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BRIEF COMMUNICATION

Presence of diadenosine polyphosphates in microdialysis samples from rat cerebellum in vivo: effect of mild hyperammonemia on their receptors

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Abstract Diadenosine triphosphate (Ap_3A), diadenosine tetraphosphate (Ap_4A), and diadenosine pentaphosphate (Ap_5A) have been identified in microdialysis samples from the cerebellum of conscious freely moving rats, under basal conditions, by means of a high-performance liquid chromatography method. The occurrence of Ap_3A in the cerebellar microdyalisates is noteworthy, as the presence of this compound in the interstitial medium in neural tissues has not been previously described. The concentrations measured for the diadenosine polyphosphates in the cerebellar dialysate were (in nanomolar) 10.5±2.9, 5.4±1.2, and 5.8±1.3 for Ap_3A, Ap_4A, and Ap_5A, respectively. These concentrations are in the range that allows the activation of the presynaptic dinucleotide receptor in nerve terminals. However, a possible interaction of these dinucleotides with other purinergic receptors cannot be ruled out, as rat cerebellum expresses a variety of P2X or P2Y receptors susceptible to be activated by diadenosine polyphosphates, such as the P2X1-4, $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{12}$ receptors, as demonstrated by quantitative real-time PCR. Also, the ecto-nucleotide pyrophosphatases/phosphodiesterases NPP1 and NPP3, able to hydrolyze the diadenosine polyphosphates and terminate their extracellular actions, are expressed in the rat cerebellum. All these evidences contribute to reinforce the role of diadenosine polyphosphates as signaling molecules in the central nervous system. Finally, we have analyzed the possible differences in the concentration of diadenosine polyphosphates in the cerebellar extracellular medium and changes in the expression levels of their receptors and hydrolyzing enzymes in an animal model of moderate hyperammonemia.

Keywords Diadenosine triphosphate · Diadenosine tetraphosphate · Diadenosine pentaphosphate · In vivo microdialysis · Cerebellum · Hyperammonemia

Introduction

Diadenosine polyphosphates, commonly abbreviated as Ap_nAs, comprise a group of compounds formed by two adenosine moieties linked by their ribose 5′-ends to a variable number of phosphates, which can range from 2 to 6. They are naturally occurring substances ubiquitously present in the cytoplasm of prokaryotic and eukaryotic cells where they are synthesized by some aminoacyl-tRNA synthetases and other enzymes [1].

Ap_nAs fulfill with the requirements to be considered as signaling molecules in the central nervous system. These dinucleotides have been found to be co-stored with ATP and classical neurotransmitters in the releasable content of storage granules in neural and neuroendocrine cells, such as the secretory granules of chromaffin cells, the cholinergic synaptic vesicles from *Torpedo marmorata* electric organ and the synaptic vesicles from rat brain nerve terminals [2–4]. Vesicular uptake of these compounds is mediated through a transporter that shows a broad range of specificity, being able to internalize a large variety of mononucleotides (such ATP ADP, AMP, UTP, etc.) as well as the diadenosine polyphosphates [5, 6]. All these secretory systems respond to depolarizing agents or secretagogues by releasing their vesicular content to the

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extracellular medium [4, 7]. In this regard, push–pull cannula experiments performed in living rats showed that after amphetamine stimulation, rat neostriatum releases diadenosine tetraphosphate, Ap_4A, and diadenosine pentaphosphate, Ap_5A, which can be detected in the perfusion samples at concentrations in the nanomolar range [8].

