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CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA TOXICOLÓGICA

INFLUÊNCIA DO ESTRESSE EM DIFERENTES PERÍODOS INICIAIS DA VIDA SOBRE O DESENVOLVIMENTO DE ANSIEDADE E PREFERÊNCIA POR MORFINA EM RATOS

DISSERTAÇÃO DE MESTRADO

Luciana Taschetto Vey

Santa Maria, RS

2015

INFLUÊNCIA DO ESTRESSE EM DIFERENTES PERÍODOS INICIAIS DA VIDA SOBRE O DESENVOLVIMENTO DE ANSIEDADE E PREFERÊNCIA POR MORFINA EM RATOS

Luciana Taschetto Vey

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Área de Concentração em Neuropsicofarmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Bioquímica Toxicológica**

Orientadora: Profª Drª Marilise Escobar Burger

Santa Maria, RS

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A Comissão Examinadora, abaixo assinada, aprova a Dissertação de Mestrado

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elaborada por
Luciana Taschetto Vey

Como requisito parcial para obtenção do grau de
Mestre em Bioquímica Toxicológica

COMISSÃO EXAMINADORA

_____
Marilise Escobar Burger, Dr.
(Presidente/Orientadora)

_____
Pâmela Billig Mello-Carpes, Ph.D. (UNIPAMPA)

_____
Sara Marchesan de Oliveira, Dr. (UFSM)

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“A mente que se abre a uma nova ideia,

jamais voltará ao seu tamanho original.”

Albert Einstein

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil.

INFLUÊNCIA DO ESTRESSE EM DIFERENTES PERÍODOS INICIAIS DA VIDA SOBRE O DESENVOLVIMENTO DE ANSIEDADE E PREFERÊNCIA POR MORFINA EM RATOS

AUTOR: LUCIANA TASCHETTO VEY
ORIENTADORA: PROFª DRª. MARILISE ESCOBAR BÜRGER
Data e Local da Defesa: Santa Maria, 17 de julho de 2015.

A adição apresenta um impacto considerável na sociedade, resultando em um dos maiores problemas de saúde pública, uma vez que assola diferentes etnias e classes sociais em todo o mundo. O Brasil é o maior consumidor de analgésicos opioides da América do Sul, apresentando, sob o ponto de vista clínico, uma situação problemática, pois a administração contínua pode levar a tolerância e dependência. Nesse sentido, o protocolo de preferência condicionada de lugar (do inglês *conditioned place preference*-CPP) tem sido amplamente utilizado para avaliar a adição relacionada à drogas de abuso. Segundo a Organização Mundial da Saúde (2010), o estresse é reconhecido por sua cronicidade, sendo identificado como o mal do século XXI. Suas repercussões estão diretamente associadas à qualidade de vida do indivíduo, da família e da sociedade. Assim, esse estudo objetivou investigar a influência de diferentes períodos de estresse (fetal e neonatal) sobre parâmetros comportamentais e de dependência à morfina em ratos jovens adultos. Animais expostos ao Post-NS mostraram menor grau de ansiedade, comportamento mais exploratório sem mostrar preferência por morfina. Em contrapartida, animais expostos ao protocolo de Pre-NS mostraram maiores níveis de corticosterona e menor ganho de peso, junto com maiores sintomas de ansiedade e preferência por morfina após três dias de abstinência. Nossos resultados indicam que o período pré-natal é mais susceptível ao estresse, cujos efeitos podem ser manifestar ao longo da vida. Embora dados demonstrem que o post-NS possa desenvolver estresse crônico ou experiências adversas, podemos verificar a partir dos nossos resultados que o post-NS pode desencadear neuroadaptações capazes de superar as consequências emocionais da vida precoce. Assim, nós hipotetizamos que o Pre-NS é capaz de modificar as respostas aos opioides ao longo da vida adulta, o que poderia facilitar o desenvolvimento de dependência a esses fármacos.

Palavras-chave: Ansiedade. Estresse. Morfina. Preferência Condicionada de Lugar.

ABSTRACT

Dissertation of Master's Degree
Postgraduate Programme in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

INFLUENCE OF STRESS IN DIFFERENT PERIODS EARLY LIFE ON THE ANXIETY OF DEVELOPMENT AND PREFERENCE BY MORPHINE IN RATS

AUTHOR: LUCIANA TASCHETTO VEY
ADVISOR: PROFª DRª. MARILISE ESCOBAR BÜRGER
Date and place of defense: Santa Maria, july, 17th, 2015

Drug addiction has exerted a considerable impact on society, resulting in one of the biggest public health problems reaching different ethnic groups and social classes worldwide. Brazil is the largest consumer of opioid analgesics in South America, presenting, from a clinical point of view, a problematic situation, because the continuous administration could lead to tolerance and dependence. In this sense, the conditioned preference place protocol (CPP) has been widely used to evaluate the addition related to drug abuse. According to the World Health Organization (2010), stress is recognized by its chronicity, being identified as the evil of the XXI century. Its effects are directly related to the quality of life of the individual, family and society. This study was performed to evaluate the influence of stress exposure in different early life periods (fetal and neonatal) on anxiety-like symptoms and emotionality, and its consequences on addiction parameters after young animals' exposure to morphine. Animals exposed to post-NS showed lesser anxiety in different behavioral paradigms as well as increased exploratory behavior, and no preference for morphine in CPP. In contrast, animals exposed to pre-NS showed increased corticosterone plasma levels together with anxiety symptoms and greater preference for morphine following three days of drug withdrawal. Our findings indicate that the prenatal period is critical for stress, whose effects may be manifest throughout life. Although data demonstrate that the post -NS can develop chronic stress or adverse experiences, we can see from our results that the post -NS can trigger neuroadaptations able to overcome emotional consequences of early life. We hypothesized that pre-NS is able to modify responses to opioids along adulthood, which may facilitate development of addiction to these drugs.

Keywords: Anxiety. Stress. Morphine. Conditioned Place Preference.

LISTA DE ILUSTRAÇÕES

INTRODUÇÃO

ARTIGO CIENTÍFICO

LISTA DE ABREVIATURAS E SIGLAS

ACTH- hormônio adrenocorticotrófico

CRH- hormônio liberador de corticotrofina

GCs- glicocorticoides

HPA- eixo hipófise-pituitária-adrenal

IN- isolamento neonatal

Post-NS- estresse pós-natal

Pre-NS- estresse pré-natal

PMA- privação materna aguda

PVN- núcleo paraventricular do hipotálamo

SMC- separação materna crônica

SNA- sistema nervoso autônomo

SUMÁRIO

APRESENTAÇÃO

Esta dissertação está estruturada nas seguintes seções: Introdução, Objetivos, Manuscrito 1, Conclusões, Perspectivas e Referências.

Os itens Materiais e Métodos, Resultados, Discussão dos resultados e Referências encontram-se inseridos no manuscrito, contido na seção **MANUSCRITO CIENTÍFICO,** representando a íntegra do estudo.

As **REFERÊNCIAS** referem-se somente às citações que aparecem no item **INTRODUÇÃO** desta dissertação.

1 INTRODUÇÃO

1.1 Adição e opioides

A adição é um quadro caracterizado pelo conjunto de sintomas que indicam o uso compulsivo de uma ou mais substâncias aditivas, ou seja, é um comportamento que foge do controle do indivíduo, o qual manifesta sintomas de disforia, ansiedade e irritabilidade quando é impedido de utilizar tais substâncias (KOOB, Le MOAL, 2008). A adição apresenta um impacto considerável na sociedade, resultando em um dos maiores problemas de saúde pública, uma vez que assola diferentes etnias e classes sociais em todo o mundo (CAMI; FARRE, 2003). Segundo o relatório da Organização das Nações Unidas (ONU), no ano de 2007 foi registrado cerca de 172 a 250 milhões de pessoas que fizeram o uso de alguma droga ilícita no mundo. Entre estas, a maconha apresenta a maior prevalência anual do uso (entre 143 a 190 milhões de pessoas), seguida pela anfetamina, cocaína e opioides.

O perigo potencial da maioria dos opioides é que o seu uso prolongado resulta em dependência física e psicológica (KALTENBACH, 1996). De acordo com um estudo publicado na revista The Lancet (KAPP, 2003), a heroína é a droga mais perigosa entre as estudadas com base em seus danos físicos ao usuário, potencial impacto negativo e total dependência na sociedade. Em 2010, houve relatos de até 2,4 milhões de pessoas nos Estados Unidos da América (EUA), com um uso problemático de opioides (ROCKVILLE, 2011). No entanto, a maioria dos opioides utilizado nos EUA são originários de uma receita médica legal prescrita para tratar a dor.

