Ectonucleotidases and Nucleotide/Nucleoside Transporters as Pharmacological Targets for Neurological Disorders

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Abstract: Extracellular nucleotide and nucleoside are signaling molecules with a wide range of actions in the central nervous system (CNS). Extracellular ATP is released by several mechanisms involving ATP binding cassette transporters, hemichannels, P2X7 receptors, or volume-sensitive chloride channels. The levels of ATP and its hydrolysis product, adenosine, in the synaptic cleft are controlled by a complex cascade of cell surface-located enzymes collectively known as ectonucleotidases. There are four major families of ectonucleotidases: ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), alkaline phosphatases, and ecto-5'-nucleotidase. Besides the production of adenosine through nucleotide hydrolysis, this neuromodulator can be released as adenosine *per se* by equilibrative and/or concentrative nucleoside transporters. In this review, the involvement of nucleotide/nucleoside transporters and ectonucleotidases in the pathophysiology of brain disorders is discussed. The identification of compounds able to modulate the activity of these players in purinergic neurotransmission and their implications in neurological disorders as potential targets for drug discovery is also highlighted.

Keywords: Ecto-5'-nucleotidase, ectonucleotidases, neurological disorders, nucleoside transporters, nucleotide transporters, nucleoside triphosphate diphosphohydrolase, nucleotide pyrophosphatase/phosphodiesterase.

INTRODUCTION

Extracellular nucleotides and nucleosides are ubiquitous signaling molecules found in central nervous system (CNS) [1]. Nucleotides are released from dying cells or by selective transport throughout the plasma membrane mediated by several mechanisms [2]. Such nucleotides exert their effects at extracellular milieu through two major receptor subfamilies: P2X, which are ligand-gated ion channels comprising a family of seven receptors, and P2Y, a group of eight G-protein coupled receptors [3, 4].

The availability of extracellular nucleotides is tightly regulated by a variety of cell surface-located enzymes named ectonucleotidases. There are four major families of ectonucleotidases, namely E-NTPDases (ectonucleoside triphosphate diphosphohydrolases), E-NPPs (ecto-nucleotide pyrophosphate/phosphodiesterases), alkaline phosphatases, and ecto-5'-nucleotidase [5, 6]. Adenosine is produced by extracellular nucleotide hydrolysis or released through bidirectional nucleoside transporters [7] and binds to the G-protein-coupled receptors A_1, A_{2A}, A_{2B}, and A_3, of which A_1 and A_{2A} are highly expressed in the brain [8].

The highly sophisticated pathway of ectonucleotidases and the existence of nucleotide/nucleoside transporters promoted a tight control of ATP and adenosine levels, which might be involved in the progression of neurological disorders and represent targets for pharmacological therapies. This review is divided in sections focusing in ATP release, the role of ectonucleotidases, and nucleoside transporters and their features in CNS.

ATP RELEASE

ATP is released from both peripheral and central neurons [9, 10] in response to hypoxia and the action of several compounds [11]. There are several hypotheses about the transport mechanism(s) involved in ATP release, including exocytosis from presynaptic terminals and diffusion through large transmembrane pores (e.g., ATP binding cassette transporters, hemichannels, P2X7 receptors, or volume-sensitive chloride channels) expressed in astroglial membranes [12]. ATP hypoxia-induced release may be in principle both neuronal and astrocytic. ATP is released from astrocytes during cell swelling, implicating membrane channels that are involved in osmoregulation or activated by membrane stretch.

Studies have demonstrated that ATP-binding cassette transporter A1 (ABCA1) reduces amyloid-beta burden in transgenic mouse models of Alzheimer's disease (AD) [13]. The overexpression of ABC transporters has been also demonstrated in the brain of patients with refractory epilepsy [14]. However, the impact of these changes in ATP release and the progression of neurological disorders remains unknown. Other mechanisms proposed for ATP release involve volume-sensitive outwardly rectifying (VSOR) chloride channel, which are found in virtually all cell types and can physically accommodate or even permeate ATP^{4-} in given experimental conditions. Pharmacological studies are controversial and argue against the actual involvement of the VSOR channel in significant ATP release [12]. Moreover,

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maxi-anion channel, which is a large-conductance anion channel, has been described and is ubiquitously expressed, representing other mechanism for ATP release [15]. It has been observed the nanoscopic maxi-anion channel pore provides sufficient room to accommodate ATP and is well suited to its function as a conductive pathway for ATP release [16]. The maxi-anion channel conducts ATP and displays a pharmacological profile similar to that of ATP release in response to osmotic, ischemic, hypoxic, and salt stresses [12, 15].

