	ATP=2MeSATP=Ap_4A> α, β-meATP>BzATP ADP inactive	PPADS>TNP-ATP> Suramin>RB 2 (μM)	Slow	Trigeminus ganglia, spinal cord, proliferative skin cells, thymus, bladder
$P2X_6$	2MeSATP≥ATP= α,β-meATP>Ap_5A >ADP	TNP-ATP>PPADS Suramin insensitive	Slow	Brain, spinal cord, sympathetic ganglia
$P2X_7$	BzATP>2MeSATP >>ATP α,β-meATP and ADP inactive	A-74003=A-438079 (rat, human) BBG (nM) (rat) PPADS=TNP-ATP (nM) KN-62 (nM) (human, mouse) Suramin (mM) (rat, human)	Fast	Retinal ganglia, immune cells, brain, spinal cord, skin, pancreas.

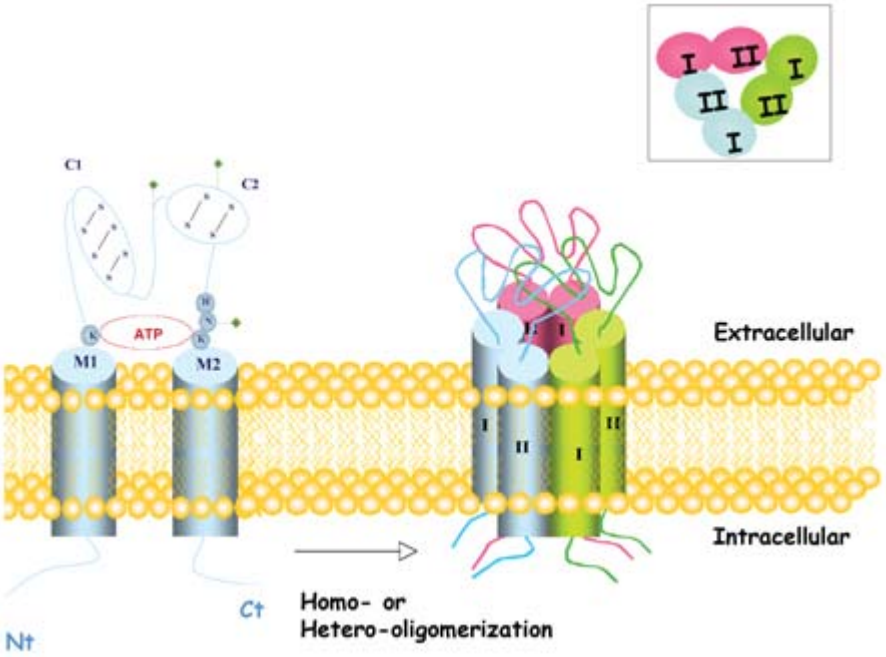

FIGURE 2. ***Transmembrane topology and oligomeric structure of P2X receptors.*** *The intracellular N- and C-termini, two transmebrane domains (M1 and M2) and the extracellular ligand-binding loop are shown. Conservatives cysteines residues, organized in two domains (C1 and C2), and the glycosilation sites are indicated. Moreover, in the extracellular loop is shown the conserved positively charged residues (K68 and K309) involved in the ATP binding. In addition, the model of oligomerization proposed for P2X receptors is shown. Transmembrane helixes II are participating mainly in the channel formation.*

PRESENCE AND FUNCTION OF P2X RECEPTORS IN SYNAPTIC TERMINALS

Synaptic terminal studies allowed us to identify the presynaptic ionotropic receptors that responded specifically to ATP and some more specific agonists and antagonists, and even link them with the presence of P2X subunits through immunohistochemistry. The results obtained point to a coexistence in a single synaptic terminal of different types of P2X and dinucleotide receptors (38-42).

Considering the great variety of synaptic terminals it is important to characterize in what types P2X receptors are preferentially found.

