UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS - NEUROCIÊNCIAS

SEPARAÇÃO MATERNA COMO POSSÍVEL FATOR DE RISCO PARA O DESENCADEAMENTO DO TRANSTORNO DE ESTRESSE PÓS-TRAUMÁTICO – UM MODELO EM ANIMAIS DE EXPERIMENTAÇÃO

Dissertação de Mestrado

Luisa Amália Diehl

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“A sabedoria da vida não está em fazer aquilo que se gosta, mas gostar daquilo que se faz.”

(Leonardo da Vinci)

Aos meus pais,
Carlos e Isolde.

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Ao meu namorado, Thi...
...sem palavras, somente amor...

SUMÁRIO

LISTA DE ABREVIATURAS

ACTH.....Hormônio Adrenocorticotrópico

ADN.....Ácido Desoxirribonucléico

ARN.....Ácido Ribonucléico

AVP.....Arginina-Vasopressina

BDNF..... Fator Neurotrófico Derivado do Encéfalo

CAT.....Catalase

CRH.....Hormônio Liberador da Corticotropina

CSF..... *Cerebrospinal Fluid*

DNA.....*Deoxyribonucleic Acid*

EROs.....Espécies Reativas de Oxigênio

GCs.....Glicocorticóides

GPx.....Glutationa Peroxidase, *Glutathione Peroxidase*

HHA.....Eixo Hipotálamo-Hipófise-Adrenal

MS.....*Maternal Separation*

TEPT.....Transtorno de Estresse Pós-Traumático

PTSD.....*Post-Traumatic Stress Disorder*

PVN.....Núcleo Paraventricular do Hipotálamo

RL.....Radicais Livres

ROS.....*Reactive Oxygen Species*

SNC.....Sistema Nervoso Central

SOD.....Superóxido Dismutase, *Superoxide Dismutase*

SR.....*Situational Reminders*

UTIs.....Unidades de Tratamento Intensivo

RESUMO

Muitas evidências indicam que exposições a eventos adversos no início da vida, como abuso e negligência, aumentam a vulnerabilidade a psicopatologias na vida adulta. Além disso, psiquiatras infantis têm sugerido que o ambiente da relação mãe-filho nas fases iniciais da vida pode mais tarde afetar fortemente o desenvolvimento mental e influenciar a taxa de prevalência de vários transtornos psiquiátricos, incluindo o Transtorno de Estresse Pós-Traumático (TEPT). Periódicas separações maternas no período neonatal têm sido usadas por vários estudiosos como um modelo animal de eventos adversos no início da vida, avaliando-se seus efeitos sobre aspectos comportamentais e fisiológicos observados na vida adulta. Assim, o objetivo deste estudo foi verificar se repetidas ações de separação materna podem tornar os animais mais susceptíveis aos efeitos de um estresse agudo na vida adulta como o modelo de TEPT, alterando diferentes aspectos como os comportamentais e neuroquímicos. Ratos Wistar foram sujeitos a repetidas separações maternas nos dia 1 ao 10 do período neonatal. Quando adultos esses animais foram submetidos ao modelo de TEPT, consistindo da exposição a um choque inescapável nas patas seguido pelos recordatórios situacionais. Na primeira parte do trabalho foram usados ratos machos e fêmeas. Foram observados efeitos duradouros em ambas as intervenções. A exposição ao choque aumentou o medo condicionado. Também houve um aumento do comportamento do tipo ansioso, mas a atividade exploratória foi diminuída por ambos os tratamentos e esses efeitos foram mais robustos em machos. Adicionalmente, o nível basal de corticosterona plasmática estava diminuído, paralelamente aos níveis observados em pacientes com TEPT. Os níveis da proteína S100B plasmática e central também foram avaliados. Os níveis plasmáticos foram correlacionados com os efeitos observados no comportamento do tipo ansioso, aumentado nos ratos machos expostos ao choque e não apresentando efeito nas fêmeas. No líquido cefalorraquidiano, os níveis da proteína S100B estavam aumentados nas fêmeas submetidas à separação materna no período neonatal. Esses resultados sugerem que, em ratos, experiências estressantes no início da vida como a separação materna podem agravar alguns efeitos da exposição a um estressor na idade adulta e que esses efeitos são sexo-específicos.

Na segunda parte desse trabalho somente foram usados ratos machos. Os animais foram expostos a uma tarefa para avaliar a memória e uma semana depois da análise comportamental os animais foram sacrificados e avaliou-se o índice de dano ao ADN hipocampal e a atividade de enzimas antioxidantes. Na análise da memória espacial através da tarefa do Labirinto Aquático de Morris os animais somente submetidos ao choque (modelo de TEPT) e os animais que foram separados no período neonatal e também submetidos ao choque na idade adulta apresentaram prejuízo nessa tarefa permanecendo mais tempo no quadrante oposto no dia do teste. Na análise do dano ao ADN através da técnica do Ensaio Cometa, os animais que foram submetidos à separação materna ou ao choque apresentaram maiores índices de dano ao ADN hipocampal. Não houve diferença significativa entre os grupos quanto às atividades das enzimas antioxidantes Superóxido Dismutase, Glutationa Peroxidade e Catalase. A separação maternal aumentou a susceptibilidade ao estresse na idade adulta (modelo de TEPT) quanto ao parâmetro cognitivo avaliado. Tanto a separação materna quanto a aplicação do choque na idade adulta foram suficientes para causar aumentos no dano ao ADN hipocampal, mas a separação não causou um aumento adicional no dano ao ADN. Possivelmente alterações neuroendocrinológicas relacionadas com a resposta a esses estresses esteja mediando tais mudanças no hipocampo e conseqüentemente alterando o desempenho na tarefa que avaliou a memória. Concluindo, a separação materna no início da vida tornou os animais mais susceptíveis a um estressor considerando os parâmetros ansiedade e memória espacial e esses efeitos parecem ser dependentes do gênero.

ABSTRACT

A large body of evidences indicates that exposure to early adverse life events in the form of childhood neglect and abuse can increase vulnerability to psychopathology in adult life. In addition, child psychiatrists have reported that the mother–child relationship and fostering environment in early childhood strongly affect later mental development and influence the prevalence rate of various psychiatric disorders including Post-Traumatic Stress Disorder (PTSD). Periodic neonatal maternal separation in the rat has been used by several investigators as a rodent model of the effects of early adverse life events on adult physiology and behavior. Therefore, the purpose of the present study was to verify if repeated long-term maternal separation would affect performance in different parameters including behavior and neurochemistry. Wistar rats were subjected to repeated maternal separation during post-natal days 1-10. When adults, rats were submitted to a PTSD model consisting of exposure to inescapable footshock, followed by situational reminders. In the first part of this work rats of both sexes were used. We observed long-lasting effects of both interventions. Exposure to shock increased fear conditioning. Anxiety-like behavior was increased and exploratory activity decreased by both treatments, and these effects were more robust in males. Additionally, basal corticosterone in plasma was decreased, paralleling effects observed in PTSD patients. Levels of S100B protein in serum and cerebrospinal fluid (CSF) were measured. Levels in serum were correlated with the effects observed in anxiety-like behavior, increasing in males exposed to shock, and presenting no effect in females. S100B in CSF was increased in females submitted to maternal separation during the neonatal period. These results suggest that, in rats, an early stress experience such as maternal separation may aggravate some effects of exposure to a stressor during adult age, and that this effect is sex-specific.

In the second part of this work the animals were exposed to a task to evaluate spatial memory and one week after the behavioral task animals were sacrificed and DNA damage and antioxidant enzymes activities in hippocampus were measured. Only male rats were used in the second part of this work. We observed that both treatments, maternal separation

during the first 10 days of life and exposure to a stressful event in adult life, presented important effects on memory and DNA damage in hippocampus. No differences were found in oxidative parameters. In this work rats exposed to shock and maternal separation plus shock presented long-lasting effects on spatial memory associated with impairments in learning and memory (they spent more time in the opposite quadrant in water maze task). Rats subjected to shock or maternal separation exhibited a higher score of DNA damage in hippocampus. Concluding, our findings showed that early adverse life events alter the susceptibility to the effects of a stressor applied in adulthood in anxiety and cognitive parameters such as spatial memory. These effects were probably sex-specific. Besides, both treatments were able to induce increased index of DNA damage.

1. Introdução

Durante o período neonatal ocorrem adaptações do recém-nascido à vida extra-uterina, esse período é um momento determinante no desenvolvimento do sistema nervoso central e conseqüentemente sobre aspectos cognitivos dos recém-nascidos. Intervenções realizadas nesse período podem causar modificações no seu desenvolvimento e tais alterações poderão ser observadas na idade adulta. Logo, a privação da figura materna nesse período, que é crítico para o desenvolvimento, pode alterar de forma definitiva como esse indivíduo se comportará na idade adulta, como por exemplo, frente a uma situação estressante.

Recém-nascidos prematuros ou com baixo peso são mais suscetíveis a situações de estresse. Mesmo com o avanço tecnológico e melhoria do atendimento em UTIs neonatais, esse grupo continua apresentando uma alta morbidade. Além disso, estudos com animais de experimentação têm demonstrado que intervenções no período neonatal podem levar a alterações nos sistemas nervoso, cardiovascular, respiratório e endócrino–metabólico, além de alterações comportamentais. A estimulação neonatal é um modelo experimental usados em animais para avaliar os efeitos que as manipulações nesse período podem acarretar nos sistemas neurais, levando a mudanças comportamentais e endócrinas que aparecerão na vida adulta.

Intervenções feitas no período neonatal influenciam na relação da mãe com os filhotes, e a mãe pode assumir determinados comportamentos que afetam o desenvolvimento do sistema nervoso dos seus filhotes. Estudos sobre o estresse neonatal têm mostrado a importância de um ambiente adequado para um desenvolvimento saudável, pois os recém-nascidos são mais vulneráveis nesse período. Assim, experimentos nesta área são de grande importância para futuramente relacionarmos eventos adversos na infância com o aparecimento de algumas patologias na vida adulta.

O Transtorno de Estresse Pós-Traumático (TEPT) é um transtorno de ansiedade que se apresenta como uma resposta inadequada a determinado estímulo (KAPLAN & SADOCK, 1999). Caracteriza-se pela experiência repetida de terror associada com um evento psicologicamente opressivo quando uma pessoa vê, tem notícia, ou é envolvida por um estressor traumático extremo. (KAPLAN & SADOCK, 2008). Por isso, a busca de suas bases biológicas se concentra em aspectos neuroendocrinológicos das reações do organismo a agressões ambientais (GRAEFF, 2003).

As estatísticas mostram que o TEPT é um problema cada vez mais presente na nossa sociedade. O TEPT atinge duas vezes mais as mulheres que os homens e os efeitos desse transtorno aparecem em aproximadamente 15% a 24% das pessoas que foram expostas a eventos traumáticos. Estima-se que a prevalência desse transtorno atinja 8% da população total, segundo os critérios do DSM-IV-TR – Manual Diagnóstico e Estatístico de Transtornos Mentais (KESSLER, 1995; KESSLER *et al*., 1999). São muitos os eventos traumáticos que podem gerar o TEPT, como por exemplo, ser testemunha e vítima de assaltos, seqüestros, abuso sexual ou físico, acidentes graves, desastres naturais, guerras e muitos outros.

Situações traumáticas geram muito sofrimento para o indivíduo que vivenciou e para a família, trazem problemas de ordem física e emocional, problemas esses que merecem atenção e tratamento. As bases biológicas do TEPT não são bem conhecidas, e por isso é importante o desenvolvimento de estudos nesta área, para que futuramente possam propiciar tratamentos mais adequados e que se reflitam numa melhor qualidade de vida para essas pessoas. Para tal, o desenvolvimento de um modelo em animais de experimentação pode ser extremamente útil para a compreensão das alterações observadas nesses pacientes.

1.1 Estresse

O cientista e médico austríaco Hans Selye definiu em 1936 o estresse como "Síndrome da Adaptação Geral", ou seja, a resposta adaptativa de um organismo à ação de agentes nocivos – os chamados agentes estressores. De acordo com os estudos de Selye, a resposta ao estresse divide-se em três estágios: um primeiro de alarme, onde o agente estressor é notado, um segundo de resistência, no qual o organismo combate o agente estressor com sucesso, e, por fim, um estado de exaustão, onde o organismo esgota sua capacidade de resposta de estresse, daí advindo os seus efeitos deletérios (SELYE, 1936, *APUD* KOPIN, 1995).

Ultimamente, a palavra "estresse" tem sido interpretada como o conjunto de respostas do organismo a um estressor. "Estressor" é definido como um desafio ao indivíduo que potencialmente pode perturbar a homeostase e, assim sendo, requer uma resposta fisiológica. Pode também ser apenas uma interpretação inadequada da situação, percebida erroneamente como ameaça, que resulta numa resposta comportamental e/ou hormonal (McEWEN, 2002; TSIGOS *et al.*, 2002).

Há dois sistemas de resposta ao estresse classicamente descritos: (a) o sistema neurovegetativo, com liberação de adrenalina pela medula adrenal; (b) os glicocorticóides, produzidos no córtex da adrenal sob estímulo hipotalâmico e hipofisário (McEWEN, 2002; TSIGOS *et aI.*, 2002). A ativação aguda desses sistemas promove principalmente aumento da disponibilidade de energia e melhora do fluxo sangüíneo para órgãos-alvo, sendo altamente adaptativa (TSIGOS *et al.*, 2002*).* Entretanto, a exposição crônica a níveis elevados de glicocorticóides pode ser danosa ao organismo (DALLMAN *et al*., 2004; MILLER *et al*., 2002).

O eixo hipotálamo-hipófise-adrenal (HHA) tem uma regulação extremamente fina no período pré e pós-natal imediato, possuindo alta plasticidade (FRANCIS *et al*., 1999). Distúrbios no padrão normal de secreção de glicocorticóides nesse período crítico podem alterar de forma definitiva as respostas do organismo ao estresse (LEVINE e*t al*., 1967).

1.2 Funcionamento do eixo Hipotálamo-Hipófise-Adrenal (HHA)

Estímulos externos têm grande impacto sobre o sistema límbico, que se relaciona com o hipotálamo. O controle central do sistema de resposta ao estresse inclui os neurônios parvocelulares do núcleo paraventricular do hipotálamo (PVN). Essas células estão sob influência de vários mecanismos intrínsecos e extrínsecos que regulam a resposta do eixo hipotálamo-hipófise-adrenal ao estresse. Aferências ao PVN são provenientes principalmente da informação sensorial, promovendo respostas do eixo a ameaças reais à homeostasia, e incluem o núcleo do trato solitário, o núcleo da rafe, o órgão subfornicial, o núcleo próprio da *stria terminalis*, o tálamo e regiões hipotalâmicas que circundam o PVN. Aferências indiretas vindas do hipocampo, amígdala, córtex pré-frontal, septo lateral e tálamo ativam os mesmos neurônios parvocelulares na ausência de desafios fisiológicos francos, mas prementes (HERMAN *et al*., 2003).

No estado de repouso (basal), o hipotálamo apresenta secreção de hormônio liberador de corticotropina (CRH) e de vasopressina (AVP) de uma maneira pulsátil, com dois ou três picos por hora (ENGLER *et al*., 1989). Durante o estresse agudo, a amplitude e a freqüência desses pulsos aumentam, resultando na liberação de adrenocorticotropina (ACTH) pela hipófise e de cortisol (corticosterona em ratos) pelo córtex da adrenal (TSIGOS *et al*., 1994). Uma série de situações pode estimular o hipotálamo, como por

exemplo, frio, infecção, hemorragia, choque, vibração, estresse emocional e/ou social, contenção, etc. (MILLER *et al*., 2002). Citocinas e outros mediadores de inflamação também são liberados nessas ocasiões e potenciam a ação dos vários componentes do eixo HHA.

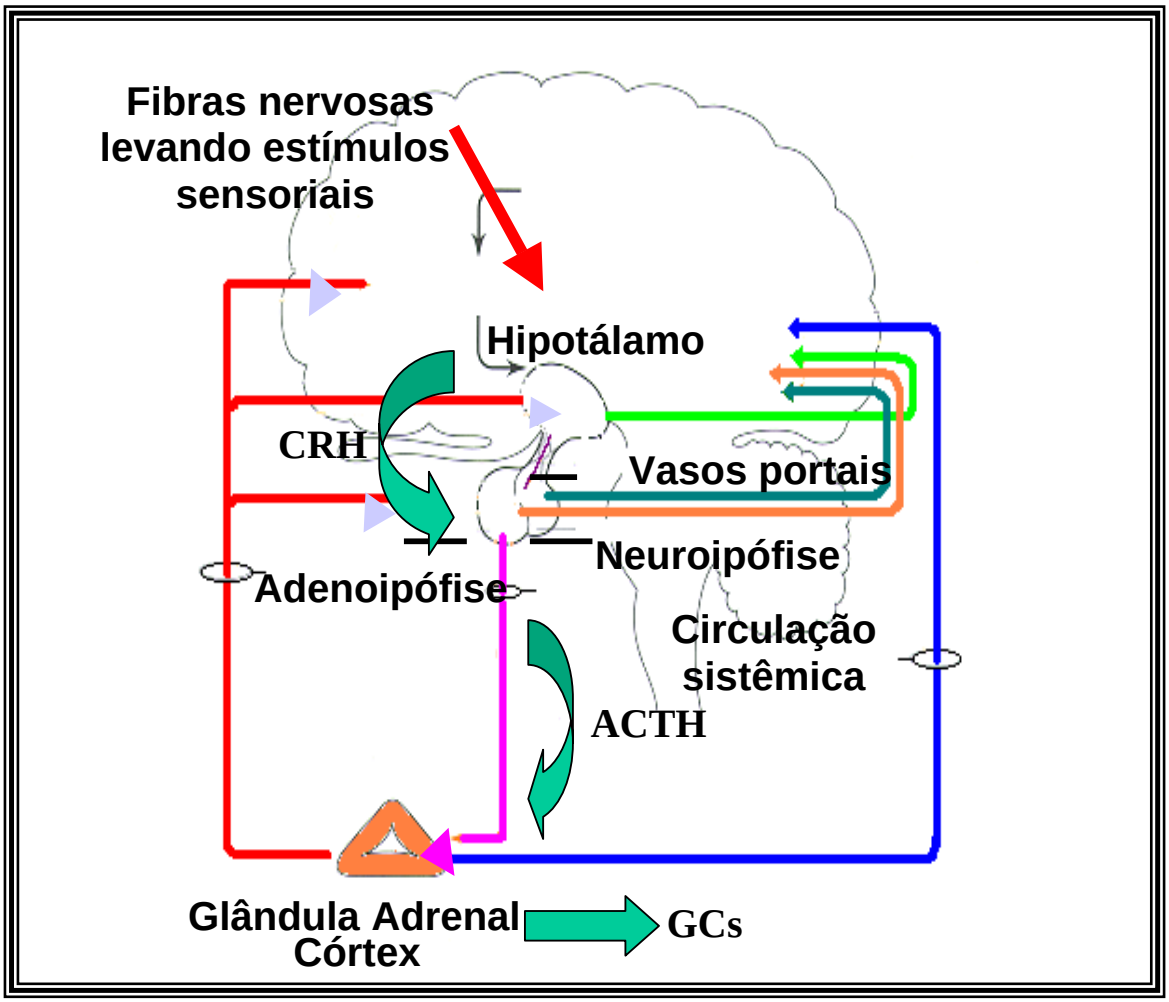

Figura 1: Eixo Hipotálamo-Hipófise-Adrenal (HHA). Estímulos ambientais externos são captados pelo sistema límbico, ativando os sistemas de resposta ao estresse, entre eles o eixo HHA.

O ACTH aumenta a síntese de glicocorticóides pelo córtex da glândula adrenal. Em situações críticas, os glicocorticóides têm ações de proteção e manutenção da homeostasia: mobilização de estoques energéticos através de gliconeogênese, lipólise e catabolismo protéico, melhora da função cognitiva, inibição da função gonadal, alteração da homeostasia do cálcio. Os glicocorticóides têm importância na própria regulação neuroendócrina, uma vez que atuam em receptores do sistema límbico (especialmente amígdala e hipocampo), do hipotálamo e da hipófise por retroalimentação negativa, encerrando a ativação (De KLOET, 1998).

Esses hormônios também inibem as citocinas, regulando a atividade do eixo HHA. A inibição causada pelos glicocorticóides limita sua própria ação, prevenindo o organismo de seus efeitos catabólicos, antirreprodutivos e imunossupressivos (TSIGOS *et al*., 2002).

1.3 Radicais Livres e Lipoperoxidação

Substâncias como o peróxido de hidrogênio e formas químicas chamadas de radicais livres compõem as espécies reativas de oxigênio (EROs), as quais estão envolvidas em dano tecidual. Os radicais livres (RL) são espécies químicas altamente reativas, caracterizadas por serem capaz de ter existência independente e possuírem um ou mais elétrons desemparelhados (HALLIWELL & GUTTERIDGE, 1984).

Tanto os RL como as demais EROs são constantemente formadas no organismo, seja através de síntese fisiológica durante processos de fagocitose, seja através de reações químicas que têm como produto a formação de radicais desta espécie, como o ânion superóxido, e os radicais hidroxila e peroxila (HALLIWELL & CROSS, 1994; PERES, 1994). As EROs são altamente reativas e capazes de danificar o ácido desoxirribonucléico

(ADN), o ácido ribonucléico (ARN), as proteínas e os lipídeos (ALLEN, 1993). O sistema nervoso central é especialmente vulnerável aos danos dos radicais livres, devido ao alto consumo de oxigênio pelo cérebro, conteúdo abundante de lipídios e insuficiência relativa de enzimas antioxidantes comparado com outros tecidos (HALLIWELL & GUTTERIDGE, 1985).

Os RL são formados por reações de oxi-redução, onde determinadas moléculas doam ou recebem um elétron, ficando desemparelhados, ou por processos de fissão homolítica de ligações covalentes. Um radical pode doar ou receber um elétron (radicais redutores e oxidantes, respectivamente), ou ainda podem unir-se a um não radical por adição, formando um radical (PERES, 1994). Assim, ocorrem reações em cadeia: um radical, para se estabilizar, produz outro radical, e somente quando dois radicais se unem é que estas reações em cadeia são terminadas (PERES, 1994). Considerando que a maioria das moléculas orgânicas são não-radicais, um único radical pode causar danos sucessivos a várias outras moléculas.

Estressores físicos e psicológicos causam liberação de glicocorticóides e essas substâncias exercem um papel adaptativo, pois mobilizam energia para os tecidos críticos nas situações de emergência e suprimem o anabolismo que não é essencial. Entretanto, há evidências de que altos níveis de glicocorticóides no cérebro podem causar dano aos neurônios. Esses danos têm sido associados ao aumento da geração de EROs (McINTOSH & SAPOLSKY, 1996).

A lipoperoxidação é um dos principais eventos provocados pelas EROs. Ela induz mudanças na fluidez e no potencial da membrana e na permeabilidade a íons. O sistema nervoso é particularmente sensível aos danos provocados pelos RL, pois é rico em

substratos oxidáveis, tem alta tensão de oxigênio e baixa capacidade antioxidante (SANDHIR *et al*., 1994).

Os danos induzidos pelas EROs são normalmente controlados por sistemas antioxidantes enzimáticos e não enzimáticos (HALLIWELL & CROSS, 1994). Essas defesas celulares diminuem as concentrações de RL e exercem reparo nos danos oxidativos às células. Os sistemas antioxidantes incluem enzimas, tais como a superóxido dismutase (SOD), que converte radicais superóxido em peróxido de hidrogênio, catalase (CAT), que tem sido apontada como responsável pela destoxificação do peróxido de hidrogênio, e a glutationa peroxidase (GPx), que diminui os níveis de peróxidos, principalmente aqueles derivados da oxidação de fosfolipídeos de membrana. A remoção do superóxido e do peróxido de hidrogênio diminui a geração de radicais hidroxila (KEHRER, 2000).

1.4 Proteína S100B

S100 é uma família de proteínas ligantes de cálcio com 21 membros já descritos, que estão implicados nas mais variadas funções, tanto intracelulares como extracelulares (ZIMMER *et al*., 1995; DONATO, 2003). No meio intracelular, a família S100 tem a função de regular a atividade de proteínas efetoras e na maioria das situações em que o cálcio está envolvido, a família S100 exerce funções regulatórias (NAGDYMAN *et al*., 2001).

Estudos demonstraram que a S100B estimula o crescimento neuronal e atua na proliferação de melanócitos. Em concentrações nanomolares, a S100B tem uma função de fator de crescimento e diferenciação, enquanto que em concentrações micromolares essa

proteína induz a apoptose. Também foram observadas astrocitose e proliferação neuronal em ratos (SCHAFER *et al*., 1996).

O mecanismo de ação da família S100 ainda não está bem descrito, mas a relação de S100B com células gliais e neurônios em concentrações nanomolares sugere uma estimulação do fluxo de cálcio e, conseqüentemente, levanta uma hipótese atraente deste íon mediar tais funções (SCHAFER *et al*., 1996).

Estudos sugerem que a S100B deva ser um importante fator contribuinte do desenvolvimento e diferenciação neuronal e do dano cerebral, e aquelas alterações de sua concentração extracelular, seguidas de injúria cerebral, podem levar a uma amplificação da lesão associada a outros fatores conhecidos na gênese de processos neurodegenerativos. Os níveis da S100B no sangue e no líquor estão aumentados em vários distúrbios agudos ou crônicos do SNC. Alterações séricas estão mais comumente relacionadas com diversos fatores que afetem sua síntese, distribuição e metabolismo no SNC.

Verificou-se que há uma relação diretamente proporcional entre os níveis de S100B no líquor e sangue e a intensidade e extensão da injúria cerebral, permitindo, então, utilizar esta proteína como marcador bioquímico de dano, ou disfunção cerebral (LARA *et al*., 2001; MACHADO-VIEIRA *et al*., 2002).

