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REVIEW **Open Access**

Enzyme histochemistry: a useful tool for examining the spatial distribution of brain ectonucleotidases in (patho)physiological conditions

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Summary. Adenosine 5'-triphosphate (ATP) and other nucleotides and nucleosides, such as adenosine, are versatile signaling molecules involved in many physiological processes and pathological conditions in the nervous system, especially those with an inflammatory component. They can be released from nerve cells, glial cells, and vascular cells into the extracellular space where they exert their function *via* ionotropic (P2X) or metabotropic (P2Y) receptors. Signaling *via* extracellular nucleotides and adenosine is regulated by cell-surface located enzymes ectonucleotidases that hydrolyze the nucleotide to the respective nucleoside. This review summarizes a histochemical approach for detection of ectonucleotidase activities in the cryo-sections of brain tissue. The enzyme histochemistry (EHC) might be used as suitable replacement for immunohistochemistry, since it gives information about both localization and activity, thus adding a functional component to a classical histological approach. With this technique, it is possible to visualize spatial distribution and cell-specific localization of ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) and ecto-5'-nucleotidase (eN/CD73) activities during brain development, after different hormonal manipulations, during neurodegeneration, etc. EHC is also suitable for investigation of microglial morphology in different (patho)physiological conditions. Furthermore, the review describes how to quantify EHC results.

Key words: Immunohistochemistry, NTPDase, Ecto-5'-nucleotidase, Hippocampus, Enzyme histochemistry, Microglia

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Introduction

Extracellular purines are ubiquitous, diffusible paracrine factors affecting a wide range of processes in the nervous system (NS) including neurotransmission, neuromodulation, proliferation, differentiation, migration, development, and regeneration (Zimmermann, 2006b, 2011b; Burnstock and Ulrich, 2011; Cavaliere et al., 2015; Oliveira et al., 2016; Grkovic et al., 2019a). Adenosine 5'-triphosphate (ATP) is the most ubiquitous purine molecule, which besides its well-known role as a main source of energy, acts as an extracellular purinergic signaling molecule that mediates communication between neuronal cells (Zimmermann, 2006a,b, 2011; Burnstock, 2007; Dale, 2008; Rebola et al., 2008; Neary and Zimmermann, 2009; Franke et al., 2012; del Puerto et al., 2013; Duster et al., 2014; Sebastiao and Ribeiro, 2015). ATP can also mediate communication between glial cells, acting as a gliotransmitter. When secreted from astrocytes *via* exocytosis or through membrane channels (connexins, volume-regulated Cl^- channels or P2X7 receptors), it can serve as an important signal for neuron-glial and glial-glial communications and interactions between different transmitter systems, by acting at subsets of purinergic P2 receptors (Khakh and North, 2012; Burnstock, 2013, 2020; Duster et al., 2014; Heine et al., 2016; Koles et al., 2016). Additionally, 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, gaminobutyric acid [GABA], noradrenalin) thus contributing to short-time actions as a neuromodulator of both excitatory and inhibitory synapses (Burnstock, 2013, 2020).

In the extracellular space, purine nucleotides (i.e. ATP) are inherently short-lived molecules, which undergo rapid enzymatic degradation by cell-surface localized ectonucleotidases (Zimmermann, 2006a,b; Yegutkin, 2008; Zimmermann et al., 2012). These enzymes are responsible for the tight control and

modulation of P2 ligand availability, and duration and degree of their activation, thus preventing their desensitization or down-regulation (Burnstock, 2007; Zimmermann et al., 2012). A final product of ATP enzymatic degradation is adenosine, a potent signaling molecule, homeostatic regulator and modulator of neural activity that affects neurotransmission, synaptic plasticity, learning and memory by acting at adenosine P1 receptors (Cunha, 2008, 2016; Gomes et al., 2011; Sperlagh and Vizi, 2011; Dias et al., 2013; Sebastiao and Ribeiro, 2015). Communication between astrocytes, microglia and neurons during neurodegenerative and neuroinflammatory conditions are severely altered and mediated *via* different signaling molecules, and ATP is one of the most ubiquitous (Sperlagh and Illes, 2007). It was shown that altered function of ectonucleotidases and dysregulation of the purinergic signaling are largely implicated in the pathophysiology of several neurological diseases (Burnstock, 2017). Understanding the pattern of catalytic activity of ectonucleotidases is essential for elucidating the control of nucleotide signaling in the brain during pathological conditions.

This review paper is focused on application of enzyme histochemistry (EHC) as a useful method for *in situ* detection of spatial pattern and orthotopic localization of ectonucleotidase activity in the rat hippocampus. This method provides insight into the localization and activity of ectonucleotidases, adding a functional component to a classical histological approach and may serve as a suitable replacement for immunohistochemistry.

Ectonucleotidases

Ectonucleotidases comprise a family of ecto-enzymes that include ecto-nucleotide pyrophosphatates/phosphodiesterases (E-NPP), alkaline phosphatases (APs), ecto-nucleoside triphosphate diphospho-hydrolases (E-NTPDases) and ecto 5' nucleotidase (eN), which differ in tissue distribution, functional and molecular properties (Yegutkin, 2008, 2014; Zimmermann et al., 2012; Grkovic et al., 2019a). The most studied ectonucleotidases in the NS belongs to APs, E-NTPDase and ecto 5' nucleotidase families.

Ecto-nucleotide pyrophosphatases/phosphodiesterases

The E-NPP family represents a versatile group of seven structurally related enzymes (NPP1-7) with pyrophosphatase and phosphodiesterase activities having a wide range of substrates (Goding et al., 2003; Zimmermann et al., 2012; Borza et al., 2022). NPP1,3,4,5 specialize on nucleotide-base substrates (e.g., diadenosine triphosphate and cyclic nucleotides), but NPP2,6,7 diverge in substrate preference, notably for lysophospholipids. A common feature is the action toward a phosphate diester bond, which remains the defining feature of the family. The thiamine pyrophosphate (TPP) is the "false" substrate mainly used for NPP identification in *in situ* activity assays (Langer et al., 2007). NPPs are optimal at highly alkaline pH values, but they also retain significant catalytic activity at pH 7.4 (Asensio et al., 2007). NPPs have multiple physiological roles, including nucleotide recycling, modulation of purinergic receptor signaling, regulation of extracellular pyrophosphate levels, stimulation of cell motility, and possible roles in regulation of insulin receptor (IR) signaling and activity of ecto-kinases (Goding et al., 2003). The membrane-bound ectoenzymes NPP1 and NPP3 and the secreted NPP2 are the most studied members.

Three members of the E-NPP family (NPP1-3) are localized in the NS, 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 (Goding et al., 2003; Vollmayer et al., 2003; Stefan et al., 2005; Bjelobaba et al., 2006; Oaknin et al., 2008; Zimmermann et al., 2012; Grkovic et al., 2019a; Onyedibe et al., 2019). NPP1 and NPP3 also hydrolyze 5' monodiester bonds in extracellular ATP, resulting in the release of AMP and inorganic pyrophosphate (PPi) (Goding et al., 2003; Stefan et al., 2005). In the rodent brain, NPP1 is indicated as the main ectoenzyme involved in the cleavage of diadenosine polyphosphates by glial cells and neurons (Bjelobaba et al., 2006; Asensio et al., 2007; Langer et al., 2008). It is also expressed by ependymal cells, in rat C6 glioma cells (Grobben et al., 1999; Claes et al., 2004), endothelial cells and together with NPP2 in the cells of the choroid plexus (Fuss et al., 1997; Bjelobaba et al., 2006) where it most likely modulates purinergic signaling that contributes to the composition and secretion of cerebral spinal fluid (Xiang and Burnstock, 2005). In glioblastoma stem-like cells, NPP1 plays an important role in balancing the pool of nucleotides, thus maintaining glioblastoma cells in an undifferentiated proliferative state (Bageritz et al., 2014). A splice variant of NPP2 has been identified during intermediate stages of rat brain oligodendrocyte differentiation, and myelin formation (Fox et al., 2004). Moreover, this enzyme can produce lysophosphatidic acid (LPA), an important molecule for cerebral maturation. In the developing brain, NPP3 is expressed in immature astrocytes (Blass Kampmann et al., 1997), while immunolabeling for NPP3 is restricted to the ependymal cells of ventricular system of adult brains (Fuss et al., 1997).

