

New insights into purinergic receptor signaling in neuronal differentiation, neuroprotection, and brain disorders

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Abstract Ionotropic P2X and metabotropic P2Y purinergic receptors are expressed in the central nervous system and participate in the synaptic process particularly associated with acetylcholine, GABA, and glutamate neurotransmission. As a result of activation, the P2 receptors promote the elevation of free intracellular calcium concentration as the main signaling pathway. Purinergic signaling is present in early stages of embryogenesis and is involved in processes of cell proliferation, migration, and differentiation. The use of new techniques such as knockout animals, in vitro models of neuronal differentiation, antisense oligonucleotides to induce downregulation of purinergic receptor gene expression, and the development of selective inhibitors for purinergic receptor subtypes contribute to the comprehension of the role of purinergic signaling during neurogenesis. In this review, we shall discuss the participation of purinergic receptors in developmental processes and in brain physiology, including neuron-glia interactions and pathophysiology.

Keywords ATP · knockout animal · neural stem cells · neurotransmitter · P19 embryonal carcinoma cells

Abbreviations

ACh	acetylcholine
AD	Alzheimer's disease
AP	alkaline phosphatase
ax-2	ataxin-2
cdks	cyclin-dependent kinases
CNS	central nervous system
DRG	dorsal root ganglia
EC	embryonal carcinoma
NTPDase2	ectonucleoside triphosphate diphosphohydrolase 2
E-NPP	ectonucleotide pyrophosphatase phosphodiesterase
EGF	epidermal growth factor
E-NTPDase	ectonucleoside triphosphate diphosphohydrolase
ES cells	embryonic stem cells
E-5'-NT	ecto-5'-nucleotidase
FGF-2	fibroblast growth factor 2
GABA	γ -aminobutyric acid
GFAP	glial fibrillary acidic protein
IFN- γ	interferon- γ
LIF	leukemia inhibitory factor
LTP	long-term potentiation
MAP-2	microtubule-associated protein-2
MAPK	mitogen-activated protein kinase
MRF	microglial response factor
MRS 2179	2'-deoxy- N^6 -methyladenosine 3', 5'-bisphosphate
NPC	neural progenitor cells
NPC	neural stem cells
NA	noradrenaline

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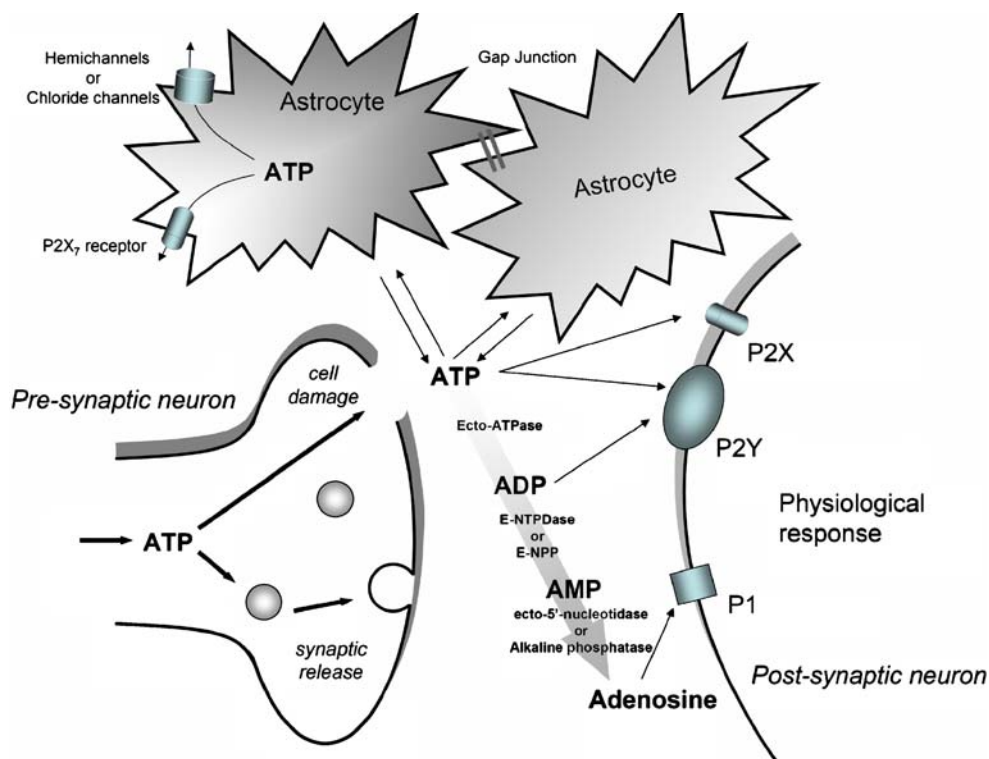
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NOS	nitric oxide synthase
NTPDase	ectonucleoside triphosphate diphosphohydrolase
NGF	nerve growth factor
OP	oligodendrocyte
oxATP	oxidized ATP
PPADS	pyridoxalphosphate-6-azophenyl-2', 4'-disulfonic acid
SVZ	subventricular zone
TGF	transforming growth factor
TNF- α	tumor necrosis factor- α
GAP-43	growth-associated protein 43
TNAP	tissue-nonspecific alkaline phosphatase
TNP-ATP	2'3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate

Introduction

During the last two decades, evidence for the participation of ATP as neurotransmitter in neuronal signaling was collected by Drs. Surprenant [1] and Silinsky [2]. Purine-sensitive receptors were first classified as P1 G-coupled receptors which are activated by adenosine and P2 receptors, responding to stimulation of ATP [3]. Based on receptor cloning and studying of receptor-induced signal transduction, P2 receptors were divided into P2X receptors as ATP-gated ion channels and P2Y G protein-coupled receptors [4].

