Cell Mol Neurobiol (2007) 27:731–743
DOI 10.1007/s10571-007-9159-8

ORIGINAL PAPER

Immunohistological Determination of Ecto-nucleoside Triphosphate Diphosphohydrolase1 (NTPDase1) and 5′-nucleotidase in Rat Hippocampus Reveals Overlapping Distribution

Ivana Bjelobaba · Mirjana Stojiljkovic · Sanja Pekovic · Sanja Dacic · Irena Lavrnja · Danijela Stojkov · Ljubisav Rakic · Nadezda Nedeljkovic

Received: 13 December 2006 / Accepted: 21 May 2007 / Published online: 6 July 2007

Abstract Distribution of two enzymes involved in the ectonucleotidase enzyme chain, ecto-nucleoside triphosphate diphosphohydrolase1 (NTPDase1) and ecto-5′-nucleotidase, was assessed by immunohistochemistry in the rat hippocampus. Obtained results have shown co-expression of the enzymes in the hippocampal region, as well as wide and strikingly similar cellular distribution. Both enzymes were expressed at the surface of pyramidal neurons in the CA1 and CA2 sections, while cells in the CA3 section were faintly stained. The granule cell layer of the dentate gyrus was moderately stained for NTPDase1, as well as for ecto-5′-nucleotidase. Glial association for ecto-5′-nucleotidase was also observed, and fiber tracts were intensively stained for both enzymes. This is the first comparative study of NTPDase1 and ecto-5′-nucleotidase distribution in the rat hippocampus. Obtained results suggest that the broad overlapping distribution of these enzymes in neurons and glial cells reflects the functional importance of ectonucleotidase actions in the nervous system.

Keywords CD39 · CD73 · Ectonucleotidase · Extracellular nucleotides · Immunohistochemistry

Introduction

Purine nucleotides and nucleosides, such as ATP and adenosine, act as important extracellular signaling molecules influencing neuronal activity. In the central nervous

I. Bjelobaba · M. Stojiljkovic · S. Pekovic · I. Lavrnja · D. Stojkov · L. Rakic
Department of Neurochemistry and Immunonology, Institute for Biological Research ''Sinisa Stankovic'', Belgrade, Serbia

M. Stojiljkovic · S. Dacic · N. Nedeljkovic (✉)
Institute of Physiology and Biochemistry, Faculty of Biology, University Belgrade, Studentski trg 3, 11001 Belgrade, Serbia
e-mail: nnedel@bf.bio.bg.ac.yu

system (CNS), ATP can be stored in synaptic vesicles together with some other classical neurotransmitter or in a separate pool of vesicles and released upon stimulation by exocytosis. Other mechanisms of ATP release, from neurons or glial cells may include gap junction hemichannels, volume-sensitive chloride channels, or dilatated P2X receptors (Pankratov et al. 2006).

ATP is a fast excitatory synaptic transmitter, acting via two types of purinergic receptors, P2X and P2Y (Illes and Norenberg 1993; Ralevic and Burnstock 1998; King et al. 1998). Numerous studies have shown that ATP was involved in astrocytic calcium wave propagation (Scemes et al. 2000), regulation of serotonine, noradrenaline, dopamine and vasopressin release (von Kugelgen et al. 1994, 1997; Song and Sladek 2005), nociception (Kennedy and Leff 1995), hippocampal long-term potentiation (Fujii 2004), regulation of blood flow and hemostasis (Enjyoji et al. 1999) and reactive astrogliosis after brain injury (Neary et al. 1999).

The events induced by extracellular ATP are under the control of ectonucleotidase enzymes pathway, which represents a general mode of terminating purinergic signaling. This enzymatic pathway includes members of ecto-nucleoside triphosphate diphosphohydrolase (NTPDase), ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), as well as the ecto-5′-nucleotidase families. There is another group of yet poorly characterized enzymes, ecto-protein kinases, which also use ATP as a substrate for the synaptic proteins phosphorylation (Ehrlich et al. 1986).

Members of E-NTPDase family hydrolyze nucleoside tri- and diphosphates. At least three known cell-surface located NTPDases (NTPDase1–3) capable of controlling the concentrations of nucleotide agonists are present in rat brain (Kegel et al. 1997; Belcher et al. 2006). Despite some structural similarities, these enzymes differ distinctly in their substrate specificity. Thus, NTPDase1 (also known as CD39, ecto-apyrase, ecto-ATP diphosphohydrolase) degrades ATP and ADP to AMP equally well, while NTPDase2 (ecto-ATPase) hydrolyzes triphosphonucleosides to respective diphosphonucleosides (Heine et al. 1999; Kukulski and Komoszynski 2003). NTPDase3 (CD39L3) is a functional intermediate that dephosphorylates ATP to AMP with a transient accumulation of ADP (Lavoie et al. 2004).

In many cells and tissues the hydrolysis of P2 receptor agonists, ATP and ADP is coupled to the hydrolysis of AMP by 5′-nucleotidase (also known as CD73) to generate adenosine. Adenosine acting at P1 adenosine receptors is very potent neurotransmitter and neuromodulator that elicit a myriad of physiological responses.

The expression of ectonucleotidase enzymes has been studied indicating wide distribution in the CNS of the rat. However, the vast majority of present information regarding regional and cellular localization of NTPDases and 5′-nucleotidase relies on biochemical analysis or on enzyme histochemical techniques that do not allow differentiation between individual members of the same enzyme family, since many hydrolyze the same substrate and can be present in the same cell type (Kegel et al. 1997; Nedeljkovic et al. 2003). Recently, immunohistochemical studies for individual members of NTPDase family were performed using isoform-specific antibodies. NTPDase1 was reported to have widespread expression in the CNS of rat (Wang and Guidotii 1998), being present in cerebral, hippocampal and cerebellar neurons, glial cells and endothelial cells. NTPDase2 protein was found in the germinal zones of rat brain, in subventricular zone and rostral migratory stream (Braun et al. 2003). This enzyme seems to be the dominant ectonucleotidase present on rat astrocytes (Wink et al. 2006). NTPDase3 was reported to be present almost exclusively in axons (Belcher et al. 2006). On the other hand, regional and cellular distribution of 5′-nucleotidase in rat brain have

been performed by using biochemical and histochemical techniques (Braun et al. 1998; Schoen et al. 1999), and no studies to date have reported comparative immunohistochemical localization of NTPDase1 and 5′-nucleotidase in the rat brain. In the present study, we performed comparative immunohistochemical analysis of NTPDase1 and 5′-nucleotidase to explore their regional and cellular distribution in the rat hippocampal area.

