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INFLUÊNCIA DO MANUSEIO NEONATAL SOBRE OS EFEITOS DO ESTRESSE EMOCIONAL E SUA INTERAÇÃO COM FÁRMACO BENZODIAZEPÍNICO EM RATOS

DISSERTAÇÃO DE MESTRADO

Nardeli Boufleur

Santa Maria, RS, Brasil
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INFLUÊNCIA DO MANUSEIO NEONATAL SOBRE OS EFEITOS DO ESTRESSE EMOCIONAL E SUA INTERAÇÃO COM FÁRMACO BENZODIAZEPÍNICO EM RATOS

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Dissertação apresentada ao Programa de Pós-Graduação em Farmacologia, Área de Concentração em Neuropsicofarmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Farmacologia.**

Orientadora: Profª. Drª. Marilise Escobar Bürger

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elaborada por
Nardeli Boufleur

como requisito parcial para obtenção do grau de
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“O segredo de progredir é começar. O segredo de começar é dividir as tarefas árduas e complicadas em tarefas pequenas e fáceis de executar, e depois começar pela primeira.”

(Mark Twain)

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Farmacologia
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INFLUÊNCIA DO MANUSEIO NEONATAL SOBRE OS EFEITOS DO ESTRESSE EMOCIONAL E SUA INTERAÇÃO COM FÁRMACO BENZODIAZEPÍNICO EM RATOS

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Data e Local da Defesa: Santa Maria, 24 de janeiro de 2012.

A exposição de roedores a estímulos como o manuseio neonatal, tem sido descrita por causar alterações comportamentais e fisiológicas benéficas na vida adulta. Já a exposição de animais adultos a ambientes estressantes, pode resultar em prejuízos emocionais e patologias neuro-psiquiátricas. Este estudo objetivou investigar a possível influência de dois tipos de manuseio neonatal, estimulação tátil (ET) e separação materna (SM), sobre o estado emocional de ratos após exposição ao estresse crônico e moderado (ECM) – superlotação, distúrbio do ciclo claro/escuro, serragem molhada entre outros. Também objetivamos avaliar se a ET neonatal poderia modificar os efeitos ansiolíticos observados com uma baixa dose de fármaco benzodiazepínico nos animais adultos. No 1º estudo, filhotes machos de ratos Wistar foram submetidos diariamente à ET ou SM desde o dia pós-natal 1 (DPN1) até o DPN21, durante 10 minutos. Os animais não manuseados (NM) permaneceram no ninho sem nenhuma manipulação. Na vida adulta (DPN67), metade dos animais de cada grupo foram expostos ao ECM durante 3 semanas e observados no teste de preferência pela sacarose (PS), labirinto em cruz elevado (LCE) e teste defensivo de cavocar (TDC), seguido de eutanásia para avaliações bioquímicas e hormonais. O ECM reduziu a PS, aumentou a ansiedade no LCE e TDC e aumentou o peso das adrenais. Alguns parâmetros de defesas antioxidants em plasma, hipocampo e córtex foram alterados pela exposição ao ECM, enquanto um aumento da oxidação proteica em hipocampo e córtex também foram observados. Ambas as formas de manuseio neonatal foram capazes de prevenir as mudanças na PS, ansiedade no TDC e peso das adrenais. Também preveniram as alterações nas defesas antioxidants em plasma, hipocampo e córtex e a oxidação proteica no hipocampo. Apenas a ET preveniu a ansiedade induzida pelo ECM no LCE e a oxidação proteica no córtex. Além disso, a ET foi associada aos menores níveis de cortisol comparado aos ratos NM antes e após a exposição ao estresse. Uma vez que a ET apresentou melhores resultados, realizamos um 2º experimento apenas com essa forma de manuseio neonatal. Na vida adulta, os animais receberam uma única administração de diazepam (DZP) (0.25 mg/Kg peso corporal-i.p.) ou veículo (V), e foram submetidos às avaliações comportamentais. O tratamento com DZP reduziu comportamentos de ansiedade no LCE e aumentou a exploração no LCE, no teste da escada e campo aberto apenas no grupo ET. Considerando os animais NM, o tratamento com DZP apenas aumentou a exploração no teste da escada. Os animais do grupo ET tratados com DZP apresentaram menor ansiedade em vários parâmetros do LCE, maior comportamento exploratório no teste da escada e campo aberto e menor imobilidade no TDC. Os resultados do presente estudo demonstraram o papel protetor do manuseio neonatal, especialmente da ET, a qual pode melhorar a habilidade para lidar com situações estressantes na vida adulta e afetar a resposta a substâncias benzodiazepínicas nesse período.

Palavras-chave: Manuseio neonatal. Estimulação tátil. Estresse crônico e moderado. Ansiedade. Diazepam.

ABSTRACT

Master Dissertation
Graduate Program in Pharmacology
Federal University of Santa Maria

INFLUENCE OF NEONATAL HANDLING ON THE EFFECTS OF EMOTIONAL STRESS AND ITS INTERACTION WITH A BENZODIAZEPINE DRUG IN RATS

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Date and place of defense: January 24th, 2012, Santa Maria.

Exposure of rodents to stimuli like neonatal handling, have been described to cause behavioral and physiological benefits in adulthood. On the other hand, exposure to adults to stressful environments can result in emotional and neuropsychiatric pathologies. This study aimed to investigate the possible influence of two forms of neonatal handling as tactile stimulation (TS) and maternal separation (MS) on the emotional status of rats exposed to chronic mils stress (CMS) – grouped housing, lights on overnight, damp sawdust and others. Furthermore, we aimed to evaluate if neonatal TS could modify anxiolytic effects observed with a low dose of a benzodiazepine drug in adult rats. In the first study, male Wistar pups were submitted daily to TS or MS, from postnatal day one (PND1) to PND21, for 10 min. Unhandled (UH) animails remained in next without any manipulation. In adulthood (PND67), half the animals of each group were exposed to the CMS for 3 weeks and observed in sucrose preference (SP), elevated plus-maze (EPM) and defensive burying test (DBT), followed by euthanasia for biochemical and hormonal assessments. CMS reduced SP, increased anxiety on EPM and DBT and increased adrenal weight. In addition, some parameters of antioxidant defenses in plasma, hippocampus and cortex were altered with exposure to CMS, whereas an increase in protein oxidation in hippocampus and cortex also were observed. In contrast, both forms of neonatal handling were able to prevent changes in SP, anxiety behavior on DBT and adrenal weight CMS-induced. Furthermore, they also prevented alterations in antioxidant defenses in plasma, hippocampus and cortex and protein oxidation in hippocampus. Only TS prevented CMS-induced anxiety symptoms on EPM and protein oxidation in cortex. Furthermore, TS was associated with lower levels of cortisol than in UH rats before and after CMS exposure. Since TS presented better results, we performed a second experiment only with this neonatal handling. In adulthood, the animals received a single administration of diazepam (DZP) (0.25 mg/kg body weight-i.p.) or vehicle (V) and were submitted to behavioral evaluations. DZP treatment reduced anxiety-like behaviors in EPM and increased exploration in EPM, staircase and open field tasks only in TS group. Considering UH animals, DZP treatment only increased exploration in staircase test. TS animals treated with DZP presented reduced anxiety-like behaviors in many parameters of EPM test, increased exploratory behavior in staircase and open field tasks and less immobility in DBT. The results of this study showed the protective role of neonatal handling, especially TS, which may enhance ability to cope with stressful situations in adulthood and affect the response for benzodiazepine substances during this period.

Keywords: Neonatal handling. Tactile stimulation. Chronic mild stress. Anxiety. Diazepam.

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LISTA DE ABREVIATURAS E SIGLAS

ACTH – hormônio adrenocorticotrófico
BDZ – benzodiazepínico
CRH – hormônio liberador de corticotropina
ECM – estresse crônico e moderado
ET – estimulação tátil
GABA – ácido gama-aminobutírico
LCE – labirinto em cruz elevado
HPA – hipotálamo-hipófise-adrenal
L-HPA – límbico-hipotálamo-pituitária-adrenal
SAN – sistema adrenérgico-noradrenérgico
SM – separação materna
SNC – sistema nervoso central
RG – receptores de glicocorticóides
RM – receptores de mineralocorticóides
TDC – teste defensivo de cavoucar

SUMÁRIO

APRESENTAÇÃO

Esta dissertação apresenta os resultados na forma de manuscritos, os quais se encontram no ítem **MANUSCRITOS CIENTÍFICOS**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se nos próprios manuscritos e representam a íntegra deste estudo.

Ao fim desta dissertação encontram-se os ítens **DISCUSSÃO** e **CONCLUSÕES**, nos quais há interpretações e comentários gerais sobre os manuscritos científicos contidos neste estudo.

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

1 INTRODUÇÃO

O sistema nervoso em desenvolvimento é altamente suscetível a alterações ambientais (CHAPILLON et al., 2002; GSCHANES et al., 1998; INAZUSTA et al., 1999; ZHANG; CAI, 2008; ZHANG et al., 2002), particularmente durante os estágios iniciais da vida (KUHN; SCHANBERG, 1998; HALL, 1998; PRYCE; FELDON, 2003). Nesse período, determinados estímulos podem influenciar o desenvolvimento dos sistemas fisiológico, emocional, cognitivo, neuroendócrino e comportamental (BARNETT; BURN, 1967; BEACH; JAYNES, 1954; FLEMING; O'DAY; KRAEMER, 1999; HOFER, 1994; LEHMANN; FELDON, 2000; LEVINE et al., 1967). Diferentes formas de manuseio neonatal têm sido utilizadas para examinar os mecanismos pelos quais variações precoces no ambiente do animal afetam o desenvolvimento de sistemas neurais e causam alterações comportamentais e neuroendócrinas (DENENBERG, 1964; LEVINE, 1962; MEERLO et al., 1999). O manuseio neonatal é constituído de uma breve separação materna (de até 15 minutos) e pode incluir a estimulação tátil realizada por um experimentador (DASKALAKIS et al., 2009). Além da própria estimulação tátil dos filhotes, vários estudos demonstraram que os efeitos do manuseio neonatal são mediados por mudanças na interação mãe-filhote, uma vez que mães de ratos estimulados permaneceram mais tempo com os filhotes (CHOU et al., 2001) e apresentaram maior número de lambidas sobre esses (CALDJI et al., 1998; LIU et al., 1997; PRYCE; BETTSCHEN; FELDON, 2001). Acredita-se que a manipulação pode ter um efeito *per se* como também pode induzir a um aumento do cuidado materno (MADRUGA, 2003). Além disso, sugere-se que os efeitos da estimulação tátil podem ser explicados pelo fato de que as vias neurais da pele para o sistema nervoso central (SNC) amadurecem mais cedo em relação a outros sistemas sensoriais (MONTAGU, 1953).

Estudos demonstraram que a estimulação neonatal causou aumento da atividade e do comportamento exploratório (FERNÁNDEZ-TERUEL et al., 1992) e diminuiu a ansiedade na tarefa do labirinto em cruz elevado em roedores (FERNÁNDEZ-TERUEL et al., 1990; McINTOSH; ANISMAN; MERALI, 1999; PLOJ et al., 1999). Alguns estudos reportaram que o manuseio neonatal acelerou a maturação de neurônios piramidais corticais (SCHAPIRO; VUKOVICH, 1970), melhorou as funções cognitivas (STAMATAKIS et al., 2008) e mostrou-se efetivo na facilitação da recuperação funcional após eventos de hipóxia-isquemia neonatal (CHOU et al., 2001; RODRIGUES et al., 2004). Além disso, a estimulação

durante o período neonatal protegeu os animais da perda neuronal associada à idade e também ao estresse (KOSTEN; LEE; KIM, 2007; MEANEY et al., 1988; PHAM et al., 1997; SAPOLSKY, 1992; STAMATAKIS et al., 2008).

Segundo Meaney e Aitken (1985), o período crítico para o manuseio neonatal abrange os primeiros 14 dias após o nascimento e a estimulação durante a primeira semana é a mais efetiva, uma vez que nesse período as experiências ambientais podem alterar a sensibilidade e a eficiência do eixo hipotálamo-hipófise-adrenal (HPA) (JUTAPAKDEEGUL et al., 2003). A estimulação neonatal causou atenuação dos níveis de hormônio adrenocorticotrófico (ACTH) e de corticosterona em resposta ao estresse agudo e aumentou a densidade dos receptores de glicocorticóides no hipocampo (LIU et al., 1997; SMYTHE; ROWE; MEANEY, 1994; WIGGER; NEUMANN, 1999). Em consonância com o papel-chave dos receptores de glicocorticóides em mediar o controle de feedback negativo do eixo HPA (De KLOET, 1991; McEWEN; De KLOET; ROSTENE, 1986), animais manuseados mostraram-se hipersensíveis aos efeitos de feedback dos corticosteróides (MEANEY et al., 1989) e apresentaram maior habilidade de adaptação a estímulos novos e/ou estressantes (MEANEY et al., 1991). O pico de liberação e o rápido retorno aos níveis basais dos hormônios glicocorticóides parecem ser extremamente adaptativos e previnem o organismo da exposição a níveis elevados de glicocorticóides que podem resultar, especialmente sob condições estressantes crônicas, em neurotoxicidade (LUPIEN et al., 1998; McEWEN; SEEMAN, 1999).

Essas mudanças nas respostas neuroendócrinas ao estresse são também acompanhadas por alterações importantes na emotividade e nos sistemas de neurotransmissores que a regulam, tais como o sistema GABAérgico (GIACHINO et al., 2007). O ácido gama-aminobutírico (GABA) é o principal neurotransmissor inibitório do SNC dos mamíferos (SIEGHART et al., 1999) e existe em 60-70% de todas as sinapses do cérebro, embora em concentrações variáveis (FANTONI; CORTOPASSI, 2002; SWINYARD; WHITE; WOLF, 1988). O desenvolvimento de substâncias com propriedades antagonistas e agonistas específicas permitiu a descoberta de dois tipos de receptores GABA, chamados de $GABA_A$ e $GABA_B$. Fármacos benzodiazepínicos, os medicamentos ansiolíticos mais amplamente utilizados (BASILE; LIPPA; SKOLNICK, 2004; NEMEROFF, 2003), modulam positivamente o GABA nos receptores $GABA_A$ através de sítios de ligação alostérica chamados receptores benzodiazepínicos (SIEGHART; SCHUSTER, 1984; SKARDA; MUIR; BEDNARSKI, 1997; SQUIRES et al., 1979). Consequentemente, essas substâncias potencializam a atividade GABAérgica através da abertura dos canais de cloreto, com uma consequente hiperpolarização das membranas e impedimento da propagação do impulso

nervoso estimulatório, gerando depressão dessa função (BRAESTRUP et al., 1984; CALDJI; DIORIO; MEANEY, 2003) (Figura 1).