Fig. 1 Purine-induced signaling pathway involves the activation of P1 adenosine and P2 purinergic receptors and purine hydrolysis by ectonucleotidases. The scheme demonstrates purinergic receptor activity present in glia-glia, neuron-glia, and neuron-neuron interaction during neurogenesis as well as in the metabolism of the adult brain



The expression of purinergic receptors has been identified during development and differentiation processes [5–10]. Nucleotides exert a synergic effect on cell proliferation in association with growth factors, chemokines, or cytokines in early stages of development [11–13] by parallel activation of the MAP kinase pathway and/or by trans-activation of growth factor receptors [14, 15].

The complete role of ATP action in developmental processes still needs to be elucidated. It is known that ATP activates purinergic receptors resulting in many cases in increases of intracellular free calcium concentration $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ are involved in several events of differentiation and the embryogenesis process [16, 17]. Spitzer et al. [18] showed that naturally occurring patterns of Ca^{2+} transients encoded neuronal differentiation. Distinct frequency patterns of $[Ca^{2+}]_i$ elevations were sufficient to promote neuronal differentiation, including physiological neurotransmitter receptors expression [19]. ATP and UTP are the main purinergic agonists activating P2X or P2Y receptors. These nucleotides can be rapidly degraded in the extracellular space by ectoenzymes to ADP or UDP, subsequently activating distinct P2Y receptors, or be finally degraded to adenosine, which is known to induce physiological responses via activation of P1 G protein-coupled receptors [20] (Fig. 1).

In this review article, we shall discuss the roles of purinergic signaling in neurogenesis such as cell cycle control during neural progenitor proliferation and differentiation as well as in maintaining physiology of neurons and

glial cells and the involvement of purinergic receptors in pathophysiology. In addition, we shall outline state-of-the-art approaches used in investigation of P2 receptor function in physiological processes such as the use of antisense oligonucleotides, generation of knockout animals, and identification of new purinergic receptor subtype-selective drugs.

Study of purinergic receptor function during *in vitro* differentiation

During the development of the mammalian nervous system, neural stem cells and their derivative progenitor cells generate neurons by asymmetric and symmetric divisions [21]. P2 receptors were shown to be one of the first functionally active membrane receptors in chick embryo cells during gastrulation, in which ATP caused rapid accumulation of inositol triphosphate and Ca^{2+} mobilization in a similar way as acetylcholine (ACh) did via activation of muscarinic acetylcholine receptors, whereas other endocrine-acting substances such as insulin and noradrenaline (NA) induced much weaker effects in terms of intracellular calcium signaling [22, 23]. The induction of transient fluctuation in $[\text{Ca}^{2+}]_i$, also denominated as calcium wave signaling allows for a coupling of spatial and temporal information. Thus, calcium waves have been proposed to play a role in mapping of neuronal networks [24] and to modulate neurogenesis during embryonic cortical development [25].

Neurotransmitters are prominent candidates for trans-cellular signals that could influence the development of embryonic neurons as they surround neural cells throughout brain development [26–29]. In addition, functional ligand-gated ionic channel receptors have been identified in neural progenitor cells prior to establishing cortical and subcortical synapses [30, 31]. In this context, the extracellular signaling mechanisms controlling the various transition steps involved in adult neurogenesis are still poorly understood. One approach used to identify the function of P2 receptors during development and differentiation is the use of *in vitro* models for neuronal and glial differentiation such as embryonic and adult neural progenitor cells (NPC), also known as neural stem cells (NSC), embryonic stem (ES), and embryonal carcinoma (EC) cells.

ES cells are obtained from the inner mass cell of the blastocyst. The differentiation of these cells closely resembles the *in vivo* process and, therefore, provides stable models for embryonic growth and development [32, 33]. ATP promotes cell proliferation acting through P2X_3 , P2X_4 , P2Y_1 , and P2Y_2 receptors in murine ES cells [34]. Tissue-nonspecific alkaline phosphatase (TNAP) was also detected in these cells and used as a marker for their undifferentiated stage [35].

The neuronal differentiation of EC cells, originated from irradiated embryo cells [36], also resembles early neuronal development *in vivo*. P19 mouse EC cells express stem cell-specific marker proteins and their phenotypic changes in specific differentiation stages are similar to those of stem cells [37]. Recently, our laboratory [38] has determined gene and protein expression of P2 receptor subtypes throughout *in vitro* neuronal differentiation of P19 cells as well as in the undifferentiated cell stage suggesting the participation of purinergic signaling in initiating and directing differentiation. Differential expression and activity of P2Y_1 , P2Y_2 , P2Y_4 , P2X_2 subtypes and P2X_6 subunits were reported during neuronal maturation of P19 cells [38, 39]. As direct evidence for participation of purinergic receptors in neuronal differentiation, the presence of the antagonists pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), reactive blue 2, or suramin during differentiation of P19 neural progenitor cells (NPC) to P19 neurons resulted in reduced activity of cholinergic and glutamate NMDA receptors in differentiated P19 cells, pointing at a participation of P2Y_1 , P2Y_2 , and P2X_2 receptors.

Other *in vitro* neuronal and glial differentiation models used to understand the purinergic signaling are neural stem cells or progenitor cells which are isolated from the subventricular region (SVZ) located in the lateral ventricles (type B cells) or in the subgranular region of the gyrus dentatus of the hippocampus (residual radial glia) or even from the subcortical parenchyma of the cerebral cortex of embryonic and adult brain [40–42]. These regions in the adult brain act as neural stem cell reservoirs. These cells are already advanced in their differentiation stage when compared to ES or EC cells. Since NSC and NPC are capable of differentiating in both functional neurons and glial cells, they possess potential therapeutic applications such as ES cells in regeneration therapy following neuronal loss.