The exocytotic release of these compounds permits them to interact with P2 receptors, both metabotropic and ionotropic. It has been shown that diadenosine polyphosphates can activate recombinant $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_{12}$, and $P2Y_{13}$ [9–13] and recombinant homomeric P2X1, P2X2, P2X3, and P2X4 receptors [14, 15]. Heteromeric P2X receptors change their sensitivity to diadenosine polyphosphates when co-assembly between different subunits occurs [16]. In addition to their interaction with P2 receptors, Ap_nAs can also activate specific receptors termed dinucleotide receptors, which are insensitive to other nucleotides or nucleosides [16]. The dinucleotide receptor is a receptor-operated Ca^{2+}-permeable channel present in synaptic terminals from different brain areas, whose activation facilitates neurotransmitter release [17–20]. Presynaptic dinucleotide receptor is under the modulation of protein kinases and protein phosphatases, which depend on second messenger cascades coupled to membrane receptors [21]. It is to be emphasized that agonistic actions on the presynaptic adenosine A_1 or $GABA_B$ receptors, which are coupled to G_i/G_0 proteins, induce a significant increase in the affinity of the dinucleotide receptors for its substrates, with EC_{50} values in the low nanomolar or even picomolar range, which confers the dinucleotide receptor the capacity to respond to more physiologically relevant Ap_nAs concentrations [22, 23]. Results obtained using very different experimental approaches support the idea that extracellular Ap_nAs, acting on either P2 or their specific dinucleotide receptors, can effectively modulate neural functions [20, 24, 25].

The extracellular actions of Ap_nAs are finished by ectonucleotidases that degrade these compounds and the subsequently generated mononucleotides, yielding adenosine as the final product, which may be recovered into the cells through transport systems. Most of these Ap_nAs-hydrolyzing enzymes display biochemical characteristics typical of members of the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family. This family contains seven members, three of which, NPP1, NPP2, and NPP3, cleave mono- and dinucleotides. Recent data point to NPP1 as the main ectoenzyme involved in the cleavage of Ap_nAs by glial cells and neurons [26].

Hyperammonemia is a metabolic disturbance characterized by an excess of ammonia in the blood. Ammonia enters the brain, where it has toxic effects leading to an impairment of cerebral function [27]. Although there are different experimental models of hyperammonemia, a diet supplemented with ammonium salts is a non-invasive method that induces a moderate increase of ammonia levels in blood [28]. Previous data showed that basal concentrations of some nucleotides, such as ATP and GMP, are modified in the extracellular medium of the cerebellum of rats made hyperammonemic by feeding them an ammonium-containing diet (unpublished results), suggesting that the purinergic system could be affected by the excess of ammonia.

In this work, by using a microdialysis technique, combined with high-performance liquid chromatography (HPLC) nucleotide detection, we have analyzed the amounts of diadenosine polyphosphates that are present in the extracellular fluid in the cerebellum of conscious freely moving rats under basal conditions, i.e., in the absence of any exogenously added stimulating substance. The presence of the Ap_nAs in detectable concentrations in the cerebellar dialysates, as a consequence of the normal brain activity of the rat, will reinforce the role of these dinucleotides as signaling molecules in the central nervous system. By means of this technique, we were able to characterize the presence of diadenosine triphosphate (Ap_3A) in the microdialysis samples, whose occurrence in the interstitial medium in neural tissues has been described here for the first time. The extracellular concentrations measured for the Ap_nAs in the cerebellum, which are in the nanomolar range, could allow these substances to activate the presynaptic dinucleotide receptor. Moreover, quantitative real-time PCR analysis demonstrated that a diversity of P2 receptors able to be activated by diadenosine polyphosphates, as well as the ectoenzymes responsible for the degradation and termination of the extracellular actions of these substances, are expressed in the cerebellum. These evidences support a physiological role for diadenosine polyphosphates as relevant extracellular messengers in this brain area. Additionally, we have analyzed whether the extracellular levels of diadenosine polyphosphates and/or the expression levels of their receptors or degrading enzymes could be altered in the cerebellum of a diet-induced model of hyperammonemic rats.

Materials and methods

Materials Ap_3A, diadenosine tetraphosphate (Ap_4A), diadenosine pentaphosphate (Ap_5A), and tetrabutylammonium hydrogen sulfate were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, HPLC grade, was purchased from Scharlau (Barcelona, Spain). Other analytical-grade reagents were obtained from Merck (Darmstadt, Germany).

Animals Male Wistar rats (150–180 g) were made hyperammonemic by feeding them an ammonium-containing diet [29] for 4–5 weeks. The experiments were performed in accordance with the European Communities Council Directive 86/609/EEC.

