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Expression of ecto-nucleoside triphosphate diphosphohydrolase3 (NTPDase3) in the female rat brain during postnatal development

Ivana Grković[a,*], Ivana Bjelobaba[b], Nataša Mitrović[a], Irena Lavrnja[b], Dunja Drakulić[a], Jelena Martinović[a], Miloš Stanojlović[a], Anica Horvat[a], Nadežda Nedeljković[c]

[a] Department of Molecular Biology and Endocrinology, VINČA Institute of Nuclear Sciences, University of Belgrade, Belgrade, Serbia
[b] Institute for Biological Research "Siniša Stanković," University of Belgrade, Belgrade, Serbia
[c] Institute for Physiology and Biochemistry, Faculty of Biology, University of Belgrade, Belgrade, Serbia

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ABSTRACT

Nucleoside triphosphate diphosphohydrolase3 (NTPDase3) is membrane-bound ecto-enzyme which hydrolyzes extracellular ATP, thus modulating the function of purinergic receptors and the pattern of purinergic signaling. Here we analyzed the developmental expression of NTPDase3 in female hypothalamus, cerebral cortex and hippocampal formation at different postnatal ages (PD7-PD90) by qRT-PCR and immunohistochemistry. In hypothalamus and hippocampus, a similar developmental profile was seen: NTPDase3 gene expression was stable during postnatal development and increased in adults. In the cortex, upregulation of NTPDase3 mRNA expression was seen at PD15 and further increase was evidenced in adults. Immunohistochemical analysis at PD7 revealed faint neuronal NTPDase3 localization in a dorsal hypothalamus. The immunoreactivity (*ir*) gradually increased in PD15 and PD20, in clusters of cells in the lateral, ventral and dorsomedial hypothalamus. Furthermore, in PD20 animals, NTPDase3-*ir* was detected on short fibers in the posterior hypothalamic area, while in PD30 the fibers appeared progressively longer and markedly varicose. In adults, the strongest NTPDase3-*ir* was observed in collections of cells in dorsomedial hypothalamic nucleus, dorsal and lateral hypothalamus and in several thalamic areas, whereas the varicose fibers traversed entire diencephalon, particularly paraventricular thalamic nucleus, ventromedial and dorsomedial hypothalamic nuclei, the arcuate nucleus and the prefornical part of the lateral hypothalamus. The presumably ascending NTPDase3-*ir* fibers were first observed in PD20; their density and the varicose appearance increased until the adulthood. Prominent enhancement of NTPDase3-*ir* in the hypothalamus coincides with age when animals acquire diurnal rhythms of sleeping and feeding, supporting the hypothesis that this enzyme may be involved in regulation of homeostatic functions.

1. Introduction

Extracellular adenosine triphosphate (ATP) acts as gliotransmitter, neurotransmitter, neuromodulator and trophic factor, controlling excitability, transmission and synaptic plasticity (Cunha and Ribeiro, 2000; Rodrigues et al., 2005) during both embryonic and postnatal development of the central nervous system (CNS) (Dias et al., 2013; Wieraszko, 1996; Zimmermann, 2006). By acting at two distinct classes of P2 receptors, ligand-gated P2X1-7 and G-protein coupled P2Y1-13 (Burnstock, 2007), extracellular ATP induces myriad of cellular actions in the CNS (for review, see Burnstock 2007; Zimmermann et al., 2012). The effects induced by ATP are under control of ectonucleotidase enzyme family, which catalyze sequential hydrolysis of extracellular ATP to ADP, AMP and adenosine (Zimmermann et al., 2012).

Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; Arch, arcuate nucleus; *chp*, choroid plexus; CM, central medial thalamic nucleus; D3V, third ventricle; DMC, compact part of dorsomedial hypothalamic nucleus; DMD, dorsomedial hypothalamic nucleus; DMV, ventral part of dorsomedial hypothalamic nucleus; Hip, hippocampus; IMD, intermmediodorsal thalamic nucleus; LH, lateral hypothalamus; LSI, lateral septal nucleus; M2, secondary motor area; MHb, medial habenular nucleus; MoDG, molecular layer of dentate gyrus; PH, posterior hypothalamic nucleus; PHD, posterior hypothalamic area; Pir, piriform cortex; PVP, paraventricular thalamic nucleus; Rh, rhomboid thalamic nucleus; VMH, ventromedial hypothalamic nucleus; VPM, ventral posteromedial thalamic nucleus; S2, secondary somatosensory cortex; slm, stratum lacunosum moleculare.

* Corresponding author at: Department of Molecular Biology and Endocrinology, VINČA Institute of Nuclear Sciences, University of Belgrade, Mike Petrovića Alasa 12-14, 11001 Belgrade, Serbia.
E-mail address: istanojevic@vinca.rs (I. Grković).

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In the rat brain, the ectonucleotidase family includes ectonucleoside triphosphate diphosphohydrolases1-3 (NTPDase1-3), which hydrolyze ATP and ADP to AMP, and ecto-5′-nucleotidase (eN), which catalyzes the final step of the conversion of AMP to adenosine. Individual members of NTPDase family differ in several respects, 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 (Smith and Kirley, 1998), leading to transient accumulation of ADP (Kukulski et al., 2005). Therefore, NTPDase2 and NTPDase3 produce agonists which act at ADP-sensitive purinoceptors, such as P2Y1, P2Y12 and P2Y13 (Abbracchio et al., 2006).

Members of ectonucleotidase family have a wide distribution in the brain, although individual members exhibit marked regional and cell-type specific localization (Bjelobaba et al., 2007; Braun et al., 2000; Langer et al., 2008; Wang and Guidotti, 1998; Wink et al., 2006). Among NTPDases expressed in the brain, the most restricted and exclusively neuronal localization is observed for NTPDase3. Somatic NTPDase3 localization was observed only in the midline regions of the brain: in the thalamus and hypothalamus, the medulla oblongata (Belcher et al., 2006) and in the spinal cord (Vongtau et al., 2011). NTPDase3-expressing neuronal fibers are abundantly present in the midline regions of the brain, while scattered NTPDase3 positive axon-like processes with prominent varicosities were also observed in the cerebral cortex, hippocampus and basal ganglia (Belcher et al., 2006; Bjelobaba et al., 2010). It should be noted that nearly all of the NTPDase3-immunoreactive (*ir*) hypothalamic neurons and the vast majority of NTPDase3-*ir* fibers express hypocretin-1/orexin-A, suggesting its role in the autonomic and hormone-regulated behaviors, such as food intake, sleep-wake cycle, and reproduction (Belcher et al., 2006). The involvement of the enzyme in the reproductive behaviors is further substantiated with the finding that in females, the expression of NTPDase3 in the sex-related regions of the medial and lateral hypothalamus can be modulated by exogenously administrated 17β-estradiol (Kiss et al., 2009). Considering that the hypothalamus, cerebral cortex and hippocampus are highly estrogen responsive brain regions, the present study has been designed to investigate the developmental profile of NTPDase3 expression in the female rat brain. We also wanted to test if ovariectomy influences the expression levels of NTPDase3 in the selected brain regions of an adult female rats. Because regional and local distribution of individual NTPDases crucially affects the type and extent of P2 receptor signaling, essential for normal brain functioning, it is important to determine whether the NTPDase3 contributes to the control of nucleotide-mediated signaling during postnatal development.

Results of our study show that, although NTPDase3 mRNA is already present at PD7, NTPDase3-*ir* that resembles the enzyme localization in the adult appears in the lateral hypothalamic areas only at the end of third postnatal week (PD20), at about the time when the animals acquire the adult-like diurnal rhythms of feeding and sleeping behavior. This implies that the developmentally regulated expression of NTPDase3 and ATP signaling may be important in the regulation of the homeostatic mechanisms involved in sleeping and feeding.