Methods

Animals

All animal treatment protocols were approved by the Belgrade University Animal Care and Use Committee. The study was performed on 3-month-old male rats of the Wistar strain (250–350 g). Animals were subjected to 12-h light-dark cycle, housed 3 per cage, with free access to food and water.

Immunohistochemistry

Following decapitation rat brains were rapidly removed and kept in 4% paraformaldehyde in 0.1 M phosphate buffer, as fixative for 12 h. After cryoprotection the brains were frozen at –70°C. The brains were cut into 16 μm thick sections in the coronal plane. Frozen sections were dried and processed for NTPDase1 and ecto-5′nucloeotidase immunohistochemistry as described previously (Nedeljkovic et al. 2006). Primary antibodies against NTPDase1/CD39 (1:1000 dilution; CD39 (A20) Santa Cruz Biotechnology, Inc.) and 5-nucleotidase/CD73 (1:1000 dilution; Santa Cruz Biotechnology, Inc. (C20)) were used. For immunoperoxidase labeling, sections were incubated for 2 h with biotinylated secondary antibody (Santa Cruz Biotechnology, Inc.) and peroxidase reaction was performed with diaminonezidine (DAB) substrate solution according to the manufacturer instructions (Sigma Chemical Co.). In the negative control experiments for both primary antibodies, omission of the primary antibodies resulted in no specific immunostaining. After dehydratation in graded ethanol, the sections were mounted with Canada Balsam (Merck) and photographed on computer-based Leica DMRB microscope.

Isolation of Crude Membrane Fraction

Crude membrane fraction was isolated as previously described (Bjelobaba et al. 2006). Briefly, tissue was homogenized in 10 volumes of 0.32 M sucrose, 10 mM Tris–HCl, pH 7.4 at 4°C and centrifuged for at 800*g* for 20 min. Resulted supernatant was centrifuged at 9,000*g* for additional 20 min. and pelleted membrane fraction was resuspended in 5 mM Tris buffer, pH 7.4 and subsequently homogenized, prior to dilution in SDS-PAGE sample buffer and immunoblot analysis.

Electrophoresis and Immunobloting Procedure

About 10 μg of crude membrane samples were separated at 12% SDS-PAGE and immunoblotted as previously described (Bjelobaba et al. 2006). After blocking with 5%

non-fat dry milk in Tris buffer saline, Tween 20, the blots were probed overnight with 1:1000 dilution of goat polyclonal antibodies against NTPDase1 and 5′-ectonucleotidise that were used in immunohistochemical analysis. Visualization procedure was performed by avidin–biotin peroxidase labeling, after incubating support membranes in anti goat IgG-horse radish peroxidase-conjugated secondary antibody (Sigma Chemical Co.).

Results

Immunoblot analysis

The specificity of the antibodies against NTPDase1 and 5′-ectonucleotidase was demonstrated by immunoblotting. Figure 1 shows representative immunoblots of crude membrane fraction probed with the antibody against NTPDase1 (Fig. 1A) and ecto-5′-nucleotidase (Fig. 1B), respectively. The antibody against NTPDase1 recognized one prominent band at about 70 kDa and another less prominent band at about 130 kDa. Antibody against 5′-nucleotidase recognized two bands, one at about 55 kDa, which matches to expected size of the enzyme and another at about double that size, which probably corresponds to the ecto-5′-nucleotidase dimmer molecule (Zimmermann 2000).

Immunolocalization of NTPDase1

Figure 2 shows NTPDase1 immunoreactivity (NTPDase1-*IR*) in the hippocampal formation. The white matter of the alveus and fimbria was moderately immunopositive

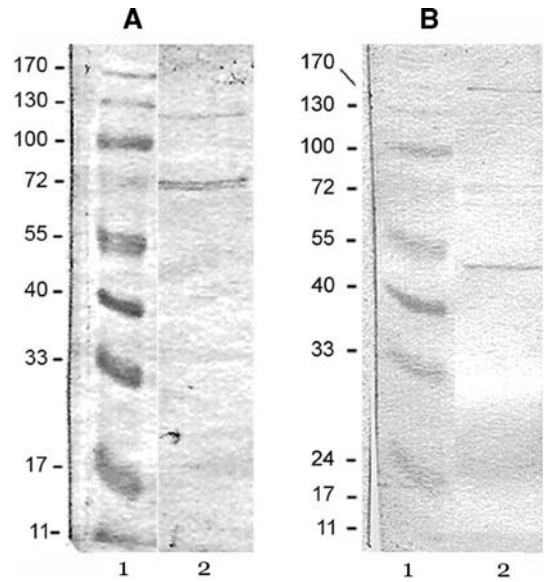


Fig. 1 Immunoblot analysis for the specificity of the antibodies against NTPDase1 (**A**) and 5′-ectonucleotidase (**B**). Lane 1 on each blot shows molecular weight markers