Mundialmente, os americanos são os maiores usuários de opioides, enquanto este tipo de adição tornou-se uma epidemia (MANCHIKANTI et al., 2012). Dentre os medicamentos opioides descritos, a morfina, oxicodona e meperidina, juntamente com a ilícita heroína, configuram as drogas mais consumidas de forma abusiva, levando a adição (POULETTY, 2002; YARGEAU et al., 2014). A morfina apresenta uso clínico no alívio de dores viscerais e/ou terminais, sendo eficaz para dor moderada a grave, e cuja eficácia vai se perdendo com o rápido desenvolvimento de tolerância. Além disso, a morfina é capaz de desencadear quadros de dependência física e psíquica, fazendo com que sua privação desencadeie um processo aversivo de grave síndrome de abstinência (O'BRIEN, 1997).

Opioides são um grupo de substâncias que podem ter origem natural (alcaloides derivadas do ópio, um exsudado extraído da papoula-*Papaver somniferum),* semissintética (produzidas a partir dos alcaloides naturais) ou sintética, as quais são quimicamente distintas, mas com atividade semelhante aos anteriores. Todos os opioides atuam em receptores fisiológicos que recebem o mesmo nome genérico "opioides", porém apresentam uma distinta classificação por letras gregas (μ, δ,κ, σ), os quais apresentam ampla distribuição em tecidos neuronais centrais e da periferia. Os opiáceos estão entre as mais antigas drogas conhecidas do mundo arqueológico, onde evidências sugerem que as sementes de papoula eram usadas desde os primórdios da humanidade (ROSENFELD; LOOSE, 2007).

Os efeitos moleculares resultantes da ativação opioide são mediados por receptores metabotrópicos, os quais são negativamente acoplados à proteína Gi (inibitória). Em outras palavras, a ativação destes receptores inibe a atividade da adenilato-ciclase, que por consequência reduz a ativação do 2º mensageiro AMPc, resultando no bloqueio da entrada de cálcio, dos canais de sódio e aumento da saída de potássio, ocorrendo assim uma hiperpolarização no espaço extra-neural que limita a produção e liberação de mediadores inflamatórios como a substância P, causadora de dor. A administração contínua da droga permite a ativação posterior de um ou mais fatores de transcrição intranucleares, ativando a codificação do gene da adenilato-ciclase, provocando uma verdadeira modificação neuroadaptativa, reduzindo a resposta celular frente à mesma dose inicial da droga opioide (SWIFT; LEWIS, 2009). Além disso, a exposição crônica aos opioides pode diminuir a neurogênese, alterando assim a transmissão sináptica hipocampal (EISCH et al, 2000). De particular importância, esta área está envolvida na informação associada à adição (NESTLER, 2001), participando de respostas relacionadas ao sistema de recompensa (REZAYOF et al., 2003; WHITE, 1996) e ao comportamento de busca pela droga (BLACK et al., 2004; VOREL et al, 2001; YANG et al., 2004). Desse modo, os opioides são bem conhecidos por sua capacidade de produzir euforia, motivando certos indivíduos a se envolver em uso recreacional (ROSENFELD; LOOSE, 2007).

O Brasil é o maior consumidor de analgésicos opioides da América do Sul (BALTIERI et al., 2004; UNODC, 2009). Conforme o segundo levantamento realizado em 2005, pelo Centro Brasileiro de Informações sobre Drogas, envolvendo 108 cidades do país, 1,3% da população fez uso de analgésicos opioides na vida em todas as faixas etárias, havendo predomínio de uso em mulheres em relação aos homens. Do ponto de vista clínico, a prescrição de opioides é problemática, pois a administração contínua pode levar a tolerância e dependência (ZHU et al., 1999).

O uso abusivo de opioides ocorre tanto por pacientes detentores de prescrição médica, quanto em profissionais da saúde. Conforme o estudo de Alves e colaboradores (2005), uma pesquisa feita com 198 médicos em tratamento por dependência química, residentes em São Paulo (90 casos), Rio Grande do Sul (25 casos), Rio de Janeiro (20 casos) e Minas Gerais (17 casos), 26,7% fizeram o uso de opioides. Os profissionais da saúde, principalmente médicos, passam por situações facilitadoras para o uso de drogas. Alguns fatores de risco para o uso de substâncias psicotrópicas entre médicos são frequentemente relatados na literatura como: acesso facilitado aos medicamentos, perda de tabu em relação a injeções, problemas emocionais, estresse no trabalho e em casa, autoadministração no tratamento para a dor e para o humor, fadiga crônica, e os de especialidade de alto risco: como anestesiologista, emergência e psiquiatria (ALVES et al., 2005; MCAULIFFE et al., 1987; WRIGHT, 1990).

Mesmo após o desaparecimento dos sintomas físicos de dependência, ex-usuários crônicos de opioides sofrem grandes taxas de recaídas ao uso dessas drogas. Isso se explica devido existência de uma interação entre o sistema opioide e de recompensa encefálico como demonstrado na figura 1 (NESTLER, 1996).

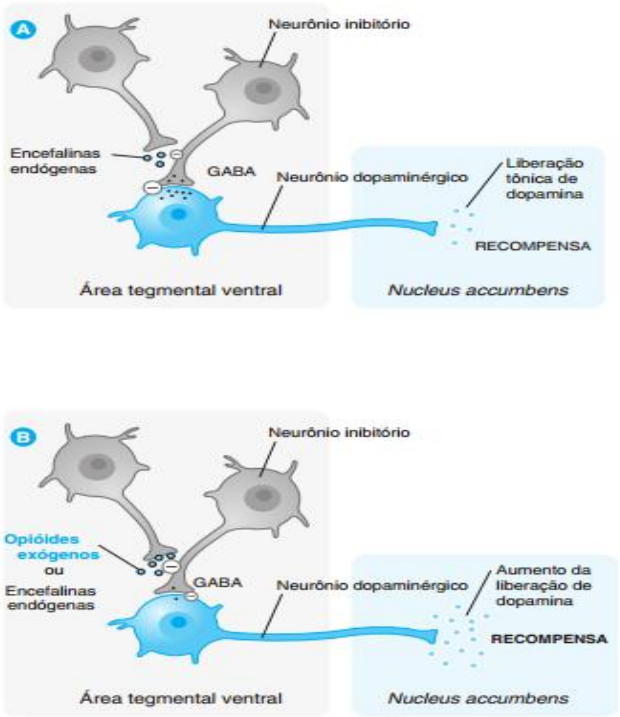

Figura 1- Papel dos opióides na via de recompensa encefálica. Fonte: DAVID e GOLAN (2009).

Um local de ação dos opioides situa-se na área tegumental ventral, onde interneurônios GABAérgicos causam a inibição tônica dos neurônios dopaminérgicos responsáveis pela

ativação da via de recompensa encefálica no *nucleus accumbens* (Figura 1A) (JOHNSON; NORTH, 1992). Esses interneurônios GABAérgicos podem ser inibidos por encefalinas endógenas, que se ligam a receptores μ-opióides nas terminações GABAérgicas (Figura 1B) (KOOB, 1992; WISE, 1990).Os opioides exógenos, como a morfina, também se ligam aos receptores μ-opióides e os ativam, um opióide exógeno administrado poderia ativar a via de recompensa encefálica mediante desinibição dos neurônios dopaminérgicos na área tegmental ventral e consequentemente o aumento liberação de dopamina (CHESSELET et al., 1983; DEVINE et al., 1993).

Os receptores opioides estão presentes em várias áreas do cérebro, e vários mecanismos podem ser afetados pela exposição aos opioides (YANAI et al., 2003). Os ratos são usados extensivamente para estudar os efeitos do desenvolvimento de dependência por opioides, porque muitas das suas respostas às drogas se assemelham as dos seres humanos (BASHORE et al., 1981).

Neste sentido, o protocolo de preferência condicionada de lugar (do inglês *conditioned place preference*-CPP) tem sido amplamente utilizado para avaliar a adição a drogas de abuso (ANTONIAZZI et al., 2014; KUHN et al., 2015; SEGAT et al., 2014; TZSCHENTKE, 2007) devido as suas inúmeras vantagens e simplicidade. Este teste baseia-se na capacidade de estímulos ambientais originalmente neutros adquirirem propriedades motivacionais positivas (preferência pelo lugar) ou negativas (aversão ao ambiente), após serem apresentados repetidamente na presença de uma substância com potencial de abuso (SANCHIS-SEGURA; SPANAGEL, 2007). Estímulos variados como cor, textura e odor podem ser usados na indução do CPP e têm sido amplamente empregado em modelos com roedores (BARDO; BEVINS, 2000). Os animais recebem a droga em um compartimento e o veículo em outro ambiente. Seguindo estes emparelhamentos, é permitido aos animais, em um estado livre da droga, o livre acesso entre os dois ambientes e a quantidade de tempo gasto no ambiente associado à droga é considerada como um índice de preferência. Em outras palavras, uma maior quantidade de tempo gasto no compartimento emparelhado com a droga indica uma maior preferência do animal, enquanto que o menor tempo indica aversão (DE WIT; STEWART, 1981; HEINRICHS et al., 2010; VOIGT et al., 2011).