Alkaline phosphatases

Alkaline phosphatases (APs) are zinc-containing dimeric membrane-bound glycoproteins that require magnesium ion (Mg^{2+}) for the hydrolysis of a wide range of phosphomonoesters. Their main functions consist of catalyzing dephosphorylation and transphosphorylation reactions on a broad spectrum of physiological and non-physiological substrates (Millan,

2005). Although optimum activity occurs at alkaline pH (9.3-10.3), they are also active at a physiological pH (Fonta et al., 2004, Langer et al., 2008). Tissue-nonspecific alkaline phosphatase (TNAP), homodimeric protein anchored to the cytoplasmic membrane *via* glycosylphosphatidyl inositol (GPI) anchor, represents the only isoform of APs expressed in the mammalian central nervous system (CNS) (Millan, 2005; Zimmermann et al., 2012; Sebastian-Serrano et al., 2015). In CNS, TNAP is associated with the blood vessel endothelium, neuronal membranes, including axonal, and dendritic processes, and synaptic cleft in the olfactory bulb, thalamus and hypothalamus, cerebral cortex, hippocampus, inferior and superior colliculi, tegmentum, dorsal and ventral medulla (Fonta et al., 2004; Langer et al., 2008). 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 (Zimmermann et al., 2012; Sebastian-Serrano et al., 2015). 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 (Fonta et al., 2004; Ermonval et al., 2009). Little is known about the mechanism that controls TNAP expression, except that it may be induced by retinoic acid (Scheibe et al., 2000), activation of the phosphatidyl inositol 3-kinase/Akt pathway (Noda et al., 2005) and 17β-estradiol (Mitrovic et al., 2017).

Ecto-nucleoside triphosphate diphosphohydrolases

The E-NTPDase family is composed of eight different members, classified in order of their discovery and classification. Each E-NTPDase member possesses different enzymatic properties and distinct cellular localization. Four of the nucleoside triphosphate diphosphohydrolases (NTPDase1, 2, 3 and 8) are cell surface-bound enzymes (Robson et al., 2006; Yegutkin, 2008, 2014; Zimmermann et al., 2012). NTPDases5 and 6 exhibit intracellular localization and undergo secretion after heterologous expression, while NTPDases 4 and 7 are intracellularly located facing the lumen of cytoplasmic organelles (Robson et al., 2006; Zimmermann et al., 2012; Yegutkin, 2014). The cell surface-expressed members of the E-NTPDase family display similar structural properties, with two transmembrane domains close to the N- and C-terminus, and a catalytic extracellular domain (Zimmermann et al., 2012). They require millimolar concentrations of Mg^{2+} and Ca^{2+} ions in order to perform substrate hydrolysis, and are active in physiological to slightly basic pH range. NTPDase1, NTPDase2, NTPDase3, and NTPDase8 hydrolyze both nucleoside triphosphates and diphosphates to nucleoside monophosphates but the range of substrates of the other enzymes is more restricted. NTPDase1 hydrolyzes ATP and ADP with almost the same efficiency, while NTPDase3 and NTPDase8 hydrolyze ATP or uridine triphosphate (UTP) more efficiently than ADP or uridine diphosphate (UDP). The NTPDase2 is the most ATP-specific NTPDase, which preferentially dephosphorylates ATP to ADP (Yegutkin, 2014).

NTPDases1, 2, and 3 are expressed in nervous tissue, while NTPDase8 has a restricted tissue distribution (Kukulski et al., 2011; Zimmermann et al., 2012; Yegutkin, 2014). As in other tissue and cells, brain NTPDase1 hydrolyzes ATP and ADP to AMP almost equally, while NTPDase2 preferentially dephosphorylates ATP (Kukulski and Komoszynski, 2003; Zimmermann et al., 2012), leading to the accumulation of 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 (Cunha, 2001a; Vorhoff et al., 2005; Zimmermann et al., 2012). Therefore, NTPDase2 and NTPDase3 produce agonists that act at ADP-sensitive purinoceptors, such as P2Y1, P2Y12, and P2Y13 (Abbracchio et al., 2006). Besides its hydrolyzing activity, NTPDase1 is reported to function as a cell adhesion molecule (Wu et al., 2006). NTPDase1 is widely expressed and associated with microglia, vascular endothelium, and neurons (Wang and Guidotti, 1998; Braun et al., 2000; Bjelobaba et al., 2007; Langer et al., 2008; Aliagas et al., 2013a; Grkovic et al., 2019a). Several studies reported that NTPDase2 is localized at neurons (Mitrovic et al., 2017; Grkovic et al., 2019a,b), astrocytes in different brain regions (Braun et al., 2003; Wink et al., 2003, 2006; Gampe et al., 2012), slow proliferating subventricular zone 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 (Braun et al., 2003; Shukla et al., 2005; Langer et al., 2007). 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 (Belcher et al., 2006; Grkovic et al., 2016). 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 were observed in the hippocampus, cortex, and other brain regions (Belcher et al., 2006; Kiss et al., 2009; Bjelobaba et al., 2010; Grkovic et al., 2016).

Ecto-5'-nucleotidase

Extracellular AMP resulting from the hydrolysis of ATP and ADP by most of the ectonucleotidases can in turn be efficiently hydrolyzed into adenosine by ecto 5'nucleotidase (eN). eN is a GPI-linked membrane-bound glycoprotein also known as cluster of differentiation 73 (CD73) (Colgan et al., 2006; Zimmermann et al., 2012; Kulesskaya et al., 2013). The enzyme displays affinity for AMP in low micromolar

range and is feed-forward inhibited by high extracellular ATP and ADP concentration, resulting in a delayed "burst-like" production of adenosine (Cunha, 2001a). It is regarded as the key enzyme in the extracellular formation of adenosine, which acts as a neuromodulator and important trophic and homeostatic factor in the brain (Cunha, 2001b). Although eN/CD73 activity is ion-independent in physiological conditions, the presence of Mg^{2+} ions *in vitro* can considerably increase its ability to hydrolyze AMP. eN is located on astrocytes, neurons, microglia, and oligodendrocytes in different brain regions (Zimmermann, 2006a,b; Bjelobaba et al., 2007, 2011; Langer et al., 2008). The functions of eN go beyond the activity of adenosine-producing enzyme (Zimmermann, 1996; Stanojevic et al., 2011; Mitrovic et al., 2016a; Adzic and Nedeljkovic, 2018; Dragic et al., 2021a). eN is relevant for intercellular adhesion, signaling, and cell migration (Zimmermann, 1996; Zimmermann et al., 2012) since it carries the epitopes implicated in the cell-cell and cell-matrix interactions and binds to the extracellular matrix components and may mediate cellular adhesion (Vogel et al., 1993; Zimmermann, 1996). Double immuno-fluorescence staining of eN and presynaptic membrane marker syntaxin provided the first direct evidence for the existence of this ecto-enzyme in the synaptic compartment (Stanojevic et al., 2011).

The highly variable levels of eN activity in health and disease raised an important question of the regulatory mechanism(s) controlling the enzyme expression at different levels. It was shown that expression of eN is under direct and/or indirect transcriptional control by various transcription factors (Spychala et al., 1999; Ille and Sommer, 2005; Salinas, 2012; Dickins and Salinas, 2013), growth factors (Kohring and Zimmermann, 1998), specific signal transduction pathways that induce *de novo* local protein synthesis (Spychala et al., 1997, 1999), and hormones (Spychala et al., 2004; Bavaresco et al., 2007; Drakulic et al., 2015; Mitrovic et al., 2016a,b).