These NPC differentiate into olfactory, cerebellar, and retinal neurons [40] in the presence of growth factors, neurotransmitters, vasoactive peptides *in vivo* [43], and growth factors such as epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), and leukemia inhibitory factor (LIF) *in vitro*. When exposed to a high concentration of FGF-2 in suspension, proliferating NPC form tridimensional cell aggregates denominated as neurospheres, which following induction to differentiation express neuronal marker proteins such as β -III-tubulin, microtubule-associated protein-2 (MAP-2), and synaptophysin [44] and express P2X_3 and P2X_7 receptors which may contribute to early $[\text{Ca}^{2+}]_i$ transients as prerequisites for further differentiation [41]. Shukla et al. [45] identified functional P2 receptors in adult mouse hippocampal progenitors *in situ* and the nucleoside triphosphate-hydrolyzing ectoenzyme (NTPDase) in type B cells of the SVZ [46] and in

hippocampal progenitor cells. In adult murine NPC of SVZ, P2Y₁ receptor activity mainly contributes to [Ca²⁺]_i transients with some participation of P2Y₂ receptors. The presence of the specific P2Y₁ receptor antagonist MRS 2179 resulted in diminished cell proliferation in neurospheres due to reductions of [Ca²⁺]_i transients. Similar results were obtained with NPC from SVZ of P2Y₁ receptor knockout mice [47]. P2Y₁ receptor-deficient mice are viable; however, they have deficits in platelet aggregation [48]. It is suggested that the purine signaling underlies autocrine or paracrine mechanisms and P2Y₁ and P2Y₂ receptors are important for NPC differentiation [47]. These models are useful tools to study the roles of P2 receptor signaling in early stages of development and differentiation. The importance of ATP release and purinergic signaling has not only been demonstrated in developmental progenitor cell expansion and neurogenesis, but also in persistent progenitor cells of the adult brain [49].

Expression of purinergic receptors during development of the central nervous system

Purinergic signaling pathways are also involved in embryonic neurogenesis in much the same way as already discussed for *in vitro* differentiation models. ATP mediates elevation of [Ca²⁺]_i and proliferation of immortalized human stem cells from embryonic telencephalon and mouse embryonic neurospheres [50, 51]. Ca²⁺ waves through radial glial cells in slices of the embryonic rat ventricular zone are mediated by P2Y₁ receptors. Disrupting Ca²⁺ waves between embryonic NPC reduced ventricular zone cell proliferation during the peak of embryonic neurogenesis [25].

ATP directly contributes to modulate network-driven giant depolarizing potentials in the rat hippocampus during early stages of postnatal development [52]. In the developing hippocampal system a trophic role of ATP and the involvement of P2 receptor subtypes in shaping interneuronal connections during neuronal differentiation have been suggested [53]. Alterations of the regulation of embryonic growth by purinergic receptors might be involved in the onset of morphological malformations [54]. During rat postnatal development ectonucleotidase activity in the cerebral cortex steadily increases, reaching maximum values at 21 days of age [55]. Several P2Y and P2X receptors were shown to be dynamically expressed in the pre- and postnatal central and peripheral nervous system [56–59]. ATP inhibited motor axon outgrowth during early embryonic neurogenesis, most likely through the P2X₃ receptor, and it was speculated that P2X₇ receptors might be involved in programmed cell death during embryogenesis [58].

From all of the studied P2X receptors, homomeric P2X₂ receptors were the first expressed in the rat central nervous system (CNS) on embryonic day 14 (E14) [56]. On E14,

heteromeric receptors were formed by P2X_{2/3} receptor subunits. P2X₃ receptor immunoreactivity was detected in cranial motor neurons as early as on E11, when neurons exited the cell cycle and started axon outgrowth, as well as postnatally on days 7 and 14 (P7 and P14) [56, 60]. Moreover, expression of P2X₃-containing heteromeric receptors and other subunits was developmentally regulated in nucleus ambiguous motoneurons [61]. From E14 onwards P2X₇ receptors were also expressed in the embryonic brain. For instance, in primary cultures of human fetal astrocytes basal levels of P2X₇ receptor mRNA transcription and protein expression were detected [62]. Sperlágh et al. [63] have demonstrated that ATP regulates glutamate release via activation of P2X₇ receptors. P2X₇ receptor-induced excessive glutamate release alters Ca²⁺ homeostasis, subsequently resulting in activation of the apoptosis-related caspase cascade [64].

P2X receptor expression was downregulated in Purkinje cells and deep cerebellar nuclei at P21 and P66 rat embryonic stages, with the exception of P2X₅ receptors whose immunoreactivity in granular cells was increased [65]. Evidence for participation of P2X receptors in different developmental processes such as neurite outgrowth (involving P2X₃ receptors), postnatal neurogenesis (related to P2X₄ and P2X₅ receptor expression), and cell death (possibly involving P2X₇ receptors) was collected. However, P2X₁ and P2X₆ receptor subunits may not play a role in neuronal development [58].

Neocortical neurons from 2-week-old rats possess a quite elaborated purine-triggered signaling system which includes both P2Y and P2X receptor activation [66]. Weissman et al. [25] showed that [Ca²⁺]_i waves and subsequent ATP release, with consequent P2Y₁ receptor activation, accompanied radial glial cell-derived neurogenesis in cultured slices of the developing rat forebrain, as mentioned above. Moreover, the importance of calcium signaling for differentiation of NPC has been studied [67, 68], and direct evidence for the participation of P2Y₁ receptor-activated pathways in the early development has been provided by Scemes et al. [69]. P2Y receptors (particularly the P2Y₁ subtype) were widely expressed in the embryonic rat brain as early as on E11 [57]. There was a marked decrease in the concentration of mRNA coding for P2Y₁ receptors and upregulation of mRNA transcription coding for P2Y₂ receptors in freshly isolated astrocytes of developing rat hippocampus [57].

