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Caren Tatiane de David Antoniazzi

INFLUÊNCIA DO MANUSEIO NEONATAL SOBRE PARÂMETROS COMPORTAMENTAIS, BIOQUÍMICOS E MOLECULARES APÓS O USO DE DROGAS PSICOESTIMULANTES EM RATOS

Santa Maria, RS
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Tese apresentada ao Curso de Pós-Graduação em Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Farmacologia**.

Orientadora: Profª Drª. Marilise Escobar Bürger

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"Amar o perdido
deixa confundido
este coração.

Nada pode o olvido
contra o sem sentido
apelo do Não.

As coisas tangíveis
tornam-se insensíveis
à palma da mão

Mas as coisas findas
muito mais que lindas,
essas ficarão."

Carlos Drummond de Andrade

RESUMO

INFLUÊNCIA DO MANUSEIO NEONATAL SOBRE PARÂMETROS COMPORTAMENTAIS, BIOQUÍMICOS E MOLECULARES APÓS O USO DE DROGAS PSICOESTIMULANTES EM RATOS

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O uso abusivo de drogas psicoestimulantes é um problema social comum em países de diferentes culturas, com incidência crescente e alarmante, cuja descontinuidade do uso está associada à síndrome de abstinência, caracterizada por quadros de ansiedade e depressão. Estudos têm mostrado a influência da exposição precoce ao estresse sobre padrões de dependência, a qual pode cumprir um importante papel sobre a vulnerabilidade para o abuso de drogas na idade adulta. Por outro lado, procedimentos como a estimulação tátil (ET) neonatal e o isolamento neonatal (IN), têm sido descritos por interferir em parâmetros comportamentais e neurofisiológicos, que podem persistir até a vida adulta. Assim, primeiramente investigou-se a influência do manuseio neonatal sobre a preferência condicionada de lugar (PCL) induzida por anfetamina (ANF), bem como sintomas de ansiedade e o status oxidativo relacionados à abstinência da droga, em ratos jovens. Filhotes machos de ratos *Wistar* foram submetidos à ET ou IN, por 10 minutos, do dia pós-natal 1 (DPN1) ao 21, enquanto a PCL com ANF ou veículo (NaCl 0.9% i.p) foi iniciada no DPN40. O condicionamento com ANF causou preferência e sintomas de abstinência em animais não manuseados, acompanhado por dano oxidativo no córtex, hipocampo e estriado, enquanto a ET favoreceu a redução da preferência pela droga, não mostrando sintomas de abstinência, a qual foi quantificada através do menor grau de ansiedade. O status oxidativo apontou ação neuroprotetora da ET ao reduzir o dano lipídico e proteico no córtex, hipocampo e estriado, aumentando alguns marcadores antioxidantes sanguíneos. Em um segundo estudo, visou-se identificar o período neonatal em que a ET exerce maior influência sobre o desenvolvimento dos animais. Filhotes machos de ratos *Wistar* foram submetidos à ET (10 min) do DPN1 ao 7, DPN8 ao 14, ou DNP15 ao 21. Na idade adulta, os animais foram avaliados através de análises comportamentais, bioquímicas e moleculares. De acordo com estes parâmetros, foi possível observar que os animais manuseados entre o DPN8 e 14 apresentaram menor índice de ansiedade, melhor memória de trabalho e maior capacidade de lidar com situações adversas. A ET na segunda semana de vida foi capaz de reduzir os níveis de corticosterona e melhorar o status oxidativo dos animais, observado pelo menor dano aos lipídios e aumento na atividade da catalase, no hipocampo. Além disso, a ET do DPN8 ao 14 aumentou o fator neurotrófico derivado do encéfalo (BDNF) no hipocampo dos animais, enquanto que os receptores de glicocorticoides foram reduzidos em todos os períodos de manuseio, exceto do DPN8 ao 14. Considerando a correlação existente entre o uso de psicoestimulantes e sintomas de depressão, no 3º estudo, os efeitos antidepressivos da ET em associação com uma dose subterapêutica de sertralina foram avaliados durante o período de abstinência de cocaína. Filhotes machos de ratos *Wistar* foram submetidos à ET (10 min) do DPN8 ao 14. Durante 14 dias consecutivos, os ratos adolescentes receberam 3 doses diárias de cocaína ou veículo (NaCl 0.9% i.p). Após 7 dias de retirada da droga, os animais receberam 0.3 mg/kg de sertralina e 30 min depois foram avaliados quanto a sintomas de depressão e ansiedade. O status oxidativo cerebral e os receptores de dopamina (D2) e de glicocorticoides (GR) também foram determinados. A ET foi capaz de proteger os animais

contra os sintomas de depressão, demonstrado através da ausência de anedonia e aumento da atividade de natação no teste do nado forçado. Os animais estimulados ainda apresentaram menores índices de ansiedade e estresse, bem como redução dos danos oxidativos no plasma e cérebros dos animais e melhor resposta do sistema de defesa antioxidante plasmático. As análises moleculares também mostraram que a ET foi capaz de alterar o imunoconteúdo de D2 e GR no estriado e hipocampo dos animais. Em resumo, a ET modificou a PCL induzida por ANF, reduzindo comportamentos de ansiedade comuns na abstinência da droga. Além disso, a ET é capaz de estimular o sistema de defesa antioxidante, e proteger áreas cerebrais intimamente relacionadas à adicção, em ratos jovens. Ainda, foi possível demonstrar que a ET pode ser mais eficiente quando aplicada no período intermediário do desenvolvimento neonatal, refletindo em emocionalidade reduzida e maior habilidade para lidar com situações estressantes na idade adulta. Finalmente, também foi possível mostrar que a ET foi capaz de otimizar os efeitos da sertralina administrada em dose subterapêutica, prevenindo contra os efeitos prejudiciais da retirada da cocaína, como depressão e ansiedade.

Palavras-chave: Estimulação tátil neonatal; Preferência condicionada de lugar; Anfetamina; Cocaína; Estresse oxidativo; Glicocorticoides; Depressão.

ABSTRACT

INFLUENCE OF NEONATAL HANDLING ON BEHAVIORAL, BIOCHEMICAL AND MOLECULAR PARAMETERS AFTER USE OF PSYCHOSTIMULANT DRUGS IN RATS

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The abusive use of psychostimulant drugs is a common social problem in countries of different cultures, with increasing and alarming incidence, whose discontinuance of use is associated with anxiety and depression cases. Studies have shown the influence of early exposure to stress on patterns of dependency, which can play an important role on the vulnerability to drug abuse in adulthood. On the other hand, procedures such as neonatal tactile stimulation (TS) and neonatal isolation (NI), have been described by interfering with behavioral and neurophysiological parameters that can persist into adulthood. Thus, we first investigated the influence of neonatal handling on conditioned place preference (CPP) induced by amphetamine (AMPH), as well as anxiety-like symptoms and oxidative status related to drug abstinence, in young rats. Male pups of *Wistar* rats were submitted to TS or NI, for 10 min, from postnatal day 1 (PND1) to 21, while the CPP with AMPH or vehicle (NaCl 0.9% i.p.) was initiated on PND40. AMPH-conditioning evoked drug-preference and abstinence-symptoms in unhandled animals, accompanied by oxidative damage in cortex, hippocampus and striatum, while TS favored a decrease on drug-preference, showing no abstinence symptoms, as observed by reduced anxiety-like symptoms. Oxidative status pointed to a neuroprotective influence of TS by reducing lipid and protein damage in cortex, hippocampus and striatum, and increasing antioxidant markers in blood. Secondly, we aimed to identify the neonatal period in which TS exerts greater influence on animals' development. Male pups of *Wistar* rats were submitted to TS (10 min) from PND1 to 7, PND8 to 14 or PND15 to 21. In adulthood, the animals were assessed using behavioral, biochemical and molecular analysis. According to these parameters, it was observed that the animals handled between PND8 and 14 had lower anxiety index, better working memory and increased ability to cope with adverse situations. TS in the second week of pups' lives was capable of reduce plasma corticosterone levels and improve the oxidative status of animals, observed by the slightest damage to lipids, and increased catalase activity in the hippocampus. In addition, TS from PND8 to 14 increased brain derived neurotrophic factor (BDNF) in the hippocampus of animals, while glucocorticoid receptors were reduced in all handling periods, except on PND8 to 14, in the same brain area. Considering the correlation between psychostimulants use and depression-like symptoms, in the 3rd study, the antidepressant effects of TS in association with a subtherapeutic dose of sertraline were evaluated during cocaine withdrawal period. Male pups of *Wistar* rats were submitted to TS (10 min) from PND8 to 14. For 14 consecutive days, adolescent rats received 3 daily doses of cocaine or vehicle (0.9% NaCl ip). Seven days after cocaine withdrawal, animals received 0.3 mg/kg of sertraline and 30 min later were evaluated for depression- and anxiety-like symptoms. Brain oxidative status and dopamine (D2) and glucocorticoid receptors (GR) were also determined. TS was able to protect animals against the depression-like symptoms, demonstrated by the absence of anhedonia and increased swimming activity in the forced swimming test. TS animals had lower levels of anxiety and stress, as well as reduction of oxidative damage in

plasma and brain of animals, besides best response of plasma antioxidant defense system. The molecular analysis also showed that TS was able to alters D2 and GR immunocontent in the striatum and hippocampus of animals. In summary, TS modified AMPH-induced preference, reducing anxiety-like behaviors that are common during drug withdrawal. Moreover, TS is able to stimulate the antioxidant defense system, and protect brain areas closely related to addiction, in young rats. Besides, it was possible to demonstrate that TS may be more effective when applied during the intermediate phase of neonatal development, reflecting reduced emotionality and improved ability to deal with stressful situations later in life. Finally, it was also possible to show that TS in association with sertraline is able to prevent against the deleterious effects of cocaine withdrawal, such as depression and anxiety.

Keywords: Neonatal tactile stimulation; Conditioned place preference; Amphetamine; Cocaine; Oxidative stress; Glucocorticoids; Depression.

LISTA DE ILUSTRAÇÕES

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LISTA DE ABREVIATURAS E SIGLAS

ACTH – hormônio adrenocorticotrófico
ANF – anfetamina
ANOVA – análise de variância
BDNF – fator neurotrófico derivado do encéfalo
DA – dopamina
D2 – receptores de dopamina do tipo 2
EO – estresse oxidativo
EROS – espécies reativas de oxigênio
ET – estimulação tátil
GR – receptores de glicocorticoides
HPA – eixo hipotálamo-pituitária-adrenal
IN – isolamento neonatal
MAO – monoamina oxidase
NE – norepinefrina
NM – não manuseados
PCL – preferência condicionada de lugar
RL – radicais livres
SM – separação materna
SNC – sistema nervoso central
THDA – transtorno da hiperatividade e déficit de atenção

SUMÁRIO

1 APRESENTAÇÃO

Esta tese está estruturada em seções dispostas da seguinte forma: Introdução (contendo Objetivos), Desenvolvimento (Referencial teórico, Artigo 1 e Manuscrito científico 1 – submetido para publicação e Manuscrito 2 – Resultados parciais), Discussão, Conclusões e Referências, Apêndice A (Manuscrito 3 – Resultados parciais).

Os itens Materiais e Métodos, Resultados, Discussão e Referências encontram-se inseridos no próprio artigo e manuscritos na seção **PRODUÇÃO CIENTÍFICA** e representam a íntegra deste estudo.

Ao fim encontram-se os itens **DISCUSSÃO e CONCLUSÕES** nos quais há interpretações e comentários gerais referentes aos manuscritos científicos e os resultados parciais contidos neste estudo.

As **REFERÊNCIAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO, DISCUSSÃO** e **CONCLUSÕES** desta tese.

O **APÊNDICE A** refere-se aos experimentos executados durante realização de Doutorado Sanduíche.

2 INTRODUÇÃO

Durante o período neonatal, o sistema nervoso central (SNC) é imaturo e por isso suscetível às intervenções ambientais (KOEHL et al., 2002; LEVINE, 1957). Nesta mesma época, as respostas adaptativas ao estresse podem ter seu desenvolvimento modificado conforme os eventos a que os neonatos estão expostos (MEANEY et al., 1993), tais como as experiências traumáticas e a exposição a ambientes adversos em períodos precoces da infância (CHAMPAGNE, de KLOET e JOELS, 2009; KNUTH e ETGEN, 2007).

Em ratos recém-nascidos, a resposta ao estresse promove morte neuronal (ZHANG et al., 2002), tornando-os mais vulneráveis à preferência por drogas (GORDON, 2002), afetando também a neurogênese, que permanece diminuída na idade adulta (LEMAIRE et al., 2000). Por ter um papel importante na cognição, emoção e memória relacionada às drogas, sugere-se que a desregulação da neurogênese esteja envolvida na diminuição da capacidade cognitiva, nos transtornos de humor e na adicção (ABROUS, KOEHL e Le MOAL, 2005; McEWEN, 2003).

Estudos recentes citam o Brasil como uma das nações emergentes em que o uso de psicoestimulantes vem aumentando, contrariando a tendência de retração observada na América do Norte e na Europa (UNODC, 2012). Entre os fatores que contribuem para esta estimativa, está o fato de que mais de um terço da população brasileira é de jovens com idades entre 15 e 34 anos (IBGE, 2010) e cerca de 45% dos usuários de cocaína no Brasil experienciaram o primeiro contato com as drogas antes dos 18 anos de idade (ABDALLA et al., 2014). A adolescência, tanto em humanos quanto em roedores, é uma fase rica em alterações neurofisiológicas (GIEDD et al., 2009; SPEAR, 2000) que podem favorecer a maior suscetibilidade ao abuso de drogas (ANDERSEN, 2003; SPEAR, 2000).

A exposição repetida à anfetamina tem sido experimentalmente empregada como um modelo animal de alterações comportamentais e neuroquímicas induzidas por psicoestimulantes em ratos (FREY et al., 2006; KAUER-SANT'ANNA et al., 2007). Tal exposição causa sensibilização comportamental, hiperlocomoção e alterações na memória dos animais (PELEG-RAIBSTEIN et al., 2009), além de levar a alterações neuroquímicas e estruturais, incluindo uma elevação de dopamina, norepinefrina e da densidade das espinhas dendríticas em diferentes regiões cerebrais (BISAGNO, FERGUSON e LUINE, 2003; ROBINSON e KOLB, 2004).

A separação materna (SM) e o isolamento neonatal (IN) são modelos animais de estresse já bem descritos na literatura e que, em certo grau, sintetizam psicopatologias que eventualmente ocorrem em humanos (SCIOLINO et al., 2010). Estes modelos têm sido associados a desordens de ansiedade (LUKKES et al., 2009) e vulnerabilidade ao abuso de

drogas (MOFFETT et al., 2007), além de favorecer alterações da morfologia cerebral (MUHAMMAD e KOLB, 2011).

Neste contexto, a estimulação tátil (ET) se destaca como uma forma positiva de estímulo sensorial aplicado sobre a pele e que, de algum modo, simula os cuidados maternos em roedores (LOVIC, FLEMING e FLETCHER, 2006). A ET funciona como um enriquecimento ao desenvolvimento cerebral (RICHARDS et al., 2012) e parece ser capaz de inibir a maioria dos prejuízos fisiológicos relacionados ao estresse neonatal (LEVINE, 2001). De modo geral, atua melhorando o aprendizado e memória (ZHANG e CAI, 2008), e a resposta ao estresse na vida adulta (BOUFLEUR et al., 2013), além de contribuir para uma menor vulnerabilidade ao abuso de drogas (ANTONIAZZI et al., 2014).

Em resumo, o IN é um modelo animal de estresse precoce relacionado a alterações neuroquímicas e comportamentais, que pode facilitar a preferência por drogas psicoestimulantes na idade adulta e a ET tem sido descrita por ter efeitos benéficos sobre a neurogênese e sobre uma maior capacidade dos animais em lidar com situações traumáticas em estágios mais avançados do desenvolvimento. Assim, a realização deste estudo torna-se relevante, pois irá determinar se a qualidade das relações interpessoais vividas na infância, pode de alguma forma alterar o desenvolvimento neuropsicomotor e influenciar padrões comportamentais que poderão determinar a vulnerabilidade ao abuso de drogas psicoativas na adolescência e idade adulta. Além disto, até o momento não se sabe em qual período do desenvolvimento a ET exerce sua maior ação, sendo necessária a determinação deste, permitindo assim a otimização do manuseio e facilitando a compreensão do exato mecanismo celular e/ou neuroendócrino envolvido no procedimento de ET.

2.1 OBJETIVOS

2.1.1 Objetivo geral

Avaliar a influência do manuseio neonatal sobre parâmetros comportamentais, bioquímicos e moleculares durante a abstinência de drogas psicoestimulantes em ratos jovens, bem como determinar o período em que a estimulação tátil (ET) neonatal exerce seus efeitos potencializados.

2.1.2 Objetivos específicos

- Avaliar os efeitos da ET e do isolamento neonatal sobre parâmetros de dependência e ansiedade em ratos jovens expostos à anfetamina;
- Quantificar parâmetros de estresse oxidativo e defesas antioxidantes em tecidos cerebrais e sanguíneo de animais submetidos ao manuseio neonatal e posteriormente expostos à anfetamina;
- Determinar o período específico do desenvolvimento dos filhotes em que a ET neonatal exerce sua melhor ação;
- Investigar a influência dos diferentes períodos da ET sobre a atividade do eixo hipotálamo-pituitária-adrenal;
- Avaliar as possíveis modificações no imunoconteúdo de fatores neurotróficos derivados do encéfalo e receptores de glicocorticoides no hipocampo de animais submetidos à ET neonatal em diferentes períodos do desenvolvimento;
- Avaliar a influência da ET sobre parâmetros de depressão e ansiedade em ratos expostos à cocaína;
- Quantificar o status oxidativo e alterações no imunoconteúdo de receptores dopaminérgicos e de glicocorticoides em áreas cerebrais dos animais submetidos à ET e posteriormente expostos à cocaína.

3 REFERENCIAL TEÓRICO

3.1 ESTRESSE NEONATAL

Experiências traumáticas e exposição a ambientes adversos em períodos precoces da infância (KNUTH e ETGEN, 2007) podem induzir alterações comportamentais e neuroendócrinas na idade adulta, tanto em humanos quanto em animais (CHAMPAGNE, de KLOET e JOELS, 2009; PLOTSKY et al., 2005).

Nos humanos, as experiências estressantes nessa fase do desenvolvimento estão associadas ao elevado risco para transtornos psicossociais agudos e crônicos na idade adulta, tais como desordens afetivas e abuso de drogas (ANAND e SCALZO, 2000; HEIM e NEMEROFF, 2001). Em animais, as experiências vividas precocemente no ninho também são críticas para o desenvolvimento e causam alterações nos sistemas fisiológico, neuroendócrino, comportamental, emocional, social e cognitivo, que irão formar a base para as respostas aos estímulos e comportamento na idade adulta (FLEMING, O'DAY e KRAEMER, 1999; LEHMANN e FELDON, 2000; LEVINE et al., 1967).

Em ratos recém-nascidos, o estresse promove morte neuronal (ZHANG et al., 2002) e torna-os mais vulneráveis à preferência por cocaína (GORDON, 2002). O estresse gestacional e neonatal também afeta a neurogênese e diminui a produção de novos neurônios na idade adulta (LEMAIRE et al., 2000). A neurogênese parece ter um papel importante na cognição, emoção e memória relacionada às drogas (ABROUS, KOEHL e Le MOAL, 2005; McEWEN, 2003). Por isso, sugere-se que sua desregulação esteja envolvida na diminuição da capacidade cognitiva, nas alterações de humor e na adicção (ABROUS, KOEHL e Le MOAL, 2005).

3.2 DEPENDÊNCIA E DROGAS PSICOESTIMULANTES

A dependência de drogas é um fenômeno com causas e consequências fisiológicas, psicológicas e sociais (NESTLER e AGHAJANIAN, 1997), que pode ser conceituada como uma síndrome em que o abuso da droga passa a ter prioridade sobre outros comportamentos considerados importantes para o indivíduo antes da sua experiência com as drogas. Em sua forma extrema, a dependência está associada ao uso compulsivo da droga (EDWARDS, ARIF e HADGSON, 1981). Considera-se como estimulante toda substância utilizada voluntariamente com a finalidade de obtenção de estados alterados de consciência,

caracterizados por estado de euforia, decorrente da estimulação do sistema nervoso central (SNC). No Brasil, fármacos proscritos como a cocaína e os compostos anfetamínicos, são utilizados com esta finalidade (CHASIN e SILVA, 2003).