In vivo microdialysis Rats were anesthetized using alothane and a microdialysis guide (CMA, Stockholm, Sweden) was

implanted stereotaxically in the cerebellum at the following coordinates (in millimeter): AP -10.2, ML -1.6, and DV -1.2, as described in [30]. After 48 h, a microdialysis probe (CMA/12; 3 mm long) was implanted in the freely moving rat. Probes were perfused at 3 μL/min with artificial cerebrospinal fluid (in millimolar): NaCl, 145; KCl, 3.0; $CaCl_2$, 2.26; buffered at pH 7.4 with 2 mM phosphate. After 2–3 h of stabilization period, samples were collected every 30 min and stored at −80 °C. When required, different samples collected in basal conditions (i.e., in the absence of any stimulating agent) from the same animal were mixed in order to perform the chromatographic analysis of Ap_nAs presence.

Chromatographic procedures Determination and quantification of diadenosine polyphosphates in the microdialysis samples (100 μL) were performed by HPLC. The chromatographic system consisted of a Waters (Milford, MA, USA) 1515 isocratic HPLC pump, a 2487 dual absorbance detector and a Reodyne injector, all managed by the Breeze software from Waters. Analysis was performed under ion-pair chromatography conditions by equilibrating the chromatographic system with the following mobile phase: 10 mM KH_2PO_4, 2 mM tetrabutylammonium hydrogen sulphate, and 16 % acetonitrile, pH 7.5. The column was a Spherisorb ODS2 (10 μm particle size, 25 cm length, and 0.46 cm internal diameter) from Waters. Detection was monitored at 260-nm wavelength. Diadenosine polyphosphates peaks were identified by their retention times and coelution with authentic standards. Quantitation was performed by comparing integrated peak areas of the samples with those of standards of known concentration. Minimum detectable amounts of Ap_nAs with our chromatographic system are in the range of 0.05 to 0.10 pmol. The purity of the standards used has been documented in a supplementary figure (Electronic supplementary material (ESM) 1). As seen in this figure, each of the diadenosine polyphosphate standards is essentially free of contamination with other Ap_nAs. Moreover, a significant presence of Ap_nAs degradation products (such as ATP, ADP, AMP, etc.) could not be detected in any of the standards used.

Quantitative real-time PCR Total RNA from rat cerebellum was extracted using RNeasy® plus mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. After digestion with TURBO DNase (Ambion, Austin, TX, USA), total RNA was quantified and reverse transcribed using M-MLV reverse transcriptase, 6 μg of random primers and 350 μM dNTPs (Invitrogen, San Francisco, CA, USA). Q-PCRs were performed using gene-specific primers (Sigma) and Taqman MGB probe (Roche, Basel, Switzerland) for the rat P2X1, P2X2, P2X3, P2X4, $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{12}$ purinergic receptors and for the rat ecto-nucleotide pyrophosphatases/phosphodiesterases NPP1 and NPP3. Fast thermal cycling was performed using a StepOnePlus® Real-Time System (Applied Biosystems, Foster City, CA, USA) as follows: denaturation, 1 cycle of 95 °C for 20 s, followed by 40 cycles each of 95 °C for 1 s and 60 °C for 20 s. Data from standard dilution series are used to generate standard curves, which are employed to calculate the absolute quantity of target in the samples. The results were normalized as indicated by parallel amplification of GAPDH. Primers and probes sequences are listed in Table 1.

Statistical analysis Results were analyzed by unpaired *t* test using GraphPad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA) and expressed as the mean±standard error of the mean (SEM). Differences were considered to be significant at $p<0.05$.

Results and discussion

Diadenosine polyphosphates are co-stored with ATP and other neurotransmitters and released in a calcium-dependent manner from neural preparations in vitro. In this sense, release of Ap_4A, Ap_5A, and Ap_6A from perfused adrenal glands and isolated chromaffin granules by the action of secretagogues has been described [7]. Moreover, Ap_4A and Ap_5A are released from brain synaptic terminals by membrane depolarization [4]. In vivo push–pull perfusion experiments allowed the detection of Ap_4A and Ap_5A that are released from the neostriatum of the conscious rat after amphetamine administration [8]. However, under basal conditions, in the absence of stimulation with amphetamine, neither Ap_4A nor Ap_5A was detectable. Therefore, the basal values were below the detection threshold of the HPLC technique available at that date.