Functional interactions between neurons and glia: a physiological overview

An increasing amount of evidence, initiated by the neuron-glia unit idea proposed by Hyden [70], indicates that glial cells, once referred to as a simple support portion in the

CNS, are now considered indispensable functional partners of neurons [71], both in physiological and pathological conditions. However, many questions remain unanswered: (1) how glia detects and interacts with neural function; (2) does neuron-glia signaling play a significant role in synaptic transmission and plasticity; and (3) how glial cells can communicate with other glial cells.

Another important subject related to the interaction between glia and neurons emerges in neurogenesis. There is now a general agreement that the adult mammalian nervous system possesses many characteristics of astrocytes. The importance of glia in neuronal development was confirmed in a recent study showing that the number of GFAP (glial fibrillary acidic protein)-containing cells was reduced following transgenic targeting of adult mouse subependymal and subgranular zones, resulting in an almost complete loss of neurogenesis [72, 73]. In addition to assisting migration of neurons to their correct position and managing neurite outgrowth to their final communication targets [74, 75], glial cells have become an essential key for understanding neuronal differentiation by promoting initial stem cell proliferation and instructing undifferentiated cells to adopt a neuronal fate [76, 77].

In the mature brain, the proximity of astrocytes to neuronal synapses or to the blood-brain barrier makes these cells appropriate to control water diffusion and ion concentration in extracellular spaces [71, 78]. In particular, astrocytes regulate homeostatic environment and neurotransmitter levels by functional syncytium, in which gap junctions and specific membrane carriers play an important role [79–81]. In addition, glial cells produce and release a vast number of neurotrophins, including fibroblast growth factor, nerve growth factor, and transforming growth factor, which directly interfere in neuron physiology and coordinate developmental processes [71, 82–85].

ATP release and degradation, connecting adenosinergic and purinergic systems

As already mentioned, it is well documented that glial cells may directly alter neuronal activity by releasing neurotrophins and consequently modulating neurotransmitter release in the synapse [86, 87]. One of the main mechanisms connecting the neuron-glia system is believed to be mediated by the release of glutamate from glial cells [88, 89]. In this context, growing evidence indicates that purinergic receptor ligands are widely involved in the cell-cell signaling mechanism by acting as neurotransmitters or neuromodulators released by glial cells to control synaptic transmission in the CNS, as part of multiple functions of astrocytes [22, 90, 91] (Fig. 1).

ATP is an ideal molecule for cell signaling due to its intrinsic properties such as its small size, diffusing molecule

rate, instability and low concentration in the extracellular environment, and impossibility to cross the plasma membrane [92, 93]. These properties imply the presence of particular pathways for ATP release that could be associated with cellular excitation/response and cell-cell signaling [94, 95]. First, ATP may be stored in synaptic vesicles alone or with other neurotransmitters and then released, as a classic synaptic mechanism in the peripheral or central nervous system [96, 97]. Second, a nonvesicular mechanism of ATP release could be observed through gap junction hemichannels, ATP-binding cassette proteins, P2X₇ receptor pores in glial cells, and via chloride channels [98–101]. Third, ATP could be released due to cytolysis or cell damage. While this is not a physiological mechanism, it takes place following biological trauma and contributes to pathological conditions [102].

Subsequent to these mechanisms, the metabolism of the released ATP is regulated by a vast number of different families of ectonucleotidases in the synaptic cleft, including the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) and the ectonucleotide pyrophosphatase phosphodiesterase (E-NPP) which catalyze the degradation of ATP to ADP or AMP. The degradation to adenosine is mediated by ecto-5'-nucleotidase (E-5'-NT) and alkaline phosphatase [91, 103] (Fig. 1). Consequently, the reaction products resulting from the ATP hydrolysis may bind to P2 receptors, in the case of ADP, or to P1 receptors in the case of adenosine [104].

The adenosinergic receptor ligand adenosine is recognized as an important regulator of cellular homeostasis in the CNS and may be involved in the prevention or induction of apoptosis [105]. The reduction of ectonucleotidase activity in certain pathological conditions provided additional evidence for the accumulation of ATP in the extracellular environment [20]. Therefore, the complexity of the communication of neural and nonneural cells expands the functional significance by the interaction of the purinergic receptors in association with a variety of neurotransmitter systems.

ATP-mediated neuron-glia signaling

Novel studies in the purinergic field began to converge with glial research as it became more widely accepted that ATP is released through synaptic vesicles and thus accessible to perisynaptic glial cells, allowing them to detect neuronal activity. In particular, glial cells are responsive to ATP, as all types of glia, such as astrocytes, oligodendrocytes, microglia, and Schwann cells, express purinergic receptors [91]. In Schwann cells and oligodendrocytes, ATP-mediated signaling predominantly occurs through P2Y receptors, which in turn trigger intracellular Ca²⁺ release [106, 107]. However, the function of P2X_{1–6} receptors in astrocytes

remains unclear, although P2X-mediated currents could be detected in astrocyte cells in culture, and P2X₇ receptors are widespread in these cells with possible contribution to pathological conditions [108, 109].

Glial cells express many types of neurotransmitter receptors and conventionally are considered to be non-excitabile [110, 111]. However, a surprising observation was reported by Dani et al. [112] that synaptic transmission may propagate to glial cells as calcium waves, inducing membrane depolarization and regulating neurotransmitter release. These properties of glial cells suggest possible rapid communications between neurons and glia during synaptic transmission. This glial communication mechanism allows the released ATP to act onto adjacent astrocytes and neurons, thus supporting the propagation of Ca²⁺ waves in glial syncytium [113]. For example, in neuronal-glia cocultures prepared from hippocampus, ATP secreted by astrocytes was shown to inhibit glutamatergic synapses through activation of P2Y receptors [114].