A cocaína, um alcalóide ativo naturalmente encontrado e extraído do arbusto da coca (Erytroxylum coca), é amplamente consumida de modo abusivo pelos humanos (JOHANSON e SCHUSTER, 1995). Classifica-se como um agente dopaminérgico de ação indireta, por ligar-se ao transportador pré-sináptico da dopamina (DA), bloqueando a sua captação para dentro do terminal pré-sináptico (O'BRIEN, 2006), e permitindo assim o acúmulo da DA na fenda sináptica. A DA livre e em concentrações aumentadas é capaz de interagir com receptores dopaminérgicos, iniciando uma sequência de eventos que modificam a atividade neuronal momentaneamente (NICOLA, SURMEIER e MALENKA, 2000). A cocaína também pode atuar reduzindo a recaptação de outras monoaminas tais como norepinefrina (NE) e serotonina, além do glutamato, os quais estão envolvidos direta ou indiretamente nos efeitos da cocaína, sendo que o conjunto de alterações momentâneas e duradouras altera a expressão do comportamento (NICOLA, SURMEIER e MALENKA, 2000; O'DONNELL, 2003).

Desta forma, os efeitos da cocaína sobre o SNC podem ser resumidos pelo desenvolvimento de sintomas como euforia, autoconfiança, aumento de atenção, redução do apetite, ansiedade, paranoia, comportamento egocêntrico, disforia, anorexia e ilusões (NNADI et al., 2005). Já os sintomas periféricos resumem-se ao aumento da atividade simpaticomimética, observada através de midríase, elevação da pressão arterial, taquicardia, arritmias e até parada cardiorrespiratória, dependendo da dose e via de administração da cocaína. Ainda, a cocaína apresenta potente propriedade anestésica local, cujo uso clínico humano foi pioneiro, servindo de modelo para a síntese dos atuais e modernos anestésicos locais (CRITS-CHRISTOPH et al., 2008).

Em humanos, o uso crônico da cocaína vem sendo correlacionado à depressão e inabilidade em expressar interesse ou prazer em estímulos recompensadores (anedonia) (BARR e MARKOU, 2005; BARR, MARKOU e PHILLIPS, 2002), sintomas estes que surgem durante a descontinuidade do uso e se assemelham aos sintomas típicos da depressão maior (MARKOU e KENNY, 2002, MARKOU, KOSTEN e KOOB, 1998). Em roedores, diversos estudos também demonstraram que a abstinência após o uso repetido de drogas de abuso está relacionada ao desenvolvimento de sintomas do tipo depressivo (ETTENBERG, RAVEN e DANLUCK, 1999; FILIP et al., 2006; PATERSON, MYERS e MARKOU, 2000). Existem ainda indícios de importante comorbidade entre depressão e uso de cocaína, uma vez que até 30% dos indivíduos com depressão maior irão apresentar algum episódio de abuso de drogas ao longo da vida, bem

como cerca de 35% dos adictos à cocaína apresentarão transtornos de humor (GAWIN e KLEBER, 1989; REGIER et al., 1990). Ao se observar que os sintomas de abstinência de cocaína reproduzem um estado depressivo, sugere-se que estas duas condições podem compartilhar bases neurobiológicas comuns, o que é reforçado pelo fato de que sintomas de abstinência de cocaína em humanos e roedores podem ser atenuados por alguns medicamentos antidepressivos (GAWIN et al., 1989; MARKOU, HAUGER e KOOB, 1992; PLIAKAS et al., 2001).

Além da cocaína, as anfetaminas são drogas psicoestimulantes sintéticas, com propriedades simpaticomiméticas (substâncias que imitam os efeitos da epinefrina e NE; ALLES, 1933; ALLES e PRINZMETAL, 1933), que em conjunto com seus derivados, constituem o grupo mais comum de fármacos psicoestimulantes causadores de euforia (BERMAN et al., 2009). Seu efeito está relacionado principalmente ao aumento da liberação de DA, NE e 5-hidroxitriptamina (serotonina) na fenda sináptica, apresentando certo predomínio sobre a NE, quando comparado às outras monoaminas (BERMAN et al., 2009; MADRAS, MILLER e FISCHMAN, 2005; ROTHMAN et al., 2001). Além disso, as anfetaminas também agem através da inibição da atividade da monoamina oxidase (MAO), que é uma enzima neuronal responsável pelo catabolismo das catecolaminas, e que apresenta papel fundamental no equilíbrio da atividade dos sistemas dopaminérgico, noradrenérgico e serotoninérgico periférico e central (ROTHMAN et al., 2001).

Conforme Ahlskog (2007), elevadas concentrações de DA no SNC podem estar envolvidas em processos neurodegenerativos, acreditando-se ainda que a metabolização do neurotransmissor seja responsável pela geração de espécies reativas de oxigênio (EROS), cuja toxicidade relaciona-se ao desenvolvimento de estresse oxidativo (CHIUEH et al., 1993; FORNSTEDT, 1990) e apoptose. Neste sentido, os radicais livres (RL) estão envolvidos no desenvolvimento de inúmeras patologias (FANTONE e WARD, 1982; SUNDSTRÖM, SVEDBERG e CARLING, 1984) e a sua produção excessiva é conhecida como estresse oxidativo (EO). O EO decorre do desequilíbrio entre mecanismos de defesa antioxidantes e a formação de EROS (DAVIES, 1995). Assim, a propriedade pró-oxidante da cocaína e da anfetamina decorre de sua atividade sobre o aumento da liberação de DA, favorecendo os elevados níveis de DA livre, a qual fica vulnerável à auto-oxidação, cujos metabólitos conhecidos são as dopamino-quinonas, precursores de RL (BOESS et al., 2000; DEVI e CHAN, 1996).

Sintetizada em 1887, mas com propriedades psicoestimulantes identificadas somente na década de 30, a anfetamina (ANF) foi então introduzida na terapêutica como descongestionante nasal e no tratamento clínico da narcolepsia (JULIEN, 1997; PRINZMETAL e BLOOMBERG, 1935), um subtipo de epilepsia caracterizada pelo sono excessivo. Posteriormente, foi utilizada para o tratamento da esquizofrenia, dependência por morfina, hipotensão arterial, enjoos e soluços

intensos (JULIEN, 1997). A partir de 1936, a mistura racêmica da ANF (mistura equivalente de dextro e levo anfetamina) passou a ser utilizada para reduzir o apetite de indivíduos obesos, no tratamento do déficit de atenção e na doença de Parkinson (SEIDEN e SABOL, 1993). Seu potencial de abuso foi reconhecido durante a Segunda Guerra Mundial, quando soldados do Eixo (Alemanha, Japão e Itália) e das forças aliadas (Estados Unidos, Reino Unido e União Soviética, entre outros países europeus e de outros continentes) utilizavam anfetaminas para aumentar a vigília, a coragem, e diminuir a fadiga, a fome e os estados de depressão, tornando-se uma droga difundida entre a sociedade no pós-guerra (CALDWELL, CALDWELL e DARLINGTON, 2003; DRUMMER e ODELL, 2001; HERNANDEZ e FERNANDEZ, 1998).

No Brasil, os últimos relatórios elaborados pelo *Escritório contra Drogas e Crimes das Nações Unidas* – UNODC (2011 e 2012) que mencionam a situação do país, alertam para o aumento progressivo do consumo de cocaína e anfetaminas nas últimas décadas, contrariamente à tendência mundial de retração. De acordo com estes relatórios, a elevada taxa de consumo de cocaína no Brasil se deve a fatores como: i) posição geográfica do Brasil, vizinha dos maiores produtores mundiais de cocaína – Peru, Colômbia e Bolívia; ii) sua população jovem, pois cerca de 35% da população brasileira tem entre 15 e 34 anos de idade (IBGE, 2010); iii) o crescimento socioeconômico ocorrido na última década no Brasil, o que representa maior poder de compra; e iv) o baixo custo da cocaína no país (UNODC, 2012).

Em um recente estudo realizado no Brasil, constatou-se que cerca de 45% dos usuários experimentaram cocaína pela primeira vez antes dos 18 anos de idade (ABDALLA et al., 2014) e vários estudos demonstram que a precocidade do uso de cocaína é um importante fator de risco para o desenvolvimento de dependência e outras doenças psiquiátricas (ADRIANI e LAVIOLA, 2004; CHUAN-YU CHEN et al., 2009). Entre os adolescentes, especialmente do sexo masculino, o uso de cocaína parece estar relacionado com a inassiduidade e abandono escolar, evidenciando a importância da prevenção e protocolos de tratamento específicos para essa faixa etária (ABDALLA et al., 2014). O neurodesenvolvimento durante a adolescência é caracterizado por profunda maturação cerebral, associada ao aumento motivacional para novas experiências e a um controle inibitório imaturo, devido à hipofuncionalidade do córtex pré-frontal medial (CASEY e JONES, 2010; CHAMBERS, TAYLOR e POTENZA, 2003). Todos esses fatores podem predispor o adolescente a uma maior impulsividade e tendência de assumir riscos, aumentando assim a probabilidade de experimentação às drogas (CHAMBERS, TAYLOR e POTENZA, 2003; SPEAR, 2000; KUHN, WILSON e SWARTZWELDER, 2013).

Em relação às anfetaminas, este aumento na taxa de consumo pode ser explicado devido ao apelo social à utilização desses fármacos para o controle da obesidade, que também são

utilizadas com a finalidade de apurar os reflexos e reduzir o cansaço, além de aumentar momentaneamente a velocidade de aprendizagem (UNODC, 2011). Dessa maneira, além de atletas, muitos estudantes, médicos, motoristas de caminhão e pilotos também fazem uso dessas substâncias (CENTRE FOR ADDICTION AND MENTAL HEALTH, 2004). Os principais objetivos dos usuários são manter o estado de alerta por maior período de tempo e aumentar a capacidade de atenção, concentração e raciocínio (YONAMINE, 2004), sem considerar os efeitos indesejáveis da droga, como elevação da pressão arterial, da temperatura corporal, da frequência cardíaca e respiratória. Em longo prazo, o uso de anfetaminas pode causar perda excessiva de peso, ansiedade, insônia, alterações de humor, além de distúrbios psicóticos, caracterizados por paranoias e alucinações (MATSUMOTO et al., 2002).

Atualmente, o uso de anfetaminas é proibido em muitos países, sendo que em alguns países europeus, também sua produção e comércio são proibidos, tornando a droga absolutamente clandestina. No Brasil, até o final de 2011, anfetamínicos inibidores do apetite eram clinicamente prescritos e dispensados em farmácias comerciais com exigência de receita médica, sendo destinados ao controle da obesidade e ao tratamento do transtorno da hiperatividade e déficit de atenção (THDA), basicamente. A partir de 9 de dezembro de 2011, os inibidores do apetite femproporex, mazindol e anfepramona tiveram seu registro cancelado e sua produção, comércio e uso proibidos no Brasil, a partir de uma resolução determinada pela Diretoria Colegiada da Agência Nacional de Vigilância Sanitária. Permanece autorizada somente a prescrição de sibutramina como um inibidor do apetite, e de metilfenidato e dextro-anfetamina, para tratar o THDA (ANVISA - RDC 52/2011).

3.3 EFEITOS DE RECOMPENSA E PREFERÊNCIA CONDICIONADA DE LUGAR

Os efeitos de recompensa de diversas drogas de abuso têm sido avaliados através da preferência condicionada de lugar (PCL) (BARDO, ROWLETT e HARRIS, 1995; HOFFMAN, 1989), um modelo experimental utilizado para promover um condicionamento através de pistas. Este paradigma é baseado na habilidade do animal em encontrar pistas associadas com a droga para descobrir o local de preferência, e que tem importantes implicações na busca de drogas e recaídas em humanos (HAND, STINUS e LE MOAL, 1989; NEISEWANDER, PIERCE e BARDO, 1990).

A PCL é um modelo animal utilizado para avaliar os efeitos hedônicos das drogas de abuso, que envolve o pareamento repetido de um compartimento com um estímulo específico (droga), enquanto o compartimento oposto é pareado com um estímulo neutro. No dia do teste,

que ocorre após o condicionamento e na ausência dos estímulos, é permitido ao animal acesso livre aos compartimentos. Um maior tempo de permanência no compartimento pareado anteriormente com a droga indica a preferência do animal pela droga, enquanto o menor tempo indica aversão (TZSCHENTKE, 1998; 2007).

3.4 SEPARAÇÃO MATERNA E ISOLAMENTO NEONATAL

Diversos estudos com roedores demonstraram que procedimentos estressantes em idade precoce promovem alteração nos padrões de abuso (HEIM e NEMEROFF, 2001; MOFFETT et al., 2007), que irão variar de acordo com a intensidade dos eventos e a idade em que ocorrerem (LEHMANN e FELDON, 2000), cumprindo assim papel significativo no desenvolvimento da vulnerabilidade ao uso excessivo de álcool e de drogas psicoestimulantes na idade adulta (FELITTI et al., 1998; SCHENK et al., 1987). Em humanos, tais estudos comprovam que os eventos adversos na infância, não só influenciam a dependência às drogas, como também aumentam a vulnerabilidade para recaídas durante períodos de abstinência, além de favorecer a manutenção do uso compulsivo de drogas (COFFEY et al., 2002; SINHA, 2001).

Dessa forma, modelos animais de estresse neonatal, como a separação materna (SM), vêm sendo desenvolvidos para explicar estas alterações neuroquímicas e comportamentais resultantes da exposição precoce a fatores estressantes (FRANCIS et al., 1999; LADD et al.,2000).

A SM consiste em afastar os filhotes da mãe por um período que pode variar de alguns minutos até várias horas por dia, durante os primeiros dias logo após o nascimento. Longos períodos de separação maternal, também chamados de isolamento neonatal – IN (IMANAKA et al., 2008), podem alterar as vias de resposta ao estresse, pois a mãe é a primeira ligação entre o filhote e o meio ambiente (FRANCIS e MEANEY, 1999) e a interferência nesta relação pode alterar a diferenciação no sistema nervoso dos filhotes (FRANCIS et al., 1996).

De acordo com Imanaka et al. (2008), o IN e a SM podem diferir no que diz respeito ao isolamento individual da ninhada já que muitos procedimentos de SM costumam retirar as mães do ninho por um breve período de tempo, enquanto os filhotes permanecem agrupados na caixa-moradia. Já no IN, obrigatoriamente os filhotes são colocados em caixas individuais menores, ficando assim isolados da mãe e da sua ninhada, por períodos de tempo mais prolongados. As ligações sociais que se formam no período pós-natal ativam sistemas neurais que fazem com que a mãe focalize o bem-estar do recém-nascido, para garantir sua saúde e sobrevivência, sendo que as alterações nesta relação, como por exemplo, o isolamento neonatal, pode induzir um comportamento parental perturbado, como abuso ou negligência (SWAIN et al., 2007).

Esta ruptura na interação entre mãe e filhote produz alterações duradouras no comportamento e na neurofisiologia da prole (HALL, 1998; KUHN e SCHANBERG, 1998), que em geral apresenta depressão, estresse psicológico crônico, dificuldade de lidar com situações estressantes na idade adulta, alterações no eixo hipotálamo-pituitária-adrenal (HPA), aumento nos quadros de ansiedade, comportamento reprodutivo alterado, comprometimento do aprendizado e resposta locomotora reduzida (CHAMPAGNE et al., 2003; HUOT et al., 2001, 2002; KALINICHEV et al., 2002).

Ainda, de acordo com Kuhn e Schanberg (1998), a SM prolongada durante os primeiros dias de vida interrompe a maturação da resposta adrenal ao estresse, altera os níveis de corticosterona, e afeta negativamente a aprendizagem dos animais adultos, a potenciação de longa duração e a organização sináptica hipocampal (MEANEY et al., 1996). Além disso, estudos mostram que os ratos que sofreram separação maternal periódica quando filhotes respondem mais intensamente a um estímulo estressante quando adultos e apresentam uma maior sensibilidade às drogas psicoativas (PLOTSKY e MEANEY, 1993).

Sabe-se que durante o desenvolvimento dos roedores, existe um período hiporresponsivo ao estresse, aproximadamente do dia 4 ao 14 pós-natal, em que a resposta adrenal ao estresse é mínima ou inexistente (LEVINE, GLICK e NAKANE, 1967; VAZQUEZ et al., 2006a; WALKER et al., 1986). Nestas duas semanas após o nascimento, o eixo HPA dos filhotes é regulado pelas mães, ou seja, ao removê-los do ninho o eixo HPA é ativado, indicando que a ausência da mãe resulta em uma liberação contínua de glicocorticoides, como a corticosterona, durante este período. Em contrapartida, a presença materna cessa a ativação deste sistema (LEVINE, 2001), demonstrando que o período hiporresponsivo ao estresse pode proteger o cérebro em desenvolvimento contra as flutuações nos níveis dos glicocorticoides circulantes (CHAMPAGNE, de KLOET e JOELS, 2009). Na ausência da mãe, em resposta até mesmo ao estresse leve, os ratos neonatos apresentam secreção aumentada de hormônio adrenocorticotrófico (ACTH) e de corticosterona (LEVINE et al., 1991; VAN OERS et al., 1998).

A privação do contato materno também diminui a expressão do fator neurotrófico derivado do encéfalo (BDNF) no hipocampo dos ratos neonatos (GRONLI et al., 2006), e em contraste, os níveis do fator de crescimento neural aumentam (CIRULLI et al., 1998). O BDNF, um fator de crescimento amplamente expressado e liberado no hipocampo, é conhecido por influenciar a sinaptogênese (YOSHII e CONSTANTINE-PATON, 2010), a sobrevivência neuronal (LIPSKY e MARINI, 2007) e a neurogênese no hipocampo adulto (SCHMIDT e DUMAN, 2007). Sugere-se que estas alterações ocorram em razão da separação materna afetar importantes sistemas de neurotransmissores cerebrais, como os receptores cerebrais de

serotonina, a expressão do transportador mesolímbico de dopamina e o desenvolvimento dos receptores $GABA_A$ e dos benzodiazepínicos centrais (BRAKE et al., 2004; MEANEY, BRAKE e GRATTON, 2002; VICENTIC et al., 2006).

Estudos demonstraram ainda que o estresse neonatal repetitivo pode produzir uma disfunção no sistema opióide endógeno, que pode ser ativado por diferentes tipos de estressores, e sugere-se que esteja envolvido em padrões de recompensa cerebral no abuso de drogas em animais. Juntamente com o sistema dopaminérgico, o sistema opióide endógeno parece influenciar a resposta ao etanol e aumentar a vulnerabilidade para iniciar o consumo excessivo de álcool na idade adulta (PLOJ et al., 2000; PLOJ, ROMAN e NYLANDER, 2003a, 2003b).

3.5 ESTIMULAÇÃO TÁTIL NEONATAL

Em razão de todas as alterações geradas pelo estresse neonatal ocasionado pela SM prolongada e pelo IN, a estimulação tátil (ET) neonatal vem se destacando como uma forma eficiente de manuseio, capaz de recuperar os déficits neonatais em ratos e reverter e prevenir certos efeitos comportamentais e fisiológicos provenientes da separação materna (BURTON et al., 2007; CHATTERJEE et al., 2007; LEVINE e OTIS, 1958; LÉVY et al., 2003).

A ET neonatal consiste em uma variedade de estímulos sensoriais externos realizados durante as primeiras semanas de vida que efetivamente aceleram a maturação dos neurônios corticais, influenciam o desenvolvimento do SNC, que irão causar alterações em uma grande variedade de processos comportamentais e fisiológicos, entre eles a resposta ao estresse, persistentes na idade adulta (CASOLINI et al., 1997; PHAM et al., 1999; SILVEIRA et al., 2005).

Sugere-se que os efeitos da ET podem ser explicados pelo fato de que as vias neurais da pele para o SNC amadurecem mais cedo do que outros sistemas sensoriais (MONTAGU, 1953). A manipulação neonatal consiste em pelo menos três estímulos diferentes: 1) breve SM, que elimina pistas olfativas bem como a ET provida pela mãe, para ser recuperada posteriormente quando mãe e filhote forem recolocados juntos (PRYCE, BETTSCHEN e FELDON, 2001); 2) ET realizada por um experimentador, durante o período de separação, que pode em certo grau imitar a estimulação proporcionada pela mãe; e 3) exposição à novidade, que consiste na oferta de novos estímulos ao filhote, neste caso, o estímulo tátil (LEVINE, 1957).

A estimulação neonatal ainda aumenta a neurogênese pós-natal, previne a perda neuronal no hipocampo associada ao estresse e envelhecimento e melhora a função cognitiva (PHAM et al., 1997; SAPOLSKY, 1992). Este estímulo também se mostrou efetivo na modulação da resposta neuroendócrina ao estresse em animais adultos, melhorando a

capacidade de lidar com eventos estressantes em estágios mais avançados do desenvolvimento (CHAMPAGNE, de KLOET e JOELS, 2009; PLOTSKY et al., 2005).