Currently we can characterize the responses in specific terminals after functional studies, using immunohistochemical techniques. The presynaptic ionotropic nucleotide receptors are functional and very abundant in cholinergic terminals, identified with double dye with the vesicle acetylcholine transporter (VAT) (43), also in GABAergic terminals characterized by the presence of the inhibitor aminoacids transporter (VIAAT) or of the neural isoform of glutamate descarboxylase (GAD65) (44, 45). Similar abundance of ionotropic nucleotide receptors is observed in aminergic terminals in striatum, characterized by the presence of monoamine vesicular transporter (VMAT-2). We should emphasize its equally abundant presence in glutamatergic terminals, detected by immunohistochemistry of the vesicular glutamate transporter, both the types 1 and 2 (46, 47).

It could be thought that in an analogous way we could characterize the terminals that store ATP and dinucleotides in their vesicles but for the moment the nucleotide vesicular transporter has not been cloned and only kinetically characterized (8, 48).

Immunocytochemical studies have proven that P2X subunit distribution in synaptic terminals is notoriously different than those present in neural soma, but for the moment it is not known how a preferential location and distribution is accomplished, and expression tests have not been yet made in polarized cells that can serve as a model. In previous work from our group it has been reported that $P2X_3$ subunit is very abundant in CNS terminals and that all of the synaptic terminals responding to α–β-methylen-ATP and ATP where afterwards immunolabeled with anti-$P2X_3$ antibodies (49). Besides, there are terminals that respond to nucleotides and do not present staining for this subunit. Recently we have reported that $P2X_7$ subunit is also present in great abundance in CNS terminals, other authors have also found this location in peripheral nerves (42, 50-53). More than 50% of isolated synaptic terminals from diverse areas of central nervous system exhibit $P2X_7$ receptors both by immunostaining and microfluorimetric techniques. Thus, all the signalling mediated by this receptor acquires a special relevance in the synaptic degenerative processes (54) and should deserve special attencion in the future. Figure 3 shows the preferential distribution of some P2X at the synaptic terminals.

P2X receptors from the presynaptic area are able to induce calcium dependent exocytotic release upon stimulation. The nucleotide effect is dose dependent and the induced release of classical neurotransmitters such as: acetylcholine, glutamate, GABA