O CPP empregado em roedores encontra validade frente à dependência em humanos, demonstrado por relatos de toxicodependentes que apresentaram fortes associações com ambientes nos quais faziam uso de drogas de abuso. Além disso, as propriedades de incentivo associados aos lugares relacionados ao uso da droga de abuso podem provocar recaída dos adictos abstinentes. Desse modo, o protocolo de CPP tem sido amplamente utilizado para

evidenciar a dependência e testar possíveis terapêuticas na recaída por drogas de abuso (ANTONIAZZI et al., 2014; KUHN et al., 2015; SEGAT et al., 2014).

1.2 Desenvolvimento neuronal e modelos animais de estresse

Segundo a Organização Mundial da Saúde (2010), o estresse é reconhecido pela cronicidade e identificado como o mal do século XXI. Suas repercussões estão diretamente ligadas à qualidade de vida do indivíduo, da família e da sociedade. Assim, o estresse é entendido como um processo complexo multidimensional no qual atuam agentes físicos e/ou psicológicos, sendo definido como um estado de homeostase ameaçado ou em desequilíbrio. Tal agressão psíquica é controlada por respostas viscerais e comportamentais que visam restaurar a homeostase perdida (CARRASCO; VAN DE KAR, 2003). As respostas ao estresse incluem a ativação do sistema nervoso autônomo (SNA) e do eixo hipotálamo-hipófise-adrenal (HPA), as quais acarretam a secreção aumentada de catecolaminas e a liberação de glicocorticoides (GCs), respectivamente (HARBUZ; LIGHTMAN, 1992; HERMAN; CULLINAN, 1997).

A resposta rápida é transmitida pelo SNA onde ocorre a liberação de norepinefrina e epinefrina. Uma resposta tardia ativa o núcleo paraventricular do hipotálamo (PVN, do inglês *paraventricular nucleus*) a liberar o hormônio liberador de corticotrofina (CRH) para a vasculatura da glândula pituitária anterior. O CRH estimula a liberação do hormônio adrenocorticotropina (ACTH), que desencadeia a libertação de GCs a partir do córtex adrenal. Os GCs exercem uma retroalimentação negativa que regula a atividade do eixo HPA (Figura 2), via seus próprios receptores (receptores de GCs e mineralocorticoides) na hipófise anterior, hipotálamo (DE KLOET et al., 2005), hipocampo e córtex pré-frontal frontal (DE VASCONCELLOS et al., 2006; FILIPOVIĆ et al., 2011; TAGLIARI et al., 2010). Neste contexto, o hipocampo e córtex pré-frontal estão profundamente envolvidos na resposta ao estresse (MCEWEN, 2008; SAPOLSKY, 2003).

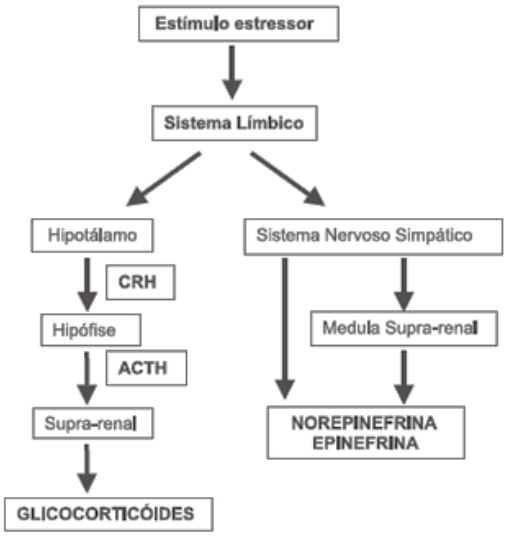

Figure 2. Ativação do sistema nervoso autonômico e neuroendócrino. Fonte: BUCKINGHAM (2000)

O aumento sérico de GCs pode desencadear alterações biológicas importantes no organismo, incluindo o desenvolvimento de disfunções psicológicas (McEWEN, 2010), que podem ser avaliadas a partir de modelos animais que reproduzem muitas das características do estresse crônico ou experiências adversas no início da vida. Tais modelos incluem a exposição ao estresse pré-natal (Pre-NS) (LEMAIRE et al., 2000), privação materna aguda (PMA) (DE KLOET et al., 2005), separação materna crônica (SMC) (HUOT et al, 2002; PLOTSKY et al, 2005; SANCHEZ et al, 2001), e isolamento neonatal (IN) como modelo de estresse pós-natal (Post-NS) (HUANG et al. 2002; LAI et al. 2006). Durante a gestação, o Pre-NS medeia mudanças na capacidade de resposta do eixo HPA fetal que já está funcional. Estudos com humanos têm demonstrado que eventos estressores no período pré-natal ou em períodos próximos ao parto aumentam a vulnerabilidade a psicopatias dos filhos na vida adulta (KOFMAN, 2002). Assim, o estresse durante a gravidez pode aumentar o risco de ocorrência de distúrbios neuropsiquiátricos como esquizofrenia, autismo, ansiedade e depressão na vida adulta (ALONSO et al., 1991; BROWN; HELENA; DERKITS, 2010; ENAYATI et al, 2012; KINNEY et al., 2008; MATRISCIANO et al, 2013; PATTERSON, 2011; VALLE et al., 1997). Evidências crescentes mostram que crianças e adolescentes, filhos de mulheres que vivenciaram eventos estressantes durante a gravidez, são mais propensos a apresentar problemas emocionais, hiperatividade, baixo rendimento escolar e déficit de atenção (BEVERSDORF et al., 2005; GLOVER et al., 2004; GUTTELING et al., 2006; TALGE; NEAL; GLOVER, 2007).

Estudos animais também identificaram estruturas cerebrais alteradas pelo pre-NS. Coe et al. (2003), estudando primatas não-humanos, demonstraram que a exposição ao ruído imprevisível, em diferentes períodos da prenhes, resultou numa diminuição do volume do hipocampo na prole. Tal estrutura cerebral tem um importante papel na memória e no controle do eixo HPA. Já em roedores, o número de receptores de GCs e mineralocorticóides no hipocampo pode ser reduzido pelo pre-NS (HENRY et al., 1994), levando a um aumento dos níveis de corticosterona (GLOVER et al, 2010; HARRIS; SECKL, 2011; VAN DEN BERGH et al., 2005). Isto poderia explicar o aumento prolongado dos níveis de corticosterona frente a um novo estressor, uma vez que há uma menor inibição da retroalimentação via corticosterona.

Outros estudos com roedores têm mostrado que os efeitos do pre-NS podem ser moderados ou até mesmo revertidos por manipulações pós-natais positivas (MACCARI et al., 1995) indicando que, embora possa haver efeitos persistentes do post-NS, a variação na natureza do cuidado materno pode ter efeitos duradouros sobre o comportamento e função do eixo HPA da prole (MEANEY et al., 1991). Filhos de mães que receberam um maior cuidado maternal se mostraram menos ansiosos e apresentaram uma resposta de corticosterona menos pronunciada (LIU et al., 1997). Confirmando esses dados, Bogoch et al. (2007) demonstraram a relação entre pre-NS e maior ansiedade em ratos de ambos os sexos e que manipulações pós-natais poderiam evitar essa emocionalidade.

Filhotes de roedores passam as suas primeiras semanas de vida no ninho materno, assim, interações dos filhotes com a mãe e com o restante da prole são essenciais para o desenvolvimento ideal do cérebro e das habilidades sociais (HUOT et al, 2002; SANCHEZ et al, 2001). A separação da ninhada por períodos prolongados (>2h) é percebida como uma ameaça pela prole e ativa o eixo HPA do neonato, elevando os níveis de corticosterona basais induzidos por estresse na idade adulta (LAJUD et al., 2012). A privação materna é um evento traumático agudo, que consiste na separação dos filhotes da mãe por um período de 24h e envolve tanto fatores nutricionais como sensoriais (SUCHECKI; ROSENFELD; LEVINE, 1993). Já a separação materna é um estressor moderado crônico que envolve diariamente separações entre a mãe e a ninhada durante as primeiras 2 ou 3 semanas de vida. Estes modelos reproduzem muitas das consequências observadas em humanos submetidos a experiências precoces adversas, tais como maus-tratos, abuso infantil e baixo nível socioeconômico (SANCHEZ et al, 2001; HUOT et al, 2002; PLOTSKY et al. , 2005).

O IN é um modelo amplamente aceito de post-NS para estudar as mudanças comportamentais de longo prazo produzidos por eventos estressores ocorridos precocemente

na vida (YOKOYAMA et al., 2006). Nesse contexto, neonatos submetidos a breves períodos de IN apresentaram um aumento do estimulo do eixo HPA, sem alteração do peso ao nascer (HUANG et al., 2002; KNUTH; ETGEN, 2005; MCCORMICK et al., 1998) e do peso e crescimento nos adultos (KEHOE et al., 1998; KEHOE; BRONZINO, 1999). Além disso, estudos animais mostram que o IN prejudica a aprendizagem e a memória (KOSTEN; LEE; KIM, 2007; MARCO et al, 2013), e aumenta a vulnerabilidade do adolescente ou adulto ao abuso de drogas (KEHOE et al., 1996; KOSTEN et al., 2006). Porém, outros estudos contestam estes resultados, mostrando que o IN melhora a memória (BUGARITH; RUSSELL, 2012; KEHOE et al, 1995; MAKENA). O estágio da vida, a espécie e o paradigma comportamental utilizados para avaliar a resposta ao IN podem explicar as discrepâncias encontradas entre os estudos. No entanto, a literatura é escassa a respeito da influência benéfica do IN sobre a resposta ao estresse (KEHOE et al., 1995; MAKENA; BUGARITH; RUSSELL, 2012).

