UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Avaliação das atividades NTPDásicas e 5'-nucleotidase em soro de ratos submetidos à administração repetida de morfina no período neonatal

Yasmine Nonose

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Dedico este trabalho
Aos meus pais,
Pelo apoio e compreensão
Ao meu irmão,
Pelo carinho e confiança
Aos colegas e amigos,
Pelas horas de trabalho e divertimento
Aos professores,
Pela paciência e entusiasmo
Ao Lucas,
Pelo companheirismo e incentivo
E
Aos ausentes
Pela inspiração.

Somos o que fazemos, mas somos, principalmente,

o que fazemos para mudar o que somos.

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Juntamente com o artigo apresentado neste Trabalho de Conclusão de Curso, encontra-se uma breve revisão bibliográfica elaborada pela autora e que foi utilizada para fins de estudo no decorrer do semestre.

Revisão bibliográfica

Dor e neonato

Historicamente, acreditava-se que recém-nascidos (RN) não sentiam dor por apresentarem o sistema nervoso central (SNC) imaturo. No entanto, nas últimas décadas, estudos têm demonstrado que esses pacientes não somente experimentam dor e estresse da mesma maneira que crianças e adultos, mas que suas respostas à estimulação dolorosa podem comprometer sua condição clínica e fisiológica [1]. Estudos têm relatado que em bebês as estruturas límbicas são desenvolvidas em estágios iniciais, estando relacionadas com a dor durante o período neonatal [2-3] e sugerindo que a codificação central da dor é um evento precoce. A partir da 16^{a} semana ocorre conexão entre neurônio sensorial e células cuneiformes da medula espinhal na presença de substância P e de opiáceos endógenos nos gânglios destas áreas. Em 24 semanas, as conexões sinápticas do córtex estão completas, e em 30 semanas existe a mielinização, tornando as vias de dor completamente prontas para serem ativadas no tálamo, onde ocorre o processamento da dor [4-8].

Estudos prévios indicam que neonatos rotineiramente experimentam dor em procedimentos invasivos [9-10] sem receberem analgesia adequada [11-12]. A dor oriunda de procedimentos invasivos ocorre com muita freqüência durante a permanência do RN na Unidade de Terapia Intensiva Neonatal (UTI). Estes RN são expostos a mais ou menos 14 procedimentos ao dia na UTI, incluindo vacinas, coleta de exames para hemograma, hemocultura e realização de punção lombar [13]. Todos estes procedimentos são extremamente dolorosos. Além disso, a dificuldade de reconhecer e de avaliar a dor no período neonatal é um dos maiores obstáculos ao seu tratamento. Isso se associa ao fato do profissional de saúde, muitas vezes, subestimar as queixas desses pacientes, desconhecer o embasamento farmacológico da prescrição analgésica e ao temor dos riscos da terapêutica [14].

Analgesia opióide em neonato

Analgésicos opióides têm sido amplamente utilizados na tentativa de diminuir a dor, sedar e atenuar o estresse em todas as faixas etárias. O grande entrave ao uso adequado desses analgésicos é a excessiva preocupação com seus efeitos adversos [15-16]. Entretanto, seu uso vem crescendo na última década em UTIs pediátricas, principalmente devido ao avanço do conhecimento

do processo fisiopatogênico, métodos de avaliação da dor e disponibilidade de novas modalidades terapêuticas [17-19].

Anand e colaboradores [20] reforçam a hipótese de que os RN apresentam melhor prognóstico clínico quando recebem analgesia adequada. A utilização de morfina em RN foi capaz de diminuir o risco de morte e a morbidade neurológica, comparado com os RN que receberam midazolam, o qual possui propriedades sedativas, mas é destituído de efeito analgésico. Os autores acreditam que o efeito benéfico observado naqueles que receberam morfina se deva à diminuição do estresse, estabilidade da pressão e melhora da oxigenação.

Sabe-se que o sistema nervoso do RN é estrutural e funcionalmente imaturo, e que mudanças significativas nos mecanismos de analgesia opióide ocorrem antes e após o nascimento [21-23]. Estudos prévios revelam que a exposição a fármacos durante o período neonatal pode desencadear conseqüências no desenvolvimento do SNC, tais como alterações permanentes na resposta farmacológica e na sinalização celular [24-26].

Vários estudos relatam a eficácia analgésica da morfina em modelo animal neonato**.** Embora os mecanismos inibitórios não estejam completamente formados antes da 3ª semana de vida [27], a morfina tem seu efeito analgésico devido à presença dos receptores opióides desde a vida intra-uterina [28]. Entretanto, estudos têm demonstrado que a exposição à analgesia opióide nesta idade pode levar a alterações em nível de respostas comportamental, como síndrome de abstinência e ansiedade, e respostas nociceptivas alteradas em longo prazo [25-26, 29-31].

Sistema Purinérgico

O conceito de neurotransmissão purinérgica foi introduzido em 1972 [32], porém a relevância da adenosina 5'-trifosfato (ATP) extracelular como uma importante molécula sinalizadora, além de seu reconhecido papel no metabolismo energético celular, levou algum tempo para ser aceita [33]. Atualmente, a sinalização purinérgica tem sido amplamente estudada e várias funções dos nucleotídeos extracelulares foram estabelecidas, tais como neurotransmissão, contração do músculo liso, resposta imunológica, inflamação, agregação plaquetária e dor [34].

Sabe-se que o ATP é co-liberado em vias simpáticas e parassimpáticas juntamente com diversos outros neurotransmissores, tais como: acetilcolina, glutamato, norepinefrina, serotonina, ácido γ-amino butírico (GABA), neuropeptídeo Y e óxido nítrico [35-36]. O ATP pode atuar como

neurotransmissor tanto nos neurônios do sistema nervoso central como no sistema nervoso periférico [37]. Além da liberação neuronal como um transmissor ou um co-transmissor, há várias outras fontes de ATP extracelular, incluindo a liberação por células danificadas ou em processo de morte [38], ou em resposta à deformação, à hipóxia ou a substâncias que não causam danos celulares, como acetilcolina, ATP e trombina [39].