Several connexins and two pannexins are expressed in neurons and astrocytes where they may function in release of ATP and glutamate [17]. Additionally, pannexin-1 appears to play a role in neuronal death. Prolonged P2X7 receptor activation by ATP in Px1/P2X7 expressing astrocytoma (1321N1 cells) activated an ionic current that was partially blocked by carbenoxolone (a Px1 blocker) [18]. In macrophages, P2X7 receptor stimulation with 3 mM ATP induced cell death and activated a dye uptake pathway that was sensitive to Px1 blockade [19]. These studies suggest that Px1 may be the 'large pore' of the P2X7 receptor and contribute to ATP-induced cell death. Studies have also demonstrated that open hemichannels in ischemia dramatically alter the permeability properties of membranes and lead to cell death through ionic dysregulation, loss of metabolites, and changes in intracellular ATP [20].

ATP and glutamate are released *via* glial hemichannels in neurodegenerative conditions and it is expected their contribution to neurotoxicity [21]. The finding that P2X(7) pores may directly mediate efflux of cytosolic glutamate, GABA, and ATP in glial cells is particularly interesting, as it provides a novel mechanism of glial transmitter release that may play important roles not only in physiological intercellular communication but also in pathological neural injury. Sustained activation of P2X7 receptors may be one of the important factors causing secondary injury following neural trauma, ischemia, or demyelinating disorders. Indeed, inhibition of P2X7 receptors reduced the excitotoxicity-based neuronal degeneration and led to an improvement in functional recovery after spinal cord injury [22]. Thus, the blockade of hemichannels expressed by glial cells and/or neurons during neuroinflammation might prevent neurodegeneration. Another study has shown that cultured astrocytes are able to release UTP either at rest or following hypoxia and that $P2Y_2$ receptor mRNA increased by 2-fold during oxygen and glucose deprivation (OGD) [23].

ECTONUCLEOTIDASES

Extracellular nucleotide and nucleoside levels in the synaptic cleft are controlled by a sophisticated and complex enzyme cascade constituted by cell surface-located proteins named ectonucleotidases. These enzymes hydrolyze nucleoside triphosphates, diphosphates, and monophosphates to their respective nucleosides and inorganic phosphate. Four major families of ectonucleotidases have been already described: ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), alkaline phosphatases, and ecto-5'-nucleotidase. The general aspects of these enzyme families, their implications in neurological disorders as potential targets for drug discovery, and the inhibitors described were presented in this review (Fig. **1**).

E-NPPs

The E-NPP (ecto-nucleotide pyrophosphatase/phosphodiesterases) members constitute a protein family, which is capable of hydrolyzing pyrophosphate and phosphodiester bonds in a wide range of substrates, such as nucleic acids, nucleotide sugars, (di) nucleotides, as well as in choline phosphate esters, and lysophospholipids [24, 25]. Evidence suggests that E-NPPs play multiple physiological roles, including nucleotide recycling, modulation of purinergic receptor signaling, regulation of extracellular pyrophosphate levels, stimulation of cell motility, regulation of insulin receptor (IR) signaling, and activity of ecto-kinases [24].

This enzyme family may be the first step of enzymatic pathway responsible for the catabolism of extracellular dinucleotides, in which adenosine and other nucleosides are the final products that are recovered by nucleoside transport systems. Seven distinct NPP-encoding genes have been described, but only three NPPs (NPP1-3) show significant (40-50%) sequence similarities at the protein level. These members are classified as type II transmembrane glycoproteins and show an intracellular N-terminal domain, a single transmembrane domain and a large extracellular domain [26]. Studies have demonstrated that this extracellular domain is composed of two somatomedin-B-like homodimerization motifs, a catalytic domain, and a putative C-terminal "EF-hand" motif [27]. NPP activity has divalent cation dependence, strong alkaline pH optimum and is inhibited by glycosaminoglycans. The K_M for the hydrolysis of synthetic substrate p-Nph-5'-TMP is in the micromolar range and ATP, ADP, and AMP competitively inhibited p-Nph-5'-TMP hydrolysis [28].

NPP1 (plasma cell differentiation antigen-1, PC-1) is expressed in the capillaries of the brain [29] and in rat C6 glioma cells [30], whereas it is not detected in neurons or glia [24]. There is a correlation between the up-regulated expression of NPP1 and the grade of the astrocytic tumor [31]. NPP2 (autotoxin, ATX, autocrine motility factor) is a secreted lysophospholipase D that hydrolyzes albumin-bound or membrane-derived lysophosphatidylcholine (LPC) to produce equimolar amounts of lysophosphatidic acid (LPA) and choline [32]. A splice variant of NPP2a has been identified during intermediate stages of rat brain oligodendrocyte differentiation and myelin formation [24]. The production of LPA by NPP2 might be important for cerebral maturation. In addition, the mRNA NPP2 expression level was detected in the hippocampus, cerebral cortex, olfactory bulb, cerebellum, and striatum throughout the development [33]. Exacerbated NPP2 expression is associated with a wide range of human diseases, such as cancer, Hodgkin's lymphoma, follicular lymphoma, glioblastoma, and fibrosis [34-36]. NPP3 ($gp130^{RB13-6}$, B10, phosphodiesterase 1β) is expressed in choroid-plexus epithelial cells [37] and might contribute to the secretion of cerebral spinal fluid. Studies have shown the elevated expression of NPP3 in solid Walker 256 mammary tumor [38] and in immature astrocytes [39]. NPP1 mRNA increases through maturation, whereas NPP3 has been demonstrated to decrease with age [33]. Studies have demonstrated E-NPP