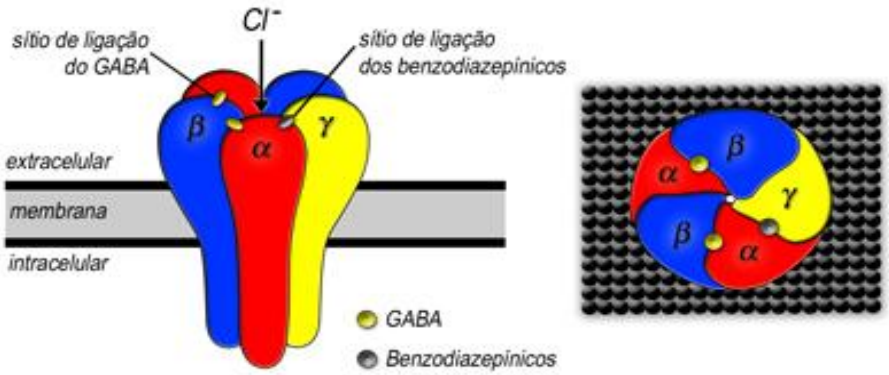

Figura 1. Receptor GABAérgico

Apesar de serem substâncias relativamente seguras, os medicamentos benzodiazepínicos podem produzir efeitos colaterais indesejáveis tais como sedação, miorrelaxamento, amnésia e um potencial considerável para induzir tolerância, dependência e abuso (ALLISON; PRATT, 2003; LADER, 1994). O GABA e seus receptores $GABA_A$ estão envolvidos na regulação de mecanismos cerebrais normais e patológicos como sono, epilepsia, memória, emoções e alguns comportamentos (KALUEFF; NUTT, 1997; NUTT; MALIZIA, 2001; SIEGHART et al., 1999). Ao mesmo tempo, disfunções do sistema GABAérgico têm sido associadas a ansiedade e depressão (NUTT; MALIZIA, 2001). Estudos demonstraram que o manuseio neonatal aumentou a densidade de receptores GABA (CALDJI et al., 2000; ESCORIHUELA et al., 1992; GIACHINO et al., 2007) e benzodiazepínicos (CALDJI et al., 2000; ESCORIHUELA et al., 1992) em algumas regiões cerebrais (CALDJI et al., 2000; ESCORIHUELA et al., 1992; GIACHINO et al., 2007) envolvidas na regulação da resposta ao estresse e comportamentos emocionais como a amígdala e o hipocampo (GIACHINO et al., 2007). Entretanto, existem poucos estudos investigando as consequências dessas alterações.

Um grande número de indivíduos convive com circunstâncias estressantes (GREENBERG; BERKTOLD, 2006; STAMBOR, 2006) e quanto mais intensas, persistentes e incontroláveis essas situações, maior a probabilidade de representarem um estímulo nocivo

(SELYE, 1976; VAN de KAR; BLAIR, 1999) e levarem ao desenvolvimento de perturbações mentais incluindo ansiedade, doenças afetivas e depressão (RAY et al., 2004). O risco elevado de doenças mentais tem sido relatado em indivíduos com altos níveis de estresse devido a baixa renda e níveis educacionais (KESSLER et al., 1994; McEWEN, 2000; REGIER et al., 1993) ou grande risco ocupacional (SAUTER et al., 1999). Adicionalmente, elevados níveis de estresse no local de trabalho ou no ambiente acadêmico predizem um maior risco para doenças mentais (DUSSELIER et al., 2005; GODIN et al., 2005). Estimativas apontam que em torno de 90% da população mundial é afetada pelo estresse (BAUER, 2002) e quando esse está presente de forma crônica, pode facilitar a ocorrência de algumas doenças (TÕNISSAAR et al., 2008), causar alterações em padrões neuroquímicos (HARRO; ORELAND, 2001) e anormalidades no sistema imune (DORIAN; GARFINKEL, 1987; O'LEARY, 1990).

Os circuitos neuronais que iniciam e mantém a resposta do organismo a um agente estressor incluem diversas estruturas nos sistemas central e periférico (PACÁK; PALKOVITS, 2001) e os principais componentes da resposta adaptativa ao estresse são o sistema adrenérgico-noradrenérgico - SAN e o sistema límbico-hipotálamo-pituitária-adrenal - L-HPA (COLEMAN, 2006). Ao aumentar o nível de catecolaminas, o hormônio liberador de corticotropina (CRH) é produzido no hipotálamo e consequentemente ocorre liberação de hormônio adrenocorticotrófico (ACTH) e corticosteróides adrenais (DE KLOET; MEIJER; VAN HAARST, 1998 apud LEVY, A. et al., 1998; KVETNANSKY et al., 1995). Certas anormalidades relativas à regulação do SAN e do eixo HPA têm sido identificadas em pacientes depressivos e ansiosos (LÚJAN et al., 2008), o que demonstra que essas doenças são precipitadas e exacerbadas pelo estresse (ALTEMUS, 2006). Uma vez que quantidades excessivas e persistentes de glicocorticóides podem ser prejudiciais ao organismo, o eixo HPA está sob forte regulação por feedback negativo através dos receptores de mineralocorticóides (RM) e de glicocorticóides (RG) (DE KLOET; MEIJER; VAN HAARST, 1998 apud LEVY, A. et al., 1998), localizados, dentre outras estruturas periféricas e centrais, em estruturas cerebrais envolvidas na regulação do medo, ansiedade, aprendizado e memória, como o hipocampo, o septo medial e a amígdala (KORTE, 2001). O hipocampo, juntamente com a amígala e outras estruturas cerebrais, controla a atividade do eixo HPA (HERMAN; CULLINAN, 1997). A amígdala, por sua vez, exerce um papel chave nas alterações do humor, nos processos de memória (CHARNEY; GRILLON; BREMNER, 1998; DAVIS; RAINNIE; CASSELL, 1994) e na mediação dos efeitos ansiolíticos de xenobióticos como os benzodiazepínicos (BURGHARDT; WILSON, 2006; KANG; WILSON; WILSON, 2000; MENARD; TREIT, 1999; PESOLD; TREIT, 1994, 1995; PETERSEN; BRAESTRUP;

SCHEEL-KRUGER, 1985; SCHEEL-KRUGER; PETERSEN, 1982; SENDERS; SHEKHAR, 1995). O estresse crônico pode promover alterações morfológicas na amígdala (VYAS et al., 2002), e mudanças nos receptores de glicocorticóides nessa estrutura estão relacionadas ao medo, à ansiedade e à depressão (SCHULKIN; GOLD; McEWEN, 1998).

Na presença de um agente estressor, ocorre liberação de aminoácidos excitatórios (glutamato e aspartato) em algumas áreas cerebrais (MOGHADDAM, 1993), o que leva à excitabilidade contínua dos neurônios, aumento de cálcio intracelular, ativação de proteases, lipases e peroxidases, que podem gerar radicais livres e inclusive levar à morte neuronal (GARCÍA-BUENO; CASO; LEZA, 2008). Além disso, os neurônios injuriados e as células gliais secretam citocinas (como interleucina-1 ou fator de necrose tumoral α) que atuam como potentes mediadores inflamatórios (DIRNAGL; IADECOLA; MOSKOWITZ, 1999). No cérebro, esses eventos levam à neuroinflamação, a qual também gera radicais livres (GARCÍA-BUENO; CASO; LEZA, 2008). Evidências mostram que a neuroinflamação contribui com algumas doenças psiquiátricas incluindo ansiedade e depressão (LUCAS; ROTHWELL; GIBSON, 2006).

É bem estabelecido que a resposta fisiológica e comportamental a um dado estímulo varia consideravelmente entre indivíduos (MEERLO et al., 1999). Parece existir uma individualidade característica na avaliação dos impactos ambientais e na resposta a situações estressantes. Essa variação entre indivíduos pode ser originada por fatores genéticos (BENUS et al., 1991; CASTANON; MORMEDE, 1994) e, além disso, existem evidências crescentes de que a reatividade ao estresse na vida adulta pode ser influenciada por fatores presentes no início do desenvolvimento (ANISMAN et al., 1998; MEANEY et al., 1991). Assim, tanto fatores genéticos quanto eventos estressantes apresentam papéis independentes no desenvolvimento de doenças afetivas, mas a fisiopatologia dessas doenças frequentemente se associa a uma ação sinérgica do estresse crônico com elevada vulnerabilidade pré-existente (KENDLER et al., 1995).

Dentre as doenças afetivas, a ansiedade, que constitui uma reação emocional desagradável produzida por um estímulo externo considerado ameaçador (VALLES; SAUCEDO, 2007 apud PEREIRA, 2009); é capaz de gerar alterações fisiológicas e comportamentais que permitem a adaptação do indivíduo às mudanças ambientais (SALOMONS et al., 2010). A ansiedade é considerada normal se for adequada às circunstâncias e aceita como um acontecimento que acompanha naturalmente o estímulo necessário para lidar com uma situação específica (PEREIRA, 2009). Entretanto, as respostas podem tornar-se disfuncionais e resultar em um estado crônico de elevada ansiedade

(SALOMONS et al., 2010), o qual pode afetar a capacidade no trabalho, estudo, bem como comprometer a interação social e a rotina diária (PEREIRA, 2009). Um estudo de Gama et al. (2008) analisou os níveis de ansiedade de 498 estudantes de uma universidade de Aracaju (SE) e demonstrou que os estudantes mais jovens do sexo feminino apresentavam níveis significativamente maiores de ansiedade. Uma das consequências da ansiedade patológica é o alto índice de consumo de medicamentos ansiolíticos, especialmente benzodiazepínicos, bem como uma pré-disposição para um quadro depressivo. De acordo com o II Levantamento Domiciliar sobre o Uso de Drogas Psicotrópicas no Brasil – um estudo envolvendo as 108 maiores cidades do País, realizado em 2005 pela Secretaria Nacional Antidrogas – Senad em parceria com o Cebrid/Unifesp – revelou que o uso de benzodiazepínicos foi maior entre a faixa etária igual ou superior a 35 anos, sendo que 3,4% dos homens e 6,9% das mulheres utilizaram esses medicamentos alguma vez na vida. Na região Sul, esse índice foi de 2,2% dos homens e 4,1% das mulheres. Já na região Sudeste, esse índice aumentou para 3,3% dos homens e 8,5% das mulheres. A média das idades em que os entrevistados utilizaram benzodiazepínico pela primeira vez foi de 30,5 anos e existiu um predomínio nítido para o sexo feminino, quando comparado ao masculino, em todas as faixas etárias (OBID, 2011).

Como descrito anteriormente, o acúmulo ou permanência de eventos estressantes podem culminar em depressão (LLYOD,1980). Post (1992) verificou que em cerca de 60% dos casos descritos na literatura, os episódios de depressão eram precedidos pela ocorrência de fatores estressantes, principalmente de origem psicossocial. Além disso, o estresse parece aumentar a probabilidade da ocorrência de depressão tanto em indivíduos de alto como baixo risco genético para tal distúrbio (KENDLER et al., 1995; LLYOD, 1980). Além disso, a falta de adaptação durante situações novas ou cotidianas também pode levar à depressão (MAES et al., 2000; MICHEL et al., 2007). A depressão grave ocupa o quarto lugar entre as dez principais causas de patologia, a nível mundial, e se as projeções estiverem corretas, até 2020 a depressão será a segunda maior causa de incapacidade e perda de qualidade de vida (WHO, 2011). Essa doença caracteriza-se por tristeza, perda de interesse nas atividades e diminuição da energia. Outros sintomas são a perda de confiança e auto-estima, o sentimento injustificado de culpa, ideias de morte e suicídio, diminuição da concentração e perturbações do sono e do apetite (WHO, 2010). Comumente chamada de doença do século ou doença da vida moderna, a depressão atinge mais de 121 milhões de pessoas no mundo e está associada a 850 mil mortes anuais, o que inclui um número elevado de suicídios (WHO, 2011). A incidência da depressão é duas vezes maior em mulheres, no entanto, não encontra relação com classe

social, etnia e nível educacional (KENDLER; KUHN; PRESCOTT, 2004; WEISSMAN et al., 1996).

Enquanto os dados em humanos claramente suportam uma correlação entre a exposição ao estresse e algumas doenças psicológicas, é difícil isolar as contribuições do estresse para a etiologia ou a gravidade de uma dessas doenças, devido às limitações associadas com estudos em humanos (DOHRENWEND et al., 1984). Dessa forma, a experimentação com animais têm mostrado que a exposição ao estresse aumenta comportamentos relacionados com ansiedade ou depressão, enquanto providencia melhor controle experimental das variáveis (MATUSZEWICH et al., 2007). O paradigma do estresse crônico e moderado (ECM) tem sido desenvolvido em animais de laboratório como modelo de irritações relativamente pequenas e imprevistas da vida cotidiana (KATZ; ROTH; CARROLL, 1981; WILLNER et al., 1987), eficaz na indução de distúrbios neurocomportamentais relevantes para os transtornos de humor (KOMPAGNE et al., 2008). Em um experimento típico, ratos (WILLNER et al., 1987; WILLNER; MUSCAT; PAPP, 1992) ou camundongos (MONLEON et al., 1994) são expostos seqüencialmente a uma variedade de estressores leves, tais como superlotação, distúrbio do ciclo claro/escuro, serragem molhada, inclinação da gaiola, isolamento entre outros, os quais mudam periodicamente, durante semanas ou meses. Roedores expostos a esse procedimento de estresse têm demonstrado comportamentos semelhantes à ansiedade (MASLOVA; BULYGINA; MARKEL, 2002; TANNENBAUM et al., 2002; ZURITA el al., 2000), redução da atividade locomotora (VOLLMAYR; HENN, 2003), perda de peso corporal (COX et al., 2011; LI et al., 2009; MUSCAT; WILLNER, 1992; WILLNER et al., 1996), diminuição no comportamento sexual e agressivo (GRONLI et al., 2005; PARDON et al., 2000), mudanças no padrão de sono (GRONLI et al., 2007) e mostraram sinais de maior atividade no eixo HPA, incluindo hipertrofia adrenal (GOUIRAND; MATUSZEWICH, 2005; MUSCAT; WILLNER, 1992) e hipersecreção de corticosterona (AYENSU et al., 1995; COX et al., 2011; LI et al., 2009).

