European Journal of Pharmacology 642 (2010) 72–76

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Neuropharmacology and Analgesia

Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral cortex of rats

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ARTICLE INFO

Article history:
Received 18 March 2010
Received in revised form 25 April 2010
Accepted 23 May 2010
Available online 8 June 2010

Keywords:
Morphine
E-NTPDase
Ecto-5′nucleotidase
Nociception
Neonatal rat

ABSTRACT

The neonate opioid system has been frequently investigated, and studies have shown that exposure to drugs in early life can have implications for nervous system development. It has been proposed that adenosine is involved in opioid antinociception, and ATP is involved in central and peripheral mechanisms of nociception. Extracellular nucleotides can be hydrolyzed by E-NTPDases and ecto-5′nucleotidase, which present the functions of removing ATP and generating adenosine. In this study, we evaluated ATP, ADP, and AMP hydrolysis in synaptosomes from spinal cord and cerebral cortex of rats at postnatal day 16 after repeated morphine exposure in early life (postnatal day 8 to 14). Additionally, we evaluated E-NTPDase (1, 2 and 3) and ecto-5′nucleotidase gene expression by semi-quantitative RT-PCR analysis. We observed an increase in ATP hydrolysis in the cerebral cortex, and a decrease in ADP hydrolysis in spinal cord. Expression levels of E-NTPDase 1 decreased in cerebral cortex and increased in spinal cord. Our findings highlight the importance of the purinergic system in young rats submitted to repeated morphine exposure by showing that in the neonatal period such exposure is capable of affecting the control system for nucleotide levels, which can promote changes in modulation or transmission of painful stimuli.

1. Introduction

The recognition of the need to adequately assess and treat pain in infants and children has led to increased use of opioids in these patients (Anand and Hall, 2006). Although the neonatal nervous system is structurally and functionally immature, significant changes in nociceptive pathways and opioid analgesic mechanisms occur before and after birth (Beland and Fitzgerald, 2001). Previous studies showed that exposure to drugs in early life can have long-lasting implications for the developing nervous system, such as permanent alterations in pharmacological responses and cell signaling (Stanwood and Levitt, 2004). Moreover, long-term administration of opioids can alter the central pain-related systems and generally results in opioid addiction (Nestler, 2004). In particular, studies with rats have shown that chronic use of morphine can promote changes in adenosine-mediated signaling pathways in several brain structures linked to the etiology of addiction (Hack and Christie, 2003) and to pain transmission (Sawynok and Liu, 2003).

Adenine nucleotides (ATP, ADP and AMP) comprise an important class of signaling molecules. They can be released from nerve, glial, and vascular cells and exert their effects via ionotropic (P2X) or metabotropic (P2Y) receptors. Similarly, the nucleoside adenosine acts via P1 receptors as a modulator of neural and vascular functions. Previous studies have shown that these nucleotides may modulate nociceptive neurotransmission. Likewise, it has been proposed that adenosine is involved in pain control and in opioid antinociception (Sawynok and Liu, 2003), possibly acting through the adenosine A_1 receptor (Keil and DeLander, 1995). The administration of morphine promotes adenosine release in the spinal cord, providing evidence to support the idea that adenosine is involved in opioid-induced analgesia (Sweeney et al., 1987a). Moreover, agonists of μ opioid and adenosine A_1 receptors produce powerful antinociception in the peripheral nervous system (Torres et al., 2003b).

Additionally, it has been accepted that ATP is involved in mechanisms of nociception. This nucleotide is released from the terminals of

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doi:10.1016/j.ejphar.2010.05.044

primary afferent neurons to act in the central pain pathway (Burnstock, 2006). The nociception mediated by ATP signaling mainly involves $P2X_3$ and $P2X_{2/3}$ receptors, because they are expressed in a subset of predominantly nociceptive sensory neurons (Burnstock, 2001).

