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

Natividade de Sá Couto Pereira

Intervenções precoces durante o desenvolvimento como fatores de resiliência/ vulnerabilidade via modulação da reconsolidação de memórias aversivas

Porto Alegre
2017

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do Grau de Doutor em Ciências Biológicas: Bioquímica

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Porto Alegre
2017

CIP - Catalogação na Publicação

Pereira, Natividade de Sá Couto
Intervenções precoces durante o desenvolvimento como fatores de resiliência/ vulnerabilidade via modulação da reconsolidação de memórias aversivas / Natividade de Sá Couto Pereira. -- 2017.
162 f.

Orientadora: Carla Dalmaz.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2017.

1. intervenções precoces. 2. comportamento materno. 3. reconsolidação de memória aversiva. 4. hipocampo. 5. amígdala. I. Dalmaz, Carla, orient. II. Título.

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

DEDICATÓRIA

Ao Carlos, que durante todo o tempo que foi necessário para a realização deste trabalho, esteve sempre presente e demonstrou amor, apoio e paciência infinitos

AGRADECIMENTOS

A todas as pessoas que contribuíram, de alguma forma, para a realização deste trabalho, o meu eterno agradecimento!

Começo pelos meus pais, por terem apoiado a criança que aos 8 anos dizia que queria ser cientista e ao invés de se assustarem com o passatempo de misturar vários produtos químicos em frascos catalogados, me ofereceram um microscópio (de brinquedo) no aniversário. Agradeço pelo incentivo para que eu buscasse sempre o melhor e não me contentasse com o "bom", por me mostrarem o mundo e me ensinarem que não existem verdades absolutas, que a vida dá inúmeras voltas e reconfigura constantemente a nossa realidade e que o desespero é a mais inútil das emoções porque tudo tem solução. Muito obrigada pelo amor, por me darem asas ainda mais fortes do que as raízes (que não são nada fracas) e pelos ensinamentos que foram essenciais para sobreviver ao doutorado!

Agradeço ao meu irmão Rui (in memoriam), que como se pressentisse que não tinha muito tempo, amadureceu tão rápido que, por vezes, mesmo sendo 5 anos mais novo, parecia meu irmão mais velho. Obrigada por teres feito parte da minha vida, por me mostrares que não há tristeza que um abraço não cure, que partilhar é importante e, com a tua partida, instigares ainda mais a minha vontade de buscar na ciência as respostas que nos faltam. À minha irmã Ana, fonte inesgotável de alegria na minha existência, minha confidente e parceira de psicoanálise, obrigada por me ouvires, pelo amor e apoio incondicionais, por me emprestares a tua roupa e reclamares depois e por seres tudo o que eu poderia querer numa irmã! A toda a minha família, avós, tios e primos, pelo carinho infindável, pelos valores que me transmitiram e pelo ambiente enriquecido que me proporcionaram, que compensou plenamente os frequentes episódios de separação materna e paterna na minha infância.

Ao Carlos, meu namorado, muito obrigada por absolutamente tudo! Pelo amor, pelo carinho e sobretudo pela paciência! Obrigada por estares sempre presente, por saberes sempre o que dizer (vide a frase mágica "depois da defesa, tu desistes"), por me fazeres querer sempre evoluir e por seres o melhor companheiro que eu

poderia querer para seguir comigo nesta grande aventura chamada vida! Estendo o meu agradecimento às famílias Michels, Scheer e Chilanti, em especial à Janice e ao Antônio, por serem a minha família aqui, tão longe de casa.

Agradeço profundamente à minha orientadora, Profa. Carla Dalmaz, pela oportunidade, pela confiança depositada em mim e por tudo o que me ensinou! Agradeço também à Profa. Maria Elisa Calcagnotto, por partilhar uma ínfima parte do seu conhecimento comigo e por estar sempre disposta a ajudar, ao Prof. Jorge Quillfeldt, por mostrar de forma bem-humorada que o que cremos ser a resposta a uma pergunta científica é na verdade o início de várias novas perguntas, e à Profa. Cristiane Matté e ao seu grupo, pela disposição constante em ajudar e pela oportunidade de trabalhar com uma técnica nova.

Não se faz ciência sozinho. Por isso, agradeço a todos os meus colegas do laboratório 37: Camilla, Grasi, Danusa, Ana, Rachel, Vivi, Charles, Marina, André, Raquelzinha, Diego, Dieguinho, Carol, por todos os auxílios técnicos e não-técnicos. Faço um agradecimento especial à Carine, por tornar os meus dias mais leves e por embarcar comigo nas muitas aventuras laboratoriais, e à Aline, por me ajudar nos comportamentos, fim-de-semana ou feriado, e por confiar em mim.

Ao Pablo Espejo, ao Dr. Víctor Molina e a todos os alunos, professores e técnicos do Instituto de Farmacología Experimental da Universidade Nacional de Córdoba, agradeço por me receberem tão bem e pelo esforço incansável para fazer com que a minha estadia tão curta tivesse tantas experiências agradáveis, que regressei com a sensação de ter vivido meses em Córdoba.

Aos amigos que fiz na Farmaco da UFCSPA, Lu, Mari, Rosane, Greice, Maurício, Felipe, as "australianas" Laísa e Pri, Yasmin e todos os ICs, agradeço imensamente por agirem como se eu nunca tivesse saído do grupo e me convidarem para todos os eventos e happy hours! Vocês foram fundamentais para a conclusão do Mestrado e continuaram sendo essenciais durante o Doutorado. À Nice, pelo carinho e prontidão em ajudar contantes.

Aos amigos Vanessa e Pedro, Cris e Dani, Paulo, Elisete, Paulinho, Camila, Marcio e Cheila, obrigada pela ótima companhia e pela amizade; ao Diego e Simone, aos amigos da dança (novos e antigos), a todas as pessoas que mesmo depois de um dia inteiro de experimentos, conseguem me fazer sorrir.

Muito obrigada a todos!

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RESUMO

O estudo dos mecanismos neurobiológicos associados a memórias aversivas tem aplicações potenciais à prevenção e ao tratamento de patologias psiquiátricas associadas a memórias traumáticas. Em particular, o processo de reconsolidação, através do qual memórias evocadas são novamente estabilizadas, tem sido visto como um possível mecanismo na origem desses transtornos e, ao mesmo tempo, um alvo para estratégias terapêuticas.

Experiências precoces influenciam o desenvolvimento e a maturação de circuitos encefálicos, e a sua interação com a carga genética do indivíduo, pode influenciar as estratégias de enfrentamento de situações aversivas ao longo da vida, gerando indivíduos resilientes ou vulneráveis ao desenvolvimento de transtornos psiquiátricos. Dentro deste contexto, o objetivo principal desta tese foi estudar diferentes experiências precoces, em modelos animais, e seu efeito sobre o processo de reconsolidação de memórias aversivas e, consequentemente, sobre o desenvolvimento de padrões de conduta de resiliência ou vulnerabilidade.

Foram utilizados dois modelos, em ratos: a manipulação neonatal e a separação materna. Os resultados obtidos mostram que ambas intervenções levaram a um aumento dos cuidados que a mãe dedica à prole, mas a separação materna induziu um comportamento mais inconsistente, que reflete menor qualidade; consistentemente, a prole de ambos os sexos exibiu alterações na secreção de corticosterona frente a um contexto previamente pareado com um estímulo aversivo, e os machos mostraram generalização do medo a um contexto novo após a experiência. A manipulação neonatal, além de gerar um aumento do cuidado materno, levou a um aumento dos níveis centrais de ocitocina nas mães. A prole do sexo masculino exibiu comportamento de congelamento diminuído no contexto condicionado a um estímulo aversivo.

Ambas intervenções geraram resistência à reconsolidação da memória aversiva condicionada, através de um mecanismo que parece envolver o hipocampo dorsal mas não a amígdala basolateral; o hipocampo ventral de ratos machos separados no período neonatal mostrou uma diminuição de espécies reativas de oxigênio e nitrogênio, sugestivo de atenuação de alguns mecanismos de plasticidade.

Estes resultados apontam que os padrões de comportamento frente a situações aversivas são afetados pelas experiências neonatais, como reportado anteriormente, e que a manipulação parece gerar uma conduta mais resiliente enquanto a separação está associada ao surgimento de um padrão de comportamento mais vulnerável. A reconsolidação de memória de medo alterada em conjunto com a generalização da memória e a inconsistência comportamental da mãe encontrados neste trabalho e as alterações estruturais e funcionais na amígdala nestes animais, reportadas anteriormente, tornam a separação materna um modelo promissor para o estudo de mecanismos neurobiológicos dos transtornos psiquiátricos associados a memórias traumáticas.

Palavras-chave: intervenções precoces, comportamento materno, memória de medo, reconsolidação, hipocampo, amígdala

ABSTRACT

The prevention and treatment of psychiatric pathologies associated with traumatic memories benefits from the study of the neurobiological mechanisms underlying aversive memories. In particular, the reconsolidation process, through which retrieved memories are restabilized, has been regarded both as a possible mechanism at the origin of these disorders and as a target for therapeutical strategies.

Early life experiences impact the development and maturation of brain circuits, and their interaction with the individual's genetic load may influence the coping mechanisms throughout life, generating individuals that are resilient or vulnerable to psychiatric disorders. Therefore, the aim of this thesis was to study, in an animal model, different early interventions and their effect on fear memory reconsolidation, and consequently the development of behavioral patterns of resilience or vulnerability.

Neonatal handling and maternal separation in rats were used as early intervention models. The results obtained show that both interventions led to an increase in the amount of care the dam provides to the offspring, but maternal separation increased behavioral inconsistency, which reflects low quality behavior; consistently, both male and female offspring exhibited changes in corticosterone secretion in response to a context that was previously paired with an aversive stimulus and males showed fear generalization to a novel context after the experience. Neonatal handling increased both maternal care and central oxytocin levels in the dam. The male offspring showed reduced freezing behavior in the context conditioned to an aversive stimulus.

Both interventions generated resistance to conditioned fear memory reconsolidation, through a mechanism that appears to involve the dorsal hippocampus but not the basolateral amygdala; the ventral hippocampus of males that were separated in the neonatal period had decreased reactive oxygen and nitrogen species, suggesting attenuated plasticity mechanisms.

These results suggest that behavioral patterns which emerge when facing aversive situations are affected by neonatal experiences, as reported previously, and that neonatal handling appears to result in resilience while maternal separation appears to be associated with a pattern of vulnerability. Changes in fear memory reconsolidation together with memory generalization and maternal behavior inconsistency, found in this work, and the structural and functional changes in the amygdala, reported earlier, suggest that maternal separation is a promising model to study the neurobiological mechanisms of psychiatric pathologies associated with traumatic memories.

Keywords: early interventions, maternal behavior, fear memory, reconsolidation, hippocampus, amygdala

LISTA DE ABREVIATURAS

AMPA	ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiónico (sigla em inglês)
BDNF	fator neurotrófico derivado do encéfalo (sigla em inglês)
BLA	complexo basolateral da amígdala (sigla em inglês)
BZD	benzodiazepínicos
CaMKII	proteína cinase dependente de cálcio/calmodulina do tipo II (sigla em inglês)
CREB	elemento de resposta a adenosina monofosfato cíclico (sigla em inglês)
ERK	cinase regulada por sinal extracelular (sigla em inglês)
GABA	ácido gama-aminobutírico (sigla em inglês)
GR	receptor de glicocorticóides (sigla em inglês)
HcD	hipocampo dorsal
HcV	hipocampo ventral
HHA	eixo hipotálamo-hipófise-adrenal
LTD	depressão de longa duração (sigla em inglês)
LTP	potenciação de longa duração (sigla em inglês)
MAPK	cinases ativadas por mitogênio (sigla em inglês)
mdz	midazolam
mPFC	córtex pré-frontal medial (sigla em inglês)
Na^+/K^+-ATPase	bomba de sódio e potássio
NF-kB	fator nuclear kappa B (sigla em inglês)
NMDA	N-metil-D-aspartato
nNOS	óxido nítrico sintase (sigla em inglês)
NO	óxido nítrico (sigla em inglês)
PKA	proteína cinase A (sigla em inglês)
SNC	Sistema Nervoso Central
TEPT	Transtorno de Estresse Pós-traumático
UPS	sistema ubiquitina-proteassoma (sigla em inglês)

1. INTRODUÇÃO

1.1 Resiliência e vulnerabilidade

> "*Adversity causes some men to break; others to break records.*"
> William Arthur Ward

Eventos adversos fazem parte da trajetória de vida de todos os seres. A forma de encarar esses eventos depende das experiências prévias do indivíduo, da sua genética, do seu estado emocional no momento, entre outros fatores; dentro do espectro complexo de respostas possíveis a um evento adverso, os indivíduos podem ser classificados, de forma simplista, em resilientes ou vulneráveis.

Apesar de não ter uma definição consensual (Southwick et al. 2014), a resiliência é vista como a capacidade de gerar uma resposta ativa e adaptativa, compreendendo os níveis biológico, psicológico e social, a um evento que, caso contrário, poderia prejudicar o funcionamento normal do indivíduo (Southwick et al. 2014; Singh-Taylor et al. 2015; Russo et al. 2012); é, em resumo, a habilidade de lidar e gerar uma resposta favorável a um evento aversivo. Estudos focados nos mecanismos neurobiológicos que medeiam a resiliência têm se tornado muito comuns na última década (Southwick et al. 2014).

A resiliência não é o oposto direto da vulnerabilidade, que se caracteriza pela baixa tolerância do indivíduo ao estresse, e portanto por uma dificuldade em manter a funcionalidade adequada do organismo em situações estressantes, facilitando o surgimento de transtornos emocionais (Zubin & Spring 1977). Assim, ser resiliente não significa ser imune ao estresse ou desconforto, nem tampouco reprimir a situação adversa, mas sim se reconfigurar para se adaptar às marcas que a experiência possa deixar, mantendo o equilíbrio

mental. Ambas as respostas, resiliência e vulnerabilidade, sofrem influência de fatores genéticos e das interações entre os genes e o ambiente (Singh-Taylor et al. 2015; Andersen 2003).

Certas fases do desenvolvimento, particularmente a gestação e os primeiros anos de vida até a adolescência, são períodos de intensa maturação encefálica, muito suscetíveis a modulação pelo ambiente externo (Gee & Casey 2015; Singh-Taylor et al. 2015; Eiland & Romeo 2013). Nestes períodos ocorrem processos de neurogênese, migração neuronal, sinaptogênese e poda sináptica que vão determinar a estrutura e o funcionamento do encéfalo no futuro (Eiland & Romeo 2013); assim, os estágios iniciais do desenvolvimento parecem ser ao mesmo tempo janelas de oportunidade e de vulnerabilidade para a ação do ambiente sobre o processo de definição das estratégias que o indivíduo adotará face a desafios, ao longo da sua vida (Andersen 2003; Dahl 2004; Buschdorf & Meaney 2016).

Experiências precoces aversivas, como abuso, negligência física e emocional, conflitos familiares, relacionamento distante com os progenitores e autoritarismo, entre outros (Anacker, O'Donnell & Meaney 2014), parecem tornar os indivíduos mais sensíveis a eventos negativos: um estudo que reuniu dados de levantamentos da Organização Mundial da Saúde, realizado em 21 países, apontou que a exposição a adversidades na infância explica quase 30% dos casos de doenças psiquiátricas na vida adulta (Kessler et al. 2010). Um exemplo destas doenças é o Transtorno de Estresse Pós-traumático (TEPT), um transtorno psiquiátrico que alguns indivíduos desenvolvem após vivenciar ou testemunhar uma situação fortemente aversiva (Yehuda 2002). O motivo pelo qual apenas 8.3% dos indivíduos expostos a uma situação traumática, segundo um levantamento norte-americano (Kilpatrick et al. 2013), desenvolve esta patologia na vida é ainda pouco conhecido, mas parece ser afetado por uma vulnerabilidade prévia (Yehuda 2002). Lanius e colaboradores (2010) apresentaram dois

possíveis modelos para explicar o surgimento deste transtorno: no primeiro, após o trauma, o indivíduo reviveria frequentemente a memória do evento, o que acarretaria sensibilização dos circuitos de processamento do medo e de outras emoções negativas, levando à desregulação emocional observada na patologia; no segundo modelo, a interação entre fatores genéticos e ambientais no início da vida, como ausência de um vínculo adequado com a(s) figura(s) parental(ais) ou maus-tratos sofridos na infância, estaria na origem da desregulação emocional que tornaria o indivíduo vulnerável ao desenvolvimento de TEPT após exposição a um trauma. Esta última hipótese está de acordo com a teoria do "estresse cumulativo", na qual se postula que sucessivas experiências adversas aumentam a probabilidade de o indivíduo se tornar vulnerável ao estresse (Taylor 2010; Daskalakis et al. 2012) e com os estudos sobre experiências precoces e patologias psiquiátricas referidos acima.

Crianças alojadas em orfanatos, onde o estabelecimento de um vínculo forte com um adulto é dificultado pela troca frequente dos cuidadores, apresentaram aceleração do amadurecimento da conectividade entre o córtex pré-frontal medial (mPFC) e a amígdala (Gee et al. 2013); outro estudo com crianças institucionalizadas mostrou um aumento da reatividade da amígdala a estímulos emocionais e ao mesmo tempo diminuição da ativação de áreas corticais, além de diminuição do contato visual numa situação de interação social (Tottenham et al. 2011), o que sugere que a maturação precoce do circuito cortico-amigdalar pode acarretar alterações funcionais duradouras; este circuito e a importância da sua regulação serão discutidos em mais detalhe na seção 1.3.1.

Por outro lado, estímulos positivos na infância, como cuidado parental adequado e estrutura familiar que forneça suporte ao indivíduo parecem estar associados ao desenvolvimento de resiliência (Southwick et al. 2014; Shai & Belsky 2016; Belsky et al. 2015). Adicionalmente, a exposição ao estresse moderado nas fases iniciais da vida também

parece exercer um efeito protetor sobre a resposta ao estresse na vida adulta. Um estudo recente mostrou que indivíduos que haviam sido expostos a adversidade moderada na infância apresentaram uma melhor capacidade de regulação emocional do que indivíduos sem essa experiência precoce, evidenciada por uma ativação diminuída da amígdala durante a exposição a mídias com conteúdo emocional negativo (Schweizer et al. 2016).

A interação entre os diversos fatores ambientais que afetam o desenvolvimento, a carga genética do indivíduo e a valência emocional da experiência a ser enfrentada configuram uma matriz complexa que torna a resultante destas interações bastante difícil de prever; de fato, alguns autores defendem que experiências aversivas no período neonatal poderiam programar o indivíduo para responder melhor ao estresse na vida adulta, enquanto a "ausência" de adversidades precoces dificultaria a adaptação do indivíduo a um ambiente hostil – "*mismatch hypothesis*" (Daskalakis et al. 2012; Buschdorf & Meaney 2016; Schmidt 2012; Schmidt 2011); a divergência entre esta teoria e a teoria do estresse cumulativo pode ter origem, em parte, na subjetividade do conceito de "resposta adequada" (Diamond & Zoladz 2015). Além disso, a forma como uma experiência precoce afeta o neurodesenvolvimento também depende do sexo do indivíduo. Ambas teorias têm em comum a hipótese que o ambiente precoce impacta a capacidade do indivíduo lidar com situações adversas.

Apesar do crescente interesse na temática da resiliência por parte da comunidade científica, ainda existem várias lacunas de conhecimento sobre os mecanismos neurobiológicos deste processo.

1.2 Modelos animais de intervenções precoces

O estudo dos mecanismos celulares e moleculares do SNC envolvidos na vulnerabilidade e na resiliência tem se beneficiado bastante do uso de modelos animais (Singh-Taylor et al. 2015; McEwen 2008; Taylor 2010; Andersen 2003; Anacker, O'Donnell

& Meaney 2014; Zhang et al. 2013). Em humanos, estudos epidemiológicos, técnicas de neuroimagem, como ressonância magnética funcional, coleta de tecido para estudos genéticos, ou dosagens periféricas de hormônios ligados à resposta ao estresse, permitiram estabelecer correlações entre experiências precoces e várias patologias psiquiátricas (Kessler et al. 2010). De forma complementar, os estudos com animais têm permitido aprofundar o conhecimento das bases neurobiológicas que determinam a resposta ao estresse, os circuitos encefálicos relevantes, além de investigar como esses mecanismos podem ser modulados para modificar a resposta final.

Dentre os vários modelos pré-clínicos, os que introduzem interferências durante o desenvolvimento em roedores têm se destacado no estudo destes mecanismos (Singh-Taylor et al. 2015; Southwick et al. 2014), tendo permitido, por exemplo, identificar alterações epigenéticas como mediadoras das consequências a longo prazo das experiências precoces (Reul 2014; Weaver et al. 2004; Anacker, O'Donnell & Meaney 2014; Zhang et al. 2013). O período neonatal em roedores, especificamente, parece se correlacionar com o final da gestação e início da infância em humanos, sobretudo na maturação de estruturas límbicas e corticais (Clancy, Darlington & Finlay 2001; Clancy et al. 2007; Eiland & Romeo 2013).

1.2.1 Intervenções no período neonatal

Ratos e outras espécies altriciais são fortemente dependentes da mãe no período pós-parto, portanto, modelos experimentais que introduzem algum tipo de perturbação sobre a díade mãe-filhote podem levar a alterações no desenvolvimento da prole (Francis & Meaney 1999).

A manipulação neonatal (*neonatal handling* ou *early handling*, em inglês) é um modelo experimental em roedores, que foi descrito inicialmente entre o final da década de

1950 e a década de 1960, a partir das observações de Levine e colaboradores que ratos que eram manipulados pelo pesquisador nos primeiros dias de vida apresentavam alterações no comportamento e nos parâmetros hormonais da resposta ao estresse na fase adulta (Levine 1967; Levine et al. 1967; Levine & Lewis 1959). Atualmente, esta intervenção consiste em uma breve separação diária da mãe e/ou a manipulação dos filhotes pelo pesquisador, nos primeiros dez a vinte dias de vida. A duração da separação é inferior a 30 minutos pois, *in natura*, é comum as mães se ausentarem do ninho por períodos desta duração. Ratos que foram manipulados no período neonatal têm respostas tipo-ansiedade diminuídas na fase adulta, em paradigmas comportamentais diversos (Kosten, Lee & Kim 2006; Macrí, Mason & Würbel 2004; Severino et al. 2004; Meerlo et al. 1999; Caldji et al. 2000), além de diminuição do comportamento tipo-depressivo (Silveira et al. 2011) e menos consequências deletérias resultantes da exposição a um estresse prolongado (Marcolin et al. 2012; Silveira et al. 2011). Acredita-se que estas alterações podem estar principalmente associadas à expressão aumentada do receptor de glicocorticóides (GR) no hipocampo destes animais, o que potencia o processo de retroalimentação negativa do eixo hipotálamo-hipófise-adrenal (HHA), permitindo o retorno mais rápido aos níveis basais de secreção de glicocorticóides após a exposição a um estressor (Liu et al. 1997; Meaney et al. 1985).

Por sua vez, os modelos experimentais que envolvem alguma forma de privação materna nestes animais têm sido muito utilizados para estudar como um forte estresse no início da vida afeta o desenvolvimento psicoemocional e a maturação encefálica. A separação materna consiste na remoção dos filhotes do ninho por períodos longos – geralmente 3 horas ou mais – durante os quais os filhotes podem ou não ser mantidos em temperatura constante. Existem outros modelos com protocolos semelhantes, como a privação materna, em que a separação dura até 24 horas, geralmente realizada uma única vez, e o isolamento neonatal, no

qual os filhotes são separados não apenas da mãe mas também do restante da ninhada. Estes modelos geram um padrão de comportamento tipo-ansioso semelhante na prole adulta (de Kloet et al. 2005), pelo que, nesta tese, se discutirá a literatura que versa sobre os seus efeitos de forma conjunta mas, para manter a clareza, será indicado qual modelo foi utilizado no período neonatal.

Vários estudos mostraram que ratos machos separados da mãe no período neonatal mostram emocionalidade aumentada em tarefas que envolvem estímulos aversivos (Diehl et al. 2014; Diehl et al. 2012; Eiland & Romeo 2013; McEwen 2008; Makena, Bugarith & Russell 2012). Além disso, tal como em humanos (Gee et al. 2013), ratos separados na fase neonatal também parecem sofrer maturação precoce dos circuitos associados ao medo, apresentando, no final do período neonatal, dificuldade em extinguir uma memória aversiva e facilitação do seu restabelecimento, características que são normalmente observadas em ratos adultos e não durante o seu desenvolvimento (Callaghan & Richardson 2011; Callaghan & Richardson 2012). Estes achados se assemelham à desregulação emocional proposta para a etiologia do TEPT (Lanius et al. 2010).

1.2.2 Comportamento materno

As intervenções no período neonatal citadas acima têm dois níveis passíveis de atuação: 1) o impacto direto da intervenção no metabolismo e na sinalização hormonal do filhote; e 2) um efeito indireto modulado pelas alterações no comportamento materno induzidas pela intervenção.

Como já referido, o cuidado parental fornecido à prole tem um grande impacto no seu desenvolvimento, como determinado em estudos em humanos (Silva 2003; McEwen 2008; Brauer et al. 2016) e em animais (McEwen 2008; Liu et al. 1997). Especificamente, em ratos,

o cuidado maternal está diretamente envolvido na programação a longo prazo da resposta ao estresse, estando associado ao desenvolvimento de resiliência na prole (Liu et al. 1997; Francis & Meaney 1999; Champagne et al. 2003; Fish et al. 2004; van Hasselt et al. 2012). No entanto, apesar da sua óbvia importância, o papel modulador do cuidado maternal alterado tem sido alvo de pouca atenção nos estudos que utilizam manipulação neonatal, separação materna ou outras intervenções precoces em roedores, e frequentemente foram encontrados resultados conflitantes (Denenberg 1999).

No caso da manipulação neonatal, acredita-se que os seus efeitos são mediados pelas alterações que a intervenção induz no cuidado da mãe, uma vez que este se apresenta aumentado na maioria dos estudos (Liu et al. 1997; Kuhn & Schanberg 1998; Stamatakis et al. 2015; Macrí, Mason & Würbel 2004) e as consequências a longo prazo nos filhotes coincidem com as da prole de mães naturalmente muito cuidadoras (Liu et al. 1997; Meaney et al. 1985; Denenberg 1999). No caso da separação materna, a abundância de relatos contraditórios em relação a aumento/ diminuição do cuidado dedicado à prole, junto com o uso de protocolos em que os efeitos da separação e do cuidado materno são dissociados (Macrí, Mason & Würbel 2004; Macrì, Chiarotti & Würbel 2008), geram controvérsias sobre o papel do cuidado materno na mediação dos efeitos da intervenção.

Em humanos, a sensibilidade materna, *i.e.* a capacidade de interagir com a prole de forma sincrônica e harmoniosa, de acordo com as necessidades e personalidade específicas de cada criança (van IJzendoorn et al. 2000; Pederson et al. 1998), mostrou estar correlacionada com parâmetros de resiliência emocional em crianças pequenas, sendo esta correlação particularmente evidente em agregados familiares vivendo em ambientes adversos (Silva 2003); de fato, a sensibilidade materna parece ser um forte fator preditivo do bom desenvolvimento cognitivo, social e emocional da criança (Belsky et al. 2015; Shai & Belsky

2016). De forma similar, o cuidado materno adequado em roedores deve estar sintonizado com as necessidades e demandas dos filhotes, pelo que quantidade e qualidade podem não estar necessariamente relacionadas (Pereira & Ferreira 2016; Pereira & Ferreira 2006). A explicação para os achados contraditórios nos estudos de cuidado materno nos modelos de intervenções neonatais pode estar relacionada com o fato de praticamente todos avaliarem apenas a frequência de comportamentos direcionados à prole, que, apesar de ser um aspecto importante, pode estar mais relacionada com fatores externos, como hora do dia, por exemplo, e pode não refletir o cenário completo do cuidado fornecido e como este é experienciado pelos filhotes. Estudos recentes com outro modelo de estresse precoce, a restrição do material do ninho, descreveram um escore para avaliar a qualidade do comportamento das mães que leva em conta a consistência do comportamento materno e que se utilizará neste trabalho (Molet, Maras, et al. 2016; Ivy et al. 2008).

Portanto, a avaliação do comportamento materno é importante porque é um dos possíveis fatores pelos quais as intervenções no período neonatal podem modificar a resposta ao estresse e levar a alterações emocionais e cognitivas na prole. Na idade adulta, estas alterações podem interferir na consolidação, evocação e reconsolidação de memórias de situações que envolvem o comportamento defensivo destes animais.

1.3 Memórias aversivas

> "*You learn emotional experiences as much as you learn cognitive experiences, except that they are more unconscious. Sometimes one represses the cognitive component of it, but it's often more difficult to repress the emotional component.*"
>
> Eric Kandel

O medo é essencial à sobrevivência, portanto os circuitos neurais de processamento desta emoção e outras emoções negativas associadas foram altamente conservados ao longo da evolução (Gross & Canteras 2012). Apesar da conservação evolutiva dos seus circuitos básicos, esta emoção em humanos é mais complexa e envolve a percepção consciente da mesma. Assim, o estudo das respostas condicionadas a ambientes aversivos em outros mamíferos, como roedores, permite o entendimento de mecanismos inconscientes envolvidos em algumas patologias psiquiátricas humanas, nomeadamente a ansiedade antecipatória e fobias específicas (Shuhama et al. 2007) e patologias que envolvem memórias traumáticas (Kosten, Kim & Lee 2012; Hartley & Phelps 2010).

Por falta de um termo mais apropriado e para seguir a nomenclatura utilizada nos trabalhos atuais desta área, os termos "memória de medo" e "memória aversiva" serão utilizados nesta tese para designar memórias relacionadas à vivência de um estímulo que promove a percepção de uma ameaça (LeDoux 2014).

Intervenções precoces em roedores, como as descritas acima, modificam processos mnemônicos de diferentes espectros emocionais. Ratos manipulados no período neonatal apresentam diminuição da resposta comportamental em paradigmas que envolvem memórias aversivas (Kosten, Lee & Kim 2006). No entanto, quando estes são treinados em paradigmas que envolvem estímulos considerados menos aversivos, como em tarefas espaciais ou de reconhecimento de objetos, ou estímulos apetitivos, os machos manipulados mostram um desempenho semelhante (Noschang et al. 2012; Noschang et al. 2010) ou superior (Kosten, Lee & Kim 2007) aos controles, sugerindo que estes animais não possuem deficits cognitivos, mas emocionalidade diminuída face a situações adversas (Kosten, Kim & Lee 2012; Caldji et al. 2000). É interessante que machos manipulados apresentaram dificuldades no aprendizado reverso de uma tarefa espacial com recompensa palatável, embora não tenham apresentado

diferenças no aprendizado dessa tarefa (Noschang et al. 2012); no entanto, outro estudo do nosso grupo mostrou melhor desempenho destes animais no aprendizado reverso de uma das dimensões de uma tarefa de atenção (Lazzaretti 2016). Ratos separados da mãe no período neonatal apresentam resultados diversos em tarefas mnemônicas aversivas, mas na maioria dos estudos parecem não apresentar diferenças em relação aos controles no tempo de congelamento face ao contexto aversivo (Diehl et al. 2007; Kosten, Lee & Kim 2006; Kosten, Kim & Lee 2012).