Presence of diadenosine polyphosphates in cerebellar microdialysates in vivo

In the present work, we have evaluated the presence of diadenosine polyphosphates in microdialysis samples from the cerebellum of the freely moving rat under basal conditions, in the absence of any stimulating agent, which could represent the amounts of these dinucleotides that are released by the normal brain activity of the rat. Cerebellum was chosen because previous studies, carried out with the dinucleotide $[^3H]Ap_4A$, revealed that this area and particularly the cerebellar cortex was one of the structures exhibiting highest levels of specific dinucleotide binding in the brain [31] and effects of diadenosine polyphosphates in modulating the function of cerebellar astrocytes and granule cell neurons have been previously described [25].

In order to detect and quantify the Ap_nAs, 100 μL of the microdyalisis samples were injected in a HPLC system as described in "Materials and methods". Ap_nAs peaks were identified by their elution times (5.8, 7.8, and 10.7 min for

Table 1 Specific primers and TaqMan MGB probes used

Target	Primer sequence	Probe sequence	Amplicon (nt)
P2X1 receptor	fw: 5′-actcaaggccattgtgcag-3′ rv: 5′-cacagttgcctgtgcgaat-3′	5′-agcccaag-3′	112
P2X2 receptor	fw: 5′-ggtggaggatggaacttctg-3′ rv: 5′-gcaatgttgccctttgaga-3′	5′-ggatgctg-3′	117
P2X3 receptor	fw: 5′-tggaggctgagaacttcacc-3′ rv: 5′-ggtgaggttaggcaggaggt-3′	5′-ggatgctg-3′	92
P2X4 receptor	fw: 5′-ttcaatgagtctgtgaagacctg-3′ rv: 5′-tttctgcagcctttaagaaagc-3′	5′-cagccacc-3′	97
$P2Y_1$ receptor	fw: 5′-gttctgcatccccctggt-3′ rv: 5′-agatcaaagctctaacaattaatccat-3′	5′-agcccaag-3′	62
$P2Y_2$ receptor	fw: 5′-agctggtgcgtttccttt-3′ rv: 5′-cacgctgatgcaggtgag-3′	5′-ggatgctg-3′	68
$P2Y_4$ receptor	fw: 5′-gtccctgggctggactaag-3′ rv: 5′-aaacctacaatcaccatctccag-3′	5′-agcccaag-3′	108
$P2Y_{12}$ receptor	fw: 5′-tttccttccgagtcaacagaa-3′ rv: 5′-tgggtgatcttgtagtctctgc-3′	5′-ggaggtgg-3′	124
NPP1	fw: 5′-cccaagtcatcccaaagaag-3′ rv: 5′-aagtccatgatcggcacaat-3′	5′-ctgtccca-3′	108
NPP3	fw: 5′-acagaagcctgtgcctcatc-3′ rv: 5′-ccgtccatggagaacaagat-3′	5′-cagccacc-3′	83
GAPDH	fw: 5′-cccctctggaaagctgtg-3′ rv: 5′-ggatgcagggatgatgttct-3′	5′-tggggcag-3′	61

Fw forward, *rv* reverse, *nt* nucleotides

Ap_3A, Ap_4A, and Ap_5A, respectively). However, to be certain that the peaks we were detecting are bona fide Ap_nAs, samples were enriched with exogenously added Ap_3A, Ap_4A, or Ap_5A (10 pmol) and the same volume (100 μL) was injected again to verify whether the putatively identified Ap_nAs peaks coeluted with the added standards. Representative chromatograms of the Ap_nAs detection in the cerebellar microdialysis samples are shown in Fig. 1.