Este modelo de manuseio também foi capaz de reverter os efeitos da SM nos comportamentos de ansiedade (VAN OERS et al., 1998) e sensibilidade à dor (STEPHAN et al., 2002) além de induzir resistência do eixo HPA ao estresse em ratos adultos. Também já demonstrou melhorar o aprendizado e o desempenho dos animais em testes cognitivos e reforçar tanto a memória espacial quanto não-espacial em ratos adultos (KOSTEN, LEE e KIM, 2007; STAMATAKIS et al., 2008). Ainda, foi capaz de facilitar a recuperação após eventos de hipóxia-isquemia neonatal (RODRIGUES et al., 2004) e lesão neonatal no córtex pré-frontal ou córtex parietal posterior (GIBB et al., 2010), sendo que tal recuperação foi correlacionada com a sinaptogênese das células piramidais no córtex intacto adjacente às lesões (GIBB et al., 2010).

Diversos estudos sugerem que a estimulação neonatal altera o comportamento maternal e a interação mãe-filhote, o que leva a diferenças no número de lambidas da mãe (LIU et al., 1997) e faz com que as mães de filhotes estimulados permaneçam mais tempo com eles, do que as mães de filhotes que não foram estimulados (CHOU et al., 2001). É este aumento das lambidas maternas durante os primeiros 10 dias de vida do filhote que geram redução nos níveis do ACTH e corticosterona em resposta ao estresse agudo, e aumentam a densidade dos receptores para glicocorticoides no hipocampo e diminuem os níveis do hormônio liberador de corticotrofina (LIU et al., 1997).

A ET neonatal é um paradigma utilizado experimentalmente em animais para testar os efeitos de estímulos externos na vida do recém-nascido em uma variedade de comportamentos e sistemas neuroendócrinos (GOMES, et al., 2005; RAINEKI et al., 2009; TODESCHIN et al., 2009; WINKELLMANN-DUARTE et al., 2007), e é muito similar à terapia através da massagem, utilizada em humanos (FIELD, 1998). Sabe-se que bebês internados em unidades de terapia intensiva neonatal estão sujeitos tanto a um ambiente altamente estressante – ruído contínuo e de alta intensidade, luz brilhante, procedimentos médicos, etc. – quanto à ausência de estímulos táteis, anteriormente experimentados no útero, e comuns durante os cuidados maternos (VICKERS et al., 2004). Assim, como a massagem pode ser aplicada tanto para diminuir o estresse quanto para fornecer estímulos táteis, ela tem sido recomendada como uma intervenção capaz de favorecer o ganho de peso e promover o desenvolvimento cerebral de neonatos prematuros (GUZZETTA et al., 2009; SCHANBERG e FIELD, 1987; VICKERS et al., 2004).

Do mesmo modo, a ET neonatal também apresenta diversos efeitos positivos, tanto em aspectos comportamentais como morfológicos (CHOU et al., 2001; FERNÁNDEZ-TERUEL et al., 1992). Entretanto, até o momento, não existe consenso na literatura sobre o período mais adequado

para sua aplicação e por quais vias de fato este procedimento exerce seus efeitos biológicos. Estudos mostram que a ET apresenta benefícios mesmo quando aplicada em diferentes períodos, seja logo após o nascimento (ANTONIAZZI et al., 2014; RICHARDS et al., 2012; RODRIGUES et al., 2004) ou até mesmo na idade adulta (GIBB et al., 2010; MUHAMMAD e KOLB, 2011). Nas diversas espécies, durante o desenvolvimento neural, existe um período transitório de crescimento acelerado e que varia de uma espécie para a outra (RICE e BARONE JR., 2000). Nos humanos, o desenvolvimento do sistema nervoso tem seu pico na fase pré-natal enquanto os roedores apresentam considerável desenvolvimento neural na fase pós-natal (DOBBING e SANDS, 1979; RICE e BARONE JR., 2000). Em geral, a sequência de eventos é comparável entre as espécies e embora as escalas de tempo/evento sejam diferentes, é possível identificar estruturas cerebrais análogas entre seres humanos e roedores (RICE e BARONE JR., 2000).

Evidentemente, o desenvolvimento cerebral ocorre de forma contínua e a valorização e o conhecimento das suas etapas têm grande importância nos estudos relacionados às hipóteses de vulnerabilidade do sistema neural às intervenções externas (DOBBING e SANDS, 1979; RICE e BARONE JR., 2000). Neste contexto, por ser a ET um enriquecimento ao desenvolvimento dos filhotes, surge o questionamento de como o eixo HPA se comportaria frente a esse manuseio, quando aplicado em diferentes fases do desenvolvimento neonatal, podendo-se determinar assim o período mais adequado para a realização da ET, a fim de estabelecer em que momento seus efeitos podem ser potencializados.

4 PRODUÇÃO CIENTÍFICA

Os resultados inseridos nesta tese apresentam-se sob a forma de Artigo 1 e Manuscritos científicos 1 e 2, os quais se encontram aqui estruturados. Os itens Materiais e Métodos, Resultados, Discussão e Referências, encontram-se no próprio artigo publicado e Manuscrito 1, submetido para publicação no periódico Progress in Neuro-Psychopharmacology & Biological Psychiatry, encontrando-se sob revisão. O Manuscrito 2 aqui apresentado contém alguns resultados parciais e encontra-se em fase de redação.

4.1 ARTIGO 1

ANTONIAZZI, C. T. D et al. Influence of neonatal tactile stimulation on amphetamine preference in young rats: Parameters of addiction and oxidative stress. **Pharmacology, Biochemistry and Behavior**, v.124, p. 341-349, 2014.

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4.2 MANUSCRITO 1 – Submetido para publicação no periódico Progress in Neuro-Psychopharmacology & Biological Psychiatry, e por isso será apresentado conforme as normas da revista.

Neonatal tactile stimulation during different developmental periods modifies hippocampal BDNF and GR, affecting memory and behavior in adult rats.

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Abstract

Recent studies have shown that tactile stimulation (TS) in pups is able to prevent and/or minimize fear, anxiety behaviors, and addiction to psychostimulant drugs in adult rats. In these studies, animals have been exposed to handling from postnatal day (PND) 1 to 21. The present study was designed to precisely establish which period of neonatal development has a greater influence of TS on neuronal development. After birth, male pups were exposed to TS from PND1 to 7, PND8 to 14, or PND15 to 21. In adulthood, the different periods of neonatal TS were assessed through behavioral, biochemical and molecular assessments. TS from PND8-14 showed lower anxiety-like symptoms, as observed by decreased Anxiety Index in elevated plus maze and increased number of center crossing in open field. This same TS period was able to improve rats' working memory by increasing the percentage of alternation rate in Y-maze, and induce better ability to cope with stressful situations, as showed in the defensive burying test by a reduced time of burying behavior. In addition, TS from PND8-14 showed lower corticosterone levels and better oxidative status, as observed by decreased lipid peroxidation and increased catalase activity in the hippocampus. BDNF immunocontent was increased in the hippocampus of animals receiving TS from PND8-14, while glucocorticoid receptors immunocontent was decreased in both TS_{1-7} and TS_{15-21}, but not TS_{8-14}. This study is the first to show TS can be more efficient if applied over the average period of neonatal development, whose beneficial influence can be reflected on reduced emotionality and increased ability to address stressful situations in adulthood.

Keywords: Stress Hyporesponsive Period; BDNF; Glucocorticoid receptor; Oxidative Stress; Working Memory; Anxiety and Fear.

1. Introduction

The neonatal period is a critical phase for the nervous system development and, likewise, the components of the hypothalamic-pituitary-adrenal (HPA) system have different ontogenetic patterns (Vazquez et al., 2006).The first two weeks of a rat's life are characterized by rapid growth, brain functional organization, neuronal proliferation, migration and differentiation, gliogenesis and myelination (Rice and Barone, 2000), whereas in humans this maturation occurs from the sixth month of pregnancy to the third year of life (Felderhoff-Mueser et al., 2004). In this sense, full development depends on external postnatal stimuli, which may modulate the brain functional maturation and determine its lifelong integrity (Andersen, 2003).

Studies demonstrate that during the first two weeks of rodents' lives, especially from days 4 to 14, adrenal response to stress is minimal, and has been designated as stress hyporesponsive period (SHRP) (Vazquez et al., 2006; for a review, see Walker et al., 2001). In this period, dams regulate HPA axis activity of their pups through mother-pup interactions such as licking, grooming and arched-back nursing, suppressing basal and stress-induced levels of circulating glucocorticoids (GCs) (de Kloet et al., 2005). Removing pups from dams promotes early HPA axis activation while their presence cease further activation of this system, indicating that their absence results in persistent release of GCs during this period (Levine, 2001). In this sense, SHRP has a biological function to protect the developing brain from fluctuations in GC levels (Champagne et al., 2009).

Prolonged elevation of GCs may cause an adverse impact on plasticity in limbic brain regions such as the hippocampus, including spine loss and dendritic atrophy (Liston and Gan, 2011), neuronal cell death (Haynes et al., 2004), and decreased neurogenesis (reviewed in Schoenfeld and Gould, 2012). Following periods of stress-induced elevations in corticosterone levels, a suppression of hippocampal brain-derived neurotrophic factor (BDNF) may occur (Gronli et al., 2006). BDNF is a growth factor robustly expressed and released in the hippocampus and is reported to strongly influence synaptogenesis and spine formation (Yoshii and Constantine-Paton, 2010), neuronal survival (Lipsky and Marini, 2007), as well as adult hippocampal neurogenesis (Schmidt and Duman, 2007).

Tactile stimulation (TS) is a favorable neonatal handling applied onto rats skin that has been related to behavioral benefits and less emotionality in the coping response to traumatic situations later in life (Silveira et al., 2005; Boufleur et al., 2013). Many studies have shown the effectiveness of TS to prevent damages from ischemia (Rodrigues et al., 2004) and cortical injury by changing dendritic morphometry and spine density in rat brain areas (Gibb et al., 2010). Neonatal TS also seems to be able to favor an attenuation of drug-induced behavioral

sensitization (Muhammad et al., 2011), and alter a variety of behaviors and neuroendocrine systems in pups' lives (Padoin et al., 2001; Raineki et al., 2009; Todeschin et al., 2009). A recent study has suggested that postnatal TS was able to completely reverse the effects of prenatal exposure to valproic acid in rats, acting as a reorganization tool that can ameliorate neuroanatomical consequences of this treatment (Raza et al., 2015).

Previous studies performed by our group have demonstrated that 21 days of TS were sufficient to prevent cocaine and amphetamine conditioned place preference (CPP; Antoniazzi et al., 2014a, 2014b), also reducing anxiety-like behavior after chronic mild stress (Boufleur et al., 2013) and improving responsiveness of rodents to benzodiazepine drugs (Boufleur et al., 2012). Current literature has shown studies involving animals exposed to TS throughout the postnatal period, i.e., from the first days after birth until weaning. Thus, we have decided to assess whether TS can act differently, according to the postnatal period in which it is applied, considering behavioral parameters of fear and anxiety, oxidative status, and molecular markers in the hippocampus, a highly plastic brain area closely related to cognitive and emotional processes.

2. Methods

2.1 Animals and experimental procedure

Seven pregnant female *Wistar* rats from the breeding facility of Universidade Federal de Santa Maria (UFSM), RS, Brazil, were housed in plexiglas cages with free access to food and water. Animals were kept in a room with controlled temperature (22-23°C), on a 12-hour light/dark cycle with lights on at 7:00 a.m. Pups' date of birth (postnatal day 0 - PND0) was monitored, and sexes distinguished by larger genital papilla and longer anogenital distance in male vs. female pups (Liu et al., 2008). Female rats were designated to other study of our laboratory.

To avoid the use of littermates in the same experimental group, male pups were randomly selected from several litters on postnatal day one (PND1) and assigned to experimental groups (n=7, each group); unhandled (UH), tactile stimulation (TS) from PND1 to 7 (TS_{1-7}), TS from PND8 to 14 (TS_{8-14}), and TS from PND15 to 21 (PND_{15-21}). All TS groups received the stimulus out of their nest for 10 minutes a day, spending the remaining time with their mothers until weaning. On PND22, litters were weaned. Groups of four male pups, one from each experimental condition, were housed and left undisturbed up to 60 days of age.

From PND60, animals were assessed in different tests every 48 hours, to avoid animal stress by excessive human handling. Thus, on PND60, anxiety-like symptoms were assessed in elevated plus maze (EPM), and on PND62 animals had their locomotor and exploratory activity quantified in the open field (OF) test. Working memory of animals was tested in the Y-maze

task 48 hours after PND64, and on PND66, anxiety-like behavior following a single shock from a novel object was observed in a defensive burying task (DBT). Following behavioral analyses, on PND68, rats were anesthetized, blood was collected (by cardiac puncture in heparinized tubes), and brain areas were removed for oxidative status estimation.

All procedures were performed in accordance with the Brazilian law nº. 11.794/2008, which is in accordance with the Policies on Use of Animals and Humans in Neuroscience Research and the Institutional and National Regulations for Animal Research (process 104/2010).

2.2 Neonatal handling

Tactile stimulation (TS) consisted of the same experimenter gently stimulating the pups' dorsal surface individually with the index finger from rostral to caudal direction for 10 min (each day) out of the nest (Antoniazzi et al., 2014a, 2014b; Boufleur et al., 2012, 2013; Rodrigues et al. 2004). TS was applied daily between 12:00 and 14:00 according to the period of each experimental group. Considering this, TS_{1-7} group received neonatal stimulus from PND1 to PND7. For TS_{8-14} group, neonatal TS was applied from PND8 until PND14 only, while TS_{15-21} received neonatal TS from PND15 to PND21. At the end of each procedure, pups were returned to their litters. UH group was only handled during the home cage regular cleaning, two times a week.

2.3 Behavioral evaluations

2.3.1 Elevated plus maze (EPM)

On PND60, animals were observed in EPM, which is based on the innate fear rodents have for open and elevated spaces (Montgomery, 1955). The apparatus was made of wood and consisted of a plus-shaped platform elevated 50 cm from the floor. Two opposite arms (50 cm×10 cm) were enclosed by 40 cm high walls whereas the other two arms had no walls. The four arms had at their intersection a central platform (10 cm×10 cm), which gave access to any of the four arms. At the beginning of each test, rats were placed on the central platform facing an open arm and allowed to explore the maze for 5 min. Arms entry was defined as entering an arm with all four paws. Behaviors assessed were time spent (in seconds) in the open arms; number of open and closed arm entries (total exploration) and head dipping frequency in the open arms. The time spent in open arms of maze was used as measure of reduced anxiety level (Hlavacova et al., 2010). Ethological measures included frequency of head dipping (exploratory movement of head/shoulders over sides of the open arms and down towards the floor), which is related to reduced fear (Hlavacova et al., 2010). Total activity on the maze (number of open

and closed arms entries) was used to calculate the anxiety index (Cohen et al., 2007; 2008; Mazor et al., 2007), as follows:

$$\text{Anxiety Index} = 1 - \left[\frac{\left(\frac{\text{Time spent in the open arms}}{\text{Total time on the maze}}\right) + \left(\frac{\text{Number of open arm entries}}{\text{Total entries on the maze}}\right)}{2}\right]$$

Anxiety index values range from 0 to 1 where an increase in the index expresses increased anxiety-like behavior (Cohen et al., 2007; 2008; Mazor et al., 2007). The apparatus was cleaned with alcohol solution 20% using wet sponge and paper towel before the introduction of each animal.

2.3.2 Open Field (OF)

On PND 62, each rat was individually placed for 5 min in the open-field center arena (40x40x30cm) subdivided into nine equal squares, as described in Kerr et al. (2005). The number of crossing (horizontal squares crossed) and rearing (vertical movements) were used as measures of locomotor activity and exploratory behavior, respectively, whereas the numbers of entries in central squares were used as measure of anxiety-like behavior (Henderson, 2004). The apparatus was cleaned with alcohol solution (20%) and paper towel between each animal.

2.3.3 Y-maze task

The Y-maze behavioral paradigm was carried out as described in Chu et al. (2012). Apparatus was made of wood and consisted of three arms 32 cm (long) ×10 cm (wide) with 26-cm walls. Briefly, on PND 64 each rat was placed in the Y-maze center and allowed to explore freely through the maze during a 5-min session for assessment of spontaneous alternating behavior. The sequence and total number of arms entered were video recorded. An entry into an arm was considered valid if all four paws entered the arm. An alternation was defined as three consecutive entries in three different arms (i.e. 1,2,3 or 2,3,1, etc.). The percentage alternation score was calculated using the following formula:

$$\left(\frac{\text{Total alternation number}}{\text{Total number of entries} - 2}\right) \text{x } 100$$

Furthermore, total number of arm entries was used as a measure of general activity in the animals. The maze was wiped clean with 70% ethanol between each animal to minimize odor cues.

2.3.4 Defensive Burying task (DBT)

On PND66 rats were submitted to the DBT task, validated as an appropriate model of animal anxiety, used to assess anxiety-like behavior following a single shock from a novel object, a shock probe. This test measures conditioned responses that require the animal to learn

that a particular stimulus is aversive through experience (Matuszewich et al., 2007). The apparatus was a modified home cage (40 x 30 x 50cm) with 4 cm of wood chip bedding material evenly distributed throughout the cage (Matuszewich et al., 2007). One end of the cage contained a shock probe with a constant current of approximately 1.0 mA (Treit et al., 1981). Each animal was placed individually into the testing apparatus facing away from the shock probe for 10 min (Gutiérrez-García et al., 2006). When an animal received a shock by making contact with the shock probe, the current was terminated so as not to provide additional shocks.

Shock and burying behavior latencies and duration of burying behavior were measured. Burying behavior was defined as any spraying or throwing of the bedding with the head or forepaws towards the shock probe, which is often used as a measure of coping strategy. After 10 min, the animal was removed and returned to his home cage, the apparatus was cleaned and new bedding was placed into the cage for the next rat.

2.4 Biochemical measurements

On PND68, animals were anesthetized with pentobarbital (80mg/kg body weight; ip) and euthanized by exsanguinations. The collected blood was centrifuged at 1300 x g for 15 min, and plasma was used for determination of corticosterone levels. Brains were immediately removed and cut coronally at the caudal border of the olfactory tubercle. Hippocampus of all rats were dissected according to Paxinos and Watson (2007), and used to determination of brain-derived neurotrophic factor (BDNF) and glucocorticoid receptor (GR) immunocontent. Hippocampus were homogenized in 10 volumes (w/v) of 10mM Tris–HCl buffer (pH7.4) for determination of lipid peroxidation (LP) levels and catalase (CAT) activity. All samples were stored at -80ºC freezer until use.

2.4.1 Corticosterone (CORT)

This assay was performed using the Corticosterone EIA Kit according to the manufacturer's instructions (Abcam® Products, San Francisco, CA, USA) by a person blind to experimental procedures. The sensitivity of the CORT assay is 0.3 ng/mL and range variation is 0.391 ng/mL – 100 ng/mL. The CORT results were shown as nanograms per milliliter (ng/mL).

2.4.2 Lipid peroxidation (LP) estimation

LP in brain tissues was evaluated by thiobarbituric acid reactive substances (TBARS) as described by Ohkawa et al. (1979). TBARS assay estimates the LP, which occurs by excessive ROS generation, and was determined through the pink chromogen produced by the

reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) at 100ºC, measured spectrophotometrically at 532 nm. Results were expressed as nmol MDA/g tissue.

2.4.3 Catalase (CAT) activity

CAT activity was spectrophotometrically quantified by the method of Aebi (1984), which involves monitoring the disappearance of H_2O_2 in the presence of cell homogenate (pH 7 at 25°C) at 240 nm for 120 s. The enzymatic activity was expressed in µmol H_2O_2/min/g tissue.

2.5 Western blotting analysis

Brains were quickly removed and unilateral hippocampus were dissected, frozen and stored at -80ºC until use for western blotting analysis. The samples were homogenized in lysis buffer, protein concentration was determined in each sample according to BCA Protein Assay Kit (Pierce, IL, USA), using bovine serum albumin (BSA) as standard. Briefly, equivalent amounts of protein samples were separated by electrophoresis on a 10% polyacrylamide gel and electrotransferred to a PVDF membrane (Millipore, MA, USA). Non-specific binding sites were blocked in Tris-buffered saline (TBS), pH 7.6, containing 5% non-fat dry milk. Membranes were rinsed in buffer (0.05% Tween-20 in TBS) and then incubated with primary antibodies. The primary antibodies were anti-actin (1:2.000), anti-BDNF (1:300), anti-GR (1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA) followed by anti-rabbit or anti-goat IgG horseradish peroxidase conjugate (1:20.000, Santa Cruz Biotechnology Inc., Santa Cruz, CA). After rinsing with buffer, the immunocomplexes were visualized by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech Inc., NJ, USA) according to the manufacturer's instructions. The film signals were digitally scanned and then quantified using ImageJ software. Actin was used as an internal control for western blotting such that data was standardized according to actin values.

2.6 Statistical Analysis

Data were analyzed by one-way ANOVA, followed by Tukey's multiple range test for all comparisons, when appropriate. $P<0.05$ was regarded as statistically significant for all comparisons made. Pearson correlation was applied for the data. Results were expressed as mean ± S.E.M.