Apesar da escassez de estudos conduzidos em fêmeas, estes mostram que as experiências precoces afetam a prole de forma diferente conforme o sexo. De forma geral, os efeitos da manipulação sobre tarefas que envolvem aprendizado são semelhantes em machos e fêmeas: em tarefas sem estímulos aversivos o seu desempenho é igual ou melhor do que as fêmeas controle, enquanto que em tarefas de condicionamento aversivo, a sua resposta comportamental é inferior (Kosten, Lee & Kim 2007; Noschang et al. 2012). Fêmeas separadas no período neonatal parecem ser diferentes dos machos submetidos à mesma intervenção, e parecem apresentar desempenho diminuído em tarefas aversivas (Kosten, Lee & Kim 2006), além de alterações menos evidentes do que os machos em tarefas que avaliam comportamento tipo-ansioso (de Kloet et al. 2005; Diehl et al. 2007).

1.3.1 Circuitos encefálicos envolvidos no processamento de memórias aversivas

Diferentes tipos de memória recrutam diferentes circuitos encefálicos. Dentro das memórias aversivas, a aquisição de memórias associadas a pistas ambientais sonoras ou olfativas parecem depender mais fortemente da amígdala (Johansen et al. 2011; Phillips & LeDoux 1992) enquanto memórias condicionadas ao contexto dependem também de processos de plasticidade sináptica no hipocampo, além da amígdala (Phillips & LeDoux

1992). Portanto, o circuito amígdala-hipocampo é responsável pelo armazenamento e evocação de memórias com componente emocional aversivo, quando o estímulo que deu origem à resposta emocional negativa foi condicionado ao ambiente no qual foi apresentado (Izquierdo, Furini & Myskiw 2016). Este circuito apresenta uma interação dinâmica, com projeções entre si, que modulam mutuamente os processos de plasticidade das duas estruturas. Estas interações sofrem influência do estresse, o que explica a modulação das memórias aversivas por estressores (Richter-Levin & Akirav 2000).

Outras estruturas encefálicas também estão envolvidas no armazenamento, processamento e expressão comportamental de memórias aversivas, como regiões corticais, núcleos do tálamo e do hipotálamo, entre outros. Excetuando o mPFC, cujo papel modulador da atividade da amígdala e do hipocampo será comentado adiante, tais estruturas não serão abordadas neste trabalho, pelo que se indica revisões bastante interessantes para o leitor interessado neste tema (ver, por exemplo, Izquierdo, Furini & Myskiw 2016, sobre memórias aversivas, ou Gross & Canteras 2012, sobre circuitos de processamento do medo).

1.3.1.1 Amígdala

A amígdala é uma estrutura encefálica chave no desenvolvimento de transtornos emocionais na vida adulta (Taylor 2010), por ter um papel essencial no processamento de estímulos de carga emocional negativa (Phelps & LeDoux 2005; Gross & Canteras 2012; Kim et al. 2011; Lalumiere 2014). Além disso, esta estrutura é essencial na formação de memórias aversivas, sendo particularmente importante para o componente emocional destas (LeDoux 2003; Kandel, Dudai & Mayford 2014).

A amígdala é composta por vários núcleos e subnúcleos que têm funções e conexões distintas (LeDoux 2007). De particular interesse para este trabalho, são o núcleo lateral e o núcleo basal que juntamente com o núcleo acessório basal compõem o complexo basolateral –

BLA (LeDoux 2007). Este complexo integra conexões sensoriais, vindas do tálamo e regiões do córtex sensorial, e do hipocampo dorsal e córtex associativo (LeDoux 2007), como resumido na Figura 1, o que o torna, entre os vários circuitos internos da amígdala, o mais fortemente envolvido com o processamento de memórias aversivas aprendidas (Gross & Canteras 2012; Izquierdo, Furini & Myskiw 2016).

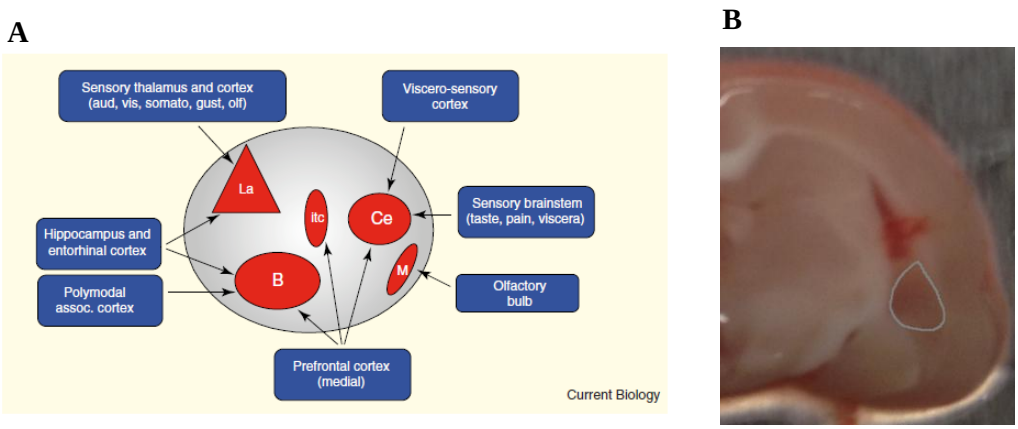

Figura 1. Complexo basolateral da amígdala (BLA). A. Conexões de outras estruturas encefálicas com os núcleos da amígdala. Fonte: LeDoux 2007, *Current Biology*, 17(20), pp. 868-874. B. Corte coronal do encéfalo de rato evidenciando a BLA do hemisfério direito e sua fácil identificação pela coloração escura do núcleo basal.

A atividade da BLA é modulada pelo mPFC; em particular, a região ventromedial foi identificada como principal no controle inibitório da BLA (Motzkin et al. 2015), através de um circuito que envolve o núcleo basomedial da amígdala (Adhikari et al. 2015). Como já mencionado acima, estresse precoce parece acelerar a maturação deste circuito e causar alterações funcionais no mesmo (Gee et al. 2013; Callaghan & Richardson 2012; Callaghan & Richardson 2011). O estresse interfere também com os circuitos inibitórios internos da amígdala (Rodríguez-Manzanares et al. 2005; Isoardi et al. 2007).

Em concordância com estes estudos, animais separados mostraram atenuação da atividade do mPFC e potenciação da atividade da amígdala em resposta à administração sistêmica de um agonista inverso parcial do receptor $GABA_A$, sugerindo que nesses animais

há diminuição do controle inibitório do mPFC sobre a BLA (Stevenson, Marsden & Mason 2008). Consistente com a hipótese de maturação precoce do circuito mPFC-BLA, alterações na plasticidade sináptica foram encontradas em animais jovens que foram submetidos à separação materna no período neonatal, nos quais se encontrou atenuação da potenciação de longa duração (LTP) e aumento da depressão de longa duração (LTD) evocadas na amígdala lateral em resposta à estimulação do mPFC (Danielewicz & Hess 2014). Além disso, observou-se também aumento do comprimento e ramificações de dendritos e da densidade pós-sináptica dos neurônios piramidais da BLA na idade adulta (Koe, Ashokan & Mitra 2016), e da atividade da bomba de sódio e potássio (Na^+/K^+-ATPase) na amígdala destes animais (Diehl et al. 2014).

Ratos manipulados na fase neonatal, por sua vez, não apresentaram aumento da excitabilidade da BLA *in vivo*, e mostraram também aumento da atividade basal do mPFC, além de não evidenciarem alterações em resposta a um agonista inverso GABAérgico (Stevenson, Marsden & Mason 2008). Este panorama é sugestivo de proteção em uma situação de estresse, o que também foi encontrado em um estudo de estresse crônico variado, no qual animais manipulados mostraram menos comportamento tipo-depressivo na tarefa de nado forçado e manutenção da atividade da Na^+/K^+-ATPase em várias estruturas encefálicas, em comparação com animais controle que mostraram diminuição da atividade desta enzima em reposta ao estresse (Silveira et al. 2011).

1.3.1.2. Hipocampo

O hipocampo está fortemente associado ao processamento cognitivo e à formação de novas memórias, estando particularmente envolvido na formação da representação contextual (Fanselow & Dong 2010; Maren, Phan & Liberzon 2013). Em relação a memórias condicionadas ao contexto, é um consenso que a associação entre o estímulo condicionado

(contexto) e o estímulo não-condicionado ou inato, como por exemplo um estímulo doloroso, ocorre na região CA1 do hipocampo (Izquierdo, Furini & Myskiw 2016), através de um processo de plasticidade que coincide com a LTP (Izquierdo et al. 2008; Whitlock et al. 2006).

Evidências anatômicas, funcionais, bioquímicas e comportamentais sugerem que o hipocampo se divide em 3 segmentos distintos (Figura 2): hipocampo dorsal - HcD, hipocampo intermediário e hipocampo ventral - HcV (Christensen et al. 2010; Moser & Moser 1998; Strange et al. 2014; Fanselow & Dong 2010). O HcD parece ser responsável pelas funções cognitivas, incluindo o reconhecimento espacial e incorporação de dicas espaciais nos traços de memória, e lesões nesta estrutura impedem a formação de memórias espaciais (Moser & Moser 1998). O HcV parece estar mais associado às emoções e à resposta ao estresse (Fanselow & Dong 2010), incluindo a regulação da ativação do eixo HHA.

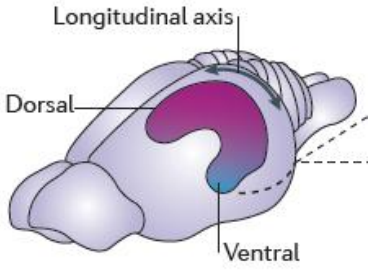

Figura 2. Representação esquemática do hipocampo de rato, evidenciando as regiões dorsal e ventral. Adaptado de Strange et al. 2014, *Nature Reviews Neuroscience*, 15(10), pp. 655-669

Embora ambas as regiões participem no processamento de memórias associativas, as suas funções e os mecanismos que são ativados divergem entre si (Donley, Schulkin & Rosen 2005). Além disso, a participação do HcV na formação de memórias contextuais parece se restringir aos casos em que existe um componente emocionalmente aversivo uma vez que lesões nesta região não prejudicaram memórias espaciais (Moser & Moser 1998). Nas

memórias aversivas condicionadas, o HcV desempenha um papel importante no armazenamento da associação entre os estímulos e a evocação deste tipo de memória induzida pela presença de pistas contextuais depende das suas projeções excitatórias para a LA e BLA, conforme proposto por Gross e Canteras (2012). Estes achados reforçam os diferentes papéis desempenhados por estas duas regiões hipocampais nas memórias aversivas.

Tal como acontece com o circuito mPFC-BLA, a separação materna também altera o curso temporal do desenvolvimento do hipocampo (Andersen & Teicher 2004). Na idade adulta, animais isolados no período neonatal, mostraram generalização de memória a um contexto novo diferente do contexto ao qual o estímulo aversivo havia sido condicionado (Sampath et al. 2014) e prejuízos na memória espacial após uma experiência fortemente aversiva (Diehl et al. 2012). Estes resultados sugerem que os animais separados podem sofrer diferentes tipos de programações nas duas regiões hipocampais; de fato, o cuidado materno, uma das variáveis envolvidas nos modelos de intervenções neonatais, influencia de forma distinta a excitabilidade e a plasticidade sináptica no HcD e no HcV (Nguyen et al. 2015), mas nenhum estudo até ao momento comparou as duas regiões hipocampais em relação a alterações neuroquímicas induzidas pela manipulação ou separação materna.

1.3.2 Reconsolidação de memórias aversivas

> "*Life is maintained by a delicate balance between continuous synthesis and degradation.*"
>
> Yoshinori Ohsumi

A teoria da consolidação, na qual se postulava que o traço mnemônico passava por um período de estabilização (consolidação), após o qual se mantinha inalterado, foi definitivamente desafiada pelo trabalho de Karin Nader e colaboradores no ano 2000 (Nader,

Schafe & Le Doux 2000), embora evidências de que a reativação deixaria a memória suscetível a interferências datassem de mais de 30 anos antes, através do trabalho de Misanin e colaboradores (Misanin, Miller & Lewis 1968). O estabelecimento e disseminação da teoria da reconsolidação deu origem a uma série de novas abordagens ao estudo da memória, com potenciais aplicações ao tratamento de transtornos psiquiátricos, sobretudo os que envolvem memórias traumáticas (Schiller et al. 2010; Fiorenza et al. 2011; Kindt & van Emmerik 2016).

Através da teoria da reconsolidação se desafia a ideia de estabilidade do traço, e se postula que após uma breve evocação da memória consolidada (reativação do traço), o traço mnemônico torna-se lábil e fica suscetível a interferências, por um período de tempo curto. O traço desestabilizado é novamente estabilizado através da reconsolidação (Duvarci & Nader 2004; Sara 2000).

A necessidade de síntese proteica *de novo* após a evocação de uma memória foi um dos primeiros achados experimentais que sugeriram a existência do processo de reconsolidação. Sabe-se hoje que este processo é essencial para a restabilização do traço. Estudos recentes têm apontado a degradação proteica via sistema ubiquitina-proteassoma (UPS), particularmente o proteassoma 26S, como o mecanismo responsável pela desestabilização inicial do traço mnemônico na amígdala (Jarome et al. 2011; Sol Fustiñana et al. 2014) e no hipocampo (Lee et al. 2008; Silva et al. 2013). Assim, a reconsolidação parece depender de um balanço entre a degradação e a síntese de proteínas.

Embora existam semelhanças entre os processos de consolidação e de reconsolidação, algumas diferenças foram reportadas, desde a ativação de diferentes vias de sinalização e fatores de transcrição (Lee, Everitt & Thomas 2004; Lee & Hynds 2013) a variações na linha temporal (Besnard, Laroche & Caboche 2014) e hoje se reconhece que a reconsolidação não é uma mera recapitulação da consolidação, mas um processo distinto (Dudai 2006; Johansen et

al. 2011; Tronson & Taylor 2007). Por exemplo, a plasticidade associada à consolidação no hipocampo coincide com o mecanismo de LTP na fase inicial e na fase tardia (Whitlock et al. 2006); no entanto, um estudo recente sobre plasticidade sináptica na reconsolidação, realizado *ex vivo*, mostrou que nas primeiras horas após a evocação, ocorre uma atenuação da LTP e um aumento de LTD, sendo estas alterações invertidas 6h após a evocação (Bhattacharya et al. 2016). Outro exemplo relaciona-se com o fator neurotrófico derivado do encéfalo (BDNF), que é essencial para a consolidação, mas parece não estar envolvido na reconsolidação (Johansen et al. 2011).

Os mecanismos moleculares que permeiam a reconsolidação têm sido alvo de estudos nos últimos anos. Apesar de existirem resultados contraditórios, já foi identificado o envolvimento de alguns sistemas de neurotransmissores e seus respectivos receptores, bem como vias de sinalização intracelular subsequentemente ativadas, nos processos que compõe a reconsolidação.

Na amígdala, a desestabilização inicial da memória parece ser dependente da ativação de receptores NMDA e β-adrenérgicos (Tronson & Taylor 2007), e independente de receptores AMPA, pelo menos na amígdala lateral (Ben-Mamou, Gamache & Nader 2006), o que pode se dever à capacidade apresentada pelos receptores NMDA desta estrutura de contribuírem para a despolarização da membrana independentemente da ativação de receptores AMPA (Li, Phillips & LeDoux 1995). No hipocampo, receptores NMDA (Fendt 2001; Lee & Hynds 2013) e canais de cálcio dependentes de voltagem do tipo L também parecem estar envolvidos na reativação (Silva et al. 2013; Suzuki et al. 2008), entre outros.

Em ambas estruturas, os receptores NMDA contribuem para o processo de reativação. Variações na composição deste receptor conferem-lhe propriedades eletrofisiológicas distintas (Cull-Candy & Leszkiewicz 2004); de fato, receptores contendo a subunidade GluN2A

medeiam correntes pós-sinápticas de maior amplitude mas os que contêm a subunidade GluN2B apresentam um decaimento mais lento (Chen, Luo & Raymond 1999), o que facilita a coincidência da abertura deste canal com outros sinais e sua integração.

Na amígdala, o influxo de cálcio para o meio intracelular mediado pelos receptores NMDA, leva à ativação da proteína cinase dependente de cálcio/calmodulina do tipo II (CaMKII), que por sua vez ativa o UPS (Jarome et al. 2016), promovendo a degradação protéica que está associada à labilização do traço. A ativação da proteína cinase A (PKA), da CaMKII e de outras vias de sinalização levam à ativação da ERK 1/2, uma das enzimas da via de sinalização das cinases ativadas por mitogênio (MAPK), que está envolvida com a reativação da memória (Besnard, Caboche & Laroche 2013; Chen et al. 2005), mas parece não ser essencial para o processo de reconsolidação subsequente no hipocampo (Besnard, Caboche & Laroche 2013; Lee & Hynds 2013). A ERK 1/2 e a PKA, na amígdala, e enzimas ativadas por outras vias de transdução de sinal, como a via do fator nuclear kappa B (NF-kB) no hipocampo (Lee & Hynds 2013), ativam fatores reguladores da transcrição no núcleo, nomeadamente o elemento de resposta a adenosina monofosfato cíclico (CREB) e Elk-1. Estes fatores são responsáveis pela indução de fatores de transcrição, como Zif268 e C/EBPβ, que controlam a síntese proteica essencial para a restabilização da memória reativada (Tronson & Taylor 2007). O Zif268, em particular, mostrou ser essencial para a reconsolidação, como mostrado em estudos em animais *knock-out* para o gene deste fator de transcrição (Besnard, Caboche & Laroche 2013; Bozon, Davis & Laroche 2003) e de silenciamento do seu transcrito (Maddox, Monsey & Schafe 2011).

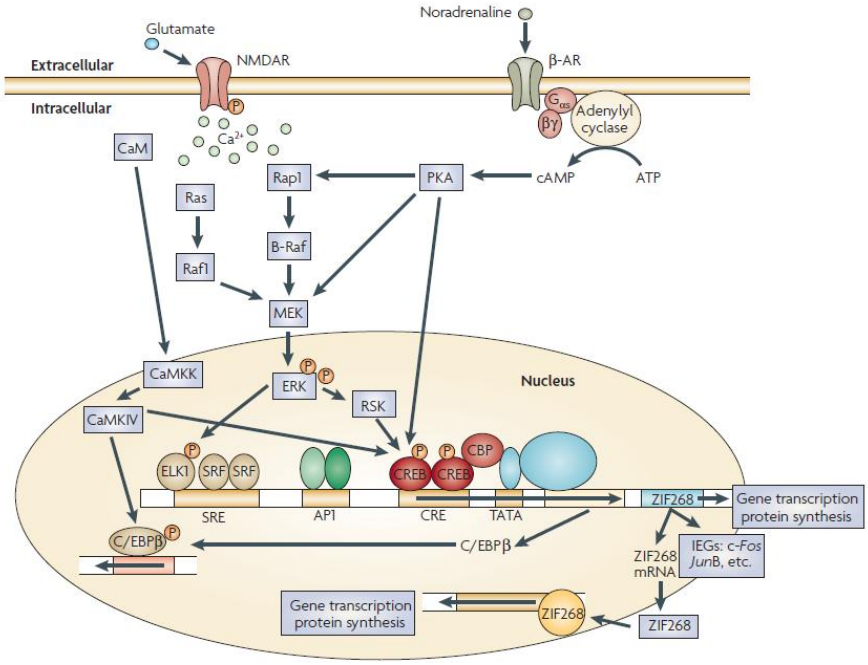

Figura 3. Mecanismos moleculares envolvidos na reconsolidação de memória, evidenciando sobretudo os receptores e vias de transdução de sinal identificados neste processo na BLA. Fonte: Tronson & Taylor 2007, *Nature Reviews Neuroscience*, 8, pp. 262-275

Uma das funções do processo de reconsolidação é manter a precisão contextual da memória e sua dependência do hipocampo, o que previne a sua generalização (de Oliveira Alvares et al. 2012). A reconsolidação também permite o fortalecimento do traço, como foi observado em roedores (Tronson & Taylor 2007; de Oliveira Alvares et al. 2013) e humanos (Forcato, Rodríguez & Pedreira 2011). Dependendo das condições em que ocorre, este processo também permite a incorporação de novas informações e a alteração da memória original (de Oliveira Alvares et al. 2013).

A ocorrência de reconsolidação é demonstrada experimentalmente pela modificação da expressão comportamental da memória após uma sessão de reativação curta ao final da qual se introduz uma interferência (Tronson & Taylor 2007). Vários tipos de interferentes são utilizados, desde agentes farmacológicos como bloqueadores da síntese proteica (Nader, Schafe & Le Doux 2000; Pedreira, Pérez-Cuesta & Maldonado 2002; Wang, de Oliveira

Alvares & Nader 2009), moduladores alostéricos positivos do receptor $GABA_A$ (Bustos, Maldonado & Molina 2006; Bustos, Maldonado & Molina 2009; Espejo et al. 2016; Zhang & Cranney 2008), antagonistas dos receptores β-adrenérgicos (Debiec & Ledoux 2004; Ortiz et al. 2015), toxinas que impedem a proliferação dos filamentos de actina (Rehberg et al. 2010), até estímulos distratores (Crestani et al. 2015) e apetitivos (Haubrich et al. 2015) e novos aprendizados (Wichert, Wolf & Schwabe 2013). Existem, no entanto, condições experimentais nas quais não é possível demonstrar a ocorrência de reconsolidação; são denominadas *boundary conditions*, em inglês, e parecem estar relacionadas, entre outras coisas, com a força e a idade da memória (Tronson & Taylor 2007; Wang, de Oliveira Alvares & Nader 2009). O conhecimento sobre estas condições limitantes tem relevância para o estudo das possibilidades de modulação de memórias aversivas, com aplicação ao tratamento de memórias traumáticas persistentes.

Como referido acima, o circuito amígdala-hipocampo inclui projeções mútuas e a "conversa" entre as duas estruturas tem um papel importante na consolidação e na reconsolidação de memórias de medo condicionadas ao contexto (Richter-Levin & Akirav 2000). Apesar da sincronia entre as duas estruturas durante os processos subjacentes à memória (Düzel, Penny & Burgess 2010; Lesting et al. 2011; Seidenbecher et al. 2003), diferentes receptores e diferentes vias de sinalização intracelular são recrutados por cada estrutura, como comentado acima. Além disso, a dinâmica temporal de ativação também difere entre o HcD e os núcleos lateral, basolateral e central da amígdala (Besnard, Laroche & Caboche 2014).

Um estudo mostrou que o isolamento neonatal modifica a plasticidade sináptica neste circuito, como evidenciado pelo aumento dos níveis tanto de LTP quanto de LTD evocados

no giro denteado em resposta a estimulação tetânica ou de baixa frequência, respectivamente, da BLA (Blaise et al. 2008).

As alterações já mencionadas na expressão comportamental de memórias aversivas em animais manipulados e separados, bem como os diferentes graus de emocionalidade e alterações estruturais e funcionais no circuito amígdala-hipocampo destes animais, sugerem que diferentes experiências precoces podem alterar o processo de reconsolidação de memória na vida adulta; no entanto, até ao momento, nenhum estudo foi realizado para testar esta hipótese. A reconsolidação de memórias associadas a emoções negativas como o medo, pode ser um dos mecanismos envolvidos na etiologia de transtornos associados a memórias traumáticas (Van Marle 2015), pela sua propriedade de fortalecer a memória original. Ao mesmo tempo, a reconsolidação permite a inserção de novas informações e a atenuação de alguns componentes da memória original e, por isso, tem sido vista como um possível alvo para a geração de estratégias terapêuticas para lidar com memórias traumáticas (Fiorenza et al. 2011; Kindt & van Emmerik 2016). Assim, o estudo da reconsolidação de memórias de medo e seus mecanismos neuroquímicos em animais submetidos a diferentes ambientes no início da vida pode contribuir para o conhecimento de como experiências precoces podem configurar-se como fatores de resiliência ou vulnerabilidade através da modulação da reconsolidação de memórias aversivas e identificar possíveis alvos moleculares para terapias que focam na reconsolidação como estratégia para a "re-significação" de memórias traumáticas.

2.1 Objetivo geral

Avaliar a influência de diferentes experiências precoces sobre a resiliência e a vulnerabilidade a patologias psiquiátricas associadas a memórias traumáticas, através de modificações no processo de reconsolidação de memória aversivas e mecanismos bioquímicos possivelmente subjacentes a tais alterações.

2.2 Objetivos específicos

2.2.1 Avaliar o papel do comportamento materno, em termos quantitativos e qualitativos, no desenvolvimento da resposta neuroendócrina da prole a um ambiente aversivo condicionado, em ratos submetidos a manipulação neonatal ou separação materna, no período neonatal;

2.2.2 Investigar o processo de reconsolidação de memória de medo condicionado ao contexto, e os mecanismos neuroquímicos de degradação e síntese proteica associados ao processo, no HcD e na BLA, em ratos machos adultos, submetidos a manipulação ou separação materna no período neonatal;

2.2.3 Investigar possíveis alterações na sinalização intracelular, através do estado redox e parâmetros mitocondriais, do HcD e HcV de ratos machos adultos, submetidos a separação materna no período neonatal.

CAPÍTULO I - Artigo intitulado "**Neonatal interventions differently affect maternal care quality and have sexually dimorphic developmental effects on corticosterone secretion"**

publicado no periódico *International Journal of Developmental Neuroscience*, 55, pp. 72–81, 2016.
DOI: 10.1016/j.ijdevneu.2016.10.001

Este trabalho atendeu ao objetivo específico **2.2.1** desta tese

Int. J. Devl Neuroscience 55 (2016) 72–81

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

Neonatal interventions differently affect maternal care quality and have sexually dimorphic developmental effects on corticosterone secretion

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ARTICLE INFO

Article history:
Received 19 July 2016
Received in revised form
15 September 2016
Accepted 3 October 2016
Available online 4 October 2016

Keywords:
Maternal care
Behavior inconsistency
Neonatal handling
Maternal separation
Central oxytocin
Corticosterone

ABSTRACT

Neonatal handling (H) and maternal separation (MS) both induce changes in maternal care, but the contribution of these changes to the behavioral and neurochemical outcomes of the offspring remains unclear, as studies often find opposite results concerning the frequency of maternal behaviors, particularly in the MS paradigm. In this study, behavior displayed by H, MS and non-handled (NH) Wistar rat dams were observed during the first 10 days after birth. A tentative assessment of the quality of maternal care was made, using a previously reported score that reflects behavior fragmentation and inconsistency. Central oxytocin levels and hippocampal synaptic plasticity markers were also evaluated in dams, immediately after litter weaning. In adulthood, male and female offspring were subjected to a contextual stress-induced corticosterone challenge to provide further information on the impact of early interventions on neuroendocrine parameters. We found that while both H and MS interventions induced an increase in the amount of pup-directed behavior, MS dams displayed a more fragmented and inconsistent pattern of care, reflecting poorer maternal care quality. Interestingly, an increase in oxytocin levels was observed only in H dams. While H offspring did not differ from NH, MS males and females showed marked differences in corticosterone secretion compared to controls. Our results suggest that briefly removing the pups from the nest alters maternal care quantity but not quality and increases central oxytocin, while long separations appear to increase low quality maternal care and change neuroendocrine responses in adult offspring in a sex-specific manner.

1. Introduction

Maternal care is an essential part of the development in many animal species. Rat pups, due to their altricial nature, display little interaction with the surrounding environment. Therefore, it is through maternal behavior, which is highly affected by environmental conditions, that pups perceive their surroundings during the early stages of development (Francis and Meaney, 1999). It has been proposed that maternal behavior prepares the offspring to respond to the environment by adequately programming their hypothalamus-pituitary-adrenal (HPA) axis response to stress (Liu et al., 1997). In fact, enhanced maternal care improves resilience in

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http://dx.doi.org/10.1016/j.ijdevneu.2016.10.001
0736-5748/

the offspring in rodents (Coutellier et al., 2008; Champagne et al., 2003; van Hasselt et al., 2012; Liu et al., 1997; Singh-Taylor et al., 2015) and humans (Brauer et al., 2016), whereas abnormal or deficient care increases the vulnerability to stress-related disorders, both in humans (Kim et al., 2016) and animals (Cirulli et al., 2003; Murgatroyd et al., 2015; Singh-Taylor et al., 2015).

In rodents, removing pups from the nest, either for brief (handling) or long periods (maternal separation), increases the frequency of pup-directed behaviors by dams (Bodensteiner et al., 2012; Macrí et al., 2004; Pryce et al., 2001), although some studies present opposite results (Aguggia et al., 2013; Boccia et al., 2007; Reis et al., 2014). It has been proposed that the long-term consequences neonatal interventions have on the offspring, particularly concerning the adaptive programming of the HPA axis by early handling in rodents, result from the changes that this intervention induces on the dams behavior (Cirulli et al., 2003; Denenberg, 1999; Kuhn and Schanberg, 1998; Liu et al., 1997; Meaney et al., 1985). However, recent studies have suggested that these effects may be linked to the novelty exposure component that comprises the handling procedure, rather than directly resulting of increased maternal care (Reeb-Sutherland and Tang, 2011; Tang et al., 2006). The impact that altered behavior in dams has on the outcomes of MS offspring was also questioned in an interesting study that used a split-litter design (Macrì et al., 2008); unfortunately, in that study, only male offspring was studied. These recent reports evaluated maternal care mostly by quantifying the frequency of pup-directed behaviors. It has been recently suggested that increased maternal care, when analyzed purely quantitatively, is not necessarily favorable to litter development (Dalle Molle et al., 2012; Murgatroyd and Nephew, 2013; Reeb-Sutherland and Tang, 2012). This idea contributes to the discussion of why MS offspring, despite having over caring mothers, have such deleterious stress responses as adults (Aisa et al., 2007; Desbonnet et al., 2008; Diehl et al., 2014, 2011; Lajud et al., 2012; Rivarola and Suárez, 2009). In addition, raises the question of how much and what maternal care features are relevant to the long time consequences of handling and maternal separation on offspring. In this scenery, despite the importance of the mother-pup interaction on offspring development, only a few studies have focused on the mother in these models, so the mechanisms underlying different forms of maternal behavior after brief or long separations between dams and pups are scarcely understood (Stamatakis et al., 2015). Since the mother and the pup form a dyad (Francis and Meaney, 1999), any manipulation of their interaction will necessarily affect both, and should be studied from both perspectives.

Maternal care in rats occurs in bouts. A maternal bout consists on a series of organized events, beginning with entering the nest and gathering the pups, followed by nursing and licking the pups and ending when the dam leaves the nest (Leon et al., 1978). Therefore, it is plausible to think that erratic and unpredictable maternal care may be a source of stress for pups (Ivy et al., 2008; Molet et al., 2016); in accordance, abnormal parental care in humans has been associated with the development of several psychopathies (Kim et al., 2016). Considering this, Ivy et al. (2008) have proposed a behavioral score to measure the quality of maternal care and showed that an early-life stress model, which results in increased anxiety and HPA axis hyperactivity in the offspring, also produced fragmented and inconsistent behavior in the dam.

The neuropeptide oxytocin is involved in mediating the bond between mother and pup in mammal species (Nagasawa et al., 2012; Pedersen and Boccia, 2003; Uvnäs-Moberg, 1997). Elevated levels of oxytocin receptor have been associated with high levels of licking and nursing in the arched-back position (Francis et al., 2000). Interestingly, H dams also exhibited increased levels of this receptor in several brain structures, including the hippocampus, medial preoptic area and central amygdala (Stamatakis et al., 2015), thus suggesting that oxytocin signaling may be involved in mediating the changes in maternal care induced by neonatal interventions.