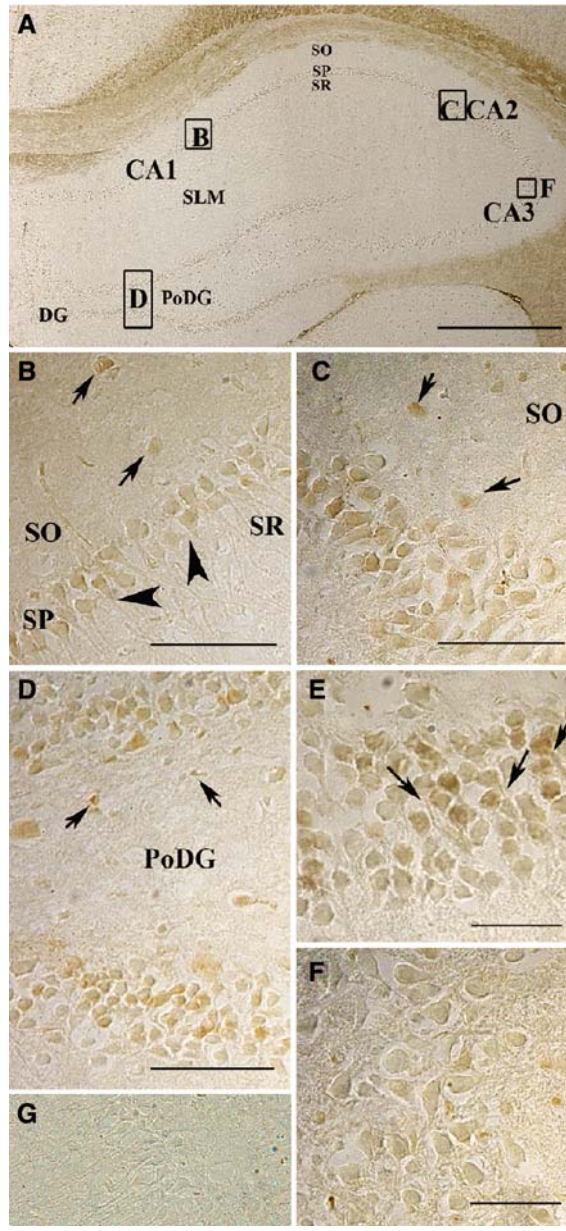


Fig. 2 Immunolocalization of NTPDase1 in the rat hippocampus. (**A**) Low-power photomicrograph of hippocampal area immunostained with the anti-NTPDase1 antibody. (**B**) High-power photomicrograph magnified from the area enclosed by a rectangle B in A, showing SP of CA1 region with marked apical dendrites (*arrowheads*). Arrows point to immunopositive neurons in SO. (**C**) Photomicrograph magnified from the area C enclosed in A, showing *IR* of pyramidal neurons in CA2 area; arrows point to interneurons in SO. (**D**) The granule cell layer of DG and PoDG, with putative glial distribution (*arrows*). (**E**) High-power photomicrograph of DG showing granule cells with marked apical dendrites (*arrows*). (**F**) Photomicrograph magnified from the area enclosed in A showing faint IR in CA3 region. (**G**) Omission of the primary antibody resulted in no specific staining. Scale bar: 500 μm in **A**; 50 μm in **B**–**D**; 25 μm in **E** and **F**

(Fig. 2A). Modest *IR* was observed in CA1 and CA2 pyramidal cell layer (Fig. 2B, C), but the most prominent labeling, was seen in the granular layer of the dentate gyrus (DG) (Fig. 2D, E). In the stratum pyramidale (SP) of CA1 and CA2, *IR* was present mostly on the surface of large neurons, while the proximal parts of apical and basal dendrites of these neurons were weakly labeled, whereas axons remained indistinguishable (Fig. 2B, C). In the granular cell layer both surface and cytoplasmic labeling was observed (Fig. 2E). Only proximal parts of apical dendrites could be seen, while mossy fibers were indistinguishable. In other hippocampal regions, particularly in CA3 (Fig. 2F) and hilus, the *IR* was faint. In the stratum oriens (SO) of all three regions, whole cell bodies of small interneurons were markedly stained (Fig. 2C). Small cell bodies of irregular shape observed in the polymorphic layer of dentate gyrus (PoDG) could be of glial origin (Fig. 2D). Omission of the primary antibody against NTPDase1 resulted in no specific immunostaining (Fig. 2G).