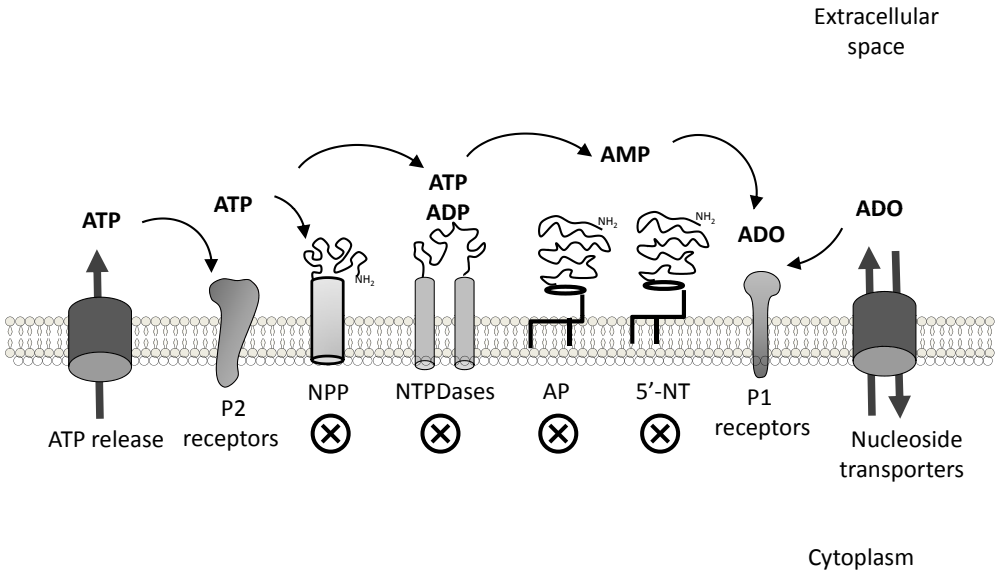

Fig. (1). Schematic representation of purinergic signaling and extracellular nucleotide-metabolizing enzymes. ATP can be released through several mechanisms (damaged cells, ATP binding cassette transporters, hemichannels, P2X7 receptors, or volume-sensitive chloride channels) in the synaptic cleft and exert its effects through P2 receptors. ATP signaling is terminated by ectonucleotidases (NPPs, NTPDases, alkaline phosphatases and 5'-nucleotidase), producing adenosine, which can act by P1 receptors. Adenosine might be also released by equilibrative and/or concentrative nucleoside transporters. The symbol ⊗ indicates potential targets for the development of drugs able to modulate nucleotide and nucleoside levels and consequently, for controlling purinergic signaling. Abbreviations: ADO, adenosine; AP, alkaline phosphatases; 5'-NT, 5'-nucleotidase; NPP, nucleotide pyrophosphatase/phosphodiesterase; NTPDases, nucleoside triphosphate diphosphohydrolases.

activity (also named Thiamine pyrophosphatase, TPPase) is associated with the neuropil, particularly in the caudoputamen, hypothalamus, and the middle cerebral peduncle [40].

The expression level of the NPP2 gene was significantly higher in Alzheimer-type dementia (ATD) cortices than in non-ATD cortices [41]. As the level of NPP expression is increased in membranes of aged rat brains (NPP1) [42], the inhibition of those enzymes has been proposed as a novel alternative for targeting neurodegenerative diseases [43]. However, NPP inhibitors have been scarcely reported (Table **1**). E-NPPP2 has been considered as an attractive therapeutic target, and studies have been performed in order to develop a selective potent inhibitor of ATX [44, 45]. Suramin (250 μM) was able to reduce the level of *p*-Nph-5′-TMP hydrolysis promoted by NPP activity [46]. However, suramin and its analogues antagonize most P2 receptors and inhibit NTPDases, not being considered specific NPP inhibitors [47]. Biscoumarin and 1, 3, 4-Oxadiazole-2(3H)-thione and their analogues were described as noncompetitive inhibitors of snake venom and human NPP1 enzymes [48, 49]. Eliahu *et al*. [50] designed diadenosine polyphosphonate derivatives as potential NPP inhibitors and one of these analogues was described as specific inhibitor of NPP1, with no activity on NPP3, NTPDase1, -2, -3, and -8, as well as ecto-5′-nucleotidase, and no significant activity toward the $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ receptors. Therefore, NPP specific inhibitors that do not alter the kinetic behavior of NTPDases and ecto-5′-nucleotidase, and not influence P2 receptor activation would be helpful as therapeutic agents for the treatment of pathological conditions, such as neurological disorders and cancer.