It has been previously reported that rats exposed to maternal separation exhibit some behavior consistent with high vulnerability to anxiety disorders, namely post-traumatic stress syndrome-like characteristics (Diehl et al., 2011). Particularly, these animals seem to process repeatedly retrieved memories differently when compared to animals that were left undisturbed in the neonatal period (Diehl et al., 2014; Zalosnik et al., 2014). Several reports have also pointed that basal and stress-induced corticosterone secretion is different in males and females that were repeatedly separated from the dam in the neonatal period (reviewed by Rees et al., 2006).

The reports regarding effects of neonatal interventions on maternal care and the effects of short and long separations from dams induce on offspring show inconsistent results. Hovewer, the evaluation of the sequence and daily distribution of dams behavior is beginning to be considered as an important tool to better understand the maternal behavior (Ivy et al., 2008; Molet et al., 2016; Peña and Champagne, 2013; Reis et al., 2014). Taking that in consideration, here we studied the quality of maternal care in Wistar rats in an attempt to contribute to the knowledge of how maternal nurturing may participate in the modulation of the offspring ability to face stressful events later in life. Additionally, parameters related to synaptic plasticity (brain derived neurotrophic factor – BDNF and synaptophysin) were measured in the dam's hippocampus, and oxytocin levels were determined in the cerebral spinal fluid (CSF) at weaning. Corticosterone circulating levels after a stress challenge were determined in the adult offspring.

2. Methods

2.1. Subjects

Primiparous pregnant Wistar rats bred at our animal facility were randomly selected (n = 27 for behavioral measurements and n = 45 for biochemical evaluations). At gestational day 17–18, they were single-housed in home cages made of Plexiglas (65 × 25 × 15 cm) with sawdust-covered floors and kept in a controlled environment (lights on between 07:00 h and 19:00 h, temperature at 22 ± 2 °C, food and water provided). All litters were randomly culled to six to eight pups within 24 h after birth. The day of birth was considered day 0 and weaning was performed on postnatal day 21 (PND 21), when offspring males and females were separated and randomly housed 3–4 per cage, and were then left undisturbed until the behavioral tests, except for cage cleaning. All animal treatments were approved by the institutional Research Ethics Committee (CEUA-UFRGS #23844) and followed the Brazilian Law regarding the use of animals (Federal Law 11.794/2008) and the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003).

2.2. Neonatal intervention models

Each litter had its own glove to be manipulated with, to avoid the spread of odors between nests. From birth to weaning, cage cleaning was performed only when necessary, similarly for all groups: dirty sawdust was carefully removed from the cage, avoiding the nest area, and replaced with clean sawdust.

Non-handled group (NH): pups and dams were left undisturbed until weaning, except for cage cleaning.

Neonatal Handling group (H): pups were gently removed from their home cages and placed into a clean cage lined with clean paper towels, inside an incubator set to 32 °C. After 10 min, pups were returned to their dams. This procedure was carried out in the first

10 days of life, between 12:00 h and 13:00 h, after which litters were left undisturbed until weaning, except for cage cleaning.

Maternal separation group (MS): same protocol as the H group, except pups remained in the incubator for 3 h (between 14:15 h and 17:30 h).

2.3. Behavioral tests

2.3.1. Maternal care observation

Maternal behavior observations were performed for all mother-litter pairs during the neonatal intervention period (PND 1–10), as described in Champagne et al. (2003). The total number of observed litters was 27 (NH: n = 10, H: n = 8, MS: n = 9). The observations were performed at regular times (cycles), twice in the dark phase (06:00 h and 20:00 h) and three times in the light phase (10:00 h, 13:00 h and 17:30 h). In each observation cycle, maternal behavior was monitored every 3 min, during 72 min, for a total of 25 observations per cycle per day for each dam, which yielded a total of 1250 observations for each dam in the 10 days of experiment.

Behavior was observed live by one of three different researchers that had previously been trained together. The computed behaviors were: licking, nursing in arched-back, blanket or supine posture, mother in or off nest, retrieving pups, nest building, mother drinking/eating. The frequency of each behavior in each cycle and day was determined and computed into a databank which was later subjected to revision. For "cycle" analyses, the sum of frequencies of each behavior for the 10 days was used (total number of observations: 250/cycle/dam). For "day" analyses, frequencies from the 5 cycles of each day were added (total number of observations: 125/day/dam).

2.3.1.1. Behavioral inconsistency. Behavioral inconsistency score was assessed as a qualitative measure of maternal care, based on Ivy et al. (2008). Maternal behavior experimental tables were analyzed for each cycle, each day; whenever behaviors changed from one observation to the next in the cycle, a grade "1" was given. Grades for each cycle were added and divided by 24 (total possible number of behavior changes between the 25 observations), resulting in a "behavioral inconsistency score" which varies between 0 and 1. The higher the score, the more fragmented and inconsistent the maternal care. Transitions between the following behaviors were considered: nursing, licking, retrieving pups, nest building, away from pups, eating/drinking.

2.3.2. Flinch-jump test

A flinch-jump test (Lehner et al., 2010) was performed on female and male adult offspring (PND 90–100) that were subjected to the neonatal interventions described here to assess possible differences in painful stimulus perception that could affect the results of the stress challenge. A total of 26 males (NH: n = 11, H: n = 8, MS: n = 7) and 22 females (NH: n = 7, H: n = 8, MS: n = 7) was used. The test was performed in a wooden lidded apparatus (28 × 26 × 23 cm), with one transparent plastic wall, and a grid floor of parallel 0.1-cm caliber stainless steel bars spaced 1.0 cm apart wired to a shock generator. Rats were placed individually in the box and allowed to habituate for 1 min, after which footshocks were delivered in ascending followed by descending order (0.1 mA, 1 s duration, 0.1–0.9 mA range), every 10s. The 'flinch' and 'jump' thresholds were defined for each rat as the average current (mA) at which the animals first (ascending series) or last (descending series) presented each behavior.

2.3.3. Stress challenge

Naïve male and female rats, aged between 90 and 100 days, subjected to the neonatal interventions described here, were used in this experiment; average weight: 364 ± 9 g (males) and 241 ± 5 g (females). Experiments took place between 9 and 12 a.m. Only one rat of each sex from the same litter was exposed to each experimental condition in this task. Animals were placed in the apparatus described above and allowed to explore it for 3 min, after which they received three 0.8 mA 1 s-duration footshocks, 30 s interval between shocks (training session); the frequency of jumps in response to each footshock was recorded for each animal. Rats remained 1 min more in the apparatus, and were then placed back in their home cages. Twenty-four hours after the exposure to the aversive stimulus, a subset of animals was re-exposed to the same context for 5 min; 15 min after the end of the challenge session, animals were quickly euthanized using a guillotine and trunk blood samples were collected for corticosterone levels evaluation. Another subset of non-challenged animals was euthanized 24 h after training and trunk blood was collected to assess basal corticosterone levels.

2.4. Biochemical analysis

2.4.1. Oxytocin assay

Immediately after litter weaning (PND 21), a different subset of dams was anesthetized using 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 a magna cistern puncture and stored at −80 °C for latter usage.

Oxytocin levels in the CSF were measured by enzyme immunoassay, using a commercial kit (Oxytocin EIA Kit, Assay designs, USA), following the manufacturer's instructions. The total number of dams used for this assay was 20 (NH: n = 7, H: n = 7, MS: n = 6). Results are expressed as pg oxytocin/ml CSF.

2.4.2. Hippocampal analysis

Immediately after litter weaning (PND 21), another subset of dams was quickly euthanized using a guillotine. Twenty-five dams were used for this experiment (NH: n = 9, H: n = 9, MS: n = 7). Hippocampi were carefully dissected on ice and stored at −80 °C until analyses. Tissue was homogenized 1:10 in a lysis buffer pH 7.9, containing 137 mM NaCl, 2.5 M KCl, 10 mM Hepes, 0.6 mm EDTA,1% SDS, 10% glycerol and 1% protease inhibitor cocktail (Roche, Switzerland) and a 15 min 4000 rpm centrifugation was performed to clear the homogenate, which was stored at −20 °C until use. Total protein content was determined using the method described by Lowry et al. (1951).

2.4.2.1. BDNF assay. For BDNF analysis, a sandwich ELISA was performed on the hippocampus homogenate, using a commercial kit (BDNF Emax® Immunoassay system, Promega, USA), as previously described (Arcego et al., 2016). Results are expressed as pg BDNF/mg protein. s

2.4.2.2. Western blot. Hippocampus homogenate samples were loaded in poliacrylamide gels (loading gel: 4.5% acrylamide; running gel: 10% acrylamide) in equal protein concentrations (40 μg of total protein/lane). After electrophoresis, proteins were transferred (XCell SureLock® Mini-Cell, Invitrogen) to nitrocellulose membranes (1 h 10 min at 50 V in transfer buffer [48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS]) and blots were then blocked for 2 h in Tris-buffered saline with 5% m/v non-fat dry milk. Blots were incubated overnight at 4 °C in blocking solution containing one of the following antibodies: anti-synaptophysin (1:200, Santa Cruz, USA) and anti-α-tubulin (1:1000, Sigma-Aldrich, USA). Secondary antibody (peroxidase-conjugated anti-rabbit IgG [1:1000, Merck-Millipore, Germany]) was diluted in blocking solution and incubated for 2 h at room temperature. Blots were developed using a chemiluminescence Amersham™ ECL kit (GE Healthcare, UK) and

exposed on a Kodak© film. The intensity of bands was quantified by densitometric analysis using the ImageJ software (National Institutes of Health, USA). Results were quantified as the ratio of the optical density (OD) of the protein of interest to that of α-tubulin (Sigma-Aldrich, USA) of the same sample in the same blot, and expressed in percentage of control (NH group). The total number of samples used for this assay was 17 (NH: n = 5, H: n = 6, MS: n = 6).

2.4.3. Corticosterone assay

Offpring serum corticosterone levels were determined as described previously (Diehl et al., 2007), from serum obtained as described in subsection 2.3.3. Briefly, corticosterone was extracted with ethyl acetate and analyzed with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Corticosterone ELISA kit, Cayman Chemical Co., USA), following the manufacturer's instructions. Results are expressed as ng corticosterone/ml serum. The total number of serum samples used for this assay was 59: non–challenged males – 15 (NH: n = 5, H: n = 4, MS: n = 6); non–challenged females – 12 (NH: n = 4, H: n = 4, MS: n = 4); challenged males – 16 (NH: n = 5, H: n = 5, MS: n = 6); challenged females – 16 (NH: n = 6, H: n = 5, MS: n = 5).

2.5. Statistical analyses

Data was analyzed using the software SPSS version 16.0. Repeated measures ANOVA (neonatal intervention as between subjects factor) was performed to compare frequencies of maternal behaviors or mean behavioral inconsistency factor throughout the intervention period (day) and throughout the day (cycle), using the Greenhouse-Geisser correction whenever data did not meet the sphericity assumption. One-way ANOVA (neonatal intervention as between subjects factor) was performed for each cycle independently whenever a statistically significant interaction between cycle and neonatal intervention was found, and also for total frequency of maternal behavior, dams' biochemical data and percentage of males in the litters. Two-way ANOVA (neonatal intervention and sex as factors) was used to analyze the flinch-jump test results. Three-way ANOVA (neonatal intervention, sex and challenge as factors) was performed for the corticosterone results, followed by one-way ANOVA to compare groups. Tukey or LSD post-hoc analyses were used when appropriate. Data is expressed as mean ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$. For the inconsistency behavior data, results were not considered for animals that presented scores that were more than 2 standard deviations from the mean (three cases, one from each group).

3. Results

3.1. Maternal care

Maternal behavior displayed by NH, H and MS dams was recorded during the first 10 days postpartum, 5 times a day. Results are presented quantitatively, first. For the frequency of pup licking, a significant interaction between neonatal intervention and observation period (cycle) was found [$F(8,96) = 5.84$, $p < 0.001$], as depicted in Fig. 1A. This behavior was more frequent when H and MS pups were returned to the nests, after their respective intervention procedure [i.e., at 13:00 h for H group ($p = 0.006$) and at 17:30 h for MS group ($p < 0.001$)], similarly to previous reports (Liu et al., 1997; Pryce et al., 2001). MS and H dams also showed an overall increase in the total frequency of licking compared to NH dams [$F(2,24) = 6.24$, $p = 0.007$; Tukey post-hoc, $p < 0.05$], but no differences between them ($p = 0.815$). A significant similar interaction was also observed for the frequency of nursing in the arched-back posture [$F(5.84,70.05) = 7.73$, $p < 0.001$]. A marked increase of this behavior was observed in MS dams in the 17:30 and 20:00 cycles ($p < 0.001$ for both), as displayed in Fig. 1B. MS dams also presented an overall increase in arched-back nursing compared to H dams [$F(2,24) = 4.85$, $p = 0.017$; Tukey post-hoc, $p = 0.019$]. While no differences were found between groups in the total frequency of nursing [$F(2,24) = 1.41$, $p = 0.26$], an interaction was observed between intervention and observation time [$F(6.16, 73.86) = 3.70$, $p = 0.003$]. MS decreased their frequency of nursing ($p = 0.005$), particularly in the 10:00 observation cycle (Fig. 1C). Total frequency of mother off nest was not different between the groups either [$F(2,24) = 0.41$, $p = 0.67$], but an interaction intervention x cycle was found [$F(5.723, 68.67) = 4.38$, $p = 0.001$]. Again, MS dams in the 10:00 observation were significantly different from controls ($p = 0.006$), which explains the decrease in nursing seen in this cycle (Fig. 1D).

As expected (Champagne et al., 2003; Leon et al., 1978; Reis et al., 2014), maternal care gradually decreased through the days (main effect of day, $p < 0.05$ for all behaviors analyzed), but no significant interactions between intervention and postpartum day were found ($p > 0.05$ for all behaviors analyzed; data not shown).

Lately attention has been given to qualitative maternal care analysis as a complimentary and rather informative tool (Ivy et al., 2008; Molet et al., 2016; Reis et al., 2014), hence we also calculated a behavioral inconsistency score that reflects the fragmentation of pup-oriented behaviors in dams, depicted in Fig. 1E. Concerning this score, a significant interaction was found between neonatal intervention and cycle [$F(8,84) = 4.04$, $p < 0.001$]. MS dams exhibited a higher score of inconsistency in the two observation cycles that followed the intervention (17:30 cycle, $p = 0.001$; 20:00 cycle, $p = 0.05$), as well as an overall increased score compared to controls [$F(2,21) = 4.41$, $p = 0.025$; Tukey post-hoc, $p = 0.024$]. H dams also had a small increase in their inconsistency score after the intervention ($p = 0.004$), but overall were not different from NH dams ($p = 0.14$). Since MS dams exhibited a very high frequency of arched-back nursing, and dams frequently exchange nursing positions (Stern, 1997), when analyzing behavioral inconsistency, we did not consider changes between nursing positions to calculate the score, thus avoiding a possible false positive result regarding MS mothers.

Dams frequently direct nurturing behavior towards male pups (Moore and Morelli, 1979) and differences have been found, not only on maternal care, but also in the offspring glucocorticoid receptor methylation status depending on the sex composition of the litter (Kosten and Nielsen, 2014). Therefore, we compared the percentage of male pups in our litters to exclude a possible effect of the litter composition on our results. No significant differences were found in the percentage of males in NH, H and MS litters ($49 \pm 6\%$, $45 \pm 7\%$, $49 \pm 7\%$, respectively; $p = 0.867$), thus eliminating this confounding factor.

Oxytocin is strongly related with maternal care (Nagasawa et al., 2012; Uvnäs-Moberg, 1997). Increased expression of oxytocin receptor have been associated with high levels of licking and nursing in the arched-back position (Francis et al., 2000) and was also found in H dams (Stamatakis et al., 2015), while intracerebroventricular infusion of an oxytocin receptor antagonist resulted in decreased licking and arched-back nursing (Pedersen and Boccia, 2003). Therefore, to provide further information about neurochemical modulation of H and MS mothers' behavior, we measured CSF levels of oxytocin in dams, at PND 21 (Fig. 2). A marked increase of oxytocin was observed in H dams when compared with both NH and MS dams [$F(2,19) = 6.635$, $p = 0.007$, Tukey post hoc $p < 0.05$].

Maternal care is essentially an innate behavior as can be demonstrated, for example, by injecting oxytocin in the cerebral ventricles of virgin females (Pedersen et al., 1982). In accordance, behavioral stability has been observed across consecutive litters of the same dam (Champagne et al., 2003). However, as demonstrated here and

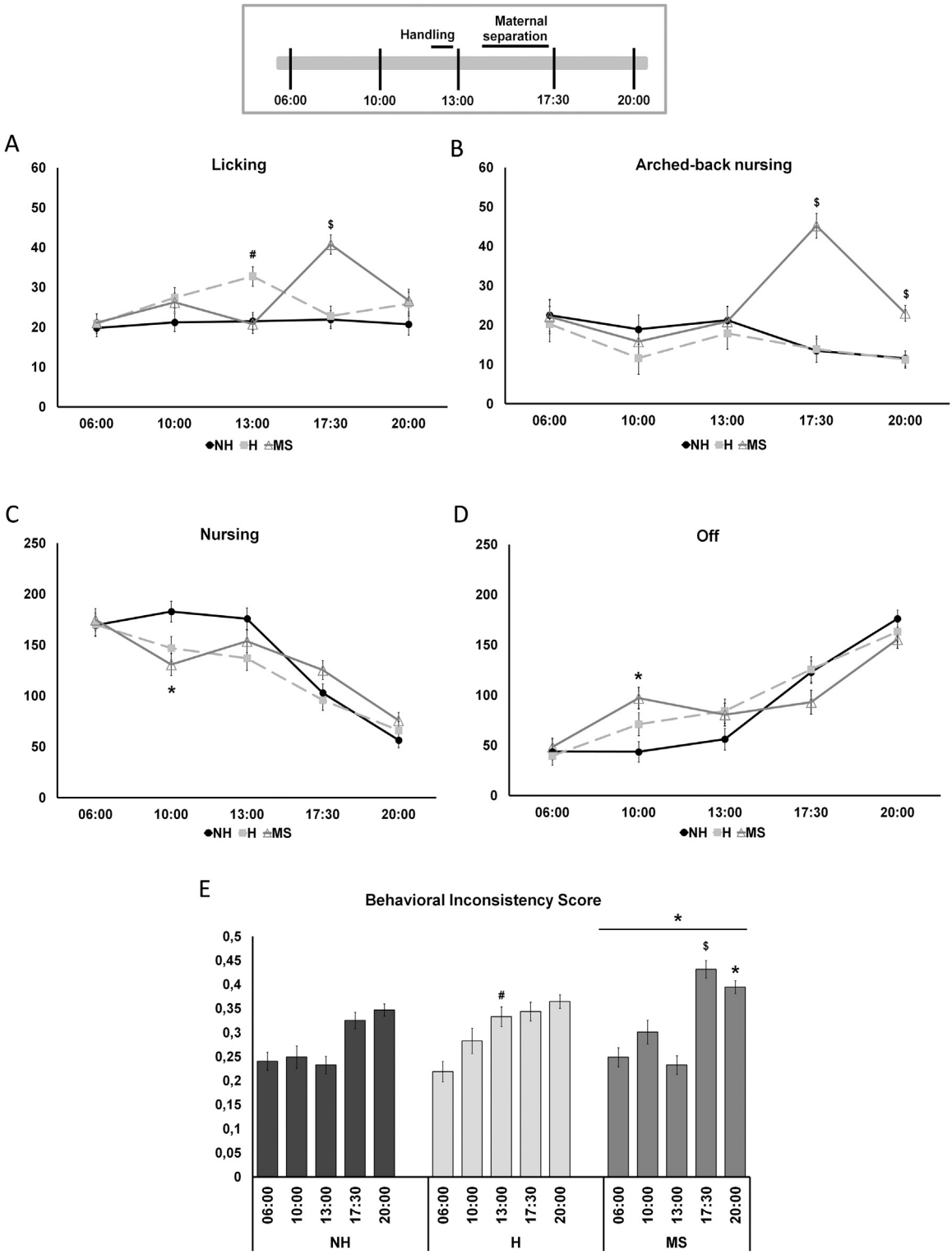

Fig. 1. Maternal care observed in dams of non-handled (NH), handling (H) and maternal separation (MS) groups, throughout the day. Frequencies of pup licking (A), arched back nursing posture (B) nursing – all postures (C), mother off nest (D) and mean behavioral inconsistency score (E) are displayed; frequencies from PND1-10 for each observation cycle were added (A–D) or averaged (E). Data is expressed as mean ± SEM and was analyzed by repeated measures ANOVA using neonatal intervention as an independent factor; one-way ANOVA was used for group comparisons and Tukey as post-hoc test; NH: n = 10; H: n = 8; MS: n = 9. * represents statistically significant difference between MS and NH groups; # represents statistically significant difference compared to NH and MS groups, in the same observation cycle; $ represents statistically significant difference compared to NH and H groups, in the same observation cycle. p Values are presented in subsection 3.1.

in other studies (Bodensteiner et al., 2012; Macrí et al., 2004; Pryce et al., 2001; Reis et al., 2014), dams adapt their behavior to meet pups' demands (Pereira and Ferreira, 2016) and to the environmental conditions. Some adaptations seem to persist to subsequent maternal experiences, even if the source of stress is removed (Wong et al., 2011), implying that a learning component also exists in maternal care (Scanlan et al., 2006). Taking that into account, we examined BDNF and synaptophysin in the hippocampus of dams

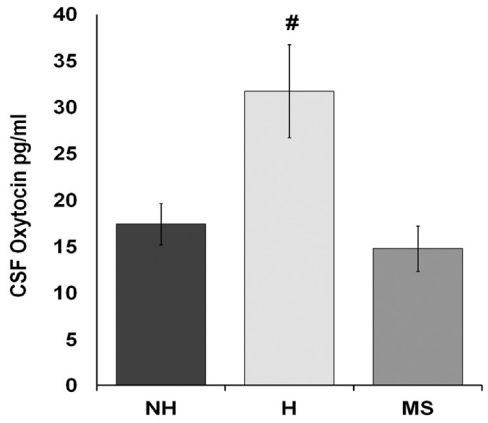

Fig. 2. Oxytocin levels (pg/ml) in the cerebral spinal fluid (CSF) of dams of non-handled (NH), handling (H) and maternal separation (MS) groups, at PND 21, immediately after litter weaning. Data is expressed as mean ± SEM and was analyzed by one-way ANOVA using neonatal intervention as an independent factor, and Tukey as post-hoc test; NH: n = 7; H: n = 7; MS: n = 6. # represents statistically significant difference compared to the other groups. p Values are presented in subsection 3.1.

subjected to the described postpartum interventions (Fig. 3). No significant differences between interventions were found either for BDNF ($p = 0.681$) or synaptophysin ($p = 0.839$). No significant differences in α-tubulin OD were found among groups either ($p = 0.617$), which assures that protein loading was similar in all samples. These results suggest that plasticity related to maternal care is not dependent on BDNF or synaptophysin in the hippocampus. However, conclusions are preliminary, since, to our knowledge, this is the first study to measure BDNF and synaptophysin levels in the hippocampus of H and MS dams.

3.2. *Effects of neonatal interventions on corticosterone secretion in adult offspring*

Males and females subjected the neonatal interventions were tested during adulthood in an attempt to provide information on the impact of maternal care on the offspring's HPA axis function, particularly how corticosterone levels would change in response to the exposure to a conditioned aversive context.

Table 1
Flinch and jump thresholds in response to electric footshocks in adult male and female offspring that were subjected to non-handled, handling and maternal separation procedures in the neonatal period.

		flinch threshold (mA)	jump threshold (mA)
males	Non-Handled	0.20 ± 0.02	0.65 ± 0.04
	Handling	0.15 ± 0.02	0.64 ± 0.04
	Maternal Separation	0.14 ± 0.02	0.61 ± 0.04
females	Non-Handled	0.14 ± 0.03	0.53 ± 0.07
	Handling	0.15 ± 0.02	0.44 ± 0.04
	Maternal Separation	0.11 ± 0.01	0.50 ± 0.06

Data is expressed mean ± SEM and was analyzed by two-way ANOVA using neonatal intervention and sex as factors, and Tukey as post-hoc test; males – NH: n = 11, H: n = 8, MS: n = 7, females – NH: n = 7, H: n = 8, MS: n = 7. Females had a lower jump threshold than males. p Values are presented in subsection 3.2.

First, a flinch-jump test (Lehner et al., 2010) was performed on the adult offspring to determine possible differences in pain sensitivity in the different groups and, consequently, differences in how the electric stimulus would be perceived and reflected as increased corticosterone levels in the stress challenge task (Table 1). No significant interaction or main effects were found for the flinch threshold in this test (interaction: $p = 0.387$), but a main effect of sex was found for the jump threshold [$F(1,36) = 10.522$, $p = 0.003$]. Since females had a slightly lower jump threshold than males, we chose a current intensity in which mainly all animals exhibited a jump response (0.8 mA), to minimize differences in stimulus sensitivity among groups.

A different subset of NH, H and MS adult offspring was submitted to the stress challenge task. A series of three 0.8 mA footshocks was applied; no significant differences between sexes in the frequency of jump in response to the three footshocks were found (one-way ANOVA: $p = 0.124$), which supported our choice of current intensity for this experiment. Twenty-four hours later, approximately half the animals were re-exposed to the footshock context for 5 min (challenged). Serum corticosterone levels were determined at a single time point (15 min after the end of the conditioned stimulus exposure) and compared with samples from animals that received the footshocks 24 h before, but were not re-exposed to the context (non-challenged). Results are depicted in Fig. 4. A signifi-

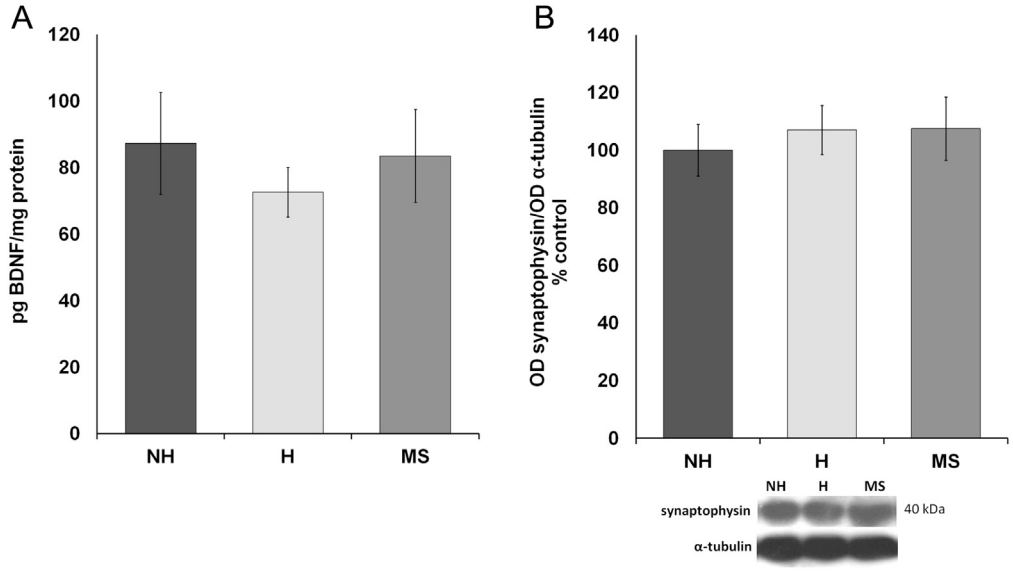

Fig. 3. BDNF (A) and synaptophysin (B) levels in the hippocampus of dams of non-handled (NH), handling (H) and maternal separation (MS) groups, at PND 21, immediately after litter weaning. No significant differences were found; BDNF – NH: n = 9, H: n = 9, MS: n = 7; synaptophysin – NH: n = 5, H: n = 6, MS: n = 6.

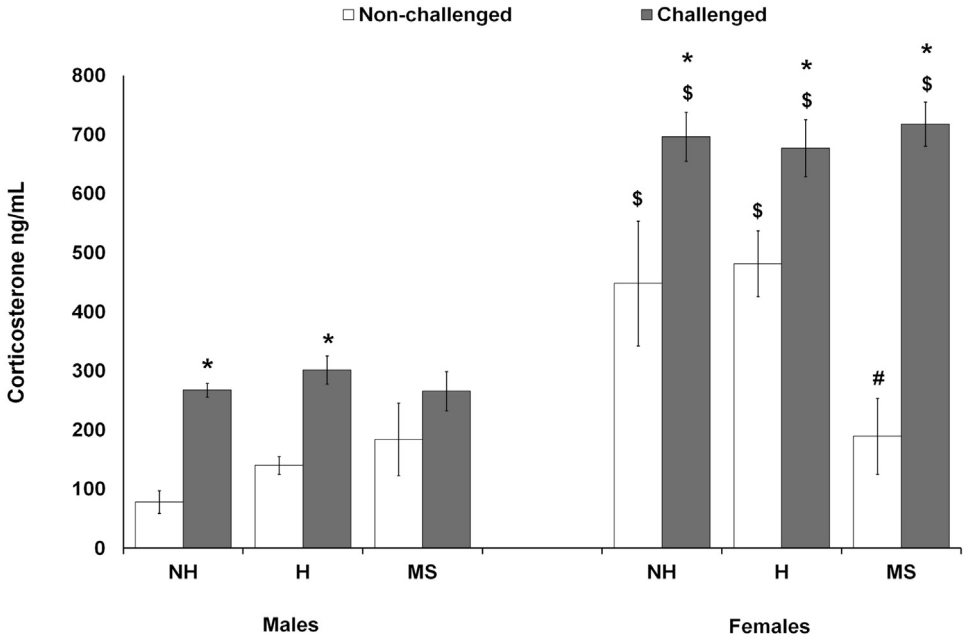

Fig. 4. Serum corticosterone levels (ng/ml) in adult male and female offspring that were subjected to non-handled (NH), handling (H) and maternal separation (MS) procedures in response to the exposure or not to a conditioned aversive context (challenge). Data is expressed as mean ± SEM and was analyzed by three-way ANOVA using neonatal intervention, sex and challenge as factors, followed by one-way ANOVA for group and LSD post-hoc test; non-challenged: 15 males (NH: n = 5, H: n = 4, MS: n = 6), 12 females (NH: n = 4, H: n = 4, MS: n = 4), challenged: 16 males (NH: n = 5, H: n = 5, MS: n = 6), 16 females (NH: n = 6, H: n = 5, MS: n = 5). * represents statistically significant difference compared to non-challenged animals of the same neonatal intervention and sex; $ represents statistically significant difference compared to males subjected to the same neonatal intervention and challenge procedure; # represents statistically significant difference compared to non-challenged NH and H females. Relevant p values are presented in subsection 3.2.

cant interaction between sex, neonatal intervention and challenge was found [$F(2,47) = 6.015$, $p = 0.005$]. In general, females and challenged animals had higher corticosterone levels. A subsequent one-way ANOVA revealed that all groups had a significant increase in corticosterone levels after being challenged ($p < 0.05$) except for MS males ($p = 0.184$); non-challenged MS females had lower corticosterone levels compared to NH and H females ($p = 0.001$ and $p < 0.001$, respectively). Interestingly, non-challenged MS males and females were not different ($p = 0.939$).

It is worthy to disclosure that our non-challenge corticosterone levels may not represent basal levels, as animals had been presented with aversive stimuli the day before and thus there is no guarantee that once removed from the vivarium, a stress response was not triggered. We did however make sure that animals were euthanized as quickly as possible once out of the vivarium (less than 5 min).