The significance of examining the activity and localization of ectonucleotidases

NTPDase1/CD39 and eN/CD73 are crucial enzymes for calibrating the duration, magnitude, and composition of the purinergic signaling. These key ectonucleotidases have been extensively investigated with regard to their molecular structures and functions (Robson et al., 2006; Knowles, 2011; Zimmermann et al., 2012). The expression and activity of both NTPDase1/CD39 and eN/CD73 undergo dynamic changes in accordance with the pathophysiological context in which they are embedded (Schetinger et al., 2007; Antonioli et al., 2013). This catabolic machinery can modulate the course and dictate the outcome of several pathophysiological conditions, such as inflammation (Antonioli et al., 2013; Eltzschig et al., 2013; Idzko et al., 2014), epilepsy (de Paula Cognato et al., 2005), infections (Sansom et al., 2008; Knowles, 2011; Mahamed et al., 2012), AIDS, autoimmune diseases, atherosclerosis, ischemia-reperfusion injury, and tumor growth, and metastasis (Stagg and Smyth, 2010; Deaglio and Robson, 2011; Michaud et al., 2011; Nikolova et al., 2011; Bonner et al., 2012; Zhang, 2012; Bastid et al., 2013; Young et al., 2014). The histochemical and cytochemical analysis of ectonucleotidase activities are of great importance in defining cell types within tissues, and even the plasma membrane domains that possess such activities (Deaglio and Robson, 2011; Nikolova et al., 2011; Bonner et al., 2012; Zhang, 2012; Bastid et al., 2013; Yegutkin, 2014). Having in mind the abovementioned, the knowledge on ectonucleotidases localization and activity is of great importance for a full understanding of the various pathophysiological conditions (Giuliani et al., 2021).

Studying ectonucleotidases using enzyme histochemistry

Enzyme histochemistry (EHC) is a morphological technique applied to functional questions in histo(patho)physiology. It constitutes a link between biochemistry, physiology and morphology (Hardonk and Koudstaal, 1976; Patrick et al., 1980; Meier-Ruge and Bruder, 2008) and provides important information complementary to conventional histology, immuno-histochemistry, and molecular analysis. On the other hand, biochemistry applied to tissue homogenates or extracts, reveals a general increase or decrease in enzyme activity measured in turnover rates. The strength of biochemistry lies in the potential for quantification: biochemistry permits exact quantification of enzymatic turnover rates, whereas until recently enzyme histochemistry had the disadvantage of a lack of reliable quantification.

Enzyme histochemistry has been a classical approach for studying the distribution of surface-located ectonucleotidases ever since the ground breaking methodological work of Wachstein and Meisel (1957) used for histochemical characterization of hepatic phosphatases (Wachstein and Meisel, 1957). EHC is feasible since ectonucleotidases maintain their hydrolyzing activity in both formalin-fixed and frozen tissues (and cells). Inorganic phosphorous (Pi) generated upon their activity in combination with a lead salt added to the reaction mixture, form brown precipitates in the enzyme-active area, allowing the visualization under light microscope. It became a suitable additional and/or replacement tool for studying nucleotidases since the classical immunohisto/cytochemistry approach was faced with several major concerns such as specificity of the antibodies, the liability of the antigen, and discrepancies between immunohistochemical and enzyme histochemical data. For example, in spite of a considerable amount of enzyme histochemical evidence, antibodies specifically directed against eN/CD73 failed to recognize the majority of sites detected by EHC in the brain (Schoen et al., 1988; Braun et al.,

1994; Zimmermann and Braun, 1996). Similarly, NTPDase1/CD39 could readily be located to cerebral blood vessels, but in brain sections, its association with microglia could be established only by enzyme histochemistry (Braun et al., 2000). As a result, there was controversy regarding the expression of individual subtypes of ectonucleotidases in neurons, astrocytes, and microglia (Zimmermann et al., 1998; Zimmermann, 2006a,b). Thanks to Professor Jean Sévigny (www.ectonucleotidases-ab.com), reliable antibodies for immunohisto- and immunocytochemical localization of ectonucleotidases have become available in the last decade. Nowadays, the overall EHC reaction of the NTPDase1/CD39 or eN/CD73 in the brain regions may be directly compared and provide novel evidence regarding their association with the neuropil and microglia.

Enzyme histochemistry technique

The distribution of ectonucleotidases can be evaluated by co-incubation of frozen tissue sections or cultured cells with lead nitrate $Pb(NO_3)_2$ and one of the following substrates: ATP, ADP and other NTPs and NDPs (for NTPDase activity); AMP (for eN/CD73); nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (serving as artificial substrates for alkaline phosphatase); thiamine pyrophosphate (in case of NPP activity); TMP and AMP (for measuring PAP-mediated nucleotide hydrolysis at acidic pH) (Langer et al., 2008; Yegutkin, 2014). The orthophosphate released in the course of the catalytic reaction is then precipitated in the presence of ammonium sulfide as a brown lead salt and can be further visualized by light microscopy.

The protocol for localization of adenosine monophosphatase (AMPase, ecto 5' nucleotidase), adenosine diphosphatase (ADPase), adenosine triphosphatase (ATPase) activities has been based on modified Wachstein-Meisel lead phosphate method described elsewhere (Braun et al., 2000; Langer et al., 2008; Dragic et al., 2019a,b, 2021a; Grkovic et al., 2019b). Following this protocol (Fig. 1), brain tissue should be fixed with 4% paraformaldehyde for 24h. Following fixation, brains need to be cryopreserved in graded sucrose (10-30% sucrose in 0.2 M phosphate buffer), cut using cryostat into 20-25 μm-thick sections, and mounted on slides. Sections should be then air-dried for 2h and stored at -20°C until further use. At the start of EHC, tissue slides need to be washed with 50 mM Tris-maleate buffer pH 7.4 at room temperature (RT), incubated for 30 min at RT with pre-incubation buffer (50 mM Tris-maleate buffer pH 7.4 containing 2 mM $MgCl_2$ and 250 mM sucrose) and then re-incubated for 1

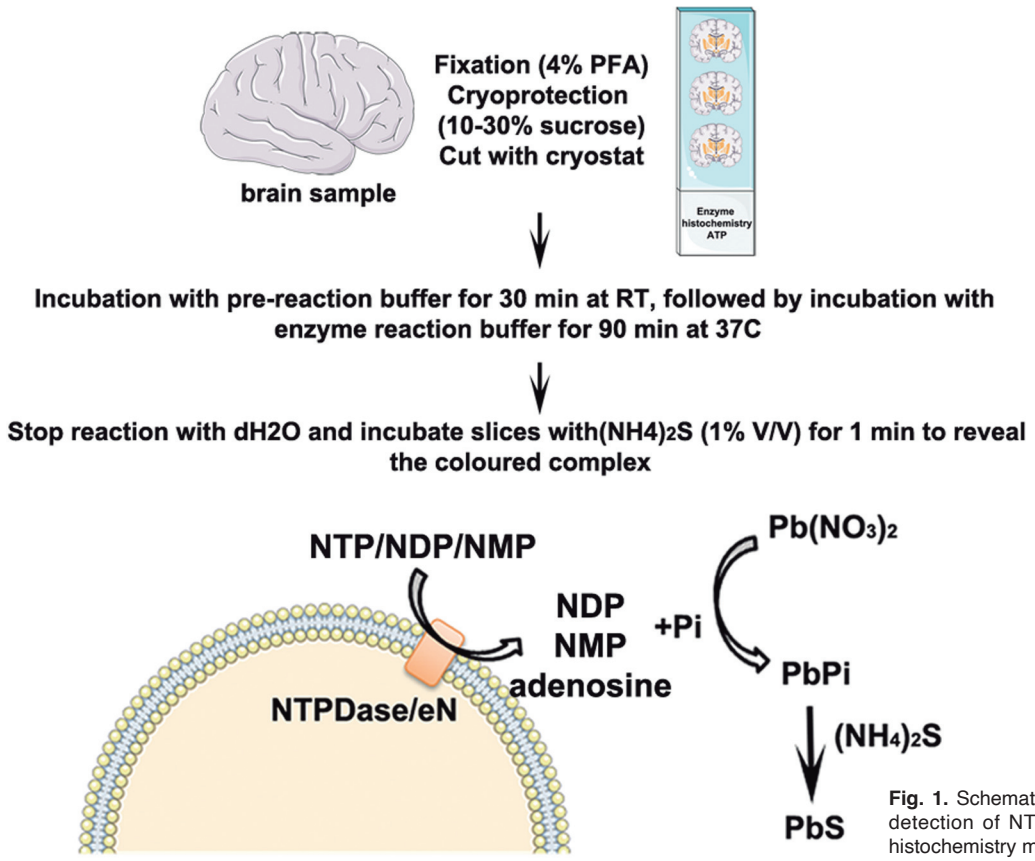

Fig. 1. Schematic illustration of the main steps for detection of NTPDase/eN activity using enzyme histochemistry method on brain cryosections.

