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REVIEW ARTICLE

Role of Ectonucleotidases in Synapse Formation During Brain Development: Physiological and Pathological Implications

Ivana Grković*, Dunja Drakulić, Jelena Martinović and Nataša Mitrović

Department of Molecular Biology and Endocrinology, VINČA Institute of Nuclear Sciences, University of Belgrade, Mike Petrovića Alasa 12-14, 11001 Belgrade, Serbia

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Abstract: ***Background*:** Extracellular adenine nucleotides and nucleosides, such as ATP and adenosine, are among the most recently identified and least investigated diffusible signaling factors that contribute to the structural and functional remodeling of the brain, both during embryonic and postnatal development. Their levels in the extracellular milieu are tightly controlled by various ectonucleotidases: ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPP), alkaline phosphatases (AP), ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) and ecto-5'-nucleotidase (eN).

***Methods*:** Studies related to the expression patterns of ectonucleotidases and their known features during brain development are reviewed, highlighting involvement of these enzymes in synapse formation and maturation in physiological as well as in pathological states.

***Results*:** During brain development and in adulthood all ectonucleotidases have diverse expression pattern, cell specific localization and function. NPPs are expressed at early embryonic days, but the expression of NPP3 is reduced and restricted to ependymal area in adult brain. NTPDase2 is dominant ectonucleotidase existing in the progenitor cells as well as main astrocytic NTPDase in the adult brain, while NTPDase3 is fully expressed after third postnatal week, almost exclusively on varicose fibers. Specific brain AP is functionally associated with synapse formation and this enzyme is sufficient for adenosine production during neurite growth and peak of synaptogenesis. eN is transiently associated with synapses during synaptogenesis, however in adult brain it is more glial than neuronal enzyme.

***Conclusion*:** Control of extracellular adenine nucleotide levels by ectonucleotidases are important for understanding the role of purinergic signaling in developing tissues and potential targets in developmental disorders such as autism.

Keywords: Brain development, ectonucleotidases, NPP, TNAP, NTPDase, ecto-5'-nucleotidase, synaptogenesis, autism.

1. INTRODUCTION

Central nervous system (CNS) development is an intricate process regulated by complex cellular systems, including extracellular matrix molecules, multiple classes of diffusible ligands and numerous receptors and co-receptor that trigger distinct signaling cascades, as well as an epigenetic gene regulation modulating developmental switches. The formation of the functional and mature CNS comprises the proliferation of early progenitors, the migration and the differentiation into neuronal cell types, including the neurite outgrowth, and finally, synaptogenesis, thus establishing highly specific connections with their target cells. Extracellular purines are among the most recently identified and least investigated diffusible signaling factors that contribute to the structural and functional remodeling of the brain, both during development and adulthood. Hence, purinergic signaling is shown to be important during neurogenesis and all cellular processes throughout development, including regulation of proliferation, differentiation and cell death and cell fate determination [1-10]. Thus, the ubiquitous purinergic signaling molecule adenosine 5'-triphosphate (ATP), a phylogenetically most ancient epigenetic factor, exerts long-term trophic effects essential for embryonic development as well as for all stages necessary for fully development and maturation of CNS. Its actions are accomplished due to control of growth and development of neural circuits [8, 11-15] and plastic

*Address correspondence to this author at the Department of Molecular Biology and Endocrinology, University of Belgrade -VINČA Institute of Nuclear Sciences, Mike Petrovića Alasa 12-14, 11001 Belgrade, Serbia;
E-mail: istanojevic@vin.bg.ac.rs

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remodeling and regeneration of the nervous system [1, 7, 16-19]. ATP is probably present in every synaptic and/or secretory vesicle at different concentrations, and can be co-stored and co-released with other neurotransmitters (glutamate, g-aminobutyric acid [GABA], noradrenaline) thus exerts short-time actions as a fast excitatory neurotransmitter and neuromodulator controlling excitatory and inhibitory synapses [11, 20-23]. It also acts as a widespread gliotransmitter. Release of ATP from astrocytes, *via* exocytosis or through membrane channels (connexins, volume-regulated Cl^- channels or P2X7 receptors), is important for neuron-glial and glial-glial communications [23, 24]. Extracellular ATP exerts biological functions by activation of purinergic P2 receptors broadly expressed by almost every cell type of CNS and involved in multiple physiological functions [9, 11, 13-15, 23, 25, 26]. It is inherently short-lived molecule which undergoes rapid enzymatic degradation by ectonucleotidases [27-30]. Ectonucleotidases include ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPP), alkaline phosphatases (APs), ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) and ecto-5'-nucleotidase (eN), which differ in functional and molecular properties [1, 27, 28, 30]. Members of all families hydrolyze extracellular adenine nucleotides and are expressed in the brain. The physiological impact of ectonucleotidases in the control of neural, glial, and vascular function has been demonstrated in numerous experiments. These enzymes are responsible for the tight control and modulation of ligand availability at P2 receptors, duration and degree of their activation, thus preventing their desensitization or downregulation [11]. They may hydrolyze ATP as agonist of P2 receptors, generating ADP as agonist of nucleoside diphosphate-sensitive P2Y receptors [11, 27]. Further, a final product of ATP enzymatic degradation is adenosine, potent signaling molecule, homeostatic regulator and modulator of neural activity [31] that affects neurotransmission, synaptic plasticity, learning and memory [15, 32-35] by acting at inhibitory A1 and facilitatory A2A adenosine receptors. Adenosine also participates in developmental processes, including cell proliferation, immature cell migration, differentiation and maturation of progenitor cells, neurite outgrowth, synapse formation and apoptosis [1, 15, 36, 37]. This paper is focused on the expression patterns of ectonucleotidases and their known features during CNS development, highlighting the involvement of these enzymes in synapse formation and maturation in normal physiological processes as well as in pathological states. We also considered ectonucleotidases as potential targets in autistic spectrum disorders (ADS).