Alkaline Phosphatases

Alkaline phosphatases (APs) are non-specific phosphomonoesterases that release inorganic phosphate from several organic compounds and degrade nucleoside 5'-triphosphates, diphosphates, and monophosphates. The mammalian APs have higher specific activity and K_M values when compared to other species. These enzymes are membrane-bound and present an optimal activity at more alkaline pH values, being inhibited by L-amino acids and peptides through an uncompetitive mechanism [51].

The tissue nonspecific alkaline phosphatase (TNAP) is anchored to the plasma membrane through a glycosylphosphatidylinositol group and is absent in the rodent brain, except for its association with endothelia of brain capillaries [52]. Some TNAP functions are well known, such as playing an essential role in osteogenesis, but its role in the central nervous system remains unknown. Studies have demonstrated that the most prominent catalytic activity of TNAP is associated with blood vessels, the choroid plexus, and the meninges [40]. It has also been demonstrated that TNAP is found in the neuropil of many brain regions, including the olfactory bulb, septum, thalamus and hypothalamus, cerebral cortex, inferior and superior colliculi, tegmentum, and dorsal and ventral medulla [40]. AP activity detected in brain tissues results from the expression of TNAP both in endothelial and neuronal cells. TNAP is involved in the promotion of the neurotoxicity induced by extracellular tau, which contributes to the development of Alzheimer's disease (AD). Intracellular phospho-tau is released in the extracellular space and is dephosphorylated by extracellular TNAP anchored in the

Table 1. Compounds with Potential Inhibitory Effects on Ectonucleotidases

Target	Inhibitor	IC50 (μM Range)	Ki (μM Range)	Reference
	1, 3, 4-Oxadiazole-2(3H)-thiones 1,3,4-thiadiazole-2 (3H)-thiones	368 66.47	360 100	[48]
	Biscoumarin derivatives	164 to > 1000	50 to 1000	[49]
	Diadenosine 5',5"- (boranated) polyphosphonate analogues	15-60	10-50	[50]
NPP2	HA130 (Boronic acid-based inhibitor)	~0.030	ND	[44]
	Suramin	250	ND	[46]
TNAP	Thiopheno-imidazo [2, 1-b] thiazole derivatives	42- 800	ND	[56]
	Arylsulfonamides	0.19-3.16	ND	[57]
	Benzo[b]thiophene derivatives	ND	85-135	[58]
	Levamisole	93	14	[58, 59]
NTPDases	Carbamazepine	20	10	[97]
	Azide	2,000-10,000	ND	[98]
	Gadolinium	3-28	29-39	[100]
	Polyoxometalates (PV4)	0.14-0.9	ND	[101]
	ARL67156	24	11-18	[102, 103]
	P2 receptor antagonists Suramin NF279 NF449 Reactive Blue	 13-300 ND ND ND	 4.3- 24 0.3-5.2 9.6->100 2.1- >100	[47]
	Uridine-5′-carboxamide derivatives	42-374	3-786	[104]
	BGO136	ND	70-150	[106, 107]
Ecto-5'-nucleotidase	APCP	0.43	0.73	[125, 126]
	Anthraquinone derivatives	ND	0.150-0.260	[127]

ND=not determined.

Abbreviations: APCP, alpha,beta-methylene adenosine-5'-diphosphate; ARL 67156, (6-N,N-diethyl-D-β,γ-dibromomethylene); BGO 136, (1-hydroxynaphthalene-3,6-disulfonate); 8-BuS-ATP, 8-thiobutyladenosine 5'-triphosphate; PV4 [hexapotassium dihydrogen monotitanoun-decatungstocobaltate(II) tridecahydrate]; suramin [8-(3-benzamido-4-methylbenzamido)naphthalene-1,3,5-trisulfonate].

cellular membrane. Extracellular dephosphorylated tau is able to interact with muscarinic receptors M1 and M3 located on the surface of neighboring cells, inducing their death [53]. Recent studies have demonstrated that TNAP is enhanced in hippocampus from both sporadic and familial AD but not in the aged brain, suggesting that such effect is related to the alterations induced by this disease. In addition, an increase in plasma TNAP was observed in AD and this change is inversely correlated with cognitive function [54]. Mice lacking TNAP at approximately two weeks after birth generated by homologous recombination develop seizures which can be fatal. Defective metabolism of pyridoxal 5'-phosphate (PLP), characterized by elevated serum PLP levels, results in reduced levels of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) in the brain [55].