h at 37°C with the enzyme reaction buffer (50 mM Tris-maleate buffer pH 7.4 supplemented with 250 mM sucrose, 2 mM $MgCl_2$, 5 mM $MnCl_2$, 2 mM $Pb(NO_3)_2$, stabilized with 3% Dextran T-250) in the presence or absence of nucleotide as substrate (e.g., ATP, ADP, AMP). The incubation time and the substrate concentration may vary depending on the experiment, but 1-2 h at 1-2 mM is generally suitable. The reaction is stopped with dH_2O. When the enzyme acts on the substrate, it yields inorganic phosphate (Pi) that reacts with the Pb^{2+} ions and precipitates "*in status nascendi*" as lead phosphate, which is insoluble in aqueous solution. As the lead phosphate is white and cannot be distinguished with the light microscope, it is necessary to develop the histochemical reaction by using a sulfide salt which will yield lead sulfide that is brown and therefore visible with the light microscope. Thus, products of enzyme activities are revealed by incubating with 1% $(NH_4)_2S$ v/v for exactly 1 min (Fig. 1). To avoid interference with AP activity, experiments need to be performed in the presence of the AP inhibitor levamisole (2.5 mM). A control slide in the absence of nucleotide, in which no reaction is expected, should be also included. Samples are then dehydrated in graded EtOH, cleared in xylene and mounted with DPX. Finally, samples are photographed under light microscope and enzyme-active sites are observed as brownish black deposits. Besides levamisole, other enzyme inhibitors might be included in both pre-incubation and enzyme reaction buffers. For example, 1 mM α,β-methylene-ADP efficiently inhibits CD73, and POM1 inhibits NTPDases (Wall et al., 2008; Bhattarai et al., 2015, 2020).

Applications of enzyme histochemistry in different experimental setups

Using this approach, tissue and cellular localization as well as substrate specificity of different ecto-enzymes often with clear overlapping with immunohisto-chemistry, had been characterized in the murine brain (Langer et al., 2008), rat brain (Braun et al., 2000; Braun et al., 2004; Grkovic et al., 2014, 2019b; Dragic et al., 2019a,b, 2021a), mouse and rat microglia and brain vessels (Braun et al., 2000, 2004; Dragic et al., 2019a; Grkovic et al., 2019b), dorsal root ganglion, spinal cord (Zylka et al., 2008; Sowa et al., 2010; Vongtau et al., 2011; Street et al., 2013; McCoy et al., 2014; Dragic et al., 2021b), and neurogenic zones (Shukla et al., 2005; Langer et al., 2007), but also in all other tissues (Kittel et al., 2004; Aliagas et al., 2010; Kauffenstein et al., 2010; Lavoie et al., 2010; Lavoie et al., 2011; Fausther et al., 2012; Mercier et al., 2012; Aliagas et al., 2013b; Trapero et al., 2019; Losenkova et al., 2020, 2022), and different cultured cells (Braun et al., 2000; Zylka et al., 2008; Street et al., 2013), etc. After slight modifications in the staining protocol, and replacement of ammonium sulfide by glutaraldehyde, this technique can also be applied for enzyme histochemical analysis using electron microscopy (Kirino et al., 2013).

Brain development

Enzyme histochemistry might be performed for investigation of the activity and topographical distribution of ecto-nucleotidases during brain development (Dalmau et al., 1998; Grkovic et al., 2014). The specific staining during embryonic and the first two weeks of postnatal development as a result of NTPase, NDPase, and NMPase activities were located on the microglial plasma membranes, as had been described by (Dalmau et al., 1998). EHC staining in the developing rat brain had revealed the presence of a heterogeneous population of glial cells belonging to the microglial lineage that are down-regulated with brain development and maturation (Dalmau et al., 1998). Furthermore, products of ATPase, ADPase and AMPase activities in the sections obtained from the rat brains at postnatal day (PD) 7 showed different patterns than in the adult sections (our unpublished results) (Fig. 2). The most interesting aspect is the specific and intensive labeling as a product of EHC observed in the subcortical white matter and corpus callosum (Fig. 2A-C,E) that probably correspond to amoeboid microglial cells (Dalmau et al., 1998) and could not be observed in the sections from adult brains (Fig. 2D,H). Nowadays techniques and availability of specific antibodies allow us closer inspection, and comparison of EHC results and fluorescent immunohistochemistry against eN/CD73 show uniform results, intensively AMPase-labeled round cells in the subcortical plate certainly express eN/CD73 (Fig. 2F,G) and could not be observed in the adult sections (Fig. 2H).

Spatial distribution of ectonucleotidases in female rat hippocampus; the influence of sex steroids

Since different methods such as enzyme assays, qPCR, immunoblots etc. indicated that ectonucleotidases are abundantly expressed in the rat hippocampus, EHC was used to examine spatial distribution of ATPase/ADPase/AMPase activity in the hippocampus of female Wistar rats (Grkovic et al., 2019b).

In the hippocampus of intact females, ATPase activity was mostly localized in synapse-rich hippocampal layers, such as *stratum oriens* (so), *stratum radiatum* (sr) and *stratum lacunosum-moleculare* (slm) of CA1-CA2, while the strongest histochemical reaction was noticeable in the interposed slm (Fig. 3A). Staining intensities in the corresponding layers of CA3 were weaker, with faintly stained *stratum lucidum* (sl). In the dentate gyrus (DG), weaker reaction is obtained in the hilus and the subgranular zone (sz). The pyramidal cell layer in CA1-CA3 fields and the granular cell layer in the DG were completely free of the reaction product.

The pattern of ADPase histochemical staining was less intense than for ATP (Fig. 3B). As for the distribution of NTPDase1, which hydrolyzes ATP and

ADP almost equally well (Robson et al., 2006; Zimmermann, 2006a; Zimmermann et al., 2012), microglia and blood vessels were depicted by ADPase histochemistry, with a faint background staining of the neuropil. Notably stronger reactions obtained with ATP than with ADP point to NTPDase2 as dominant ectonucleotidase in the hippocampal neuropil, which preferentially hydrolyses ATP (Heine et al., 1999; Grkovic et al., 2019b). This assumption was supported with the expression analysis data showing that NTPDase2-mRNA is, by far, the most abundant ectonucleotidase transcript expressed in the hippocampal tissue (Grkovic et al., 2019b). Selective ADPase reaction (not obtained in the presence of ATP) was observed in scattered cells in the granule cell layer of DG. The staining delineated glial cell processes, which traversed the pyramidal and DG cell layers (Grkovic et al., 2019b).

