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

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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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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