1.5 Estimulação e Separação Materna no Período Neonatal e o Desenvolvimento Neural

Interações genéticas e ambientais regulam os mecanismos neurais envolvidos no desenvolvimento de determinados comportamentos. Experiências sensoriais no início da vida pós-natal podem afetar o desenvolvimento neural e o comportamento de um animal

adulto. Estímulos estressantes são algumas das influências ambientais que podem modificar o desenvolvimento neural (GONZÁLES *et al*., 1990).

A estimulação neonatal em ratos consiste tipicamente na “manipulação” dos filhotes por alguns minutos, na maioria dos estudos no período que abrange as primeiras duas semanas de vida. Essa manipulação em geral é realizada durante poucos minutos por dia no período neonatal. Por outro lado, a separação (ou privação) materna consiste da separação dos filhotes, que neste caso permanecem longe da mãe durante períodos mais prolongados (mais de 30 min; LIU *et al*., 1997).

A manipulação tem como conseqüência na vida adulta uma série de alterações comportamentais e endócrinas que se caracterizam por uma diminuição do medo a novos ambientes. Além disso, esses animais, na idade adulta, apresentam uma resposta menos acentuada da secreção de glicocorticóides pela adrenal quando expostos a estímulos estressores. Portanto, os ratos estimulados na infância apresentam uma secreção de corticosterona menor frente a novos estímulos estressantes (LEVINE, 1993; MEANEY *et al*., 1993). Contudo, os níveis basais de corticosterona de animais manipulados e não manipulados não diferem entre si quando adultos, mas as diferenças entre eles parecem ser devidas a uma sensibilidade diferencial do sistema nervoso central ao mecanismo de retroalimentação negativa da adrenal (LEVINE, 1994). Foi encontrada também uma maior concentração de receptores glicocorticóides no hipocampo (MEANEY *et al*., 1989) e um aumento da inibição mediada pelo hipocampo e diminuição da excitação mediada pela amígdala na resposta neuroendócrina do eixo HHA nos animais que sofreram estresse neonatal (De KLOET *et al*., 1998).

As duas primeiras semanas após o nascimento representam um período crítico para o desenvolvimento neural em ratos. Conseqüentemente, a estimulação precoce atua sobre o

desenvolvimento do sistema nervoso e induz uma variedade de mudanças neuroquímicas e comportamentais no adulto.

1.6 Período Hiporresponsivo ao Estresse

As duas primeiras semanas de vida de um rato constituem o chamado *período hiporresponsivo ao estresse* (SAPOLSKY *et al*., 1986). Durante essa fase, a resposta do eixo hipotálamo-hipófise-adrenal (HHA) a estímulos nocivos é reduzida (HALTMEYER *et al*., 1966; BARTOVA, 1968), ou seja, há uma exacerbação do mecanismo de retroalimentação negativa dos glicocorticóides na hipófise e diminuição da sensibilidade da adrenal ao hormônio adrenocorticotrópico (ACTH) (YOSHIMURA *et al*., 2003).

1.7 A relação mãe-filhote

Vários estudos demonstraram que a manipulação ou qualquer outro tipo de estimulação do animal no período neonatal provoca um distúrbio da relação mãe-filho. As mães de filhotes manipulados lambem mais a sua prole do que mães de filhotes não manipulados. Este comportamento da mãe em relação ao filhote afeta o desenvolvimento do sistema nervoso deste (LEVINE, 1994). Acredita-se que a perturbação dessa relação é que induziria o padrão comportamental e endócrino observado na vida adulta do rato manipulado no período neonatal.

O distúrbio dessa interação mãe-filho promove uma série de respostas comportamentais e fisiológicas que incluem mudanças na temperatura corporal, na locomoção, na freqüência cardíaca e na reação emocional (HINDE & SPENCER-BOOTH,

1971). Foi demonstrado que, além de mudanças fisiológicas, há alterações bioquímicas em animais que sofreram privação materna, tais como redução da atividade da ornitina descarboxilase e dos níveis do fator neurotrófico derivado do encéfalo (BDNF) em certas regiões cerebrais (SCHANBERG & KUHN, 1985).

A estimulação pós-natal aumenta a expressão de receptores para glicocorticóides no hipocampo e córtex frontal, ou seja, duas regiões que estão envolvidas na regulação, por meio de um sistema de retroalimentação, da atividade do eixo HHA (FRANCIS *et al*., 1996). No entanto, períodos de separação maternal mais prolongada, por cerca de 180 minutos, produzem uma diminuição da concentração dos receptores de glicocorticóides no hipotálamo, córtex frontal e hipocampo (LIU *et al*., 1997).

Os estudos acima nos indicam que a diferenciação dos sistemas de receptores para glicocorticóides em determinadas regiões do sistema nervoso é sensível a uma variedade de sinais ambientais durante o período pós-natal, ou seja, o ambiente na fase neonatal pode determinar a responsividade do eixo HHA ao estresse por toda a vida do animal.

Figura 2: Rata lactante com seus filhotes neonatos.

1.8 Transtorno de Estresse Pós-Traumático (TEPT)

Síndromes relacionadas à exposição ao estresse agudo ou crônico, que aparecem após graves traumas físicos e emocionais, constituem importante problema de saúde pública e possuem significativas implicações médico-legais. As estatísticas mostram que o TEPT é um problema cada vez mais presente na nossa sociedade. O TEPT atinge duas vezes mais as mulheres que os homens, sendo que os efeitos deste transtorno aparecem em aproximadamente 15% a 24% das pessoas que foram expostas a eventos traumáticos. Estima-se que a prevalência desse transtorno atinja 8% da população total, segundo os critérios do DSM-IV-TR – Manual Diagnóstico e Estatístico de Transtornos Mentais (KESSLER, 1995; KESSLER *et al.*, 1999).

O TEPT é um transtorno de ansiedade e, como tal, representa uma resposta inadequada a um determinado estímulo. Caracteriza-se pela experiência repetida de terror associado com um evento psicologicamente opressivo. É uma condição que se desenvolve quando uma pessoa vê, tem notícia, ou é envolvida por um estressor traumático extremo (KAPLAN & SADOCK, 2008). Este transtorno consiste em revivência do trauma através de sonhos e de pensamentos durante a vigília, evitação persistente de coisas que lembrem o trauma e hiperexcitação persistente. Em uma pessoa portadora de TEPT são comuns depressão, ansiedade e dificuldades cognitivas. A duração mínima dos sintomas é de mais de um mês para ser considerado como TEPT (KAPLAN & SADOCK, 2008).

Entre os sintomas neurovegetativos, disfóricos e cognitivos se caracterizam fenômenos como reação exagerada de medo, dificuldade de concentração, prejuízo da memória, sentimentos de culpa, dificuldades no sono, hipervigilância, evitação fóbica de situações que despertem lembranças do trauma, intrusão e recorrência das recordações,

sonhos ou pesadelos recorrentes, exacerbação dos sintomas quando o paciente é exposto a situações semelhantes às do evento traumático, diminuição do interesse, sentimento de estar desligado ou estar estranho aos demais e acentuada limitação de respostas emocionais. Além desses aspectos essenciais, que são os responsáveis pelo diagnóstico da doença, podem aparecer aspectos associados como: sintomas depressivos, inquietude, nervosismo, tremor, irritabilidade, labilidade emocional, cefaléias, vertigens e abuso de álcool ou de drogas (KAPLAN & SADOCK, 2008).

Diversos tipos de estressores contribuem para o desenvolvimento do TEPT. O estressor precisa ser bastante violento e estar fora do campo de experiência humana geralmente considerada normal, como por exemplo, agentes estressantes como calamidades da natureza (inundações, terremotos), desastres provocados pelo homem (acidentes de automóvel, incêndios). Contudo, nem todos experimentam o transtorno após um evento traumático. Deve ser considerado também fatores individuais preexistentes biológicos e psicossociais e acontecimentos que tenham ocorrido antes e depois do traumatismo. No TEPT o trauma pode envolver um fator físico, mas sempre envolve um componente psicológico que provoca significativo choque emocional (KAPLAN & SADOCK, 2008). O tratamento do TEPT consiste em reduzir os sintomas estabelecidos, prevenção da incapacidade crônica e a reabilitação ocupacional e social através de técnicas comportamentais (terapias de relaxamento e dessensibilização progressiva na superação de sintomas fóbicos), medicação (uso de inibidores da recaptação da serotonina, como sertralina e paroxetina – como tratamento de primeira linha e outros medicamentos que podem ser benéficos incluem os inibidores da monoaminoxidase como a fenelzina e trazodona) e várias formas de psicoterapia [casos leves e agudos respondem à psicoterapia de apoio, os casos crônicos e graves exigem modalidades de psicoterapia com ênfase no

aumento de auto-conscientização (*insight),* catarse e ab-reação] (KAPLAN & SADOCK, 2008).

Pesquisas neuroendocrinológicas dos sistemas fisiológicos de pacientes com TEPT evidenciam hiperfunção do eixo simpático-adrenal, em conjunto com uma redução da atividade do eixo HHA. A vulnerabilidade ao desenvolvimento do TEPT parece estar associada a uma resposta prejudicada do cortisol aos estressores (GRAEFF, 2003). A consolidação das memórias traumáticas e a indevida generalização para outras situações estressantes podem ser promovidas por um excesso de catecolaminas, sem o pareamento do aumento dos corticóides. Alguns estudos de neuroimagem evidenciaram uma redução do volume hipocampal neste transtorno, que tem sido relacionada a alterações cognitivas e anormalidades do eixo HHA encontrados no TEPT. Há muitas evidências mostrando que o eixo simpático-adrenal ligado à reação de defesa está hiperativado no TEPT. Um exemplo disso é um estudo em pacientes com TEPT, avaliados muitos anos depois de iniciado o transtorno, que mostra que a freqüência cardíaca está mais elevada e sua variabilidade diminuída, o que indica uma ativação do sistema simpático e uma redução do tônus vagal (COHEN *et al*., 1997). Também foram avaliadas outras medidas psicofisiológicas, que mostraram, entre outras alterações, maior reatividade da condutância elétrica da pele e resposta ocular de sobressalto amplificada em presença de estímulos condicionados aversivos (SHALEV *et al*., 1992).

O hormônio liberador da corticotropina (CRH) extra-hipotalâmico pode mediar a hiperativação do sistema de defesa no TEPT. O CRH, além de ser sintetizado no núcleo paraventricular do hipotálamo e ser liberado no sistema hipofisário, participando da cadeia hormonal do eixo HHA, é produzido por uma extensa rede de neurônios, onde ele funciona como neurotransmissor. Na amígdala, no *locus coeruleus* e na matéria cinzenta

periaquedutal estão os corpos celulares e/ou os terminais destes neurônios. Foi verificado que a injeção de CRH no interior dos ventrículos cerebrais de animais de laboratório resulta em alterações comportamentais como aumento da ansiedade e depressão, além da ativação do sistema simpático-adrenal (LENZ *et al*., 1987). A partir disso, chegou-se à conclusão de que o CRH inicia as respostas características do estresse. Pacientes com transtorno do pânico foram estudados e observou-se, mas não é consenso, que eles apresentavam níveis elevados de CRH no líquido cefalorraquidiano, os quais refletem o funcionamento do sistema extra-hipotalâmico (BREMNER *et al*., 1997). A ativação dos neurônios extra-hipotalâmicos que contêm CRH pode não ser concomitante à das células neurossecretoras do hipotálamo. Estudos moleculares evidenciaram que a expressão dos genes do CRH no núcleo central da amígdala e no núcleo próprio da estria terminal está dissociada daquela do núcleo paraventricular, compatibilizando os achados de hiperativação simpático-adrenal com os de hipofunção do eixo HHA no TEPT (GRAEFF, 2003).

Mesmo com os pacientes apresentando uma reação anormalmente intensa e prolongada a um estressor, podendo indicar uma ativação maior que o normal do eixo HHA, achados experimentais apontam para a direção oposta, mostrando uma associação entre redução dos níveis de cortisol e maior vulnerabilidade ao TEPT. Uma alça de retroalimentação (*feedback*) negativa é responsável pelo controle da secreção de cortisol, que é ativada pela ação do mesmo circulante. O cortisol atua sobre receptores de glicocorticóides situados no hipocampo, onde a concentração desses receptores é alta, bem como no hipotálamo e hipófise, inibindo a secreção de ACTH. No TEPT, os pacientes apresentam hiperativação simpático-adrenal associada à hipofunção do eixo HHA; quanto mais reduzida a função deste, maior o risco de desenvolver TEPT. O cortisol tem a função de terminar a fase de alarme da reação ao estresse, caracterizada pela ativação simpático-

adrenal. Desse modo, foi proposto que a deficiência de cortisol, verificada no TEPT, faria com que os pacientes ficassem estagnados na fase da reação de defesa (GRAEFF, 2003).

O TEPT cria alterações no campo da memória. Memórias traumáticas tornam-se indeléveis e resistentes à extinção. Estudos em animais de laboratório e em seres humanos têm mostrado que memórias de conteúdo emocional são mais facilmente lembradas que as neutras, devido à ação de hormônios e neurotransmissores, que são liberados por ocasião das emoções e que promovem a consolidação dos traços de memória. No TEPT, o excesso de catecolaminas devido ao trauma, não refreado pelos corticóides, levaria à consolidação excessiva das memórias traumáticas. Devido à implicação do hipocampo na aquisição da memória declarativa, este vem sendo muito estudado no TEPT. Por meio da morfometria aplicada a imagens de ressonância magnética nuclear, foi descoberta uma diminuição do volume do hipocampo, também constatada na doença de Cushing e no transtorno depressivo maior, onde o nível de cortisol está aumentado (SAPOLSKY *et al*., 2000).

Através de estudos em animais de laboratório, foi evidenciado que os corticóides potencializam o efeito neurotóxico de aminoácidos excitatórios, então foi criada a hipótese de que a atrofia hipocampal fosse resultante do aumento de corticóides promovido pelo estresse. Hipótese aceita para a depressão, mas contraditória no TEPT, pois os níveis de cortisol circulante estão reduzidos. Algumas explicações ainda a serem estudadas são de que os níveis de corticóides no momento do trauma poderiam estar elevados, de que alterações de neurotransmissores, como a serotonina, o ácido gama-aminobutírico (GABA), o glutamato e fatores de crescimento possam participar de processos de neurogênese e neurodegeneração. Esse último caso poderia ser fator de vulnerabilidade, posto que o hipocampo tem importante papel na regulação do eixo HHA (GRAEFF, 2003).

2. Objetivos

2.1 Objetivo Geral:

Estudar se o estresse neonatal por separação materna pode ser um fator de risco para o desencadeamento de características relacionadas ao Transtorno de Estresse Pós-Traumático, quando os animais forem submetidos a um estressor (utilizado como modelo de TEPT) na idade adulta.

2.2 Objetivos Específicos:

2.2.1 – Artigo 1

Estudos comportamentais:

a. Analisar o efeito da separação materna no período neonatal sobre o medo condicionado, após os animais serem ou não submetidos a um estressor (choque inescapável) na idade adulta, em ratos machos e fêmeas. Para tal, usaremos o tempo de imobilidade durante recordatórios situacionais.
b. Analisar o efeito da separação materna no período neonatal sobre a atividade motora e a ansiedade após os animais serem ou não submetidos a um estressor (choque inescapável, seguido de recordatórios situacionais) na idade adulta, em ratos machos e fêmeas. Para tal, usaremos a tarefa de exposição ao Campo Aberto (*Open Field*).

Estudos Neuroquímicos:

c. Analisar o efeito da separação materna no período neonatal sobre os níveis de corticosterona no plasma dos animais submetidos ou não a um estressor (choque inescapável) na idade adulta, em ratos machos e fêmeas.
d. Analisar o efeito da separação materna no período neonatal sobre os níveis de S100B no líquor e no plasma dos animais submetidos ou não a um estressor (choque inescapável) na idade adulta, em ratos machos e fêmeas.

2.2.2 – Artigo 2

Estudos comportamentais:

a. Analisar o efeito da separação materna no período neonatal sobre a memória na tarefa do Labirinto Aquático de Morris (*Water Maze*) após os animais serem ou não submetidos a um estressor (choque inescapável, seguido de recordatórios situacionais) na idade adulta, em ratos machos.

Estudos Neuroquímicos:

b. Analisar o efeito da separação materna no período neonatal sobre parâmetros bioquímicos relacionados ao estresse oxidativo [determinação da atividade das enzimas antioxidantes glutationa peroxidase (GPx), superóxido dismutase (SOD) e catalase (CAT)] em hipocampo dos animais submetidos ou não a um estressor (choque inescapável) na idade adulta, em ratos machos.

c. Analisar o efeito da separação materna no período neonatal sobre lesão ao ADN hipocampal de animais submetidos ou não a um estressor (choque inescapável) na idade adulta, em ratos machos. Para tal, realizaremos o Ensaio Cometa.

3. Métodos e Resultados

Os materiais e métodos e os resultados desta dissertação estão apresentados a seguir, da seguinte forma:

Capítulo 1: **Artigo publicado**: Luisa Amalia Diehl, Patrícia Pelufo Silveira, Marina C. Leite, Leonardo Machado Crema, Andre Krumel Portella, Mauro Nör Billodre, Edelvan Nunes, Thiago P. Henriques, Linda Brenda Fidelix-da-Silva, Marta D. Heis, Carlos Alberto Gonçalves, Jorge Alberto Quillfeldt, Carla Dalmaz. "Long lasting sex-specific effects upon behavior and S100b levels after maternal separation and exposure to a model of post-traumatic stress disorder in rats." *Brain Research*, **1144** (2007) 107-116.

Capítulo 2: **Artigo a ser submetido**: Luisa Amalia Diehl, Lucas de Oliveira Álvares, Cristie Grazziotin Noschang, Douglas Engelke, Leonardo Machado Crema, Rachel Krolow, Ana Cristina Andreazza, Carlos Alberto Gonçalves, Jorge Alberto Quillfeldt, Carla Dalmaz. "Early adverse life events alter the susceptibility to the effects of a stressor applied in adulthood: cognitive parameters and DNA damage to the hippocampus."

CAPÍTULO 1

ARTIGO 1

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Research Report

Long lasting sex-specific effects upon behavior and S100b levels after maternal separation and exposure to a model of post-traumatic stress disorder in rats

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ABSTRACT

This study was undertaken to verify if repeated long-term separation from dams would affect the development of parameters related to post-traumatic stress disorder (PTSD) after animals are subjected to inescapable shock when adults. Wistar rats were subjected to repeated maternal separation during post-natal days 1–10. When adults, rats from both sexes were submitted to a PTSD model consisting of exposure to inescapable footshock, followed by situational reminders. We observed long-lasting effects of both interventions. Exposure to shock increased fear conditioning. Anxiety-like behavior was increased and exploratory activity decreased by both treatments, and these effects were more robust in males. Additionally, basal corticosterone in plasma was decreased, paralleling effects observed in PTSD patients. Levels of S100B protein in serum and cerebrospinal fluid (CSF) were measured. Levels in serum correlated with the effects observed in anxiety-like behavior, increasing in males exposed to shock, and presenting no effect in females. S100B in CSF was increased in females submitted to maternal separation during the neonatal period. These results suggest that, in rats, an early stress experience such as maternal separation may aggravate some effects of exposure to a stressor during adult age, and that this effect is sex-specific. Additionally, data suggest that the increased S100B levels, observed in serum, have an extracerebral origin, possibly mediated by an increase in the noradrenergic tonus. Increased S100B in brain could be related to its neurotrophic actions.

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Abbreviations: PTSD, post-traumatic stress disorder; CSF, cerebrospinal fluid; MS, maternal separation; SR, situational reminders

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1. Introduction

The first 2 weeks of life are a critical period for neural development in rats, and postnatal rearing conditions are known to influence the development of hypothalamic–pituitary–adrenal (HPA) responses to stress. Among the many paradigms of neonatal intervention studied, brief postnatal handling (around 15 min) has been shown to dampen HPA responsivity to stress and to lead to reduced anxiety-like behavior (Liu et al., 2000; Kalinichev et al., 2002; Ladd et al., 2004; Severino et al., 2004), while prolonged periods of maternal separation (e.g., 3 h or more) usually have the opposite effect, and may increase stressor reactivity during adulthood (for a review, see De Kloet et al., 2005), and enhance anxiety-like behavior (Neumann et al., 2005; Kalinichev et al., 2002; Ladd et al., 2004).

Manipulation of the environment during postnatal development appears to be one approach for the identification of potential animal models for psychiatric disorders, since early stress may interact with later ability to cope with stress in adulthood. For example, prenatal stress in female rats has been associated with higher conditioned fear in an animal model of posttraumatic stress disorder (Louvart et al., 2005a).

Post-traumatic stress disorder (PTSD) is the pathological replay of the emotional memory formed in response to painful, life-threatening, or horrifying events. This psychopathology affects a vulnerable subpopulation of individuals confronted with a stressful experience that exceeds their capacity to adapt, and one possible factor in this different vulnerability could be their different life histories. PTSD induces long-lasting symptomatic clusters: re-experiencing, avoidance of trauma-like situations, and hyperarousal (Vieweg et al., 2006). At the endocrine level, these patients frequently exhibit reduced activity of the hypothalamo–pituitary–adrenal (HPA) axis (Yehuda, 2005; Risbrough and Stein, 2006). PTSD also manifests a high co-morbidity with anxiety disorders.

Animal models of posttraumatic stress disorder have been suggested. Pynoos et al. (1996) have proposed a model in which the animal is subjected to a stressor (a brief electric shock), followed by repeated situational reminders, based on findings that exposure to stress reminders contribute to the reactivity and the chronic aspects of this disorder. They observed an abnormal response in a plus maze and increased startle reflex and aggressive behavior (Pynoos et al., 1996). Using a similar model, Louvart et al. (2005b) have observed that behavioral disturbances lasted more than 1 month after the footshock administration (the criteria for

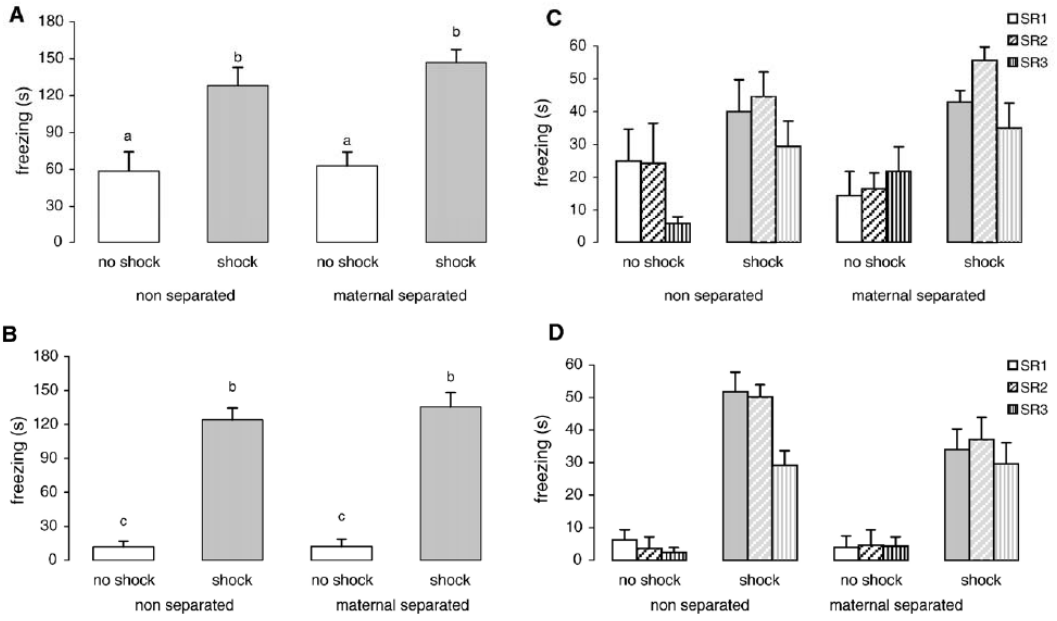

Fig. 1 – Effect of maternal separation during the first 10 days of life on conditioned fear during exposure to situational reminders in animals subjected or not to an inescapable shock as a PTSD model in adult age. Data expressed as mean+SEM of time spent in freezing. (A) Males, total time spent in freezing. (B) Females, total time spent in freezing. (C) Males, freezing behavior during the 3 SR. (D) Females, freezing behavior during the 3 SR. There was a significant effect of exposure to shock and a significant interaction between shock and sex (ANOVA, $P<0.001$ in both cases). Groups labeled with the same letters are not different. Groups labeled with different letters indicate differences between groups ($P<0.05$; Duncan's multiple range test). N=8–16 animals/group.

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an animal model of PTSD include biological alterations that persist over time; see Yehuda and Antelman, 1993). Additionally, it has been observed that gender might be a key factor impacting the effects induced by an intense stress (Louvart et al., 2006).

The neural signaling related to the mechanism of response to physical and/or psychological stress involves changes in cytokine secretion. S100B is an astrocyte-derived cytokine, whose expression and/or secretion appears to be modulated by several factors, including ACTH (Suzuki et al., 1987), a mediator of the stress response. Its release is regulated by serotonin (Whitaker-Azmitia et al., 1990). Blood levels of S100B have been reported to increase after exposure to stress (Scaccianoce et al., 2004), while cerebrospinal fluid (CSF) levels may increase or decrease, presenting a temporal course of variation (Margis et al., 2004). S100B levels both in CSF and in serum have also been reported to change in several psychiatric disorders (Rothermundt et al., 2004; Zimmer et al., 2005). Considering the possible extracerebral sources of S100B, the investigation of its levels in cerebrospinal fluid and in blood may be of interest.

Since different types of psychosocial stressors, including adverse conditions during early life, have been recognized as potential agents in PTSD in humans (Shea et al., 2005; Imanaka et al., 2006), the aim of the present study is to verify if maternal separation in rats alters the susceptibility to the effects of a stressor applied in adult age, verifying its long-lasting effects on conditioned fear during situational reminders, anxiety-like behavior, motor activity, plasma corticosterone (CORT) and S100B levels, as well as CSF S100B levels, using both male and female animals.