Extracellular nucleotides (ATP and ADP) may be hydrolyzed by members of the ecto-nucleoside triphosphate diphosphohydrolase family (E-NTPDases), and AMP may be hydrolyzed by the ecto-5′-nucleotidase to produce adenosine (Abbracchio et al., 2009). In this way, E-NTPDases control the availability of ligands for both nucleotide and nucleoside receptors and, consequently, the duration of receptor activation (Chen and Guidotti, 2001). Therefore, this is an enzymatic pathway with the double function of eliminating one signaling molecule, ATP, and generating another, adenosine. These enzymes may also exert a protective function by keeping extracellular ATP/ADP and adenosine within physiological concentrations (Agteresch et al., 1999).

Neonatal exposure to analgesic drugs is inevitable in some cases, thus the evaluation of possible physiological effects of such exposure is an area of scientific interest. Considering the close relationship between opioid and purinergic systems in the control and processing of nociceptive transmission we investigated the activity and expression of E-NTPDases after early morphine exposure.

2. Materials and methods

2.1. Animals

Male Wistar rats were housed in home cages made of Plexiglas material (65×25×15 cm), with the floor covered with sawdust. Animals were maintained on a standard 12-h dark/light cycle (lights on between 7.00 h and 19.00 h) at room temperature (22±2 °C). The animals had free access to food and water. Litters were culled to eight pups per dam, and rat pups were randomly cross-fostered on the day of birth. The Institutional Research Committee approved all animal procedures, and measures were taken to minimize pain and discomfort.

2.2. Reagents

Nucleotides (ATP, ADP, and AMP), Percoll, Trizma base, Coomassie Brilliant Blue G, EDTA, and HEPES were purchased from Sigma, St. Louis, MO, USA. Morphine sulfate (Dimorf® 10 mg/ml) was purchased from Cristália, Porto Alegre, RS, Brazil. All other reagents were of analytical grade.

2.3. Morphine administration

The rats were divided into two groups: saline-control (n=18) and morphine-treated (n=18). The morphine or saline treatment was performed from postnatal day 8 to 14. Animals on postnatal day 8 were chosen because it is accepted that at this point rats are at a similar stage of neurological development to that of a human newborn, presenting developmental changes in the brain and plasticity of the pain system (Bishop, 1982; Fitzgerald and Anand, 1993; Rabinowicz et al., 1996). Each animal received saline or morphine (5 μg s.c. in the mid-scapular area) from postnatal day 8, once a day for seven days (Rozisky et al., 2008). Morphine sulfate 1 ml (Dimorf® 10 mg/ml, Cristália) was dissolved in 9 ml of 0.9% saline, and animals were treated at the same time each day (11:00 h). The experimental procedures were performed two days after the end of treatment (postnatal day 16). The animals were killed and the chosen structures were removed for enzyme assays and analysis of gene expression. The enzyme assays were performed on spinal cord (Control: n=6; Morphine: n=6) and cerebral cortex (Control: n=6; Morphine: n=6). Gene expression was analyzed in spinal cord (Control: n=3; Morphine: n=3) and cerebral cortex (Control: n=3; Morphine: n=3).

2.4. Subcellular fractionation

The animals were killed by decapitation and the spinal cord and cerebral cortex were rapidly removed and gently homogenized in 10 vols. of ice-cold medium containing 320 mM sucrose, 0.1 mM EDTA, and 5.0 mM HEPES, pH 7.5, with a motor driven Teflon-glass homogenizer. Synaptosomes were then isolated as described previously (Nagy et al., 1984). Briefly, 0.5 ml of the crude mitochondrial fraction was mixed with 4.0 ml of 8.5% Percoll solution and layered onto an isosmotic Percoll sucrose discontinuous gradient (10/20% for spinal cord and 10/16% for cerebral cortex). The synaptosomes that banded at the 10/20% and 10/16% Percoll interface were collected with a wide-tip disposable plastic transfer pipette. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

2.5. Enzyme assays

The reaction medium used to assay ATP and ADP hydrolysis was essentially as described previously (Battastini et al., 1991). The medium contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μl. The synaptosomal fraction (10–20 μg protein) was added to the reaction mixture and pre-incubated for 10 min at 37 °C. The reaction was then initiated by the addition of ATP or ADP to a final concentration of 1.0 mM and was stopped by the addition of 200 μl 10% trichloroacetic acid. The samples were chilled on ice for 10 min, and 100 μl samples were taken for the assay of released inorganic phosphate (Pi) (Chan et al., 1986).