Uma vez que o estresse persistente pode levar ao desenvolvimento de perturbações mentais, alterações comportamentais, neuroendócrinas e bioquímicas, e que o manuseio neonatal é capaz de produzir inúmeros benefícios, estudos sobre o impacto da estimulação neonatal sobre o estresse na vida adulta tornam-se importantes. O presente estudo investigou se o manuseio de filhotes de ratos no início da vida seria capaz de alterar as respostas ao modelo de estresse crônico e moderado, já que a reatividade ao estresse na vida adulta pode ser influenciada por fatores presentes no início do desenvolvimento. Além dos inúmeros

benefícios comportamentais, morfológicos e neuroendócrinos do manuseio neonatal, especialmente da estimulação tátil, alguns estudos demonstraram que o manuseio neonatal foi capaz de aumentar a densidade de receptores GABA e benzodiazepínicos em algumas regiões cerebrais (CALDJI et al., 2000; ESCORIHUELA et al., 1992; GIACHINO et al., 2007) envolvidas na regulação de comportamentos emocionais. Dessa forma, investigamos se a estimulação tátil neonatal seria capaz de alterar parâmetros comportamentais ao ser associada a uma dose baixa de diazepam, um agonista $GABA_A$. Com isso, objetivamos observar se um adequado manuseio neonatal poderia influenciar a resposta a medicamentos ansiolíticos administrados em uma dose menor do que a usualmente utilizada em estudos com animais.

2 OBJETIVOS

2.1 Objetivo geral

Investigar a influência do manuseio neonatal sobre os efeitos deletérios emocionais induzidos por um modelo de estresse crônico e moderado (ECM) em ratos. Avaliar a influência da estimulação tátil neonatal sobre os efeitos de um fármaco benzodiazepínico na vida adulta.

2.2 Objetivos específicos

- Avaliar os efeitos do manuseio neonatal sobre comportamentos de ansiedade após exposição de ratos ao ECM;

- Investigar os efeitos do manuseio neonatal sobre a atividade do eixo HPA nos animais expostos ao ECM;

- Avaliar os efeitos do manuseio neonatal sobre parâmetros de estresse oxidativo e defesas antioxidantes enzimáticas em córtex e hipocampo dos animais expostos ao ECM;

- Avaliar os efeitos do manuseio neonatal sobre os níveis plasmáticos de vitamina C nos animais expostos ao ECM;

- Investigar a possível influência da estimulação tátil neonatal sobre os efeitos de uma baixa dose de diazepam sobre a ansiedade, exploração e locomoção em ratos.

3 MANUSCRITOS CIENTÍFICOS

Os resultados inseridos nesta dissertação apresentam-se sob a forma de manuscritos científicos, os quais se encontram aqui estruturados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios manuscritos. O 1º manuscrito foi submetido para publicação na revista Stress e encontra-se sob revisão. O 2º manuscrito encontra-se em fase final de redação.

Manuscrito 1

3.1 Neonatal handling prevent anxiety-like symptoms in rats exposed to chronic mild stress: behavioral and oxidative parameters

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Neonatal handling prevent anxiety-like symptoms in rats exposed to chronic mild stress: behavioral and oxidative parameters

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Abstract

This study investigated the influence of neonatal handling on behavioral and biochemical changes in rats submitted to chronic mild stress (CMS) in adulthood. Male pups were submitted daily to tactile stimulation (TS) or maternal separation (MS), from postnatal day one (PND1) to postnatal day 21 (PND21), for 10 min. In adulthood, half the animals were exposed to the CMS for 3 weeks and observed concerning sucrose preference and elevated plus-maze (EPM) and defensive burying tests (DBT), followed by euthanasia for biochemical assessments. CMS reduced sucrose preference, increased anxiety on EPM and DBT and increased adrenal weight. In addition, CMS decreased plasmatic vitamin C levels and increased protein carbonyl (PC) levels, catalase (CAT) activity in hippocampus and cortex, and superoxide dismutase (SOD) levels in cortex. In contrast, both forms of neonatal handling were able to prevent reduction in sucrose preference, anxiety behavior on DBT and of CMS-induced adrenal weight increase. Furthermore, they were also able to prevent plasma vitamin C reduction, hippocampal PC levels increase, CAT activity increase in hippocampus and cortex and SOD levels increase in cortex following CMS. Only TS was able to prevent CMS-induced anxiety symptoms on EPM and PC levels in cortex. Furthermore, TS was also associated with lower levels of plasma cortisol than in unhandled rats before and after CMS exposure. Taken together, these findings show the protective role of neonatal handling, especially TS, which may enhance ability to cope with stressful situations in adulthood.

Keywords: Chronic mild stress, Neonatal handling, Tactile stimulation, Maternal separation, Anxiety, Oxidative stress

1. Introduction

Many people live with chronic stressful situations, and a failure of adaptive mechanisms can lead to stress-related illnesses such as anxiety, depression and affective disorders (Matuszewich et al., 2007). The chronic mild stress (CMS) paradigm has been experimentally used as an animal model that mimics unanticipated irritations of everyday life (Kompagne et al., 2008). Its protocol is effective in inducing neurobehavioral disturbances significant to mood disorders (Kompagne et al., 2008) and has been related to increased metabolic rates and generation of reactive oxygen species (ROS) (Zhang et al., 2009). In this sense, when concentration of ROS exceeds the body's capacity to neutralize them, a process of oxidative stress (OS) is initiated, resulting in damage to cells, tissues and organs (Zhang et al., 2009) often involved in the pathophysiology of different diseases, including anxiety and depression (Hovatta et al., 2010).

Stress exposure stimulates the hypothalamic–pituitary–adrenal (HPA) axis by increasing blood levels of glucocorticoids (Cox et al., 2011; Shoji & Mizoguchi, 2010) and adrenal glands weight (Gouirand & Matuszewich, 2005), while impairment of the HPA axis has been related to occurrence of major depression (Vedder et al., 2007). The developing brain is very susceptible to environmental stimuli (Inazusta et al., 1999) and during the neonatal period, interventions in the environment can modify the nervous system formation (Zhang & Cai, 2008). Some studies have related pups' early life experiences to changes in their behavioral and neuroendocrine functions in adulthood (Lehman et al., 2002; Champagne et al., 2009). For instance, it has been shown that repetitive brief early handling leads to better development of the neuroendocrine system and improved ability to cope with stressful situations (Champagne et al., 2009; Levine et al., 1957).

Neonatal tactile stimulation (TS) is able to accelerate maturation of cortical neurons (Schapiro & Vukovich, 1970), improve postnatal neurogenesis, enhance cognition, and prevent deleterious effects of stress and aging on hippocampal neurons loss (Rodrigues et al., 2004; Zhang & Cai, 2008). So, this neonatal handling can also be related to a lower response of the HPA axis after stressful conditions (Meerlo et al., 1999). Another form of neonatal handling is maternal separation (MS). Studies showed that after brief periods of MS, pups presented less anxiety (McIntosh et al., 1999), better spatial cognition (Stamatakis et al., 2008) and lower release of glucocorticoid after stress exposure (Lehmann et al., 2002). Beneficial effects of neonatal handling on brain insults were studied by Rodrigues et al.

(2004), who found protective effects of TS and MS on hippocampal morphological changes in rats exposed to neonatal ischemia.

Considering that CMS can induce neuroendocrine and behavioral disturbances relevant to mood disorders and that neonatal handling is able to produce beneficial effects, we proposed to investigate whether TS and MS can improve the ability to cope with stressful situations during adulthood, as well as interfering in the oxidative parameters related to CMS.

2. Material and methods

2.1 Animals

Fourteen pregnant female Wistar rats from the breeding facility of Universidade Federal de Santa Maria (UFSM), RS, Brazil, were kept in plexiglas cages with free access to food and water in a room with controlled temperature (22–23°C) and on a 12 h-light/dark cycle with lights on at 7:00 a.m. The day of birth was monitored and litters were culled to 8 pups (5 males and 3 females) to ensure adequate nutritional status. At postnatal day one (PND1) male pups were weighed and randomly assigned to one of three experimental groups (n=14): unhandled (UH), tactile stimulation (TS) and maternal separation (MS), remaining together with their mothers until weaning. At PND22, litters were weaned and weighed; male pups from the same condition (only one per litter for each handling procedure) were housed by group of three (±1) and left undisturbed up to 60 days old. All procedures were in accordance with the rules of the Committee on Care and Use of Experimental Animal of the UFSM, which follows international rules (NIH Publication No. 80-23; revised 1978).

2.2 Neonatal handling

Neonatal handling was applied daily from PND1 to PND21, between 8:00 and 10:00 a.m. Rats were submitted to two distinct forms of handling: TS or MS. For TS, pups were removed from the nest, held gently by experimenter and stroked with the index finger on the dorsal surface, in the rostral caudal direction, for 10 min (Rodrigues et al., 2004). For MS, pups were removed from the nest, held gently by experimenter and put in an individual plastic box lined with soft paper and warmed with an incandescent lamp for 10 min (microenvironment temperature was close to 32º C, similar to nest temperature). UH group remained in their nest without any touch by human hand.

2.3 General procedure

At 60 days of age (PND60), animals were habituated to receive 1% (w/v) sucrose/water solution and tap water in their home cage until PND66. On PND67, half the animals of each group (n=7) were submitted to the chronic mild stress (CMS) procedure for 3 weeks (until PND87). One day after the end of CMS, rats were submitted to behavioral assessments (PND88 to PND91). At PND92, between 8:00 and 10:00 a.m., animals were weighed, anesthetized with sodium pentobarbital (80mg/kg, i.p.) and euthanized by exsanguination. Blood was collected with heparin and brain was removed and used for biochemical determinations.

2.4 Chronic mild stress procedure

A modified version of the CMS protocol first described by Willner et al. (1987) was used. Rats were subjected daily to two or three different stressors following a semi-randomized schedule that included: damp sawdust, grouped housing, cage tilting (45^{o}), lights on overnight, isolation, switching cages, and foreign object in cage for 3 weeks (Kompagne et al., 2008). The stress procedure did not involve any food or water deprivation (Kompagne et al., 2008).

2.5 Behavioral evaluations

2.5.1 Sucrose preference test

From PND60 to PND66, 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. One day after finishing the CMS protocol (PND88), animals were individually tested on a sucrose preference test (Kompagne et al., 2008) at the start of the dark cycle (19:00 p.m.). They were deprived of food and water for 5 h, and then presented with two bottles containing either tap water or the sweet solution. One-hour intake was measured by weighing bottles before and after the test. Sucrose preference (SP) was calculated according to the following equation:

SP= [(sucrose intake) / (sucrose intake + water intake)] x 100.

2.5.2 Elevated plus maze test (EPM)

On PND89, 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×13.5 cm), which gave access to any of the four arms. At the beginning of each test, the rat was placed in the central platform facing an open arm. Time spent and entries number in the open arms, head dipping frequency and closed arms entries number were registered during the five-minute test. Time spent (expressed as a percentage of the total test duration) and entries number in the open arms of the maze were used as measures of the anxiety level (Hlavacova et al., 2010), while the number of closed arms entries were used as index of locomotor activity (Rodgers and Dalvi, 1997). Ethological measures included the frequency of head dipping (exploratory movement of head/shoulders over sides of the open arms and down towards the floor). The apparatus was cleaned with alcohol solution 20% using wet sponge and paper towel before the introduction of each animal.

2.5.3 Defensive burying task (DBT)

On PND91 rats were submitted to the DBT task, used to measure 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 and 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 a 10 min test. When the animal received a shock by making contact with the shock probe, current was terminated so as not to provide additional shocks. Duration of burying behavior and freezing time were measured. Burying behavior was defined as any spraying or throwing of the bedding with the head or forepaws in the direction of the shock probe, which is often used as a measure of coping. In exchange, freezing time (standing on four feet with body and head motionless) is typically recognized as the anxiety measure in this test (Matuszewich et al., 2007). After 10 min, the animal was removed and returned to his home cage, apparatus was cleaned and new bedding was placed into the cage for the next rat.

2.6 Biochemical measurements

Plasma was obtained after centrifugation (1300 ×*g* for 15 min) of blood collected by cardiac puncture, and used for cortisol and vitamin C (VIT C) determination. The brains were immediately excised and put on ice. Cortex and hippocampus were removed and

homogenized in 10 volumes (w/v) of 10mM tris-HCl buffer (pH 7.4). One part of homogenates was centrifuged (1800 ×*g* for 15 min), and the supernatants were used for measurements of enzymatic antioxidant defenses (catalase- CAT activity and superoxide dismutase-SOD levels). In this time was possible to measure SOD only in cortex, due to the small volume of the hippocampus. Another part of homogenates was not centrifuged and was stored for determination of protein carbonyl (PC), which estimates oxidative damages to proteins.

2.6.1 Cortisol measurement

Although corticosterone is more frequently determined in rats, both corticosterone and cortisol are regulated in the same way and released in parallel (Saito et al.,1992), which allows cortisol to stand as a general stress measure for adrenocortical function (Milanes et al., 1991). Different laboratories (Issa et al., 2010; Prasad et al., 2006; Radahmadi et al., 2006) have used cortisol to estimate stress development in rats, whose level was quantified in the present study, using a commercially available EIA kit (EIAgenTM Cortisol, Adaltis Italy S.p.A).The specificity of the test was evaluated by comparing the parallelism between the standard curve and serial dilutions in PBS (pH 7.4) of plasma samples.