O ATP, juntamente com a adenosina 5'-difosfato (ADP), a adenosina 5'-monofosfato (AMP) e a adenosina, produtos da sua hidrólise, são importantes moléculas sinalizadoras responsáveis por promover múltiplos efeitos biológicos, tais como: neuromodulação, neurotransmissão, e proliferação e crescimento celular [39].

Os nucleotídeos extracelulares, como o ATP e ADP, e o nucleosídeo adenosina exercem seus efeitos biológicos através dos purinoreceptores, que compreendem os receptores do tipo P2, subdivididos em P2X para ATP (P2X1-7, ionotrópicos permeáveis ao Na^{+}, K^{+} e Ca^{+2}) e P2Y para nucleosídeos tri e difosfatos, como o ATP e o ADP ($P2Y_{1,\ 2,\ 4,\ 6,\ 11\text{-}14}$, acoplados a proteína-G), e os receptores do tipo P1 para adenosina (A_1, A_{2A}, A_{2B} e A_3, acoplados à proteína-G) [40] (Figura 1). Os receptores purinérgicos são amplamente distribuídos no organismo e estão presentes em diversos órgãos e tecidos, tais como: encéfalo, medula espinhal, coração, pulmão, musculatura lisa, terminais nervosos autônomos, entre outros. Além disso, diversos tipos de células expressam estes receptores, incluindo astrócitos, plaquetas, células da microglia, imunes, epiteliais e endoteliais [39].

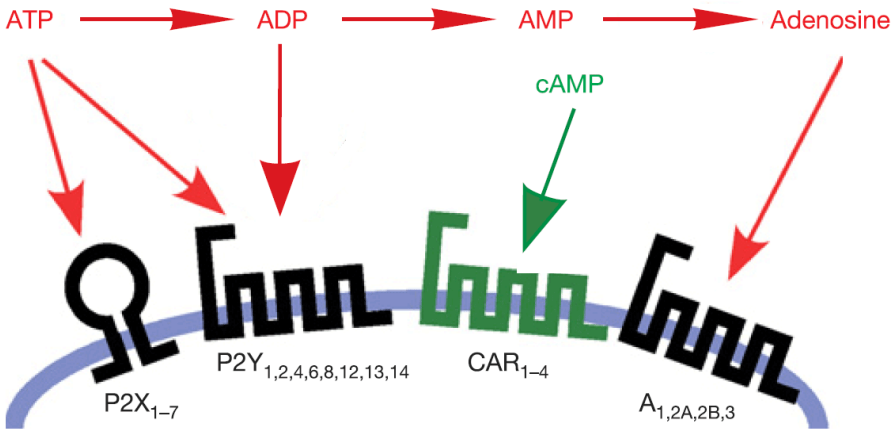

Figura 1. Receptores de nucleotídeos (receptores P2) e de adenosina (receptores P1). Adaptado de Khakh e North, 2006 [41].

Os efeitos dos nucleotídeos extracelulares na nocicepção são complexos e dependem do subtipo de receptor ativado. Sabe-se que o ATP é um neurotransmissor liberado pelos neurônios aferentes primários (NAP) na medula espinhal para atuar na sinalização central da dor [33, 42], estando envolvido em mecanismos centrais e periféricos da nocicepção. Os receptores purinérgicos P2X localizam-se em tecidos periféricos [43] e em neurônios sensoriais de pequeno diâmetro do gânglio da raiz dorsal e em terminais pré-sinápticos de NAP no corno dorsal da medula espinhal. Testes eletrofisiológicos mostram que o ATP aplicado perifericamente causa um marcado aumento na descarga dos neurônios sensoriais [42, 44] e que a ativação de receptores P2X pelo ATP provoca a liberação espontânea de glutamato. A ação nociceptiva do ATP ocorre principalmente por meio da ligação aos receptores purinérgicos A_2, P2X3 e P2X2/3, os quais estão amplamente expressos em NAP [42]. Os receptores de adenosina de subtipo A_1 estão associados a efeito modulatório na transmissão da dor em medula espinhal [45]. Agonistas A_1 parecem atuar pré-sinapticamente, inibindo a liberação de neurotransmissores, ou pós-sinapticamente, reduzindo a excitabilidade neuronal [46-48].

Estudo prévio do grupo de pesquisa demonstrou que a administração periférica de agonista de receptor A_1 e de bloqueador de transporte de adenosina (dipiridamol) desencadeia antinocicepção, enquanto que antagonista de receptor A_1 reverte este efeito [49]. Por outro lado, a atuação de adenosina em receptores A_2 aumenta a liberação de neurotransmissores excitatórios, aumentando a excitabilidade celular e promovendo um efeito pró-nociceptivo [50]. Associado a isto, um estudo relata que a administração de morfina produz liberação de adenosina e em sinaptossomas de medula espinhal envolvendo transportadores de nucleosídeo sensíveis a dipiridamol, mas insensíveis a nitrobenziltioinisina [51]. O processo de transporte do nucleosídeo (adenosina) pode ter papel na regulação dos seus níveis endógenos no SNC. A inibição de transportadores de nucleosídeo pode aumentar significativamente a concentração de adenosina extracelular, provavelmente devido à inibição da captação de adenosina resultante da hidrólise de nucleotídeos. A inibição da captação pode aumentar os níveis de adenosina na fenda sináptica e subseqüentemente aumentar a ativação de receptores extracelulares de adenosina, o que pode levar à resposta antinociceptiva via receptor A_1 [52].