The reaction medium used to assay the AMP hydrolysis contained 10 mM $MgCl_2$, 0.1 M Tris–HCl, pH 7.0 and 0.15 M sucrose in a final volume of 200 μl (Heymann et al., 1984). First, the synaptosome preparation (10–20 μg protein) was pre-incubated for 10 min at 37 °C. The reaction was then initiated by the addition of AMP to a final concentration of 1.0 mM and stopped by the addition of 200 μl of 10% trichloroacetic acid; 100 μl samples were taken for the assay of released inorganic phosphate (Pi) (Chan et al., 1986).

For both enzyme assays, incubation times and protein concentration were chosen in pilot studies to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct for non-enzymatic hydrolysis of the substrates. All samples were run in triplicate, and protein was measured by the Coomassie Blue method (Bradford, 1976), using bovine serum albumin as standard. Enzyme activities were expressed as nmol of inorganic phosphate released per min per milligram of protein ($nmolPi.min^{-1}\,mg^{-1}$ of protein).

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

Analysis of E-NTPDase expression (E-NTPDase 1, E-NTPDase 2 and E-NTPDase 3) and that of ecto-5′nucleotidase was carried out with a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. Total RNA was extracted from spinal cord or cerebral cortex with the TRIzol reagent (Invitrogen Corporation, Carlsbad, USA) according to the manufacturer's instructions. Afterwards, cDNA species were synthesized with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) from 3 μg of total RNA and oligo dT, in accordance with the supplier's protocol. RT reactions were performed for 50 min at 42 °C. cDNA (0.1 μl) was used as a template for RT-PCR, with specific primers for all enzymes analyzed (primer sequences described below). β-actin PCR was performed as a control for cDNA synthesis. PCR reactions were performed (total volume of 25 μl) using 0.4 μM of each primer and 1 U Platinum® Taq DNA polymerase (Invitrogen) in the supplied reaction buffer. To improve the conditions for the E-NTPDase 1 PCR 5% glycerol was added to the reaction medium. Conditions for E-NTPDase PCRs were as follows: initial 1 min denaturation step at

94 °C, 1 min at 94 °C, 1 min annealing step at 65 °C (E-NTPDase 1 and E-NTPDase 3) or 66 °C (E-NTPDase 2), 1 min extension step at 72 °C for 35 cycles and a final 10 min extension at 72 °C (Oses et al., 2007). Conditions for β-actin PCR were as follows: initial 1 min denaturation step at 94 °C, 1 min at 94 °C, 1 min annealing step at 58.5 °C, 1 min extension step at 72 °C for 35 cycles and a final 10 min extension at 72 °C. PCR products were separated by electrophoresis with a 1% agarose gel. Band intensities were analyzed in a semi-quantitative manner using the software ImageJ. The following primers were used: E-NTPDase 1: Primer 1 — 5′-GAT CAT CAC TGG GCA GGA GGA AGG-3′, Primer 2 — 5′-AAG ACA CCG TTG AAG GCA CAC TGG-3′, E-NTPDase 2: Primer 1 — 5′-GCT GGG TGG GCC GGT GGA TAC G-3′, Primer 2 — 5′-ATT GAA GGC CCG GGG ACG CTG AC-3′, E-NTPDase 3: Primer 1 — 5′-CGG GAT CCT TGC TGT GCG TGG CAT TTC TT-3′, Primer 2 — 5′-TCT AGA GGT GCT CTG GCA GGA ATC AGT-3′; 5′-Nucleotidase (CD73): Primer 1 — 5′-CCC GGG GGC CAC TAG CAC CTC A-3′, Primer 2 — 5′-GCC TGG ACC ACG GGA ACC TT-3′, β-actin: Primer 1 — 5′-TAT GCC AAC ACA GTG CTG TCT GG-3′, Primer 2 — 5′-TAC TCC TGC TTC CTG ATC CAC AT-3′. The expected amplification products were: E-NTPDase 1: 543 bp, E-NTPDase 2: 331 bp, E-NTPDase 3: 267 bp, 5′-Nucleotidase: 405 bp and β-actin: 210 bp.