The concentrations measured for the Ap_nAs in the cerebellar dialysate under basal conditions were (in nanomolar): 10.5±2.9, 5.4±1.2, and 5.8±1.3 (mean±SEM) for Ap_3A (n=12), Ap_4A (n=12), and Ap_5A (n=8), respectively (Fig. 2). It is necessary to note that the quantity of an analyte collected by microdialysis most often represents a fraction of the actual extracellular concentration. At usual perfusate flow rates the ratio between the actual extracellular concentration of an analyte and its dialysate concentration is typically <40 % [32]. For instance, the recovery of cyclic GMP through the microdialysis probe, under the same experimental conditions used here (i.e., at perfusion rate of 3 μL/min) was around 11 to 15 % [30], although a higher recovery value (~56 %) was obtained for ammonia [33]. Regarding the diadenosine polyphosphates, the in vitro recovering with a perfusion rate of 3 μL/min was 6.1, 6.8, and 4.3 % for Ap_3A, Ap_4A, and Ap_5A, respectively Thus, the actual concentrations of Ap_nAs in the cerebellar interstitial fluid could be higher than those reported here. Additionally, because of the dimensions of microdialysis probes, microdialysis does not sample neurotransmitters directly from the synaptic cleft but detects compounds relatively far away from the site of release, detection being thus affected by factors such as diffusion and clearance from the extracellular space. So dialysate levels do not exactly matches the local concentration that a neurotransmitter can reach at the synaptic cleft after release. In spite of these considerations, the extracellular concentrations measured here for the Ap_nAs could be of physiological relevance. The presence of dinucleotide receptors, activated by diadenosine polyphosphates but not by ATP, has been described in synaptic terminals isolated from guinea pig cerebellum [16, 34]. As mentioned before, presynaptic dinucleotide receptors are regulated by G_i/G_0 protein-coupled adenosine or GABA receptors that coexist in the same nerve terminal. Activation of $GABA_B$ or adenosine A_1 and A_2 receptors by their specific ligands dramatically changed the affinity of the dinucleotide receptor that becomes sensitive to very low concentrations of the diadenosine polyphosphates, being able to respond to Ap_nAs concentrations in the nanomolar or even the picomolar range [22, 23]. Moreover, the diversity of actions that have been described for the diadenosine polyphosphates on both cerebellar astrocytes and granule neurons [25] must also imply the interaction of these dinucleotides with P2X or P2Y receptors.

Cerebellar expression of receptors and ectoenzymes related to Ap_nAs function

On the basis of previous considerations, we have analyzed the expression in the cerebellum, at the transcriptional level, of a diversity of receptors susceptible to be activated by diadenosine polyphosphates. In order to characterize the

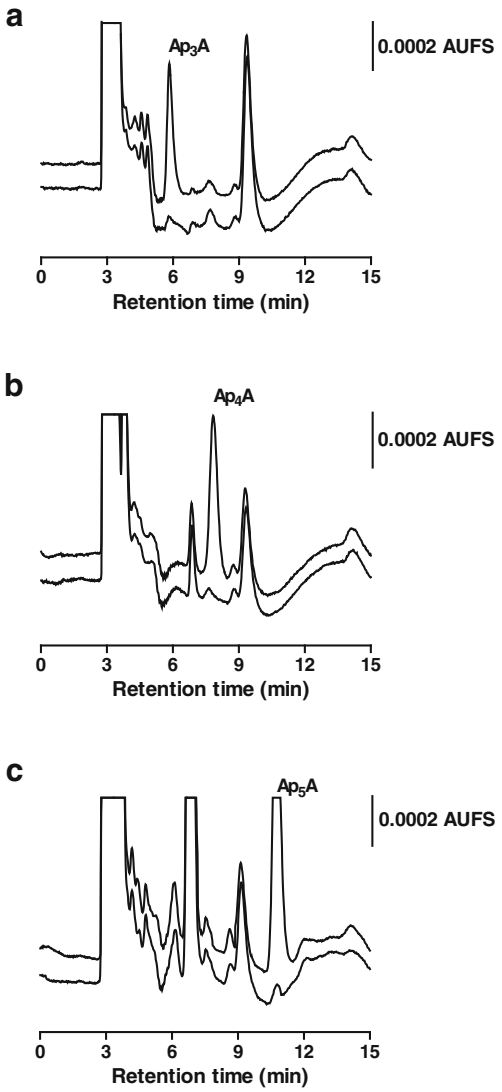

Fig. 1 Detection of Ap_nAs in the cerebellar microdialysis samples. One hundred microliters of cerebellar dialysates were injected in a HPLC system in order to detect and quantify the diadenosine polyphosphates, as described in the "Materials and methods" section. Peaks corresponding to the Ap_nAs were identified by their retention times. Representative chromatograms for the cerebellar microdialysis samples showing the presence of Ap_nAs peaks are shown in **a**, **b**, and **c** (*lower traces*). The identification of the Ap_nAs was confirmed by injecting the same volume of the samples (100 μL) enriched with 10 pmol of exogenously added Ap_3A (**a**, *upper trace*), Ap_4A (**b**, *upper trace*), or Ap_5A (**c**, *upper trace*) and verifying that the presumptive Ap_nAs peaks coeluted with the corresponding standard