Enzimas NTPDases e 5'-nucleotidase

O ATP extracelular, bem como os demais nucleotídeos de adenina e pirimidina, pode ser rapidamente hidrolisado pela ação das ecto-nucleotidases presentes na superfície celular, solúveis no meio intersticial ou nos fluidos biológicos [53]. As nucleotidases desempenham uma função essencial na sinalização purinérgica, controlando a disponibilidade e os níveis extracelulares de ATP, ADP, AMP e adenosina [54] e, consequentemente, regulando as respostas mediadas pelos purinoreceptores [55]. Assim, a defosforilação completa do ATP ocorre pela ação conjunta das enzimas denominadas "ecto-nucleotidases", que incluem as ecto-nucleosídeo-trifosfato-difosfohidrolases (NTPDases), as ecto-nucleotídeo pirofosfatase/fosfodiesterase (E-NPPs), as fosfatases alcalinas, e a 5'-nucleotidase [53].

Desta forma, a primeira etapa de degradação dos nucleotídeos (ATP e ADP) pode ser catalisada por enzimas da família das NTPDases (Figura 2). Em mamíferos, foram identificados 8 membros desta família: NTPDase1-8, as quais catalisam a primeira etapa de degradação do ATP extracelular até AMP. As NTPDases1, 2, 3 e 8 são proteínas transmembrana, localizadas na superfície da membrana plasmática celular, com o sítio catalítico voltado para o meio extracelular. A NTPDase1, enzima que hidrolisa igualmente bem o ATP e ADP com uma razão de aproximadamente 1:1, tem sido o membro mais estudado da família das NTPDases. Já a NTPDase2 apresenta clara preferência pelos nucleotídeos trifosfatados em proporção de 30:1, por esta razão também é chamada de ATPase [53]. Esta característica pode ser importante em situações patológicas e injúrias onde as células são expostas a elevadas concentrações de ATP extracelular [39]. As NTPDases3 e 8 preferem o ATP ao ADP, em proporções de 3:1 e 2:1, respectivamente. As NTPDases4, 5, 6 e 7 estão localizadas intracelularmente, ancoradas nas membranas de organelas intracelulares, com o sítio catalítico voltado para o lúmen de compartimentos [56], tais como aparelho de Golgi [57], vacúolos lisossomais ou retículo endoplasmático [58]. Sua atividade catalítica máxima requer a presença dos cátions divalentes Ca^{2+} e Mg^{2+}, sendo inativas na ausência destes íons [59]. Além disso, as NTPDase5 e 6 podem sofrer clivagem proteolítica e serem secretadas em uma forma solúvel [60].

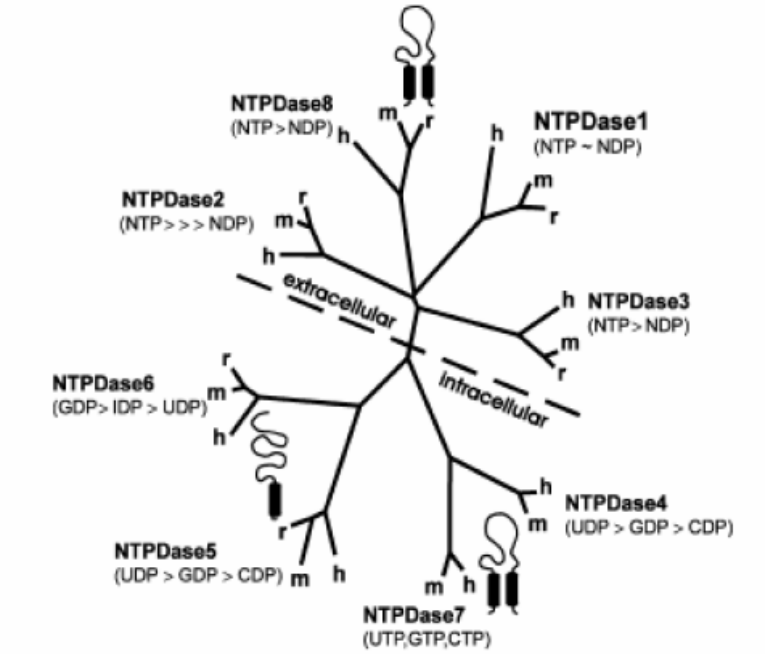

Figura 2. Árvore filogenética hipotética derivada dos 22 membros selecionados da família das E-NTPDases (1-8) de rato (*r*), humano (*h*) e camundongo (*m*), seguindo o alinhamento da sequência de aminoácidos. A linha tracejada separa os tipos de E-NTPDases que apresentam sítio catalítico voltado para o meio extra ou intracelular. Adicionalmente, representa-se a preferência aos substratos de cada enzima e a topografia de membrana para cada grupo de enzimas (um ou dois domínios transmembrana, indicados com barris). Adaptado de Robson *et al.*, 2006 [56].

O AMP resultante da hidrolise do ATP e do ADP pela ação das NTPDases é subsequentemente hidrolisado pela ação da 5'-nucleotidase até adenosina. A 5'-nucleotidase é classificada em quatro grupos de acordo com sua localização celular e propriedades bioquímicas: uma ecto-5'-nucleotidase ancorada à membrana plasmática, uma forma solúvel, e duas formas citoplasmáticas [61]. A ecto-5'-nucleotidase está ligada à membrana plasmática por uma âncora de glicosilfosfatidilinositol (GPI) [62], ligação que pode ser clivada por fosfolipase C, resultando em sua forma solúvel (Figura 5) [63-64].

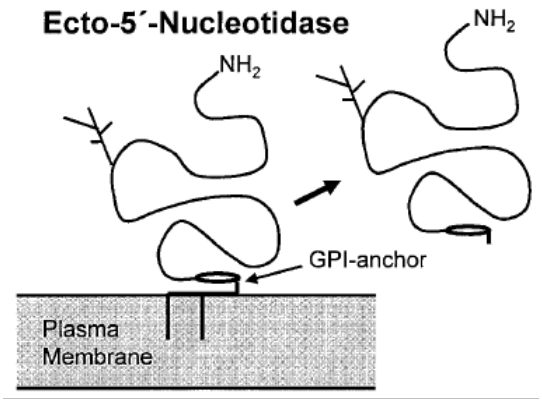

Figura 3. Estrutura da enzima (ecto)-5'-nucleotidase. A enzima pode estar ancorada a membrana plasmática ou ser liberada através de clivagem proteolítica. Adaptado de Zimmermann, 2000 [53].