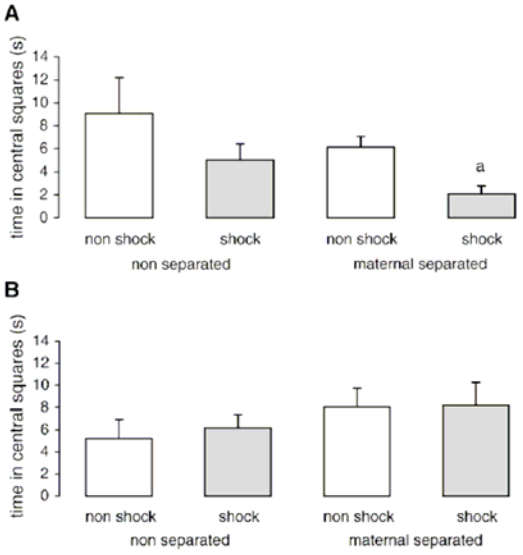

Fig. 2 – Effect of maternal separation during the first 10 days of life on anxiety-like behavior in animals subjected or not to an inescapable shock as a PTSD model in adult age. Data expressed as mean+SEM of time spent in the central area of an open field. (A) Males. (B) Females. There were significant interactions between maternal separation and sex and between exposure to shock and sex (ANOVA, $P<0.05$ in both cases). (a) Different from non-shock non-separated group ($P<0.05$; Duncan's multiple range test). $N=8$–16 animals/group.

2. Results

The effect of an exposure to inescapable shock, used as a PTSD model in animals subjected or not to maternal separation during the first 10 days of life, on conditioned fear is shown in Fig. 1A and B, which displays the time spent freezing during the three situational reminders. A three-way analysis of variance showed a significant effect of inescapable shock [$F(1,101)=141.54$, $P<0.001$], and a significant effect of sex [$F(1,101)=11.96$, $P<0.001$], denoted by a lower time spent freezing in females not exposed to the shock, as well as a significant interaction between sex and shock [$F(1,101)=6.30$, $P<0.02$], due to the fact that the increase in time spent freezing was higher in females exposed to shock when compared to males, reaching similar values for both sexes after shock. No interaction shock×sex×MS was observed [$F(1,101)=0.011$, $P>0.10$]. Freezing behavior during the 3 SR are shown in Fig. 1C and D. A repeated measures analysis of variance using SR as within subjects factors showed a significant effect of SR [$F(1,101)=18.04$, $P<0.001$], and a significant interaction between SR and shock [$F(1,101)=5.64$, $P<0.05$].

The animals were exposed to the open field 1 month after being subjected to the shock. The time spent in the central area was used to evaluate anxiety-like behavior (Prut and Belzung, 2003) and is presented in Fig. 2. Percentages of time spent in central area in relation to the control (non-separated non-shock) group were, for males: 70% (shocked group), 69% (maternal separated group), and 26% (separated+shocked group); for females: 122% (shocked group), 161% (maternal separated group), and 110% (separated+shocked group). Significant interactions were observed between maternal separation and sex [three-factor ANOVA, $F(1,63)=5.61$, $P=0.02$], and between exposure to shock and sex [$F(1,63)=4.17$, $P<0.05$]; no interaction was observed between shock and sex and maternal separation [$F(1,63)=0.029$, $P>0.10$]. While in male animals both maternal separation and exposure to shock increased anxiety-like behavior in females no effect was observed.

Fig. 3 displays the effects observed on exploratory activity 1 month after inescapable shock. Percentages of crossings in relation to the control (non-separated non-shock) group were, for males: 42% (shocked group), 83% (maternal separated group), and 41% (separated+shocked group); for females: 94% (shocked group), 109% (maternal separated group), and 77% (separated + shocked group). Significant effects of shock [three-way ANOVA, $F(1,101)=15.49$, $P<0.001$], and sex [$F(1,101)=9.72$, $P<0.01$] were observed in the number of crossings, as well as a marginally significant interaction between shock and sex [$F(1,101)=3.30$, $P=0.07$]. No interaction shock×sex×MS was observed [three-way ANOVA, $F(1,101)=1.46$, $P>0.1$]. Exposure to shock decreased motility in the open

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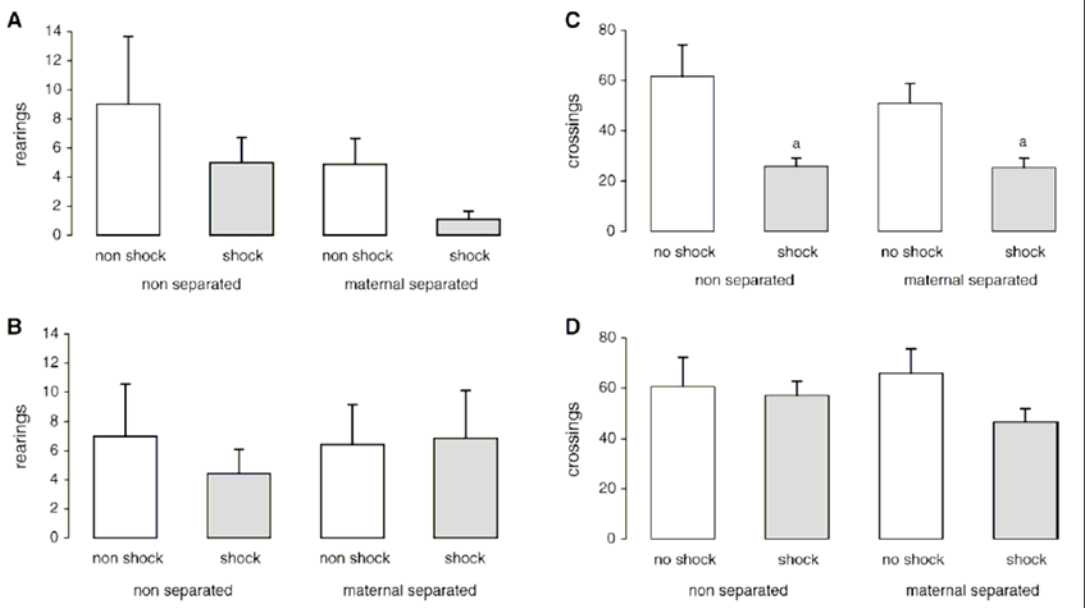

Fig. 3 – Effect of maternal separation during the first 10 days of life on exploratory activity 1 month after subjecting the animals to an inescapable shock as a PTSD model in adult age. Data expressed as mean+SEM of crossings and rearings in the open field. (A) Number of rearings in males. (B) Number of rearings in females. (C) Number of crossings in males. (D) Number of crossings in females. There were significant effects of shock both on rearings ($P<0.05$) and crossings ($P<0.001$), and an effect of sex ($P<0.01$) on crossings, as well as a marginally significant interaction between shock and sex on crossings ($P=0.07$). (a) Groups labeled with this letter are significantly different from non-labeled groups ($P<0.05$; Duncan's multiple range test). $N=8$–16 animals/group.

field in male animals, while no effect was observed in females. A significant effect of shock was observed also on the number of rearings [three-way ANOVA, $F(1,101)=3.89$, $P=0.05$]. Percentages of rearings in relation to the control (non-separated non-shock) group were, for males: 55% (shocked group), 54% (maternal separated group), and 12% (separated+shocked group); for females: 63% (shocked group), 92% (maternal separated group), and 98% (separated+shocked group).

Basal plasma corticosterone was measured and the results are shown in Fig. 4. Significant effects of maternal separation [three-way ANOVA, $F(1,38)=14.50$, $P<0.001$], and exposure to shock [$F(1,38)=19.33$, $P<0.01$] were observed, as well as significant interactions between shock and sex [$F(1,38)=5.30$, $P<0.05$] and between exposure to shock, maternal separation and sex [$F(1,38)=7.91$, $P<0.001$]. Both interventions (maternal separation and exposure to inescapable shock) decreased plasma corticosterone levels; however, this effect was more pronounced in males.

S100B was measured in plasma and in CSF (Fig. 5). In plasma, we observed an effect of exposure to shock [three-way ANOVA, $F(1,47)=3.82$, $P=0.05$], and a significant interaction between shock and sex [$F(1,47)=6.42$, $P<0.02$]. Exposure to inescapable shock increased plasma S100B levels; however, this effect was observed only in males. On the other hand, with regard to CSF S100B levels, no effect was observed in male animals. In females, however, maternal separation caused a significant increase in CSF S100B levels [two-way ANOVA, $F(1,40)=5.31$, $P=0.026$]. Exposure to shock had no effect on CSF S100B levels and no interaction was observed between maternal separation and shock.

3. Discussion

PTSD is a debilitating anxiety disorder, and its symptoms include anxiety and hyperarousal, as well as cognitive impairments. Cortisol levels have been observed to be in the low range of normal in these patients, and a hypersensitivity of the HPA-axis feedback or an increased tissue sensitivity to glucocorticoids has been suggested (Yehuda et al., 2004; Shea et al., 2005). Not all individuals exposed to severe traumatic experiences develop PTSD, and risk factors may include individual neurobiology as well as past experiences. Early adverse experiences are considered a major risk factor for the development of anxiety disorders. Therefore, this study sets out to investigate the effects of maternal separation (MS), in male and female rats, on the outcome of exposing them, as adults, to a stressful experience. Behavioral observations were made at least 1 month after the stressful experience (the inescapable shock, in this case), in an attempt to model long-term effects of this exposure to a severe stressor, as observed in humans with PTSD.

We observed that both treatments, maternal separation during the first 10 days of life and exposure to a stressful event

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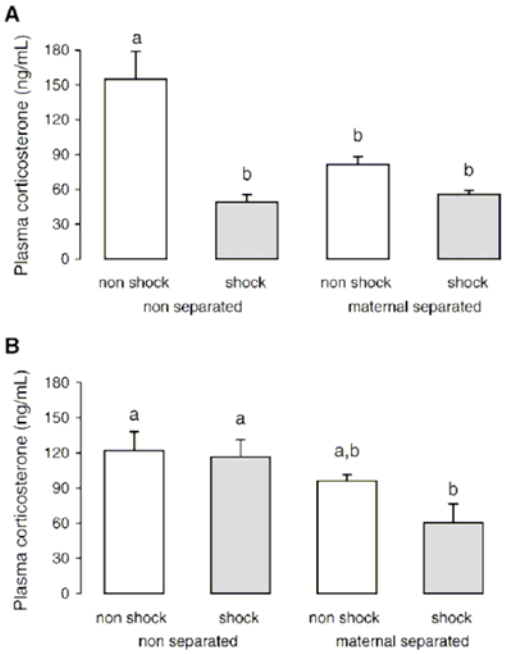

Fig. 4 – Effect of maternal separation during the first 10 days of life on plasma corticosterone levels in the adulthood 2 months after subjecting the animals to an inescapable shock as a PTSD model in adult age. Data expressed as mean + SEM. (A) Plasma corticosterone in males. (B) Plasma corticosterone in females. There were significant effects of both maternal separation and exposure to shock ($P<0.001$ in both cases) and interactions between sex and shock ($P<0.05$), and between sex, maternal separation and shock ($P<0.01$). $N=5–7$ animals/group. Groups labeled with different letters indicate differences between groups ($P<0.05$; Duncan's multiple range test).

in adult life, presented important effects on anxiety-like behavior and exploratory behavior in a new environment, but mainly in male animals. Both male and female animals subjected to shock presented long-lasting effects on fear conditioning, while maternal separation presented no effect in this parameter. In contrast, males exposed to maternal separation and to shock exhibited decreased exploratory activity in the open field; this activity was further decreased in male animals subjected to both interventions. No difference was observed in female rats with regard to the effects on exploratory activity.

Our results agree with other reports from the literature, in which locomotor hypoactivity was observed in male rats exposed to a severe stressor (Van Dijken et al., 1992; Van den Berg et al., 1998). Using this model of PTSD, no difference was observed in mice (Pynoos et al., 1996); therefore, this result may be species-specific. Louvart et al. (2005b) observed that female rats exposed to shock exhibited locomotor hipoactivity; however, this evaluation was made in the plus maze, a task considerably more anxiogenic than an open field. Effects of maternal separation in decreasing exploratory activity have also been reported (Kalinichev et al., 2002). Therefore, the present results agree with the literature with regard to the effects of exposure to maternal separation (MS) and to a severe stressor on exploratory activity, suggesting, furthermore, that MS may aggravate the outcome of exposure to a PTSD model in this parameter, at least in males.

Anxiety-like behavior, as evaluated by the time spent in the central area of the open field (Prut and Belzung, 2003), presented the same general results, suggesting an aggravation of the anxiety-like index (i.e., reduction of the time spent in central squares) in MS males, when exposed to a stressor, and with no effect observed in females.

Therefore, an important observation of the present study was the higher susceptibility of male animals to both interventions used (maternal separation and stress exposure at an adult age) to the development of increased anxiety-like levels. Several studies examining the effects of maternal separation in male and female offspring have observed sex-dependent effects, with males more susceptible than females (Wigger and Neumann, 1999; Kalinichev et al., 2002; Renard et al., 2005; Lehmann et al., 1999). For example, maternal separation was observed to present a more robust effect on anxiety-like behavior in males than in females when evaluated by the plus maze (Wigger and Neumann, 1999; Kalinichev et al., 2002; Renard et al., 2005), and by ultrasonic vocalizations (Kalinichev et al., 2002). Maternal separation effects on fear-potentiated startle have also been observed to be more pronounced in males than in females (De Jongh et al., 2005).

The studies mentioned above are in agreement with our data, concerning more robust effects of maternal separation in males than in females on anxiety-like behavior. On the other hand, the fact that early adverse experience affects males with more intensity is surprising, considering the fact that women have a higher prevalence of anxiety disorders than men, and that early adverse experiences are a major risk factor for the development of anxiety disorders (Lehmann et al., 1999). However, it is important to point out that any attempt to consider sex differences in the susceptibility to early adverse experiences when using animal models must take into account species-specific differences, including dams' behavior, and the fact that these animals usually come from mixed litters, differently from humans.

When measuring basal plasma corticosterone, no significant difference between non-separated non-shocked males and females was observed. Female rats have been observed to present higher levels of corticosterone than males (Kitay, 1961; Weinstock et al., 1998), and these sexual differences are dependent on estrous cycle stage (Atkinson and Waddell, 1997). At the time of the circadian rhythm considered here, however, males and females do not show differences in plasma corticosterone levels in Wistars (Atkinson and Waddell, 1997) and other strains (Critchlow et al., 1963).

Basal plasma corticosterone was reduced in animals subjected to maternal separation or to a PTSD model, and these changes were more notable in male rats. In relation to the literature, different paradigms of maternal separation have been used, and the effects of MS on HPA axis in adult rats are not consistent among studies. Reports have shown that maternal separated animals present increased response to

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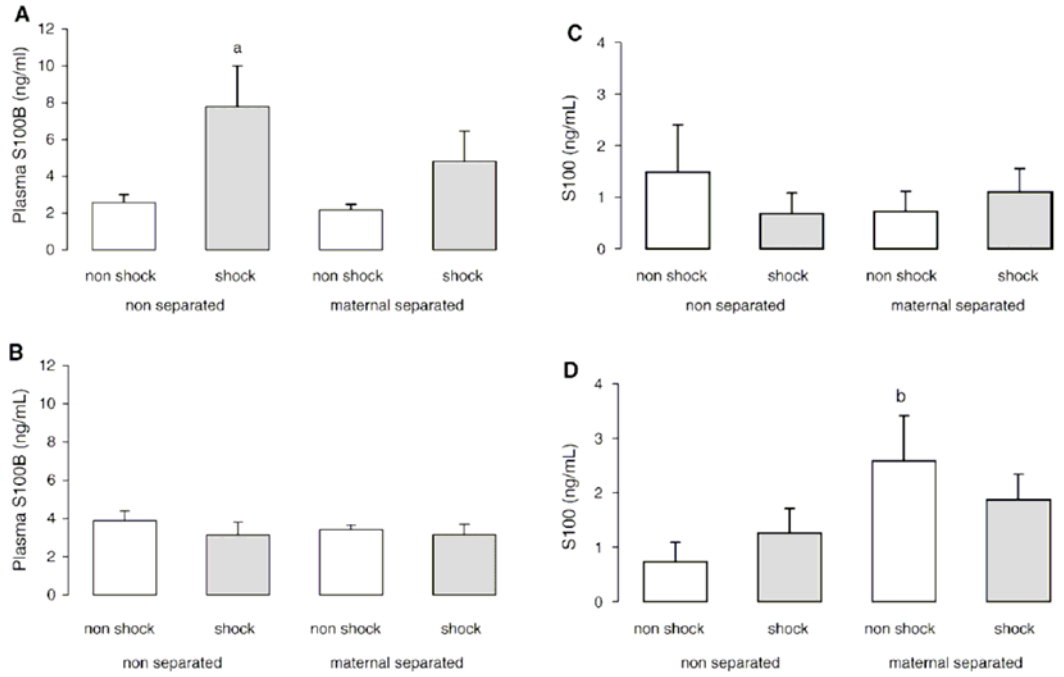

Fig. 5 – Effect of maternal separation during the first 10 days of life on plasma and CSF S100B levels in the adulthood 2 months after subjecting the animals to an inescapable shock as a PTSD model in adult age. Data expressed as mean + SEM. (A) Plasma S100B in males. (B) Plasma S100B in females. (C) CSF S100B in males. (D) CSF S100B in females. In plasma, there was an effect of exposure to shock [three-way ANOVA, $F(1,47)=3.82$, $P=0.05$], and a significant interaction between shock and sex [$F(1,47)=6.42$, $P<0.02$]. In CSF, there was a significant effect of maternal separation in females [two-way ANOVA, $F(1,40)=5.31$, $P<0.05$]. Measurements in plasma: $N=5–8$ animals/group. Measurements in CSF: $N=5–12$ animals/group. (a) Different from both groups not subjected to shock (Duncan's multiple range test, $P<0.05$). (b) Different from non-shock non-separated females (Duncan's multiple range test, $P<0.05$).

stress, although, in general no differences are observed in basal corticosterone levels (Plotsky and Meaney, 1993; Wigger and Neumann, 1999; Kalinichev et al., 2002; Francis et al., 2002), and decreased reactive feedback have been suggested to contribute to this different response to an acute stressor (Ladd et al., 2004). On the other hand, there is recent published data showing that maternal separation leads to decreased basal plasma corticosterone levels and decreased adrenal glands weight (Slotten et al., 2006), and reduced response of corticosterone to stress has also been shown in MS rats (Ogawa et al., 1994; Kim et al., 2005). These different observations suggest that distinct MS conditions influence its long-term effects, and it has recently been shown that the length and the time of the day when maternal separation is performed are both important factors for these effects (Yoshihara et al., 2005).

In an animal PTSD model, Harvey et al. (2003, 2006) observed reduced corticosterone levels and Louvart et al. (2006), using female rats and a similar model of PTSD, observed blunted corticosterone response to stress, although no difference was observed in basal levels. Cohen et al. (2006) observed that, following exposure to a severe stressor, behavioral changes were more prevalent in rats with a blunted HPA axis response. This is in agreement with the more pronounced effects observed in males, especially when previously subjected to maternal separation.

Endocrinic changes, as observed by reduced plasma corticosterone levels, presented by animals subjected to both treatments parallel those observed in some PTSD patients (Yehuda, 2005; Yehuda et al., 2006): in PTSD patients, a hypersensitivity of the HPA-axis negative feedback system is suggested to be involved in the excessive suppression of glucocorticoid release, since these patients present increased negative feedback to dexamethasone (Yehuda et al., 1993).

S100B serum levels have been associated with a number of pathological conditions (Rothermundt et al., 2003), and have been reported to increase after exposure to stress (Scaccianoce et al., 2004). This effect of stress on S100B levels was independent of glucocorticoid hormones (Scaccianoce et al., 2004). In the present study, exposure to shock induced a marked and persistent increase in serum S100B in males, while no effect was observed in females. These results correlate with those observed in exploratory and anxiety-like behavior, when shock effects were much more robust in males. This serum S100B change was not observed in CSF, indicating an absence of correlation in the S100B contents

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between these two compartments. These results suggest that the increase observed in serum S100B levels does not have a central origin, and extracerebral sources must be considered. A possible explanation is that the effect of these interventions on serum S100B levels in males was mediated by an increase in the noradrenergic tonus. In fact, epinephrine has been reported to stimulate S100B release by adipocytes (Netto et al., 2006a). It is interesting to observe that, in PTSD patients, increased noradrenergic tonus has been suggested (Cohen et al., 2000a,b; Young and Breslau, 2004). Human subjects exposed to a traumatic event and with increased heart rate are considered to be at high risk for developing PTSD (Shalev et al., 1998), and administration of exogenous catecholamines to intensive care unit patients favors the evocation of traumatic memories (Schelling et al., 2004). Moreover, CSF S100B was increased in females exposed to maternal separation and, again, without a correlation to serum values. Taken together, these results indicate that extracellular levels of S100B (released from cerebral and extracerebral sources) in rats appear to be gender-dependent.

S100B protein in brain is produced primarily by astrocytes, and has been used as a marker for brain injury (e.g., Netto et al., 2006b; Foerch et al., 2005; Chen and Zhu, 2005). This protein has also been shown to be neurotrophic and neuroprotective (Donato, 2001; Van Eldik and Wainwright, 2003; Willoughby et al., 2004). It presents a trophic activity on neurons, increasing survival and promoting neurite extension in vitro (Nishi et al., 2000). Given the neurotrophic actions of S100B, we could speculate that its increased secretion in the CNS of maternal deprived females could be protecting these animals against the effects of additional stressors applied, as was observed in the present study.

Our findings of increased serum S100B, which seems to be related to increased anxiety-like levels, may be of some relevance in the study of PTSD neurobiology. Obviously, these data may not be extrapolated to humans; however, they reinforce the necessity to investigate the role of this protein in neuropsychiatric disorders. Further studies on S100B levels in PTSD patients could add more significance to this hypothesis.

In summary, exposure to a severe stressor in adult life, which is considered a model of PTSD, had enduring effects on behavior and physiology, mainly in male rats, increasing anxiety-like behavior and conditioned fear, and caused a reduction in exploratory behavior; increased serum S100B levels paralleled these behavior changes. Some of these effects were further increased in male rats exposed to maternal separation in a critical period of the development, but not in females.

4. Experimental procedure

4.1. Subjects

Pregnant Wistar rats bred at our own animal facility were randomly selected. Animals were housed alone in home cages made of Plexiglas (65×25×15 cm) with the floor covered with sawdust and were maintained in a controlled environment until offspring: lights on between 07:00 h and 19:00 h, temperature of 22±2 °C, cage cleaning once a week, food and water provided. All litters were culled within 24 h to eight pups and were maintained intact unless for maternal separation procedures, which were carried out between 10:00 h and 14:00 h.

Weaning was on postnatal day 21. A maximum of two male and two female pups were used per litter per experiment. Rats were housed four to five per cage, and separated by sex. Fifty one experimental male rats and fifty eight female rats were used in the different experiments, derived from 15 different litters. Rats had free access to food (standard lab rat chow) and water, except during the period when the behavioral tasks were applied. Tasks were performed between 13:00 h and 16:00 h, after animals had reached adult life.

All animal treatments were approved by the Institutional Ethical Committee and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS).

4.2. Maternal separation

4.2.1. Non-separated group

Pups were left undisturbed with the dam until weaning. It was stated on the cage that these animals should not be touched, not even for cage cleaning. Dirty sawdust was carefully removed from one side of the cage, without disturbing the mother and the nest, and replaced by clean sawdust at that side by the principal researcher.

4.2.2. Maternal separation

Pups were removed from their home cage and were placed into a clean cage lined with clean paper towel, inside an incubator at 34 ° C next to the dam's cage. After 3 h, pups were returned to their dams. This procedure was carried out for the first 10 days of life, after which pups were left undisturbed until the 21st day of life.

4.3. Exposure to a stressor during adulthood

After reaching 60 days of age, the animals were subdivided into two other groups: no shock and shock (adapted from Pynoos et al., 1996), which consisted of a single exposure to footshock, followed by 3 weekly exposures to a situational reminder (SR).

The apparatus consisted of a 50×25×25 cm box, which was divided in two equal compartments, both compartments with a frontal glass wall. The first compartment presented a linoleum floor, and the second compartment presented a grid floor consisting of 1 mm bronze bars spaced 10 mm apart. The animals were gently held by their body and lowered in the first compartment, with their nose pointing to the rear left corner. After 2 min, a guillotine door was opened until the animal crossed to the second compartment. The door was then closed and a 1 mA 60 Hz footshock was delivered during 20 s. The no shock group was subjected to the same treatment, but no shock was delivered.

4.4. Exposure to situational reminders

For the exposure to situational reminders (SR), 1 week after exposure to the apparatus described above, the animals were

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replaced in the box for 2 min, but just in the first compartment. During the SR, the time spent in freezing was scored, as a measure of conditioned fear. This procedure was repeated during 3 weeks, with a seven-day interval between each SR.

4.5. *Exposure to the open field*

A 50-cm high, 40×60-cm open field made of wood with a frontal glass wall was used (Silveira et al., 2005). The floor was subdivided with white lines into 12 equal 13.3- by 15.0-cm rectangles. The animals were submitted to this task 1 week after the third SR, i.e., 4 weeks after shock exposure, and were gently placed facing the left corner and allowed to explore the arena for 5 min. The performance of rearings, line crossings, and the time spent in central squares was counted. The number of rearings and crossings was used as a measure of exploratory behavior, while time spent in central squares was considered to evaluate anxiety-like behavior (Prut and Belzung, 2003).

4.6. *Biochemical measurements*

CSF collection was made between 3 and 4 weeks after the behavioral experiments. Rats were transported to another room and anesthetized with 120 mg/kg ketamine HCl (Dopalen: Agribrands, Campinas, SP, Brazil) and 16 mg/kg xylazine (Anasedan: Agribrands, Campinas, SP, Brazil). CSF samples were obtained by magna cistern puncture and stored at −70 °C for S100B measurement. One week later, animals were sacrificed between 13:00 and 16:00 h, and the trunk blood was collected into heparinized tubes, centrifuged at 4 °C at 3000×*g*, and plasma separated and stored at −20 °C until analysis. All animals were sacrificed within this interval of time in a random order considering groups and genders.