3. Results

3.1 Anxiety-like symptoms assessed in elevated plus maze (EPM) (Figure 1):

One-way ANOVA revealed a significant influence of handling on time spent in open arms [F (3,24) =7.68, P=0.0013], number of head dipping [F (3,24) =5.89, P=0.0047], and anxiety index [F (3,24) =7.38, P=0.0016]. Tukey's post hoc test showed increased time spent in open arms in both TS_{8-14} and TS_{15-21}, but this increase was not observed in TS_{1-7}, whose time spent in open arms was comparable to UH group (Fig 1A). Additionally, TS_{8-14} group showed a higher number of head dipping in open arms than both TS_{1-7} and $TS_{15-21,}$ which had similar results when compared to UH group (Fig. 1B). Furthermore, TS_{8-14} group showed reduced anxiety index (AI) in relation to all other groups (UH, TS_{1-7} and TS_{15-21}), which showed similar AI (Fig. 1C).

3.2 Locomotor and exploratory performance assessed in open field (OF) task (Figure 2):

One-way ANOVA revealed a significant influence of handling on crossing, rearing and central squares crossing in OF [F (3,24) =24.39, 20.89, and 19.42; *P*=0.000 for all], respectively. Post hoc test showed that neonatal TS was able to increase the crossing number of both TS_{8-14} and TS_{15-21} in relation to UH group, whose crossing number was significantly lower than TS_{8-14}, and comparable to TS_{1-7} (Fig. 2A). TS also increased rearing number (Fig. 2B) and crossing number in central squares (Fig. 2C) of all handled groups in relation to UH group.

3.3 Working memory measured by spontaneous alternating behavior in Y-maze task (Figure 3):

One-way ANOVA revealed a significant effect of handling on number of total arm entries and alternating behavior [F (3,24) =23.83, *P*=0.000; and 4.04, *P*=0.020, respectively]. Tukey's post hoc test showed that TS increased counts of total arm entries in TS_{8-14} and TS_{15-21} in relation to both UH and TS_{1-7} groups. Rats from TS_{1-7} group did not show increased values on this behavioral parameter when compared to UH group (Fig. 3A). Similarly, tactile stimulation increased alternating behavior only in TS_{8-14} group when compared to all other experimental groups, while UH, TS_{1-7} and TS_{15-21} presented similar alternating behavior (Fig. 3B).

3.4 Anxiety-like symptoms observed in defensive burying test (DBT) (Figure 4):

One-way ANOVA revealed a significant effect of handling on shock latency as well as on latency and duration of burying behavior [F (3,24) =6.57, *P*=0.0028; 16.65, *P*=0.000 and 28.18, *P*=0.000, respectively]. Post hoc test showed an increase in shock latency in both TS_{1-7} and TS_{15-21} when compared to UH and TS_{8-14} groups, which had similar shock latency to each other (Fig. 4A). When compared to UH, neonatal TS decreased burying behavior latency of all

experimental groups, which were similar to each other (Fig. 4B). Burying behavior duration was increased in TS_{1-7} and decreased in TS_{8-14} when compared to UH group, while TS_{15-21} showed no difference from UH (Fig. 4C).

3.5 Biochemical measurements

3.5.1 Corticosterone (CORT) measurement in plasma (Figure 5):

One-way ANOVA revealed a significant effect of handling on plasma levels of CORT [$F(3,24) = 20.27$, $P = 0.000$]. Tukey's test showed a decrease in CORT levels of TS_{8-14} and TS_{15-21} groups, when compared to UH. TS_{1-7} and TS_{15-21} showed similar CORT measures, while TS_{8-14} presented significantly lower CORT levels than both TS_{1-7} and TS_{15-21} (Fig. 5).

3.5.2 Lipid peroxidation (LP) estimation and catalase (CAT) activity in the hippocampus (Figure 6):

One-way ANOVA revealed a significant effect of handling on LP levels in the hippocampus [$F(3,24) = 12.46$, $P = 0.000$]. Neonatal handling also exerted significant influence on CAT activity in the hippocampus [$F(3,24) = 37.67$, $P < 0.000$]. Post hoc test showed that TS_{8-14} presented a decrease in LP (Fig. 6A) and an increase in CAT activity in the hippocampus (Fig.6B) when compared to UH. Also, UH, TS_{1-7} and TS_{15-21} showed similar LP levels and CAT activity.

3.6 Western blot analysis in the hippocampus (Figure 7):

One-way ANOVA revealed a significant effect of handling on brain-derived neurotrophic factor (BDNF) and glucocorticoid receptor (GR) immunocontent [$F(3,24) = 15.65$ and 30.34, $P = 0.000$, respectively]. Hippocampal BDNF imunnocontent was increased only in TS_{8-14}, decreased in TS_{15-21} and not altered in TS_{1-7}, whose value was similar to UH (Fig. 7A). This same brain area showed a decrease in GR immunocontent in TS_{1-7} and TS_{15-21}, while TS_{8-14} showed no difference when compared to UH group (Fig. 7B). Besides, GR immunocontent for TS_{8-14} showed an increased value than those observed in both TS_{1-7} and TS_{15-21}, which also were different from each other.

4. Discussion

Our study has examined the most favorable neonatal period for holding tactile stimulation (TS) in rats, in order to define in which period handling could act to improve cognitive and motor development and reduce anxiety-like symptoms later in life. In general, we found that TS applied between postnatal days 8 and 14 was able to modify anxiety, improving memory parameters. Besides, this handling reduced plasma corticosterone and hippocampal

lipid peroxidation, increasing catalase activity in the same brain area, modifying both BDNF and GR immunocontent in the hippocampus.

Several studies have demonstrated that TS is a model of positive early experiences in developing rats (Horiquini-Barbosa and Lachat, 2016; Antoniazzi et al., 2014a, 2014b; Richards et al., 2012), exerting solid influence on brain development (Chiba et al., 2012). Regarding this, our current findings indicate that animals exposed to TS during the second week of life (PND8-14) showed reduced anxiety-like behavior, as observed by: i) increased time spent in open arms of elevated plus maze (EPM), resulting in a lower anxiety index (AI) in this same paradigm; and ii) reduction of their cumulative burying time in defensive burying test (DBT), which is a type of strategy associated with the active behavior of burying shock probe (Matuszewich et al., 2007). In addition to modifying the classical spatiotemporal measures of anxiety-like symptoms (Hlavacova et al., 2010), TS in different postnatal periods was able to improve ethological parameters related to exploratory behavior, as observed by: i) higher frequency of head dips, in a manner consistent with an anxiolytic outcome in EPM; ii) increased rearing and center crossing number in open field (OF); and iii) increased total entries in arms of Y-maze, confirming the natural tendency of animals in a new environment, which is to explore it, despite stress and conflict (Henderson, 2004). These results are in accordance with previous studies which state that increased locomotor activity in animals may reflect a status of tranquility (Boufleur et al., 2012; Shoji and Mizoguchi, 2010), while decreased center crossing number in OF could be related to increased anxiety-like behavior (File, 1997).

On the other hand, neonatal TS in the first week of pups' life ($TS_{1\text{-}7}$ group) evidenced: i) longer latency to contact shock probe in DBT, an indicator of potential changes in locomotor or exploratory activity (Gutiérrez-García et al., 2006); and ii) longest cumulative burying time in DBT, which is a conditioned response task that requires the animal to learn through experience that a stimulus is aversive (De Boer and Koolhaas, 2003). In this sense, the total amount of time spent in burying is directly related to their anxiety (Boufleur et al., 2013; Matuszewich et al., 2007; De Boer and Koolhaas, 2003), likewise, the time elapsed between the first shock received and the first attempt at burying (latency) inversely indicates rats' reactivity (Gutiérrez-García et al., 2006). Taken together, these parameters reveal a state of anxiety in this experimental group, in accordance with other authors who have shown an increase in cumulative burying after different stressful situations (Gutiérrez-García et al., 2006; Boufleur et al., 2013; Vey et al., 2016).

In a Y-maze task, spontaneous alternation behavior requires attention (Katz and Schmaltz, 1980) and working memory (Sarter et al., 1988), and only TS during the second week of life was able to increase the alternation rate. This alternation ability has been interpreted in

terms of working memory (Beninger et al., 1986), which requires rats to remember their choice and tendency to enter the arm of the maze which was least recently explored (Sarter et al., 1988). In this sense, working memory refers to recalling recent events and is vulnerable to interfering effects (Olton et al., 1980).

Furthermore, an increase in the metabolic rate is expected as a physiological response to different stressful events, by increasing the generation of oxidative products and leading to an imbalance between reactive species (RS) and antioxidant defenses (Zhang et al., 2009). Here, induced lipid peroxidation (LP) as result from RS action, which is damaging to cell membrane phospholipids (Stadtman et al., 1991), was minimized in the hippocampus of animals handled from PND8-14, indicating that TS can prevent oxidative damages when applied in this specific period of pups' lives. On the other hand, the antioxidant defense system, represented here by catalase (CAT) activity, is responsible for removing RS by controlling their deleterious oxidative damage (Gopinath et al., 2004). Our findings showed increased CAT activity in animals from $TS_{8\text{-}14}$ group, what once more reflects the positive influence of TS on the antioxidant defense system especially during this period. In addition, oxidative events in rat brain structures may play a role in the pathogenesis of anxiety and depression (Eren et al., 2007), since Michel et al. (2007) have reported a connection between oxidative markers and depression in humans. Interestingly, here we show that TS was able to attenuate LP by increasing CAT activity in $TS_{8\text{-}14}$ group, what could be closely related to preservation of cysteine terminal groups present in this enzyme. The current findings are in line with previous reports by our group, reinforcing the involvement of oxidative parameters in anxiety-like and stress symptoms (Antoniazzi et al., 2014a, 2014b; Boufleur et al., 2013), what confirms the protective role of TS, suggesting it may be useful to reduce individual risks of developing depression and anxiety-like symptoms. In contrast, a study by Marcolin et al. (2012) has shown that a brief period of neonatal stress may cause imbalance in the antioxidant system putting some brain structures at risk. Lastly, the biochemical results obtained here may be related to decreased symptoms of anxiety and fear, and improved working memory observed in behavioral tasks, reinforcing the importance of previous life events when studying behavioral and psychological disorders.

It is well known that plasma corticosterone (CORT) in rats is a sensitive marker of emotional state (Biggio et al., 2014), in which elevated levels of circulating glucocorticoids may be considered markers of stress (Heiderstadt et al., 2000), while manipulations in early life can affect adrenocortical function in adult rats (Daskalakis et al., 2009). In this context, our findings showed that TS applied from PND8 to 14 and from PND15 to 21 was able to decrease

CORT plasma levels, confirming beneficial influence of this procedure on neuroendocrine function, especially during the second week of pups' lives, when this hormonal decrease was more evident. Taking into account the reduction in CORT levels and the behavioral results obtained in EPM, OF and DBT, we can state that TS is able to reduce emotionality, anxiety and fear-like symptoms during adult life by regulating HPA axis activity. These results are in accordance with a recent study by our laboratory in which TS has shown its beneficial effect by reducing CORT and cortisol, which are regulated in the same way and simultaneously released in animals submitted to a depression-like protocol (Freitas et al., 2015).

Some authors have shown that exposure to elevated CORT levels in the hippocampus may reduce neurotrophic support in this brain area, impairing synaptic function and spatial memory (Wosiski-Kuhn et al., 2014). Specifically, the suppression of brain-derived neurotrophic factor (BDNF) in the hippocampus may occur following chronic stress-induced elevations in CORT levels (Gronli et al., 2006; Wosiski-Kuhn et al., 2014), which are sufficient to evoke cognitive and synaptic deficits. In the current study, we show for the first time that TS acts reducing CORT levels and consequently increasing BDNF when applied from PND8 to 14, what may explain the better performance of this experimental group in behavioral paradigms, once it is well known that BDNF plays an important role in activity-dependent sculpting and refinement of synapses (reviewed in Kuczewski et al., 2009), contributing to functional plasticity (Wosiski-Kuhn et al., 2014). Some authors have shown that repeated neonatal handling increases BDNF levels in the hippocampus of male rats and it is associated with an improvement in spatial memory (Garoflos et al., 2005). On the other hand, Reis et al. (2014) found BDNF increase in pups handled from PND1-7, whose result was attributed to a cumulative effect of increased maternal licking over previous days. However, it is important to consider that neonatal handling procedures are different and tissue collection for their experiment occurred on PND7, while our analysis was performed on PND68, reinforcing that TS benefits can last into adulthood.

Moreover, there is a significant consensus surrounding the negative regulation of BDNF by prolonged activation of glucocorticoid receptors (GR) (Jöhren et al., 2007; Campbell et al., 2010). Excessive amounts of glucocorticoids can be harmful to the organism (Luján et al., 2008), although HPA axis is tightly regulated by negative feedback of mineralocorticoid and glucocorticoid receptors located in different brain regions including the hippocampus (Herman and Cullinan, 1997). By contrast, studies have demonstrated that handled animals have higher levels of GR in the hippocampus and the frontal cortex (O'Donnell et al., 1994; Stamatakis et al., 2008), suggesting that HPA axis glucocorticoid feedback is enhanced and provides better response against stress

(Pham et al.,1997). Here, TS was able to maintain GR immunocontent in those animals handled from PND 8 to 14, while TS during first and third weeks of life (PND1-7 and 15-21, respectively) was harmful in this matter, showing once more that there is an ideal period to perform TS. Furthermore, a recent study has shown that a decrease of GR in male rodents increases depression and anxiety-like behaviors and reduces explorative behaviors (Wisłowska-Stanek et al, 2013), a similar result to the one found in TS group handled from PND1-7. On the contrary, other procedures, i.e. environmental enrichment, enhanced GR immunocontent in the hippocampus of rats, what may be related to decreased plasma levels of CORT, and lead to an attenuated adrenocortical response, providing positive impact on cognitive and anxiety-related behaviors in future situations (Vivinetto et al., 2013; Zhang et al., 2013; Lin et al., 2011; Fernández-Teruel et al., 2002). As described before, TS is a positive approach which has been used in several animal models, however the mechanism through which TS induces GR immunocontent is uncertain. As well as the environmental enrichment procedure, it is likely that TS increases monoaminergic activity, and GR immunocontent consequently modulates both hippocampal function and behavioral responses (Rasmuson et al., 1998; Del Arco et al., 2007). Interestingly, these outcomes would support the hypothesis of a better regulation of anxiety behaviors through high GR immunocontent in the hippocampus as suggested by Sampedro-Piquero et al. (2014). Considering these findings, we may confirm that TS is able to mimic antidepressant and anxiolytic drug effects as recently shown (Freitas et al., 2015; Boufleur et al., 2012).

Regarding the results obtained in this study, literature data suggest that the second week of life is a critical period of neuroplasticity and rats handled from PND 3 to 14 maintain the effects beyond puberty (Avishai-Eliner et al., 2001; Fenoglio et al., 2006). During these first two weeks of life, referred to as stress hyporesponsive period (SHRP), CORT concentrations remain at low levels (Levine, 2001) as well as the concentrations of pituitary adrecorticotropin (ACTH) and hypothalamic corticotropin-releasing (CRH) hormones; and stressors that would cause CORT elevations in adult rats only elicit a minimal adrenal response during SHRP (Walker et al., 2001). Furthermore, these animals handled during SHRP show decreased anxiety and minor HPA responses in adulthood (Avishai-Eliner et al., 2001; Fenoglio et al., 2006). This is in line with our findings and may be a hypothesis for the results obtained, since behavioral, biochemical and molecular improvements were found in animals handled from PND8 to 14.

The beneficial influence of TS on anxiety parameters, as observed here, is well known since previous studies performed by our group evidenced similar behaviors in rats exposed to TS (Freitas et al., 2015; Antoniazzi et al., 2014a, 2014b; Boufleur et al., 2013), reinforcing reduced emotionality of adult animals and anxiolytic properties of this procedure, also improving their

ability to cope with stressful situations. Besides, we can hypothesize that TS is able to improve working memory when applied in a specific period of development, since alternation behavior may reflect a primitive working memory ability, suggesting that TS could have some interaction with a cholinergic system involved in memory processes. A recent study has shown that TS is able to modify hippocampal neurogenesis, spatial learning and memory in prenatally stressed rats (de Los Angeles et al., 2016). The main explanation is related to the production of trophic factors such as nerve growth factor, a key to hippocampal cognitive processes, induced by TS (Pham et al., 1997), thus favoring hippocampal synaptic plasticity (Roggeri et al., 2008). Moreover, the better performance of rats exposed to TS has been attributed to an increase in arborization and dendrite length caused by this procedure (Richards et al., 2012), and these structural changes have been associated with increased synaptic protein expression, which is involved in the generation of new dendritic synapses and spines (Kozorovitskiy et al., 2005).

5. Conclusion

In conclusion, we have demonstrated for the first time (to the best of our knowledge) that neonatal tactile stimulation influences adult rats differently, considering the period when it is applied. This study shows that TS applied during the second week of rats' lives is able to reduce emotionality, fear and anxiety-like behaviors, by modulating HPA axis response. TS from PND8 to 14 prevents induced oxidative damage and reflects a better antioxidant defense mechanism confirming its protective role, also inducing neuroplasticity effects on BDNF immucontent and regulating GR immunocontent in the hippocampus. These results suggest that TS is able to exert beneficial influences on neural development during the neonatal period, especially when applied during the intermediate phase of neonatal period, and can also minimize or prevent the development of neuropsychiatric disorders throughout adult life.

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Disclosure

The authors declare no conflict of interest. None of the authors have received compensation for professional services in any of the previous years, or anticipate receiving such compensation in the near future.

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Figures:

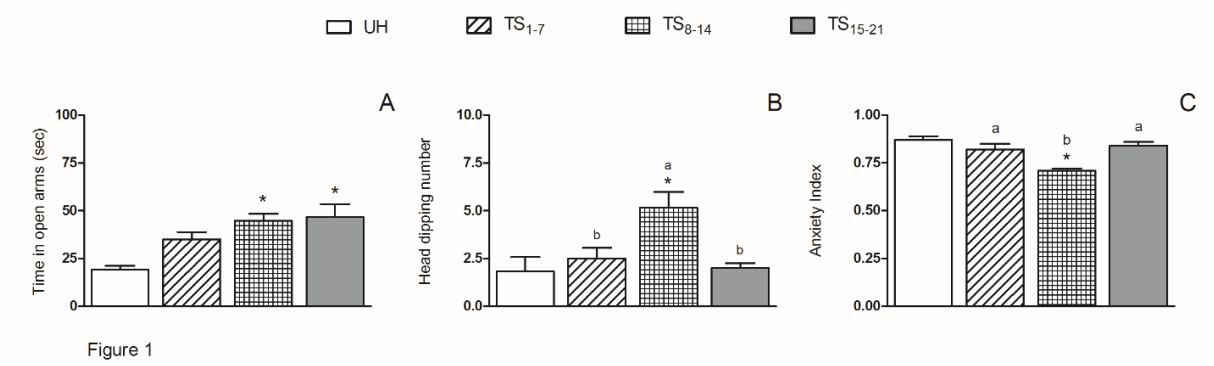

Figure 1 – Influence of tactile stimulation on anxiety-like symptoms evaluated in elevated plus maze (EPM) on PND60. Anxiety-like symptoms were evaluated by time in open arms (A), head dipping number (B) and anxiety index (C). Data are expressed as mean±S.E.M (n=7). *indicates significant difference from UH group to different periods of TS (P<0.05). Different lowercase letters indicate significant difference (P<0.05) among different periods of TS (TS_{1-7}, TS_{8-14}, and TS_{15-21}). Abbreviations: UH: unhandled; TS: tactile stimulation.

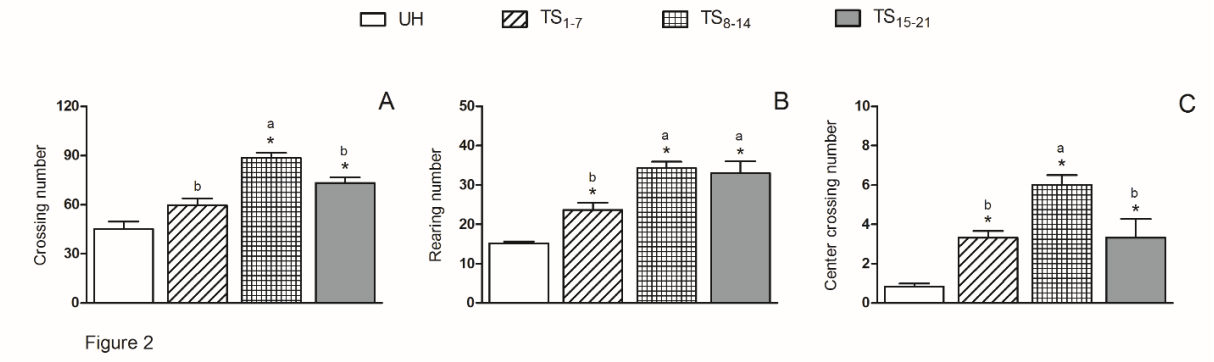

Figure 2 – Influence of tactile stimulation on locomotor and exploratory performance evaluated in open field (OF) on PND62. Locomotor and exploratory activity were evaluated by crossing (A), rearing (B), and center crossing (C) numbers. Data are expressed as mean±S.E.M (n=7). *indicates significant difference from UH group to different periods of TS (P<0.05). Different lowercase letters indicate significant difference (P<0.05) among different periods of TS (TS_{1-7}, TS_{8-14}, and TS_{15-21}). Abbreviations: UH: unhandled; TS: tactile stimulation.

