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Expression mapping of ectonucleotide pyrophosphatase/phosphodiesterase 1-3 (E-NPP1–3) in different brain structures during rat development

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Abstract

Ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPPs) are membrane-bound ecto-enzymes involved in the modulation of purinergic signaling. Important physiological roles related to brain development have been associated to purinergic neurotransmission. NPP1, two splice isoforms of NPP2, and NPP3 have already been identified in adult rat brain. However, there are no studies evaluating the mRNA expression of these NPP members during the brain development. The effort of the present study was to map NPP gene expression pattern in olfactory bulb, hippocampus, cerebral cortex, striatum, and cerebellum at crucial ages for rat development (7, 14, 21, 60, and 150 days old) by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) strategy. Our results demonstrated an increase in the relative expression of NPP1 throughout the aging in all structures analyzed, except in hippocampus, where the higher expression has been detected in 14 days old rats. Both NPP2 isoforms have shown a similar pattern of expression among all structures. The relative expression of NPP3 decreased during the aging mainly on cerebellum, hippocampus, and olfactory bulb. Altogether, the different patterns of NPP gene expression during rat brain development reinforce the idea that each enzyme may play a distinct role on modulating the purinergic signaling throughout aging.

Keywords: Developing brain; E-NPP; LPA; Autotoxin; Purinergic signaling; ATP

1. Introduction

The inactivation of extracellular nucleotides signaling represents a crucial control of purine-mediated functions in the nervous system. Nucleotides are released from glial cells or neurons upon activation and can act in two types of purinergic receptors: P2X (ligand-gated ionic channels receptors) and P2Y (G-protein coupled receptors) (Ralevic and Burnstock, 1998). Once released, ATP can exert a variety of physiological responses, including a trophic factor function during development (Burnstock, 2007). This nucleotide is sequentially degraded by ecto-nucleotidases (Robson et al., 2006), resulting in the formation of adenosine as a principle metabolite (Todorov et al., 1997). This nucleoside exerts its neuromodulatory effects through its own receptor subtypes (P1) (Fredholm et al., 1994) or serve as a salvage product of cellular purine metabolism.

Several members of ecto-nucleotidase families can contribute to extracellular nucleotide metabolism. The currently known ecto-nucleotidases include the ectonucleotide pyrophosphatase/phosphodiesterase family (E-NPP), ectonucleoside triphosphate diphosphohydrolase family (E-NTPDase), 5′-nucleotidase, and alkaline phosphatases (Zimmermann, 2006a).

The E-NPP members represent a family of ubiquitous and conserved proteins that are expressed as transmembrane ecto-enzymes, which are able to hydrolyze 5′-phosphodiester bounds in nucleotides, resulting in the release of 5′-nucleotide monophosphates (Bollen et al., 2000; Goding et al., 2003; Cimpean et al., 2004). Mammalian genomes contain at least seven distinct NPP-encoding genes, but only three NPPs

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(NPP1–3) show a range of 40–50% similarities at the protein level. These members are classified as type II transmembrane glycoproteins, with an intracellular N-terminal domain, a single transmembrane domain and a large domain faced to extracellular space (Cimpean et al., 2004). Previous studies demonstrated that this extracellular domain is consisted by two somatomedin-B-like homodimerization motifs, a catalytic domain, a ''nuclease-like'' sequence and a putative C-terminal ''EF-hand'' motif, which still has an uncertain role (Gijsbers et al., 2003; Sakagami et al., 2005). Although molecular and structural enzyme properties of all ecto-nucleotidase members have been investigated, the functional role of E-NPPs and their distribution in brain are still controversial.