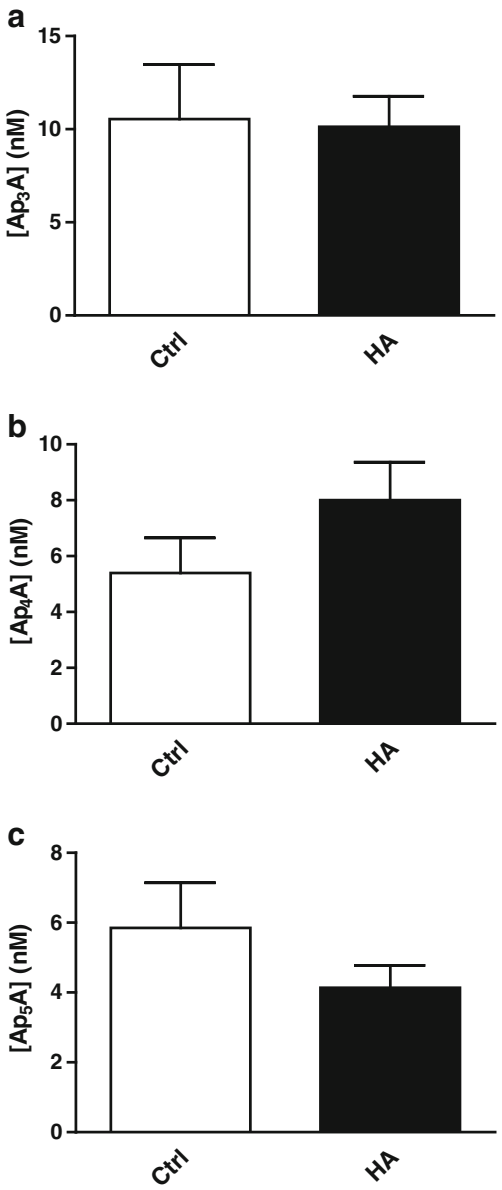

Fig. 2 Concentration of the diadenosine polyphosphates in cerebellar dialysates of hyperammonemic vs control rats. The amounts of Ap_3A (**a**), Ap_4A (**b**), and Ap_5A (**c**) were quantified in microdialysis samples from the cerebellum of rats made hyperammonemic by feeding them with an ammonium supplemented diet (*HA*) or control animals (*Ctrl*). *Bars* represent the mean±SEM of samples obtained from the cerebellum of 12 (**a**, **b**) or 8 (**c**) different animals

relative amount of each transcript, quantitative real-time PCR assays were performed. Among the great diversity of nucleotidic receptors, homomeric P2X1, P2X2, P2X3, P2X4, and also $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_{12}$, and $P2Y_{13}$ receptors have been described as possible targets for dinucleotides [9–15]. As it can be observed in Fig. 3, mRNAs coding for P2X1, P2X2, P2X3, and P2X4 subunits were found in the rat cerebellum, with P2X4 transcripts being the most abundant. Regarding P2Y receptors, we analyzed the expression of the $P2Y_1$ and $P2Y_{12}$ subtypes, as examples of ADP-preferring receptors, and the $P2Y_2$ and $P2Y_4$, as pyrimidine-sensitive subtypes. As shown in Fig. 3a, all these receptors are present in the cerebellum, $P2Y_1$ showing the higher level of expression among the P2Y subtypes analyzed. We could also detect the presence in the cerebellum of mRNAs coding for the diadenosine polyphosphate-hydrolyzing enzymes NPP1 and NPP3, which showed comparable levels of expression (Fig. 3b). A similar analysis could not be done with the