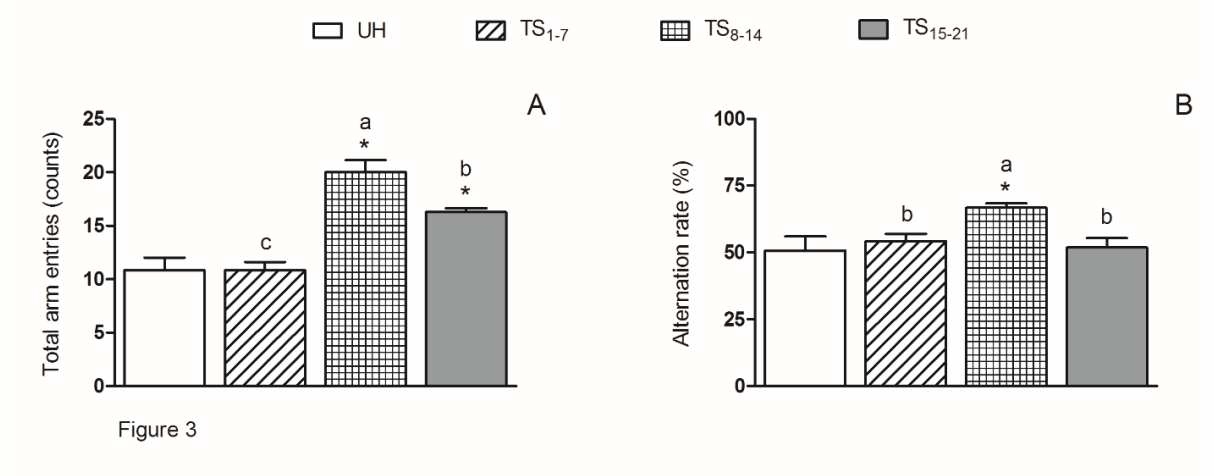

Figure 3 – Influence of tactile stimulation on working memory measured by spontaneous alternating behavior in Y-maze task on PND64. Working memory was evaluated by total arm entries (A) and alternation rate (B). Data are expressed as mean±S.E.M (n=7). *indicates significant difference from UH group to different periods of TS (P<0.05). Different lowercase letters indicate significant difference (P<0.05) among different periods of TS (TS_{1-7}, TS_{8-14}, and TS_{15-21}). Abbreviations: UH: unhandled; TS: tactile stimulation.

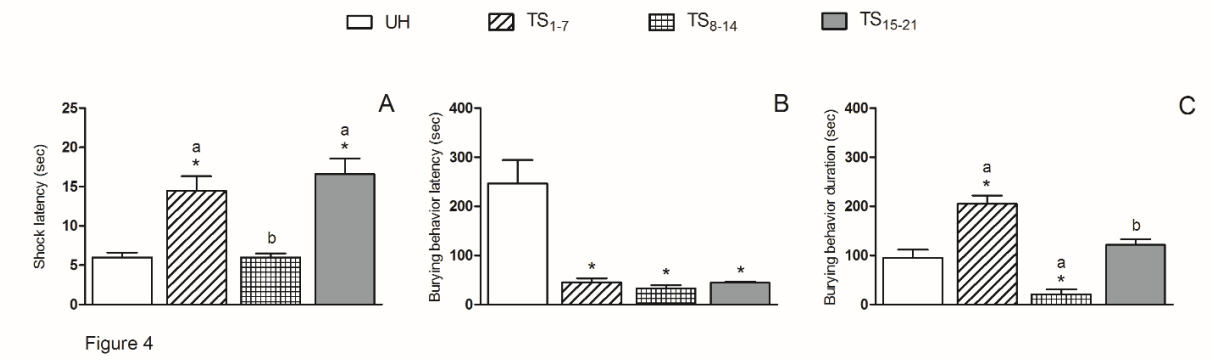

Figure 4 – Influence of tactile stimulation on anxiety-like behavior following a single shock observed in defensive burying task (DBT) on PND66. Anxiety-like behavior was evaluated by shock latency (A), burying behavior latency (B) and duration (C). Data are expressed as mean±S.E.M (n=7). *indicates significant difference from UH group to different periods of TS (P<0.05). Different lowercase letters indicate significant difference (P<0.05) between different periods of TS (TS_{1-7}, TS_{8-14}, and TS_{15-21}). Abbreviations: UH: unhandled; TS: tactile stimulation.

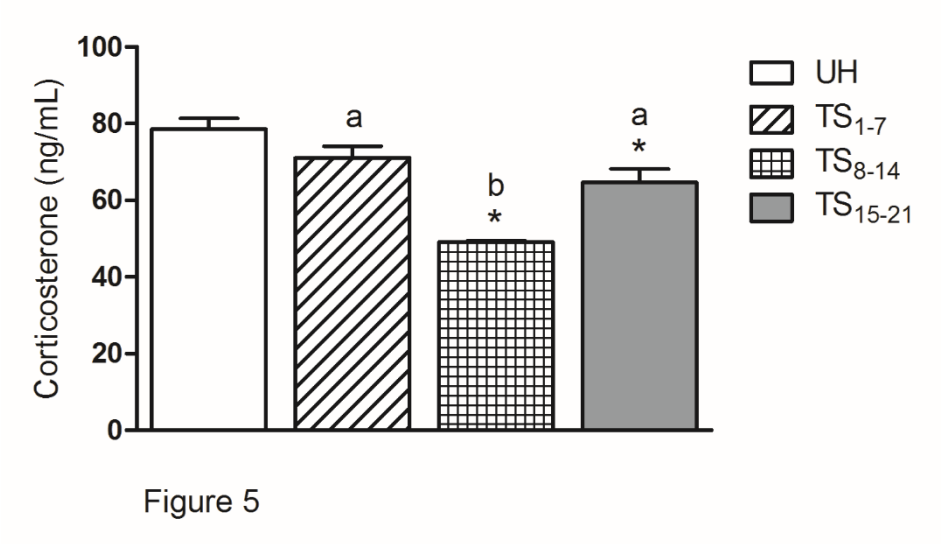

Figure 5 - Influence of tactile stimulation on corticosterone (CORT) levels in plasma. Data are expressed as mean±S.E.M (n=7). *indicates significant difference from UH group to different periods of TS (P<0.05). Different lowercase letters indicate significant difference (P<0.05) between different periods of TS (TS_{1-7}, TS_{8-14}, and TS_{15-21}). Abbreviations: UH: unhandled; TS: tactile stimulation.

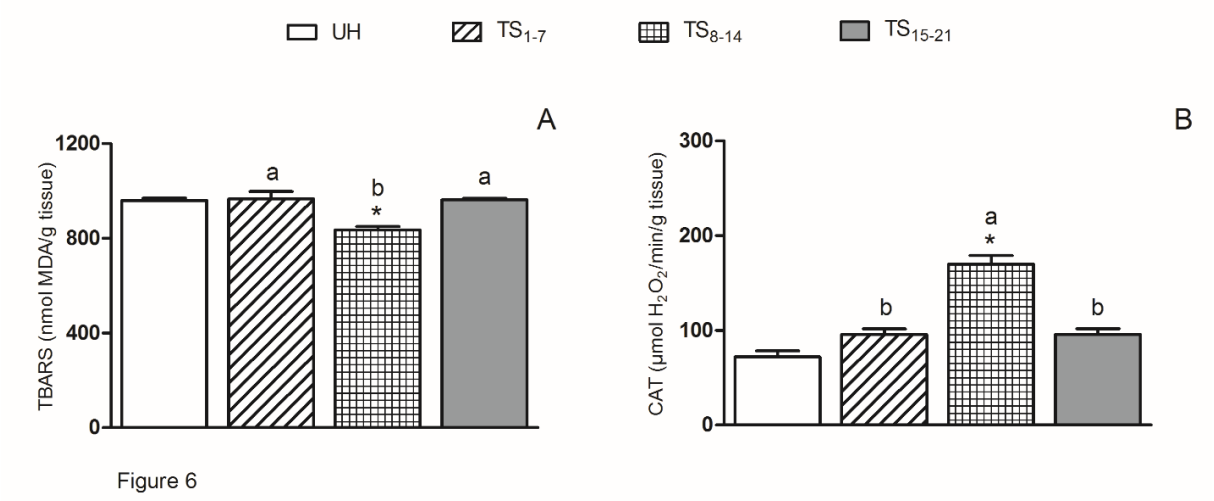

Figure 6 - Influence of tactile stimulation on lipid peroxidation (TBARS) estimation (A) and catalase (CAT) activity (B) in hippocampus. Data are expressed as mean±S.E.M (n=7). *indicates significant difference from UH group to different periods of TS (P<0.05). Different lowercase letters indicate significant difference (P<0.05) between different periods of TS (TS_{1-7}, TS_{8-14}, and TS_{15-21}). Abbreviations: UH: unhandled; TS: tactile stimulation.

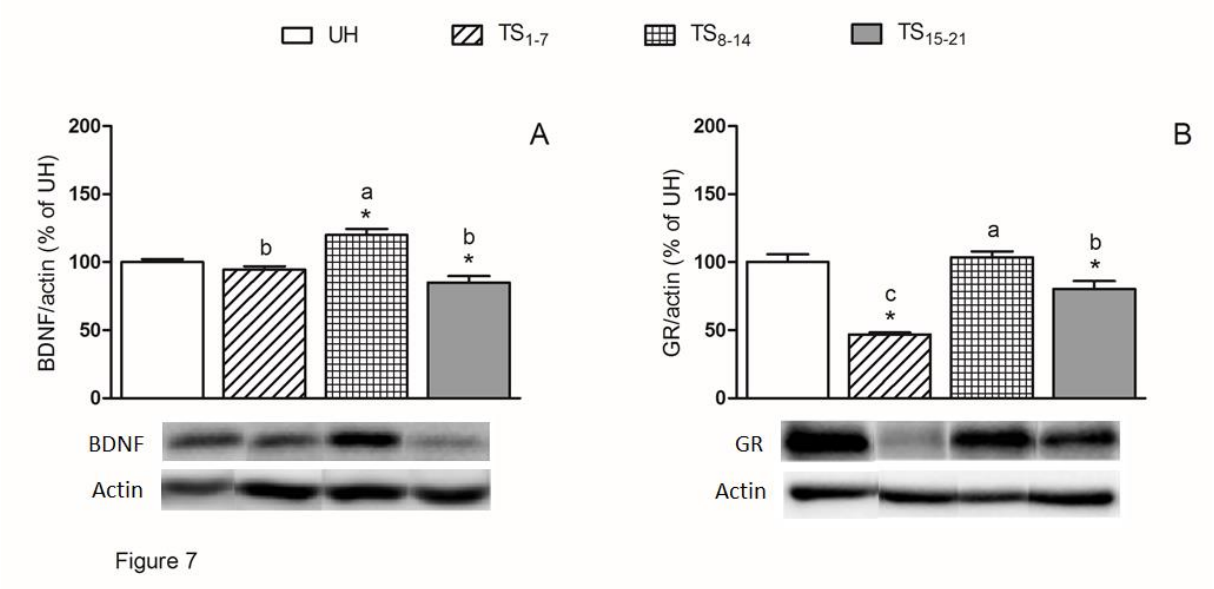

Figure 7

Figure 7 - Influence of tactile stimulation on brain-derived neurotrophic factor (BDNF) immunocontent (A) and glucocorticoid receptor (GR) immunocontent (B) in hippocampus. Data are expressed as mean±S.E.M (n=7). *indicates significant difference from UH group to different periods of TS (P<0.05). Different lowercase letters indicate significant difference (P<0.05) between different periods of TS (TS_{1-7}, TS_{8-14}, and TS_{15-21}). Abbreviations: UH: unhandled; TS: tactile stimulation.

4.3 MANUSCRITO 2 – Este manuscrito encontra-se em fase de redação, e por isso será apresentado na forma de resultados parciais, composto de materiais e métodos, e resultados.

Tactile stimulation reduces cocaine-induced depressive-like behavior during withdrawal period by altering monoaminergic system in rats

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Abstract

1. Introduction

2. Methods

2.1 Animals and experimental procedure (Figure 1)

Seven pregnant female *Wistar* rats from the breeding facility of Universidade Federal de Santa Maria (UFSM), RS, Brazil, were housed in plexiglas cages with free access to food and water. The animals were kept on a 12-hour light/dark cycle with lights on at 7:00 a.m., in a room with controlled temperature (22-23°C). The pups date of birth (postnatal day 0 - PND0) was monitored, and sexes distinguished by larger genital papilla and longer anogenital distance in male vs. female pups (Liu et al., 2008). Male pups were taken from several litters, to avoid the use of littermates in the same experimental group, and at postnatal day one (PND1) they were randomly assigned to experimental groups (n=14, each group) known as unhandled (UH) or tactile stimulation (TS). TS group received stimulus from PND8 to 14 out of the nest for 10 minutes per day, staying the remaining time with their mothers until weaning.

On PND22, litters were weaned and groups of four male pups, were housed and left undisturbed up to PND33, when animals were habituated to receive 1% (w/v) sucrose/water solution and tap water in their home cage until PND39. On PND40 half of animals (n=7) of each group (UH and TS) were treated following a binge pattern administration of cocaine, adapted from Alves et al. (2014), from PND40 to PND53.

This administration period was selected to match the onset of mid-adolescence and extend into the late adolescence period, during which intense reorganization of the mesocorticolimbic dopamine system is occurring (Andersen, 2003; Varlinskaya and Spear, 2008). These animals received three daily administrations of 15mg/kg of cocaine, administrated in a volume of 1mL/kg and injected intraperitoneally every hour between 9:00 and 11:00 a.m. This dosing schedule was selected to mimic a frequent pattern of cocaine self-administration in humans (Quinones-Jenab et al., 2000). Moreover, the daily dose of 3x15 mg/kg of cocaine was previously demonstrated to induce neurobiological and behavioral alterations in rats (Sarnyai et al., 1998; Schlussman et al., 2002; Tsukada et al., 1996; Zhou et al., 2005). Control animals received equal doses of NaCl vehicle (0.9% w/v) following the same protocol of administration. Cocaine hydrochloride was supplied by Merck (Germany). The assessment of cocaine effects was performed 7 days after withdrawal, a time-point that matches the beginning of adulthood (Andersen, 2003). Furthermore, persistent effects of exposure to drugs of abuse are often reported after a 10-day withdrawal period (Kimmel et al., 2003; Sarnyai et al., 1998; Zhou et al., 1996).

Thus, on PND60, rats were submitted to sucrose preference (SP) test, which evaluates their anhedonic state, followed by 15 min of training session on forced swimming test (FST). This procedure works as a stressor which is thought to induce a state of behavioral despair (Castagne et al., 2009; Porsolt et al., 1978) related to a "depressive state" (Borsini and Meli, 1988). At PND61 all animals received a single sub-therapeutic dose of sertraline (SERT, 0.3mg/kg body weight i.p.; Freitas et al., 2015). SERT (Pharmanostra, India) was dissolved in NaCl 0.9% plus 0.05 mL Tween 80 (Sigma–Aldrich, Brazil) and was the antidepressant drug chosen because it has been frequently reported in studies involving animal models (Kaygisiz et al., 2014; Mikail et al., 2012; Ulloa et al., 2010). Thirty minutes following the injections, animals were submitted to FST and one hour after, anxiety-like symptoms were observed in elevated plus maze (EPM). Following behavioral analysis, on PND62 all animals were weighed, anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and blood was collected (by cardiac puncture in heparinized tubes). Brain areas were dissected for oxidative status estimation and molecular analysis.

All procedures were carried out in accordance with Brazilian law nº. 11.794/2008, which is in agreement with the Policies on the Use of Animals and Humans in Neuroscience Research and with the Institutional and National Regulations for Animal Research (process 104/2010).

2.2 Neonatal handling

Tactile stimulation (TS) was applied daily between 12:00 and 14:00 p.m. from PND8 to PND14 (Freitas et al., 2015), and consisted of gently stimulating the pups' dorsal surface individually, by the same experimenter, with the index finger from rostral to caudal direction for 10 min daily (Antoniazzi et al., 2014; Boufleur et al., 2013). At the end of procedures, pups were returned to their litters. The UH group remained in their nest and were handled during the home cage regular cleaning, two times a week.

2.3 Behavioral evaluations

2.3.1 Sucrose preference (SP) test

From PND33 to PND39, rats were allowed to consume 1% (w/v) sucrose/water solution or tap water in their home cage. The position of the bottles was switched twice a day. Seven days after finishing the cocaine administration protocol (PND60), animals were individually tested on a sucrose preference (SP) test (Kompagne et al. 2008) at the start of the dark cycle (19:00 pm). They were deprived of food and water for 5 h and then presented with two bottles containing either tap water or sweet solution. One-hour intake was measured by weighing bottles before and after the test. SP was calculated according to the following equation:

$$\text{SP} = \left(\frac{\text{Sucrose intake}}{\text{Sucrose intake} + \text{Water intake}}\right) \text{x } 100$$

2.3.2 Forced swimming test (FST)

Behavioral responses related to depression-like symptoms are experimentally assessed in FST, which method has been described in several studies (Porsolt et al., 1978; Wieland and Lucki, 1990; Castagne et al., 2009). On the first day (PND60) rats were forced to swim for a 15-min period (training session), and carefully dried before returning to their home cages. Twenty-four hours following the test (PND61), all rats were injected with SERT, as described above, and submitted to FST, for 5 min. Immobility, climbing and swimming times were quantified by trained raters blinded to handling and treatment. Immobility is considered as no additional activity other than the required to keep the head above water, whereas climbing is defined as upward struggling movements of the forepaws at the side of the cylinder. Movements around the swimming cylinder (Porsolt et al., 1978) are indicative of swimming time.

2.3.3 Elevated plus maze (EPM)

One hour after FST on PND61, animals were observed in EPM, which is based on the innate fear rodents have for open and elevated spaces (Montgomery, 1955). The apparatus was made of wood and consisted of a plus-shaped platform elevated 50 cm from the floor. Two opposite arms (50 cm×10 cm) were enclosed by 40 cm high walls whereas the other two arms had no walls. The four arms had at their intersection a central platform (10 cm×10 cm), which gave access to any of the four arms. At the beginning of each test, rats were placed on the central platform facing an open arm and allowed to explore the maze for 5 min. Arm entry was defined as entering an arm with all four paws. Behaviors assessed were time spent (in seconds) in open arms; number of open and closed arm entries (total exploration on the maze); and head dipping frequency in open arms. Time spent in open and closed arms of the maze were used as measures of reduced and increased anxiety level, respectively (Hlavacova et al., 2010). Ethological measures included frequency of head dipping (exploratory movement of head/shoulders over sides of the open arms and down towards the floor), which is related to reduced fear, in a manner consistent with an anxiolytic outcome (Hlavacova et al., 2010). Total activity on the maze (total arm entries) and number of open and closed arms entries was used as anxiety index (Cohen et al., 2007; 2008; Mazor et al., 2007). Anxiety index was calculated as follows:

$$\text{Anxiety index} = 1 - \left[\frac{\left(\frac{\text{Time spent in the open arms}}{\text{Total time on the maze}}\right) + \left(\frac{\text{Number of open arm entries}}{\text{Total entries on the maze}}\right)}{2}\right]$$

Anxiety index values range from 0 to 1 where an increase in the index expresses increased anxiety-like behavior (Cohen et al., 2007; 2008; Mazor et al., 2007). The apparatus was cleaned with alcohol solution 20% using wet sponge and paper towel before the introduction of each animal.

2.4 Biochemical measurements

On PND62, animals were anesthetized with pentobarbital (80mg/kg body weight; ip) and euthanized by exsanguinations. The collected blood was centrifuged at 1300 x g for 15 min, and plasma was used for lipid peroxidation (LP) and non proteic thiol (NPSH) determination. Adrenal glands were removed and weighed. Brains were immediately removed and cut coronally at the caudal border of the olfactory tubercle. Striatum and hippocampus of all rats were dissected according to Paxinos and Watson (2007), and used to determination of dopamine receptor (D2) and glucocorticoid receptor (GR) immunocontent. Prefrontal cortex, hippocampus and striatum were homogenized in 10 volumes (w/v) of 10mM Tris–HCl buffer (pH7.4) for determination of lipid peroxidation (TBARS) levels. All samples were stored in eppendorfs at -80ºC freezer until use.