Three NPP members (NPP1–3) have been localized in the central nervous system. Studies have shown that NPP1 is expressed in the brain capillaries (Harahap and Goding, 1988) and in rat C6 glioma (Grobben et al., 1999; Claes et al., 2001) whereas it is not detected in neurons or glia (Goding et al., 2003). NPP2 (also known as autotaxin) and NPP3 are expressed in the choroid-plexus epithelial cells (Fuss et al., 1997) and, possibly, the modulation of purinergic signaling is able to contribute to the secretion of cerebral spinal fluid (CSF) (Xiang and Burnstock, 2005). NPP2 has been correlated with intermediate stages of rat brain oligodendrocyte differentiation and myelin formation (Fuss et al., 1997). Moreover, this enzyme can produce lysophosphatidic acid (LPA), an important molecule to cerebral maturation. In the developing brain, NPP3 is expressed in immature astrocytes (Blass-Kampmann et al., 1997). Considering that NPP1–3 act in synergy to modulate purinergic signaling and also that this regulation is essential to the brain development, it became important to investigate the expression profile of NPPs during the rat cerebral maturation. Thus, our aim was to map the gene expression pattern of NPP1–3 members in rat brain regions to verify whether these genes can contribute differently for the control of nucleotide-mediated signaling during development.

2. Experimental procedures

2.1. Chemicals

Trizol reagent, SuperScript™ III First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) kit, and Taq DNA polymerase were purchased from Invitrogen. All other reagents were of analytical grade.

2.2. Animals

Male Wistar rats of different ages (7, 14, 21, 60, and 150 days) from our breeding stock were used in all experiments ($n = 3$ per age). The animals had access to water and food *ad libitum* and were kept on a 12-h light/dark cycle (lights on at 7:00 am) at a temperature of 23 ± 1 °C. Adult rats (60 and 150 day-old-rats) received rat chow (Nutrilab1, Brazil) and water *ad libitum*. The younger rats (7, 14, and 21 days old) were kept with their dams until the experimental procedure. The care and use of animals were adopted according to the regulations of Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council).

2.3. Analysis of gene expression by semi-quantitative RT-PCR

The expression analysis of NPP1, NPP2, and NPP3 was carried out by a semi-quantitative RT-PCR assay. Naive rats (aged 7, 14, 21, 60, and 150 days; $n = 3$ per age) were sacrificed by decapitation, the brains were removed and placed into ice-cold RNAse free water. Olfactory bulb, hippocampus, striatum, cerebellum, and cerebral cortex of both hemispheres were dissected and immediately frozen with liquid nitrogen for storage in −80 °C freezer. The total RNA extraction of all brain structures was performed using TRIzol reagent (Invitrogen, USA) in accordance with the manufacturer instructions. The cDNA species were synthesized with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) from 1 μg of total RNA and oligo (dT) primer following the suppliers. RT reactions were performed for 50 min at 50 °C. cDNA (1 μL) was used as a template for PCR with specific primers for NPP1, NPP2, and NPP3. The NPP1 and β-actin primers were described previously (Vollmayer et al., 2001; Cognato et al., 2007). DNA sequences encoding to NPP1–3 were retrieved from GenBank database and aligned using ClustalX program. Regions with low scores of similarity among the sequences were used for searching specific primers, which were designed using the program Oligos 9.6. NPP2 splice isoforms were identified using primers localized upstream and downstream of the splice junction resulting in two different PCR products. NPP2 isoform 1 PCR product had a fragment length of 587 bp (with the intron) and NPP2 isoform 2 PCR product had 512 bp (without the intron). In order to confirm the primer specificity, each primer was blasted against rat genome and it was able to recognize only its specific target sequence. Thus, the strategy adopted to construct the primers did not allow cross-amplification. The primer sequences, GenBank accession numbers and PCR products of all NPP members analyzed and β-actin are shown in Table 1. PCR reactions were performed (total volume of 25 μL) using a concentration of 0.4 μM of each primer and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer. The conditions for all PCRs were carried out as follows: initial 1 min denaturation step at 94 °C, 1 min at 94 °C, 1 min annealing step (NPP1: 60 °C; NPP2: 67 °C; NPP3: 65 °C; β-actin: 58.5 °C), 1 min extension step at 72 °C for 35 cycles and a final 10 min extension at 72 °C.