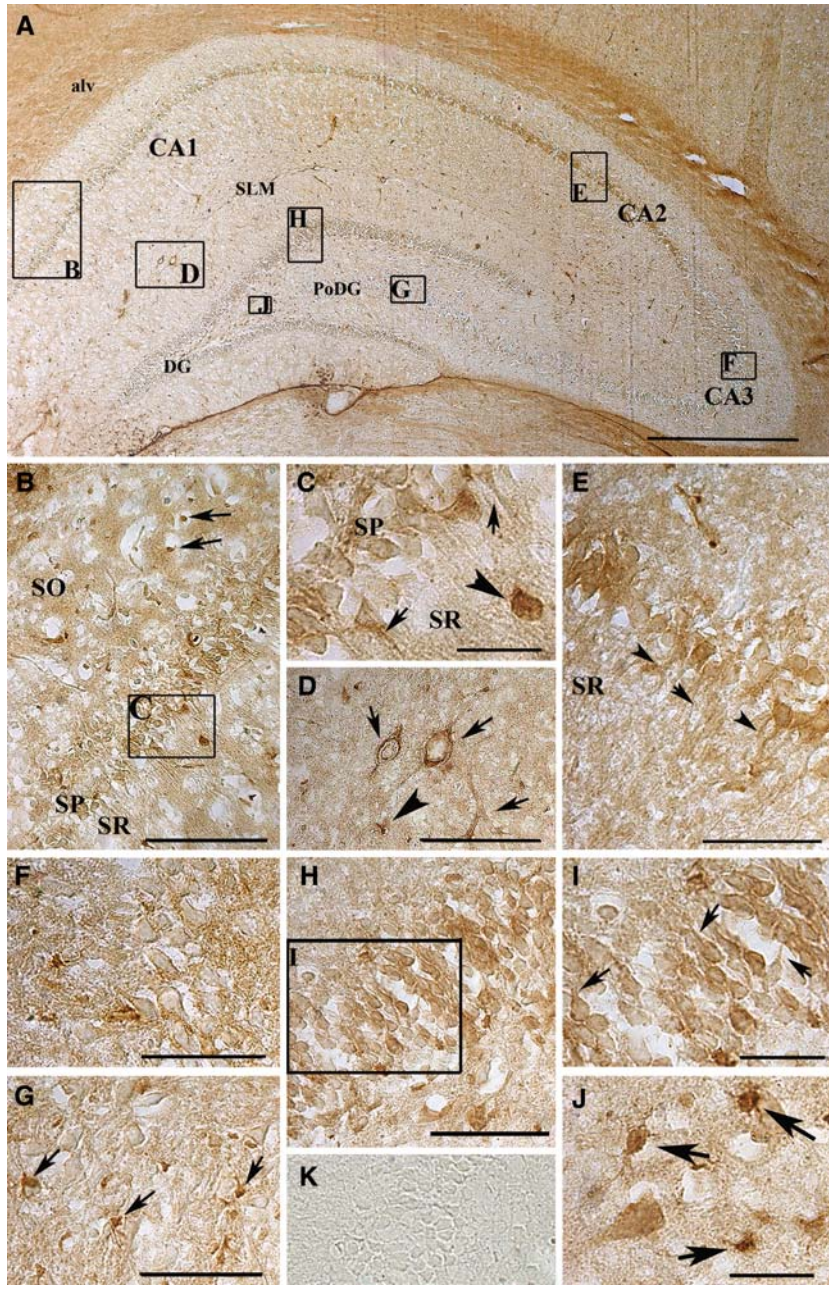
Immunolocalization of 5′-nucleotidase

Figure 3 shows immunohistochemical distribution of 5′-nucleotidase in Amon's horn and dentate gyrus of the hippocampal formation. Low-power microscopic observation revealed most intensive relative labeling in the pyramidal cell layer in CA1 and CA2 and in the granule cell layer of DG (Fig. 3A). Alveus and fimbria were moderately labeled. Individual cell bodies were more intensively stained in fields CA1–CA2 (Fig. 3B, C, E) than in CA3 and hilus (Fig. 3F, G). CA2 pyramidal cells appeared with conspicuous apical dendrites that projected to the stratum radiatum (SR) (Fig. 3E). In few cells *IR* was concentrated around the cell bodies producing marked outline, but cytoplasmic labeling was also prominent. Large neuronal cell bodies in SR were intensively stained (Fig. 3C). Immunoreaction at the small interneurons was also observed in the SO of all hippocampal fields. Individual granule cells of DG showed prominent *IR*, particularly at proximal parts of apical dendrites, while mossy fibers were not distinguishable (Fig. 3H, I). Strong *IR* was also observed at astrocytic cell bodies in stratum lacunosum moleculare (SLM) (Fig. 3D) and their marked processes interposed between the cells of the hilus (Fig. 3G), in the PoDG (Fig. 3J) and in other areas as well. Capillaries and larger blood vessels were also immunopositive (Fig. 3D). Substantial background staining was observed throughout the tissue, and this pattern was not observed when the primary antibody was omitted (Fig. 3K).

Fig. 3 Immunolocalization of ecto-5′-nucleotidase in the rat hippocampus. (**A**) Low-power photomicrograph of hippocampal area immunostained with the anti-5′-nucleotidase antibody. (**B**) Photomicrograph magnified from the area enclosed by a rectangle B in A, showing the *IR* in the CA1 area. Arrows point to the immunoreactive interneurons in SO. (**C**) High-power micrograph magnified from B, showing pyramidal cells with marked apical dendrites (*arrows*) and large neuron in SR (*arrowhead*). (**D**) Capillaries and larger blood vessels in SLM showed positive on ecto-5′-nucleotidase (*arrows*). Astrocytes were also detected (*arrowheads*). (**E**) Pyramidal neurons in CA2 region with pronounced apical dendrites (*arrowheads*). (**F**) Pyramidal cells in CA3 region, enlarged photomicrograph from A. (**G**) The pyramidal cells and strongly positive astrocytes interposed in hilus (*arrows*). (**H**) The granular cell layer of DG. (**I**) High-power photomicrograph enlarged from H, showing the pattern of staining of granule cells, with pronounced apical dendrites (*arrows*). (**J**) High-power photomicrograph enlarged from A showing astrocytes with pronounced processes in PoDG. (**K**) Omission of the primary antibody resulted in no specific immunostaining. Scale bar: 500 μm in **A**; 100 μm in **B** and **D**; 50 μm in **E**–**H**; 25 μm in **C** and **J**

Discussion

Most of the data regarding regional distribution and cellular localization of NTPDase and ecto-5′-nucleotidase in the brain rely on biochemical or enzyme histochemistry techniques. These techniques are based on the addition of the substrate, ATP or AMP,



to the membrane preparation or tissue section and formation of either soluble product or a precipitate formed with lead or cesium salt, respectively. Thus, neither of the two approaches allow conclusion about which of the NTPDase family member is present since many hydrolyze the same substrate.

To elucidate cellular localization of two key enzymes involved in the extracellular metabolism of purine nucleotides in the rat hippocampus, i.e., NTPDase1/CD39 and ecto-5′-nucleotidase/CD73, we have performed a comparative immunohistochemical study.