2.6.2 Vitamin C (VIT C) levels

Plasmatic VIT C was estimated as described by Galley et al. (1996) with some modifications (Jacques-Silva et al., 2001). Fresh isolated plasma was precipitated with 5% trichloroacetic acid solution and centrifuged. Supernatants were mixed with 2,4-dinitrophenylhydrazine (4.5 mg/mL) and 13.3% trichloroacetic acid, and incubated (3h at 37 ºC). Sulfuric acid solution (65%) was added and samples were measured at 520 nm. A standard curve using ascorbic acid was used to calculate the content of VIT C and expressed as μg VIT C/mL plasma.

2.6.3 Protein carbonyl (PC) quantification

PC was quantified by the method of Yan et al. (1995), with some modifications. Soluble protein was mixed with 2,4-dinitrophenylhydrazine (DNPH; 10mM in 2M HCl) or HCl (2 M) and incubated at room temperature for 1 h. Denaturing buffer (150mM sodium phosphate buffer, pH 6.8, with 3% sodium dodecyl sulfate), ethanol (99.8%) and hexane (99.5%) were added, mixed by shaking and centrifuged. The protein isolated from the interface was washed twice with ethyl acetate/ethanol 1:1 (v/v) and suspended in denaturing

buffer. Each DNPH sample was read at 370nm in a spectrophotometer against the corresponding HCl sample (blank). The results were expressed as nmol carbonyl/g tissue.

2.6.4 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.

2.6.5 Superoxide dismutase (SOD) levels

SOD was assayed spectrophotometrically as previously described by Misra and Fridovich (1972), which involves inhibition of auto-oxidation of epinephrine in presence of tissue homogenate at 480 nm, for 120s at intervals of 15s. SOD levels were expressed as units of SOD (U) (1U=amount of enzyme required to produce 50% inhibition at 30°C).

2.7 Statistical analysis

Data were analyzed by two-way ANOVA followed by Duncan's Post Hoc tests when appropriate. (Software package Statistica 8.0 for Windows was used). Values of $P<0.05$ were considered statistically significant for all comparisons made.

3. Results

3.1 Sucrose preference

Analysis of variance (ANOVA) followed by Duncan's test showed that CMS significantly reduced sucrose preference in UH group and both forms of neonatal handling prevented this reduction (Table 1).

3.2 Elevated plus maze (EPM)

Duncan's test showed that TS *per se* increased time spent in open arms in relation to other neonatal handlings (UH and MS groups), which were similar to each other. CMS protocol reduced percentage of time spent in open arms of all groups (UH, TS and MS), whose value was significantly higher for TS than for those others (Figure 1A).

Post hoc test showed that TS-CMS group showed a higher number of open arms entries in relation to the other groups (Figure 1B), while the number of closed arms entries was not change by handling or CMS exposure (data not shown).

Post hoc test showed that TS *per se* increased number of head dippings in relation to UH rats. CMS exposure did not change this behavioral parameter, which was higher in TS group than in UH and MS groups (Figure 1C).

3.3 Defensive burying

CMS exposure increased burying time in UH rats as compared to TS and MS (Figure 2A). In addition, CMS protocol also increased the freezing time of UH group in relation to handled groups (Figure 2B).

3.4 Biochemical measurements in plasma

TS reduced *per se* plasma cortisol levels in relation to UH animals. CMS exposure did not cause changes in cortisol levels, but across different handlings, hormone levels were lower in TS group than in UH rats, while MS group showed a tendency for this effect. CMS exposure increased adrenal weight and adrenal weight/body weight ratio of UH animals, but not of TS and MS groups. In fact, after CMS exposure animals treated with TS and MS presented lower adrenal weight and adrenal weight/body weight ratio than UH group. CMS exposure caused a reduction of VIT C levels in UH rats, but not in TS and MS groups. In fact, after CMS exposure, plasma levels of VIT C of UH rats were lower than in TS and MS animals (Table 2).

3.5 Biochemical measurements in hippocampus

Post hoc test showed a tendency of lower PC levels in hippocampus of TS rats than in UH animals (P=0.080). CMS exposure increased this oxidative parameter in hippocampus of UH animals and both forms of neonatal handling prevented this increase (Figure 3A).

CMS exposure also increased hippocampal CAT activity in UH animals and both forms of neonatal handling prevented this increase (Figure 3B).

3.6 Biochemical measurements in cerebral cortex

Between animals not submitted to CMS protocol, cortical PC levels in MS rats were higher than UH and TS groups. CMS exposure increased cortical PC levels in UH animals

and TS prevented this increase. After CMS exposure, MS and UH animals showed similar levels of PC, which were higher than in TS group (Figure 4A).

CMS exposure increased CAT activity in cerebral cortex of UH rats and both forms of neonatal handling prevented this increase (Figure 4B).

CMS exposure also increased SOD levels in cortex of UH rats and both forms of neonatal handling prevented this increase (Figure 4C).

4. Discussion

The chronic mild stress (CMS) paradigm used in this study has been employed by others, and is considered an stress animal model that induces anhedonia (Willner et al., 1987), anxiety-like and stress symptoms (Matuszewich et al., 2007; Tõnissaar et al., 2008), as well as hormonal (Cox et al., 2011; Luján et al., 2008), and biochemical changes, particularly important for the antioxidant defense system (Lucca et al., 2009). In this study, CMS exposure reduced sucrose intake, indicating an anhedonic state of the animals that validates the protocol employed. In this sense, anhedonia can be observed by decreasing consumption and preference for palatable solutions (Li et al., 2010), experimentally observed after stress exposure (Kompagne et al., 2008), and has been used as an index of efficacy of the CMS model (Willner, 2005). In our study, neonatal handling was able to prevent anhedonia symptoms, leading to the idea that both TS and MS can reduce susceptibility to depression and stress. In addition, our study aimed to evaluate the influence of neonatal handling on the development of anxiety as well as on markers of oxidative damages and stress, which were induced by CMS exposure. Our findings showed that CMS exposure caused anxiety symptoms in UH animals, as observed by lower time spend and lower entries number in the open arms and lower head dipping in the EPM, which are not an artifact of locomotor activity, since all experimental groups showed the same entries number in the closed arms of EPM. Besides modifying the classical spatiotemporal measures of anxiety, TS also improved the ethological parameters related to exploration behavior, observed by higher frequency of head dipping in both animals exposed and not exposed to the stress protocol, in a manner consistent with an anxiolytic outcome (Hlavacova et al., 2010). In addition, the increased anxiety-like symptoms of UH-CMS animals was also observed in our findings through of higher time of burying- and freezing- time in the DBT, which are types of strategies related to active and passive behavior, respectively.

At this time it is possible to hypothesize that neonatal TS is related to less emotionality of adult animals exposed to stressful situations. In this sense, neonatal handling consist of different manipulations: while TS consists of an additional stimulus by the experimenter, in MS pups remain separate for 10 min, which is also considered an incentive because they receive more licking and grooming from their mothers than UH animals (Liu et al., 1997; Rodrigues et al., 2004). As TS presents an additional stimulus compared to MS, we believe that this may be responsible for their better results observed in our study. Thus, under basal conditions, much of the handling behaviors are similar to those of UH animals, but in threatening situations, the differences between behaviors become clearer (Meerlo et al., 1999), as we demonstrated here by the beneficial effects of the neonatal handlings on CMS-induced behavioral effects.

These beneficial effects of neonatal handling on emotionality and stress development may be explained by early maturation of neural pathways from skin to CNS (Montagu, 1953), and responses can be reflected as decreased anxiety (Levine et al., 1957) as well as reduced corticosteroid levels in stressful situations (Lehmann et al., 2002; Meerlo et al., 1999). In addition, different studies have shown that early stimuli are able to increase neurogenesis and reduce hippocampal neuron loss related to aging and stress (Pham et al., 1997; Rodrigues et al., 2004). In this sense, it is well known that stress acts as a predisposing or precipitating factor in the beginning of depression and anxiety disorders (Hovatta et al., 2010). Clinically, many patients exhibit both anxiety and depression in the form of comorbidities, which have shown a causal relationship, i.e., high levels of anxiety may become a risk factor for the occurrence of mood disorders (Ducottet & Belzung, 2005).

Under different stressful situations, a common physiological response is the increased metabolic rate, which results in oxidative stress when occurs an imbalance between ROS and antioxidant defenses (Zhang et al., 2009). Thus, while proteins and other cellular components are subject to attack by free radicals, antioxidants are responsible for removing them, controlling their deleterious oxidative damage (Gopinath et al., 2004). Our findings showed that CMS exposure increased PC levels in cortex and hippocampus of UH rats, in line with results recently shown by Lucca et al. (2009) reinforcing the involvement of oxidative damages in anxiety-like and stress symptoms.

In fact, Sahin & Gümüslü (2004) also have shown an interaction between stress models and oxidative damages development in animal brain tissues. In addition, oxidative stress in rat brain structures may play a role in the pathogenesis of anxiety and depression (Eren et al., 2007), since a link between markers of oxidative stress and depression has

already been reported in humans (Michel et al., 2007). Interestingly, our findings also showed that CMS-induced oxidative damages were attenuated by TS (in both cortex and hippocampus) and MS (in hippocampus), suggesting that oxidative stress may be involved in the pathophysiology of depression and anxiety. So, TS and MS seems to have preventative properties for stress induced changes, such as oxidative stress, and these handlings in turn, may be useful to reduce individual's risks to develop depression and anxiety. Supporting this hypothesis, antioxidant defenses of UH animals exposed to CMS protocol were also observed here, with findings of lower plasma levels of VIT C and increase of SOD levels in cortex and of CAT activity in cortex and hippocampus.

Hovatta et al. (2010) reported a reduction of blood levels of non-enzymatic antioxidant defenses in anxiety and depression situations, suggesting its depletion, while Eren et al. (2007) showed lower VIT C levels in cortex of rats exposed to CMS. On the other hand, Sahin & Gümüslü (2004) found increased CAT and SOD activities in brain of animals exposed to stress. Similar changes were also observed in our study, indicating that CMS protocol is able to interfere on the antioxidant defense system, here represented by VIT C, CAT and SOD, whose changes were effectively prevented by both forms of neonatal handling.

So far, we do not know the exact mechanism for this, but a similar result was clinically observed in patients with major depression, which presented high antioxidant enzymatic activity, including plasma SOD (Bilici et al., 2001). In general, antioxidant defenses appear increased in anxiety and depression (Hovatta et al., 2010), so it’s possible to suggest that in these situations, the higher production of ROS in brain tissues stimulates an increase of antioxidant enzymes activity, which can work effectively to remove them (Sahin & Gümüslü, 2004). Moreover, the increment in cortical levels of SOD suggests that CMS can increase the rate of O2–• formation and subsequently, H_2O_2 formation (Sahin & Gümüslü, 2004), which can be removed by CAT, whose activity was also increased in our study.

Stress is an environmental factor able to induce behavioral changes in individuals, whose activation of the HPA axis elevates glucocorticoid secretion from adrenals and promotes physiological changes that help the organism deal with stress (Shoji & Mizoguchi, 2010). In stressful events, excessive amounts of glucocorticoid 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 & Cullinan, 1997). In this sense, adrenal gland weight is considered as an indicator of stress (Heiderstadt et al., 2000) and the CMS protocol has been

reported to cause adrenal hypertrophy (Gouirand & Matuszewich, 2005). These reports were confirmed by the present study, when CMS exposure also caused hypertrophy of adrenals in UH animals, while both neonatal handling forms, TS and MS, were able to prevent this. This result is not considered as false-positive, mainly because CMS exposure did not change the final body weight of animals, either handled or not, confirming our findings. These results are in agreement with those of Zhang et al. (2009), who did not find alterations in body weight of stress-exposed animals either. Some studies showed an initial loss of body weight in stress-exposed rats (Matuszewich et al., 2007; Tõnissaar et al., 2008), but this effect was temporary due to adaptation of animals to the procedure (Tõnissaar et al., 2008), while others observed less weight gain by stress-exposed animals, whose protocol included food and water deprivation among stressors (Li et al., 2010), and therefore, do not contradict our findings. In line with this, our study also showed that TS *per se* decreased cortisol plasma levels, which remained decreased following exposure of the animals to the CMS protocol, although it was not sufficient to affect plasma cortisol levels in any group of handling. Studies demonstrated that handled animals have higher levels of glucocorticoid receptors in the hippocampus and frontal cortex (O'Donnell et al., 1994; Stamatakis et al., 2008), showing that their HPA axis feedback to glucocorticoids is enhanced, affording better response against stress (Pham et al., 1997). Therefore, neonatal handling, mainly TS, may be considered as a form of prevention against HPA axis imbalance caused by common stress situations in adulthood, observed in our study by adrenal weight changes and cortisol levels.

In summary, the present study indicated that whereas CMS protocol caused behavioral, biochemical and neuroendocrine changes in adult rats, different neonatal handlings, such as TS, and to a less extent MS, were able to improve animal ability to cope with stressful situations. In other words, adequate neonatal handling in early life may play an important preventive role on development of anxiety and depression, as well as their deleterious pro-oxidant effects in brain tissues, common in adulthood. To our knowledge, this is the first study that investigated preventive effects of neonatal handling on the oxidative insults and antioxidant defenses related to anxiety-like symptoms. Currently, we are studying the possible effects of neonatal handling on the neurobiological changes and their interaction with anxyolitic drugs**.**

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Declaration of interest

The authors report no conflicts of interest

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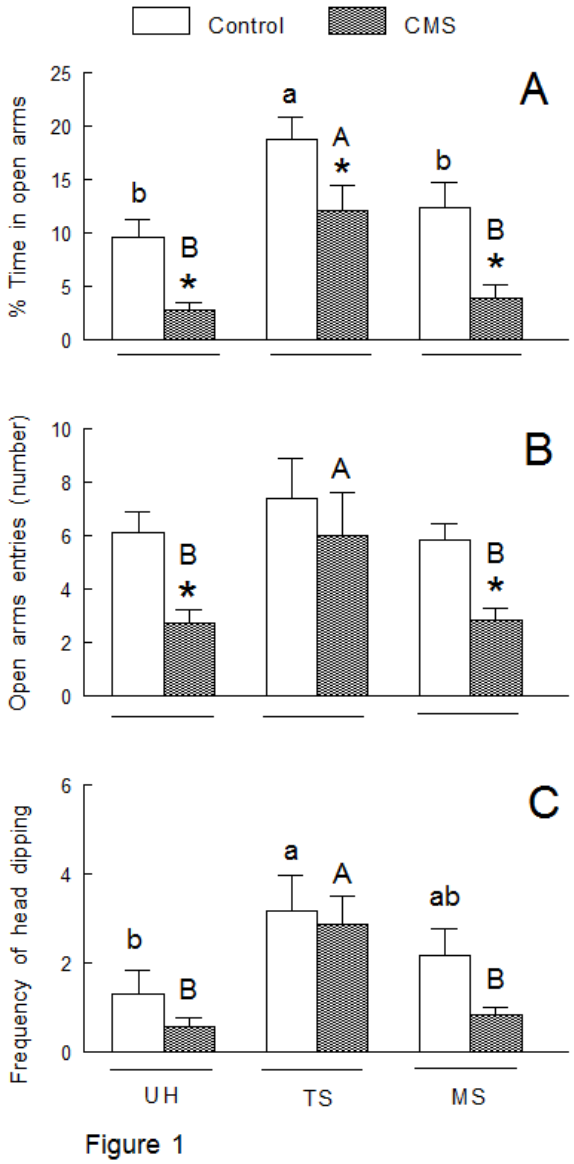

Figure 1. Effects of neonatal handlings on elevated plus maze (EPM) task, performed two days after the last exposure of adult rats to chronic mild stress (CMS).