Curtos períodos de separação materna nas primeiras semanas de vida mostraram um "fenótipo benéfico" na idade adulta, com melhores adaptações fisiológicas e comportamentais ao estresse, além de um aumento da plasticidade cerebral e menor ansiedade (LIU et al., 1997, 2000). O cuidado materno após protocolos de separação materna mostrou resultado fenotípico benéfico (LEVINE; MODY, 2003; MCINTOSH; ANISMAN; MERALI, 1999; MEANEY et al., 1991; NEISEWANDER; PEARTREE; PENTKOWSKI, 2012), embora algumas discrepâncias têm sido relatadas (ANISMAN et al., 1998; LEVINE, 2000; MACRÌ; CHIAROTTI; WÜRBEL, 2008; WALKER et al., 1991).

Considerando que há uma maior expressão de receptores opioides nas primeiras semanas de vida (AUGUY-VALETTE et al., 1978; CLENDENNIN et al., 1976; PETRILLO et al., 1987) e que o estresse modifica a resposta do eixo HPA em diferentes estágios da vida (GLOVER et al, 2010; HARRIS; SECKL, 2011; VAN DEN BERGH et al., 2005), o presente estudo avaliou a influência do estresse em diferentes períodos de vida sobre parâmetros de ansiedade e emocionalidade, assim como suas consequências na dependência por morfina em ratos.

2 OBJETIVOS

2.1 Objetivo Geral

Investigar a influência do estresse aplicado durante os períodos fetal e neonatal sobre sintomas de ansiedade e emocionalidade, e suas consequências sobre parâmetros de adição após a exposição dos ratos à morfina durante a adolescência.

2.2 Objetivos Específicos

- Investigar a influência do estresse aplicado durante os períodos fetal e neonatal sobre os níveis plasmáticos de corticosterona e ganho de peso dos filhotes;

- Avaliar a influência do estresse aplicado durante os períodos fetal e neonatal sobre parâmetros de ansiedade e medo nos ratos machos adultos jovens;

- Investigar a influência do estresse aplicado durante os períodos fetal e neonatal sobre a atividade locomotora, e exploratória dos ratos jovens adultos;

- Avaliar a influência do estresse aplicado durante os períodos fetal e neonatal sobre a preferência condicionada de lugar com morfina em ratos jovens adultos.

3 MANUSCRITO CIENTÍFICO

Os resultados inseridos nesta dissertação apresentam-se sob a forma de manuscrito científico, o qual se encontra aqui estruturado. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências, encontram-se no próprio manuscrito. O manuscrito foi submetido para publicação na revista Behavior Brain Research, e encontra-se sob revisão.

Manuscrito Científico

3.1 Stress during the prenatal period modifies pups' emotionality parameters and favors preference for morphine in adolescence

Luciana Taschetto Vey, Higor Zuquetto Rosa, Raquel Cristine Silva Barcelos, Hecson Jesser Segat, Vinícia Metz, Verônica Tironi Dias, Thiago Duarte, Marta M. M. F. Duarte, Marilise Escobar Burger

Stress during the prenatal period modifies pups' emotionality parameters and favors preference for morphine in adolescence

Luciana Taschetto Vey[a], Higor Zuquetto Rosa[1], Raquel Cristine Silva Barcelos[b], Hecson Jesser Segat[a], Vinícia Metz[1], Verônica Tironi Dias[b], Thiago Duarte[b], Marta M. M. F. Duarte[2b], Marilise Escobar Burger[1ab]

[a] Programa de Pós Graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria (UFSM), Av. Roraima, 1000, Prédio 18, Cidade Universitária, CEP 97105-900 Santa Maria, RS, Brazil

[b] Programa de Pós Graduação em Farmacologia UFSM, Av. Roraima, 1000, Prédio 21, Cidade Universitária, CEP 97105-900 Santa Maria, RS, Brazil

[1] Departamento de Fisiologia e Farmacologia, UFSM, Av. Roraima, 1000, Prédio 21, Cidade Universitária, CEP 97105-900 Santa Maria, RS, Brazil

[2] Lutheran University of Brazil (ULBRA), Santa Maria, Brazil

*Corresponding author:

Prof. Dr. Marilise Escobar Bürger

Departamento de Fisiologia e Farmacologia-CCS

Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, BRAZIL

Phone/FAX:+055-55 3220 8676

E-mail: mariliseeb@yahoo.com.br

Abstract

Experimental animal studies have shown that early life periods are highly vulnerable to environmental factors, which may exert prolonged impact on HPA axis function and on subsequent neurochemical and behavioral responses in adulthood. Here we evaluated the influence of stress exposure in early life (pre- and postnatal periods) on development of anxiety-like symptoms, locomotor and exploratory activities, and morphine-conditioned place preference (CPP), which is indicative of addiction. Animals exposed to postnatal stress (post-NS) showed lesser anxiety in different behavioral paradigms (elevated plus maze - EPM and defensive burying test - DBT) as well as increased exploratory behavior (open-field task - OFT), and no preference for morphine in CPP. In contrast, animals exposed to prenatal stress (pre-NS) showed increased corticosterone plasma levels together with anxiety symptoms and greater preference for morphine following three days of drug withdrawal. Our findings indicate that the prenatal period is critical for stress, whose effects may be manifest throughout life. On the other hand, post-NS can trigger neuroadaptations able to overcome emotional consequences of early life. We hypothesized that pre-NS is able to modify responses to opioids along adulthood, which may facilitate development of addiction to these drugs.

Keywords: Addiction, Anxiety, Pre-natal stress, Post-natal stress, Morphine

1. Introduction

Drug addiction has exerted a considerable impact on society, resulting in one of the biggest public health problems reaching different ethnic groups and social classes worldwide [1]. In 2007, the United Nations Organization (UNO) reported that about 172 to 250 million people have used an illicit drug worldwide. Among these drugs, marijuana has the highest annual prevalence of use (143-190 million people), followed by amphetamine, cocaine and opioids [2].

Over the past two decades, the use of opioid drugs has increased following different non-cancer chronic pains [3]. It has also been shown that only one in 6, or 17.3% of users of non-therapeutic opioids reported that they received the drugs through a medical prescription (NSDUH, 2010) [4]. Morphine, oxycodone and meperidine, besides heroin, which is illegal in most countries, are the opioids most commonly abused , and their continual use favors the development of addiction [5-6]. Morphine is clinically used in relieving moderate to severe pain, but its effectiveness is gradually lost with the rapid development of tolerance. In addition, chronic use of morphine leads to physical and psychological dependence, and its withdrawal triggers aversive symptoms known as abstinence syndrome [7].

Against this background, stressful experiences appear to have a strong influence on susceptibility to drug-taking behavior [8-9]. Adversities during pregnancy can compromise the development of the fetus, changing physiological and behavioral aspects of the offspring. In particular, prenatal stress (pre-NS) has been shown to produce behavioral and neuroendocrine changes, which may result in enhanced release of corticosterone due to maternal stress [10-11]. There is growing evidence that repeated exposure to stress early in life results in profound changes in the brain, some of which appear to be long lasting and possibly permanent. In humans, early stress in the form of childhood abuse and neglect has

been associated with increases in stress-reactivity and vulnerability to several psychiatric disorders and substance abuse later in adult life [12]. Experimental studies have confirmed clinical evidence of both prenatal and early postnatal perturbations, which may exert prolonged impact on hypothalamic-pituitary-adrenal (HPA) axis function, and on subsequent neurochemical and behavioral responses to stress [13-14]. Consequences from adverse experiences in early postnatal life may be assessed through animal models, which include exposure to maternal separation and/or neonatal isolation [15]. Early life experiences, particularly parental care, could program the development of HPA axis responses to stress [16-19] and result in persistent consequences on the neurobehavioral development. However, the protective or therapeutic effects of early intervention on the negative impact of interruptions to the parent–pup relationship have been poorly understood.

This study was performed to evaluate the influence of stress exposure in different early life periods (fetal and neonatal) on anxiety-like symptoms and emotionality, and its consequences on addiction parameters after young animals' exposure to morphine.

2. Methods

2.1 Animals

Twelve female pregnant Wistar rats from the breeding facility of Universidade Federal de Santa Maria (UFSM), RS, Brazil, were individually kept in plexiglas cages with free access to food and water in a room with controlled temperature (22–23°C) and on a 12 h-light/dark cycle with lights on at 7:00 a.m.. This study was approved by the Animal Ethical Committee of Universidade Federal de Santa Maria (027132-UFSM), affiliated to the Council for the Control of Animal Experiments (CONCEA), following international norms of animal care and maintenance.