2.4.1 Non proteic thiol (NPSH) content

NPSH content in plasma was determined after reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). The yellow color developed was read at 412nm, in accordance with Boyne and Ellman (1972) after modifications (Jacques-Silva et al., 2001). A standard curve using glutathione was constructed in order to calculate the NPSH content, expressed as nmol NPSH/mL plasma.

2.4.2 Lipid peroxidation (LP) estimation

LP in brain tissues was evaluated by thiobarbituric acid reactive substances (TBARS) as described by Ohkawa et al. (1979). TBARS assay estimates the LP, which occurs by excessive ROS generation, and was determined through the pink chromogen produced by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) at 100ºC, measured spectrophotometrically at 532 nm. In plasma, TBARS was estimated by the method described by Lapenna et al. (2001). Results were expressed as nmol MDA/mL plasma and nmol MDA/g tissue, for brain areas.

2.5 Western blot analysis

Brains were quickly removed and unilateral striatum and hippocampus were dissected, frozen and stored at -80ºC until use for western blot analysis. The samples were homogenized in lysis buffer, protein concentration was determined in each sample according to BCA Protein Assay Kit (Pierce, IL, USA), using bovine serum albumin (BSA) as standard. Briefly, equivalent

amounts of protein samples were separated by electrophoresis on a 10% polyacrylamide gel and electrotransferred to a PVDF membrane (Millipore, MA, USA). Non-specific binding sites were blocked in Tris-buffered saline (TBS), pH 7.6, containing 5% non-fat dry milk. Membranes were rinsed in buffer (0.05% Tween-20 in TBS) and then incubated with primary antibodies. The primary antibodies were anti-actin (1:2.000), anti-D2 (1:500), anti-GR (1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA) followed by anti-rabbit IgG horseradish peroxidase conjugate (1:40.000, Santa Cruz Biotechnology Inc., Santa Cruz, CA). After rinsing with buffer, the immunocomplexes were visualized by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech Inc., NJ, USA) according to the manufacturer's instructions. The film signals were digitally scanned and then quantified using ImageJ software. Actin was used as an internal control for western blot such that data was standardized according to actin values.

2.6 Statistical Analysis

Data were analyzed by two-way ANOVA, followed by Newman-Keuls multiple range test for all comparisons, using Statistica 8.0 software. $P<0.05$ was regarded as statistically significant for all comparisons made and results were expressed as mean ± S.E.M.

3. Results

3.1 Sucrose preference (SP)

Two-way ANOVA revealed a significant influence of tactile stimulation (TS) [$F(1,24)=39.26$, $P<0.001$] and TS x treatment interaction [$F(1,24)=6.84$, $P<0.05$] on sucrose preference (SP). TS *per se* increased sucrose intake in vehicle-injected rats when compared to unhandled (UH) group, in the same treatment. Cocaine administration reduced SP of UH animals, but did not alter preference of TS cocaine-injected group, when compared to their respective vehicle-injected groups (Fig. 2).

3.2 Forced swimming test (FST)

Two-way ANOVA revealed a significant influence of TS, treatment and TS x treatment interaction on climbing time [$F(1,24)=13.58$, $P<0.001$; $F(1,24)=24.64$, $P<0.001$ and $F(1,24)=3.98$, $P<0.05$, respectively] and swimming time [$F(1,24)=22.07$, $P<0.001$; $F(1,24)=24.75$, $P<0.001$ and $F(1,24)=9.92$, $P<0.05$, respectively], as well as a significant influence of TS and treatment on immobility time [$F(1,24)=60.90$, $P<0.001$ and $F(1,24)=19.05$, $P<0.001$, respectively]. Post hoc test showed that TS *per se* increased climbing (Fig. 3A) and reduced immobility times (Fig. 3C) when compared to UH group, while showed no difference

in swimming time (Fig. 3B). Cocaine administration increased climbing and reduced swimming times in UH group, but did not alter these behavioral parameters in TS group, when compared to their respective vehicle-injected groups (Fig. 3A; 3B). Also, UH and TS cocaine-injected rats reduced immobility time, compared to vehicle-injected groups (Fig. 3C).

3.3 Elevated plus maze (EPM)

Two-way ANOVA revealed a significant main effect of TS on time spent in open arms [$F(1,24)=22.66$, $P<0.001$], open arm entries [$F(1,24)=12.24$, $P<0.001$], head dipping frequency [$F(1,24)=13.04$, $P<0.001$], and anxiety index (AI) [$F(1,24)=13.63$, $P<0.001$]. Newman Keuls' test showed that TS *per se* increased time spent (Fig. 4A) and open arm entries (Fig. 4B), as well as the head dipping frequency (Fig. 4C), and decreased AI (Fig. 4D) of vehicle-injected rats compared to UH group, in the same treatment. Cocaine administration increased time spent in open arms of UH animals, which was not altered in TS group (Fig. 4A) when compared to their respective vehicle-injected groups. Also, cocaine did not modify the open arm entries in cocaine-injected animals from both UH and TS groups (Fig. 4B), and was able to reduce head dipping frequency of TS animals, compared to its vehicle-injected group (Fig. 4C). Regarding AI, cocaine administration reduced this index only in TS group, when compared to UH, in the same treatment (Fig. 4D).

3.4 Adrenal gland weight, body weight and adrenal/body weight ratio

Two-way ANOVA revealed a significant influence of TS on adrenal gland weight [$F(1,24)=4.95$, $P<0.05$], and a significant main effect on TS, treatment and TS x treatment interaction on body weight [$F(1,24)=66.79$; $F(1,24)=41.44$, and $F(1,24)=12.24$, respectively, $P<0.001$ for all] and adrenal/body weight ratio [$F(1,24)=72.11$, $P<0.001$; $F(1,24)=37.51$, $P<0.001$ and $F(1,24)=8.54$, $P<0.05$, respectively]. Post hoc test showed no difference on adrenal weight among experimental groups (Table 1), and no difference on body weight of rats from UH groups. However, TS *per se* increased rats' body weight, and cocaine administration decreased this parameter in the same handling. Moreover, TS *per se* was able to decrease the adrenal/body weight ratio, while cocaine administration increased this ratio in both, UH and TS groups, when compared to their respective vehicle-injected groups. Also, TS reduced this adrenal/body weight ratio in cocaine-injected rats when related to UH cocaine-injected group (Table 1).

3.5 Non proteic thiol (NPSH) content and lipid peroxidation (LP) levels evaluated in plasma

Two-way ANOVA revealed a significant main effect of TS and treatment on plasma NPSH content [$F(1,24)=66.33$, $P<0.001$ and $F(1,24)=7.54$, $P<0.05$, respectively], and on

plasma LP levels [F(1,24)=222.39, P<0.001 and F(1,24)=60.06, P<0.001, respectively]. Post hoc test showed that TS *per se* increased NPSH content and decreased LP level in relation to UH vehicle-treated group (Fig. 5). Cocaine was able to increase NPSH in TS group when compared to UH, but TS and UH cocaine-treated groups were not different from their respective vehicle-treated groups (Fig. 5A) Moreover, cocaine decreased LP in UH and TS groups, compared to their respective vehicle-treated groups, and also TS cocaine-treated showed reduced LP levels in relation to UH in same drug treatment (Fig. 5B).

3.6 Lipid peroxidation (LP) levels in prefrontal cortex (PFC), hippocampus and striatum

Two-way ANOVA revealed a significant main effect of TS, treatment and TS x treatment interaction on LP levels in prefrontal cortex [F(1,24)=47.60, P<0.001; F(1,24)=5.80, P<0.05 and F(1,24)=4.55, P<0.05, respectively], and a significant influence of TS and treatment on LP levels in hippocampus [F(1,24)=21.77, P<0.001 and F(1,24)=5.79, P<0.05, respectively] and striatum [F(1,24)=26.83, P<0.001 and F(1,24)=9.36, P<0.05, respectively]. Newman-Keuls' test showed that TS *per se* decreased LP levels in PFC, hippocampus and striatum, when compared to UH group. Besides, TS cocaine-injected animals showed reduced LP levels in all evaluated brain areas, compared to UH cocaine-injected group. Moreover, UH cocaine-injected group showed reduced LP levels when compared to UH vehicle-injected group, in PFC (Fig. 6A), and TS cocaine-injected also reduced LP levels compared to TS vehicle-injected rats, in hippocampus (Fig. 6B) and striatum (Fig. 6C).

3.7 Dopamine receptor (D2) and glucocorticoid receptor (GR) immunocontent in striatum and hippocampus

Two-way ANOVA revealed a significant main effect of TS, treatment and TS x treatment interaction on D2 immunocontent in striatum [F(1,24)=73.66, P<0.001; F(1,24)=11.33, P<0.05 and F(1,24)=12.35, P<0.001, respectively], and a significant influence of TS and TS x treatment interaction on GR immunocontent, in the same brain area [F(1,24)=4.15, P<0.05 and F(1,24)=31.58, P<0.001, respectively]. In hippocampus, Two-way ANOVA revealed a significant main effect of treatment on D2 immunocontent [F(1,24)=7.87, P<0.05], and a significant influence of treatment, and TS x treatment interaction on GR immunocontent [F(1,24)=81.54, P<0.001 and F(1,24)=99.37, P<0.001, respectively]. On striatum, post hoc test showed that TS *per se* decreased D2 and increased GR immunocontent, in relation to UH group. Cocaine administration was able to increase D2 in UH group when compared to TS in the same treatment and compared to its vehicle-treated groups, while TS

cocaine-treated group showed no difference from its vehicle (Fig. 7A). Also, cocaine administration increased GR immunocontent in UH group and decreased this parameter in TS group, in relation to their respective vehicle-treated groups (Fig. 7C). On hippocampus, D2 immunocontent in vehicle-treated UH and TS showed no difference to each other, while cocaine administration increased this molecular marker only in UH group (Fig. 7B). Regarding GR, TS *per se* increased this parameter when compared to UH group. Cocaine administration increased GR in UH group but did not alter this marker in TS group, when compared to their respective UH and TS vehicle-treated groups. Besides, TS cocaine-treated group showed decreased GR immunocontent when compared to UH cocaine-treated (Fig. 7D).

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Figures:

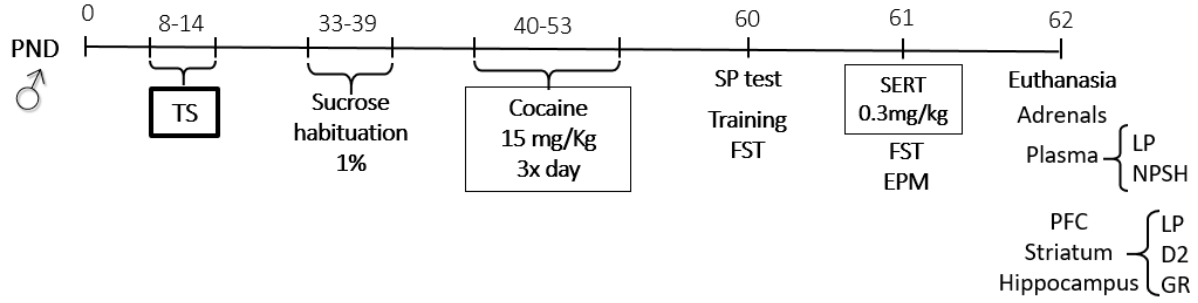

Figure 1 – Experimental design.

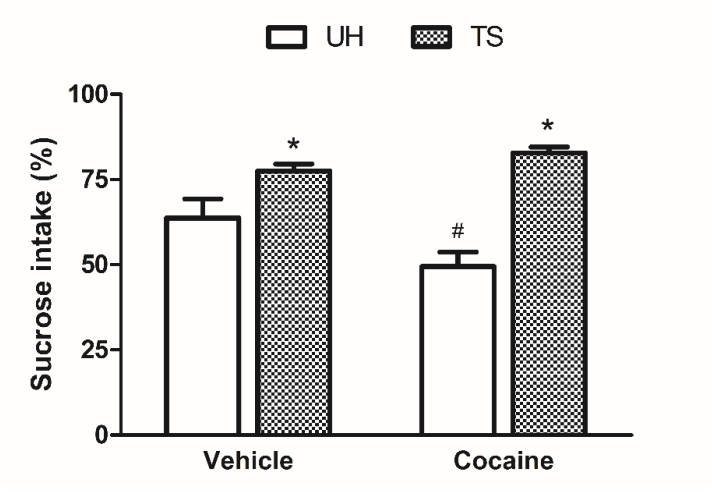

Figure 2 – Influence of tactile stimulation on anhedonia assessed in sucrose preference test during cocaine withdrawal in rats. Data are expressed as mean ± S.E.M. (n=7). *indicates significant difference (P<0.05) between UH and TS, in the same treatment (vehicle- or cocaine-injection). #indicates significant difference between vehicle and cocaine, in the same handling. Abbreviations: UH: unhandled; TS: tactile stimulation.

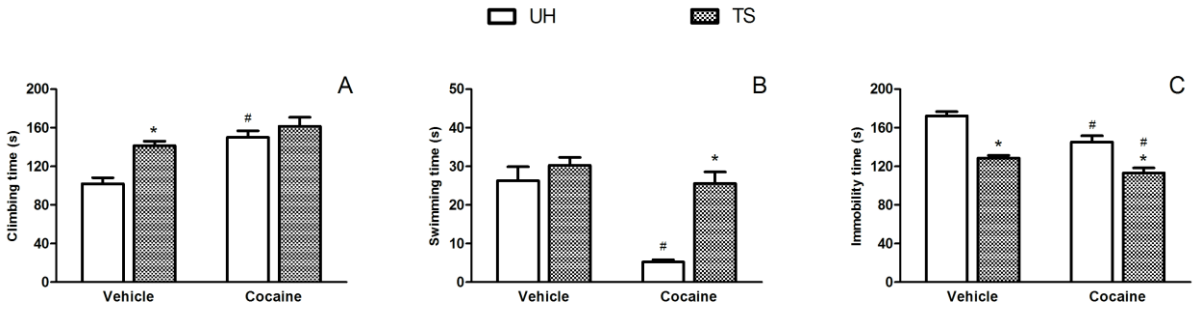

Figure 3 – Influence of tactile stimulation on depressive-like behavior assessed in forced swimming test during cocaine withdrawal in rats. Depressive-like behavior were evaluated by climbing time (A), swimming time (B), and immobility time (C). Data are expressed as mean ± S.E.M. (n=7). *indicates significant difference (P<0.05) between UH and TS, in the same treatment (vehicle- or cocaine-injection). #indicates significant difference between vehicle and cocaine, in the same handling. Abbreviations: UH: unhandled; TS: tactile stimulation.

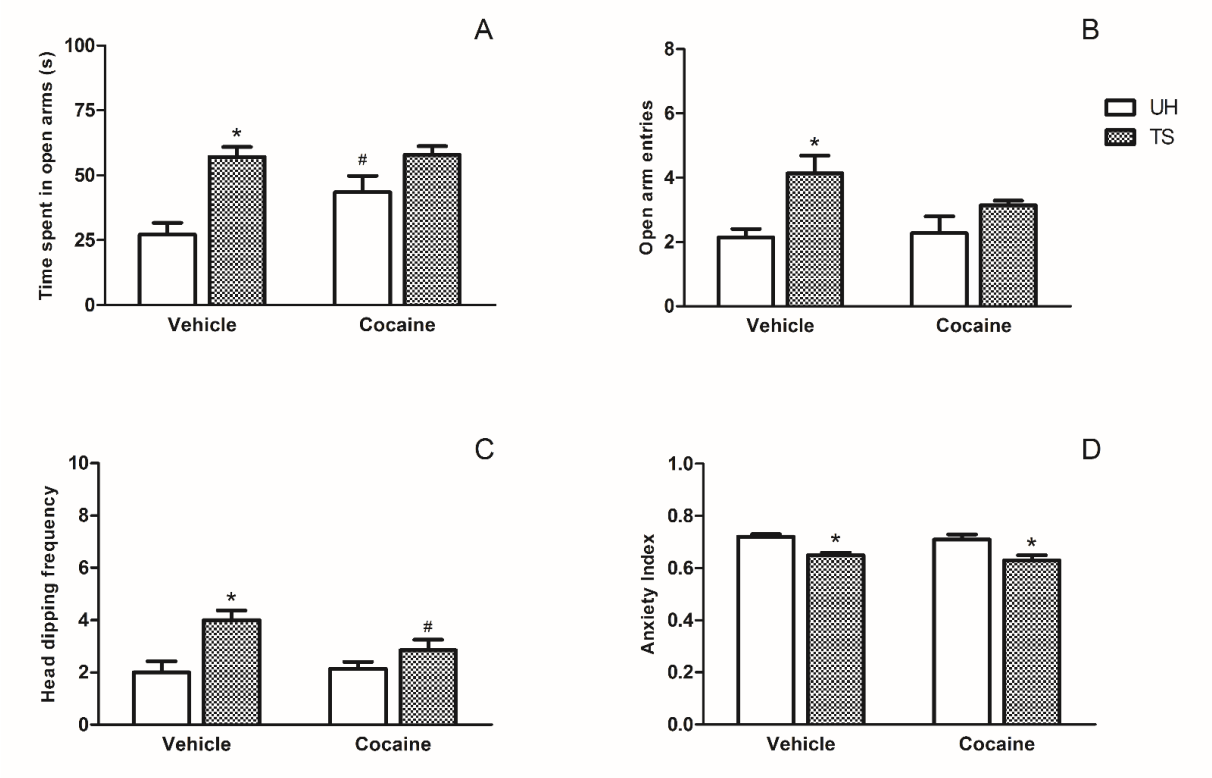

Figure 4 – Influence of tactile stimulation on anxiety-like behavior assessed in elevated plus maze during cocaine withdrawal in rats. Anxiety-like symptoms were evaluated by time spent in open arms (A), open arm entries (B), head dipping frequency (C), and anxiety index (D). Data are expressed as mean ± S.E.M. (n=7). *indicates significant difference (P<0.05) between UH and TS, in the same treatment (vehicle- or cocaine-injection). #indicates significant difference between vehicle and cocaine, in the same handling. Abbreviations: UH: unhandled; TS: tactile stimulation.

Table 1 – Influence of tactile stimulation on adrenal/body weight ratio obtained by adrenal weight and body weight during cocaine withdrawal in rats. Data are expressed as mean ± S.E.M.

	UH+Vehicle	**TS+Vehicle**	**UH+Cocaine**	**TS+Cocaine**
Adrenal weight (g)	0.074±0.001	0.068±0.001	0.072±0.001	0.071±0.002
Body weight (g)	271.85±5.43	331.42±7.13*	256.85±2.09	280.71±4.41#
Adrenal/Body weight ($x10^{-4}$g)	2.72±0.05	2.14±0.06*	2.88±0.03#	2.60±0.06*,#

*indicates significant difference (P<0.05) between UH and TS, in the same treatment (vehicle- or cocaine-injection). #indicates significant difference between vehicle and cocaine, in the same handling. Abbreviations: UH: unhandled; TS: tactile stimulation.

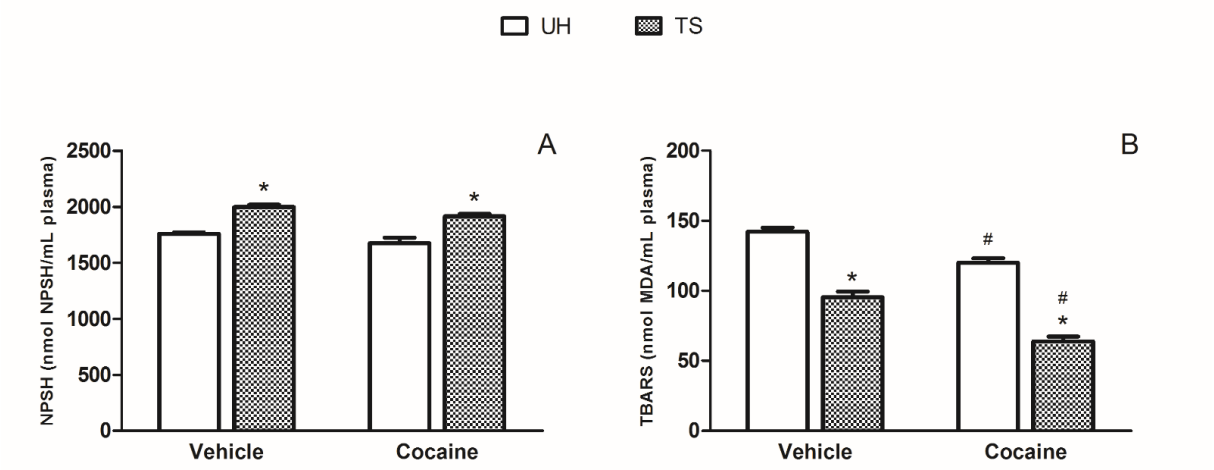

Figure 5 – Influence of tactile stimulation on oxidative status in plasma obtained by non proteic thiol (NPSH) content (A) and lipid peroxidation (TBARS) levels (B) during cocaine withdrawal in rats. Data are expressed as mean ± S.E.M. (n=7). *indicates significant difference (P<0.05) between UH and TS, in the same treatment (vehicle- or cocaine-injection). #indicates significant difference between vehicle and cocaine, in the same handling. Abbreviations: UH: unhandled; TS: tactile stimulation.