S100B content was measured both in plasma and CSF by ELISA, as described previously (Tramontina et al., 2000). Briefly, 50 μl of sample (diluted 1:4 and 1:50 in case of sample of CSF and serum, respectively) plus 50 μl of barbital buffer was incubated for 3 h on a microtiter plate previously coated with monoclonal anti-S100B (SH-B1, Sigma). Peroxidase-conjugated anti-S100 (from DAKO) was then incubated for 1 h. The color reaction with o-phenylenediamine was measured at 492 nm in a plate reader.

For corticosterone determination, plasma was extracted with ethyl acetate, and the extract evaporated and dissolved for the hormone evaluation with an ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA). The sensitivity of the assay is 24 pg/ml and the intraassay coefficient of variation was 15%.

4.7. *Statistical analysis*

Data were expressed as mean+standard error of the mean, and were analyzed by a three-factors ANOVA, using maternal separation, exposure to shock, and sex as factors, followed by the Duncan multiple range test when the *F* test was significant. The significance level was accepted as different when the *P* value was equal or less than 0.05. Sample size varies in each experiment and is shown individually in the Results section.

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REFERENCES

Atkinson, H.C., Waddell, B.J., 1997. Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: sexual dimorphism and changes across the estrous cycle. Endocrinology 138, 3842–3848.

Chen, D.Q., Zhu, L.L., 2005. Dynamic change of serum protein S100b and its clinical significance in patients with traumatic brain injury. Chin. J. Traumatol. 8, 245–248.

Cohen, H., Benjamin, J., Geva, A.B., Matar, M.A., Kaplan, Z., Kotler, M., 2000a. Autonomic dysregulation in panic disorder and in post-traumatic stress disorder: application of power spectrum analysis of heart rate variability at rest and in response to recollection of trauma or panic attacks. Psychiatry Res. 96, 1–13.

Cohen, H., Kotler, M., Matar, M., Kaplan, Z., 2000b. Normalization of heart rate variability in post-traumatic stress disorder patients following fluoxetine treatment: preliminary results. Isr. Med. Assoc. J. 2, 296–301.

Cohen, H., Zohar, J., Gidron, Y., Matar, M.A., Belkind, D., Loewenthal, U., Kozlovsky, N., Kaplan, Z., 2006. Blunted HPA axis response to stress influences susceptibility to posttraumatic stress response in rats. Biol. Psychiatry 59, 1208–1218.

Critchlow, V., Liebelt, R.A., Bar-Sela, M., Mountcastle, W., Lipscomb, H.S., 1963. Sex difference in resting pituitary-adrenal function in the rat. Am. J. Physiol. 205, 807–815.

de Jongh, R., Geyer, M.A., Olivier, B., Groenink, L., 2005. The effects of sex and neonatal maternal separation on fear-potentiated and light-enhanced startle. Behav. Brain Res. 161, 190–196.

de Kloet, E.R., Sibug, R.M., Helmerhorst, F.M., Schmidt, M.V., 2005. Stress, genes and the mechanism of programming the brain for later life. Neurosci. Biobehav. Rev. 29, 271–281.

Donato, R., 2001. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. Int. J. Biochem. Cell Biol. 33, 637–668.

Foerch, C., Singer, O.C., Neumann-Haefelin, T., du Mesnil de Rochemont, R., Steinmetz, H., Sitzer, M., 2005. Evaluation of serum S100B as a surrogate marker for long-term outcome and infarct volume in acute middle cerebral artery infarction. Arch. Neurol. 62, 1130–1134.

Francis, D.D., Diorio, J., Plotsky, P.M., Meaney, M.J., 2002. Environmental enrichment reverses the effects of maternal separation on stress reactivity. J. Neurosci. 22, 7840–7843.

Harvey, B.H., Naciti, C., Brand, L., Stein, D.J., 2003. Endocrine, cognitive and hippocampal/cortical 5HT 1A/2A receptor changes evoked by a time-dependent sensitisation (TDS) stress model in rats. Brain Res. 983, 97–107.

Harvey, B.H., Brand, L., Jeeva, Z., Stein, D.J., 2006. Cortical/hippocampal monoamines, HPA-axis changes and aversive behavior following stress and restress in an animal model of post-traumatic stress disorder. Physiol. Behav. 87, 881–890.

Imanaka, A., Morinobu, S., Toki, S., Yamawaki, S., 2006. Importance of early environment in the development of post-traumatic stress disorder-like behaviors. Behav. Brain Res. 173, 129–137.

Kalinichev, M., Easterling, K.W., Plotsky, P.M., Holtzman, S.G., 2002. Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long–Evans rats. Pharmacol. Biochem. Behav. 73, 131–140.

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Kim, H.J., Lee, J.H., Choi, S.H., Lee, Y.S., Jahng, J.W., 2005. Fasting-induced increases of arcuate NPY mRNA and plasma corticosterone are blunted in the rat experienced neonatal maternal separation. Neuropeptides 39, 587–594.

Kitay, J.I., 1961. Sex differences in adrenal cortical secretion in the rat. Endocrinology 68, 818–824.

Ladd, C.O., Huot, R.L., Thrivikraman, K.V., Nemeroff, C.B., Plotsky, P.M., 2004. Long-term adaptations in glucocorticoid receptor and mineralocorticoid receptor mRNA and negative feedback on the hypothalamo–pituitary–adrenal axis following neonatal maternal separation. Biol. Psychiatry 55, 367–375.

Lehmann, J., Pryce, C.R., Bettschen, D., Feldon, J., 1999. The maternal separation paradigm and adult emotionality and cognition in male and female Wistar rats. Pharmacol. Biochem. Behav. 64, 705–715.

Liu, D., Caldji, C., Sharma, S., Plotsky, P.M., Meaney, M.J., 2000. Influence of neonatal rearing conditions on stress-induced adrenocorticotropin responses and norepinepherine release in the hypothalamic paraventricular nucleus. J. Neuroendocrinol. 12, 5–12.

Louvart, H., Maccari, S., Darnaudery, M., 2005a. Prenatal stress affects behavioral reactivity to an intense stress in adult female rats. Brain Res. 1031, 67–73.

Louvart, H., Maccari, S., Ducrocq, F., Thomas, P., Darnaudery, M., 2005b. Long-term behavioural alterations in female rats after a single intense footshock followed by situational reminders. Psychoneuroendocrinology 30, 316–324.

Louvart, H., Maccari, S., Lesage, J., Leonhardt, M., Dickes-Coopman, A., Darnaudery, M., 2006. Effects of a single footshock followed by situational reminders on HPA axis and behaviour in the aversive context in male and female rats. Psychoneuroendocrinology 31, 92–99.

Margis, R., Zanatto, V.C., Tramontina, F., Vinade, E., Lhullier, F., Portela, L.V., Souza, D.O., Dalmaz, C., Kapczinski, F., Gonçalves, C.A., 2004. Changes in S100B cerebrospinal fluid levels of rats subjected to predator stress. Brain Res. 1028, 213–218.

Netto, C.B., Conte, S., Leite, M.C., Pires, C., Martins, T.L., Vidal, P., Benfato, M.S., Giugliani, R., Gonçalves, C.A., 2006a. Serum S100B protein is increased in fasting rats. Arch. Med. Res. 37, 683–686.

Netto, C.B., Portela, L.V., Felix, T.M., Souza, D.O., Goncalves, C.A., Giugliani, R., 2006b. Serum S100B levels in patients with neural tube defects. Clin. Chim. Acta 364, 275–278.

Neumann, I.D., Wigger, A., Kromer, S., Frank, E., Landgraf, R., Bosch, O.J., 2005. Differential effects of periodic maternal separation on adult stress coping in a rat model of extremes in trait anxiety. Neuroscience 132, 867–877.

Nishi, M., Kawata, M., Azmitia, E.C., 2000. Trophic interactions between brain-derived neurotrophic factor and s100beta on cultured serotonergic neurons. Brain Res. 868, 113–118.

Ogawa, T., Mikuni, M., Kuroda, Y., Muneoka, K., Mori, K.J., Takahashi, K., 1994. Periodic maternal deprivation alters stress response in adult offspring: potentiates the negative feedback regulation of restraint stress-induced adrenocortical response and reduces the frequencies of open field-induced behaviors. Pharmacol. Biochem. Behav. 49, 961–967.

Plotsky, P.M., Meaney, M.J., 1993. Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Brain Res. Mol. Brain Res. 18, 195–200.

Prut, L., Belzung, C., 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur. J. Pharmacol. 463, 3–33.

Pynoos, R.S., Ritzmann, R.F., Steinberg, A.M., Goenjian, A., Prisecaru, I., 1996. A behavioral animal model of posttraumatic stress disorder featuring repeated exposure to situational reminders. Biol. Psychiatry 39, 129–134.

Renard, G.M., Suarez, M.M., Levin, G.M., Rivarola, M.A., 2005. Sex differences in rats: effects of chronic stress on sympathetic system and anxiety. Physiol. Behav. 85, 363–369.

Risbrough, V.B., Stein, M.B., 2006. Role of corticotropin releasing factor in anxiety disorders: a translational research perspective. Horm. Behav. 50, 550–561.

Rothermundt, M., Peters, M., Prehn, J.H., Arolt, V., 2003. S100B in brain damage and neurodegeneration. Microsc. Res. Tech. 60, 614–632.

Rothermundt, M., Ponath, G., Arolt, V., 2004. S100B in schizophrenic psychosis. Int. Rev. Neurobiol. 59, 445–470.

Scaccianoce, S., Del Bianco, P., Pannitteri, G., Passarelli, F., 2004. Relationship between stress and circulating levels of S100B protein. Brain Res. 1004, 208–211.

Schelling, G., Roozendaal, B., De Quervain, D.J., 2004. Can posttraumatic stress disorder be prevented with glucocorticoids? Ann. N. Y. Acad. Sci. 1032, 158–166.

Severino, G.S., Fossati, I.A., Padoin, M.J., Gomes, C.M., Trevizan, L., Sanvitto, G.L., Franci, C.R., Anselmo-Franci, J.A., Lucion, A.B., 2004. Effects of neonatal handling on the behavior and prolactin stress response in male and female rats at various ages and estrous cycle phases of females. Physiol. Behav. 81, 489–498.

Shalev, A.Y., Sahar, T., Freedman, S., Peri, T., Glick, N., Brandes, D., Orr, S.P., Pitman, R.K., 1998. A prospective study of heart rate response following trauma and the subsequent development of posttraumatic stress disorder. Arch. Gen. Psychiatry 55, 553–559.

Shea, A., Walsh, C., Macmillan, H., Steiner, M., 2005. Child maltreatment and HPA axis dysregulation: relationship to major depressive disorder and post traumatic stress disorder in females. Psychoneuroendocrinology 30, 162–178.

Silveira, P.P., Portella, A.K., Clemente, Z., Gamaro, G.D., Dalmaz, C., 2005. The effect of neonatal handling on adult feeding behavior is not an anxiety-like behavior. Int. J. Dev. Neurosci. 23, 93–99.

Slotten, H.A., Kalinichev, M., Hagan, J.J., Marsden, C.A., Fone, K.C., 2006. Long-lasting changes in behavioural and neuroendocrine indices in the rat following neonatal maternal separation: gender-dependent effects. Brain Res. 1097, 123–132.

Suzuki, F., Kato, K., Kato, T., Ogasawara, N., 1987. S-100 protein in clonal astroglioma cells is released by adrenocorticotropic hormone and corticotropin-like intermediate-lobe peptide. J. Neurochem. 49, 1557–1563.

Tramontina, F., Karl, J., Gottfried, C., Mendez, A., Goncalves, D., Portela, L.V., Goncalves, C.A., 2000. Digitonin-permeabilization of astrocytes in culture monitored by trypan blue exclusion and loss of S100B by ELISA. Brain Res. Protoc. 6, 86–90.

Van den Berg, C.L., Lamberts, R.R., Wolterink, G., Wiegant, V.M., Van Ree, J.M., 1998. Emotional and footshock stimuli induce differential long-lasting behavioural effects in rats; involvement of opioids. Brain Res. 799, 6–15.

van Dijken, H.H., Mos, J., van der Heyden, J.A., Tilders, F.J., 1992. Characterization of stress-induced long-term behavioural changes in rats: evidence in favor of anxiety. Physiol. Behav. 52, 945–951.

Van Eldik, L.J., Wainwright, M.S., 2003. The Janus face of glial-derived S100B: beneficial and detrimental functions in the brain. Restor. Neurol. Neurosci. 21, 97–108.

Vieweg, W.V., Julius, D.A., Fernandez, A., Beatty-Brooks, M., Hettema, J.M., Pandurangi, A.K., 2006. Posttraumatic stress disorder: clinical features, pathophysiology, and treatment. Am. J. Med. 119, 383–390.

Weinstock, M., Razin, M., Schorer-Apelbaum, D., Men, D., McCarty, R., 1998. Gender differences in sympathoadrenal activity in rats at rest and in response to footshock stress. Int. J. Dev. Neurosci. 16, 289–295.

Whitaker-Azmitia, P.M., Murphy, R., Azmitia, E.C., 1990. Stimulation of astroglial 5-HT1A receptors releases the

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serotonergic growth factor, protein S-100, and alters astroglial morphology. Brain Res. 528, 155–158.
Wigger, A., Neumann, I.D., 1999. Periodic maternal deprivation induces gender-dependent alterations in behavioral and neuroendocrine responses to emotional stress in adult rats. Physiol. Behav. 66, 293–302.
Willoughby, K.A., Kleindienst, A., Muller, C., Chen, T., Muir, J.K., Ellis, E.F., 2004. S100B protein is released by in vitro trauma and reduces delayed neuronal injury. J. Neurochem. 91, 1284–1291.
Yehuda, R., 2005. Neuroendocrine aspects of PTSD. Handb. Exp. Pharmacol. 169, 371–403.
Yehuda, R., Antelman, S.M., 1993. Criteria for rationally evaluating animal models of posttraumatic stress disorder. Biol. Psychiatry 33, 479–486.
Yehuda, R., Southwick, S.M., Krystal, J.H., Bremner, D., Charney, D.S., Mason, J.W., 1993. Enhanced suppression of cortisol following dexamethasone administration in posttraumatic stress disorder. Am. J. Psychiatry 150, 83–86.
Yehuda, R., Golier, J.A., Yang, R.K., Tischler, L., 2004. Enhanced sensitivity to glucocorticoids in peripheral mononuclear leukocytes in posttraumatic stress disorder. Biol. Psychiatry 55, 1110–1116.
Yehuda, R., Yang, R.K., Buchsbaum, M.S., Golier, J.A., 2006. Alterations in cortisol negative feedback inhibition as examined using the ACTH response to cortisol administration in PTSD. Psychoneuroendocrinology 31, 447–451.
Yoshihara, T., Otsuki, Y., Yamazaki, A., Honma, S., Yamasaki, Y., Honma, K., 2005. Maternal deprivation in neonatal rats alters the expression of circadian system under light–dark cycles and restricted daily feeding in adulthood. Physiol. Behav. 85, 646–654.
Young, E.A., Breslau, N., 2004. Cortisol and catecholamines in posttraumatic stress disorder: an epidemiologic community study. Arch. Gen. Psychiatry 61, 394–401.
Zimmer, D.B., Chaplin, J., Baldwin, A., Rast, M., 2005. S100-mediated signal transduction in the nervous system and neurological diseases. Cell. Mol. Biol. 51, 201–214.

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CAPÍTULO 2

ARTIGO 2

ARTIGO A SER SUBMETIDO

EARLY ADVERSE LIFE EVENTS ALTER THE SUSCEPTIBILITY TO THE EFFECTS OF A STRESSOR APPLIED IN ADULTHOOD: COGNITIVE PARAMETERS AND DNA DAMAGE TO THE HIPPOCAMPUS

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Abstract

Adverse early life events, such as periodic maternal separation, may alter the normal pattern of brain development and subsequently the vulnerability to a variety of mental disorders in adulthood. For example, patients with a history of early adversities present higher frequency of post-traumatic stress disorder (PTSD). This study was undertaken to verify if repeated long-term separation from dams would affect the development of cognitive and biochemical effects after exposure to an animal model of PTSD. Nests of Wistar rats were divided in intact and subjected to maternal separation (incubator at 32°C, 3h/day) during post-natal days 1 to 10. When adults, the animals were subdivided into exposed or not to a traumatic event (inescapable shock) and to three situational reminders. One month after exposure to the shock, the animals were exposed to a task to evaluate memory (Morris water maze) and another month later animals were sacrificed and DNA damage and oxidative parameters (antioxidant enzymes activities) were measured in hippocampus. Rats exposed to shock or maternal separation plus shock presented long-lasting effects on spatial memory, spending more time in the opposite quadrant of the water maze; this effect was higher in animals subjected to both maternal separation and shock. Animals subjected to both shock and maternal separation exhibited a higher score of DNA damage in hippocampus. No differences were observed on antioxidant enzymes activities. In conclusion, periodic maternal separation may increase the susceptibility to the effects of a stressor applied in adulthood in performance in the water maze. Increased DNA damage in hippocampus was induced by both, maternal separation and exposure to shock.

Key words: maternal separation; post-traumatic stress disorder; comet assay; memory; oxidative stress; superoxide dismutase; glutathione peroxidase; catalase; antioxidant enzymes.

Abbreviations: PTSD, post-traumatic stress disorder; MS, maternal separation; SR, situational reminders; ROS, Reactive Oxygen Species; HPA, hypothalamic–pituitary–adrenocortical; ACTH, adrenocorticotropin; DNA, deoxyribonucleic acid; SOD superoxide dismutase; CAT, catalase; GPx glutathione peroxidase; brain-derived neurotrophic factor, BDNF.

INTRODUCTION

The neonatal stage from approximately days 3 to 14 of life has been described as a "stress hyporesponsive period" that is critical for the maturation of the hypothalamic–pituitary–adrenocortical (HPA) axis in the rat. This period is characterized by a low responsiveness of the adrenocortical system to stressors, reduced sensitivity of the adrenals to adrenocorticotropin (ACTH) and amplified negative feedback action of corticosterone (WIGGER and NEUMANN, 1999). The HPA axis is one of the major neuroendocrine systems activated in response to actual or perceived environmental challenges. Therefore, early environmental manipulations exerts long-lasting effects on behavioral parameters related to coping with stress (WIGGER & NEUMANN, 1999). One of these manipulations, long-term separation from the dam is considered to be one of the most potent naturally occurring stressors to which rat pups can be exposed during this period and results in an acute disinhibition of the HPA axis. In this procedure, neonatal rats are removed from the mother for several hours daily during the first 2 weeks of life (MEANEY, *et al*., 1993; WIGGER & NEUMANN, 1999; KALINICHEV *et al*., 2002). When tested as adults, maternally separated (MS) adult offspring exhibit a cluster of behavioral and neuroendocrine signs similar to those observed in patients with depression and anxiety disorders (LADD *et al*., 2000; DIEHL *et al*., 2007).

In the last years, there has been an increased interest in the topic of early environmental influences on physiology and behavior. Many evidences indicate that exposure to early adverse life events in the form of childhood neglect and abuse can increase vulnerability to psychopathology in adult life (CALDJI *et al*., 2000), such as post-traumatic stress disorder (PTSD). PTSD is a serious and debilitating anxiety disorder in

which a person exposed to a traumatic event (or events), such as natural disasters, road accidents, violent assault, torture or wars develops symptoms in three domains: avoiding stimuli associated with the trauma, experiencing symptoms of increased autonomic arousal and reexperiencing the trauma. The reexperiencing of the traumatic event occurs persistently in recurrent and intrusive recollections of the event and recurrent dreams (VIEWEG *et al*., 2006). Studies in samples of both general population and treatment individuals document high rates of psychiatric comorbidity among subjects with PTSD (KESSLER, 1995; BRESLAU, 2000). This disorder consists in pathological replay of the emotional memory formed in response to painful, life-threatening, or horrifying events (VIEWEG *et al*., 2006). At the endocrine level, these patients frequently exhibit reduced activity of the HPA axis and also manifest a high co-morbidity with anxiety disorders (YEHUDA, 2005; RISBROUGH & STEIN, 2006). Imaging studies in PTSD patients have demonstrated volume reductions in the hippocampus that seems to be correlated with illness severity and the degree of cognitive deficit (BREMNER, 1999, 2002).

The hippocampus is a vulnerable brain structure that is susceptible to damage during aging and stress (SAPOLSKY, 1992). Altered activities of the antioxidant defense system enzymes and levels of free radical scavengers, as well as other parameters of oxidative stress, have been found to be correlated with conditions related to stress exposure (DERIN *et al*., 2006; FONTELLA *et al*., 2005), suggesting that the stress response leads to increased production of free radicals (MCINTOSH *et al*., 1998; LIU *et al*., 1996).

In order to understand the neurobiology of PTSD, animal models of the disorder have been used, in which different aspects of this condition may be studied. The exposure to uncontrollable stressors, such as inescapable footshock, produces many behavioral changes, and this paradigm has been proposed as model of depression and of anxiety-

related disorders such as PTSD (PYNOOS *et al*., 1996). Some authors use a re-exposure to a traumatic stressor (VAN DER KOLK *et al*., 1989; GARRICK *et al*., 1997) or repeated exposures to situational reminders (SAWAMURA *et al*., 2004; LOUVART *et al*., 2005; MAIER, 2001), which are believed to induce re-experiencing of the aversive event.

The aim of the present study is to verify if maternal separation in rats alters the susceptibility to the effects of a intense stressor applied in adult age, verifying its long-lasting effects on cognitive aspects (spatial memory, evaluated by the performance in Morris water maze task) when adults and also verifying DNA damage, and antioxidant enzymes activities in the hippocampus.

EXPERIMENTAL PROCEDURE

Subjects:

Pregnant Wistar rats bred at our own animal facility were randomly selected. Animals were housed alone in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust and were maintained in a controlled environment until offspring: lights on between 07:00h and 19:00h, temperature of 22 $\pm$ 2°C, cage cleaning once a week, food and water provided. All litters were culled within 24 h to eight pups and were maintained intact unless for maternal separation procedures, which were carried out between 10:00h and 14:00h.

Weaning was on postnatal day 21. A maximum of two male pups were used per litter per experiment. Rats were housed four to five per cage, and separated by sex. Fifty-three experimental male rats were used in the different experiments, derived from 10

different litters. Rats had free access to food (standard lab rat chow) and water, except during the period when the behavioral tasks were applied. Tasks were performed between 13:00h and 16:00h, after animals had reached adult life.

All animal treatments were approved by the Ethical Committee of our university and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS).

Maternal separation:

Non-separated group – Pups were left undisturbed with the dam until weaning. It was stated on the cage that these animals should not be touched, not even for cage cleaning. Dirty sawdust was carefully removed from one side of the cage, without disturbing the mother and the nest, and replaced by clean sawdust at that side by the principal researcher.

Maternal separation group – Pups were removed from their home cage and were placed into a clean cage lined with clean paper towel, inside an incubator at 32° C next to the dam's cage. After 3 hours, pups were returned to their dams. This procedure was carried out for the first ten days of life, after which pups were left undisturbed until the 21st day of life.

Exposure to a stressor during adulthood:

After reaching 60 days of age, the animals were subdivided into two other groups: no shock and shock (adapted from PYNOOS *et al*., 1996), which consisted of a single exposure to a footshock, followed by three weekly exposures to a situational reminder (SR).

The apparatus consisted of a 50x25x25 cm box, which was divided in two equal compartments, both compartments with a frontal glass wall. The first compartment presented a lowered floor, and the second compartment presented a grid floor consisting of 1 mm bronze bars spaced 10 mm apart. The animals were gently held by their body and lowered in the first compartment, with their nose pointing to the rear left corner. After 2 minutes, a guillotine door was opened until the animal crossed to the second compartment. The door was then closed and a 1 mA 60 Hz footshock was delivered during 20 seconds. The no shock group was subjected to the same treatment, but no shock was delivered.

Exposure to situational reminders:

For the exposure to situational reminders (SR), one week after exposure to the apparatus described above, the animals were replaced in the box for two minutes, but just in the first compartment. This procedure was repeated during three weeks, with a seven-days interval between each SR.

Morris water maze:

One week after the last SR, rats were submitted to spatial memory testing in the Morris water maze. The maze consisted of a black circular pool with 180 cm in diameter filled with water (temperature 22 °C, depth 40 cm) situated in a room with visual cues on the walls. A transparent platform with 10 cm in diameter was submerged in the water (2 cm below the water surface). The pool was conceptually divided in four quadrants and had four points designed as starting positions (N, S, W or E). Rats received five training days (sessions) and a probe trial in the 6th day. Each session consisted of four trials with a 10 min intertrial interval. A trial began when the rat was placed in the water at one of the

four starting positions, chosen at random, facing the wall. The order of starting position varied in every trial and any given sequence was not repeated on acquisition phase days. The rat was given 60 s to locate the platform; if the animal did not succeed, it was gently guided to the platform and left on it for 20 s. Rats were dried and returned to their home cages after each trial. The probe trial consisted of a single trial, with the platform removed. The time spent in the target (where the platform used to be), as well as in the opposite quadrants, were measured (VASCONCELLOS *et al.*, 2003).

Biochemical measurements:

Preparation of the Samples

One month after the behavioral procedure animals were sacrificed between 10:00 and 14:00 hours and the hippocampus was dissected and used to assess DNA damage through the comet assay, or frozen at -70^{o} C until evaluation of antioxidant enzymes acitivies. All animals were sacrificed within this interval of time in a random order considering groups.

Single cell gel electrophoresis — comet assay

A standard protocol for comet assay preparation and analysis was adopted (TICE *et al*., 2000). After the sacrifice of the animals by decapitation, the hippocampus was immediately dissected out and gently homogenized in phosphate-buffered saline solution (PBS) pH 7.4. The slides were prepared by mixing 20 µl of hippocampus homogenate (in cold PBS), with 80 µl low melting point agarose (0.75%). The mixture (cells-agarose) was added to a microscope slide coated with a layer of 500 µl of normal melting agarose (1%). After solidification, the cover slip was gently removed and the slides were placed in lysis

solution (2.5M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.5, with freshly added 1% Triton X-100 and 10% DMSO) for one day. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 10 min. The DNA was electrophoresed during 20min at 25V (0.90 V/cm) and 300mA. Afterwards, the slides were neutralized with Tris buffer (0.4 M; pH 7.5). Finally, the DNA was stained with ethidium bromide. After electrophoresis, neutralized and stained nuclei (from random 100-cells fields) were blindly analyzed by fluorescence microscopy with visual inspection (200x). Cells were scored from zero (undamaged) to 4 (maximal damage), according to the tail intensity (size and shape), resulting in a single DNA damage score for each cell, and, consequently, for each group. Therefore, a group damage index could range from zero (all cells no tail, 100 cellsx0) to 400 (all cells with maximally long tails, 100 cellsx4) (COLLINS *et al*., 1997). The DNA damage index was calculated by multiplying the number of cells by its respective index score and than summing up.