The glial communication mechanism based on Ca²⁺ wave propagation could be inhibited by P2 receptor blockers or enzymes that rapidly hydrolyze extracellular ATP [115]. A stimulation applied to a single astrocyte in cocultures of rat forebrain astrocytes and associated neurons caused an elevation of [Ca²⁺]_i and induced Ca²⁺ wave propagation in dorsal spinal cord through P2Y₁ receptor activation [116] and glutamate release [117]. This new finding provided a parallel mechanism of intercellular communication that could allow astrocytes to detect synaptic function, propagate the information through neighboring glial cells, and then influence synaptic function in a distant part of the nervous system.

Purinergic receptor-calcium signaling in glial cells plays important roles during CNS development. P2X₁, P2X₄, and P2X₇ receptors were expressed in microglia at rat embryonic stage (E16) [59]. Moreover, changes in P2X₄ receptor expression in microglial cells during postnatal development of the rat cerebellum have been reported. P2X₅ receptor immunoreactivity was also upregulated in microglia and granular cells. Both P1 and P2 receptors contribute to the modulation of oligodendrocyte (OP) development, since they have been shown to exert similar effects on OP proliferation and differentiation [118].

The majority of the studies of ATP action have been concerned with the short-term P2 receptor signaling that occurs in neurotransmission and in secretion [119]. Furthermore, there is increasing evidence that purines and pyrimidines can have trophic roles in neuritogenesis [120, 121], regeneration [122], and proliferation [123]. However, some purines by themselves have limited trophic effects in a few types of cells; they appear to be much more effective as neuritogenic agents when they are combined with other trophic factors, such as NGF. For instance, inosine and 5'

AMP alone do not elicit neurite extensions in PC12 cells [124].

Heine et al. [53] demonstrated that P2 receptor activation induced fiber outgrowth in organotypic cocultures in rat hippocampus. Fiber outgrowth was inhibited in the presence of the purinergic antagonist PPADS, suggesting the involvement of P2 receptors. In another study, the synergistic interaction between bFGF and ATP was reported on DNA synthesis in primary cultures of rat cortical astrocytes. ATP and bFGF induced a twofold and tenfold incorporation of [³H]thymidine into astrocytes, respectively, but when ATP and bFGF were added at the same time a 50-fold increase in [³H]thymidine incorporation was observed [12].

Neuroprotection

ATP can activate P2X₇ receptors in astrocytes to release glutamate, GABA, and also ATP which might regulate the excitability of neurons in certain pathological conditions [125]. It has been suggested that astrocytes can sense the severity of damage in the CNS by the amount of ATP released from damaged cells and that extracellular ATP concentration and the corresponding subtype of activated astrocytic P2 receptor modulate the tumor necrosis factor- α (TNF- α)-mediated inflammatory response [126]. After mechanical brain injury, the administration of PPADS facilitated the recovery of pathologically changed electroencephalograms [127]. These results suggest that interference with the ATP-induced excitatory responses could provide neuroprotection and possible therapeutic consequences.

Evidence for a neuroprotective role was also found for the adenosine A₁ receptor in hippocampus. This cerebral region is highly sensitive to hypoxia and ischemia. The study of the action of hypoxia on synaptic transmission in hippocampal slices has suggested that substances being released during hypoxia, such as GABA, ACh, and even glutamate, may also play neuroprotective roles. However, the actions of these neurotransmitters become evident only when activation of P1 receptors is impaired, suggesting a critical role for this receptor during hypoxic events. These substances can operate in a redundant or even overprotective manner, acting as a substitute for some adenosine actions when the nucleoside is not operative [128].

Neuroimmune interactions

Microglia, the immune cells of the CNS, can be activated by purines and pyrimidines to release inflammatory cytokines such as IL-1, IL-6, and TNF- α . However, hyperstimulation of the immune reaction in the brain may accelerate neuronal damage. The P2X₇ receptor is considered to have a potentially pivotal role in the regulation of

various inflammatory conditions. ATP selectively suppresses the synthesis of the inflammatory protein microglial response factor through calcium influx via P2X₇ receptors in microglia [129], which also leads to enhancement of interferon- γ (IFN- γ)-induced type II nitric oxide synthase (NOS) activity [130, 131]. P2X₇ receptor activity also participated in ATP-induced IL-1 release from macrophages and microglia that had been primed with substances such as bacterial endotoxin [132] and was shown to stimulate the transcription of nuclear factor κ B, TNF- α [133], the stress-activated protein kinases (SAPK)/JNK pathway [134], and the production of 2-arachidonoylglycerol, which is also involved in inflammation induction by microglial cells.

P2Y rather than P2X₇ receptors seem to have a major role in the IL-6 production by microglial cells [135]. ATP evoked the release of plasminogen [136] and IL-6 [135]. The stimulation of microglia by either ATP or BzATP revealed neurotoxic properties and the involvement of the P2X₇ receptor has been reported in excitotoxic/necrotic and apoptotic degeneration [109].