2.7. Statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.M.). Comparisons among groups were made by Student's *t* test, considering $P<0.05$ as significant.

3. Results

3.1. Ectonucleotidase activities in spinal cord and cerebral cortex synaptosomes after repeated morphine exposure in rat pups

After the daily morphine exposure, from postnatal day 8 to 14, the ectonucleotidase activities from spinal cord were compared between the saline-control and morphine-treated groups at postnatal day 16. Our results demonstrate a significant decrease in ADP hydrolysis in the morphine group when compared to the control group (Control = 82.55 ± 1.64 nmol Pi $min^{-1} mg^{-1}$ of protein, Morphine = 51.85 ± 10.32 nmol Pi $min^{-1} mg^{-1}$ of protein; Student's *t* test, $P<0.05$; Fig. 1A). There was no difference in the hydrolysis of other nucleotides (ATP: Control = 155.59 ± 10.05 nmol Pi $min^{-1} mg^{-1}$ of protein, Morphine = 142.35 ± 14.05 nmol Pi $min^{-1} mg^{-1}$ of protein; AMP: Control = 8.59 ± 1.97 nmol Pi $min^{-1} mg^{-1}$ of protein, Morphine = 6.54 ± 1.14 nmol Pi $min^{-1} mg^{-1}$ of protein; Student's *t* test, $P>0.05$; Fig. 1A).

The ectonucleotidase activities from cerebral cortex were also compared between the saline-control and morphine-treated groups at postnatal day 16. Our results demonstrate a significant increase in ATP hydrolysis in the morphine group when compared to the control group (Control = 161.65 ± 27.52 nmol Pi $min^{-1} mg^{-1}$ of protein, Morphine = 213.03 ± 30.88 nmol Pi $min^{-1} mg^{-1}$ of protein; Student's *t* test, $P<0.05$; Fig. 1B). There was no difference in the hydrolysis of other nucleotides (ADP: Control = 95.88 ± 19.55 nmol Pi $min^{-1} mg^{-1}$ of protein, Morphine = 104.33 ± 17.67 nmol Pi $min^{-1} mg^{-1}$ of protein; AMP: Control = 12.44 ± 1.97 nmol Pi $min^{-1} mg^{-1}$ of protein, Morphine = 14.53 ± 2.85 nmol Pi $min^{-1} mg^{-1}$ of protein; Student's *t* test, $P>0.05$; Fig. 1B).

3.2. Ectonucleotidase gene expression in spinal cord and cerebral cortex after repeated morphine exposure in rat pups

After the daily morphine exposure, from postnatal days 8 to 14, the gene expression patterns of ectonucleotidases from the spinal cord were compared between the groups at postnatal day 16. We found that the morphine-treated group presented a significant increase (23%) in E-NTPDase 1 mRNA transcript levels when compared to the control group (optical density for Control: 120.61, and for Morphine: 148.26; Student's *t* test, $P<0.05$). There were no differences in mRNA transcript levels for other E-NTPDases (2 and 3) and ecto-5′ nucleotidase after the repeated morphine exposure (Fig. 2A.1, A.2).