The pattern of AMPase histochemical reaction clearly differed from those obtained with ATP and ADP as substrates, and for the most part, could not be assigned to individual cell types (Fig. 3C) (Grkovic et al., 2014, 2019b; Dragic et al., 2019b, 2021a). The strongest reaction product was observed in the so and sr of CA1-CA2, in the molecular layer and slm, while apparently there was less intense reaction in *stratum moleculare* (sm). The moderate reaction was noticed in the *stratum lucidum* (sl) and the hilus of the DG. Other CA3 and DG layers were faintly stained. The pyramidal cell layer of CA1-CA3 and granular cell layer were completely spared of staining. The results are in accordance with data obtained from Western blot analysis which revealed that hippocampal subcellular fractions such as purified synaptosomal fraction (SYN) expresses the whole enzyme chain, NTPDase1/NTPDase2/NTPDase3/eN (Grkovic et al., 2014; Mitrovic et al., 2016a, 2017; Grkovic et al., 2019b),

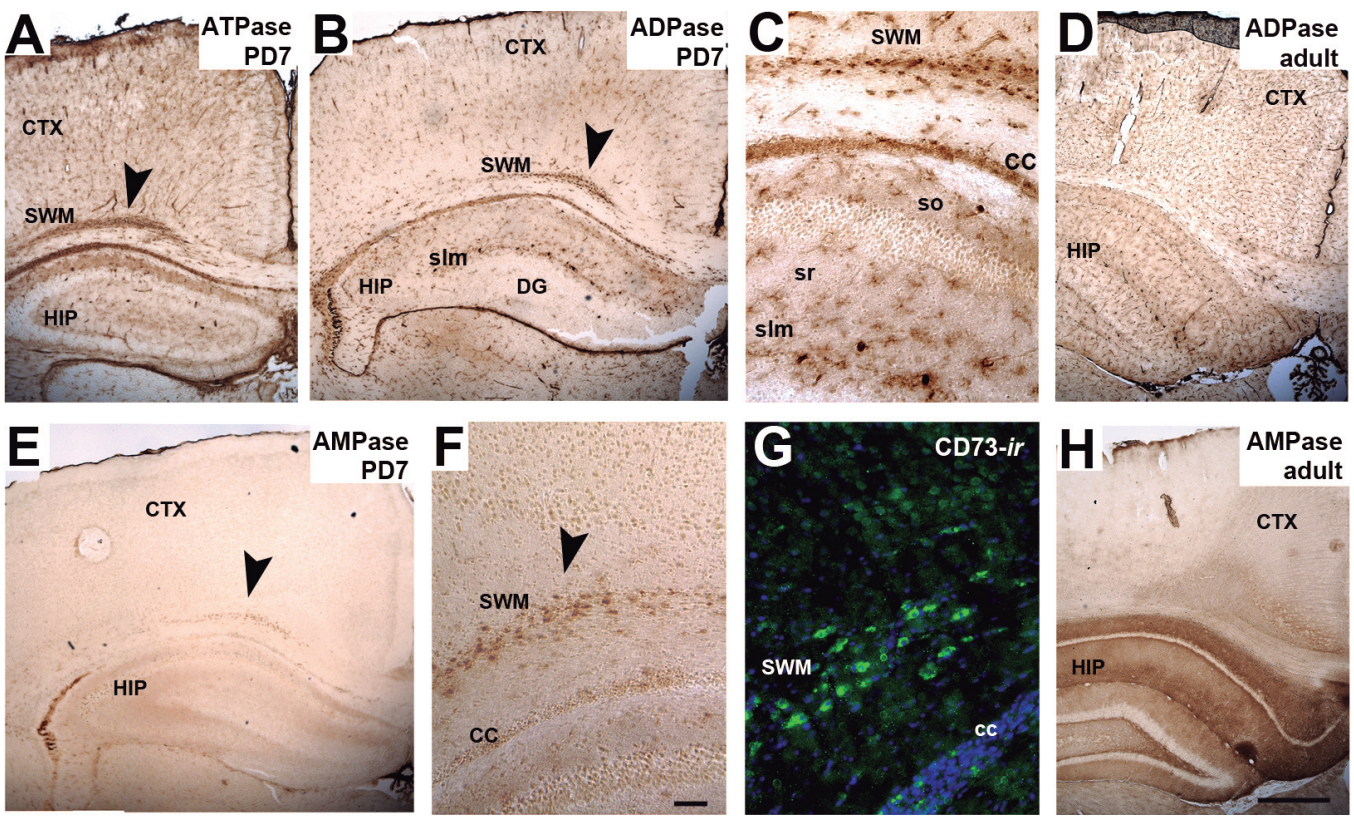

Fig. 2. Enzyme histochemistry in the postnatal rat brain. Sections obtained from the rat brains at postnatal day 7 PD7 **(A-C, E-G)** and adult **(D, H)**. ATPase **(A)** and ADPase **(B, C)** activity in the sensorimotor cortex and the hippocampus in sections obtained from the rat brains at postnatal day 7 (PD7). By means of ADPase histochemistry **(B, C)**, clearly labeled microglial cells are demonstrated in the subcortical white matter (SWM; arrowheads), in the different cortical layers, including the subpial molecular layer cells as well as in the corpus callosum. Also, clearly labeled microglial cells might be observed in the hippocampus subregions, such as stratum oriens (so), stratum radiatum (sr) and the most numerous in the stratum lacunosum moleculare (slm) but without staining in the dentate gyrus (DG). In contrast, ADPase histochemistry of adult sections **(D)** uniformly labeled blood vessels and microglial cells. By means of AMP histochemistry at PD7 that represent eN activity **(E, F)**, staining intensity is almost absent in the dentate gyrus and very weak in other subregions of the hippocampal and cortical areas. Cells from the microglial lineage are basically observed in the subcortical white matter (SWM; arrowheads) and in corpus callosum. This is confirmed with immunofluorescence labeling with antibodies against eN **(G)**. eN-immunoreactivity (eN-ir) is green while dapi staining is blue. In the adult sections **(H)**, the staining was uniformly present in all hippocampal regions and exceptions were the pyramidal and granule cell layers, which were labelled faintly as well as cortical area. Scale bars: A, B, D, E, H, 500 µm; C, F, G, 50 µm.

while gliosomes (GLIO, parts of astrocyte cells) express NTPDase2/eN as dominant ectonucleotidases (Grkovic et al., 2016, 2019b).

These enzymes are modulated/regulated by ovarian hormones (Mitrovic et al., 2016a,b, 2017, 2019), thus EHC was also used for investigation of hormonal manipulations impact of on ecto-enzymes (Grkovic et al., 2019b). Surgically-suppressed circulating ovarian hormones by OVX markedly reduced overall ATPase, ADPase, and AMPase histochemical reactions, whereas E2 restored the histochemical reactions to the level of control (Grkovic et al., 2019b). These findings have been further supported by expression analysis data showing that OVX down-regulates expression of NTPDase1-, NTPDase2-, and eN-mRNA, while E2 restores NTPDase2-mRNA and up-regulates NTPDase1- and eN-mRNA. OVX had no effect on NTPDase3 expression in the hippocampus (Grkovic et al., 2016, 2019b).

Spatial distribution of ectonucleotidase activities in the rat models of neurodegenerative diseases

Altered function of NTPDase1/CD39 and eN/CD73, and dysregulation of purinergic signaling are largely implicated in the pathophysiology of several neurological diseases, including Alzheimer's and Parkinson disease, multiple sclerosis, and astroglioma (Burnstock, 2017), but their cell-specific localization during neurodegeneration is rarely explored. Thus, Dragic and coworkers (2021) recently applied a trimethyltin (TMT)-induced model of hippocampal neurodegeneration and gliosis to characterize the pattern of the ectonucleotidase enzyme activity (Dragic et al., 2019a, 2019b). At the early stage of TMT-induced neurodegeneration, lead-phosphate depositions delineated reactive microglia that covered the strata but also entered the neuronal layers (Dragic et al., 2021a). At the late stage of TMT-induced neurodegeneration, activated microglia accounted for most of the enhanced ATPase/ADPase activities, revealing strong staining of CA strata, while DG was mostly without reaction (Fig. 4C, D). The obtained patterns of ATP/ADP enzyme activities closely corresponded to Iba1-ir and NTPDase1/CD39-ir (Fig. 4E-G), suggesting that reactive microglial cells up-regulated NTPDase1/CD39 after the exposure to TMT.

eN/CD73 activity and localization in response to TMT were determined using AMP-based EHC (Fig. 5A,B). During TMT-induced neurodegeneration,