Neurological disorders

Epilepsy Several anti-epileptic agents reduce the ability of astrocytes to transmit Ca²⁺ waves, raising the possibility that blockade of ATP-induced [Ca²⁺]_i transients in astrocytes by purinergic receptor antagonists could offer new treatments for epileptic disorders. Antiepileptic effects of adenosine are mostly due to the well-known inhibitory actions of P1 receptors on synaptic transmission in the hippocampus. However, as recently pointed out, adenosine actions are not limited to presynaptic actions on glutamate release [137]. The intraventricular injection of high doses of ATP in rats evoked severe chronic-tonic convulsions, whereas lower doses of ATP or adenosine elicited a kinetic state with muscle weakness [138]. P2X₂ and P2X₄ receptor expression in the hippocampus of seizure-prone gerbils was significantly reduced compared with that of normal gerbils [139]. GABA_A receptors mediated modulation of expression of both P2X₂ and P2X₄ receptors, which may play an important role in the regulation of seizure activity in the gerbil hippocampus [139]. P2X₇ receptors are thought to play a definite, but not yet well defined role in epilepsy. Treatment with the GABA_B receptor agonist baclofen and antagonist phaclofen resulted in increased and decreased P2X₇ receptor expression in hippocampus, respectively [140]. These purinergic receptor responses were interpreted as compensatory responses to the modulation of GABA_B receptor function [140]. It is noteworthy to mention that this positive relationship between P2X and GABA_A receptors was also reported for the spinal cord [141] and dorsal root ganglia (DRG) [142]. In these populations of neurons, ATP-mediated P2X receptor function may partic-

ipate in neuronal transmission accompanied by GABA-mediated actions [139].

Pain The heteromeric channel comprised of P2X₂ and P2X₃ subunits was expressed almost exclusively in a subset of primary afferents implicated in nociception [143–145]. It has been observed that mechanical allodynia is reduced in mice with deleted P2X₃ receptor genes [146, 147] in agreement with data obtained in rats that have been treated with intrathecal antisense oligonucleotides reducing expression of P2X₃ receptors [148] or with the selective antagonist for P2X₃ and P2X_{2/3} receptors A-317491 [148, 149]. P2X₃ receptor knockout mice showed additional defects in afferent pathways.

The P2X₄ receptor is also implicated in pain sensation. Activation of dorsal horn microglia and tactile allodynia developing several days after ligation of a spinal nerve were greatly reduced when gene expression of P2X₄ receptor in the dorsal horn had been inhibited by the presence of intrathecal antisense oligonucleotides [150]. Accordingly, intraspinal administration of microglia following induction of expression and activity of P2X₄ receptors produced tactile allodynia in naive rats. Intrathecal administration of cultured brain microglia produced allodynia, but only when the cells had been pretreated with ATP [150]. The inhibition of P2X₄ receptor activity in microglia might be a new therapeutic strategy for pain induced by nerve injury.

Alzheimer's disease Alzheimer's disease (AD) is caused by extracellular deposition of amyloid β -peptide, which can damage neurons, leading to their dysfunction and death [151]. ATP and, in particular, aluminum-ATP promoted the formation of thioflavin T-reactive fibrils of β -amyloid and an unrelated amyloidogenic peptide, which could be blocked by suramin [152].

Microglial cells are believed to contribute to the progression of AD and are known to release proinflammatory neurotoxic substances. Extracellular ATP, acting through the P2X₇ receptor, can alter β -amyloid peptide-induced cytokine secretion from human macrophages and microglia and thus may be an important modulator of neuroinflammation in AD [153]. P2X₇ receptors mediate superoxide production in primary microglia, and the expression of this receptor subtype was specifically upregulated around β -amyloid plaques in a transgenic mouse model of AD [154].

In contrast to the control human brain, the P2Y₁ receptor was colocalized with a number of characteristic AD structures such as neurofibrillary tangles, neuritic plaques, and neuropil threads in the hippocampus and cortex [155]. In general, control brain tissue exhibited a greater and more abundant level of P2Y₁ receptor immunostaining than AD tissue did, probably due to severe neuronal cell degenera-

tion in most AD brains. The intense P2Y₁ receptor staining observed over pathological AD structures might imply that this receptor is involved either directly or indirectly in signaling events mediating neurodegeneration of pyramidal cells. Alternatively, P2Y₁ receptors might have other diverse signaling roles, possibly involved in the production of intracellular tau deposits or might even serve to stabilize these tangle structures in some way [156].

Ischemia/hypoxia Under pathological conditions of hypoxia or ischemia, extracellular purine nucleotides leak from damaged cells and thereby may reach high concentrations in the extracellular space [157]. A direct participation of extracellular ATP and P2 receptors in ischemic stress has been reported in various cellular systems [157–160]. For example, P2X₂ and P2X₄ receptor expression in neurons and microglia, respectively, in the hippocampus of gerbils was upregulated following transient global ischemia [161]. Increased P2X₇ receptor expression in astrocytes, microglia, and neurons appears to contribute to the mechanisms of cell death caused by *in vivo* and *in vitro* ischemia [162, 163]. Following induction of ischemia P2X₇ receptor mRNA transcription and protein expression were elevated in cultured cerebellar granule neurons and organotypic hippocampal cultures [163]. Hence, the P2X₇ receptor is apparently an important element in the mechanisms of cellular damage induced by hypoxia/ischemia. In many cell types, the activation of the P2X₇ receptor led to rapid cytoskeletal rearrangements, such as membrane blebbing and cell lysis [164]. P2Y₁ receptors are intensely expressed in Purkinje cells in deep layers of the cerebral cortex and in ischemia-sensitive areas of the hippocampus [165]. In conclusion, extensive evidence demonstrates a postischemic time- and region-dependent upregulation of P2X_{2,4,7} and P2Y₁ receptor subtypes in neurons and glial cells and suggests a direct role of P2 receptors in the pathophysiology of cerebral ischemia *in vitro* and *in vivo*.