4. Discussion

Disrupting the mother-pup interaction results in changes in maternal care in rats, and early interventions such as neonatal handling and maternal separation, have been studied to assert the influence of the environment on the pup's development, especially concerning the functioning of the HPA axis (Lajud et al., 2012; Liu et al., 1997; Macrí et al., 2004). The outcomes that these two interventions have on the offspring have been intensively studied, but their impact on maternal care and how this contributes to the consequences observed on the offspring are not clear, particularly concerning the paradigms that involve long pup separations. Here, we showed, for the first time, that MS Wistar rat mothers present high fragmentation of maternal care, while H dams only show a small and single increase in behavioral inconsistency, when compared to NH mothers. We also evaluated central oxytocin and hippocampal BDNF and synaptophysin, and we found increased CSF oxytocin in dams whose pups were handled. Additionally, contextual stress-induced corticosterone was evaluated on the adult offspring and group and sex-specific responses to stress were observed.

The circadian distribution of maternal care in NH mothers was very constant, and the frequency of behaviors that are strongly correlated with improved care, like licking and arched-back nursing, hardly changed throughout the day. H dams exhibited similar daily patterns, except for an increase in pup licking, which is in accordance with previous reports (Liu et al., 1997; Stamatakis et al., 2015). Other slight variations in maternal behavior pattern presented by H dams did not reach significance, but were similar to those observed by Reis et al. (2014). As for MS mothers, their behavior changed considerably compared to NH dams, with intervention-induced high increases in licking and arched-back nursing and increased time spent away from pups and consequently less time spent nursing in the morning (10:00 observation). The observed increase in arched-back nursing in MS mothers did not imply increased total frequency of nursing, but a switch from the more common blanket posture to the arched-back posture, probably induced by the demanding pups (Stern, 1997). In summary, long separations seem to induce an acute increase in maternal care (Pryce et al., 2001) that is not maintained throughout the day, and more importantly, alter the circadian pattern of pup-related behaviors, particularly in the light phase. The transient nature of the increase in MS dams care and the fact that it occurs in the light phase may possibly explain why some studies reported a decrease in nurturing frequency in these dams (Boccia et al., 2007). Apart from the sustained increase in arched-back nursing in MS mothers observed in the 20:00 cycle, no differences between groups were observed in the two nocturnal observations (6:00 and 20:00), which was expected since nursing is more common during the light phase in rats (Champagne et al., 2003).

The sequence and pattern of maternal care seem to be relevant factors to assess the quality of this behavior and its impacts on the offspring development (Ivy et al., 2008; Molet et al., 2016; Reis

et al., 2014). Based on this fact, we also performed a qualitative analysis of maternal care of dams, by scoring the inconsistency and fragmentation of their behavior (Ivy et al., 2008). In H dams, we found only a small increase in behavioral inconsistency in the cycle after the procedure, which could be explained by the findings of Liu et al. (1997) that H dams had shorter but more frequent nest bouts than controls. MS mothers, on the other hand, showed higher fragmentation of maternal behavior, which also occurred in mothers subjected to the stress paradigm reported by Ivy et al. (2008). The increase in behavior fragmentation is not due to alternations between nursing postures, which frequently occur in rat as reflex responses to pup demands (Stern, 1997), since we did not consider them to determine the inconsistency score. Frequent intervals between licking bouts may contribute to this result, since rat dams usually exhibit rather short licking bouts (Champagne et al., 2003), which could count as behavior changes in our analysis and increase the inconsistency score. However, the total frequency of licking was similar among all groups in the late night observation, and the inconsistency score remained high for MS mothers in this period. Inconsistent care displayed by these dams may result from a conflict between attending demanding pups and dealing with its own stress and, despite being quantitatively high, may not be tuned with the pups needs (Pereira and Ferreira, 2016). We should consider that NH mothers show an increase in fragmentation of their maternal behavior late in the afternoon. As such, the increase in fragmentation in the MS mothers could be an exacerbation of this behavior. It would be interesting to perform further studies addressing the point of handling late in the afternoon.

Several studies have shown that oxytocin is involved in the mediation of maternal behavior, particularly by enhancing the dam's motivation to respond to pups (Bridges, 2015). We reported here that brief separations from pups induce a high and long lasting increase in the levels of this neuropeptide in the dams CSF, which could be related to the increase in maternal care observed in this group. One limitation of this study is that, since behavior was recorded at PND1-10, and biochemical analyses were performed on PND21, it is possible that some biochemical effect could have changed by the time of euthanasia. However, Stamatakis et al. (2015) have shown an increase in the expression of oxytocin receptors in several brain areas in H dams, which lasted up to PND 22. Oxytocin levels appear to be inversely correlated with depressive-like symptoms in the postpartum period, in humans (Moura et al., 2016). MS mothers in our study did not exhibit an increase in oxytocin production at the analysis day, despite their higher frequency of maternal activities, a neurobehavioral pattern that has been previously referred to as "attenuated nursing efficiency" by Murgatroyd and Nephew (2013), regarding another model of maternal stress. Rat mothers that experienced long separations from their pups exhibit increased anxiety-like (Aguggia et al., 2013; Maniam and Morris, 2010) and depression-like behaviors (Boccia et al., 2007; Maniam and Morris, 2010; von Poser Toigo et al., 2012). Together with the inconsistent maternal care and the absence of increased oxytocin levels reported here (compared to H dams), our study offers further support to the suggestion that maternal separation may correlate with postpartum depression in humans (Boccia et al., 2007; von Poser Toigo et al., 2012). Human mothers also show an association between poor childcare and depression/anxiety (Kim et al., 2016).

Changes in the HPA axis reactivity have already been reported in adult MS and H animals (Colman et al., 2015; Diehl et al., 2007; Kalinichev et al., 2002; Lajud et al., 2012; Llorente-Berzal et al., 2012; Rees et al., 2006), with a variety of responses that range from no differences to either increase or decrease in the intervention groups. Such inconsistencies may result from a dependency of the HPA response on the MS protocol used, age, strain and sex of the animals and the type of stressor (reviewed by Rees et al., 2006). Although a similar approach was used by Pryce et al. (2003) on early deprivation (isolation) Wistar rat offspring, to our knowledge, this is the first report of changes in MS circulating corticosterone levels in response to the exposure not to a stressor *per se*, but to a conditioned aversive context, which represents a psychological stressor. These changes may have implications on how males and females that were separated from the mother during the neonatal period retrieve and reconsolidate aversive contextual memories, since glucocorticoids strongly affect these processes (Cai et al., 2006).

Our results concerning circulating levels of corticosterone appear to coincide with the quality of care our dams offered to their offspring. H mothers did not show marked differences compared to NH, except for the increased licking behavior, which consequently results in a small increase in the behavior inconsistency score in the period when it was observed. Similarly, H adult offspring did not differ from controls in their corticosterone response, either when non-challenge or when exposed to the aversive environment, which is in accordance with previous studies which also submitted H animals (Wistar and Long-Evans rats) to different stressors (Colman et al., 2015; Kalinichev et al., 2002). It is important to mention that animals that were handled in the neonatal period do show several behavioral and neurochemical alterations (Liu et al., 1997; Marcolin et al., 2012; Singh-Taylor et al., 2015), but context-induced endocrine stress response was not altered here. On the other hand, a strong increase in behavioral fragmentation was observed in MS dams, and the offspring also exhibited very different corticosterone secretion patterns compared to controls. Male MS rats did not increase corticosterone production in response to the aversive context exposure, since their basal levels were already high; this may correlate with the increase in anxiety-like behavior and memory processing changes previously reported in MS male Wistar rats (Aisa et al., 2007; Diehl et al., 2011, 2014). MS females, on the other side, had lower basal corticosterone levels than controls, as previously reported also in Wistar rats (Diehl et al., 2007; Llorente-Berzal et al., 2012), and showed a marked increase after the challenge.

It is worthy to state that only one corticosterone measure in response to a specific type of stressor was used in this report, so the conclusions we draw concerning neuroendocrine responses in H and MS animals are limited. However, changes reported here are consistent with other studies (Colman et al., 2015; Diehl et al., 2007; Kalinichev et al., 2002). In addition, our goal was to study the contribution of maternal care to the long-term consequences of neonatal interventions, to which our experiment provided interesting insights.

Two important factors interact in the MS procedure: first, the pups lose tactile contact with the mother and are kept without a nutrition source for 3 h per day (in our MS model, heating is assured and the pups are not separated from their siblings, as occurs in some deprivation studies); second, the dam increases the quantity and decreases the quality of care it provides to the litter, as shown here. Conditions listed in the first item have been shown to affect the HPA long-term regulation (Suchecki et al., 1993) and other aspects of MS pups development have been attributed specifically to the lack of tactile stimulation during the separation period (Kuhn and Schanberg, 1998). Interestingly, our adult MS females and males, which received the same neonatal treatment, exhibited very different corticosterone secretion patterns when challenged. Even when non-challenged, these animals show opposite differences towards their respective controls. This could be an effect that arose later in life as a consequence of modulation of corticosterone synthesis by sexual hormones, which are modified by early life interventions (Viveros et al., 2010) or it could be a result of estrogen effects on neurodevelopment during the neonatal phase, as some studies with maternal deprivation have suggested (Oomen et al., 2009). While the work by Oomen et al. (2009) appeared to exclude the impact

of maternal care on their results, Macrì et al. (2008) proposed that increased maternal care in MS dams could partially compensate the deleterious effects of the separation. Hence, the role of altered maternal care on MS offspring neurodevelopment remains controversial. Again, a purely quantitative approach was taken in these studies. The amount of licking rat mothers perform on pups has predictive value when applied to handling studies (Liu et al., 1997; van Hasselt et al., 2012; Macrì et al., 2008; Meaney et al., 1985). However, there appears to be a ceiling effect in the benefits of tactile stimulation to the pups, as determined by studies that use artificial rearing (Lomanowska and Melo, 2016), so the stronger increases in this behavior observed in MS dams may not necessarily be advantageous to the offspring; also, the pattern of care, rather than just the frequency, appears to be important, and may better correlate with neurobehavioral outcomes of neonatal interventions.

Quality assessment of maternal care in this report points that H mothers, in addition to providing higher care for their pups, did so without creating a high disruption in the pattern of "normal" maternal care; they also had a marked increase in the central production of oxytocin. Their adult offspring did not show differences in aversive context-induced corticosterone secretion compared to controls. On the contrary, the increase in maternal behavior observed in MS mothers was not accompanied by an increase in oxytocin production and occurred in a fragmented and inconsistent manner, which we propose, could be an additional source of stress for MS pups and contribute to the long-term negative consequences observed in these animals in this and other studies. Future studies should better address the interaction between pup sex and the quality of maternal care. This could contribute to a better understanding of sexual dimorphic long-term effects of early interventions.

Funding

This work was financially supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The funding agencies had no involvement in data collection/analysis or in the preparation/submission of this manuscript.

Acknowledgement

The authors wish to deeply thank Dr. Maria Elisa Calcagnotto for her assistance with language editing and proofreading of the manuscript.

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CAPÍTULO II - Artigo intitulado "**Early life interventions affect aversive memory reconsolidation in male rats"**

a ser submetido ao periódico *Neurobiology of Learning and Memory*

Fator de Impacto (2015): 3,439

Este capítulo atendeu ao objetivo específico **2.2.2** desta tese

Early life interventions affect aversive memory reconsolidation in male rats

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Abstract

Our aim was to study the effect of early life experiences on aversive memory reconsolidation, in male rats. Litters were assigned to handling (H) – pups were separated from dams for 10 min, or maternal separation (MS) – same procedure for 3 hours, from PDN1-10; non-handled (NH) litters, left undisturbed with their dams, were used as controls. Adult animals were trained in a contextual fear conditioning task; 24h later, a short reactivation session was conducted in the conditioned context (A) or in a novel context (B), followed by administration of either saline or midazolam 3 mg/kg (mdz); a test was performed 24h after. Another subset of animals was euthanized 15 or 60 min after memory reactivation to assess the immunocontent of relevant molecular markers of reconsolidation in the dorsal hippocampus (HcD) and basolateral amygdale complex (BLA). In the test session, mdz-treated NH males, but not H or MS, exhibited decreased freezing, consistent with reconsolidation impairment. Also, MS males showed increased freezing in the novel context compared to H, suggesting fear generalization. Increased levels of Zif268, GluN2B and k48-linked polyubiquitinated proteins in the BLA suggests that memory reconsolidation was triggered in this structure, in all groups. In the dHc, only NH showed increased Zif268 levels after memory retrieval. We showed here that the reconsolidation process of a contextual fear memory is insensitive to the interference of a GABAergic drug in H and MS rats and that at a molecular level, the dHc, contrary to the BLA, does not seem to undergo reconsolidation after memory retrieval in these animals. This shows a hippocampal-dependent mechanism for reconsolidation resistance in rodent models of early life experiences that is different from most studies on the effects of stress on memory reconsolidation.

Keywords: neonatal handling; maternal separation; fear memory; reconsolidation; dorsal hippocampus; basolateral amygdala

1. Introduction

Reconsolidation of fear memories has been regarded as an opportunity for the treatment of aversive memories (Hartley & Phelps, 2010; Kindt & van Emmerik, 2016; Schiller et al., 2010; Seidenbecher, Laxmi, Stork, & Pape, 2003). The ability to disrupt memory reconsolidation and hence modify its emotional valence has important implications for the treatment of disorders linked to traumatic memories, such as post-traumatic stress disorder (Akirav & Maroun, 2013). Concerning this mental disorder, a theoretical model has been proposed to explain its pathophysiology, in which aversive early life experiences induce an emotional dysregulation that renders individuals more susceptible to develop the disorder after exposure to a traumatic event (Lanius, Frewen, Vermetten, & Yehuda, 2010). Hence, rodent models of early life interventions may be useful to study the mechanisms that influence resilience or vulnerability responses to aversive events, particularly regarding fear memory reconsolidation and its boundary conditions (Tronson & Taylor, 2007).

Neonatal interventions change memory processing and its behavioral expression, in rats (Kosten, Kim, & Lee, 2012). Specifically for aversive memory tasks, the majority of studies on brief early manipulations such as handling (H), show an impairment on aversive conditioning, while both impairment and enhancement have been reported for maternal separation (MS) rats (Kosten et al., 2012). We had previously reported that while non-handled (NH) and MS rats showed no differences in the expression of a context-evoked memory (Diehl et al., 2007), rats that were separated during the neonatal period showed a remarked increase in total freezing time when the retrieval session was increased to 20 min (Diehl et al., 2014) as well as impaired spatial memory (Diehl et al., 2012) and increased anxiety-like behavior (Diehl et al., 2007) after a strongly aversive experience that was followed by repetitive situational remainders.

While the effects of early interventions on memory acquisition, recall and extinction have been extensively studied in rodents (Kosten et al., 2012), to our knowledge, there are no studies investigating the aversive memory reconsolidation process in rats that were exposed to early manipulations.

Memory reconsolidation seems to comprise two distinct but entangled processes. First, the reactivated memory is destabilized and the trace becomes again labile; this process appears to depend on protein degradation via the ubiquitin-proteasome system - UPS (Artinian et al., 2008; Jarome, Ferrara, Kwapis, & Helmstetter, 2016; Jarome, Werner, Kwapis, & Helmstetter, 2011; Lee et al., 2008; Sol Fustiñana, Federman, Freudenthal, & Romano, 2014). NMDA receptors (NMDAR) activity is required for memory destabilization in the BLA, as shown by the administration of selective antagonists (Ben-Mamou, Gamache, & Nader, 2006; Milton, Lee, Butler, Gardner, & Everitt, 2008). Further studies have shown that GluN2B-containing NMDA receptors are specifically involved with protein degradation via the UPS through activation of the calcium–calmodulin dependent protein kinase II (CaMKII), which in turn, activates the UPS (Jarome et al., 2016; Mao, Lin, & Gean, 2008).

The reconsolidation theory postulates that memory destabilization is followed by a restabilization phase that depends on protein synthesis (Akirav & Maroun, 2013; Artinian et al., 2008; Nader, Schafe, & Le Doux, 2000; Pedreira, Pérez-Cuesta, & Maldonado, 2002). Hence, activity-inducible transcription factors, such as Zif268, have been shown to be necessary for memory reconsolidation (Besnard, Caboche, & Laroche, 2013; Bozon, Davis, & Laroche, 2003; Maddox, Monsey, & Schafe, 2011).

Retrieval-induced labilization renders the memory susceptible to external or internal interferents, which may disrupt or update the original memory. Benzodiazepines (BZD), $GABA_A$ receptor ($GABA_AR$) positive allosteric modulators, have long been known for their

amnestic properties (Malkani & Rosen, 2000), and their use as reconsolidation interferents has brought some interesting insights about the process (Makkar, Zhang, & Cranney, 2010). In particular, midazolam (mdz), a rapid absorption BZD, has been applied in studies that focus on stress-modulatory effects on memory reconsolidation (Bustos, Giachero, Maldonado, & Molina, 2010; Espejo, Ortiz, Martijena, & Molina, 2016; Ortiz, Giachero, Espejo, Molina, & Martijena, 2015; Zhang & Cranney, 2008). These studies have shown that stress previous to training render aversive memories resistant to reconsolidation (Bustos et al., 2010; Espejo et al., 2016; Hoffman et al., 2015; Ortiz et al., 2015), hypothetically by increasing memory strength, a boundary condition of memory reconsolidation that has been attributed to a decrease in NMDAR-mediated glutamatergic neurotransmission, particularly to the GluN2B subunit (Wang, de Oliveira Alvares, & Nader, 2009), in the basolateral amygdala complex – BLA (Espejo et al., 2016; Ortiz et al., 2015). These observations are in accordance with the essential role the amygdala plays in processing the emotional content of memories (LeDoux, 2003). In addition to the amygdala, the hippocampus, particularly its dorsal region – dHc, also has a relevant part in encoding and retrieving context-conditioned emotional memories (Phillips & LeDoux, 1992; Richter-Levin & Akirav, 2000).

MS induces long-lasting morphologic effects on pyramidal neurons of the basolateral nucleus of the amygdala (BLA), such as dendritic hypertrophy and increased spine density (Koe, Ashokan, & Mitra, 2016), besides increasing Na+/K+-ATPase activity in the amygdala (Diehl et al., 2014); in accordance, two recent studies using different early interventions, one that administered corticosterone in the neonatal period in mice (Koppensteiner et al., 2014) and another in rats that were socially isolated from PND21 (Rau, Chappell, Butler, Ariwodola, & Weiner, 2015), both found increased basal excitability in BLA pyramidal neurons. Consistently, BLA firing rate in vivo was increased in MS rats after the

administration of a GABAergic inverse agonist, while H animals showed both no increased excitability in the BLA and increased firing rate in the medial prefrontal cortex (mPFC), a region involved with inhibitory control of the amygdala (Stevenson, Marsden, & Mason, 2008). The hippocampus development is also affected by early life experiences (Andersen & Teicher, 2004; Lajud, Roque, Cajero, Gutiérrez-Ospina, & Torner, 2012).

2. Methods

2.1 Subjects

Primiparous pregnant Wistar rats bred at our animal facility were used. At gestational day 17-18, they were single-housed in home cages made of Plexiglas (65 x 25 x 15 cm) with sawdust-covered floors and kept in a controlled environment (lights on between 07:00 h and 19:00 h, temperature at 22 ± 2°C, food and water provided ad libitum). The day of birth was considered postnatal day 0 (PND 0). All litters were randomly culled to 6-8 pups within 24 h after birth and litters were randomly assigned to one of the neonatal interventions described below. Weaning was performed on PND 21: males were separated and randomly housed 3-4 per cage, and then remained under standard animal facility conditions until the beginning of the experiments. To avoid the influence of genetic load on results, for behavioral experiments no more than two males from the same litter were assigned to the same drug/reactivation protocol and for biochemical experiments, only 1 male per litter was used in the same reactivation protocol. Females were assigned to other experiments.

All animal treatments were approved by the institutional Research Ethics Committee (CEUA-UFRGS #23844) and followed the Brazilian Law regarding the use of animals (Federal Law 11.794/2008) and the Guidelines for the Care and Use of Mammals in

Neuroscience and Behavioral Research (National Research Council 2003). Care was taken to minimize animal suffering during the experiments.

2.2 Neonatal intervention models

Non-handled group (NH): pups and dams were left undisturbed until weaning, except for cage cleaning.

Neonatal Handling group (H): from PND 1 to PND 10, once a day, pups were gently removed from their home cages and placed together in a clean box lined with a paper towel, inside a warm bath set to 32° C, where they remained for 10 minutes. After this period, pups were returned to their respective cages. This procedure was performed during the lights-on cycle, between 12:00 h and 13:00 h.

Maternal separation group (MS): same protocol as the H group, except pups remained in the warm bath for 3 hours and this intervention was conducted between 14:15 h and 17:30 h.

Each litter had its own glove to be manipulated with, to avoid the spread of odors between nests. Dams remained in the warm bath room during the interventions, so they could listen to the pups' vocalizations.

From birth to weaning, cage cleaning was performed only when necessary, similarly for all groups: dirty sawdust was carefully removed from the cage, avoiding the nest area, and replaced with clean sawdust.

2.3 Contextual fear conditioning

The contextual fear conditioning task was performed on male rats, aged PND 90-100, weighing 404±38 g (mean±standard deviation), that were subjected to the neonatal

interventions described above. Experiments took place between 9 a.m. and 1 p.m. Two days before the beginning of the contextual fear conditioning task, animals were taken to the experiment room and remained there for two hours for acclimation; at the end of this period, they where gently handled and weighted by the Experimenter, one at a time.

Conditioning was performed in a wooden lidded apparatus (220x280x260 mm), with a transparent plastic wall, and a grid floor of parallel 0.1-cm caliber stainless steel bars spaced 1.0 cm apart (context A). In the training session, all rats were placed in the chamber and were allowed 3 minutes of context exploration, to assure that they would form and consolidate a coherent representation of the context (Fanselow & Dong, 2010); animals then received three 1s-duration footshocks of 0.8 mA, 30s-interval, and one minute later they were removed from the apparatus and placed in clean home cages, to avoid contact with untrained animals. This current intensity was chosen based on the results of a flinch-jump test we had previously performed on H and MS offspring (Couto-Pereira et al., 2016).

Groups were subdivided in order to be exposed to two different Reactivation (React) sessions, which were conducted 24 hours after training. In the React A session, 55 animals (NH – 20; H – 17; MS – 18) were re-exposed to context A, for 5 minutes. In the pseudo React B, 47 animals (NH – 15; H – 16; MS – 16) were exposed to an unfamiliar context (B) for 5 minutes, which consisted of a plastic transparent box (400x220x260 mm) with smooth floor and walls, placed in a different room with dim light; the aim was to test memory precision and the specificity of the reconsolidation interferent. Immediately after the end of both React sessions, animals received an intraperitoneal injection (i.p.) of either sterile saline solution – sal 1 mL/kg or mdz ("Dormonid", Produtos Roche Químicos e Farmacêuticos, Brazil) diluted in sterile saline solution to a concentration of 3 mg/mL/kg. Twenty four hours later, a Test was conducted on all animals, in context A, for 5 min. All React and Test sessions were

recorded; the training session of 39 animals (NH – 13; H – 13; MS – 13) was record for basal assessment. Freezing duration, defined as the total absence of body and head movement except for that associated with breathing (Blanchard & Blanchard, 1969) was later scored by a single experimenter, whose analysis was compared with another experimenter for inter-reliability (Intraclass correlation coefficient = 0.988). Freezing is expressed in percentage of total session time.

2.4 Biochemical analyses

To evaluate the reconsolidation process at a molecular level in NH, H and MS adult males, animals were euthanized at two different timepoints: 15 and 60 min after the end of a 5-min Reactivation session in the context A (React), with trained but non re-exposed animals animals as controls (No React). The 60 min post React experiment was divided in two experiments to obtain the cytosolic fraction (cyt) and, in a different subset of animals, a synaptossomal membrane fraction (synapt). Adult male animals, weighing 381±35 g (mean±standard deviation), which were subjected to the neonatal interventions described above, were divided as follows: 15 min cyt – NH: 13 (React – 6; No React – 7), H: 13 (React – 8; No React – 5), MS: 14 (React – 7; No React – 7); 60 min cyt - NH: 16 (React – 8; No React – 8), H: 12 (React – 6; No React – 6), MS: 14 (React – 6; No React – 6); 60 min synapt - NH: 13 (React – 6; No React – 7), H: 12 (React – 6; No React – 6), MS: 11 (React – 6; No React – 5). Experiments were performed between 9 and 12 a.m.

2.4.1 Brain dissection

Fresh brain tissue was dissected on an ice cold petri dish. To dissect the dHc and BLA, coronal brain slices of 2mm were cut using an acrylic brain matrix to maintain consistency in tissue collection. Structure boundaries were identified using a rat brain atlas (Paxinos &

Watson, 1998): the dorsal portion of the hippocampus from slices until approximately bregma -4.5 mm was considered dHc and slices between approximately bregma -2 mm and -4 mm were used for the BLA, which after localization, was dissected using a 2 mm-diameter punch. Immediately after dissection, samples were immersed in liquid nitrogen and later stored at -80ºC until further analysis.

2.4.2 Protein extraction

2.4.2.1 Cyt fraction

BLA and dHc tissue samples were thawed on ice and homogenized in 1:10 (w:v) hypotonic 10 mM Hepes buffer, containing 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1mM DTT, protease inhibitor (#11697498001, Roche, Germany) and phosphatase inhibitor (#88667, Pierce, ThermoFisher Scientific, USA) cocktails, pH =7.9. Samples were incubated on ice for 15 min to allow cell swelling; Nonidet P-40 (#E109, Amresco, USA) was then added to a final concentration of 0.6% and samples were placed on ice for 5 min more, with agitation every 15 s. Samples were centrifuged at 10.000 g, for 10 minutes, at 4ºC, and the supernatant containing the cytosolic proteins was collected. Total protein content was determined using the PierceTM BCA Protein Assay kit (#23227, ThermoFisher Scientific, USA).

2.4.2.2 Synapt fraction

Synapt fraction of BLA and dHc was obtained as described previously (Dunah & Standaert, 2001; Jarome et al., 2011) . This extraction method yields a fraction that is rich in synaptic membrane receptors and synapse associated proteins (Dunah & Standaert, 2001). Briefly, samples were thawed on ice and then homogeneized in 1:10 (m/v) TEVP+sucrose buffer [10 mM Tris-HCl, 5 mM NaF, 1 mM EDTA, 1mM EGTA, phosphatase inhibitor cocktail (#88667, Pierce, ThermoFisher Scientific, USA), 320 mM sucrose, pH 7.4], using a

pestle and a glass tissue grinder. Homogenates were centrifuged at 1000 g for 10 min, at 4 ºC. The supernatant was collected and centrifuged at 10,000 g for 10 min, at 4 ºC. The resulting pellet was ressuspended in 1/5 detergent containing Lysis buffer [in 10 ml ultra-pure H_2O: 0.0605 g Tris-HCl, 0.025 g sodium deoxycholate, 0.0876 g NaCl, 1 ml 10% SDS solution, protease inhibitor (#11697498001, Roche, Germany)] and then centrifuged at 15,000 g for 5 min, 4 ºC. The supernatant containing synaptossomal membrane proteins was collected and total protein content was determined using the Pierce™ BCA Protein Assay kit (#23227, ThermoFisher Scientific, USA).

2.4.3 Western blot

Denatured samples were loaded (40 µg of total protein/lane) on NuPAGE® precast 4-12% gradient polyacrylamide gels (#NP0323BOX, Life Technologies, ThermoFisher Scientific, USA); groups were counterbalanced in gels of the same experiment, so that all contained an equivalent number of samples of each group. A 12-225 kDa molecular weight marker (#RPN800E, Amersham, GE Healthcare, UK) was loaded on all gels. Electrophoresis and electrotransfer were performed on a XCell SureLock® Mini-Cell and XCell II™ Blot Module, respectively (#EI0002, Invitrogen, ThermoFisher Scientific, USA). Proteins were transferred to nitrocellulose membranes (1h50min at 50 V in transfer buffer [48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS]) and blots were then blocked for 2 h in Tris-buffered saline containing tween and 5% (m/v) non-fat dry milk or 5% (m/v) BSA for phosphorylated proteins detection. Blots were incubated overnight, at 4ºC, with one of the following primary antibodies: anti-Zif268 (1:1000, #4154 - EGR-1, Cell Signaling Technology, USA), anti-ERK 1/2 (1:4000, #ABS44 – MAPK 1/2, Millipore, Germany), anti-pERK 1/2 (1:2000, #9101 - Phospho-p44/42 MAPK, Cell Signaling Technology, USA), anti-GluN2A (1:1000, #M264 - NR2A, Sigma-Aldrich, USA), anti-GluN2B (1:2000, #06600 -

NR2B, Millipore, Germany), anti-pGluN2B (pNR2B, 1:1000, #M2442, Sigma-Aldrich, USA), anti-$GABA_AR$ α1-6 (1:500, #sc-376282, Santa Cruz Biotechnology, USA), anti-synaptophysin (1:2000, #AB9272, Millipore, Germany), anti-ubiquitin k48-specific (1:500, #05-1307, Merck-Millipore, Germany), anti-α-tubulin (1:4000, #T6074, Sigma-Aldrich, USA) or anti-β-actin (1:3500, #8457, Cell Signaling Technology, USA). Secondary peroxidase-conjugated anti-rabbit antibody (1:1000, #AP132P, Merck-Millipore, Germany, or 1:2500, Jackson ImmunoResearch, USA) or anti-mouse antibody (1:1000, #402335, Calbiochem, Merck-Millipore, Germany) was incubated for 2h at room temperature. Blots were developed using a chemiluminescence ECL kit (#RPN2209, Amersham, GE Healthcare, UK) and images were acquired using the digital camera system ImageQuant LAS 4000 (GE Healthcare Bio-Sciences AB, Sweden); in the k48-linked polyubiquitination experiment, x-ray films were used to acquire blots' chemiluminescence.. Antibody stripping was performed using 1M sodium hydroxide and the stripping efficiency was confirmed by incubating blots with the respective secondary antibody, followed by chemiluminescence detection.

Optical density was determined using the software ImageJ (National Institutes of Health, USA). Results were quantified as the ratio of the optical density (OD) of the protein of interest to the loading control OD, and are expressed in percentage of control (NH No Reactivation group).

For BLA synaptosome blots, α-tubulin was used as a loading control since a strong trend towards reactivation-induced increase in β-actin OD was found in these blots [β-actin: $F(2,36)= 3.975$, $p=0.053$, 2w-ANOVA, main effect of Reactivation, data not shown; α-tubulin: no significant interaction nor effects, $p>0.05$]; this effect is in accordance with the report that arresting actin filaments in the mouse BLA 30 minutes after reactivation of a contextual fear memory disrupts its reconsolidation (Rehberg, Bergado-Acosta, Koch, &

Stork, 2010). No significant interaction or main effects were found regarding β-actin OD in 15 min, 60 min, 60 min polyubiquitin assays or dHc synaptosome blots ($p>0.05$, 2w-ANOVA), so this protein was used as loading control in the mentioned experiments.

2.5 Statistical analysis

Data was analyzed using the software SPSS version 16.0. Levene's test of equality of error variances was used to test the homogeneity of group variances. Two-way analysis of variance (2w-ANOVA), with neonatal intervention and drug as factors, or one-way ANOVA (1w-ANOVA), with neonatal intervention as factors were performed for behavioral results. 2w-ANOVA with neonatal intervention and reactivation as factors was used for biochemical results. Tukey post-hoc test was performed to compare groups when appropriate. Data is expressed as mean ± standard error of the mean (SEM). Statistical significance was set at $p<0.05$. Rats were excluded from behavioral experiments only if their freezing level was more than 2 standard deviations from the group mean.