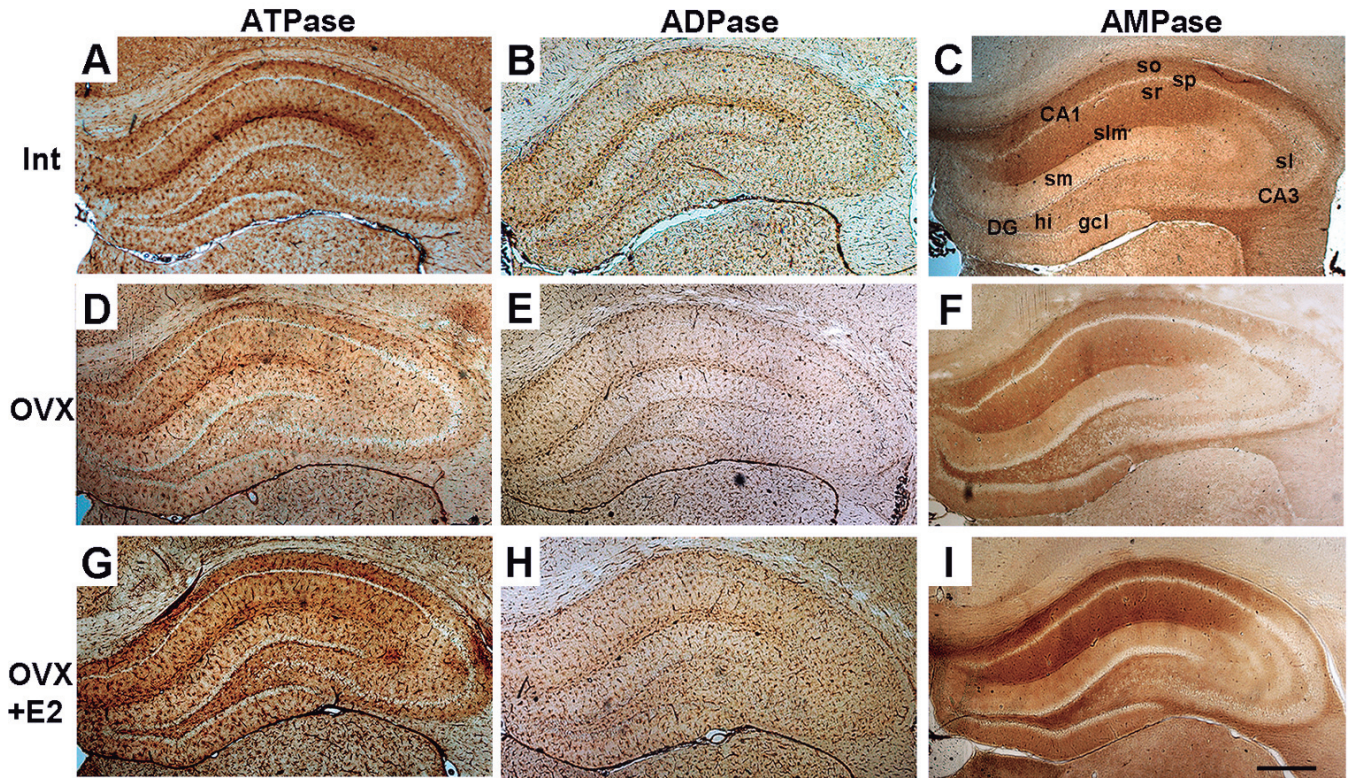

Fig. 3. Distribution of ectonucleotidases in the hippocampal region. Ectonucleotidase histochemistry in the presence of ATP **(A, D, G)**, ADP **(B, E, H)**, and AMP **(C, F, I)**, in hippocampal region of intact control (Int), ovariectomized animals (OVX), and the animals treated with E2 (OVX + E2). C: Abbreviations indicate the position of hippocampal layers-Cornu Amonis (CA), stratum oriens (so), pyramidal cell layer (sp), stratum radiatum (sr), stratum lacunosum moleculare (slm), stratum lucidum (sl), stratum moleculare (sm), granule cell layer of dentate gyrus (gcl), hilus (hi), and dentate gyrus (DG). Published in (Grkovic et al., 2019b). Scale bar: 500 μm

products of AMPase activity were accumulated in the neuronal strata, infiltrating within neuronal cell layers (Dragic et al., 2021a). AMPase depicted individual round-shaped elements which represent amoeboid microglial cells (Fig. 5F-H) that covered neuronal layers mostly noticeable at the late stage of TMT-induced neurodegeneration (Dragic et al., 2019a, 2021a).

Similar upregulation of AMPase activity that corresponds to eN/CD73-ir was found in the spinal cord after induction of experimental autoimmune encephalomyelitis (EAE, Fig. 5) (Dragic et al. 2021b), the most commonly used experimental model for the

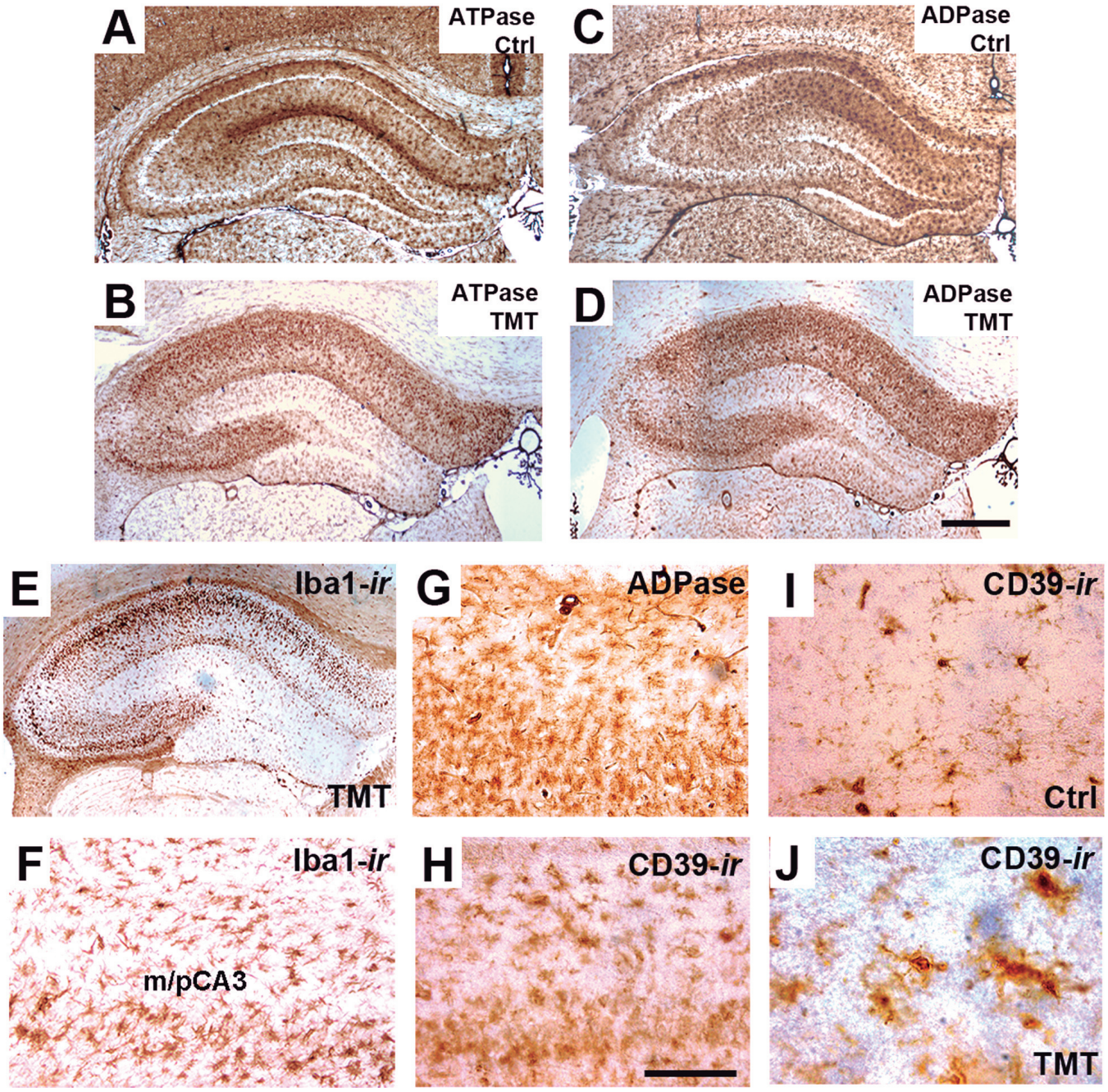

Fig. 4. Activity of NTPDase1/CD39 based on ATP and ADP enzyme histochemistry in the hippocampal region after TMT exposure. In the presence of ATP **(A, B)** or ADP **(C, D, G)** as substrate, enzyme histochemistry labeled cells and structures correspond to ectonucleotidase activities in the hippocampal region of control (Ctrl) and TMT-exposed animals. Microglial cells were clearly labeled by ATP and ADP enzyme histochemistry and closely corresponded to Iba1-ir **(E, F)**, and NTPDase1/CD39-ir **(H-J)**. Scale bars: A, B, D, E, 500 µm; F-I, 50 µm.