Trauma and axotomy P2 receptors are suggested to be involved in neuronal reactions after axotomy. Colocalization and temporal coactivation of purinergic and nitric markers support this idea, indicating possible interactions between these two systems [166]. Following peripheral nerve lesions, P2X₃ receptor expression in DRG neurons was changed [167]. The increased expression of P2X₃ receptor mRNA in intact neurons indicates a role of this subtype in the post-injury pathomechanism in primary sensory neurons [167]. After spinal cord injury, large regions of the peritraumatic zone were characterized by a sustained process of pathologically high ATP release [168]. Spinal cord neurons express P2X₇ receptors, and exposure to ATP led to high-frequency spiking, irreversible increases in [Ca²⁺]_i and cell death. The administration of P2 receptor

antagonists (PPADS, oxATP) after acute impact injury significantly improved functional recovery and diminished cell death in the peritraumatic zone [168]. The involvement of P2X₁ and P2X₂ receptors in neuronal reactions after hemispherectomy was also described [169]. Furthermore, neuronal NOS and P2 receptors were colocalized and showed temporal coactivation after cerebellar lesions, indicating a close relationship between these two systems [166]. In addition, in this mixed model of differentiation and axotomy, the colocalization of ataxin-2 (ax-2, involved in resistance to degeneration phenomena, which may be lost after mutation)-immunopositive cells and P2X₂ receptors was demonstrated in neurons, and post-lesional induction of P2X₁ receptor and ax-2 immunoreactivity was reported as well [170]. *In vivo* treatment of P2Y₂ receptor-expressing sciatic nerves with ATP-γS increased expression levels of the growth-associated protein 43 (GAP-43) as a marker for axonal growth in wild-type but not in P2Y₂ ^{-/-} mice [171].

Possible therapeutic manipulations to modulate astrocytic proliferation and to diminish glial scar formation in the adult brain and during development include the use of drugs known to interfere with nucleotide synthesis. Pekovic et al. [172] showed that treatment with the purine nucleoside analogue ribavirin (Virazole; 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) downregulates the process of reactive gliosis after sensory motor cortex lesion of the adult brain and facilitates re-establishing synaptic connections with the denervated cells at the lesion site. This may be a useful approach for improving neurological recovery from brain damage. The antiproliferative effect of ribavirin is due to the inhibition of *de novo* nucleic acid synthesis after depletion of GTP and dGTP pools with consequent impairment of specific transduction pathways.

ATP-induced effects on cell cycle progression

There is evidence showing that extracellular ATP enhances the expression of cell cycle regulating proteins [173, 174]. Progression of the cell cycle is highly controlled. Cyclins are synthesized and degraded in a synchronous way due to changing transcription or proteolysis rates, thereby directing the periods of the cellular cycle. Cyclins interact with cyclin-dependent kinases (cdks) resulting in activation of their kinase activity, phosphorylating their targets and themselves, and regulating the specific progression of the cell cycle through checkpoints [175].

Proliferation rates in mammals are largely determined during the G1 phase of the cell cycle. The relevant proteins include three D-type cyclins (D1, D2, and D3) that, in different combinations, bind to and allosterically regulate one of two cdk subunits, cdk4 and cdk6, as well as the E-type

cyclins (E1 and E2), which govern the activity of a single catalytic subunit, cdk2 [176]. Various combinations of D-type cyclins are expressed in different cell types, whereas cyclin E-cdk2 complexes are ubiquitously expressed [177].

Two families of cdk inhibitors regulate the activity of G1-type cyclins-cdks complexes: the Ink4 family (p16, p15, p18, and p19), which blocks the activity of cyclin D-cdk4-6 complexes, and the Cip/Kip family (p21, p27, and p57), which preferentially inhibits cyclin E-cdk2 complexes and also acts as a scaffold for the catalytically active cyclin D-cdk4-6 complexes. In addition to cyclins and cdks, mitogen-activated protein kinase (MAPK) is also believed to have a role in induction of cell proliferation. Therefore, cyclin D-dependent kinases may play a role in controlling the cell cycle of embryonic and maybe neural progenitor cells. In addition MAPK is also believed to have a role in induction of cell proliferation. Extracellular ATP induces Ca^{2+} -dependent MAPK activation via stimulation of P2 receptors in neonatal rat astrocytes [178]. On the other hand, cell proliferation is associated with activation of diverse proteins. Positive regulators include cyclins and their partners with catalytic activity (cdks), which are essential for progression of the cells through each phase of the cell cycle and various cell cycle checkpoints [179, 180]. The regulation of cyclin D1 expression is also mediated by the Ras/ERK signaling pathways [181, 182]. Raf/MEK/ERK and PI3-K/Akt signaling pathways can act in synergy to promote the G1-S phase cell cycle progression in both normal and cancer cells [183, 184]. The promoter for cyclin D1 contains an AP-1 site, and the ectopic expression of either *c-fos* or *c-jun* induces cyclin D1 mRNA expression [185, 186]. In many cell types, phosphatidylinositol (PI)-3-kinase-dependent signaling pathways also regulate cyclin D1 expression [187]. It was also reported that the control of the cell cycle regulatory proteins was dependent on PI3-kinase and p44/42 MAPK pathways, indicating that extracellular ATP alone is sufficient to induce cell cycle progression beyond the G1 phase of the cell cycle. These findings also suggest that, once P2 receptors are activated, protein kinase C (PKC) transmit signals to the nucleus through one or more of the MAPK cascades, which may include Raf-1, MEK, and ERK, and stimulate transcription factors such as myc, max, fos, and jun. Moreover, MAPKs are upstream regulators of cdk2 and cdk4 expression. It has been reported that p44/42 MAPK phosphorylation is essential and sufficient for the increase in cdk2 [188, 189] and decrease in p27Kip1 expression [190, 191]. However, Delmas et al. [192] provided evidence that p44/p42 MAPK activation triggers p27Kip1 degradation independently from cdk2/cyclin E in NIH 3T3 cells. As described above, ATP regulation of the MAPK and cdk-cyclin complex has not been elucidated in other types of cells [193].

