UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

EFEITO DO TRATAMENTO REPETIDO COM MORFINA NO PERÍODO NEONATAL: IMPLICAÇÕES NA ONTOGÊNESE AVALIADAS POR MECANISMOS BIOQUÍMICOS E COMPORTAMENTAIS

Joanna Ripoll Rozisky

Porto Alegre, RS

Outubro de 2012

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

EFEITO DO TRATAMENTO REPETIDO COM MORFINA NO PERÍODO NEONATAL: IMPLICAÇÕES NA ONTOGÊNESE AVALIADAS POR MECANISMOS BIOQUÍMICOS E COMPORTAMENTAIS

Joanna Ripoll Rozisky

Profª. Drª. Iraci Lucena da Silva Torres

(Orientadora)

Tese apresentada ao Programa de Pós-Graduação em Medicina: Ciências Médicas da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Doutor.

Porto Alegre, RS

Outubro de 2012

CIP - Catalogação na Publicação

Ripoll Rozisky, Joanna
EFEITO DO TRATAMENTO REPETIDO COM MORFINA NO PERÍODO NEONATAL: IMPLICAÇÕES NA ONTOGÊNESE AVALIADAS POR MECANISMOS BIOQUÍMICOS E COMPORTAMENTAIS / Joanna Ripoll Rozisky. -- 2012.
251 f.

Orientadora: Iraci Lucena da Silva Torres.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Programa de Pós-Graduação em Medicina: Ciências Médicas, Porto Alegre, BR-RS, 2012.

1. morfina. 2. ratos neonatos. 3. nocicepção. 4. glutamato. 5. BDNF. I. Lucena da Silva Torres, Iraci, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

FONTES DE FINANCIAMENTO

Este trabalho foi financiado pelo Fundo de Incentivo à Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE – HCPA; projeto número 08-345), pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (I.L.S. Torres, número 307772/2008-0/2008) e pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) através da bolsa de doutorado concedida à aluna Joanna Ripoll Rozisky.

A todas as pessoas que me ajudaram

neste trabalho, em especial

a minha família.

AGRADECIMENTOS

Em primeiro lugar, a Deus por estar sempre presente em minha vida guiando-me no caminho da sabedoria.

A minha família, em especial aos meus pais, Gilson e Sandra, por abdicarem de seus sonhos para a realização dos meus e pela formação necessária para que eu continuasse meus estudos até o doutorado. E a minha irmã, Renata, pelo apoio e incentivo constantes em busca dos meus objetivos.

Ao meu noivo, Douglas, pelo amor, paciência, companheirismo, amizade e palavras de incentivo, motivando-me sempre a seguir em frente.

A minha querida orientadora, Iraci Torres, pela oportunidade, dedicação, confiança, amizade, e pelos conselhos e sugestões fundamentais para a realização deste trabalho. Agradeço por estar sempre ao meu lado em toda a minha trajetória acadêmica científica.

Ao professor Wolnei Caumo, pela co-orientação, sugestões, ensinamentos, dedicação e pela confiança depositada em mim. E principalmente pelo fato de admirá-lo como pesquisador ético e responsável.

A todos os colegas e amigos do laboratório de Farmacologia da Dor e Neuromodulação – Modelos Animais, por serem fundamentais para a realização deste trabalho, em especial aos colegas Gabriela, Lauren, Isabel, Liciane e Cléverson pela amizade, incansável ajuda, pelas risadas e pelo ótimo convívio durante todos esses anos.

Ao meu querido aluno de iniciação científica, Vinicius, pela amizade e intensa dedicação em todas as etapas deste trabalho. A "japinha" Yasmine, pelo companheirismo e valiosa contribuição em alguns dos protocolos experimentais. Aos bolsistas Paulo, Fernanda e Stefânia, pelas ajudas e boa convivência no laboratório.

A professora Carla Bonan, pelo incentivo, apoio e amizade desde o mestrado.

As professoras Carla Dalmaz e Deusa Vendite, pela oportunidade, sugestões e orientação de alguns protocolos experimentais. E aos alunos do laboratório 37 do

Departamento de Bioquímica, em especial a Cristie e a Rachel, pela parceria nos experimentos.

A professora Ana Maria Battastini, pela oportunidade, sugestões e discussões de protocolos experimentais. E aos alunos do laboratório 22 do Departamento de Bioquímica, pelos auxílios e amizade.

A professora Ionara Siqueira, pelas dicas, sugestões e disponibilidade para discussão de alguns resultados deste trabalho. E aos alunos do laboratório de Psicofarmacologia, em especial a Claudia, Karine e Gisele, pela amizade, compreensão e pelos bons momentos de descontração quando dividíamos o laboratório.

A todos os meus amigos fora do meio acadêmico, pela amizade, paciência, incentivo e compreensão de minha ausência durante esta jornada.

Aos membros da banca do Exame Geral de Qualificação, professora Ana Maria Battastini, professora Izabel Custódio, Dr. André Schmidt e Dr. Rafael Vercelino, pela disponibilidade, críticas e valiosas sugestões ao projeto apresentado.

A Vera e aos demais funcionários da secretaria do Programa de Pós-Graduação em Medicina – Ciências Médicas, pelas informações e ajuda quando solicitadas.

Aos funcionários da Unidade de Experimentação Animal do Hospital de Clínicas, Vera, Eduardo e Rosa e, em especial, a veterinária Fabíola e a chefe Marta, pela preocupação e inúmeras ajudas prestadas durante a realização de alguns experimentos. Ao funcionário do GPPG Everaldo, que sempre atendeu prontamente às solicitações de encomendas de reagentes e materiais indispensáveis para a realização deste trabalho.

Aos funcionários do CREAL, em especial a Dona Geni, pela intensa preocupação com as ninhadas solicitadas.

Aos funcionários do Departamento de Farmacologia Dona Jane, Ieda e Ângela, pelo convívio durante esses anos.

A UFRGS, pela oportunidade.

Ao FIPE do Hospital de Clínicas, pelo financiamento deste projeto, e ao GPPG, pelas revisões dos artigos científicos.

A CAPES, pela bolsa de doutorado.

A PROPESQ e a FAPERGS, pelas bolsas concedidas aos alunos de iniciação científica que participaram deste trabalho.

“Bom mesmo é ir a luta com determinação, abraçar a vida e viver com paixão, perder com classe e vencer com ousadia, pois o triunfo pertence a quem se atreve e a vida é muito bela para ser insignificante.”

Charlie Chaplin

É do buscar e não do achar que nasce o que eu não conhecia.

Clarice Lispector

SUMÁRIO

PARTE II

PARTE III

RESUMO

A dor pediátrica tem sido o foco de estudo de muitos pesquisadores nas últimas décadas devido à constatação de que neonatos apresentam menor limiar para estímulos nocivos e inócuos em comparação aos adultos. Em decorrência disto, o uso de analgésicos tem sido frequente em ambiente hospitalar. A morfina é um dos analgésicos opióides mais utilizados para sedação e analgesia nestes pacientes. Este opióide apresenta maior potência analgésica no período neonatal, sendo o receptor μ mais expresso em neurônios medulares com pico de densidade em P7 e diminuindo até o P21, atingindo então níveis de adulto. Nosso grupo mostrou que ratos neonatos submetidos ao tratamento repetido com morfina não apresentam tolerância. Porém, permanecem maior tempo em analgesia ao final do tratamento do que no 1º dia. Além disto, após dois dias do término do tratamento os animais apresentaram alterações na atividade e expressão gênica da NTPDase 1 (enzima que hidrolisa ATP até adenosina) em medula espinal e córtex cerebral, sugerindo modulação nos níveis extracelulares de nucleotídeos, o que pode levar alterações na resposta nociceptiva. Além do sistema purinérgico outro importante sistema no processamento da resposta nociceptiva é o glutamatérgico. Desde o período neonatal o glutamato é o responsável pelos estímulos nociceptivos em medula espinal, e transportadores no terminal pré-sináptico ou glia são responsáveis pela captação do glutamato liberado, controlando seus níveis. A exposição à morfina também leva a mudanças comportamentais, conhecidas por sensibilização comportamental, que são dependentes, em parte, da ativação do receptor dopaminérgico D2 no sistema límbico. Além disso, esta exposição pode alterar os níveis de BDNF em estruturas relacionadas à nocicepção. Levando em consideração a carência de estudos focados nos efeitos da exposição repetida a opióides em neonatos, o objetivo deste estudo foi verificar os efeitos a curto (P16), médio (P30) e longo prazo (P60) do tratamento com 5 μg de morfina, uma vez ao dia, do P8 ao P14, sobre comportamento nociceptivo; captação de glutamato em medula espinal; comportamentos exploratórios e do tipo ansioso, avaliando o envolvimento do receptor D2; níveis de BDNF e TNF-α, e estresse oxidativo em hipocampo; atividades de nucleotidases solúveis em soro; e além disso avaliar a ação antinociceptiva da melatonina nas respostas nociceptivas alteradas. Os animais que receberam morfina demonstraram em P30 e P60: aumento da resposta nociceptiva que foi revertida por antagonista do receptor NMDA; diminuição da captação de glutamato em medula espinal; aumento dos níveis de BDNF em hipocampo, e diminuição da atividade da SOD no P60; alterações nas atividades das nucleotidases solúveis; aumento do comportamento exploratório no P16 e P30; efeito antinociceptivo da melatonina na hiperalgesia. Estes dados demonstram a necessidade de pesquisas que sejam focadas nos efeitos do tratamento com morfina no período neonatal ao longo da vida, bem como buscar alternativas terapêuticas que possam reverter possíveis alterações.

Palavras-Chave: morfina; ratos neonatos; nocicepção; captação de glutamato; comportamento exploratório; BDNF; superóxido dismutase; hipocampo; melatonina.

ABSTRACT

The study of pediatric pain has been the focus of many researchers in recent decades due to the fact that neonates have a lower threshold for innocuous and noxious stimuli compared to adults. As a result, the use of analgesics has been frequent in hospitals. Morphine is an opioid analgesic commonly used for sedation in these patients. This opioid presents greater analgesic potency in the neonatal period in which the μ opioid receptor is over expressed in neurons of the spinal cord, with peak binding on day 7 and decreasing until day 21, reaching adult levels then. Our research group has shown that neonate rats subjected to repeated treatment with morphine not present tolerance. However, they remain with more time of analgesia at the end of treatment than on first day. Moreover, two days after the end of treatment the animals showed changes in activity and gene expression of NTPDase 1 (enzyme that hydrolyzes ATP to adenosine) in spinal cord and cerebral cortex, suggesting modulation of nucleotides extracellular levels which can alters nociceptive response. Besides the purinergic system another important system associated with nociceptive pathways is glutamatergic. Since the neonatal period glutamate is responsible for nociceptive stimulus in spinal cord, being its level controlled by transporters in the presynaptic terminal or glia responsible for the uptake of glutamate released. Importantly, studies have shown that morphine administration in adult animals can lead to behavioral changes, known as behavioral sensitization, dependent of activation of the limbic dopamine system, with the D2 dopamine receptor is associated with these changes. Moreover, such exposure may lead to altered levels of BDNF on the nociception related structures. Considering the lack of studies focused on the effects of repeated exposure to opioids in neonates, the objective of this study was to assess the short-(P16), medium (P30) and long term (P60) effects of treatment with 5 μg of morphine, once a day, from 8 to 14 days old, on nociceptive behavioral responses; glutamate uptake in the spinal cord; exploratory and type anxiously behavioral responses assessing the involvement of the dopamine receptor D2; levels of BDNF and TNF-α, and oxidative stress in the hippocampus; activities of serum soluble nucleotidases, and furthermore evaluate the antinociceptive action of melatonin on nociceptive responses changed. The results obtained with this study show that in P30 and P60 the animals that had received morphine: increase of the nociceptive response that was reversed by NMDA receptor antagonist, decreased glutamate uptake in spinal cord; increasing levels of BDNF in hippocampus, and decreased activity of the antioxidant enzyme SOD in P60; changes in nucleotide hydrolysis; increased exploratory behavior in P16 and P30; antinociceptive effect of melatonin on nociceptive responses increased. These data demonstrate the need for further research that are focused on the effects of morphine treatment in the neonatal period lifelong and seek alternative therapies that can reverse any changes.

Keywords: morphine; neonate rats; nociceptive response; glutamate uptake; exploratory behavior; BDNF; superoxide dismutase; hippocampus; melatonin.

LISTA DE ABREVIATURAS

ADP adenosina 5'-difosfato

ADN ácido desoxirribonucleico

AMP adenosina 5'-monofosfato

AMPc adenosina 5'-difosfato cíclico

ATP adenosina 5'-trifosfato

AMPA α-amino-3-hidróxi-5-metil-4-isoxazolepropionato

ANOVA Análise de Variância (*Analysis of Variance*, em inglês)

BDNF fator neurotrófico derivado do cérebro (*brain derived neurotrophic factor*, em inglês)

CAT catalase

COX-2 Ciclo-Oxigenase 2

EAAC-1 transportador-1 de aminoácidos excitatórios

E-NTPDASE Ecto-nucleosídeo trifosfato difosfohidrolase

ERO espécies reativas de oxigênio

ERN espécies reativas de nitrogênio

GABA ácido Gama-amino-butírico

GLAST transportador de aspartato e glutamato

GLT-1 transportador-1 de glutamato

GPx glutationa peroxidase

GTs transportadores de glutamato

LDH lactato desidrogenase

NAc Núcleo Accumbens

NADPH nicotinamida adenina dinucleotídeo fosfato

NGF fator de crescimento neural (*neural growth factor*, em inglês)

NMDA *N*-metil – D – aspartato

nNOS Óxido nítrico sintase neuronal **(***neuronal Nitric Oxide Synthase*, em inglês**)**

NTs neurotrofinas

P0 dia do nascimento

P1 1º dia pós-natal

P7 7º dia pós-natal

P8 8º dia pós-natal

P10 10º dia pós-natal

P14 14º dia pós-natal

P16 16º dia pós-natal

P30 30º dia pós-natal

P60 60º dia pós-natal

P120 120º dia pós-natal

P180 180º dia pós-natal

RN recém-nascido

s.c. subcutânea

SEM Erro Padrão da Média (*Standard Error of Mean*, em inglês)

SN sistema nervoso

SNC sistema nervoso central

SNP sistema nervoso periférico

SOD superóxido dismutase

TNF-α fator de necrose tumoral alfa (*tumor necrosis factor alpha*, em inglês)

trK tirosina Cinases (*tirosine kinases*, em inglês)

UDP uridina 5'-difosfato

UTIN Unidade de Terapia Intensiva Neonatal

UTIs Unidades de Terapia Intensiva

LISTA DE TABELAS

LISTA DE FIGURAS

APRESENTAÇÃO

Esta Tese está estruturada em 3 partes: Parte I - Introdução, Revisão da Literatura, Objetivos e Referências Bibliográficas; Parte II - Materiais e Métodos, Resultados e Discussão na forma de artigos científicos; Parte III – Considerações Finais, aprovação do comitê de ética e a produção acadêmica durante o período de doutoramento.

O item Referências Bibliográficas refere-se somente às referências contidas nos itens Introdução, Revisão da Literatura e Considerações Finais.

Detalhes técnicos mais precisos sobre a metodologia empregada em cada um dos trabalhos apresentados podem ser encontrados nos trabalhos científicos.

PARTE I

1 INTRODUÇÃO

O estudo da dor pediátrica vem crescendo intensamente nas últimas décadas em decorrência da constatação de que neonatos não somente sentem dor como apresentam um menor limiar para estímulos nocivos e inócuos que adultos (Grunau et al., 1994; Johnston et al., 1996). Isto se deve ao fato de que a maturação das vias descendentes inibitórias ocorre somente após a completa maturação das vias excitatórias, sendo que a transmissão do estímulo nociceptivo através do corno dorsal da medula espinal ocorre desde a 16ª semana gestacional através da liberação de neurotransmissores excitatórios como, por exemplo, o glutamato (Simons e Tibboel, 2006).

Dessa forma, a dor no neonato é um fenômeno complexo e envolve diferentes sistemas biológicos do sistema nervoso em desenvolvimento (Smith et al., 2000; Fitzgerald e Beggs, 2001). Vários estudos têm documentado os efeitos deletérios de longa duração resultantes da exposição repetida a procedimentos dolorosos em Unidades de Terapia Intensiva Neonatal (UTIN). Tais procedimentos levam a respostas comportamentais subjetivas de sensibilidade à dor, como hiperalgesia (Johnston e Stevens, 1996; De Lima et al., 1999; Whitfield e Grunau, 2000; Grunau et al., 2001) e respostas de estresse, identificando alterações do eixo hipotálamo-hipófise-adrenal (Grunau et al., 2004). Aliado a estes estudos, e levando em conta que o alívio da dor é uma das missões médicas primordiais, o uso de anestésicos e analgésicos vem crescendo em pacientes nesta faixa etária (De Lima et al., 1996; Suresh e Anand, 2001, El Sayed et al., 2007).

A morfina é um dos analgésicos mais utilizados em ambiente hospitalar. Atualmente, os neonatos são rotineiramente tratados com morfina para o alívio da dor, especialmente com exposição crônica para sedação para permitir ventilação mecânica

nas UTIs pediátricas (Anand et al., 1999). Estudo de Anand e colaboradores (1999) demonstrou que os recém-nascidos que receberam morfina apresentaram menor risco de morte e menor morbidade neurológica comparado com os RN que receberam midazolam que, embora possua propriedades sedativas, é destituída 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.

Estudos em modelos animais permitiram investigar a presença dos receptores opióides desde o nascimento. Desde o 1º dia pós-natal (P1), os receptores opióides já se encontram amplamente distribuídos no Sistema nervoso central (SNC), sendo que os receptores μ e k são os que predominam neste período. O pico de densidade do receptor μ ocorre no sétimo dia pós-natal e declina gradualmente até a terceira semana quando, então, estabelece os níveis do adulto (Beland e Fitzgerald, 2001; Kar e Quirion, 1995). Dessa forma, alguns autores sugerem que a potência analgésica da morfina é maior no neonato e declina com o avançar da idade (Nandi e Fitzgerald, 2005). Previamente nosso grupo demonstrou que animais que receberam morfina no período neonatal, do P8 ao P14, apresentam maior tempo de analgesia no último dia de tratamento que no primeiro dia (Rozisky et al., 2008). Possivelmente, a variação na expressão de receptor opióide e sua sensibilidade aos agonistas explique as flutuações na susceptibilidade à analgesia e ao desenvolvimento de tolerância ao longo do desenvolvimento. Apesar destes animais não apresentarem tolerância no final do tratamento, quando submetidos a uma dose aguda na idade adulta eles apresentam maior tempo de analgesia que os demais animais. Estes dados refletem as possíveis alterações em longo prazo induzidas

pela morfina em estruturas do sistema nervoso central que modulam as respostas nociceptivas (Rozisky et al., 2008).

Sintomas de dependência também são amplamente estudados após o uso crônico com analgésicos opióides. Em animais experimentais estes fármacos são conhecidos por desenvolverem sintomas comportamentais que dependem, pelo menos em parte, de mudanças no sistema dopaminérgico (Kuribara, 1995). Um dos sintomas conhecidos é a sensibilização comportamental evidenciada pelo aumento da resposta locomotora após a administração de morfina (Vries et al., 1998). Em um estudo recente, Yang e colaboradores (2011) demonstraram que estes efeitos são modulados pelo receptor dopaminérgico D2, principalmente pelo fato de que o haloperidol (antagonista do receptor dopaminérgico D2) foi capaz de atenuar os sintomas de tolerância e dependência da morfina em animais adultos (Yang et al., 2011). Entretanto, pouco se sabe sobre comportamentos de retirada após o tratamento com morfina no período neonatal e da possível relação com este receptor dopaminérgico. Aqui neste estudo abordaremos alguns comportamentos e o envolvimento do receptor D2.

Alguns estudos também relatam alterações neuroquímicas induzidas pela exposição à morfina no hipocampo. Em um estudo pesquisadores demonstraram que a exposição à morfina no período pré-natal diminui os níveis de fator neurotrófico derivado do cérebro (BDNF) em hipocampo (Schrott et al., 2008). O BDNF é uma neurotrofina amplamente expressa em várias estruturas do SNC e apresenta elevados níveis no hipocampo, regula a sobrevivência, o crescimento e a diferenciação neuronal (Davies, 1994). Possivelmente as alterações provocadas pela morfina durante o período pré-natal tenham impacto sobre processos de plasticidade sináptica ao longo do desenvolvimento (Schrott et al., 2008)

Um dos sistemas que tem sido estudado e que é capaz de regular as ações da morfina é o sistema purinérgico. Estudos em animais têm identificado uma ligação entre os receptores opióides μ e A1 adenosinérgicos, uma vez que o receptor opióide, quando ativado, induz a liberação de adenosina endógena, contribuindo para o efeito antinociceptivo dos opióides (Sweeney et al., 1987; Cahill et al., 1995). Nosso grupo demonstrou que animais que receberam doses baixas de morfina durante o período neonatal foram capazes de alterar as atividades de hidrólise dos nucleotídeos em sinaptossoma de medula espinal (diminuição da hidrólise de ADP) e córtex cerebral (aumento da hidrólise de ATP), bem como a expressão gênica da NTPDase 1 (enzima que hidrolisa o ATP até adenosina) em ambas estruturas (aumento na medula e diminuição no córtex cerebral) (Rozisky et al., 2010). Estes resultados demonstram uma relação entre sistema purinérgico e tratamento repetido com morfina, uma vez que este sistema de controle de níveis de nucleotídeos está alterado após o tratamento, podendo estar envolvido em alterações na modulação ou na transmissão de estímulos dolorosos. As enzimas estudadas foram as E-NTPDases e a Ecto-5'-nucleotidase, as quais, além de estarem amplamente expressas no sistema nervoso central e periférico, são também expressas em células endoteliais e hematopoiéticas, e são consideradas as maiores reguladoras da sinalização purinérgica no sistema cardiovascular (Zimmermann, 1999; Robson et al., 2005).

Considerando que fármacos opióides são amplamente utilizados em UTIs neonatais e pouco se sabe a respeito de seus efeitos ao longo da vida, o objetivo geral desta tese foi investigar o papel da administração repetida de morfina em ratos neonatos sobre parâmetros nociceptivos, respostas comportamentais e alterações bioquímicas em curto, médio e longo prazo.

2. REVISÃO DA LITERATURA

2.1 Dor

A dor é considerada uma das grandes preocupações da humanidade e, sendo sintoma comum a muitos quadros clínicos, continua sendo a principal razão da procura de auxílio médico. De acordo com a Associação Internacional para Estudos da Dor (*International Association for Study of Pain* - IASP), a dor é uma experiência sensorial e emocional desagradável, relacionada com dano tecidual real ou potencial ou descrita em termos deste tipo de dano (IASP, 1979). A partir deste conceito distinguem-se dois componentes envolvidos na dor: a sensação dolorosa propriamente dita ou nocicepção, e a reatividade emocional à dor. O termo nocicepção, derivado de *noci* (dano ou lesão em latim), é utilizado para descrever a resposta neural aos estímulos nocivos, os quais podem ser exógenos (mecânicos, químicos, físicos e biológicos) ou endógenos (por exemplo: inflamação, isquemia cerebral). Além disso, compreende a recepção dos estímulos nocivos por receptores específicos (nociceptores), condução até o sistema nervoso central por meio de vias sensoriais e a integração da sensação dolorosa em níveis talâmico e cortical (Loeser et al., 2001). A reatividade emocional à dor corresponde à interpretação afetiva da dor, de caráter individual e influenciada por estados ou traços psicológicos, experiências prévias e fatores sociais, culturais e ambientais (Jones, 1993; Wall e Melzack, 1994). Sendo assim, a dor é sempre uma manifestação subjetiva. Em modelos animais fala-se de nocicepção, uma vez que a dor é avaliada indiretamente por meio de respostas comportamentais (Millan, 1999).

Os estímulos nociceptivos associados à experiência subjetiva de dor têm o objetivo de manter a integridade corporal afastando a região acometida desse ou outros estímulos. A percepção de estímulos nocivos ocorre por meio da ativação de receptores

sensoriais especializados chamados de nociceptores, e são representados por terminações nervosas periféricas livres de fibras sensoriais que conduzem impulsos elétricos até a medula espinal. Estas fibras estão amplamente distribuídas na pele e tecidos mais profundos e são axônios de neurônios aferentes primários localizados no corno dorsal da medula espinal (Loeser et al., 2001). São classificadas de acordo com o estímulo recebido em: fibras Aδ (A delta) - são mielínicas (condução rápida), ativadas por estímulos térmicos de temperatura extrema (maior que 45°C e menor que 5°C) ou por estímulos mecânicos; e fibras C - amielínicas (condução lenta), ativadas tanto por estímulos químicos, térmicos ou mecânicos de alta intensidade (Basbaum e Jessel, 2000). As projeções destas fibras entram no corno dorsal, ascendem por segmentos pelo trato pósterolateral (Trato de Liassauer) e terminam nas lâminas superficiais do corno dorsal (Cervego e Iggo, 1980). No corno dorsal da medula espinal tais fibras fazem sinapse com neurônios aferentes secundários, interneurônios e neurônios do corno ventral motor (Morgan e Mikhail, 1996; Basbaum e Jessel, 2000; Loeser et al., 2001). A lâmina I (lâmina marginal) recebe somente aferências nociceptivas, enquanto que a lâmina II (substância gelatinosa) é formada por interneurônios excitatórios e inibitórios, os quais respondem às estimulações nocivas e não nocivas (Basbaum e Jessel, 2000).

A condução da dor da medula espinal até estruturas supraespinhais ocorre através de dois tipos de vias: Neoespinotalâmica e Paleoespinotalâmica (Basbaum e Jessel, 2000; Loeser et al., 2001). A primeira tem poucas estações sinápticas e se projeta no núcleo pósterolateral do tálamo, e a partir deste até o córtex somatossensorial, onde ocorre a percepção da qualidade, localização, intensidade e duração da dor (Morgan e Mikhail, 1996). A via Paleoespinotalâmica possui várias estações sinápticas e se projeta do tálamo medial de onde partem projeções para o córtex de ambos os hemisférios

cerebrais. Algumas dessas fibras se projetam para o sistema límbico, responsável pelas respostas de luta e fuga e emocionais desagradáveis ao estímulo nociceptivo (Morgan e Mikhail, 1996; Loeser et al., 2000; Basbaum e Jessel, 2001).

2.1.1 Dor em Neonato

Neonatos são submetidos a uma série de procedimentos dolorosos tais como entubação, acesso venoso, coleta de exames por punção arterial, punção lombar, aspiração de tubo orotraqueal, ventilação mecânica, drenagem de tórax, punção de calcanhar, entre outros processamentos repetidos de dor (Barker et al., 1996; Porter et al., 1997, 1999; Johnston et al., 1997). No entanto, pouco se sabe sobre de que forma estes eventos dolorosos podem interferir no desenvolvimento destas crianças (Johnston e Stevens, 1996; Grunau, 2000).

Estudos iniciais do desenvolvimento humano difundiram a crença de que o feto e o recém-nascido não sentiam dor ou não a percebiam como adultos (McGraw 1943, Levy, 1960). Devido a este conceito pouca ou nenhuma importância foi dada à prevenção e tratamento da dor em neonatos até a década de 80. Entretanto, o desenvolvimento científico permitiu compreender melhor a neurofisiologia da dor no recém-nascido determinando que estes pacientes não somente sentem dor como também são capazes de responder aos estímulos dolorosos. Desde então, tem havido um crescimento em estudos focados no sistema neurobiológico da dor em neonatos humanos (Dixon et al., 1984; Franck, 1986; Johnston e Strada, 1986; Anand e Hickey, 1987).

A dor no neonato é um fenômeno complexo e envolve diferentes sistemas neurobiológicos em desenvolvimento (Smith et al., 2000; Fitzgerald e Beggs, 2001). O

processo nociceptivo envolve alterações neurobiológicas sequenciais induzidas pela ativação de nociceptores periféricos com consequente modulação central (Basbaum e Jessen, 2000). No entanto, se a dor no neonato for de longa duração ou recorrente, o desenvolvimento do sistema nociceptivo pode ser modificado de forma permanente, resultando em alterações a nível espinal e supraespinal (Anand, 1998; Anand e Scalzo, 2000). Vários estudos têm documentado os efeitos deletérios de longa duração resultantes da exposição repetida a procedimentos dolorosos em Unidades de Terapia Intensiva Neonatal (UTIN), como hiperalgesia (Johnston e Stevens, 1996; De Lima et al., 1999; Whitfield e Grunau, 2000; Grunau et al., 2001) e estresse (Grunau et al., 2004). Desta forma, estudos em animais têm colaborado enormemente nas investigações dos efeitos deletérios do estímulo doloroso resultante de procedimentos invasivos (Rahman et al., 1997; Ruda et al., 2000; Beland e Fitzgerald, 2001).

2.1.2 Aspectos Neurobiológicos do desenvolvimento da dor

Com o reconhecimento da importância do tratamento da dor no neonato e na infância, pesquisadores têm focado sua atenção no estudo dos mecanismos neurobiológicos do desenvolvimento das vias nociceptivas. Estudos indicam que tanto crianças quanto animais de menor faixa etária são capazes de responder a estímulos dolorosos indicativos de dor (Falcon et al., 1996; Guinsburg et al., 2000), uma vez que o processo de maturação das vias nociceptivas inicia na gestação e continua após o nascimento.

Ratos recém-nascidos apresentam maturação das vias nociceptivas semelhantes às de um feto humano de 24 semanas de gestação (Marsh et al., 1997); com uma semana de vida, estas vias no rato se assemelham às de um humano recém-nascido a termo, e

com 21 dias são semelhantes a uma criança de um ano de idade (Fitzgerald e Anand, 1993). Há algumas décadas, o sistema nociceptivo era considerado totalmente imaturo em humanos no período neonatal. Estimava-se que eles não sentiam dor por serem incapazes de descrevê-la ou demonstrar os fenômenos subjetivos relacionados. Contudo, estudos histológicos demonstram que as propriedades eletrofisiológicas dos nociceptores no neonato são semelhantes às do adulto. O processamento nociceptivo começa a ser traçado a partir da presença dos nociceptores próximos a epiderme e, gradualmente, eles descem para as camadas mais profundas de acordo com o desenvolvimento epitelial (Anand e Hickey, 1987; Anand e Carr, 1989; Fitzgerald, 1991). Durante a 16ª semana de gestação, os receptores sensoriais cutâneos encontram-se presentes, e na 30ª semana de gestação as vias nociceptivas talâmicas e espinhais já se encontram mielinizadas, e as vias aferentes nociceptivas atingem o córtex entre a 20ª e 26ª semanas de gestação (para revisão ver Simons e Tibboel, 2006). A partir da 26ª semana de gestação o feto já apresenta considerável maturação das vias ascendentes periféricas, espinhais e supraespinhais. Entretanto, a maturação das vias inibitórias descendentes ocorre somente após as vias ascendentes estarem pré-estabelecidas (para revisão ver Berde e Sethna, 2000).

O desenvolvimento dos reflexos sensoriais é precedido por uma intensa sinaptogênese entre as fibras aferentes e neurônios sensoriais na medula espinal. As fibras mielinizadas Aβ (A beta) e Aδ são as primeiras a se desenvolverem na medula espinal e a formar conexões com as camadas mais profundas do corno dorsal. Dessa forma, o estímulo nociceptivo na vida fetal e em prematuros é conduzido pelas fibras mielinizadas até a maturação das conexões das fibras C não mielinizadas (Desphande e Anand, 1996). O desenvolvimento do corno dorsal, dos diferentes tipos de neurônios

com seu arranjo laminar e conexões interneurais, começa em humanos no 1º trimestre e está praticamente completo na 32ª semana gestacional. Inicialmente, os campos receptivos dos neurônios do corno dorsal são grandes e com extensa sobreposição entre os campos receptivos dos neurônios adjacentes. Com o processo de maturação, os campos receptivos individuais diminuem progressivamente e podem ser mais precisamente definidos (Anand e Hickey, 1987; Anand e Carr, 1989; Fitzgerald, 1991).

A transmissão do estímulo nociceptivo através do corno dorsal da medula espinal ocorre através da liberação de neurotransmissores excitatórios presentes já na 16ª semana gestacional, tais como substância P (SP), glutamato, peptídeo relacionado ao gene da calcitonina (CGRP), peptídeo intestinal vasoativo (VIP), neuropeptídeo Y, e somatostatina. Entretanto, a maturação das vias descendentes inibitórias ocorre somente após a completa maturação das vias excitatórias. Os axônios dos neurônios de projeção descendente do tronco cerebral não são funcionalmente eficazes até o 10º dia pós-natal em ratos (Fitzgerald e Koltzenburg 1986; Praag e Frenk 1991). Isto pode ser resultante de deficiência de neurotransmissores, como a serotonina (5-HT) e noradrenalina, e/ou da lenta maturação de interneurônios inibitórios. Tem sido sugerido que a inibição descendente é dependente da atividade da fibra C, aferente, uma vez que ratos neonatos tratados com capsaicina reduziram respostas inibitórias quando adultos (Cervero e Plenderleith, 1985).

A transmissão inibitória rápida no corno dorsal da medula espinal é mediada pelos neurotransmissores ácido-gama-amino-butírico (GABA) e glicina. Ao longo do desenvolvimento do animal, os receptores GABA apresentam mudanças na expressão das suas subunidades e respondem de diferentes maneiras, de acordo com o estímulo.

No neonato, o sistema GABAérgico parece ser o responsável pela maior parte das sinapses inibitórias (Bacei e Fiztgerald, 2004).

2.2 Sistema Opióide

O ópio é conhecido desde a antiguidade, e tem sido usado pelo homem muito provavelmente antes da história escrita. Diversos estudos demonstram que a maioria dos povos antigos já conhecia e utilizava esta substância, incluindo os assírios, árabes, egípcios, gregos, romanos, chineses e persas. Em 1680, o botânico inglês, Thomas Sydenham, após estudar as variedades da papoula, introduz o *Laudanum Sydenham*, um composto de ópio, recomendando-o como poderoso analgésico e antidiarreico, citando: "entre todos os remédios que o poderoso Deus achou conveniente dar ao homem para aliviar seus sofrimentos, nenhum é tão difundido e eficaz como o ópio" (Wright, 1961; Baraka, 1982; Smale, 2001). Mas foi em 1806 que a morfina foi primeiramente isolada do ópio por Setürner, e devido às suas características sedativas e alucinantes este nome lhe foi conferido em homenagem ao Deus Grego dos sonhos Morfeu (para revisão ver Howard e Huda, 2001). Alguns anos mais tarde, em 1827, a companhia Merck & Co, na Alemanha, começa a produção comercial de morfina.

Em meados da década de 1960, Paul Janssen sintetizou pela primeira vez o fentanil em seu laboratório e, nos anos 1970, foram isolados e purificados os primeiros polipeptídeos endógenos (encefalinas e β-endorfinas) (Pasternak et al., 1976). A descoberta dos receptores opióides ocorreu alguns anos após, em 1973, através de estudos farmacológicos com antagonistas e agonistas em que foram identificados os receptores μ (mu) e k (kappa) (Pert e Snyder, 1973). Na década de 1980, outro grupo de

polipeptídeos endógenos foi identificado e foi chamado de dinorfinas (Brownstein, 1993). Mais tarde, Kosterlitz e col. (1983), utilizando-se de *vas deferens* de ratos, determinou um novo tipo de receptor e, seguindo a mesma regra de nomenclatura, denominou δ (delta), com isso, perfazendo um total de três receptores opióides (Brownstein, 1993; Pert e Snyder, 1973). Os receptores opióides estão amplamente expressos em várias estruturas do sistema nervoso central e no sistema nervoso periférico. Desde o 1º dia pós-natal (P1) os receptores já se encontram amplamente distribuídos no SNC, sendo que os receptores μ e k são os que predominam neste período. Em particular, na medula espinal o receptor δ está presente em pouca densidade e o pico ocorre na terceira semana pós-natal. Por outro lado, o pico de densidade do receptor μ ocorre no sétimo dia pós-natal e declina gradualmente até a terceira semana quando, então, estabelece os níveis do adulto (Beland e Fitzgerald, 2001; Kar e Quirion, 1995).

As ações dos opióides dependem da afinidade pelo subtipo de receptores e da localização dos mesmos no sistema nervoso. Os receptores δ são responsáveis primariamente pela analgesia, mas também modulam funções cognitivas e de dependência física. Estão localizados nos núcleos pontinos, amígdalas, bulbo olfatório, córtex cerebral profundo e nos neurônios sensitivos periféricos (Dhawan et al., 1996). Os receptores κ estão localizados no hipotálamo, substância cinzenta periaquedutal, substância gelatinosa na medula espinal, além de neurônios sensitivos periféricos e também estão envolvidos em resposta nociceptiva, mas também na termorregulação, no controle de diurese e em secreção neuroendócrina (Dhawan et al., 1996). Os receptores μ regulam o processos nociceptivo, o ciclo respiratório e o trânsito intestinal, estando

localizados no córtex cerebral, no tálamo, substância cinzenta periaquedutal, substância gelatinosa e trato gastrintestinal (Dhawan et al., 1996).

Os receptores opióides presentes nas membranas celulares são acoplados à proteína G inibitória e, quando estimulados por um agonista opióide, promovem inibição da enzima adenilato ciclase, levando à diminuição do nível de adenosina monofosfato cíclico (AMPc) intracelular. Com isso ocorre fechamento dos canais de cálcio voltagem dependentes nas terminações pré-sinápticas, reduzindo a liberação de neurotransmissores excitatórios, como o glutamato. A estimulação dos receptores opióides pós-sinápticos abre canais de K^+, promovendo hiperpolarização neuronal, reduzindo a excitabilidade evocada pelas fibras Aδ e C, e bloqueando parcialmente a transmissão do estímulo doloroso (Jordan e Devi, 1999).

2.2.1 Morfina em neonato

Até a década de 80, a dor não era mencionada nos livros didáticos de medicina pediátrica devido a noções equivocadas de que lactentes e crianças não sentiam dor, considerando a imaturidade do sistema nervoso central e periférico. A partir de estudos neurobiológicos do desenvolvimento da dor, observou-se que os pacientes nesta faixa etária não só sentem dor, como apresentam o limiar da dor diminuído; portanto, um aumento da resposta fisiológica ao estímulo nocivo e inócuo em relação às crianças de maior idade e aos adultos (Grunau et al., 1994; Johnston et al., 1996). Associado a isto, tem-se o reconhecimento de que as primeiras experiências dolorosas podem ter efeitos em longo prazo sobre as respostas nociceptivas (Johnston et al., 1996; Grunau et al.,1994, 2006; Oberlander et al., 2000; Ruda et al, 2000). Devido a estas importantes

descobertas e com o reconhecimento dos efeitos de longo prazo da dor subtratada em lactentes e crianças, tem ocorrido um aumento da utilização de analgésicos nesta população de pacientes (De Lima et al., 1996; Suresh e Anand, 2001, El Sayed et al., 2007).

Os opióides são considerados "Padrão ouro" entre os fármacos analgésicos em ambiente hospitalar. Atualmente, bebês e crianças são rotineiramente tratados com opióides para o alívio da dor, especialmente com exposição crônica para sedação para permitir ventilação mecânica nas UTIs pediátricas (Anand et al., 1999). Anand e colaboradores (1999) reforçam a hipótese de que os recém-nascidos que receberam morfina apresentaram menor risco de morte e menor morbidade neurológica, comparado com os RN que receberam midazolam que, embora possua propriedades sedativas, é destituída 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.

A eficácia analgésica da morfina em ratos neonatos já tem sido demonstrada. Alguns autores sugerem que a sua potência analgésica é maior no neonato e declina com o avançar da idade (Nandi e Fitzgerald, 2001). Outros postulam que o efeito da morfina aumenta com a idade devido à: a) proliferação dos receptores opióides (Auguy-Valette et al., 1978; Zhang e Pasternak, 1981); b) maturação dos mecanismos inibitórios de modulação na terceira semana após o nascimento (Nandi e Fitzgerald, 2001). Além das evidências relacionadas à maturidade do sistema neurobiológico, a mudança de resposta ao efeito da morfina também tem sido demonstrada a partir de estudos comportamentais. Vários estudos sugerem que a morfina produz antinocicepção de

modo dose-dependente em ratos adultos; porém, animais mais jovens demonstram maior sensibilidade aos opióides (Bouwmeester et al., 2003).

Quando administrada em neonatos humanos, a morfina apresenta início de ação rápida, de aproximadamente 5 minutos, e o pico do efeito analgésico é de 15 minutos. É metabolizada pelo fígado pela enzima uridina 5′-difosfato glucoroniltransferase em dois compostos: morfina-3- glucuronídeo (M3G) e morfina-6 glucuronídeo (M6G), e o *clearance* ocorre através da eliminação dos seus metabólitos pela urina (Widt et al., 1999). Dentre os efeitos adversos da morfina destacam-se depressão respiratória, náuseas e vômitos e retenção urinária, comuns a todos os opióides. A morfina desencadeia também liberação de histamina, a qual está relacionada a prurido e broncoespasmo, este último especialmente em neonatos portadores de doença pulmonar crônica. Além disso, liberação histamínica e supressão do tônus adrenérgico são responsáveis pelo aparecimento de hipotensão arterial, mais prevalente em pacientes hipovolêmicos.

Tolerância e síndrome de abstinência podem ser observadas, dependendo do tempo de utilização do fármaco e da estratégia empregada para a sua suspensão (Arnold et al., 1990; Guinsburg, 1999). A tolerância é definida como alterações da resposta fisiológica ou celular à exposição repetida a opióides levando a diminuição do efeito analgésico, e a dependência representa a manifestação dos sintomas que ocorrem após a retirada opióide (Richardson et al., 2006). Alguns autores sugerem que o desenvolvimento de tolerância depende do grau de ligação do fármaco ao receptor (Hovav e Weinstock, 1987). Anand e colaboradores (1999) dizem que a tolerância depende da idade e do regime de dosagem em que o opióide está sendo administrado. Em animais adultos, a exposição continuada de opióide por 4 horas dá início ao

processo de tolerância; no entanto, em animais mais jovens é difícil notar efeitos que sugerem tolerância antes de 72 horas de infusão contínua (Anand et al., 1999). Estudos do nosso grupo de pesquisa têm evidenciado que em ratos com oito dias de vida, que receberam morfina por sete dias consecutivos (do P8 ao P14), na dose de 5 µg, não desenvolveram tolerância (Rozisky et al., 2008). Adicionalmente, no último dia de tratamento, os animais apresentaram maior duração do efeito analgésico da morfina comparado ao efeito observado no primeiro dia de uso. Nossos achados corroboram evidências de outros estudos, em que tolerância ao uso repetido de morfina em ratos infantes é menos pronunciada do que em adultos (Praag and Frenk, 1991). Possivelmente, a variação na expressão de receptor opióide e sua sensibilidade aos agonistas explique as flutuações na susceptibilidade à analgesia e ao desenvolvimento de tolerância ao longo do desenvolvimento. Apesar destes animais não apresentarem tolerância no final do tratamento, quando submetidos a uma dose aguda na idade adulta, eles apresentam maior tempo de analgesia que os demais animais. Estes dados refletem as possíveis alterações em longo prazo induzidas pela morfina em estruturas do sistema nervoso central que modulam as respostas nociceptivas (Rozisky et al., 2008).

O tratamento com opióide durante o período neonatal também pode desencadear alterações comportamentais após a retirada do fármaco (síndrome de abstinência). Mais de 48% dos bebês e crianças que recebem doses terapêuticas de opióides intravenosos demonstram sintomas de retirada e dependência aos opióides, tais como sinais disfóricos e aversivo, os quais são também demonstrados através de modelos animais (Arnold et al., 1990; French e Nocera, 1994; Franck e Vilardi, 1995; Franck et al., 1998). Estudos desenvolvidos em nosso grupo de pesquisa demonstraram que animais com 14 dias submetidos à administração de fentanil (opióide sintético) associado à

cetamina (antagonista do receptor NMDA) apresentaram redução no comportamento do tipo ansioso, enquanto o isoflurano (anestésico geral inalatório) aumentou a atividade locomotora na vida adulta (Medeiros et al., 2011). Além disso, quando os dois fármacos foram administrados separadamente, observamos que o fentanil, um potente agonista opióide, foi o responsável pelas alterações comportamentais observadas na associação de fentanil e cetamina utilizada no estudo prévio (Medeiros et al., 2012). Fármacos opióides, como a morfina, são conhecidos por desenvolverem sintomas comportamentais que dependem, pelo menos em parte, de mudanças no sistema dopaminérgico (Kuribara, 1995).

2.3. Sistema Dopaminérgico

A dopamina é o neurotransmissor catecolaminérgico predominante no cérebro de mamíferos, onde controla uma variedade de funções incluindo atividade locomotora, cognição, emoção, ingestão de alimento e regulação endócrina. Na periferia atua como moduladora da função vascular, liberação de catecolaminas, secreção hormonal, tônus vascular, função renal, e mobilidade gastrointestinal (para revisão ver Missale et al., 1998). Em relação aos efeitos comportamentais, evidências sugerem que o sistema dopaminérgico encontra-se funcional desde o período pré-natal (Moody et al., 1993).