NTPDase1 Regional and Cellular Distribution

Up to date, three cell-surface NTPDases (NTPDase1–3) have been localized in the mammalian brain. According to the deduced amino acid sequence and predicted secondary structure they all have transmembrane domains at the N- and at the C-terminus. Highly related NTPDase1 and NTPDase2 share little sequence identity between the two transmembrane regions at the N- and C-terminal end of the sequence (Kegel et al. 1997) and additionally NTPDase2 lacks the short intracellular chain of amino acid residues present at the N-terminus of NTPDase1 protein (Kegel et al. 1997; Zimmermann 2000). Since the antibody used in this study specifically recognizes the epitope near the N-terminus, it does not bind to NTPDase2 protein. On the other hand, NTPDase3 having the same membrane topography and intracellular domain at N-terminus (Zimmerman 2000), shares only 35.1% identity in primary structure with NTPDase1 (Vorhoff et al. 2005). We confirmed by immunoblotting that the antibody was useful as a probe for NTPDase1 in crude membrane preparation and as a tool for detecting NTPDase1 protein in hippocampal tissue. The antibody recognized two bands on immunoblots, one at about 70 kDa corresponding to the expected size of NTPDase1 from rat brain membrane (Braun et al. 2000) and another, less prominent band at about 130 kDa probably corresponding to the NTPDase1 dimer molecule. It is known that NTPDase1 can exist as homooligomers, dimers, trimers, or even tetramers (Smith and Kirley 1999).

Our immunohistochemical study revealed that NTPDase1 is predominantly associated with neuronal cell bodies and dendritic processes in all hippocampal areas. However, putative glial association was also observed. In addition, many NTPDase1 positive interneurons in SO and SLM of all hippocampal fields were detected. Although the surrounding white matter displayed strong NTPDase1-*IR*, axons of pyramidal cells and mossy fibers were indistinguishable.

The overall pattern of immunoreactivity obtained in this study was consistent with the pattern of immunoreactivity obtained previously with the use of another antibody specific for NTPDase1 (Wang and Guidotti 1998) and with the enzyme histochemistry based on formation of cerium phosphate precipitate (Zinchuk et al. 1999). Yet another study reported the presence of NTPDase1-*IR* at cultured pyramidal neurons of rat hippocampus (Boeck et al. 2002). However, immunohistochemical approaches based on lead precipitation protocols has shown that the NTPDase1 was restricted to microglia and the vasculature of murine brain (Braun et al. 2000). These variances can be explained by interspecies variations in enzyme distribution. On the other hand, lead staining protocol revealed faint staining of hippocampal pyramidal cell layer and granule cell layer of DG, with slightly enhanced lead precipitation in SLM and intense staining of the brain capillaries endothelium (Braun et al. 1998). Mismatches between lead and cerium histochemical methods were previously observed and explained with

the fact that lead, in contrast to cerium, interferes with the medium components, making the results of detection hardly interpretable (Zinchuk et al. 1999).

5′-Nucleotidase Regional and Cellular Distribution

Immunoblot analysis for the specificity of the antibody against 5′-nucleotidase revealed two bands, one at about 55 kDa and another at about double that size that probably corresponds to a dimer. Single gene has been identified in vertebrates (Zimmermann 2000) and the enzyme occurs mainly as a dimer and the apparent molecular weight of the monomer in the rat hippocampal membranes is 64 kDa (Cunha et al. 2000).

In the present study, immunohistochemical analysis of hippocampal coronal sections revealed that, at cellular level, enzyme was associated both with neurons and astrocytes. Pyramidal cells, in CA1 and CA2 areas were moderately labeled as well as the granule cell layer, while neurons in CA3 and hilus were very lightly labeled. Giant neuronal cells in SR, now established as a type of principal excitatory cells in the hippocampus (Gulyás et al. 1998), were clearly labeled. Similarly to NTPDase1, ecto-5′-nucleotidase could not be recognized on the axons of pyramidal cells or mossy fibers. However, significant background staining was always present on the sections, a pattern never observed when the primary antibody was omitted or in the case of NTPDase1 immunoreactivity. This finding strongly implies the presence of soluble form of ecto-5′-nucleotidase in the extracellular matrix throughout the brain tissue that was already reported (Zimmermann 1992).

Several immunohistochemical studies regarding ecto-5′-nucleotidase were carried out in the past. Yet localization of this enzyme in the brain is still controversial. According to Zimmermann et al. (1993), a band corresponding to the innervation area of mossy fiber terminals within area CA3 was selectively labeled, while CA1 and the DG were negative. In some earlier studies performed by using the antibody raised against liver 5′-nucleotidase, the enzyme was assigned to the surface of glial elements like Bergman glia, astrocytes (Schoen et al. 1987, 1988) and also in association with myelinated nerve fibers (Cammer et al. 1986). Neuronal localization of ecto-5′-nucleotidase was rarely observed (Kreutzberg et al. 1986; Nacimiento and Kreutzberg 1990) and it was proposed to be associated with migrating nerve cells during development (Schoen et al. 1988). In contrast, enzyme histochemical (Bernstein et al. 1978; Franco et al. 1986) and biochemical studies (Richardson et al. 1987; Cunha et al. 1992) implied that ecto-5′-nucleotidase was associated with nerve terminals in the rat brain. Other studies based on enzyme histochemical procedures revealed strong positive reaction in SR, SO and SLM of CA1 hippocampal field in the rat (Lee et al. 1986; Braun et al. 1998), whereas low levels were observed in other species examined, including mouse, pig, and gerbil (Lee et al. 1986).

Functional Consideration

Results presented herein show the wide distribution of NTPDase1 and ecto-5′-nucleotidase in the rat hippocampus, suggesting their involvement in the control of the purinergic signaling. Several studies confirmed the presence of highly efficient cascade, capable of extracellular nucleotide hydrolysis in the hippocampus (Cunha et al. 1992, 1996; Dunwiddie et al. 1997). It was reported that ATP elicited inhibition of synaptic transmission in Shaffer fibers/CA1 pyramid synapses was actually caused by adenosine,

the final product of ectonucleotidase pathway (Cunha et al. 1998). Ectonucleotidases also mediate depression of epileptiform activity in an in vitro model (Ross et al. 1998) and it was shown that these inhibitory effects are mediated mostly through the A1 receptors (Masino et al. 2002; Kukley et al. 2004), which are widely present in the hippocampus (Ochiishi et al. 1999).