(A) % of time spent in the open arms; (B) Number of entries in the open arms; (C) Frequency of head dipping. C- control; UH- unhandled; TS- tactile stimulation; MS- maternal separation. Data are expressed as mean±S.E.M. *Significant difference from C group at the same neonaltal handling (P<0.05). Different lowercase indicates significant differences between neonatal handlings of animals exposed to CMS protocol (P<0.05); Different uppercase indicates significant differences between neonatal handlings of control animals (non-exposed to CMS protocol) (P<0.05). (A) Main effect of handling [$F_{(2,36)}$=14.50; P<0.001] and stress [$F_{(1,36)}$=24.97; P<0.001]; (B) Main effect of handling [$F_{(2,36)}$=3.39; P<0.05] and stress [$F_{(1,36)}$=9.63; P<0.05]; (C) Main effect of handling [$F_{(2,36)}$=8.10; P<0.05].

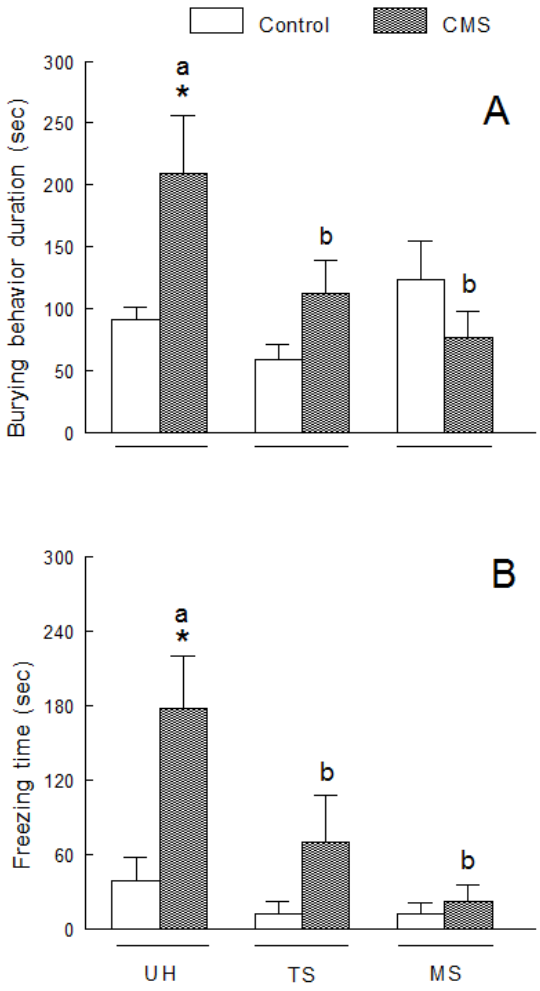

Figure 2

Figure 2. Effects of neonatal handlings on defensive burying test, performed four days after the last exposure of adult rats to chronic mild stress (CMS).

(A) Duration of burying behavior and (B) Freezing time. C- control; UH- unhandled; TS-tactile stimulation; MS-maternal separation. Data are expressed as mean±S.E.M. *Significant difference from C group at the same neonatal handling ($P<0.05$). Different lowercase indicates significant differences between neonatal handlings of animals exposed to CMS protocol ($P<0.05$). (A) Significant handling x stress interaction [$F_{(2,36)}=4.47$; $P<0.05$]; (B) Main effect of handling [$F_{(2,36)}=7.15$; $P<0.05$], stress [$F_{(1,36)}=11.78$; $P<0.05$] and handling x stress interaction [$F_{(2,36)}=3.43$; $P<0.05$].

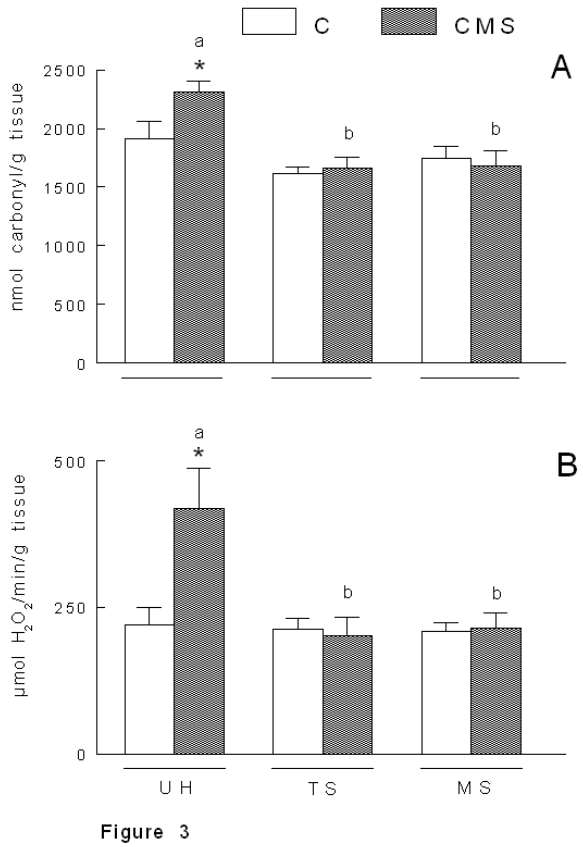

Figure 3. Effects of neonatal handlings on biochemical evaluations performed in hippocampus of rats submitted or no to chronic mild stress (CMS).

(A) protein carbonyl levels; (B) catalase activity. C- control; UH- unhandled; TS- tactile stimulation; MS- maternal separation. Data are expressed as mean±S.E.M. *Significant difference from C group at the same neonaltal handling (P<0.05). Different lowercase indicates significant differences between neonatal handlings of animals exposed to CMS protocol (P<0.05). (A) Main effect of handling [$F_{(2,36)}$=11.57; P<0.001] and tendency to handling x stress interaction (P=0.083); (B) Main effect of handling [$F_{(2,36)}$=6.07; P<0.05]; stress [$F_{(1,36)}$=4.83; P<0.05] and a significant handling x stress interaction [$F_{(2,36)}$=5.1; P<0.05].

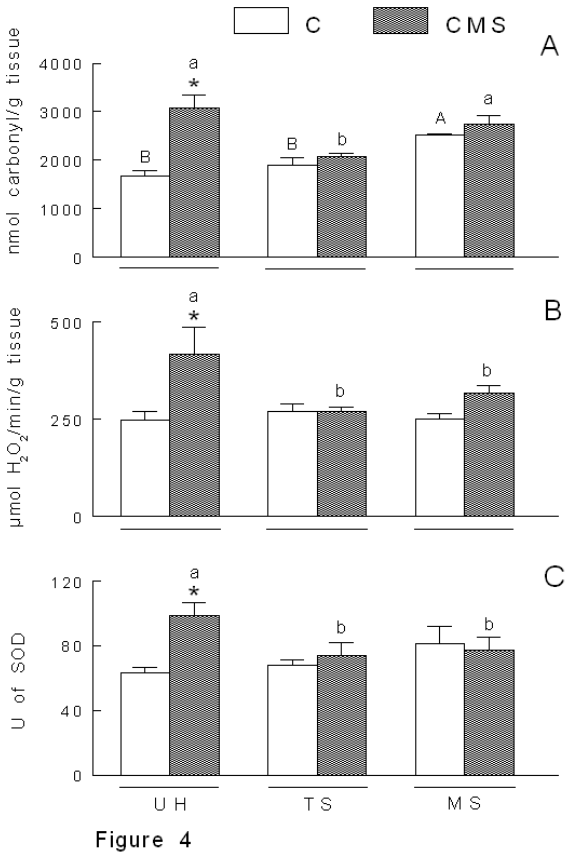

Figure 4. Effects of neonatal handlings on biochemical evaluations performed in cortex of rats submitted or no to chronic mild stress (CMS).

(A) protein carbonyl levels; (B) catalase activity; (C) superoxide dismutase levels. C- control; UH- unhandled; TS- tactile stimulation; MS- maternal separation. Data are expressed as mean±S.E.M. *Significant difference from C group at the same neonaltal handling (P<0.05). Different lowercase indicates significant differences between neonatal handlings of animals exposed to CMS protocol (P<0.05); Different uppercase indicates significant differences between neonatal handlings of control animals (no exposed to CMS protocol) (P<0.05). (A) Main effect of handling [$F_{(2,36)}$=8.31; P<0.05], stress [$F_{(1,36)}$=20.54; P<0.001] and a significant handling x stress interaction [$F_{(2,36)}$=9.08; P<0.001]; (B) Main effect of stress [$F_{(1,36)}$=8.44; P<0.05] and a significant handling x stress interaction [$F_{(2,36)}$=3.28; P<0.05]; (C) Main effect of stress [$F_{(1,36)}$=4.07; P<0.05] and a significant handling x stress interaction [$F_{(2,36)}$=3.85; P<0.05].

Table 1- Effects of neonatal handlings on sucrose preference of rats exposed to chronic mild stress (CMS).

Handling	Stress	Sucrose preference (%)
UH	C	97.15±2.33
	CMS	84.46±1.75*****
TS	C	90.21±3.26
	CMS	91.64±3.41
MS	C	91.00±4.55
	CMS	92.56±5.06

C=control; UH=unhandled; TS=tactile stimulation; MS=maternal separation.
Data are expressed as mean±S.E.M.
*****Significant difference from C group at the same neonatal handling (P<0.05).

Table 2- Effects of neonatal handlings on plasma levels of cortisol and vitamin C (VIT C), adrenal weight (AW), final body weight (BW) and adrenal weight/body weight ratio of rats exposed to chronic mild stress (CMS).

Handling	Stress	Cortisol (µg/dL)	Adrenals Weight (g)	Body Weight (g)	AW/BW (10^{-3})	VIT C (µg/mL plasma)
UH	C	7.16±0.42^{A}	0.050±0.001	330.71±6.47	0.159±0.008	24.64±1.85
	CMS	8.17±0.43^{a}	0.069±0.003*^{a}	342.14±10.69	0.198±0.007*^{a}	14.41±1.61*^{b}
TS	C	5.19±0.90^{B}	0.053±0.001	324.02±14.67	0.163±0.008	22.87±2.53
	CMS	5.53±0.33^{b}	0.053±0.005^{b}	337.00±6.70	0.162±0.014^{b}	22.22±2.06^{a}
MS	C	6.33±0.93^{AB}	0.049±0.002	327.28±5.40	0.154±0.008	25.21±1.66
	CMS	6.56±0.17^{ab}	0.056±0.002^{b}	330.01±6.39	0.169±0.008^{b}	25.30±2.09^{a}

C-control; UH-unhandled; TS-tactile stimulation; MS-maternal separation.

Data are expressed as mean±S.E.M. *Significant difference from C group at the same neonaltal handling (P<0.05). Different lowercase indicates significant differences between neonatal handlings of animals exposed to CMS protocol (P<0.05); Different uppercase indicates significant differences between neonatal handlings of control animals (P<0.05). Cortisol: Main effect of handling [$F_{(2,36)}$=7.26; P<0.05]; Adrenals weight: Main effect of handling [$F_{(2,36)}$=3.79; P<0.05]; stress [$F_{(1,36)}$=14.89; P<0.001] and a significant handling x stress interaction [$F_{(2,36)}$=5.41; P<0.05]; AW/BW: Main effect stress [$F_{(1,36)}$=6.77; P<0.05]; Vit C: Main effect of handling [$F_{(2,36)}$=3.56; P<0.05]; stress [$F_{(1,36)}$=4.44; P<0.05] and a significant handling x stress interaction [$F_{(2,36)}$=3.54; P<0.05].

Manuscrito 2

3.2 Neonatal tactile stimulation improves anxiety-like behavior and ameliorates the responsivity to diazepam

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Neonatal tactile stimulation improves anxiety-like behavior and ameliorates the responsivity to diazepam

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Abstract

In this study we evaluated the influence of neonatal tactile stimulation (TS) on behavioral effects related to a low dose of diazepam (DZP). Male pups were handled (TS) daily, from PND1 to PND21, for 10 min, while unhandled (UH) animals were not touched. In adulthood, half of the animals of each group receveid a single administration of diazepam (0.25 mg/kg body weight-i.p.) or vehicle, and were submitted to behavioral evaluations. In TS group, DZP administration reduced anxiety-like symptoms in different behavioral paradigms (elevated plus maze –EPM; staircase, open-field and defensive burying test –DBT) and increased exploratory behavior. These findings show that neonatal TS increased DZP pharmacological responses that were observed by reduced anxiety-like symptoms in relation to UH animals. Here we are showing by the first time that neonatal TS is able to change the sensitivity for benzodiazepine drugs and provide better pharmacological responses in novel situations.

Keywords: Neonatal handling, Tactile stimulation, Anxiety, Diazepam.

1. Introduction

Environmental changes originate individual adaptations, whose responses may generate a dysfunctional state of high anxiety and result in neuropsychiatric disorders (Salomons et al., 2010). These pathologies include insomnia, mood disorders, panic and others, often developed in high anxious persons who tend to present impaired adaptive capacity (Beck et al., 1985) and slow recovery after stressful situations (Hoehn-Saric and McLeod, 1988).