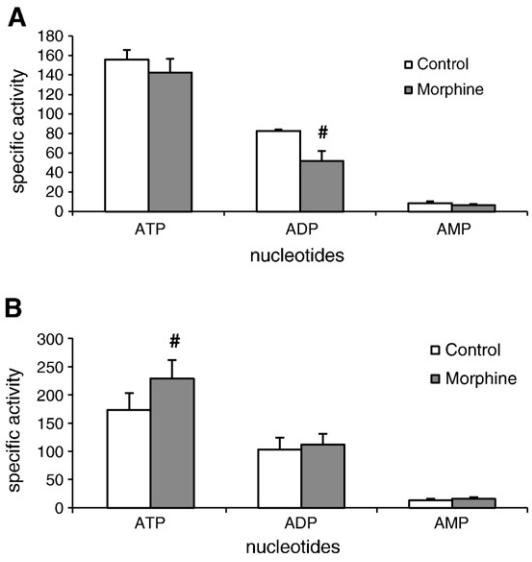

Fig. 1. A: ATP, ADP, and AMP hydrolysis in synaptosomes from spinal cord of male rats at 16 days of age. Values are mean ± S.E.M. Specific enzyme activities were expressed as nmol Pi $min^{-1} mg^{-1}$ of protein. There was a significant difference in ADP hydrolysis between the groups. (Student's *t* test, $P>0.05$). # indicates a significant difference between the control and the morphine-treated groups. B: ATP, ADP, and AMP hydrolysis in synaptosomes from cerebral cortex of male rats at 16 days of age. Values are mean ± S.E.M. Specific enzyme activities were expressed as nmol Pi $min^{-1} mg^{-1}$ of protein. There was a significant difference in ATP hydrolysis between the groups. (Student's *t* test, $P>0.05$). # indicates a significant difference between the control and the morphine-treated groups.

The gene expression patterns of ectonucleotidases from cerebral cortex were also compared between the groups at postnatal day 16. Our results demonstrated a significant decrease (28%) in E-NTPDase 1 mRNA transcript levels when compared to the control group (optical density for Control: 230.08, and for Morphine: 165.36; Student's *t* test, $P<0.05$). There were no differences in other E-NTPDases (2 and 3) and ecto-5′nucleotidase mRNA transcript levels after the repeated morphine exposure (Fig. 2B.1, B.2).

4. Discussion

In this study, after the repeated, daily exposure to morphine beginning on postnatal day 8 and continuing until postnatal day 14, we observed a decrease in ADP hydrolysis in the synaptosomes from spinal cord and an increase in ATP hydrolysis in the synaptosomes from cerebral cortex when these were analyzed at postnatal day 16, two days after the end of treatment with morphine. Furthermore, when the expression of E-NTPDases was analyzed by RT-PCR, it was observed that only E-NTPDase 1 mRNA transcript levels were altered in both structures, presenting an increase (28%) in spinal cord and a decrease (23%) in cerebral cortex after morphine treatment.

Previous studies have shown that ectonucleotidase activities differ over the course of development in some structures of the central nervous system, including spinal cord and cerebral cortex (de Paula Cognato et al., 2005; Torres et al., 2003a). For example, synaptosomes obtained from the cerebral cortex present significantly higher nucleotide hydrolysis at postnatal day 16 when compared with later stages of postnatal development (de Paula Cognato et al., 2005). This early period coincides with an intense synaptogenesis and augmentation of the activities of several enzymes involved in neurotransmitter metabolism and neuronal functions (Fiedler et al., 1987).

In this study we observed that morphine treatment during an early postnatal period altered nucleotide hydrolysis from spinal cord and