Antioxidant enzyme activities

For evaluating antioxidant enzyme activities, the hippocampus was stored at minus 70ºC until analysis, when it was homogenized in 10 vol (w:v) ice-cold 50mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA for determination of Superoxide Dismutase (SOD) activity, and Glutathione Peroxidase (GPx) activity. To determinate catalase (CAT) activity, samples were homogenized in 10vol (w:v) ice-cold potassium phosphate buffer 10mM (pH 7,0). The homogenate was centrifuged at 3,000 rpm for 10min at 4ºC and the supernatant was used.

Superoxide Dismutase Activity - SOD activity was determined using a RANSOD kit (Randox Labs., USA) which is based on the procedure described by DELMAS-BEAUVIEUX *et al*. (1995). This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a formazan dye that is assayed spectrophotometrically at 492 nm at 37ºC. The inhibition in production of the chromogen is proportional to the activity of SOD present in the sample.

Catalase Activity - Catalase is an enzyme able to degrade peroxides, including hydrogen peroxide (H_2O_2), and its activity assessment is based upon establishing the rate of H_2O_2 degradation spectrophotometrically at 240 nm at 25ºC (AEBI, 1984). CAT activity was calculated in terms of micromoles of H_2O_2 consumed per minute per milligram of protein, using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$.

Glutathione Peroxidase Activity - GPx activity was determined according to WENDEL (1981), with modifications. The reaction was carried out at 37ºC in 200 μL of solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione and 0.4 U glutathione reductase. The activity of GPx was measured taking tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as pmol NADPH oxidized per minute per mg protein.

Protein Assay

The total protein concentrations were determined using the method described by LOWRY *et al*. (1951) using bovine serum albumin as the standard.

Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean, and were analyzed by a two factors ANOVA, using maternal separation and exposure to shock as factors, followed by the Duncan multiple range test, when indicated. The significance level was accepted as different when the *P* value was equal or less than 0.05. Sample size varies in each experiment and is shown individually in the Results section.

RESULTS

Water Maze task

Two months after exposure to footshock, rats were submitted to a spatial memory task, using the Morris water maze. Two-way ANOVA showed differences in the time spent in the target and opposite quadrants (Figure 1). Animals that were subjected to shock spent less time in the target quadrant [F (1, 28) = 8,702; P<0,01]. Significant effects of shock [F (1, 28) = 33,01; P<0,001] and a significant interaction shock x maternal separation [F (1, 28) = 5,407; P<0,05] were observed in time spent in the opposite quadrant, since exposure to shock impaired the performance in this parameter and this effect was further increased by maternal separation.

Comet assay

A higher score of DNA damage was observed in hippocampus of animals subjected to shock or maternal separated. A two-way ANOVA showed a significant effects of shock [F (1, 23) = 5,503; P<0,05] and maternal separation [F (1, 23) = 35,473; P<0,001], as displayed in Figure 2.

Antioxidants Enzymes Activity - Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx)

No differences were found among the groups on the antioxidant enzymes activities (SOD, CAT, GPx) (two-way ANOVA; P>0.05; Figure 3).

DISCUSSION

Environmental conditions during the neonatal period may affect adult behavioral, cognitive and neuroendocrine responsiveness via modulation of normal growth of neuronal processes or synaptic development, as well as through alterations in neurogenesis or apoptosis, and the interaction of genetic and environmental factors in the pathogenesis of psychiatric disorders appears to be firmly established. It is known, for example, that traumatic experiences can alter the brain and can damage it (SAPOLSKY, 2000). In this sense, risk factors for Post-Traumatic Stress Disorder (PTSD) may include individual neurobiology as well as past experiences, and early adverse experiences are considered a major risk factor for the development of anxiety disorders. Therefore, this study investigated the effects of maternal separation (MS), in male rats, on the outcome of

exposing them, as adults, to a stressful experience. Behavioral observations were made at least 1 month after the stressful experience, in an attempt to model long-term effects of this exposure to a severe stressor, as observed in humans with PTSD. We observed that both treatments, maternal separation during the first 10 days of life and exposure to a stressful event in adult life, presented important effects on memory and DNA damage in hippocampus. No differences were found in antioxidant enzymes activities.

Studies of structural brain abnormalities in PTSD have focused in particular on the hippocampus, a grey matter structure in the limbic system that is critically involved in memory (SQUIRE, 1992; ELDRIDGE *et al*., 2000). The hippocampus has also a neuroendocrine role in hypothalamic-pituitary adrenal (HPA) axis. Furthermore, dysregulations of the HPA axis and of glucocorticoid release in PTSD are well described, but while increased plasma cortisol levels occur immediately after or during the stress, studies have showed a marked suppression of plasma cortisol in PTSD, suggesting a hypersensitivity of the inhibitory elements of the HPA-axis (HARVEY *et al.,* 2003). Because of its critical role in learning and memory as well as in stress regulation, alterations in the hippocampus have been proposed as contributing to the etiology of PTSD (BREMNER, 2001; SAPOLSKY, 2000), and many studies indicate that PTSD is associated with atrophy of the hippocampus (BREMNER *et al*., 1995; SAPOLSKY, 2000). Glucocorticoids, the adrenal hormones secreted during situations of the stress, can damage the hippocampus, but the mechanisms that explain trauma-related hippocampal atrophy are not clear (SAPOLSKY, 2000).

In PTSD, the patients can develop memory and concentration impairments (JOHNSEN *et al.,* 1997; HARVEY *et al.,* 2003). In this work, rats exposed to a PTSD model showed long-lasting effects suggesting impairments in learning and memory, since

they spent more time in the opposite quadrant in the water maze task, while spending less time in the target quadrant. Besides, in the first of these parameters, rats subjected to maternal separation and to this model of PTSD (shock) spent more time in the opposite quadrant, suggesting that maternal separation, while not having an effect by itself, worsened the impairment observed after exposure to shock. In addition, both situations induced a higher score of DNA damage in hippocampus.

In agreement with the present results of DNA damage to the hippocampus in animals subjected to a PTSD model (inescapable shock exposure with situational reminders) and correlated impairments in spatial memory, deficits in learning and memory have been associated with damage to the hippocampus, which may be caused by stress (McEWEN *et al.*, 1992; SAPOLSKY, 1996; MALBERG *et al.*, 2000; ARBEL *et al.*, 1994; LUINE *et al.*, 1994). Mechanisms that have been proposed for these effects include stress-induced elevations in glucocorticoids with associated glutamatergic toxicity (SAPOLSKY, 1996), decreased brain-derived neurotrophic factor (BDNF; SMITH *et al.*, 1995; RASMUSSON *et al.*, 2002), increased levels of excitatory amino acids such as glutamate (MOGHADDAM *et al.*, 1997), alterations in serotonin (McEWEN *et al.*, 1997) and hippocampal effects of corticotropin releasing factor (BRUNSON *et al.*, 2001). Several of these alterations have been suggested to affect memory as well. For example, a role for BDNF and TrkB, a protein-tyrosine kinase receptor for BDNF, in learning and memory processes is suggested by their function in activity dependent synaptic long-term-potentiation (LTP), the transcription-dependent electrophysiological correlate of long-term memory (NGUYEN & KANDEL, 1996; ROSENBLUM *et al.*, 2002; YING *et al.*, 2002).

Stress hormones released during emotionally arousing experiences regulate explicit /declarative memory storage especially in the hippocampus, but also in other brain regions.

Thus, emotion can significantly modify the accuracy and retention of new explicit memory, as for example in the interpretation of incoming sensory information critical for spatial orientation in the Morris Water Maze. Severe anxiety/panic attacks, impairment of memory function, and reduced cortisol levels, are core findings of PTSD. Moreover, clinical findings suggest that low cortisol levels in PTSD may constitute a vulnerability marker related to chronic PTSD (HARVEY *et al.,* 2003). Evidence from other animal model leading to reduced plasma cortisol levels and memory impairments in the water maze (as measured 7-days post-stress) suggests a correlation between memory effects and neurochemical effects in hippocampus (HARVEY *et al.,* 2003). In the present study, we observed long-lasting effects of exposure to a shock and to situational reminders, both on behavior and on hippocampal DNA damage index.

Neonatal maternal separation of rat pups leads to a stress hyperresponsive phenotype characterized by increased basal levels of corticotropin releasing factor (CRF) mRNA in the hypothalamic and extra-hypothalamic nuclei, increased hypothalamic CRF release, and enhanced responses to psychological stressors (HUOT *et al*., 2002). It is known that the majority of hippocampal granule neurons develops and extends their axons between days 1 and 21 of life (AMARAL & DENT, 1981; BAYER *et al*., 1982; SCHLESSINGER *et al*., 1975). This peak period of neurogenesis overlaps the stress hyporesponsive period (days 4–14), and exposure to elevated levels of corticosterone during the neonatal period may affect hippocampal development (HUOT *et al*., 2002).

Glucocorticoids may be capable of affecting hippocampal development by directly or indirectly influencing the balance between neurogenesis and apoptosis of granule neurons throughout life in many species (BOHN, 1980; GOULD, 1994). In the present study, maternal separated rats exhibited a higher score of DNA damage in hippocampus.

However, strand breaks in DNA arise from oxidative damage, but also from the process of DNA repair (HALLIWELL & WHITEMAN, 2004), and this increased index of DNA breaks, as observed here, does not mean these breaks are permanent.

Chronic neonatal maternal separation has been shown to produce significant impairments in learning and memory (HUOT *et al*., 2002). In the present study, although it worsened the effects of another stressor, maternal separation did not had appreciable effect per se on memory. These differences could be attributed to different different rat lineages or different MS schedules.

Concluding, our findings showed that early adverse life events may enhance the susceptibility to the effects of a stressor applied in adulthood. Long-lasting effects of exposure to a shock and to situational reminders were observed both on behavior and on hippocampal DNA damage index, and these effects could be further enhanced by previous maternal separation during the neonatal period.

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REFERENCES

AEBI, H. Catalase in vitro. Methods Enzymol. 1984, 105:121-126.

AMARAL, D.G., Dent, J.A. Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. J. Comp. Neurol. 1981, 195, 51–86.

ARBEL, I., Kadar, T., Silberman, M., Levy, A. The effects of long-term corticosterone administration on hippocampal morphology and cognitive performance of middle-aged rats. Brain Res. 1994, 657: 227–235.

BAYER, S.A., Yackel, J.W., Puri, P.S. Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life, Science. 1982, 216 890–892.

BOHN, M.C. Granule cell genesis in the hippocampus of rats treated neonatally with hydrocortisone, Neuroscience. 1980, 5, 2003–2012.

BREMNER, D.; Randall, P., Scott, T. M.; Bronem, R. A.; Seibyl, J. P.; Southwick, S. M. MRI-based measurement of hippocampal volume in patients with combat-related posttraumatic stress disorder. Am J Psychiatry. 1995; 1, 54, p. 624-629.

BREMNER, J.D. Does stress damage the brain? Biol. Psyciatry. 1999; 45, 797-805.

BREMNER, J.D. Hypotheses and controversies related to effects of stress on the hippocampus: an argument for stress-induced damage to the hippocampus in patients with posttraumatic stress disorder. Hippocampus. 2001; 11, 75–81 Discussion 82–74.

BREMNER, J.D. Neuroimaging studies in post traumatic stress disorder. Curr. Psychiatry rep. 2002; 4, 254-263.

BRESLAU, N.; Davis, G.C.; Peterson, E.L.; Schultz, L.R. A second look at comorbidity in victims of trauma: the posttraumatic stress disorder-major depression connection. Biol Psychiatry. 2000, Nov; 1; 48(9): 902-9.

BRUNSON, K. L., Eghbal-Ahmadi, M., Bender, R., Chen, Y., Baram, T.Z. Long-term, progressive hippocampal cell loss and dysfunction induced by early life administration of corticotropin-releasing hormone reproduce the effects of early life stress. Proc Natl Acad Sci USA. 2001, 98: 8856–8861

CALDJI, C.; Diorio, J.; Meaney, M.J. Variations in maternal care in infancy regulate the development of stress reactivity. Biol Psychiatry. 2000, Dec; 15; 48(12):1164-74.

COLLINS, A., Dusinská, M., Franklin, M., Somorovská, M., Petrovská, H., Duthie, S. *et al.* Comet assay in human biomonitoring studies: reliability, validation, and applications. Environ Mol Mutagen. 1997; 30:139-46.

DELMAS-BEAUVIEUX, M.C., Peuchant, E., Dumon, M.F. *et al*. Relationship between red blood cell antioxidant enzymatic system status and lipoperoxidation during the acute phase of malaria. Clin. Biochem. 1995, 28:163-169.

DERIN, N., Aydin, S., Yargicoglu, P. *et al*. Changes in visual evoked potentials, lipid peroxidation and antioxidant enzymes in rats exposed to restraint stress: effects of l-carnitine. Int. J. Neurosci. 2006; 116:205--221.

DIEHL, L.A., Silveira, P.P., Leite, M.C., Crema, L.M., Portella, A.K., Billodre, M.N., Nunes, E., Henriques, T.P., Fidelix-da-Silva, L., Heis, M.D., Gonçalves, C.A., Quillfeldt, J.A., Dalmaz, C. Long lasting sex-specific effects upon behavior end S100b levels after maternal separation and exposure to a model of post-traumatic stress disorder in rats. Brain Research. 2007; 1144, 107-116.

ELDRIDGE, L.L., Knowlton, B.J., Furmanski, C.S., Bookheimer, S.Y., Engel, S.A. Remembering episodes: a selective role for the hippocampus during retrieval. Nature Neuroscience. 2000; 3, 1149–1152.

FONTELLA, F.U., Siqueira, I.R., Vasconcellos, A.P. *et al*. Repeated restraint stress induces oxidative damage in rat hippocampus. Neurochem. Res. 2005, 30:105--111.

GARRICK, T., Morrow, N., Eth, S., Marciano, D., Shalev, A. Psychophysiologic parameters of traumatic stress disorder in rats. Ann N Y Acad Sci. 1997, Jun; 21;821:533-7.

GOULD, E. The effects of adrenal steroids and excitatory input on neuronal birth and survival, Ann. NY Acad. Sci. 1994, 743; 73–93.

HALLIWELL, B., Whiteman, M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol. 2004; 142:231-55.

HARVEY, B.H., Naciti, C., Brand, L., Stein, D.J. Endocrine cognitive and hippocampal/cortical 5HT1A/2A receptor changes evoked by a time dependent sensitization (TDS) stress model in rats. Brain Research. 2003; 983, 97-107.

HUOT, R.L., Plotsky, P.M., Lenox, R.H., McNamara, R.K. Neonatal maternal separation reduces hippocampal mossy fiber density in adult Long Evans rats. Brain Research. 2002, 950 52–63.

JOHNSEN, B.H., Eid, J., Løvstad, T., & Michelsen, L.T. Posttraumatic stress symptoms in non exposed, victims, spontaneous rescuers after an avalanche. Journal of Traumatic Stress. 1997, 10(1), 133–140.

KALINICHEV, M., Easterling, K.W., Plotsky, P.M., Holtzman, S. Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats. Pharmacol Biochem Behav. 2002, 73 (1), p. 131-140.

KESSLER, R. C., Sonnega, A., Bromet, E., Hughes, M., Nelson, C. B. Posttraumatic stress disorder in the National Comorbidity Survey. Arch Gen Psychiatry. 1995, Oct; 52, 12, p. 1048-1060.

LADD, C.O., Huot, R.L., Thrivikraman, K.V., Nemeroff, C.B., Meaney, M.J., Plotsky, P.M. Long-term behavioral and neuroendocrine adaptations to adverse early experience. Prog Brain Res. 2000; 122:81-103.

LIU, J., Wang, X., Shigenaga, M.K. *et al*. Immobilization stress causes oxidative damage to lipid, protein, and DNA in the brain of rats. FASEB J. 10:1 1996, 532--1538.

LOWRY, O.H., Rosebrough, N.J., Farr, A.L. *et al*. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951, 193:265--275.

LOUVART, H., Maccari, S., Ducrocq, F., Thomas, P., Darnaudery, M. Long-term behavioral alterations in female rats after a single intense footshock followed by situational reminders. Psychoneuroendocrinology. 2005; 30, 316-324.

LUINE, V., Villages, M., Martinex, C., McEwen, B.S. Repeated stress causes reversible impairments of spatial memory performance. Brain Res. 1994, 639: 167–170.

MALBERG, J.E., Eisch, A.J., Nestler, E.J., Duman, R.S. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci. 2000, 20: 9104–9110.

McEWEN, B.S., Gould, E.A., Sakai, R.R. The vulnerability of the hippocampus to protective and destructive effects of glucocorticoids in relation to stress. Br J Psychiatry Suppl. 1992, (15):18-23.

McEWEN, B.S., Conrad, C.D., Kuroda, Y., Frankfurt, M., Magarinos, A.M., McKittrick, C. Prevention of stress-induced morphological and cognitive consequences. Eur Neuropsychopharmacol. 1997, *7*: S322–328.

McINTOSH, L.J., Cortopassi, K.M., Sapolsky, R.M. Glucocorticoids may alter antioxidant enzyme capacity in the brain: kainic acid studies. Brain Res. 1998, 791:215--222.

MEANEY, M.J., Bhatnagar, S., Larocque, S., McCormick, C., Shanks, N., Sharma, S., Smythe, J., Viau, V., Plotsky, P.M. Individual differences in the hypothalamic-pituitary-adrenal stress response and the hypothalamic CRF system. Annals of the New York Academy of Sciences. 1993, Oct; 29, 697, p. 70-85.

MOGHADDAM, B., Adams, B., Verma, A., Daly, D. Activation of glutamatergic neurotransmission by ketamine: a novel step in the pathway from NMDA receptor blockade to dopaminergic and cognitive disruptions associated with the prefrontal cortex. J Neurosci. 1997, 17: 2912–2127.

NGUYEN, P.V., Kandel, E.R. A macromolecular synthesisdependent late phase of long-term potentiation requiring cAMP in the medial perforant pathway of rat hippocampal slices. Journal of Neuroscience. 1996, 16, 3189–3198.

PYNOOS, R.S., Ritzmann, R.F., Steinberg, A.M., Goenjian, A., Prisecaru, I. A behavioral animal model of posttraumatic stress disorder featuring repeated exposure to situational reminders. Biol Psychiatry. 1996, Jan; 15, 39, (2), p. 129-134.

RASMUSSON, A.M., Shi, L., Duman, R. Downregulation of BDNF mRNA in the hippocampal dentate gyrus after re-exposure to cues previously associated with footshock. Neuropsychopharmacology. 2002, 27, 133–142.

RISBROUGH, V.B., Stein, M.B. Role of corticotropin releasing factor in anxiety disorders: A translational research perspective. Horm. Behav. 2006; 50, 550-561.

ROSENBLUM, K., Futter, M., Voss, K., Erent, M., Skehel, P.A., French, P., Obosi, L., Jones, M.W., Bliss, T.V. The role of extracellular regulated kinases I/II in late-phase long-term potentiation. Journal of Neuroscience. 2002, 22, 5432–5441.

SAPOLSKY, R.M. Do glucocorticoid concentrations rise with age in the rat? Neurobiol. Aging. 1992, 13, 171–174.

SAPOLSKY, R.M. Why stress is bad for your brain. Science. 1996, 273: 749–750.

SAPOLSKY, R.M. Glucorticoids and hippocampal atrophy in neuro psychiatric disorders, Arch. Gen. Psychiatry. 2000; 57, 925–935.

SAWAMURA, T., Shimizu, M., Wakizono, T., Suzuki, G., Tsunoda, T., Takahashi, Y., Nomura, S. Effect of paroxetine on a model of posttraumatic stress disorder in rats. Neuroscience Letters. 2004; 357, 37-40.

SCHLESSINGER, A.R., Cowan, W.M., Gottlieb, D.I. An autoradiographic study of the time of origin and the pattern of granule cell migration in the dentate gyrus of the rat, J. Comp. Neurol. 1975, 159, 149–176.

SMITH, M.A., Makino, S., Kvetnansky, R., Post, R.M. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. Journal of Neuroscience. 1995, 15, 1768–1777.

SQUIRE, L.R. Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. Psychological Reviews. 1992. 99, 195–231.

TICE, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen. 2000; 35(3):206-21.

VAN DER KOLK, B.A., VAN DER HART, O. Pierre Janet and the breakdown of adaptation in psychological trauma. Am J Psychiatry. 1989 Dec; 146(12):1530-40.

VASCONCELLOS, A.P., Tabajara, A.S., Ferrari, C., Rocha, E., Dalmaz, C. Effect of chronic stress on spatial memory in rats is attenuated by lithium treatment. Physiol Behav. 2003; 79(2):143-9.

VIEWEG, W.V., Julius, D.A., Fernandez, A., Beatty-Brooks, M., Hettema, J.M., Pandurangi, A.K. Posttraumatic stress disorder: clinical features, pathophysiology, and treatment. Am. J. Med. 2006; 119, 383-390.

WENDEL, A. Glutathione peroxidase. Meth. Enzymol. 1981 77:325--333.

WIGGER, A., Neumann, I.D. Periodic maternal deprivation induces gender-dependent alterations in behavioral and neuroendocrine responses to emotional stress in adult rats. Physiol Behav. 1999, Apr; 66(2): 293-302.

YEHUDA, R. Neuroendocrine aspects of PTSD. Handb. Exp. Pharmacol. 2005; 169, 371-403.

YING, S.W., Futter, M., Rosenblum, K., Webber, M.J., Hunt, S.P., Bliss, T.V., Bramham, C.R. Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. Journal of Neuroscience. 2002, 22, 1532–1540.

FIGURE LEGENDS

Figure 1. Performance in the Morris Water Maze of the no-shock, shock, non-maternal separation and maternal separation groups. Data are shown as mean ± S.E.M. of the time (in seconds) spent in the target (A) and opposite (B) quadrants during the 60 seconds exposure to the maze in the test session. N= 7-10 animals per group. A two-way ANOVA showed a significant effect of exposure to shock.

*Different from the non-shock non-separated group (Duncan multiple range test, $P<0.05$).

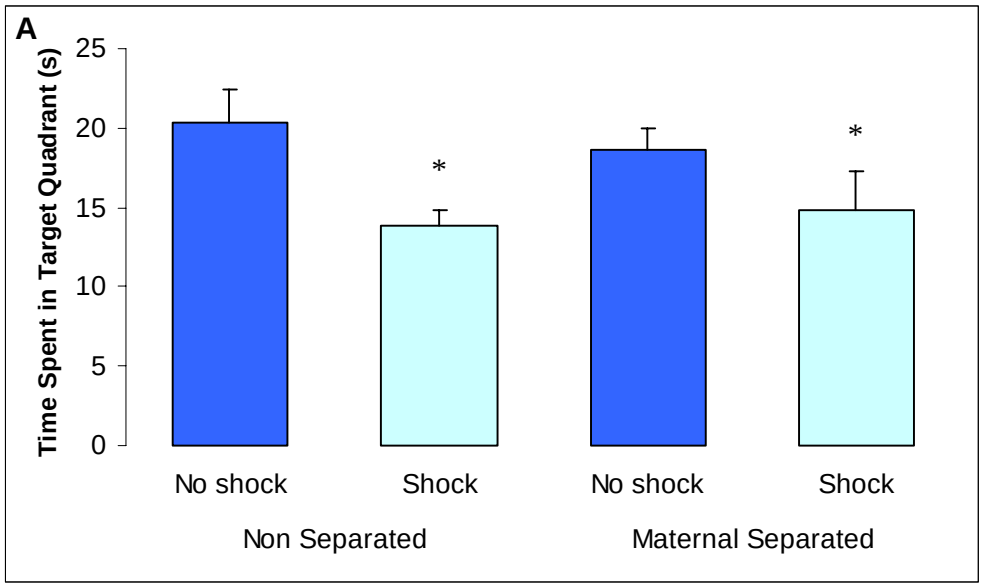
[a, b]Different from all the other groups (Duncan multiple range test, $P<0.05$).

Figure 2. Effect of shock and maternal separation on DNA damage in hippocampus two months after subjecting the animals to an inescapable shock as a PTSD model. Data expressed as mean ± SEM. N=6-8 animals/group. A two-way ANOVA showed significant effects of both maternal separation and exposure to shock.

(*) Different from the other groups (Duncan multiple range test, $p>0,05$).

Figure 3. Effects of shock and maternal separation on antioxidant enzymes activities, (A) SOD, (B) CAT and (C) GPx, in the hippocampus of rats. Data are expressed as mean ± S.E.M. N = 6/group. Two-way ANOVA showed no differences between the groups ($P>0.05$).

Figure 1.