Studies have described TNAP inhibitors as potential therapeutic agents to target several diseases involving soft tissue calcification, such as thiopheno-imidazo [2, 1-b] thiazole derivatives [56], arylsulfonamides [57], and benzo [b] thiophenes [58]. However, specific inhibitors for AP in central nervous system have not been described until now. Studies have shown that the hydrolysis of ATP to adenosine in neuron-glia signaling is inhibited by levamisole, a TNAP inhibitor, but not by ARL67156, an ecto-ATPase inhibitor, in olfactory bulb [59], suggesting that TNAP is physiologically relevant in nucleotide-mediated signaling (Table **1**).

E-NTPDases

The protein family of ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDase, CD39) hydrolyzes nucleoside 5'-triphosphates and nucleoside 5'-diphosphates with wide preference, different tissue distribution, and cellular location [60]. The GDA1_CD39 superfamily comprises NTPDases with common motifs in their protein sequences and this family was named due to two proteins: the yeast GDPase (GDA1) and a lymphoid cell activation antigen, CD39 [61]. In this review, we highlight ecto-NTPDases (E-NTPDases), present on the cell surface and involved in the control of the purinergic signaling. All cell-surface members of E-NTPDase family are highly glycosylated proteins with molecular masses ~70-80 kDa,

which show close immunological cross-reactivity and may exist either in monomeric or in higher homooligomeric (dimeric to tetrameric) states [62, 63]. These enzymes contain two predicted transmembrane domains at the N- and C-terminus with a large extracellular loop containing a more central hydrophobic region with five highly conserved sequence domains known as "apyrase conserved regions" (ACR). ACR1 and ACR4 share common sequence homology with members of the actin/ HSP70/sugar kinase superfamily [60, 64].

E-NTPDases have an alkaline optimum pH and millimolar concentrations of either Ca^{2+} or Mg^{2+} can stimulate the catalytic activity of this enzyme family [65]. However, the literature relates a range of physiological or pathological stimuli capable of modifying the E-NTPDase activity [6]. These stimuli are critical in mammalian central nervous system (CNS), and their responses frequently vary throughout development. Examples of events that evoke these modifications include seizures and epilepsy [66, 67], hormonal alterations [68, 69], stress [70], and nociceptive response [71, 72].

According to the current nomenclature, at least eight different members of the NTPDase family (NTPDases 1-8) have been discovered, cloned, and functionally characterized [60, 61]. NTPDase1-3 and 8 possess similar amino acid sequences and membrane topology with two membrane spanning domains and catalytic sites facing the extracellular milieu [73]. NTPDase1 hydrolyses ATP and ADP equally well, while NTPDases3 and 8 prefer to hydrolyze ATP over ADP. NTPDase 2 has high hydrolyzing activity toward nucleoside triphosphates [60]. Histochemical investigations demonstrated the hydrolysis of nucleoside tri- and diphosphates in all cell types of the nervous system [74]. E-NTPDases1, 2, and 3 are expressed in the mammalian brain and mediate the termination of ATP signaling in the synaptic cleft [65, 75].

E-NTPDase1 is localized at the surface of endothelial vessels in the CNS and is strongly expressed in microglia [76]. E-NTPDase2 is associated with progenitor cells in the adult rodent brain [77, 78] and is expressed in muscularised vessels [79], cultured astrocytes [80], non-myelating Schwann cells, and other glial cells of central and peripheral nervous systems [60]. E-NTPDase3 is expressed in multiple brain regions and at the surface of PC12 cells as the predominant ectonucleotidase [81]. Immunohistochemistry studies have revealed that this enzyme is strongly associated with axon-like neuronal structures in the brain, where it may act as a pre-synaptic regulator of extracellular ATP levels, and coordinates multiple homeostatic systems, including feeding and sleep-wake behaviors [82]. E-NTPDase8 expression is very low in the brain and is highly expressed in the liver, kidney, and jejunum [83].

The involvement of NTPDase activities in memory processing has been demonstrated. A decrease in the ATP and ADP hydrolysis was observed in hippocampal synaptosomes of rats trained and sacrificed immediately after and 180 min after training in an inhibitory avoidance task, but only ATP hydrolysis remained inhibited at 30 minutes after training [84, 85]. These results indicate that these ecto-enzymes may be involved in mechanisms of acquisition and modulation of memory processing. Changes in ATP and ADP hydrolysis were observed in other brain structures, such as entorhinal cortex, anterior and posterior cingulate cortex, and medial precentral area after training in an inhibitory avoidance task [85, 86]. When other forms of memory were evaluated, there were significant changes in NTPDase activities immediately after and 60 min after the test session in an open field task, without changes after the training session [87]. This study has revealed the involvement of ectonucleotidase activities in different learning paradigms during memory processing.