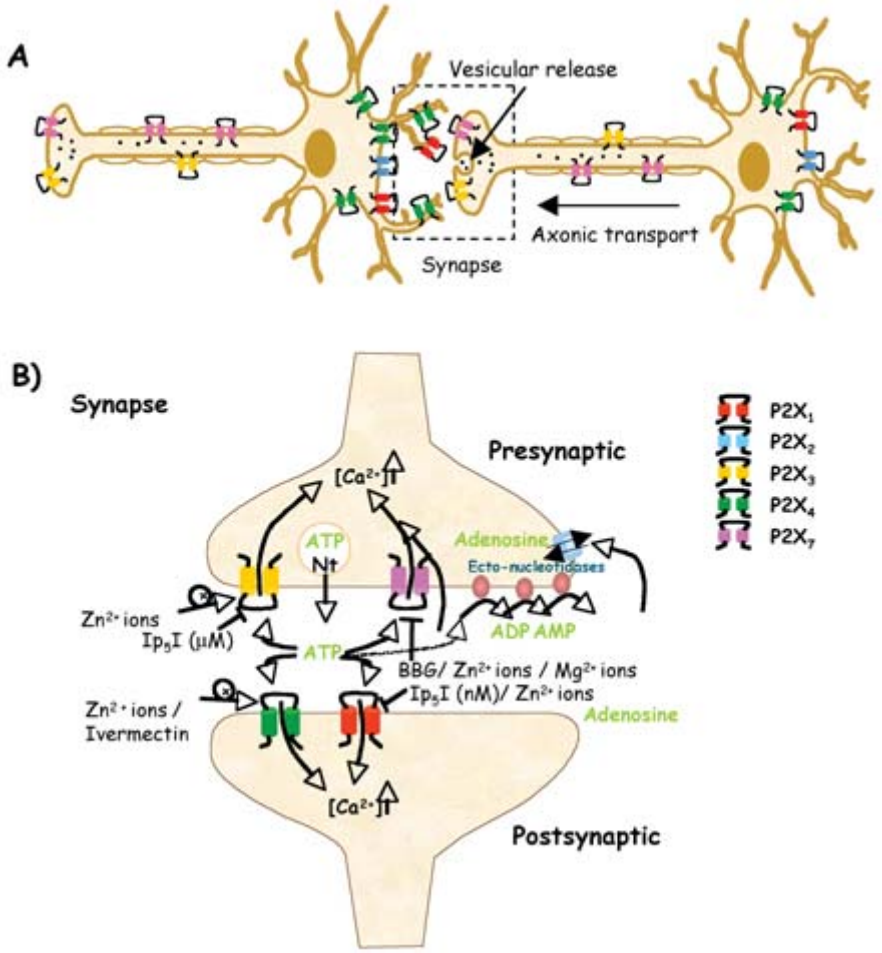

FIGURE 3. ***Experimental based distribution of P2X receptors in CNS.*** *P2X receptors exhibit a specific distribution along the axodendritic fibres (A). $P2X_1$ (red) and $P2X_4$ (green) are preferently located at the dendrites and somas. $P2X_3$ (yellow) and $P2X_7$ (pink) are mainly at the axons. $P2X_2$ subunit expression (blue) is restricted to the somas. The activation of the different P2X receptors produces an increase in intracellular calcium levels and activates different signalling mechanisms.*

or catecholamines has been reported (55-61). Figure 4 shows the exocytotic release from rat midbrain synaptic terminals, after stimulation. It can be observed that the quantity of neurotransmitter released by mg of protein from synaptosomal preparation mirrors the relative abundance of eah specific type of terminal, being glutamatergic the most abundant. The dose response curves reported in Figure 4 have been obtained with ATP in the presence of Mg^{2+} ion and correspond in a great extent to secretion mediated by $P2X_3$ presynaptic receptors. Later the secretory responses mediated by $P2X_3$ compared to $P2X_7$ will be reported in cultured granule neurons.

Presynaptic ionotropic receptors functionality is something that has recently appeared and very insistently, as the mean to regulate in a drastic way the secretory capacity of terminals and essentially all ionotropic receptors described in neural soma have been now characterized in the neural secreting area (55-63). This could be the case for NMDA, AMPA, $GABA_A$ and especially nicotinic and, nucleotidic neural receptors wich role on the control of synaptic terminals secretion, synaptic plasticity, recovery and stability, or long term depression or potentiation, has to be settled (57, 58, 60, 61, 64-

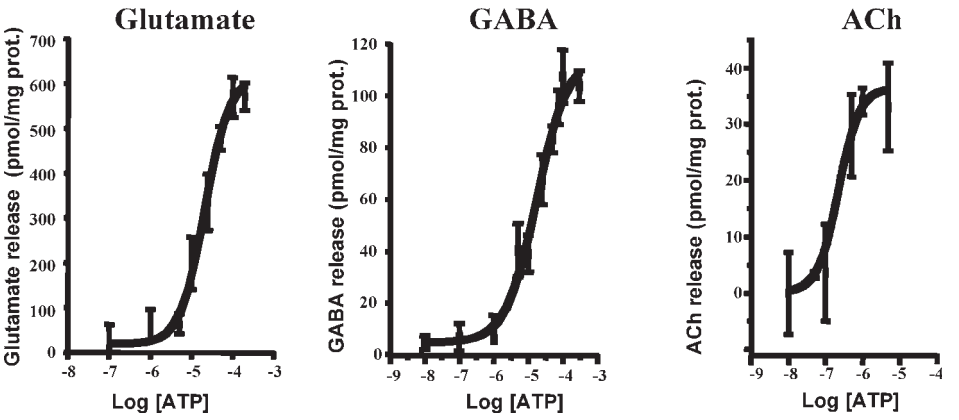

FIGURE 4. ***P2X receptor activation induces glutamate, GABA and acetylcholine release from presynaptic terminals.*** *Synaptic terminals isolated from rat midbrain were stimulated with increasing ATP concentrations. The amount of glutamate and GABA in the extrasynaptosomal media were measured by HPLC. Extrasynaptosomal acetylcholine was quantified by the luminometric protocol described by Israel and Lesbats (85).*

66). The presence at the presynaptic level of a large variety of ionotropic and metabotropic receptors suggests the possibility of multiple interactions and cross-talk, an attempt to understand some of them is described in next paragraph.