2.2 Experimental procedures

Six dams were exposed to unpredictable stress protocol (prenatal stress), which was held for two weeks. On postnatal day (PND) 1, male pups were separated and two animals of each mother were assigned to each experimental group, respectively, one pup for control (n=6) and one pup for morphine (n=6) group. Animals were not handled until behavioral tests (PND 38). Another experimental group of six dams was not exposed to the stress protocol during the gestational period. One day after birth (PND 1) of the offspring, four male pups of each dam were randomly assigned to two experimental groups: unhandled (UH) and postnatal stress (Post-NS). Each of these groups (UH and post-NS) was re-assigned to control (one pup of each offspring, totaling n=6) and morphine (one pup of each mother, totaling n=6) groups. Pups body weight was measured at PND1 and PND-9, in order to assess body weight variation in this period. On PND 38, animals were initially subjected to behavioral assessments to evaluate anxiety parameters, locomotion and exploration, which were followed by the morphine-conditioned place preference (CPP) protocol, as shown in Figure 1.

2.3 Prenatal stress (Pre-NS) procedure

Six pregnant rats were exposed daily to stress between gestational days 7 and 20. A modified version of the chronic moderate stress (CMS) protocol first described by Willner et al. [20] was adopted. The stress protocol consisted of two or three different stressors following a semi-randomized schedule, including: damp sawdust, grouped housing, cage tilting (45º), lights on overnight, isolation, switching cages, and foreign object in cage for 14 consecutive days. This protocol did not involve any food or water deprivation [21].

2.4 Postnatal stress (Post-NS) procedure

Newborn litters found before 5 p.m. were considered to be born on that day (Day 0). Pups were randomly assigned to one of two treatments: neonatal isolation or unhandled (UH) (n=16). The treatments were distributed between litters to avoid differential maternal treatment within a litter and were carried out between 9 a.m. and 1 p.m.. For neonatal isolation, pups were removed from the nest one at a time, marked for identification, and weighed. Each pup was placed individually into a clean cup in a temperature and humidity controlled chamber maintained at 30°C. After 1 h, the pups were returned to the home cage, where they stayed untouched until PND 9. Pups from the UH group were left undisturbed and not handled in any way throughout the treatment period except for weekly cage cleaning [22] and weight assessment at PND 9.

2.5 Weight gain

A morphometric analysis was performed to evaluate a possible influence of pre- and/or post-natal stress on pups' body weight. Animals of all experimental groups were weighed at PND1 and PND9 in digital balance and then returned to their home cage.

2. 6 Measurement of plasma corticosterone (CORT)

Corticosterone plasma levels were determined in pups of each experimental group (Pre-NS, Post-NS and UH; n=4), following decapitation on PND9. Blood aliquots were centrifuged at 4° C at 1500 × g for 15 min (n=4). Plasma aliquots were frozen at -80 °C until their analysis. Corticosterone plasma levels were analyzed through enzyme immunoassay (ELISA) using a commercial kit according to the manufacturer's instructions (Immuno Biological Laboratories).

2.7 Behavioral testing

2.7.1 Open field task

Behavioral measures relevant to rodent models can be taken during exploration of an open field, as there is a natural tendency for an animal in a new environment to explore it, despite stress and conflict [23]. Each pup was placed individually for 5 min at the center of the open-field arena (40x40x30 cm) [24]. The crossing (horizontal movements) and rearing (vertical movements) numbers were used as measures of locomotor and exploratory activities, respectively, whereas the number of entries in the central squares was used as a measure of anxiety [23]. The open field apparatus was cleaned with alcohol solution (20%) and dried with paper towel between each test.

2.7.2 Elevated plus maze (EPM) task

To evaluate anxiety-like symptoms, animals were observed in the EPM, which is based on the innate fear rodents have for open and elevated spaces [25]. The apparatus consists in a platform elevated 50 cm from the floor. Forty-centimeter (40cm) high walls enclose two opposite arms (50x10cm) whereas the other two arms have no walls. All of the arms have a central intersection (10x10 cm). At the beginning of the test, the rat was placed at the central intersection facing the open arm. Time and entries number spent in the closed and open arms, as well as head dipping frequency in the open arms were quantified for 5 minutes in the EPM. We also calculated the anxiety index, which integrates the EPM behavioral measures as demonstrated in the following formula:

Anxiety index= 1

$$- \left[\frac{\left(\frac{\text{Time spent in the open arms}}{\text{Total time on the maze}} \right) + \left(\frac{\text{Number of entries to the open arms}}{\text{Total exploration on the maze}} \right)}{2} \right]$$

Anxiety index values range from 0 to 1, where an increase in the index expresses increased anxiety-like behavior [26-27]. The apparatus was cleaned with alcohol solution (20%) and dried with paper towel before the introduction of each animal.

2.7.3 Defensive burying behavior

Anxiety-like behavior following a single shock from a novel object, a shock probe, can be assessed in this test [28]. The apparatus was a modified home cage (40x30x50cm) with 4 cm of wood chip bedding material evenly distributed throughout the cage [28]. One end of the cage contained a shock probe with a constant current of approximately 1.0 mA [29]. Each animal was placed individually into the testing apparatus facing away from the shock probe for a 5-min test [30-31]. When the animal received a shock by touching the probe, the current was terminated so as not to provide additional shocks. After 5 minutes, the animal was removed and returned to its home cage, the apparatus was cleaned and new bedding was placed into the cage for the next rat. Behaviors measured were: latency to first contact with the probe and to be shocked, latency to initiate burying (defined as pushing bedding material with the snout or forelimbs forward in the direction of the prod) from first contact, height of buried bedding and duration of burying behavior over the entire test period [32]. In addition, the duration and frequency of immobility were assessed (standing on four feet with body and head motionless) [28].

2.8 Drugs

Morphine sulfate (10 mg/mL Itapira-São Paulo) was diluted in 0.9% saline to a previously standardized final concentration of 4 mg/kg (data not shown, adapted from Ma et al) [33].

2.9 Conditioned place preference (CPP)

2.9.1 Apparatus

The CPP apparatus had three compartments that are separated by manual guillotine doors: two boxes of equal size (45 × 45 × 50 cm) and equivalent intensity of light, but with different textures. One compartment had a smooth white floor and striped walls, while the other had a striped floor and smooth white walls. These two lateral compartments were accessible by a central compartment (18×36×50cm), which was gray with a smooth floor. The boxes were indirectly illuminated by incandescent light (60 W) of equal intensity at all times. The apparatus was cleaned with alcohol 20% using wet sponge and paper towel before the introduction of each animal. This experimental paradigm was performed as described by Thanos et al. [34].

2.9.2 Development and expression of CPP

CPP methods have been described in detail previously [35]. CPP was performed through the following steps: habituation, pre-test, conditioning, and test. For phase habituation, at PND 40, animals were placed in the central compartment for 15 minutes in order to demonstrate any natural preference of each animal [35]. The aim of this procedure is to eliminate exploratory behavior, which is common in new environments, for both pre-testing and conditioning phases, thereby avoiding misinterpretations. After habituation, animals were subjected to pretest at PND 41, when they were placed at the center chamber with the guillotine doors removed to allow access to the entire apparatus for 15 min. The amount of time spent in each chamber was monitored and used to assess natural preferences. Animals showing some natural preference were excluded from the experiment. The next day, PND 42, rats were assigned to receive saline or morphine (4mg/kg) paired with one of the two

conditioning environments in a counterbalanced manner (the 'unbiased' procedure). All animals were allocated to stay for a period of 45 min in the lateral chambers twice daily (9 a.m. and 3 p.m.) for 4 days, with the saline group receiving 0.9% sodium chloride injections on both sides of the boxes, whereas the morphine group received morphine injection on one side and saline on the other side. Morphine-paired sides were counterbalanced among all groups. The center choice chamber was never used during conditioning and was blocked by guillotine doors. After the 4-day conditionings, rats were placed at the center choice chamber with access to the entire apparatus for 15 min to perform the conditioned place preference test. The time spent in each chamber was recorded.

2.9 Statistical analyses

Levene's test was applied to verify data homogeneity. Behaviors related to anxiety, emotionality, locomotion and exploration were analyzed by one-way ANOVA because they were assessed before morphine conditioning. Behaviors of preference for morphine observed in CPP task (test and extinction) were analyzed by two-way ANOVA [3 (UH, pre-NS and post-NS) x 2 (morphine, vehicle)]. Both one- and two-way analysis were followed by Duncan's multiple range test, when appropriate (software package Statistica 8.0 for Windows was used). All the data are expressed as means ± SEM. Values of $p<0.05$ were considered statistically significant for all comparisons made.