Table 1
GenBank accession number, primers sequences, and PCR products of NPP1–3 and β-actin

Protein	GenBank accession number	Primers sequences	PCR product
NPP1[a]	NP_445987	F 5′-GAATTCTTGAGTGGCTACAGCTTCCTA-3′ R 5′-CTCTAGAAATGCTGGGTTTGGCTCCCGGCA-3′	410 bp
NPP2 (1)[b] NPP2 (2)	Q64610	F 5′-CCATGCCAGACGAAGTCAGCCGACC-3′ R 5′-CCAAACACGTTTGAAGGCGGGGTAC-3′	587 bp, 512 bp
NPP3	AAH97326	F 5′-GAGAAGACAAATTTGCCATTTGGGAGG-3′ R 5′-TCTCATTATTTCCTTTGATTGCGGGAG-3′	301 bp
β-Actin	NP_112406	F 5′-TATGCCAACACAGTGCTGTCTGG-3′ R 5′-TACTCCTGCTTCCTGATCCACAT-3′	210 bp

[a] Primer sequences were obtained from Vollmayer et al., 2001.
[b] Represent the splice isoform of NPP2.

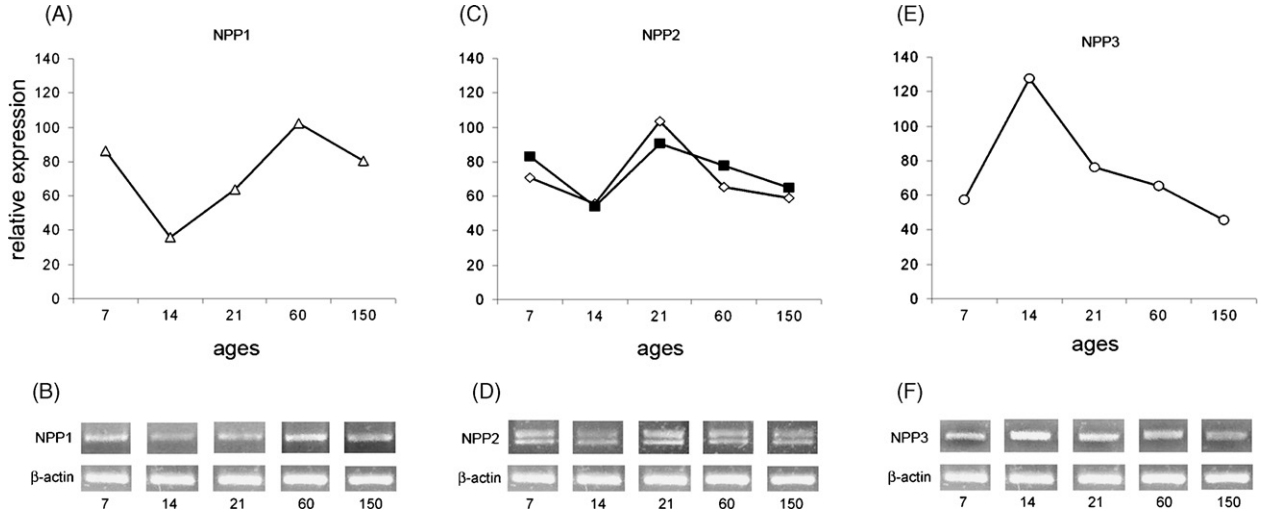

Fig. 1. Gene expression patterns of NPP1 (A and B), NPP2 isoforms 1 (◇) and 2 (■) (C and D), and NPP3 (E and F) on olfactory bulb of rats at post-natal day 7, 14, 21, 60, and 150. At least three independent experiments (PCRs) were performed, with entirely consistent results.

The fragments length of PCR reactions was confirmed with Low DNA Mass Ladder (Invitrogen, USA) and β-actin was carried out as an internal standard. The relative abundance of each mRNA versus β-actin was determined in the brain structures studied by densitometry using the freeware ImageJ 1.37 for Windows.