A dopamina atua em diferentes tipos de receptores, os quais são amplamente expressos no sistema nervoso central e periférico, bem como em vários outros órgãos e tecidos. Seus receptores dividem-se em 5 tipos (D1-5) de acordo com a localização e mecanismo de ação (Missale et al., 1998). Os receptores D1 e D2 são os mais estudados em relação aos efeitos comportamentais relacionados ao uso de fármacos. Estudo

recente demonstrou que as expressões de ambos receptores variam de acordo com o tratamento, ou seja, em uma exposição aguda à morfina há o aumento da expressão gênica do receptor D1 no NAc, enquanto que o tratamento repetido leva ao aumento da expressão gênica do receptor D2 na mesma estrutura (Enoksson et al., 2012). Em um estudo recente, Yang e colaboradores (2011) demonstraram uma forte relação entre os sintomas de dependência da morfina e receptor dopaminérgico do tipo D2. Este estudo mostrou que o haloperidol (antagonista do receptor dopaminérgico D2) foi capaz de atenuar os sintomas de tolerância e de dependência à morfina em animais adultos (Yang et al., 2011). Outros pesquisadores têm apontado a forte interação entre os sistemas opióide e dopaminérgico. E drogas antipsicóticas, como o haloperidol, têm sido utilizadas para bloquear os efeitos da atividade dopaminérgica (Adamus et al., 1981; Cheido e Idova, 2007; Unterwald e Cuntapay, 2008).

Administração repetida de morfina pode induzir um fenômeno chamado de sensibilização comportamental, o qual é evidenciado por um aumento da locomoção após o término do tratamento (Vries et al., 1998). Este efeito ocorre via sistema dopaminérgico presente em estruturas límbicas, o qual se origina na área tegmental ventral e se projeta ao núcleo accubens (NAc) e outras áreas, tais como hipocampo e amígdala (Vanderschuren e kalivas, 2000). O desenvolvimento da sensibilização comportamental à morfina é conhecido por envolver repetida estimulação do receptor opióide μ (Kuribara, 1995), levando ao aumento da liberação de dopamina no sistema límbico (Devine e Wise, 1994; Everitt et al., 2001). Por exemplo, injeção intra-NAc de antagonista do receptor opióide μ (D-Pen-Cys-Tyr-D-Trp- Orn-Thr-Pen-Thr-NH2 - CTOP) bloqueia o desenvolvimento da sensibilização locomotora à morfina (Johnson e Napier, 2000). Entretanto, os comportamentos evocados pela exposição repetida à

morfina refletem não somente ações neuroquímicas nos receptors opióides, mas em outros neurotransmissores como glutamato e GABA, que estão sobre influência direta dos receptores opióides (Yoon et al., 2007).

2.4 Sistema Glutamatérgico

O sistema glutamatérgico é amplamente distribuído pelo sistema nervoso central e seu neurotransmissor é o glutamato, considerado o principal neurotransmissor excitatório. O glutamato desempenha suas funções através de receptores específicos presentes na membrana plasmática de neurônios e de células gliais e são classificados em metabotrópicos e ionotrópicos. Os primeiros agem por meio de segundos mensageiros, ativados pela proteína G; já os segundos agem por meio do fluxo de íons e são subdivididos em receptores não-NMDA representados pelos receptores AMPA (α-amino-3-hidróxi-5-metil-4-isoxazolepropionato) e Cainato ativados por Na^{+} e K^{+} (Li et al., 1994; Ozawa et al., 1998; You et al., 2003), e receptores NMDA (N-metil-D-aspartato) bloqueados por Mg^{+2} (Steinhauser e Gallo, 1996; Shelton e McCarthy, 1999; Bergles et al., 2000).

O glutamato está envolvido na sinalização do estímulo nociceptivo no corno dorsal da medula espinal. Além deste importante papel na transmissão nociceptiva, o glutamato está envolvido em inúmeros aspectos do funcionamento normal do cérebro como, por exemplo, cognição, memória e aprendizado (para revisão ver Fonnum, 1984; Ottersen e Storm-Mathisen, 1984; Collingridge e Lester, 1989; Headley e Grillner, 1990). Além disso, também apresenta papel importante no desenvolvimento do sistema nervoso central, incluindo sinaptogênese, migração, diferenciação e morte celular (Urch

et al., 2001). Quando é liberado mediante um estímulo nociceptivo agudo e estímulos repetidos, o glutamato liga-se em receptores AMPA e NMDA, respectivamente (Mello e Dickenson, 2008). Após estimulação persistente das fibras C, ocorre a amplificação da resposta nociceptiva dos neurônios no corno dorsal da medula espinal, resultante da ativação dos receptores NMDA. Porém, quando um estímulo nociceptivo de baixa frequência ocorre, como na dor aguda, o receptor NMDA encontra-se bloqueado por níveis normais de Mg^{+2}. Para que este desbloqueio ocorra é necessária despolarização da membrana neuronal, permitindo que ocorra a ativação dos receptores NMDA e consequente abertura dos canais de Ca^{+2}.

O nível de glutamato extracelular é regulado por meio da recaptação por transportadores (GTs) presentes na membrana plasmática de neurônios e células gliais (Niederberger et al., 2003, 2006). Os três subtipos de GTs presentes na medula espinal, GLAST (transportador de aspartato e glutamato) e o GLT1 (transportador-1 de glutamato) e EAAC1 (transportador-1 de aminoácidos excitatórios) são considerados essenciais para a manutenção dos níveis de glutamato em níveis menores que 1 mM e para prevenir a hiperestimulação dos receptores glutamatérgicos (Mennerick et al., 1999). Notavelmente, estes transportadores estão concentrados na superfície do corno dorsal da medula espinal e somam mais de 80% do total da captação de glutamato nesta estrutura (Danbolt, 2001). Esta regulação é crucial para a transmissão nociceptiva e para a prevenção de neurotoxicidade induzida pelo glutamato. Mudanças no processo de captação de glutamato têm sido implicadas em dano do sistema nervoso periférico (Kiryu et al., 1995; Mawrin et al., 2003), dor neuropática (Sung et al., 1998) e hiperalgesia (Weng et al., 2005, 2006).

No período perinatal correntes de glutamato são evidentes no corno dorsal de ratos provenientes de sua liberação pelos terminais aferentes primários. Estas correntes aumentam no período pós-natal, especialmente entre os dias pós-natais 5 (P5) e 10 (P10) (Patinzon e Fitzgerald, 2004). Os receptores AMPA e Cainato também já se encontram pré-estabelecidos durante o desenvolvimento e são amplamente expressos na medula espinal desde o 1º trimestre gestacional (Akesson et al., 2000), e diminuem ao longo das primeiras três semanas após o nascimento (Jacowec et al., 1995). Adicionalmente, o receptor NMDA é amplamente expresso em medula espinal durante o período neonatal em comparação ao adulto e participa intensamente nos mecanismos de reorganização das sinapses e na plasticidade sináptica durante o crescimento (Loftis e Janowsky, 2003).

No adulto os receptores NMDA estão envolvidos no processo de sensibilização central, no qual há uma intensa ativação das fibras nociceptivas, principalmente tipo C, efeito claramente observado no teste da formalina em ratos (Chapman et al., 1995).

2.5 Sistema Purinérgico

Estudos de nosso grupo de pesquisa têm demonstrado uma correlação entre os sistemas opióide e purinérgico. Em um estudo com animais que receberam morfina durante o período neonatal observamos alterações nas atividades de hidrólise dos nucleotídeos em sinaptossoma de córtex cerebral e medula espinal, bem como a expressão gênica da NTPDase 1 [enzima que hidrolisa o ATP (adenosina 5'-trifosfato) até adenosina] em ambas estruturas (Rozisky et al., 2010). Além disso, em outro experimento observamos que a administração de agonistas opióides e adenosinérgicos

por via intratecal induz analgesia em animais adultos (Torres et al., 2003). Esta ação agonista modula a liberação de neurotransmissores excitatórios atuando na transmissão do estímulo doloroso no sistema nervoso central e periférico (Torres et al., 2003). Em outro estudo demonstramos que as atividades das enzimas ectonucleotidases mudam em função da idade avaliadas em sinaptossomas de medula espinal (P30, P60, P120 e P180), demonstrando a importância ontogênica destas enzimas em medula espinal na regulação dos níveis de nucleotídeos e nucleosídeo em SNC (Torres et al., 2003).

A introdução do conceito de sinalização purinérgica por Burnstock (1972) revelou que o ATP, além de exercer seu reconhecido papel no metabolismo celular, representa também uma importante molécula sinalizadora no meio extracelular, atuando em processos como neuromodulação, neurotransmissão, contração do músculo liso, inflamação, resposta imunológica, agregação plaquetária e nocicepção (Ralevick e Burnstock, 1998), bem como regulação de mecanismos celulares de proliferação, diferenciação e apoptose (White e Burnstock, 2006). O ATP pode ser co-liberado em vias simpáticas e parassimpáticas juntamente com outros neurotransmissores (Burnstock, 1999, 2004; Cunha e Ribeiro, 2000), ou ainda por células danificadas ou em processo de morte (Bodin e Burnstock, 2001). Após serem liberados, os nucleotídeos ATP e ADP [adenosina 5'-difosfatoproduto da hidrólise do ATP), e o nucleosídeo adenosina [produto da hidrólise do AMP (adenosina 5'-monofosfato)], exercem seus efeitos biológicos através dos purinoreceptores, que compreendem os receptores do tipo P2, subdivididos em P2X para ATP ($P2X_{1\text{-}7}$, ionotrópicos) e P2Y para o ATP e o ADP ($P2Y_{1,\ 2,\ 4,\ 6,\ 11\text{-}14}$, metabotrópicos), e os receptores do tipo P1 para o nucleosídeo adenosina (A_1, A_{2A}, A_{2B} e A_3, metabotrópicos) (Burnstock, 2006). Estes receptores são

amplamente distribuídos em diversos tipos de células, dentre elas astrócitos, plaquetas, células da microglia, imunes, epiteliais e endoteliais (Burnstock, 2008).

Em relação ao sistema cardiovascular, o ATP, ADP e adenosina exercem importantes funções: o ATP induz agregação plaquetária, vasoconstrição ou vasodilatação da musculatura lisa, ações que dependem do tipo de receptor a que ele irá se ligar (Burnstock, 2006); o ADP é potente agregador plaquetário e induz vasocontrição (Kunupuali e Daniel, 1998; Furukoji et al., 2008); a adenosina induz vasodilatação e é considerada molécula cardioprotetora (Jacobson e Gao, 2006).

Após exercerem seus efeitos, o ATP e ADP podem ser hidrolisados por membros da família E-NTPDase (Ecto-nucleosídeo trifosfato difosfohidrolase), enquanto que o AMP é hidrolisado pela Ecto 5'-nucleotidase (Zimmermann, 2001). As E-NTPDases representam uma família composta por 8 membros, sendo que as NTPDases 1, 2, 3 e 8 são proteínas transmembrana com o sítio catalítico voltado para o meio extracelular, enquanto que as NTPDases 4, 5, 6 e 7 estão localizadas intracelularmente, ancoradas nas membranas de organelas com o sítio catalítico voltado para o lúmen ou compartimentos (Robson et al., 2006). Estas enzimas apresentam diferentes características cinéticas, sendo que a NTPDase 1 hidrolisa ATP e ADP na razão aproximada de 1:1; NTPDases 2, 3 e 8 hidrolisam nas proporções de 30:1, 3:1 e 3:2 respectivamente; NTPDase 4 prefere o UDP; a 5 e a 6 preferem nucleotídeos difosfatados, sendo que podem sofrer clivagem proteolítica e serem secretadas em forma solúvel (Zimmermann, 2001).

A Ecto 5'-nucleotidase é classificada em 4 grupos de acordo com a localização celular e propriedades bioquímicas: uma ancorada à membrana plasmática, uma forma solúvel, e duas formas citoplasmáticas (Kawashima et al., 2000). As NTPDases e 5'-

nucleotidase são amplamente expressas em células endoteliais e hematopoiéticas e são consideradas as maiores reguladoras da sinalização purinérgica no sistema cardiovascular (Zimmermann, 1999; Robson et al., 2005).

Entretanto, ao contrário do que se pensava inicialmente em que a metabolização do ATP era mediada somente pelas nucleotidases ligadas à membrana plasmática, foi demonstrado que nucleotidases solúveis, liberadas por terminações simpáticas, também estão envolvidas (Todorov et al., 1997). Tais 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 (Agteresch et al., 1999; Oses et al., 2004).

2.6 Melatonina

Um grande corpo de evidências sugere diferentes interações entre a melatonina e o sistema opióide. Por exemplo, ratos parecem ser menos sensíveis à dor e o efeito analgésico da morfina é maior durante a noite, em que o nível de melatonina é mais elevado (Frederickson et al., 1977; Lakin et al., 1981; Kavaliers et al., 1983). Além disso, a pinealectomia em ratos induz mudanças nos níveis e ritmo circadiano de opióides endógenos, como a encefalina e β-endorfina, envolvidos na regulação da sensibilidade à dor no hipotálamo (Ebadi et al., 1998). Essas mudanças nas concentrações cerebrais dos opióides endógenos possivelmente são provocadas por um mecanismo induzido pela melatonina para a mediação da modulação da sensibilidade dolorosa. Além disso, tem sido demonstrado que os opióides estimulam os pinealócitos a secretar melatonina, aumentando o nível de melatonina no sangue do rato (Ebadi et

al., 1998). Adicionando-se às evidências experimentais, recente ensaio clínico randomizado duplo-cego controlado por placebo, demonstrou que o uso de melatonina pré-operatória reduziu os níveis de dor e o consumo de morfina no pós-operatório (Caumo et al., 2007).

O efeito analgésico da melatonina também pode envolver a ação em receptores de melatonina MT1 e MT2, expressos na medula espinal e em outras estruturas do sistema nervoso central, levando à redução do estímulo doloroso via hiperpolarização de neurônios do corno dorsal (Laurido et al., 2002; Lewy et al., 2006). O mecanismo antinociceptivo da melatonina também foi estudado em ratos diabéticos usando um agente bloqueador de receptor MT2, onde foi observada diminuição do efeito antinociceptivo da melatonina. Assim, isto demonstra que receptores MT2, em medula espinal, podem estar envolvidos na antinocicepção induzida pela melatonina (Arreola-Espino et al., 2007). Estudos também demonstraram os efeitos da melatonina na excitabilidade neuronal atuando em receptores GABAérgicos, promovendo hiperpolarização (Wu et al., 1999), e em receptores NMDA, reduzindo a atividade da óxido nítrico sintase neuronal (nNOS) e regulando sítios redox do receptor NMDA em diversas estruturas do sistema nervoso central (Yamamotto e Tang, 1998; El-Scherif et al., 2002; Escames et al., 2004).

Associado ao efeito analgésico, a melatonina apresenta efeito anti-inflamatório diminuindo a produção de ácido araquidônico e a expressão da COX-2, atuando como modulador negativo da expressão da enzima fosfolipase A2 (Li et al., 2000). Em nível de sistema imunológico, a melatonina atua diretamente sobre os linfócitos e macrófagos inibindo a síntese do fator nuclear kappa B (NFkB), a produção de citocinas pró-inflamatórias como o fator de necrose tumoral α (TNF α), interleucina 1 (IL-1) e

radicais livres (Reiter et al., 2001; Li e Verma, 2002; Konturek et al., 2007). A melatonina desempenha um papel importante na ativação de defesas antioxidantes como a superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GSHPx), a glutationa redutase (GSH-Rd) e a glicose-6-fosfato desidrogenase (G6PD) (Lewy et al; 2006). Estes efeitos permitem diminuir a formação de espécies reativas de oxigênio (ROS), auxiliando em patologias que cursam com estresse oxidativo (Yoon et al, 2008).

É interessante salientar que estudos ontogenéticos identificaram que a síntese de melatonina pineal começa a partir do 5º dia pós natal e, após a primeira semana várias mudanças ocorrem concomitante a maturação da inervação adrenérgica (Stehle et al., 1995). A partir deste momento um ritmo circadiano de secreção é detectável, e ao fim da terceira semana a atividade máxima da N-acetiltransferase noturna e o pico de melatonina são atingidos (Rubio et al., 1993; Ribelayga et al., 1998).

2.7. Fator Neurotrófico Derivado do Cérebro (BDNF)

Alguns estudos têm demonstrado que a exposição à opióide leva a alterações nos níveis de BDNF. Um estudo com animais demonstrou que a exposição à morfina no período perinatal diminui os níveis de BDNF no hipocampo (Schrott et al., 2008). Além disso, estudo clínico com pacientes adictos de heroína demonstrou que estes pacientes apresentavam níveis séricos de BDNF diminuídos (Angelucci et al., 2007).

O BDNF é um membro da família da neurotrofinas (NTs) ou fatores neurotróficos conhecidos originalmente por regularem a sobrevivência, o crescimento e a diferenciação neuronal (Davies, 1994). Além do BDNF, as neurotrofinas são representadas pelos seguintes membros: fator de crescimento neural (NGF), neurotrofina 3 (NT-3) e neurotrofina 4/5 (NT-4/5). Todas as NTs têm baixa afinidade

por receptores transmembrana p75, mas alta afinidade por um dos subtipos de receptor de tirosina quinase (trk): NGF em trkA, BDNF e NT4/5 em trkB, e NT3 em trkC.

O BDNF é amplamente expresso no sistema nervoso periférico e central durante o desenvolvimento, sendo que no hipocampo apresenta níveis elevados (Leibrock et al., 1989; Hohn et al., 1990; Ernfors et al., 1992). Depois de se ligar ao receptor TrkB, regula a sobrevivência neuronal, promove o crescimento de neuronal e mantém a conectividade sináptica no sistema nervoso adulto (Zhang e Ko, 2009). Outros estudos têm demonstrado que o BDNF possui ação neuromoduladora na aprendizagem, na memória, na depressão (Altar et al., 1992) e na dependência de drogas (Grimm et al., 2003; McGough et al., 2004; Filip et al., 2006). Há evidências de que o BDNF tem um papel significativo em processos de plasticidade sináptica (Li et al., 2005) e sensibilização locomotora (Liang et al., 2011) após a retirada de opióides.

2.8 Estresse oxidativo

A exposição de ratos à morfina no período pós-natal e na vida adulta também tem sido associada com a geração de estresse oxidativo devido a um desequilíbrio entre os altos níveis celulares de espécies reativas de oxigênio (ERO) e de nitrogênio (ERN) e defesas antioxidantes celulares (Halliwell e Gutteridge, 2000; Guzmán et al., 2006). O balanço redox das células é feito por um sistema antioxidante enzimático e outro não-enzimático. O sistema antioxidante enzimático é composto por enzimas como superóxido dismutase (SOD), que converte o radical superóxido em peróxido de hidrogênio; a catalase, que promove a degradação do peróxido de hidrogênio; e a glutationa peroxidase (GPx), que promove a degradação dos superóxidos, especialmente os derivados da oxidação dos fosfolipídios de membrana (Kehrer, 2000).

As ERO são produzidas em baixos níveis nas células de mamíferos por vários processos metabólicos, como a cadeia respiratória na mitocôndria, a atividade da NADPH oxidase e o metabolismo oxidativo do ácido araquidônico (Kehrer, 2000). Quando ocorre um desequilíbrio entre a produção de ERO e atividade das enzimas antioxidantes, ocorre o fenômeno conhecido como estresse oxidativo. Neste processo, a célula perde a capacidade de detoxificar o excesso de ERO, induzindo a dano em componentes celulares, como proteínas, ADN (ácido desoxirribonucleico), lipídios e morte celular (Kroemer et al., 1997). Outra consequência da produção de ERO é a indução da biossíntese de citocinas pró-inflamatórias, tais como o fator de necrose tumoral alfa (TNF-α), sugerindo uma interação complexa entre processo inflamatório no sistema nervoso central e ERO (Turchan-Cholewo et al., 2009).

3. JUSTIFICATIVA

Estudos na área do desenvolvimento neurobiológico dos processos nociceptivos vêm crescendo nas últimas décadas com o descobrimento de que neonatos e crianças de menor idade não somente sentem dor como também apresentam menor limiar para os estímulos dolorosos comparados aos adultos. Sendo assim, o uso de fármacos analgésicos, especialmente opióides**,** vêm crescendo como consequência dos avanços nos tratamentos da dor nestes pacientes de menor idade (Suresh e Anand, 2001; El Sayed et al., 2007). Entretanto, ainda existe uma lacuna em pesquisas que investiguem os efeitos sobre sistemas relacionados à nocicepção, sobre respostas comportamentais e parâmetros bioquímicos ao longo da vida destes pacientes expostos à analgesia opióide durante o período neonatal. O uso de modelos experimentais permite-nos investigar e consolidar o conhecimento da ontogênese do sistema nociceptivo. Este foco de pesquisa é de particular relevância por abordar mecanismos neurobiológicos no processo de desenvolvimento do sistema nociceptivo e de que forma intervenções precoces podem influenciar o curso do desenvolvimento. Esta questão se reveste de importância ainda maior, em vista de que a dor intensa ou persistente no período neonatal pode alterar a ontogênese das vias neurobiológicas produzindo alterações duradouras no comportamento do tipo ansioso, locomoção e na percepção da dor (Rozisky et al, 2008; Medeiros et al, 2011). Hipotetiza-se que alterações nas vias nociceptivas aconteçam pela demanda excessiva deste sistema em desenvolvimento com consequentes alterações nestas vias e em suas conexões sinápticas. Espera-se que a compreensão do efeito de terapêuticas farmacológicas no período perinatal permita aprimorar o conhecimento dos mecanismos neuroquímicos e moleculares da dor.

Dessa forma, esta tese pretende contribuir com a ampliação do conhecimento da fisiopatogênia da dor e de possíveis alterações bioquímicas e comportamentais decorrentes da exposição a analgésicos opióides em indivíduos de menor idade.

4. OBJETIVOS

Objetivo Geral

Considerando a importância deste tema e da realização de estudos com modelos animais que estejam focalizados em sistemas envolvidos com mecanismos de dor que contribuam para o melhor entendimento da resposta à analgesia opióide em neonatos, o objetivo desta tese foi investigar o efeito da administração repetida de morfina em ratos neonatos em parâmetros comportamentais e bioquímicos em curto, médio e longo prazos.

Objetivos Específicos

Avaliar o efeito de uma administração diária de 5µg de morfina por sete dias, por via s.c., do 8º ao 14º dias de vida sobre:

(a) a resposta nociceptiva através do teste da formalina no 16º, 30º e 60º dias de vida, investigando o papel do receptor glutamatérgico NMDA nas respostas alteradas;

(b) a captação de glutamato em medula espinal no 30º e 60º dias de vida.

(c) os níveis de BDNF e TNF- α, atividades de enzimas antioxidantes e viabilidade e morte celular no hipocampo no 16º, 30º e 60º dias de vida.

(e) atividades das enzimas NTPDase e 5´-nucleotidase séricas no 16º, 30º e 60º dias de vida.

(d) respostas comportamentais nos testes de campo aberto e labirinto em cruz

elevado no 16º e 30º dias de vida, investigando o possível envolvimento do receptor dopaminérgico D2 nos comportamentos alterados.

Avaliar o papel antinociceptivo da melatonina no 30º e 60º dias de vida frente às respostas comportamentais nociceptivas induzidas pelo tratamento repetido com 5µg de morfina por sete dias, do 8º ao 14º dias.

Referências Bibliográficas

Adamus A, Melzacka M, Vetulani J. Behavioral and pharmacokinetic interaction between morphine and haloperidol in the rat. Pol J Pharmacol Pharm 1984; 36(1):51-8.

Agteresch HJ, Dagnelie PC, van den Berg JW, Wilson JH. Adenosine triphosphate: established and potential clinical applications. Drugs. 1999;58(2): 211-232.

Akesson E, Kjaeldgaard A, Samuelsson EB, Seiger A, Sundström E. Ionotropic glutamate receptor expression in human spinal cord during first trimester development. Brain Res Dev Brain Res. 2000; 119(1):55-63.

Altar CA, Boylan CB, Jackson C, Hershenson S, Miller J, Wiegand SJ, et al. Brain-derived neurotrophic factor augments rotational behavior and nigrostriatal dopamine turnover in vivo. Proc Natl Acad Sci U S A. 1992; 89:11347-51.

Anand KJ, Barton BA, McIntosh N, Lagercrantz H, Pelausa E, Young TE, Vasa R. Analgesia and sedation in preterm neonates who require ventilatory support: results from the NOPAIN trial. Neonatal Outcome and Prolonged Analgesia in Neonates. Arch Pediatr Adolesc Med. 1999; 153(4):331-8.

Anand KJS, Hickey PR. Pain and its effects in the human neonate and fetus. N Engl J Med. 1987;317:1321–1329.

Anand KJS, Hickey PR. Pain and its effects in the human neonate and fetus. N Engl J Med 1987;317:1321e9.

Anand KJS, Scalzo FM. Can adverse neonatal experiences alter brain development and subsequent behavior? Biol Neonate 2000;77:69–82.

Anand KJS. Clinical importance of pain and stress in preterm neonates. Biol Neonate. 1998;73:1–9.

Anand KJ, Carr DB. The neuroanatomy, neurophysiology, and neurochemistry of pain, stress, and analgesia in newborns and children. Pediatr Clin North Am. 1989;36(4):795-822.

Angelucci F, Ricci V, Pomponi M, Conte G, Mathé AA, Attilio Tonali P, Bria P. Chronic heroin and cocaine abuse is associated with decreased serum concentrations of the nerve growth factor and brain-derived neurotrophic factor. J Psychopharmacol. 2007;21(8):820-5.

Arnold JH, Truog RD, Orav EJ, Scavone JM, Hershenson MB. Tolerance and dependence in neonates sedated with fentanyl during extracorporeal membrane oxygenation. Anesthesiology. 1990;73(6):1136-40.

Arreola-Espino R, Urquiza-Marín H, Ambriz-Tututi M, Araiza-Saldaña CI, Caram-Salas NL, Rocha-González HI, Mixcoatl-Zecuatl T, Granados-Soto V. Melatonin reduces formalin-induced nociception and tactile allodynia in diabetic rats. Eur J Pharmacol. 2007;577(1-3):203-10.

Auguy-Valette A, Cross J, Gouarderes C, Gout R, Pontennier G. Morphine analgesia and cerebral opiate receptors: a developmental study. Br J Pharmacol 1978; 63:303-308.

Baccei ML, Fitzgerald M. Development of GABAergic and glycinergic transmission in the neonatal rat dorsal horn. J Neurosci. 2004;24(20):4749-57.

Baraka A. Historical aspects of opium. Middle East J Anaesthesiol 1982;6(5):289-302.

Barker DP, Rutter N. Stress, severity of illness, and outcome in ventilated preterm infants. Arch Dis Child Fetal Neonatal Ed. 1996;75:F187–F190.

Basbaum AI, Jessel TM. The Perception of Pain. In: Kandel ER, Schartz JH, Jessel TM (eds). Principles of Neural Science. 4ed. New York: McGraw-Hill, 2000:472-491.

Beland B, Fitzgerald M. Mu- and delta-opioid receptors are downregulated in the largest diameter primary sensory neurons during postnatal development in rats. Pain 2001; 90(1-2):143-50.

Berde CB, Sethna NF. Drug therapy: analgesics for the treatment of pain in children. N Eng J Med 2002; 347:1094-1103.

Bergles DE, Roberts JD, Somogyi P, Jahr CE. Glutamatergic synapses on oligodendrocyte precursor cells in the hippocampus. Nature. 2000; 405(6783):187-91.

Bodin P and Burnstock G. Purinergic signalling: ATP release. Neurochem Res.2001;26(8-9): 959-69.

Bouwmeester NJ, Van Den Anker JN, Hop WC, Anand KJ, Tibboel D. Age- and therapy-related effects on morphine requirements and plasma concentrations of morphine and its metabolites in postoperative infants. Br J Anaesth 2003; 90:642-52.

Brownstein MJ. A brief history of opiates, opioid peptides, and opioid receptors. Proc Natl Acad Sci USA 1993;15;90(12):5391-3.

Burnstock G. Purinergic nerves. Pharmacol Rev 1972; 24(3):509-81.

Burnstock G. Purinergic cotransmission. Brain Res Bull.1999;50(5-6): 355-7.

Burnstock G. Cotransmission. Curr Opin Pharmacol.2004;4(1): 47-52.

Burnstock G. Purinergic signalling--an overview. Novartis Found Symp.2006;276: 26-48; discussion 48-57, 275-81.

Burnstock G. Purinergic signalling and disorders of the central nervous system. Nat Rev Drug Discov 2008;7(7): 575-90.

Cahill CM, White TD, Sawynok J. Spinal opioid receptors and adenosine release: neurochemical and behavioral characterization of opioid subtypes. J Pharmacol Exp Ther. 1995; 275(1):84-93.

Caumo W, Torres F, Moreira NL Jr, Auzani JA, Monteiro CA, Londero G, Ribeiro DF, Hidalgo MP. The clinical impact of preoperative melatonin on postoperative outcomes in patients undergoing abdominal hysterectomy. Anesth Analg. 2007;105(5):1263-71.

Cervero F, Iggo A. The substancia gelatinosa of the spinal cord. A critical review. Brain 1980; 103:717-772.

Cervero F, Plenderleith MB. C-fibre excitation and tonic descending inhibition of dorsal horn neurones in adult rats treated at birth with capsaicin. J Physiol. 1985;365:223-37.

Chapman V, Honoré P, Buritova J, Besson JM. The contribution of NMDA receptor activation to spinal c-Fos expression in a model of inflammatory pain. Br J Pharmacol. 1995; 116(1):1628-34.

Collingridge GL, Lester RA. Excitatory amino acid receptors in the vertebrate central nervous system. Pharmacol Rev. 1989; 41(2):143-210.

Cheido MA, Idova GV. The differential contribution of dopamine D(1) and D (2) receptors to mu-opioidergic immunomodulation. Neurosci Behav Physio 2007; 37(7):721-4.

Cunha RA, Ribeiro JÁ. ATP as a presynaptic modulator. Life Sci 2000;68(2):119-37.

Danbolt NC. Glutamate uptake. Prog Neurobiol 2001; 65:1–105.

Davies AM. The role of neurotrophins during successive stages of sensory neuron development. Prog Growth Factor Res. 1994; 5(3):263-89.

De Lima J, Alvares D, Hatch DJ, Fitzgerald M. Sensory hyperinnervation after neonatal skin wounding: effect of bupivacaine sciatic nerve block. Br J Anaesth. 1999;83:662–664.

De Lima J, Lloyd-Thomas AR, Howard RF, Sumner E, Quinn TM. Infant and neonatal pain: anaesthetists' perceptions and prescribing patterns. BMJ. 1996 ;313 (7060):787.

Devine DP, Wise RA. Self-administration of morphine, DAMGO, and DPDPE into the ventral tegmental area of rats. J Neurosci 1994; 14:1978-1984.

De Wildt SN, Kearns GL, Leeder JS, van den Anker JN.Glucuronidation in humans. Pharmacogenetic and developmental aspects. Clin Pharmacokinet 1999; 36: 439–52.

Deshpande JK, Anand KJS. Basic aspects of acute pediatric pain and sedation. In: Deshpande JK, Tobias JD (eds). The Pediatric Pain Handbook. St. Louis: Mosby-Years Book, Inc., 1996. p.1-48.

Dhawan BN, Cesselin F, Raghubir R, et al. International Union of Pharmacology. XII. Classification of opioid receptors. Pharmacol Rev 1996;48(4):567-92.

Dixon S, Snyder J, Holve R, Bromberger P. Behavioral effects of circumcision with and without anesthesia. J Dev Behav Pediatr. 1984;5:246–250.

Ebadi M, Govitrapong P, Phansuwan-Pujito P, Nelson F, Reiter RJ. Pineal opioid receptors and analgesic action of melatonin. J Pineal Res. 1998;24(4):193-200.

El Sayed MF, Taddio A, Fallah S, De Silva N, Moore AM. Safety profile of morphine following surgery in neonates.J Perinatol. 2007; 27(7):444-7.

Falcon M, Guendellman D, Stolberg A, Frenk H, Urca G. Development of thermal nociception in rats. Pain. 1996:67(1):203-8.

Enoksson T, Bertran-Gonzalez J, Christie MJ. Nucleus accumbens D2- and D1-receptor expressing medium spiny neurons are selectively activated by morphine withdrawal and acute morphine, respectively. Neuropharmacology. 2012; 62(8):2463-71.

Ernfors P, Merlio JP, Persson H. Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. Eur J Neurosci 1992; 4:1140-1158.

Escames G, León J, López LC, Acuña-Castroviejo D. Mechanisms of N-methyl-D-aspartate receptor inhibition by melatonin in the rat striatum. J Neuroendocrinol. 2004;16(11):929-35.

Everitt BJ, Dickinson A, Robbins TW. The neuropsychological basis of addictive behavior. Brain Res Rev 2001; 36:129-138.

Filip M, Faron-Górecka A, Kuśmider M, Gołda A, Frankowska M, Dziedzicka-Wasylewska M. Alterations in BDNF and trkB mRNAs following acute or sensitizing cocaine treatments and withdrawal. Brain Res 2006; 1071:218-225.

Fitzgerald M, Anand KJ. Developmental neuroanatomy and neurophysiology of pain. In: Schechter NL, Berde CB, Yaster M (eds). Pain in infant, children, and adolescents. Baltimore: Williams & Wilkins, 1993. p. 11-31.

Fitzgerald M, Beggs S. The neurobiology of pain: developmental aspects. Neuroscientist. 2001;7:246–257.

Fitzgerald M, Koltzenburg M. The functional development of descending inhibitory pathways in the dorsolateral funiculus of the newborn rat spinal cord. Brain Res. 1986; 389(1-2):261-70.

Fitzgerald M. Development of pain mechanisms. Br Med Bull. 1991;47(3):667-75.

Franck LS. A new method to quantitatively describe pain behavior in infants. Nurs Res. 1986;35:28–31.

Fonnum F. Glutamate: a neurotransmitter in mammalian brain. J Neurochem. 1984; 42(1):1-11.

Franck L, Vilardi J. Assessment and management of opioid withdrawal in ill neonates. Neonatal Netw. 1995;14(2):39-48.

Franck LS, Vilardi J, Durand D, Powers R. Opioid withdrawal in neonates after continuous infusions of morphine or fentanyl during extracorporeal membrane oxygenation. Am J Crit Care. 1998;7(5):364-9.

Frederickson RC, Burgis V, Edwards JD. Hyperalgesia induced by naloxone follows diurnal rhythm in responsivity to painful stimuli. Science. 1977;198(4318):756-8.

French JP, Nocera M. Drug withdrawal symptoms in children after continuous infusions of fentanyl. J Pediatr Nurs. 1994;9(2):107-13.

Furukoji E, Tanaka N, Yamashita A et al. Ecto-nucleoside triphosphate diphosphohydrolase inhibits ATP- and ADP-induced vasoconstriction. Thromb Res 2008; 121:583–585.

Grimm JW, Lu L, Hayashi T, Hope BT, Su TP, Shaham Y. Time-dependent increases in brain-derived neurotrophic factor protein levels within the mesolimbic dopamine system after withdrawal from cocaine: implications for incubation of cocaine craving. J Neurosci 2003; 23: 742-7.

Grunau RE, Oberlander TF, Whitfield MF, Fitzgerald C, Lee SK. Demographic and therapeutic determinants of pain reactivity in very low birth neonates at 32 weeks' postconceptional age. Pediatrics. 2001;107:105–112.

Grunau RE, Weinberg J, Whitfield MF. Neonatal procedural pain and preterm infant cortisol response to novelty at 8 months. Pediatrics. 2004;114:77–84.

Grunau RE. Long-term consequences of pain in human neonates. In: Anand KJS, Stevens BJ, McGrath PJ, eds. Pain in Neonates. Amsterdam, Netherlands: Elsevier Science; 2000: 55–76.

Grunau RV, Whitfield MF, Petrie JH, Fryer EL. Early pain experience, child and family factors, as precursors of somatization: a prospective study of extremely premature and fullterm children. Pain 1994;56:353e9.

Guinsburg R. Assessing and treating pain in the newborn. J Pediatr (Rio J) 1999;75(3):149-60.

Guinsburg R, de Araújo Peres C, Branco de Almeida MF, de Cássia Xavier Balda R, Cássia Berenguel R, Tonelotto J, Kopelman BI. Differences in pain expression between male and female newborn infants. Pain. 2000;85(1-2):127-33.

Guzmán D, Vázquez I, Brizuela N, Alvarez R, Mejía G, García E, et al. Assessment of oxidative damage induced by acute doses of morphine sulfate in postnatal and adult rat brain. Neurochem Res 2006; 31:549–554.

Halliwell B, Gutteridge JM. Free radicals and antioxidants in the year 2000. A historical look to the future. Annals of the New York Acad of Sci 2000; 899:136–147.

Headley PM, Grillner S. Excitatory amino acids and synaptic transmission: the evidence for a physiological function. Trends Pharmacol Sci. 1990; 11(5):205-11.

Hohn A, Leibrock J, Bailey K, Barde YA. Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature 1990; 344: 339-41.

Hovav E, Weinstock M. Temporal factors influencing the development of acute tolerance to opiates. J Pharmacol Exp Ther 1987; 242(1):251-256.

Howard BG, Huda A. Analgésicos Opióides. In: Alfred Goodman Gilman, Joel G. Hardman, Lee E. Limbird (eds). *The pharmacological basis of therapeutics*, 11ed. United States of America: McGRAW-HILL, Medical Publishing Division. 2001. p. 569-617.

International Association for the Study of Pain (IASP). Subcomittee on Taxonomy. Pain term: A list with definitions and notes on useage. Pain 1979; 6(2):249.

Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. Nat Rev Drug Discov. 2006;5(3):247-64.

Jakowec MW, Fox AJ, Martin LJ, Kalb RG. Quantitative and qualitative changes in AMPA receptor expression during spinal cord development. Neurosci 1995; 67(4):893-907.

Johnson PI, Napier TC. Ventral pallidal injections of a mu antagonist block the development of behavioral sensitization to systemic morphine. Synapse 2000; 38: 61-70.

Johnston CC, Collinge JM, Henderson SJ, Anand KJS. A cross-sectional survey of pain and pharmacological analgesia in Canadian neonatal intensive care units. Clin J Pain. 1997; 13:308–312

Johnston CC, Stevens BJ. Experience in a neonatal intensive care unit affects pain response. Pediatrics. 1996;98:925–930.

Johnston CC, Strada ME. Acute pain response in infants: a multidimensional description. Pain. 1986;24:373–382.

Jones SE. Effects of psychological processes on chronic pain. Brit J Nurs 1993; 2:463-467.

Jordan BA, Devi LA. G-protein-coupled receptor heterodimerization modulates receptor function. Nature 1999;399(6737):697-700.

Kar S, Quirion R. Neuropeptide receptors in developing and adult rat spinal cord: an in vitro quantitative autoradiography study of calcitonin gene-related peptide, neurokinins, mu-opioid, galanin, somatostatin, neurotensin and vasoactive intestinal polypeptide receptors. J Comp Neurol. 1995; 354(2):253-81.

Kavaliers M, Hirst M, Teskey GC. Ageing, opioid analgesia and the pineal gland. Life Sci. 1983; 32(19):2279-87.

Kavashima Y, Toshiro N, Ninomiya H. Contribution of ecto-5'-nucleotidase to the inhibition of the platelet aggregation by human endothelial cells. Blood 2000; 96:2157-2162.

Kehrer JP. The Haber-Weiss reaction and mechanisms of toxicity. Toxicology 2000; 149:43-50.

Kiryu S, Yao GL, Morita N, Kato H, Kiyama H. Nerve injury enhances rat neuronal glutamate transporter expression: identification by differential display PCR. J Neurosci 1995; 15(12):7872-8.

Kosterlitz HW, Lord JA, Paterson SJ, et al. Effects of changes in the structure of enkephalins and of narcotic analgesic drugs on their interactions with mu- and delta-receptors. Br J Pharmacol 1980;68(2):333-42.

Kroemer G. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat Med 1997; 3:614–620.

Kunapuli SP, Daniel JL. P2 receptor subtypes in the cardiovascular system. Biochem J 1998; 336:513–523.

Kuribara H. Modification of morphine sensitization by opioid and dopamine receptor antagonists: evaluation by studying ambulation in mice. Eur J Pharmacol 1995; 275:251-8.

Lakin ML, Miller CH, Stott ML, Winters WD. Involvement of the pineal gland and melatonin in murine analgesia. Life Sci. 1981; 29(24):2543-51.

Laurido C, Pelissie T, Soto-Moyano R, Valladares L, Flores F, Hernández A. Effect of melatonin on rat spinal cord nociceptive transmission. Neuroreport. 2002; 13: 89-91.

Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P, et al. Molecular cloning and expression of brain-derived neurotrophic factor. Nature 1989; 341:149-52.

Lewy AJ, Emens J, Jackman A, Yuhas K. Circadian uses of melatonin in humans. Chronobiol Int. 2006; 23(1-2):403-12.

Liang J, Zheng X, Chen J, Li Y, Xing X, Bai Y. Roles of bdnf, dopamine d(3) receptors, and their interactions in the expression of morphine-induced context-specific locomotor sensitization. Eur Neuropsychopharmacol 2011; 21:825–834.

Li P, Tong C, Eisenach JC, Figueroa JP. NMDA causes release of nitric oxide from rat spinal cord in vitro. Brain Res. 1994; 637(1-2):287-91.

Li Y, Jia YC, Cui K, Li N, Zheng ZY, Wang YZ, Yuan XB. Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. Nature 2005; 43:4 894– 898.

Loeser JD, Butler SH, Chapman CR, Turk DC (eds). Bonica's Management of Pain. 3ed. Philadelphia: Lippincott Williams & Wilkins, 2001.

Loftis JM, Janowsky A. The N-methyl-D-aspartate receptor subunit NR2B: localization, functional properties, regulation, and clinical implications. Pharmacol Ther 2003; 97(1):55-85.

Marsh DF, Hatch DJ, Fitzgerald M. Opioid system and the newborn. Br J Anaesth 1997:79:787-95.

Millan MJ. The induction of pain: an integrative review. Prog Neurobiol 1995; 57(1): 1-164.

Mawrin C, Pap T, Pallas M, Dietzmann K, Behrens-Baumann W, Vorwerk CK. Changes of retinal glutamate transporter GLT-1 mRNA levels following optic nerve damage. Mol Vis 2003; 9:10-3.

McGough NN, He DY, Logrip ML, Jeanblanc J, Phamluong K, Luong K, et al. RACK1 and brain-derived neurotrophic factor: a homeostatic pathway that regulates alcohol addiction. J Neurosci 2004; 24:10542-52.

Medeiros LF, Rozisky JR, de Souza A, Hidalgo MP, Netto CA, Caumo W, Battastini AM, Torres IL. Lifetime behavioural changes after exposure to anaesthetics in infant rats. Behav Brain Res. 2011; 218(1):51-6.

Medeiros LF, de Souza AC, de Souza A, Cioato SG, Scarabelot VL, Caumo W, Fernandes LC, Torres IL. Fentanyl administration in infant rats produces long-term behavioral responses. Int J Dev Neurosci. 2012; 30(1):25-30.

Mello R, Dickenson AH. Spinal cord mechanisms of pain. Br J Anaesth. 2008;101(1):8-16

Mennerick S, Shen W, Xu W, Benz A, Tanaka K, Shimamoto K, Isenberg KE, Krause JE, Zorumski CF. Substrate turnover by transporter curtails synaptic glutamate transients. J Neurosci 1999; 19(21):9242-51.

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG. Dopamine receptors: from structure to function. Physiol Rev. 1998;78(1):189-225

Moody CA, Robinson SR, Spear LP, Smotherman WP. Fetal behavior and the dopamine system: activity effects of D1 and D2 receptor manipulations. Pharmacol Biochem Behav 1993; 44(4):843-50.

Morgan GE, Mikhail MS. Pain Management. In: Morgan GE, Mikhail MS (eds). Clinical Anesthesiology. 2ed. Stamford: Appleton & Lange, 1996: 274-316.

Nandi R, Fitzgerald M. Opioid analgesia in the newborn. Eur J Pain. 2005; 9(2):105-8.

of the 8th World Congress on Pain. Seattle, WA: IASP Press; 1997:783–794.

Niederberger E, Schmidtko A, Coste O, et al, The glutamate transporter GLAST is involved in spinal nociceptive processing. Biochem Biophys Res Commun 2006; 346:393–399.

Niederberger E, Schmidtko A, Rothstein JD, et al, Modulation of spinal nociceptive processing through the glutamate transporter GLT-1. Neurosci 2003; 116:81–87.

Oberlander TF, Grunau RE, Whitfield MF, Fitzgerald C, Pitfield S, Saul JP. Biobehavioral pain responses in former extremely low birth weight infants at four months' corrected age. Pediatrics. 2000;105(1).

Oses JP, Cardoso CM, Germano RA et al. Soluble NTPDase: an additional system of nucleotide hydrolysis in rat blood serum. Life Sci 2004; 74:3275–3284.

Ottersen OP, Storm-Mathisen J. Glutamate- and GABA-containing neurons in the mouse and rat brain, as demonstrated with a new immunocytochemical technique. J Comp Neurol. 1984; 229(3):374-92.

Ozawa S, Kamiya H, Tsuzuki K. Glutamate receptors in the mammalian central nervous system. Prog Neurobiol. 1998; 54(5):581-618.

Pasternak GW, Simantov R, Snyder SH. Characterization of an endogenous morphine-like factor (enkephalin) in mammalian brain. Mol Pharmacol 1976;12(3):504-13.

Pattinson D, Fitzgerald M. The neurobiology of infant pain: Development of excitatory and inhibitory neurotransmission in the spinal dorsal horn. Reg Anesth Pain Med 2004; 29:36-44.

Pert CB, Snyder SH. Opiate receptor: demonstration in nervous tissue. Science 1973;9;179(4077):1011-4.

Porter FL, Wolf CM, Gold J, Lotsoff D, Miller JP. Pain and pain management in newborn infants: a survey of physicians and nurses. Pediatrics. 1997;100:626–632.

Porter FL, Wolf CM, Miller JP. Procedural pain in newborn infants: the influence of intensity and development. Pediatrics 1999;104(1).