3. Results

3.1 Mdz disrupts memory reconsolidation in NH but not in H and MS adult male rats

To study the effects of different neonatal interventions on aversive memory reconsolidation, we established a protocol that triggered contextual fear memory reconsolidation in male adult rats. We used mdz, a GABAergic drug known to interfere with memory reconsolidation (Bustos, Maldonado, & Molina, 2006), to provide behavioral evidence that the protocol used was inducing reconsolidation. Animals were trained to associate an unconditioned stimulus (foothshock), with the context. The following day, immediately after a 5 min non-reinforced React session in the conditioned context (A), animals received either sal or mdz 3mg/kg i.p., and 24 hours later were re-exposed to context

A, for 5 min (Fig. 1C). Freezing duration was measured during both sessions period, as a behavioral indicator of fear memory (Blanchard & Blanchard, 1969). A significant interaction between neonatal intervention and drug was found in the Test session [$F(2,48)=4.108$, $p=0.023$, 2w-ANOVA]. Post-hoc analysis revealed that NH animals that were administered mdz 3 mg/kg after React had freezing levels significantly lower than NH rats that received sal ($p=0.040$, 1W-ANOVA, Tukey post-hoc test), showing that mdz administered during the reconsolidation window successfully disrupted memory in NH rats and providing evidence that the experimental conditions employed here successfully induced memory reconsolidation in control animals.

Futhermore, neonatal interventions appeared to affect this process; there were no differences in freezing between sal or mdz-treated H and MS rats ($p>0.05$), suggesting that memory in these animals was resistant to interference by a $GABA_AR$ positive allosteric modulator, after reactivation.

3.2 H animals exhibit less freezing when re-exposed to the conditioned context

In the React A session (Fig. 1B), a significant effect of neonatal intervention was found [$F(2,52)= 3.448$, $p=0.039$, 2w-ANOVA, no interaction]; Tukey post-hoc revealed that H animals exhibited significantly less freezing than NH ($p=0.030$), but no differences were detected between MS and NH rats ($p>0.05$), which is in accordance with previous studies on aversive memory consolidation in H and MS rats (Arnett et al., 2015; Diehl et al., 2007; Kosten, Lee, & Kim, 2006; Ladd et al., 2004; Meerlo, Horvath, Nagy, Bohus, & Koolhaas, 1999).

Context-induced freezing may be influenced by the emotional valence of the aversive experience or by pain sensitivity. Regarding training strength, we have previously reported

that 0.8 mA is a current intensity that generates similar behavioral responses and corticosterone secretion levels after conditioned context exposure in NH, H and MS male rats; also, no differences on footshock sensitivity were observed in these groups in the flinch-jump test (Couto-Pereira et al., 2016).

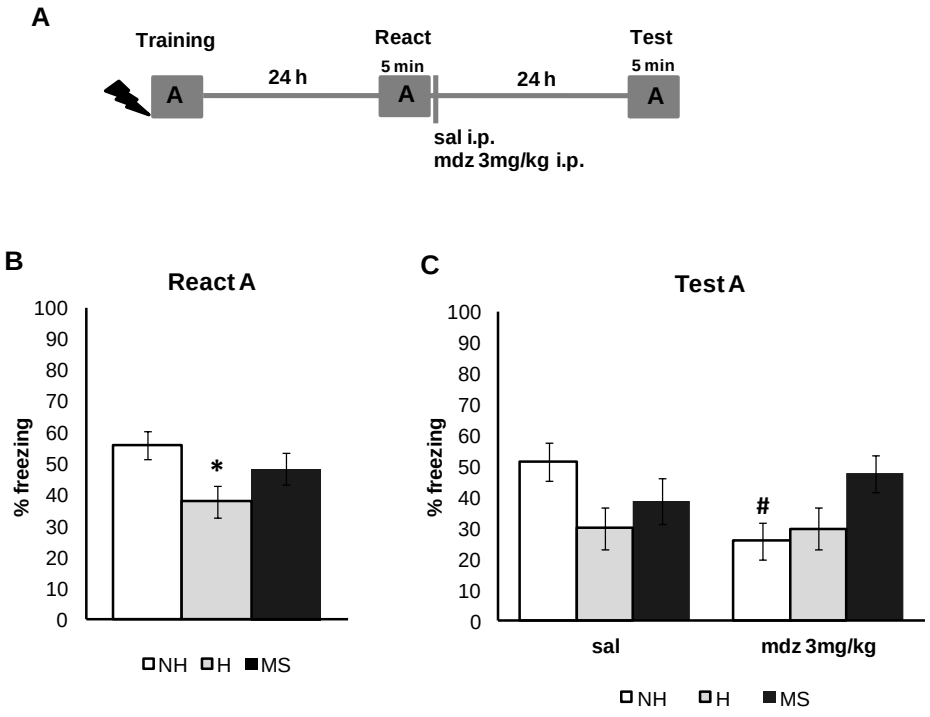

Fig. 1 – Effect of midazolam (mdz) injection after memory reactivation by re-exposure to context A in adult male rats that were non-handled (NH) or subjected to handling (H) or maternal separation (MS) in the neonatal period. A. Schematic diagram of the experimental design; B. Freezing in context A, in the Reactivation (React) session, n=17-20/group; C. Freezing in context A, in the Test session, of animals that received either sal or mdz 3mg/kg after the React session, n=8-10/group. Data is expressed as mean ± SEM, as percentage of total session duration. 2w-ANOVA (neonatal intervention and reactivation as factors) was used for statistical analyses; * represents statistically significant difference compared to NH; # represents statistically significant difference compared to NH sal. Statistics results are presented in detail in subsections 3.1 and 3.2.

3.3 MS rats generalize the fear response to novel environments

Memory precision was tested by exposing the animals to an unfamiliar context 24 hours after training –*pseudo* React in context B (Fig. 2B). A significant effect of neonatal intervention was detected [F(2,44)=4.102, p=0.027, 2w-ANOVA, no interaction]; post-hoc

analysis showed that MS rats freezing was significantly increased in the new context compared to H animals ($p=0.020$), suggesting that MS induces generalization of fear memory, at least in comparison to H.

Anxiety could be a confounding factor for this result, since several studies reported that MS animals show increased anxiety-like behaviors in unfamiliar environments (Makena, Bugarith, & Russell, 2012); hence, freezing in context B could be the result of increased novelty anxiety. To test this hypothesis, we examined freezing in a subset of animals, in the training session, during the 3 minutes pre-shock, when animals were exploring a context that was new to them at that moment. Consistent with our previous report (Diehl et al. 2014), neonatal interventions did not induce changes in freezing in response to a new environment, before conditioning: NH – 1.3%±0.5, H – 0.9%±0.5, MS – 1.0%±0.5. Since no differences were found at this point [$p>0.05$, 1w-ANOVA], it is valid to assume that the aversive experience was necessary to induce the generalization of fear to unconditioned environments observed in MS rats.

3.4 Mdz disrupting effect on reconsolidation requires properly reactivated memories

The *pseudo* React B session was also performed to evaluate the specificity of the effect of mdz on memory reconsolidation. After exposure to context B, sal or mdz 3 mg/kg i.p. was injected and 24 hours later, animals were re-exposed to the conditioned context (A) for 5 minutes (Fig. 2C). No significant interaction or main effects were detected ($p>0.05$, 2w-ANOVA) so, consistently with previous reports (Bustos et al., 2006; Bustos, Maldonado, & Molina, 2009), mdz only impaired memory when it was properly reactivated in the

conditioned context, providing further evidence of the specific effect of the drug in disrupting memory reconsolidation after retrieval.

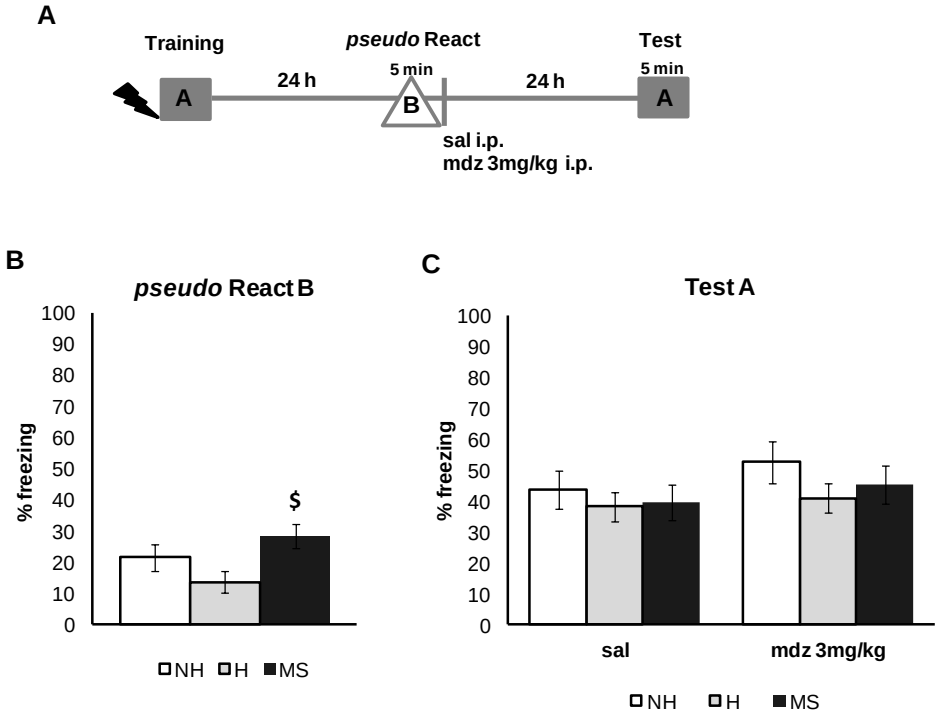

Fig. 2 –Effect of midazolam (mdz) injection after a *pseudo* reactivation in context B in adult male rats that were non-handled (NH) or subjected to handling (H) or maternal separation (MS) in the neonatal period. A. Schematic diagram of the experimental design; B. Freezing in context B, in the *pseudo* Reactivation (React) session, n=15-17/group; C. Freezing in context A, in the Test session, of animals that received either sal or mdz 3mg/kg after the *pseudo* React B session, n=7-9/group. Data is expressed as mean ± SEM, in percentage of total session duration. 2w-ANOVA (neonatal intervention and reactivation as factors) was used for statistical analyses; $ represents statistically significant difference compared to H group. Statistics results are presented in detail in subsections 3.3 and 3.4.

3.5 ERK 1/2 activity and Zif268 levels were not changed in the BLA 15 min after aversive memory reactivation

To better understand the resistance to reconsolidation interference in H and MS rats, a different subset of animals was trained and re-exposed to the conditioned apparatus A (React) 24 h later and dHc and BLA were collected 15 or 60 min after the end of the session; as controls of this experiment, animals that were trained but not re-exposed to the context (No

React) were used. Freezing was determined in React animals: NH – 47.7%±4.8; H – 41.0%±5.0; MS – 47.1% ±5.3; results were similar to React A in the mdz experiment, but no significant differences were detected ($p>0.05$, 1w-ANOVA).

ERK 1/2 levels, as well as its activation by phosphorylation at residues T202/Y204, were evaluated by Western blot, 15 min after the end of the Reactivation session, in the BLA of NH, H and MS adult males (Fig 3B-D,F). No significant interaction or main effects were found for ERK 1/2 or its phosphorylated form ($p>0.05$, 2w-ANOVA). BLA ERK 1/2 activation happens later than in the dHc, reaching a peak at 30 min post-reactivationn, but a small non-significant increase at 15 min could be observed previously (Besnard, Laroche, & Caboche, 2014).

Zif268 induction has been implicated in the memory reconsolidation process in several reports (Besnard et al., 2013; Bozon et al., 2003; Espejo et al., 2016; Hall, Thomas, & Everitt, 2001; Lee, Everitt, & Thomas, 2004; Maddox et al., 2011). Here, we did not find any significant differences in Zif268 levels at this timepoint (Fig. 3E,F; $p>0.05$, 2w-ANOVA).

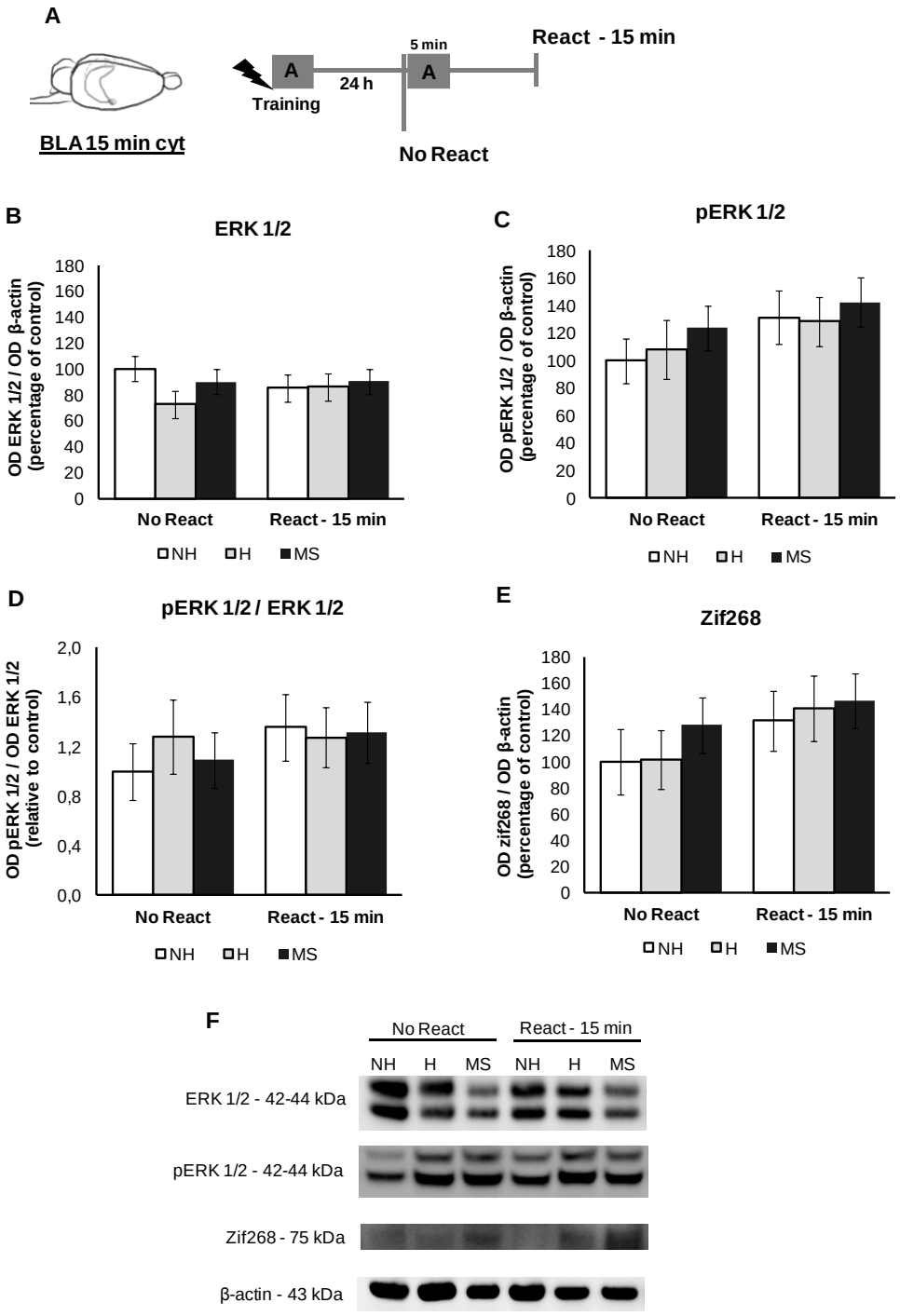

Fig 3. –ERK 1/2, pERK 1/2 and Zif268 cytosolic levels (cyt) in the basolateral amygdale complex (BLA) of adult male rats that were non-handled (NH) or subjected to handling (H) or maternal separation (MS) in the neonatal period, 15 min after Reactivation (React) compared to trained animals that were not re-exposed to the training context (No React). A. Schematic diagram of the experimental design; B. ERK 1/2 immunocontent; C. pERK 1/2

immunocontent; D. calculated ratio of pERK 1/2 per ERK 1/2 immunocontent; E. Zif268 immunocontent; F. representative Western blot bands. Data is expressed as mean ± SEM. n = 5-7/group. 2w-ANOVA (neonatal intervention and reactivation as factors) was used for statistical analyses. Statistics results are presented in detail in subsection 3.5.

3.6 Zif268 levels increase in the BLA, 60 min after aversive memory reactivation

The immunocontent of Zif268 was also evaluated at 60 min post-reactivation (Fig. 4B,D), a timepoint at which higher increases in the BLA have been reported (Besnard et al., 2014). NH, H and MS rats that were exposed to the conditioned context had significantly increases levels of Zif268 in the BLA, at this timepoint [$F(1,35)= 19.965$, $p<0.01$, 2w-ANOVA, main effect of Reactivation].

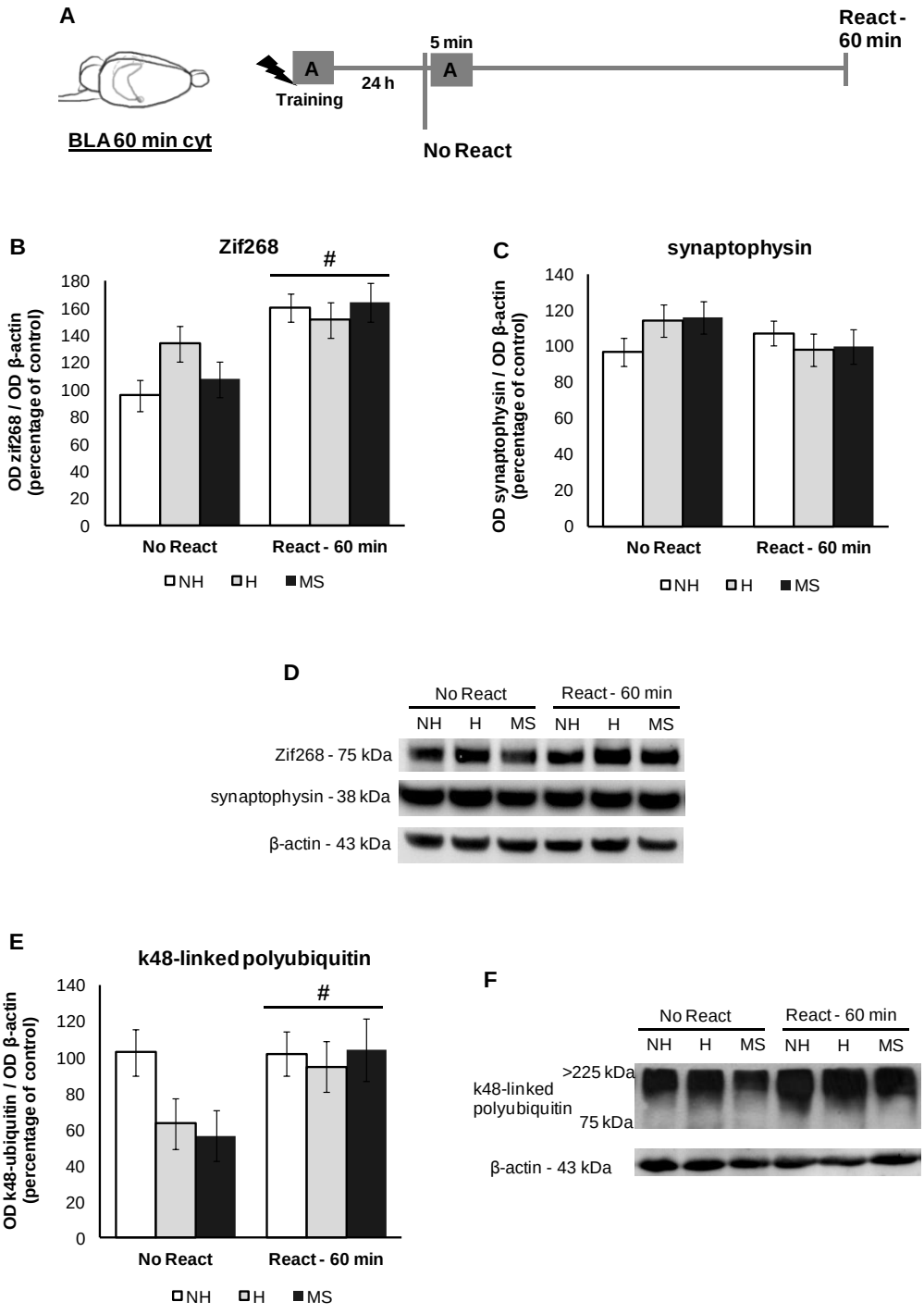

Fig 4. – Cytosolic levels (cyt) of Zif268, synaptophysin and k48-linked polyubiquitinated proteins in the basolateral amygdala complex (BLA) of adult male rats that were non-handled (NH) or subjected to handling (H) or maternal separation (MS) in the neonatal period, 60 min after Reactivation (React) compared to trained animals that were not re-exposed to the training context (No React). A. Schematic diagram of the experimental design; B. Zif268 immunocontent; C. synaptophysin immunocontent; D. representative Western blot bands; E.

k48-linked polyubiquitin immunocontent; F. representative Western blot bands. Data is expressed as mean ± SEM. n = 5-8/group. 2w-ANOVA (neonatal intervention and reactivation as factors) was used for statistical analyses; # represents statistically significant difference between React and No React (main effect of reactivation factor). Statistics results are presented in detail in subsections 3.6, 3.7 and 3.10.

3.7 Memory reactivation induces changes in receptor composition at the BLA synapses

NMDAR subunits GluN2A and GluN2B (total and phosphorylated) and α1-6 subunits of the $GABA_AR$ were measured in the BLA synapt fraction, 60 min post-reactivationn (Fig. 5).

The immunocontent of GluN2B, pGluN2B, GluN2A and $GABA_AR$ α1-6 subunits was significantly increased in the synapt fraction by memory reactivation, but no differences between neonatal groups were detected [GluN2B: $F(1,25)= 12.861$, $p=0.001$; pGluN2B: $F(1,25)= 8.311$, $p= 0.009$; GluN2A: $F(1,25)= 4.225$, $p=0.050$; $GABA_AR$ α1-6: $F(1, 30)= 5.815$, $p= 0.022$; 2w-ANOVA, main effect of reactivation, no interaction]. For GluN2A, a trend towards an interaction was also found ($F(2,25)=2.575$, $p=0.098$, 2w-ANOVA).

Phosphorylation of NR2B subunit was assessed using an antibody that recognizes this protein phosphorylated at Y1472, its major phosphorylation site (Chen & Roche 2007). While both total and phosphorylated forms of GluN2B were significantly increased by reactivation, no interaction or main effects were found for their ratio ($p>0.05$, 2w-ANOVA), which means that the increase in GluN2B at synapses was accompanied by its phosphorylation. A ratio of the synapt immunocontent of GluN2A per that of GluN2B was calculated since it has been shown that increased GluN2A/GluN2B synaptic ratio in the BLA inhibits retrieval-dependent memory destabilization (Holehonnur et al., 2016). No significant interaction or main effects were found for this ratio ($p>0.05$, 2w-ANOVA, data not shown).

Since a $GABA_AR$ positive allosteric modulator was used as a memory interferent in this work, it was important to assess this receptor concentration at synapses. To do so, we used an antibody that recognizes all 1-6 α-type subunits of the $GABA_AR$. Since all $GABA_AR$ possess two α subunits in their composition (Olsen & Sieghart, 2009), measurement of total levels of this subunit should provide an approximate determination of total receptor content. While a reactivation effect was observed, no interaction or neonatal intervention effect was observed for this measure ($p>0.05$, 2w-ANOVA), suggesting that $GABA_AR$ levels were not altered in the BLA synapses of H, NH and MS rats before exposure to context. This does not necessarily represent basal levels, since No Reactivation rats had been trained in an aversive task 24 h earlier.

To detect a possible confounding effect on our BLA synapt results that could arise from a variation in the number or activity of synapses in our neonatal intervention groups, we measured synaptophysin levels in the BLA cyt fraction (Andersen & Teicher, 2004). Synaptophysin levels were determined in the cyt fraction and not directly in the synapt fraction since by loading equal amounts of total synaptic proteins on the gels, we could be diluting possible differences between groups. No significant interaction or main effects were found concerning synaptophysin levels in the BLA of NH, H and MS adult rats [Fig. 4C,D, $p>0.05$, 2w-ANOVA].

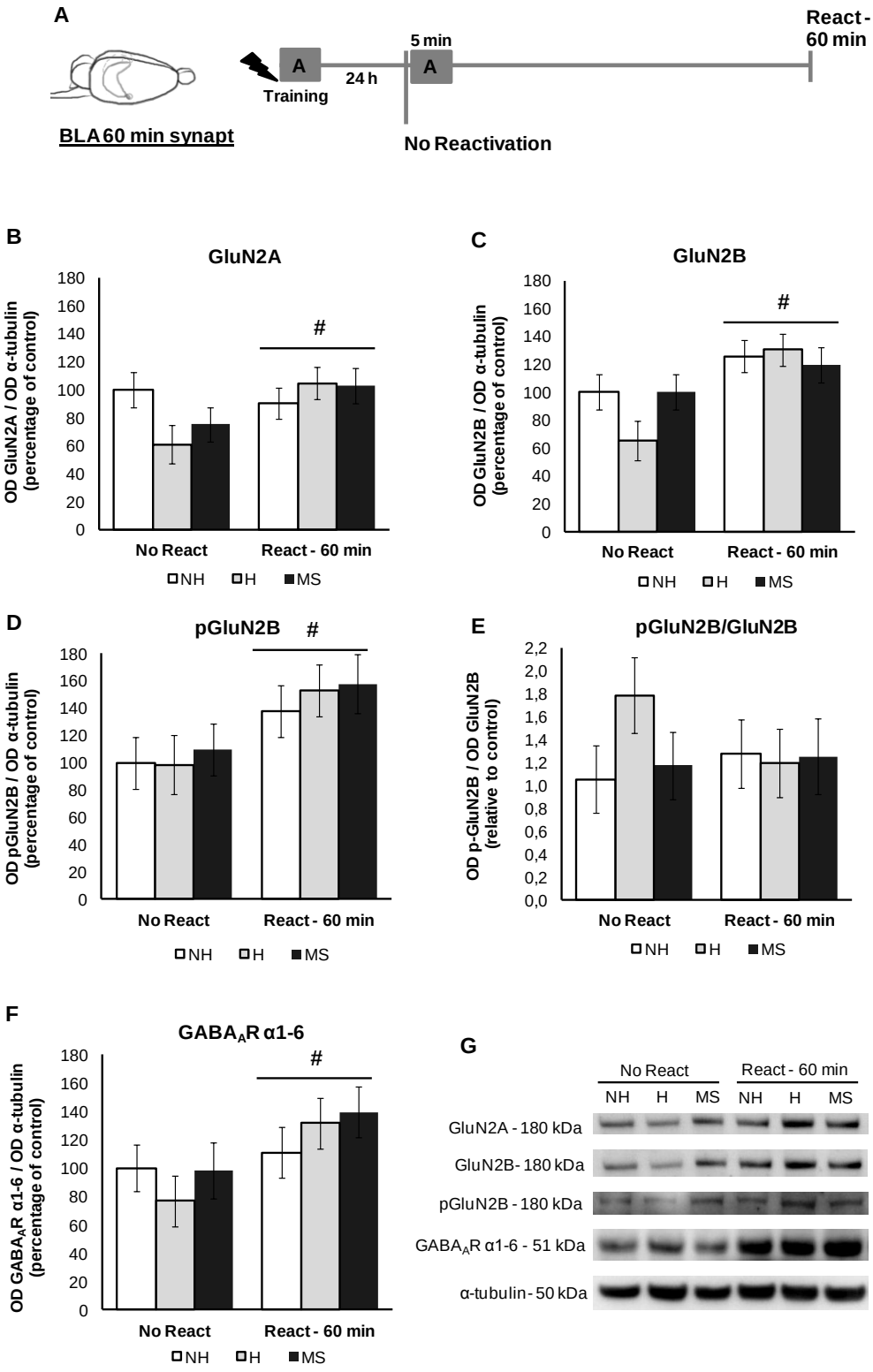

Fig 5. – Synaptosome membrane (synapt) levels of the NMDAR subunits GluN2A and GluN2B (total and phosphorylated) and GABA$_A$R α1-6 subunits in the basolateral amygdala complex (BLA) of adult male rats that were non-handled (NH) or subjected to handling (H) or

maternal separation (MS) in the neonatal period, 60 min after Reactivation (React) compared to trained animals that were not re-exposed to the training context (No React). A. Schematic diagram of the experimental design; B. GluN2A immunocontent; C. GluN2B immunocontent; D. pGluN2B immunocontent; E. calculated ratio of pGluN2B per GluN2B immunocontent; F. $GABA_AR$ α1-6 subunits immunocontent; G. representative Western blot bands. Data is expressed as mean ± SEM. n = 5-7/group. 2w-ANOVA (neonatal intervention and reactivation as factors) was used for statistical analyses. # represents statistically significant difference between React and No React (main effect of reactivation factor). Statistics results are presented in detail in subsection 3.7.

3.8 Neonatal interventions change ERK 1/2 activation in the dHc, 15 min after aversive memory reactivation

ERK 1/2 levels, as well as its activation by phosphorylation, were evaluated by Western blot, 15 min after the end of the reactivation session, in the dHc of NH, H and MS adult males (Fig. 6B-D, F).

A significant interaction was found for ERK 1/2 levels in this structure [$F(2,31)= 3.905$, $p=0.031$, 2w-ANOVA, neonatal intervention x reactivation]. It appears that early after reactivation, ERK 1/2 decreases in H and MS, but increases in NH animals. Phosphorylated ERK 1/2 was lower in MS animals [$F(2,31)= 3.589$, $p=0.040$, 2w-ANOVA, main effect of neonatal intervention, no interaction]. A ratio of pERK 1/2 per total ERK 1/2 levels was calculated to evaluate changes in the relative phosphorylation status of ERK 1/2. A significant interaction was found for this ratio [$F(2,31)= 4.590$, $p=0.018$, 2w-ANOVA, neonatal intervention x reactivation].

Increases in Zif268 immunocontent in hippocampal areas have been reported as early as 15 min post-reactivation (Besnard et al., 2014). Here, we did not find any significant differences in Zif268 levels at this timepoint (Fig. 6E,F; $p>0.05$, 2w-ANOVA). The lack of a significant effect may be attributed to the lower sensitivity of the Western blot technique compared to immunohistochemistry, which was used in the mentioned study.