2. ECTONUCLEOTIDASES DURING CNS DEVELOPMENT

2.1. Ecto-nucleotide Pyrophosphatase/Phosphodiesterase Family

Three members of E-NPP family (NPP1-3) are localized in the CNS (Fig. **1**), where in alkaline pH hydrolyze pyrophosphate or phosphodiester bonds in a variety of extracellular compounds including nucleotides, (lyso)phospholipids and choline phosphate esters, acting in synergy to modulate purinergic signaling [27, 38-40]. These enzymes in association with NTPDases and eN are able to promote the complete hydrolysis of dinucleotides to adenosine in the synaptic cleft [41]. NPP1-3 also hydrolyze 5′-monodiester bonds in extracellular ATP, resulting in the release of AMP and inorganic pyrophosphate (PPi) [38, 39]. NPP1 is widely spread in the adult rat forebrain, particularly in midline regions of hypothalamus and thalamus [42, 43], and is indicated as the main ectoenzyme involved in the cleavage of diadenosine

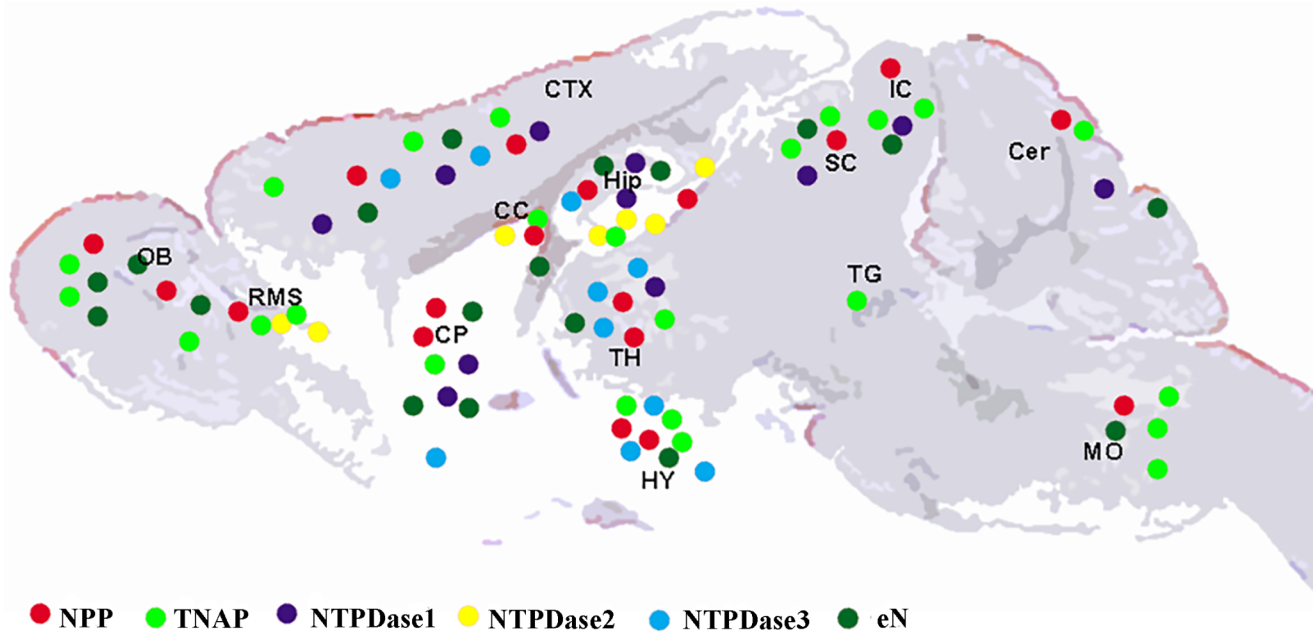

Fig. (1). Regional distribution of ectonucleotidases in the adult rodent brain. OB, olfactory bulb; RMS, rostral migratory stream; Ctx, cortex; Hip, hippocampus; CC, corpus callosum; CP, caudoputamen; TH, thalamus; HY, hypothalamus; SC, superior colliculus; IC, inferior colliculus; TG, tegmentum; Cer, cerebellum; MO, medulla oblongata. (*The color version of the figure is available in the electronic copy of the article*).

polyphosphates by glial cells and neurons [44]. It is also expressed by ependymal cell, endothelial cells and together with NPP2 in the cells of the choroid plexus [42, 45], where most likely modulates purinergic signaling that contributes to the composition and secretion of cerebral spinal fluid [46]. Immunolabeling for NPP3 is restricted to the ependymal cells of ventricular system [45].

Analysis of NPP1 expression pattern at critical stages during postnatal brain development (postnatal days, PD7, 14, 21, 60) shows an age-related NPP1-mRNA increase in the olfactory bulb, cerebral cortex, striatum, and cerebellum, except in the hippocampus, where the highest expression is detected at PD14 [47]. NPP2 was detected already at embryonic day (E) 8.5 in the anterior folds of the neural tube and at the most posterior region of the midbrain; but thereafter, expression is displaced in the floor plate of the neural tube [48]. At E13.5, NPP2 expression is dominant in mesenchymal tissues and choroid plexus epithelia [48, 49], where remains highly expressed until birth [48]. During later brain development, NPP2 is correlated with intermediate stages of rat brain oligodendrocyte differentiation and myelin formation and a brain-specific NPP2 variant (PD-Iα/ATXγ) is found almost exclusively on the oligodendrocytes [45]. It participates in the myelination by altering integrin-dependent focal adhesion assembly and consequently oligodendrocyte extracellular matrix interactions [50, 51]. NPP2 (but not NPP1 and NPP3) also targets lipids, exerting lysophospholipase D activity. The involvement of NPP2 in oligodendrocyte development may mainly result from its lysophospholipase D activity and an additional functional domain mediating the modulation of oligodendrocyte remodeling and focal adhesion organization [52], a property independent of its catalytic activity [52, 53].The importance of NPP2 in brain development is underscored in chick embryos studies, where it regulates the maintenance of the diencephalon–mesencephalon boundary and neuroepithelial cell proliferation [54]. Interestingly, the chick embryonic brain expresses a C-terminally truncated NPP2 splice variant that lacks catalytic activity. NPP2 can also produce lysophosphatidic acid (LPA), a lipid mediator important for cortical neurogenesis and pattern formation and the vascular development of immature brain [55, 56]. The NPP2-LPA signaling "axis" has been implicated in neural development [57], neuropathic pain [58], fetal hydrocephalus [59]. Fotopolou and coworkers reported that neurite outgrowth is compromised in $Enpp2^{(-/-)}$ embryo explants, and could be rescued by LPA addition [57]. $Enpp2^{(-/-)}$ mice embryos (at E8.5) show malformations in the neural tube and headfolds (*i.e*. the future forebrain) [60, 61]. At E9.5, the majority of mutant embryos had not initiated axial turning, which may reflect generally retarded development. Furthermore, NPP2-deficient embryos show defects in neural tube closure [57, 62].