Praag HV, Frenk H. Evidence for opiate tolerance en newborn rats. Dev Brain Res 1991; 60:99-102.

Rahman W, Fitzgerald M, Aynsley-Green A, Dickenson AH. The effects of neonatal exposure to inflammation and/or morphine on neuronal responses and morphine analgesia in adult rats. In: Jensen TS, Turner JA, Wiesenfeld-Hallin Z, eds. Proceedings

Ralevic V, Burnstock G. Receptors for purines and pyrimidines. *Pharmacol Rev* 1998; 50(3):413-492.

Ribelayga C, Gauer F, Pe´vet P et al. Ontogenesis of hydroxyindole-O-methyltransferase gene expression and activity in the rat pineal gland. Dev Brain Res 1998; 110:235–239.

Robson SC,Wu Y, Sun XF, Knosalla C, Dwyer K, Enjyoji K. Ectonucleotidases of CD39 family modulate vascular inflammation and thrombosis in transplantation. Semin Thromb Hemost 2005; 31:217–23.3

Robson SC, Sévigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signalling 2006; 2:409-430.

Rozisky JR, da Silva RS, Adachi LS, Capiotti KM, Ramos DB, Bogo MR, Bonan CD, Sarkis JJ, Torres IL.. Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral cortex of rats. Eur J Pharmacol 2010; 642(1-3):72-6.

Rozisky JR, Dantas G, Adachi LS, Alves VS, Ferreira MB, Sarkis JJ, Torres IL. Long-term effect of morphine administration in young rats on the analgesic opioid response in adult life. Int J Dev Neurosci. 2008; 26(6):561-5.

Rozisky JR, da Silva RS, Adachi LS, Capiotti KM, Ramos DB, Bogo MR, Bonan CD, Sarkis JJ, Torres IL. Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral cortex of rats. Eur J Pharmacol 2010; 642(1-3):72-6.

Ribelayga C, Gauer F, Pe´vet P et al. Ontogenesis of hydroxyindole-O-methyltransferase gene expression and activity in the rat pineal gland. Dev Brain Res 1998; 110:235–239.

Rubio A, Guerrero JM, Reiter RJ et al. Involvement of a- and b-adenergic receptors in the regulation of rat pineal N-acetyltransferase activity during development. Endocinology 1993; 132:393–398.

Ruda MA, Ling QD, Hohmann AG, Peng YB, Tachibana T. Altered nociceptive neuronal circuits after neonatal peripheral inflammation. Science. 2000;289:628–631.

Schrott LM, Franklin LM, Serrano PA. Prenatal opiate exposure impairs radial arm maze performance and reduces levels of BDNF precursor following training. Brain Res. 2008; 1198:132-40.

Simons SH, Tibboel D. Pain perception development and maturation. Semin Fetal Neonatal Med. 2006;11(4):227-31.

Smale R. Addiction and creativity: from laudanum to recreational drugs. J Psychiatr Ment Health Nurs 2001;8(5):459-63.

Smith RP, Gitau R, Glover V, Fisk NM. Pain and stress in the human fetus. Eur J Obstet Gynecol Reprod Biol. 2000;92:161–165.

Shelton MK, McCarthy KD. Mature hippocampal astrocytes exhibit functional metabotropic and ionotropic glutamate receptors in situ. Glia. 1999; 26(1):1-11.

Stehle JH, Foulkes NS, Pe´vet P et al. Developmental maturation of pineal gland function: synchronized CREM inductibility and adrenergic stimulation. Mol Endocrinol 1995; 9:706–716.

Steinhäuser C, Gallo V. News on glutamate receptors in glial cells. Trends Neurosci. 1996;19(8):339-45.

Stehle JH, Foulkes NS, Pe´vet P et al. Developmental maturation of pineal gland function: synchronized CREM inductibility and adrenergic stimulation. Mol Endocrinol 1995; 9:706–716.

Sung B, Na HS, Kim YI, Yoon YW, Han HC, Nahm SH, Hong SK. Supraspinal involvement in the production of mechanical allodynia by spinal nerve injury in rats. Neurosci Lett 1998; 246(2):117-9.

Suresh S, Anand KJ. Opioid tolerance in neonates: a state-of-the-art review. Paediatr Anaesth. 2001; 11(5):511-21.

Sweeney MI, White TD, Jhamandas KH, Sawynok J. Morphine releases endogenous adenosine from the spinal cord in vivo. Eur J Pharmacol 1987; 141: 169–170.

Todorov LD, Mihaylova-Todorova S, Westfall TD, Sneddon P, Kennedy C, Bjur RA, et al. Neuronal release of soluble nucleotidases and their role in neurotransmitter inactivation. Nature.1997;387(6628): 76-9.

Torres IL, Battastini AM, Buffon A, Fürstenau CR, Siqueira I, Sarkis JJ, Dalmaz C, Ferreira MB. Ecto-nucleotidase activities in spinal cord of rats changes as function of age. Int J Dev Neurosci. 2003; 21(8):425-9.

Turchan-Cholewo J, Dimayuga VM, Gupta S, Gorospe RM, Keller JN, Bruce-Keller AJ. NADPH oxidase drives cytokine and neurotoxin release from microglia and macrophages in response to HIV-Tat. Antioxid Redox Signal 2009; 11:193-204.

Unterwald EM and Cuntapay M. Dopamine-opioid interactions in the rat striatum: a modulatory role for dopamine D1 receptors in delta opioid receptor-mediated signal transduction. Neuropharmacology 2000; 39:372-381.

Urch CE, Rahman W, Dickenson AH. Electrophysiological studies on the role of the NMDA receptor in nociception in the developing rat spinal cord. Brain Res Dev Brain Res. 2001;126(1):81-9.

Vanderschuren LJ, Kalivas PW. Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: A critical review of preclinical studies. Psychopharmacology (Berl.) 2000; 151: 99-120.

Vries DTJ, Schoffelmeer AN, Binnekade R, Mulder AH, Vanderschuren LJ. Drug-induced reinstatement of heroin- and cocaine-seeking behavior following long-term extinction is associated with expression of behavioral sensitization. Eur J Neurosci 1998; 10: 3565-3571.

Wall PD, Melzack R. Textbook of Pain. 3ed. Edinburg: Churchill Livingstone1994: 1524.

Weng HR, Aravindan N, Cata JP, Chen JH, Shaw AD, Dougherty PM. Spinal glial glutamate transporters downregulate in rats with taxol-induced hyperalgesia. Neurosci Lett 2005; 386(1):18-22.

Weng HR, Chen JH, Cata JP, Inhibition of glutamate uptake in the spinal cord induces hyperalgesia and increased responses of spinal dorsal horn neurons to peripheral afferent stimulation. Neurosci 2006;138(4):1351-60.

White N, Burnstock G. P2 receptors and cancer. Trends Pharmacol Sci 2006; 27(4):211-7.

Whitfield MF, Grunau RE. Behavior, pain perception, and the extremely low-birth weight survivor. Clin Perinatol. 2000;27:363–379.

Wright AD. The history of opium. Trans Stud Coll Physicians Phila 1961;29(1):22-7.

Yang C, Chen Y, Tang L, Wang ZJ. Haloperidol disrupts opioid antinociceptive tolerance and physical dependence. J Pharmacol Exp Ther 2011; 338(1):164-72.

Yang C, Chen Y, Tang L, Wang ZJ. Haloperidol disrupts opioid antinociceptive tolerance and physical dependence. J Pharmacol Exp Ther 2011; 338(1):164-72.

Yoon SS, Lee BH, Kim HS, Choi KH, Yun J, Jang EY, et al. Potential roles of GABA receptors in morphine self-administration in rats. Neurosci Lett 2007; 428:33-37.

You HJ, Mørch CD, Chen J, Arendt-Nielsen L. Role of central NMDA versus non-NMDA receptor in spinal withdrawal reflex in spinal anesthetized rats under normal and hyperexcitable conditions. Brain Res. 2003; 981(1-2):12-22.

Zhang AZ, Pasternak GW. Ontogeny of opioid pharmacology and receptors: high and low affinity site differences. Eur J Pharmacol 1981; 73:29-40.

Zhang HN, Ko MC. Seizure activity involved in the up-regulation of BDNF mRNA expression by activation of central mu opioid receptors. Neurosci 2009;161: 301-10.

Zimmermann H, Braun N. Ecto-nucleotidases–molecular structures, catalytic properties, and functional roles in the nervous system. Prog Brain Res 1999; 120:371–385.

Zimmermann H. Ecto-nucleotidases. Purinergic and pyrimidergic signalling. In: Abbracchio MP, Williams M (eds). Handbook of experimental pharmacology. New York: Springer, Berlin Heidelberg; 2001. p. 209-250.

PARTE II

ARTIGO 1

Morphine exposure in early life increases nociceptive behavior in a rat formalin tonic pain model in adult life.

Brain Research 2011; 1367:122-129

Morphine exposure in early life increases nociceptive behaviour in a rat formalin tonic pain model in adult life.

JOANNA RIPOLL ROZISKY[1,2,4], LICIANE FERNANDES MEDEIROS[2,4], LAUREN SPEZIA ADACHI[2], JANAÍNA ESPINOSA[2], ANDRESSA DE SOUZA[1,2,4], ALBERTO SETTE NETO[2], CARLA DENISE BONAN[3], WOLNEI CAUMO[1,2], IRACI LUCENA DA SILVA TORRES[1,2,4*]

[1]Programa de Pós-Graduação em Medicina – Ciências Médicas – Universidade Federal do Rio Grande do Sul – CEP 90035-003 – Porto Alegre – Brazil

[2]Laboratório de Farmacologia, Dor e Neuromodulação – Departamento de Farmacologia – Instituto de Ciências Básicas da Saúde - Universidade Federal do Rio Grande do Sul – CEP 90050–170 – Porto Alegre – Brazil

[3]Laboratório de Neuroquímica e Psicofarmacologia – Departamento de Biologia Celular e Molecular – Faculdade de Biociências - Pontifícia Universidade Católica do Rio Grande do Sul, CEP 90619-900 – Porto Alegre – Brazil.

[4]Unidade de Experimentação Animal e Grupo de Pesquisa e Pós-Graduação – Hospital de Clínicas de Porto Alegre – CEP 90035-003 – Porto Alegre – Brazil.

24 pages, 8 figures

[*]CORRESPONDENCE ADDRESS:

IRACI LUCENA DA SILVA TORRES

Departamento de Farmacologia - ICBS, UFRGS.

Rua Sarmento Leite, 500 sala 202.

90050-170 - Porto Alegre, RS, Brazil.

Phone: 0055-51 3316 3183; Fax: 0055-51 3316 3121.

E-mail: iracitorres@gmail.com

Abstract

Considering the importance of a deeper understanding of the effect throughout life of opioid analgesia at birth our objective was to determine whether morphine administration in early life, once a day for seven days in 8-day-old rats, alters the nociceptive response over the short- (P16), medium- (P30), and long-term (P60), and to evaluate which system is involved in the altered nociceptive response. The nociceptive responses were assessed by the formalin test and the behaviour analyzed was the total time spent in biting and flicking of the formalin-injected hindpaw, recorded during the first 5 min (phase I) and from 15-30 min (phase II). The morphine group showed no change in nociceptive response at P16, but at P30 and P60 the nociceptive response was increased in phase I, and in both phases, respectively. At P30 and P60 the animals received a non-steroidal anti-inflammatory drug (indomethacin) or NMDA receptor antagonist (ketamine) 30 min before the formalin test. The increase in the nociceptive response was completely reversed by ketamine, and partially by indomethacin. These results indicate that early morphine exposure causes an increase in the nociceptive response in adult life. It is possible that this lower nociception threshold is due to neuroadaptations in nociceptive circuits, such as the glutamatergic system. Thus, this work demonstrates the importance of evaluating clinical consequences related to early opioid administration and suggests a need for a novel design of agents that may counteract opiate-induced neuroplastic changes.

Section: Neurophysiology, Neuropharmacology and other forms of Intercellular Communication

Keywords: analgesic response; formalin test; morphine; nociception; neonate rats.

1. **Introduction**

Opioid analgesics, such as morphine, are the most effective and frequently used substances for the relief of moderate to severe pain. The use of these analgesics has increased in the Neonatal Intensive Care Unit over the last few decades as a consequence of changes and advances in the understanding, identification, and treatment of pain in children (De Lima et al., 1996; El Sayed et al., 2007; Suresh & Anand, 2001). In addition, improvements in short- and long-term clinical outcomes of critically ill neonates have necessitated the widespread use of opioid drugs for analgesia and sedation (Suresh & Anand, 2001). However, the consequences for the development of neurophysiological systems remain unknown.

The efficacy of morphine in reducing pain in neonatal animals has already been demonstrated (Nandi & Fitzgerald, 2005; Rozisky et al., 2008). Although descending inhibitory mechanisms are not completely formed until the third week of life (Nandi & Fitzgerald, 2005), morphine and other opioid receptor agonists are effective analgesics during the early neonatal period due to the presence of spinal opioid receptors from birth (Rahman & Dickenson, 1999). In a previous study by our group, using the tail-flick test (a measure of the pain threshold at the spinal level), we observed that animals in the second week of life showed an increased response to repeated morphine administration without developing tolerance. However, at P80 rats showed 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 the same age (P80) (Rozisky et al., 2008). These results indicate that early morphine exposure lead to the development of an altered opioid analgesic response that may be

expressed into adulthood. Although such effects are described in the literature, the precise mechanisms that underlie the long-term consequences of chronic opioid treatment in the neonatal period have not been thoroughly investigated, and the use of young animal models to evaluate the long-term effect of morphine on nociceptive systems has not been widely explored.

As described above, previous tests carried out by our group demonstrated an altered nociceptive response in the tail-flick test in animals that received morphine in the second week of life, but it is important to further evaluate the nociception in these animals using other nociceptive tests. To investigate the possible mechanisms underlying this response we selected one of the most widely-used animal models to assess the response generated by injured tissue which mimics some features of post-injury pain and is thus considered to be more relevant to clinical pain states than phasic pain, bridging the gap between acute and chronic pain (Tjølsen et al., 1992).

Considering the relevance of the subject, the aim of this study was to investigate whether repeated morphine exposure during early life alters the neurogenic and inflammatory pain in the short- (P16), medium- (P30) and long-term (P60) using the formalin test, as well as to investigate the possible mechanisms involved in these changes.

2. Results

2.1. Short, medium and long-term effects of repeated morphine administration on the nociceptive behaviour induced by formalin:

After daily morphine exposure, from P8 to P14, the nociceptive behaviours were compared between the control and morphine groups at P16, P30 and P60. The subcutaneous injection of 2% formalin into the plantar region of the hindpaw of animals of all ages and in all groups resulted in behavioural responses, such as biphasic licking, biting, and flicking of the injected paw. At P16, two days after the end of repeated morphine exposure, there were no differences between the groups of animals for either phase (phase I: F= 0.69; phase II: F= 0.05, Student's t test, P>0.05 for both phases; Fig. 2A). At P30, the morphine group showed a stronger nociceptive response in phase II (phase I: F=1.16, Student's t test, P>0.05; phase II: F=1.21, Student's t test, P<0.05; Fig. 2B). At P60, the morphine group showed a stronger nociceptive response in both phases of the formalin test (phase I: F= 0.018; phase II: F= 0.035, Student's t test, P<0.05 for both phases; Fig. 2C).

Insert Figures 2A, 2B and 2C here

2.2. Effects of indomethacin administration on behaviour in the formalin test at P30 and P60 after repeated morphine administration:

After daily morphine exposure, from P8 to P14, we investigated whether an injection of indomethacin 30 min before the formalin test was able to reverse the increased nociceptive behaviour at P30 and P60 in the morphine group compared to the control group. Our results demonstrated that at P30 the control-indomethacin (C-Indomethacin) and morphine-indomethacin (M-indomethacin) animals experienced a decrease in the nociceptive response in both phases of the test when compared to control-vehicle I (C-vehicle I) and morphine-vehicle I (M-vehicle I) (phase I: F=29.0, phase II: F=22.65,

one-way ANOVA, Bonferroni's test, $P<0.05$ for both phases; Fig. 3A). However, the morphine-indomethacin group presented a more intense nociceptive response when compared to control-indomethacin in both phases of the test (one-way ANOVA, Bonferroni's test, $P<0.05$; Fig. 3A). The morphine-vehicle I group showed no difference in the nociceptive response compared to the control-vehicle I group in phase I (one-way ANOVA, $P>0.05$), but it presented a more intense nociceptive response in phase II when compared to all groups (one-way ANOVA/Bonferroni's test $P<0.05$, Fig. 3A).

At P60, we observed a pattern of nociceptive behaviour similar to the responses recorded at P30 for all groups in both phases (phase I: $F=6.4$, phase II: $F=12.52$, one-way ANOVA, Bonferroni's test, $P>0.05$, Fig. 3B). However, the morphine-vehicle I group presented a more marked nociceptive response in phases I and II when compared to other groups (one-way ANOVA/Bonferroni's test, $P<0.05$, Fig. 3B).

Insert Figures 3A and 3B here

2.3. Effects of ketamine administration on the formalin test at P30 and P60 after repeated morphine administration:

The administration of ketamine 30 min before the formalin test prevented the higher nociceptive response observed in the morphine group compared to the control group, at P30 and P60. Our results show that at P30, the control-ketamine (C-ketamine) and morphine-ketamine (M-ketamine) groups presented decreased nociceptive responses in both phases of the test when compared to the control-vehicle II (C-vehicle

II) and morphine-vehicle II (M-vehicle II) groups (phase I: F=7.97, phase II: F= 79.28, one-way ANOVA, Bonferroni's test, P<0.05 for both phases; Fig. 4A). However, the morphine-ketamine group exhibited a less marked nociceptive response when compared to the control-ketamine group in both phases of the test (one-way ANOVA, Bonferroni's test, P<0.05; Fig. 4A). The morphine-vehicle II group, in turn, presented a similar nociceptive response to that of the control-vehicle II group in phase I (one-way ANOVA, P>0.05), but a higher nociceptive response in phase II when compared to all groups (one-way ANOVA/Bonferroni's test P<0.05, Fig. 4A).

At P60, we observed a pattern of nociceptive response similar to that seen at P30 for all groups in both phases (one-way ANOVA, Bonferroni's test, P<0.05, Fig. 4B). However, the morphine-vehicle II group presented a more intense nociceptive response than all other groups in phase I and phase II (phase I: F=5.63, phase II: F=11.92, one-way ANOVA/Bonferroni's test, P<0.05 for both phases, Fig. 4B).

Insert Figures 4A and 4B here

---

3. Discussion

In this study, we demonstrated that rats that received morphine during the second week of life showed an increase in nociceptive behaviour in phase II of the formalin test at P30. This increased response was partially reversed by a non-steroidal anti-inflammatory drug (indomethacin) and completely reversed by an NMDA receptor antagonist (ketamine). Moreover, at P60 the morphine-treated animals showed an

increase in the nociceptive response in both phases of the formalin test (representing the neurogenic and inflammatory pain responses), which was also partially reversed by indomethacin and completely reversed by ketamine.

These results indicate that exposure to drugs in early life can have long-lasting implications for the development of the nervous system, such as permanent changes in pharmacological responses and cell signalling (for a review, see Stanwood & Levitt, 2004). Other investigations have uncovered the neurobiological consequences of repeated opioid administration and revealed possible neuroplastic adaptations that likely underlie opioid-induced paradoxical pain (Vanderah et al., 2000, 2001; Gardell et al., 2002). This may be a consequence of the structural and functional immaturity of the neonatal nervous system, and the significant changes in opioid analgesic mechanisms that occur before and after birth (Beland & Fitzgerald, 2001; Marsh et al., 1997; Rahman et al., 1998).

In the formalin test the rodent hindpaw presents a characteristic biphasic nociceptive response using both weighted pain measures (Dubuisson & Dennis, 1977) and continuous scoring systems (Wheeler-Aceto & Cowan, 1991). The transient early phase (occurring in the first 5–10 min) is interpreted as reflecting direct activation of nociceptive sensory afferents by formalin, while the tonic phase (expressed from 20 to 90 min) is regarded as depending on an ensuing inflammatory response, associated with central sensitization (Tjølsen et al., 1992; Coderre et al., 1993). Formalin can also activate central processes that lead to longer term events (over 3–4 weeks), such as the expression of immediate-early genes and activation of microglia, providing in this context a model of chronic pathological pain (Sawynok & Liu, 2004). Thus, the increase in formalin-induced nociceptive behaviour observed in this study suggests a

central hyperexcitability of the ascending second-order dorsal horn neurons induced by previous sustained exposure to morphine, and this is a long-term effect. Our results agree with those of Zissen and colleagues (2006 & 2007), who have demonstrated that while infant rats (P5 to P8) are more sensitive to the long-term changes in formalin-induced pain and mechanical thresholds following continuous exposure to morphine, when compared to young rats (P19 to P21) they are also better able to compensate for changes in mechanical thresholds following intermittent administration of morphine, given twice a day for 3 days. It is possible that short bouts of morphine withdrawal-induced excitation may off-set morphine-induced inhibition in infants, but not in young rats, and thus, may better maintain the balance of activity and inactivity during this crucial developmental phase.

Ossipov and colleagues (2005) showed that opioids can produce hyperalgesia under many circumstances, and that such effects might contribute to the drawbacks of acute and chronic administration of these drugs. Although the mechanisms of this phenomenon have not yet been fully clarified, research has shown that chronic exposure to opioids induces a change in the function of spinal cord neurons that can be manifested as neuronal hyperactivity during opiate withdrawal (Rohde et al., 1997; Vanderah et al., 2001; Gardell et al., 2006). Other studies have reported that repeated opioid exposure can lead to spinal cord neuroplasticity (for review, see Mao & Mayer, 2001) and these adaptations may involve changes in supraspinal pain modulatory circuits (Ossipov et al., 2005). Consistent with these results, we suggest that exposure to morphine in early life might lead to drug-induced adaptations in the excitatory pain pathways, such as neuroplastic changes at the receptor level and/or in the synthesis of

algesic substances (Yaksh et al., 1986) which may produce secondary hyperalgesic effects that increase the intensity of the pain (Celerier et al., 1999; Larcher et al., 1998).

The effect of ketamine seen here may be explained by activation of the glutamatergic system in opioid–mediated hyperalgesia (Sanford & Silverman, 2009). It is well accepted that persistent activation of the NMDA receptor by excitatory amino acids released from primary afferent terminals results in the sensitization of spinal neurons (Baranauskas & Nistri, 1998), and such NMDA receptor-mediated central sensitization is believed to drive enhanced nociception in chronic pain states and opioid-induced abnormal pain (Larcher et al., 1998; Laulin et al., 1999; Mao & Mayer, 2001). The involvement of excitatory neurotransmitters, mainly glutamate, in inflammatory nociception is supported by the increase in levels of these neurotransmitters in the dorsal root ganglion and dorsal horn, elicited by chronic inflammation (Wimalawansa, 1996; Löfgren et al., 1997; Ossipov et al., 2005). In addition, peripheral inflammation is capable of increasing the expression of subunits of the NMDA receptor and enhancing neurotransmitter release in CNS structures related to nociception (Zhuo, 2002; Zhao et al., 2006). Therefore, it is possible that the animals that received morphine in early life presented central sensitization in the medium- and long-term induced by changes in the glutamatergic system, and this may be responsible, at least in part, for the increase in nociceptive behaviour in phase II of the formalin test (which represents the inflammatory pain response) observed in this study. This explanation for the latter result is supported by the fact that an NMDA receptor antagonist (ketamine) completely eliminated the hyperalgesia induced by morphine exposure in early life.

In addition, indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), was unable to completely reverse the hyperalgesia resulting from early morphine treatment.

This suggests that there is an inflammatory component involved, but we can not discard other mechanisms that may contribute to the hyperalgesia observed in this study. Following on from previous studies which found that pre-treatment with an NSAID may increase spinal cord levels of kynurenic acid (an endogenous excitatory amino acid antagonist) (Edwards et al., 2000) and partially inhibit increases in spinal cord levels of *c-fos* (an immediate-early genetic marker of nociceptive activity) stimulated by application of the rat tail ischemia-reperfusion acute model of hyperalgesia (Lin et al., 2000), Grace and colleagues (2001) reported that subcutaneous injection of NSAIDs completely eliminated the hyperalgesic response elicited in rats by ischemic stimulation of the tail and suppressed the increased prostaglandin formation in the brains of the animals. However, the relief of hyperalgesia was short-lived and corresponded only to the first phase of the (spontaneous) hyperalgesia (Scheuren et al., 1997). In addition, PGE2 has been found in microdialysate of the spinal cord after injection of formalin in the paw of the rat (Malmberg & Yaksh, 1995; Scheuren et al., 1997), and its production was antagonized by systemic injection of paracetamol (Muth-Selbach et al., 1999) or by intrathecal injection of other NSAIDs (Malmberg & Yaksh, 1992). Direct evidence for a spinal antinociceptive action of NSAIDs derives from observations made in patients and animal experiments. It has been reported that intrathecal injection of acetylsalicylic acid, salicylic acid and indomethacin depressed the nociceptive activity that was evoked in thalamic neurons of rats by electrical stimulation of afferent C-type fibres in the sural nerve (Jurna et al., 1992).

The development of nociceptive pathways is an activity-dependent process (Fitzgerald and Jennings, 1999, Fitzgerald and Beggs, 2001, Beggs et al., 2002) and thus, abnormal activity such as that generated by early opioid exposure may alter

normal synaptic development producing changes in somatosensory processing and behaviour that would not occur in similarly exposed adults. Our group has demonstrated that neonatal rats may be more sensitive to low doses of morphine because there is extensive re-modelling of opioid receptor expression in the first 3 postnatal weeks (Rahman et al., 1998, Rahman and Dickenson, 1999, Beland and Fitzgerald, 2001). For example, at P14 spinal μ-opioid receptors (μORs) are limited to the dorsal horn, whereas they appear throughout the spinal grey matter at P7, and the density of binding is seen to decrease in the first three postnatal weeks, with peak binding at P7 that then falls to the adult level by P21. This abundance of μORs in early postnatal life could explain why exposure to morphine for 7 days, from P8 to P14, produces analgesia instead of tolerance (Rozisky et al., 2008). Thus, the greater expression of μORs at P7 in comparison to adult rats suggests a more widespread effect of morphine, acting both directly within the spinal cord and indirectly through larger termination profiles of primary afferents (Nandi et al., 2004). This, coupled with the over-expression of excitatory amino acid receptors, at the primary afferent-spinal cord synapse, supports a potential role for μORs in the normal maturation of nociceptive circuitry, and hence, disruption of this by exogenous administration of opioid agonists may have detrimental consequences for the maturation of pain circuitry (Thornton & Smith, 1998; Thornton et al., 2000). Activity-dependent processes drive the maturation of nociceptive C-fibres during this period of neurodevelopment (Beggs et al., 2002; Fitzgerald et al., 1994). In adult rats, Aδ and C, but not Aβ primary afferent fibres transmit painful stimuli. In contrast, in P7 rats Aβ primary afferents can also transmit such stimuli (Fitzgerald & Jennings, 1999). It has been hypothesized that increased activity in Aβ-fibres early in development may be modulated by sub-threshold C-fibre depolarisation that primes the

spinal cord for Aβ-fibre input (Dickenson & Rahman, 1999). Functionally, in adult rodents opioid agonists selectively inhibit Aδ- and C-fibre nociceptors but not Aβ-fibres (Dickenson et al., 1987; Rahman & Dickenson, 1999). In contrast, in young rats morphine can inhibit Aβ- and C-fibre-mediated activity in the lumbar spinal cord (Rahman et al., 1998) which parallels expression of μORs in both small (Aδ and C) and large (Aβ) diameter cell bodies in the dorsal root ganglion. Based on the results of our study, we suggest that at P16 the animals do not exhibit increased nociceptive behaviour in the formalin test because repeated exposure to a μOR agonist has influenced the development of C-fibres during maturation. However, we did observe that following the formalin test the treated animals presented an inflammation-like oedema in the formalin-injected hindpaw, which was measured and compared to the volume of the non-injected hindpaw by plethysmometry. It is interesting to note that there were no differences between the volume of formalin-injected hindpaws in the morphine and control groups (data not shown).

Taking into account the importance of a deeper understanding of the effects throughout life of opioid analgesia at birth, and that previous results from our group showed that morphine exposure in early life lead to changes in the analgesic response in adult life (Rozisky et al., 2008), we hypothesized that the use of opioids in early life can induce persistent changes in nociceptive and opioid analgesic responses. We conclude from the present results that the altered nociceptive response induced by repeated morphine exposure can change in an age-dependent manner. In addition, the altered nociceptive response was expressed until adulthood, and this effect was partially reversed by indomethacin and completely reversed by an NMDA receptor antagonist. However, it should be noted that the response is complex and unlikely to be

predominantly caused by any single mediator. Taken together, our data indicate that opioids elicit glutamatergic adaptations at the system level. Finally, the behavioural changes seen in response to repeated exposure to morphine during early life illustrate the need to examine nociceptive processing in neonatal patients who have been exposed to therapeutic morphine; moreover, this indicates the importance of evaluating the clinical consequences of long-term opioid administration. These findings also highlight the need for further studies involving the design of pharmacological approaches that may counteract opioid-induced neuroadaptations and subsequently prevent abnormal pain states.

4. Experimental procedure

4.1. Animals

Eight-day-old male Wistar rats were divided into two groups: saline-control (C) and morphine-treated (M). Naive animals were housed 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 (lights 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 P8 were chosen because it is accepted that animals of this age are at a similar stage of neurological development to that of a human newborn (Fitzgerald & Anand, 1993). It is also accepted that they are in a physiologically immature state (Pattinson & Fitzgerald, 2004) since this period is characterized by major developmental changes in the brain and plasticity of the

developing pain system (Bishop, 1982; Kim et al., 1996; Rabinowicz et al., 1996). Animal handling and all experiments were performed in accordance with international guidelines for animal welfare. The protocol of this experimental study was approved by the Ethics Committee of the institution where the work was conducted.

4.2. Pharmacological treatment

Each animal received saline (Control group) or morphine (5 μg s.c. in the mid-scapular area; Morphine group) starting at P8, then once a day for 7 days. This dose had been chosen based on a previous study by Rozisky and colleagues (2008, 2010), 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 millilitre of morphine sulphate (Dimorf® 10 mg/ml, obtained from Cristália, Porto Alegre, Rio Grande do Sul, Brazil) was diluted in 9 ml of 0.9% NaCl (saline). The formalin test was performed in 16-, 30-, and 60-day-old rats (Fig. 1). The number of animals used per group was 8 to 15.

At the ages where we observed significant differences in the nociceptive behaviour in the formalin test, the control and morphine groups were subdivided into four groups, each one designed to evaluate the effect of i.p. administration of an NMDA receptor antagonist or non-steroidal anti-inflammatory drug (NSAID), applied 30 minutes before the formalin test: 1) non-steroidal anti-inflammatory drug: 10 mg/kg of indomethacin (Indomethacin®, obtained from Sigma-Aldrich, São Paulo, Brazil) (Bastos et al., 2004) diluted in 1.29% sodium bicarbonate solution (control-indomethacin, morphine-indomethacin); 2) Vehicle for indomethacin (vehicle I): 10 mg/kg of 1.29% sodium bicarbonate solution (pH=7.4) (control-vehicle I, morphine-vehicle I); 3) NMDA receptor antagonist: 30 mg/kg of ketamine (Cetamine®, obtained

from Hospital de Clínicas de Porto Alegre, Brazil) (Campos et al., 2006) diluted in 0.9% saline (control-ketamine, morphine-ketamine); 4) Vehicle for ketamine (vehicle II): 30 mg/kg of saline (control-vehicle II, morphine-vehicle II) (Fig. 1). The number of animals used per group was 6 to 9.

Insert Figure-1 here

4.3. Formalin test

The formalin test was performed as previously described (Tjølsen et al., 1992; Tai et al., 2006) with minor modifications. Twenty-four hours before the test each animal was placed in the chamber for ten minutes to familiarize them with the procedure, since the novelty of the apparatus itself can induce antinociception (Netto et al., 2004). The animals were injected s.c. on the plantar surface of the left hindpaw with 0.17 ml/kg of a 2% formalin solution (Formaldehyde P.A.®, obtained from Sigma-Aldrich, São Paulo, Brazil) diluted in 0.9% NaCl (saline). Each animal was observed in a varnished wood cage, measuring 60 x 40 x 50 cm, with the inside lined with glass, and the nociceptive response was recorded for a period of 30 min. This test produces two distinct phases of nociceptive behaviour: an early, transient phase (phase I; up to 5 min after the injection) and a late, persistent phase (phase II; 15-30 min after the injection). Phase I has been considered to reflect direct stimulation of primary afferent fibres, predominantly C-fibres (neurogenic pain) (Martindale et al., 2001), whereas phase II is dependent on peripheral inflammation (inflammatory pain) (Dubuisson & Dennis, 1977; Shibata et al., 1989; Tjølsen et al., 1992). The total time (seconds) spent in licking,

biting, and flicking of the formalin-injected hindpaw was recorded in phases I and II. The test was performed once only in each rat.

4.5. Statistical analysis

Data were expressed as means ± standard error of the mean (SEM). Depending on the experiment, Student's *t* test or one-way ANOVA was performed, followed by a multiple comparisons test (Bonferroni's test) when indicated. Differences were considered statistically significant if $P < 0.05$.

Acknowledgements: This work was supported by the following Brazilian funding agencies: Graduate Research Group (GPPG) at Hospital de Clínicas de Porto Alegre (Dr ILS Torres; Grant no. 08345); Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (ILS Torres); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (JR Rozisky); Fundação de Amparo à Pesquisa do Rio Grande do Sul - FAPERGS (LN Adachi) and Pró-Reitoria de Pesquisa - Universidade Federal do Rio Grande do Sul - PROPESQ-UFRGS (AS Neto). We would like to thanks to Dr Gareth Cuttle for the English correction and editing of the manuscript.

References

Baranauskas, G., Nistri, A., 1998. Sensitization of pain pathways in the spinal cord: cellular mechanisms. Prog Neurobiol. 54, 349-365.

Bastos, L.C., Veiga, A.B.G., Guimarães, J.A., Tonussi, C.A., 2004 Nociceptive and edematogenic responses elicited by a crude bristle extract of Lonomia obliqua caterpillars. Toxicon. 43, 273–278.

Beggs, S., Torsney, C., Drew, L.J., Fitzgerald, M., 2002. The postnatal reorganization of primary afferent input and dorsal horn cell receptive fields in the rat spinal cord is an activity-dependent process. Eur. J. Neurosci. 16, 1249–1258.

Beland, B., Fitzgerald, M., 2001. Mu- and delta opioid receptors are down-regulated in large primary sensory neurons during postnatal development in rats. Pain 90, 143-150.

Bishop, B., 1982. Neural plasticity. Part 2. Postnatal maturation and function induced plasticity. Phys Ther. 62, 1132–1143.

Campos, A.R., Santos, F.A., Rao, V.S., 2006 Ketamine-Induced Potentiation of Morphine Analgesia in Rat Tail-Flick Test: Role of Opioid-, a2-Adrenoceptors and ATP-Sensitive Potassium Channels. Biol. Pharm Bull. 29, 86-89.

Celerier, E., Laulin, J., Larcher, A., Le Moal, M., Simonnet, G., 1999. Evidence for opiate-activated NMDA processes masking opiate analgesia in rats. Brain Res. 847, 18-25.

Coderre, T.J., Fundytus, M.E,, McKenna, J.E., Dalal, S., Melzack, R.. 1993. The formalin test: a validation of the weighted-scores method of behavioural pain rating. Pain 54, 43-50.

De Lima, J., Lloyd-Thomas, A.R., Howard, R.F., Sumner, E., Quinn, T.M., 1996. Infant and neonatal pain: anaesthetists' perceptions and prescribing patterns. BMJ. 313, 787.

Dickenson, A.H., Sullivan, A.F., Knox, R., Zajac, J.M., Roques, B.P., 1987. Opioid receptor subtypes in the rat spinal cord: electrophysiological studies with mu- and delta-opioid receptor agonists in the control of nociception. Brain Res. 413, 36–44.

Dickenson, A., Rahman, W., 1999. Mechanisms of chronic pain and the

developing nervous system, in: P. McGrath (Ed.), Chronic and Recurrent Pain in children and Adolescents, vol. 13, IASP Press, Seattle, pp. 5–38.

Dubuisson, D., Dennis, S.G., 1977. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 4, 161-74.

Edwards, S.R., Mather, L.E., Lin, Y., Power, I., Cousins, M.J., 2000. Concurrent determination of glutamate and kynurenate in the rat central nervous system following treatment with diclofenac or tail ischaemia. J Pharm Pharmacol. 52, 1-8.

El Sayed, M.F., Taddio, A., Fallah, S., De Silva, N., Moore, A.M., 2007. Safety profile of morphine following surgery in neonates. J Perinatol. 27, 444-7.

Fitzgerald, M., Anand, K.J., 1993. Developmental neuroanatomy and neurophysiology of pain. In: Schechter, N.L., Berde, C.B., Yaster, M., editors. Pain in infants, children, and adolescents. ed. Williams & Wilkins, Baltimore, pp. 11-31.

Fitzgerald, M., Butcher, T., Shortland, P., 1994. Developmental changes in the laminar termination of A fibre cutaneous sensory afferents in the rat spinal cord dorsal horn. J. Comp. Neurol. 348, 225–233.

Fitzgerald, M., Jennings, E., 1999. The postnatal development of spinal sensory processing, Proc. Natl. Acad Sci. U.S.A. 96, 7719–7722.

Fitzgerald, M., Beggs, S., 2001. The neurobiology of pain: developmental aspects. Neuroscientist 7, 246–257.

Gardell, L. R., Wang, R., Burgess, S. E., Ossipov, M. H., Vanderah, T. W., Malan, T. P. Jr., Lai, J., Porreca, F.J., 2002. Sustained morphine exposure induces a spinal dynorphin-dependent enhancement of excitatory transmitter release from primary afferent fibres. Neurosci. 22, 6747-6755.

Gardell, L.R., King, T., Ossipov, M.H., Rice, K.C., Lai, J., Vanderah, T.W., Porreca, F., 2006. Opioid receptor-mediated hyperalgesia and antinociceptive tolerance induced by sustained opiate delivery. Neurosci Lett. 396, 44-49.

Grace, .F., Lin, Y., Edwards, S.R., Power, I., Mather, L.E., 2001. Effects of diclofenac in the rat tail ischemia–reperfusion injury model of acute hyperalgesia. Pain 89, 117–125.

Jurna, I., Spohrer, B., Bock, R., 1992. Intrathecal injection of acetylsalicylic acid, salicylic acid and indomethacin depresses C fibre-evoked activity in the rat thalamus and spinal cord. Pain 49, 249–256.

Kim, J.J., Foy, M.R., 1996. Thompson RF. Behavioral stress modifies hippocampal plasticity through N-methyl-D-aspartate receptor activation. Proc Natl Acad Sci U.S.A. 93, 4750–4753.

Laulin, J.P., Celerier, E., Larcher, A., Le Moal, M., Simonnet, G., 1999. Opiate tolerance to daily heroin administration: an apparent phenomenon associated with enhanced pain sensitivity. Neurosci. 89, 631-636.

Larcher, A., Laulin, J.P., Celerier, E., Le Moal, M., Simonnet, G., 1998. Acute tolerance associated with a single opiate administration: involvement of N-methyl-D-aspartate-dependent pain facilitatory systems. Neurosci. 84, 583-589.

Lin, Y., Mather, L.E., Power, I., Cousins, M.J., 2000. Effect of diclofenac on expression of spinal cord c-fos-like immunoreactivity following ischemia-reperfusion induced hyperalgesia in the rat tail. Anesth Analg. 90, 1411-1415.

Löfgren, O., Yu, L.C., Theodorsson, E., Hansson, P., Lundeberg, T., 1997. Intrathecal CGRP (8-37) results in a bilateral increase in hindpaw withdrawal latency in rats with a unilateral thermal injury. Neuropeptides 31, 601-607.

Malmberg, A.B., Yaksh, T.L., 1992. Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. J Pharmacol Exp Ther. 263, 136–146.

Malmberg, A.B., Yaksh, T.L., 1995. Cyclooxygenase inhibition and the spinal release of prostaglandin E2 and amino acids evoked by paw formalin injection: a microdialysis study in unanesthetized rats. J Neurosci. 15, 2768–2776.

Mao, J., Mayer, D.J., 2001. Spinal cord neuroplasticity following repeated opioid exposure and its relation to pathological pain. Ann N Y Acad Sci. 933, 175-184.

Marsh, D.F., Hatch, D.J., Fitzgerald, M., 1997. Opioid system and the newborn. Br J Anaesth. 79, 787-795.

Martindale, J., Bland-Ward, P.A., Chessell, I.P., 2001. Inhibition of C-fibre mediated sensory transmission in the rat following intraplantar formalin. Neurosci Lett. 316, 33-36.

Muth-Selbach, U.S., Tegeder, I., Brune, K., Geisslinger, G. 1999. Acetaminophen inhibits spinal prostaglandin E2 release after peripheral noxious stimulation. Anaesthesiology 91, 231-239.

Nandi, R., Beacham, D., Middleton, J., Koltzenburg, M., Howard, R.F., Fitzgerald, M., 2004. The functional expression of mu opioid receptors on sensory neurons is developmentally regulated; morphine analgesia is less selective in the neonate. Pain 111, 38–50.

Nandi, R., Fitzgerald, M., 2005. Opioid analgesia in the newborn. Eur J Pain. 9, 105-108.

Netto, C.A., Siegfried, B., Izquierdo, I., 2004. Analgesia induced by exposure to a novel environment in rats: effect of a concurrent and post-training stressful

stimulation. Behav Neural Biol. 48.304-309.

Ossipov, M.H., Lai, J., King, T., Vanderah, T.W., Porreca, F., 2005. Underlying mechanisms of pronociceptive consequences of prolonged morphine exposure. Pept Sci. 80, 319-325.

Pattinson, D., Fitzgerald, M., 2004. The neurobiology of infant pain: Development of excitatory and inhibitory neurotransmission in the spinal dorsal horn. Reg Anesth Pain Med. 29, 36-44.

Rabinowicz, T., de Courten-Myers, G.M., Petetot, J.M., Xi, G., de los Reyes, E., 1996. Human cortex development: estimates of neural numbers indicate major loss late during gestation. J Neuropathol Exp Neurol. 55, 320–328.

Rahman, W., Dashwood, M.R., Fitzgerald, M., Aynsley-Green, A., Dickenson, A.H., 1998. Postnatal development of multiple opioid receptors in the spinal cord and development of spinal morphine analgesia. Dev Brain Res. 108, 239-254.

Rahman, W., Dickenson, A.H., 1999. Development of spinal opioid systems. Reg Anesth Pain Med. 24, 383-385.

Rohde, D.S., McKay, W.R., Abbadie, C., Basbaum, A.I., 1997. Contribution of sacral spinal cord neurons to the autonomic and somatic consequences of withdrawal from morphine in the rat. Brain Res. 745, 83-95.

Rozisky, J.R., Dantas, G., Adachi, L.S., Alves, V.S., Ferreira, M.B.C., Sarkis, J.J., Torres, I.L.S., 2008. Long-term effect of morphine administration in young rats on the analgesic opioid response in adult life. Int J Dev Neurosci. 26, 561-565.

Rozisky, J.R., Souza, R.S., Adachi, L.S., Capiotti, K.M., Ramos, D.B., Bogo, M.R., Bonan, C.D., Sarkis, J.J.F., Torres, I.L.S., 2010. Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral

cortex of rats. Eur J Pharmacol. 642, 72-76.

Sanford, M., Silverman, M.D., 2009. Opioid Induced Hyperalgesia: Clinical Implications for the Pain Practitioner. Pain Physician 12, 679-684.

Sawynok, J., Liu, X.J., 2003. Adenosine in the spinal cord and periphery: release and regulation of pain. Prog Neurobiol. 69, 313-340

Scheuren, N., Neupert, W., Ionac, M., Neuhuber, W., Brune, K., Geisslinger, G., 1997. Peripheral noxious stimulation releases spinal PGE2 during the first phase in the formalin assay of the rat. Life Sci. 60, 295–300.

Shibata, M., Ohkubo, T., Takahashi, H., Inoki, R., 1998. Modified formalin test: characteristic biphasic pain response. Pain 38, 347-52.

Stanwood, G.D., Levitt, P., 2004. Drug exposure early in life: functional repercussions of changing neuropharmacology during sensitive periods of brain development. Curr Opin Pharmacol. 4, 65-71.

Suresh, S., Anand, K.J., 2001. Opioid tolerance in neonates: a state-of-the-art review. Paediatric Anaesth. 11, 511-551.

Tai, Y.H., Wang, Y.H., Wang, J.J., Tao, P.L., Tung, C.S., Wong, C.S., 2006. Amitriptyline suppresses neuroinflammation and up-regulates glutamate transporters in morphine-tolerant rats. Pain 124, 77-86.

Thornton, S.R., Smith, F.L., 1998. Long-term alterations in opiate antinociception resulting from infant fentanyl tolerance and dependence. Eur. J. Pharmacol. 363, 113–119.

Thornton, S.R., Lohmann, A.B., Nicholson, R.A., Smith, F.L., 2000. Fentanyl self-administration in juvenile rats that were tolerant and dependent to fentanyl as infants. Pharmacol. Biochem. Behav. 65, 563–570.

Tjølsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H., Hole, K., 1992. The formalin test: an evaluation of the method. Pain 51, 5-17.

Vanderah, T.W., Gardell, L.R., Burgess, S.E., Ibrahim, M., Dogrul, A., Zhong, C.M., Zhang, E.T., Malan, T.P. Jr., Ossipov, M.H., Lai, J., Porreca, F., 2000. Dynorphin promotes abnormal pain and spinal opioid antinociceptive tolerance. J Neurosci. 20, 7074-7079.