3. Results

3.1. NPP1 relative expression

It was observed an age-dependent increase in the relative expression of NPP1 in all brain structures analyzed here, except in hippocampus, where the higher relative expression was found in 14-day-old rats (62.5%) (Fig. 2A and B). The relative expression of NPP1 in olfactory bulb has begun in 7-day-old rats (86.5%) followed by a dramatic reduction in 14-day-old rats (36.2%). The relative expression of NPP1 in olfactory bulb has returned to high levels in 60-day-old rats (102.6%, Fig. 1A and B). In cerebral cortex, NPP1 presented the highest relative expression in 150-day-old rats (65.3%) and the lowest in 21-day-old rats (37%, Fig. 3A and B). A strong increase in the relative expression of NPP1 was observed in striatum (from 16% in 7-day-old rats to 71% in 60-day-old rats, Fig. 4A and B) and cerebellum (from 30% in 7-day-old rats to 92.5% in 60-day-old rats, Fig. 5A and B).

3.2. NPP2 relative expression

Using the primers designed to discriminate both splice isoforms of NPP2 (Table 1), it was possible to detect the relative expression of each enzyme in all brain regions and ages analyzed here. The expression pattern of NPP2 splice isoforms was similar in the course of the rat aging. The relative expressions of NPP2 splice isoforms in olfactory bulb of 14-day-old rats were 55.6% (isoform 1) and 54.3% (isoform 2). The relative expression of both isoforms of NPP2 in olfactory bulb of 21-day-old rats presented a higher expression (90.6% and 103.6% for isoforms 1 and 2, respectively, Fig. 1C and D).

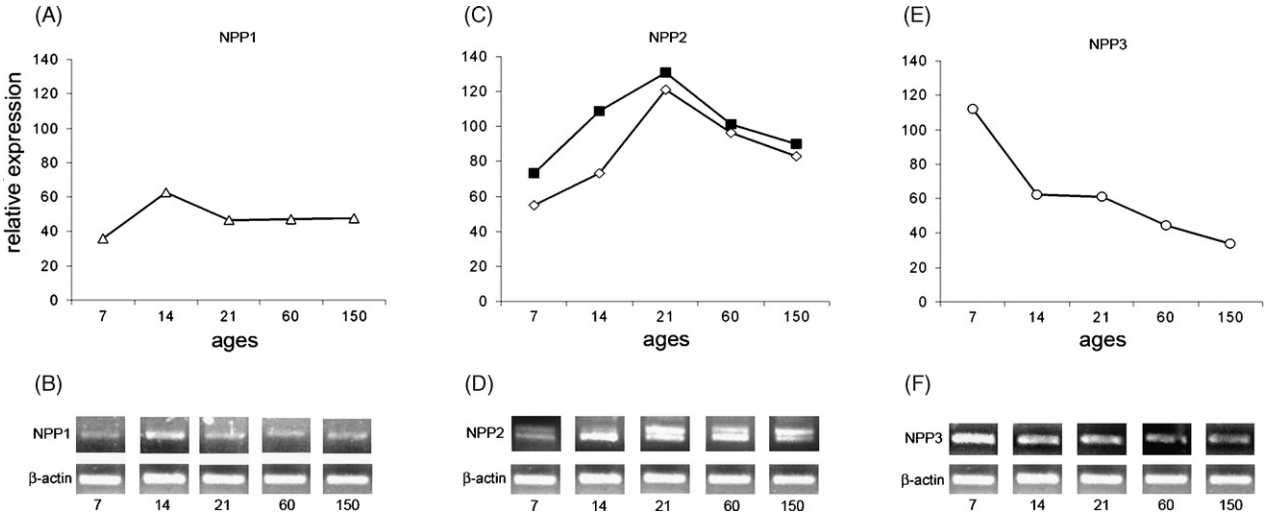

Fig. 2. Gene expression patterns of NPP1 (A and B), NPP2 isoforms 1 (◇) and 2 (■) (C and D), and NPP3 (E and F) on hippocampus of rats at post-natal day 7, 14, 21, 60, and 150. At least three independent experiments (PCRs) were performed, with entirely consistent results.