Inicialmente, pensava-se que a metabolização do ATP fosse mediada somente pelas nucleotidases ligadas à membrana plasmática (ecto). No entanto, foi demonstrado que nucleotidases solúveis, provavelmente liberadas por terminações simpáticas, também estão envolvidas [65]. As nucleotidases solúveis juntamente com ecto-nucleotidases nas plaquetas e na membrana plasmática de células endoteliais são responsáveis pela manutenção de nucleotídeos de adenina e adenosina dentro dos níveis fisiológicos. Portanto, a cascata das nucleotidases é uma via enzimática com dupla função de remover o sinal (ATP) e gerar um segundo sinalizador (adenosina).

Levando em consideração a íntima relação entre os sistemas purinérgico e opióide, este trabalho de conclusão de curso teve por objetivo verificar se o tratamento repetido com morfina durante o período neonatal altera as atividades das nucleotidases em curto, médio e longo prazo em soro de ratos.

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MORPHINE TREATMENT IN EARLY LIFE ALTERS NTPDASE ACTIVITY IN RAT BLOOD SERUM

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Running title: Neonatal morphine modulates NTPDase activity

Abstract

We have shown that morphine exposure in early life promotes alterations in E-NTPDase activity and gene expression pattern in central nervous structures of rats. The E-NTPDases hydrolyze ATP and ADP, while 5′-nucleotidase hydrolyzes AMP to adenosine. These enzymes are the major regulators of purinergic signaling in the blood. It has been shown that ATP stimulates a nociceptive response, although the adenosine mediates a component of morphine analgesia. The aim of this study was to evaluate whether morphine exposure in early life, from postnatal day 8 until postnatal day 14, alters NTPDases and 5´-nucleotidase activities in the short, medium and long term in blood serum of rats. At P16, we did not observe any difference in nucleotides hydrolysis. However, at P30 the morphine group exhibited an increase in ATP hydrolysis and at P60 a decrease in ADP hydrolysis in blood serum. It is probable that the two different NTPDases are carrying out the same function, one hydrolyzing preferentially ATP and the other hydrolyzing ADP slowly. The nucleotide hydrolysis profile may lead to an increase in the ADP availability at the peripheral level. Our findings highlight the importance of NTPDases in regulating nucleotide levels in rats exposed to morphine.

Keywords: NTPDase; 5'-nucleotidase; morphine; neonate; rat blood serum

1. INTRODUCTION

The recognition of the need to adequately assess and treat pain in children and infants has led to increasing use of opioids in these patients. Opioids such as morphine remain the most common treatment for severe acute and chronic pain, and are often used for sedation in the intensive care setting (1-2). Currently, human infants and children are routinely treated with opioids for pain relief and for the purposes of sedation to permit mechanical ventilation (3-4). Unfortunately, more than 48% of infants and children administered therapeutic doses of intravenous opioids in the intensive care unit develop symptoms of opiate withdrawal (5-9). Previous studies by our group showed that exposure to drugs in early life can have implications in the developing nervous system, such as long-lasting altered behavior after anesthesia exposure (10). Moreover, other studies showed permanent alterations in pharmacological responses and cell signaling (11). Likewise, we have shown that morphine exposure in early life promotes a hyperalgesic response to noxious events in adult life (12). Additionally, another study has shown that long-term administration of opioids can alter the central pain-related systems and results in opioid addiction (13). In particular, studies in rats have shown that the chronic use of morphine can promote changes in adenosine-mediated signaling pathways in several brain structures related to the etiology of addiction (14) and to pain transmission (15).

In other work our laboratory has shown that early morphine exposure alters E-NTPDase activity and gene expression patterns in spinal cord and cerebral cortex of rats (16). The enzymes of the ecto-nucleoside triphosphate diphosphohydrolase family (NTPDases) and ecto-5'-nucleotidase are responsible for inactivation of extracellular adenine nucleotides (17). The NTPDases hydrolyze ATP and ADP, while ecto-5′-nucleotidase hydrolyzes AMP to adenosine (18). These enzymes are

ubiquitously coexpressed in endothelial and hematopoietic cells and are considered to be the major regulators of purinergic signaling in the blood (19-20). Previous studies suggested the presence of soluble NTPDases in rat blood serum (21) as well as in human blood (22). Furthermore, the presence of NTPDases associated with circulating plasma microparticles was observed (23).

The relationship between the purinergic and nociceptive systems was reviewed by Donnelly-Roberts and colleagues (2007) (24). It has been shown that ATP stimulates cellular excitability, promotes the release of excitatory amino acids, initiates a nociceptive response and can induce apoptosis (25-26). By other hand, the nucleoside adenosine decreases nociception, inflammation and cellular excitability (27), and is a component of spinal analgesia after morphine and serotonin injection (28). It is known that morphine administration promotes adenosine release in the spinal cord and brain, providing evidence to support the idea that adenosine is involved in opioid-induced analgesia (15), possibly acting through the adenosine A_1 receptor (29-30). Moreover, intradermal co-injections of μ opioid and A_1 receptor agonists with the inflammatory mediator PGE2 show a bi-directional cross-tolerance to peripheral antinociception, suggesting a common cellular role for the μ opioid and A_1 receptors on primary afferent nociceptors (31). Likewise, cross-tolerance and cross-withdrawal studies have proposed that a μ opioid, α2 adrenergic, A_1 receptor complex mediates antinociception in the periphery (32). These considerations involving the activities of ecto-nucleotidases and pain modulation are target of interest, since plasma and cerebrospinal fluid levels of adenosine are reduced by half in subjects with neuropathic pain (33).

Considering the close relationship between opioid and purinergic systems, the aim of this study was to investigate whether morphine exposure in early life alters

NTPDases and 5´-nucleotidase activities in the short, medium and long term in blood serum of rats.