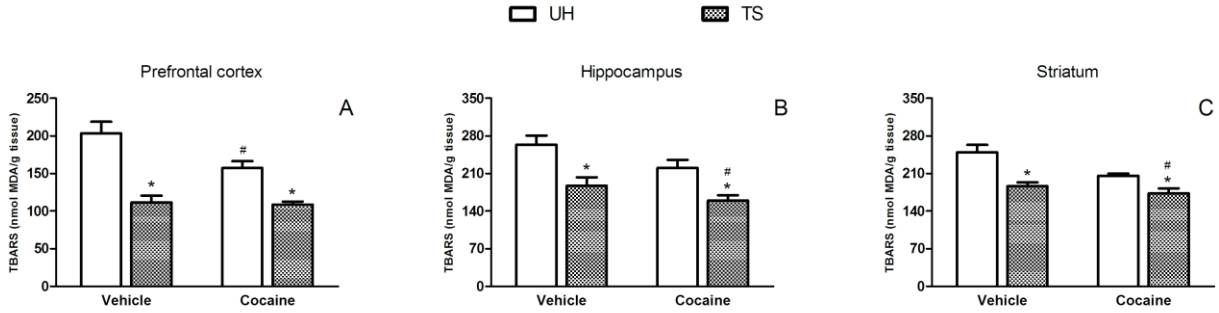

Figure 6 – Influence of tactile stimulation on lipid peroxidation levels in prefrontal cortex (A), hippocampus (B), and striatum (C) during cocaine withdrawal in rats. Data are expressed as mean ± S.E.M. (n=7). *indicates significant difference (P<0.05) between UH and TS, in the same treatment (vehicle- or cocaine-injection). #indicates significant difference between vehicle and cocaine, in the same handling. Abbreviations: UH: unhandled; TS: tactile stimulation.

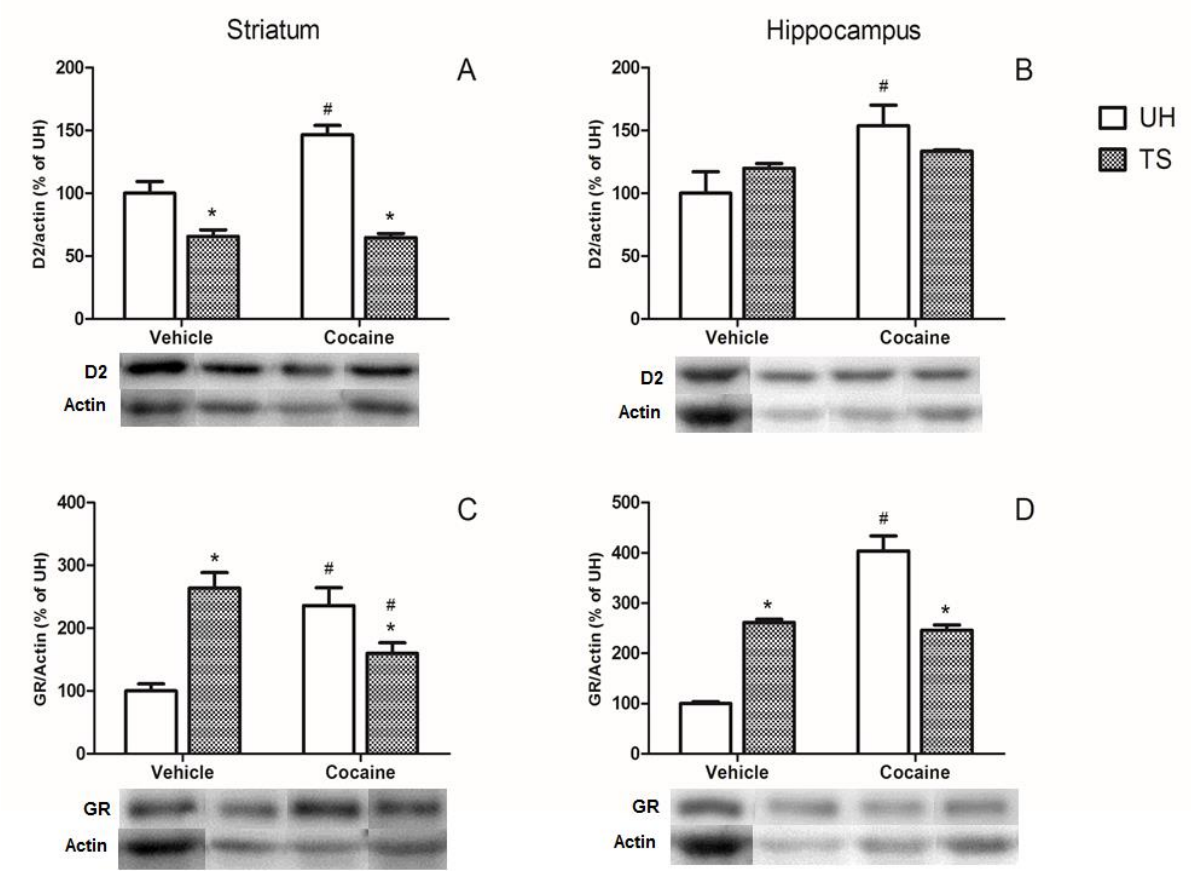

Figure 7 – Influence of tactile stimulation on dopamine receptor (D2) in striatum (A) and hippocampus (B), and on glucocorticoid receptor (GR) immunocontent in the same brain areas (C, D, respectively) during cocaine withdrawal in rats. Data are expressed as mean ± S.E.M. (n=7). *indicates significant difference ($P<0.05$) between UH and TS, in the same treatment (vehicle- or cocaine-injection). #indicates significant difference between vehicle and cocaine, in the same handling. Abbreviations: UH: unhandled; TS: tactile stimulation.

5 DISCUSSÃO

Nos últimos anos, nosso grupo de pesquisa vem estudando a influência de diferentes manuseios, em especial da estimulação tátil (ET) neonatal, sobre a resposta dos roedores aos benzodiazepínicos (BOUFLEUR et al., 2012), capacidade dos animais em lidar com situações de estresse na idade adulta (BOUFLEUR et al., 2013), suscetibilidade para desenvolver preferência por drogas, como a cocaína (ANTONIAZZI et al., 2014) e efeitos antidepressivos (FREITAS et al., 2015).

No presente estudo, avaliou-se, em um primeiro momento, a influência do manuseio neonatal sobre a preferência condicionada de lugar (PCL) induzida por anfetamina, o consequente comportamento relacionado à abstinência, bem como o status oxidativo cerebral e sanguíneo em ratos jovens. Além de melhorar a habilidade para enfrentar situações estressantes e reduzir comportamentos de ansiedade, a ET foi capaz de reduzir a preferência por anfetamina (ANF) tanto 24 quanto 96h após a última administração da droga. Estes dados permitem-nos propor que a ET é capaz de modificar sistemas de recompensa (WEINSTOCK, 2001) e afetar a vulnerabilidade para o abuso de drogas (KOSTEN et al., 2000; SCHENK et al., 1987), visto que este grupo mostrou menor preferência e menores sintomas de ansiedade.

Até o momento, os mecanismos moleculares envolvidos na influência da ET sobre a preferência às drogas psicoestimulantes podem apenas ser hipotetisados. Neste sentido, a ET neonatal seria capaz de aumentar a neurogênese (PHAM et al., 1997; SAPOLSKY, 1992), tornando indispensável uma investigação mais aprofundada desta influência sobre a neuroplasticidade e responsividade frente ao abuso de drogas na idade adulta.

Este estudo também permite propor que o manuseio neonatal pode modificar comportamentos relacionados aos sintomas de ansiedade (VAN OERS et al., 1998), como observado no labirinto em cruz elevado (LCE) durante o período de abstinência de anfetamina. De modo geral, os animais submetidos à ET mostraram melhor desempenho nesta tarefa, onde o manuseio neonatal preveniu o desenvolvimento de sintomas de medo, demonstrando melhor capacidade de enfrentar tais situações (CASOLINI et al., 1997; MEANEY et al., 1991; PHAM et al., 1999). Sugere-se que estes efeitos benéficos da ET sobre os sintomas de ansiedade e medo podem estar relacionados com a maturação precoce das vias neurais da pele para o sistema nervoso central (MONTAGU, 1953), a qual pode ser refletida sobre a emocionalidade reduzida (LEVINE et al., 1957) e menor liberação de corticosterona (LEHMANN et al., 2002; MEERLO et al., 1999). Em conjunto, estas alterações mostram uma influência positiva da ET sobre a funcionalidade do eixo hipotálamo-pituitária-adrenal (HPA).

Além disso, a ET representa um estímulo adicional em relação ao isolamento neonatal (IN), pois além de receberem maior atenção e movimentos de limpeza maternos, a qual busca compensar a ausência da cria, os filhotes também recebem o estímulo tátil proveniente do experimentador. Por outro lado, no IN os filhotes não recebem o estímulo do experimentador e a atenção materna no retorno ao ninho é bastante reduzida, representando uma perda da interação mãe-filhote, o que pode refletir no amadurecimento precoce do eixo HPA e sobre a neurogênese (CHOU et al., 2001; LIU et al., 1997; RODRIGUES et al., 2004). Assim, este estudo propõe que estas relações maternas também exerçam influência sobre as respostas positivas da ET, gerando menor preferência a psicoestimulantes e menores sintomas de ansiedade, uma vez que isto não foi observado nos animais submetidos ao IN.

Adicionalmente, o estudo mostrou que animais submetidos à ET e condicionados com ANF apresentaram menor geração de metabólitos oxidativos, associados à melhor performance do sistema de defesa antioxidante, indicando que a ET pode exercer influência preventiva sobre o dano induzido por anfetamina. A partir disto, pode-se sugerir que a ET é capaz de prevenir os danos oxidativos, relacionados tanto ao estresse decorrente da abstinência quanto pela administração de ANF, preservando o sistema de defesa antioxidante no sangue e em áreas do cérebro intimamente envolvidas no desenvolvimento de ansiedade e comportamentos de busca pela droga.

Estudos prévios desenvolvidos pelo nosso grupo demonstraram que 21 dias de ET foram suficientes para prevenir e melhorar diversos aspectos nos animais avaliados (ANTONIAZZI et al., 2014; BOUFLEUR et al., 2012; 2013; FREITAS et al., 2015). A partir disto, decidiu-se investigar se a ET pode atuar de maneira diferente, de acordo com o período pós-natal em que é aplicada. Assim, os resultados relacionados ao período pós-natal mais adequado para a realização da ET sugerem que, além do período compreendido do dia pós-natal 1 (DPN1) ao 21, que adotamos até então, o período entre o DPN8 e DPN14 também se mostrou bastante promissor, visto que os benefícios da ET ficaram mais evidentes nesta fase do desenvolvimento. Em geral, a ET aplicada do DPN8 ao 14 foi capaz de reduzir ansiedade e melhorar parâmetros de memória, além de reduzir corticosterona no plasma e a peroxidação lipídica no hipocampo. Ainda, a ET realizada na segunda semana de vida foi capaz de aumentar a atividade antioxidante e modificar tanto o BDNF quanto os receptores de glicocorticoides (GR), também no hipocampo dos animais.

Esses menores índices de ansiedade verificados no LCE, associados à maior capacidade de lidar com uma situação de estresse, avaliada no teste defensivo de cavoucar, e à maior atividade locomotora, refletem um status de tranquilidade (BOUFLEUR et al., 2012; SHOJI e MIZOGUCHI, 2010), confirmando que a tendência natural do animal de explorar ambientes novos e enfrentar o conflito (HENDERSON, 2004) é mais acentuada nos animais estimulados

do DPN8 ao 14. Além disso, foi neste mesmo período que os animais apresentaram melhor memória de trabalho, atividade que requer ao animal lembrar de suas escolhas recentes e que é vulnerável às interferências do meio (OLTON, BECKER e HANDELMANN, 1980; SARTER, BODEWITZ e STEPHENS, 1988).

Em relação ao status oxidativo, espera-se que ocorra um aumento da taxa metabólica como resposta fisiológica aos diferentes eventos estressantes, gerando um desequilíbrio entre geração de espécies oxidativas e defesas antioxidantes (ZHANG et al., 2009). Aqui, a ET realizada do DPN8 ao 14 foi capaz de prevenir os danos e melhorar a performance do sistema de defesa antioxidante no hipocampo. Estudos em humanos relataram uma conexão entre o aumento de marcadores oxidativos e a depressão (MICHEL et al., 2007), do mesmo modo que eventos oxidativos em estruturas cerebrais de roedores podem desempenhar importante papel na patogênese da ansiedade e depressão (EREN, NAZIROGLU e DEMIRDAS, 2007). Estudos do nosso grupo também reforçam o envolvimento de parâmetros oxidativos nos sintomas de ansiedade e estresse (ANTONIAZZI et al., 2014; BOUFLEUR et al., 2012), e confirmam o papel protetor da ET, sugerindo que ela pode ser útil em reduzir os riscos para depressão e ansiedade. Tomados em conjunto, os resultados bioquímicos obtidos aqui podem ser relacionados com os sintomas de medo e ansiedade diminuídos, e com a melhoria da memória de trabalho, observados nos testes comportamentais, reforçando a importância de conhecer os eventos prévios de vida quando se estuda transtornos comportamentais e psicológicos.

A corticosterona plasmática é um importante marcador do estado emocional em ratos (BIGGIO et al., 2014) e a elevação do seu nível pode ser considerado um marcador de estresse (HEIDERSTADT et al., 2000). Durante a segunda semana de vida dos animais, a ET foi capaz de produzir uma redução mais evidente nos níveis de corticosterona, confirmando a influência benéfica desse procedimento sobre a função neuroendócrina e regulação do eixo HPA, corroborando o resultado recentemente demonstrado pelo nosso laboratório, em que a ET foi eficiente em reduzir corticosterona e cortisol, em animais submetidos a um protocolo de depressão (FREITAS et al., 2015). Contrariamente, uma elevação nos níveis de corticosterona no hipocampo, por exemplo, pode reduzir o suporte neurotrófico nessa região e prejudicar a função sináptica e a memória espacial (WOSISKI-KUHN et al., 2014).

No presente estudo, a ET reduziu os níveis de corticosterona no período compreendido entre o DPN8 e 14 e consequentemente aumentou o fator neurotrófico derivado do encéfalo (BDNF), o que pode explicar o melhor desempenho deste grupo de animais nas avaliações comportamentais, uma vez que o BDNF está relacionado com o refinamento das sinapses e com a plasticidade funcional (KUCZEWSKI et al., 2009; WOSISKI-KUHN et al., 2014). Além

disso, existe um consenso de que o BDNF é negativamente regulado pela ativação prolongada dos GR (CAMPBELL et al., 2010; JÖHREN et al., 2007). Alguns estudos demonstraram que animais manuseados apresentam níveis mais elevados de GR no hipocampo e córtex frontal (O'DONNELL et al., 1994; STAMATAKIS et al., 2008), sugerindo que o feedback do eixo HPA é reforçado e proporciona melhor resposta contra o estresse (PHAM et al., 1997). Porém aqui, a ET manteve o imunoconteúdo do GR nos animais manuseados na segunda semana de vida, enquanto que na primeira e terceira semanas a ET foi prejudicial neste aspecto, demonstrando mais uma vez que existe um período ideal para a realização do manuseio. Como descrito anteriormente, a ET é um procedimento com efeitos positivos e que tem sido utilizado em diversos modelos animais, entretanto o mecanismo através do qual a ET altera o imunoconteúdo do GR é desconhecido. Um estudo recente mostrou que a diminuição do GR em roedores contribui para o aumento de comportamentos de ansiedade e depressão (WISŁOWSKA-STANEK et al, 2013) enquanto que o aumento do GR no hipocampo de ratos pode ser relacionado a uma diminuição da corticosterona plasmática, gerando uma resposta adrenocortical atenuada e promovendo melhores resultados cognitivos e comportamentais (FERNÁNDEZ-TERUEL et al., 2002; LIN et al., 2011; VIVINETTO, SUÁREZ e RIVAROLA, 2013; ZHANG et al., 2013). Com base nisto, é possível confirmar que a ET é capaz de mimetizar os efeitos de drogas ansiolíticas e dos antidepressivos, como mostrado recentemente (BOUFLEUR et al., 2012; FREITAS et al., 2015).

Considerando os resultados obtidos até aqui, diversos estudos sugerem que a segunda semana de vida é um período importante para a neuroplasticidade, e que ratos manuseados do DPN3 ao 14 mantém os efeitos até além da puberdade (AVISHAI-ELINER et al., 2001; FENOGLIO et al., 2006). Essas duas semanas inicias de vida são conhecidas como o período hiporresponsivo ao estresse, em que os níveis de corticosterona permanecem reduzidos, e quando manuseados nesse período, os animais apresentam ansiedade diminuída e menor resposta do eixo HPA na idade adulta (AVISHAI-ELINER et al., 2001; FENOGLIO, CHEN e BARAM, 2006). Este fato está de acordo com os resultados obtidos neste estudo e pode ser uma hipótese para justificar o que foi encontrado até aqui, uma vez que o melhor desempenho comportamental, bioquímico e molecular foi apresentado pelos animais manuseados do DPN8 ao 14. Além disso, um estudo recente mostrou que a ET pode modificar aprendizado, memória e neurogênese hipocampal de ratos submetidos ao estresse pré-natal (DE LOS ANGELES et al., 2016). A principal explicação seria o fato de a ET induzir a produção de fatores de crescimento neural (PHAM et al., 1997), favorecendo a plasticidade sináptica no hipocampo (ROGGERI et al., 2008), além de aumentar a arborização e comprimento dos dendritos, o que

contribui para a melhor performance dos animais estimulados nas tarefas desempenhadas (RICHARDS et al., 2012).

Como citado anteriormente, a ET mostrou-se eficaz em prevenir a PCL por cocaína em ratos jovens, bem como reduzir os sintomas de ansiedade relacionados à abstinência da droga (ANTONIAZZI et al., 2014). O uso crônico e compulsivo da cocaína pode ser associado a sintomas típicos da depressão maior, que surgem especialmente durante a abstinência, tanto em humanos quanto em roedores (FILIP et al., 2006; MARKOU e KENNY, 2002, MARKOU, KOSTEN e KOOB, 1998; PATERSON, MYERS e MARKOU, 2000). No entanto, as recaídas são recorrentes durante este período e antidepressivos, como a sertralina, têm sido usados para atenuar a ansiedade e depressão associadas à abstinência de cocaína. (GAWIN et al., 1989; MARKOU, HAUGER e KOOB, 1992; PLIAKAS et al., 2001).

Resultados adicionais relacionados aos efeitos antidepressivos da ET em associação com uma dose subterapêutica de sertralina (ver FREITAS et al., 2015) durante o período de abstinência de cocaína em ratos sugerem que a ET é capaz de proteger os animais contra os sintomas típicos da depressão que se manifestam durante a retirada da droga. Isso pôde ser comprovado pela ausência de anedonia, verificada pela maior ingestão de sacarose e através do comportamento pró-ativo demonstrado no teste de nado forçado. Os animais submetidos a ET ainda apresentaram menor índice de ansiedade, verificado no LCE, e menor relação peso das adrenais/peso do corpo, indicando melhor habilidade de lidar com o estresse. Em relação ao status oxidativo, a ET foi capaz de reduzir os danos no plasma e no cérebro dos animais, e melhorar a performance do sistema de defesa antioxidante no plasma. Além disso, as análises moleculares mostraram que a ET foi capaz de alterar o imunoconteúdo dos receptores de dopamina (D2) e GR em estriado e hipocampo. Tais análises ainda se encontram em andamento, dificultando uma maior interpretação relacionada ao 3º protocolo experimental descrito nesta tese.

Entretanto, os resultados obtidos até aqui estão mostrando que a cocaína é capaz de produzir alterações duradouras na função do eixo HPA, resultando em respostas comportamentais alteradas, como as observadas no teste do nado forçado e no LCE, prejudicando o status oxidativo cerebral. Neste contexto, a ET em associação com uma dose subterapêutica de sertralina foi capaz de reduzir ou até mesmo evitar os efeitos nocivos da retirada da cocaína. Isso poderia estar fundamentado em uma função dopaminérgica alterada no estriado e hipocampo, nos permitindo sugerir que a ET neonatal poderia alterar os sistemas monoaminérgico, serotoninérgico e opioide. Neste contexto, a ET também poderia se tornar um agente benéfico para prevenir e/ou reduzir os efeitos da retirada causados pelas drogas psicoestimulantes, prevenindo os episódios de recaída.