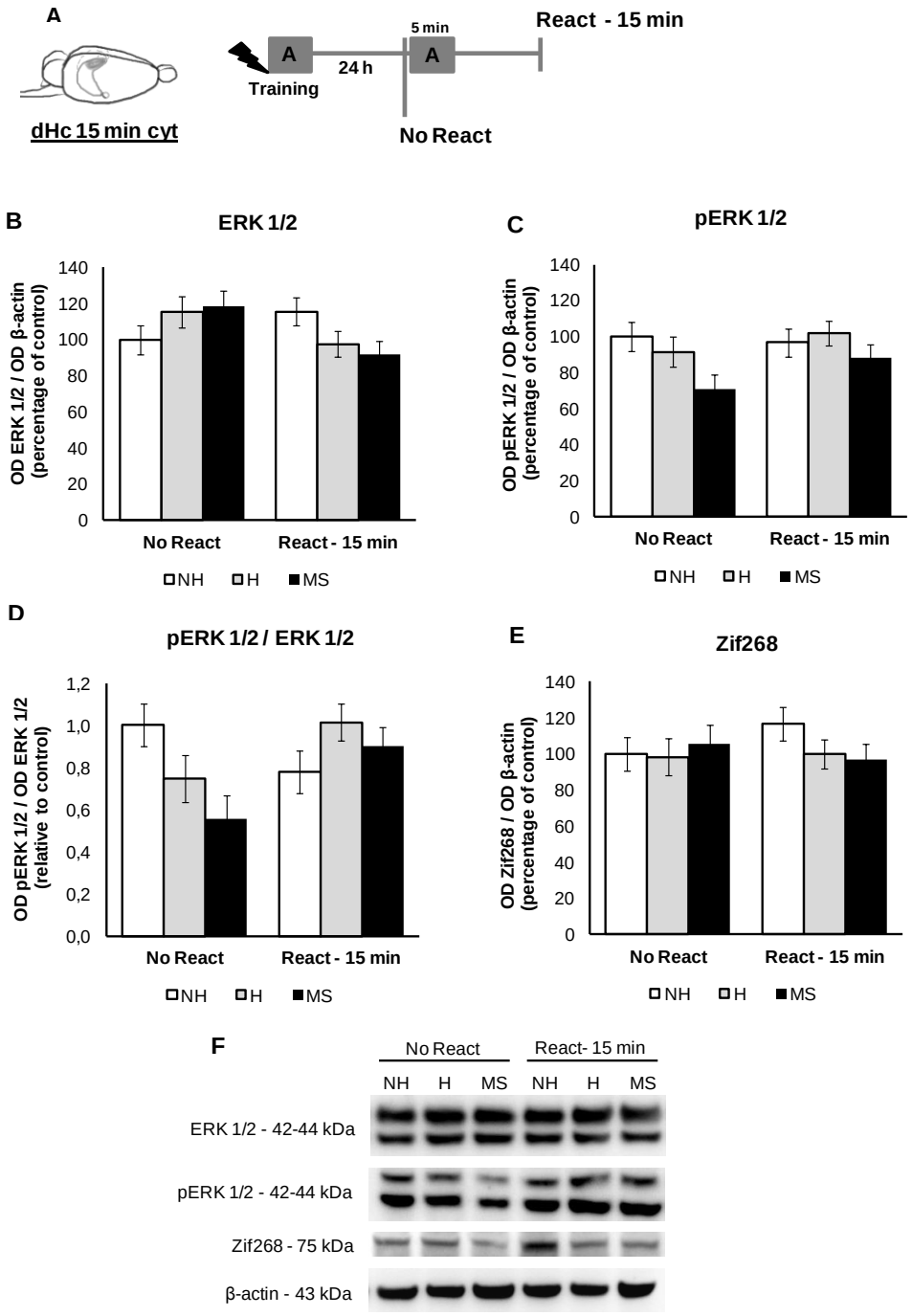

Fig 6. –ERK 1/2, pERK 1/2 and Zif268 cytosolic levels (cyt) in the dorsal hippocampus (dHc) of adult male rats that were non-handled (NH) or subjected to handling (H) or maternal separation (MS) in the neonatal period, 15 min after Reactivation (React) compared to trained animals that were not re-exposed to the training context (No React). A. Schematic diagram of the experimental design; B. ERK 1/2 immunocontent; C. pERK 1/2 immunocontent; D.

calculated ratio of pERK 1/2 per ERK 1/2 immunocontent; E. Zif268 immunocontent; F. representative Western blot bands. Data is expressed as mean ± SEM. n = 5-8/group. 2w-ANOVA (neonatal intervention and reactivation as factors) was used for statistical analyses. Statistics results are presented in detail in subsection 3.8.

3.9 Zif268 levels increase in the dHc of NH, but not H or MS, 60 min after aversive memory reactivation

Zif268 levels steadily increase in dentate gyrus during the reconsolidation window (Besnard, Laroche & Caboche 2014). Hence, we also evaluated Zif268 immunocontent at 60 min post-reactivation (Fig. 7B,C). A significant interaction was found [$F(2,29)= 5.361$, $p=0.010$, 2w-ANOVA, neonatal intervention x reactivation]; Tukey post-hoc showed that memory reactivation in NH induced a significant increase in the immunocontent of this transcription factor, compared to No React NH ($p=0.001$). React H and MS were not different from their respective controls ($p>0.05$).

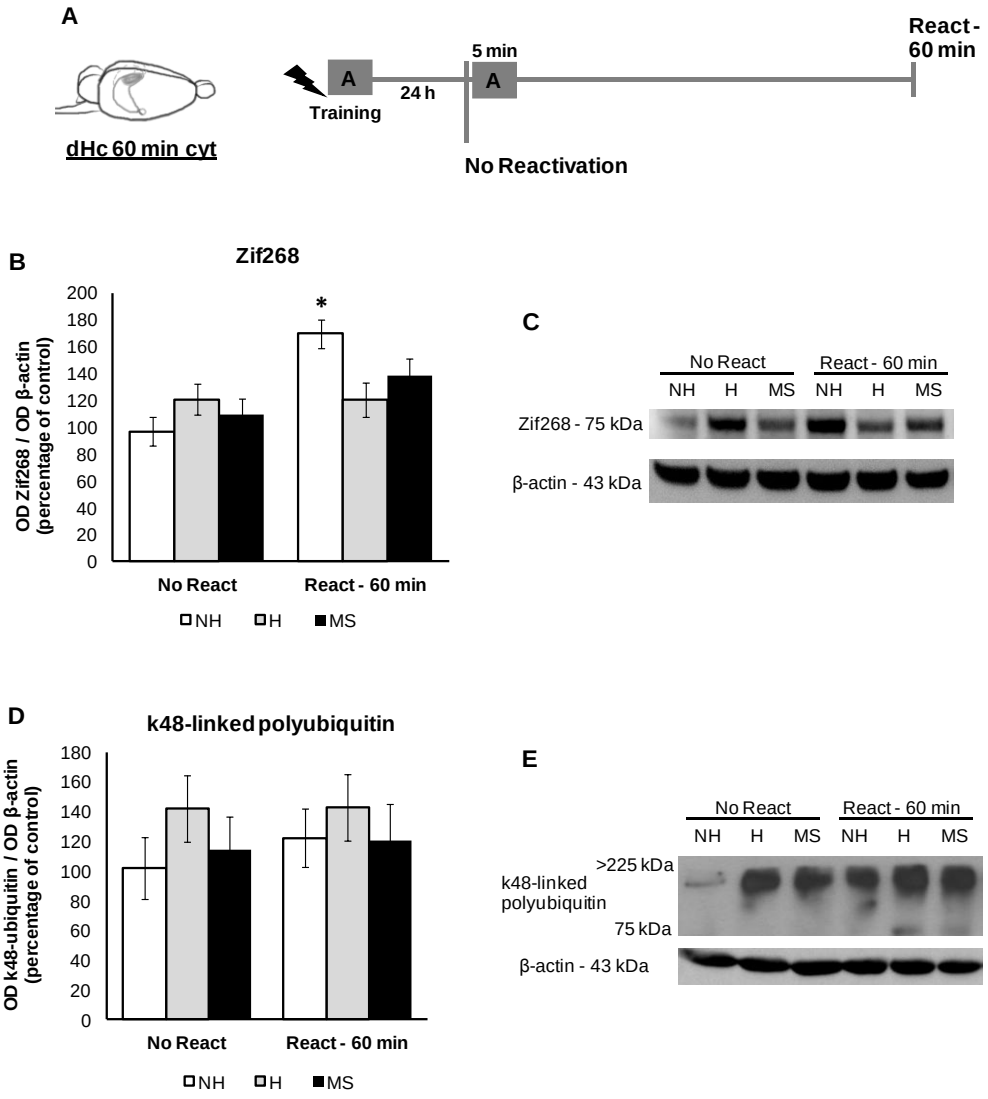

Fig 7. – Cytosolic levels (cyt) of Zif268 and k48-linked polyubiquitinated proteins in the dorsal hippocampus (dHc) of adult male rats that were non-handled (NH) or subjected to handling (H) or maternal separation (MS) in the neonatal period, 60 min after Reactivation (React) compared to trained animals that were not re-exposed to the training context (No React). A. Schematic diagram of the experimental design; B. Zif268 immunocontent; C. representative Western blot bands; D. k48-linked polyubiquitin immunocontent; E. representative Western blot bands. Data is expressed as mean ± SEM. n = 6-8/group. 2w-ANOVA (neonatal intervention and reactivation as factors) was used for statistical analyses; * represents statistically significant difference between React NH and No React NH. Statistics results are presented in detail in subsections 3.9 and 3.10.

3.9 Synaptic NMDA and $GABA_A$ receptors subunits were not changed by memory reactivation in the dHc

NMDAR subunits GluN2A and GluN2B and α1-6 subunits of the $GABA_AR$ were also measured in the dHc synapt fraction, at 60 min post-reactivation (Fig. 8).

No significant changes were found in the immunocontent of GluN2A or GluN2B subunits ($p>0.05$, 2w-ANOVA). For $GABA_AR$ α1-6 subunits, a trend towards a reactivation effect was found ($p=0.09$, 2w-ANOVA), but no significant interaction or main effect of neonatal intervention were detected ($p>0.05$, 2w-ANOVA), which as mentioned before, suggests no significant differences in $GABA_AR$ content at dHc synapses.

As in the BLA, no significant interaction or main effects were found for the GluN2A/GluN2B synaptic ratio ($p>0.05$, 2w-ANOVA, data not shown).

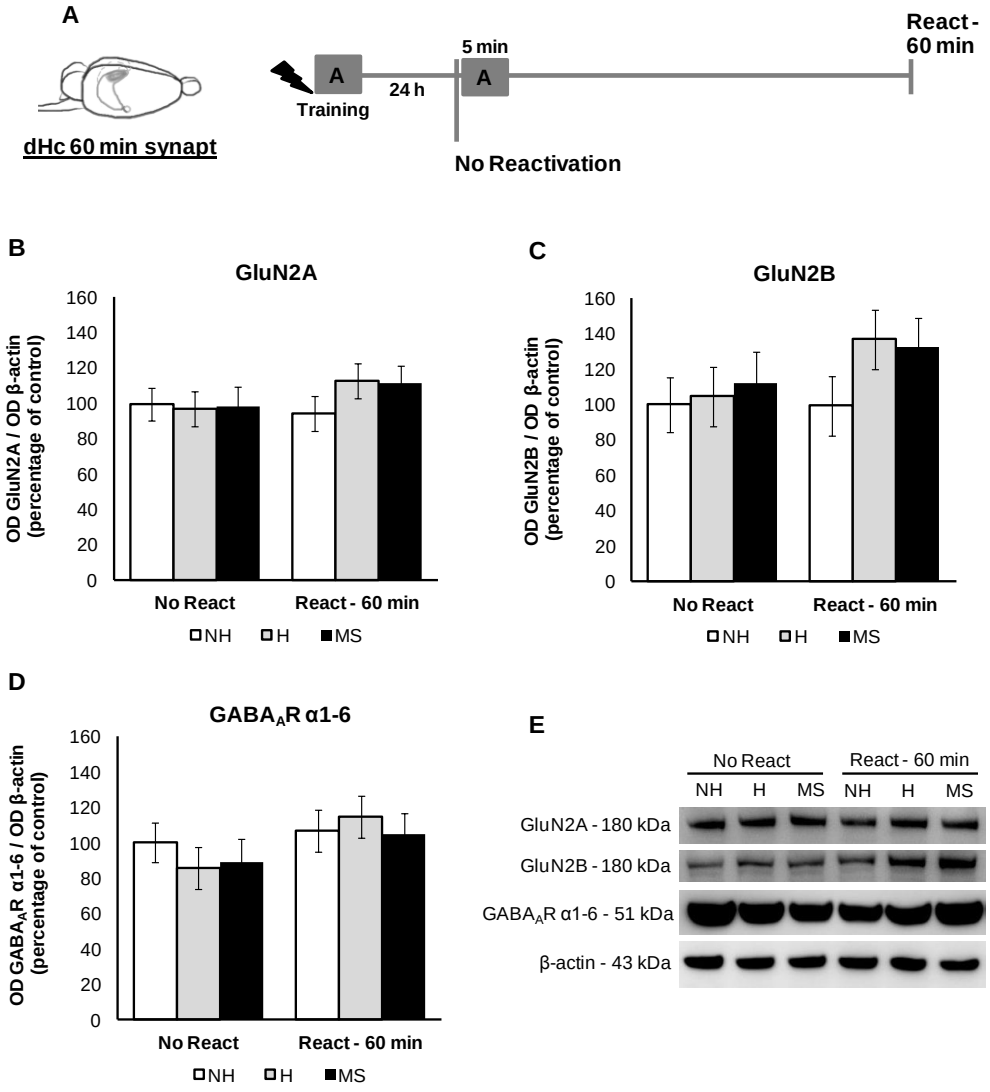

Fig 8. – Synaptosome membrane (synapt) levels of the NMDAR subunits GluN2A and GluN2B and $GABA_AR$ α1-6 subunits in the dorsal hippocampus (dHc) of adult male rats that were non-handled (NH) or subjected to handling (H) or maternal separation (MS) in the neonatal period, 60 min after Reactivation (React) compared to trained animals that were not re-exposed to the training context (No React). A. Schematic diagram of the experimental design; B. GluN2A immunocontent; C. GluN2B immunocontent; D. $GABA_AR$ α1-6 subunits immunocontent; E. representative Western blot bands. Data is expressed as mean ± SEM. n = 5-7/group. 2w-ANOVA (neonatal intervention and reactivation as factors) was used for statistical analyses. Statistics results are presented in detail in subsection 3.9.

3.10 k48-linked polyubiquitin levels were increased by reactivation in the BLA, but not in dHc

k48-linked polyubiquitination was assessed 60 min after memory reactivation, since it has been demonstrated that at this timepoint, retrieval-induced UPS activation significantly increases both in the hippocampus and amygdala (Jarome et al., 2011; Lee et al., 2008). A significant increase was detected in Reactivation animals in the BLA [Fig. 4E,F; $F(1,31)= 5.041$, $p= 0.032$, 2w-ANOVA, main effect of Reactivation, no interaction]. In the dHc, no interaction or main effects were detected (Fig. 7D,E; $p>0.05$, 2w-ANOVA). k48-linked polyubiquitination is an indirect measure of protein degradation, that has been used successfully in the amygdala (Jarome et al., 2011), but in the hippocampus, more direct techniques have been used to link protein degradation to retrieval-induced memory destabilization, including UPS inhibition (Artinian et al., 2008; Lee et al., 2008; Sol Fustiñana et al., 2014) and sample purification using the 26S proteasome subunit S5a before Western blot experiments (Lee et al., 2008), so it is possible that the method used here lacks sensitivity for hippocampal analysis of protein degradation.

4. Discussion:

Neonatal interventions change memory consolidation and retrieval (Kosten et al., 2012). Here, we showed for the first time that they also affect aversive memory reconsolidation. Unlike NH rats, H and MS animals showed resistance to reconsolidation disruption by mdz, a GABAergic drug, administered after memory retrieval. At a molecular level, both groups showed changes similar to the controls in the BLA after memory reactivation, but their dHc appeared to respond differently. Furthermore, behavioral expression of the aversive memory was very different in the two groups: while H animals exhibited significant less freezing to the conditioned context presentation, MS rats generalized their fear response to a new and unconditioned context.

Decreased retrieval-induced freezing shown by H rats is consistent with reduced emotional reactivity and the proposed increased inhibitory control of the amygdala by mPFC (Stevenson et al., 2008), since amygdala nuclei are involved in fear expression through its excitatory projections to the periaqueductal gray – PAG (Gross & Canteras, 2012). It could also be the result of cortical or hippocampal dysfunction and consequently impairments in context recognition. However, H males perform normally in memory tasks that are coupled with neutral or appetitive stimuli (Kosten, Lee, & Kim, 2007; Noschang et al., 2012; Noschang et al., 2010) so the cognitive impairment hypothesis has been disregarded (Kosten et al., 2012).

Aversive memory generalization is a less investigated type of memory impairment, which can be observed when animals freeze in response to unspecific cues. Fear generalization to a new context has already been shown in the adult neonatal isolation offspring (Sampath et al., 2014) and in adult rats that had been stressed 24 hours before training (Bustos et al., 2010). MS rats not only exhibited higher freezing in the new context (B), compared to H, but also responded similarly to the new and to the conditioned apparatus the following day, which suggests fear memory generalization (Sampath et al., 2014; Winocur, Frankland, Sekeres, Fogel, & Moscovitch, 2009; Yang, Huang, & Hsu, 2011). Novelty anxiety impact on this result was ruled out by showing that before receiving footshocks, MS did not freeze more than the other groups in an unfamiliar environment.

Fear generalization in neonatal isolation rats was attributed to enhanced theta synchronization in the hippocampus–amygdala–cortical loops during REM sleep (Sampath et al., 2014); the dHc has an important role in storing the contextual component of the fear memory and this structure malfunction has been implicated in fear generalization (Fanselow & Dong, 2010; Yang et al., 2011). In agreement with this hypothesis, a previous study from

our group reported that an aversive experience subsequently impaired spatial memory in MS rats (Diehl et al., 2012); together with the results reported here, it seems that MS hippocampal function may be impaired, at least after the animals have been through a stressful situation.

Since H and MS rats showed resistance to mdz interfering effect on memory reconsolidation, we further investigated the molecular pathways involved with this process in the BLA and dHc. Both these brain regions are involved with the processing of contextual aversive memories, as pointed earlier (Phillips & LeDoux, 1992), but play different roles. ERK 1/2 intracellular signaling cascade has been implicated in the molecular mechanism of memory reconsolidation (Besnard et al., 2013, 2014; Tronson & Taylor, 2007), but in different manners in the dHc and BLA. Here, we reported that 15 min after reactivation, ERK 1/2 activation was altered in the dHc, but not in the BLA, possibly because in the latter, pERK 1/2 levels have been reported to increase 30 min after reactivation (Besnard et al., 2014). ERK1/2 activation in hippocampal areas is both time and region-specific: following retrieval, the number of p-ERK1/2 positive cells rapidly increased in the dentate gyrus, but returned to values lower than control at 15' post-reactivation, while at this time point, non-significant increases were observed in CA1 and CA3 (Besnard et al., 2014). Since whole dHc tissue was analyzed here, it is possible that the opposing effects were diluted, resulting in the unaltered levels of ERK1/2 phosphorylation found here for NH rats; another study also failed to find increases in pERK 1 in the dHc, 15 min after reactivation (Lee & Hynds, 2013). Also, it is important to reinforce that results presented here refer to cytosolic protein levels. Upon activation, pERK 1/2 translocates to the nucleus, where it phosphorylates downstream transcription factors (Treisman, 1996); the nuclear translocation of activated ERK 1/2 has been shown to be essential for LTP in the dentate gyrus (Davis, Vanhoutte, Pagès, Caboche, & Laroche, 2000). Hence, to further elucidate these results, it would be interesting to analyze

ERK 1/2 levels in the nuclear fraction or the activation of its nuclear substrates, such as the cAMP response element-binding protein (CREB) or Elk-1.

Zif268, an inducible transcription factor which expression is also regulated by ERK 1/2 signalling (Davis et al., 2000; Tronson & Taylor, 2007), is necessary for reconsolidation (Besnard et al., 2013; Bozon et al., 2003; Lee et al., 2004), and has been shown to be increased in the hippocampus (Besnard et al., 2014; Hall et al., 2001) and amygdala (Espejo et al., 2016; Hall et al., 2001; Maddox et al., 2011), after contextual memory retrieval. We reported here a significant increase in Zif268, 60 min after the reactivation session in the BLA of all animals, independently of the neonatal treatment. In contrast, in the dHc, only NH rats showed a significant increase in Zif268 levels, but not H or MS animals.

Increases in GluN2A, GluN2B subunit and its phosphorylated form were also observed in the BLA synap fraction, 60 min after reactivation; whether these changes result from increased synthesis, as reported for GluN2B in previous studies that investigated the reconsolidation process in amygdala nuclei (Espejo et al., 2016), or increased trafficking of receptor subunits to the synapse or both remains unanswered; it does not seem to result from decreased endocytosis, since no changes in the ratio of pGluN2B to total GluN2B were found. Together with the upregulation of Zif268, these results strongly support that a reconsolidation-like process happened in the BLA of the three groups. Interestingly, an increase in GABAAR α1-6 subunits was also found in the BLA after memory reactivation, which to our knowledge, had not been reported previously.

Altered number of synapses or synaptic activity could have an impact on our results, so we analyzed the levels synaptophysin in the BLA. Synaptophysin is the most abundant synaptic vesicle membrane protein and seems to be involved with the endocytosis of synaptic vesicles (Kwon & Chapman 2011) and activity-dependent formation of new synapses (Tarsa

& Goda, 2002) and it is commonly used as pre-synaptic terminal marker. Structural and functional changes in postsynaptic terminals of MS rats BLA have been reported, including dendrite hypertrophy and increased spine density (Koe et al., 2016), as well as increased firing rate in the BLA in vivo, when a GABAAR inverse agonist was administered (Stevenson et al., 2008). In the present study, no significant differences were found concerning synaptophysin in the BLA, which is in accordance with a previous study in MS rats (Andersen & Teicher, 2004); this suggests that there are no changes in terms of presynaptic terminals in the BLA of NH, H and MS adult rats.

In the dHc synapt, no changes in NMDA receptor or GABAAR subunits were found. NMDA antagonism in the dHc prevented the reconsolidation-induced update of an aversive memory (Crestani et al., 2015; Haubrich et al., 2015), but this does not seem to have been the mechanism by which memory reconsolidation was apparently not triggered in the dHc of H and MS rats.

Memory reactivation by exposure to contextual cues brings the trace back to a labile state which has been attributed to protein degradation via UPS (Jarome et al., 2016, 2011; Lee et al., 2008; Sol Fustiñana et al., 2014). Retrieval-induced UPS activation depends on NMDA receptor-mediated calcium influx and subsequent activation of CaMKII, in the amygdala (Jarome et al., 2016, 2011). A significant increase in the levels of polyubiquitinated k48-linked proteins was found 60 min after retrieval, in the BLA of all groups studied here. Ubiquitin polymeric chains linked through lysine residue 48 are involved with targeting proteins for degradation by the UPS (Mattiroli & Sixma, 2014) and the amount of k48-polyubiquitinated proteins detected in the amygdala have been shown to be correlated with proteasome activity in the amygdala (Jarome et al., 2011). This suggests that memory destabilization occurred in the BLA.

The involvement of protein degradation in memory destabilization has been less studied in the hippocampus; while, as in the BLA, it has been suggested as the mechanism underlying memory destabilization (Lee et al., 2008), inhibition of the UPS in hippocampal areas has been shown to produce the same effects as protein synthesis inhibitors in spatial memory (Artinian et al., 2008); furthermore, memory destabilization in the hippocampus has been shown to be dependent on ERK 1/2 activation (Besnard et al., 2013) but not CaMKII activity (Silva, Cardoso, Bonini, Benetti, & Izquierdo, 2013). Here, we could not detect any significant changes in k48-linked polyubiquitin levels in the dHc.

Why did H and MS animals fail to change their behavioral responses to the aversive context after receiving the GABAergic drug mdz, following memory retrieval? The simplest explanation is that the GABAergic system is altered in these animals, so that the drug does not achieve the same effect as in NH rats. In accordance, stressful experiences in rats can change GABAergic transmission in the BLA afferents and internal circuits (Caldji, Francis, Sharma, Plotsky, & Meaney, 2000; Rodríguez-Manzanares, Isoardi, Carrer, & Molina, 2005; Stevenson et al., 2008) and may decrease the effect of mdz on memory reconsolidation (Ortiz et al., 2015). In adult H males, increased binding of a non-bzd GABAAR agonist was reported in amygdala nuclei, compared to NH and MS (Caldji et al., 2000). In the dentate gyrus of adult animals that were exposed to a single maternal separation event, decreased neuronal GABAAR-mediated inhibitory currents were found, as well as changes in α subunits transcription (Hsu et al., 2003). Here, we found no significant differences in the total content of α subunits in the dHc and BLA synapt membrane fraction, suggesting no changes in the total GABAAR density at the synapses; furthermore, if altered GABAergic transmission were the main explanation for the findings reported here, at least H rats would be expected to respond to mdz treatment, since evidences point to an increase in overall GABAergic function

in these animals amygdala. The possibility that a floor effect was reached by these animals is not excluded, since they exhibit low freezing on all sessions, which where equivalent to NH freezing after memory impairment by mdz. It would be interesting to test reconsolidation impairment in these animals in response to a different drug type.

More relevant to answer this question is that our results suggest that H and MS rats BLA underwent a process very similar to what is currently believed to be reconsolidation, in terms of Zif268 upregulation, GluN2B increased insertion at the synaptic membrane and increased protein polyubiquitination, but their dHc did not present the investigated molecular markers of reconsolidation, including the increased Zif268 levels that were observed in NH; our results are similar to the another recent study on memory reconsolidation resistance in rats subjected adult rats to a strong and prolonged stress as adults (Hoffman et al., 2015). Nevertheless, H and MS rats did show increases in ERK 1/2 activation in the dHc, 15 min post-reactivationn, a timepoint at which NH rats did not appear to. In the hippocampus, ERK 1/2 signaling is involved with memory retrieval but not directly with reconsolidation (Besnard et al., 2013). Retrieval as a process independent of reconsolidation has been reported previously (Cammarota, Bevilaqua, Medina, & Izquierdo, 2004) and is now beginning to be more extensively investigated (Gisquet-Verrier & Riccio, 2012).

Most studies on reconsolidation boundary conditions related to memory strenght have identified impairments in the BLA retrieval-reconsolidation process (Espejo et al., 2016; Ortiz et al., 2015; Wang et al., 2009). In fact, downregulation of GluN2B (Wang et al., 2009) or the related increased GluN2A/ GluN2B ratio (Holehonnur et al., 2016) in the BLA have been proposed to be the mechanism that prevents strong memories from becoming labile and undergoing reconsolidation. Despite the non-significant but consistent trend towards decreased levels of both GluN2A and GluN2B subunits in the BLA synapt of H rats, no

differences were found regarding the ratio of the two subunits. Also, Zif268 increased expression has been shown to be dependent on NMDA receptors activity (Malkani & Rosen, 2001; Mokin & Keifer, 2005) and H animals had an increase in the levels of this transcription factor. It is plausible to think that despite the proposed differences in basal and stress-induced excitability of the amygdala resulting from different early experiences (Koe et al., 2016; Koppensteiner et al., 2014; Rau et al., 2015; Stevenson et al., 2008), the NH, H and MS BLA appear to have the same capacity to reconsolidate a retrieved contextual fear memory. Hence, our results point to the hippocampus or to its interaction with the amygdala as the possible origin of their differences in memory reconsolidation.

The dHc is responsible for detecting novelty in the context where memory is retrieved (Rossato et al., 2007) and a mismatch between the expectation the animal has when it is exposed to the context and reality is a condition that has been shown to be necessary to trigger reconsolidation (Pedreira, Pérez-Cuesta, & Maldonado, 2004). Futhermore, hippocampal plasticity mechanisms have been implicated in memory update after retrieval (Crestani et al., 2015; de Oliveira Alvares et al., 2013; Haubrich et al., 2015) and blocking protein synthesis in the dHc after memory retrieval impaired subsequent freezing to multiple contextual cues, while the same procedure in the BLA only impaired freezing to an auditory cue (Yang et al., 2011). The BLA-dHc circuit presents a dual-dynamic interaction (Richter-Levin & Akirav, 2000) and orchestrated processing by the two structures in memory reconsolidation has been reported (Besnard et al., 2014; Wang et al., 2009), including enhanced theta synchronization in this circuit during retrieval (Lesting et al., 2011; Seidenbecher et al., 2003). If decreased dHc plasticity was the mechanism responsible for the failure in memory update in H and MS rats, it could be the result of differential BLA modulation of dHc in H and MS rats or partial

failure in enhancing theta synchronization between the two structures during reactivation. The apparent timeshift in dHc ERK 1/2 activation in H and MS rats supports this hypothesis.

In summary, our results suggest that neonatal interventions in rodents are interesting models to study boundary conditions that appear to be arising from impairments in dHc reconsolidation mechanism; plus due to the two very different patterns of freezing after conditioning, together with the proposed differences in amygdala excitability, these models may also help to extend the study on dHc-BLA crosstalk in memory reconsolidation; finally, understanding how early experiences modulate fear memory reconsolidation in rodents may provide interesting insights on the neurobiological mechanisms and new therapeutical approaches for pathologies associated with traumatic memories, such as PTSD.

Funding: This work was financially supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The funding agencies had no involvement in data collection/analysis or in the preparation/submission of this manuscript.

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CAPÍTULO III - Artigo intitulado "**Differential long-term effects of maternal separation on dorsal and ventral hippocampus redox and mitochondrial parameters"**

a ser submetido ao periódico *Hippocampus*

Fator de Impacto (2015): 4,074

Este capítulo atende ao objetivo específico **2.2.3** desta tese

Differential long-term effects of maternal separation on dorsal and ventral hippocampus redox and mitochondrial parameters

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Abstract

The dorsal (dHc) and ventral (vHc) regions of the hippocampus differ in their anatomical and functional connectivity and their development, in rats, is differently affected by early experiences. Considering the involvement of the two regions in memory encoding and retrieving, the fact that early life stress, such as maternal separation (MS), show hippocampal-related memory impairments, and the involvement of reactive oxygen (ROS) and nitrogen species, such as nitric oxide (NO), in intracellular signaling involved in plasticity mechanisms and memory, we investigated the redox state and mitochondrial function in the dHc and vHc of MS adult offspring. Wistar rat litters were assigned to MS (3h separation, from PND 1-10) or non-handled – NH (left undisturbed with dams) procedures. In the dHc and vHc of adult male offspring, ROS, NO, mitochondrial mass, potential and superoxide were determined by flow cytometry; antioxidant enzymes activity and total thiol content were also determined. Our results show that dHc and vHc of adult MS rats differ significantly from NH. The dHc showed increased mitochondrial mass and potential and decreased mitochondrial superoxide levels, while the vHc showed decreased levels of ROS and NO, which together with no changes in mitochondrial function or antioxidant enzymes activity, suggests decreased signaling via NMDA receptor, and possible changes in plasticity mediated by this receptor. This study contributes to the understanding of the impact of MS on hippocampal regions development and negative emotional memory impairments induced by early life stress.

Keywords: early life stress; mitochondria; reactive oxygen species; nitric oxide; memory; plasticity

1. Introduction

The importance of the hippocampus on cognitive processing, particularly on memory, has long been established (O'Keefe and Dostrovsky, 1971; Fanselow, 1990; Moser and Moser, 1998; Whitlock et al., 2006). Recent behavioral, anatomical, functional and gene expression evidences (Banasr et al., 2005; Christensen et al., 2010; O'Leary and Cryan, 2014; Strange et al., 2014; Sigurdsson and Duvarci, 2016) have supported the initial observation made by Moser and Moser in rats (1998) that the hippocampus is not a unitary structure, and can be divided in regions or zones along its dorsal-ventral axis, with distinct functions and connections. Hence, the hippocampus is now starting to be regarded as a set of separate structures with a dorsal region (dHc), posterior in humans, responsible for cognitive function and a ventral region (vHc), anterior in humans, implied in emotional processing, with a separating intermediate region that has only partly overlapping characteristics with its neighbors (Fanselow and Dong, 2010).