Vanderah, T.W., Suenaga, N.M., Ossipov, M.H., Malan, T.P. Jr., Lai, J., Porreca, F., 2001. Tonic descending facilitation from the rostral ventromedial medulla mediates opioid-induced abnormal pain and antinociceptive tolerance. J Neurosc. 21, 279-286.

Wimalawansa, S.J., 1996. Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology, and therapeutic potentials. Endocr Rev. 17, 533-585.

Wheeler-Aceto, H., Cowan, A., 1991. Standardization of the rat paw formalin test for the evaluation of analgesics. Psychopharmacology. 104, 35-44.

Yaksh, T.L., Harty, G.J., Onofrio, B.M., 1986. High dose of spinal morphine produce a nonopiate receptor-mediated hyperesthesia: clinical and theoretic implications. Anaesthesiology 64, 590-597.

Zhao, M.G., Ko, S.W., Wu, L.J., Toyoda, H., Xu, H., Quan, J., Li, J., Jia, Y., Ren, M., Xu, Z.C., Zhuo, M., 2006. Enhanced presynaptic neurotransmitter release in the anterior cingulated cortex of mice with chronic pain. J Neurosci. 26, 8923-8930.

Zhuo, M., 2002. Glutamate receptors and persistent pain: targeting forebrain NR2B subunits. Drug Discov Today 7, 259-267.

Zissen, M.H., Zhang, G., Kendig, J.J., Sweitzer, S.M., 2006. Acute and chronic morphine alters formalin pain in neonatal rats. Neurosci Lett. 400, 154-7.

Zissen, M.H., Zhang, G., McKelvy, A., Propst, J.T., Kendig, J.J., Sweitzer, S.M., 2007. Tolerance, opioid-induced allodynia and withdrawal associated allodynia in infant and young rats. Neurosci. 144, 247-62.

Legends

Figure 1: Experimental design.

Figure 2: Changes in the formalin-induced neurogenic and inflammatory pain after repeated morphine administration in early life:

A: at P16, two days after termination of repeated morphine exposure, the animals did not show differences between groups in either phase (Student's *t* test, $P > 0.05$); B: at P30, the animals did not show differences between the groups in phase I (Student's *t* test, $P > 0.05$), but during phase II they presented a significant difference (Student's *t* test, $P < 0.05$); C: at P60, there was a significant difference between the animals in both phases of the formalin test (Student's *t* test, $P < 0.05$, Panel C).

[#] significant difference from control group.

Figure 3: Effects of indomethacin administration 30 min before the formalin test at P30 and P60 after repeated morphine administration in early life: A: at P30 the control-indomethacin and morphine-indomethacin groups were significantly different from all groups in phase I (one-way ANOVA/Bonferroni's test, $P<0.05$); in phase II, all groups were significantly different from each other (one-way ANOVA/Bonferroni's test $P<0.05$). B: at P60, all groups were significantly different from each other in both phases of the test (one-way ANOVA/Bonferroni's test, $P<0.05$).

[#] significant difference from control-vehicle I group; ** significant difference between groups.

Figure 4: Effects of ketamine administration 30 min before the formalin test at P30 and P60 after repeated morphine administration in early life: A: at P30, the control-

ketamine and morphine-ketamine groups were similar, but they were significantly different from other groups (control-vehicle II and morphine-vehicle II) in phase I (one-way ANOVA/Bonferroni's test, $P<0.05$). In phase II all groups were significantly different from each other (one-way ANOVA/Bonferroni's test, $P<0.05$). B: at P60 all groups were significantly different from each other in both phases of the test (one-way ANOVA/Bonferroni's test, $P<0.05$).

[#] significant difference from control-vehicle II group; ** significant difference between groups.

Fig. 1

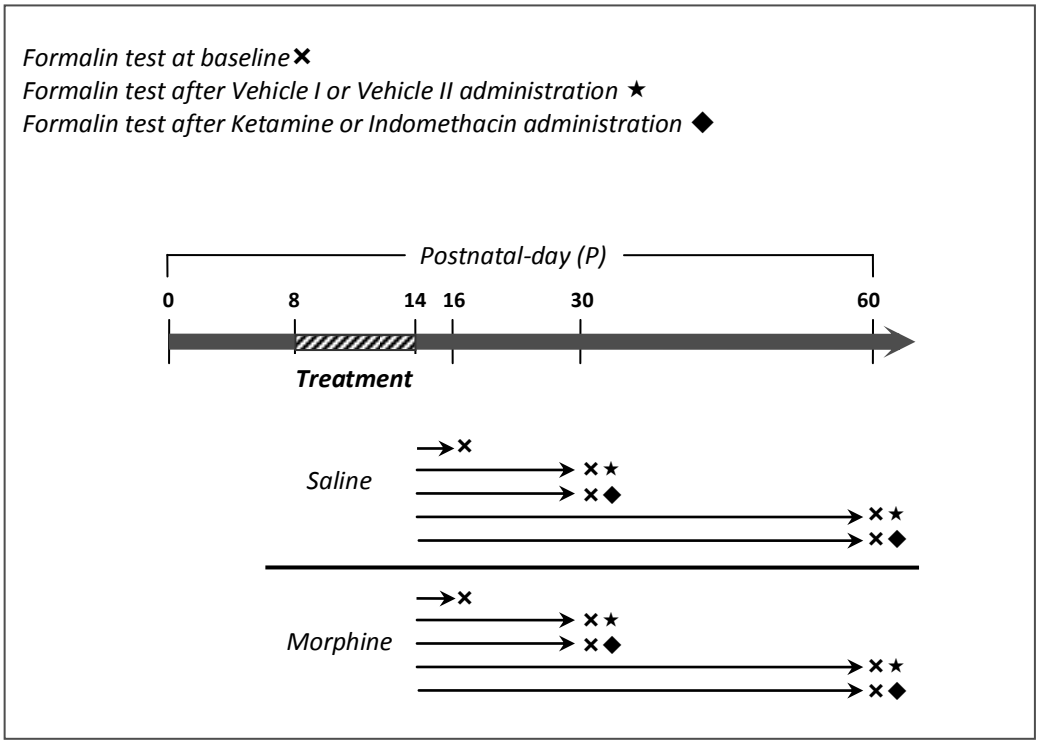

Formalin test at baseline ×
Formalin test after Vehicle I or Vehicle II administration ★
Formalin test after Ketamine or Indomethacin administration ◆
Postnatal-day (P)
0
8
14
16
30
60
Treatment
Saline
Morphine

FIG. 2A

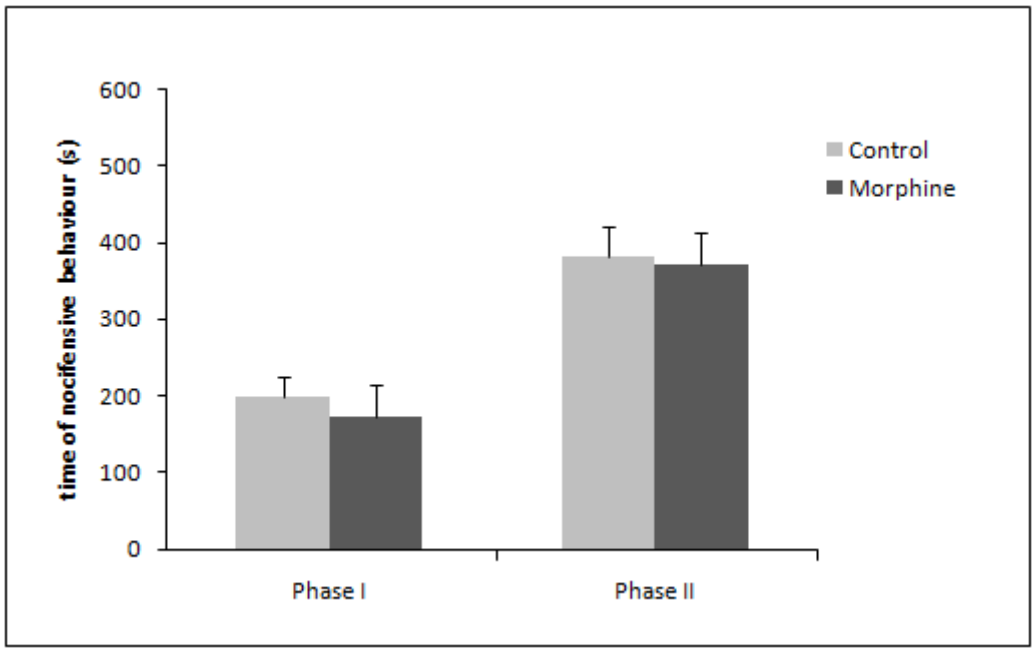

time of nocifensive behaviour (s)
600
500
400
300
200
100
0
Control
Morphine
Phase I
Phase II

FIG. 2B

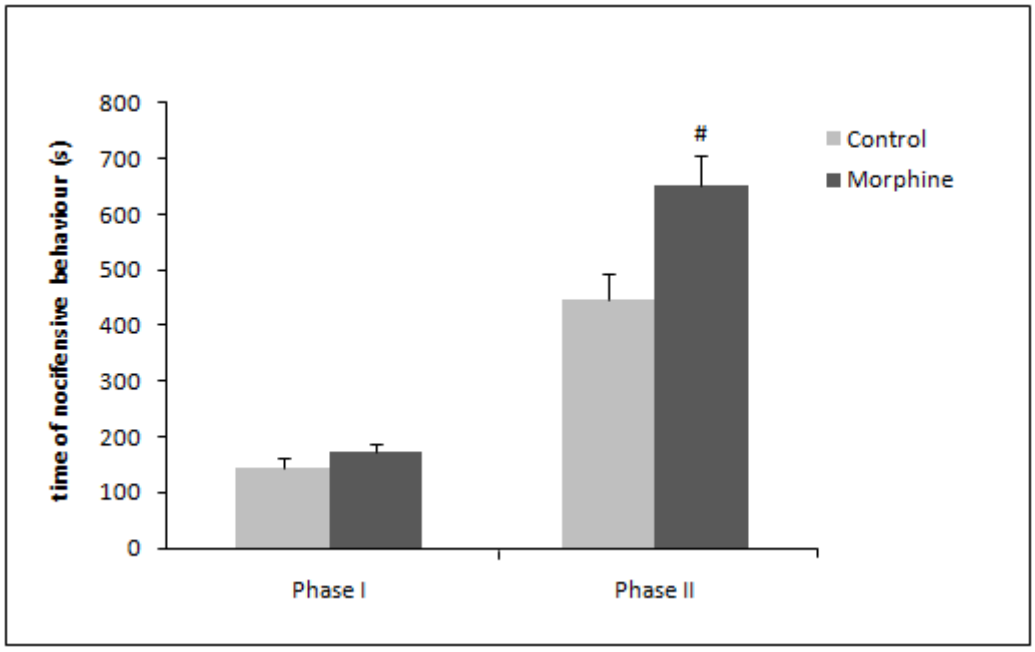

time of nocifensive behaviour (s)
800
700
600
500
400
300
200
100
0
#
Control
Morphine
Phase I
Phase II

FIG. 2C

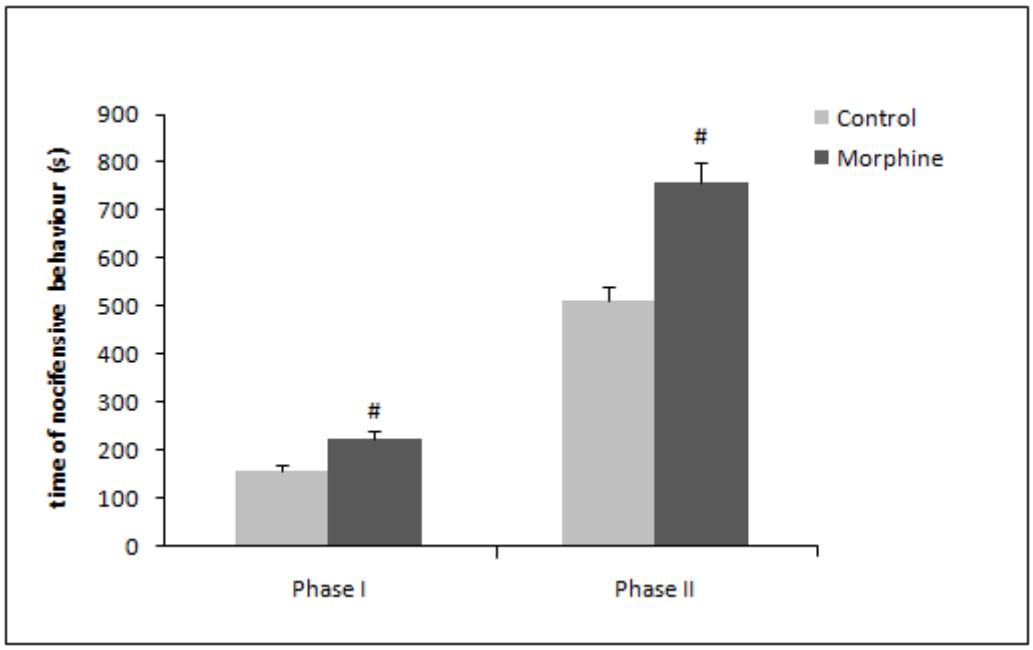

time of nocifensive behaviour (s)
900
800
700
600
500
400
300
200
100
0
#
#
Control
Morphine
Phase I
Phase II

FIG. 3A

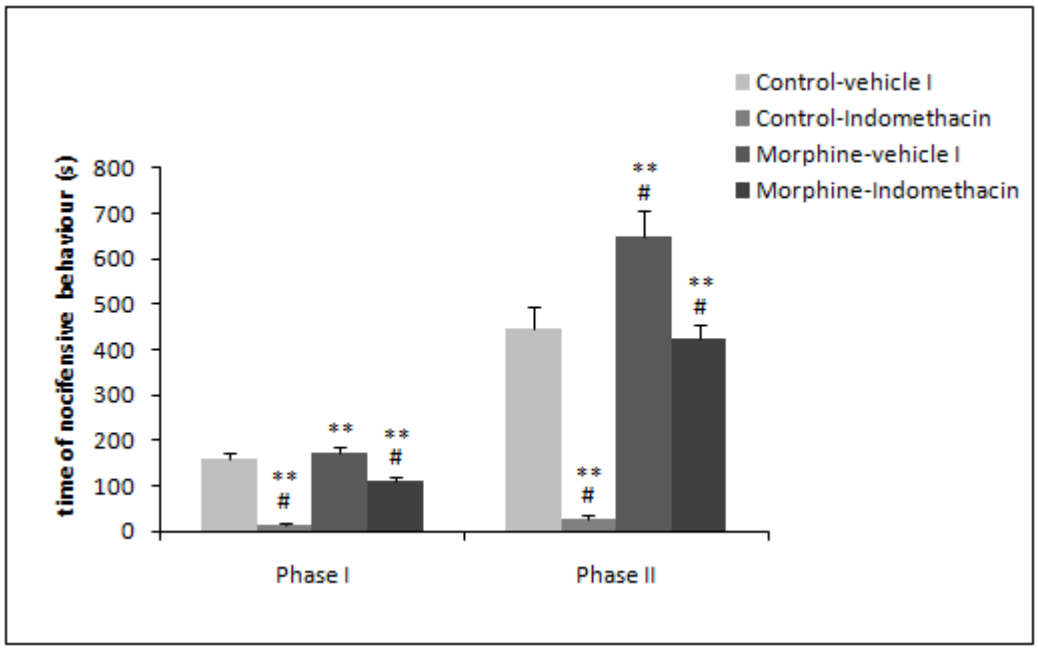

Control-vehicle I
Control-Indomethacin
Morphine-vehicle I
Morphine-Indomethacin
time of nocifensive behaviour (s)
800
700
600
500
400
300
200
100
0
** #
** #
** #
** #
** #
Phase I
Phase II

FIG. 3B

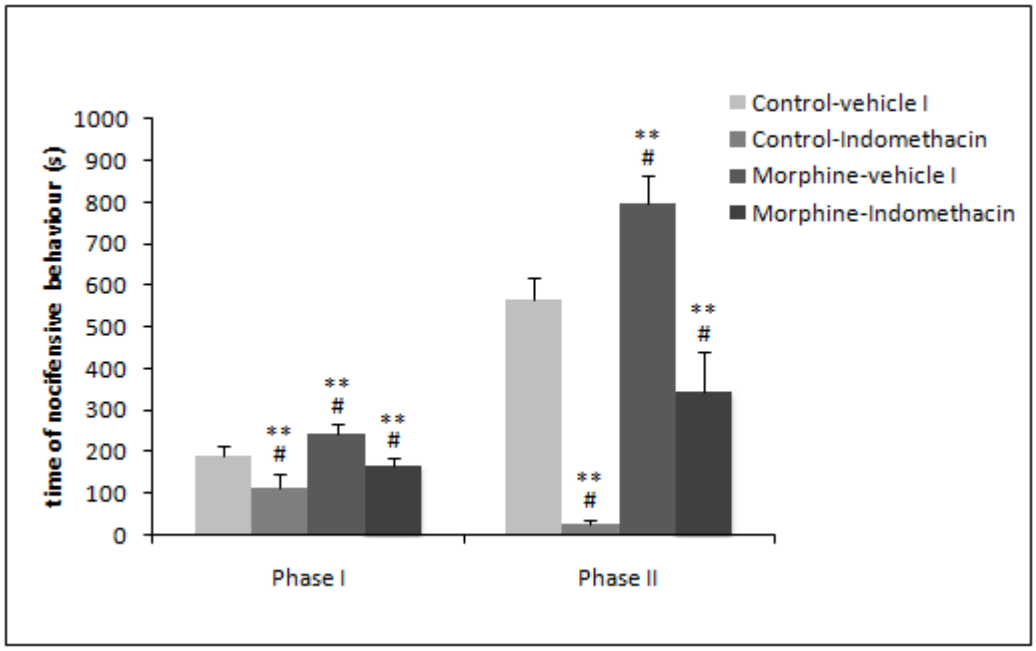

Control-vehicle I
Control-Indomethacin
Morphine-vehicle I
Morphine-Indomethacin
time of nocifensive behaviour (s)
1000
900
800
700
600
500
400
300
200
100
0
** #
** #
** #
** #
** #
Phase I
Phase II

FIG. 4A

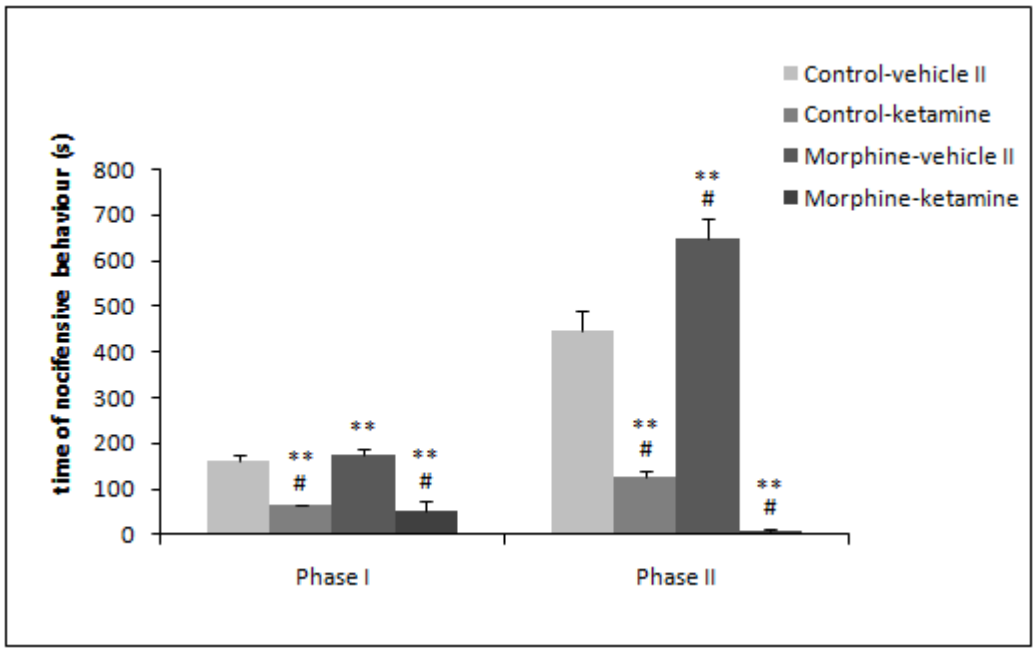
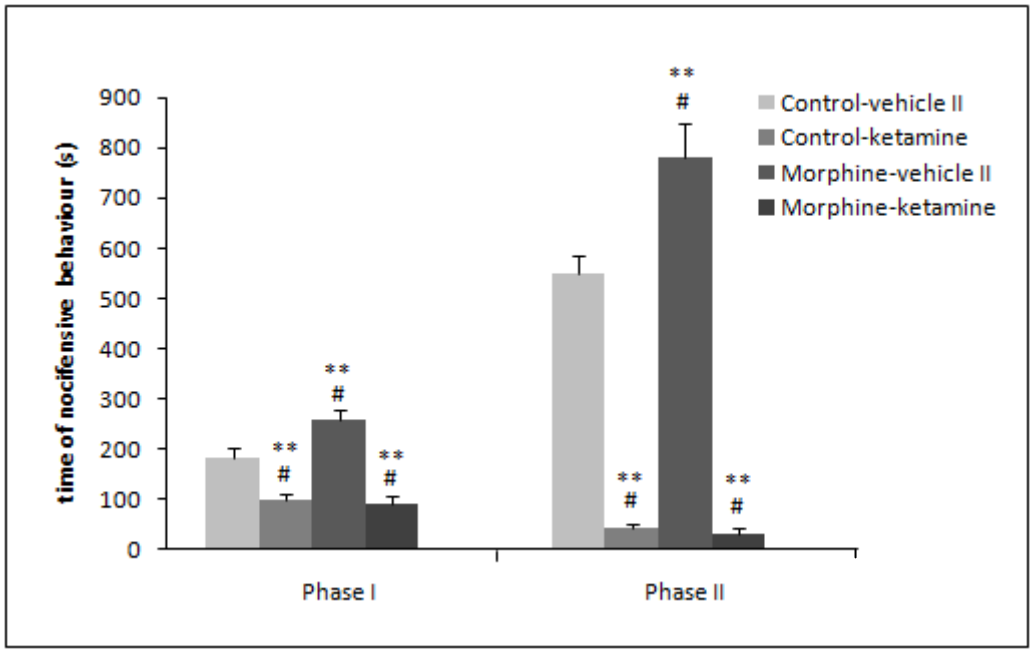

time of nocifensive behaviour (s)
800
700
600
500
400
300
200
100
0
Control-vehicle II
Control-ketamine
Morphine-vehicle II
Morphine-ketamine
** #
Phase I
Phase II

FIG. 4B


time of nocifensive behaviour (s)
900
800
700
600
500
400
300
200
100
0
Control-vehicle II
Control-ketamine
Morphine-vehicle II
Morphine-ketamine
** #
Phase I
Phase II

ARTIGO 2

Morphine treatment in early life alters glutamate uptake in the spinal synaptosomes of adult rats

Neuroscience Letters 2012; 529:51-54

MORPHINE TREATMENT IN EARLY LIFE ALTERS GLUTAMATE UPTAKE IN THE SPINAL SYNAPTOSOMES OF ADULT RATS

JOANNA RIPOLL ROZISKY[a,b,d], DEUSA VENDITE[c], FERNANDA URRUTH FONTELLA[c,d], YASMINE NONOSE[a,d], GABRIELA LASTE[a,b,d], CARLA DALMAZ[c], WOLNEI CAUMO[a,b], IRACI L. S. TORRES[a,b,d*]

[a] Laboratory of Pharmacology of Pain and Neuromodulation: Animal Models, Department of Pharmacology – Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul – Porto Alegre, RS 90050-170, Brazil.

[b] Postgraduate Program in Medical Sciences, Universidade Federal do Rio Grande do Sul – Porto Alegre, RS 90035-003, Brazil.

[c] Department of Biochemistry – Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul – Porto Alegre, RS 90035-003, Brazil.

[d] Animal Experimentation Unit, Graduate Research Group – Hospital de Clínicas de Porto Alegre, RS 90035-003, Brazil.

[*]CORRESPONDING AUTHOR: Iraci Lucena da Silva Torres, Departamento de Farmacologia – ICBS, UFRGS. Rua Sarmento Leite, 500 sala 202, Zip Code: 90050-170, Phone: 0055-51 3308 3183; FAX: 0055-51 3308 3121. Porto Alegre, RS, Brazil. E-mail: iracitorres@gmail.com

ABSTRACT

Morphine exposure during the neonatal period can promote changes in pain signaling pathways that can be expressed as an increased nociceptive response in adult life. Glutamate is the major excitatory neurotransmitter in primary afferent terminals and plays a critical role in normal spinal excitatory synaptic transmission. Considering the importance of a better understanding of the mechanisms that underlie nociceptive changes throughout the life course, the aim of this study was investigate the effects of repeated morphine administration at postnatal days 8 (P8) to 14 (P14) on glutamate uptake in spinal synaptosomes at P30 and P60. The morphine group showed decreased [3H]-glutamate uptake as compared to control groups in both P30 and P60. These findings suggest that morphine exposure in early life leads to changes in glutamatergic signaling at least until the 60th day of age, which may lead to increased levels of glutamate in the spinal synaptic cleft and, consequently, an increased nociceptive response in adult life. Thus, this study highlights the importance of conducting research in this field to provide a comprehensive knowledge of the long-term effects of early-life morphine treatment on nociceptive pathways.

KEYWORDS: morphine; neonate; glutamate uptake; spinal synaptosomes

Introduction

Opioids are frequently used in critically ill children and neonates for sedation and analgesia in the intensive care setting [1,6]. However, prolonged therapy often leads to tolerance and withdrawal symptoms when opioids are weaned or discontinued [16,43,47]. Crosstalk between the pathways underlying opioid tolerance and hyperalgesia has also been proposed, suggesting that a common cellular mechanism may be causal in both conditions [23,26].

Some rat studies have shown that morphine exposure during the neonatal period can promote changes in pain signaling pathways [39,44]. Sweitzer et al. [44] demonstrated that acute administration in young rats produces spontaneous or precipitated withdrawal-associated pain hypersensitivity. Recently, our group showed that repeated morphine exposure in early life promotes a hyperalgesic response to noxious events in the adult life of rats; this increased nociceptive response was reversed by a NMDA receptor antagonist [39], suggesting that early morphine exposure reduced the nociceptive threshold due to possible adaptations in the glutamatergic system.

Glutamate is a major excitatory neurotransmitter in primary afferent terminals and plays a critical role in normal spinal excitatory synaptic transmission [11,22,40,50,53]. The homeostasis of extracellular glutamate is tightly regulated by reuptake carried out by glutamate transporters (GTs) in the plasma membranes of both neurons and glia [29,30], and it is crucial for nociception transmission and for the prevention of glutamate-induced neurotoxicity. Changes in glutamate uptake have been implicated in peripheral nervous system damage [19,25], neuropathic pain [42] and hyperalgesia [51,52].

Taking into account that morphine exposure in early life can lead to changes in nociceptive behavior response in adult life, and that this effect is reversed by administration of a glutamatergic receptor antagonist, this study sought to assess the long-term effect of neonatal repeated morphine treatment on glutamate uptake in the spinal synaptosomes of rats in adulthood.

Material and Methods

At birth, litters were standardized to contain up to 8 pups per dam, and pups remained with their mothers until 21 days of age. Eight-day-old male Wistar rats were divided into two groups: saline control (C) and morphine-treated (M). Rats at P8 were chosen because it is accepted that animals in this age are at a similar stage of neurological development to that of a human newborn [13]. It is also accepted that they are in a physiologically immature state [32], since this period is characterized by major developmental changes in the brain and plasticity of the developing pain system [7,18, 33]. Animal handling and all experiments were performed in accordance with international guidelines for animal welfare. Each animal received saline (control group) or morphine (5 μg s.c. in the mid-scapular area; morphine group) starting at P8, then once daily for 7 days. This dose was chosen on the basis of a previous study by Rozisky et al. [37,38,39]. The experimental assays were performed when the animals were 30 and 60 days old.

Two (P30) and 5 weeks (P60) after the end of neonatal morphine treatment the animals were decapitated. The spinal cord was rapidly removed, dissected out on ice, and gently homogenized in 10 vol. of ice-cold medium consisting of 320 mM sucrose, 1 mM EDTA, and 0.25 mM dithiothreitol, pH 7.4, with a motor-driven Teflon-glass

homogenizer. The synaptosomal fraction was isolated on a discontinuous Percoll gradient, according to Dunkley et al. [12]. Protein concentration was measured using the Lowry method [21]. The entire spinal cord was used and a pool from two rats was used for each one synaptosomes assay, due to low yield of the enriched fraction in viable synaptosomes. The material was prepared fresh daily and kept at 0–4°C throughout the experiment.

Determination of Na+- dependent high-affinity glutamate uptake was performed as described by Leal et al. with minor modifications [20]. Synaptosomal preparations were washed twice through resuspension in 3 vol. of 300 mM sucrose with 15 mM Tris/acetate buffer (pH 7.4) and centrifugation at 13000*g* for 15 min at 4°C. The final pellet was resuspended in 300 mM sucrose with 15 mM Tris/acetate buffer (pH 7.4) and incubated in HBSS (Hepes/Cl buffered salt solution) pH 7.4 (containing 27 mM HEPES, 133 mM NaCl, 2.4 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 12 mM glucose, 1.0 mM CaCl2) in the presence of 2 µM of L-[3H]glutamic acid (PerkinElmer, Inc., Massachusetts, USA, specific activity 1.48-2.96TBq/mmol) for 1 min at 37°C. The reaction was stopped by filtration through GF/B glass microfiber filters (Whatman). The filters were washed three times with 3 ml of ice-cold 15 mM Tris/acetate buffer (pH 7.4) in 155 mM ammonium acetate, and the radioactivity retained on the filters was measured in a Wallac scintillation counter. Specific [3H]glutamate uptake was calculated as the difference between uptake obtained in the incubation medium as described above and uptake obtained with a similar incubation medium containing choline chloride instead of NaCl (nonspecific uptake). Na+-independent uptake was less than 10% of the total. All measurements were made in triplicate. The results were expressed as percentage of [3H] glutamate uptake obtained on control group, which was

considered as 100%. The between-group comparisons were performed with Student's *t*-test for independent samples. Differences were considered statistically significant if $P<0.05$.

Results

After repeated morphine treatment in early life, from P8 to P14, [3H]-glutamate uptake in spinal synaptosomes was assessed at P30 and P60. In both ages, animals that had received morphine exhibited decreased [3H]-glutamate uptake as compared with control animals that had received saline (P30: C=100.0±29.0% of [3H] glutamate uptake, M=12.96±3.66% of [3H] glutamate uptake obtained on control group, Student's t-test, $P=0.019$; P60: C= 100.0±22.63% of [3H] glutamate uptake, M=31.37±7.06% of [3H] glutamate uptake obtained on control group; Student's t-test, $P=0.04$, Fig. 1 Panels A and B).

----------insert Fig 1 about here----------

Discussion

In this study, once-daily, low-dose morphine administration in early life, from P8 to P14, decreased [3H]-glutamate uptake in spinal synaptosomes P30 and P60. This result suggests that opioid exposure during development of the central nervous system leads to long-lasting adaptations in the glutamatergic system.

Our group has suggested that newborn rats may be more sensitive to low doses of morphine [38] because there is extensive remodeling of opioid receptor expression in the first 3 postnatal weeks [5,34,35]. At P14, spinal μ-opioid receptors (μORs) are

restricted to the dorsal horn, whereas they appear throughout the spinal grey matter at P7, and the density of binding decreases in the first 3 postnatal weeks, with peak binding at P7, that then falls to adult levels by P21 [5]. This abundance of μORs in early postnatal life could explain why exposure to morphine for 7 days, from P8 to P14, produces analgesia instead of tolerance [38]. Thus, the greater expression of μORs at P7 in comparison to adult rats suggests a more widespread effect of morphine, acting both directly within the spinal cord and indirectly through larger termination profiles of primary afferents (for a review, see [28]). This, coupled with the overexpression of excitatory amino acid receptors at the primary afferent-spinal cord synapse, supports a potential role for μORs in the normal maturation of nociceptive circuitry; hence, disruption of this by administration of exogenous opioid agonists may have detrimental consequences for the maturation of pain circuitry [45,46].

Some studies have shown that repeated opioid administration leads to possible neural adaptations that may underlie opioid-induced paradoxical pain [17,48,49]. Recently, we showed that early morphine treatment increases nociceptive behavioral responses at P30 and P60 in a rat formalin tonic pain model. Remarkably, these behavioral changes were reverted by administration of a NMDA receptor antagonist in both ages, suggesting direct involvement of the glutamatergic system in opioid-induced nociception [39].

The homeostasis of extracellular glutamate is tightly regulated by glutamate transporters (GTs) in the plasma membranes of both neurons and glia [27]. The three glutamate transport protein subtypes isolated in the spinal cord (GLAST, GLT-1, EAAC1) are considered essential to the maintenance of low resting levels of glutamate (<1 mM) and the prevention of overstimulation of glutamate receptors [27]. Notably, these glutamate transporters are concentrated in the superficial dorsal horn of the spinal cord and

account for >80% of total glutamate transport [10]. Several studies indicate that GTs play a crucial role in the prevention of glutamate neurotoxicity under both physiological and pathological conditions [27, for review see 10]. The competitive opioid antagonist naloxone decreases GLT-1 mRNA in brain regions and is involved in the expression of morphine withdrawal symptoms [31].

Recently, another study showed that morphine exposure in adult rats leads to downregulation of spinal glutamate GLT-1 transporter and is involved in the generation of opioid-induced hyperalgesia [9]. In addition, it bears stressing that synaptosomes have been widely used to investigate glutamate uptake; but the specific glutamate transporters present in the synaptosomal preparation are still a matter of controversy. However, pharmacological analysis using different types of transporter blockers suggests that this uptake aligns most closely with that of the GLT-1 transporter [41] (for a review, see [8,10]). Thus, one might suggest that the decreased [3H]-glutamate reuptake observed in spinal synaptosomes after neonatal repeated morphine treatment (P30 and P60) would lead to increased extracellular glutamate levels in the spinal cord synaptic cleft. This, in turn, may increase excitatory amino acid receptor activation (including NMDA). These considerations can explain, at least in part, the increase in nociceptive behavioral responses at P30 and P60 induced by early-life morphine treatment in this rat model.

In conclusion, the findings presented here added new insights to our previous studies [39], suggesting that repeated morphine exposure in early life leads to long-lasting adaptations in glutamatergic signaling, due to a decrease in [3H]-glutamate uptake in spinal synaptosomes at P30 and P60. This result may be associated with increased

levels of excitatory amino acids and potential hyperactivity of glutamatergic receptors, such as the NMDA receptor, which, in turn, leads to a hyperalgesic response in adult life. Furthermore, this study highlights the importance of continuing research in this field to provide comprehensive knowledge of the long-term effects of morphine administration in early life.

Acknowledgements: This study was supported by the Brazilian National Council for Scientific and Technological Development – CNPq (I.L.S. Torres- Process 307772/2008-0/2008, W.Caumo, C. Dalmaz); the National Coordination for the Development of Higher Education Personnel – CAPES (J.R. Rozisky, G. Laste; F.U. Fontella) and the Graduate Research Group (GPPG) of Hospital de Clínicas de Porto Alegre (I.L.S. Torres – Grant 08345).

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Ethical standard: Animal handling and all experiments were performed in accordance with Brazilian Law No. 11.794 of October 8, 2008, on the use of animals for scientific research and conformed to the Guide Laboratory for the care and use of animals 8th ed 2011. All efforts were made to minimize animal suffering and use only the number of animals necessary to produce reliable scientific data. All experiments and procedures were approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre (protocol number: GPPG-08345).

References

1. K.J. Anand, Pharmacological approaches to the management of pain in the neonatal intensive care unit, J. Perinatol. 27 (2007) Suppl 1:S4-S11

2. S.L. Andersen, Trajectories of brain development: point of vulnerability or window of opportunity?, Neurosci. Biobehav. Rev. 27(1-2) (2003) 3-18.

3. J.L. Arriza, M.P. Kavanaugh, W.A. Fairman, Y.N. Wu, G.H. Murdoch, R.A. North, S.G. Amara, Cloning and expression of human neutral amino acid transporter with structural similarity to the glutamate transporter gene family, J. Biol. Chem. 268(21) (1993) 15329-32.

4. S. Beggs, C. Torsney, L.J. Drew, M. Fitzgerald, The postnatal reorganization of primary afferent input and dorsal horn cell receptive fields in the rat spinal cord is an activity-dependent process, Eur. J. Neurosci. 16 (2002) 1249–1258.

5. B. Beland, M. Fitzgerald, Mu- and delta opioid receptors are down-regulated in large primary sensory neurons during postnatal development in rats, Pain 90 (2001) 143–150.

6. C.B. Berde, N.F. Sethna, Analgesics for the treatment of pain in children, N. Engl. J. Med. 347(14) (2002) 1094–1103.

7. B. Bishop, Neural plasticity. Part 2. Postnatal maturation and function induced plasticity, Phys. Ther. 62 (1982) 1132–1143.

8. R.J. Bridges, M.P. Kavanaugh, A.R. Chamberlin, A pharmacological review of competitive inhibitors and substrates of highaffinity, sodium-dependent glutamate transport in the central nervous system, Curr. Pharm. Des. 5 (1999) 363–379.

9. Z. Chen, Y. He, Z.J. Wang, The beta-lactam antibiotic, ceftriaxone, inhibits the development of opioid-induced hyperalgesia in mice, Neurosci. Lett. 509(2) (2012) 69-71.

10. N.C. Danbolt, Glutamate uptake, Prog. Neurobiol. 65 (2001) 1–105.

11. A.H. Dickenson, V. Chapman, G.M. Green, The pharmacology of excitatory and inhibitory amino acid-mediated events in the transmission and modulation of pain in the spinal cord, Gen. Pharmacol. 28 (1997) 633–638.

12. P.R. Dunkley, J.W. Heath, S.M. Harrison, P.E. Jarvie, P.J. Glenfield, J.A.P. Rostas, A rapid Percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: homogeneity and morphology of subcellular fractions, Brain Res. 441 (1988) 59–71.

13. M. Fitzgerald, K.J. Anand, Developmental neuroanatomy and neurophysiology of pain, in: N.L. Schechter, C.B. Berde, M. Yaster (Eds.), Pain in infants, children, and adolescents, Williams & Wilkins, Baltimore, 1983, pp. 11–31.

14. M. Fitzgerald, S. Beggs, The neurobiology of pain: developmental aspects, Neuroscientist 7 (2001) 246–257.

15. M. Fitzgerald, E. Jennings, The postnatal development of spinal sensory processing, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 7719–7722.

16. L.S. Franck, J. Vilardi, D. Durand, R. Powers, Opioid withdrawal in neonates after continuous infusions of morphine or fentanyl during extracorporeal membrane oxygenation, Am. J. Crit. Care. 7(5) (1998) 364–369.

17. L.R. Gardell, R. Wang, S.E. Burgess, M.H. Ossipov, T.W. Vanderah, T.P. Malan Jr., J. Lai, F.J. Porreca, Sustained morphine exposure induces a spinal dynorphin-

dependent enhancement of excitatory transmitter release from primary afferent fibres. Neurosci, 22 (2002) 6747–6755.

18. J.J. Kim, M.R. Foy, R.F. Thompson, Behavioral stress modifies hippocampal plasticity through N-methyl-D-aspartate receptor activation, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 4750–4753.

19. S. Kiryu, G.L. Yao, N. Morita, H. Kato, H. Kiyama, Nerve injury enhances rat neuronal glutamate transporter expression: identification by differential display PCR, J. Neurosci. 15(12) (1995) 7872-8.

20. M.B. Leal, T. Emanuelli, L.D. Porciuncula, D.O. Souza, E. Elisabetsky, Ibogaine alters synaptosomal and glial glutamate release and uptake. Neuroreport 12 (2001) 263–267.

21. O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin-phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

22. A.B. Malmberg, T.L. Yaksh, Cyclooxygenase inhibition and the spinal release of prostaglandin E2 and amino acids evoked by paw formalin injection: a microdialysis study in unanesthetized rats, J. Neurosci. 15 (1995) 2768–2776.

23. J. Mao, D.D. Price, D.J. Mayer, Mechanisms of hyperalgesia and morphine tolerance: a current view of their possible interactions, Pain 62 (1995) 259–74.

24. D.F. Marsh, D.J. Hatch, M. Fitzgerald, Opioid system and the newborn. Br. J. Anaesth. 79 (1997) 787–795.

25. C. Mawrin, T. Pap, M. Pallas, K. Dietzmann, W. Behrens-Baumann, C.K. Vorwerk, Changes of retinal glutamate transporter GLT-1 mRNA levels following optic nerve damage, Mol. Vis. 9 (2003) 10-3.

26. D.J. Mayer, L. Mao, J. Holt, D.D. Price, Cellular mechanisms of neuropathic pain, morphine tolerance, and their interactions, Proc. Natl. Acad. Sci. U S A 96 (1999) 7731–6.

27. S. Mennerick, W. Shen, W. Xu, A. Benz, K. Tanaka, K. Shimamoto, K.E. Isenberg, J.E. Krause, C.F. Zorumski, Substrate turnover by transporter curtails synaptic glutamate transients. J. Neurosci. 19(21) (1999) 9242-51.

28. R. Nandi, D. Beacham, J. Middleton, M. Koltzenburg, R.F. Howard, M. Fitzgerald, The functional expression of mu opioid receptors on sensory neurons is developmentally regulated; morphine analgesia is less selective in the neonate, Pain 111 (2004) 38–50.

29. E. Niederberger, A. Schmidtko, O. Coste, et al, The glutamate transporter GLAST is involved in spinal nociceptive processing, Biochem. Biophys. Res. Commun. 346 (2006) 393–399.

30. E. Niederberger, A. Schmidtko, J.D. Rothstein, et al, Modulation of spinal nociceptive processing through the glutamate transporter GLT-1, Neurosci. 116 (2003) 81–87.

31. T. Ozawa, T. Nakagawa, K. Shige, M. Minami, M. Satoh, Changes in the expression of glial glutamate transporters in the rat brain accompanied with morphine dependence and naloxone-precipitated withdrawal, Brain Res. 905(1-2) (2001) 254-8.

32. D. Pattinson, M. Fitzgerald, The neurobiology of infant pain: development of excitatory and inhibitory neurotransmission in the spinal dorsal horn, Reg. Anesth. Pain Med. 29 (2004) 36–44.

33. T. Rabinowicz, G.M. de Courten-Myers, J.M. Petetot, G. Xi, E. de los Reyes, Human cortex development: estimates of neural numbers indicate major loss late during gestation. J. Neuropathol. Exp. Neurol. 55 (1996) 320–328.

34. W. Rahman, M.R. Dashwood, M. Fitzgerald, A. Aynsley-Green, A.H. Dickenson, Postnatal development of multiple opioid receptors in the spinal cord and development of spinal morphine analgesia, Dev. Brain Res. 108 (1998) 239–254.

35. W. Rahman, A.H. Dickenson, Development of spinal opioid systems. Reg. Anesth. Pain Med. 24 (1999) 383–385.

36. M.B. Robinson, L.A. Dowd, Heterogeneity and functional properties of subtypes of sodium-dependent glutamate transporters in the mammalian central nervous system, Adv. Pharmacol. 37 (1997) 69-115.

37. J.R. Rozisky, R.S. da Silva, L.S. Adachi, K.M. Capiotti, D.B. Ramos, M.R. Bogo, C.D. Bonan, J.J. Sarkis, I.L. Torres, Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral cortex of rats, Eur. J. Pharmacol. 642(1-3) (2010) 72-6.

38. J.R. Rozisky, G. Dantas, L.S. Adachi, V.S. Alves, M.B. Ferreira, J.J. Sarkis, I.L. Torres, Long-term effect of morphine administration in young rats on the analgesic opioid response in adult life, Int. J. Dev. Neurosci. 26(6) (2008) 561-5.

39. J.R. Rozisky, L.F. Medeiros, L.S. Adachi, J. Espinosa, A. de Souza, A.S. Neto, C.D. Bonan, W. Caumo, I.L. Torres, Morphine exposure in early life increases nociceptive behavior in a rat formalin tonic pain model in adult life. Brain Res. 1367 (2011) 122-9.

40. K.A. Sluka, W.D. Willis, Increased spinal release of excitatory amino acids following intradermal injection of capsaicin is reduced by a protein kinase G inhibitor, Brain Res. 798 (1998) 281–286.

41. S.K. Suchak, N.V. Baloyianni, M.S. Perkinton, et al, The 'glial' glutamate transporter, EAAT2 (Glt-1) accounts for high affinity glutamate uptake into adult rodent nerve endings. J. Neurochem. 84 (2003) 522–532.

42. B. Sung, H.S. Na, Y.I. Kim, Y.W. Yoon, H.C. Han, S.H. Nahm, S.K. Hong, Supraspinal involvement in the production of mechanical allodynia by spinal nerve injury in rats, Neurosci. Lett. 246(2) (1998) 117-9.

43. S. Suresh, K.J.S. Anand, Opioid tolerance in neonates: a state-of-the-art review, Paediatr. Anaesth. 11(5) (2001) 511–521.

44. S.M. Sweitzer, C.P. Allen, M.H. Zissen, J.J. Kendig, Mechanical allodynia and thermal hyperalgesia upon acute opioid withdrawal in the neonatal rat, Pain. 110(1-2) (2004) 269-80.