A
Time Spent in Target Quadrant (s)
25
20
15
10
5
0
*
*
No shock
Shock
No shock
Shock
Non Separated
Maternal Separated

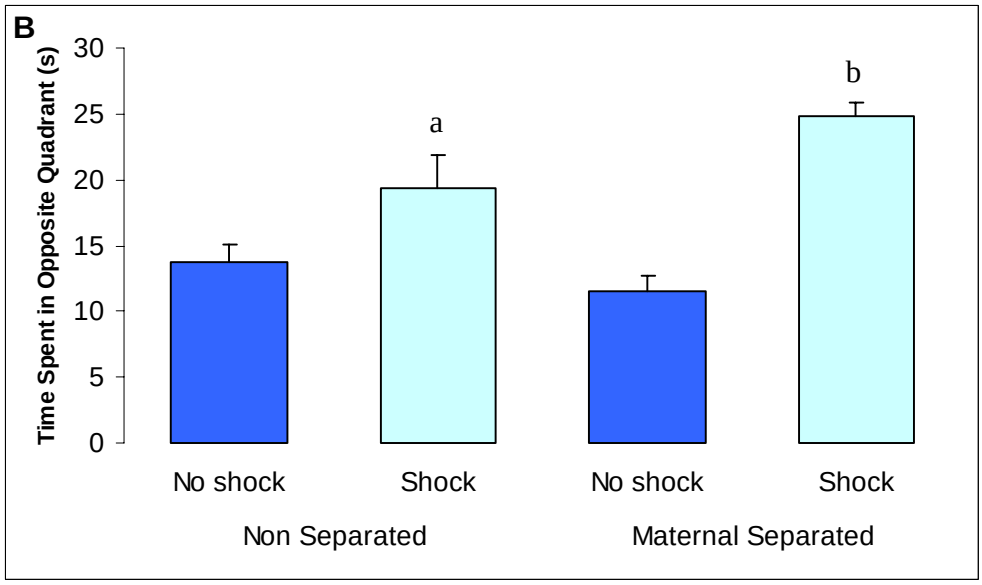

B
Time Spent in Opposite Quadrant (s)
30
25
20
15
10
5
0
a
b
No shock
Shock
No shock
Shock
Non Separated
Maternal Separated

Figure 2.

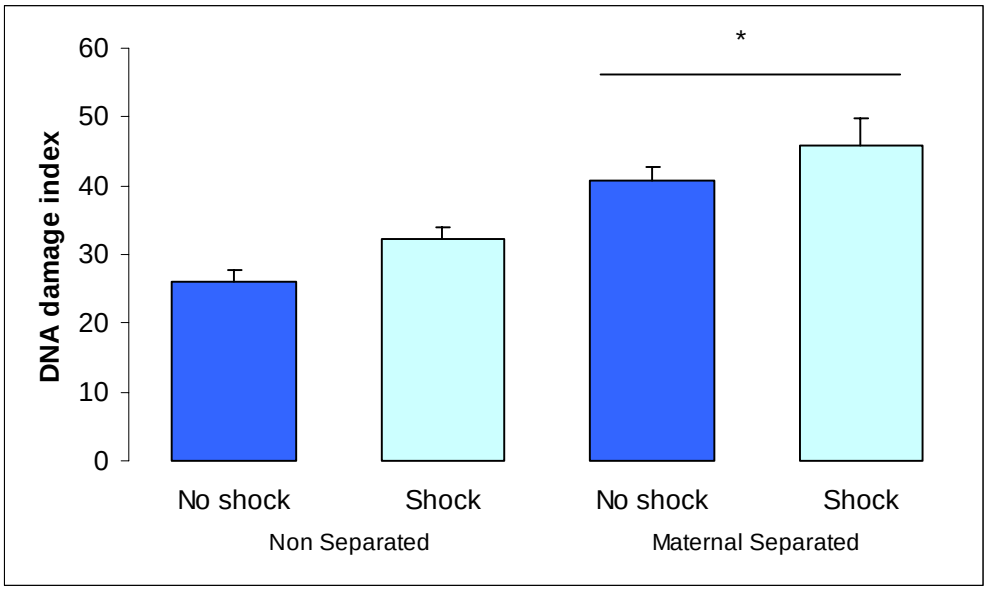
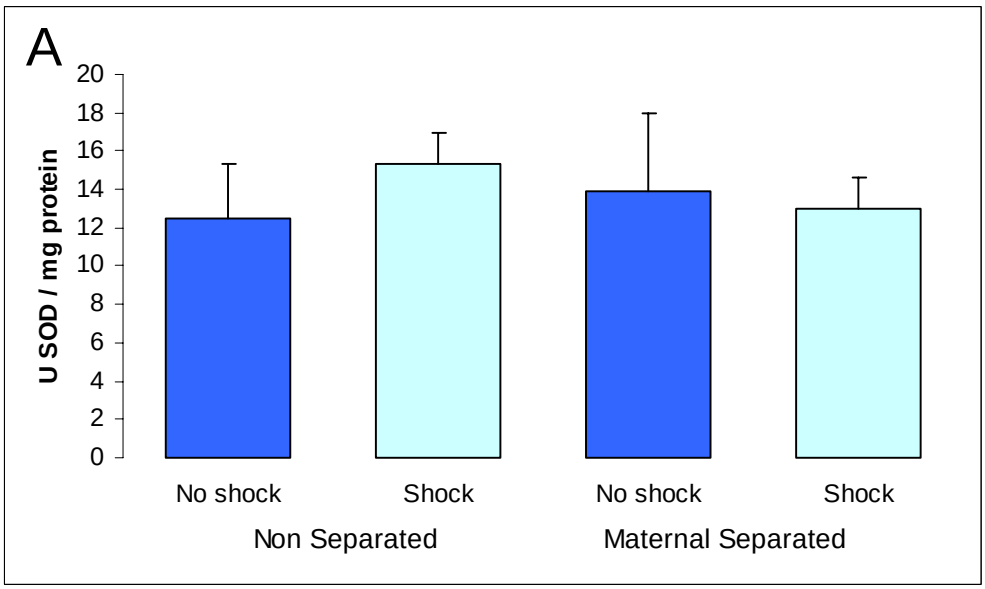
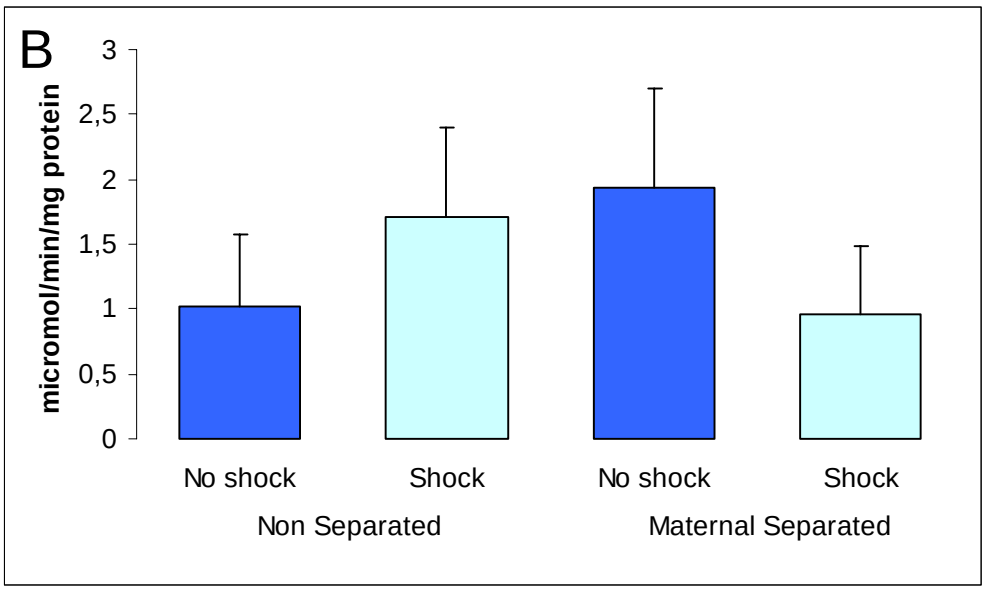
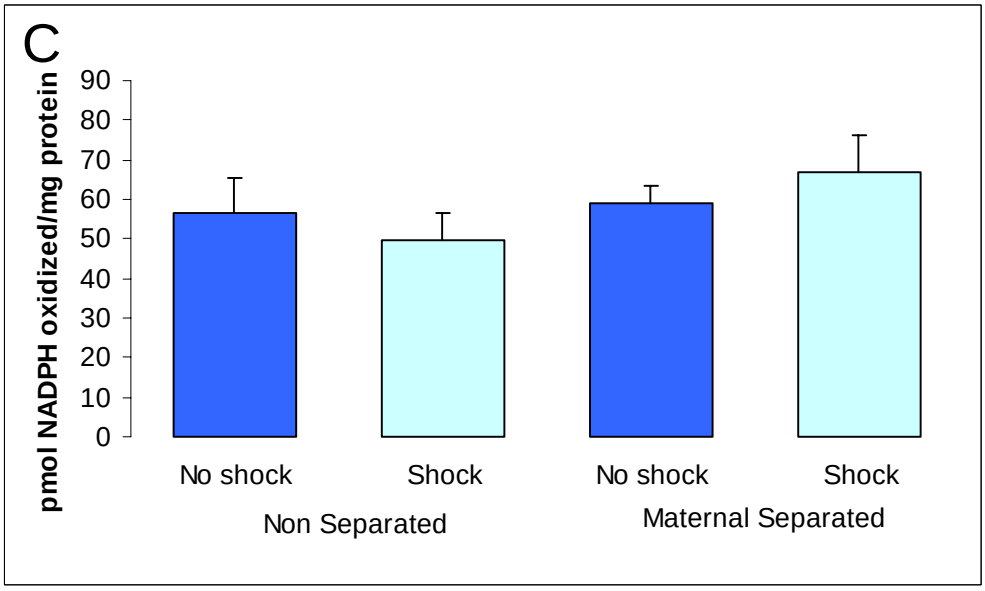


Figure 3.




B
micromol/min/mg protein
3
2,5
2
1,5
1
0,5
0
No shock
Shock
No shock
Shock
Non Separated
Maternal Separated


C
pmol NADPH oxidized/mg protein
90
80
70
60
50
40
30
20
10
0
No shock
Shock
No shock
Shock
Non Separated
Maternal Separated

4. Discussão Geral

Neste trabalho encontramos resultados muito relevantes. Alguns aspectos encontrados nos levaram a formular novas perguntas dando seguimento a uma série de experimentos com o intuito de tentar esclarecer tais questionamentos.

Diferentes estressores podem desencadear o Transtorno do Estresse Pós-Traumático. Pacientes com uma história de adversidades precoces apresentam maior freqüência do transtorno, além de maior incidência de comorbidades (POST *et al*., 2001). O desenvolvimento de modelos animais desse transtorno é de grande importância para a compreensão da fisiopatologia da doença e para o desenvolvimento de terapias farmacológicas.

Experiências precoces podem resultar em alteração da relação mãe-filhote. Em animais de experimentação, utiliza-se a intervenção no período neonatal, com manipulação dos animais ou separação da mãe por períodos diversos. A separação da mãe por períodos curtos leva a uma diminuição na resposta ao estresse na idade adulta, enquanto a separação por períodos mais longos, como a utilizada no presente trabalho, leva a uma resposta exagerada ao estresse (LEVINE, 2005).

No capítulo 1 deste trabalho, comparamos machos e fêmeas, uma vez que a incidência do TEPT é reconhecidamente maior em mulheres (KAPLAN & SADOCK, 1999). É importante observar que as fêmeas utilizadas para as observações comportamentais estavam ciclando ao acaso, pois o controle diário do ciclo poderia representar um estresse adicional para as fêmeas na idade adulta. Assim, não há relação entre esses resultados e a fase do ciclo estral.

Após a exposição ao choque (utilizada neste trabalho como uma situação de trauma), os animais foram submetidos a recordatórios (nova exposição ao ambiente onde haviam sido colocados antes de passarem ao ambiente em que lhes era aplicado o choque). Durante

os recordatórios, o tempo em que o animal permaneceu imóvel (*freezing*) foi utilizado para avaliar o medo condicionado, que representa a memória de um ambiente relacionado à experiência de choque inescapável. A exposição ao choque aumentou o tempo de imobilidade em todos os grupos (memória aversiva), mas de forma diferente em machos e fêmeas; estas últimas apresentaram um aumento proporcionalmente maior no tempo de imobilidade após a exposição ao choque (Fig. 1, pág. 27). Trabalhos da literatura relatam que fêmeas mostram maior potenciação da resposta de sobressalto (uma medida de medo), o que está de acordo com os presentes dados (JONGH *et al*., 2005). No entanto, não houve diferença entre animais separados ou não neste parâmetro. Esse aumento mais acentuado do medo condicionado nas fêmeas, conforme avaliado pelo tempo de imobilidade, poderia ser secundário a uma maior reatividade ao choque, visto que o peso corporal dos machos é consideravelmente maior que o das fêmeas. No entanto, a reatividade ao choque não foi avaliada no presente estudo.

Um dos sintomas do TEPT é uma maior ansiedade (KAPLAN & SADOCK, 1999). Assim sendo, avaliamos os níveis de ansiedade dos animais mais de 30 dias após a exposição ao choque. Para tal, foi utilizada a tarefa do Campo Aberto e o tempo despendido pelos animais nos quadrados centrais foi avaliado (SILVEIRA *et al*., 2005). Esta tarefa baseia-se na sugestão de que, quanto maior a ansiedade, mais o animal procura por lugares onde se sinta protegido, evitando situações de risco, onde estaria mais exposto a um predador, por exemplo. Diferenças em parâmetros relacionados a medo e ansiedade entre fêmeas e machos submetidos à separação maternal foram observados por outros autores (JONGH *et al*., 2005; KALINICHEV *et al*., 2002).

Por exemplo, Kalinichev e colaboradores (2002) observaram que ratos machos submetidos à separação materna, em um modelo semelhante ao do presente estudo,

apresentaram maior amplitude do reflexo do sobressalto, quando adultos, enquanto as fêmeas submetidas à separação materna no período neonatal não foram diferentes das fêmeas do grupo controle. Estes dados, juntamente com os dados deste trabalho, nos sugerem uma maior susceptibilidade dos machos submetidos à separação materna a um evento traumático.

Essa maior susceptibilidade dos ratos machos submetidos à separação materna também é observada quando se compara o tempo gasto nos quadrados centrais do campo aberto, outra medida de ansiedade. Também neste caso, machos separados da mãe no período neonatal permaneceram menos tempo nos quadrados centrais, sugerindo maior ansiedade (Fig. 2, pág. 28). É importante lembrar que essas tarefas comportamentais foram realizadas mais de 30 dias após o evento traumático, mostrando que a indução de ansiedade nestes animais é um fenômeno de longa duração. As fêmeas, por outro lado, comportaram-se de forma diferente, e não apresentaram aumento de ansiedade neste período após a exposição ao choque.

Está bem determinado que períodos mais curtos (10 min) de separação da mãe podem causar efeitos sobre o comportamento sexual e sobre a capacidade reprodutiva, tanto em machos quanto em fêmeas (PADOIN *et al*., 2001, GOMES *et al*., 2005). Por sua vez, os esteróides gonadais influenciam a ansiedade (e.g., ANDRADE *et al*., 2005), entre outros comportamentos, sendo que estrógenos apresentam efeitos ansiolíticos. As diferenças comportamentais observadas entre sexos neste trabalho quanto aos efeitos da separação materna podem ter relação com mudanças em esteróides gonadais. Se os períodos de separação materna nos primeiros dias de vida determinam marcas que irão no futuro afetar estrógenos ou testosterona circulantes, esses hormônios poderiam estar relacionados às alterações comportamentais observadas de forma dependente do gênero. Por exemplo, a

enzima 3-α-hidroxiesteróide desidrogenase, que participa da produção de esteróides neuroativos reduzindo o anel A de hormônios esteróides precursores, apresenta uma diferente expressão em ratos machos e fêmeas que são submetidos à separação materna no período neonatal (MITEV *et al*., 2003). Os produtos dessa enzima possuem ações ansiolíticas, atuando sobre receptores $GABA_A$. O estresse neonatal no trabalho citado afetou os ratos machos, enquanto as fêmeas somente apresentaram alterações comportamentais após gonadectomia, sugerindo que secreções fisiológicas de hormônios gonadais protejam as fêmeas de alterações resultantes de experiências adversas no período neonatal (MITEV *et al*., 2003).

Outra explicação para os efeitos da separação materna sobre o comportamento na idade adulta relaciona-se aos níveis de corticosterona. A separação materna influencia a resposta ao estresse e, assim, os níveis deste esteróide (VAN OERS *et al*., 1998); não está claro, porém, se tais efeitos seriam observados de forma diferente em machos e fêmeas.

Por outro lado, está bem determinado que mães que são separadas de seus filhotes apresentam alteração nos cuidados dispensados aos mesmos (MACRI *et al*., 2004). O cuidado maternal tem sido proposto como um dos principais fatores responsáveis pelas alterações comportamentais observadas nesses filhotes posteriormente na vida (SILVEIRA *et al*., 2005). Assim sendo, as diferenças comportamentais observadas entre os sexos no presente estudo podem ser também devidas a diferentes graus de cuidados maternos. Estudos têm mostrado que as ratas apresentam diferentes graus de cuidados com seus filhotes em função do sexo dos mesmos: por exemplo, mães com ninhadas contendo apenas machos apresentam maiores índices de cuidados maternos, como manutenção do ninho,

lambidas, etc (ALLEVA *et al*., 1989). Esses dados sugerem que filhotes machos e fêmeas estão expostos desde o período neonatal a diferentes padrões de comportamento materno.

Em um modelo animal de TEPT (PYNOOS *et al*., 1996), alteração da atividade locomotora foi observada após a exposição a um trauma (choque), porém essa alteração não permaneceu 3-6 semanas após o choque. Em nosso estudo, verificamos a atividade locomotora 30 dias após a exposição ao evento traumático. Observamos que animais expostos ao choque apresentaram uma redução da atividade motora (Fig. 3, pág, 29). Esse efeito também foi mais evidente em machos que em fêmeas, como pode ser observado nos nossos resultados. Além disso, independentemente da separação materna, as fêmeas apresentaram maior atividade motora que os machos. Esses resultados estão de acordo com outros estudos da literatura (SILVEIRA *et al*, 2005). A redução na atividade locomotora apresentada pelos machos expostos ao choque pode estar relacionada aos maiores níveis de ansiedade, conforme verificado nos testes discutidos acima.

Quanto aos níveis basais de cortisol circulante, sabe-se que indivíduos com o Transtorno de Estresse Pós-Traumático apresentam baixos níveis de cortisol devido a uma hipersensibilidade do sistema de retroalimentação negativa do eixo HHA (YEHUDA, 2005). Neste trabalho encontrou-se níveis basais de corticosterona reduzidos nos animais submetidos a separação materna ou ao modelo de TEPT e essas mudanças são mais notáveis em machos (Fig. 4, pág. 30).

Alterações séricas na concentração da proteína S100B estão mais comumentes relacionadas a diversos fatores que afetem sua síntese, distribuição, e metabolismo no SNC, incluindo injúria astrocítica e alterações na barreira hematoencefálica. Aumento de seus níveis plasmáticos têm sido encontrados após exposição a estressores e esses aumentos

tanto centrais quanto plasmáticos também têm sido relacionados a vários transtornos psiquiátricos (MACHADO-VIEIRA *et al*., 2002).

Neste estudo, quanto aos níveis plasmáticos e centrais da proteína S100B, observamos que a exposição ao choque induziu um aumento da S100B plasmática em machos enquanto nas fêmeas esse efeito não foi observado (Fig. 5, pág. 31). Esse dado pode ser correlacionado aos encontrados nas atividades exploratória e do tipo comportamento ansioso, que também são mais robustos nos machos. Isso, no entanto, não é observado nos níveis centrais de S100B. Assim, esses dados sugerem que um aumento nos níveis da proteína S100B no plasma em machos tenha uma origem extracerebral, possivelmente mediada por um aumento do tônus noradrenérgico (COHEN *et al*., 2000a,b).

Sabe-se que a adrenalina tem sido reportada como estimuladora da secreção de proteína S100B por adipócitos (NETTO, *et al*, 2006) e que pacientes com TEPT têm um aumento do tônus noradrenérgico (COHEN *et al*., 2000a,b). Estudos têm mostrado alterações dos níveis de S100B tanto periféricas quanto centrais em transtornos psiquiátricos. Em nosso trabalho, os níveis centrais de S100B estão aumentados nas fêmeas separadas das mães, o que não ocorreu nos níveis plasmáticos (Fig. 5 pág. 31). Esse aumento central parece ser dependente do gênero. Assim, esses níveis aumentados nas fêmeas separadas das mães podem estar relacionados com ações neurotróficas, que as protejam de agentes estressores adicionais como o modelo de TEPT.

Outra característica do TEPT são as alterações cognitivas. Há freqüentemente um déficit de memória, apesar da memória relacionada ao evento estressante estar extremamente bem preservada, sendo resistente à extinção; essas memórias traumáticas são muitas vezes intrusivas, interrompendo os pensamentos (GRAEFF, 2003). Neurotransmissores e hormônios relacionados ao estresse, incluindo a adrenalina e a

corticosterona, são sabidamente importantes para a consolidação das memórias (McGAUGH, *et al*., 2002). Assim sendo, no 2º capítulo desse trabalho, avaliamos o desempenho desses animais separados das mães no período neonatal e submetidos a um modelo do Transtorno de Estresse Pós-Traumático na tarefa do Labirinto Aquático de Morris, analisando a memória do tipo espacial, sendo um comportamento que está fortemente relacionado com a função hipocampal.

No intuito de relacionarmos possíveis alterações no desempenho cognitivo, avaliamos no hipocampo parâmetros relacionados ao estresse oxidativo, pela medida das atividades das enzimas antioxidantes (Superóxido Dismutase, Glutationa Peroxidade e Catalase). Pela técnica do Ensaio Cometa analisamos o índice de dano ao ADN hipocampal.

Animais que foram submetidos ao choque (modelo de TEPT) ou que foram separados da mãe no período neonatal e na idade adulta também submetidos ao choque, apresentaram prejuízo na memória espacial passando mais tempo no quadrante oposto no dia do teste na tarefa do Labirinto Aquático de Morris.

Anormalidades estruturais no hipocampo tem sido o foco de estudos em paciente com TEPT e acredita-se que alterações nessa estrutura contribuam para a etiologia dessa doença. O hipocampo está criticamente envolvido nos processos de memória explícita (declarativa), memória de trabalho e memória episódica (SQUIRE, 1992; ELDRIDGE *et al*., 2000). Por sua vez, glicocorticóides, hormônios secretados durante situações de estresse, podem danificar o hipocampo (McEWEN *et al.*, 2002; SAPOLSKY, 1996) e causar inibição de fatores de crescimento (MALBERG *et al.*, 2000) com déficits no aprendizado e memória (ARBEL *et al.*, 1994; LUINE *et al.*, 1994), mas os mecanismos que explicam essa relação com atrofia hipocampal não estão muito claros.

A exposição a um estresse ou trauma nos períodos iniciais da vida pode alterar a formação e a função de vias encefálicas e em particular circuitos do sistema límbico (AVITAL *et al*., 2006). Esses achados, juntamente com os nossos resultados, mostram que a ativação em resposta ao estresse no período neonatal pode levar a alterações hipocampais observadas na idade adulta (HARVEY *et al.,* 2003). Os mecanismos que têm sido propostos para esses efeitos incluem elevações nos níveis de glicocorticóides induzida pelo estresse, toxicidade glutamatérgica, diminuição nos níveis de BDNF (Fator Neurotrófico Derivado do Encéfalo) e alterações nos níveis de serotonina (McEWEN *et al.*, 1997).

A emoção também pode significativamente modificar a percepção e a retenção de novas memórias explícitas, como por exemplo, a interpretação de informações sensoriais críticas para a orientação em tarefas como do Labirinto Aquático de Morris e altos níveis de ansiedade podem levar a prejuízos na memória (HARVEY *et al.,* 2003). A separação materna no período neonatal em ratos leva a um fenótipo caracterizado por uma hiperreatividade ao estresse acompanhado por um aumento basal dos níveis de ARNm do Fator Liberador de Corticotropina nos núcleos hipotalâmicos e extra-hipotalâmicos, aumento da liberação do Hormônio Liberador de Corticotropina hipotalâmico e aumento da liberação do Hormônio Adrenocorticotrópico e corticosterona em resposta ao estresse (HUOT *et al*., 2002).

Dessa maneira, tendo em vista os resultados aqui observados, podemos relacionar eventos estressantes no início da vida com uma maior susceptibilidade às consequências da exposição a um estressor na idade adulta, tais como prejuízos cognitivos, como o observado na tarefa do Labirinto Aquático de Morris. Essa associação nos faz acreditar que eventos traumáticos no início da vida exerçam grande influência em alterações comportamentais, neuroquímicas e estruturais observadas na idade adulta.

5. Conclusões

Modelos animais são importantes para a compreensão da fisiopatologia da doença e para o desenvolvimento de terapias farmacológicas;

A exposição ao choque aumentou o medo condicionado em todos os grupos (memória aversiva), mas de forma diferente em machos e fêmeas; estas últimas apresentaram um aumento proporcionalmente maior no tempo de imobilidade após a exposição ao choque, mas não houve diferença entre animais separados ou não neste parâmetro;

Machos separados da mãe no período neonatal permaneceram menos tempo nos quadrados centrais na tarefa do Campo Aberto, sugerindo maior ansiedade;

As fêmeas, por outro lado, comportaram-se de forma diferente, e após a exposição ao choque não houve diferença entre separadas ou não nos níveis de ansiedade;

Observamos que animais expostos ao choque apresentaram uma redução da atividade motora. Esse efeito também foi mais evidente em machos que em fêmeas. Além disso, independentemente da separação materna, as fêmeas apresentaram maior atividade motora que os machos;

Os machos submetidos à separação materna no período neonatal e expostos ao choque quando adultos apresentaram menor habituação na exposição ao campo aberto, indicativo de menor memória para essa tarefa;

Neste trabalho encontrou-se níveis basais de corticosterona reduzidos nos animais submetidos a separação materna ou ao modelo de TEPT e essas mudanças são mais notáveis em machos;

A exposição ao choque induziu um aumento da S100B plasmática em machos enquanto nas fêmeas esse efeito não foi observado e os níveis centrais de S100B estão

aumentados nas fêmeas separadas das mães o que não ocorreu nos níveis plasmáticos e esse aumento central parece ser dependente do gênero;

Na tarefa do Labirinto Aquático de Morris, os animais submetidos ao choque (modelo de TEPT) na idade adulta ou separados da mãe no período neonatal e também expostos ao choque, apresentaram prejuízo no desempenho nesta tarefa, permanecendo mais tempo no quadrante oposto no dia do teste;

Na análise de dano ao ADN hipocampal através da técnica do Ensaio Cometa os animais submetidos a separação materna no período neonatal ou ao choque na idade adulta apresentaram o índice de dano aumentado;

Não houve diferença entre os grupos quanto à atividade das enzimas antioxidantes Superóxido Dismutase, Glutationa Peroxidade e Catalase.