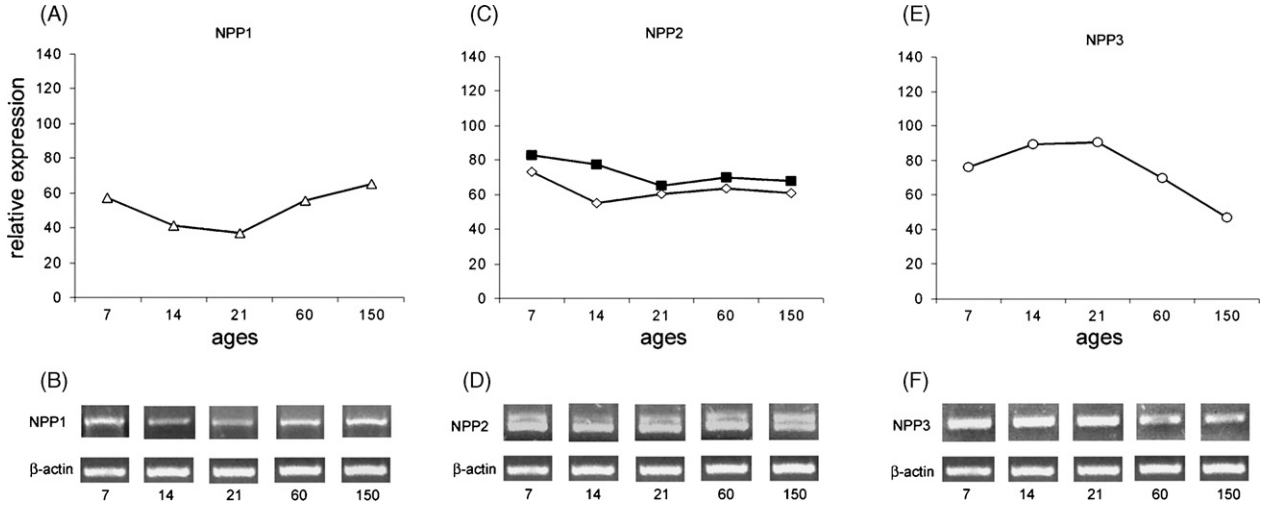

Fig. 3. Gene expression patterns of NPP1 (A and B), NPP2 isoforms 1 (◇) and 2 (■) (C and D), and NPP3 (E and F) on cerebral cortex of rats at post-natal day 7, 14, 21, 60, and 150. At least three independent experiments (PCRs) were performed, with entirely consistent results.