Clinical and preclinical evidence link GABAergic system dysfunctions in development of anxiety symptoms (Millan, 2003). Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the adult mammalian central nervous system (CNS), and is involved in regulation of physiological functions, emotion, cognition and behavior (Vekovischeva et al., 1999). Benzodiazepines (BZ) are a prototypical class of drugs widely prescribed to relieve anxiety symptoms (Carlini, 2003; Gallager and Primus, 1993; Woods et al., 1992), whose action mechanism consists in modulate the functionality of the GABAergic system and enhance chloride ion flux through $GABA_A$ receptors at a number of limbic areas (Caldji et al., 2003; Gonzalez et al. 1996; Pesold and Treit 1995; Thomas et al. 1985; Gray, 1987). Despite showing higher selectivity than others CNS depressant drugs, the continued use of high doses has been related to sedation, memory loss and amnesia (Woods et al., 1992), especially in older persons.

It is widely known that during early development, the CNS presents great plasticity and can be very sensitive to even moderate environmental interventions (Gschanes et al., 1998; Inazusta et al., 1999; Sternberg and Ridgway, 2003; Zhang and Cai, 2008). Some studies showed that behavioral and physiological processes at adult rodents can be a result from exposure to distinct stimuli during the first weeks of life (Casolini et al., 1997; Pham et al., 1999). Studies demonstrated that neonatal tactile stimulation (TS), as one kind of external sensory stimuli, influences physiological and behavioral processes, like acceleration of cortical neuron maturation (Schapiro and Vukovich, 1970), improvement of passive avoidance response in adulthood (Zhang and Cai, 2006) and increase of pups weight gain (Levine and Otis, 1958). Moreover, this neonatal handling is related to less hypothalamic–pituitary–adrenal (HPA) response to stressful situations, since handled animals have lower plasma corticosterone levels after novel situations (Levine et al., 1967; Meaney et al., 1991,1992). Furthermore, neonatal manipulations have been used to study neurobiological changes associated with psychiatric disorders (Cirulli et al., 2003).

Beyond of physiological, neuroendocrine and morfological effects of neonatal handling, some studies have shown that such stimulations during infancy cause significant changes on fear-related behavior in adult animals (Bodnoff et al., 1987; Hilakivi-Clarke et al., 1991; Núñez et al., 1996). Diferent studies also have reported less anxiety-behavior in the elevated plus-maze – EPM test (Fernandez-Teruel et al., 1990; Núñez et al., 1995; Vallee et al., 1997; Wakshlak and Weinstock, 1990). Furthermore, it has been shown that early-life experiences influence the development of the GABA complex in brain regions that mediate stress reactivity and change the expression of fearfulness in rats (Caldji et al., 2000; Giachino et al., 2007). So, it is possible to hypothesize that alteration in $GABA_A$ receptor density may affect the threshold for responsiveness to agonist drugs acting in the BZ site (Cirulli et al., 2010).

Since some studies demonstrated changes in the GABAergic system after neonatal handling and little is known about the possible influence of these manipulations with positive modulators of this system, more studies about this are necessary. So, we decided to investigate the influence of neonatal TS on behavioral parameters associated to diazepam (DZP) administration, a $GABA_A$ receptor agonist. Furthermore, we also investigated the effects of this association TS-DZP on development of anxiety-like symptoms that were observed in different behavioral paradigms.

2. Material and methods

2.1 Animals

Seven pregnant female Wistar rats from the breeding facility of Universidade Federal de Santa Maria (UFSM), RS, Brazil, were individually kept in plexiglas cages with free access to food and water in a room with controlled temperature (22–23°C) and on a 12 h-light/dark cycle with lights on at 7:00 a.m. All procedures were in accordance with the rules of the Committee on Care and Use of Experimental Animal of the UFSM, which follows international rules (NIH Publication No. 80-23; revised 1978).

2.2 Neonatal handling

At postnatal day (PND) 1 (PND1) male pups were randomly distributed to one of two experimental groups (n=14): unhandled (UH, not touched) and tactile stimulation (TS). Neonatal handling was applied from PND1 to PND21, between 1 and 3 p.m. Pups submitted to TS were removed from the nest, held gently by experimenter and stroked with the index

finger on the dorsal surface, in the rostral caudal direction, during 10 min (Rodrigues et al., 2004). The entire litter remained together with their mother until weaning and experimental groups consisted of pups from all litters. At PND22 the animals were weaned, separated by sex and left undisturbed until 70 days of age, when they were again subdivided into four experimental groups: UH + V (vehicle), UH + DZP (diazepam), TS + V and TS + DZP.

2.3 Drugs and experimental procedure

Diazepam (DZP) (Deg, São Paulo-SP, Brazil) was dissolved in physiological saline with one drop of Tween 80 (Sigma Aldrich, Brazil); vehicle (V) consisted of physiological saline containing one drop of Tween 80. Thirty minutes before behavioural evaluation, the animals received a single low dose of DZP (0.25 mg/kg body weight-i.p.) that was not related to locomotor alterations and was reached by pilot studies conducted in our laboratory (data not shown), or vehicle.

2.4 Behavioral testing

2.4.1 Elevated plus maze (EPM) test

This test 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×13.5 cm) that gave access to any of the four arms (Pellow et al., 1985). At beginning of each test, the rat was placed in the central platform facing an open arm and evaluated during 5 minutes. Behaviour scores comprised traditional spatiotemporal and ethological measures. Spatiotemporal measures were the number of entries and time spent in both arms of the maze (expressed as a percentage of the total test duration). Time spent in the arms and numbers of entries in open arms were used as measures of anxiety level (Hlavacova et al., 2010; Reis and Canto-de-Souza, 2008) and closed arms entries were used as index of locomotor activity (File, 1992; Lister, 1987; Rodgers and Dalvi, 1997). Ethological measures included frequency of head dipping (exploratory movement of head/shoulders over sides of the open arms and down towards the floor), stretched-attend postures (SAP; when the animal stretches to its full length with the forepaws and turns back to its original position without moving forward) and rearing. SAP are interpreted in terms of risk assessment and considered highly responsive for anxiety-like behavior assessment (Rodgers and Dalvi, 1997; Setem et al., 1999). These categories were defined according to previous studies (Albrechet-Souza et

al., 2007; Griebel et al, 2002; Hlavacova et al., 2010; Lepicard et al., 2000; Rodgers and Dalvi, 1997). The apparatus was cleaned with alcohol solution (20%) and paper towel before introduction of each animal.

2.4.2 Staircase test

This is a simple and rapid test used to study some components of exploratory behavior in rodents. The procedure was first proposed by Molinengo and Ricci-Gamalero (1970) and the apparatus comprised an enclosed staircase made of wood with five steps. Each step was 2.5 cm in height, 7.5 cm in length, and 10 cm in width, such that the staircase rose to a height of 12.5 cm at the top step. The total length of the apparatus was 45 cm and it was surrounded by walls 12.5 cm in height at one end, rising to 25 cm at the other. Rats were subsequently individually placed on the floor of the box, facing away from the stairs. The scores of rearing and number of steps climbed in a 3-min period were recorded (Thiébot et al., 1973). The apparatus was cleaned with alcohol solution (20%) and paper towel between each animal.

2.4.3 Open field

Behavioral measures relevant for rodent models can be assessed during exploration of an open field and the natural tendency of the animal in a new environment is to explore it, despite stress and conflict (Henderson et al., 2004). Each rat was individually placed for 5 min in the center of the open-field arena (40x40x30 cm) subdivided into nine equal squares, as described elsewhere (Kerr et al., 2005). The number of crossings (horizontal squares crossed) and rearings (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 (Henderson et al., 2004). The apparatus was cleaned with alcohol solution (20%) and paper towel between each animal.

2.4.4 Defensive burying behavior

Anxiety-like behavior following a single shock from a novel object, a shock probe, can be assessed in this test (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, Pinel and Fibiger, 1981). Each animal was placed individually into the testing apparatus facing away from the shock probe for a 10 min test (Gutiérrez-García et al., 2006). When the animal received a shock by making contact with the

schock probe, current was terminated so as not to provide additional shocks. After 10 min, the animal was removed and returned to his home cage, apparatus was cleaned and new bedding was placed into the cage for next rat. Duration of burying behavior was measured and this conduct was defined as any spraying or throwing of the bedding with the head or forepaws in the direction of the shock probe. In addition, the duration of immobility was assessed (standing on four feet with body and head motionless) (Matuszewich et al., 2007).

2.5 Statistical analysis

Results were expressed as mean ± S.E.M. Data were analyzed by Two-way ANOVA followed by Duncan's Post Hoc tests when appropriated. (*Statistica* software package for Windows version 8.0 was used). Values of $P<0.05$ were considered statistically significant for all comparisons made.

3. Results

3.1 Elevated plus maze (EPM)

Duncan's test showed that TS increased *per se* open arms entries number in relation to UH animals. DZP administration increased both entries number and time spent in open arms of animals exposed to TS, but not in UH animals. Rats submitted to TS and treated with DPZ also spent less time in closed arms, when compared to UH + DZP group. This result cannot be considered false-positive, mainly because the animals of both experimental groups showed similar entries number in closed arms that are indicative of locomotor activity in EPM (File, 1992; Lister, 1987; Rodgers and Dalvi, 1997). DZP and TS modified not only the classical spatiotemporal measures of anxiety, but also the ethological parameters related to exploration and risk assessment behavior in a manner consistent with an anxiolytic outcome. TS group *per se* showed higher head dipping number compared to UH group. Regarding DZP treatment, TS animals treated with DZP presented increased head dipping number compared to UH + DZP group. DZP reduced frequency of SAP in TS group and TS animals treated with DZP showed reduced SAP compared to UH + DZP group. Moreover, DZP increased rearing number in handled animals, and this experimental group presented higher rearing number than no handled and treated with DZP rats (Table 1).

3.2 Staircase test

Duncan's test showed that TS group *per se* presented higher number of steps climbed in relation to UH group. DZP increased this parameter in UH animals, but TS animals treated with DZP presented higher number of stairs climbed compared to UH + DZP group. DZP treatment increased rearing number only in TS group (Figure 1).

3.3 Open field

Post hoc test showed that TS group *per se* showed higher crossing number in relation to UH group. Regardinz DZP treatment, it increased rearing number and central squares crossings in TS group. TS animals treated with DZP presented higher crossing, rearing and central squares numbers compared to UH + DZP group (Figure 2).

3.4 Defensive burying

Duncan's test showed no differences in burying behavior duration, indicating that the different treatments did not changed this behavioral parameter. In relation to immobility or freezing time, TS reduced *per se* this behavior when compared to UH animals. Furthermore, animals submitted to TS and treated with DZP showed lower immobility time in relation to UH animals treated with DZP (Figure 3).

4. Discussion

It is well known that early manipulations of the infant-mother interaction can induce neurochemical, physical and psychological changes in the offspring (Cirulli et al., 2003; Imanaka et al., 2008; Pryce and Feldon, 2003; Weaver et al., 2004) and might result in prolonged behavioral effects in adulthood (Cameron et al., 2005; Giachino et al., 2007). Mild and brief (3-15 min) neonatal daily manipulations exert persistent effects on behavior of adults including decreased fearfulness to novelty and reduced emotionality (Bodnoff et al., 1987; Denenberg, 1964; Levine, 1957, 1962), enhanced curiosity and exploratory behavior (Caldji et al., 2000; Denenberg and Smith, 1963; Levine, 1960). Animals handled during neonatal development also have shown beneficial changes in the functionality of the HPA axis (Levine, 1957) and so, their adaptation to novel and/or stressful stimuli can be significantly increased (Meaney et al., 1991). These beneficial effects of neonatal handling may be explained by early maturation of neural pathways from skin to CNS (Montagu, 1953), and also by changes on maternal behavior patterns (Cirulli et al., 2000, Levine, 1994) since mothers of handled pups exhibit increased levels of licking/grooming and arched-back

nursing behavior (Caldji et al., 1998; Lee and Williams, 1975; Liu et al., 1997; Pryce et al., 2001). Moreover, handled animals also may present important alterations in those neurotransmitter systems involved in the regulation of emotionality, such as the GABAergic one (Giachino et al., 2007). Most of the brain $GABA_A$ receptor alterations in rat occur during early-life period, prior to 20 days of age (Laurie et al., 1992). It has been previously shown that neonatal handling increases GABAergic interneurons densities in hypothalamus and amygdale that are brain regions related to stress response and emotional behavior (Giachino et al., 2007). In addition, high levels of maternal care also were related to increase of $GABA_A$/benzodiazepine receptor density in *locus coeruleus* of the offspring (Caldji et al., 2000, 2003).

In the present study, we evaluated the influence of neonatal TS on anxiety, locomotor and exploratory behaviors of adult rats treated with a low dose of a $GABA_A$ receptor agonist (DZP) in comparison to UH animals. Exposure of rodents to a novel ambient can induce both exploratory and fear behaviors, thus creating an approach-avoidance conflict on animals (Montgomery, 1955). EPM test is based on the conflict between an innate aversion to exposed spaces and a tendency for rodents to explore any new environment (Lister, 1987). In our findings, rats submitted to TS and treated with DZP showed a greater ability to overcome the natural fear and exploit this new environment, which was evidenced by higher time spent and greater entries number in open arms, as well as by lower time spent in closed arms. In this sense, we can hypothesize that TS was able to modify the pharmacological response to DZP, since animals subjected only to neonatal handling (without DZP) presented less behavioral benefits.

DZP treatment, when associated to neonatal TS, reduced anxiety-like symptoms by modifying the classical spatiotemporal measures of anxiety, and also the ethological parameters related to exploration and risk assessment behavior. The risk assessment, mainly observed by SAP movements in EPM (Lepicard et al., 2000; Rodgers and Johnson, 1995; Shepherd et al., 1994), is positively correlated with anxiety-like behavior and is consider one of the parameters more responsive for assessment of this behavior in EPM (Rodgers and Dalvi, 1997; Setem et al., 1999). Our findings clearly showed lower SAP frequency of rats submitted to TS and treated with DZP, confirming that risk assessment behaviors are sensitive to anxiolytic drugs (Cole and Rodgers, 1993; Handley, 1991). In line with this, full agonists of BZ receptors, like chlordiazepoxide and diazepam, typically increased exploratory head dipping of rodents (Cole and Rodgers, 1993, 1995), and this effect we also observed in rats handled and treated with DZP. With respect to exploratory behavior, inhibition of this

conduct is often related to high emotionality or anxiety (Archer, 1973). Furthermore, studies showed that some anxiolytic drugs at low doses increased the number of steps climbed and rearing in the staircase test (Jacobson and Cryan, 2008; Lepicard et al., 2000; Stéru et al., 1987). In fact, our findings showed that animals handled and treated with DZP presented higher exploratory behavior observed by rearing number in EPM, open field and staircase test, as well as greater number of stairs climbed in staircase test.