Small subset of precursor cells distributed in a specific spatio-temporal pattern in the germinal layers of the ventricular zone of immature rat brain transiently expresses NPP3 during development [63]. Transfection of NPP3 into several cell lines induced cell aggregation, cell motility, and invasion into collagen I, inducing co-expression of both glial and neuronal markers [64]. Further, it may be an important factor in the process of glial cell proliferation and migration [1], and promote differentiation and invasion of glial cells [64] since it is expressed in immature astrocytes in the prenatal and neonatal rats [63]. During postnatal development, the relative mRNA expression of NPP3 begins to decline from the PD7 or PD14 depending of investigated brain structure [47]. Both NPP1 and NPP3 are expressed in Neuro-2a neuroblastoma cells and their expression levels dramatically decrease when cells differentiate into a neuronal-like phenotype. Subsequent activity assays carried out with differentiated cells showed that the dinucleotide hydrolytic activity largely depends on the NPP1 isozyme, since NPP3 protein is almost completely loss [65].

2.2. Alkaline Phosphatase Family

Members of alkaline phosphatases (APs) family degrade nucleoside 5'-tri-, -di-, and –monophosphates, releasing inorganic phosphate from a large variety of organic compounds and share optimum alkaline pH. Tissue-nonspecific alkaline phosphatase (TNAP), homodimeric protein anchored to the cytoplasmic membrane *via* glycosylphosphatidyl inositol (GPI) anchor [66, 67], represents the only isoform of APs expressed in the mammalian CNS [27, 66, 67]. In the adult CNS (Fig. **1**), TNAP is associated with the blood vessel endothelium, neuronal membranes, including axonal and dendritic processes and the synaptic cleft in the olfactory bulb, thalamus and hypothalamus, cerebral cortex, hippocampus, inferior and superior colliculi, tegmentum and dorsal and ventral medulla [68, 69]. This enzyme has been described as an ectonucleotidase, since it is able to hydrolyze all extracellular adenine nucleotides and finally produces adenosine, influencing the purinergic signaling [27]. It is implicated in the regulation of neurotransmission and metabolism of different neurotransmitters, such as GABA or serotonin as well as in developmental plasticity, activity-dependent cortical functions and homeostasis [69, 70]. Currently, little is known about the mechanism that controls TNAP expression, except that it may be induced by retinoic acid [71] and by activation of the phosphatidyl inositol 3-kinase/Akt pathway [72]. Interestingly, besides a close functional interrelation with P2X7 receptors (R) whereby TNAP prevents P2X7R activation by hydrolyzing ATP in the immediate environment of the receptor, the inhibition of P2X7R is shown to reduce TNAP expression, whereas the addition of AP enhances P2X7R expression [73].

Embryonic stem cells express the AP and react to ATP with increased proliferation [74]. TNAP also regulates the differentiation of neurons and/or oligodendrocytes *in vitro* [75]. Strong expression of TNAP in the neuroendothelial cells of the neural tube at embryonic day E8.5 [76] and in migrating primordial germ cells [77-79] indicates its contribution to early embryonic development of the CNS. Thereafter, TNAP activity is observed between the mesencephalon and the rhombencephalon, along the entire spinal cord and the cranial nerves emerging from the myelencephalon, suggesting an association with pioneer growth cones, while at E14.5, it is considerably reduced and very low in the adult brain [76]. Furthermore, a significant activity of TNAP is found around E14 in ventricular and subventricular zones (VZ and SVZ) where neural precursors are placed [80].

In the rat cerebellar cortex, a peak of AP activity is observed in the proliferative external granular cells until PD7 [81]. In the postnatal marmoset, expression of TNAP is developmentally regulated both in gray and white matter [82], while its activity exerts a complementary pattern as myelin staining and gradually disappears as myelination proceeds during the development of the white matter in the primate brain [82]. Hanics and coworkers emphasize that TNAP activity is necessary for the normal development of myelin as well for the processes regulating maturation of synaptic transmission and axonal conduction [83]. Mice lacking TNAP do not show abnormalities in brain development, but they develop fatal seizures at approximately 2 weeks after birth, which is attributed to a defective metabolism of pyridoxal 5′-phosphate and subsequent reduced levels of the inhibitory neurotransmitter GABA [84, 85].

2.3. Ecto-nucleoside Triphosphate Diphosphohydrolase Family

In the CNS, the extracellular levels of ATP are tightly regulated *via* E-NTPDase family members, which include cell surface-bound nucleoside triphosphate diphosphohydrolase1-3 (NTPDase1-3). These individual enzymes differ in several aspects, including their substrate preferences. Specifically, NTPDase1 uses ATP and ADP equally well to produce AMP, while NTPDase2 preferentially dephosphorylates ATP to ADP. NTPDase3 is the functional intermediate between the previous two, as it hydrolyzes both ATP and ADP with a molecular ratio of about 1:0.3, leading to transient accumulation of ADP [27, 86, 87]. Therefore, NTPDase2 and NTPDase3 produce agonists which act at ADP-sensitive purinoceptors, such as P2Y1, P2Y12 and P2Y13 [88]. Besides its hydrolyzing activity, NTPDase1 is reported to function as cell adhesion molecule [89].