INTERACTION OF P2X AND OTHER IONOTROPIC AND METABOTROPIC RECEPTORS AT THE PRESYNAPTIC LEVEL

Ionotropic receptors interaction

Previous publications from our group reported the coexistence of ionotropic ATP and dinucleotide receptors with neuronal nicotinic receptors (41, 60), both being functional when are separatedly stimulated. This fact posses a series of questions: 1) whether a synergism between them exists, maybe leading to an exhaustion of the terminals, 2) whether they exclude each other and to what extent, and 3) whether their own subunits interacts with each other. This last possibility has been outlined in an interesting work by Khakh et al. (67), in which they show that the coexpression of ATP homomeric $P2X_2$ and acetylcholine receptors, nicotinic α3-β4 in oocytes, interfere mutually in the ion intake responses. If this happens in particular terminals it is something important, since α3, α4 and α7 nicotinic subunits have been involved, when they are located at a presynaptic level, in Alzheimer's disease, and constitute nowadays a preferential target in the new pharmacology to alleviate said disease. This is only an example of how complex are the interactions between different ionotropic receptors, in which nucleotide receptors widely participate, and that beyond this complexity it is necessary to undertake, if we pretend to understand how is actually functioning the presynaptic area.

The specific situation of nicotinic and nucleotidic ionotropic receptors colocalizing at the same terminal exhibits relevant functional characteristics, because the activation of nicotinic receptors inhibits in a high extent the ionotropic P2X, and dinucleotide receptors calcium responses. This reduction in the calcium entrance also results in a decrease in the acetylcholine

release induced by nucleotides. What is the mechanism responsible for the effect? This is a question with relevant physiological implications. In our experimental model of cholinergic synaptic terminals the inhibitory effect of nicotinic receptors on P2X receptors appears to be mediated by the activation of Calcium calmoduline kinase II, CaMKII, as this enzyme becomes activated by the intrasynaptosomal calcium increase upon receptor activation. The key role played by this enzyme in nerve terminals is confirmed by using the enzyme inhibitor KN62 wich completely prevents the inhibitory effect induced through nicotinic receptors agonists. This undoubtedly will allow us to take another step in the synaptic knowledge and avoid over-stimulation (60, 68). Figure 5 sumarises the modulation of P2X receptors by ionotropic (nicotinic) and metabotropic ($GABA_B$) receptors.

Ionotropic nucleotide receptors modulation by metabotropic receptors

Presynaptic ionotropic nucleotide receptors are also under the influence of a large variety of metabotropic receptors located in the presynaptic area. To this respect we have described dramatic effects caused by protein kinase and phosphatase action, when they are directly activated or inhibited, or through a more physiological way of presynaptic receptors stimulation. The receptor's affinity for their nucleotide agonists can be decreased in situations where kinase activity is increased, both by cAMP dependent kinases, PKA, or members of the PKC family (69). The oposite situation also occurs and an inhibition of protein kinases, or agonistic action on Gi coupled metabotropic receptors, results in a bigger affinity of P2X receptors, and also an increase in calcium maximal response. There are remarkable examples such as those reported for A1 adenosine receptors, and also $GABA_B$ receptors, both coupled to Gi. In the presence of these receptors agonists, the affinity values of nucleotides for P2X are increased in 3-5 orders of magnitude, going from mM to nM or pM (70). An especial mention should be given to $GABA_B$ receptors, since they are ubiquitous in CNS and especially at a presynaptic level, given the abundance of nucleotide signalling and presence of $P2X_3$ subunits in GABAergic terminals, the study of the

modulation of P2X receptors by GABA metabotropic receptors can give a clue to understand neuropathic pain, in which insufficient $GABA_B$ signalling has been involved (55, 57, 59). Although the A1 adenosine and $GABA_B$ receptors are among the most abundant located at a presynaptic level, the presence and effect of other Gi coupled receptors among the dopamine family (D2, D3, D4) and glutamatergic metabotropic family (group II and group III) should be explored in specific brain areas.