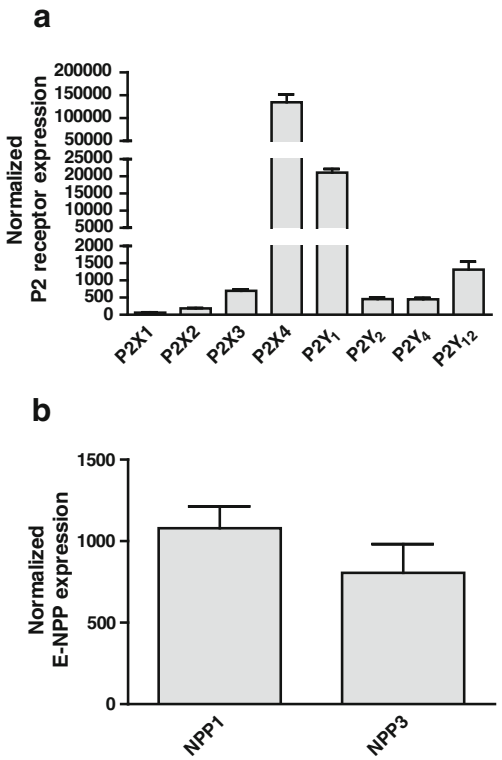

Fig. 3 Rat cerebellum expresses ecto-nucleotide pyrophosphatases/phosphodiesterases and several purinergic receptors susceptible to be activated by diadenosine polyphosphates. **a** Quantitative real-time PCR for ionotropic P2X1, P2X2, P2X3, and P2X4 receptors, and metabotropic $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{12}$ receptors in rat cerebella. Values were normalized by the content of GAPDH transcript. The results are the mean±SEM of six animals. **b** Quantitative real-time PCR for NPP1 and NPP3 ecto-enzymes in rat cerebella. Values were normalized by the content of GAPDH transcript. The results are the mean±SEM of six animals

dinucleotide receptor, as its molecular identity has not yet been elucidated, and there are only pharmacological evidences of its presence in cerebellar nerve terminals [16, 34]. Taken together, all these evidences (i.e., the occurrence of the Ap_nAs in the extracellular medium, the existence of receptors able to respond to these substances, which has been demonstrated by pharmacological or molecular techniques, and the existence of ecto-enzymes able to hydrolyze the diadenosine polyphosphates and terminate their extracellular actions) contribute to reinforce the role of these dinucleotides as extracellular messengers in the cerebellum and confirms their function as signaling molecules in the central nervous system.

It is also relevant to highlight the presence of Ap_3A in the microdialysis samples, whose occurrence in the extracellular medium in neural tissues has been described here for the first time. Previous assays on the release of Ap_nAs from brain synaptic terminals induced by depolarizing agents and the push–pull perfusion experiments in the rat neurostriatum stimulated with amphetamine, failed to show the presence of this dinucleotide [4, 8]. However, Ap_3A is stored, along with other Ap_nAs, ATP, ADP, and serotonin, in the granule dense of platelets, and is released to the extracellular medium during platelet aggregation acting as a signaling molecule in the vascular system [35, 36]. In addition to its interaction with the dinucleotide receptor, Ap_3A also behaves as a good agonist at the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors [12–14], suggesting that a sensitivity to Ap_3A is a common feature of ADP receptors. The $P2Y_4$ pyrimidinoreceptor is also sensitive to Ap_3A, but the maximal effect of this dinucleotide is only 20–25 % that of UTP [11]. Regarding P2X receptors, Ap_3A is a partial agonist, although with a higher potency than ATP, at the homomeric P2X3 receptor. Moreover, Ap_3A does not exert any agonistic effect but potentiates the responses elicited by ATP on the P2X4 receptor [15]. All of the above-mentioned receptors are abundantly expressed in the cerebellum (Fig. 3a) and other brain areas [37], suggesting that, as occurs in the vascular system, Ap_3A could also behave as a relevant signaling compound in the central nervous system.