Some of the ecto-5′-nucleotidase positive cells, like astrocytes and giant cells of SR which project to SO, had shown cytoplasmatic labeling which could be explained by cell recycling (Van den Bosch et al. 1988) and may reflect intensive extracellular nucleotide metabolism and high adenosine production. Our study also shows the same cellular allocation of NTPDase1 and ecto-5′-nucleotidase in interneurons of SO and SLM. ATP-induced excitation of interneurons and activation of astrocytes in the CA1 lead to synaptic inhibition through the activation of P2Y1 receptors and subsequent release of GABA (Kawamura et al. 2004). Therefore, regulation of synaptic networking could also depend on ectonucleotidase activity.

It is also important to note that another ATP-hydrolyzing enzyme, ecto-protein kinase is involved in the process of long-term potentiation (LTP) in CA1 neurons (Chen et al. 1996; Fujii 2004; Martin and Buño 2005). Thus, a competition for the same substrate could occur between NTPDase1 and ecto-protein kinase. On the other hand, NTPDase1, which hydrolyzes ADP with the same efficiency as ATP (Kukulski and Komoszynski 2003), could, in concert with ecto-5′-nucleotidase, promote the activity of ecto-protein kinase by removing its molecular inhibitors, ADP and AMP (Volonte et al. 1994). Therefore, it could be speculated that ectonucleotidases control the substrate availability for ecto-protein kinase, thus indirectly modulating the process of synaptic plasticity. In addition to above considerations, both NTPDase1 and ecto-5′-nucleotidase posses, non-enzymatic features related to cell–cell interaction and cell adhesion (Kansas et al. 1991; Zimmermann and Braun 1999).

Since hippocampus is involved in the process of memory consolidation and retrieval our findings raise questions about importance of ectonucleotidase pathway in biochemical events related to these phenomena. It was previously evidenced that formation of aversive memory was associated with learning-specific, time-dependent decrease in the ATP and AMP hydrolysis (Bonan et al. 1998, 2000), whereas significant increase in the ATP hydrolyzing activity have been observed following habituation to an open field (Pedrazza et al. 2007). These findings indicate that different learning paradigms involve purinergic system. Indeed both ATP, acting through P2 receptors (Almeida et al. 2003) or as a substrate of ecto-protein kinases (Chen et al. 1996), as well as adenosine (de Mendonca et al. 2002) modulate long-term synaptic plasticity phenomena, such as long-term potentiation, long-term depression and depotentiation, thus concerted action of ectonucleotidase enzymes is necessary for the proper execution of memory formation and retrieval.

In summary our results indicate that NTPDase1 and ecto-5′-nucleotidase are present at same cell types in rat hippocampus. Overlapping distribution has been already noticed for different members of ectonucleotidase enzyme families in various tissues (see Zimmermann 2000). Additionally, we have previously observed the similar hippocampal distribution for E-NPP1, member of E-NPP family (Bjelobaba et al. 2006). The involvement of multiple enzyme species in extracellular nucleotide hydrolysis suggests the existence of well-organized control over nucleotide signaling and underlines the significance of these processes in proper brain functioning.

Acknowledgements The authors are grateful to Dr Stanko Stojilkovic, National Institute for Health, Bethesda, MD, USA for his valuable suggestions and help. The study was supported by Serbian Ministry of Science and Environmental Control Grant No. 143005.

References

Almeida T, Rodrigues RJ, de Mendonca A, Ribeiro JA, Cunha RA (2003) Purinergic P2 receptors trigger adenosine release leading to adenosine A2A receptor activation and facilitation of long-term potentiation in rat hippocampal slices. Neuroscience 122:111–121

Belcher SM, Zsarnovszsky A, Crawford PA, Hemani H, Spurling L, Kirley TL (2006) Immunolocalization of ecto-nucleoside triphosphate diphosphohydrolase 3 in rat brain: implications for modulation of multiple homeostatic systems including feeding and sleep-wake behaviors. Neuroscience 137:1331–1346

Bernstein HG, Weiss J, Luppa H (1978) Cytochemical investigations on the localization of 5′-nucleotidase in the rat hippocampus with special reference to synaptic regions. Histochemistry 55:261–267

Bjelobaba I, Nedeljkovic N, Subasic S, Lavrnja I, Pekovic S, Stojkov D, Rakic L, Stojiljkovic M (2006) Immunolocalization of ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) in the rat forebrain. Brain Res 1120:53–64

Boeck CR, Sarkis JJ, Vendite D (2002) Kinetic characterization and immunodetection of ecto-ATP diphosphohydrolase (EC 3.6.1.5) in cultured hippocampal neurons. Neurochem Int 40:449–453

Bonan CD, Dias MM, Battastini AM, Dias RD, Sarkis JJ (1998) Inhibitory avoidance learning inhibits ectonucleotidases activities in hippocampal synaptosomes of adult rats. Neurochem Res 23:977–982

Bonan CD, Roesler R, Pereira GS, Battastini AM, Izquierdo I, Sarkis JJ (2000) Learning-specific decrease in synaptossomal ATP diphosphohydrolase activity from hippocampus and entorhinal cortex of adults rats. Brain Res 31:253–256

Van den Bosch RA, du Maine APM, Geuze HJ, van der Ende A, Strous GJ (1988) Recycling of 5′-nucleotidase in a rat hepatoma cell line. EMBO J 7:3345–3351

Braun N, Zhu Y, Krieglstein J, Culmsee C, Zimmermann H (1998) Upregulation of the enzyme chain hydrolysing extracellular ATP after transient forebrain ischemia in the rat. J Neurosci 18:4891–4900

Braun N, Sevigny J, Robson SC, Enjyoji K, Guckelberger O, Hammer K, Di Virgilio F, Zimmermann H (2000) Assignment of ecto-nucleoside triphosphate diphosphohydrolase-1/cd39 expression to microglia and vasculature of the brain. Eur J Neurosci 12:4357–4366