Desse modo,

- A separação materna em um período crítico do desenvolvimento pode funcionar como um fator agravante nas alterações comportamentais, neuroendócrinas e bioquímicas induzidas pela exposição a um trauma na idade adulta, em ratos;
- Observamos diferenças dependentes do gênero nesses efeitos, sendo as fêmeas mais resistentes aos efeitos da exposição a traumas na idade adulta, no que concerne à indução de ansiedade.

Observações:

Esses dados não podem ser extrapolados para humanos; os efeitos a longo prazo das experiências precoces em humanos são difíceis de estudar, devido ao grande número de variáveis necessariamente adicionadas;

Não é possível determinar com clareza a gravidade da exposição a essas experiências no período neonatal;

Apesar dessas limitações, há grande concordância com relação a essa hipótese de que intervenções realizadas num período crítico do desenvolvimento, como o período neonatal, podem contribuir para o surgimento de psicopatologias na idade adulta;

Como a eficácia dos tratamentos atualmente disponíveis para o TEPT é limitada, alimenta-se a esperança de que o conhecimento mais aprofundado de suas bases biológicas possa levar a melhores instrumentos terapêuticos.

6. Referências

ALLEN, R.G. Free radicals and differentiation: the interrelationship of development and aging. In: Yu. B.P., Ed. Free radicals in aging, Boca Raton. 1993. p: 12-23.

ALLEVA, E., Caprioli, A., Laviola, G. Litter gender composition affects maternal behavior of the primiparous mouse dam (Mus musculus). J Comp Psychol. 1989. 103 (1), p. 83-87.

ANDRADE, T.G., Nakamuta, J.S., Avanzi, V., Graeff, F.G. Anxiolytic effect of estradiol in the median raphe nucleus mediated by 5-HT1A receptors. Behav. Brain Res. 2005. 30, 163 (1), p. 18-25.

ARBEL, I., Kadar, T., Silberman, M., Levy, A. The effects of long-term corticosterone administration on hippocampal morphology and cognitive performance of middle-aged rats. Brain Res. 1994. 657: 227–235.

AVITAL, A., Ram, E., Maayan, R., Weizman, A., Richter-Levin, G. Effects of early-life stress on behavior and neurosteroid levels in the rat hypothalamus and entorhinal córtex. Brain Research Bulletin. 2006. 68; 419–424.

BARTOVA, A. Functioning of the hypothalamo-pituitary-adrenal system during postnatal development in rats. Gen Comp Endocrinol. 1968. 10, 2, p. 235-239.

BREMNER, D., Licinio, J., Darnell, A., Cristal, J.H., Owens, M.J., Southwick, S.M. Elevated CSF corticotropi-releasing factor concentrations in post-traumatic stress disorder. Am. J. Psychiatry. 1997. 154, p. 624-629.

COHEN, H., Kotler, M., Matar, M.A., Kaplan, Z., Miodownik, H., Casuto, Y. Power spectral análisis of Herat rate variability in post-traumatic stress disorder patients. Biol Psychitry. 1997. 41, p. 627-629.

COHEN, H., Benjamin, J., Geva, A.B., Matar, M.A., Kaplan, Z., Kotler, M. Autonomic dysregulation in panic disorder and in post-traumatic stress disorder: application of power spectrum analysis of heart rate variability at rest and in response to recollection of trauma or panic attacks. Psychiatry Res. 2000a. 96, 1–13.

COHEN, H., Kotler, M., Matar, M., Kaplan, Z. Normalization of heart rate variability in post-traumatic stress disorder patients following fluoxetine treatment: preliminary results. Isr. Med. Assoc. J. 2000b. 2, 296–301.

DALLMAN, M.F., Akana, S.F., Scribner, K.S., Pecoraro, N., La Fleur, S.E., Houshyar, H., Gomez, F. Chronic stress-induced effects of corticosterone on brain: direct and indirect. Annals of the New York Academy of Sciences. 2004. 1018, p.141-150.

De KLOET, E.R., Vreugdenhil, E., Oitzl, M.S., Joels, M. Brain corticosteroid receptor balance in health and disease .Endocr. Rev. 1998. 19, 3,p. 269-301.

DONATO, R. Intracellular and cellular roles of S100 proteins. Microscopy Research And Technique. 2003. 60: 40-51.

ELDRIDGE, L.L., Knowlton, B.J., Furmanski, C.S., Bookheimer, S.Y., Engel, S.A. Remembering episodes: a selective role for the hippocampus during retrieval. Nature Neuroscience. 2000. 3, 1149–1152.

ENGLER O., Pham, T., Fullenon, M.J., Ooi, G., Funder, J.W., Clarke, I.J. Studies of the secretion of corticotropin releasing factor and arginin vasopressin into hypophyseal portal circulation of the concius sheep. Neuroendocrinol. 1989. 49, p. 367-381.

FRANCIS, D., Diorio, J., LaPlante, P., Weaver, S., Seckl, J.R., Meaney, M.J. The role of early environmental events in regulating neuroendocrine development. Moms, pups, stress, and glucocorticoid receptors. Annals of the New York Academy of Sciences. 1996. 20, 794, p. 136-152.

FRANCIS, D.D., Meaney, M.J. Maternal care and the development of stress responses. Cur Op Neurobiol. 1999. 9, (1), p. 128-134.

GOMES, C.M., Raineki, C., Ramos de Paula, P., Severino, G.S., Helena, C.V., Anselmo-Franci, J.A., Franci, C.R., Sanvitto, G.L., Lucion, A.B. Neonatal handling and reproductive function in female rats. J Endocrinol. 2005. 184, (2), p. 435-445.

GONZALEZ, A.S., Rodriguez Echandia, E.L., Cabrera, R., Foscolo, M.R., Fracchia, L.N. Neonatal chronic stress induces subsensitivity to chronic stress in adult rats. I. Effects on forced swim behavior and endocrine responses. Physiology & Behavior. 1990. 47, 4, p. 735-741.

GRAEFF, F.G. Biological basis of posttraumatic stress disorder Revista Brasileira de Psiquiatria, 25, 1, p. 21-24, Jun, 2003.

HALLIWELL, B., Gutteridge J.M. Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy. Lancet. 1984. 23; 1(8391):1396-7.

HALLIWELL, B., Gutteridge, J.M. Trends Neurosci. 1985. 8:22-6.

HALLIWELL, B., Cross, C.E. Oxygen-derived species: their relation to human disease and environmental stress. Environ Health Perspect. 1994.102 Suppl 10:5-12. Review.

HALTMEYER, G.C., Denenberg, V.H., Thatcher, J., Zarrow, M.X. Response of the adrenal cortex of the neonatal rat after subjection to stress. Nature. 1966. 17, 212, p. 1371-1373.

HARVEY, B.H., Naciti, C., Brand, L., Stein, D.J. Endocrine cognitive and hippocampal/cortical 5HT1A/2A receptor changes evoked by a time dependent sensitization (TDS) stress model in rats. Brain Research. 2003. 983, 97-107.

HERMAN J.P., Figueiredo, H., Mueller, N.K., Ulrich-Lai, Y., Ostrander, M.M., Choi, D.C., Cullinam, W.E. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol. 2003. 24, (3), p. 151-180.

HINDE, R.A., Spencer-Booth, Y. Effects of brief separation from mother on rhesus monkeys. Science. 1971. 9, 992, p. 111-118.

HUOT, R.L., Plotsky, P.M., Lenox, R.H., McNamara, R.K. Neonatal maternal separation reduces hippocampal mossy fiber density in adult Long Evans rats. Brain Research. 2002. 950 52–63.

JONGH, R., Geyer, M.A., Olivier, B. The effects of sex and neonatal maternal separation on fear-potentiated and light-enhanced startle. Behav Brain Res. 2005. 20, 161, (2), p. 190-196.

KALINICHEV, M., Easterling, K.W., Plotsky, P.M., Holtzman, S. Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats. Pharmacol Biochem Behav. 2002. 73 (1), p. 131-140.

KAPLAN, H.I., Sadock, B.J., Grebb, J. A. Manual Conciso de Psiquiatria. 2.ed. Porto Alegre: Artes Médicas. 2008. 688p. Cap. 13, p. 258-264.

KEHRER, J.P. The Haber-Weiss reaction and mechanisms of toxicity. Toxicology. 2000. Aug 14; 149(1):43-50. Review.

KESSLER, R.C., Sonnega, A., Bromet, E., Hughes, M., Nelson, C.B. Posttraumatic stress disorder in the National Comorbidity Survey. Arch Gen Psychiatry. 1995. 52, 12, p. 1048-1060.

KESSLER, R.C., Breslau, N., Peterson, E.L., Schultz, L.R. Short screening scale for DSM-IV posttraumatic stress disorder. Am J Psychiatry. 1999. 156, 6, p. 908-911.

KOPIN, I.J. Definitions of stress and sympathetic neuronal responses. Ann. New York Acad. Sciences. 1995. 771, p. 19-30.

LARA, D.R., Gama, C.S., Belmonte de Abreu P, Portela LV, Gonçalves C, Hauck S *et al*. Increased serum S100B protein in schizofrenia: a study in medication-free patients. J Psychiat Res. 2001. 35:11-14.

LENZ, H.J., Raedler, A., Greten, H., Brown, M.R. CRF initiates biological actions in the brain that are observed in response to stress. Am J Physiol. 1987. 252, p. 34-39.

LEVINE, S., Haltmeyer G.C., Karas, G.C., Denenberg, V.H. Physiological and behavioral effects of infantile stimulation. Physiol Behav. 1967. 2, p. 55-59.

LEVINE, S. The psychoendocrinology of stress. Annals of the New York Academy of Sciences. 1993. 29, 697, p. 61-69.

LEVINE, S. The ontogeny of the hypothalamic-pituitary-adrenal axis. The influence of maternal factors. Annals of the New York Academy of Sciences. 1994. 30, 746, p. 275-288.

LEVINE, S. Developmental determinants of sensitivity and resistance to stress. Psychoneuroendocrinology. 2005. 30, (10), p. 939-946.

LIU, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., Sharma, S., Pearson, D., Plotsky, P.M., Meaney, M.J. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science. 1997. 12, 5332, p.1659-1662.

LUINE, V., Villages, M., Martinex, C., McEwen, B.S. Repeated stress causes reversible impairments of spatial memory performance. Brain Res. 1994. 639: 167–170.

MACHADO-VIEIRA, R., Lara, D.R., Portela, L.V., Gonçalves, C.A., Soares, J.C., Kapczinski, F. *et al*. Elevated serum S100B protein in drug-free bipolar patients during first manic episode: a pilot study. European Neuropsychopharmacology. 2002. 12:269-72.

MACRI, S., Mason, G.J., Wurbel, H. Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats. Eur J Neurosci. 2004. 20, (4), p. 1017-1024.

MALBERG, J.E., Eisch, A.J., Nestler, E.J., Duman, R.S. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci. 2000. 20: 9104–9110.

McEWEN, B.S., Conrad, C.D., Kuroda, Y., Frankfurt, M., Magarinos, A.M., McKittrick, C. Prevention of stress-induced morphological and cognitive consequences. Eur Neuropsychopharmacol. 1997. *7*: S322–328.

McEWEN, B.S. Sex, stress and the hippocampus allostasis, allostatic load and the aging process. Neurobiol Aging. 2002. 23, p. 921-939.

McGAUGH, J.L., Roozendaal, B. Role of adrenal stress hormones in forming lasting memories in the brain. Curr Opin Neurobiol. 2002. 12, (2), p. 205-210.

McINTOSH, L.J., Sapolsky, R.M. Glucocorticoids increase the accumulation of reactive oxygen species and enhance adriamycin-induced toxicity in neuronal culture. Exp Neurol. 1996. Oct; 141(2):201-6.

MEANEY, M.J., Aitken, D.H., Viau, V., Sharma, S., Sarrieau, A. Neonatal handling alters adrenocortical negative feedback sensitivity and hippocampal type II glucocorticoid receptor binding in the rat. Neuroendocrinology. 1989. 50, 5, p. 597-604.

MEANEY, M.J., Bhatnagar, S., Larocque, S., McCormick, C., Shanks, N., Sharma, S., Smythe, J., Viau, V., Plotsky, P.M. Individual differences in the hypothalamic-pituitary-adrenal stress response and the hypothalamic CRF system. Annals of the New York Academy of Sciences. 1993. 29, 697, p. 70-85.

MILLER, D.B., O'Callaghan J.P. Neuroendocrine aspects of the response to stress. Metabolism. 2002. 51, (6), p. 5-10.

MITEV, Y.A., Darwish, M., Wolf, S.S., Holsboer, F., Almeida, O.F., Patchev, V.K. Gender differences in the regulation of 3 alpha-hydroxysteroid dehydrogenase in rat brain and sensitivity to neurosteroid-mediated stress protection. Neuroscience. 2003. 120, (2), p. 541-549.

MOSES, S.N., Sutherland, R.J., McDonald, R.J. Differential involvement of amygdala and hippocampus in responding to novel objects and contexts. Brain Res Bull. 2002, 15, 58, (5), p. 517-527.

NAGDYMAN, N., Komen, W., Ko, H.K., Muller, C., Obladen, M. Early biochemical indicators of hypoxic-eschemic encephalopathy after birth asphyxia. Pediatric Research. 2001. 49: 502-506.

NETTO, C.B., Conte, S., Leite, M.C., Pires, C., Martins, T.L., Vidal, P., Benfato, M.S., Giugliani, R., Gonçalves, C.A. Serum S100B protein is increased in fasting rats. Arch. Med. Res. 2006. 37, 683–686.

PADOIN, M.J., Cadore, L.P., Gomes, C.M., Barros, H.M., Lucion, A.B. Long-lasting effects of neonatal stimulation on the behavior of rats. Behav Neurosci. 2001. 115, (6), p. 1332-1340.

PERES, W. Radicais livres em níveis biológicos. 1 ed., Pelotas: EDUCAT, 1994.

POST, R.M., Leverich, G.S., Xing, G., Weiss, R.B. Developmental vulnerabilities to the onset and course of bipolar disorder. Dev Psychopathol. 2001. 13, (3), p. 581-598.

PYNOOS, R.S., Ritzmann, R.F., Steinberg, A.M., Goenjian, A., Prisecaru, I. A behavioral animal model of posttraumatic stress disorder featuring repeated exposure to situational reminders. Biol Psychiatry. 1996. 15, 39, (2), p. 129-134.

SANDHIR, R., Julka, D., Gill, K.D. Lipoperoxidative damage on lead exposure in rat brain and its implications on membrane bound enzymes. Pharmacol Toxicol. 1994. Feb; 74(2):66-71.

SAPOLSKY, R.M., Meaney, M.J. Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. Brain Research Reviews. 1986. 396, 1, p. 64-76.

SAPOLSKY, R.M. Why stress is bad for your brain. Science. 1996. 273: 749–750.

SAPOLSKY, R. M. Gluocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch Gen Psychiatry. 2000. 57, p. 925-935.

SCHAFER, B.W, Heinzman, C.W. The S100 family of EF-hand calcium binding proteins: functions and patology. Tibs 21, 1996. 134-39.

SELYE, H. A Syndrome produced by diverse nocious agents. Nature. 1936. 138:32.

SHALEV, A.Y., Orr, S.P., Peri, T., Schreiber, S., Pitman, R.K. Physiologic responses to loud tones in Israeli patients with posttraumatic stress disorder. Arch Gen Psychiatry. 1992. 49(11):870-5.

SHAMBERG, S.M., Kuhn, C.M. The Biochemical effects of tactile deprivation in neonatal rats. Perspectives on Behavioral Medicine. 1985. 2, p. 133-148.

SILVEIRA, P.P., Portella, A.K., Clemente, Z., Gamaro, G.D., Dalmaz, C. The effect of neonatal handling on adult feeding behavior is not an anxiety-like behavior. Int J Dev Neurosci. 2005. 23, (1), p. 93-99.

SQUIRE, L.R. Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. Psychological Reviews. 1992. 99, 195–231.

TSIGOS, C., Chrousos, G.P. Physiology of the hypothalamic-pituitary-adrenal axis in health and dysregulation in psychiatric and autoimmune disorders. Endocrinol Metab Clin North Am. 1994. 23, p. 451-466.

TSIGOS, C., Chrousos, G.P. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res. 2002. 53, p. 865-871.

VAN OERS, H.J., de Kloet, E.R., Li, C., Levine, S. The ontogeny of glucocorticoid negative feedback: influence of maternal deprivation. Endocrinology. 1998. 139, (6), p. 2838-2846.

YEHUDA, R. Neuroendocrine aspects of PTSD. Handb. Exp. Pharmacol. 2005. 169, 371–403.

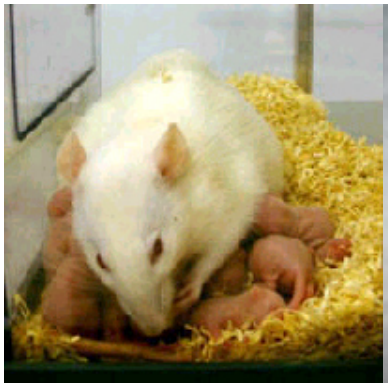
YOSHIMURA, S., Sakamoto, S., Kudo, H., Sassa, S., Kumai, A., Okamoto, R. Sex-differences in adrenocortical responsiveness during development in rats. Steroids. 2003. 68, 5, p. 439-445.

ZIMMER, D., Cornwall, E.H., Landar, A., Song, W. The S100 protein family: history, function, and expression. Brain Research Bulletin. 1995. 37(4):417-29.

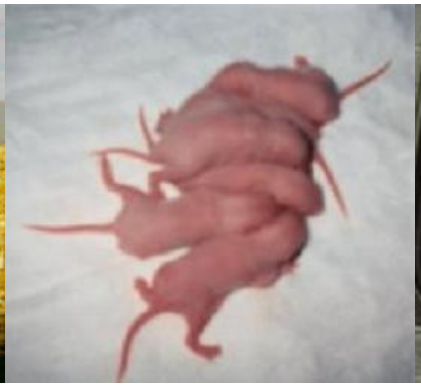
7. ANEXOS

7.1 Ilustrações dos Procedimentos Práticos

7.1.1 Estresse Neonatal:



Rata lactante com os filhotes



Ninhada

Ninhadas numa incubadora a 34ºC

7.1.2 Aparato utilizado como modelo para o Transtorno de Estresse Pós-Traumático:

7.1.3 Aparato utilizado para verificar comportamento do tipo ansioso e atividade locomotora – Campo Aberto:

7.1.4 Tarefa utilizada para avaliar memória espacial - Labirinto Aquático de Morris:

7.2 Resultados Adicionais – Projetos realizados em colaboração com outros laboratórios:

DISRUPTION OF TRAUMATIC MEMORY BY MIDAZOLAM AND ITS LONG-TERM CONSEQUENCES

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Abstract

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric disorder that can result from extreme stressful experiences. It is characterized by hyperarousal, anxiety and amnesic symptoms. In this study, we examined the effect of midazolam infused after a situational reminder (memory reactivation) for a traumatic memory. We investigated the effect of this treatment on the persistence of the fear memory, and on other long-term consequences in this PTSD model, such as anxiety, cognitive deficit and corticosterone levels. Rats were submitted to a PTSD model consisting of a strong inescapable footshock protocol and 4 situational reminders (SR) sessions. In order to evaluate some permanent sequelae of this model, and a possible reversion by the midazolam (MDZ) treatment, the animals were tested in plus maze, water maze and assays to measure glucocorticoid levels

were carried out. Our results showed that MDZ disrupted the fear memory regardless of the memory age. Further, MDZ treated groups also reverted the high anxiety and spatial memory deficit. Consistent with previous data in the literature, plasmatic glucocorticoids levels were reduced in animals subjected to the PTSD model, without recover by the MDZ. This work suggests that even after strong aversive past event, there is the possibility of attenuation by midazolam right after the recall, and probably for this reason, improve some comorbidities with a close relationship with the trauma.

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INTRODUCTION

Post-traumatic stress disorder (PTSD) is a serious and debilitating medical condition, characterized by the replay of the emotional memory formed in response to a severe traumatic event. The patient develops symptoms in three domains: reexperiencing the trauma, avoiding stimuli associated with the trauma and experiencing increased autonomic arousal (Harvey *et al*., 2003; Sawamura *et al*., 2004; Vieweg *et al*., 2006). Although a hypersensitivity of the HPA-axis feedback or an increased tissue sensitivity to glucocorticoids has been proposed to occur in this disorder (Yehuda *et al*., 2004; Shea *et*

al., 2005; Diehl *et al*., 2007), reports of cortisol levels in patients vary widely (Bonne *et al*., 2003a; Hawk *et al*., 2000; Lipschitz *et al*., 2003; Carrion *et al.,* 2002; Lemieux and Coe 1995; Lindley *et al*., 2004). In animal models of PTSD, reduced corticosterone levels are observed (Diehl *et al*., 2007; Harvey *et al*., 2003; 2006).

Problems with memory and lack of concentration characterize most of the patients treated for PTSD. Both the elevated arousal levels and the intrusive memories related to PTSD have been hypothesized to interfere with ongoing cognitive processing and thus produce memory impairment (Kolb, 1987; Bremner, 1999; Bremner *et al*., 1999, Bremner, 2002; Elzinga, *et al*., 2002; McF.wen, 1999; Sapolsky, 2000; Van der Kolk, 1994). In addition, PTSD also shows a high co-morbidity with anxiety disorders, with anxiety symptoms that may be stimulus-related (avoidance of feared situations) or generalized, such as worry, disturbed sleep, tremor and hyper-vigilance (Cohen *et al*., 2000).

Exposure to uncontrollable stressors, such as inescapable shock, produces a set of behavioral changes such as learned helplessness, and this paradigm has been proposed to be a model of depression and of anxiety-related disorders such as PTSD (Pynoos *et al*., 1996). In order to maintain the initial trauma, some authors use a re-exposure to a traumatic stressor or repeated exposures to situational reminders (Louvart *et al*., 2005), which are believed to induce re-experiencing of the aversive event. These animal models appear to produce some behavioral changes analogous to those seen in patients with PTSD, and so may be useful in studying the different aspects of this disorder, and in testing potential treatments (Pynoos *et al*., 1996).

In the last years, some studies have shown that, when previously consolidated memories are reactivated, they become transiently labile again, in a process called reconsolidation (Nader *et al*., 2000; Suzuki *et al*., 2004). The discovery of this transient

window of lability following reactivation raises the possibility of pharmacologically altering memory after the consolidation period; a maladaptive memory, such as a trauma could potentially be weakened pharmacologically by reactivating the memory, and subsequently treating the animal with drugs that interfere with the reconsolidation process.

Bustos and colleagues showed that the benzodiazepine midazolam (MDZ) impairs reconsolidation of aversive memories when administered after reactivation (Bustos *et al.*, 2006). This benzodiazepine is widely utilized in humans without significant side effects. Therefore, the aim of the present study is to extend the findings of Bustos et al by verify if MDZ administered after the reactivation of a traumatic event can revert some persistent consequences of the stressor in an animal model of PTSD. We evaluated anxiety, contextual fear and spatial memory and corticosterone levels.

MATERIAL AND METHODS

Subjects:

The subjects were 58 male Wistar rats (Universidade Federal do Rio Grande do Sul, Brazil) weighing 250–350 g, aged 8 weeks. Experimentally naive animals were housed in groups of four or five rats in home cages made of Plexiglas (65 cm · 25 cm · 15 cm) with the floor covered with sawdust. They were maintained under a standard dark– light cycle lights on between 7:00 a.m. and 7:00 p.m.), with a room temperature of 22 ± 2_C.

All procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behavior Guidelines for care and use of laboratory animals, which

comply with international laws and policies, and were approved by the Ethics Committee of our University.

Exposure to a stressor and situational reminders:

At 60 days of age, the animals were exposed to a model of PTSD (adapted from Pynoos *et al*., 1996), which consisted of a single exposure to footshock, followed by four exposures to situational reminders (SR).

The apparatus consisted of a 50x25x25 cm box, which was divided in two equal compartments, both compartments with a frontal glass wall. The first compartment had a linoleum floor, and the second compartment had a grid floor consisting of 1 mm bronze bars spaced 10 mm apart. The animals were gently held by their body and lowered into the first compartment, with their nose pointing to the rear left corner. After 2 minutes, a guillotine door was opened until the animal crossed to the second compartment. The door was then closed and a 1 mA 60 Hz footshock was delivered lasting 20 seconds. The no-shock group was subjected to the same treatment, but no shock was delivered (naïve group).

For the exposure to situational reminders (SR), one week after exposure to the apparatus described above, the animals were replaced in the box for 150 seconds, but were confined to the first compartment only. This procedure was repeated three times over the course of three weeks, with a seven-day interval between each SR. The test (a last SR) took place one month after the third SR. During the SR, the time spent freezing (total immobility except for breathing) was scored by experienced observers as a measure of fear memory.

Elevated plus-maze test:

The elevated plus maze (EPM) test was conducted using a standard plus maze apparatus kept 80 cm above the floor, consisting of four arms arranged in the shape of a plus sign (arms measured 50 ×10 cm). The four arms were joined at the center by a 10 cm square platform. Two of the arms, opposite to each other, were surrounded by a 1 cm high Plexiglas ledge (open arms), and two other arms (closed arms) were enclosed by a 40-cm-high wall. The behavioral test was conducted in an observational room illuminated by a red light. The animal was placed in the center of the plus maze, facing one of the open arms, and remained in the apparatus for 5 min. The number of entries and the time spent in the open or enclosed arms were analyzed (Silveira, *et al*., 2004). A rat was considered to have entered one arm of the maze when all four feet were within the arm. The ratio "time spent in the open arms/time spent in all (i.e., open and closed) arms" was calculated and multiplied by 100, to yield the percentages of time spent in open arms. This parameter is considered to reflect fear-induced inhibition from entering the open arms and can be related to the "anxiety" level experienced by the animal.