PRESENCE AND FUNCTION OF P2 RECEPTORS IN CULTURED NEURONS

In previous works by our group we proved that P2Y metabotropic receptors can coexist with P2X ionotropic receptors in neurons, like Purkinje, neurochromaffin cells from adrenal medulla and in cerebellar granule neurons (52, 71-74). In endothelial cells and

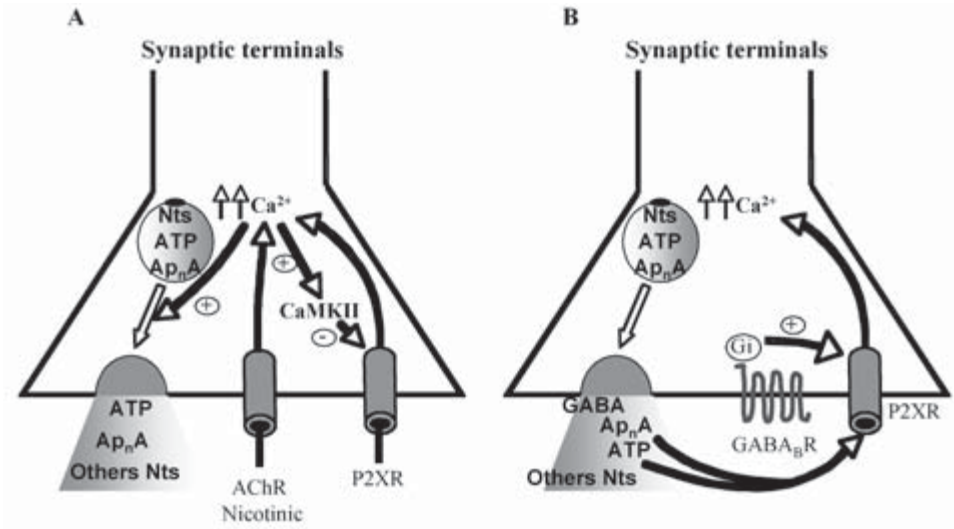

FIGURE 5. ***P2X receptor modulation by ionotropic and metabotropic receptors. A)*** *Co-existence of nicotinic and P2X receptors. Agonistic effect on acetylcholine nicotinic receptors induces intracellular calcium increase, activation of CaMKII and as a result inhibition of P2X responses in calcium entrance.* ***B)*** *Co-existence of metabotropic $GABA_B$ and P2X receptors. Agonistic effect on $GABA_B$ receptors coupled to Gi results in an affinity and calcium increase mediated by P2X receptors agonists.*

astrocytes multiple subtypes of P2Y receptors coexist. One of the first references about this coexistence is our group's work in vascular endothelial cells (75). One data of extreme complexity that can reach the nucleotide signalling system in neuronal cells is proved by the presence in cerebellar granular neurons in culture, of most of the P2Y and P2X receptors cloned so far, furthermore, with preferentially somatic or axonic/dendritic distribution depending on the receptor subtype (51, 52). The presence of P2 receptors and their function has also been studied in neuroblastoma and other tumoral cell lines from neural origin, where the expression of diverse members of P2Y and P2X family has also been shown. The complex neural architecture is also an element to keep in mind concerning the toxicity, stress and neurodegeneration aspects, since the topographic distribution of P2X and P2Y receptors is extremely complex and could generate different responses in neural maintenance and recuperation. Cerebellar granule neurons have been extensively studied by our group and a brief summary concerning the presence and signalling of P2X and P2Y is reported below.

Presence and function of P2X receptors from cultured cerebellar granule neurons

The presence, distribution and function of P2X receptors have been extensively studied in the experimental model of cultured granule neurons obtained from young mice cerebellum. The relative expression levels of P2X receptors have been analysed by real time PCR, at 9 div, the most abundant are: $P2X_4$, $P2X_7$, $P2X_3$, $P2X_2$, and $P2X_1$, and by that order. The mRNA levels correlate well with the Western blot protein levels, detected with specific antibodies (76). Microfluorimetric techniques to study the calcium responses with specific P2X agonists and antagonists show the presence of functional receptors, being $P2X_7$ and $P2X_3$ the ones most relevant at the axodendritic fibers, in agreement with their immunocytochemical localization. Both receptors are able to induce calcium entrance and calcium dependent exocytotic release, in this case due to the glutamatergic nature of granule cells, the released neurotransmitter is glutamate. The intracellular calcium increase also results in cytoskeletal reorganization mediated by activation of the calcium

calmoduline kinase II, CaMKII. This enzyme phosphorylates synapsin allowing the approaching of neurosecretory vesicles to the docking secretory areas of the presynaptic plasma membrane (61, 77). Figure 6 shows cultured granule neurons loaded with Fura-2 responding to the $P2X_7$ agonist, and to a massive depolarization induced by K^+ ion, to compare the magnitude of both events.