Early life stress, like maternal separation (MS) in rats, results in memory impairments (Kosten et al., 2012). Specifically concerning hippocampus-dependent memories, increased freezing compared to non-handled (NH) in a long re-exposure session of a contextual fear conditioning task (Diehl et al., 2014), spatial memory impairments after a strong aversive event in MS adult rats (Diehl et al., 2012) and fear memory generalization to a novel unconditioned context in neonatal isolation adult rats (Sampath et al., 2014) have been reported.

The development of the dHc and the vHc has been shown to be differently modulated by early life experiences (Katsouli et al., 2014; Nguyen et al., 2015). The amount of maternal care received in the neonatal period differently affects synaptic plasticity in the two hippocampal regions: low pup licking exhibited by the dam was associated with suppressed

long-term potentiation (LTP) in the adult offspring dHc, but enhanced LTP and excitability in the vHc (Nguyen et al., 2015). LTP is a plasticity process that has been shown to underlie memory consolidation in the hippocampus (Whitlock et al., 2006; Izquierdo et al., 2016). MS increases maternal care in terms of quantity, but decreases the quality of the care provided (Couto-Pereira et al., 2016), so it is reasonable to believe that this intervention probably also modulates the dHc and vHc functionality in a different manner. Glutamatergic neurotransmission is involved in synaptic plasticity processes, such as LTP and long term depression (LTD); interestingly, the transcription of glutamate receptors AMPA and NMDA subunits have been shown to be reduced in the hippocampus of MS rats (Pickering et al., 2006).

Reactive oxygen species (ROS) have been implicated in a number of important biological processes, ranging from immunity, to cell growth and cell signaling (Serrano and Klann, 2004; Kawakami-Mori et al., 2012). ROS can be originated in a number of cellular reactions; two of the main sources are the oxidative respiratory process in the mitochondria and NMDA receptors activation (Dikalov, 2011), which have been shown to increase the production of ROS through activation of the membrane-cytoplasmic NADPH oxidase complex - NOX (Brennan et al., 2009; Brennan-Minnella et al., 2013). Genetic or pharmacological inhibition of NOX 2, the neuronal isoform of this enzyme, prevented the induction of NMDA receptor-dependent LTP in the hippocampus (Kishida et al., 2006).

NMDA receptor-mediated calcium influx has also been shown to activate neuronal Nitric Oxide Synthase (nNOS) by increasing the affinity of calmodulin to this enzyme (Cho et al., 1992); uncoupled nNOS subunits can also produce superoxide, another ROS. Nitric oxide (NO) can act as retrograde messenger and has been shown to be involved in the modifications of the presynaptic terminal that occur in memory consolidation (Schafe et al., 2005).

Specifically, activation of guanylyl cyclase by NO leads to the subsequent activation of cGMP-dependent protein kinase (PKG), which through the activation of the MAPK signaling cascade, increases the expression of the transcription factor Zif268, resulting in increased levels of synaptophysin and synapsin, two synaptic vesicles-associated proteins (Overeem et al., 2010) and putative facilitated neurotransmitter release by the presynaptic terminal.

Synaptic plasticity events are essential for optimal neuronal functioning and are involved in memory processing; oxygen and nitrogen-derived reactive species therefore play important roles in cell functioning in the Central Nervous System (CNS). Unbalanced production of reactive species, however, can lead to oxidative stress, which has several deleterious effects. The CNS is particularly vulnerable to damage because of its high use of glucose and oxygen for energy production and high concentration of lipids susceptible to peroxidation. Oxidative stress has been implicated in the pathological mechanism of neurodegenerative diseases and other CNS malfunctions (Floyd and Hensley, 2002), including memory impairment (Knapp and Klann, 2002). It is clear that the levels of oxygen and nitrogen reactive species in the CNS need to be finely tuned with the metabolic requirements, and both their over accumulation or under production can be detrimental.

Very few studies have compared the redox state of dorsal and ventral regions of the hippocampus in response to environmental challenges. One study using glutathione (GSH) knockout mice showed that the ventral hippocampus seems to be more sensitive to redox inbalance, as shown by the greater loss of parvalbumin-positive gabaergic interneurons in this region (Steullet et al., 2010). Two recent studies have performed proteomics analysis on the vHc of Sprague–Dawley rats that were subjected to MS and found some interesting differences in proteins involved in oxidative stress and synaptic plasticity, among others (Marais et al., 2009; Daniels et al., 2012).

Here, we propose to investigate the effects of early life stress in rats on mitochondrial function and redox state in the context of intracellular signaling and plasticity processes, on the dorsal and ventral hippocampal regions.

2. Matherials and Methods

2.1 Subjects

Primiparous pregnant Wistar rats bred at our animal facility were used (n= 10). At gestational day 17-18, they were single-housed in home cages made of Plexiglas (65 x 25 x 15 cm) with sawdust-covered floors and kept in a controlled environment (lights on between 07:00 h and 19:00 h, temperature at 22 ± 2°C, food and water provided *ad libitum*). The day of birth was considered postnatal day 0 (PND 0). All litters were randomly culled to six to eight pups within 24 h after birth and litters were randomly assigned to one of the neonatal interventions described below. Weaning was performed on PND 21: males were separated and randomly housed 3-4 per cage, and were then left undisturbed until adulthood. Females were assigned to other experiments.

All animal treatments were approved by the institutional Research Ethics Committee (CEUA-UFRGS #23844) and followed the Brazilian Law regarding the use of animals (Federal Law 11.794/2008) and the NIH Guide for the Care and Use of Laboratory Animals.

2.2 Neonatal interventions

Non-handled group (NH): pups and dams were left undisturbed until weaning, except for cage cleaning.

Maternal separation group (MS): from PND 1 to PND 10, once a day, pups were gently removed from their home cages and placed in a clean container lined with a paper

towel, in a water bath at 32° C, where they remained for 3 hours. After this period, pups were returned to their respective cages. This procedure was performed during the lights-on cycle, between 13:00 h and 17:00 h. Each litter had its own glove to be manipulated with, to avoid the spread of odors between them. Dams remained in the same room, inside their home cages, during the whole separation procedure, so they could listen to the pups' vocalizations.

From birth to weaning, cage cleaning was performed when necessary, similarly for both groups: dirty sawdust was carefully removed from the cage, avoiding the nest area, and replaced with clean sawdust.

2.3 Dorsal (dHc) and ventral (vHc) hippocampus dissection

Male adult Wistar rats (PND 120-130) that were subjected to the neonatal interventions described above were used. No more than two animals from the same litter were used for the same range of techniques to avoid the influence of the genetic load on results.

Thirty-three animals were quickly euthanized by a trained researcher. Hippocampi were carefully dissected on an ice-cold Petri dish and transversally cut on the medial line (Christensen et al., 2010) to separate the upper part (dorsal region – dHc) and the lower part (ventral and intermediate regions, here referred to as ventral – vHc, as in Banasr et al. 2005; Moser and Moser 1998; Christensen et al. 2010), according to the literature on rodent hippocampal anatomic organization (Moser and Moser, 1998; Banasr et al., 2005; Christensen et al., 2010; O'Leary and Cryan, 2014; Strange et al., 2014; Sigurdsson and Duvarci, 2016). Twenty animals (NH – 9; MS – 11) were used for flow cytometry, antioxidant enzymes activity and thiol content assays: dHc and vHc right hemisphere samples were immediately placed in 1 ml phosphate-buffered saline (PBS) for flow cytometry analysis; the left hemisphere tissue obtained from these animals was stored at -80ºC and 7 days later used for

the enzymatic and thiols assays. Pools of the right and left hippocampal regions of 13 animals (NH – 6; MS – 7) were stored at -80ºC for further western blot analyses. An estimated 2-3 minute period was to necessary to complete the euthanasia-dissection process.

2.4 Flow cytometry

Fresh tissue samples, dissected as described in subsection 2.3, were dissociated in 1 mL PBS, pH 7.4, containing 0,01 mg/mL collagenase IV (Sigma-Aldrich, Germany) and 0.05 mg/mL DNase (Sigma-Aldrich, Germany), using a fire-polished glass Pasteur pipette. Samples were then filtered into a clean 50 mL Falcon tube (BD Biosciences, USA) through a 40 µm nylon cell filter strainer (BD Biosciences, USA) to ensure single cell suspensions and kept on ice until incubation (Stone et al., 2014; Ferreira et al., 2015).

Incubation with the probes was performed in cytometry tubes, at 37ºC, protected from light, using 50 µL of sample for each probe. At the end of the incubation period, 500 µL of pre-cooled PBS buffer was added to all samples both to slow down the reaction and to dilute them.

Flow cytometry was performed using a FACScalibur flow cytometer (BD Biosciences, USA); 20,000 events per sample were evaluated. A gate to include only cells with forward-scattered light (FSC) >50 was defined to exclude cell debris and was applied to all samples. Negative controls (samples without stain) were run to set up the instrument voltages. Data was acquired using the software CELLQuest Pro data acquisition (Becton–Dickinson, USA) and analyzed using the software FlowJo^{TM} (FlowJo, LCC, USA). Data was plotted by density as a single-parameter histogram that shows the relative fluorescence on the x-axis (logarithmic scale) and the number of events (cell count) on the y-axis. The median fluorescence intensity (MFI) was then determined for each sample.

Specific incubation and fluorescence detection details of each probe are described below.

2.4.1 Mitochondrial superoxide

Superoxide generation by mitochondria was determined using the probe MitoSOX™ Red mitochondrial superoxide indicator (#M36008, Molecular Probes, ThermoFisher Scientific, USA); this reagent is permeant to live cells and selectively targets the mitochondria, where it reacts with superoxide and becomes fluorescent by binding to DNA (Robinson et al., 2006). Samples were incubated with the MitoSOX™ probe at a final concentration of 3 µM, for 10 minutes.

2.4.2 Mitochondrial mass and membrane potencial

Samples were incubated with the probes MitoTracker® Green (#M7514, Molecular Probes, ThermoFisher Scientific, USA) and MitoTracker® Red (#M22425, Molecular Probes, ThermoFisher Scientific, USA), at a final concentration of 0.1 µM, for 45 minutes, to assess mitochondrial mass and potential, respectively (Cottet-Rousselle et al., 2011). MitoTracker® Green is taken up electrophoretically into mitochondria, where chloromethyl groups form covalent adducts with thiol groups of mitochondrial matrix proteins; MitoTracker® Red is a cationic dye that is attracted to the mitochondrial membrane potential, so that loss of membrane potential results in release of MTR from mitochondria and a subsequent decrease in fluorescence (Poot et al., 1996).

2.4.3 Reactive oxygen species (ROS)

Reactive oxygen/nitrogen species levels were evaluated by 2'-7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) oxidation (LeBel et al., 1992). $DCFH_2$-DA enters the cell where it is cleaved by intracellular esterases and oxidized to the fluorescent DCF, mainly by hydrogen peroxide but also by peroxynitrite, lipid hydroperoxides, and, to a

lesser extent, superoxide; since most of its oxidants are oxygen derivative molecules, in this report, we will refer to DCF assay results as a general indicator of ROS levels (Dikalov, 2011). Samples were incubated with $DCFH_2$-DA (Sigma-Aldrich, Germany) at a final concentration of 10 µM, for 30 minutes.

2.4.1 Nitric oxide (NO)

NO levels were measured using the probe DAF-FM Diacetate (#D-23844, Molecular Probes, ThermoFisher Scientific, USA), based on the reaction between 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate and NO, which yields a fluorescent benzotriazole derivative (Kojima et al., 1999). Samples were incubated with the DAF-FM probe at a final concentration of 1 µM, for 60 minutes.

2.5 Enzyme kinetics assays

Dorsal and ventral hippocampi, dissected as described in subsection 2.3, were homogenized in 1:10 (w:v) ice-cold 50 mM potassium phosphate buffer pH 7.4, containing 1 mM EDTA. The homogenates were centrifuged at 3000 rpm for 10 min at 4 ºC and the supernatant was collected and used for the enzymatic assays. Total protein content in each sample was determined using the Lowry method (Lowry et al., 1951), with bovine serum albumin as standard.

2.5.1 Superoxide dismutase (SOD)

Determination of SOD activity was performed using the commercial kit RANSOD (#SD125, Randox Laboratories Ltd., UK), according to the manufacturer's instructions. Due to the extraction protocol and the detection reaction employed here, specifically SOD 1 (cytosolic isoform) activity is measured by this method. Xanthine and xanthine oxidase are used to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-

phenyltetrazolium chloride (INT) to form a red formazan dye; spectrophotometric readings at 492 nm, 37 ºC, determine the inhibition in the production of the chromogen, which is proportional to the activity of SOD present in the sample; one unit of SOD causes 50 % inhibition of the rate of reduction of INT under the assay conditions. Results are expressed as SOD milliunits (mU) per mg of total protein in the sample.

2.5.2 Glutathione peroxidase (GPx)

GPx activity was determined using the commercial kit RANSEL (#RS504, Randox Laboratories Ltd., UK), according to the manufacturer's instructions. GPx catalyses the oxidation of Glutathione, which is again reduced in the presence of Glutathione Reductase with NADPH as an electron donor. The decrease in the known concentration of NADPH was monitored by spectrophotometry at 340 nm, 25 ºC. The contribution of spontaneous NADPH oxidation was subtracted from the overall reaction ratio. GPx activity is expressed as nmol NADPH oxidized per minute per mg of total protein in the sample.

2.5.2 Catalase (CAT)

Evaluation of CAT activity was based on establishing the rate of H_2O_2 degradation by spectrophotometric analysis at 240 nm, 25ºC (Aebi, 1984). CAT activity was calculated in terms of micromoles of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$.

2.6 Total thiol groups content

This assay detects both protein and non-protein thiols; the latter is mainly represented by the reduced form of glutathione and the method is based in the reduction of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) by thiol groups, which become oxidized to disulfide, yielding a yellow compound (TNB), which absorption is measured by spectrophotometric analysis at

412 nm (Aksenov and Markesbery, 2001). The sulfhydryl content of a tissue is correlated with reduced thiol groups (including reduced glutathione). Results are reported as nmol SH/mg protein.

2.7 Western blot

The immunocontent of synaptophysin and nNOS was measured by Western blot in the dHc and vHc, respectively, which were dissected as described in subsection 2.3. Samples were thawed on ice and homogenized in 1:10 (w:v) hypotonic 10 mM Hepes buffer, containing 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1mM DTT, protease inhibitor (#11697498001, Roche, Germany) and phosphatase inhibitor (#88667, Pierce, ThermoFisher Scientific, USA) cocktails, pH=7.9. Nonidet P-40 (#E109, Amresco, USA) was then added to a final concentration of 0.6% and cells were allowed to swell on ice for 5 minutes, with agitation. Samples were centrifuged at 10.000 g, for 10 minutes, at 4ºC, and the supernatant containing the cytosolic proteins was collected. Total protein content was determined using the Pierce™ BCA Protein Assay kit (#23227, ThermoFisher Scientific, USA). Denatured samples were loaded (40 µg of total protein/lane) on NuPAGE® precast 4-12% gradient polyacrylamide gels (#NP0323BOX, Life Technologies, ThermoFisher Scientific, USA); a 12-225 kDa molecular weight marker (#RPN800E, Amersham, GE Healthcare, UK) was loaded on all gels. Electrophoresis and electrotransfer were performed on an XCell SureLock® Mini-Cell and an XCell II™ Blot Module, respectively (#EI0002, Invitrogen, ThermoFisher Scientific, USA). Proteins were transferred to nitrocellulose membranes (1h50min at 50 V in transfer buffer [48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS]) and blots were then blocked for 2 h in Tris-buffered saline containing tween and 5% (m/v) non-fat dry milk. Blots were incubated overnight, at 4ºC, with one of the following

primary antibodies: anti-synaptophysin (1:2000, #AB9272, Millipore, Germany), anti-nNOS (1:2000, #SAB4502010, Sigma-Aldrich, Germany) and anti-β-actin (1:3500, #8457, Cell Signaling Technology, USA). Secondary peroxidase-conjugated anti-rabbit antibody (1:1000, #AP132P, Merck-Millipore, Germany) was incubated for 2 h at room temperature. Blots were developed using a chemiluminescence ECL kit (#RPN2209, Amersham, GE Healthcare, UK) and images were acquired using the digital camera system ImageQuant LAS 4000 (GE Healthcare Bio-Sciences AB, Sweden). The optical density of bands was determined using the software ImageJ (National Institutes of Health, USA). Results were quantified as the ratio of the optical density (OD) of the protein of interest to that of β-actin of the same sample, and are expressed in percentage of control (NH group).

2.8 Statistical Analysis

Data was analyzed using the software SPSS version 16.0. Student t-test for independent samples was performed to compare NH and MS animals for all parameters analyzed. Welch-Satterthwaite method for adjustment was applied when group variances failed to meet the homogeneity assumption. Statistical significance was set at $p<0.05$. Data is expressed as mean ± standard error of the mean (SEM).

3. Results

3.1 Mitochondrial parameters

Mitochondrial mass, potential and superoxide generation were determined by flow cytometry in the dHc (Fig. 1A-F) and vHc (Fig. 2A-F) of both controls (NH) and animals that were separated from dams in the neonatal period (MS), in adulthood. In the dHc, all mitochondrial parameters evaluated in this study were found altered by maternal separation:

mitochondrial superoxide was significantly decreased in MS rats [t(18)= 2.451, p=0.025, Student t test], while mitochondrial membrane potential [t(17)= 2.245, p=0.038, Student t test] and mass [t(17)= 2,487, p=0.024, Student t test] were both significantly increased. In contrast, in the vHc, mitochondrial parameters were unchanged by early life stress: mitochondrial superoxide, membrane potential and mass were not significantly different between NH and MS animals (p>0.05 for all assays, Student t test).

3.2 ROS and NO levels

ROS and NO levels were also measured by flow cytometry in the dHc (Fig. 1G-J) and vHc (Fig. 2G-J) of NH and MS adult rats. In this report, we will refer to DCF assay results as an indicative of ROS levels, as explained in section 2.4.3. No significant differences were found regarding ROS or NO levels in the dHc (p>0.05 for both assays, Student t test). On the other hand, in the vHc, both ROS [t(17)= 2.132, p=0.048, Student t test] and NO levels [t(16)= 2.350, p=0.032, Student t test] were found significantly decreased in MS compared to NH rats.

3.3 Antioxidant enzymes activity and total thiols content

The activity of the antioxidant enzymes SOD, GPx and CAT was assessed in the two hippocampal regions (Table 1). In the dHc, no significant differences were found between neonatal interventions regarding the activity of GPx or CAT (p>0.05 for both assays, Student t test), but a trend was found towards a decrease in SOD activity [t(16)= 2.032, p=0.059, Student t test]. Similar results were obtained for the vHc: the activity of SOD, GPx and CAT also did not differ between NH and MS males (p>0.05 for all assays, Student t test).

Total thiols content of the two hippocampal regions studied here are also presented in Table 1. In the dHc, no significant differences were found in thiols content between NH and

MS rats ($p>0.05$, Student t test). In the vHc, a significant small increase (5.5%) in thiols content was found in MS compared to NH animals [$t(16.082)= 2.603$, $p=0.019$, Student t test, Welch-Satterthwaite adjustment].

3.4 dHc synaptophysin

Increased mitochondrial mass and potential in the dHc suggests increased biogenesis, which could be related to altered number of synapses; to test this, we determined synaptophysin levels of NH and MS animals in the dHc, by Western blot (Fig. 3). No significant differences were found ($p>0.05$, Student t test). Furthermore, no significant differences between groups were detected regarding β-actin OD ($p>0.05$, Student t test), indicating that this was a suitable loading control for this experiment.

3.5 vHc nNOS

To investigate if decreased NO levels in the vHc could be linked to decreased levels of the one of the enzymes responsible for its synthesis, nNOS immunocontent was measured by Western blot, in the vHc of NH and MS animals (Fig. 4). No significant differences were found ($p>0.05$, Student t test). Also, no significant differences were found in β-actin OD ($p>0.05$, Student t test), indicating that this was a suitable loading control for this experiment.

4. Discussion

To our knowledge, this is the first study to compare the dHc and vHc of NH and MS adult males concerning redox and mitochondrial parameters. We reported here that early life stress altered mitochondrial organization and function in the dHc, by increasing both mass and membrane potential in a correlated way and decreasing superoxide production, but did

not seem to affect the number of presynaptic terminals, as assessed by synaptophysin immunocontent. Oxygen and nitrogen reactive species were also not altered in the dHc. The vHc was affected in a very different way by MS: while no mitochondria-related aspects were changed by the intervention, significant decreases in ROS and NO were observed, suggesting changes in neurotransmission in this hippocampal region. nNOS immunocontent was not modified by MS. Considering that the hippocampus is strongly involved with memory consolidation, evocation, reconsolidation and extinction (Izquierdo et al., 2016), and that its dorsal and ventral portions play different roles in memory processes (Donley et al., 2005), our results may contribute to the understanding of the impact early life interventions have on memories, particularly those that include emotional content.

Since the frontier between dorsal and ventral hippocampus is not consensual among the literature, here we choose to divide the hippocampus medially so that the dorsal region contained the septal portion, which projects to the lateral nuclei of amygdala, and the ventral region contained the temporal hippocampus, with efferences to the basal part of the amygdala, and the main part of the intermediate hippocampus.

Our results in the dHc suggest that, in MS rats, cells in this region have an increased amount of mitochondria, which are both active and more efficient, as suggested by the increased mitochondrial mass and membrane potential and decreased superoxide generation, respectively. It is worthy to disclosure that staining by the MitoTracker® probes used here may be slightly affected by changes in mitochondrial potential and ROS outbursts (Xiao et al., 2016). However, mitochondrial superoxide production was found decreased and no significant differences in ROS were found in the dHc, minimizing the possible impact of ROS on our results concerning the mitochondria; besides, MitoTracker® probes have been

regarded as suitable to assess mitochondrial mass and membrane potential (Cottet-Rousselle et al., 2011).

Mitochondria move easily along the microtubules network in neurons, and are captured by high metabolically-active sites, particularly synapses (Chang et al., 2006). Mitochondria motility is higher in axons than in dendrites, so that the correlation between activity and mitochondria accumulation tends to be more proportionate in the presynaptic terminals (Jeanneteau and Arango-Lievano, 2016). Here, we found no differences between NH and MS rats in synaptophysin levels in the dHc, as reported previously (Makena et al., 2012). Synaptophysin is the most abundant protein in the synaptic vesicle membrane; while its function has not been fully clarified yet, in the hippocampus, it has been implicated in the endocytosis of synaptic vesicles (Kwon and Chapman, 2011) and in activity-dependent synapse formation (Tarsa and Goda, 2002). Due to its abundance in the axon terminal, it is commonly used as pre-synaptic terminal marker (Kwon and Chapman, 2011). Our result then suggests that there is no increase in the number of pre-synaptic terminals in the dHc of MS animals, despite having increased mitochondrial mass in this brain structure.

Due to motility constrains, mitochondria traffic to the synaptic spines is not proportional to the spines' activity and it is common that the same mitochondrion serves multiple postsynaptic terminals; hence, mitochondrial mass is not necessarily proportional to the number of dendritic spines and a greater proportion of highly charged, metabolically active mitochondria has been found in dendrites, compared to axons (Jeanneteau and Arango-Lievano, 2016). It has also been reported that mitochondria undergo structural modifications to adapt to energy demands, such as fission, fusion and elongation (Jeanneteau and Arango-Lievano, 2016). The increased mitochondrial mass and potential in the dHc of MS rats, reported here, together with no changes in synaptophysin levels, may suggest that the

metabolic activity in postsynaptic terminals of the dHc is modified by early life stress, but further studies need to be performed to better elucidate this question.

A previous study from our group had already reported decreased ROS levels in the whole hippocampus of MS males (Ferreira et al., 2015). Here, we found the same result in the vHc but not in the dHc, showing a region-specific effect of MS on ROS production in the hippocampus.

No differences regarding antioxidant enzymes activity were found between NH and MS rats both in the vHc and in the dHc, which is also in accordance with previous reports from our group, analyzing the hippocampus as a whole structure (Diehl et al., 2012; Ferreira et al., 2015). In the vHc of MS rats, an increase in SOD 1 protein levels was reported previously (Daniels et al., 2012), however according to our results, this does not seem to reflect on increased activity of the enzyme. The lack of changes in the enzymatic antioxidant rates suggests that the decrease in ROS found here is probably due to decreased generation. The main source of ROS in the cell is the mitochondria; surprisingly, no decrease in superoxide production by mitochondria, nor changes in mitochondrial mass or potential, were found in the vHc, which suggests that decreased ROS in this structure of MS rats is not linked to changes in mitochondrial function. Another important source of ROS in neurons is the activation of NMDA receptors, which subsequently activate NOX 2 (Brennan et al., 2009; Brennan-Minnella et al., 2013). Changes in NMDA receptor functionality, either due to altered expression or posttranslational modifications, could be the mechanism responsible for the lower ROS levels detected in MS vHc.

In addition to decreased ROS, we also found decreased NO levels in the vHc of MS adult animals. NO is an easily diffusing gas, that may act as a retrograde messenger. The activation of signaling cascades by NO in the presynaptic neuron leads to changes in the

expression of Zif268, an immediate early gene that has been thoroughly implicated in memory processes (Bozon et al., 2003; Lee et al., 2004; Besnard et al., 2013), and ultimately results in increased expression of neurotransmitter synaptic vesicle proteins (Overeem et al., 2010), which could lead to facilitated presynaptic neurotransmitter release. In accordance, in another model of early life interventions in rats, handling, NO levels were found decreased in the hippocampus of adult females, together with impairments in spatial memory in the Morris water maze (Noschang et al., 2010). NO was also implicated in the behavioral expression of a conditioned fear memory, at least in the dHc (Spiacci et al., 2016).

NO is synthesized by three isoforms of NOS. Particularly relevant to this work is the neuronal isoform; nNOS is a constitutive protein, but its immunocontent has been shown to be altered by chronic stress in the hippocampus of rats (Gądek-Michalska et al., 2012; Gadek-Michalska et al., 2015) and mice (Palumbo et al., 2007) and by MS in the distal colon (Tjong et al., 2011). Despite the decrease in NO levels in MS vHc, no significant differences in immunocontent of nNOS were found here. To our knowledge, no previous studies have evaluated nNOS levels in the hippocampus of MS rats. Our result does not exclude the possibility that this enzyme may have its activity reduced or that the other isoforms of may be involved. Interestingly, the contribution of inducible and endothelial NOS to the decrease in total activity of NOS in the hippocampus of mice that underwent chronic mild stress was found irrelevant (Palumbo et al., 2007).

Like NOX 2, nNOS activity can also be modulated by NMDA receptors activation. The increase in intracellular calcium concentration resulting of NMDA channel opening allows the binding of calmodulin to nNOS, which activates the enzyme. Although calmodulin levels have been found increased in the vHc of MS rats (Daniels et al., 2012), its binding and

consequent activation of nNOS depends on the intracellular concentration of calcium (Cho et al., 1992).

Our results concerning decreased ROS and NO in the vHc suggest that MS rats may have a decrease in NMDA receptor activity in this region. In accordance, the transcription of the GluN2B subunit of NMDA receptors was found decreased, while mRNA levels for GLAST, an important glutamate transporter with rapid clearance activity at the synapse, were found increased in the hippocampus of adult rats that were separated from dams for 6 hours, compared to handled ones (Pickering et al., 2006). In another study (N. S. Couto-Pereira et al., unpublished observations), we analyzed the immunocontent of NMDA receptor subunits GluN2A and GluN2B in the dHc synaptossomes of MS and control rats and found no differences between them, so it is possible that the results found by Pickering et al. (2006) could be mainly attributed to differences in the vHc. GluR1 and GluR2 subunits of AMPA receptor were also found downregulated in the same study (Pickering et al., 2006); AMPA receptors activation is responsible for the initial depolarization of the postsynaptic neuron membrane that releases the blockade of NMDA receptors and allows their further activation. Overall, our results support the hypothesis that there is a decrease in glutamatergic transmission in the ventral region of MS rats hippocampus. Interestingly, another study found decreased number and length of dendritic spines in vHc neurons of adult MS rats (Monroy et al., 2010), further pointing to changes in postsynaptic function.

Based on the lower levels of ROS and NO, both pre and postsynaptic signaling mechanisms may be impaired in the vHc of rats that were separated from dams in the neonatal period (Knapp and Klann, 2002). The vHc is involved in fear memory retrieval (Cox et al., 2013), possibly through modulation of the amygdala excitability (Gross and Canteras, 2012), and NMDA receptor activity in the dentate gyrus is necessary for pattern separation, a process

which is thought to allow context-specificity during memory encoding and retrieving (McHugh et al., 2007). Hence, our results in the vHc could contribute to explain the fear memory generalization to new environments observed in MS rats after an aversive experience (Sampath et al., 2014).

We also found a small increase in the total thiol content in the vHc of MS rats. This could be explained by the lower levels of ROS in this structure, which would result in fewer attacks to protein sulfhydryl groups.

In conclusion, dorsal and ventral hippocampal regions in adult rats are differentially affected by MS. The dHc shows increased mitochondrial function, while the ventral hippocampus appears to have decreased glutamatergic activity and decreased signaling via oxygen and nitrogen reactive species. This study contributes to the understanding of the altered emotionality and negative emotional memory impairments of adult rats that were separated from dams in the neonatal period.