45. S.R. Thornton, A.B. Lohmann, R.A. Nicholson, F.L. Smith, Fentanyl self-administration in juvenile rats that were tolerant and dependent to fentanyl as infants, Pharmacol. Biochem. Behav, 65 (2000) 563–570.

46. S.R. Thornton, F.L. Smith, Long-term alterations in opiate antinociception resulting from infant fentanyl tolerance and dependence. Eur. J. Pharmacol. 363 (1998) 113–119.

47. J.D. Tobias, Tolerance, withdrawal, and physical dependency after long-term sedation and analgesia of children in the pediatric intensive care unit, Crit. Care Med. 28(6) (2000) 2122–2132.

48. T.W. Vanderah, L.R. Gardell, S.E. Burgess, M. Ibrahim, A. Dogrul, C.M. Zhong, E.T. Zhang, T.P. Malan Jr., M.H. Ossipov, J. Lai, F. Porreca, Dynorphin promotes

abnormal pain and spinal opioid antinociceptive tolerance, J. Neurosci. 20 (2000) 7074–7079.

49. T.W. Vanderah, N.M. Suenaga, M.H. Ossipov, T.P. Malan Jr., J. Lai, F. Porreca, Tonic descending facilitation from the rostral ventromedial medulla mediates opioid-induced abnormal pain and antinociceptive tolerance, J. Neurosci. 21 (2001) 279–286.

50. G. Vetter, G. Geisslinger, I. Tegeder, Release of glutamate, nitric oxide and prostaglandin E2 and metabolic activity in the spinal cord of rats following peripheral nociceptive stimulation, Pain 92 (2001) 213–218.

51. H.R. Weng, N. Aravindan, J.P. Cata, J.H. Chen, A.D. Shaw, P.M. Dougherty, Spinal glial glutamate transporters downregulate in rats with taxol-induced hyperalgesia, Neurosci. Lett. 386(1) (2005) 18-22.

52. H.R. Weng, J.H. Chen, J.P. Cata, Inhibition of glutamate uptake in the spinal cord induces hyperalgesia and increased responses of spinal dorsal horn neurons to peripheral afferent stimulation, Neurosci. 138(4) (2006) 1351-60.

53. M. Yoshimura, T. Jessell, Amino acid-mediated EPSPs at primary afferent synapses with substantia gelatinosa neurones in the rat spinal cord, J. Physiol. 430 (1990) 315–335.

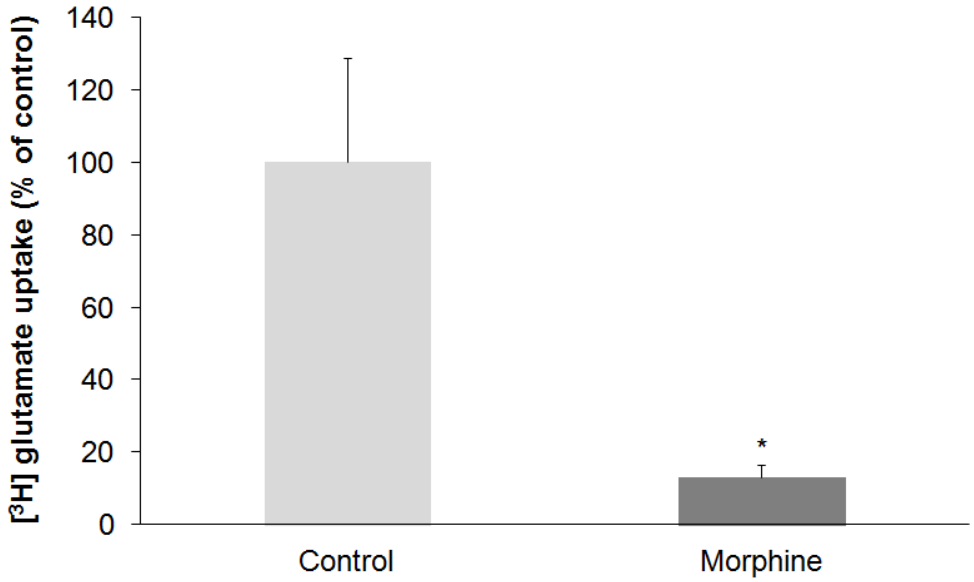
Legend:

Figure 1 shows that repeated low-dose (5µg/day) morphine treatment from P8 to P14 decreased [3H]-glutamate uptake in spinal cord synaptosomes at P30 (Panel A) and P60 (Panel B) (Student's t-test, P= 0.019 for P30 and P= 0.04 for P60, n= 3-4 synaptosomes/group).

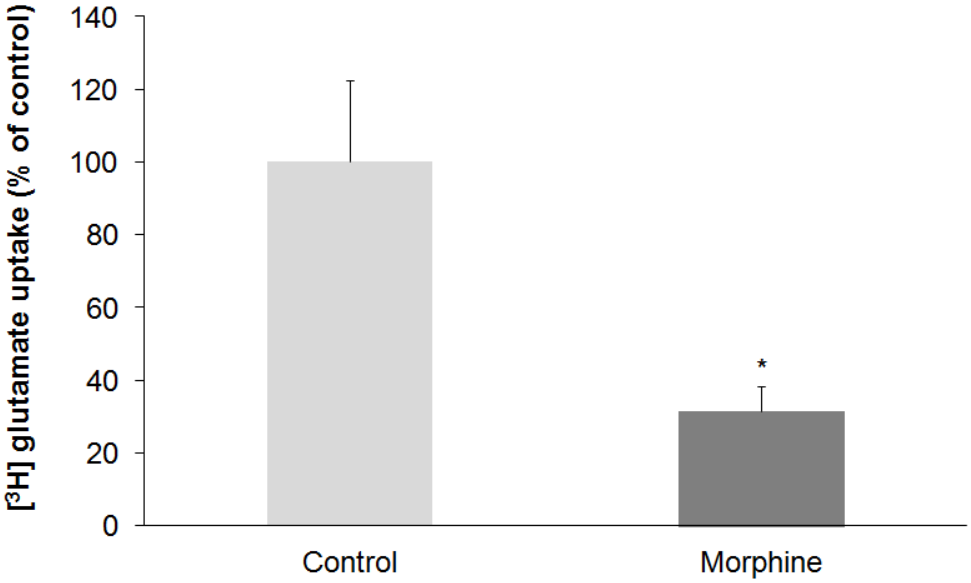
Bars represent mean ± SEM of glutamate uptake (percentage of control group) in spinal cord synaptosomes.

represents a significant difference from control group

Panel A



Panel B



ARTIGO 3

Neonatal morphine treatment induces hyperalgesic behavior in adult rats: reversal by acute melatonin administration

Submetido na revista Neuroreport

Neonatal morphine treatment induces hyperalgesic behavior in adult rats: reversal by acute melatonin administration

JOANNA RIPOLL ROZISKY[a,b,c#], GABRIELA LASTE[a,b,c#], CLEVERSON DE OLIVEIRA[a,b,c], ALÍCIA DEITOS[a,b,c], WOLNEI CAUMO[a,b,c], IRACI L S TORRES[a,b,c*]

[a] Pain Pharmacology and Animal Models of Neuromodulation Laboratory, Department of Pharmacology – Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul – Porto Alegre, RS 90050-170, Brazil.

[b]Postgraduate Program in Medical Sciences, Universidade Federal do Rio Grande do Sul – Porto Alegre, RS 90035-003, Brazil.

[c]Animal Experimentation Unit, Graduate Research Group – Hospital de Clínicas de Porto Alegre, RS 90035-003, Brazil.

[#] Both are the first authors

Conflicts of interest: None declared

[*]CORRESPONDING AUTHOR:

Iraci Lucena da Silva Torres

Departamento de Farmacologia - ICBS, UFRGS.

Rua Sarmento Leite, 500 sala 202.

90050-170 - Porto Alegre, RS, Brazil.

Phone: 0055-51 3308 3183; FAX: 0055-51 3308 3121.

E-mail: iracitorres@gmail.com

Running title: Melatonin reverses opioid hyperalgesia

ABSTRACT

Human infants are routinely treated with opioids in the intensive care unit setting for pain relief and sedation to permit mechanical ventilation. However, opioids can produce paradoxical-hyperalgesia, and such effects might contribute to the drawbacks of acute and chronic administration of these drugs. Considering the relevance of the topic, this study used the formalin test to assess the medium and long-term effects of melatonin administration on hyperalgesia induced by repeated morphine exposure in early life. Newborn rats were divided into two groups, which received saline (control group) or morphine 5 μg s.c. (experiment group) in the mid-scapular area once daily for 7 days, from postnatal day 8 (P8) until P14. Nociceptive responses were assessed by the formalin test. The behaviors analyzed were total time spent biting and flicking the formalin-injected hind paw, recorded during the first 5 min (phase I) and from 15–30 min (phase II). At P30 and P60, both groups received melatonin or inert vehicle 30 min before the formalin test. Initially, animals in the morphine/vehicle group showed increased nociceptive behavior in phase II of the formalin test at P30 and in phases I and II alike at P60. These increased nociceptive responses were fully reversed by melatonin administration at both ages. These findings suggest that melatonin could be useful to counteract opiate-induced hyperalgesia.

Keywords: morphine; neonate rats; formalin test; hyperalgesia; melatonin.

Introduction

Human infants are routinely treated with opioids in the intensive care unit setting for pain relief and sedation to permit mechanical ventilation [1]. However, opioids can produce paradoxical-hyperalgesia, and such effects might contribute to the drawbacks of acute and chronic administration of these drugs [2]. Researchers have addressed the neurobiological consequences of sustained opioid administration and showed possible neuroplastic adaptations involved with the mechanism of paradoxical pain opioid-induced. These adaptations may involve changes in supraspinal pain modulatory circuitry [2]. Other studies have reported that repeated opioid exposure can lead to spinal cord neuroplasticity [3] and it induces a change in the function of spinal cord neurons that can be manifested as neuronal hyperactivity during opiate withdrawal [4]. Recently, our group demonstrated that repeated low-dose morphine in neonatal period produced hyperalgesia in adult life, which was reversed by NMDA receptor antagonists [5]. Consistent with this evidence, we suggest that repeated exposure to morphine during the central nervous system development might lead to adaptations in excitatory pain pathways; possibly producing secondary hyperalgesic effects during the life course [6]. Thus, it is important to explore strategies to change this impact on pain response after morphine exposure.

Melatonin, (N-acetyl-5-methoxytryptamine) a derivative of serotonin (5-HT), is a neurohormone that is primarily produced by the pineal gland, and it has been implicated in the control of the wake-sleep cycle [7]. Recently, evidence has been mounting to support the role of melatonin in pain regulation [8]. Ray et al. (2004) demonstrated that melatonin could exert an antinociceptive effect on the formalin pain response, and that modulation of enkephalin and endorphin levels may be involved [9]. Considering the

relevance of the topic and the fact that, in our previous study, we demonstrated that newborn rats subjected to repeated morphine administration show increased nociceptive behavior in the formalin test in the medium and long term [5], this study sought to ascertain the effect of melatonin on hyperalgesic behavior induced by this morphine treatment regimen.

Materials and Methods

Animals

Eight-day-old male Wistar rats were divided into two groups: saline control (C) and morphine-treated (M). Animals were housed in polypropylene home cages (49 cm x 34 cm x 16 cm) with sawdust-covered floors. All animals were kept on a standard 12-hour light/dark cycle (lights on at 07:00 a.m. and lights off at 07:00 p.m.) in a temperature-controlled environment (22±2°C). Animals had access to water and chow *ad libitum*. At birth, 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 P8 were chosen because it is accepted that animals of this age are at a similar stage of neurological development to that of a human newborn [10]. All experiments were performed in accordance with Brazilian Law No. 11.794 of October 8, 2008 and Guide for the Care and Use of Laboratory Animals 8th edition (2011). Animal handling and all experiments were performed in accordance with international guidelines for animal welfare and measures were taken to minimize animal pain and discomfort and use only the number of animals necessary to produce reliable scientific data. All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA

protocol No. 08-345) To control the possible effect of outliers, we excluded rats which did not present any response on behavioral testing.

Pharmacological treatment

Each animal received saline (control group) or morphine (5 μg s.c. in the mid-scapular area; morphine group) starting at P8, then once a day for 7 days. This dose was chosen on the basis of previous studies by our group [5,11-13], and it produced analgesia in all animals subjected to the tail-flick test. All treatments were administered at the same time each day (1100h). One milliliter of morphine sulfate (Dimorf® 10 mg/mL, obtained from Hospital de Clínicas de Porto Alegre, Rio Grande do Sul, Brazil) was diluted in 9 ml of 0.9% NaCl (saline solution).

The formalin test was performed in 30 and 60-day-old rats, once only in each rat, where we observed significant between-group differences in nociceptive behavior [5]. The control and morphine groups were subdivided into two groups, each designed to evaluate the effect of i.p. administration of melatonin 30 min before the formalin test: 1) 50 mg/kg of melatonin [14,15] (obtained from Sigma-Aldrich, São Paulo, Brazil) diluted in 1% ethanol in saline (control/melatonin = CMt, morphine/melatonin = MMt), this dose reverted the hyperalgesia induced by chronic inflammatory pain in previous experiments conducted by our group (Laste et al. 2012; personal communication); 2) 1% ethanol in saline (control/vehicle= CV, morphine/vehicle = MV). This dose also was choosing based in our previous experiments which melatonin-dose response was evaluated in formalin test, and we verified that this was a minor dose able to promote analgesic effect in formalin test (data not shown) without causing sedation. The number of animals used per group was 5 to 7.

Formalin test

The formalin test was performed as previously described [16,17] with minor modifications. Twenty-four hours before the test, each animal was placed in the chamber for ten minutes to familiarize them with the procedure, since the novelty of the apparatus itself can induce antinociception [18]. The animals were injected subcutaneously on the plantar surface of the left hindpaw with 0.17 ml/kg of a 2% formalin solution (Formaldehyde P.A.®, obtained from Sigma-Aldrich, São Paulo, Brazil) diluted in 0.9% NaCl (saline). Each animal was observed in a varnished wood cage, measuring 60 x 40 x 50 cm, with the inside lined with glass, and the nociceptive response was recorded for a period of 30 min. This test produces two distinct phases of nociceptive behavior: an early, transient phase (phase I; up to 5 min after injection) and a late, persistent phase (phase II; 15–30 min after injection). Phase I has been considered to reflect direct stimulation of primary afferent fibers, predominantly C-fibers (neurogenic pain) [19], whereas phase II is dependent on peripheral inflammation (inflammatory pain) [16]. The total duration (seconds) of licking, biting, and flicking of the formalin-injected hindpaw was recorded in phases I and II.

Statistical analysis

Data were expressed as means ± standard error of the mean (SEM). One-way ANOVA was performed, followed by a multiple comparisons test (Student–Newman–Keuls method) when indicated. Differences were considered statistically significant if $P<0.05$.

Results

After daily morphine exposure, from P8 to P14, we investigated whether an injection of melatonin 30 min before the formalin test was able to reverse increased nociceptive behavior at P30 and P60 in the morphine group as compared to the control group. Initially, this study showed that the morphine group, which received the melatonin vehicle before the formalin test, exhibited a hyperalgesic behavior response at P30 and P60, as found in a previous study by our group (Rozisky et al., 2011). At P30, the morphine/vehicle group showed an increase in nociceptive behavior only in phase II of the formalin test. In phase I, melatonin promoted a marked antinociceptive response, decreasing nociceptive behavior in both groups (control/melatonin and morphine/melatonin) as compared to the control/vehicle and morphine/vehicle groups (CV= 137.4±15.8 s; MV= 178.3±24.9 s; CMt= 69±11.3 s; MMt= 52.2±9.4 s; $F_{(3,23)}$=10.939, one-way ANOVA/SNK, P<0.05; Fig. 1). In phase II, the morphine/vehicle group showed an increased nociceptive behavior response when compared to the other groups, and melatonin reversed this hyperalgesic behavior response, since this effect was similar to control/vehicle and control/melatonin (CV= 530.6±24.8 s; MV= 828.6±13 s; CMt= 551.8±88.8 s; MMt= 367.3±97.8 s; $F_{(3,23)}$= 10.276, one-way ANOVA/SNK, P<0.05).

At P60, the morphine/vehicle group showed an increased nociceptive behavior response in phases I and II alike. In phase I, melatonin administration promoted a marked decrease of nociceptive behavior only in the morphine group; there were no significant differences between the control/vehicle and control/melatonin groups (phase I: CV= 132±20.27 s; MV= 219.8±18 s; CMt= 150.33±9.60; MMt= 51.67±16.72; $F_{(3,21)}$=17.243; one-way ANOVA/SNK, P<0.05, Fig. 2). In phase II, the morphine/vehicle group

showed increased nociceptive behavior, and both groups that received melatonin showed a more marked decrease in the nociceptive behavior response as compared to the control/vehicle and morphine/vehicle groups (CV= 493±50.6 s; MV= 755.8±44.7 s; CMt= 43.50±15.16 MMt: 59.60±23.6 s; $F_{(3,21)}$=85.523, one-way ANOVA/SNK, P<0.05, Fig. 2).

Discussion

In this study, we confirmed that morphine administration in the neonatal period induced hyperalgesia in formalin test in adult life, according to the model described by Rozisky et al. (2011) [5]. In addition we showed that the melatonin administration before formalin test was able to completely reverse this hyperalgesia. At P30 the analgesic effect was observed in both groups (control/melatonin and morphine/melatonin) in phase I (neurogenic pain response) and in phase II (inflammatory pain response). At P60 melatonin administration promoted a marked analgesia only in the morphine group in phase I, and both groups that received melatonin showed an analgesic response in phase II.

Repeated opioid exposure in the neonatal period is known to lead to spinal cord neuroplasticity with neuronal hyperactivity [3] and changes in supraspinal pain modulatory circuits [2]. In this line, we have shown that this altered nociceptive response, expressed until adulthood, was partially reversed by indomethacin and completely reversed by an NMDA receptor antagonist, indicating that opioids induce adaptations at the glutamatergic system level [5]. The NMDA receptors in the spinal cord play a major role in pain transmission at the dorsal horn level, and are involved in the potentiation of nociceptive synaptic transmission in the spinal cord, a phenomenon

known as “wind-up” [22]. In this process, there is a repetitive increase in the response of dorsal horn neurons due to an increase in the intensity of C fibers stimulation. Using C fibers stimulation to initiate the spinal wind-up effect Laurido et al. (2002) found that melatonin decreased spinal cord wind-up [23]. The authors suggested that this effect of melatonin may have been due to the intracellular influence of melatonin on NMDA receptor signaling [23].

In addition, in the formalin test, the rodent hindpaw presents a characteristic biphasic nociceptive response on both weighted pain measures [19]. The transient early phase (Phase I) is interpreted as reflecting direct activation of nociceptive sensory afferents by formalin and an intense increase in the spontaneous activity of C fibers [19], while the tonic phase (Phase II) is regarded as depending on an ensuing inflammatory response, associated with central sensitization [16]. It is known that increased activation of C fibers results from activation of $TRPA_1$ gated channels [20] by heat and protons. These channels play a key role in the sensitization of nociceptors in response to inflammation, and decrease interneuron inhibitory synapse efficacy by reducing GABA transport [21]. Therefore, it is biologically plausible that at least part of the marked antihyperalgesic effect of melatonin is mediated by its effects on the GABAergic system and its attenuation of glutamate-mediated Ca^{2+} influx, which constitutes part of the central sensitization cascade.

The effect of melatonin seen here at P30 may be explained, in part, by inhibition of the glutamatergic activity in opioid-mediated hyperalgesia [24], since that both control and morphine groups that received melatonin showed analgesic effect in phase I and reversal the hyperalgesic response of morphine group in phase II. On the other hand, the involvement of glutamate in inflammatory nociception is supported by the increase in

levels of these neurotransmitters in the dorsal root ganglion and dorsal horn, elicited by chronic inflammation [2]. Apart from glutamate action in the inflammatory phase of the formalin test (phase II) there is increased vascular permeability and leukocyte migration. Thus, the effectiveness of melatonin in this phase could be attributed to its ability to inhibit nitric oxide (NO) and peroxynitrite formation [25]. Recently other study indentified that rats with opioid-mediated hyperalgesia showed upregulated protein kinase C (PKC) activity and increase in the cycle adenosine monophosphate (cAMP) levels and these effects were significantly inhibited by melatonin when administered with morphine in adult rats [26]. Therefore, it is important to note that at P60 melatonin promotes analgesic effect only in morphine group in phase I and in both control and morphine group in phase II. These results can be suggested by inhibition of C fibers activity and anti-inflammatory effect of melatonin, since that phase II is more dependent of inflammatory response. Moreover to decrease of hyperalgesic behavior of morphine group, the lack of significant melatonin-mediated antinociception in phase I of the control-melatonin group of P60 might suggest that the dose of melatonin used was too low for get a significant antinociceptive response, as other studies have reported more consistent antinociception with higher doses of melatonin [9,26] .

In summary, the present findings suggest that melatonin is able to reverses hyperalgesic behavior in the formalin test in adulthood induced by repeated morphine exposure in neonatal period. Additionally, this study suggests that melatonin may be useful as an adjunct in pain therapy, especially when neurogenic and inflammatory components are involved.

Acknowledgments: This study was supported by the Brazilian National Council for Scientific and Technological Development – CNPq (Dr. I.L.S. Torres and Dr. W. Caumo), the Graduate Research Group (GPPG) of Hospital de Clínicas de Porto Alegre (Dr. I.L.S. Torres, Grant no. 08-345), and the National Coordination for the Development of Higher Education Personnel – CAPES (J.R. Rozisky, A. Deitos and G. Lates).

References

[1] Franck LS, Vilardi J, Durand D, Powers R. Opioid withdrawal in neonates after continuous infusions of morphine or fentanyl during extracorporeal membrane oxygenation. Am. J. Crit. Care. 1998; 7:364–369.

[2] Ossipov MH, Lai J, King T, Vanderah, TW, Porreca F. Underlying mechanisms of pronociceptive consequences of prolonged morphine exposure. Pept. Sci. 2005; 80:319–325.

[3] Mao J, Mayer DJ. Spinal cord neuroplasticity following repeated opioid exposure and its relation to pathological pain. Ann. NY Acad. Sci. 2001; 933:175–184.

[4] Gardell LR, King T, Ossipov MH, Rice KC, Lai J, Vanderah TW, et al. Opioid receptor-mediated hyperalgesia and antinociceptive tolerance induced by sustained opiate delivery. Neurosci. Lett. 2006; 396:44–49.

[5] Rozisky JR, Medeiros LF, Adachia LS, Espinosa J, de Souza A, Neto AS, et al. Morphine exposure in early life increases nociceptive behaviour in a rat formalin tonic pain model in adult life. Brain Res. 2011; 1367:122–129.

[6] Celerier E, Laulin J, Larcher A, Le Moal M, Simonnet G. Evidence for opiate-activated NMDA processes masking opiate analgesia in rats. Brain Res. 1999; 847:18–25.

[7] Pandi-Perumal SR, Trakht I, Srinivasan V, Spence DW, Maestroni GJ, Zisapel N, et al. Physiological effects of melatonin: role of melatonin receptors and signal transduction pathways. Prog. Neurobiol. 2008; 85:335–353.

[8] El-Shenawy SM, Abdel-Salam OM, Baiuomy AR, El-Batran S, Arbid MS. Studies on the anti-inflammatory and antinociceptive effects of melatonin in the rat. Pharmacol. Res. 2002; 46:235–243.

[9] Ray M, Mediratta PK, Mahajan P, Sharma KK. Evaluation of the role of melatonin in formalininduced pain response in mice. Indian J. Med. 2004; 58:122–130.

[10] Fitzgerald M, Anand KJ. Developmental neuroanatomy and neurophysiology of pain. In: Schechter NL, Berde CB, Yaster M. Pain in infants, children, and adolescents. Williams & Wilkins: Baltimore. 1993; pp. 11–31.

[11] Rozisky JR, Dantas G, Adachi LS, Alves VS, Ferreira MBC, Sarkis JJ, et al. Long-term effect of morphine administration in young rats on the analgesic opioid response in adult life. Int. J. Dev. Neurosci. 2008; 26:561–565.

[12] Rozisky JR, Souza RS, Adachi LS, Capiotti KM, Ramos DB, Bogo MR, et al. Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral cortex of rats. Eur. J. Pharmacol. 2010; 642:72–76.

[13] Rozisky JR, Nonose, Y, Laste, G, Santos VS, Macedo, IC, Battastini A, et al. Morphine treatment alters nucleotidase activities in rat blood serum. Journal of Experimental Pharmacology (in press) 2012.

[14] Wilhelmsen M, Amirian I, Reiter RJ, Rosenberg J, Gögenur I. Analgesic effects of melatonin: a review of current evidence from experimental and clinical studies J. Pineal Res. 2011; 51:270–277.

[15] Laste G, Macedo IC, Rozisky, JR, Silva FR, Caumo, W, TORRES ILS. Melatonin administration reduces inflammatory pain in rats. Journal of Pain Research (in press) 2012.

[16] Tjølsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. Pain. 1992; 51: 5–17.

[17] Tai YH, Wang YH, Wang JJ, Tao PL, Tung CS, Wong CS. Amitriptyline suppresses neuroinflammation and up-regulates glutamate transporters in morphine-tolerant rats. Pain. 2006; 124:77–86.

[18] Netto CA, Siegfried B, Izquierdo I. Analgesia induced by exposure to a novel environment in rats: effect of a concurrent and post-training stressful stimulation. Behav. Neural Biol. 2004; 48:304–309.

[19] Martindale J, Bland-Ward PA, Chessell IP. Inhibition of C-fibre mediated sensory transmission in the rat following intraplantar formalin. Neurosci. Lett. 2001; 316:33–36.

[20] McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, et al. TRPA1 mediates formalin-induced pain. Proc. Natl. Acad. Sci. USA. 2007; 104:13525–13530.

[21] Shigetomi E, Xiaoping T, Kwan KY, Corey D, Khakh BS. TRPA1 channels regulate astrocyte resting calcium and inhibitory synapse efficacy through GAT-3. Nat Neurosci. 2011; 15:70–80.

[22] Dickenson AH, Sullivan AF. Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurones following C fibre stimulation. Neuropharmacology. 1987; 26:1235– 8.

[23] Laurido C, Pelissie T, Soto-Moyano R, Valladares L, Flores F, Hernández A. Effect of melatonin on rat spinal cord nociceptive transmission. Neuroreport. 2002; 13: 89-91.

[24] Sanford M, Silverman MD. Opioid Induced Hyperalgesia: Clinical. Implications for the Pain Practitioner. Pain Physician. 2009; 12:679-684.

[25] Tamura EK, Cecon E, Monteiro AWA, Silva CLM, Markus RP. Melatonin inhibits LPS-induced NO production in rat endothelial cells. J. Pineal Res. 2009; 46:268–274.

[26] Xin W, Chun W, Ling L, Wei W. Role of melatonin in the prevention of morphine-induced hyperalgesia and spinal glial activation in rats: protein kinase C pathway involved. Int J Neurosci. 2012; 122:154-63.

Legends

Figure 1: Effects of melatonin administration on nociceptive behavior in the formalin test at P30 after repeated morphine administration in early life.

Data are presented as mean ± standard error of the mean (SEM) of total time (seconds) spent in nocifensive behavior.

#significant decrease of total time in nocifensive behavior from the control/vehicle group (one-way ANOVA, Student–Newman–Keuls test; $P<0.05$; n=5-7 animals per group).

**significant difference from the control/vehicle group (one-way ANOVA, Student–Newman–Keuls test; $P<0.05$; n=5-7 animals per group).

Figure 2: Effects of melatonin administration on nociceptive behavior in the formalin test at P60 after repeated morphine administration in early life.

Data are presented as mean ± standard error of the mean (SEM) of total time (seconds) spent in nocifensive behavior.

significant difference from the control/vehicle group.

**Significant increase from all groups (one-way ANOVA, Student–Newman–Keuls test; $P<0.05$; n=5-6 animals per group).

Figure 1

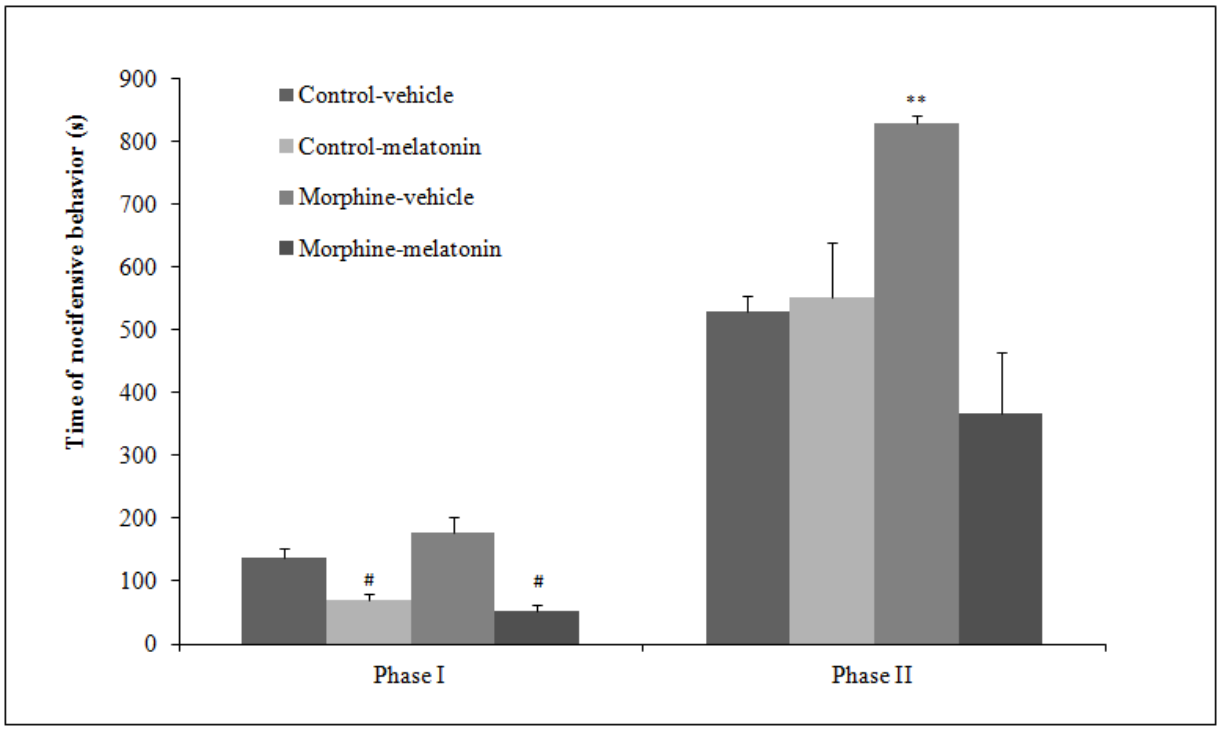
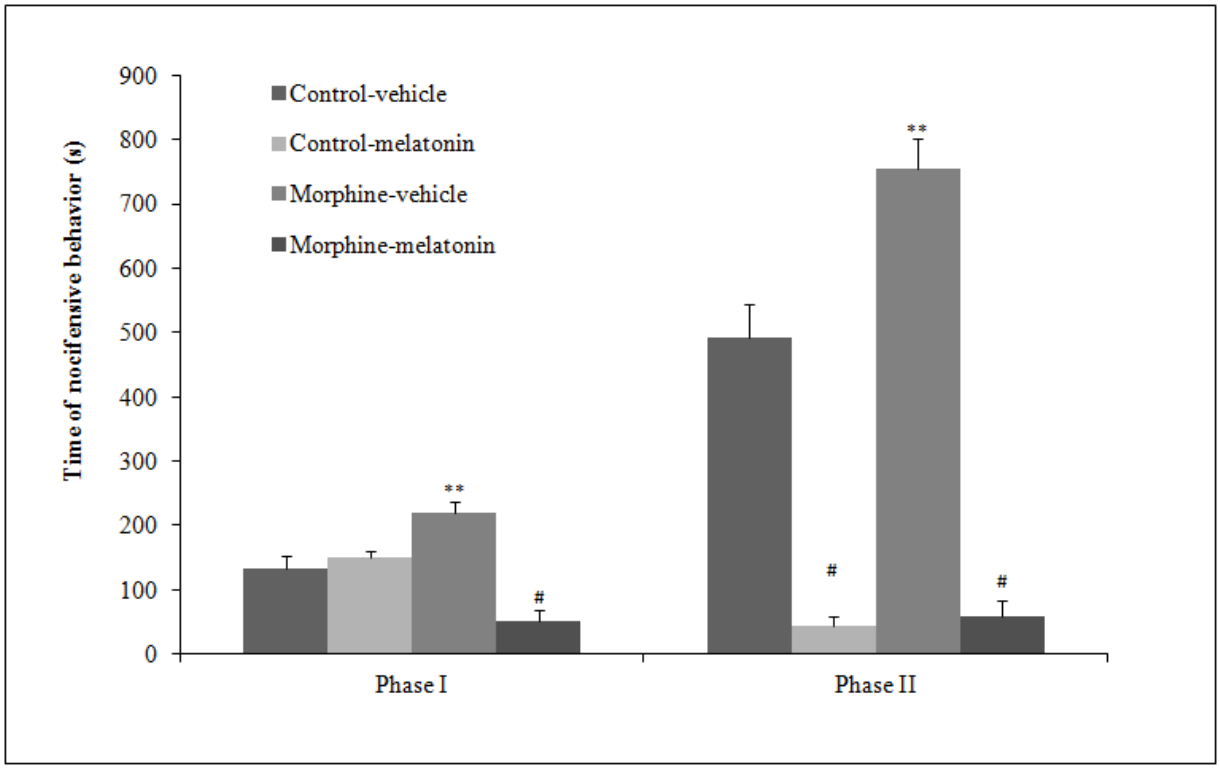


Figure 2



ARTIGO 4

Neonatal morphine administration leads to changes in hippocampal BDNF levels and antioxidant enzyme activity in the adult life of rats

Neurochemical Research (*in press*)

Neonatal morphine administration leads to changes in hippocampal BDNF levels and antioxidant enzyme activity in the adult life of rats

J R Rozisky[1,2,5], G Laste[1,2,5], I C de Macedo[1,5], V S Santos[1,5], R Krolow[4], C Noschang[4], C Vanzella[3,4], K Bertoldi[3], G A Lovatel[3], I C C de Souza[1,2,5], I R Siqueira[3], C Dalmaz[4], W Caumo[1,2,5], I L S Torres[1,2,5*]

[1]Pain Pharmacology and Neuromodulation Laboratory: Animal Models, Department of Pharmacology, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

[2]Postgraduate Program in Medical Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

[3]Neuropsychopharmacology Laboratory, Department of Pharmacology, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

[4]Department of Biochemistry, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

[5]Animal Experimentation Unit, Graduate Research Group – Hospital de Clínicas de Porto Alegre, Brazil.

CORRESPONDENCE: Iraci Lucena da Silva Torres, Departamento de Farmacologia – ICBS, UFRGS. Rua Sarmento Leite, 500 sala 202, Zip Code: 90050-170, Porto Alegre, RS, Brazil. E-mail: iracitorres@gmail.com

Abstract It is know that repeated exposure to opiates impairs spatial learning and memory and that the hippocampus has important neuromodulatory effects after drug exposure and withdrawal symptoms. Thus, the aim of this investigation was to assess hippocampal levels of BDNF, oxidative stress markers associated with cell viability, and TNF-α in the short, medium and long term after repeated morphine treatment in early life. Newborn male Wistar rats received subcutaneous injections of morphine (morphine group) or saline (control group), 5 μg in the mid-scapular area, starting on postnatal day 8 (P8), once daily for 7 days, and neurochemical parameters were assessed in the hippocampus on postnatal days 16 (P16), 30 (P30), and 60 (P60). For the first time, we observed that morphine treatment in early life modulates BDNF levels in the medium and long term and also modulates superoxide dismutase activity in the long term. In addition, it was observed effect of treatment and age in TNF-α levels, and no effects in lactate dehydrogenase levels, or cell viability. These findings show that repeated morphine treatment in the neonatal period can lead to long-lasting neurochemical changes in the hippocampus of male rats, and indicate the importance of cellular and intracellular adaptations in the hippocampus after early-life opioid exposure to tolerance, withdrawal and addiction.

Keywords: Morphine; Neonate rats; BDNF; superoxide dismutase.

Abbreviations: BDNF, brain-derived neurotrophic factor; DCF, dichlorofluorescein; DCFH, dichlorodihydrofluorescein; DMSO, dimethyl sulfoxide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GPx, glutathione peroxidase; HEPES, 2-[4-(2-

hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; INT, 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride; LDH, lactate dehydrogenase; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; PBS, phosphate buffered saline; ROS, reactive oxygen species; RNS, reactive nitrogen species; SNK, Student–Newman–Keuls test; SOD, superoxide dismutase; TMB, 3,3',5,5'-tetramethylbenzidine; TNB, 2-nitro-5-thiobenzoic acid; TNF-α, tumour necrosis factor alpha; TrkB, tyrosine kinase B receptor.

Introduction

Morphine has been widely used in ventilated neonates because of its potent analgesic effect, prolonged duration of action, and relatively low potential for tolerance [1, 2]. Evidence suggests that the tolerance to repeated injections of morphine in pups is less pronounced than in adults, since this tolerance could be masked by several processes [3]. In addition, our group showed that animals submitted to repeated morphine exposure in early life (5µg sc/dia/7dias from P8) did not developed to classic opioid tolerance, and they presented longer analgesia duration at P14 that was observed at least until P80 [4]. These results also agree with another study that suggests that the development of opioid system occurs within the first 2 weeks of life [5]. Thus, there are increasing concerns that opiates may have detrimental effects on neurodevelopment outcomes. Therefore, it is important to understand the effects of chronic opiate exposure on the immature body.

Studies have shown that prolonged exposure to opiates in the adult rat produces distinct biochemical and physiological changes in neurons and in entire interacting circuits of neurons, which, in turn, can result in long-term alterations in cellular communication [6-9]. The opioid exposure can decrease spine density, neurogenesis, and alter synaptic transmission in the hippocampus [10-13]. In contrast, other studies show that opioids can modulate some processes in the hippocampal formation, including adult neurogenesis and development of neonatal transmitter systems [14]. Prolonged exposure to opiates also reduces hippocampal long-term potentiation (LTP) [15, 16] which is related to spatial learning and memory prejudice [17]. On the other hand, our group has shown that exposure of newborn rats to morphine alters nociceptive parameters until adult life [4, 18] and affects some exploratory and anxiolytic-like

behaviours in the short and medium term (Rozisky *et al*., personal communication). These effects are possibly related to drug withdrawal. At a cellular level, other researchers have shown that morphine treatment in the neonatal period alters gene expression in the hippocampus [19] and its cellular composition [20], producing long-term neurobehavioral deficits in rodents [21, 22]. In addition, exposure of rats to this drug in the postnatal period and adult life has also been associated with generation of free radicals due to an imbalance between high cellular levels of reactive oxygen and nitrogen species (ROS and RNS) and cellular antioxidant defences, namely oxidative stress [23, 24]. Both reactive species can induce mitochondrial dysfunction [25], damage neuronal precursors and impair neurogenesis [26]. A consequence of the production of ROS is induction of biosynthesis of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), suggesting a complex interplay between CNS inflammation and oxidative stress [27].

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, is widely expressed in the developing central and peripheral nervous system [28, 29], and in high levels in the hippocampus [30]. After binding to the TrkB (tyrosine kinase B receptor) on the cell surface of neurons, BDNF regulates neuronal survival, promotes neurite outgrowth, and maintains synaptic connectivity in the adult nervous system [31]. Other studies have reported neuromodulatory effects of BDNF on learning and memory, depression [32], and drug addiction [33-35]. There is evidence that BDNF plays a significant role in brain effects, such as synaptic plasticity [36] and locomotor sensitization [37] after opioid withdrawal.

Considering the dearth of studies on the neurochemical effects of neonatal morphine exposure and the involvement of the hippocampus in neuroplasticity process

after drug exposure, the aim of this study was to verify whether repeated morphine administration in early life alters hippocampal BDNF and TNF-α levels, as well as oxidative stress markers and the susceptibility to H_2O_2-induced oxidative damage in hippocampal slices.

Methods

Animals

Animals and housing conditions

Neonate male Wistar rats, 8-day-old at the beginning of the experiment, were used. They were in home cages made of polypropylene (49x34x16 cm) with the floor covered with sawdust, with their mothers, maintained on a standard 12-h dark/light cycle (lights on at 07:00 a.m. and lights off at 07:00 p.m.) at room temperature (22 ± 2°C). The animals had free access to food and water. All experiments in this study were conducted in male rats because the nociceptive process and drug responses are altered by modulations in hormone state [38]. At birth, the male litters were standardized in according Silveira et al. [39, 40] with minor modifications, containing 8 male pups per dam. Studies that assesses the development of offspring during lactation, is the current procedure of adjustment litter in the first days of birth in order to homogenization nutritional conditions for all puppies [41]. Infant rats (at P8) were divided into two groups: saline control (C) and morphine-treated (M). Rats at P8 were chosen because it is accepted that animals of this age have a similar neurological development to that of a human newborn [42]. These animals are in a physiologically immature state [43] since this period is characterized by major developmental changes in the brain and plasticity of the developing pain system [44, 45, 46].

Several reports suggest that the ability to induce long-term potentiation in area CA1 of hipocampal slices appears between the first and second week after birth [47, 48]. In the neonatal hippocampus, glutamatergic LTP is not readily observed until P14 [48, 49] and LTD has not been revealed earlier than P6 [49, 50, 51].

At 21 days of age, the animals were separated from their mothers. Animal handling and all experiments were performed in accordance with Brazilian Law No. 11.794 of October 8, 2008, on the use of animals for scientific research, and performed in accordance with the Guide for the Care and Use of Laboratory Animals 8th edition (2011). All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol No. 08345). And, measures were taken to minimize animal pain and discomfort. The experiment used the minimum number of animals required to produce reliable scientific data.

Drug administration

Each animal received saline (control group) or morphine (5 μg s.c. in the mid-scapular area; morphine group) starting at P8, then once daily for 7 days. This dose was chosen on the basis of previous studies by our research group [4, 18, 52, 53, 54], and is known to produce analgesia in all animals subjected to the tail-flick test. All treatments were administered at the same time each day (11.00 h). One millilitre of morphine sulfate was diluted in 9 ml of 0.9% NaCl (normal saline solution). After two days (P16), two weeks (P30) and six weeks (P60) of the end of treatment, the rats were decapitated and their hippocampi were rapidly dissected out for cell viability analysis or stored at -70^{o} C until BDNF and TNF-α assays and analysis of oxidative stress parameters. The assays

were performed at P16 (n=4-5/group/assay of rats), P30 (n=5-7/group/assay of rats), and P60 (n=4-5/group/assay of rats).

Morphine sulfate (Dimorf® 10 mg/ml, Cristália Ltda., São Paulo, Brazil) and other chemicals were provided by Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil.

Analysis of BDNF immunocontent

BDNF levels were determined by enzyme-linked immunosorbent assay (ELISA) using the ChemiKine kit (Millipore), according to manufacturer recommendations. Briefly, hippocampi were individually homogenised (1:10 w/v) in a lysis buffer containing 1M NaCl, 100 mM Tris–HCl (pH 7.0), 4mM disodium edetate, 2% Triton X-100, and the protease inhibitors (Sigma) aprotinin (5 μg/mL), antipain (0.5 μg/mL), benzamidine (157 μg/mL), pepstatin A (0.1 μg/mL), and phenylmethanesulfonyl fluoride (PMSF, 17 μg/mL). Homogenates were centrifuged at 14000×*g* for 30 minutes at 4°C. The resulting supernatants were used for the BDNF assay. Samples were incubated on 96-well flat bottom plates previously coated with anti-BDNF polyclonal antibody at 2–8°C overnight. After this first incubation, biotinylated mouse anti-BDNF monoclonal antibody was added to each well and incubated for 2-3 h and streptavidin-horseradish peroxidase conjugate for 1 h. TMB/E Substrate was added to each well for 15 minutes until the 500 pg/mL standard reached a deep blue colour. The reaction was stopped by addition of stop solution (HCl) to each well, the blue colour changing to yellow. The colour reaction was quantified immediately by spectrophotometry in a plate reader at 450 nm. The standard BDNF curve was performed on each plate, ranging from 0 to 500 pg/mL.

Analysis of TNF-α immunocontent

The analysis of TNF-α in hippocampus homogenates was performed only at the ages where there were significant differences in BDNF levels (P30 and P60). TNF-α level was determined with a commercially available enzyme-linked immunosorbent assay (ELISA) kit for rat TNF-α (Uscn, Life Science Inc.), according to manufacturer protocols. Briefly, hippocampi were individually homogenised (1:10 w/v) in phosphate buffered saline (PBS). The homogenates were centrifuged at 5000×*g* for 5 minutes at 4°C. The resulting supernatants were used for the TNF-α assay. Samples were incubated on 96-well flat bottom plates previously coated with anti-TNF-α antibody for 2 h at 37°C. After this first incubation, a TNF biotin-conjugated antibody preparation specific for TNF-α was added to each well and incubated for 1 h and avidin-conjugated horseradish peroxidase for 30 minutes at 37°C. TMB/E Substrate was added to each well for 15–25 minutes at 37°C until the 1,000 pg/mL standard reached a deep blue colour. The reaction was stopped by addition of stop solution (H_2SO_4) to each well (the blue colour changing to yellow). The colour reaction was quantified immediately by spectrophotometry in a plate reader at 450 nm. The standard TNF-α curve was performed on each plate, ranging from 0 to 1,000 pg/mL.

Assessment of oxidative stress parameters

Structures were thawed and homogenised in 10 vol (w/v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA. The homogenate was centrifuged at 1000×*g* for 10 min at 4^{o} C and the supernatant was used for assays of oxidative stress.