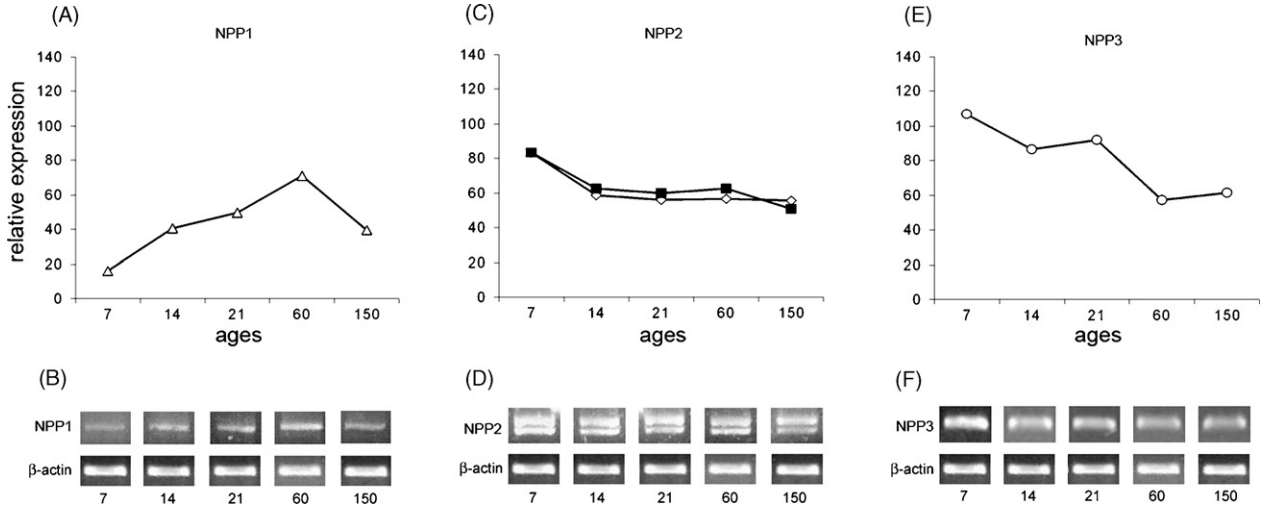

Fig. 4. Gene expression patterns of NPP1 (A and B) NPP2 isoforms 1 (◇) and 2 (■) (C and D) and NPP3 (E and F) on striatum of rats at post-natal day 7, 14, 21, 60, and 150. At least three independent experiments (PCRs) were performed, with entirely consistent results.

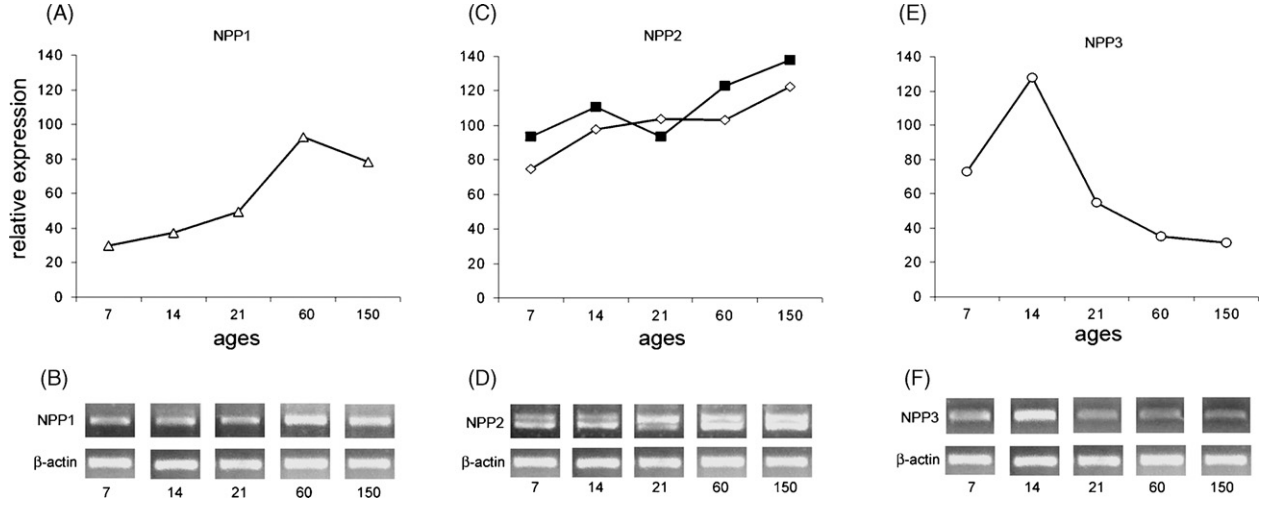

Fig. 5. Gene expression patterns of NPP1 (A and B) NPP2 isoforms 1 (◇) and 2 (■) (C and D) and NPP3 (E and F) on cerebellum of rats at post-natal day 7, 14, 21, 60, and 150. At least three independent experiments (PCRs) were performed, with entirely consistent results.

In hippocampus, the relative expression of both isoforms has also been highest in 21-day-old rats (121% and 131% for isoforms 1 and 2, respectively) when compared to all other ages tested, mainly in 7-day-old rats (54.6% and 73.3% for isoforms 1 and 2, respectively, Fig. 2C and D). The NPP2 isoforms presented a constant pattern of expression in cerebral cortex and striatum, remaining in the range of 60% for NPP2 isoform 1 and 70% for NPP2 isoform 2 in both brain regions (Figs. 3 and 4C and D). In cerebellum, NPP2 isoforms show an interesting expression pattern, which differs from the profiles presented by other brain regions. The relative expression has increased from 74% (NPP2 isoform 1) and 93% (NPP2 isoform 2) in 7-day-old rats to 122.3% (NPP2 isoform 1) and 138% (NPP2 isoform 2) in 150-day-old rats (Fig. 5C and D).