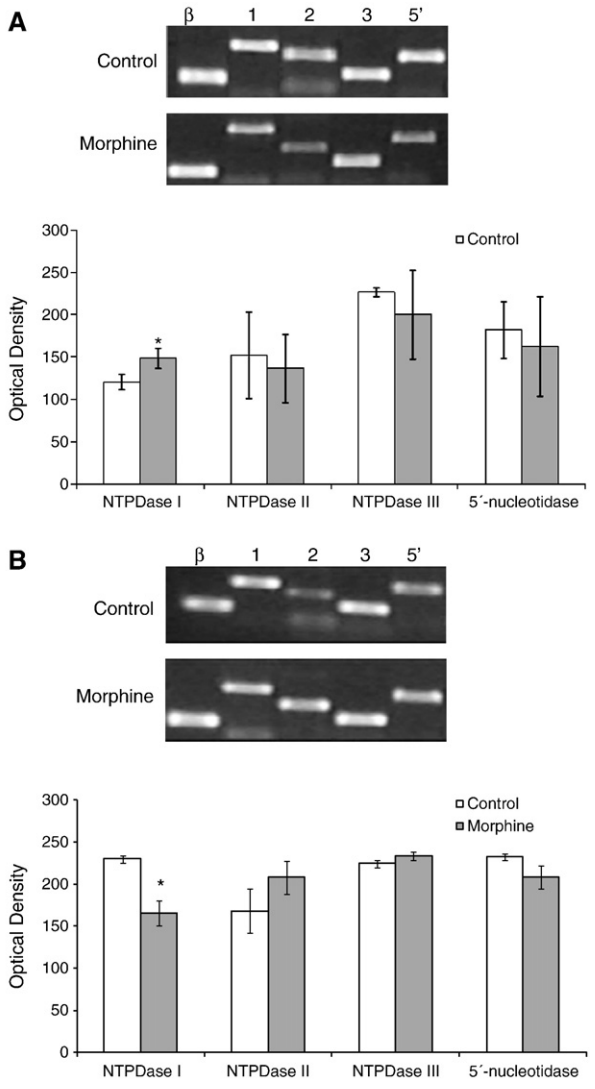

Fig. 2. A: Effect of repeated morphine exposure, from postnatal day 8 until postnatal day 14, on E-NTPDase 1, 2, and 3, and 5′-nucleotidase mRNA transcript levels from spinal cord of male rats at 16 days of age. (1) Representative agarose gel electrophoresis and (2) Bars showing mean value ± S.E.M. from optical densitometry analysis of three independent experiments, with entirely consistent results. * represents a significant difference at $P<0.05$ in relation to control rats. B: Effect of repeated morphine exposure, from postnatal day 8 until postnatal day, on E-NTPDase 1, 2, and 3, and 5′-nucleotidase mRNA transcript levels from cerebral cortex of male rats at 16 days of age. (1) Representative agarose gel electrophoresis and (2) Bars showing mean value ± S.E.M. from optical densitometry analysis of three independent experiments, with entirely consistent results. * represents a significant difference at $P<0.05$ in relation to control rats.

cerebral cortex associated with different gene expression of E-NTPDase 1 in both structures. E-NTPDases 1, 2, and 3 differ in their catalytic properties, whereby E-NTPDase 1 hydrolyzes ATP and ADP about equally well, E-NTPDase 2 has a high (about 20- to 30-fold) preference for ATP over ADP, and E-NTPDase 3 has a three- to five-fold preference for ATP (Robson et al., 2006). Although the mRNAs for the cell surface-located E-NTPDases 1, 2, and 3 have been identified in the central nervous system, the cellular location of each member of this enzyme family is less clear. Several studies have identified the different gene expression patterns of E-NTPDases in central nervous system structures. For example, E-NTPDase 1 presents a wide distribution on the surface of all cell types from the central nervous system (Zimmermann 2006; Langer et al., 2008); meanwhile, E-NTPDase 2 is expressed in embryonic rat brain (Zimmermann 2006; Langer et al., 2008).

The increased activity observed in this study in cerebral cortex synaptosomes, after the repeated exposure to morphine, may be important in modulating ATP signaling. Although we know that ATP facilitates nociceptive transmission through binding to $P2X_3$ receptors, these receptors are co-localized with the $P2Y_1$ inhibitory receptors involved in nociceptive transmission (Ruan and Burnstock, 2003). Previous studies have shown that P2Y activation by ADP may decrease the excitatory effect of primary inputs on secondary sensory neurons and thereby partly counterbalance the algogenic effect of ATP (Gerevich et al., 2004). Although this hypothesis was not tested in the present study, we could suggest that the increase in ATP hydrolysis seen in cerebral cortex synaptosomes has the function of terminating the ATP signal and generating a second signal, mediated by ADP.

E-NTPDase 1 stands out from other members of the family due to its high preference for ATP and ADP. The conversion of extracellular ATP to AMP by E-NTPDase 1 produces no appreciable ADP products, and the inhibition of E-NTPDase 1 can increase ATP availability to other members of the E-NTPDase family. E-NTPDases with higher affinity for ATP can produce relative stability of ADP and in this case the AMP that constitutes the substrate for ecto-5′nucleotidase may be reduced. Here, we observed decreased ADPase activity in the spinal cord of morphine-treated animals that could contribute to ADP accumulation in sensory neurons of the spinal cord. On the other hand, since no change was observed in the ecto-5′nucleotidase activity it is difficult to determine whether this result, which was obtained in vitro, would or would not result in increased extracellular adenosine in vivo. Conversely, the ATP hydrolysis did not change probably due to an up-regulation of an ecto-ATPase (E-NTPDase 2) that is co-expressed with the ATP diphosphohydrolase (E-NTPDase 1) in the central nervous system (Kegel et al., 1997).