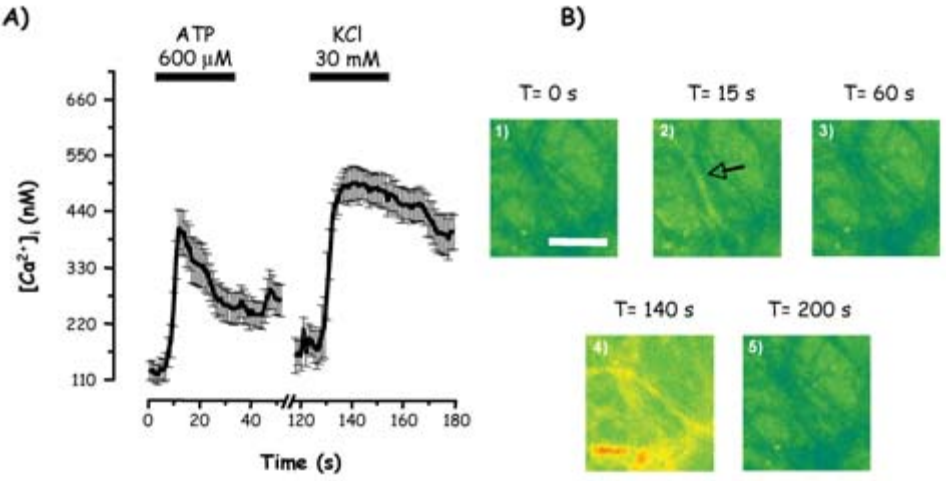

FIGURE 6. ***ATP effect on cultured granule neurons from mouse cerebellum.*** ***A)*** *Cultured neurons (9 days in vitro) were stimulated with ATP 600 µM for 30 seconds. At the end of the experiment cells were stimulated with KCl to asses its neuronal nature.* ***B)*** *Microphotographs of the granule neurons at t = 0 s (1); t = 15 s, during ATP stimulation (2); t = 60 s, before ATP stimulation (3); t = 140 s, during the masive depolarization induced by KCl (4); t = 200 s, in the presence of the perfussion medium. The axodendritic fibres that respond to ATP and to KCl are shown in yellow. The arrow point a responding fibre to ATP. Scale bar = 5 µm.*

Presence and function of P2Y receptors from cultured cerebellar granule neurons

Cerebellar granule neurons exhibit a great heterogeneity in nucleotide responses when studies in single cell are carried out. These responses concern both the ionotropic and metabotropic responses of P2X and P2Y receptors. These responses all became modified during the course of granule cells differentiation, not only

at the level of the number of responding cells but also in the magnitude of the response to nucleotides (51). These *in vitro* developmental changes were more significant in metabotropic responses to pyrimidine nucleotides, UTP and UDP, which were down and up regulated respectively, during the time in culture. These changes correlated with changes in the mRNA expression levels for $P2Y_4$ and $P2Y_6$ receptors.

$P2Y_1$ receptor is present in almost all, if not all, granule neurons when single cell microfluorimetric experiments are carried out, and cells challenged with 2MeSATP, that is a full synthetic agonist of this receptor. In most cells the calcium responses are completely inhibited, or significantly reduced, in the presence of MRS2179, that is the best antagonist of $P2Y_1$ available. The mRNA expression and the presence of the protein measured by western blot, for $P2Y_1$ receptor, increases with time in culture, but in a more moderate way compared with the $P2Y_4$ and $P2Y_6$ receptors.