The NTPDases have a broad distribution in the brain, although individual enzymes exhibit marked regional and cell-type specific localization (Fig. **1**). In the adult brain, NTPDase1 is widely expressed and associated with microglia, vascular endothelium and neurons of the cerebral cortex, cerebellum and hippocampus with particularly high expression in the thalamic, caudoputamen and hippocampal neuropil [68, 90-93]. Several studies reported that NTPDase2 is localized at astrocytes in different brain regions [94-97], slow proliferating SVZ precursor stem cells (type B cells), glial tube cells of the rostral migratory stream, tanycytes of the third ventricle and at neuronal precursor cells of the hippocampus [80, 94, 98].

In the rodent brain, NTPDase2 is expressed from E17 onward and is associated with progenitor cells [94, 98]. It gradually becomes expressed along the VZ and SVZ, followed by retraction to the adult expression pattern at PD21 [94]. Shortly before birth, the cortical radial glia is selectively marked by NTPDase2 [98]. In addition, NTPDase2 is selectively associated with progenitor cells of the mouse dentate gyrus, beginning with the early migration of progenitor cells into the dentate gyrus anlage during embryonic development [98]. By PD23, the immunostaining corresponds to the adult stage, when the NTPDase2-positive cells represent “residual radial glia”, the presumptive adult progenitors of hippocampal granule neurons [98]. Results of Langer and coworkers showed that NTPDase2 is barely detectable at the lateral ventricles at E18, and it is initially associated rather with the septal walls of the lateral ventricles. NTPDase2 was exclusively associated with SVZ border cells, which are also weakly stained for TNAP activity. This pattern was maintained during the first postnatal week (until PD7). Later (PD14), NTPDase2 is associated with cells that originally border the SVZ, move to the subependymal layer and finally form glial tubes (type B cells) unsheathing migrating neuroblasts, while from PD21 its expression closely corresponds to the adult stage [80].

The most restricted and exclusively neuronal localization is found for NTPDase3. In the rodent brain, somatic NTPDase3 localization is detected only in the midline regions: in the thalamus, hypothalamus, and the medulla oblongata [99, 100]. NTPDase3-expressing neuronal fibers are abundantly present in the midline regions of the brain, while scattered NTPDase3 positive axon-like processes with prominent varicosities, including both dendrites and axons, are also observed in the cerebral cortex, hippocampus and basal ganglia [99-102]. Much of the ATPase activity in the hypothalamus, hippocampus, and cortex presumably is attributable to NTPDase3, which is shown to be widely associated with hypocretin-1/orexin-A-positive neurons [99]. In the humans, NTPDase3 is detected in striatal gray matter [93].

Developmental pattern of entpd3 expression shows that the mRNA is already expressed at PD7, when NTPDase3-immunoreactivity (ir) is observed sporadically at neuronal cell bodies in the restricted diencephalic and limbic structures, such as sub-regions of the hypothalamus and the septohippocampal nucleus, while fibers expressing NTPDase3 are not observable [100]. Although the expression of the NTPDase3-mRNA was stable from PD7 to PD30, significant NTPDase3 labeling is detected after PD20 when the somatic NTPDase3-ir gradually increases until the end of the third postnatal week, particularly in the collections of cells in the lateral, ventral and dorsomedial hypothalamus. At PD20, short, marginally varicose NTPDase3 containing fibers appears in the posterior hypothalamic area, while at PD30 the fibers became longer with prominent varicosities and extended along the midline of the whole diencephalon. From PD7, and during maturation and in adulthood, prominent NTPDase3-ir is detected at cells of the choroid plexus along with strong enzyme histochemical staining of this area [80, 96, 100] where it may regulate the function of choroid plexus and cerebrospinal fluid composition [46]. As in the adults, expression pattern of NTPDase3 is also consistent with the hypocretin/orexin expression in the hypothalamus during postnatal development [103, 104] that could correspond to the developmental shift in two important autonomic functions, feeding and sleep/wake behavior.

2.4. Ecto-5'-nucleotidase

Adenosine regulates several functions during CNS development. The main pathway of extracellular adenosine synthesis in the rodent CNS is from the enzymatic breakdown of AMP by ecto-5'-nucleotidase (CD73; eN) [105], an ectoenzyme that is anchored to the extracellular surface of cell membrane through a glycosyl phosphatidylinositol (GPI)

linkage [27]. The enzyme is located on astrocytes, neurons, microglia and oligodendrocytes in different brain regions, including olfactory bulb, caudoputamen, hippocampus, cingular cortex and cerebellum (Fig. **1**) [1, 68, 92, 106, 107]. Being the last member of the enzyme cascade which degrades extracellular ATP, it is thought that the main role of eN is the production of adenosine. However, there are substantial data indicating that functions of eN go beyond the activity of adenosine-producing enzyme [29]. eN carries the epitopes implicated in the cell-cell and cell-matrix interactions [108], binds to the extracellular matrix components and may mediate cellular adhesion [29], thus, it is relevant for intercellular adhesion, signaling and cell migration [27, 29, 106].

Data obtained from postnatal cerebellum showed that eN is associated with the surface of migrating and immature nerve cells [109, 110]. eN is found with a subset of synapses during synaptogenesis and synaptic remodeling in the postnatal cerebellum, the adult olfactory bulb, lesion induced sprouting within the adult dentate gyrus [1, 106], and also during reactive synaptogenesis in the human brain [111]. Several studies clearly documented up to five-fold increase of eN activity in different brain regions during ontogeny [87, 112-116]. Since similar increase is not observed in tissues other than the brain [117], the finding implies that age-dependent increase in eN activity represents developmental phenomenon unique to the brain, rather than some common mechanism of cell aging. Moreover, it is found that expression and function of eN significantly increases in the hippocampal region from birth to adulthood, whereby eN-mRNA markedly increases from PD7 to PD20, reaching a plateau, while the enzyme activity continued to increase beyond this age. Namely, at PD7, the eN gene is weakly expressed concomitant with poor enzyme activity in most hippocampal fields, while in some subregions the activity was completely absent [114]. Further analysis also reveals that layers rich in synapses generally displays the highest levels of eN activity, while in neuronal cell bodies layers, the enzyme expression is weak or completely absent.