It is documented in the literature that purinergic receptor inhibitors interfere with the S phase of the cell cycle. Neurospheres treated with the purinergic receptor antagonists reactive blue 2 or suramin are mostly in S phase ($5.7 \pm 0.3\%$ or $8.4 \pm 2.3\%$) when compared to untreated control neurospheres with $16.4 \pm 1.8\%$ of the cells being in S phase. Moreover, neurosphere cultures treated with suramin or reactive blue 2 showed an increase in the expression of the tumor suppressor p27 as a strong regulator of cell division [49].

The discussed findings led to the suggestion that extracellular ATP plays an important physiological role during mammalian embryonic development by stimulating proliferation of ES cells, and therefore P2 receptor agonists and antagonists might provide novel and powerful tools for modulating embryonic cell functions. In conclusion, P2X and P2Y purinergic receptors can promote proliferation of ES cells as well as of progenitor cell types by a mechanism by that ATP induces increases in $[\text{Ca}^{2+}]_i$, leading to activation of PKC, PI3-kinase/Akt, p38, and p44/42 MAPK, followed by an alteration in the cdk-cyclin complex with p21 and p27, which are involved in stimulation of cell proliferation.

Pharmacological approaches

Most purinergic receptors do not have specific inhibitors. Therefore, P2 receptor agonists and antagonists acting on most of the purinergic receptor subtypes are widely used in experimental approaches to study biological functions of these receptors. Such approaches are feasible, since these compounds mostly have higher affinities to some P2 receptor subtypes than to other ones. As an example, we have used suramin, PPADS, and reactive blue 2 to study the participation of P2Y₁, P2Y₂, and P2X₂ receptors in neuronal differentiation of P19 EC cells [38].

One possible approach towards a subtype-specific inhibitor would be based on results from P2 receptor structure determination. Using site-directed mutagenesis it has been possible to understand which amino acids are involved in ATP binding and to identify allosteric sites in purinergic receptors. The knowledge obtained on location and structural features of ligand and inhibitor binding sites is used in rational based drug design of selective purinergic subtype antagonists. Alternatively, combinatorial libraries formed by vast amounts of possible ligands can be employed for discovery of subtype-specific inhibitors.

A-317491 was identified as a specific inhibitor for P2X_{2/3} and P2X₃ receptors. In the presence of A-317491 both thermal hyperalgesia and mechanical allodynia were attenuated after chronic nerve constriction injury in which P2X₃ homomeric and P2X_{2/3} heteromeric receptor activities were involved. Although active in chronic pain models, A-317491 was ineffective in reducing nociception in animal

models of acute postoperative pain and visceral pain indicating that P2X₃ and P2X_{2/3} receptor activation may not be a major mediator of acute postoperative or visceral pain [149]. MRS 2179 (2'-deoxy-N⁶-methyladenosine 3',5'-bisphosphate) was discovered as a specific inhibitor of P2Y₁ receptor activity [194]. This compound has an efficient antithrombotic action in which P2Y₁ receptors are involved [195].

Based on structure design or combinatorial library approaches specific agonists or antagonists may be discovered for other purinergic receptor subtypes. For instance, the SELEX (systematic evolution of ligands by exponential enrichment) technique provides a particularly promising approach for the discovery of such compounds. This technique is based on the reiterative presentation of a partial random RNA or single-stranded DNA library to a protein preparation containing a particular purinergic receptor subtype. RNA or DNA molecules bound to a target site on the receptor are displaced from the receptor and eluted by addition of an excess concentration of an unspecific purinergic receptor antagonist and amplified by reverse transcription polymerase chain reaction (PCR) or PCR to restore the library used for the next in vitro selection cycle. Using this approach, it was possible to identify inhibitors specific for isoforms of a target protein [196]. Our group prepared membrane protein fractions of 1321N1 cells stably transfected with rat P2X₂ receptors and coupled them onto an immobilized artificial membrane (IAM) as matrix for affinity chromatography. The equilibrium binding to the receptor and competition between ATP and the purinergic antagonists suramin and 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP) were analyzed by a chromatographic assay using [α -³²P]-ATP as a radioligand. Our data indicate that suramin does not compete with ATP for the ligand binding site and TNP-ATP is a competitive antagonist, as already shown by Trujillo et al. [197]. Moreover, this chromatographic assay can be used in in vitro selection procedures for RNA aptamers binding to P2X₂ receptors from a combinatorial SELEX RNA library [198]. The development of a subtype-specific P2X receptor antagonist by using the SELEX technique or another combinatorial library-based approach shall serve as proof of principle and encourage further works to obtain such specific antagonists for all P2 receptor subtypes as tools for elucidating their biological functions and for possible therapeutic applications.

Conclusion

P2 receptor function is involved in most physiological processes and participates in neurotransmission in the CNS. Results obtained with mouse ES and P19 EC and neural

progenitor cells suggest an important role of purinergic signaling in early embryogenesis, especially in cell proliferation, migration, and differentiation, with different subtypes of receptors participating in these processes. Our understanding of the biological functions of specific P2 receptor subtypes during CNS development and in the adult brain has increased due to the availability of knockout animals and specific inhibition of gene expression or activity of purinergic receptor subtypes. The importance of P2 receptor signaling in neuroprotection, neuroimmunity, and guiding neuronal differentiation, especially in glial and microglial cells, has been related to purinergic receptor expression. Most importantly, specific agonists and antagonists for individual P2 receptor subtypes are both needed for studying their involvement in biological processes. The discovery of such selective compounds will elucidate yet unknown biological functions of P2 receptor subtypes as well as open new avenues for therapeutic approaches to disease states in which purinergic receptor activity is involved.

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