human inflammatory demyelinating disease, multiple sclerosis (MS). The diffuse histochemical reaction produced by eN/CD73-catalyzed hydrolysis of AMP was dominantly observed in the control spinal cord gray matter, whereas the white matter was faintly stained (Fig. 5I). In EAE sections, an increased reaction was observed in both gray (Fig. 5J), and white matter with numerous fiber-like structures that traverse through white matter (Fig. 5J). This prominent increase in AMPase activity in EAE corresponds to upregulated CD73-ir mainly associated with Iba-1-ir (Fig. 5K).

Labeling of microglia by enzyme histochemistry

For years, NDPase histochemistry has been used for the selective labeling of microglial cells in the CNS of different species including fishes and even humans (Castellano et al., 1991; Jensen et al., 1994; Vela et al., 1995; Salvador-Silva et al., 2000; Almolda et al., 2009, 2013). NDPase histochemistry may stain not only amoeboid and ramified microglia in the normal intact nervous system (Dalmau et al., 1998; Almolda et al., 2013), but also reactive microglia in the experimentally lesioned brain (Jørgensen et al., 1993) and in neurological diseases such as Alzheimer's disease and other disorders (Vostrikov, 1985; Almolda et al., 2009; Dragic et al., 2021a). Compared to immunohistochemical methods, NDPase histochemistry is a faster and cheaper technique that can be performed not only on histological sections but also in cell cultures (Dalmau et al., 1996) allowing the fast identification of microglial cells, providing information about both activity and morphology. Moreover, it almost completely overlaps with Iba1- and NTPDase1/CD39-ir (Fig. 6). Thus,

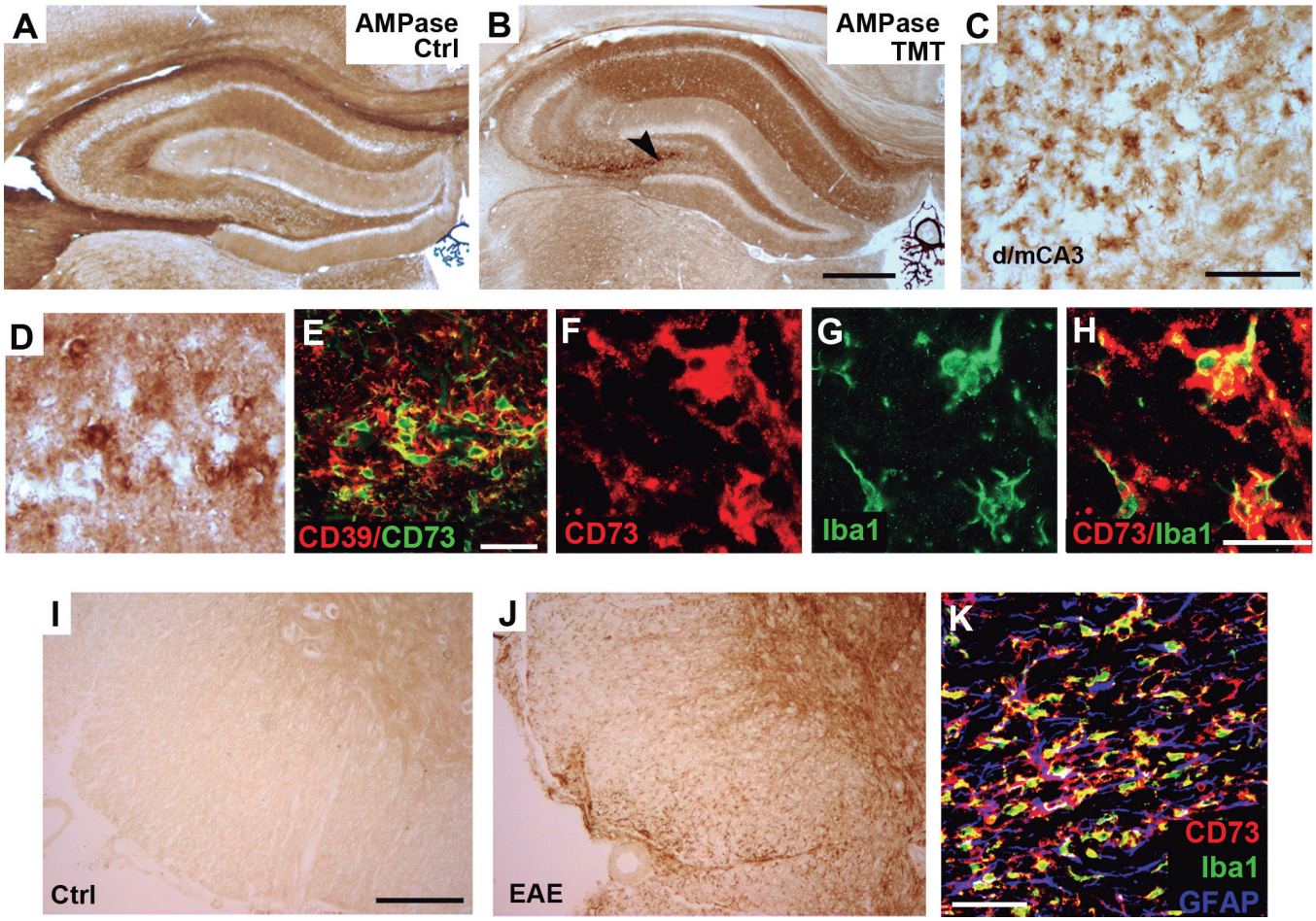

Fig. 5. Activity of eN/CD73 based on AMP enzyme histochemistry in the hippocampal region after TMT exposure **(A-H)** and after induction of EAE **(I-K)**. Following TMT, enzyme histochemistry products of AMPase activity were accumulated in the neuronal strata, infiltrating within neuronal cell layers **(B)**. AMPase depicted individual round-shaped elements which represent amoeboid microglial cells that covered neuronal layers **(C, D)**, and were most noticeable at the late stage of TMT-induced neurodegeneration that was confirmed by double immunofluorescent labeling of Iba-1 positive cells (red) and eN/CD73 (green) **(E-H)**. In the Control sections of spinal cord **(I)** diffuse staining patterns localized mainly in gray matter were observed while white matter was devoid of staining. Spinal cord of EAE animals **(J)** revealed a marked increase in AMPase activity localized in gray and white matter **(F)**. In EAE sections, a marked increase in eN/CD73 staining co-localized with GFAP+ and IBA-1+ cells. Scale bars: A, B, 500 µm; C-K, 50 µm.