The highly variable levels of eN activity in health and disease raise an important question of the regulatory mechanism(s) controlling the enzyme expression at different levels. Among the several regulatory elements present in the promoter region of eN gene [118], there is a binding site for TCF1/LEF transcription factor, which is the nuclear target of Wnt signaling [119], crucially involved in dendritogenesis, axon guidance and synaptogenesis [120, 121]. Thus, developmental expression of eN is under direct and/or indirect transcriptional control by various transcription factors [118], growth factors [122] specific signal transduction pathways which induce *de novo* local protein synthesis [118, 123] and hormones [124-126].

Currently known data of ectonucleotidases expression patterns and their roles in the context of main developmental events are summarized in Table **1**.

3. IMPLICATION OF ECTONUCLEOTIDASES IN SYNAPSE FORMATION, MATURATION AND FUNCTION

After proliferation of neural precursors and migration to their correct position, the next essential event during CNS

Table 1. Summary of ectonucleotidase expression and role during rodent brain development.

	Period of Development	Investigated Region	Cell Type	Developmental Process	Function
NPP1	PD7-Adult ↑	Olfactory bulb, cerebral cortex, striatum, cerebellum, hippocampus	Neurons and glial cells	n.d.	Enzymatic
NPP2	E8.5 E13.5-PD0 PD7-Adult ↑	Neural tube, midbrain Choroid plexus Whole brain	Epithelial cells Olygodendrocytes	Neural tube closure Oligodendrocyte differentiation and myelination	Non-enzymatic
NPP3	E13-PD7 PD7-Adult1 ↓	Germinal layers of the ventricular zone Olfactory bulb, cerebral cortex, striatum, cerebellum, hippocampus	Precursor cells, immature astrocytes	Glial cell proliferation and migration, astrocytes differentiation	n.d
TNAP	E8.5 E9-E15 E15-Adult ↓	Neural tube Between mesencephalon and rhombencephalon, spinal cord and cranial nerves, myelencephalon, around ventricular and subventricular zone	Endothelial cells, primordial germ cells Pioneer growth cones, neural precursors	Proliferation Synaptogenesis, neurogenesis, myelination,	Enzymatic
NTPDase2	E17-Adult	Along the ventricular and subventricular zone, dentate gyrus of the hippocampus	Progenitor cells, radial glia	Neurogenesis/migration	Enzymatic
NTPDase3	PD7-Adult ↑	Diencephalic and limbic structures; cerebral cortex, hippocampus, thalamus, hypothalamus, choroid plexus	Neurons	n.d	Enzymatic
eN	PD6-PD28 PD7-Adult ↑	Cerebellum Hippocampus	Neurons Neurons	Migration, differentiation, maturation, synaptogenesis	Non-enzymatic Enzymatic

The shown data bring together results of different methods, *e.g.* immunohistochemistry, enzyme histochemistry, RT-PCR analysis *etc*. ″↑″ increased during indicated period; ″↓″ decreased during indicated period. E, embryonic day; PD, postnatal day.

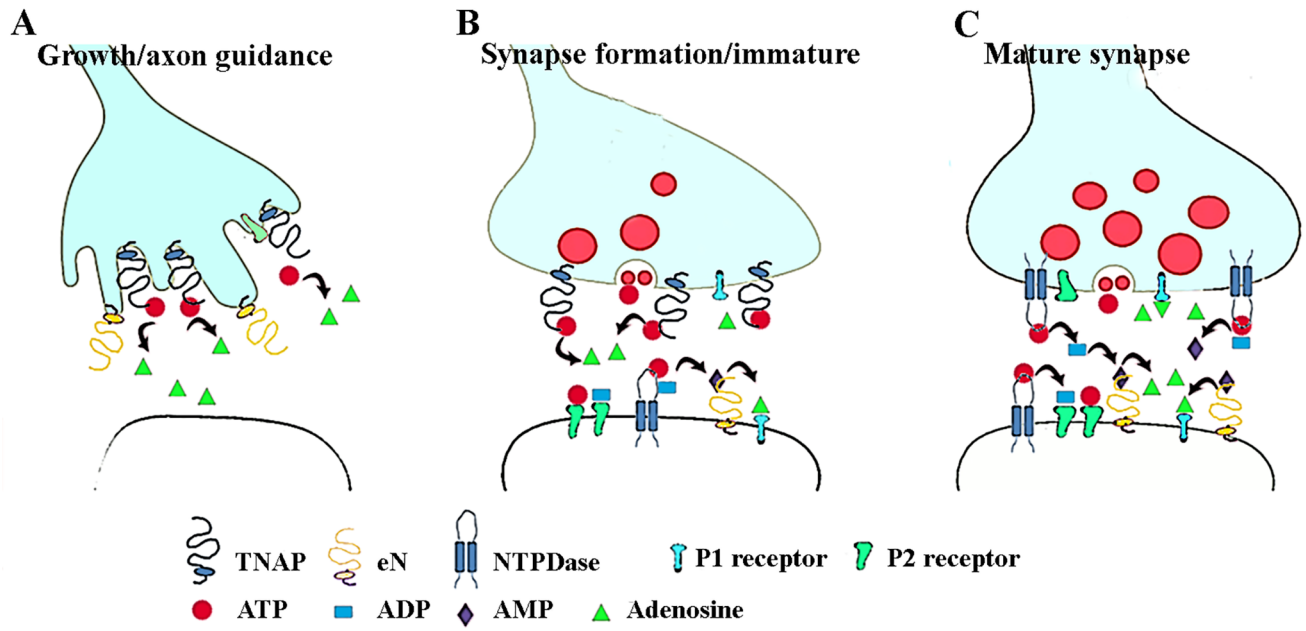

Fig. (2). Role of ectonucleotidases during axonal growth, in immature and mature synapses. A) ATP negatively controls axonal growth and branching. TNAP hydrolyzes ATP directly to adenosine which induces axonal elongation, while eN acts as adhesion protein. **B**) During synapse maturation, synaptic TNAP abundance is decreased and replaced with NTPDases and eN. **C**) NTPDases and eN are main enzymes responsible for ATP/ADP dephosphorylation and adenosine formation in the synaptic cleft of mature synapses. (*The color version of the figure is available in the electronic copy of the article*).