3. Results

3.1 Pups weight gains and corticosterone plasma levels are shown in Table 1:

One-way ANOVA revealed a significant influence of stress on weight gain and corticosterone levels [($F=35.17$ and 13.80; $P<0{,}000$), respectively]. Duncan's test showed that pre-NS decreased weight gain and increased corticosterone plasma levels, when compared to

UH and post-NS. In fact, pups exposed to post-NS showed decreased corticosterone levels in relation to UH group (Table 1).

3.2 Behavioral measurements

3.2.1 Anxiety-like symptoms evaluated in Elevated Plus Maze (EPM) are shown in Figure 2.

One-way ANOVA of EPM revealed a significant influence of stress on time spent in open arms, number of entries in open arms, head dipping number and anxiety index [(F=10.72, *P*<0.000; 14.84, *P*<0.000; 9.12, *P*<0.001 and 3.15, *P*<0.05), respectively].

Post-hoc test showed that pre-NS decreased the time spent in the open arms of the EPM in relation to both UH and post-NS groups. This behavioral parameter was increased in post-NS when compared to UH group (Fig. 2A). The open arms entries number was increased in both experimental stress-exposed groups (pre-NS and post-NS) in relation to UH group. This increase was greater in post-NS than in pre-NS (Fig. 2B). Head dipping frequency was increased only in post-NS group in relation to both UH and pre-NS, which were similar to each other (Fig. 2C). Pre-NS increased anxiety index in relation to both UH and post-NS, which had comparable values with each other (Fig. 2D).

3.2.2 Locomotor index evaluated in Open Field (OF) task is shown in Figure 3.

One-way ANOVA of OF task revealed a significant influence of stress on crossing, rearing and central squares number [(F=13.69, *P*<0.000; 24.63, *P*<0.000 and 6.75, *P*<0.05, respectively].

Duncan's test showed that animals exposed to post-NS presented increased crossing (Fig. 3A) and rearing (Fig. 3B) numbers, as well as increased central squares crossing in the OF (Fig. 3C), when compared to both UH and pre-NS groups, whose activities in the OF were comparable to each other.

3.2.3 Emotionality symptoms evaluated in Defensive Burying Task (DBT) are shown in Figure 4:

One-way ANOVA of DBT revealed a significant influence of stress on latency time to bury, burying behavior time, time and frequency of freezing [(F=7.42, $P<0.05$; 14.09, $P<0.000$; 10.23, $P<0.000$ and 3.91, $P<0.05$), respectively]. Post-hoc test showed that pre-NS increased latency time to burying after the shock in comparison to both UH and post-NS, which were comparable to each other (Fig. 4A). Freezing time was also higher in Pre-NS than in UH and post-NS, although in the latter it was smaller than in UH (Fig. 4C). This behavioral paradigm also showed that post-NS-exposed animals presented greater time of burying (Fig. 4B), besides lower frequency of freezing (Fig. 4D), as compared to both UH and pre-NS, whose values were similar to each other.

3.2.4 Dependence parameters related to Morphine-Conditioned Place Preference (morphine-CPP) paradigm is shown in Figure 5:

Two-way ANOVA of morphine conditioning revealed a significant main effect of drug and drug x stress interaction [F(2,36)=22.55, $P<0.000$ and 3.91; $P<0.05$], respectively. Post-hoc test revealed that after 24h and 72h of morphine conditioning pre-NS and UH groups rats spent more time in the drug-conditioned place, as post-NS group only showed no preference for either side of CPP (Fig.5B and 5C). Similarly to pre-test day (Fig. 5A), between vehicle-injected groups, all handling groups (UH, pre-NS and post-NS) showed similar preference for both sides of the apparatus after 24 (Fig. 5B) and 72 (Fig.5C) hours.

4. Discussion

The current experimental protocol included morphometric analysis of the offspring following stress protocols in order to consider possible alterations caused by pre- and post-natal stress exposure. Our findings indicated that the life period of stress exposure was an interesting influence factor on pups' body weight gain. Indeed, when the stress protocol was applied in the prenatal period, pups gained less body weight than UH and post-NS groups did. From this finding it can be hypothesized that: i) fetuses are significantly sensitive to maternal environment when mothers are exposed to stress during pregnancy (prenatal stress), as blood flow in the uterine arteries is reduced [36]. This event is able to decrease the nutritional supply to the fetus, influencing development [37-39]; (ii) gestational stress reduces breast milk production, decreasing nutritional provision to the litter, delaying their development [40]; (iii) exposure to stress-mediated endogenous corticosteroids could increase leptin release, decreasing neuropeptide Y mRNA levels and leading to smaller body weight gain [41].

Stress exposure can affect the neuroendocrine system and consequently increase levels of circulating hormones. When the trigger of this activation remains, damage begins to occur, mainly to the nervous system. In this context, our findings showed that pre-NS favored increased corticosterone plasma levels alongside anxiety-like symptoms, which were evidenced by decreased time spent in the open arms and higher anxiety index in the EPM, as well as increased latency time to burying following shock and freezing duration in the DBT. In contrast, post-NS-exposed animals showed decreased corticosterone plasma levels, besides tranquility-like symptoms, as observed by increased time spent and entries number in open arms of the EPM and increased head dipping frequency, together with increased burying behavior and decreased frequency and time of freezing in the DBT. A recent study suggested that burying behavior and immobility duration could be indicators of increased anxiety [42], but other studies have shown that hyperlocomotion/arousal and stress are not always

correlated, indicating that different responses may be involved with neurotransmitter systems [43]. In addition, the post-NS group also showed higher locomotor index and lowest emotionality, as observed by increased crossing and rearing numbers and increased crossing number in the central squares of the open field task (OF), respectively. In turn, other studies had shown that post-NS-exposed animals presented greater safety in new environments, thus favoring exploration and locomotion in these [44-45]. In this sense, the OF task has also been used to estimate locomotor and exploratory activities, as well the emotionality associated to a new environment. Regarding the behavioral tests employed here, the EPM paradigm is able to explore rodents' aversion to open spaces, as well their tendency to explore a new environment [46-47]. Here, the EPM task was used to assess possible influences of stress in different life periods on anxiety-like behaviors. In line with our findings, recent studies using EPM task have shown that pre-NS exposure was related to anxiety-like symptoms in the offspring [48-52].

Taken together, findings obtained from different behavioral paradigms indicated that pre-NS was able to increase anxiety parameters. Therefore, there are two noteworthy outcomes: i) animals exposed to pre-NS showed increased anxiety-like behavior; ii) early postnatal manipulation was able to prevent anxiety-like symptoms. These data are consistent with Bogosh et al. [53], who demonstrated that anxious behavior was more predominant in pre-natally stressed animals, while its prevention may be related to neonatal handling protocols. Moreover, functionality of the HPA axis can be beneficially changed in animals handled during neonatal development [54], increasing their adaptation to novel and/or stressful stimuli [55].

Besides anxiety and fear-like symptoms, the current study also included parameters of preference for morphine, which was developed in adolescence. It is well known that animals in this life period show increased sensitivity to stress, as well increased predisposition to

consumption of addictive drugs [56]. In fact, this age is strongly linked to increased risk for novelty seeking, which may result in addiction [57], making this the most prone life period to drug searching [58]. Different experimental studies have employed the CPP paradigm to assess both preference for addictive drugs and relapse symptoms [58-62]. Here we found that UH and more evidently pre-NS groups showed morphine-conditioned place preference, while post-NS showed no drug preference. In fact, the latter experimental group did not show anxiety-like symptoms or increased corticosterone plasma levels, which may have contributed to the absence of morphine preference. In this context, some type of neonatal handling has shown beneficial influences in the functionality of the HPA axis [54] and so, their adaptation to novel and/or stressful stimuli can be significantly increased [55]. Our current findings are not contradictory with a previous study from our group showing the impairing influence of neonatal isolation [59], where this was applied for 21 consecutive days. Here, pups were exposed to a similar protocol for nine days only, leading us to assume that besides length of exposure, the timing of stress is crucial in determining any harmful or beneficial influence for pups. In line with this, additional studies have shown that post-natal handling could increase the glucocorticoid receptor density in different brain areas [63], indicating that the HPA axis feedback to glucocorticoids can be enhanced, affording better response against stressful situations [64]. Furthermore, when mothers are separated from pups, mother's licking and grooming behaviors and arched-back nursing with their pups are increased, as compared to mothers of non-handled pups [65].

Particularly relevant to our findings, although opiates are known to influence the HPA axis by increasing plasma levels of ACTH, β-endorphin and corticosterone [66], an increased release of opioids such as β-endorphin [67] and met-encephalin [68] was observed in response to stress. Additionally, corticosterone plasma levels, ACTH and β-endorphin were significantly elevated in rats exposed to restraint stress [69], confirming that stress triggers the

release of opioids in the brain [70] Even though our experimental protocol did not include opiate plasma levels, we are showing for the first time that pre-NS was able to modify pups' HPA axis, modifying their responses to opioid exposure. Taken together, these hormonal and behavioral changes appear to have facilitated the development of morphine-preference in CPP, as observed in pre-NS-exposed animals. Our outcomes lead to some hypotheses to explain the higher morphine-preference observed in the pre-NS group: i) pre-NS exposure stimulates HPA axis, thus increasing corticosterone plasma levels ; ii) HPA axis activation favors anxiety and fear behaviors; iii) pre-NS exposure modifies the endogenous opioid system, thus affecting the density or responsivity of receptors, as well endogenous agonists activity; iv) when in contact with exogenous opiates, individuals previously exposed to pre-NS may show increased preference for this addictive drug, facilitating addiction to opiates.