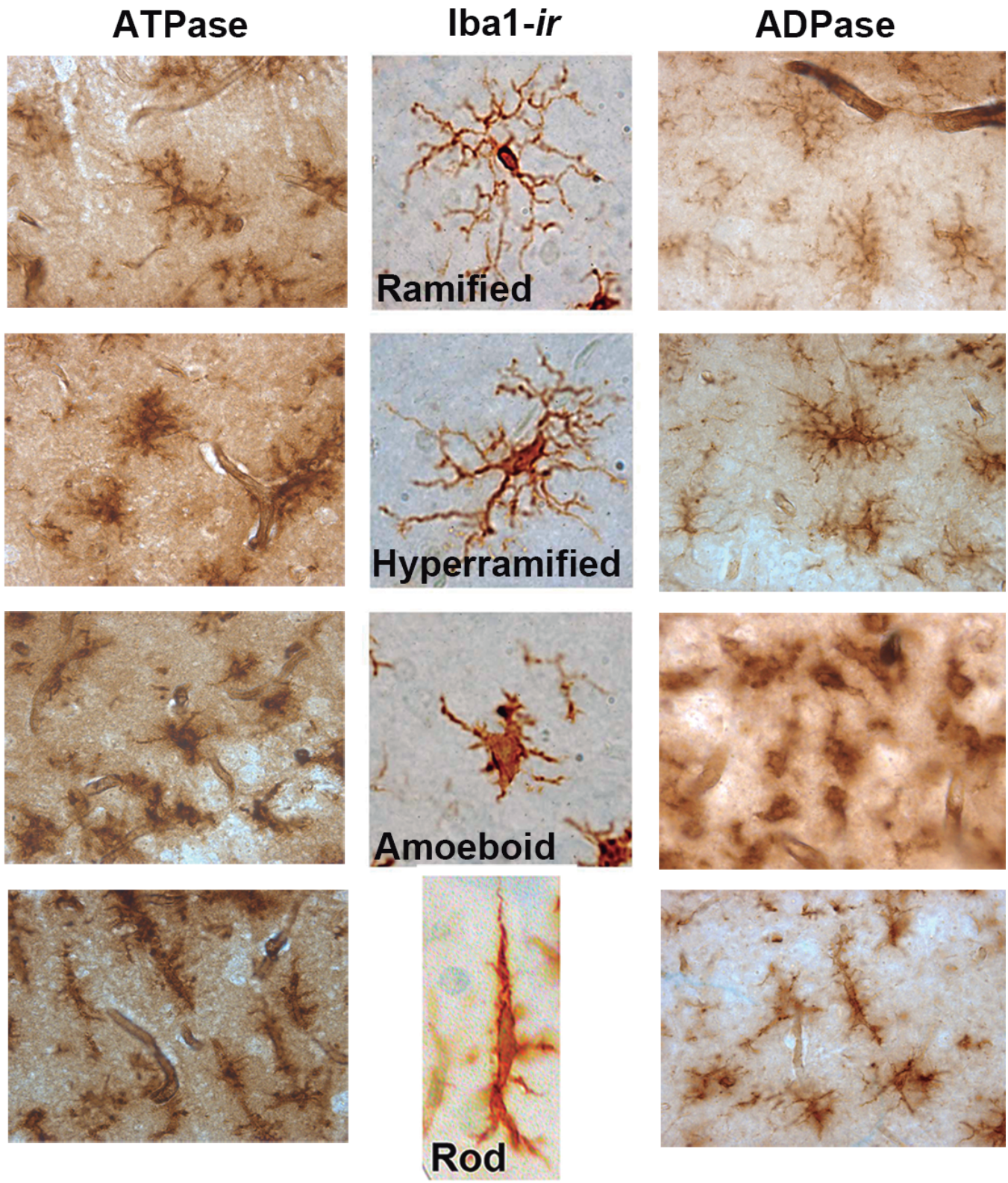

Fig. 6. Representative images of different Iba1-ir morphological phenotypes observed in control and after TMT exposure labeled with ATP and ADP enzyme histochemistry. In control animals, highly ramified Iba1-immunoreactive (ir) cells exhibit typical small ovoid cell body with radially oriented ramified processes. After exposure to neurotoxicant, microglia changes morphology from hyperramified to bushy/amoeboid gradually transformed to rod Iba1-ir cells. Those cells overlap with ATPase and ADPase enzyme histochemistry staining that represents the product of NTPDase1/CD39 activity. Published in Dragic et al. (2021a).

different Iba1-ir morphological phenotypes might be observed using EHC.

Combining immunolabeling and in situ nucleotidase activity experiments

Enzyme histochemistry might be combined with other histochemical methods to identify both the activity (*in situ* histochemistry/or cytochemistry) and the enzyme localization (immunohistochemistry or immunofluorescence) on the same slide. Almolda and coworkers (2013) described a combination of this histochemical technique with other immunohistochemical methods thus allowing the visualization of astrocytes and processing of NDPase-labeled sections for electron microscopy analysis (Almolda et al., 2013). For instance, double labeling of microglia and astrocytes can be obtained by combining NDPase with immunohistochemical detection of GFAP (an astrocyte marker) or Iba1 (microglial marker) (Almolda et al., 2013). To accomplish that, after the histoenzymatic incubation and before the treatment with sulfide, the process is interrupted and followed with immunohistochemical staining. When immunohistochemical staining is finished, the development of the histoenzymatic reaction is done (Almolda et al., 2013). As the final product of the histochemical technique is brownish black, it is necessary to develop the immunohistochemical reaction in a completely different color (e.g., light blue) to distinguish both labels which could be obtained by usage of AP-conjugated secondary antibodies. Furthermore, the EHC technique might be combined with immunofluorescent staining against different proteins as described (Aliagas et al., 2010; Villamonte et al., 2018; Trapero et al., 2019).

Quantification of enzyme histochemistry results

Until recently, EHC could be used only as a qualitative method since it has been shown that lead can precipitate even without nucleotides in the reaction mixture, producing a reactional artifact (Rosenthal et al., 1969). However, each micrograph possesses a set of unique textural features, which are used for pattern quantification by texture analysis. Texture analysis emerged as an excellent tool for image quantification of subtle differences reflected in both spatial discrepancies and gray level values of pixels. This is also a promising tool for quantification of EHC. Since EHC results in specific patterns of brown precipitate, several textural features could be computed based on a distribution of different combination of pixels, each having a specified position relative to each other in the analyzed image (Lubner et al., 2015). By comparing the extracted features of two analyzed EHC micrographs, it is possible to acquire information about differences in complexity, homogeneity/heterogeneity and uniformity which all stem from specific patterns of lead precipitations (Dragic et al., 2019a). Therefore, texture analysis could provide an additional tool for EHC assessment, adding a quantitative dimension to the method, but also serving as a means of further analysis and interpretation in light of regional or cellular changes in pattern of ectonucleotidases activity (Stankovic et al., 2016; Dragic et al., 2019a). Literary data argue that texture analysis could be used for the determination and quantification of fine differences in the enzyme activities with the possibility to ascribe those differences to regions or specific cell types (Dragic et al., 2019a). However, due to the possibility of artifact formation, the results of EHC quantification should be analyzed with caution.

Perspective

The understanding of catalytic activity pattern of ectonucleotidases is essential for elucidating the control of nucleotide signaling in the NS. As described in this review article, the distribution of the activity pattern attributable to the NTPDases and eN in the targeted brain regions could be investigated using EHC, a valuable experimental and resourceful histopathological method, which provides simultaneous information about function and spatial localization of the enzyme activity. Enzyme histochemistry remains an unrivaled histochemical technique for studying ectonucleotidases activity *in situ*. Clear overlapping of EHC and immunohistochemistry staining against NTPDase1 and eN/CD73, and the use of texture analysis as a method for quantification of this descriptive technique makes EHC a useful tool for determination of spatial localization of ectonucleotidases in both physiological and pathological conditions. However, with recent development of advanced tools such as tissue clearing-enhanced three-dimensional (3D) techniques, a new platform has been created which allows cell-level analysis in volumetric dimension and permits cell-level analysis of cell positioning in the context of macroscale tissue structure (Paris et al., 2018; Li et al., 2019; Liang and Luo, 2021; Vieites-Prado and Renier, 2021; Losenkova et al., 2022). If coupled with existing, well-established techniques for studying ectonucleotidases i.e. EHC, it could provide a more complete, physiological picture, adding useful information about both localization and volumetric distribution of certain NTPDase and purinergic receptors in various cells, as well as their interactions (Losenkova et al., 2022).

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