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined using the RANSOD kit (Randox Labs, USA), which is based on the procedure described by Delmas-Beauvieux [55]. This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a formazan dye that is assayed spectrophotometrically at 492 nm at 37^{o} C. The inhibition in chromogen production is proportional to the activity of SOD present in the sample; one unit of SOD causes 50% inhibition of the rate of INT reduction under the conditions of the assay.

Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was determined as described by Wendel [56], with some modifications. The reaction was carried out at 37^{o}C in a solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione, and 0.4 U glutathione reductase. GPx activity was measured with *tert*-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as nmol of NADPH oxidized per minute per mg of protein.

Evaluation of reactive species production by chemical oxidation of dichlorodihydrofluorescein (DCFH)

Samples were incubated with 2',7'-dichlorodihydrofluorescein diacetate (100 μM) at 37°C for 30 minutes. The formation of the oxidized fluorescent derivative

dichlorofluorescein (DCF) was monitored by excitation and emission wavelengths of 488 and 525 nm respectively, using a SpectraMax M5 plate reader. The formation of reactive oxygen/nitrogen species was quantified using a DCF standard curve and results were expressed as nmol of DCF formed per mg of protein [57].

Determination of total thiol

This assay is based on the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) by thiol groups, which becomes oxidized (disulfide), yielding a yellow compound (2-nitro-5-thiobenzoic acid, TNB) whose absorption is measured spectrophotometrically at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol of TNB per mg of protein [58].

Preparation of hippocampal slices and susceptibility to H_2O_2-induced cell damage

Rats were killed by decapitation on postnatal days 16 (P16), 30 (P30) and 60 (P60), and hippocampi were quickly dissected out and transverse sections (400 µm) prepared using a McIlwain tissue chopper. Slices of the hippocampus of each animal were divided in two sets (control condition and *in vitro* H_2O_2-induced injury), placed into separate 24-well culture plates and pre-incubated for 30 min in a medium with the following composition: 120 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM HEPES, 1 mM KH_2PO_4, and 10 mM glucose, adjusted to pH 7.4. After pre-incubation, the medium in the control plate was replaced with another medium solution and incubated for 60 min at 37°C.

In the H_2O_2-induced injury plate, the medium was changed and 1mM H_2O_2 was added for 60 min at 37°. The control and H_2O_2 experiments were run concomitantly, using

four slices of the same animal in each plate [59]. At the end of the recovery period, cellular viability (mitochondrial activity) and cellular damage (membrane lyses) were evaluated.

Cellular damage

Cellular damage was quantified by measuring lactate dehydrogenase (LDH) released into the culture medium (lacking serum) [60]. After the recovery period, LDH activity was determined using a commercially available kit (Doles Reagents, Goiania, Brazil). Each experiment was normalized by subtracting the background levels of LDH produced from the control wells. Samples were quantified using a standard curve; the optical density was measured at 490 nm. Results were expressed as percentage of control.

Cellular viability

Mitochondrial activity was evaluated by the colorimetric MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma Chemicals) method. Hippocampal slices were incubated for 30 min at 37°C in the presence of MTT (5 mg/ml). Active mitochondrial dehydrogenases of living cells cause cleavage and reduction of the soluble yellow MTT dye to insoluble purple formazan, which was extracted in dimethyl sulfoxide (DMSO); the optical density was measured at 560 and 630 nm [61]. Results were expressed as percentage of control.

Protein assay

Total protein concentration was determined using the Lowry method [62], with bovine serum albumin as the standard.

Statistical analysis

The results were expressed as the mean ± standard error of the mean (S.E.M.). The data analysis and interactions were evaluated using two-way ANOVA (morphine, age, morphine*age) followed by the Student–Newman–Keuls (SNK) method when indicated. The between group differences were considered significant at $P<0.05$.

Results

Hippocampal BDNF and TNF-α levels after morphine exposure in early life

The two way ANOVA showed no effect of age ($P= 0.327$). However, there is effect of morphine ($P= 0.042$), and interaction between age and morphine treatment ($P= 0.049$) in BDNF levels (Fig 1 Panel A).

In the TNF-α levels, the two way ANOVA showed effects of age ($P= 0.036$), of morphine treatment ($P= 0.011$), and no interaction between age and morphine treatment ($P= 0.275$) (Fig 1 Panel B). TNF-α levels were analyzed only at the ages where differences in BDNF were observed (i.e. P30 and P60).

Antioxidant enzyme activities, total thiol levels, and reactive species production in the hippocampus after morphine exposure in early life

Table 1 shows oxidative stress parameters after morphine exposure in newborn rats, from P8 through P14, analyzed at the short (P16), medium (P30), and long term (P60). There is effect of morphine treatment ($P= 0.01$), however it was not observed

effect of age (*P*= 0.127) and no interaction between age and morphine treatment in the SOD activity (*P*= 0.144) (two way ANOVA for all).

In the GPx activity, it was observed effect of age (*P*< 0.001), no effects of morphine treatment (*P*= 0.624) and no interactions between age and morphine treatment (*P*=0.164) (two way ANOVA for all).

In the thiol levels was also observed effect of age (*P*= 0.041), no effects of morphine treatment (*P*= 0.178) and no interactions between age and morphine treatment (*P*=0.254) (two way ANOVA for all).

In the analysis of reactive species production (by DCF technique) were observed effect of age (*P*< 0.001), no effects of morphine treatment (*P*= 0.541) and no interactions between age and morphine treatment (*P*=0.715) (two way ANOVA for all).

In the SOD/GPx ratio was observed effect of age (*P*= 0.031), effect of treatment (*P*= 0.033) and marginal interactions between age and morphine treatment (*P*= 0.06) (two way ANOVA for all).

Susceptibility to oxidative damage in hippocampal slices after morphine exposure in early life

In order to analyse the effect of morphine treatment in early life on H_2O_2-induced injury, we employed lactate dehydrogenase (LDH) released into the media as a marker of cell damage or lysis, and reduction of the soluble yellow MTT dye as a marker of living cells.

Table 2 shows cell damage (expressed by released LDH) and cellular viability (measured by the MTT assay) H_2O_2-induced injury cell at P16, P30 and P60. In the MTT assay, there are no interactions between age and morphine treatment and H_2O_2-

induced cellular damage (P= 0.887), morphine and age (P= 0.880), morphine and H_2O_2-induced cellular damage (P= 0.200), and morphine treatment (P= 0.526) (two way ANOVA for all). However, the adjustment for multiple comparisons (Bonferroni's test) showed an effect of H_2O_2-induced cellular damage (P< 0.001), interaction between age and H_2O_2-induced cellular damage (P= 0.034), and effect of age showed significant difference between P16 and P60 (P=0.009).

The following results were observed for LDH analysis: there are no interactions between age and morphine treatment and H_2O_2-induced cellular damage (P= 0.303), between age and H_2O_2-induced cellular damage (P= 0.502), and morphine treatment and H_2O_2-induced cellular damage (P= 0.664), and not effects of age (P= 0.433) and of morphine treatment (P= 0.611) (two way ANOVA for all). However, the adjustment for multiple comparisons (Bonferroni's test) showed effect of H_2O_2-induced cellular damage (P< 0.001).

Discussion

In this study, we showed that morphine treatment in newborn rats alters BDNF levels and oxidative stress markers in the hippocampus, finding, for the first time, that morphine treatment in early life modulates BDNF levels by age. Furthermore, neonatal morphine exposure negatively modulates superoxide dismutase activity. It bears stressing that this finding—and the parallel modulation in SOD/GPx activity ratio may lead to lower concentrations of H_2O_2, as this ratio has been used as a marker of peroxide overload challenge [63]. In addition, were observed effect of treatment and age in levels of the pro-inflammatory cytokine TNF-α. On the other hand, we observed that both the

control and morphine groups exhibited hippocampal oxidative damage after exposure to H_2O_2 in both ages analyzed under the conditions of this study.

A previous study demonstrated that BDNF is involved as a modulator of morphine withdrawal symptoms, increasing locomotor sensitization, for instance [37]. Consistently with these findings, we have shown that similar treatment with morphine induces some exploratory-like behaviour related to opioid withdrawal in the medium (P30) and long term (P60) (Rozisky *et al*., personal communication). There is evidence that chronic exposure to psychotropic drugs can lead to increased BDNF levels in mesolimbic system-related structures, such as the ventral tegmental area in rats [64]. Moreover, BDNF infusions in this structure dramatically enhance several behavioural effects of drugs, including psychomotor sensitization [64].

Our group recently showed that this morphine treatment promotes a hyperalgesic response in the medium and long term (at P30 and P60), which was reverted by administration of an NMDA receptor antagonist [18] and behavioural sensitization in the short and medium term (at P16 and P30) (Rozisky et al., personal communication). Thus, we can suggest that morphine treatment in early life could lead to increased BDNF levels in the hippocampus, associated with possible central sensitization in the medium and long term. Viewed as a whole, our findings suggest that BDNF could modulate withdrawal symptoms in the medium and long term after morphine exposure in early life, since this neurotrophin might operate as a modulator of synaptic transmission, and this could be a cellular correlate for information processing of withdrawal symptoms in the mammalian brain after exposure to opioids. The spontaneous withdrawal by cessation of morphine administration resulted in selective and specific neuronal and glial cell damage in the cerebral cortex, hippocampus,

brainstem, thalamus and hypothalamus [11-14]. Wan et al. [65] demonstrated that chronic morphine exposure leads to up-regulation of BDNF mRNA and protein in areas associated with rewarding, such as the hippocampus. Yu et al. [66] showed that BDNF could protect neurons, and suppress morphine dependence and withdrawal formation by increasing BDNF gene expression in the hippocampus, *locus coeruleus*, and prefrontal cortex. In addition to these studies, spontaneous morphine withdrawal resulted in selective and specific neuronal and glial cell damage in hippocampus and in other structures related to nociceptive transmission and behavioral sensitization [67, 68, 69].

In this study, we also observed a decrease in superoxide dismutase activity and SOD/GPx ratio after morphine treatment in early life, and only effects of age in other oxidative stress parameters. SOD is considered an important antioxidant enzyme that protects from the damage caused by superoxide radicals [70]. Imbalances in SOD activity and a decrease of the SOD/GPx ratio could lead to increased superoxide anion ($O_2^{\bullet-}$) and a decrease of H_2O_2, and, thus, inhibition of production of the hydroxyl radical ($OH^{\bullet}$), through a reaction with iron or copper (Fenton chemistry) [71], since this radical is the most powerful oxidant molecule. A previous study showed that morphine-induced superoxide production could occur as a result of activation of μ-opioid receptors, leading to activation of the phospholipase D pathway, an increase in intracellular Ca^{2+} and generation of superoxide anion, which can elicit oxidative stress and apoptosis [72]. In addition, evidence suggests that SOD activity is decreased when cells or tissues undergo oxidative stress [73, 74]. Despite this effect on oxidative stress parameters, we observed no differences in cell damage or cell viability between the control and morphine groups when using hippocampal slices. However, when these slices were incubated with H_2O_2, increased LDH release and MTT reduction was observed in both

groups and at all three points in time (P16, P30 and P60) after morphine treatment. Nevertheless, further studies are required to ascertain whether morphine treatment in early life can promote neuronal death or plasticity in the medium and long term.

Additionally, studies have shown that neuroinflammation and expression of pro-inflammatory cytokines, such as TNF-α, are associated with development of morphine tolerance [75], pain transmission and the generation of inflammatory and neuropathic pain [76]. Although these pro-inflammatory cytokines have been shown to be involved in modulation of morphine withdrawal symptoms, we found effects of age and of morphine treatment in hippocampal TNF-α levels. Thus, we suggest that repeated low doses of morphine in early life lead to changes in inflammatory processes in the hippocampus, and it can be related to BDNF changes after morphine withdrawal.

The limitation of the animal studies concerns the potential for their results to be translated. For instance, all experiments in this study were conducted in male rats, complicating the translation of the results to both genders in humans, particularly because the nociceptive process is altered by modulations in hormone state [38]. Our limitations also could be related to it was not evaluated the binding and expression of receptors, this will be the next step of study.

In conclusion, our findings show that repeated morphine treatment in the neonatal period can lead to long-lasting neurochemical changes in the hippocampus of male rats, as demonstrated by increased BDNF levels in the medium and long term and inhibition of SOD activity and decreased SOD/GPx ratio in the long term. Thus, this study indicates the importance of knowledge of cellular and intracellular adaptations that occur in the hippocampus after opioid exposure in early life and the potential importance of these adaptations to withdrawal. In addition, this knowledge may prove

beneficial in limiting the side effects of morphine in clinical practice and in the development of strategies for treatment and prevention of opioid addiction after exposure in the neonatal period.

Acknowledgements: This study was supported by the Brazilian National Council for Scientific and Technological Development – CNPq (I.L.S. Torres, W. Caumo, I.R. Siqueira, C. Dalmaz); the National Coordination for the Development of Higher Education Personnel – CAPES (J.R. Rozisky, G. Laste, I.C. de Macedo) and the Graduate Research Group (GPPG) of Hospital de Clínicas de Porto Alegre (I.L.S. Torres – Grant 08345).

Conflict of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Menon G, Anand KJ, Mdntosh N (1998) Practical approach to analgesia and sedation in the neonatal intensive care unit. Semin Perinatol 22: 417-424

2. Anand KJ, Hall RW (2006) Pharmacological therapy for analgesia and sedation in the newborn. Arch Dis Child Fetal Neonatal Ed 91(6): F448-53

3. Van Praag H, Frenk H (1991) Evidence for opiate tolerance in newborn rats. Brain Res Dev Brain Res 60(1):99-102

4. Rozisky JR, Dantas G, Adachi LS, Alves VS, Ferreira MBC, et al (2008) Long-term effect of morphine administration in young rats on the analgesic opioid response in adult life. Int J Dev Neurosci 26: 561–565

5. Nandi R, Fitzgerald M (2005) Opioid analgesia in the newborn. Eur J Pain 9: 105–108

6. Koob GF, Bloom FE (1988) Cellular and molecular mechanisms of drug dependence. Science 242: 715-23

7. Ingram SL, Vaughan CW, Bagley EE, Connor M, Christie MJ (1998) Enhanced opioid efficacy in opioid dependence is caused by an altered signal transduction pathway. J Neurosci 18: 10269-76

8. Carlezon WA Jr, Boundy VA, Haile CN, Lane SB, Kalb RG, Neve RL, Nestler EJ (1997) Sensitization to morphine induced by viral-mediated gene transfer. Science 277: 812-4

9. Abul-Husn NS, Annangudi SP, Ma'ayan A, Ramos-Ortolaza DL, Stockton SD Jr, Gomes I, et al. (2011) Chronic morphine alters the presynaptic protein profile: identification of novel molecular targets using proteomics and network analysis. PLoS One 6: e25535

10. Corrigall WA (1983) Opiates and the hippocampus: a review of the functional and morphological evidence. Pharmacol Biochem Behav 18: 255-62

11. Simonato M (1996) The neurochemistry of morphine addiction in the neocortex. Trends Pharmacol Sci 17: 410-5

12. Gao H, Xiang Y, Sun N, Zhu H, Wang Y, Liu M, et al (2007) Metabolic changes in rat prefrontal cortex and hippocampus induced by chronic morphine treatment studied ex vivo by high resolution 1H NMR spectroscopy. Neurochem Int 50: 386-94

13. Eisch AJ, Barrot M, Schad CA, Self DW, Nestler EJ (2000) Opiates inhibit neurogenesis in the adult rat hippocampus. Proc Natl Acad Sci U S A 97: 7579-84

14. Drake CT, Chavkin C, Milner TA (2007) Opioid systems in the dentate gyrus. Prog Brain Res 163: 245-63

15. Bao G, Kang L, Li H, Li Y, Pu L, Xia P, Ma L, Pei G (2007) Morphine and heroin differentially modulate in vivo hippocampal LTP in opiate-dependent rat. Neuropsychopharmacology 32(8):1738-49

16. Pu L, Bao GB, Xu NJ, Ma L, Pei G (2002) Hippocampal long-term potentiation is reduced by chronic opiate treatment and can be restored by re-exposure to opiates. J Neurosci 22(5):1914-21

17. Guerra D, Sole A, Cami J, Tobena A (1987) Neuropsychological performance in opiate addicts after rapid detoxification. Drug Alcohol Depend 20:261–270

18. Rozisky JR, Medeiros LF, Adachi LS, Espinosa J, de Souza A, Neto AS, et al (2011) Morphine exposure in early life increases nociceptive behavior in a rat formalin tonic pain model in adult life. Brain Res 1367: 122-9

19. Vien TN, Gleason CA, Hays SL, McPherson RJ, Chavkin C, Juul SE (2009) Effects of neonatal stress and morphine on kappa opioid receptor signaling. Neonatology 96: 235-43

20. Juul SE, Beyer RP, Bammler TK, Farin FM, Gleason CA (2011) Effects of neonatal stress and morphine on murine hippocampal gene expression. Pediatr Res 69: 285-92

21. McPherson RJ, Gleason C, Mascher-Denen M, Chan M, Kellert B, Juul SE (2007) A new model of neonatal stress which produces lasting neurobehavioral effects in adult rats. Neonatology 92: 33-41

22. Boasen JF, McPherson RJ, Hays SL, Juul SE, Gleason CA (2009) Neonatal stress or morphine treatment alters adult mouse conditioned place preference. Neonatology 95: 230-9

23. Halliwell B, Gutteridge JM (2000) Free radicals and antioxidants in the year 2000. A historical look to the future. Annals of the New York Acad of Sci 899: 136–147

24. Guzmán D, Vázquez I, Brizuela N, Alvarez R, Mejía G, García E, et al. (2006) Assessment of oxidative damage induced by acute doses of morphine sulfate in postnatal and adult rat brain. Neurochem Res 31: 549–554

25. Papadopoulos MC, Koumenis IL, Dugan LL, Giffard RG (1997) Vulnerability to glucose deprivation injury correlates with glutathione levels in astrocytes. Brain Res 748: 151–156

26. Kroemer G (1997) The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat Med 3: 614–620

27. Turchan-Cholewo J, Dimayuga VM, Gupta S, Gorospe RM, Keller JN, Bruce-Keller AJ (2009) NADPH oxidase drives cytokine and neurotoxin release from microglia and macrophages in response to HIV-Tat. Antioxid Redox Signal 11: 193-204

28. Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P, et al (1989) Molecular cloning and expression of brain-derived neurotrophic factor. Nature 341: 149-52

29. Hohn A, Leibrock J, Bailey K, Barde YA (1990) Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature 344: 339-41

30. Ernfors P, Merlio JP, Persson H (1992) Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. Eur J Neurosci 4: 1140-1158

31. Zhang HN, Ko MC (2009) Seizure activity involved in the up-regulation of BDNF mRNA expression by activation of central mu opioid receptors. Neuroscience 161: 301-10

32. Altar CA, Boylan CB, Jackson C, Hershenson S, Miller J, Wiegand SJ, et al (1992) Brain-derived neurotrophic factor augments rotational behavior and nigrostriatal dopamine turnover in vivo. Proc Natl Acad Sci U S A. 89: 11347-51

33. Grimm JW, Lu L, Hayashi T, Hope BT, Su TP, Shaham Y (2003) Time-dependent increases in brain-derived neurotrophic factor protein levels within the mesolimbic dopamine system after withdrawal from cocaine: implications for incubation of cocaine craving. J Neurosci 23: 742-7

34. McGough NN, He DY, Logrip ML, Jeanblanc J, Phamluong K, Luong K, et al (2004) RACK1 and brain-derived neurotrophic factor: a homeostatic pathway that regulates alcohol addiction. J Neurosci 24: 10542-52

35. Filip M, Faron-Górecka A, Kuśmider M, Gołda A, Frankowska M, Dziedzicka-Wasylewska M (2006) Alterations in BDNF and trkB mRNAs following acute or sensitizing cocaine treatments and withdrawal. Brain Res 1071: 218-225

36. Li Y, Jia YC, Cui K, Li N, Zheng ZY, Wang YZ, Yuan XB (2005) Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. Nature 43: 4 894– 898

37. Liang J, Zheng X, Chen J, Li Y, Xing X, Bai Y (2011) Roles of bdnf, dopamine d(3) receptors, and their interactions in the expression of morphine-induced context-specific locomotor sensitization. Eur Neuropsychopharmacol 21: 825–834

38. Ribeiro S, Yang P, Reyes-Vazquez C, Swann A, Dafny N (2005) Sex differences in tail-flick latency of non-stressed and stressed rats. Int J Neurosci 115(10):1383-95

39. Silveira PP, Portella AK, Assis SACN, Nieto FB, Diehl LA, Crema LM, Peres W, et al. (2010) Early life experience alters behavioral responses to sweet food and accumbal dopamine metabolism. Int J Dev Neurosci 28:111-118

40. Silveira PP, Portella AK, Benetti C da S, Zugno AI, Scherer EB, Mattos CB, Wyse AT, et al. (2011) Association between Na+,K+-ATPase activity and the vulnerability/resilence to mood disorders induced by early life experience. Neurochem Res 36(11):2075-82

41. Tanaka T (2004). The relationships between litter size, offspring weigth and behavioral development in laboratory mice. Mammal Study, Tokio, 29:147-153

42. Fitzgerald M, Anand KJ (1993) Developmental neuroanatomy and neurophysiology of pain. In: Schechter NL, Berde CB, Yaster M (Eds), Pain in infants, children, and adolescents. Williams & Wilkins, Baltimore, pp 11–31

43. Pattinson D, Fitzgerald M (2004) The neurobiology of infant pain: development of excitatory and inhibitory neurotransmission in the spinal dorsal horn. Reg Anesth Pain Med 29: 36–44

44. Bishop B (1982) Neural plasticity. Part 2. Postnatal maturation and function induced plasticity. Phys Ther 62: 1132–1143

45. Kim JJ, Foy MR, Thompson RF (1996) Behavioral stress modifies hippocampal plasticity through N-methyl-D-aspartate receptor activation. Proc Natl Acad Sci USA 93: 4750–4753

46. Rabinowicz T, de Courten-Myers GM, Petetot JM, Xi G, de los Reyes E (1996) Human cortex development: estimates of neural numbers indicate major loss late during gestation. J Neuropathol Exp Neurol 55: 320–328

47. Baudry M, Arst D, Oliver M, Lynch G (1981) Development of glutamate binding sites and their regulation by calcium in rat hippocampus. Dev Brain Res 1:37-48

48. Harris KH, Teyler TJ (1984) Developmental onset of long-term potentiation in area CA1 of the rat hippocampus. J Physiol (Lond.), 346:27-48

49. Dudek SM, Bear MF (1993) Bi-directional long-term modification of synaptic effectiveness in the adult and immature hippocampus. J Neurosci 13:2910-2918

50. McLean H, Caillard O, Ben-Ari Y, Gaiarsa JL (1996) Bidirectional plasticity expressed by GABAergic synapses in the neonatal rat hippocampus. J Physiol 496: 471-477

51. Bolshakov VY, Siegelbaum SA (1994) Postsynaptic induction and presynaptic expression of hippocampal long-term depression. Science 264: 1148-1152

52. Rozisky JR, Souza RS, Adachi LS, Capiotti KM, Ramos DB, Bogo MR, et al (2010) Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral cortex of rats. Eur J Pharmacol. 642: 72–76

53. Rozisky JR, Vendite D, Fontella FU, Nonose Y, Laste G, Dalmaz C, Caumo W, Torres IL (2012) Morphine treatment in early life alters glutamate uptake in the spinal synaptosomes of adult rats. Neurosci Lett. (in press).

54. Rozisky JR, Nonose Y, Laste G, Santos VS, de Macedo IC, Battastini AM, Caumo C, Torres IL (2012) Morphine treatment alters nucleotidase activities in rat blood serum. J Exp Pharmacol (in press).

55. Delmas-Beauvieux MC, Peuchant E, Dumon MF, Receveur MC, Le Bras M, Clerc M (1995) Relationship between red blood cell antioxidant enzymatic system status and lipoperoxidation during the acute phase of malaria. Clinical Biochemistry 28: 163–169

56. Wendel A (1981) Glutathione peroxidase. Methods in Enzymology 77: 325–333

57. Sriram K, Pai KS, Boyd MR, Ravindranath V (1997) Evidence for generation of oxidative stress in brain by MPTP: in vitro and in vivo studies in mice. Brain Research 21: 44 –52

58. Aksenov MY, Markesbery WR (2001) Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease. Neurosci Lett 302: 141–145

59. Siqueira IR, Elsner VR, Leite MC, Vanzella C, Moysés F dos S, Spindler C, et al (2011) Ascorbate uptake is decreased in the hippocampus of ageing rats. Neurochem Int 58: 527-32

60. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55-63

61. Koh JY, Choi DW (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J Neurosci Methods 20: 83-90

62. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265–275

63. De Oliveira MR, Silvestrin RB, Souza TM, Moreira JCF (2007) Oxidative stress in the hippocampus, anxiety-like behavior and decreased locomotory and exploratory activity of adult rats. Effects of sub acute vitamin A supplementation at therapeutic doses. Neurotoxicology 28: 1191–1199

64. Bolaños CA, Nestler EJ (2004) Neurotrophic mechanisms in drug addiction. Neuromolecular Med 51: 69-83

65. Wan L, Xie Y, Su L, Liu Y, Wang Y, Wang Z (2011) RACK1 affects morphine reward via BDNF. Brain Research 1416: 2 6–3 4

66. Yu H, Hu H, Meng H, Deng W, Fu Y, Luo Q (2011) Brain-derived neurotrophic factor and Bcl-2 expression in rat brain areas following chronic morphine treatment. Neural Regen Res 6(7):528-533

67. Sharma HS, Sjöquist PO, Ali SF (2010) Alterations in blood-brain barrier function and brain pathology by morphine in the rat. Neuroprotective effects of antioxidant H-290/51. Acta Neurochir Suppl 106: 61-66

68. Sharma HS, Sjöquist PO, Ali SF (2007) Drugs of abuse-induced hyperthermia, blood-brain barrier dysfunction and neurotoxicity: neuroprotective effects of a new antioxidant compound H-290/51. Curr Pharm Des 13(18): 1903-1923

69. Sharma HS, Lundstedt T, Boman A, Lek P, Seifert E, Wiklund L, Ali SF (2006) A potent serotonin-modulating compound AP-267 attenuates morphine withdrawal-induced blood-brain barrier dysfunction in rats. Ann. N. Y. Acad. Sci. 1074: 482-496

70. Freeman BA and Crapo JD (1982) Biology of disease. Free radicals and tissue injury. Lab Invest 47: 412-426

71. Halliwell B (2006) Oxidative stress and neurodegeneration. Where are we now? Journal of Neurochemistry 97: 1634–1658

72. Salvemini D (2009) Peroxynitrite and opiate antinociceptive tolerance: a painful reality. Arch Biochem Biophys 484: 238-44

73. Payabvash S, Ghahremani MH, Goliaei A, Mandegary A, Shafaroodi H, Amanlou M, et al (2006) Nitric oxide modulates glutathione synthesis during endotoxemia. Free Radic Biol Med 41: 1817-28

74. Das SK, Mukherjee S, Gupta G, Rao DN, Vasudevan DM (2010) Protective effect of resveratrol and vitamin E against ethanol-induced oxidative damage in mice: biochemical and immunological basis. Indian J Biochem Biophys 47: 32-7

75. Tai YH, Wang YH, Wang JJ, Tao PL, Tung CS, Wong CS (2006) Amitriptyline suppresses neuroinflammation and up-regulates glutamate transporters in morphinetolerant rats. Pain 124:77–86

76. Tsai RY, Jang FL, Tai YH, Lin SL, Shen CH, Wong CS (2008) Ultra-low-dose naloxone restores the antinociceptive effect of morphine and suppresses spinal neuroinflammation in PTX-treated rats. Neuropsychopharmacology 33:2772–82

Legends:

Table 1

There is effect of morphine treatment (P= 0.01), no effect of age (P= 0.127) and no interaction between age and morphine treatment in the SOD activity (P= 0.144); effect of age (P< 0.001), no effects of morphine treatment (P= 0.624) and no interactions between age and morphine treatment in the GPx activity (P=0.164); in the thiol levels was also observed effect of age (P= 0.041), no effects of morphine treatment (P= 0.178) and no interactions between age and morphine treatment (P=0.254); there is effect of age (P< 0.001), no effects of treatment (P= 0.541) and no interactions between age and morphine treatment in the analysis of reactive species production (P=0.715). In the SOD/GPx ratio was observed effect of age (P= 0.031), effect of treatment (P= 0.033) and marginal interactions between age and morphine treatment (P= 0.06).

The data analysis and interactions were evaluated using two-way ANOVA followed by Bonferroni's method. The results are expressed as the mean ± S.E.M.

Abbreviations: SOD, superoxide dismutase; GPx, glutathione peroxidase.

Table 2

In the MTT assay, there are no interactions between age and morphine treatment and H_2O_2-induced cellular damage (P= 0.887), between morphine and age (P= 0.880) and between morphine and H_2O_2-induced cellular damage (P= 0.200). However, there is effect of H_2O_2-induced cellular damage (P< 0.001), and interaction between age and H_2O_2-induced cellular damage (P= 0.034), and no effect of morphine treatment (P= 0.526). There is significant difference between P16 and P60 (P=0.009).

In the LDH assay, there are no interactions between age and morphine treatment and H_2O_2-induced cellular damage (P= 0.303), between age and H_2O_2-induced cellular damage (P= 0.502), and morphine treatment and H_2O_2-induced cellular damage (P= 0.664). However, there is effect of H_2O_2-induced cellular damage (P< 0.001), but there are not effects of age (P= 0.433) and morphine treatment (P= 0.611).

The data analysis and interactions were evaluated using two-way ANOVA followed by Bonferroni's method. The results are expressed as percentage of MTT reduced or LDH released obtained on control group, which is considered as 100%.

Abbreviations: MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase.

Fig. 1. Effect of morphine administration in early life on TNF-α and BDNF levels at postnatal days 16, 30 and 60.

There is effect of morphine (P= 0.042), interaction between age and morphine treatment (P= 0.049), and no effect of age (P= 0.327) in BDNF levels (Panel A).

In the TNF-α levels, there are effects of age (P= 0.036), of morphine treatment (P= 0.011), and no interaction between age and morphine treatment (P= 0.275) (Panel B). TNF-α levels were analyzed only at the ages where differences in BDNF were observed (i.e. P30 and P60).

The data analysis and interactions were evaluated using two-way ANOVA followed by Bonferroni's method. Bars represent mean ± SEM. BDNF (Panel A) and TNF-α (Panel B) levels are expressed as $pg.mL^{-1}$ of hippocampus tissue homogenates.

Abbreviations: BDNF, brain-derived neurotrophic factor; TNF-α, tumour necrosis factor alpha.

Table 1 Effect of morphine administration in early life on antioxidant enzyme activity, total thiol levels, and reactive species production in the hippocampus at postnatal days 16, 30 and 60.

Oxidative Stress Parameters	Postnatal day 16		Postnatal day 30		Postnatal day 60	
	Control	*Morphine*	*Control*	*Morphine*	*Control*	*Morphine*
SOD	8.54 (±1.85)	5.44 (±0.63)	5.03 (±0.38)	5.04 (±0.15)	8.0 (±1.32)	4.68 (±0.37)
GPx	19.04 (±0.42)	22.11 (±1.33)	17.42 (±0.77)	15.76 (±0.79)	29.13 (±1.81)	25.74 (±2.8)
Thiol	46.75 (±4.9)	48.17 (±4.14)	60.94 (±1.68)	57.75 (±1.09)	62.3 (±6.89)	49.26 (±4.41)
DCF	2.35 (±0.13)	2.47 (±0.27)	5.36 (±1.77)	4.47 (±0.38)	14.78 (±1.0)	14.59 (±0.87)
SOD/GPx	0.45 (±0.11)	0.255 (±0,04)	0.29 (±0.02)	0.324 (±0.02)	0.27 (±0.02)	0.18 (±0.01)

Table 2 Effect of morphine administration in early life on lactate dehydrogenase levels and cellular viability before and after oxidative damage (H_2O_2) in hippocampal slices at postnatal days 16, 30 and 60.

Age	*Parameter*	no cell damage induced - H2O2		with cell damage induced - H2O2	
		Control	*Morphine*	*Control*	*Morphine*
Postnatal day 16	LDH released (% of control)	94.22 (±2.44)	94.97 (±2.78)	115.63 (±5.12)	109.14 (±3.03)
	MTT reduction (% of control)	100 (±7.12)	97.48 (±4.2)	53.94 (±2.35)	51.93 (±6.6)
Postnatal day 30	LDH released (% of control)	97.90 (±2.84)	95.89 (±2.97)	105.47 (±0.98)	110.05 (±1.98)
	MTT reduction (% of control)	101.43 (±3.76)	98.76 (±6.22)	61.44 (±3.71)	61.61 (±2.72)
Postnatal day 60	LDH released (% of control)	97.44 (±3.74)	101.77 (±2.06)	118.15 (±3.47)	116.13 (±4.3)
	MTT reduction (% of control)	102.91 (±4.63)	98.34 (±2.8)	70.6 (±3.49)	72.84 (±5.15)

Fig 1 Panel A

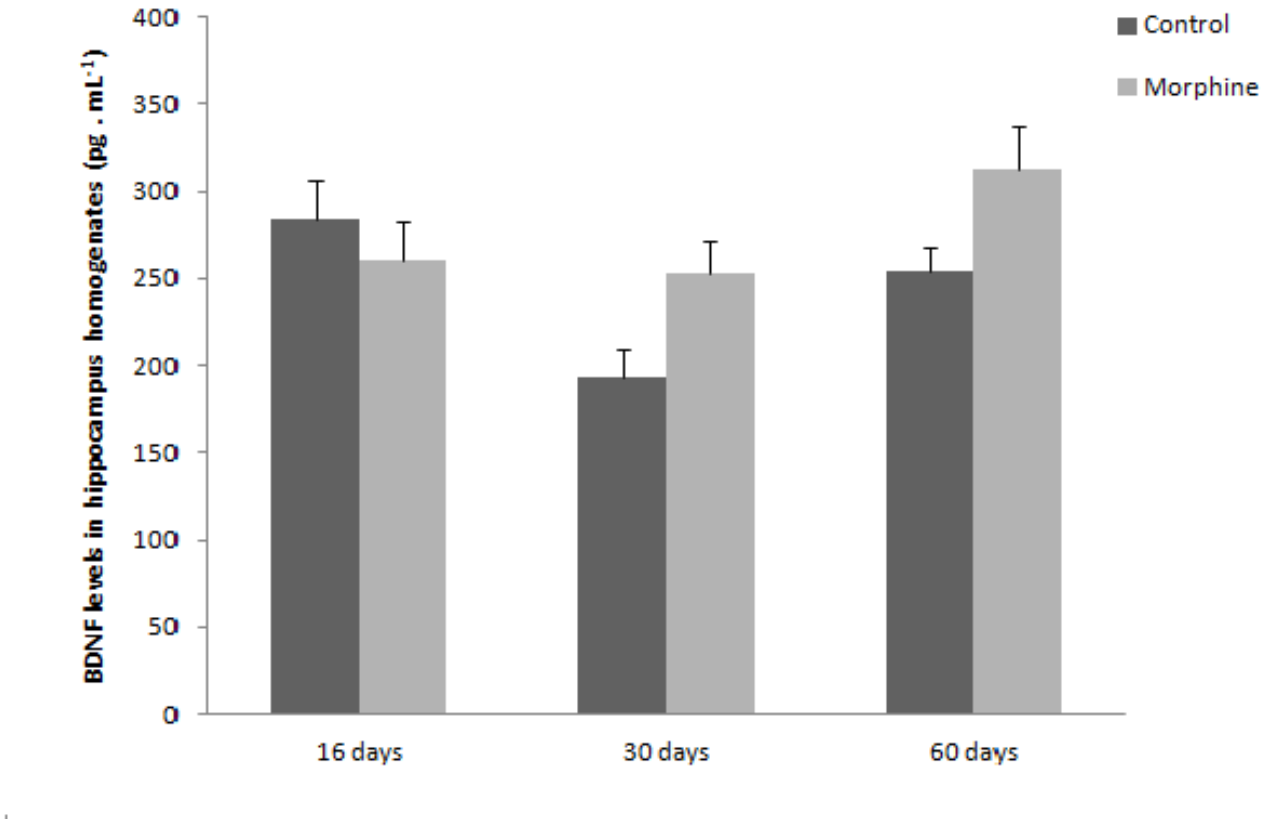
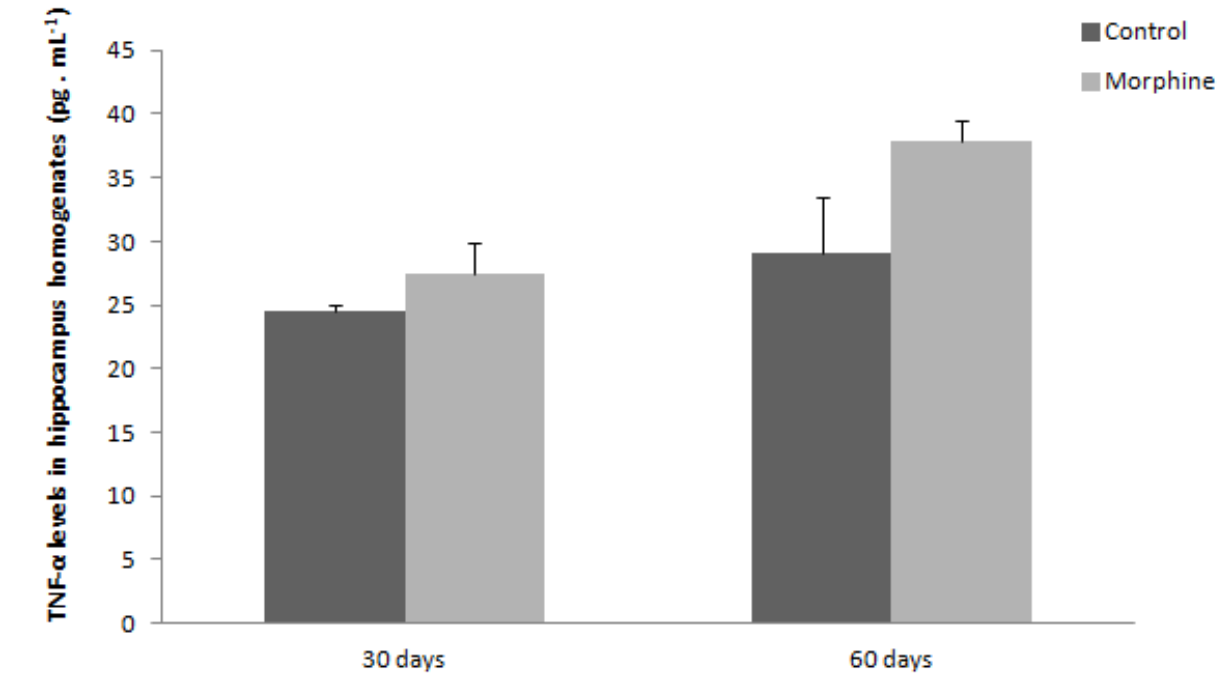

BDNF levels in hippocampus homogenates (pg . mL-1)
400
350
300
250
200
150
100
50
0
16 days
30 days
60 days
Control
Morphine

Fig 1 Panel B


TNF-α levels in hippocampus homogenates (pg . mL-1)
45
40
35
30
25
20
15
10
5
0
30 days
60 days
Control
Morphine

ARTIGO 5

Morphine treatment in neonate rats increases exploratory activity: reversal by antagonist D2 receptor

Submetido na revista Physiology & Behavior

Morphine treatment in neonate rats increases exploratory activity: reversal by antagonist D2 receptor

Joanna R Rozisky[a,b,c], Liciane F Medeiros[b,c], Vinicius S dos Santos[b,c], Gabriela Laste[a,b,c], Isabel C de Macedo[b,c], Lauren S Adachi[a,b,c], Andressa de Souza[a,b,c], Janaina Espinosa[b], Wolnei Caumo[a,b], Iraci L S Torres[a,b,c]

[a] Postgraduate Program in Medical Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 90035-003, Brazil.

[b]Pain Pharmacology and Neuromodulation: animals models Laboratory: Department of Pharmacology, Institute of Basic Health Sciences – Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 90050-170, Brazil.

[c]Animal Experimentation Unit and Graduate Research Group, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS 90035-003, Brazil.

Corresponding author:

I. L.S. Torres

Pain Pharmacology and Neuromodulation: animals models Laboratory: Department of Pharmacology, Institute of Basic Health Sciences – Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 90050-170, Brazil. Street Sarmento Leite, 500 room 202. CEP 90050-170 - Porto Alegre, RS, Brazil.

Phone: 00 55-51 33083894; FAX: 00 55-51 3308 3121.

E-mail: iracitorres@gmail.com

Running head: D_2 antagonist receptor reverses behavioral changes after early morphine exposure

Conflict of Interest: There was no financial relationship between any of the authors or any commercial interest in the outcome of this study.

This work was supported by the Brazilian funding agencies: National Council for Scientific and Technological Development - CNPq (I.L.S. Torres, W. Caumo); Committee for the Development of Higher Education Personnel - CAPES (J.R. Rozisky, G. Laste; A. Souza, L. N. Adachi, L.F. Medeiros); Graduate Research Group (GPPG) of Hospital de Clínicas de Porto Alegre - HCPA (I.L.S. Torres, Grant no. 08-345).

Abstract

Morphine treatment in early life is a practice widely used in pediatric intensive care units. However, the consequences of this treatment on behavioral responses throughout life have been poorly studied. Our objective was to evaluate whether 5 µg morphine administration, once daily for 7 days in 8-day-old rats (P8), alters behavior responses over short- (P16) and medium-term (P30) periods in the open-field (OF) and elevated-plus maze (EPM) tests, and to verify the involvement of the D_2 receptor in these behaviors. In the OF, an increase in grooming at P16 was seen, as well as increases in exploratory behaviors at P30 in the morphine group. In the EPM, the percentage of open arms behaviors, and non-protected head dipping was increased at P30. At the ages at which differences in behaviors were observed, the control and morphine groups received D2 antagonist receptor (haloperidol) 30 min before each test. All behaviors changes seen in the morphine group at P30 were totally reversed by haloperidol administration. Our findings demonstrate that morphine treatment in neonate period promotes behavioral changes in OF and EPM at P16 and P30. However, only alterations observed at P30 depend, at least in part, of dopaminergic system, particularly of the D_2 receptor.

Keywords: morphine; open-field; elevated-plus-maze; exploratory-like behavior; D_2 receptor; neonate rat.

Introduction

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 (Howard, 2003). Unfortunately, more than 48% of infants and children that receive therapeutic doses of intravenous opioids in the intensive care unit demonstrate symptoms of opiate withdrawal (French and Nocera, 1994; Franck et al., 1998). Our research group has been studied the tolerance and dependence effects of opioid treatment in neonate rats. We observed a somewhat unexpected behavioral outcome in neonate rats undergoing spontaneous opioid withdrawal. Specifically, 8-day-old rats that received morphine administration for 7 days promote an altered nociceptive response in the tail-flick test in adulthood (Rozisky et al., 2008), including changes in nociceptive response as evaluated by formalin test until the age of 60 days (Rozisky et al., 2011). In addition, infant rats treated with fentanyl plus S(+)-ketamine exhibited altered behavioral responses in the open-field and in the plus-maze tests (Medeiros et al., 2011). However, when administered separately, we found that each drug produces behavioral changes in both tests (Medeiros et al., 2012). These findings highlight the importance of continue the investigations on the effect of opioid administration in young rats into adulthood and the mechanisms that underling the behavioral changes.

Development of behavioral symptoms after morphine exposure is presumed to depend on certain changes in the dopaminergic system (Kuribara, 1995). In a study recently published by Yang and colleagues (2011), there is a strong evidence of the involvement of D2 receptor in the opioid tolerance and physical dependence. This study demonstrated that haloperidol (D2 antagonist receptor) was able to attenuate opioid antinociceptive tolerance and physical dependence in adult animals (Yang et al., 2011).

Other researches also showed the interactions between the dopamine and opioid systems (Adamus et al., 1981; Cheido and Idova, 2007; Unterwald and Cuntapay, 2008), and they have employed typical antipsychotic drugs to block the dopamine activity.

Take into account the importance of study the short and long term-effects of opioid exposure in early life, our objective was to evaluate the effect of morphine administration during neonatal period in behavioral responses in open field and elevated plus maze tests, as well as to investigate whether D_2 antagonist receptor reverses the behavioral changes.

Methods

Animals and housing conditions

Eight-day-old male Wistar rats were utilized in this study. The animals were housed in home cages made of Polypropylene material (49 x34x16cm) with the floor covered with sawdust. And, they were maintained in a standard 12:12 light-dark cycle (lights on at 07:00 a.m. and lights off at 07:00 p.m.) in a controlled environment (22±2°C). Animals had *ad libitum* access to water and chow. At birth, the litters were standardized in according Silveira et al. (2010, 2011) with minor modifications, containing 8 male pups per dam. Studies that assesses the development of offspring during lactation, is the current procedure of adjustment litter in the first days of birth in order to homogenization nutritional conditions for all puppies (Tanaka, 2004). At 21 days of age, the animals were separated from their mothers. Rats at P8 were chosen because it is accepted that animals of this age have a similar neurological development to that of a human newborn (Fitzgerald and Anand, 1993).

These animals are in a physiologically immature state (Pattinson and Fitzgerald, 2004) since this period is characterized by major developmental changes in the brain and plasticity of the developing pain system (Bishop, 1982; Kim et al., 1996; Rabinowicz et al., 1996).