Therefore, nucleotide hydrolysis in morphine-treated animals could be carrying out the same function, promoting increased ATP hydrolysis in the cerebral cortex, or decreased ADP hydrolysis in the spinal cord, and thus increasing the ADP level in the synaptic cleft. We found that such effects persisted for a short time, within which the activities were already normalized in animals aged 30 and 60 days (data not shown). These results may suggest that ADP acts as a neuromodulator in the opioid withdrawal process due to the sustained exposure to morphine in early life. Although our laboratory previously demonstrated that rats do not present tolerance to morphine at postnatal day 14 (Rozisky et al., 2008), we have found that they do present some symptoms of morphine withdrawal (Rozisky et al., personal communication). Thus, it is probable that early morphine exposure is able to induce differential E-NTPDase 1 expression in the tissues tested. In the spinal cord, we observed a decrease in ADP hydrolysis, where E-NTPDase 1 is up-regulated, while in the cerebral cortex we observed an increase in ATP hydrolysis, where this enzyme is down-regulated. We could suggest that these different expression levels in two structures related to nociceptive transmission are modulated by the concentrations of nucleotides and, therefore, promote a counteradaptive response. Furthermore, studies have demonstrated that the peak of enzyme protein expression does not always coincide with enzyme activity (Banjac et al., 2001; Nedeljkovic et al., 2005). In fact, high protein levels of NTPDase 1 from synaptic plasma membrane from cerebral cortex did not match the peak of enzymatic activity (Nedeljkovic et al., 2005). These findings indicate that changes in enzyme activity are not necessarily reflected by similar changes in expression levels.

Several studies have demonstrated the cross-talk between purinergic and opioid systems (Fink et al., 1992; Schiffmann and Vanderhaeghen, 1993; Yao et al., 2006). In addition, a study in spinal cord from A_{2A} knock-out mice has shown a reduction of [3H]-deltorphin-I binding to δ-opioid receptors and an increase in [3H]-Cl-977 binding to κ-opioid receptors (Bailey et al., 2002) associated with functional changes in opioid antinociception. The spinal antinociceptive effects of morphine appear to be mediated, at least in part, by the release of endogenous adenosine and subsequent activation of A_1 and A_2 receptors (Sweeney et al., 1987b, 1991). Furthermore, the blockade of adenosine metabolism by adenosine

kinase inhibitors (Kaplan and Coyle, 1998) and the administration of adenosine agonists (Kaplan and Sears, 1996) decrease the severity of morphine abstinence, whereas adenosine antagonists increase the expression of morphine withdrawal symptoms in rats (Salem and Hope, 1997). An analysis of purinergic receptors expression and functionality could provide an additional insight into the functional consequences of early morphine administration on purinergic transmission.

It is important to note that to our knowledge this is the only study to date of E-NTPDase activity and gene expression patterns in structures related to nociception. Thus, since there are no reports on the ontogeny of different expression levels of E-NTPDases in the spinal cord it is difficult to say whether the expression seen in the spinal cord of 16-day old animals may or may not change with increasing age.

5. Conclusion

In conclusion, our findings highlight the importance of the purinergic system of young rats submitted to repeated exposure to morphine. The changes in E-NTPDase activities described may constitute one of the mechanisms that mediate the development of some of the side effects, such as the opioid withdrawal syndrome, associated with repeated exposure to this drug in early life. Further studies are required to investigate the kinetic profiles of ectonucleotidases and the role of ADP function following repeated opioid administration in young rats.

Acknowledgments

This work was supported by the National Research Council of Brazil (CNPq), Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS), and Pró-Reitoria de Pesquisa, Universidade Federal do Rio Grande do Sul (PROPESQ-UFRGS). We would like to thank Dr Gareth Cuttle for the English correction and editing of the manuscript.

Dedication

This work is dedicated to our dear professor, João José Freitas Sarkis (*in memorium*), who taught us the importance of advancing research associated with ectonucleotidases.

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