Braun N, Sévigny J, Mishra SK, Robson SC, Barth SW, Gerstberger R, Hammer K, Zimmermann H (2003) Expression of the ecto-ATPase NTPDase2 in the germinal zones of the developing and adult rat brain. Eur J Biochem 17:1355–1364

Cammer W, Tansey FA, Sacchi R (1986) Antibody against mouse liver 5′-nucleotidase immunostains white matter in the adult mouse central nervous system. J Neurol Sci 73:155–167

Chen W, Wieraszko A, Hogan MV, Yang H-A, Kornecki E, Ehrlich YH (1996) Surface protein phosphorilation by ecto-protein kinase is required for the maintenance of hippocampal long-term potentiation. Proc Natl Acad Sci USA 93:8688–8693

Cunha RA, Sebastiao AM, Ribeiro JA (1992) Ecto-5′-nucleotidase is associated with cholinergic nerve terminals in the hippocampus but not in the cerebral cortex of the rat. J Neurochem 59:657–666

Cunha RA, Vizi SE, Ribeiro JA, Sebastiao AM (1996) Preferential release of ATP and its extracellular catabolism as a source of adenosine upon high- but not low-frequency stimulation of rat hippocampal slices. J Neurochem 67:2180–2187

Cunha RA, Sebastiao AM, Ribeiro JA (1998) Inhibition by ATP of hippocampal synaptic transmission requires localized extracellular catabolism by ecto-nucleotidases into adenosine and channeling to adenosine A1 receptors. J Neurosci 18:1987–1995

Cunha RA, Brendel P, Zimmermann H, Ribeiro JA (2000) Immunologically distinct isoforms of ecto-5′-nucleotidase in nerve terminals of different areas of the rat hippocampus. J Neurochem 74:334–338

Dunwiddie TV, Diao L, Proctor WR (1997) Adenine nucleotides undergo rapid, quantitative conversion to adenosine in the extracellular space in rat hippocampus. J Neurosci 17:7673–7682

Ehrlich YH, Davis TB, Bock E, Kornecki E, Lenox RH (1986) Ecto-protein kinase activity on the external surface of neural cells. Nature 320:67–70

Enjyoji K, Sévigny J, Lin Y, Frenette P, Christie PD, Esch JSA, Imai M, Edelberger JM, Rayburn H, Lech M, Beeler DM, Csizmadia E, Wagner DD, Robson SC, Rosemberg RD (1999) Targeted disruption of CD39/ATP diphosphohydrolase results in disordered hemostasis and tromboregulation. Nature Med 5:1010–1017

Franco R, Canela EI, Bozal J (1986) Heterogeneous localization of some purine enzymes in subcellular fractions of rat brain and cerebellum. Neurochem Res 11:423–435
Fujii S (2004) ATP- and adenosine-mediated signaling in the central nervous system: the role of extracellular ATP in hippocampal long-term potentiation. J Pharmacol Sci 94:103–106
Gulyás AI, Toth K, McBain CJ, Freund TF (1998) Stratum radiatum giant cells: a type of principal cells in the rat hippocampus. Eur J Neurosci 10:3813–3822
Heine P, Braun N, Heilbronn A, Zimmermann H (1999) Functional characterization of rat ecto-ATPase and ecto-ATP diphosphohydrolase after heterologous expression in CHO cells. Eur J Biochem 262:102–107
Illes P, Norenberg W (1993) Neuronal ATP receptors and their mechanism of action. Trends Pharmacol Sci 14:50–54
Kansas GS, Wood GS, Tedder TF (1991) Expression, distribution, and biochemistry of human CD39. Role in activation-associated homotypic adhesion of lymphocytes. J Immunol 146:2235–2244
Kawamura M, Gachet C, Inoue K, Kato F (2004) Direct excitation of inhibitory interneurons by extracellular ATP mediated by P2Y1 receptors in the hippocampal slice. J Neurosci 24:10835–10845
Kegel B, Braun N, Heine P, Maliszewski CR, Zimmermann H (1997) An ecto-ATPase and an ecto-ATP diphosphohydrolase are expressed in rat brain. Neuropharmacology 36:1189–1200
Kennedy C, Leff P (1995) Painful connection for ATP. Nature 3:385–386
King BF, Townsend-Nicholson A, Burnstock G (1998) Metabotropic receptors for ATP and UTP: exploring the correspondence between native and recombinant nucleotide receptors. Trends Pharmacol Sci 19:506–514
Kreutzberg GW, Heymann D, Reddington M (1986) 5′-nucleotidase in the nervous system. In: Kreutzberg GW, Reddington M, Zimmermann H (eds) Cellular biology of ectoenzymes. Springer-Verlag, Berlin, pp 147–164
von Kugelgen I, Spath L, Starke K (1994) Evidence for P2-purinoceptormediated inhibition of noradrenaline release in rat brain cortex. Br J Pharmacol 113:815–822
von Kugelgen I, Koch H, Starke K (1997) P2-receptor-mediated inhibition of serotonin release in the rat brain cortex. Neuropharmacology 36:1221–1227
Kukley M, Stausberg P, Adelmann G, Iain PC, Dietrich D (2004) Ecto-nucleotidases and nucleoside transporters mediate activation of adenosine receptors on hippocampal mossy fibers by P2X7 receptor agonist. J Neurosci 24:7128–7139
Kukulski F, Komoszynski M (2003) Purification and characterization of NTPDase 1 (ecto-apyrase) and NTPDase 2 (ecto-ATPase) from porcine brain cortex synaptosomes. Eur J Biochem 270:3447–3454
Lavoie EG, Kukulski F, Levesque SA, Lecka J, Sevigny J (2004) Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-3. Biochem Pharmacol 67:1917–1926
Lee KS, Schubert P, Reddington M, Kreutzberg GW (1986) The distribution of adenosine A1 receptors and 5′-nucleotidase in the hippocampal formation of several mammalian species. J Comp Neurol 246:427–434
Martin E, Buño W (2005) Stabilizing effects of extracellular ATP on synaptic efficacy and plasticity in hippocampal pyramidal neurons. Eur J Neurosci 21:936–944
Masino SA, Diao I, Illes P, Zahniser NR, Larson GA, Johanson B, Fredholm BB, Dunwiddie TV (2002) Modulation of hippocampal glutaminergic transmission by ATP is dependant on adenosine A1 receptors. J Pharmacol Exp Ther 303:356–363
de Mendonca A, Costenla AR, Ribeiro JA (2002) Persistence of the neuromodulatory effects of adenosine on synaptic transmission after long-term potentiation and long-term depression. Brain Res 5:56–60
Nacimiento W, Kreutzberg GW (1990) Cytochemistry of 5′-nucleotidase in the superior cervical ganglion of the rat: effects of pre- and postganglionic axotomy.Exp. Neurology 109:362–373
Neary JT, Kang Y, Bu Y, Yu E, Akong K, Peters CM (1999) Mitogenic signaling by ATP/P2Y purinergic receptors in astrocytes: involvement of a calcium-independent protein kinase C, extracellular signal-regulated protein kinase pathway distinct from the phosphatidylinositol-specific phospholipase C/calcium pathway. J Neurosci 19:4211–4220
Nedeljkovic N, Banjac A, Horvat A, Stojiljkovic M, Nikezic G (2003) Ecto-ATPase and ecto-ATP-diphosphohydrolase are co-localized in rat hippocampal and caudate nucleus synaptic plasma membranes. Physiol Res 52:797–804
Nedeljkovic N, Bjelobaba I, Subasic S, Lavrnja I, Pekovic S, Stojkov D, Vjestica A, Rakic L, Stojiljkovic M (2006) Up-regulation of ectonucleotidase activity after cortical stab injury in rats. Cell Biol Int 30:541–546