5. Conclusion

Our study is the first to show that pre-NS exerted deleterious influence on pups, as evidenced by smaller body weight gain, increased corticosterone levels, anxiety-like symptoms and morphine-preference symptoms. On the other hand, post-NS was related to protective influences on pups, as observed by less anxiety-like symptoms and weaker morphine-induced CPP. This innovative study is a precursor of future molecular studies contributing to new preventative approaches regarding opiate addiction. So far, our findings indicate that pre-NS stress may be determinant on the neuropsychiatric and neurobiological systems because it is related to disruption of the adrenal response maturation to stress [71] especially during adolescence. Additional studies at molecular and epigenetic level should be conducted.

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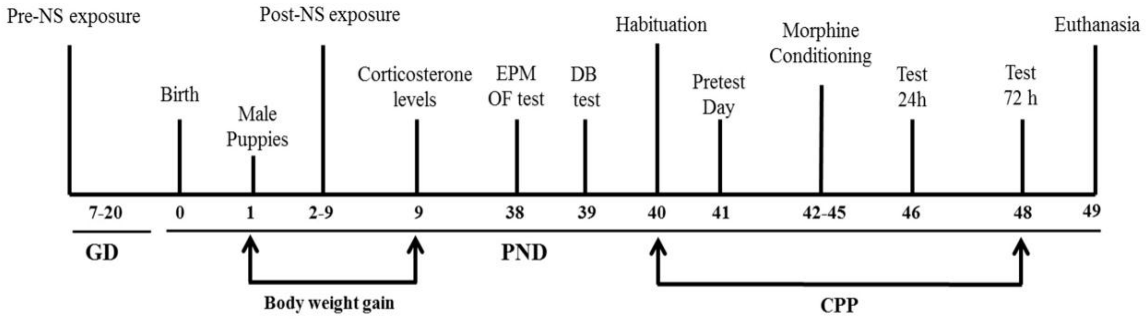

Figure 1

Figure 1: Experimental design. Influence of prenatal and postnatal stress on parameters of anxiety, emotionality and locomotion, and preference for morphine in adolescent male rats. Abbreviations: GD: gestational day; PND: Post-natal day; Pre-NS: prenatal stress; Post-NS: post-natal stress; OF: open field; EPM: elevated plus-maze; DB: defensive burying test; CPP: conditioned place preference.

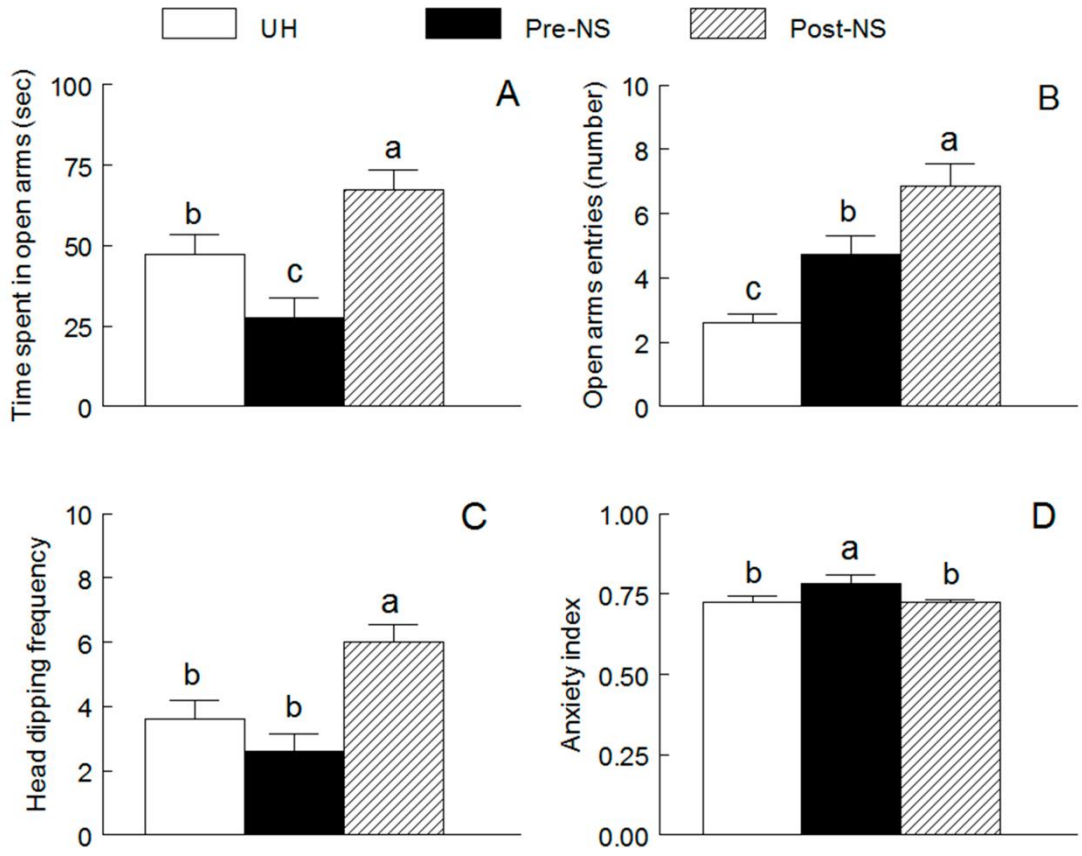

Figure 2

Figure 2: Influence of prenatal and postnatal stress on anxiety symptoms in elevated plus maze (EPM) task. Anxiety-like symptoms were evaluated by time spent (A), entries number (B) and head dipping frequency (C) in the open arms of EPM, besides anxiety index (D). Data are expressed as mean ± S.E.M (n=12). Different lowercase ^{a,b,c} indicates significant difference between the experimental groups ($P<0.05$). Abbreviations: UH: unhandled; Pre-NS: prenatal stress; Post-NS: postnatal stress.

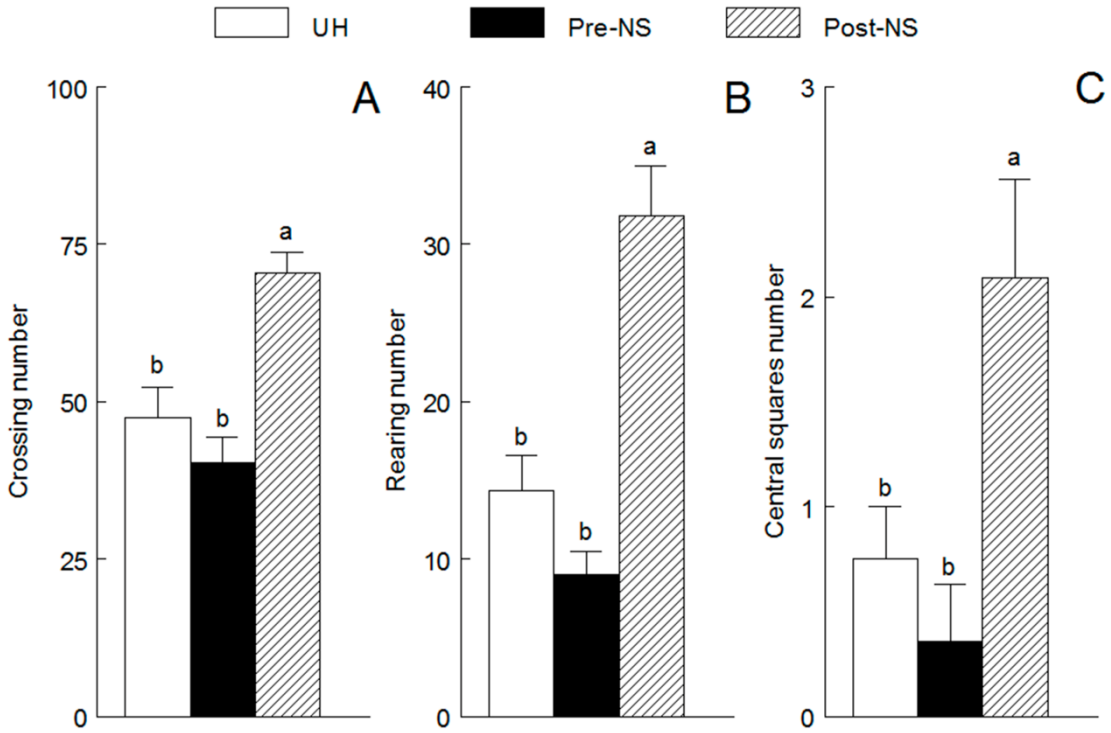

Figure 3

Figure 3: Influence of prenatal and postnatal stress on locomotor parameters in open field (OF) task, evaluated by crossing number (A), rearing number (B) and central squares entry number (C). Data are expressed as mean ± S.E.M (n=12). Different lowercase [a,b,c] indicates significant difference between the experimental groups (*P<0.05*). Abbreviations: UH: unhandled; Pre-NS: prenatal stress; Post-NS: postnatal stress.