Effects of mild hyperammonemia on Ap_nAs

Finally, we have analyzed whether the extracellular concentrations of the Ap_nAs and/or the expression levels of their receptors and hydrolyzing enzymes are modified in a model of hyperammonemic rats. Hyperammonemia leads to alterations of multiple neurotransmitter systems in the brain. In this sense, there are evidences that the excess of ammonia affects the glutamatergic neurotransmission in the cerebellum by impairing the glutamate-nitric oxide (NO)-cGMP pathway, such impairment being responsible for a reduced ability to learn some types of tasks [38]. Additionally, the GABAergic tone appears to be augmented in the cerebellum of rats with chronic hyperammonemia, which could be a consequence of an increase in extracellular GABA [39]. Regarding the purinergic system, preliminary data show that hyperammonemia induces changes in the basal concentrations of ATP and GMP in the

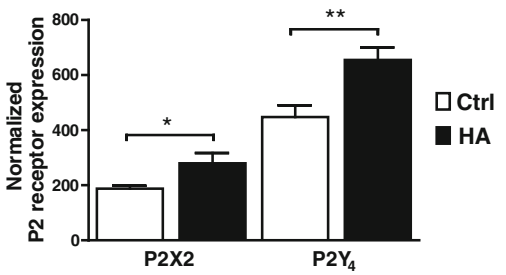

Fig. 4 Changes in purinergic receptors expression in the cerebella of hyperammonemic rats. Quantitative real-time PCR for P2X2 and $P2Y_4$ receptors in control (*Ctrl*) and hyperammonemic (*HA*) rat cerebella. Values were normalized by the content of GAPDH transcript. The results are the mean±SEM of six to seven animals. Statistical significance versus corresponding control, $*p<0.05$; $**p<0.01$ (Student's *t* test)

cerebellar interstitial medium (unpublished results). However, no differences were observed when the extracellular levels of Ap_3A were measured in the cerebellum of hyperammonemic (10.1±1.6 nM) and control rats (10.5±2.9 nM), as shown in Fig. 2a. Regarding Ap_4A, its extracellular concentration appears to be slightly increased in the cerebellum of the hyperammonemic rats (8.0±1.4 nM) when compared to control animals (5.4±1.2 nM), although the differences were not statistically significant when analyzed by means of the Student's *t* test (Fig. 2b). Similar result was obtained with Ap_5A (Fig. 2c), whose concentration was not significantly modified in the cerebellum of the hyperammonemic animals (4.1±0.6 vs 5.8±1.3 nM in control rats). Regarding the purinergic receptors and Ap_nAs-hydrolyzing enzymes, we only found differences in the expression of the P2X2 and $P2Y_4$ receptor subtypes, whose transcripts are increased in the cerebellum of the hyperammonemic animals (Fig. 4) whereas no significant changes in the expression of the P2X1, P2X3, P2X4, $P2Y_1$, $P2Y_2$, and $P2Y_{12}$ receptors or the NPP1 and NPP3 ectoenzymes were observed (data not shown). It is necessary to take into account that the model of hyperammonemia used here (rats fed with a high-ammonia diet) shows a moderate increase of ammonia in the brain: Ammonia levels in striatum are 0.38±0.01 μmol/g tissue in control rats and are increased in hyperammonemic animals to 0.55±0.01 μmol/g tissue [40]. Similar differences should occur in other brain areas. From the results presented here, it seems that mild hyperammonemia, as occurs in the model used here, only induce slight changes, which are non-statistically significant, in the amounts of Ap_nAs released from the cerebellum under basal conditions, although alterations in the extracellular levels of diadenosine polyphosphates in other models of stronger hyperammonemia cannot be discarded. However, even moderate hyperamonemia induced changes in the expression of the P2X2 subunit and the $P2Y_4$ receptor in the cerebellum, which indicates that the purinergic transmission, as occurs with other neurotransmitter systems, can be also affected by the excess of ammonia. More work on the cellular localization and function of these receptors will be necessary to fully understand the physiological significance of these changes.

The experimental data reported here confirm the presence of diadenosine polyphosphates in the extracellular medium in brain. Not to neglect that they are physiological agonists of a large number of P2 receptors. Thus, their contribution to purinergic signaling should be considered when studying the physiology and pathology of the central nervous system.

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