development is differentiation, including axonal growth and guidance essential for establishing precise connectivity patterns, which are highly coordinated processes controlled by a variety of extracellular and intracellular signaling pathways. Once the axon reaches the proper target, it establishes synaptic contacts, *i.e*. synapses, which mature over the first 3 weeks during the postnatal period in rodents and through adolescence in monkeys and humans. Synaptogenesis consists of biochemical and morphological changes in both pre- and postsynaptic elements whose physicochemical compatibility and correct timing with competitive exclusion of inappropriate connections are essential for the maturation of synaptic connections. It is demonstrated that extracellular ATP negatively controls axonal growth and branching in cultured hippocampal neurons [127]. Moreover, ATP reduces neurite extension from motoneuron-containing neural tube explants of the rat embryos at E12 [128]. In contrast, extracellular adenosine-induced neurite elongation in human neuroblastoma cells [129] while the expression of eN is found to be mandatory for neurite extension *in vitro* [130]. Since TNAP can catalyze hydrolysis of all extracellular adenine nucleotides, it may scavenge or produce ligands of P2 receptors and finally produce adenosine as the ligand of P1 receptors. Hence, it could be assumed that this enzyme is sufficient for the adenosine production during neurite growth and peak of synaptogenesis, while eN acts as an adhesion protein and attending in migration (Fig. **2A**). In favor of that assumption is founding that TNAP is involved in the metabolism of extracellular nucleotides modulating purinergic signaling during early development [131]. An *in vitro* study showing that TNAP is engaged in the control of axon extension and branching of hippocampal neurons, adds evidence for the role of TNAP in the maturation of neural connections and pathways [73]. The enzyme probably induces axonal elongation by hydrolyzing ATP in the immediate environment of the receptors, thus preventing the activation of P2X7R [73].

To date, there is only scarce information concerning the role of ATP, other nucleotides as well as expression pattern of individual ectonucleotidases in the development of synaptic network activity, although it is shown by several studies that synaptic compartments comprise ectonucleotidases, *i.e*. NTPDase1–3, eN, [68, 87, 100, 114, 115, 126, 132-134]. It is also found that TNAP is selectively expressed in the synaptic cleft of sensory cortical areas in adult primates [69] and in humans [135] and its activity is regulated by sensory experience in the primate visual cortex [82]. In the rodent cortex, high activity of TNAP coincides with the peak of synaptogenesis [82, 83], suggesting a functional involvement of TNAP in synapse formation while its actions are declined in mature synapses, when developmental synaptogenesis is terminated [83]. On the other hand, synaptic NTPDases activity is increased from birth to the end of the third postnatal week (PD21), when synaptic formation is completed. In the purified synaptosomes or synaptic membranes obtained *e.g*. from the rat cerebral cortex or hippocampus, ATPase and ADPase activity increases steadily from birth, reaching maximum values at PD21 [136-138] while relative abundance of NTPDase1 is highest at PD15 [139, 140]. This leads to the assumption that NTPDase reaches full expression and activity only in the mature synapses (Fig. **2B**, **C**). In accordance is the finding that significant NTPDase3 protein expression in the varicose fibers, which partially overlaps with pre- and postsynaptic membranes, is detectable after PD20 [100]. Diferent studies point to NTPDase3 as the main NTPDase present in the synaptic compartment [28, 86, 87,