A recent study showed that increased locomotor activity in animals may reflect an anxiolytic status (Shoji and Mizoguchi, 2010), while a decrease in central squares crossed in open field can demonstrate increased anxiety-like behavior (File, 1997). Since exploratory behavior is hard to dissociate from general and locomotor activity, both types of conduct had to be considered in experimental studies (Lepicard et al., 2000). Here, TS increased crossing number in the open field, revealing increased locomotor activity. On the other hand, only animals submitted to TS and treated with DZP showed increased rearing and number of central squares crossed, revealing higher exploration and lower fear symptoms, respectively.

With respect to DBT, the increased anxiety-like behavior in this test can be observed through of two types of strategies: the active behavior associated with burying the shock probe and the passive behavior associated with immobility or freezing (De Boer and Koolhaas, 2003; Matuszewich et al., 2007). Our study demonstrated differences in freezing time, which is a passive behavior that reflects extreme anxiety (De Boer and Koolhas, 2003; Saavedra et al., 2006). Our results showed that TS decreased immobility behavior in both groups treated or not with DZP, whose animals explored more the apparatus and presented a quieter behavior during the test.

Taken together, animals submitted to TS and treated with DZP presented reduced anxiety-like behavior in all behavioral assessments, increased locomotion and exploration, confirming that this neonatal handling was able of favorably modify the anxyolitic effects of a low dose of DZP. On the other hand, in UH animals, this same dose of DZP was probably not enough to improve the effects of a novel environment. Our results are in line with those showing that neonatal handling alters GABAergic system (Caldji et al., 2000; Giachino et al., 2007) and confirm that this handling affects the responsiveness to BZ agonists, like proposed by Cirulli et al. (2010). A study of Bodnoff et al. (1987) showed that neonatal handling was related to increased levels of forebrain central benzodiazepine (CBZ) receptor, which is a component of the $GABA_A$ receptor complex (Caldji et al., 2000). When an agonist binds to the CBZ site, the $GABA_A$ receptor enhance his affinity for GABA, and thus, CBZ activation is related to increased inhibition of fear and anxiety mediated by GABAergic system (Caldji

et al., 2000). At this time it is possible to hypothesize that neonatal TS is related to less emotionality of adult animals exposed to novel environments. Moreover, these findings are also consistent with human data showing decreased CBZ receptor sensitivity in panic disorder patients (Roy-Byrne et al., 1996).

5. Conclusion

In summary, the present study indicated that a reduced dose of DZP was able to prevent just a minor anxiety-like behavior in UH animals, but when associated with neonatal TS, this low dose showed clear and relevant anxiolytic effects, as demonstrated in all behavioral assessments. Our date suggest that an adequate neonatal handling in early life provides better responses in novel situations and environments and also allows reduction in BZ doses, if such pharmacological intervention is required. In future studies, we intend to investigate the influence of TS on the interaction GABA-BZ receptor, as well as the number of connections and their consequent hyperpolarization.

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Table 1 - Effects of neonatal handlings on elevated plus maze task performed in adult rats.

Behavior	UH+V	UH+DZP	TS+V	TS+DZP
% time in OA	5.35±2.4	5.00±1.4^{b}	5.33±1.5	18.8±4.6*^{a}
% time in CA	78.61±3.7	81.09±2.1^{a}	69.83±5.8	65.26±2.3^{b}
OA entries number	1.16±0.4^{B}	2.66±0.4^{b}	4.16±0.4^{A}	6.83±1.5*^{a}
CA entries number	7.16±1.0	6.85±0.4	6.71±0.6	6.66±0.9
Rearing number	14.42±0.3	14.85±1.0^{b}	13.85±0.8	18.00±1.0*^{a}
Head dipping number	2.57±0.7^{B}	2.14±0.8^{b}	6.0±1.4^{A}	8.33±1.6^{a}
SAP	9.00±0.8	10.00±0.9^{a}	10.00±0.4	5.42±0.7*^{b}

UH=unhandled; TS=tactile stimulation; V=vehicle; DZP=diazepam; OA=open arms; CA=closed arms; SAP=stretched-attend postures.
Data are expressed as mean±S.E.M., (n=7)
*****Significant differences from vehicle at the same neonatal handling (P<0.05).
Different lowercase indicates significant differences between neonatal handlings of animals treated with diazepam (P<0.05); Different uppercase indicates significant differences between neonatal handlings of animals treated with vehicle (P<0.05).

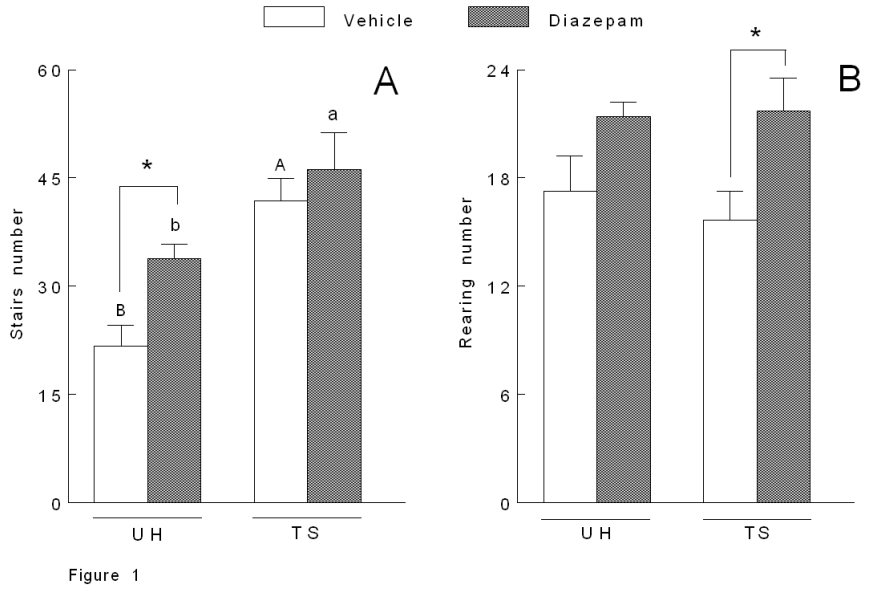

Figure 1. Effects of neonatal handling on staircase test.

(A) Number of stairs climbed (Effect of handling and DZP [F(1,22)=21.7, *P*<0.001 ; 5.6; *P*<0.05]); (B) Rearing number (Effect of DZP [F(1,22)=9.9; *P*<0.05]). UH- unhandled ; TS- tactile stimulation. Data are expressed as mean±S.E.M. *Significant difference from vehicle group at the same neonaltal handling (*P*<0.05). Different lowercase indicates significant differences between neonatal handlings of animals treated with diazepam (*P*<0.05); Different uppercase indicates significant differences between neonatal handlings of animals treated with vehicle (*P*<0.05).

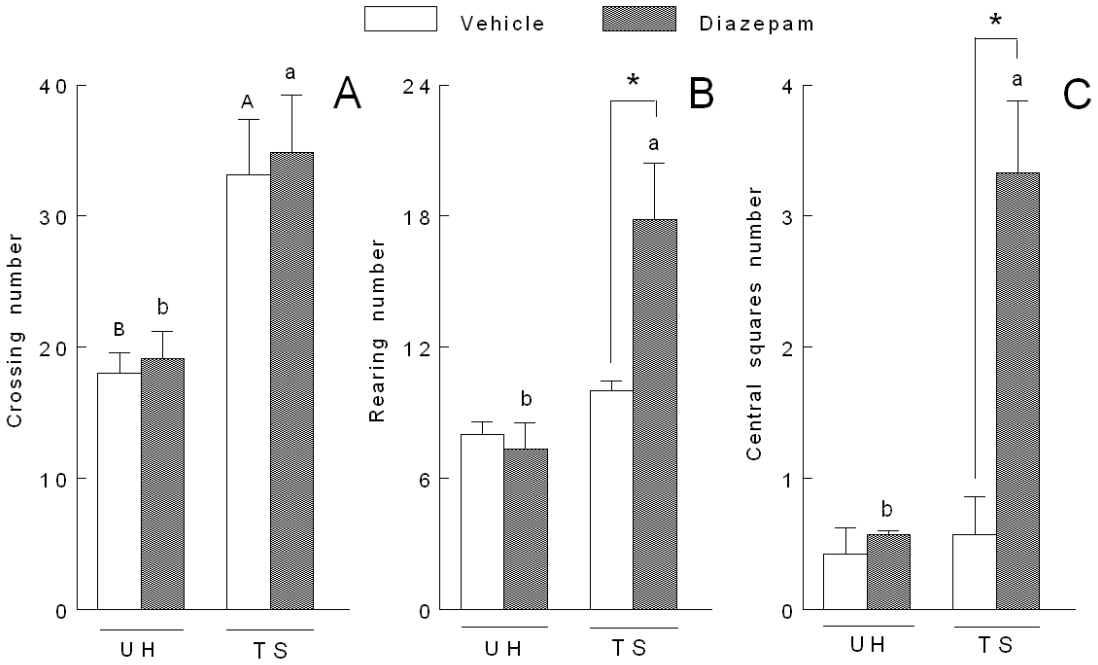

Figure 2. Effects of neonatal handlings on open field test.

(A) Number of crossings (Effect of handling [F(1,22)=20,4; *P*<0.001]); (B) Number of rearings (Effect of handling, DZP and handling x DZP interaction [F(1,22)=19.3; 6.35; and F(2,22)=8.9; *P*<0.05, respectively]). (C) Number of central squares crossed (Effect of handling, DZP and handling x DZP interaction [F(1,22)=17.6; 17.6; and F(2,22)=14.3; *P*<0.001]). UH- unhandled; TS- tactile stimulation. Data are expressed as mean±S.E.M. *Significant difference from vehicle group at the same neonaltal handling (*P*<0.05). Different lowercase indicates significant differences between neonatal handlings of animals treated with diazepam (*P*<0.05); Different uppercase indicates significant differences between neonatal handlings of animals treated with vehicle (*P*<0.05).

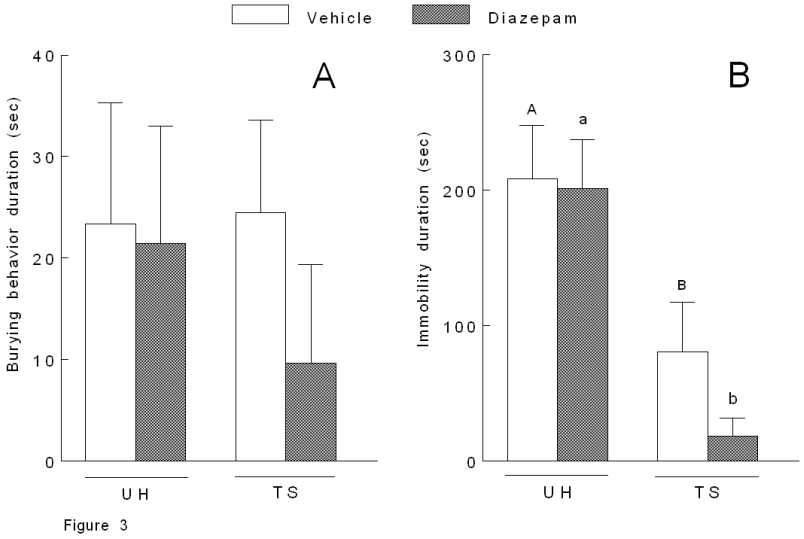

Figure 3. Effects of neonatal handlings on defensive burying test.

(A) Duration of burying behavior; (B) Duration of immobility behavior (Effect of handling [$F(1,22)=23.6$; $P<0.001$]). UH- unhandled; TS-tactile stimulation. Data are expressed as mean±S.E.M. Different lowercase indicates significant differences between neonatal handlings of animals treated with diazepam ($P<0.05$); Different uppercase indicates significant differences between neonatal handlings of animals treated with vehicle ($P<0.05$).

4 DISCUSSÃO

Os dados obtidos no presente estudo servem para confirmar os efeitos benéficos do manuseio neonatal, especialmente na forma de ET. Além de melhorar a habilidade para enfrentar situações estressantes, a ET foi capaz de reduzir comportamentos ansiosos ao ser associada a uma baixa dose de diazepam, o que demonstra que um adequado manuseio neonatal pode influenciar a densidade dos receptores $GABA_A$ e/ou aumentar a resposta a substâncias benzodiazepínicas na vida adulta.

Algumas experiências no início da vida são críticas para o desenvolvimento normal da maioria dos sistemas funcionais do organismo, os quais formarão a base para o comportamento e respostas a determinados estímulos na idade adulta (BARNETT; BURN, 1967; BEACH; JAYNES, 1954; FLEMING; O'DAY; KRAEMER, 1999; HOFER, 1994; LEHMANN; FELDON, 2000; LEVINE et al., 1967). Uma experiência positiva nesse período é o manuseio neonatal, que consiste em uma variedade de estímulos sensoriais capazes de influenciar o desenvolvimento do SNC (ZHANG; CAI, 2008), melhorar o desenvolvimento do sistema neuroendócrino (VAN OERS et al., 1998) e causar alterações em vários processos comportamentais e fisiológicos, entre eles, a resposta ao estresse (CASOLINI et al., 1997; PHAM et al., 1999).

Por outro lado, a exposição ao estresse em qualquer período da vida altera o funcionamento normal do organismo. Agentes estressores são normalmente considerados estímulos que perturbam a homeostasia corporal, ou o equilíbrio dinâmico do organismo, cuja intensidade e persistência aumentam a probabilidade de serem prejudiciais (SELYE, 1976; VAN de KAR; BLAIR, 1999). Um resultado potencialmente negativo associado à exposição ao estresse crônico é o desenvolvimento de desordens psicológicas, entre elas, ansiedade, desordens psicoafetivas e depressão (RAY et al., 2004), além de mudanças em padrões neuroquímicos no organismo (HARRO; ORELAND, 2001). Nossos resultados estão de acordo com esses dados, uma vez que a exposição ao estresse na vida adulta promoveu várias alterações prejudiciais nos animais.