2. MATERIALS AND METHODS

2.1. Animals

Eight-day-old male Wistar rats were divided into two groups: saline-control (C) and morphine-treated (M). Naive animals were placed in home cages made of Plexiglas (65 cm x 25 cm x 15 cm) with sawdust covering the floor. Animals were maintained on a standard 12-h dark/light cycle (light on between 7.00 h and 19.00 h) at room temperature (22 $\pm$ 2°C). The animals had free access to food and water. At birth, the litters were standardized to contain up to 8 pups per dam, and the pups remained with their mothers until 21 days of age. Rats at postnatal day 8 (P8) were chosen because it exhibit a neurological development similar to a human newborn (34). It is also accepted that they are in a physiologically immature state (35) since this period is characterized by major developmental changes in the brain and plasticity of the developing pain system (36-38). Animal handling and all experiments were performed in accordance with international guidelines of animal care. The protocol of the present study was approved by the Ethics Committee of the institution where the work was conducted.

2.2. Reagents

Nucleotides (ATP, ADP and AMP), Trizma base and Coomassie Brilliant Blue G were purchased from Sigma Chemical Co. (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 treatment

Each animal received saline (Control group) or morphine (Morphine group; total dose of 5 μg s.c. in the mid-scapular area) once a day for 7 days. This dose had been chosen based on previous studies by Rozisky and colleagues (12, 16, 39), and it produced analgesia in all animals submitted to the tail-flick test. All treatments were administered at the same time each day (11:00 h). One milliliter of morphine sulfate was diluted in 9 ml of 0.9% NaCl (saline). At the end of each stage of the experiment rats were decapitated and troncular blood was collected. The samples were centrifuged in plastic tubes for 5 min at 5000 x *g* at room temperature (40). Serum was obtained and stored at –20°C until the analyses were performed. The enzyme assays were carried out on blood serum at P16 (Control: n=5; Morphine: n=4), P30 (Control: n=11; Morphine: n=11), and P60 (Control: n=7; Morphine: n=7) (Fig. 1).

Insert Fig. 1 about here

2.4. Enzymatic assay

ATP and ADP hydrolysis were determined using a modification of the method described by Oses and colleagues (2004) (21). The reaction mixture containing 0.5 to 1.0 mg serum protein in 112.5 mM Tris–HCl, pH 8.0 was preincubated for 10 min to equilibrate the mixture. The reaction was started by the addition of ATP or ADP (final concentration of 3.0 mM). The mixture was incubated at 37°C in a final volume of 200 µL, for 40 min. The reaction was stopped by the addition of 200 µL 10% trichloroacetic acid (TCA). All samples were centrifuged at 5000 x *g* for 5 min to eliminate precipitated protein and the supernatant was used for the colorimetric assay. The inorganic phosphate (Pi) released was measured by the Malachite green method (41). AMP hydrolysis was quantified essentially as described above for ATP and ADP hydrolysis. The reaction mixture, containing 3.0 mM AMP as substrate in 100 mM Tris–HCl, pH 7.5, was incubated with 0.5 to 1.0 mg serum protein at 37°C in a final volume of 200 µL. All other procedures were the same as described above for ATP and ADP hydrolysis.

The incubation times, substrate and protein concentrations were chosen in order to ensure the linearity of the reactions. All samples were run in triplicate. In order to correct for non-enzymatic hydrolysis, we performed controls by adding the serum after the reaction was stopped with TCA. The protein concentration was measured by the Coomassie Blue method using bovine serum albumin as standard (42). Enzyme activities were expressed as nmol of inorganic phosphate released *per* minute *per* milligram of protein (nmol Pi/min/mg protein).

2.5. Statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using Student's *t* test. Differences between groups were considered significant at $P < 0.05$.

3. RESULTS

After the daily morphine exposure, from postnatal day 8 to 14, the NTPDase and 5´-nucleotidase activities in blood serum were compared between the saline-control and morphine-treated groups at P16, P30 and P60. At P16 the groups did not show any differences in nucleotide hydrolysis (ATP: Control = 1.63 $\pm$ 0.27 nmol $Pi.min^{-1}.mg^{-1}$ of protein, Morphine = 1.41 $\pm$ 0.39 nmol $Pi.min^{-1}.mg^{-1}$ of protein; ADP: Control = 1.21 $\pm$ 0.15 nmol $Pi.min^{-1}.mg^{-1}$ of protein, Morphine = 1.21 $\pm$ 0.37 nmol $Pi.min^{-1}.mg^{-1}$ of protein; AMP: Control = 1.32 $\pm$ 0.22 nmol $Pi.min^{-1}.mg^{-1}$ of protein, Morphine = 1.04 $\pm$ 0.1 nmol $Pi.min^{-1}.mg^{-1}$ of protein, Student's *t* test, $P > 0.05$; Fig. 2A).

By contrast, at P30 the morphine group demonstrated a significant increase in ATP hydrolysis when compared to the control group (Control = 2.18 $\pm$ 0.21 nmol $Pi.min^{-1}.mg^{-1}$ of protein, Morphine = 2.6 $\pm$ 0.49 nmol $Pi.min^{-1}.mg^{-1}$ of protein; Student's *t* test, $P < 0.05$; Fig. 2B), although there was no difference in the hydrolysis of other nucleotides (ADP: Control = 3.22 $\pm$ 0.3 nmol $Pi.min^{-1}.mg^{-1}$ of protein, Morphine = 3.28 $\pm$ 0.41 nmol $Pi.min^{-1}.mg^{-1}$ of protein; AMP: Control = 1.78 $\pm$ 0.39 nmol $Pi.min^{-1}.mg^{-1}$ of protein, Morphine = 2.08 $\pm$ 0.28 nmol $Pi.min^{-1}.mg^{-1}$ of protein; Student's *t* test, $P > 0.05$; Fig. 2B).