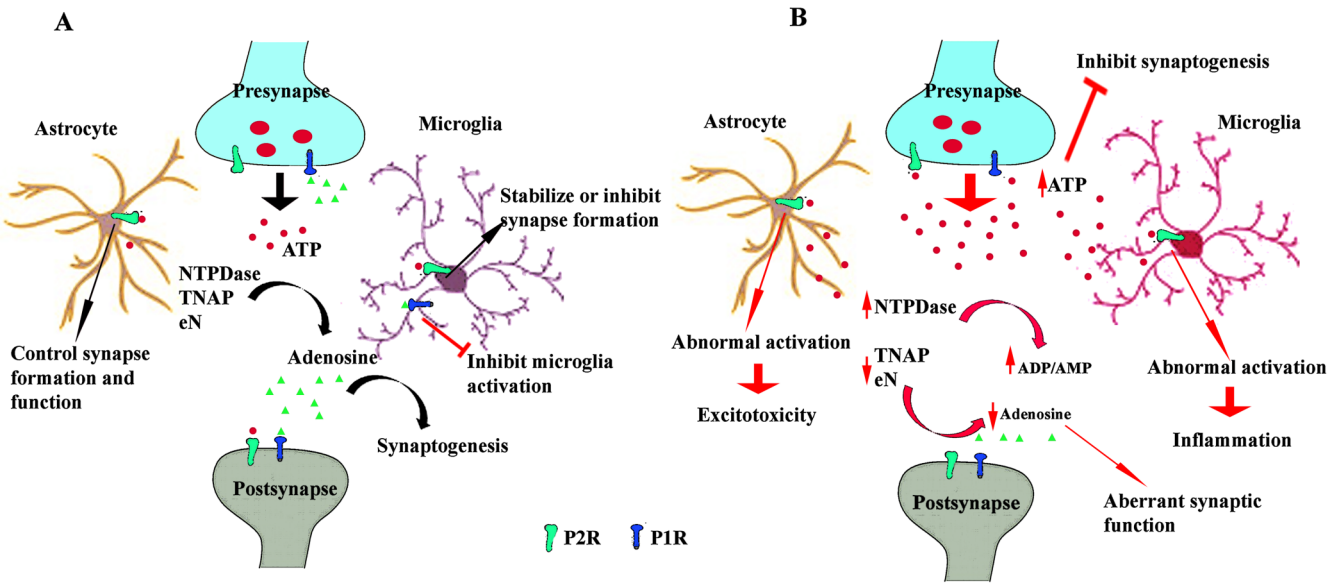

Fig. (3). Putative role of purinergic signaling in the pathophysiological mechanisms of autism. A) In physiological conditions, ATP is released as co-transmitter at GABAergic and glutamatergic synapse and binds to P2 receptors on astrocytes, microglia and postsynaptic neuron to exert numerous effects on cellular function. Astrocytes also release ATP into synaptic cleft, controlling synaptic function. Microglial cells monitor synaptic activity and respond to ATP to either stabilize or inhibit synapse formation. NTPDases, TNAP and eN rapidly terminate ATP signaling, producing adenosine, which acts through its pre- and postsynaptic P1 receptors, fine-tuning synaptic transmission and promoting synaptogenesis. Adenosine may also act on neighboring microglia, where inhibits their conversion to a reactive phenotype, preventing microglial activation. **B)** If increased efflux of purines occurs, whichc results in pathological concentrations of ATP in the extracellular space during vulnerable periods of brain development, ATP acts as a damage-associated molecular pattern, triggering innate immunity and inflammation, alters synapse formation and maturation, and contributes to excitotoxicity. Excessive release of ATP over activates microglial P2 receptors, activating microglia and promoting neuroinflammation. Since in different studies is found excess synapses in autistic brain, it is also possible that NTPDases are abnormally expressed and/or activated, leading to accumulation of ADP and/or AMP in the synaptic cleft of immature synapses. During synapse maturation, the main adenosine producing enzyme eN, is not fully expressed or does not have enzymatic role, thus generating lover adenosine levels in the synaptic cleft, inducing aberrant synaptic function. (*The color version of the figure is available in the electronic copy of the article*).

133]. Further, synaptic NTPDase activity is accompanied with biochemical activity profile of eN, the last and the rate-limiting enzyme of the extracellular ATP metabolism [27]. As mentioned above, eN activity increases during postnatal development. The most prominent rise of NTPDase and eN activity corresponds to the end of synaptogenesis, suggesting that the adenosine-mediated signaling may have an important role in mature synapses. It is shown that synaptically eN-mediated formation of extracellular adenosine is responsible for the local activation of A2A receptors [32, 134]. Accordingly, the levels of A2A receptors in the newborn are very low, gradually increasing during the postnatal period and at PD25, the levels of these receptors are already as high as in older animals [141].

3.1. Synaptic Ectonucleotidase Activity in Pathology Conditions

Although the synaptic components of purinergic signaling, NTPDases and eN, are essential for normal postnatal development as well as functioning of the adult brain, there are just a few studies implicating them with the onset and progress of different pathological conditions. For instance, disorders of thyroid gland function are associated with the modulation of ectonucleotidase activities, expression of adenosine A1 receptors and transport of neuromodulator adenosine [142]. Hyper and hypothyroidism are shown to affect distinct biochemical events, however both thyroid diseases are able to influence the adenosine production in brain synaptosomes [143, 144]. Namely, T4 treatment inhibits ATP, ADP and AMP hydrolysis by about 14-52% in both hippocampal and cortical synaptosomes from 5 to 60 days-old animals. Hence, hyperthyroidism consequently interferes in the balance of extracellular nucleotides and affects the complete enzyme cascade responsible for the hydrolysis of ATP to adenosine throughout the development [144]. On the other hand, in the neonatal period, thyroid dysfunction in the form of hypothyroidism leads to abnormal development of the CNS, enhancing the metabolism of adenine nucleotides in astrocyte cultures from rat brain [145] and also eN activity in the synaptosomes from hippocampus and cerebral cortex in the rats at different stages of postnatal development [143].

Epilepsy, a neurological chronic disorder, is characterized by unprovoked, recurrent seizures. These sudden rushes of electrical activity in the brain are episodes that can vary from brief and nearly undetectable to long periods of vigorous shaking, frequently resulting in physical injuries. In pilocarpine model of epilepsy, in the synaptosomes purified from the cerebral cortex and hippocampus, ATP and ADP hydrolysis are not modulated during postnatal development [112]. However, eN activity is enhanced in the cortical syn-

aptosomes at PD27-30, indicating that the plastic events induced by pilocarpine promote alterations in this enzyme activity as well as in the adult rats, where it induces an augmentation of ectonucleotidase activities in the synaptosomes of both brain regions. These findings also support differences between the expression pattern of NTPDases and eN of developing and adult brains. Furthermore, in a different model of epilepsy, a systemic administration of kainic acid on PD7 leads to memory impairment in the adult rats accompanied by an increased ATP hydrolysis in the hippocampal synaptosomes, suggesting that observed effects on NTPDases responsible for ATP hydrolysis are not due to an increased synthesis of these proteins [146]. The neonatal exposure to analgesic drugs can have long-lasting implications for the developing nervous system, such as permanent modulation in pharmacological responses and cell signaling [147], alteration in the central pain related systems and generally resulting in opioid addiction [148]. In particular, chronic use of morphine promotes changes in adenosine-mediated signaling pathways in several brain structures linked to the etiology of addiction [149]. Further, it is shown that after the daily morphine exposure from PD8 to PD14, at PD16, ntpd1 mRNA level and ATP hydrolysis in the synaptosomes from cerebral cortex are significantly increased [150].

Several studies highlight that chronically administered caffeine has beneficial effects against a number of neurological disorders, like stroke, Alzheimer's and Parkinson's diseases [151]. However, chronic exposure to caffeine in early life, either as a therapeutic drug or food, affects adenosinergic tonus and must be carefully evaluated, due to the physiological environment of the immature brain [152, 153]. For instance, caffeine exposure during gestational and lactational periods stimulates hippocampal AMP hydrolysis in 7-days-old rats, while at PD7 and PD14 it inhibits ATP hydrolysis and at PD21 enhances it [154]. In parallel, the expression level of NTPDase1 was decreased, correlating it with decrease in ATP hydrolysis in the hippocampal slices from the caffeine-treated 14-days-old rats, although this enzyme has equal preference for hydrolyzing ATP/ADP [154]. Thus, caffeine alters hippocampal nucleotides hydrolysis during the first 3 weeks of postnatal life, which could be a result of adjustment in adenosine receptors expression and nucleotide availability after chronic caffeine exposure, and also, be related to differential expression of ectonucleotidases and adenosine receptors during the intense phase of development until reaching the adult patterns.