There is evidence demonstrating NTPDase involvement in neurodegenerative disorders. It has been shown changes in extracellular nucleotide hydrolysis from striatal slices at 4 weeks after the induction of 6-hydroxydopamine model of Parkinson's disease [88]. Most studies have indicated that NTPDase activities may be involved in epileptogenesis mechanism. A reduced ATP hydrolysis has been observed in the rat cerebral cortex during prolonged *Status epilepticus* (SE) induced by the sequential administration of lithium and pilocarpine [89]. However, extracellular ATP and ADP hydrolysis significantly increased at 48-52 h, 7-9 days, and 45-50 days in synaptosomes from rat hippocampus and cerebral cortex after the induction of SE by pilocarpine or kainic acid [90]. These findings suggest that an important adaptive plasticity of the ectonucleotidase pathway could occur to decrease ATP levels, which is an excitatory neurotransmitter, and increase adenosine levels, a neuroprotective compound. Furthermore, rats showing greater resistance to Pentylenetetrazole (PTZ) kindling presented increased ATP hydrolysis in hippocampal and cerebral cortex synaptosomes [91]. A marked enhancement of adenine and guanine nucleotide hydrolysis was also observed in rat cerebrospinal fluid after PTZ-kindling [92]. Changes in ectonucleotidase activities were not seen after a single convulsant PTZ injection at any given time (immediately, 1h, 24 h, and 5 days). Similarly, there were no differences in ATP, ADP, and AMP hydrolysis immediately after a single PTZ exposure (for 20 min) in zebrafish [93]. These findings corroborate the hypothesis that recurrent seizures could lead to late and prolonged changes in ectonucleotidase activities as a consequence of an adaptive plasticity induced by recurrent seizures. A recent study has shown memory impairment in adult rats previously submitted to one single seizure episode in neonatal period, which is accompanied by an increased ATP hydrolysis in hippocampal synaptosomes [94].

Classical antiepileptic drugs (AEDs) may modulate purinergic signalling in CNS. Phenytoin and carbamazepine can prevent the increase in ectonucleotidase activities elicited by pilocarpine in brain synaptosomes, but only sodium valproate can inhibit the increase of ATP and ADP hydrolysis in hippocampal synaptosomes [95]. Carbamazepine decreased ATP hydrolysis at 1000 μM in zebrafish brain membranes [96], suggesting that AED-induced effects on ectonucleotidases are related to anchorage form. Such findings are in agreement with previous results, demonstrating that carbamazepine can inhibit *in vitro* ecto-ATPase activity in rat brain synaptosomal plasma membranes [97].

The complexity of NTPDase family has impaired the development of specific inhibitors for these enzymes. Some

NTPDase inhibitors have been described and characterized, such as azide [98], 8-thiobutyladenosine 5'-triphosphate (8-BuS-ATP) [99], gadolinium [100], polyoxometalates (PV4) [101], ARL67156 [102,103], P2 receptor antagonists [47], Uridine-5′-carboxamide derivatives [104], specific NTPDase3 monoclonal antibodies (hN3-B3s and hN3-H10s) [105] and BGO136, which is a new and selective NTPDase 1 and 2 blocker recently commercially available [106, 107] (Table **1**). However, the lack of specific inhibitors for each NTPDase family member does not allow the determination of their precise function and makes the drug design difficult, using these enzymes as pharmacological targets.

Several other compounds have induced modulatory effects on NTPDase activities, but there is no evidence about their specificity for each member of this enzyme family. Several classical drugs used in the therapy for neuropsychiatric disorders have been shown to alter NTPDase activities, such as antipsychotics [108], antidepressants [109], and anxiolytics [97, 110].

Ecto-5'-Nucleotidase

Ecto-5'-nucleotidase (lymphocyte surface protein CD73; E.C. 3.1.3.5) is a glycosylphosphatidylinositol (GPI)-anchored enzyme that catalyzes the hydrolysis of the phosphoric ester bond of 5'-ribonucleotides to the corresponding ribonucleoside and phosphate [111, 112]. AMP is the most successfully hydrolyzed nucleotide, since the K_M values are in the low micromolar range (50 μM). One of the most important actions of ecto-5'-nucleotidase is the formation of adenosine from extracellular AMP and the subsequent activation of P1 adenosine receptors [5]. This enzyme works in an orchestrated manner, finishing the nucleotide signaling, which is mediated by activation of P2X and P2Y receptors. Like other surface-located enzymes, ecto-5'-nucleotidase has been implicated in non-enzymatic functions such as T-cell activation and cell-cell adhesion [113]. Ecto-5'-nucleotidase is abundant in synaptic membranes from hippocampus, cerebellum, and medulla oblongata [114]. In addition, there is evidence that this enzyme is transiently active within synaptic clefts during development and regeneration [40, 115].