At P60, the morphine group demonstrated a significant decrease in ADP hydrolysis when compared to the control group (Control = 2.56 $\pm$ 0.51 nmol $Pi.min^{-1}.mg^{-1}$ of protein, Morphine = 1.74 $\pm$ 0.21 nmol $Pi.min^{-1}.mg^{-1}$ of protein; Student's *t* test, $P < 0.05$; Fig. 2C), while there was no difference in the hydrolysis of other nucleotides (ATP:

Control = 2.39 ± 0.46 nmol $Pi.min^{-1}.mg^{-1}$ of protein, Morphine = 2.28 ± 0.39 nmol $Pi.min^{-1}.mg^{-1}$ of protein; AMP: Control = 1.83 ± 0.35 nmol $Pi.min^{-1}.mg^{-1}$ of protein, Morphine = 1.38 ± 0.16 nmol $Pi.min^{-1}.mg^{-1}$ of protein; Student's *t* test, $P > 0.05$; Fig. 2C).

Insert Fig. 2 about here

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4. DISCUSSION

In this study, after the repeated, daily exposure to morphine beginning on P8 and continuing until P14, we observed an increase in ATP hydrolysis at P30, and a decrease in ADP hydrolysis at P60 in blood serum of rats.

It is well known that the neonatal nervous system is structurally and functionally immature and significant changes in nociceptive pathways and opioid analgesic mechanisms occur before and after birth (43-45). Exposure to analgesic opioids during early life can have short- and long-lasting consequences in the development and function of some neurotransmitter systems in the central nervous system (CNS), such as glutamatergic and dopaminergic systems (12, 46-47). In the developing nervous system, these effects are different from those on the mature system (11). In a previous study published by our group, using a measure of the pain threshold at the spinal level, we observed that animals in the second week of life showed an increased analgesic response to repeated morphine administration without developing tolerance. However, at P80 rats showed a greater morphine

analgesia and a classic tolerance effect. In addition, the animals that received morphine from P8 until P14 displayed a longer duration of morphine analgesia at P80 (39). These results indicate that early morphine exposure promotes alterations in the opioid analgesic response that may be expressed into adulthood. In addition, we found that rats which received the same morphine treatment showed an increase in nociceptive behavior in response to formalin test at P30 and at P60. This response was partially reversed by a non-steroidal anti-inflammatory drug (indomethacin) and completely reversed by a NMDA receptor antagonist (ketamine), suggesting that this early exposure can lead to neuroplastic changes in the nociceptive circuits, such as the glutamatergic system (12).

Recently, our group showed that treatment with morphine in early life alters the hydrolysis of nucleotides in CNS structures (16). We showed that the synaptosomes from P16 animals exhibited a decrease in ADP hydrolysis and an increase in ATP hydrolysis. The expression of E-NTPDase1 mRNA transcripts was increased in spinal cord and decreased in cerebral cortex after the treatment (16).

The difference in activities observed after morphine withdrawal in this study are possibly due to different types of NTPDases present in blood serum, since our results showed a change in the ATPase and ADPase activities at P30 and at P60, respectively. NTPDase1, also known as apyrase, is notable for its high preference for nucleoside triphosphates and nucleoside diphosphates. This enzyme presents a wide distribution on the surface of all the cell types of the CNS (48-50) and also is expressed in the endothelium, endocardium, and to a lesser extent by vascular smooth muscle (51-52). NTPDase1 together with NTPDase2 represents the dominant ecto-nucleotidases expressed by vascular endothelial cells and accessory vascular cells, such as the adventitia of vessels and microvascular pericytes (17).

NTPDase2 stands out for its high preference for nucleoside triphosphates over nucleoside diphosphates, and thus not only inactivates ligands for nucleoside triphosphate-sensitive receptors but also generates ligands for nucleoside diphosphate-sensitive receptors (53). When NTPDase1 is active, extracellular ATP is converted to AMP and then to adenosine by 5'-nucleotidase, and ADP is not an appreciable product. However, when NTPDase1 is inhibited, as is the case at P60, ATP is converted to ADP by other ATPases, and ADP will be relatively stable. In this case, the AMP which is the substrate for 5'-nucleotidase may be reduced and ADP may accumulate in the bloodstream due to decreased ADPase activity. However, no change was observed in the 5'-nucleotidase activity making it difficult to infer whether this outcome, which was measured in vitro, will or will not result in decreased extracellular adenosine in vivo.

Extracellular nucleotides (ATP and ADP) have been shown to act as signaling molecules in the central and peripheral nervous systems by binding to two types of P2 receptors: P2X (ligand-gated cationic channels) and P2Y (G-protein-coupled receptors) (54-55). The increased enzymatic activity at P30 observed in this study, after the repeated morphine exposure, may be responsible for modulating the magnitude of the ATP signal in the periphery. A previous study showed that a single subplantar injection into the hindpaw with α,β-methylene ATP (selective P2X receptor agonist) activates P2X receptors present on peripheral sensory neurons, and results in nociception in animals (56). It is well known that ATP facilitates nociceptive transmission through binding to P2X3 receptors; however, these receptors are co-localized with the P2Y inhibitory receptors involved in nociceptive transmission (57). ADP is a potent agonist of P2Y receptors (53,58), which are widely expressed in peripheral sensory neurons, and are capable of inhibiting nociceptive signaling in

isolated neurons and reducing hyperalgesia in vivo (59). Anti-nociceptive actions resulting from activation of these receptors appear to be antagonized by the Gq-coupled ADP receptor, $P2Y_1$, which is required for the full expression of inflammatory hyperalgesia (59). Activation of Gi-coupled receptors in sensory neurons is often associated with inhibition of N-type Ca^{+2} channels and attenuation of neurotransmitter release, which is the principle mechanism for the inhibition of peripheral nociceptive signaling by μ opioid receptor agonists (60-61). Expression of Gi-coupled nucleotide receptors is regulated in response to inflammation, indicating that changes in $P2Y_{Gi}$ expression contribute to the neuronal response to inflammatory injury. Results described by Malin and Molliver (2010) (59) indicate that ADP acts on both the Gq-coupled $P2Y_1$ and the Gi-coupled $P2Y_{12}$ and $P2Y_{13}$ receptors on sensory neurons, and this suggests that the integration of these antagonistic pathways is an important mechanism for the modulation of nociceptor sensitivity. Furthermore, the same authors provided evidence that $P2Y_1$ has pronociceptive actions in sensory neurons and participates in inflammatory sensitization, while Gi-coupled receptors, also expressed in sensory neurons, are dynamically upregulated in response to inflammation and inhibit excitatory signaling in sensory neurons, including capsaicin-responsive nociceptors (59). Although purinergic receptors were not the focus of this study, we could suggest that the increase in ATP hydrolysis at P30, which was measured in the periphery, has the function of removing the ATP signal and generating a second signal in the form of ADP.