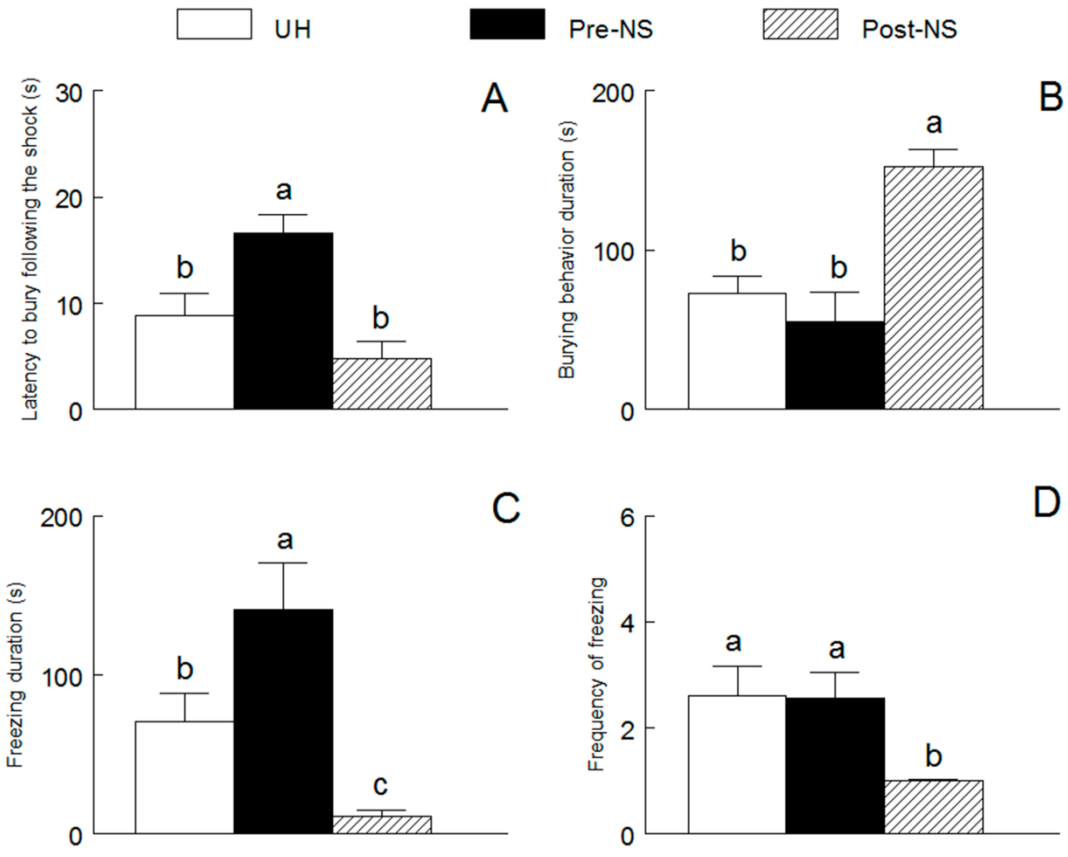

Figure 4

Figure 4: Influence of prenatal and postnatal stress on defensive burying test, where emotionality-like symptoms were evaluated by latency to burying following electrical shock (A), burying behavior duration (B), freezing duration (C) and freezing frequency (D). Data are expressed as mean ± S.E.M (n=12). Different lowercase ^{a,b,c} indicates significant difference between the experimental groups (*P<0.05).* Abbreviations: UH: unhandled; Pre-NS: prenatal stress; Post-NS: postnatal stress.

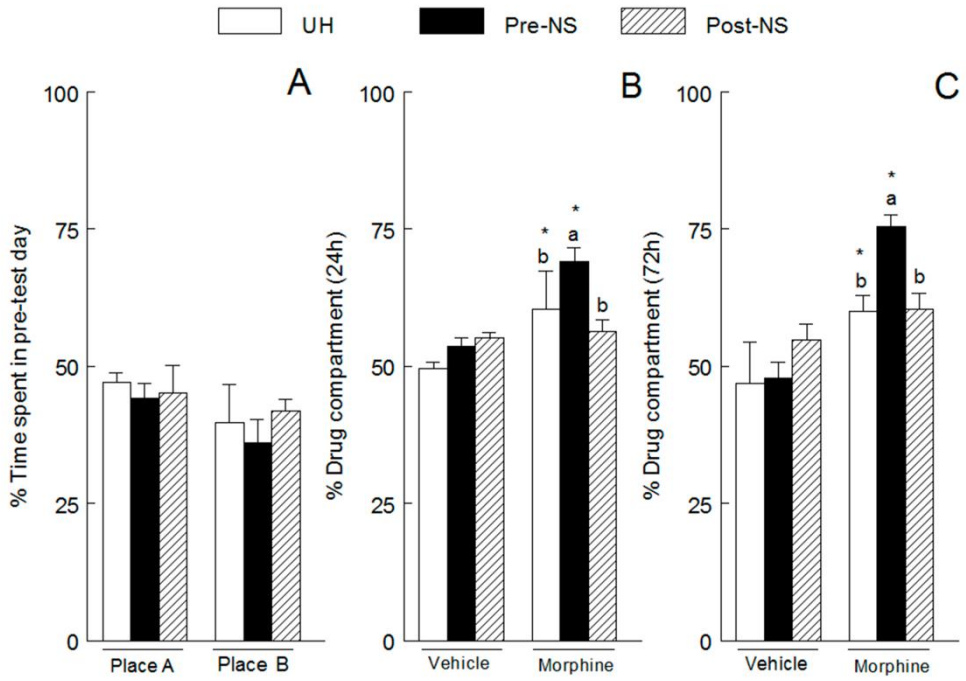

Figure 5

Figure 5: Influence of prenatal and postnatal stress on conditioned place preference (CPP) induced by morphine (4 mg.kg^{-1}, i.p. for 4 days). Behavioral observations were made before morphine conditioning (pre-test day) (A) and 24h (B) and 72h (C) after morphine conditioning. Data are expressed as mean ± S.E.M. (n=6). Different lowercase ^{a,b,c} indicates significant difference between the experimental groups ($P<0.05$); * indicates a significant difference between morphine and vehicle-injected groups ($P<0.05$). UH: unhandled; Pre-NS: prenatal stress; Post-NS: postnatal stress.

Table 1- Influence of prenatal stress and postnatal stress on morphometric analysis and corticosterone levels in males offspring.

Groups	Body Weight gain (g)	Costicosterone (ng/mL)
UH	13.12 ± 0.34^{a}	139.3 ± 8.00^{b}
Pre-NS	8.68 ± 0.47^{b}	159.3 ± 2.39^{a}
Post-NS	12.62 ± 0.40^{a}	115.7 ± 5.77^{c}

UH: unhandled; Pre-NS: prenatal stress; Post-NS: postnatal stress.
Data are expressed as mean ± S.E.M. (n=4). Different lowercase ^{a,b,c} indicates significant difference between the experimental groups ($P<0.05$).

4 CONCLUSÕES

Através dos resultados experimentais obtidos, podemos chegar as seguintes conclusões:

- ✓ O pre-NS aumentou os níveis plasmáticos de corticosterona, e diminuiu o ganho de peso corporal dos filhotes;

- ✓ Em contrapartida, o post-NS reduziu os níveis plasmáticos corticosterona quando comparados aos animais não manuseados;

- ✓ Ratos expostos ao pre-NS apresentaram maior emocionalidade e sintomas de ansiedade, enquanto os animais submetidos ao post-NS não apresentaram tais sintomas;

- ✓ Animais expostos ao protocolo de post-NS apresentaram maior atividade locomotora e exploratória quando comparados aos animais não manuseados;

- ✓ Ratos expostos ao pre-NS mostraram preferência condicionada de lugar com morfina nos dois tempos (24 e 72h) avaliados, enquanto animais expostos ao post-NS não apresentaram preferência de lugar nestes mesmos tempos avaliados.

- ✓ A partir do presente estudo pode-se concluir que o estresse sofrido durante o período gestacional apresenta significativas implicações emocionais que persistem ao longo da vida, podendo modificar a atividade do eixo HPA, e possivelmente a resposta farmacológica de drogas opioides, o que poderá favorecer o desenvolvimento de dependência à tais drogas.

5 PERSPECTIVAS

Na continuidade dos estudos, uma investigação relacionada aos diferentes períodos de exposição ao estresse deverá ser conduzida, com a finalidade primária em avaliar os parâmetros moleculares e histológicos envolvidos no sistema de recompensa ativado por opioides, além de investigar a neurotransmissão dopaminérgica resultante como também as vias de sinalização celular.

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