Alterations were also observed for ecto-5'-nucleotidase activity from CNS in pathotological and physiological situations, indicating the relevance of this enzyme for brain pathophysiology [6, 67]. Age-related alterations were also observed for ecto-5'-nucleotidase activity from CNS. For example, ecto-5'-nucleotidase activity was 5-fold higher in the hippocampus of aged rats compared to young rats [116]. Furthermore, there was a significant age-related increase in ecto-5′-nucleotidase activity and expression in regions containing the vertical and horizontal limb of the diagonal band of Broca, ventrolateral preoptic area, tuberomammillary nucleus, dorsal raphe and locus coeruleus as well as in the cerebral cortex of old and intermediate-aged rats [117]. It has been demonstrated that striatal adenine- and guanine-based purine hydrolysis is increased in a 6-hydroxydopamine rat model of Parkinson's disease with a 25% decrease in ecto-5'-nucleotidase activity [88].

Previous studies have demonstrated that ecto-5´-nucleotidase activity in synaptosomes of the rat hippocampus and cerebral cortex significantly increased at 48-52 h, 7-9 days and 45-50 days after the induction of SE by pilocarpine or kainic acid models [90]. However, only ecto-5´-nucleotidase activity remained elevated at 100-110 days after treatment with kainic acid [90]. There were no changes in AMP hydrolysis in female and male rats subjected to pilocarpine model at different ages, even though a significant increase in AMP hydrolysis (71%) was observed in cerebral cortex synaptosomes from male rats at 27-30 days [66]. The different sensitivity of developing rats may be related to the immaturity of neuronal networks engaged in the generation and spread of seizure activity. Increased ecto-5´-nucleotidase staining in the hippocampus during the silent and chronic phases has also been shown [118]. The presence of 5´-nucleotidase in mossy fibers of the rat dentate gyrus has been detected after kainate injection and induction of kindling [119]. Studies have demonstrated that phenytoin *in vitro* increased AMP hydrolysis at 500 μM (65%) and 1000 μM (64.8%) in zebrafish brain membranes [96]. In addition, carbamazepine and phenytoin can prevent the pilocarpine-induced increase on AMP hydrolysis in synaptosomes from hippocampus and cerebral cortex [95].

Other models of brain damage, such as the rat model of cortical stab injury (CSI), induced biphasic changes in ecto-5'-nucleotidase activity, promoting an immediate decrease in this activity [120], followed by an up-regulation of the expression and activity of this enzyme during 2 weeks after the injury [121]. The loss of the enzyme from neuronal membranes accounted for a rapid decrease in the total protein level, whereas an increase in ecto-5'-nucleotidase-positive astrocytes in the damaged brain area are directly related to the increase in total protein levels [122].

The participation of ecto-5'-nucleotidase as a factor involved in the control of cell proliferation and adhesion has been previously proposed [123, 124]. Treatment with 1μM APCP, a competitive ecto-5'-NT/CD73 inhibitor [125, 126], caused a significant reduction of 30% in glioma cell proliferation [123]. Ecto-5'-nucleotidase inhibitors have potential as novel drugs and a set of 55 anthraquinone derivatives has been developed as inhibitors of this enzyme with a competitive inhibition mechanism [127] (Table **1**). Inhibition of ecto-5'-nucleotidase has been also induced by natural compounds or plant extracts, such as *Casearia sylvestris* [128], *Ilex paraguariensis* [129], and quercetin [130].

NUCLEOSIDE TRANSPORTERS

Adenosine can be produced from extracellular ATP hydrolysis promoted by ectonucleotidase pathway or be released *per se* through nucleoside transporters. This transport is mediated by bidirectional equilibrative processes driven by chemical gradients and unidirectional concentrative processes driven by sodium (and proton) electrochemical gradients, which are distributed in the brain [131, 132]. Nucleoside transporters are able to regulate synaptic levels of purinergic messengers and, consequently, influence physiological processes [133, 134].

Four types of equilibrative nucleoside transporters (ENT) have been characterized: ENT1, ENT2, ENT3, and ENT4. ENT1 and ENT2 are the well characterized transporters and

appear to be present on all cell types, including neurons and glia [131]. These transporters are directly involved in the cellular influx or efflux of adenosine, with the direction of movement dependent upon the extracellular and intracellular concentrations of this nucleoside [132]. hENT1 and hENT2 transport purine and pyrimidine nucleosides and show different sensitivities to inhibition by nitrobenzylmercapto-purine ribonucleoside (NBMPR), because hENT1 is NBMPR-sensitive [135]. It has been demonstrate that recombinant human NT2 and rat ENT2 transport purine and pyrimidine bases [135]. Human ENT1 also transports thymine and adenine and, to a lesser extent, uracil and guanine [136]. ENT3 and ENT4 are less studied and have low affinity for adenosine transport [137].