These results also could suggest that ADP acts as a peripheral and central neuromodulator during the opioid withdrawal process due to the sustained morphine exposure in early life. Our laboratory has demonstrated that animals with early

exposure to morphine do not present tolerance to the drug (39), but they present increased nociceptive behavior in the formalin test at P30 and P60 (12). It is possible that ADP may modulate this opioid-induced hyperalgesia in these animals through P2Y receptors present in the periphery, more specifically on nociceptors. This nucleotide can modulate inflammatory and nociceptive responses by inhibiting excitatory signaling in sensory neurons (59). Thus, it is probable that these results are consequences of opioid modulation of different NTPDase activities in the CNS (16) and in blood serum. It is probable that NTPDase1 and NTPDase2 are carrying out the same function, one hydrolyzing ATP more quickly (NTPDase2 at P30), and the other hydrolyzing ADP more slowly (NTPDase1 at P60), and in both situations this may lead to an increase in the ADP availability at the peripheral level. These effects are long-lasting, since they persist for up to 45 days after the end of treatment. Accordingly, these changes in NTPDase activities may constitute one of the mechanisms that mediate the development of some of the long-lasting effects of morphine treatment in early life, such as hyperalgesia at P30 and P60 (12).

In summary, our findings highlight the importance of NTPDases in regulating the levels of nucleotides, and consequently the level of nucleotide receptor activation in rats exposed to morphine. Moreover, we propose that the hyperalgesia induced by morphine (12) might be modulated by ADP signaling in the periphery. Nevertheless, further studies are required to elucidate the exact mechanisms by which ADP acts in the periphery after the end of treatment with morphine.

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LEGENDS

Figure 1. Experimental design

Figure 2. Effect of morphine administration in early life upon NTPDases and 5´-nucleotidase in rat blood serum at P16, P30 and P60:

A) At P16 no difference was observed in nucleotide hydrolysis (Student's *t* test, $P > 0.05$).

B) At P30 the morphine group showed an increase in ATP hydrolysis in comparison to control group (Student's *t* test, $P < 0.05$).

C) At P60 the morphine group showed a decrease in ADP hydrolysis in comparison to control group (Student's *t* test, $P < 0.05$).

Values are mean $\pm$ S.E.M. Specific enzyme activities were expressed as nmol of $Pi.min^{-1}.mg^{-1}$ protein. # indicates difference between the control (white bars) and morphine-treated group (black bars).

Figure 1

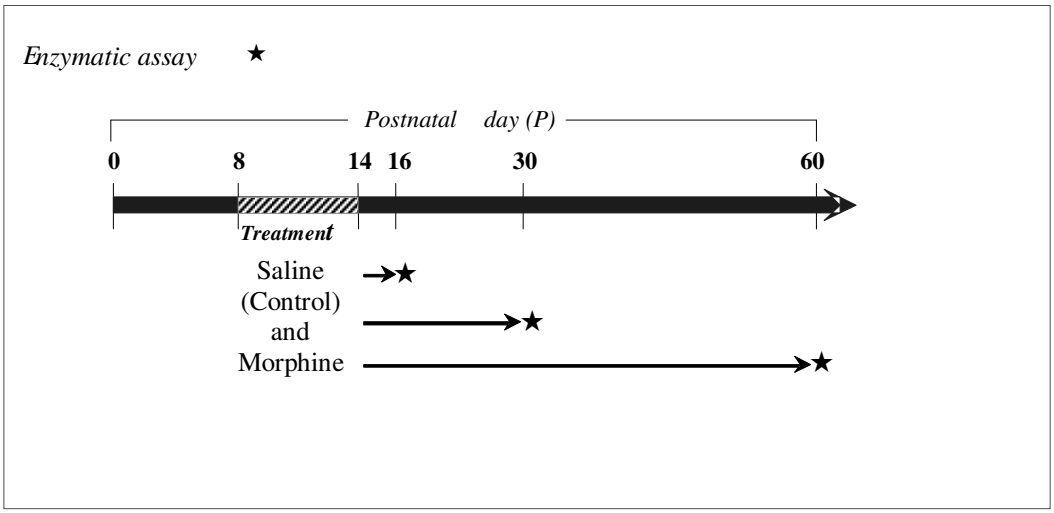
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Figure 2

A)

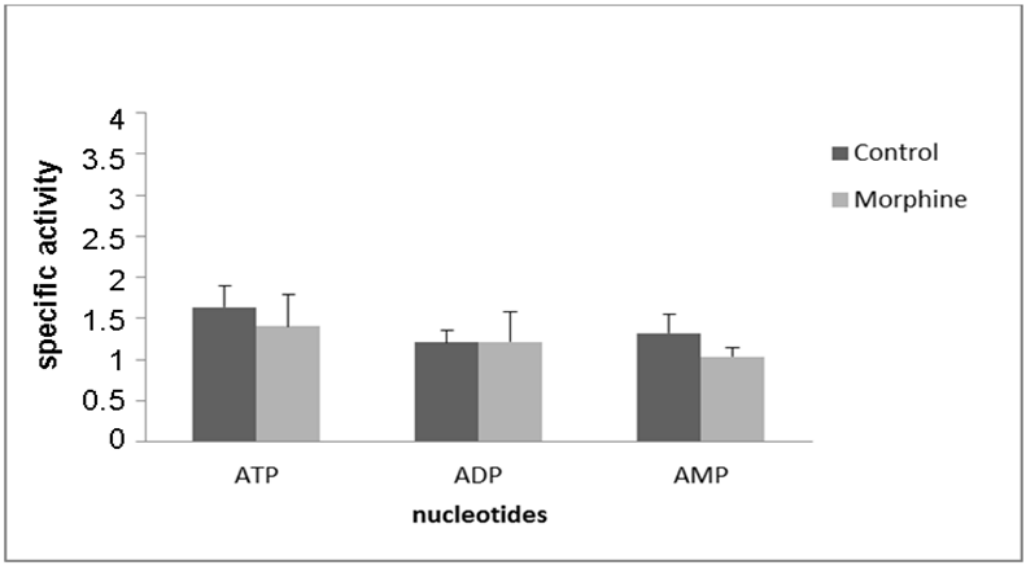
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Figure 2