Ochiisshi T, Chen L, Yukawa A, Saitoh Y, Sekino Y, Arai T, Nakata H, Miyamoto H (1999) Cellular localization of adenosine A1 receptors in rat forebrain: immunohistochemical analysis using adenosine A1 receptor-specific monoclonal antibody. J Comp Neurol 411:301–316
Pankratov Y, Lalo U, Verkhratsky A, North RA (2006) Vesicular release of ATP at central synapses. Pflugers Arch Eur J Physiol 452:589–597
Pedrazza EL, Riboldi GP, Pereira GS, Izquierdo I, Bonan CD (2007) Habituation to an open field alters ecto-nucleotidase activities in rat hippocampal synaptosomes. Neurosci Lett 413:21–24
Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492
Richardson PJ, Brown SJ, Bailyes EM, Luzio JP (1987) Ectoenzymes control adenosine modulation of immunoisolated cholinergic synapses. Nature 327:232–234
Ross FM, Brodie MJ, Stone TW (1998) Adenosine monophosphate as a mediator of ATP effects at P1 purinoceptors. Brit J Pharmacol 124:818–824
Scemes E, Suadicani SO, Spray DC (2000) Intercellular communication in spinal cord astrocytes: fine tuning between gap junctions and P2 nucleotide receptors in calcium wave propagation. J Neurosci 20:1435–1445
Schoen SW, Graeber MB, Reddington M, Kreutzberg GW (1987) Light and electron microscopical immunocytochemistry of 5′-nucleotidase in rat cerebellum. Histochemistry 87:107–113
Schoen SW, Graeber MB, Toth L, Kreutzberg GW (1988) 5′-Nucleotidase in postnatal ontogeny of rat cerebellum: a marker for migrating nerve cells? Brain Res 467:125–136
Schoen SW, Ebert U, Loscher W (1999) 5′-Nucleotidase activity of mossy fibers in the dentate gyrus of normal and epileptic rats. Neuroscience 93:519–526
Smith TM, Kirley TL (1999) Glycosylation is essential for functional expression of a human brain ecto-apyrase. Biochemistry 38:1509–1516
Song Z, Sladek CD (2005) Does conversion of ATP to adenosine terminate ATP-stimulated vasopressin release from hypothalamo-neurohypophyseal explants? Brain Res 1047:105–111
Volonte C, Merlo D, Ciotti MT, Calissano P (1994) Identification of an ectokinase activity in cerebellar granule primary neurons. J Neurochem 63:2028–2037
Vorhoff T, Zimmermann H, Pelletier J, Sevigny J, Braun N (2005) Cloning and characterization of the ecto-nucleotidase NTPDase3 from rat brain: predicted secondary structure and relation to other members of the E-NTPDase family and actin. Purinergic Signal 1:259–270
Wang T-F, Guidotti G (1998) Widespread expression of ecto-apyrase (CD39) in the central nervous system. Brain Res 790:318–322
Wink MR, Braganhol E, Tamajusuku AS, Lenz G, Zerbini LF, Libermann TA, Sevigny J, Battastini AM, Robson SC (2006) Nucleoside triphosphate diphosphohydrolase-2 (NTPDase2/CD39L1) is the dominant ectonucleotidase expressed by rat astrocytes. Neuroscience 138:421–432
Zimmermann H (1992) 5′-Nucleotidase: molecular structure and functional aspects. Biochem J 285:345–365
Zimmermann H (2000) Extracellular metabolism of ATP and other nucleotides. Nanyn-Schmiedeberg's Arch Pharmacol 362:299–309
Zimmermann H, Braun N (1999) Ecto-nucleotidases: molecular structures, catalytic properties, and functional roles in the nervous system. Prog Brain Res 120:371–385
Zimmermann H, Vogel M, Laube U (1993) Hippocampal localization of 5′-nucleotidase as revealed by immunocytochemistry. Neuroscience 55:105–112
Zinchuk VS, Okada T, Kobayashi T (1999) Detection of ecto-ATPase activity in synaptic plasma membranes for studying extracellular ATP induced signal transduction. Brain Res Prot 4:258–265