2. Materials and methods

2.1. Animals

Female rats of Wistar strain from a local colony of VINČA Institute of Nuclear Science breeding stock were used in all experiments. All procedures were carried out in accordance with the principles from Guide for Care and Use of Laboratory Animals (NIH publication no. 80-23), and the Ethical Committee for the Use of Laboratory Animals of VINČA Institute of Nuclear Sciences, University of Belgrade, Belgrade, Serbia, (02/11) approved the protocols. Animals were housed under standard conditions: 12 h light/dark regime, constant ambient temperature (22 ± 2 °C) and free access to food and water.

Females were organized in the following groups: postnatal 7- (PD7), 15- (PD15), 20- (PD20), 30- (PD30) and 90- (adult) days old rats. Rats in PD7, PD15 and PD20 groups were kept in the litter, while the animals in PD30 and adults were weaned at the 21st postnatal day and kept 3/cage. At each postnatal group, rats (n = 9) were selected from at least five separate litters (reducing litter size uniformly).

Adult rats were divided into 3 groups: OVX group (n = 9/group) was subjected to bilateral ovariectomy through one dorsal incision under ketamine (50 mg/kg) and xylazine (5 mg/kg) anesthesia. The same procedure was conducted on the sham group (n = 6/group), without removal of ovaries. Intact (n = 14) animals taken in diestrus phase of the estrous cycle were used as a control. Vaginal lavage was performed between 9 and 10 am during two weeks and the stage was determined by evaluating relative proportion of epithelial nucleated cells, squamous cells and leucocytes in vaginal smears. The presence of leukocytes in the smear indicated diestrus. Only those animals with regular 4–5 days cycle were included. OVX and sham animals were sacrificed 3 weeks after the surgery. Since sham operation had not induced any significant effect on the expression of NTPDase3, those data were not discussed or included in the graphs.

2.2. Preparation of subcellular fractions

Subcellular localization of NTPDase3 and specificity of the antibodies used in the immunohistochemical study was tested by Western blot analysis, after resolving sample proteins by SDS-PAGE electrophoresis. Crude membrane fraction (P2), gliosomes, synaptosomes, purified synaptic plasma membranes, synaptic mitochondria and the cytosolic fractions were isolated from whole cerebral cortex and hippocampal formation of adult female rats by differential centrifugation on discontinuous Percoll gradient (Dunkley et al., 2008; Moutsatsou et al., 2001). Due to small sample size, the only fraction prepared from the dissected hypothalamic tissue was the P2 fraction.

Cortices, hippocampi and hypothalami were dissected and homogenized in 10 vols of ice cold isolation buffer (0.32 M sucrose, 5 mM Tris–HCl, pH 7.4) in a Teflon/glass homogenizer (clearance 0.20 mm) at 900 rpm. Crude nuclear fraction and cell debris were removed by centrifugation at 1000*g* for 10 min. Supernatants were collected and centrifuged at 17000*g* for 20 min in order to obtain crude membrane fraction (P2). The P2 pellet isolated from the hypothalamus was lysed in hypo-osmotic solution (5 mMTris–HCl, pH 7.4), aliquoted and kept on −80 °C until use. P2 fractions isolated from the cortices and hippocampi were resuspended in the isolation buffer, placed on a discontinuous Percoll (Sigma-Aldrich, Munich, Germany) gradient (2, 6, 15, and 23% v/v of Percoll in 0.32 M sucrose and 1 mM EDTA, pH 7.4) and centrifuged at 35,000*g* for 5 min. The bands containing gliosomal (Glio) and synaptosomal (Syn) fractions were removed from 2 to 6% and 15–23% Percoll interface, respectively, diluted in the isolation buffer and pelleted by centrifugation at 14,000*g* for 20 min at 4 °C to remove myelin. The synaptosomal fraction was re-suspended in 5 mMTris–HCl, pH 7.4, centrifuged at 15,000*g* for 20 min at 4 °C. Supernatant was collected as synaptic cytosol (Cit), while the pellet was placed on a discontinuous sucrose gradient (0.32, 0.8, 1.0, and 1.2 M in 5 mM Tris–HCl, pH 7.4) and centrifuged at 90,000*g*