Morris water maze:

One week after the last SR, rats were subjected to spatial memory testing in the Morris water maze. The maze consisted of a black circular pool 180 cm in diameter filled with water (temperature around 23 °C, depth 40 cm) situated in a room with visual cues on the walls. A transparent platform with 10 cm in diameter was submerged 2 cm below the water surface. The pool was conceptually divided in four quadrants and had four points

designated as starting positions (N, S, W or E). Rats were subjected to five training days (sessions) and a probe trial in the 6th day. Each training session consisted of four trials with a 10 min inter-trial interval. A trial began when the rat was placed in the water at one of the four starting positions, chosen at random, facing the wall. The order of starting position varied in every session and any given sequence was not repeated during the acquisition phase. The rat was given 60 s to locate the platform; if the animal did not succeed, it was gently guided to the platform and left on it for 20 s. Rats were dried and returned to their home cages after each trial. The probe trial consisted of a single trial, with the platform removed. Here, the time spent in the target, as well as in the opposite quadrants, were measured and used as an index of memory for the task (Pereira, *et al.,* 2006).

Drugs:

MDZ (Dormonid®, Roche, Brazil) was diluted in sterile isotonic saline (SAL) (0.9% w/v) to a concentration of 3mg/ml. MDZ was administered intraperitoneally (i.p.) at a dose of 3mg/kg. The total volume of the drug or of SAL was 1ml/kg in all cases. The dose of MDZ used has been previously shown to impair memory reconsolidation in our lab. Immediately after each SRs, the animals were treated with MDZ or saline.

Corticosterone measurements:

Animals were sacrificed between 10:00 and 12:00 hours and the trunk blood was collected into heparinized tubes, centrifuged at 4^{o} C at 3,000g, and plasma separated and stored at -20^{o} C until analysis. Blood collection was made between 3 and 4 weeks after the

behavioral experiments. For corticosterone determination, plasma was extracted with ethyl acetate, and the extract evaporated and dissolved for the hormone evaluation with an ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA).

Experimental groups:

The animals were divided into five groups:

Group 1 (saline): immediately after each SRs, the animals were treated with saline;

Group 2 (MDZ 1): immediately after SR1 (first situational reminder), the animals were treated with MDZ; these animals received saline after SR2 and SR3;

Group 3 (MDZ 123): immediately after each SR, the animals were treated with MDZ;

Group 4 (MDZ 3): the animals received saline immediately after SR1 and SR2, and were treated with MDZ immediately after SR3;

Group 5 (no shock): this group did not receive footshock and were treated with saline after each SRs.

For the experiments evaluating spatial memory (water maze) and anxiety (elevated plus maze), data from the MDZ-treated animals (groups 2, 3 and 4) was pooled. This resulted in only three experimental groups: saline, no shock, and a pool of the MDZ treated animals. For a general summary of the experimental procedures, see Figure 1.

Statistical analysis:

Data were expressed as mean ± standard error of the mean, and were analyzed ~~by~~ using a One-way ANOVA, followed by the Duncan Post-Hoc test. Results were considered significant when the *P* value was equal or less than 0.05.

RESULTS

Conditioned Fear

The effect of an injection of MDZ immediately after a situational reminder, in a PTSD model, on conditioned fear is show in Figure 2, which displays the time spent freezing during the three situational reminders and in the test (1 month after SR3). One-way ANOVA showed differences in the time spent in freezing in SR1 [F (4, 45)= 5,676; P<0,001], SR2 [F (4, 45)= 22,483; P<0,001], SR3 [F (4, 45)= 14,361; P<0,001] and during the test session [F (4, 45)= 18,047; P<0,001]. Animals subjected to the shock and that received MDZ exhibited reduced freezing when compared to the groups that were exposed to shock but were not administered MDZ.

Elevated Plus Maze task

The animals were exposed to the elevated plus maze apparatus 2 months after being subjected to the shock. The percentage of time spent in the open arms was used to evaluate anxiety-like behavior. One-way ANOVA showed differences in the time spent in the open arms between groups [F (2, 25)= 3,429; P<0,05] as is displayed in Fig. 3. While animals exposed to the PTSD model decreased their percentage of time in the open arms, MDZ-treated animals presented a behavior similar to that of the non-shock group.

Water Maze task

Two months after foot-shock, rats were submitted to a spatial memory task, using the Morris water maze. One-way ANOVA showed differences in the time spent in the target [F (2, 18)= 6,169; P<0,05] and opposite quadrants [F (2, 18)= 4,108; P<0,05], as displayed in Fig. 4. Animals that were subjected to shock and were administered i.p. MDZ shortly after a SR performed comparably to animals not subjected to shock. On the other hand, the group which was exposed to shock and treated with saline showed impairment in memory for this task.

Basal plasma corticosterone

Basal plasma corticosterone was measured and the results are shown in Fig. 5. No significant differences between groups were observed One-way ANOVA, [F (4, 35)= 1,079658; P>0,05].

DISCUSSION

Consistent with previous studies, we observed significant long-term effects on anxiety-like behavior and memory following exposure to a strong aversive procedure (Diehl *et al*., 2007; Pynoos *et al*., 1996). Exposure to inescapable shock had long-lasting effects on anxiety as evaluated using an elevated plus maze and on spatial memory. Additionally, the administration of an amnestic agent such as MDZ when the animals were reexposed to cues of the stressor (SR) was able to attenuate or completely reverse the consequences of the aversive event on anxiety and memory.

In contrast to the work of Suzuki and colleagues, the age of the memory did not appear to be an important factor, since the administration of MDZ immediately after memory reactivation 7 or 21 days after the inescapable shocks had the same effect. These results suggest that an aversive memory can be disrupted even long after the event that originated this memory. Further, only one administration of MDZ was sufficient to decrease the strength of the memory. The group that received three MDZ injections expressed comparable levels of fear to the groups treated once (see Figure 2).

One issue in reconsolidation research that deserves attention is whether the disruption of reconsolidation is transient or permanent (Lattal and Abel 2004; Tronson and

Taylor 2007). In the present study, inhibition of memory reconsolidation appears to be permanent, at least at the times tested. We should emphasize that, even when the animals were tested several times, memory expression remained reduced, and this effect was observed several weeks after disruption of reconsolidation.

In another study, with a similar scope, Cohen and collaborators (2006) did not find effect of anisomycin (one protein synthesis inhibitor) administered after the SR (reactivation) and subsequent anxiety-like behavior and startle response. This conflicting result may be due to the fact that the aversive event used was different: In our study, we used a learned fear (associating a footshock with a context), while in the cited work the animals were exposed to a predator odor (that is, an innate fear).

PTSD is characterized by paradoxical effects on memory: usually there is an enhancement in the formation of memories associated with fear (Kim and Baxter 2001; Rau *et al*., 2005), and impairment in the acquisition of other memories (Quervain et al., 1998; Bremner, 2007). One possible explanation for the observed memory deficits might be that the traumatic experience results in hippocampal damage which impairs memory processes. On the other hand, overstimulation of the amygdala (Bremner, 2007) may explain the enhancement of fear learning. In line with the hypothesis of hippocampal damage following traumatic stress, animals submitted to our PTSD model exhibited an impairment of spatial memory in the Morris Water Maze task. Treatment with MDZ reversed this effect (suggesting that effects of traumatic stress on hippocampal function are reversible).

Our study demonstrates a significant long-term anxiogenic effect of a strong aversive event on the EPM. This result agrees with several other studies (e.g., Matar *et al*., 2006; Cohen *et al*., 2006). Again, animals treated with MDZ showed normal anxiety-like behavior (similar to the animals not subjected to the shock). On the other hand, no effect

was observed in plasma corticosterone levels, indicating that some consequences of traumatic stress may not be reversible through pharmacological interference with reconsolidation.

In conclusion, the present results show that it is possible to weaken maladaptive memories, as well as some associated long-term consequences, such as cognitive deficits and anxiety, by applying pharmacological treatments immediately after the reactivation of a traumatic memory. These findings point to possible treatment of PTSD patients using pharmacotherapy associated with reconsolidation of traumatic memories. Further studies are warranted.

LITERATURE REFERENCES

Bonne, O., Gilboa, A., Louzoun, Y., Brandes, D., Yona, I., Lester, H., Barkai, G., Freedman, N., Chisin, R., Shalev, A.Y. Resting regional cerebral perfusion in recent posttraumatic stress disorder.Biol Psychiatry. 2003 Nov 15;54(10):1077-86.

Bremner, J.D. 1999. Does stress damage the brain? Biol. Psyciatry 45, 797-805.

Bremner, J.D. 2002. Neuroimaging studies in post traumatic stress disorder. Curr. Psychiatry rep. 4, 254-263.

Bremner, J.D. 2007. Neuroimaging in Posttraumatic Stress Disorder and Other Stress-Related Disorders.Neuroimaging Clin N Am.17(4):523-538.

Bremner, J.D. Traumatic memories lost and found, in: Williams, L.M., Banyard, V.L. (F.ds.), Trauma and Memory, Sage, C.A., USA, 1999, pp. 217-227.

Bustos, S.G., Maldonado, H., Molina, V.A. 2006. Midazolam disrupts fear memory reconsolidation. Neuroscience. 139(3):831-842.

Carrion, V.G., Weems, C.F., Ray, R.D., Glaser, B., Hessl, D., Reiss, A.L. Diurnal salivary cortisol in pediatric posttraumatic stress disorder. Biol Psychiatry. 2002 Apr 1;51(7):575-82.

Cohen, H., Benjamin, J., Kaplan, Z., Kotler, M. 2000. Administration of high-dose ketoconazole, an inhibitor of steroid synthesis, prevents posttraumatic anxiety in an animal model. Europeam Neuropsychopharmacology. 10, 429-435.

Cohen, H., Kaplan, Z., Matar, M.A., Loewentha,l U., Kozlovsky, N., Zohar, J. 2006. Anisomycin, a protein synthesis inhibitor, disrupts traumatic memory consolidation and attenuates posttraumatic stress response in rats. Biol Psychiatry.1;60(7):767-776.

de Quervain, D.J., Roozendaal, B., McGaugh, J.L. 1998. Stress and glucocorticoids impair retrieval of long-term spatial memory. Nature 394(6695):787-90.

Diehl, L.A., Silveira, P.P., Leite, M.C., Crema, L.M., Portella, A.K., Billodre, M.N., Nunes, E., Henriques, T.P., Fidelix-da-Silva, L., Heis, M.D., Gonçalves, C.A., Quillfeldt, J.A., Dalmaz, C. 2007. Long lasting sex-specific effects upon behavior end S100b levels after maternal separation and exposure to a model of post-traumatic stress disorder in rats. Brain Research, 2007 May 4;1144:107-16.

Elzinga, B.M., Bremner, J.D. 2002. Are the neural substrates of memory the final common pathway in post traumatic stress disorder PTSD? J. Affect. Disord. 70, 1-17.

Harvey, B.H., Brand, L., Jeeva, Z., Stein, D.J. 2006. Cortical/hippocampal monoamines, HPA-axis changes and aversive behavior following stress and restress in an animal model of post-traumatic stresse disorder-like behaviors. Physiol. Behav. 87, 881-890.

Harvey, B.H., Naciti, C., Brand, L., Stein, D.J. 2003. Endocrine cognitive and hippocampal/cortical 5HT1A/2A receptor changes evoked by a time dependent sensitization (TDS) stress model in rats. Brain Research 983, 97-107.

Hawk, L.W., Dougall, A.L., Ursano, R.J., Baum, A. Urinary catecholamines and cortisol in recent-onset posttraumatic stress disorder after motor vehicle accidents. Psychosom Med. 2000 May-Jun;62(3):423-34.

Kim, J.J., Baxter, M.G. 2001. Multiple brain-memory systems: the whole does not equal the sum of its parts.Trends Neurosci. 24(6):324-30.

Lattal, K.M., Abel, T. 2004. Behavioral impairments caused by injections of the protein synthesis inhibitor anisomycin after contextual retrieval reverse with time. Proc Natl Acad Sci U S A 101(13):4667-72.

Lemieux, A.M., Coe, C.L. Abuse-related posttraumatic stress disorder: evidence for chronic neuroendocrine activation in women. Psychosom Med. 1995 Mar-Apr; 57(2):105-15.

Lindley, S.E., Carlson, E.B., Benoit, M. Basal and dexamethasone suppressed salivary cortisol concentrations in a community sample of patients with posttraumatic stress disorder. Biol Psychiatry. 2004 May 1;55(9):940-5.

Lipschitz, D.S., Rasmusson, A.M., Yehuda, R., Wang, S., Anyan, W., Gueoguieva, R., Grilo, C.M., Fehon, D.C., Southwick, S.M. Salivary cortisol responses to dexamethasone in adolescents with posttraumatic stress disorder. J Am Acad Child Adolesc Psychiatry. 2003 Nov;42(11):1310-7.

Louvart, H., Maccari, S., Ducrocq, F., Thomas, P., Darnaudery, M., 2005. Long-term behavioral alterations in female rats after a single intense footshock followed by situational reminders. Psychoneuroendocrinology 30, 316-324.

Matar M.A., Cohen, H., Kaplan, Z., Zohar, J. 2006. The effect of early poststressor intervention with sertraline on behavioral responses in an animal model of post-traumatic stress disorder.Neuropsychopharmacology. 31(12):2610-2618.

McF.wen, B.S. 1999. Stress and hippocampal plasticity. REVISTA. 22, 105-122.

Nader, K., Schafe, G.E., Le Doux, J.E. 2000. Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. Nature. 406(6797):722-726.

Pereira, L.O., Arteni, N.S., Petersen, R.C., da Rocha, A.P., Achaval, M., Netto, C.A. Effects of daily environmental enrichment on memory deficits and brain injury following neonatal hypoxia-ischemia in the rat. Neurobiol Learn Mem. 2007 Jan;87(1):101-8.

Pynoos, R.S., Ritzmann, R.F., Steinberg, A.M., Goenjian, A., Prisecaru, I., 1996. A behavioral animal model of posttraumatic stress disorder featuring repeated exposure to situational reminders. Biol. Psychiatry 39, 129-134.

Rau, V., DeCola, J.P., Fanselow, MS. 2005. Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. Neurosci Biobehav Rev. 29(8):1207-1223.

Risbrough, V.B., Stein, M.B., 2006. Role of corticotropin releasing factor in anxiety disorders: A translational research perspective. Horm. Behav. 50, 550-561.

Sapolsky, R. 2000. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch. Gen. Psychiatry. 57, 925-935.

Sawamura, T., Shimizu, M., Wakizono, T., Suzuki, g., Tsunoda, T., Takahashi, Y., Nomura, S. 2004. Effect of paroxetine on a model of posttraumatic stress disorder in rats. Neuroscience Letters 357, 37-40.

Shea, A.Y., Walsh, C., Macmillan, H., Steiner, M. 2005. Child maltreatment and HPA axis dysregulation: relationship to major depressive disorder and post traumatic stress disorder in females. Psychoneuroendocrinology. 30, 162-178.

Silveira, P.P., Portella, A.K., Clemente, Z., Bassani, E., Tabajara, A.S., Gamaro, G.D., Dantas, G., Torres, I.L., Lucion, A.B., Dalmaz, C. Neonatal handling alters feeding behavior of adult rats. Physiol Behav. 2004 Feb;80(5):739-45.

Suzuki, A., Josselyn, S.A., Frankland, P.W., Masushige, S., Silva, A,J, Kida, S. 2004. Memory reconsolidation and extinction have distinct temporal and biochemical signatures. J Neurosci. 24(20):4787-4795.

Tronson, N.C., Taylor, J.R. 2007. Molecular mechanisms of memory reconsolidation. Nat Rev Neurosci. 8(4):262-75

Van der Kolk, B. 1994. The body keeps the score: memory and the evolving psychobiology of posttraumatic stress. Harvard rev. Psychiatry. I, 253-265.

Vieweg, W.V., Julius, D.A., Fernandez, A., Beatty-Brooks, M., Hettema, J.M., Pandurangi, A.K. 2006. Posttraumatic stress disorder: clinical features, pathophysiology, and treatment. Am. J. Med. 119, 383-390.

Yehuda, R., Golier, J.A., Yang, R.K., Tischler, L. 2004. Enhanced sensitivity to glucocorticoids in peripheral mononuclear leukocytes in posttraumatic stress disorder. Bio. Psychiatry. 55, 1110-1116.

FIGURE LEGENDS

Fig. 1. General summary of the experimental procedures.

Fig. 2. Effect of MDZ on conditioned fear during exposure to situational reminders in animals subjected or not to an inescapable shock as a PTSD model. Data expressed as mean ± S.E.M. of time spent in freezing. N= 9-11 animals per group. (*) No-shock is different from other groups. (#) MDZ SR1, MDZ SR123 and MDZ SR3 are different from saline. (P<0,05; Duncan's multiple range test)

Fig. 3. Performance on the elevated Plus Maze of the no-shock, saline and MDZ-treated animals. Mean ± S.E.M. of the time spent in the open arms during the 5-min exposure to the elevated Plus Maze N= 8-12 animals per group. (*) MDZ and no-shock group are different from saline group, which spent less time in the open arms (P<0,005; Duncan's multiple range test).

Fig. 4. Performance in the Morris Water Maze of the no-shock, saline and MDZ-treated animals. Time (s) spent in the target (TQ) and opposite quadrants (OQ). Mean ± S.E.M. of the time spent in the quadrants during the 60 seconds exposure to the Maze in the test session. N= 6-8 animals per group. (*) different from saline group (P<0.05; Duncan's multiple range test).

Fig. 5. Effect of MDZ on plasma corticosterone levels 2 months after subjecting the animals to an inescapable shock as a PTSD model. Data expressed as mean + SEM. There was no effect of MDZ ($p>0,05$; Duncan's multiple range test). N=8 animals/group.

Fig. 1

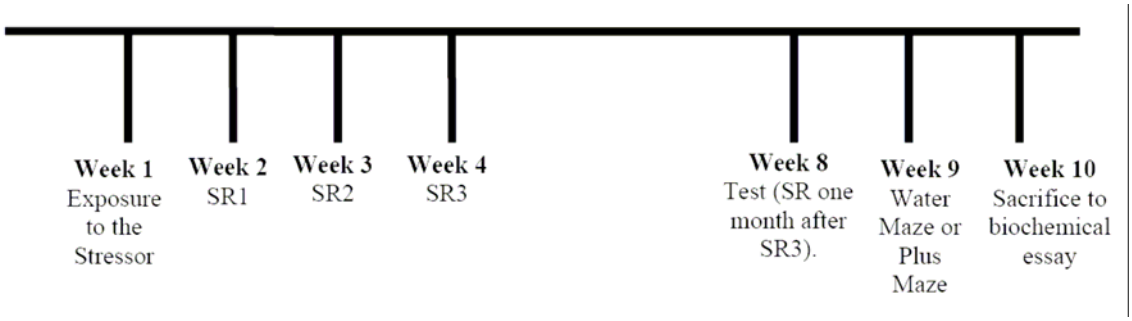


Fig. 2

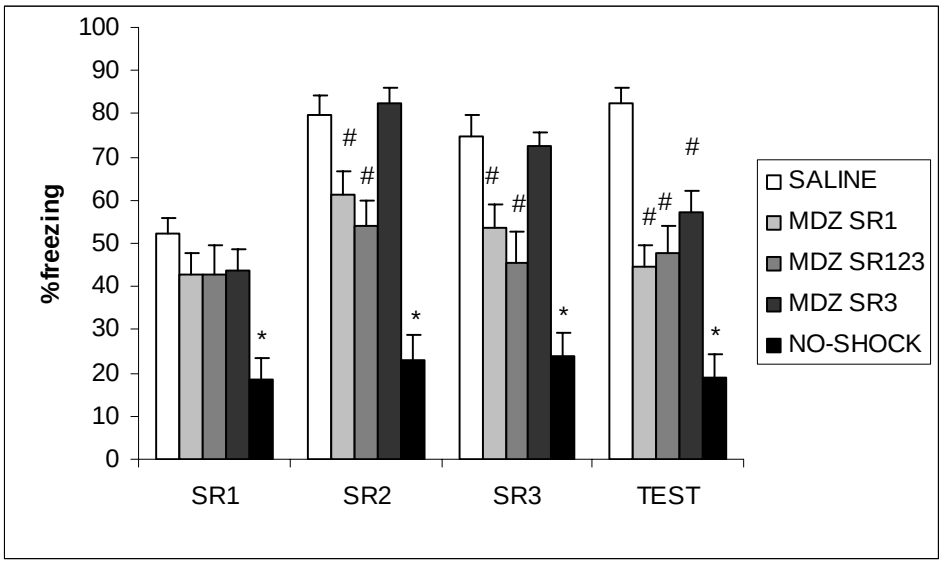


Fig. 3

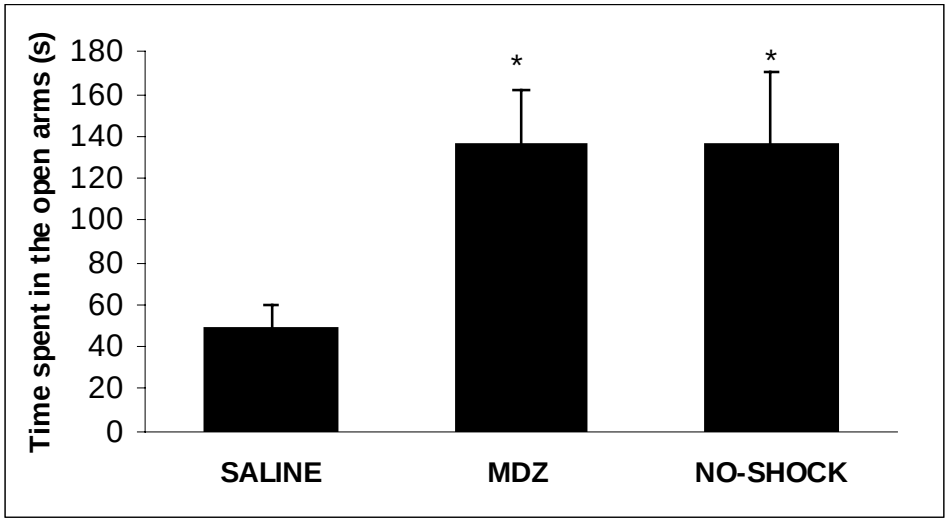

Time spent in the open arms (s)
180
160
140
120
100
80
60
40
20
0
*
*
SALINE
MDZ
NO-SHOCK

Fig. 4

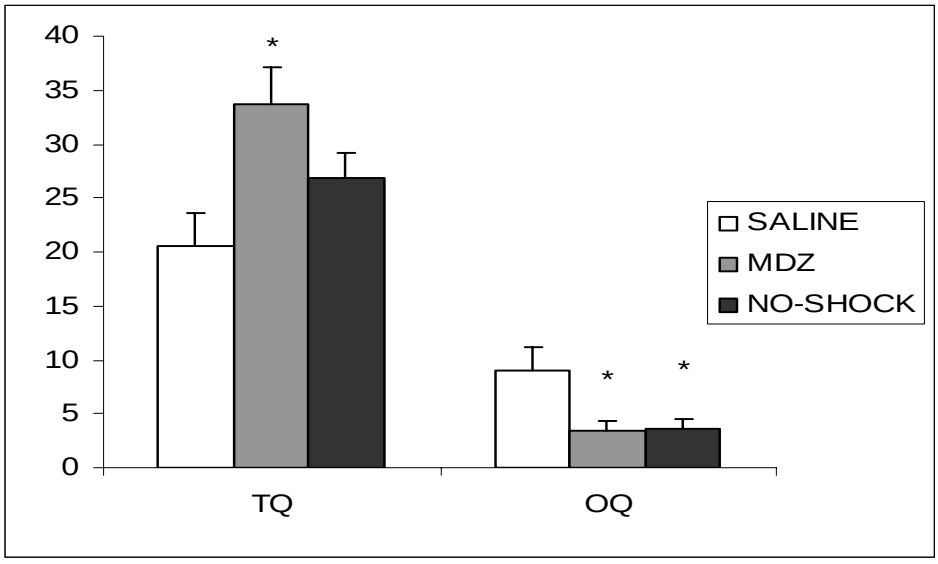
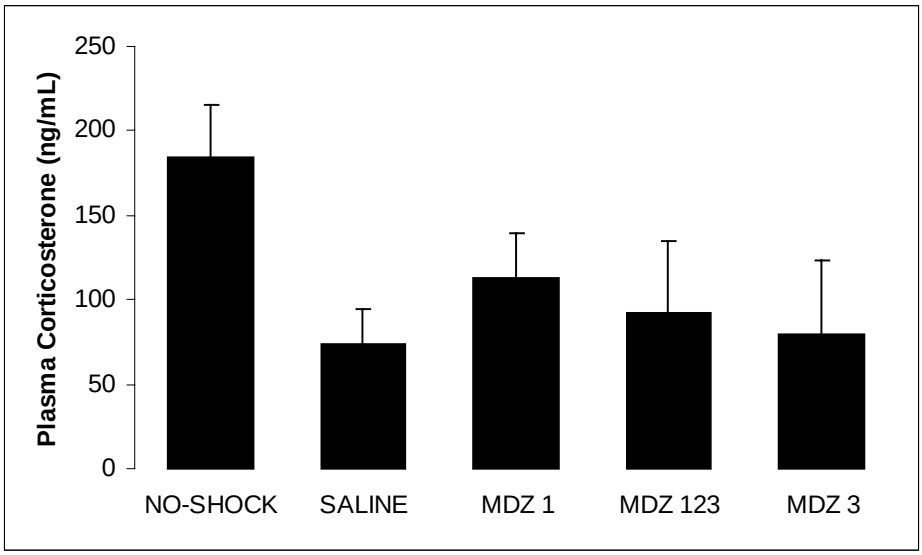

40
35
30
25
20
15
10
5
0
*
*
*
SALINE
MDZ
NO-SHOCK
TQ
OQ

Fig. 6


Plasma Corticosterone (ng/mL)
250
200
150
100
50
0
NO-SHOCK
SALINE
MDZ 1
MDZ 123
MDZ 3