Pharmacological treatment:

Each animal received saline (control group) or morphine (5 μg s.c. in the mid-scapular area; morphine group) starting at P8, then once daily for 7 days. This dose was chosen based on previous studies by Rozisky et al. (2008, 2010, 2011, 2012). All treatments were administered at the same time each day (1100 h). One milliliter of morphine sulphate (Dimorf® 10 mg/ml, obtained from the Hospital de Clínicas de Porto Alegre, Brazil) was diluted in 9 ml of 0.9% NaCl (saline). The open-field and elevated-plus-maze tests were performed in 16- and 30-day-old rats. The number of animals used per group was 14 to 16 for the open-field test, and 8 to 9 for the elevated-plus-maze test (first experiment).

At the ages where we observed significant differences in behavior responses in both tests, the control and morphine groups were subdivided into three groups, each one designed to evaluate the effect of i.p. administration of a dopamine D2 antagonist receptor (Haloperidol®, obtained from the Hospital de Clínicas de Porto Alegre, Brazil) (Yang et al., 2011), applied 30 min before each test: (1) 0.2 mg/kg of haloperidol; (2) 0.5 mg/kg of haloperidol (control-haloperidol, morphine-haloperidol); (3) Vehicle for haloperidol: 0.5 mg/kg of saline (control-vehicle, morphine-vehicle). The number of animals used per group was 5 to 8 (second experiment).

Open-Field Test

Behavioral assessments were performed in a varnished wood cage, measuring 60cm×40cm×50 cm, with the inside lined with glass. The floor was covered with linoleum and divided with dark lines into 12 squares of 13 cm × 13 cm. Each animal was gently placed in the back left corner, and allowed to explore the surroundings for 5 min (Bianchin et al., 1993; Carlini et al., 2002). The number of line crossings performed by each animal was taken as a measure of locomotor activity (Roesler et al., 1999), while the latency to leave the first quadrant was taken as a measure of anxiety (Britton and Britton, 1981; Lister, 1990). Rearing was defined as the moment the rat rose up on its hind legs, ending when one or both front paws touched the floor again (Wells et al., 2009); this was evaluated as exploratory activity (Silveira et al., 2005). Grooming was defined as licking/washing of the head and body and therefore was regarded as a biological function of caring for the body surface (Spruijt et al., 1992). The start of a trial occurred immediately after the rat was placed in the environment for scoring purposes. In this test, the animal was recorded as entering a new area when all four paws crossed the boundary into a different marked-out area. Four measurements were taken during the 5-min test sessions: (1) Number of line crossings (i.e. horizontal activity) in the OF; (2) latency to leave the first quadrant; (3) grooming (time in seconds); and (4) number of rearing behaviors (i.e. vertical activity). The box was cleaned in between each trial. The test was performed once for each rat.

Elevated-plus-maze test

The elevated-plus-maze is a rodent model of anxiety that is used as a screening test for putative anxiolytic compounds and as a general research tool in neurobiological

anxiety research. The model is based on the rodents' aversion of open spaces. This aversion leads to a behavior termed, thigmotaxis, which involves the avoidance of open areas by confining movements to enclosed spaces or to the edges of a bound space (Rodgers, 1997; Carobrez and Bertoglio, 2005).

The maze was constructed from black PVC synthetic material and elevated to a height of 50 cm above floor level. The apparatus comprised two open arms and two closed arms (50 cm × 40 cm × 10 cm), which extended from a common central platform (10 cm × 10 cm). The animal was placed in the central area of the EPM, facing one of the open arms, and the following behavioral measurements were recorded during the 5-min test sessions: number of protected head-dipping movements (PHD) and of non-protected head-dipping movements (NPHD); the percentage of number of entries into open arms and of total time spent in the open arms. Protected head dips involved dipping the head over the sides of the maze from within the central platform or a closed arm, whereas unprotected head dips were considered to occur when the animal dipped its head over the sides of the maze while in an open arm. In the EPM, entering a new area was recorded when all four paws crossed into a new arm or into the central area. The percentages of open arm entries and of open arm time (Buckman et al., 2009) were calculated, as the standard anxiety indices, as follows: (a) %OAT (the ratio of time spent in the open arms to the total time spent in any arm×100); (b) %OAE (the ratio of entries in open arms compared to total entries×100). After each test, the apparatus was cleaned to remove any animal scent. Both behavioral tests were performed between 09:00 and 16:00h, and the animals were evaluated only once in each test environment.

Statistical analysis

Data was expressed as mean ± standard error of the mean (SEM). In the first experiment, Student's *t* test was used, and in the second experiments one-way ANOVA was used followed by Student-Newman-Keuls multiple comparisons test (SNK) when indicated. Differences were considered statistically significant if $P<0.05$.

Results

The data are presented in two different sections according to the experimental design:

First experiment: the animals exposed to morphine treatment from P8 to P14 were evaluated in the open-field and elevated-plus-maze tests at P16 and P30.

Second experiment: at the ages at which significant differences in behavioral responses were observed in first experiment the both control and morphine groups were subdivided into three groups, which received vehicle (saline) or haloperidol in different doses (0.2 or 0.5 mg/kg) 30 min before each test.

First Experiment: Effects of morphine treatment in early life on behavior in the open-field and elevated-plus-maze tests at P16 and P30

At P16, 2 days after the end of repeated morphine treatment, the morphine group showed only increase grooming time (Student's *t* test, $P=0.001$), and did not present any differences in other behaviors in the open-field (Student's *t* test, $P>0.05$ for all behaviors, Table 1). In the elevated-plus-maze test morphine and control groups did not show differences in all behaviors analyzed (Student's *t* test, $P>0.05$, Table 2).

In contrast, at P30 the morphine group showed the following behaviors compared to control group in open-field test: an increased number of crossings and

rearing, and a decreased grooming time (Student's *t* test, $P<0.05$ for all behaviors, Table 1). No differences was observed for time of latency (Student's *t* test, $P>0.05$, Table 1). In the elevated-plus-maze test the following behaviors were analyzed in morphine group: an increased NPHD, %OAT and %OAE compared to values recorded for the control group (Student's *t* test, $P<0.05$ for both behaviors, Table 2). No differences in PHD was observed between groups (Student's *t* test, $P>0.05$, Table 2).

---------- Insert Tables 1 and 2 about here ----------

Second experiment:

Effects of haloperidol administration on behavior in the open-field test at P16 and P30 after morphine treatment in early life

After daily morphine exposure, from P8 to P14, we investigated whether an injection of 0.2 or 0.5 mg/kg of haloperidol at 30 min before the open-field test could reverse the increased exploratory and locomotor activities at P16 and P30 in the morphine group, compared to those of the control group. Our results demonstrated that at P16, after haloperidol administration, morphine group showed increased grooming time compared to control-vehicle and control-haloperidol, but similar to that morphine-vehicle group, i.e., haloperidol in both doses failed to reverse the increase of this behavior at this age (one-way ANOVA/SNK, $P<0.05$; Table 3). Haloperidol administration increased the latency time and decrease the rearing and crossing behaviors in both groups (control-haloperidol and morphine-haloperidol) compared to groups that received vehicle (control-vehicle and morphine-vehicle) (one-way ANOVA/SNK, $P<0.001$ for both behaviors; Table 3).

At P30, morphine-vehicle group exhibited increased rearing and crossings behaviors (one-way ANOVA/SNK, $P<0.005$ for both behaviors; Table 3) and decreased grooming time compared to all groups (one-way ANOVA/SNK, $P<0.05$; Table 3). However, haloperidol in both doses (0.2 and 0.5 mg/kg) was able to reverse the all behavior changes (increased rearing and crossing, and decreased grooming) observed in morphine-vehicle group (one-way ANOVA/SNK, $P<0.05$; Table 3).

---------- Insert table 3 about here----------

Effects of haloperidol administration on behavior in the elevated-plus-maze test at P30 after morphine treatment in early life

As there were no significant differences in P16 in all the behaviors analyzed in the elevated-plus-maze we did not perform the administration of haloperidol in the control and morphine groups before this test.

However, we investigated the effects of 0.2 or 0.5 mg/kg of haloperidol administration at 30 min before the elevated-plus-maze test at P30. It was observed that the morphine-vehicle group exhibited increased NPHD, %OAE and %OAT when compared to all groups (one-way ANOVA/SNK, $P<0.01$; Table 4). However, haloperidol in both doses was able to reverse these behavioral alterations observed in morphine-vehicle group, i.e., the morphine-haloperidol exhibited the similar NPHD, %OAE and %OAT behaviors to control-haloperidol and control-vehicle. In addition, both groups that received 0.5 mg/kg of haloperidol (control-haloperidol and morphine haloperidol) showed decreased PHD compared to groups that received vehicle (control-vehicle and morphine-vehicle) (one-way ANOVA/SNK, $P<0.01$; Table 4). Morphine groups that received both doses of haloperidol showed totally decreased of PHD

compared to other groups. However, control group-haloperidol groups showed partially decreased of PHD, since that they are similar to morphine-haloperidol groups and control-vehicle and morphine-vehicle (one-way ANOVA/SNK, $P<0.01$; Table 4)

---------- Insert table 4 about here----------

Discussion

This study showed that the animals treated with low doses of morphine during neonatal period, from P8 until P14, showed some exploratory-like behaviors after at least two weeks following the end of treatment (P30). At P16, the morphine group showed an increased grooming time in the open-field test, which was not reversed by haloperidol, and no differences were observed in the behaviors examined in the elevated-plus-maze. At P30 the animals showed increased exploratory behaviors in the open-field (crossing and rearing) and in the elevated-plus-maze (NPHD, %OAT and %OAE), which all were reversed by haloperidol.

Several studies have identified some expected behaviors after morphine withdrawal, such as increased locomotor activity and anxiolytic-like behaviors (Cadoni and Di Chiara, 1999; Buckman et al., 2009; Rezayof et al., 2009; Le Marec et al., 2010). Our results corroborate those previous studies, since we showed that young animals present some exploratory and anxiolytic-like behaviors at two days after morphine withdrawal and at 30 days of age. Although we observed different behaviors, such as increased grooming at P16 and increased rearing, crossing, % of time spent in the open arm, % of number of entries in the open arm and NPHD at P30, it is probable that the treated animals develop age-dependent behavioral responses. It is well known that 16-day-old animals have immature nervous systems and that they are not capable of

showing exploratory behaviors (Arakawa, 2005), such as rearing and crossing. A previous study showed that an increase in the active exploratory pattern is inhibited by the establishment of social relationships among adult rats, while a decrease in activity is a primarily effect of subordination (Arakawa, 2005). Moreover, social interactions have been shown to be rewarding for male juvenile rats (Douglas et al., 2004), which is likely to facilitate the neural maturation of mesolimbic dopamine systems during puberty (Andersen et al., 1997; Vanderschuren et al., 1997). This neural maturation of dopamine systems may contribute to the age-specific behavior in prepubertal male rats, which show marked propensities for risk-taking, novelty seeking, and active exploration (Bardo et al., 1996; Spear, 2000, Palanza et al., 2001). Indeed, basal dopamine synthesis in the nucleus accumbens is lower in juvenile rats than in adults (Andersen et al., 1997). These studies may indicate why the animals at P16 showed no exploratory behavior in open-field and plus-maze tests. On the other hand, the animals treated with morphine showed an increased grooming that was not reversed by haloperidol. The use of a grooming paradigm has been considered quite useful in the investigation of neural abnormalities resulting from pharmacological manipulations in animals (Aude et al., 2006). Modulation of spontaneous grooming has been shown to involve the dopaminergic system, but not at the dopamine D2 receptor. Systemic administration of D1 agonists enhances grooming in rats (Berridge and Aldridge, 2000; Eilam et al., 1992; Van Wimersma Greidanus et al., 1989; Wachtel et al., 1992), whereas systemic administration of D1 antagonists suppresses this behavior (van Wimersma Greidanus et al., 1989). Thus, these considerations may explain why the increase in grooming is not reverted by haloperidol. In contrast, haloperidol in both doses (0.2 and 0.5 mg/kg) increased the time of latency to leave the first quadrant, and consequently decreased the

exploratory behaviors in the open-field test in both groups (control and morphine). It is probable that haloperidol can act on other dopaminergic receptors rather than D2 receptor in this age.

At P30, the morphine group showed characteristic exploratory behaviors, as evidenced by the increase in the number of rearings and crossings in open-field test, which were reversed by haloperidol administration. Interestingly, in association with increased of exploratory behavior, the grooming time was decreased in the morphine group, and this was also reversed by both doses of haloperidol. These behavioral changes occurred synchronously, as the increased exploratory behavior consequently would have decreased the time of grooming, during which the animal remains in the same position but with movements of self-cleaning. As previously described, grooming have been more related to activation of D1 receptor that D2 (Arakawa, 2005). With this we believe that when D2 receptor was blocked by haloperidol, the neurotransmitter dopamine can be able to bind more in D1 receptor, reversing the effect observed at P30, and finding in increase of grooming behavior. In addition, the elevated-plus-maze test demonstrated that the morphine group exhibited an increase in anxiolytic-like behaviors, which were reversed by haloperidol. Considering that these effects were specific for animal treated with morphine, we believe that the behavioral alterations reversed by haloperidol have a relationship with modulation induced by morphine treatment in dopaminergic system, at least in part, at P30 in the D2 receptor. In addition, we suggest that both exploratory and anxiolytic-like behaviors analyzed in open-field and plus-maze tests are different for each age analyzed.

It well established that the dopaminergic pathway has a crucial role in the locomotor and anxiolytic-like effects induced by morphine. Morphine is known to

stimulate dopaminergic transmission, particularly in two areas, the dorsolateral caudate-putamen and nucleus accumbens (Cadoni and Di Chiara, 1999). This effect is regarded as the substrate for their motor stimulant effects (Babbini and Davis, 1972; Di Chiara and Imperato, 1988; Koob, 1992; Wise and Bozarth, 1987; Vanderschuren and Kalivas, 2000). Katz (1980) also showed that low doses of opioid, such as morphine, produced two age related effects: behavioral activation which increased with age, and catalepsy which decreased with age. It might be speculated that the maturational differences in behavior reflect perhaps a concomitant change in the availability of some mediating transmitter, or perhaps altered receptor number or sensitivity (Katz, 1980). Whatever the cause of these effects, however, they suggest that opiate systems or systems associated with the latter, show maturational changes which last up to the adulthood of the rat (Katz, 1980).

Although the neural control of motor behavior has been studied in detail in the adult rat, the ontogeny of dopamine system that mediated the motor activity has been examined much less thoroughly. The dopaminergic system is functional in modulating behavior activity as early as the prenatal period (Moody et al. 1993). Some studies have related that systemic administration of a dopamine agonist produces quantitatively greater behavioral effects in adulthood than during the pre weanling or adolescent periods (for reviews, see Spear and Brake, 1983; Carlezon et al., 2003). It might due the development of this neurochemical system, which rapidly develops during the infantile period, particularly during the second postnatal week of life. The density of D2 receptors in nucleus accumbens is significantly higher in 14-day-old rats than in 7-day-olds rats (Tarazi and Baldessarini, 2000). By the second postnatal week of life there is evidence that the dopaminergic system participates in locomotor activity and stereotypic

behavior (McDougall et al., 1990) and modulates locomotor and motivational effects induced by psychostimulants (McDougall et al., 1992, 1994; Pruitt et al., 1995). On the other hand, the pattern of μ-opioid receptor expression also changes after birth in several central nervous systems structures, such as spinal cord (Marsh et al., 1997; Rahmann et al., 1998), mesencephalon, thalamus and midbrain (Kornblum et al., 1987). Besides the studies of μ-opioid receptors ontogeny, early activation of opioid receptors by exogenously administered agonists may induce long-term changes in receptor number and distribution (Tsang and Ng, 1980), and it can lead to concomitant behavioral changes after birth (Zagon and McLaughlin, 1978). These data, together with those of our study, suggest that the changes in the dopamine and opioid receptors after birth might be critical for the long-term effects of morphine. It is likely that the exploratory and anxiolytic-like behaviors induced by morphine treatment in neonatal period may be related to the alterations in dopamine release and / or in number or sensitization of dopamine receptors in the central nervous system areas that regulates these behaviors.

Some other factors have been shown to influence locomotor activity after morphine exposure, such as type of treatment and regimen - chronic or intermittent, and the time at which the behaviors were observed (Vanderschuren et al., 1997; Vanderschuren and Kalivas, 2000; Le Maerc et al., 2010). In contrast to most of studies, which usually measure exploratory behaviors at one or two time points after morphine treatment, we followed the behavior of animals from two days after the end of treatment (P16), 2 weeks (P30) and 6 weeks (P60). However, during this last stage, we did not observe differences between the morphine and control groups (data not shown). Thus,

our results demonstrate that a low dose and intermittent regimen of morphine in early life can alter the exploratory and anxiolytic-like behaviors.

In conclusion, these studies showed that early exposure to morphine results in the subsequent development of altered behavioral responses that depend, at least in part, of the dopamine D_2 receptor. These data also suggest that these behavioral responses could change in an age-dependent manner, and thus the mechanisms involved in the behavioral changes after opioid withdrawal in the younger differ from those in adult animals. In addition, these findings indicate the importance of evaluating the clinical consequences of long-term opioid administration and highlight the need for novel studies involving the design of agents that may counteract opiate-induced behavioral adaptations.

Acknowledgments: This work was supported by the Brazilian funding agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Dr. I.L.S. Torres, Dr W.Caumo), Graduate Research Group (GPPG) at Hospital de Clínicas de Porto Alegre (Dr. I.L.S. Torres, Grant no. 08-345), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We would like to thanks to Dr Nicola Conran for the English correction and editing of the manuscript.

Ethical standard: The experimental protocol was approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol No: 08345). All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), Brazilian Community's Council Directive of October 8, 2008 (Law No. 11.794) and conformed to the Guide Laboratory for the care and use of animals 8thed 2011. Animal handling and all experiments were performed in accordance with Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003) for animal welfare and measures were taken to minimize animal pain and discomfort. The experiment used the number of animals necessary to produce reliable scientific data. To control the possible effect of outliers we exclude those rats which did not present anything response in the behavioral tests. All the experimenters were blinded to condition treatment during the post treatment behavioral test.

Authors' contributions: J. Rozisky carried out the design of the study, and performed the experimental assays and statistical analysis; V. Santos, L. Medeiros, G. Laste, L. Adachi, I.C, Macedo, A. Souza and J. Espinosa carried out the experimental assays; W.

Caumo participated in the design of the study; I. Torres coordinated the study, performed the statistical analysis, and helped to draft the manuscript. All authors read and approved the final manuscript.

References

Adamus A, Melzacka M, Vetulani J (1984). Behavioral and pharmacokinetic interaction between morphine and haloperidol in the rat. *Pol J Pharmacol Pharm* 36(1):51-8.

Andersen SL, Dumont NL, Teicher MH (1997). Developmental differences in dopamine synthesis inhibition by (±)-7-OH-DPAT. *Naunyn-Schmiedeberg's Arch Pharmacol* 356: 173–181.

Arakawa H (2005). Interaction between isolation rearing and social development on exploratory behavior in male rats. *Behav Processes* 70(3):223-34.

Aude MC, Goulet S, Doré FY (2006). Repeated subchronic exposure to phencyclidine elicits excessive atypical grooming in rats. *Behav Brain Res* 167:103–11.

Babbini M, Davis WM (1972). Time-dose relationships for locomotor activity effects of morphine after acute or repeated treatment. *Br J Pharmacol* 46(2):213-24.

Bardo MT, Donobew RL, Harrington NG (1996). Psychobiology of novelty seeking and drug seeking behavior. Behav. *Brain Res* 77: 23–43.

Berridge KC, Aldridge JW (2000). Super-stereotypy I: enhancement of a complex movement sequence by systemic dopamine D1 agonists. *Synapse* 37:194–204.

Bianchin M, Walz R, Ruschel AC, Zanatta MS, Da Silva RC, Bueno e Silva M, et al. (1993). I. Memory expression is blocked by the infusion of CNQX into the hippocampus and/or the amygdala up to 20 days after training. *Behav Neural Biol* 59(2):83-6.

Bishop B (1982). Neural plasticity. Part 2. Postnatal maturation and function induced plasticity. *Phys Ther* 62(8):1132–1143.

Britton DR, Britton KT (1981). A sensitive open field measure of anxiolytic drug activity. *Pharmacol Biochem Behav* 15(4):577-82.

Buckman SG, Hodgson SR, Hofford RS, Eitan S (2009). Increased elevated plus maze open-arm time in mice during spontaneous morphine withdrawal. *Behav Brain Res* 197(2):454-6.

Cadoni C, Di Chiara G (1999). Reciprocal changes in dopamine responsiveness in the nucleus accumbens shell and core and in the dorsal caudate-putamen in rats sensitized to morphine. *Neuroscience* 90(2):447-55.

Carlezon WA Jr, Mague SD, Andersen SL (2003). Enduring behavioral effects of early exposure to methylphenidate in rats. *Biol Psychiatry* 54(12):1330-7.

Carlini VP, Monzón ME, Varas MM, Cragnolini AB, Schiöth HB, Scimonelli TN, et al. (2002). Ghrelin increases anxiety-like behavior and memory retention in rats. *Biochem Biophys Res Commun* 299(5):739-43.

Carobrez AP, Bertoglio LJ (2005). Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. *Neurosci Biobehav Rev* 29(8):1193-205.

Cheido MA, Idova GV (2007). The differential contribution of dopamine D(1) and D (2) receptors to mu-opioidergic immunomodulation. *Neurosci Behav Physio* 37(7):721-4.

Di Chiara G, Imperato A (1988). Opposite effects of mu and kappa opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of freely moving rats. *J Pharmacol Exp Ther* 244(3):1067-80.

Douglas LA, Varlinskaya EI, Spear LP (2004). Rewarding properties of social interactions in adolescent and adult male and female rats: impact of housing social

versus isolate housing of subjects and partners. *Dev Psychobiol* 45:153–162.

Eilam D, Talangbayan H, Canaran G, Szechtman H (1992). Dopaminergic control of locomotion, mouthing, snout contact, and grooming: opposing roles of D1 and D2 receptors. *Psychopharmacology (Berl)* 106:447–54.

Fitzgerald M, Anand KJ (1993). Developmental neuroanatomy and neurophysiology of pain. In: Schechter NL, Berde CB, Yaster M (ed). Pain in infant, children, and adolescents. Williams & Wilkins, Baltimore, pp 11-31.

Franck LS, Vilardi J, Durand D, Powers R (1998). Opioid withdrawal in neonates after continuous infusions of morphine or fentanyl during extracorporeal membrane oxygenation. *Am J Crit Care* 7:364-9.

French JP, Nocera M (1994). Drug withdrawal symptoms in children after continuous infusions of fentanyl. *J Pediatr Nurs* 9:107-13.

Howard RF (2003). Current status of pain management in children. *JAMA* 290:2464-9.

Johnson SW, North RA (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J Neurosci* 12(2):483-8.

Katz RJ (1980). Ontogenetic determinants of behavioral responses to morphine in rats. *Drug Alcohol Depend* 5(2):129-33.

Kim JJ, Foy MR, Thompson RF (1996). Behavioral stress modifies hippocampal plasticity through N-methyl-D-aspartate receptor activation. *Proc Natl Acad Sci U.S.A.* 93(10):4750–4753.

Koob GF (1992). Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci* 13(5):177-84.

Kornblum HI, Hurlbut DE, Leslie FM (1987). Postnatal development of multiple opioid receptors in rat brain. *Brain Res* 465(1-2):21-41.

Kuribara H (1995). Modification of morphine sensitization by opioid and dopamine receptor antagonists: evaluation by studying ambulation in mice. *Eur J Pharmacol* 275:251-8.

Le Marec T, Marie-Claire C, Noble F, Marie N (2011). Chronic and intermittent morphine treatment differently regulates opioid and dopamine systems: a role in locomotor sensitization. *Psychopharmacology (Berl)* 216(2):297-303.

Lister RG (1990). Ethologically-based animal models of anxiety disorders. *Pharmacol Ther* 46:321–40.

Marsh DF, Hatch DJ, Fitzgerald M (1997). Opioid systems and the newborn. *Br J Anaesth* 79(6):787-95.

McDougall SA, Arnold TF, Nonneman AJ (1990). Ontogeny of locomotor activity and grooming in the young rat: role of dopamine D1 and D2 receptors. *Eur J Pharmacol* 186(2-3):223-30.

McDougall SA, Crawford CA, Nonneman AJ (1992). Reinforced responding of the 11-day-old rat pup: synergistic interaction of D1 and D2 dopamine receptors. *Pharmacol Biochem Behav* 42(1):163-8.

McDougall SA, Duke MA, Bolanos CA, Crawford CA (1994). Ontogeny of behavioral sensitization in the rat: effects of direct and indirect dopamine agonists. *Psychopharmacology (Berl)* 116(4):483-90.

Medeiros LF, Rozisky JR, de Souza A, Hidalgo MP, Netto CA, Caumo W, Battastini AM, Torres IL (2011). Lifetime behavioural changes after exposure to anaesthetics in infant rats. *Behav Brain Res* 218(1):51-6.

Medeiros LF, Souza AC, Souza A, Cioato SG, Scarabelot VL, Caumo W, Fernandes LC, Torres IL (2012). Fentanyl administration in infant rats produces long-term

behavioral responses. *Int J Dev Neurosci* 30(1):25-30.

Moody CA, Robinson SR, Spear LP, Smotherman WP (1993). Fetal behavior and the dopamine system: activity effects of D1 and D2 receptor manipulations. *Pharmacol Biochem Behav* 44(4):843-50.

Palanza P, Gioiosa L, Parmigiani S (2001). Social stress in mice: gender differences and effects of estrous cycle and social dominance. *Physiol Behav* 73: 411–420.

Pattinson D, Fitzgerald M (2004). The neurobiology of infant pain: Development of excitatory and inhibitory neurotransmission in the spinal dorsal horn. *Reg Anesth Pain Med* 29(1):36-44.

Pruitt DL, Bolanos CA, McDougall SA (1995). Effects of dopamine D1 and D2 receptor antagonists on cocaine-induced place preference conditioning in preweanling rats. *Eur J Pharmacol* 283(1-3):125-31.

Rabinowicz T, de Courten-Myers GM, Petetot JM, Xi G, de los Reyes E (1996). Human cortex development: estimates of neural numbers indicate major loss late during gestation. *J Neuropathol Exp Neurol* 55(3):320–328.

Rahman W, Dashwood MR, Fitzgerald M, Aynsley-Green A, Dickenson AH (1998). Postnatal development of multiple opioid receptors in the spinal cord and development of spinal morphine analgesia. *Brain Res Dev Brain Res* 108(1-2):239-54.

Rezayof A, Hosseini SS, Zarrindast MR (2009). Effects of morphine on rat behaviour in the elevated plus maze: the role of central amygdala dopamine receptors. *Behav Brain Res* 202(2):171-8.

Rodgers RJ (1997). Animal models of 'anxiety': where next? *Behav Pharmacol* 8:477-96.

Roesler R, Walz R, Quevedo J, de-Paris F, Zanata SM, Graner E, et al. (1999). Normal inhibitory avoidance learning and anxiety, but increased locomotor activity in mice devoid of PrP^{C}. *Mol Brain Res* 71:349–53.

Rozisky JR, Dantas G, Adachi LS, Alves VS, Ferreira MB, Sarkis JJ, Torres IL (2008). Long-term effect of morphine administration in young rats on the analgesic opioid response in adult life. *Int J Develop Neurosci* 26:561-5.

Rozisky JR, da Silva RS, Adachi LS, Capiotti KM, Ramos DB, Bogo MR, et al. (2010). Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral cortex of rats. *Eur J Pharmacol* 642(1-3):72-6.

Rozisky JR, Medeiros LF, Adachi LS, Espinosa J, de Souza A, Neto AS, Bonan CD, Caumo W, Torres IL (2011). Morphine exposure in early life increases nociceptive behavior in a rat formalin tonic pain model in adult life. *Brain Res* 1367: 122-9.

Rozisky JR, Nonose Y, Laste G, Santos VS, Macedo IC, Battastini AMO, Caumo W, Torres ILS (2012). Morphine treatment alters nucleotidase activities in rat blood serum. *J Exper Pharmacol (in press).*

Silveira PP, Portella AK, Clemente Z, Gamaro GD, Dalmaz C (2005). The effect of neonatal handling on adult feeding behavior is not an anxiety-like behavior. *Int J Dev Neurosci* 23(1):93-9.

Silveira PP, Portella AK, Assis SACN, Nieto FB, Diehl LA, Crema LM, Peres W, et al. (2010). Early life experience alters behavioral responses to sweet food and accumbal dopamine metabolism. *Int J Dev Neurosci* 28:111-118.

Silveira PP, Portella AK, Benetti C da S, Zugno AI, Scherer EB, Mattos CB, Wyse AT, et al. (2011). Association between Na+,K+-ATPase activity and the vulnerability/resilence to mood disorders induced by early life experience. *Neurochem*

Res 36(11):2075-82.

Spear LP, Brake SC (1983). Periadolescence: age-dependent behavior and psychopharmacological responsivity in rats. *Dev Psychobiol* 16(2):83-109.

Spear LP (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci Biobehav Rev* 24: 417–463.

Spruijt BM, van Hoof JA (1992). Gispen W.H. Ethology and neurobiology of grooming behavior. *Physiol Rev* 72:825-52.

Tanaka T (2004). The relationships between litter size, offspring weigth and behavioral development in laboratory mice. *Mammal Study, Tokio,* 29:147-153.

Tarazi FI, Baldessarini RJ (2000). Comparative postnatal development of dopamine D(1), D(2) and D(4) receptors in rat forebrain. *Int J Dev Neurosci* 18(1):29-37.

Tsang D, Ng SC (1980). Effect of antenatal exposure to opiates on the development of opiate receptors in rat brain. *Brain Res* 188(1):199-206.

Tzschentke TM (2001). Pharmacology and behavioral pharmacology of the mesocortical dopamine system. *Prog Neurobiol* 63(3):241-320.

Unterwald EM and Cuntapay M (2000). Dopamine-opioid interactions in the rat striatum: a modulatory role for dopamine D1 receptors in delta opioid receptor-mediated signal transduction. *Neuropharmacology* 39:372-381.

Van Wimersma Greidanus TB, Maigret C, Torn M, Ronner E, Van der Kracht S, Van der Wee NJ, et al (1989). Dopamine D-1 and D-2 receptor agonists and antagonists and neuropeptide-induced excessive grooming. *Eur J Pharmacol* 173:227–31.

Vanderschuren LJ, Kalivas PW (2000). Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacology (Berl)* 151(2-3):99-120.

Vanderschuren LJ, Tjon GH, Nestby P, Mulder AH, Schoffelmeer AN, De Vries TJ (1997). Morphine-induced long-term sensitization to the locomotor effects of morphine and amphetamine depends on the temporal pattern of the pretreatment regimen. *Psychopharmacology (Berl)* 131(2):115-22.

Wachtel SR, Brooderson RJ, White FJ (1992). Parametric and pharmacological analyses of the enhanced grooming response elicited by the D1 dopamine receptor agonist SKF 38393 in the rat. *Psychopharmacology (Berl)* 109:41–8.

Wells CE, Krikke B, Saunders J, Whittington A, Lever C (2009). Changes to open field surfaces typically used to elicit hippocampal remapping elicit graded exploratory responses. *Behav Brain Res* 197(1):234-8.

Wise RA, Bozarth MA (1987). A psychomotor stimulant theory of addiction. *Psychol Rev* 94(4):469-92.

Yang C, Chen Y, Tang L, Wang ZJ (2011). Haloperidol disrupts opioid antinociceptive tolerance and physical dependence. *J Pharmacol Exp Ther* 338(1):164-72.

Zagon IS, McLaughlin PJ (1978). Perinatal methadone exposure and its influence on the behavioral ontogeny of rats. *Pharmacol Biochem Behav* 9(5):665-72.

Legends

Table 1.

At P16 morphine group showed a significant difference in grooming time compared to control group (Student's *t* test, $P<0.05$) and no differences were observed between groups for latency time, number of rearing and crossing (Student's *t* test, $P> 0.05$). At P30 morphine group showed increase in number of rearing and crossing, and a decrease of grooming time compared to control group (Student's *t* test, $P<0.05$). No difference were observed in latency time (Student's *t* test, *P*> (Student's *t* test, $P> 0.05$).

The data were expressed by mean ± SEM.

[#] significantly different from control group.

Table 2.

No differences were observed at P16 in between groups in all behaviors analyzed (Student's *t* test, $P>0.05$). However, at P30 morphine group showed an increase in % of open arm time (%OAT) and in open arm entries (%OAE), and in Non-protected head dipping (Student's *t* test, $P< 0.05$).

The data were expressed by mean ± SEM, with a significant difference if $P<0.05$.

[#] significantly different from control group.

Table 3:

At P16 both groups that received 0.2 or 0.5 mg/kg of haloperidol (control-haloperidol and morphine-haloperidol) showed decreased rearing and crossings, and increased latency compared to groups that received vehicle (control-vehicle and morphine-

vehicle) (one way ANOVA, $P<0.01$). On the other hand, morphine-vehicle and morphine-haloperidol (0.2 and 0.5 mg/kg) groups showed increased of grooming time compared to other groups (one way ANOVA, $P<0.01$).

At P30 morphine-vehicle group showed increase in rearing and crossing behaviors, and decreased grooming time compared to all groups, since that both doses of haloperidol administration reverses this effect (morphine-haloperidol is statistically similar to control-vehicle and control-haloperidol groups (one way ANOVA, $P<0.01$). No differences between groups were observed for latency to leave the first quadrant (one way ANOVA, $P>0.05$).

The data were expressed by mean $\pm$ SEM.

* significantly different from the control-vehicle group.

Table 4:

Morphine-vehicle group showed increased % of open arm time, % of open arm entries and number of NPHD compared to other groups (one way ANOVA/SNK, $P<0.01$), since that haloperidol in both doses reverses these behavioral parameters. In addition, morphine groups that received haloperidol in both doses (0.2 and 0.5 mg/kg) showed totally decreased PHD compared to control-vehicle group, and control-haloperidol group showed partially decreased of this parameter compared to control-vehicle, since that both groups that received haloperidol in both doses are statistically similar, but only morphine-haloperidol groups are different to morphine-vehicle and control-vehicle groups (one way ANOVA/SNK, $P< 0.01$).

significantly different from the control-vehicle and morphine-vehicle groups.

* significantly different from control-vehicle group.

Table 1. Effects of repeated morphine administration in early life on behavioral responses in the open-field test at P16 and P30.

Behaviors Parameters in Open Field test	Postnatal day 16		Postnatal day 30	
	Control	*Morphine*	*Control*	*Morphine*
Latency to leave the first quadrant (s)	25.43 (±3.39)	23.37 (±2.59)	7.06 (±1.11)	5.78 (±1.02)
Grooming (s)	16.71 (±1.05)	26.87* (±2.45)	10.66 (±1.83)	6.0* (±1.06)
Rearing (counts)	13.71 (±1.43)	15.81 (±1.04)	34.33 (±2.44)	47.28* (±3.79)
Crossing (counts)	35.92 (±4.14)	41.18 (±3.86)	70.86 (±3.85)	98.35* (±4.12)

Table 2. Effects of repeated morphine administration in early life on behavioral responses in the elevated plus-maze test at P16 and P30.

Behaviors Parameters in Elevated Plus Maze test	Postnatal day 16		Postnatal day 30	
	Control	*Morphine*	*Control*	*Morphine*
Non-protected head dipping (counts)	1.37 (±0.62)	2.0 (±1.0)	0.5 (±0.3)	6.14* (±1.9)
Protected head dipping (counts)	0.63 (±0.32)	0.43 (±0.29)	4.13 (±1.2)	3.14 (±0.45)
Open arm time (%)	8.7 (±3.0)	11.94 (±4.2)	2.72 (±1.04)	10.43* (±1.54)
Open arm entries (%)	25.0 (±9.4)	24.05 (±9.6)	14.54 (±5.5)	38.24* (±4.64)

Table 3. Effects of haloperidol administration on behaviors in the open fiel test at P16 and P30 after repeated morphine administration

Age	*Behaviors Parameters in Open Field test*	Vehicle		Haloperidol 0.2 mg/kg		Haloperidol 0.5 mg/kg		*F*
		Control	*Morphine*	*Control*	*Morphine*	*Control*	*Morphine*	
Postnatal day 16	Latency to leave the first quadrant (s)	26.92 (±3.53)	31.0 (±4.55)	300 (±0)*	293.75 (±6.25)*	260.75 (±40)*	233.2 (±40.98)*	49.56
	Grooming (s)	18.25 (±0.94)	29.63* (±2.44)	17.25 (±2.62)	23.33 (±3.38)*	13.33 (±2.07)	28.75 (±7.75)*	5.18
	Rearing (counts)	15.38 (±1.58)	13.92 (±1.2)	2.25 (±1.1)*	3.25 (±1.1)*	3.63 (±0.8)*	6.28 (±3.1)*	11.22
	Crossing (counts)	33.23 (±4.02)	31.14 (±4.53)	0*	0.75 (±0.75)*	2.75 (±2)*	6.57 (±3.47)*	13.29
Postnatal day 30	Latency to leave the first quadrant (s)	5 (±0.57)	4.66 (±0.74)	2.6 (±0.6)	4 (±0.85)	3.87 (±0.72)	2.87 (±0.72)	1.60
	Grooming (s)	15.22 (±1.56)	4.1 (±0.95)*	15.16 (±2.72)	14.33 (±3.7)	18.8 (±2.74)	16.16 (±2.28)	6.52
	Rearing (counts)	32.2 (±1.86)	49.92 (±3.45)*	22.5 (±3.5)	19.5 (±3.65)	30.12 (±5.72)	28.25 (±3.75)	9.57
	Crossing (counts)	66.26 (±2.92)	98.35 (±4.12)*	71.5 (±7.14)	56.5 (±8.34)	63.12 (±6.08)	70.62 (±4.59)	10.49

Table 4. Effects of haloperidol administration on behaviors in the elevated plus-maze test at P30 after repeated morphine administration

Age	*Behaviors Parameters in Elevated Plus Maze test*	Vehicle		Haloperidol 0.2 mg/kg		Haloperidol 0.5 mg/kg		*F*
		Control	*Morphine*	*Control*	*Morphine*	*Control*	*Morphine*	
Postnatal day 30	Non-protected head dipping (counts)	0.5 (±0.37)	6.14 (±1.94)*	1.75 (±0.8)	1.75 (±1.0)	0.75 (±0.41)	1.71 (±0.8)	4.26
	Protected head dipping (counts)	3.14 (±0.91)	3.14 (±0.45)	1.2 (±0.37)#	0.6 (±0.6)*	1.25 (±0.41)#	0.5 (±0.26)*	5.01
	Open arm time (%)	4.29 (±2.85)	36.97 (±5.32)*	9.9 (±6.6)	6.25 (±3.6)	0	8.9 (±5.8)	10.08
	Open arm entries (%)	0.37 (±0.25)	17.49 (±5.82)*	4.27 (±1.6)	3.5 (±2.2)	0.41 (±0.41)	2.13 (±1.0)	6.05

ARTIGO 6

Morphine treatment alters nucleotidase activities in rat blood serum

Journal of Experimental Pharmacology 2012; 4:187-193

MORPHINE TREATMENT ALTERS NUCLEOTIDASE ACTIVITIES IN RAT BLOOD SERUM

JOANNA RIPOLL ROZISKY[1,2,4,#], YASMINE NONOSE[1,4,#], GABRIELA LASTE[1,2,4], VINICIUS SOUZA DOS SANTOS[1,4], ISABEL CRISTINA DE MACEDO[1,2,4], ANA MARIA OLIVEIRA BATTASTINI[2,3], WOLNEI CAUMO[2,4], IRACI L. S. TORRES[1,2,4,*]

[1] Laboratório de Farmacologia da Dor e Neuromodulação: Modelos Animais – Departamento de Farmacologia – Instituto de Ciências Básicas da Saúde - Universidade Federal do Rio Grande do Sul, CEP 90050-170 - Porto Alegre – Rio Grande do Sul – Brazil

[2] Programa de Pós-Graduação em Medicina – Ciências Médicas - Universidade Federal do Rio Grande do Sul – CEP 90035-003 – Porto Alegre – Rio Grande do Sul – Brazil

[3] Departamento de Bioquímica – Instituto de Ciências Básicas da Saúde – Universidade Federal do Rio Grande do Sul, Prédio Anexo CEP 90035-003 – Porto Alegre – Rio Grande do Sul – Brazil.

[4] Unidade de Experimentação Animal e Grupo de Pesquisa e Pós-Graduação – Hospital de Clínicas de Porto Alegre – CEP 90035-003 – Porto Alegre – Rio Grande do Sul – Brazil.

[#] Both authors contributed equally to this work.

[*]CORRESPONDING AUTHOR:

Iraci Lucena da Silva Torres

Departamento de Farmacologia - ICBS, UFRGS.

Rua Sarmento Leite, 500 sala 202.

90050-170 - Porto Alegre, RS, Brazil.

Phone: 0055-51 3308 3183; FAX: 0055-51 3308 3121.

E-mail: iracitorres@gmail.com

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

Running header: Neonatal morphine treatment and NTPDase activity

Abstract

Morphine has been widely used in neonatal pain management. However, this treatment may produce adaptive changes in several physiologic systems. Our laboratory has demonstrated that morphine treatment in neonate rats alters nucleoside triphosphate diphosphohydrolase (NTPDase) activity and gene expression in central nervous system structures. Considering the relationship between the opioid and purinergic systems, our aim was to verify whether treatment with morphine from postnatal days 8 (P8) through 14 (P14) at a dose of 5 µg per day alters NTPDase and 5´-nucleotidase activities in rat serum over the short, medium and long term. We also investigated the ontogenic profile of these soluble nucleotidases. Morphine group showed increased hydrolysis of all nucleotides at P30, and a decrease in ADP (adenosine 5'-diphosphate) hydrolysis at P60. Moreover, we found that nucleotidase activities change with age: ADPase activity was lower at P16, and AMPase activity was higher at P60. These changes are very important, because these enzymes are the main regulators of blood nucleotide levels and, consequently, nucleotide signaling. Our findings showed that morphine treatment alters nucleotide hydrolysis in rat blood serum, which suggests that purine homeostasis can be influenced by opioid treatment in the neonatal period.

1. INTRODUCTION

Opioids, such as morphine, are widely used in neonatal pain management because of reported benefits to neonatal behavior and positive outcomes from opioid-based analgesia and anesthesia.[1-3] However, this practice may produce adaptive changes in the opioid system and associated signaling systems, leading to neuronal plasticity in brain regions and in other tissues and cells expressing opioid receptors, such as in the vascular and cardiac systems.[4-6]

Adenosine 5′-triphosphate (ATP) and its breakdown products, adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), and adenosine, produce marked effects upon a range of biological processes, including neurotransmission and neuromodulation, and play an important role in cardiovascular system regulation.[7-9] ATP and ADP are released into the bloodstream from vascular smooth muscle, endothelium, and circulating blood cells, and via outflow upon cell lysis. When released, they promote a range of effects on platelets, endothelial tissue, and vascular smooth muscle.[10] Inactivation of extracellular adenine nucleotides is performed by the enzymes of the nucleoside triphosphate diphosphohydrolase family (NTPDases), nucleotide phosphate/phosphodiesterase family (NPP), alkaline phosphatases, and 5′-nucleotidase. The NTPDases hydrolyze ATP and ADP, while 5′-nucleotidase hydrolyzes AMP to adenosine.[11] These enzymes are ubiquitously coexpressed in endothelial and hematopoietic cells and are the main regulators of purinergic signaling in the blood.[12] Previous studies have demonstrated the presence of soluble NTPDases in rat blood serum as well as in human blood.[13,14] Our group has also demonstrated the presence of NTPDase and 5'-nucleotidase in rat blood serum.[15-21] Furthermore, the

presence of NTPDases associated with circulating plasma microparticles has been noted.[22] These circulating soluble nucleotidases play a role in controlling the availability of ATP, ADP, AMP and adenosine, keeping extracellular levels within physiological limits and forming an enzymatic pathway with the dual function of removing one signal in the form of ATP and generating another in the form of adenosine.[23] Thus, soluble NTPDase and 5'-nucleotidase are expressed in mammals, with the important role of maintaining homeostasis.

Our laboratory has investigated the effects of morphine treatment in early life upon E-NTPDase activities in central nervous system structures. We demonstrated that morphine administration in early life alters E-NTPDase activity and gene expression in the rat spinal cord and cerebral cortex.[24] These relationships between the opioid and purinergic systems have been also studied by other researchers, who have shown that morphine administration promotes adenosine release in the central nervous system.[25] Likewise, cross-tolerance and cross-withdrawal studies have led to the proposal that a μ-opioid, α2-adrenergic, A1-adenosine receptor complex mediates antinociception in the periphery.[26] Moreover, morphine has also been shown to stimulate cardiovascular release of adenosine, a well-documented cardioprotective agent, in hypotension.[27]

Considering this close relationship between the opioid and purinergic systems, the present study investigated the activities of NTPDases and 5'-nucleotidase in rat blood serum after repeated morphine exposure in early life. We also investigated the ontogenic profile of these soluble nucleotidases.

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 housed in Plexiglas home cages (65 cm x 25 cm x 15 cm) with sawdust-covered floors. Animals were kept on a standard 12-hour dark/light cycle (lights on between 0700 h and 1900 h), at room temperature (22 + 2°C), with free access to food and water. At birth, 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 is accepted that animals of this age are at a similar stage of neurological development to that of a human newborn.[28] It is also accepted that they are in a physiologically immature state.[29] since this period is characterized by major developmental changes in the brain and plasticity of the developing pain system.[30-32] Animal handling and all experiments were performed in accordance with international guidelines for animal welfare. The protocol of this experimental study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre, 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 provided by Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil. All other reagents were analytical grade.