In addition to $P2Y_1$, the presence of $P2Y_{12}$ and $P2Y_{13}$ has been recently reported in granule neurons, all of them being members of the ADP responding nucleotide receptors. Thus the metabotropic cascades activated by ADP require to be carefully studied to properly asignate the function to one of the receptors families. Recently our group has found that Glycogen synthase kinase-3 (GSK-3) a multifaceted enzyme involved in development, neurogenesis, and survival at the CNS was induced to phosphorylation and subsequent inhibition of its catalytic activity by 2-methyl-thio-ADP (2MeSADP). This compound could be acting through several P2Y-ADP receptors expressed in granule neurons, as RT-PCR expression was found for $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors. As the effect on GSK-3 phosphorylation was sensitive to pertussis toxin and was unaffected by specific antagonists of $P2Y_1$ and $P2Y_{12}$ receptors, such as MRS2179 and 2-methyl-thio-AMP, respectively, the pharmacological data fitted well with a Gi-coupled $P2Y_{13}$ receptor. The signalling cascade from $P2Y_{13}$ after activation through 2MeSADP is relatively complex as it was able to phosphorylate and activate extracellular signal-regulated kinase (ERK)-1,2 and Akt proteins, but its effect on GSK-3 phosphorylation was primarily dependent on the phosphatidyl inositol-3 kinase (PI3-K)/Akt pathway, as it was abolished by the PI3-K inhibitor wortmannin. GSK-3 inactivation by 2MeSADP in granule

neurons resulted in nuclear translocation of its substrate β-catenin, which functions as a transcriptional regulator, this effect being lost with wortmaninn (78). Figure 7 summarises the signalling cascade from $P2Y_{13}$ to GSK-3 and nuclear β-catenin. Now, further work is necessary to understand the physiological meaning of this signalling pathway in CNS development, maintenance and senescence.

PRESENCE AND FUNCTION OF P2 RECEPTORS IN CULTURED ASTROCYTES

Cultured astrocytes from rat cerebellum express almost all the P2Y receptors described so far, and there are also evidence for the presence and peculiar functionality of some P2X receptors.

The mRNA expression was studied by PCR and presence of $P2Y_{1,2,4,6,11,12,13}$ metabotropic nucleotide receptors was detected. At the same time and if specific antibodies were available, studies of immunocytochemistry or western blot were accomplished. Not always the presence of mRNA corresponded with protein detection by western blot or immunocytochemistry, or later on with functional responses from astrocytes. For example, $P2Y_1$ receptor correlates well the mRNA expression, protein detection and cellular responses, but that is not the case for $P2Y_{12}$ receptor that in spite of mRNA presence, there was no specific labelling of astrocytes with anti-$P2Y_{12}$ receptor antibody, whereas a specific staining was observed, as positive control in platelets.

Studies of the P2X subunits expression was carried out by RT-PCR techniques. In these assays high levels of mRNA codifying for $P2X_1$, $P2X_3$, $P2X_4$ and $P2X_7$ subunits were detected. In contrast, $P2X_2$, $P2X_5$ and $P2X_6$ subunits appeared to be nearly absent in astrocytes cultures. In most cases the mRNA detection does not correlate with the presence of the protein. In fact, by immunocytochemistry using specific antibodies only $P2X_4$ and $P2X_7$ subunits were detected. These data are relevant because it is possible that $P2X_7$ and $P2X_4$ form functional hetero-oligomers (37) in cerebellar astrocytes.

P2Y functional responses in astrocytes

Calcium responses measured using fura-2 microfluorimetric techniques suggest that different P2Y subtypes could be responsible