O estresse crônico é um fator ambiental capaz de induzir alterações no eixo hipotálamo-pituitária-adrenal, as quais estão relacionadas com a ocorrência de desordens do humor (VEDDER et al., 2007). Nossos resultados demonstraram um aumento no peso das adrenais nos animais expostos ao estresse, o que reflete uma maior atividade no eixo

hipotálamo-pituitária-adrenal nesses animais e está de acordo com outro estudo (MUSCAT; WILLNER, 1992) que também relatou maior peso das adrenais após exposição ao estresse. Por outro lado, as duas formas de manuseio neonatal preveniram a hipertrofia adrenal induzida pelo estresse, o que pode estar relacionado aos melhores padrões comportamentais observados nos animais manuseados. Adicionalmente, os animais do grupo ET apresentaram menores níveis de cortisol plasmático em relação aos animais não manuseados antes e após exposição ao estresse.

O modelo de estresse crônico e moderado também tem sido relacionado a um aumento na produção de espécies reativas de oxigênio (ZHANG et al., 2009). Quando essas espécies excedem a capacidade de neutralização do organismo, podem causar danos teciduais (ZHANG et al., 2009), além de estarem relacionadas a diversas doenças entre elas, ansiedade e depressão (EREN; NAZIROGLU; DEMIRDAS, 2007; HOVATTA; JUHILA; DONNER, 2010). Neste sentido, o estresse aumentou a oxidação de proteínas no córtex e hipocampo dos animais e este resultado está de acordo com o estudo de Lucca et al. (2009), o que reforça o envolvimento dos danos oxidativos com eventos estressantes. A exposição ao estresse também pode causar alterações nos perfis antioxidantes, como demonstrado em nosso estudo pelos menores níveis de vitamina C plasmáticos e maiores níveis da enzima superóxido dismutase no córtex, além de maior atividade da enzima catalase no hipocampo e córtex dos animais. Uma vez que a atividade enzimática apresentou-se elevada em diferentes estudos com ratos expostos ao estresse (BONDARENKO et al., 1999; SAHIN; GÜMÜSLÜ, 2004), é possível sugerir que uma maior atividade enzimática seria uma tentativa do organismo em neutralizar os níveis elevados de espécies reativas de oxigênio presentes em situações de estresse (SAHIN; GÜMÜSLÜ, 2004). Em relação às defesas antioxidantes não-enzimáticas, a vitamina C é um capturador de radicais livres de amplo espectro e apresenta uma importante função detoxificante em compartimentos aquosos do organismo (PACKER; SLATER;WILSON, 1979), além de regenerar a vitamina E e impedir a peroxidação de lipídios plasmáticos (PADH, 2005). Estudos demonstraram uma redução nos níveis sanguíneos (HOVATTA; JUHILA; DONNER, 2010) e cerebrais (EREN; NAZIROGLU; DEMIRDAS, 2007) de defesas antioxidantes não-enzimáticas em situações de ansiedade e depressão (HOVATTA; JUHILA; DONNER, 2010) ou exposição ao modelo de estresse crônico e moderado (EREN; NAZIROGLU; DEMIRDAS, 2007). Uma vez que as doenças do humor geralmente estão relacionadas a alterações nas defesas antioxidantes (HOVATTA; JUHILA; DONNER, 2010), o papel protetor do manuseio neonatal em relação a essas defesas

pode ter contribuído com os melhores parâmetros comportamentais observados nesses animais.

Em relação aos testes comportamentais, o teste de preferência pela sacarose pode fornecer dados importantes a respeito da sensibilidade à recompensa, uma vez que a redução no consumo de sacarose reflete um estado anedônico dos animais (WILLNER, 1985) e pode ser utilizado como um índice de eficácia do modelo de estresse crônico e moderado (WILLNER, 2005). A anedonia é definida como uma diminuição no interesse ou perda da reatividade a estímulos agradáveis (AMERICAN PSYCHIATRIC ASSOCIATION, 1994) e é característica de pacientes depressivos (WILLNER, 1985). Nosso estudo demonstrou que os animais expostos ao estresse apresentaram redução na preferência pela sacarose, o que comprova que o protocolo foi adequado. Por outro lado, ambas as formas de manuseio neonatal, ET e SM, preveniram a redução na preferência pela sacarose induzida pelo estresse, o que demonstra os benefícios do manuseio neonatal em relação às desordens do humor, da mesma forma que o estudo de Anisman et al. (1998), o qual relatou aumento nos níveis de serotonina no hipocampo em animais manuseados precocemente (ANISMAN et al., 1998).

A exposição ao estresse também produziu sintomas de ansiedade nos animais não manuseados no labirinto em cruz elevado e teste defensivo de cavocar, o que confirma que a exposição de roedores a esse modelo de estresse pode resultar em perfis comportamentais indicativos de psicopatologias humanas (BUWALDA et al., 2005; D'AQUILA; BRAIN; WILLNER, 1994; WILLNER, 1984; ZURITA et al., 2000). Além disso, esses resultados estão de acordo com outros estudos que demonstraram aumento dos comportamentos de ansiedade após exposição ao estresse crônico e moderado (GRIEBEL et al., 2002; LUJÁN et al., 2008; MATUSZEWICH et al., 2007; TANNENBAUM et al., 2002; TÕNISSAAR et al., 2008; ZURITA et al., 2000). Por outro lado, ambas as formas de manuseio neonatal preveniram os comportamentos de ansiedade no teste defensivo de cavocar, o que confirma a melhor capacidade dos animais manuseados em enfrentarem situações estressantes (CASOLINI et al., 1997; MEANEY et al., 1991; PHAM et al., 1999). No entanto, apenas a ET foi capaz de prevenir os sintomas de ansiedade no labirinto em cruz elevado, o que demonstra os maiores benefícios dessa forma de manuseio neonatal. Adicionalmente, a ET foi capaz de reduzir comportamentos de ansiedade em vários testes comportamentais, ao ser associada a uma dose baixa de diazepam. Essa forma de manuseio também aumentou a atividade exploratória dos animais, o que está de acordo com os resultados dos comportamentos de ansiedade, uma vez que a inibição da exploração está relacionada a níveis elevados de ansiedade (ARCHER, 1973). Uma vez que nos animais não manuseados a injeção

de diazepam apenas causou alteração na atividade exploratória em um dos testes comportamentais, nossos resultados demonstraram que a ET foi efetivamente capaz de reduzir comportamentos de ansiedade em associação a uma dose baixa de diazepam, enquanto nos animais não manuseados, essa dose provavelmente não foi suficiente para produzir respostas comportamentais satisfatórias. Além dos benefícios da ET em associação ao diazepam, os animais do grupo ET tratados com veículo também apresentaram melhores padrões comportamentais em relação aos animais não manuseados e tratados com veículo em alguns parâmetros dos testes comportamentais. Isso demonstra um efeito *per se* da ET e está de acordo com os resultados do primeiro experimento em relação aos benefícios da ET sobre parâmetros de ansiedade.

Os resultados da associação da ET com diazepam confirmaram a hipótese de Cirulli et al. (2010), o qual propôs que o manuseio neonatal poderia afetar a resposta de agonistas atuantes no sítio benzodiazepínico, uma vez que estudos descreveram um aumento dos receptores do ácido gama-aminobutírico (GABA) (CALDJI et al., 2000; ESCORIHUELA et al., 1992; GIACHINO et al., 2007) e de benzodiazepínicos (CALDJI et al., 2000; ESCORIHUELA et al., 1992) no cérebro de animais manuseados. Nesse sentido, nosso estudo demonstrou resultados interessantes e inovadores em relação à associação da ET neonatal com uma dose baixa de diazepam em ratos adultos e demonstra que um adequado manuseio neonatal poderia permitir a redução na dose de certos medicamentos ansiolíticos, caso esse tratamento fosse necessário. Sabe-se que os medicamentos benzodiazepínicos são amplamente prescritos para o tratamento da ansiedade (CARLINI, 2003; GALLAGER; PRIMUS, 1993; WOODS, KATZ, WINGER, 1992), mas podem causar efeitos indesejados, tais como sedação, miorrelaxamento, amnésia e inclusive, dependência (ALLISON; PRATT, 2003; LADER, 1994). Dessa maneira, reduções nas doses terapêuticas são interessantes para minimizar esses potenciais efeitos colaterais.

Nossos resultados demonstraram que o manuseio neonatal é capaz de promover benefícios em longo prazo, uma vez que as avaliações comportamentais e bioquímicas foram realizadas na vida adulta dos animais. Outros estudos demonstraram que os efeitos do manuseio neonatal persistem durante a maturidade, uma vez que animais manuseados precocemente apresentaram menor perda de neurônios piramidais e receptores de glicocorticóides no hipocampo e demonstram melhores resultados no aprendizado espacial em relação a animais não manuseados (MEANEY et al., 1988). Dessa forma, o manuseio precoce previne a perda de neurônios no hipocampo, o aumento nos níveis basais de glicocorticóides e a redução nos receptores de glutamato tipicamente associados a animais senescentes ou

cronicamente estressados (KOSTEN; LEE; KIM, 2007; PHAM et al., 1997; SAPOLSKY, 1992; SAPOLSKY; KREY; McEWEN, 1985; STAMATAKIS et al., 2008).

Os inúmeros benefícios promovidos pelo manuseio neonatal podem estar relacionados ao aumento do cuidado materno (CALDJI et al., 1998; LEE; WILLIAMS, 1975; LIU et al., 1997; PRYCE; BETTSCHEN; FELDON, 2001) que ocorre após breves períodos de separação dos filhotes. Além disso, as vias neurais da pele ao SNC sofrem maturação precoce em relação aos outros sistemas sensoriais (MONTAGU, 1953), o que pode explicar os maiores benefícios obtidos com a ET, uma vez que essa forma de manuseio confere um estímulo tátil adicional promovido pelo experimentador.

Estudos realizados em animais têm demonstrado que a ET apresenta inúmeros benefícios, tanto em aspectos comportamentais como morfológicos (CHOU et al., 2001; FERNÁNDEZ-TERUEL et al., 1992), assim como a terapia através da massagem utilizada em humanos (FIELD, 1998). Em bebês, a massagem promove um adequado ganho de peso (VICKERS et al., 2004), reduz o estresse pós-natal, melhora o crescimento e desenvolvimento do recém-nascido e aumenta a mineralização óssea em bebês pré-termo (FIELD, 2002; FIELD et al., 2006; HERNANDEZ-REIF; DIEGO; FIELD, 2007; MOYER-MILEUR et al., 1995, SCAFIDI; FIELD; SCHANBERG , 1993).

De modo geral, pode-se concluir que o manuseio neonatal apresentou inúmeros efeitos benéficos, uma vez que melhorou a capacidade comportamental, endócrina e bioquímica dos animais em lidarem com situações de estresse crônico na vida adulta. Além do mais, a ET mostrou-se efetiva em reduzir comportamentos de ansiedade ao ser associada a uma baixa dose de diazepam, a qual não foi capaz de produzir efeitos significativos nos animais não manuseados. Dessa maneira, podemos sugerir que a ET neonatal pode aumentar a sensibilidade do receptor GABA às substâncias benzodiazepínicas. Tendo em vista a escassez de estudos nessa área e a partir dos dados promissores da ET associada ao diazepam, estudos sobre os efeitos do manuseio neonatal em associaçao a outras substâncias psicoativas poderão ser interessantes.

5 CONCLUSÕES

Através dos resultados experimentais obtidos podemos chegar às seguintes conclusões:

1. A exposição dos animais ao ECM reduziu a preferência pela sacarose e aumentou os sintomas de ansiedade no labirinto em cruz elevado (LCE) e teste defensivo de cavocar (TDC). Por outro lado, ambas as formas de manuseio neonatal preveniram a redução na preferência pela sacarose e comportamentos de ansiedade no TDC, entretanto, apenas a ET foi capaz de prevenir os sintomas de ansiedade no LCE.

2. A exposição dos animais ao ECM causou hipertrofia das glândulas adrenais, demonstando maior atividade do eixo HPA. Ambas as formas de manuseio neonatal preveniram esse aumento. Adicionalmente, a ET foi associada a menores níveis de cortisol plasmático em relação aos animais não manuseados antes e após exposição ao ECM.

3. A exposição dos animais ao ECM reduziu os níveis de vitamina C plasmáticos e causou danos oxidativos e alterações nas enzimas antioxidantes em hipocampo e córtex. Pelo contrário, ambas as formas de manuseio neonatal preveniram a redução dos níveis de vitamina C plasmática, o dano oxidativo hipocampal e as alterações enzimáticas cerebrais. Apenas a ET foi capaz de prevenir os níveis aumentados de oxidação de proteínas no córtex.

4. De uma maneira geral, a ET foi o manuseio neonatal que apresentou os melhores benefícios.

5. Em animais não manuseados, o tratamento com diazepam apenas aumentou a atividade exploratória no teste da escada.

6. Já nos animais do grupo ET, o tratamento com diazepam reduziu comportamentos de ansiedade no LCE, teste da escada, campo aberto e TDC e aumentou a atividade exploratória no LCE, teste da escada e campo aberto.

7. O grupo da ET tratado com diazepam demonstrou menor ansiedade em relação ao grupo não manuseado tratado com diazepam em todos os testes comportamentais, maior atividade exploratória no LCE, teste da escada e campo aberto, além de maior atividade locomotora em campo aberto.

8. Um adequado manuseio neonatal nos estágios iniciais do desenvolvimento, como a ET, promove melhores respostas em situações e ambientes novos. Além disso, é capaz de alterar a resposta ao diazepam, possivelmente por alterar a sensibilidade do complexo GABA a essa substância.

6 PERSPECTIVAS

Investigar os mecanismos de ação da ET neonatal em nível central, através de estudos relacionados à interação com o receptor GABA-BDZ e possíveis alterações na sensibilidade a substâncias atuantes nesses sítios.

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