3.2. Ectonucleotidases as Potential Target in ASD

Autism spectrum disorders (ASD), neurodevelopmental conditions characterized by deficits in social interaction, anxiety, impaired communication, behavioral abnormalities, and restricted repetitive behaviors or interests may appear due to alterations in the development of neurotransmitter systems and synaptic proteins. Similar features are also indicated in a variety of neurodevelopmental disorders, including epilepsy, Rett syndrome, and Fragile X syndrome, which are characterized by dysfunctions in the balance between excitatory and inhibitory neurotransmissions [155, 156]. Evidence indicates an important role of the extracellular ATP and purinergic signaling in ASD [157-159], while abnormalities in purine metabolism and a relationship between adenosine and autism in terms of symptoms and behavior have been reported [160, 161] *e.g* insufficient adenosine levels may be related to some symptoms such as poor eye contact or repetitive movements [162, 163]. Since locomotor activity and social contacts are behavioural patterns normally controlled by eN activity, main adenosine-producing enzyme in the CNS [105, 134, 164] it is not surprising that the changes in these behavioural patterns contribute to endophenotypes of ASD [165], while eN may be a tool for pharmaceutical intervention in behavioural disorders. Furthermore, several studies suggest the therapeutic potential of adenosine in relation to autism while the interventions generating an increase in adenosine levels are an important strategy to alleviate symptoms related to autism [162, 166-169]. Synaptically eN-mediated formation of extracellular adenosine is responsible for the local activation of A2A receptors [32, 134], which are involved in the locomotion, anxiety, inhibition of excitatory neuronal activity, sleep regulation [169] and thus in perseverative behaviors. Thus, A2A receptor is a promising candidate for genetic association studies in ASD [162, 166] as well as new target for the treatment of repetitive behaviors in autism [167, 169]. Naviaux and coworkers hypothesized that ″antipurinergic therapy″ with suramin corrected numerous multisystem abnormalities that defined the ASD-like phenotype in mice models. This includes correction of the core social deficits and sensorimotor coordination abnormalities, prevention of cerebellar Purkinje cell loss, correction of the ultrastructural synaptic dysmorphology, hypothermia, P2Y2 and P2X7 receptor expression, and signal transduction abnormalities [170]. Thereafter, it is also reported that treatment with suramin in different animal models of ASD restores normal social behavior, and improves metabolism and brain synaptosomal structure [171, 172]. It is known that the pharmacological components of suramin allow binding against purinergic P2X receptors without release, serving as an nonselective antagonist of these receptors [173]. Synaptosomal NTPDases are also noncompetitively inhibited by suramin, which preferentially inhibit ecto-ATPase and does not affect the ADPase activity [132, 174-177], generating increased extracellular AMP levels, substrate for adenosine-producing enzyme eN in the synaptic cleft, and thus increasing the level of adenosine. Therefore, it is possible that suramin acts as inhibitor of ATPase component of abnormally expressed and/or activated NTPDases in rodent models of ASD. Thus, ectonucleotidases, ATP- and adenosine-metabolizing enzymes, may be involved in the pathophysiological mechanisms of ASD. Since current data are scarce, it is of great importance to examine the exact expression profile and activity of ectonucleotidases, especially main enzymes responsible for ATP/ADP dephosphorylation and adenosine formation, NTPDase1-3 and eN, both during pre and postnatal CNS development as well as in different animal models of ASD. An understanding of how these ectonucleotidases are involved in regulating cellular responses is crucial for the therapeutic approach with potent and selective ectonucleotidase inhibitors [178-181]. Fig. (**3**) illustrates role of purinergic signaling in the cells which are involved in normal synapse formation and potential contribution of ectonucleotidases in the pathophysiological mechanisms of autism. From the perspective of brain development and func-

tion, purinergic signaling represents an important new area for study in ASD as it has direct effects on pathways implicated in ASD [182].

CONCLUSION

Bearing in mind the importance of purinergic signaling in embryonic neurogenesis and all cellular developmental processes, understanding the patterns of catalytic activity and expression of specific ectonucleotidases during CNS development and maturation is essential for elucidating the control of nucleotide signaling in the brain. This signaling pathway is unique, as the extracellular hydrolysis of adenine nucleotide ligands includes a large number of ectonucleotidases with different catalytic properties. Depending on the ligand, the subtype of nucleotide receptor, and the type of ectonucleotidase present, extracellular nucleotide hydrolysis can result in the inactivation of a ligand, the production of an additional or alternative ligand, or the production of a nucleoside. Moreover, noncatalytic functions of the enzymes should not be excluded. The components of the purinergic signaling pathways *i.e*. P1 and P2 receptors and various types of ectonucleotidases are expressed early during embryonic development of the CNS and vary with developmental stage or is transient, suggesting affection of stage-specific developmental processes. E-NPPs are expressed at early embryonic days, but the expression of E-NPP3 is reduced and restricted to ependymal area in adult brain. Specific brain alkaline phosphatase is functionally associated with synapse formation and maturation. NTPDase2 is dominant ectonucleotidase expressed in the progenitor cells during development as well as dominant astrocytic NTPDase in adult CNS, since NTPDase3 is fully expressed after third postnatal week, almost exclusively on varicose fibers with a role in feeding and sleeping. eN is transiently associated with synapses during synaptogenesis and became a main adenosine-producing enzyme in the adult CNS. Thus, it is important to investigate the type of ectonucleotidase(s) that can potentially contribute to the control of adenine nucleotide signaling in distinct brain regions during CNS development and physiological settings. Further work needs to define the enzyme subtypes involved and the mechanisms underlying the regulation of ectonucleotidase expression, both during development and in adulthood. Disturbance of normal stimuli during critical periods of neural development can be an important factor to understand different responses to disorders between immature and mature nervous system. Since NTPDase3 and eN are ectonucleotidases included in the behavioral patterns which are altered in ASD, together with their final product adenosine and its receptors, it is important to investigate how they operate in neuronal development and pathophysiology conditions spatially and temporally. Thus, detection of ectonucleotidase responsible for metabolizing extracellular adenine nucleotides in different developing onset might provide new target for ASD treatment.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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