2.3. Morphine treatment

Each animal received saline (control group) or morphine (5 μg s.c. in the mid-scapular area; morphine group) starting at postnatal day 8 (P8), then once a day for 7 days, such that at start of treatment (P8) the morphine's dose was 0.33mg/kg since the animals weighed in average about 15 g, and at the end of treatment (P14) the dose administered was 0.23 mg/kg since animals weighed in average about 22 g. This dose was chosen on the basis of the first study conducted by our group in this line of research, because it was the lowest dose able to induce analgesia in neonate rats.[33] Moreover, our group has demonstrated that this dose, when administered for 7 days in the postnatal period, induces changes in NTPDase 1 activity and its mRNA expression pattern in the rat spinal cord and cerebral cortex two days after the end of treatment, which constitutes a short-term effect.[24] In other studies, we found that this treatment regimen induces hyperalgesic behavior in the medium and long term.[34] All treatments were administered at the same time each day (1100 h). One milliliter of morphine sulfate was diluted in 9 ml of 0.9% NaCl (saline solution). At the end of each stage of the experiment, rats were decapitated for collection of blood samples. Enzyme assays were performed at postnatal day 16 (P16) (n=4-5 per group), postnatal day 30 (P30) (n=5-7 per group), and postnatal day 60 (P60) (n=4-5 per group).

2.4. Isolation of blood serum fraction

Blood samples were drawn after decapitation, as described previously, and were soon centrifuged in plastic tubes at 5000*g* for 5 minutes at room temperature, without any additives or anticoagulant.[14] After centrifugation, the supernatant (serum) was separated and kept frozen at −20 °C until the assays were performed.

2.5. Enzyme assay (determination of NTPDase and 5'-nucleotidase activity)

ATP and ADP hydrolysis were determined using a modification of the method described by Oses and colleagues.[13] 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) and 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*g* for 5 min to eliminate precipitated protein and the supernatant was used for colorimetric assay. The inorganic phosphate (Pi) released was measured by the Malachite green method.[35] 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.

For all enzyme assays, 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. Protein concentration was measured by the Coomassie Blue method, using bovine serum albumin as standard.[36] Enzyme activity was expressed as nmol of inorganic phosphate released per minute per milligram of protein (nmol of $Pi.min^{-1}.mg^{-1}$ protein).

2.6. Statistical analysis

The between-group comparisons were performed with Student's *t*-test for independent samples or one-way ANOVA followed by the Student–Newman–Keuls test, as indicated in the figure legends. The results were expressed as mean ± standard error of the mean (SEM). Differences were considered significant at $P < 0.05$.

3. RESULTS

3.1. Effect of morphine treatment on nucleotide hydrolysis in rat blood serum

We verified the effects of morphine administration in early life on nucleotide hydrolysis in rat serum at different ages. It was performed after daily morphine exposure from postnatal days 8 through 14, and NTPDase and 5´-nucleotidase activities in serum was analyzed at postnatal days 16, 30 and 60. In this assay, we found no significant between-group differences at P16 (ATP: C=1.63±0.26; M=1.60±0.37; ADP: C=1.2±0.15; M=1.21±0.38; AMP: C=1.32±0.22; M=1.03±0.1; Student's *t* test, *P*>0.05; Fig. 1). However, at P30, the morphine group exhibited a significant increase in hydrolysis of all nucleotides when compared to the control group (ATP: C=2.14±0.2; M=4.22±0.56; ADP: C=2.45±0.16; M=3.71±0.47; AMP: C=0.9±0.22; M=1.85±0.3; Student's *t* test, *P*<0.05; Fig. 1). At P60, the morphine group exhibited a significant decrease in ADP hydrolysis when compared to the control group (ADP C= 3.45±0.52; M=1.97±0.2; Student's *t* test, *P*<0.05), while there was no difference in the hydrolysis of other nucleotides (ATP: C=2.77±0.71; M=2.72±0.38; AMP: C=2.29±0.51; M=1.5±0.2; Student's *t* test, *P*>0.05; Fig. 1).

---------- Insert Fig. 1 about here ----------

In order to verify the presence of soluble NTPDases in serum after morphine exposure, we analyzed the ratio of ATP/ADP hydrolysis in all ages after morphine treatment. A value of ~1:1 was observed, with no significant between-group differences in all ages (P16: C=1.06±0.13; M=1.31±0.1; P30: C=0.93±0.15; M=1.2±0.22; P60: C=0.96±0.3; M=1.23±0.14; Student's *t* test, $P>0.05$, Fig. 2). These results indicate the presence of NTPDase 1, which hydrolyzes ATP and ADP equally well.[37]

----------Insert Fig. 2 about here ----------

3.2. Effect of aging on nucleotide hydrolysis in rat blood serum:

We also assessed whether nucleotide hydrolysis changes with age. We found that ADP hydrolysis was lower at P16 as compared to P30 and P60 ($F_{(2,13)} = 11.847$; one way ANOVA, SNK, $P<0.05$, Fig. 3a). On the other hand, 5'-nucleotidase activity was higher at P60 than at P16 or P30 ($F_{(2,13)} = 5.044$; one way ANOVA, SNK, $P<0.05$, Fig. 3a). No changes in ATP hydrolysis were observed between these ages ($F_{(2,13)} = 2.010$; one way ANOVA, $P>0.05$, Fig. 3a). The ~1:1 ratios of ATP/ADP hydrolysis suggest the presence of NTPDase 1 at all analyzed ages. The ratio between ATPase and ADPase activity remained constant at all analyzed ages ($F_{(2,13)} = 0.218$; one-way ANOVA, $P>0.05$, Fig. 3b).

---------- Insert Fig. 3a and 3b about here ----------

4. DISCUSSION

In this study, we have shown that ATPase, ADPase and AMPase activities in rat serum are sensitive to morphine administration in neonatal period. We observed that this treatment positively modulates the hydrolysis of all nucleotides at the medium term (P30). Meanwhile, in the long term (P60), morphine administration negatively modulates ADP hydrolysis. These results show a long-term effect of morphine treatment in early life upon soluble NTPDases and 5'-nucleotidase in serum. Notably, the parallel increase in ATP/ADP hydrolysis seen at P30 suggests a possible effect on the activity of the soluble NTPDase 1-like enzyme, since parallel behavior for the hydrolysis of these substrates is a characteristic of this enzyme.

Previous studies by our group have evidenced the presence of soluble NTPDases and 5'-nucleotidase in rat blood serum.[15-21] Under baseline conditions, vascular endothelial NTPDases and 5'-nucleotidase, acting in concert, are the enzymes that regulate nucleotide homeostasis in the vasculature.[38,12] NTPDase-1 is expressed on the cell surface of vascular endothelial tissue and smooth muscle cells, and it may be released through proteolytic cleavage of membrane-bound NTPDase or co-released with ATP and norepinephrine via sympathetic stimulation.[38-40] Ecto-5'-nucleotidase, which hydrolyzes AMP to adenosine, is attached to the cell surface, and may occur also in a soluble form via cleavage of its glycosyl-phosphatidylinositol (GPI)-anchor by phospholipase C.[41] Likewise, it is able to reduce infarct size and improve cardioprotection both in wild-type and CD73−/− mice during acute myocardial ischemia, and also to attenuate tissue damage and improve survival during acute lung injury.[42,43]

NTPDase 1 is notable for its high preference for nucleoside triphosphates and nucleoside diphosphates.[44,45] When NTPDase 1 is active, extracellular ATP is converted

to AMP and then to adenosine by 5'-nucleotidase, and ADP is not an appreciable product. Thus, we can suggest that the difference in ATP and ADP hydrolysis observed at P30 is possibly due to an increase in NTPDase 1-like activity in serum, since our results showed a ~1:1 ratio of ATPase/ADPase.[45] On the other hand, at P60 ADP hydrolysis was decreased. In this way ATP is converted to ADP by other ATPases, such as NTPDase 2, NPPs and alkaline phosphatase, and ADP concentrations will be relatively stable. In this case, AMP (which is the substrate for 5'-nucleotidase) may be reduced, and ADP may accumulate in the bloodstream. However, no change in 5'-nucleotidase activity was observed, making it difficult to infer whether this outcome will result in decreased extracellular adenosine *in vivo*. Moreover, decreased activity of NTPDase 1 may prolong the effect of ATP and ADP at their respective receptors.[46] In our experimental conditions, we did not rule out a potential role of NTPDase 5 and 6 in addition to NTPDase 1 and 2.

In a previous study, we demonstrated that ecto-ATPase, ecto-ADPase and ecto-5'-nucleotidase activities in rat spinal cord synaptosomes change in relation to gender and age.[47] It is likely that these ecto-nucleotidases, regulating the concentration of ATP and adenosine in the synaptic cleft, play an important role in central nervous system development.[48] Corroborating these findings, we verified in the present study that the nucleotide hydrolysis profile in the serum of control animals also changes with age, with lower ADPase activity at P16 and higher AMPase activity at P60. This change is very important, because these enzymes are regarded as the major regulators of purinergic signaling in blood.[49]

The relationships between the purinergic and vascular systems have been widely studied. ATP can be released into the circulation with norepinephrine from sympathetic

nerves, causing vasoconstriction via purinergic P2X receptors on smooth muscle cells.[50,51] In addition, there is growing evidence for the role of ATP as a neurotransmitter in perivascular non-adrenergic/non-cholinergic nerves, which promote vasodilatation through direct actions on vascular smooth muscle, or indirectly through their effects on the endothelium, mediated by purinergic P2Y receptors.[50] ADP is a potent platelet aggregator and it also promotes vasoconstriction.[10,52] In contrast, adenosine—besides other effects—inhibits platelet aggregation.[52] Therefore, soluble nucleotidases also may play an important role in the maintenance of homeostasis and thromboregulation, as they reduce excess levels of these nucleotides in the bloodstream.[38] This may partly explain why ADPase activity is lower in young life and the AMPase activity is higher in adult life in naïve animals. It is well known that, at P16, the rat cardiovascular system is still immature, and that modulation of nucleotide levels is very important to the development of cardiac and vascular functions and contributes to the regulation of vascular tone.[53] In addition, the regulation of ATP-metabolizing enzymes in blood probably plays a significant role in the normal function of this tissue at different ages. Conversely, 5'-nucleotidase activity is higher in adult life. This can lead to increased blood levels of adenosine, which can thus exert its vascular and cardioprotective effects.[27]

Studies about relationship between opioid and cardiovascular systems are well know, Chronic opioid-μ receptor stimulation can decrease muscle sympathetic nerve activity and plasma levels of norepinephrine.[54] Furthermore, low doses of morphine have been found to elicit cardioprotective effects against myocardial ischemia-reperfusion through modulation of sympathetic nervous system activity, thus suggesting an additional role of the central nervous system in the cardioprotective effect of opioid drugs.[55] In a

previous study, we demonstrated that norepinephrine increases soluble NTPDase 1-like activity in blood serum by a direct stimulatory effect.[20] This positive modulatory effect suggests a new role for circulating norepinephrine in regulation of the nucleotidases (probably soluble NTPDase-1) pathway, where it may induce a decrease in extracellular ATP and ADP in the circulation.[20] Taking into account that the morphine treatment in early life changes soluble nucleotidase activities in rat serum in medium and long term, observed as increased NTPDase 1-like and 5'nucleotidase at P30, and decreased NTPDase 1-like at P60, we can suggest that in these different ages soluble nucleotidases modulate the cardiovascular tonus by control the levels of nucleotides in blood serum, such as increase of adenosine levels at P30 and increase ADP levels at P60. Although adenosine is well know by inhibits platelet aggregation and ADP induces platelet aggregation and vasconstriction, we do not know whether nucleotides actually exert the mentioned functions in medium and long-term after morphine treatment in early life, as several other factors are very important in determining the direction of the vascular response elicited by adenine nucleotides, such as the presence and nature of the purinoceptor subtype involved and its location in relation to the structural components of the vascular wall. Other studies concerning the functional relevance of these changes are warranted.

In light of the above, we suggest that the results of the changes on nucleotidase activities were of medium and long-term after morphine treatment, i.e., after 2 and 6 weeks after end of treatment. In this way, we believe that there are not circulating morphine in blood serum and it was no able to act directly on nucleotidase structures. Thereby, morphine treatment during neonatal period could act at transcriptional level, at the similar manner showed by our previous work when were observed these changes in

central nervous system structures.[24] In addition, we emphasize that the peak of enzyme protein expression does not always coincide with enzyme activity.[56,57] For example, high protein levels of NTPDase 1 from synaptic plasma membrane from cerebral cortex did not match the peak of enzymatic activity.[57] These findings indicate that changes in enzyme activity are not necessarily reflected by similar changes in expression levels.

In summary, our findings showed that morphine treatment in neonatal period altered nucleotide hydrolysis in rat serum, which suggests that purine homeostasis can be influenced by opioid treatment in the neonatal period. This modulator effect of morphine could involve the maintenance of extracellular nucleotide levels in medium and long-term and, consequently, contribute to the inhibition of platelet aggregation and thrombus formation induced by ATP and ADP. Moreover, we propose that this change in the nucleotide pathway could be considered a new pathway of side effects in the medium and long term after discontinuation of morphine treatment.

Acknowledgements: This study was supported by the Brazilian National Council for Scientific and Technological Development – CNPq (I.L.S. Torres); the National Coordination for the Development of Higher Education Personnel – CAPES (J.R. Rozisky, G. Laste, I.C. de Macedo); Fundação de Amparo à Pesquisa do Rio Grande do Sul – FAPERGS (V. S dos Santos; PRONEM 03/2011); the Graduate Research Group (GPPG) of Hospital de Clínicas de Porto Alegre (I.L.S. Torres – Grant 08345); and the Office of the Vice-Provost for Research, Universidade Federal do Rio Grande do Sul – PROPESQ-UFRGS (Y. Nonose).

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Ethical standard All experiments were performed in accordance with Brazilian Law No. 11.794 of October 8, 2008. All efforts were made to minimize animal suffering and use only the number of animals necessary to produce reliable scientific data. The experimental protocol was approved by the Hospital de Clínicas de Porto Alegre Research Ethics Committee.

REFERENCES

1. Arnold JH, Truog RD, Orav EJ, Scavone JM, Hershenson MB. Tolerance and dependence in neonates sedated with fentanyl during extracorporeal membrane oxygenation. *Anesthesiology*. 1990;73(6):1136-40.

2. Franck L, Vilardi J. Assessment and management of opioid withdrawal in ill neonates. *Neonatal Netw.* 1995;14(2):39-48.

3. Franck LS, Vilardi J, Durand D, Powers R. Opioid withdrawal in neonates after continuous infusions of morphine or fentanyl during extracorporeal membrane oxygenation. *Am J Crit Care.* 1998;7(5):364-9.

4. Nestler EJ, Aghajanian GK. Molecular and cellular basis of addiction. *Science.* 1997;278(5335):58-63.

5. Ueda H. Locus-specific involvements of anti-opioid systems in morphine tolerance and dependence. *Ann NY Acad Sci*. 2004;1025:376–382.

6. Pugsley MK. The diverse molecular mechanism responsible for the actions of opioids on the cardiovascular system. *Pharmacol Ther*. 2002;93:51–57.

7. Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev.* 2007;87:659–797.

8. Burnstock G. Purinergic signalling and disorders of the central nervous system. *Nat Rev Drug Discov*. 2008;7:575–590.

9. Erlinge D, Burnstock G. P2 receptors in cardiovascular regulation and disease. *Purinergic Signal.* 2008;4(1):1-20.

10. Kunapuli SP, Daniel JL. P2 receptor subtypes in the cardiovascular system. *Biochem J*. 1998;336:513–523.

11. Zimmermann H. Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol*. 2000;362(4-5):299-309.

12. Robson SC, Wu Y, Sun X, Knosalla C, Dwyer K, Enjyoji K. Ectonucleotidases of CD39 family modulate vascular inflammation and thrombosis in transplantation. *Semin Thromb Hemost*. 2005;31:217–233.

13. Oses JP, Cardoso CM, Germano RA, et al. Soluble NTPDase: an additional system of nucleotide hydrolysis in rat blood serum. *Life Sci*. 2004;74:3275–3284.

14. Yegutkin GG. Kinetic analysis of enzymatic hydrolysis of ATP in human and rat blood serum. *Biochemistry (Mosc)*. 1997;62:619–622.

15. Torres IL, Buffon A, Dantas G, et al. Chronic stress effects on adenine nucleotide hydrolysis in the blood serum and brain structures of rats. *Pharmacol Biochem Behav*. 2002;74:181-186.

16. Torres IL, Fürstenau CR, Rossi G, et al. Methylprednisolone administration alters adenine nucleotide hydrolysis in rat blood serum. *Eur J Pharmacol*. 2007;560:212-215.

17. Böhmer AE, Fürstenau CR, Torres IL, et al. The effect of stress upon hydrolysis adenine nucleotides in blood serum of rats. *Pharmacol Biochem Behav*. 2003;75: 467-471.

18. Siqueira IR, Elsner VR, Rilho LS, et al. A neuroprotective exercise protocol reduces the adenine nucleotide hydrolysis in hippocampal synaptosomes and serum of rats. *Brain Res*. 2010;1316:173-180.

19. Detanico BC, de Souza A, Medeiros LF, et al. 24-hour temporal pattern of NTPDase and 5'-nucleotidase enzymes in rat blood serum. *Chronobiol Int*. 2004;27(9-10):1751-1561.

20. Detanico BC, Rozisky JR, Battastini AM, Torres IL. Physiological level of norepinephrine increases adenine nucleotides hydrolysis in rat blood serum. *Purinergic Signal*. 2011;7(4):373-379.

21. Souza A, Detanico BC, Medeiros LF, et al. Effects of restraint stress on the daily rhythm of hydrolysis of adenine nucleotides in rat serum. *J Circadian Rhythms*. 2011;28:9-7.

22. Banz Y, Beldi G, Wu Y, Atkinson B, Usheva A, Robson SC. CD39 is incorporated into plasma microparticles where it maintains functional properties and impacts endothelial activation. *Br J Haematol*. 2008;142(4):627-37.

23. Agteresch HJ, Dagnelie PC, van den Berg JW, Wilson JH. Adenosine triphosphate: established and potential clinical applications. *Drugs*. 1999;58(2): 211-232.

24. Rozisky JR, da Silva RS, Adachi LS, et al. Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral cortex of rats. *Eur J Pharmacol*. 2010;642(1-3):72-76.

25. Sawynok J, Liu XJ. Adenosine in the spinal cord and periphery: release and regulation of pain. *Prog Neurobiol*. 2003;69(5):313-340.

26. Aley KO, Levine JD. Multiple receptors involved in peripheral alpha 2, mu, and A1 antinociception, tolerance, and withdrawal. *J Neurosci*. 1997;17(2):735-744.

27. Calignano A, Persico P, Mancuso F, Sorrentino L. Adenosine release in morphine-induced hypotension in rats. *Gen Pharmacol*. 1992;23(1):7-10

28. Fitzgerald M, Anand KJ. Developmental neuroanatomy and neurophysiology of pain. In: Schechter NL, Berde CB, Yaster M, editors. *Pain in infant, children, and adolescents*. Baltimore: Williams & Wilkins; 1993:11-31.

29. Pattinson D, Fitzgerald M. The neurobiology of infant pain: development of excitatory and inhibitory neurotransmission in the spinal dorsal horn. *Reg Anesth Pain Med*. 2004;29(1):36-44.

30. Bishop B. Neural plasticity: Part 2. Postnatal maturation and function-induced plasticity. *Phys Ther*. 1982;62(8):1132-1143.

31. Kim JJ, Foy MR, Thompson RF. Behavioral stress modifies hippocampal plasticity through N-methyl-D-aspartate receptor activation. *Proc Natl Acad Sci*. U S A. 1996;93(10):4750-4753.

32. Rabinowicz T, de Courten-Myers GM, Petetot JM, Xi G, de los Reyes E. Human cortex development: estimates of neuronal numbers indicate major loss late during gestation. J Neuropathol. *Exp Neurol*. 1996;55(3):320-328.

33. Rozisky, J.R., Dantas, G,, Adachi, L.S., Alves, V.S., Ferreira, M.B., Sarkis, J.J., Torres, I.L., 2008. Long-term effect of morphine administration in young rats on the analgesic opioid response in adult life. Int. J Dev. Neurosci. 26 (6), 561-5.

34. Rozisky JR, Medeiros LF, Adachi LS, et al. Morphine exposure in early life increases nociceptive behavior in a rat formalin tonic pain model in adult life. *Brain Res*. 2011;1367:122-129.

35. Chan KM, Delfert D, Junger KD. A direct colorimetric assay for Ca2+ -stimulated ATPase activity. *Anal Biochem*. 1986;157(2):375-380.

36. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248-254.

37. Kukulski F, Levesque SA, Lavoie EG, et al. Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. *Purinergic Signal*. 2008;1:193–204.

38. Marcus AJ, Broekman MJ, Drosopoulos JH, et al. Metabolic control of excessive extracellular nucleotide accumulation by CD39/ecto-nucleotidase-1: implications for ischemic vascular diseases. *J Pharmacol Exp Ther*. 2003;305(1): 9-16.

39. Mihaylova-Todorova ST, Todorov LD, Westfall DP. Enzyme kinetics and pharmacological characterization of nucleotidases released from the guinea pig isolated vas deferens during nerve stimulation: evidence for a soluble ecto-nucleoside triphosphate diphosphohydrolase-like ATPase and a soluble ecto-5´-nucleotidase-like AMPase. J *Pharmacol Exp Ther.* 2002;302:992–1001.

40. Todorov LD, Mihaylova-Todorova S, Westfall TD, et al. Neuronal release of soluble nucleotidases and their role in neurotransmitter inactivation. *Nature* 1997;387:76–79.

41. Robson SC, Sévigny J, Zimmermann H. The ENTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic Signal*. 2006; 2(2):409–430.

42. Eckle T, Krahn T, Grenz A, et al. Cardioprotection by ecto-5'-nucleotidase (CD73) and A2B adenosine receptors. *Circulation* 2007a;115:1581–1590.

43. Eckle T, Fullbier L, Wehrmann M, et al. Identification of ectonucleotidases CD39 and CD73 in innate protection during acute lung injury. *J Immunol*. 2007b;178:8127–8137.

44. Zimmermann H. Ecto-nucleotidases. In: Abbrachio MP, Williams M. editors. *Handbook of experimental pharmacology. Purinergic and pyrimidergic signalling.* New York: Springer; 2001:209-250.

45. Zimmermann H. Ectonucleotidases in the nervous system. *Novartis Found Symp*. 2006;276:113-128; discussion 128-30, 233-7, 275-81.

46. Gendron FP, Benrezzak O, Krugh BW, Kong Q, Weisman GA, Beaudoin AR. Purine signaling and potential new therapeutic approach: possible outcomes of NTPDase inhibition. *Curr Drug Targets* 2002;3(3):229-245.

47. Torres IL, Battastini AM, Buffon A, et al. Ecto-nucleotidase activities in spinal cord of rats changes as function of age. *Int J Dev Neurosci*. 2003;21(8):425-429.

48. Ralevic V. Purines as neurotransmitters and neuromodulators in blood vessels. *Curr Vasc Pharmacol*. 2009; 7:3–14.

49. Yegutkin GG. Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signaling cascade. *Biochim Biophys Acta*. 2008;1783: 673–694.

50. Westfall DP, Todorov LD, Mihaylova-Todorova ST. ATP as a cotransmitter in sympathetic nerves and its inactivation by releasable enzymes. *J Pharmacol Exp Ther*. 2002;303:439–444

51. Furukoji E, Tanaka N, Yamashita A et al. Ecto-nucleoside triphosphate diphosphohydrolase inhibits ATP- and ADP-induced vasoconstriction. *Thromb Res*. 2008;121:;583–585.

52. Zimmermann H, Braun N. Ecto-nucleotidases–molecular structures, catalytic properties, and functional roles in the nervous system. *Prog Brain Res*. 1999;120:371–385.

53. Kojima M, Sperelakis N, Sada H. Ontogenesis of transmembrane signaling systems for control of cardiac Ca2+ channels. *J Dev Physiol*. 1990;14(4):181-219.

54. Kienbaum P, Heuter T, Michel MC, Scherbaum N, Gastpar M, Peters J. Chronic μ-opioid receptor stimulation in humans decreases muscle sympathetic nerve activity. *Circulation*. 2001;103:850–855.

55. Groban L, Vernon JC, Butterworth J. Intrathecal morphine reduces infarct size in a rat model of ischemia-reperfusion injury. *Anesth Analg.* 2004;98(4):903-909.

56. Banjac A, Nedeljkovic N, Horvat A, Kanazir D, Nikezic G. Ontogenic profile of ecto-ATPase activity in rat hippocampal and caudate nucleus synaptic plasma membrane fractions. *Physiol. Res*. 2001;50:411–418.

57. Nedeljkovic N, Banjac A, Horvat A, Stojiljkovic M, Nikezic G. Developmental profile of NTPDase activity in synaptic plasma membranes isolated from rat cerebral cortex. *Int J Dev Neurosci.* 2005;23:45–51.

LEGENDS

FIG. 1. Effect of morphine administration in early life on NTPDases and 5´-nucleotidase in rat blood serum at P16, P30 and P60. At P16, no between-group differences in ATP, ADP or AMP hydrolysis were observed (Student's *t* test, $P > 0.05$). At P30, however, the morphine group showed an increase in hydrolysis of all nucleotides as compared to the control group (Student's *t* test, $P<0.05$). At P60, ADP hydrolysis was decreased in the morphine group (Student's *t* test, $P<0.05$), while there were no differences in the hydrolysis of other nucleotides (Student's *t* test, $P>0.05$ for ATP and AMP).

Bars represent mean ± SEM. Specific enzyme activities expressed as nmol of $Pi.min^{-1}.mg^{-1}$ protein. *denotes a difference between the control and morphine-treated groups.

FIG. 2. Ratio of ATPase/ADPase activity following morphine administration in early life. Bars represent mean ± SEM. Specific enzyme activities expressed as nmol of Pi.min-1.mg-1 protein. There were no between-group differences in the ratio of ATP/ADP hydrolysis at any age (Student's *t* test, $P>0.05$).

FIG. 3. Effect of aging on adenine nucleotide hydrolysis in rat blood serum (A) and on ratio of ATPase/ADPase activity (B). At P16 it was observed a decrease of ADP hydrolysis in comparison to P30 and P60, while at P60 it was observed an increase of AMP hydrolysis in comparison to P16 and P30 (one way ANOVA, $P>0.05$ for ATP; one way ANOVA, SNK, $P<0.05$ for ADP and AMP). The ratio of ATP/ADP hydrolysis did not change with age (one way ANOVA, $P>0.05$).

Bars represent mean ± SEM. Specific enzyme activities expressed as nmol of $Pi.min^{-1}.mg^{-1}$ protein. * denotes a difference between ages.

Figure 1

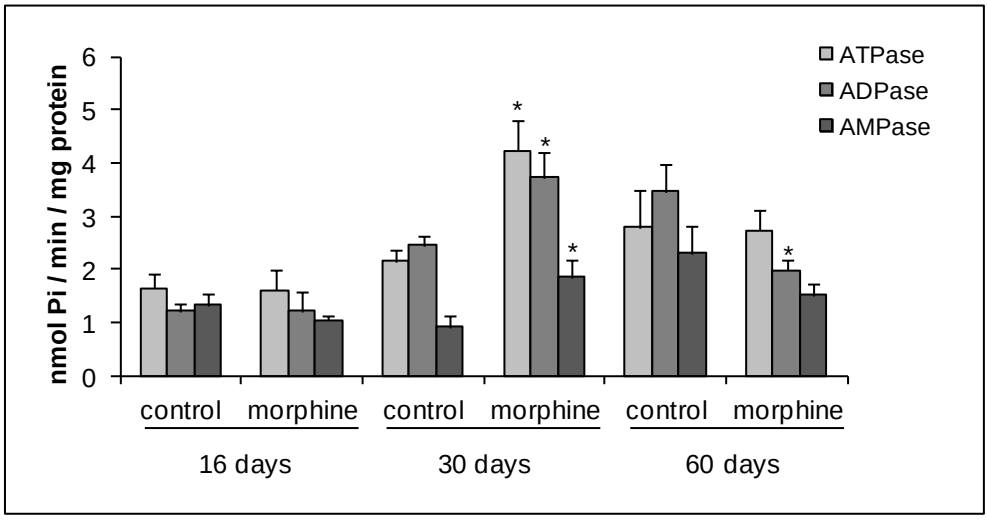

nmol Pi / min / mg protein
6
5
4
3
2
1
0
ATPase
ADPase
AMPase
*
*
*
*
control
morphine
control
morphine
control
morphine
16 days
30 days
60 days

Figure 2

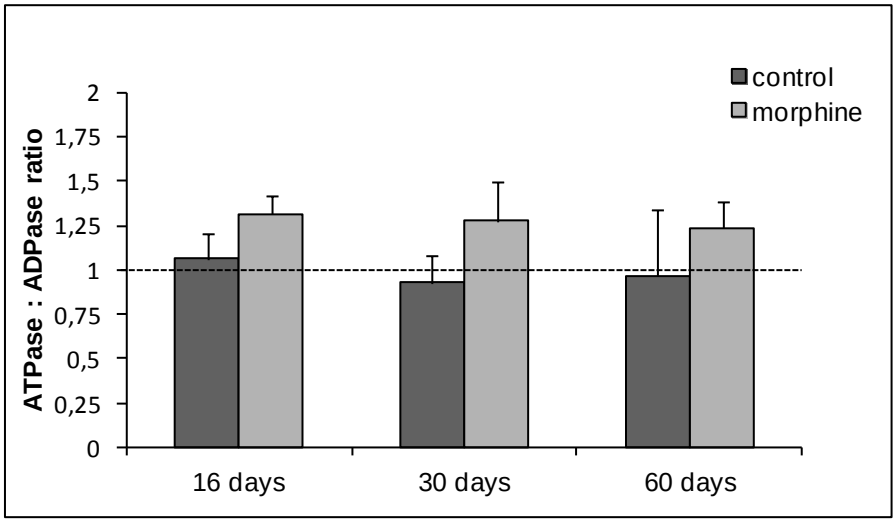

control
morphine
ATPase : ADPase ratio
2
1,75
1,5
1,25
1
0,75
0,5
0,25
0
16 days
30 days
60 days

Figure 3a

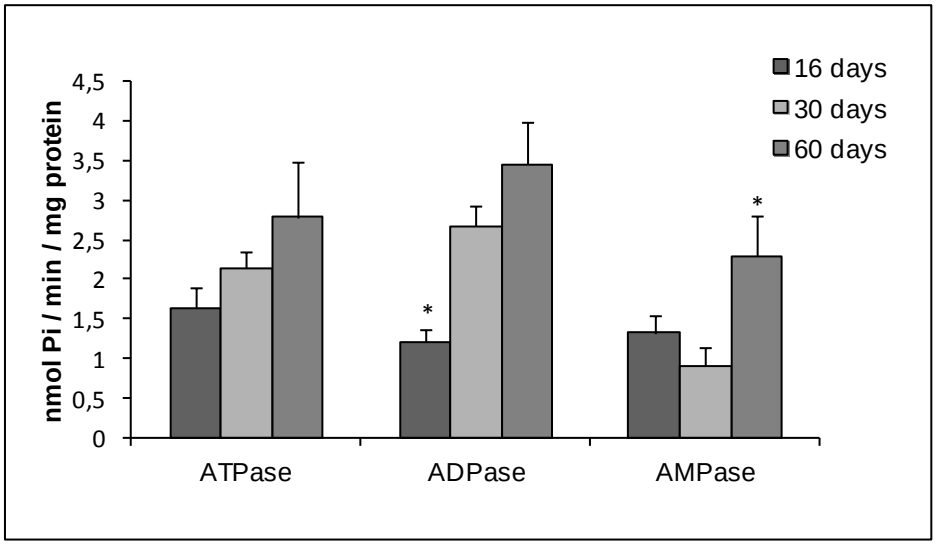
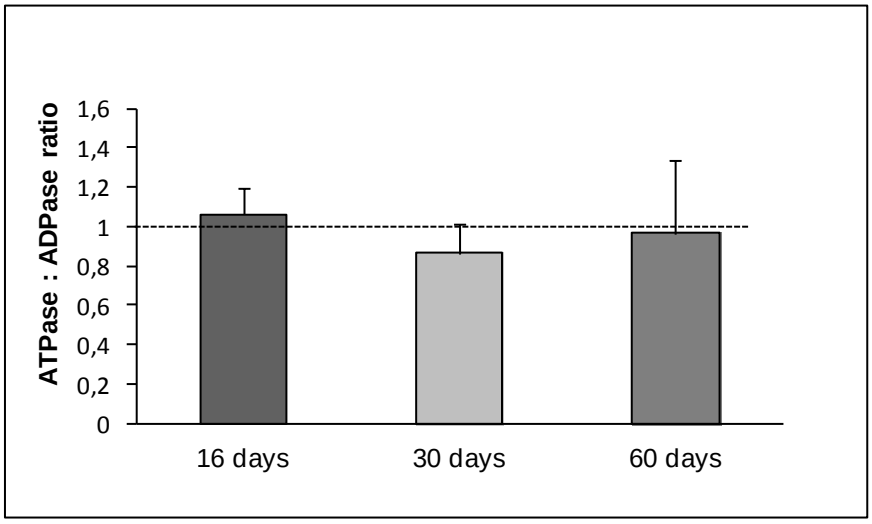

16 days
30 days
60 days
nmol Pi / min / mg protein
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
*
*
ATPase
ADPase
AMPase

Figure 3b


ATPase : ADPase ratio
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
16 days
30 days
60 days

PARTE III

CONSIDERAÇÕES FINAIS

Considerando que a morfina possui propriedades analgésicas potentes no período neonatal e que tem sido amplamente utilizada em pacientes neonatos em ambiente hospitalar tanto para sedação como analgesia, a carência de estudos que estejam focados nas alterações comportamentais e bioquímicas após o tratamento nos levou a estudar e aprofundar os conhecimentos nesta importante área da farmacologia da dor em neonatos. Os resultados obtidos com esta tese demonstram que animais que receberam tratamento com morfina na dose de 5 μg, do 8º ao 14º dias pós-natal, apresentam uma série de alterações tanto comportamentais como bioquímicas por pelo menos até 45 dias após o fim do tratamento. Primeiramente observou-se aumento de comportamentos relacionados à nocicepção no teste da formalina a médio (P30) e longo prazo (P60). No P30, os animais apresentaram aumento da resposta nociceptiva na fase II do teste, enquanto que no P60 a resposta nociceptiva encontrou-se aumentada em ambas as fases I (neurogênica) e II (inflamatória). Estas respostas foram totalmente revertidas por antagonista do receptor glutamatérgico NMDA (cetamina) e parcialmente revertidas por anti-inflamatório não esteroidal (indometacina). O efeito da cetamina pode ser explicado pela ativação do sistema glutamatérgico na hiperalgesia induzida pela morfina (Stanford e Silverman, 2009). Neste fenômeno há ativação persistente dos receptores NMDA pelo glutamato liberado pelas fibras aferentes primárias levando à hipersensibilização dos neurônios do corno dorsal da medula espinal (Baranauskas e Nistri, 1998). Além disso, o envolvimento dos neurotransmissores excitatórios, principalmente o glutamato, na dor inflamatória, é justificado pelo aumento dos níveis deste neurotransmissor no gânglio da raiz dorsal e na medula espinal em modelos

experimentais de dor inflamatória (Wimalawansa, 1996; Löfgren et al., 1997; Ossipov et al., 2005).

Estes dados demonstram uma forte evidência dos efeitos do tratamento repetido com morfina no período neonatal com consequentes alterações no sistema glutamatérgico. Considerando a importância do controle da concentração de glutamato no espaço extracelular após sua liberação e, consequentemente, da ativação dos receptores glutamatérgicos, foi então avaliada a captação de glutamato em sinaptossomas de medula espinal. Observamos que tanto no P30 quanto no P60 os animais que receberem o tratamento com morfina apresentaram menor captação de glutamato comparado aos animais do grupo controle. Os transportadores de glutamato parecem ter um papel mais refinado do que a simples remoção do neurotransmissor, uma vez que regulam a extensão da ativação dos receptores glutamatérgicos e, desta forma, participam na regulação da transmissão sináptica. Acredita-se que as alterações na atividade dos transportadores de glutamato e propriedades funcionais, e em especial as suas densidades e localizações, podem ter implicações importantes para o funcionamento normal do sistema nervoso (para revisão ver Danbolt, 2001). Com o fato de que a exposição à morfina no período neonatal altera a resposta nociceptiva em P30 e P60, a diminuição da captação de glutamato pode estar relacionada com altas concentrações deste neurotransmissor na fenda sináptica.

De acordo com as evidências de que os animais que receberam morfina no período neonatal apresentam adaptações na circuitaria nociceptiva, observado primeiramente por aumento da resposta nociceptiva no teste da formalina no P30 e P60, então nós conduzimos uma investigação de estratégias terapêuticas objetivando reverter estas respostas alteradas. Escolhemos para este estudo a melatonina devido ao seu

potencial efeito antinociceptivo e anti-inflamatório. Observamos que a administração de melatonina antes do teste da formalina foi capaz de reverter o aumento da resposta nociceptiva nos animais que receberam morfina no período neonatal. Aos 30 dias, o efeito antinociceptivo foi observado em ambos os grupos morfina e controle em ambas as fases do teste em comparação aos animais que receberam veículo da melatonina. Aos 60 dias a melatonina promoveu um efeito antinociceptivo mais pronunciado somente no grupo morfina na fase I; porém, na fase II ambos os grupos controle e morfina apresentaram efeito antinociceptivo em comparação aos grupos que receberam veículo. Assim, estes resultados sugerem que a melatonina é capaz de reverter o aumento da resposta nociceptiva induzida pelo tratamento com morfina no período neonatal e pode ser útil como terapia adjuvante em processos dolorosos, principalmente quando há envolvimento dos componentes neurogênico e inflamatório.

Além das alterações nas respostas nociceptivas, observamos que os animais tratados com morfina apresentaram respostas comportamentais alteradas, como aumento de comportamento exploratório e diminuição do comportamento do tipo ansioso em P16 e P30 nos testes de campo aberto e labirinto em cruz. Estes comportamentos foram revertidos pela administração de antagonista dopaminérgico D2 no P30. Considerando que estes efeitos foram específicos para os animais tratados com morfina no período neonatal, podemos sugerir que este tratamento modulou o sistema dopaminérgico, mais especificamente em nível de receptor D2 no P30, uma vez que no P16 os comportamentos não foram revertidos com a administração de haloperidol. Adicionalmente, sugerimos que os comportamentos analisados em ambos os testes sejam específicos para cada idade analisada. A participação de outros sistemas nestas alterações comportamentais não pode ser descartada; porém, novos estudos são

necessários para elucidar os mecanismos e sistemas envolvidos em cada idade.

Ao analisarmos possíveis alterações neuroquímicas no hipocampo, observamos que os animais que receberam morfina no período neonatal apresentaram aumento dos níveis de BDNF no P30 e P60. Adicionalmente, observamos uma menor atividade da enzima antioxidante SOD com paralela diminuição da razão SOD/GPx em P60 em comparação ao grupo controle. Estes dados sugerem que a exposição à morfina no período neonatal leva a alterações de longa duração no hipocampo. O BDNF pode estar atuando em processos de modulação da transmissão sináptica e, dessa forma, mediar os efeitos de retirada em médio e longo prazo após exposição repetida à morfina no período neonatal. Por outro lado, a diminuição da atividade da SOD pode levar ao aumento da formação de peroxinitrito (ERO), podendo desencadear disfunções mitocondriais levando a danos oxidativos nas células do hipocampo. Porém, não foram observadas alterações na viabilidade e morte celular no hipocampo. Mas, após insulto com peróxido de hidrogênio, observamos uma menor viabilidade e maior morte celular no hipocampo em ambos os grupos. No entanto, novos estudos devem ser feitos para verificar se o tratamento repetido com morfina leva a alterações celulares e plasticidade sináptica no hipocampo.

Nas análises dos efeitos deste tratamento sobre as atividades das nucleotidases solúveis no soro sanguíneo, observamos que o grupo morfina apresentou aumento da atividade de hidrólise de ATP, ADP e AMP no P30, e uma diminuição na atividade de hidrólise do ADP em P60. Estes dados são muito importantes, uma vez que estas enzimas são as principais reguladoras das concentrações de nucleotídeos no sangue e consequentemente da sinalização purinérgica. Dessa forma, sugerimos que as nucleotidases possam estar modulando o tônus vascular por controlar os níveis de

nucleotídeos após o tratamento repetido com morfina no período neonatal. Entretanto, outros estudos devem ser realizados para investigar os efeitos deste tratamento sobre o sistema vascular e a relação destas mudanças com as alterações vistas aqui no sistema purinérgico.

Concluindo, os resultados apresentados nesta tese demonstram que o tratamento repetido com morfina no período neonatal de ratos leva a alterações em médio e longo prazo em parâmetros nociceptivos associado a mudanças no sistema glutamatérgico; em curto e médio prazo a mudanças comportamentais, sendo as mudanças em médio prazo possivelmente associadas a alterações no sistema dopaminérgico; a alterações neuroquímicas no hipocampo, como mudanças a médio e longo prazo nos níveis de BDNF e em longo prazo na atividade da SOD; a alterações em médio e longo prazos nas atividades das nucleotidases solúveis séricas.

Estes dados demonstram a necessidade de novas pesquisas focando os efeitos do tratamento com morfina no período neonatal em alterações comportamentais e neuroquímicos, bem como a necessidade de se buscarem alternativas terapêuticas que possam reverter tais alterações.

ANEXOS

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
Grupo de Pesquisa e Pós-Graduação
COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

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

Lista de artigos científicos publicados em periódicos internacionais indexados durante o período do Doutorado (2008 – 2012)

- ✓ Rozisky JR, Dantas G, Adachi LS, Alves VS, Ferreira MBC, Sarkis JJ, Torres ILS. Long-term effect of morphine administration in young rats on the analgesic opioid response in adult life. International Journal of Developmental Neuroscience, v. 26, p. 561-565, 2008.
- ✓ Rozisky JR, Silva RS, Adachi LNS, Capiotti KM, Ramos DB, Bogo MR, Bonan CD, Sarkis JJ, Torres ILS. Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral cortex of rats. European Journal of Pharmacology, v. 642, p. 72-76, 2010.
- ✓ Siqueira IR, Elsner VR, Sulzbach L, Bahlis MG, Bertoldi K, Rozisky JR, Battastini AMO, Torres ILS. A neuroprotective exercise protocol reduces the adenine nucleotide hydrolysis in hippocampal synaptosomes and serum of rats. Brain Research, v. 1316, p. 173-180, 2010
- ✓ Detanico BC, Souza A, Medeiros LF, Rozisky JR, Hidalgo MP, Caumo W, Battastini AMO, Torres ILS. 24-Hour Temporal Pattern of NTPDase and 5 - nucleotidase enzymes in rat blood serum. Chronobiology International, v. 27, p. 1561-1571, 2010.
- ✓ Medeiros LF, Rozisky JR, Souza A, Netto CA, Hidalgo MP, Caumo W, Torres ILS. Lifetime behavioural changes after exposure to anaesthetics in infant rats. Behavioural Brain Research, v. 218, p. 51-56, 2011.

- ✓ Detanico BC, Rozisky JR, Battastini AMO, Torres ILS. Physiological level of norepinephrine increases adenine nucleotides hydrolysis in rat blood serum. Purinergic Signalling (Print), v. 7, p. 373-79 2011.
- ✓ Souza A, Detanico BC, Medeiros LF, Rozisky JR, Caumo W, Hidalgo MP, Battastini AMO, Torres ILS. Effects of restraint stress on the daily rhythm of hydrolysis of adenine nucleotides in rat serum. Journal of Circadian Rhythms, v. 9, p. 7, 2011.
- ✓ Stefani LC, Torres ILS, Souza ICC, Rozisky JR, Fregi F, Caumo W. BDNF as an effect modifier for gender effects on pain thresholds in healthy subjects. Neuroscience Letters (Print), v. 514, p. 62-6, 2012.
- ✓ Laste G, Caumo W, Adachi LS, Rozisky JR, Macedo IC, Marques PR, Partata W, Fregni F, Torres ILS. After-effects of consecutive sessions of transcranial direct current stimulation (tDCS) in a rat model of chronic inflammation. Experimental Brain Research, v. 221, p. 75-83, 2012.
- ✓ Laste G, Macedo IC, Rozisky JR, Silva FR, Caumo W, Torres ILS. Melatonin administration reduces inflammatory pain in rats. Journal of Pain Research, v. 5, p. 359-362, 2012.
- ✓ Macedo IC, Medeiros LF, Oliveira C, Oliveira CM, Rozisky JR, Scarabelot V, Souza A, Da Silva FR, Souza VS, Cioato SG, Caumo W, Torres ILS. Cafeteria Diet-induced obesity plus chronic stress alters serum leptin levels. Peptides (New York, N.Y. 1980), v. 38, p. 189-96, 2012.
- ✓ Spezia Adachi LN, Caumo W, Laste G, Fernandes Medeiros L, Ripoll Rozisky J, de Souza A, Fregni F, Torres IL. Reversal of chronic stress-induced pain by

transcranial direct current stimulation (tDCS) in an animal model. Brain Research v. 1489, p. 17-26, 2012.

- ✓ Laste G, Rozisky JR, Macedo IC, Souza ICC, Souza VS, Caumo W, Torres ILS. Spinal cord bdnf levels increase after dexamethasone treatment in male rats with chronic inflammation. Neuroimmunomodulation (Basel), 2012 (*in press*).