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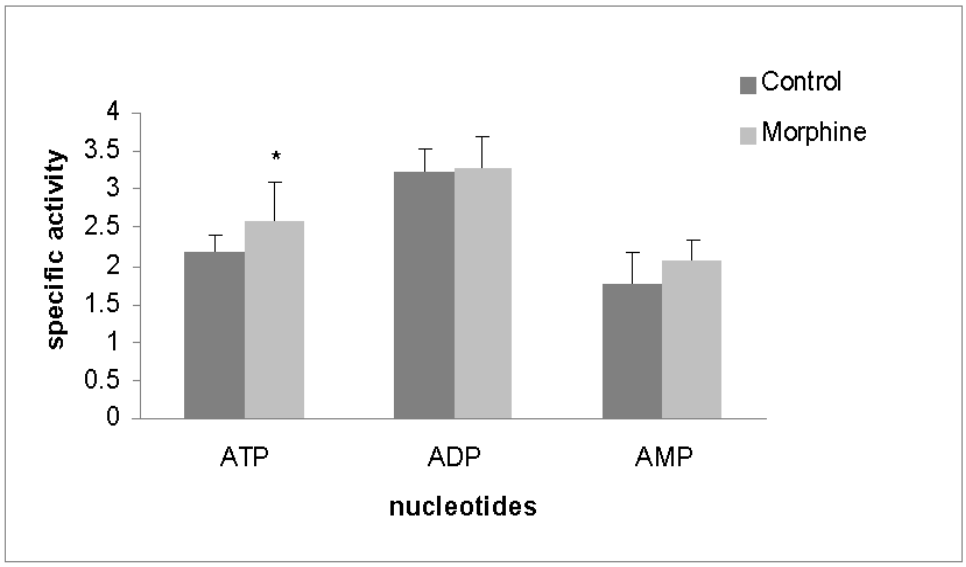
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Figure 2

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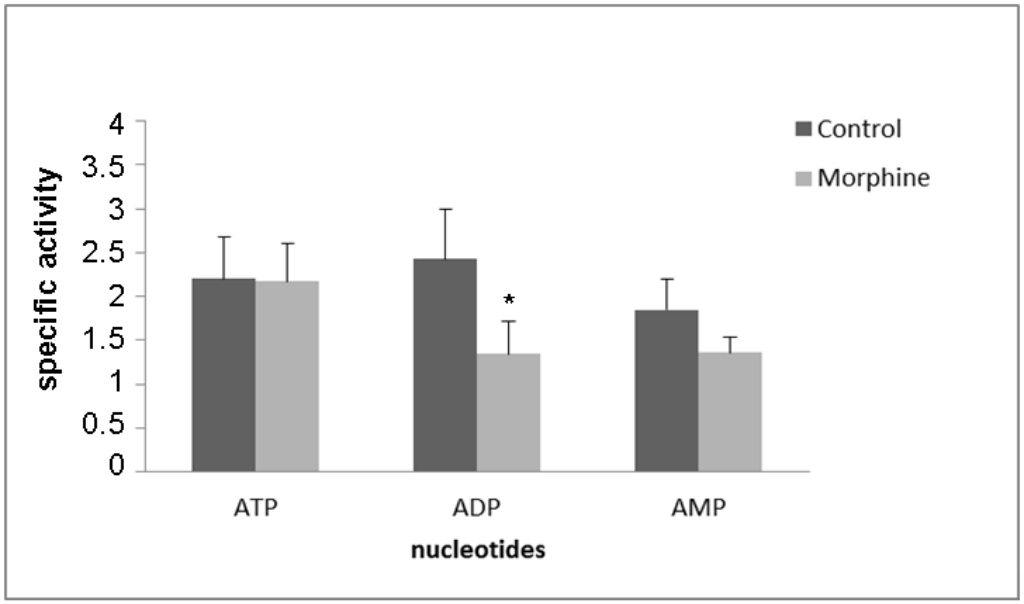
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A Comissão Científica e a Comissão de Pesquisa e Ética em Saúde, que é reconhecida pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS como Comitê de Ética em Pesquisa do HCPA e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institucional Review Board (IRB0000921) analisaram o projeto:

Projeto: 08-345

Pesquisadores:
IRACI LUCENA DA SILVA TORRES
WOLNEI CAUMO
JOANNA RIPOLL ROZISKY

Título: TRATAMENTO REPETIDO COM MORFINA DURANTE O PERÍODO NEONATAL: IMPACTO SOBRE SISTEMAS DE NEUROTRANSMISSÃO E AVALIAÇÃO DE PARÂMETROS NOCICEPTIVOS E COMPORTAMENTAIS

Este projeto foi Aprovado em seus aspectos éticos e metodológicos, de acordo com as Diretrizes e Normas Internacionais e Nacionais, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde. Toda e qualquer alteração do Projeto deverá ser comunicada ao CEP/HCPA.
Os membros do CEP/HCPA não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

Porto Alegre, 19 de agosto de 2008.

Profª Nadine Clausell
Coordenadora do GPPG e CEP-HCPA

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