3.3. NPP3 relative expression

In all brain regions analyzed, it has been found a decrease in relative expression of NPP3 through rat aging. In hippocampus and striatum, the relative expression of NPP3 in 7-day-old rats has declined from 112% and 107% to 34% and 61% in rats with 150-day-old, respectively (Figs. 2 and 4E and F). This expression pattern was also observed in olfactory bulb and cerebellum, in which the relative expression in 14-day-old rats (127.6% and 127.5%, respectively) has diminished in relation to 150-day-old rats (45.75% and 31.6%, respectively, Figs. 1 and 5E and F). In cerebral cortex, the first three ages evaluated here (7, 14, and 21 days) have presented a similar relative expression of NPP3 (76%, 89.6%, and 90.6%, respectively) whereas 60- and 150-day-old rats have shown a diminished expression pattern (69% and 47%, respectively, Fig. 3E and F).

4. Discussion

The distribution of NPP1 in the adult rat brain has already been evaluated. Bjelobaba et al. (2006) have observed a widespread distribution of NPP1 in the rat forebrain, mainly in hippocampus and cerebral cortex. The results presented here show that NPP1 relative expression is long-standing for these brain regions during the lifetime. Another study revealed NPP1 has been expressed in the capillaries of the brain (Harahap and Goding, 1988). It was previously described that CNS is mainly vascularized by angiogenesis, the sprouting of capillaries from preexisting vessels, during the development (Marin-Padilla, 1985) whereas it is almost absent in adult tissues (Plate et al., 1994). In accordance to these studies, the relative expression of NPP1 in olfactory bulb, striatum and cerebellum increased until 60 days old and then decreased at 150 days old.

It has been suggested endogenous release of ATP starts to enhance the synaptic activity in Purkinje neurons by the end of the second post-natal week (Casel et al., 2005). The increase in relative expression of NPP1 and NPP2 through aging may reflect that these enzymes act in synergy hydrolyzing ATP during brain developing. Moreover, NPP2 also can produce LPA, a lipid mediator with a wide variety of biological functions, including important actions for brain development, such as cortical neurogenesis and pattern formation (Kingsbury et al., 2003). LPA is also implicated in the vascular development of immature brain. van Meeteren et al. (2006) have shown that NPP2-deficient mice embryos die at embryonic day 9 with severely impaired vessel formation. Since NPP2 is the ecto-nucleotidase responsible for modulating LPA levels through the hydrolysis of lysophosphatidylcholine (Tokumura et al., 2002), the presence of these enzymes during brain maturation is at least essential.

It has been hypothesized that NPP3 may represent an important factor in the process of glial cell proliferation which is accompanied by migration of glial precursors (Zimmermann, 2006b). In fact, it was reported NPP3 has been only detected on immature astrocytes (Blass-Kampmann et al., 1997). Our results support this data since we have observed a decreased relative expression of NPP3 during brain maturation in all areas investigated. Interestingly, mRNA expression of NPP3 in hippocampus and striatum begins to decline from the age of 7 days old. This pattern differs from cerebellum and olfactory bulb, since the relative expression of NPP3 in those brain areas starts to diminish from the age of 14 days old. These results indicate that NPP3 is differently altered during the brain development, which could influence the process of glial cell proliferation.

In summary, the present study demonstrated a widespread mRNA relative expression for NPPs, which are capable of hydrolyzing important molecules for proper maturation of the brain (for example, ATP and LPA). Altogether, the different patterns of NPP gene expression during rat brain development reinforce the idea that each enzyme may play a distinct role on modulating the purinergic signaling throughout aging.

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