There is evidence that ENT1 allelic variations could be linked to a genetic risk in human alcoholism [138]. Homozygous transgenic mice with neuronal expression of human equilibrative nucleoside transporter 1 (hENT1) presented increased ENT1 gene expression and protein abundance relative to heterozygous. Transgenic mice showed a greater response to ethanol and a reduced response to caffeine than wild type littermates [139]. The absence of ENT1 is associated with reduced anxiety-like behavior in mice as well as the microinjection of NBMPR, a specific ENT1 inhibitor, into the amygdala of mice also reduced anxiety-like behavior, suggesting that the phenotype of reduced anxiety-like behavior in ENT1 null mice is due to absence of ENT1 in the adult brain [140]. Neuronal ENTs decrease adenosine levels after hypoxia and ischemia, which indicates that adenosine transporters may be a target for the treatment of cerebral ischemia [141]. In addition, decreased ENT1 expression in the superior temporal gyrus has been observed in patients with schizophrenia [142].

The therapeutic utility of dipyridamole, a potent ENT inhibitor, is impaired by binding to the serum protein α_1-acid glycoprotein (AGP). Recently, it has been demonstrated the effect of prodrugs, such as monophenyl carbamate and mono-4-methoxyphenyl carbamate, that exhibited the best ENT-inhibitory activity and reduced AGP binding relative to dipyridamole [143]. Recently, a new drug able to activate adenosinergic system named N (6)-(4-hydroxybenzyl) adenine riboside (designated T1-11) was developed, which activates the adenosine A_{2A} receptor and ENT1 [144]. This dual function drug activates adenosinergic system and delay the progression of Huntington disease [144].

Three members of the sodium-coupled CNT have been cloned and characterized: CNT1, CNT2 (also called the sodium-dependent purine nucleoside transporter SPNT), and CNT3, that differ in their cation coupling, stoichiometries and tissue distribution [137, 145]. These membrane-bound proteins transport nucleosides and nucleoside analogs actively into cells by coupling transport to the inwardly directed sodium gradient [145, 146]. There is no known disease states associated with this family of nucleoside transporters. Up to the present, there is no characterization of potent inhibitors of the CNT family, but antibodies against the CNT2 transporter have been developed [146]. The mRNA of high-affinity Na^+-dependent purine nucleoside transporter CNT2 is widespread in rat brain including the striatum and it has been also reported the presence of CNTs on synaptic vesicles [147]. A possible role of CNT2 in the control of adenosine concentrations in the sleep wakefulness cycle has been suggested, since a decrease in CNT2 mRNA levels was induced by sleep deprivation [148].

Several studies have already described that nucleoside transporter drugs and inhibitors constitute a relevant contribution as therapeutical alternatives for cancer and protozoan parasitoses [131]; however, their use for CNS disorders remains unclear.

CONCLUDING REMARKS

The co-existence of several mechanisms able to promote a fine-tuning regulation of purinergic messenger levels reinforces the influence of these molecules in signaling pathways in physiological conditions. However, there is growing evidence that these control mechanisms are altered in pathological conditions, especially in neurological disorders. The presence of several mechanisms involved in ATP release and the mapping of possible changes induced by neurodegenerative and psychiatric disorders will allow a better understanding about the impact of nucleotide release in the progression of such diseases. This aspect of purinergic signaling deserves more attention and may represent a promising field for investigation for new pharmacological targets. Moreover, the ectonucleotidase pathway is a sophisticated route that plays a role managing the appearance and inactivation of extracellular agonists for purinoceptors on the cell surface. Modulation of this enzyme pathway in memory processing, aging, seizures, epilepsy, Parkinson's disease, and cancer indicate that these enzymes may play a role in these processes as well as in the progression of the neurological disorders. Therefore, identifying changes induced by neurological disorders and the exact mechanisms by which these enzymes regulate local nucleotide and nucleoside concentrations will be an area of intense interest in the future. As mentioned above, some compounds are able to inhibit these enzyme activities. The presence of several members in ectonucleotidase family with a high structural similarity make the development of specific inhibitors for each member difficult, which represents a limitation to be overcome for the drug design targeting these enzyme activities. The action of these enzymes promotes the extracellular ATP degradation and the consequent production of adenosine, which is a nucleoside with a wide spectrum of functions triggered by the activation of P1 purinoceptors. The control of adenosine levels might be also promoted by nucleoside transporters; however, there is no clear evidence about the contribution of these transporters in the development of CNS disorders. Up to the present moment, it is possible to assume that these studies have generated a lot of hypotheses though a few certainties. On the other hand, such studies may open up further research to assess the potential therapeutic and diagnostic applications for the mechanisms of appearance and inactivation of purinergic messengers and clearly suggest a more active role in neurological disorders than it was previously suspected.

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

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