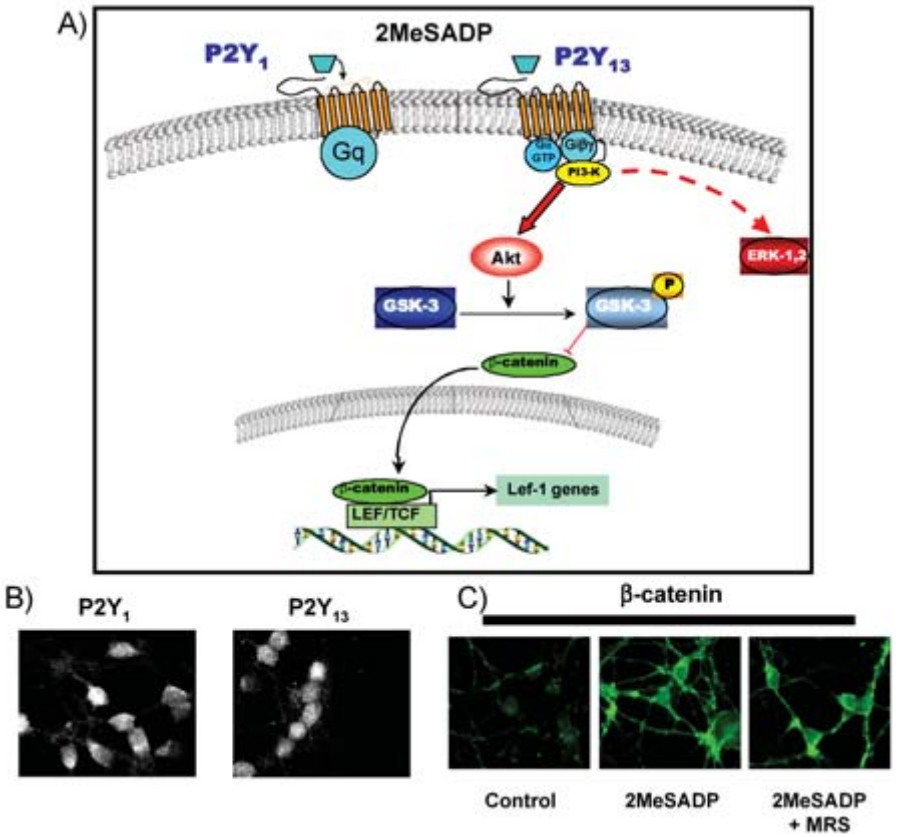

FIGURE 7. ***A)*** *Signalling pathways activated by 2MeSADP in cerebellar granule neurons. 2MeSADP, activating a $P2Y_{13}$ receptor, induces an increase in GSK-3 phosphorylation and inhibition of its catalytic activity. This phosphorylation occurs through a PI3K/Akt-dependent pathway. The lack of activity of GSK-3 towards its substrate β-catenin avoids its phosphorylation and degradation, and allows its translocation to the nucleus, where it functions as a transcriptional regulator.* ***B)*** *Immunostainnig of the main P2Y-ADP receptors expressed in cerebellar granule neurons, $P2Y_1$ and $P2Y_{13}$ receptors.* ***C)*** *Fluorescence images of β-catenin immunostaining in cerebellar granule neurons that were stimulated for 10 minutes with 1 μM 2MeSADP. The increase in β-catenin levels induced by 2MeSADP treatment was maintained in presence of the specific antagonist of $P2Y_1$ receptor, MRS-2179.*

for ATP metabotropic calcium responses in single type 1 astrocytes. All tested astrocytes responded to ATP and UTP stimulations evoking similar calcium transients. Most astrocytes also responded to 2-MeSADP and ADP challenges. The agonist potency and cross-desensitization experiments demonstrated the presence of functional $P2Y_1$ and $P2Y_2$ or $P2Y_4$ receptors. In addition, the existence of an astrocyte subpopulation that express functional $P2Y_6$ was demonstrated, this accounting for 30-40% of the total.

In addition to phospholiphase C activation, PLC, and subsequent calcium increase from internal stores, $P2Y_{1,2,4,6}$ receptors, can also activate the ERKs signalling cascade as they increase in western blot the phosphorylated forms of ERK1 and ERK2 (79).

P2X functional responses in astrocytes

Recent data from our group support the presence of functional ionotropic $P2X_7$ nucleotide receptors in cerebellar astrocytes. Although both $P2X_4$ and $P2X_7$ receptors were expressed at the protein level, the responses found with the specific agonist BzATP are in agreement with the activation of a $P2X_7$ type, which exhibits a distinctive behaviour in astrocytes. BzATP-induced calcium increases are sustained, and not transient, as those obtained with the metabotropic agonists 2MeSADP or UTP and far from inducing cell lysis as described for $P2X_7$ receptors from macrophages. Moreover, stimulation of $P2X_7$-like receptor induces significant morphological changes in astrocytes and leads to differentiation (84).

CONCLUSIONS

Nucleotide receptors are very abundant at the central nervous system, in all types of neural cells. Their physiological roles are far from being elucidated and only fragmentary data are available. However, there are increasing evidence concerning their involvement in neuropathogenesis due to aging and neurotrauma. The specific distribution and signalling cascades asociated with their neuronal location, as soma, dendritic or axonal subcellular distribution, should

be considered to analyse in depth their physiological function, the possible diseases asociated to their disfuction and the pharmacological perspectives for their specific agonists and antagonists.

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