Dados adicionais contidos no item Apêndice A desta tese foram desenvolvidos em estudos de doutorado sanduíche, cujos resultados refletem a importância de se estudar a influência da ET paterna na carga epigenética da prole. Estudos anteriores já demonstraram que o enriquecimento ambiental parental foi capaz de causar modificações no cérebro, no comportamento e no epigenoma da ninhada (MYCHASIUK et al., 2012), bem como o estresse paterno antes mesmo da concepção é capaz de alterar a metilação do DNA e o comportamento dos filhotes (MYCHASIUK et al., 2013), e ainda alterar a morfologia dos dendritos e a conectividade cerebral de filhotes machos e fêmeas (HARKER et al., 2015). Até o momento, somente dados comportamentais referentes à ET aplicada aos pais foram processados e pôde-se perceber que os filhotes provenientes de pais estimulados apresentam melhor desempenho nas atividades que avaliam o aprendizado do neonato, como a geotaxia negativa, e o desenvolvimento motor, como o teste do campo aberto para filhotes. Em filhotes avaliados após o desmame, foi possível verificar que a ET aplicada nos pais repercutiu em atividade locomotora diminuída e menor índice de ansiedade dos filhotes, quando comparados aos filhotes de pais não-manuseados.

Em resumo, nossos resultados mostraram que o manuseio neonatal é capaz de promover benefícios em longo prazo, uma vez que o condicionamento de preferência às drogas psicoestimulantes e os testes comportamentais foram realizados quando os animais estavam próximos à idade adulta. A ET causou benefícios, melhorando a performance comportamental dos animais ao lidar com situações de estresse, atuando de forma preventiva sobre a PCL e retirada/abstinência de drogas psicoestimulantes. De fato, tal resposta não foi observada nos grupos não manuseados e IN, sugerindo assim que a ET pode modificar as respostas frente a situações novas, ou seja, o manuseio neonatal adequado pode representar um procedimento promissor na prevenção da ansiedade e depressão associados ao abuso de drogas, atualmente tratados com farmacoterapia pouco preconizada e psicoterapia de grupo. Além disso, também foi possível demonstrar que a ET neonatal influencia os animais na idade adulta de maneira diferente, considerando o período em que é aplicada. Quando aplicada durante a segunda semana de vida dos animais, a ET foi capaz de reduzir medo e comportamentos de ansiedade através da modulação da resposta do eixo HPA, induzindo efeitos de neuroplasticidade sobre o BDNF e regulando o GR no hipocampo. Assim, a ET pode exercer efeitos benéficos no desenvolvimento neural dos filhotes e pode ainda minimizar ou prevenir o desenvolvimento de transtornos neuropsiquiátricos ao longo da vida adulta.

6 CONCLUSÕES

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

1. No condicionamento com anfetamina, a ET se mostrou mais efetiva em reduzir a preferência pela droga durante a abstinência de 24h e 96h, enquanto o IN mostrou maior prejuízo 24h após a última administração de anfetamina.
2. O manuseio neonatal mostrou-se efetivo ao modificar comportamentos relacionados aos sintomas de medo e ansiedade verificados no LCE durante o período de abstinência de anfetamina.
3. O manuseio neonatal foi capaz de produzir benefícios em longo prazo, uma vez que a PCL e os parâmetros comportamentais foram realizados quando os animais estavam próximos à idade adulta.
4. A ET mostrou sua influência protetora sobre o status oxidativo, através de reduzida peroxidação lipídica e carbonilação proteica em córtex, hipocampo e estriado, e aumento das defesas antioxidantes do sangue.
5. A ET pode ser mais efetiva se realizada em períodos específicos do desenvolvimento neonatal.
6. Quando aplicada no período adequado, a ET é capaz de reduzir sintomas de ansiedade, melhorar a capacidade de lidar com o estresse, melhorar a memória, status oxidativo e promover a neuroplasticidade dos animais, quando comparada a grupos não-manuseados.
7. O manuseio neonatal adequado, como a ET, em estágios iniciais do desenvolvimento promove melhores respostas em situações e ambientes novos, e pode representar uma medida promissora na prevenção da ansiedade e da depressão.
8. A ET neonatal em associação com antidepressivos pode ser uma importante ferramenta no combate à depressão associada à abstinência de drogas psicoestimulantes, contribuindo para a prevenção dos episódios de recaída.
9. Os efeitos positivos da ET realizada em animais machos adultos podem ser transmitidos para os filhotes, refletindo em melhor desenvolvimento e menos ansiedade dos filhotes.

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APÊNDICE A – MANUSCRITO 3

O manuscrito científico 3 refere-se aos estudos desenvolvidos durante a realização de estágio de Doutorado Sanduíche junto ao Canadian Centre for Behavioural Neuroscience (CCBN), no laboratório de pesquisa da Profª. Drª. Robbin Gibb, da University of Lethbridge, na cidade de Lethbridge/Canadá.

Os resultados processados até o momento e parcialmente apresentados a seguir estão estruturados nos itens Materiais e Métodos, e Resultados.

MANUSCRITO 3 – Este manuscrito encontra-se em fase de redação, e por isso será apresentado na forma de resultados parciais, composto de materiais e métodos, e resultados.

Paternal tactile stimulation alters rat offspring epigenome, affecting sensorimotor development and behavior.

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Abstract

1 Introduction

2 Experimental procedures

2.1 Animals

All procedures were conducted in accordance with the Canadian Council of Animal Care and were approved by the University of Lethbridge Animal Care and Use Committee. Ten female Long-Evans rats were mated with 10 male Long-Evans rats (five paternal tactile stimulation (TS) and five non-tactile stimulation (NTS)). All pairs successfully mated resulting in 117 pups (61 from TS and 56 from NTS). Male pups (29 TS and 30 NTS) were used for the current experiment and the remaining female pups were used in parallel experiments. Animals were given access to food and water ad libitum and were maintained on a 12-h light/dark schedule (lights on from 07:30 to 19:30 h) in a temperature controlled (21º) breeding room. All pups were tested in a negative geotaxis (at postnatal day 9 (P9) and P10) and open field test (at P10-13 and P15). On postnatal day 22 (P22), pups were euthanized for morphological analysis of neurons in Golgi-cox staining (n=6 for each group) and global DNA methylation (n=7 for TS and n=8 for NTS). The remaining pups were submitted to an exploratory behavioural task, the activity box, at P25 and elevated plus maze at P26.

2.2 Paternal Tactile Stimulation

Paternal TS was applied a total of 48 consecutive days prior to the mating session in five adult male rats. TS was performed three times a day (09:00,13:00, and 16:00) for 15 min intervals. Five TS and five NTS (control) adult males were utilized, where male and female rat pups, in each litter, were assigned to the TS and NTS groups, as the paternal handling. The animals were transported in cleaned home cage to a testing room for the TS sessions. A baby hairbrush was used to stimulate the rats of TS group, individually, on the experimenter's lap. At the end of each session, rats were returned to their cages and transported back to the breeding room. Control males were only handled during the home cage regular cleaning, two times a week.

Following the 48 days of TS, paternal TS and control males were immediately mated with females. This was the only exposure that female dams had with the handled male rats. Subsequent to mating, each 2 or 3 female dams in the same experimental condition (ex. control–control vs. paternal TS–paternal TS) were housed in shoe-box cages. Female dams were separated and housed individually prior to birth of pups and remained individually housed following the birth of their litter. Pups did not receive any kind of neonatal handling, other than that required for home cages regular cleaning.

2.3 Behavioral assessments

2.3.1 Negative geotaxis

Pups were tested on the negative geotaxis task at P9 and P10. At this point, pups' eyes are still closed. Pups were individually placed facing downward on a Plexiglas® board set to a 40◦ angle. Pups were filmed for 60s each day. If the pup slid off the board, they were replaced on the board facing in the downward direction. A pup was considered to be in an upward position when its head crossed the horizontal plane. Pups were scored for the amount of time they spent in the upward direction by a research associate blinded to the experimental parameters.

2.3.2 Early open field

Pups were tested on the open field task from P10 to P13 and P15. Pups are individually placed in the centre of a transparent Plexiglas® box (16cm×20cm×20cm). The base of the open field box was divided into roughly 130 squares (2cm×2cm). Pups were filmed for 60s on each testing day and scored for exploratory behaviour by calculating the total number of novel squares their front paws entered. The box was cleaned with vircon in between the filming of each pup. A research who was blind to the treatment groups scored each video. As we are aware of the negative consequences associated with maternal separation (Monroy et al., 2010), we wanted to limit the time pups were away from their mothers and therefore tested them for 1 min/day. This would ensure pups were only separated from their mothers for brief periods of time.

2.3.3 Activity box

On P25, the exploratory behavior of the animals was recorded. Rats were individually placed in Accusan® activity-monitoring boxes. Each Plexiglas® box measured 41cm×41cm×30.5cm, which recorded the movements of each rat. Rats were introduced to the activity box for 10 min and their activity level was recorded in two 5-min intervals, using the VersaMax™ computer software. The intervals were combined and the overall activity/distance traveled was determined.

2.3.4 Elevated plus maze

Testing on the EPM occurred on P26. The EPM apparatus consisted of 3 main components: a base (94cm), two open arms (10cm×40cm), and two closed arms (10cm×40cm×40cm). The apparatus, made of black Plexiglas, was housed in a well-lit empty testing room. On testing day, rats were introduced to the maze, where their forepaws were placed in the center square of the maze facing a closed arm. Time spent in the open and closed

arms were scored, as well as the entries number in each arm. An animal was deemed to be occupying an arm when the first half of their body entered the arm. Total activity on the maze (number of open and closed arms entries) was used to calculate the anxiety index (Cohen et al., 2007; 2008; Mazor et al., 2007), as follows:

$$\text{Anxiety Index} = 1 - \left[\frac{\left(\frac{\text{Time spent in the open arms}}{\text{Total time on the maze}}\right) + \left(\frac{\text{Number of open arm entries}}{\text{Total entries on the maze}}\right)}{2}\right]$$

Anxiety index values range from 0 to 1 where an increase in the index expresses increased anxiety-like behavior (Cohen et al., 2007; 2008; Mazor et al., 2007). Animals were video recorded for 5 min, with the camera elevated at the end of an open arm. The apparatus was cleaned with vircon solution using paper towel before the introduction of each animal.

2.6 Statistical Analysis

Data were analyzed by one-way ANOVA (handling), except for negative geotaxis and early open field, which were analyzed by repeated measures analysis of variance (handling x age), followed by Tukey's multiple range test for all comparisons, when appropriate, using Statistica 8.0 software. $P<0.05$ was regarded as statistically significant for all comparisons made and results were expressed as mean ± S.E.M.

3 Results

3.1 Negative geotaxis

Repeated measures analysis of variance revealed a significant main effect of handling, age, and handling x age interaction on average percentage of time upwards [$F(1,57)=4.78$, $P<0.05$; $F(1,57)=31.53$, $P<0.001$ and $F(1,57)=4.49$, $P<0.05$, respectively]. Tukey's post hoc test showed no difference between NTS and TS groups on P9. On P10, TS increased the average percentage of time upwards when compared to NTS group on the same day, and in relation to itself on P9 (Fig. 1).

3.2 Early open field

Repeated measures analysis of variance revealed a significant main effect of age on average number of novel squares entered [$F(4,220)=65.67$, $P<0.001$] and no effect of handling, and handling x age interaction [$F(4,220)=0.06$, $P=0.79$ and $F(4,220)=1.84$, $P=0.12$, respectively]. Post hoc test showed a progressive increase on average number of novel squares entered throughout the days. On P15, NTS group showed an increased number of squares

entered when compared to P10, P11, P12 and P13 in the same handling (Fig. 2A). The same increase was observed on TS group at P15, which was statistically different from P10, P11, P12 and P13. NTS and TS groups showed no significant difference between them (Fig. 2A).

One-way ANOVA revealed a significant influence of handling on average number of novel squares summed for all five testing days [$F(1,57)=5.62$, $P<0.05$]. Tukey's post hoc test showed that TS group increased the average number of novel squares when compared to NTS group (Fig. 2B).

3.3 Activity box

One-way ANOVA revealed a significant main effect of handling on horizontal activity [$F(1,30)=14.21$, $P<0.001$], horizontal movements [$F(1,30)=32.05$, $P<0.001$], total distance [$F(1,30)=14.95$, $P<0.001$], margin distance [$F(1,30)=12.36$, $P<0.001$], margin time [$F(1,30)=5.46$, $P<0.05$], centre distance [$F(1,30)=12.80$, $P<0.001$], and centre time [$F(1,30)=5.45$, $P<0.05$]. Tukey' post hoc test showed that TS was able to decrease horizontal activity, horizontal movements, total distance, margin distance, centre distance and centre time, and increase margin time when compared to NTS group (Table 1).

3.4 Elevated plus maze

One-way ANOVA revealed a significant influence of handling on time spent in open arms [$F(1,30)=6.38$, $P<0.05$] and on anxiety index [$F(1,30)=19.09$, $P<0.001$]. No handling effect was observed on open arm entries [$F(1,30)=1.91$, $P=0.17$] and on total entries number [$F(1,30)=0.80$, $P=0.37$]. Post hoc test showed that TS group increased the time spent in open arms (Fig. 3A) and decreased the anxiety index (Fig. 3D), when compared to NTS group. NTS and TS groups showed no significant difference to each other on open arm entries (Fig. 3B) and total entries number (Fig. 3C).

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Figures

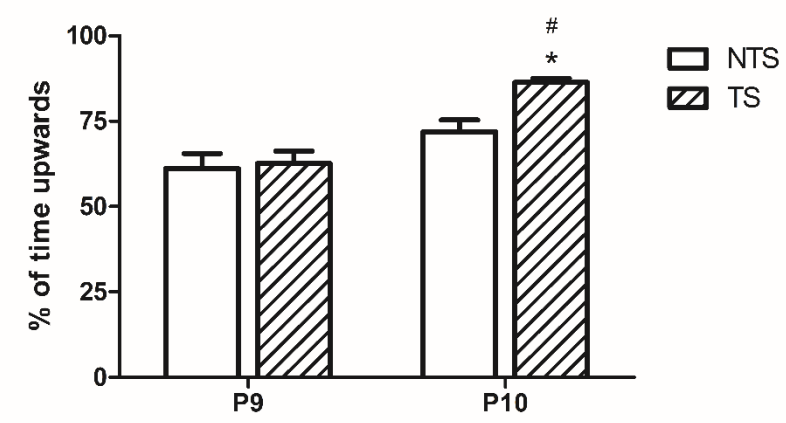

Figure 1 – Average percentage of the total time male offspring spent in the upwards direction on the negative geotaxis task. Data are expressed as mean ± S.E.M. ($P<0.05$); * indicates significant difference between TS and NTS groups at the same age. # indicates significant difference of TS at different ages. Abbreviations: NTS: non-tactile stimulation; TS: tactile stimulation.

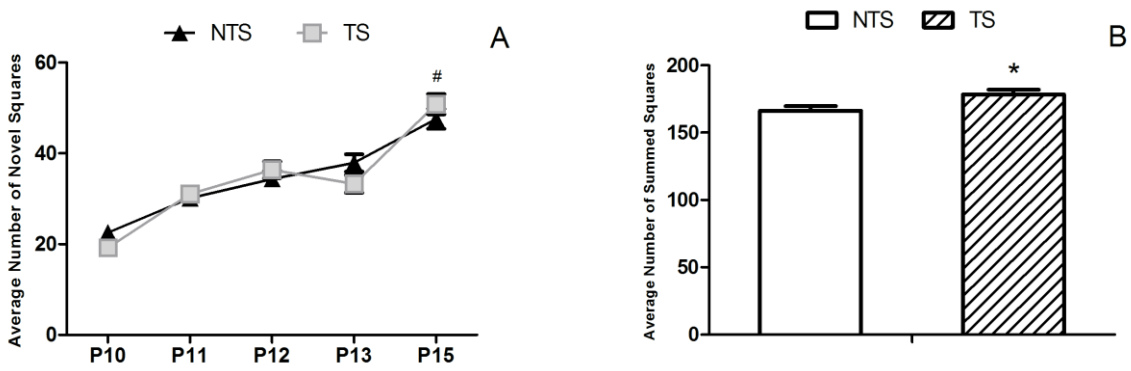

Figure 2 – Open field: (A) Average number of novel squares entered by male offspring over the five testing days, (B) average number of novel squares offspring entered summed for all 5 testing days (P10-P13 and P15). Data are expressed as mean ± S.E.M. ($P<0.05$); * indicates significant difference between TS and NTS groups. # indicates difference of the same handling at P15 from all ages. Abbreviations: NTS: non-tactile stimulation; TS: tactile stimulation.

Table 1 – Exploratory behavior assessed in activity box, on postnatal day 25 of male offspring.

Activity box measures	**NTS**	**TS**
Horizontal activity (s)	3188.25±269.87	2067.50±124.52*
Horizontal movements	126.06±5.16	90.56±3.55*
Total distance (cm)	1761.25±226.72	860.68±53.19*
Margin distance (cm)	1496.93±208.81	748.37±41.45*
Margin time (s)	554.95±9.46	580.23±5.24*
Centre distance (cm)	264.18±36.62	112.75±21.20*
Centre time (s)	45.04±9.46	19.76±5.24*

* indicates significant difference between TS and NTS groups.
Data are expressed as mean ± S.E.M. ($P<0.05$).
Abbreviations: NTS: non-tactile stimulation; TS: tactile stimulation

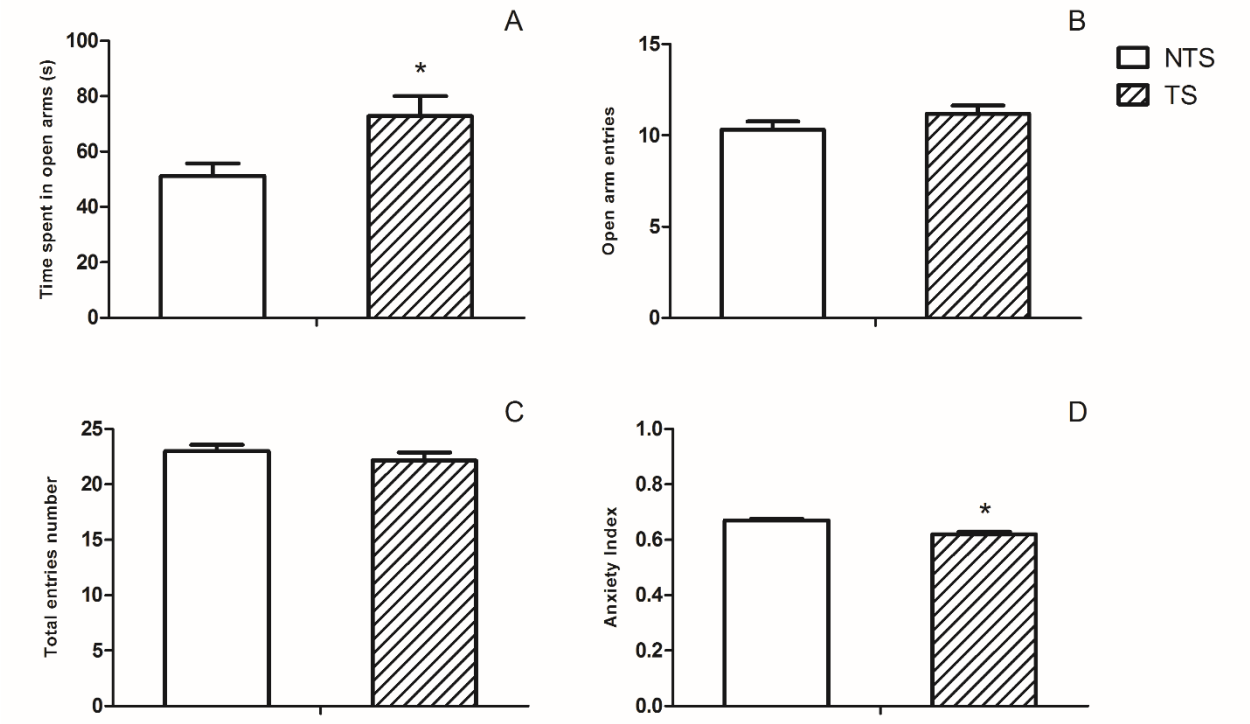

Figure 3 – Anxiety-like symptoms assessed in elevated plus maze, on postnatal day 26, by time spent in open arms (A), open arms entries (B), total entries number (C), and anxiety index (D). Data are expressed as mean ± S.E.M. ($P<0.05$); * indicates significant difference between TS and NTS groups at the same age. Abbreviations: NTS: non-tactile stimulation; TS: tactile stimulation.