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Figures and captions

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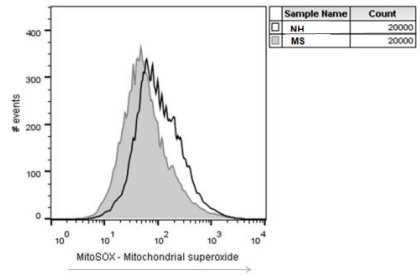

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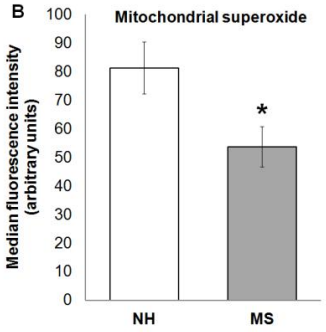

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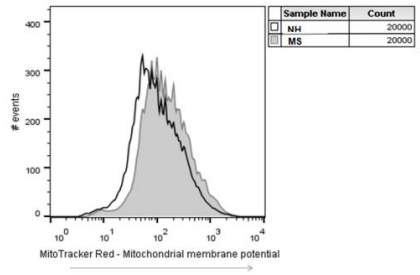

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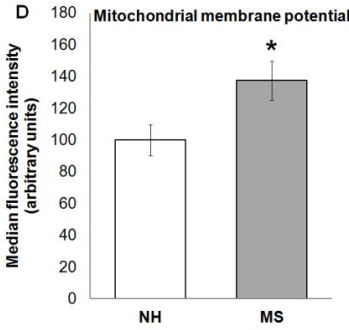

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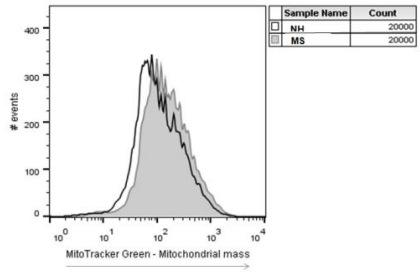

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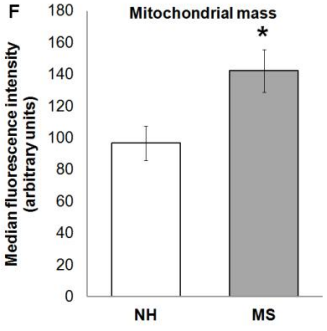

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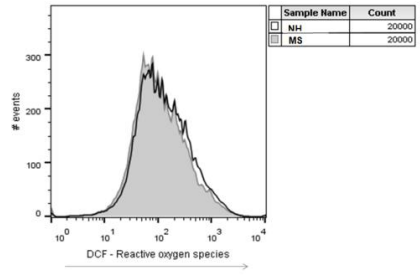

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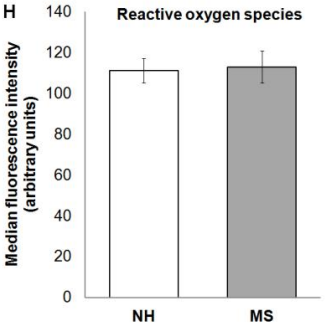

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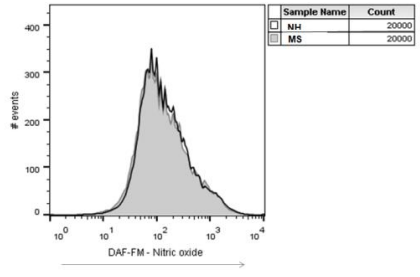

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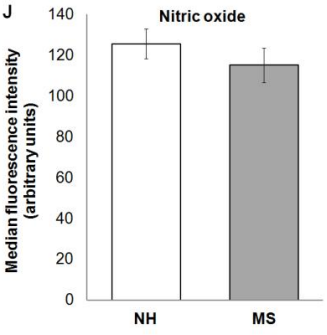
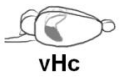
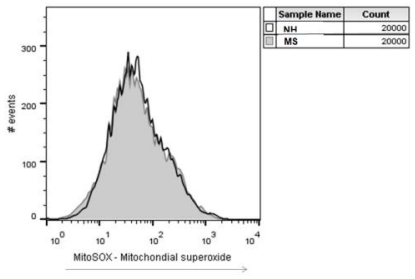


Fig. 1 – Mitochondrial parameters (mass, potential and superoxide production) and reactive oxygen species (ROS) and nitric oxide (NO) levels in the dorsal portion of the hippocampus (dHc) of adult male rats that were non-handled (NH) or subjected to maternal separation (MS) in the neonatal period, determined by flow cytometry. A, B - Mitochondrial superoxide; C, D – Mitochondrial membrane potential; E, F – Mitochondrial mass; G, H – Reactive oxygen species (ROS) levels; I, J – Nitric oxide (NO) levels. Panels on the left (A, C, E, G, I) show representative histograms and panels on the right (B, D, F, H, J) represent combined median fluorescence intensity (MFI) of both groups. MFI data is expressed as mean ± SEM. NH: n=8-9, MS: n= 11. Student t-test for independent samples was used to compare groups; *represents statistically significant difference between NH and MS. Statistics results are presented in detail in subsections 3.1 and 3.2.



vHc

A



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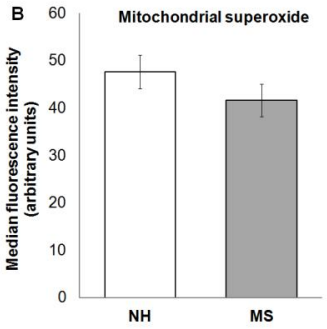

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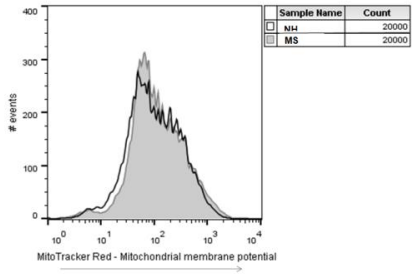

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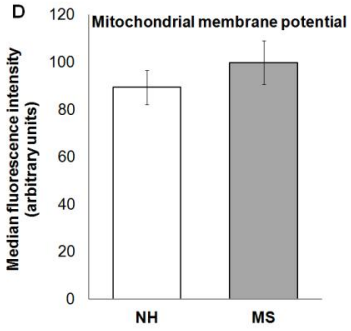

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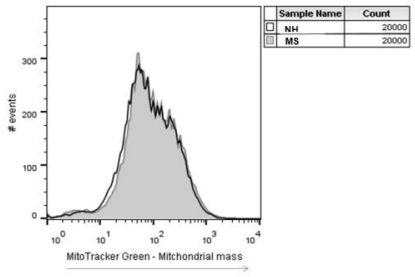

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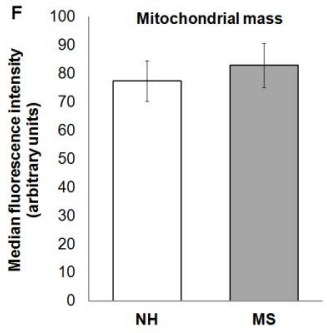

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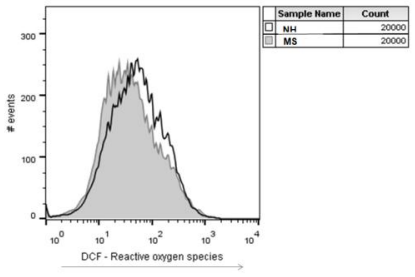

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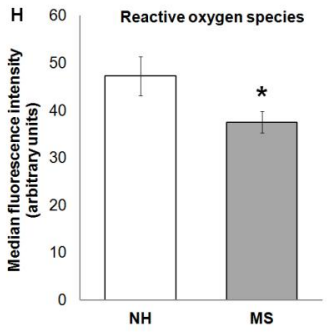

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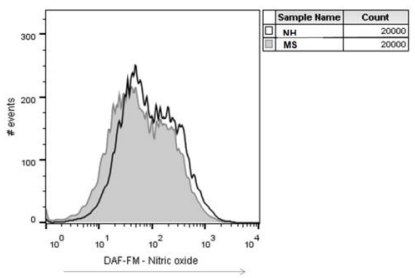

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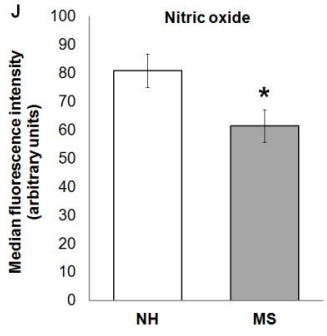

Fig. 2 – Mitochondrial parameters (mass, potential and superoxide production) and reactive oxygen species (ROS) and nitric oxide (NO) levels in the ventral portion of the hippocampus (vHc) of adult male rats that were non-handled (NH) or subjected to maternal separation (MS) in the neonatal period, determined by flow cytometry. A, B - Mitochondrial superoxide; C, D – Mitochondrial membrane potential; E, F – Mitochondrial mass; G, H – Reactive oxygen species (ROS) levels; I, J – Nitric oxide (NO) levels. Panels on the left (A, C, E, G, I) show representative histograms and panels on the right (B, D, F, H, J) represent combined median fluorescence intensity (MFI) of both groups. MFI data is expressed as mean ± SEM. NH: n=8-9, MS: n= 10-11. Student t-test for independent samples was used to compare groups; *represents statistically significant difference between NH and MS. Statistics results are presented in detail in subsections 3.1 and 3.2.

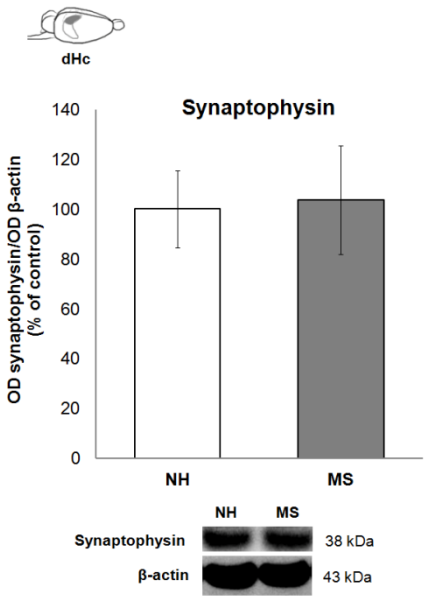

Fig. 3 – Immunocontent of synaptophysin in the dorsal hippocampus (dHc) of non-handled (NH) and maternal separation (MS) groups (representative bands of each group depicted). No significant differences between groups were found; NH: n=6, MS: n=7.

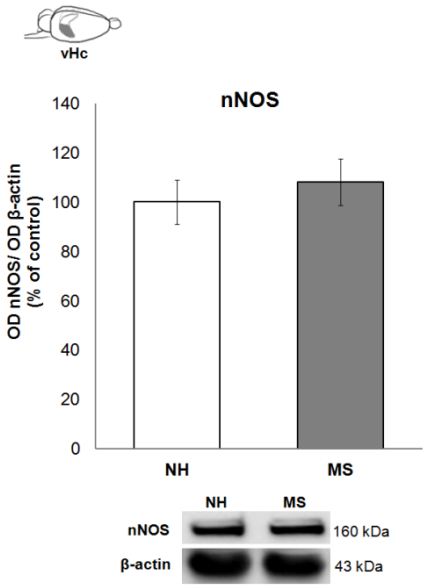

Fig. 4 – Immunocontent of neuronal Nitric Oxide Synthase (nNOS) in the ventral hippocampus (vHc) of non-handled (NH) and maternal separation (MS) groups (representative bands of each group depicted). No significant differences between groups were found; NH: n=6, MS: n=7.

Tables

Table 1 – Antioxidant enzymes activity and total thiols content of dorsal and ventral hippocampus of adult male rats that were non-handled (NH) or subjected to maternal separation (MS) in the neonatal period

	Catalase (µmol H_2O_2/min/mg protein)	**GPx** (nmol NADPH/min/mg protein)	**SOD** (mU/mg protein)	**Thiols** (nmol SH/mg protein)
		Dorsal Hippocampus		
NH	1.64±0.36	63.3±6.4	6.70±0.40	49.3±1.6
MS	1.67±0.17	68.3±4.4	5.49±0.42	50.3±1.7
		Ventral Hippocampus		
NH	0.99±0.05	62.5±2.7	4.66±0.23	47.2±0.5
MS	0.96±0.07	59.3±5.4	4.50±0.24	49.8±0.8 *****

Data is expressed as mean ± SEM; NH: n=6-9, MS: n=7-11. Student t-test for independent samples was used to compare groups; *represents statistically significant difference between NH and MS. Statistics results are presented in detail in subsection 3.3.

DISCUSSÃO

A prevalência de patologias psiquiátricas associadas a memórias traumáticas, a sua relação com experiências prévias e o desafio de desenvolver tratamentos eficientes para estas condições constituem a justificativa desta tese, cujo objetivo principal foi investigar se diferentes experiências precoces, em ratos, poderiam contribuir para o desenvolvimento de padrões de resiliência ou vulnerabilidade a patologias psiquiátricas através da modulação do processo de reconsolidação de memórias aversivas.

Neste trabalho, estudaram-se dois tipos de intervenção precoce em roedores, amplamente utilizados em estudos científicos da área: a manipulação neonatal e a separação materna, que produzem alterações duradouras na emocionalidade e na memória da prole adulta (Kosten, Kim & Lee 2012). Em suma, os resultados apresentados nos Capítulos I, II e III mostram que:

1) ambas intervenções estudadas neste trabalho aumentam a frequência de comportamentos direcionados às ninhadas, conforme relatos anteriores (Liu et al. 1997; Pryce, Bettschen & Feldon 2001; Macrì, Chiarotti & Würbel 2008), mas as mães das ninhadas separadas têm um comportamento mais inconsistente do que as restantes e uma alteração do padrão circadiano de cuidado, além de não apresentarem aumento dos níveis de ocitocina no liquor, como ocorreu nas mães das ninhadas manipuladas; este cenário pode configurar uma fonte adicional de estresse para a prole separada;
2) a separação materna no período neonatal modifica, de forma dependente do sexo, a ativação do eixo HHA em resposta à exposição a um ambiente previamente condicionado a um estímulo aversivo, na prole adulta: machos não aumentam a secreção de corticosterona 15 minutos após a reativação da memória aversiva, mas

apresentam níveis semelhantes aos outros grupos de machos, cuja secreção aumentou comparado com o nível basal, enquanto fêmeas separadas no período neonatal têm níveis basais inferiores às outras fêmeas e apresentam um aumento considerável após a experiência; a manipulação neonatal não afeta a secreção de corticosterona após um desafio contextual, no tempo estudado;

3) a memória contextual aversiva de ratos machos adultos que foram manipulados ou separados no período neonatal é resistente à administração de um modulador alostérico positivo do receptor $GABA_A$, um fenômeno que parece se dever a alterações na reativação/ reconsolidação da memória no HcD, uma vez que a BLA de todos os animais apresentou alterações induzidas pela exposição ao contexto aversivo coincidentes com o que se conhece do processo de reconsolidação, enquanto que o HcD dos animais que sofreram intervenções apresenta evidências moleculares de reativação mas não de reconsolidação, pelo menos no momento estudado (1 hora pós-reativação);

4) apesar da semelhança na resistência a uma droga interferente da reconsolidação, ratos manipulados e separados evidenciaram comportamentos opostos: ratos manipulados exibem comportamento de congelamento diminuído frente ao contexto associado ao estímulo aversivo, comparado com os controles, enquanto ratos separados mostraram congelamento aumentado, comparado com os manipulados, num contexto novo, não pareado, sugerindo generalização da memória de medo;

5) o HcD e o HcV dos ratos separados no período neonatal apresentam perfis diferentes, entre si e comparados com os controles, na idade adulta, no que se relaciona à atividade mitocondrial e geração de espécies reativas de oxigênio e nitrogênio; o maior número de mitocôndrias aparentemente funcionais dos animais separados no HcD, junto com a ausência de alterações na expressão de sinaptofisina, uma proteína associada a vesículas

de neurotransmissores no terminal pré-sináptico, sugere uma alteração de atividade nos terminais pós-sinápticos; a diminuição dos níveis de espécies reativas de oxigênio e de NO no HcV dos ratos separados pode representar capacidade diminuída de sinalização e plasticidade.

As intervenções neonatais afetam a prole e a mãe, criando uma dinâmica cujos componentes são difíceis de analisar isoladamente. Alguns estudos foram realizados com este objetivo (Macrì, Chiarotti & Würbel 2008; Macrí, Mason & Würbel 2004; Tang et al. 2006), no entanto, todos utilizaram a frequência do cuidado materno como indicador deste comportamento. O cuidado da prole é um comportamento complexo e dinâmico, cujos efeitos dependem do seu ajuste às necessidades momentâneas da ninhada (Pereira & Ferreira 2016). Buscando elucidar melhor o componente materno nas intervenções neonatais, nesta tese utilizou-se uma ferramenta previamente desenvolvida em outro modelo de estresse precoce (Ivy et al. 2008), para avaliar o comportamento materno de forma qualitativa, durante o período em que foram realizadas as intervenções, conforme se mostra no Capítulo I. Esta análise evidencia o grau de inconsistência/ fragmentação do cuidado provido à ninhada, com base na ideia de que os episódios de cuidado materno "naturais" não ocorrem de forma aleatória, seguem uma ordem lógica (Leon, Croskerry & Smith 1978) e a fragmentação e a imprevisibilidade do comportamento materno parecem prever o desfecho da intervenção precoce em termos de desenvolvimento emocional da prole (Molet, Heins, et al. 2016).

O grau de inconsistência comportamental nas mães cuja prole foi submetida à separação materna mostrou-se aumentado em comparação com as mães controle; as mães das ninhadas manipuladas evidenciaram um pequeno aumento no período de observação após a intervenção, que foi atribuído ao aumento no comportamento de lamber a prole, uma vez que

os episódios deste comportamento são tipicamente curtos (Champagne et al. 2003). O padrão circadiano de cuidado também se mostrou alterado nas mães das ninhadas separadas em comparação com os controles. De forma geral, as duas intervenções induziram um aumento na frequência de comportamentos direcionados às ninhadas mas, na separação materna, este ocorreu de forma inconsistente. Além disso, apenas nas mães das ninhadas manipuladas se encontrou um aumento significativo nos níveis de ocitocina no líquido cefalo-raquidiano, um efeito que seria previsto observar também nas mães das ninhadas separadas, devido à forte associação deste neuromodulador com o comportamento materno (Bridges 2015).

As alterações à qualidade do cuidado materno pareceram refletir-se na prole adulta em termos de resposta do eixo HHA, de forma mais evidente do que as alterações quantitativas. Enquanto machos e fêmeas manipulados mostraram um aumento semelhante aos controles nos níveis circulantes de corticosterona após exposição a um contexto previamente condicionado a um estímulo doloroso, animais separados da mãe no período neonatal evidenciaram padrões de secreção de corticosterona diferentes dos controles, e de forma dependente do sexo: machos separados não apresentaram aumento após exposição ao contexto aversivo, uma vez que os seus níveis basais já eram elevados, ao contrário das fêmeas, cujos níveis basais eram inferiores e tiveram um aumento relativo muito superior às fêmeas controle e manipuladas. Vários estudos mostraram que estas intervenções precoces modificam o funcionamento do eixo HHA e que estas alterações dependem do sexo (de Kloet et al. 2005). No caso particular da manipulação, o aumento da expressão do GR no hipocampo facilita a retroalimentação negativa do eixo e promove um retorno mais rápido aos níveis basais de glicocorticóides, após um desafio, mas não modifica necessariamente os níveis de hormônio no pico da resposta, que costuma ser observado entre 15 a 30 minutos após a exposição ao estresse, em ratos (Grota, Bienen & Felten 1997). Assim, a ausência de

diferenças entre manipulados e controles aqui relatada, que reflete apenas um momento específico após o desafio contextual, não significa que não existam diferenças na resposta ao estresse entre estes animais.

A influência de glicocorticóides e do seu efeito mediado pelo receptor GR na consolidação e na reconsolidação de memórias aversivas não é linear, uma vez que foi reportado que tanto a administração de corticosterona quanto de antagonistas do GR interferem com o processamento de diferentes tipos de memórias com conteúdo emocional negativo (Akirav & Maroun 2013).

A resposta endócrina ao estresse de ser exposto a um contexto aversivo, modificada nos animais separados (ausência de aumento), juntamente com o aumento do comportamento tipo-ansioso (Makena, Bugarith & Russell 2012; Romeo et al. 2003), e as alterações estruturais e funcionais observadas na amígdala (Koe, Ashokan & Mitra 2016; Diehl et al. 2014; Stevenson, Marsden & Mason 2008), sugerem que a saliência do estímulo aversivo apresentado no condicionamento pode ser mais forte, levando a alterações na codificação da memória da experiência. Memórias mais "fortes", independentemente do mecanismo usado para manipular a "força" do treino, parecem ser resistentes à reconsolidação (Wang, de Oliveira Alvares & Nader 2009; Gazarini et al. 2014). De fato, como mostrado no Capítulo II, animais separados evidenciaram resistência à administração de um interferente da reconsolidação. Embora não tenham sido encontradas alterações na secreção de corticosterona nos animais manipulados, estudos prévios sobre a excitabilidade na amígdala e o seu *freezing* diminuído em resposta ao contexto condicionado parecem sugerir o oposto nestes animais. Inesperadamente, estes animais também se mostraram resistentes ao midazolam (mdz) administrado após a reativação da memória.

Como discutido no referido Capítulo, não fica totalmente esclarecido se a resistência à droga administrada se deve a uma resistência *per se* aos benzodiazepínicos, por possíveis alterações na neurotransmissão GABAérgica (Caldji et al. 2000), ou se deve de fato a uma alteração no mecanismo de reconsolidação. Os estudos anteriores sobre estresse como uma condição limitante para a reconsolidação, em que se utilizou benzodiazepínicos para mostrar experimentalmente esta condição, identificaram ausência de labilização da memória na amígdala como o mecanismo responsável pela resistência à reconsolidação induzida pelo estresse, uma vez que essa resistência foi revertida pela administração de D-cicloserina, um agonista parcial do receptor NMDA, antes da reativação (Bustos, Maldonado & Molina 2009; Espejo et al. 2016; Ortiz et al. 2015). Em um desses estudos (Ortiz et al. 2015), a ausência de efeito do mdz se deveu à expressão diminuída da subunidade α1 do receptor $GABA_A$ na membrana, na BLA, resultante do tipo de estresse utilizado nesse estudo (abstinência após uso prolongado de álcool); ainda assim, nesse estudo, o uso de outro agente interferente (propranolol – antagonista do receptor β–adrenérgico), mostrou que a resistência à reconsolidação se devia à dificuldade de labilização da memória na BLA. No presente estudo, os ratos separados e manipulados não evidenciaram nenhum indicativo de ausência de labilização da memória na BLA: a razão GluN2A/GluN2B não se encontrou alterada, o que caso contrário poderia prejudicar a labilização (Wang, de Oliveira Alvares & Nader 2009; Holehonnur et al. 2016), e os níveis de proteínas marcadas para degradação pelo UPS aumentou 1 hora após a reativação; além disso, o processo de reconsolidação nesta estrutura parece ter sido engajado em todos os grupos, como evidenciado pela indução de Zif268 e aumento dos níveis de NR2B na fração sinaptossomal. Estas várias evidências sugerem que diferente dos estudos que simulam exposição a estresse na vida adulta, a separação e a manipulação no período neonatal não parecem interferir com o processo de reconsolidação na

BLA. Isto não significa que os animais sujeitos a estas intervenções não formem memórias com valências emocionais distintas, como se pode deduzir pelo seu comportamento oposto frente ao contexto condicionado e ao contexto novo.

A ocorrência do processo de labilização-reconsolidação na BLA dos animais que sofreram as intervenções neonatais poderia, em teoria, tornar a memória suscetível à interferência administrada. A disrupção da memória durante a reconsolidação modifica a valência do estímulo aversivo da memória original, resultando no comportamento de congelamento diminuído que se observou nos controles. De novo, não se exclui a possibilidade de resistência ao efeito do mdz por alterações no sistema GABAérgico. No entanto, os animais manipulados parecem ter atividade GABAérgica aumentada na amígdala (Caldji et al. 2000), coincidente com animais que receberam cuidado materno aumentado (Caldji, Diorio & Meaney 2003), e não foram encontradas alterações no conteúdo total de subunidades do receptor $GABA_A$ nos sinaptossomas de nenhum dos grupos. Assim, esta hipótese não explica a resistência à interferência na reconsolidação exibida por manipulados e separados.

O HcD tem um papel essencial na reconsolidação de memória, sendo responsável, entre várias outras funções, por detectar novidades em um contexto conhecido (Rossato et al. 2007); a existência de discrepância entre a expectativa do animal e a realidade é uma condição necessária para que ocorra reconsolidação (Pedreira, Pérez-Cuesta & Maldonado 2004).

Os resultados obtidos no HcD parecem sugerir que nesta estrutura não ocorreu reconsolidação, conforme se entende como um processo que depende da síntese de proteínas e que depende da indução do fator de transcrição Zif268 nesta estrutura (Besnard, Caboche & Laroche 2013; Maddox, Monsey & Schafe 2011; Hall, Thomas & Everitt 2001; Besnard, Laroche & Caboche 2014; Bozon, Davis & Laroche 2003). A ausência do processo de

reconsolidação no HcD pode explicar porque a droga interferente não conseguiu produzir um efeito sobre a memória condicionada.

É interessante que ratos manipulados e separados evidenciaram um aumento na ativação da ERK1/2 15 minutos após a reativação, o que não foi observado nos controles. Como sugerido no Capítulo II, o resultado nos controles pode se dever à menor sensibilidade da técnica utilizada ou ao fato de a ativação da via de sinalização da ERK1/2 ocorrer com maior intensidade nos primeiros momentos após a reativação (ou durante), no hipocampo (Besnard, Laroche & Caboche 2014). O fato de ter sido detectada nos manipulados e separados sugere que esta ativação foi mais significativa nestes animais ou ocorreu mais tarde do que nos controles, sugerindo uma alteração temporal na decorrência do processo de reativação no HcD. No hipocampo, a via de sinalização da ERK1/2 parece estar envolvida com a reativação da memória, mas um estudo mostrou que não é necessária para a fase de reconsolidação (Besnard, Caboche & Laroche 2013). Alguns autores têm sugerido que a reativação pode ocorrer sem que necessariamente seja seguida de reconsolidação dependente da síntese de proteínas (Gisquet-Verrier & Riccio 2012). Assim, ratos manipulados e separados podem ter reativado a memória a nível do hipocampo, mas pode hipoteticamente ter ocorrido uma falha no sincronismo temporal entre a reativação no HcD e na BLA.

Durante a consolidação (Düzel, Penny & Burgess 2010; Popa et al. 2010), a evocação (Seidenbecher et al. 2003; Lesting et al. 2011; Narayanan, Seidenbecher, Kluge, et al. 2007) e a reconsolidação (Narayanan, Seidenbecher, Sangha, et al. 2007) de uma memória contextual aversiva, ocorre um aumento da sincronização em ritmo teta entre a região CA1 do hipocampo e da amígdala lateral, sendo este sincronismo atualmente tido como o mecanismo de interação entre regiões encefálicas que possibilita o armazenamento e reativação do traço mnemônico dentro de um circuito (Düzel, Penny & Burgess 2010; Fell & Axmacher 2011).

Em um estudo com animais isolados no período neonatal, verificou-se que estes apresentam maior duração de sono REM, durante o qual se encontrou um aumento do sincronismo na via amígdala-hipocampo-mPFC; usando a análise de causalidade de Granger, os autores identificaram que o fluxo aumentado de informação de CA1 para o mPFC ocorre em frequências altas, sugestivas de incorporação de novas informações em circuitos já existentes, e que o aumento do fluxo de CA1 para a amígdala lateral e da amígdala lateral para o mPFC, em ritmo teta, é unidirecional (Sampath et al. 2014). Os autores encontraram também generalização da resposta de medo a um contexto novo, tal como foi relatado no presente trabalho. Outro estudo em animais isolados neste período encontrou níveis aumentados de LTP e LTD evocados, mas não basais, no giro denteado, em resposta a estimulação da BLA, quando os animais estavam acordados (Blaise et al. 2008). Os dados sobre o sono REM foram obtidos em ratos separados que não foram submetidos à tarefa de memória, portanto não refletem necessariamente o processo de sincronização durante a evocação; no entanto, fica claro que uma intervenção precoce similar à separação materna modifica a interação e a plasticidade no circuito hipocampo-amígdala. Estudos futuros poderiam elucidar melhor esta questão avaliando a sincronização de oscilações entre o HcD e BLA durante a reativação de memórias aversivas, em ratos manipulados e separados.

Dentro do circuito hipocampo-amígdala, o HcV é uma região essencial para a codificação de memórias aversivas condicionadas ao contexto. Acredita-se que a associação entre os estímulos contextuais e o estímulo sensorial pode ser armazenada principalmente no HcV e esta região está fortemente envolvida na evocação de memórias aversivas (Gross & Canteras 2012; Cox et al. 2013), controlando a sua expressão comportamental através de projeções para a amígdala (Maren, Phan & Liberzon 2013; Gross & Canteras 2012).

A estimulação dos axônios de neurônios piramidais de CA1 do HcV, também em frequência teta, induziu hiperpolarização dos neurônios piramidais da amígdala lateral, através da ativação de interneurônios GABAérgicos, no entanto, o mesmo tipo de estímulo nas fibras gerou uma atenuação da inibição GABAérgica, com consequente facilitação de LTP na via amígdala lateral-basal (Bazelot et al. 2015). Este estudo mostra que a atividade do HcV modula os mecanismos de inibição da amígdala, de uma forma dinâmica e finamente ajustada, que pode ter relevância para entender porque os animais separados no período neonatal generalizam a expressão comportamental da memória aversiva a um contexto que não representa uma ameaça, comparado com os manipulados.

No presente trabalho, mostrou-se que o HcV dos animais separados apresenta níveis menores de espécies reativas de oxigênio e nitrogênio. Como discutido no Capítulo III, estes resultados, aliados à ausência de diferenças nos parâmetros mitocondriais e na atividade das enzimas antioxidantes, sugerem uma diminuição da atividade dos receptores NMDA nesta estrutura, o que está de acordo com um estudo anterior que analisou a expressão de subunidades deste receptor no hipocampo total de ratos separados (Pickering et al. 2006). Além disso, o NO é importante para o processos de consolidação (Overeem et al. 2010; Schafe et al. 2005) e reconsolidação (Bal et al. 2017) de memória aversiva. O NO difunde-se facilmente para o meio extracelular e pode atuar como mensageiro retrógrado em neurônios aferentes; a sua ação como mediador das mudanças funcionais e estruturais que ocorrem na consolidação de memória foi mostrada na BLA em uma tarefa de medo condicionado a um estímulo auditivo, através de um mecanismo que envolve a indução da expressão de Zif268 (Overeem et al. 2010).

Os nossos resultados não permitem identificar quais neurônios (ou células) têm níveis de espécies reativas alterados, mas são sugestivos de diminuição de plasticidade mediada por

receptores NMDA nesta região e possível prejuízo de um dos mecanismos envolvidos com a consolidação; tendo em conta a importância do HcV no armazenamento da associação contexto-estímulo aversivo, estes achados podem ajudar a entender a generalização de medo observada nos separados.

Um estudo anterior do nosso grupo (Ferreira et al. 2015) analisou o hipocampo total em ratos separados e manipulados e encontrou níveis de espécies reativas de oxigênio diminuídos nos separados, como relatado aqui no HcV, mas não em manipulados. Além disso, encontrou também potencial mitocondrial diminuído em ratos manipulados, um efeito oposto ao relatado aqui no HcD de ratos separados. Estes achados evidenciam as diferenças a nível do funcionamento hipocampal nos animais submetidos às duas intervenções estudadas neste trabalho, pelo que estudos futuros direcionados ao processo de reconsolidação no hipocampo destes animais, como os estudos de eletrofisiologia *in vivo* sugeridos acima, trariam informações importantes para a compreensão da influência de intervenções neonatais sobre este processo mnemônico.

A separação materna em roedores parece ser um bom modelo pré-clínico das experiências precoces que geram um cenário de desregulação emocional (Lanius et al. 2010), como o que se postulou ser uma das vias que levam à vulnerabilidade ao desenvolvimento de TEPT. Estudos anteriores do nosso laboratório haviam sugerido o mesmo (Diehl et al. 2012; Diehl et al. 2007; Diehl et al. 2014). Nesta intervenção, os animais foram expostos a um cuidado materno exacerbado mas inconsistente, que poderia estar correlacionado com sensibilidade materna reduzida em humanos (Belsky et al. 2015); além disso, a separação materna induz um aumento do comportamento tipo-ansioso (Maniam & Morris 2010) e tipo-depressivo nas mães (Toigo et al. 2012; Boccia et al. 2007; Maniam & Morris 2010), que em humanos também se mostrou estar associado a cuidado materno de menor qualidade (Kim,

Strathearn & Swain 2016). Juntos, estes fatores podem configurar, em roedores, uma experiência correlacionável com o ambiente precoce empobrecido sugerido no referido modelo em humanos. A experiência vivenciada pelas ninhadas manipuladas parece ser bastante distinta, como discutido no Capítulo I, e é sugestiva de um ambiente neonatal enriquecido. É importante ressaltar, no entanto, que este trabalho se focou em animais no início da vida adulta e usando uma tarefa de memória com carga emocional negativa; diferentes resultados poderiam ter sido encontrados em animais mais velhos ou se outros tipos de memórias como, por exemplo, memórias relacionadas à interação social tivessem sido investigados (Raineki, Lucion & Weinberg 2014; Zinn et al. 2016).

O mecanismo através do qual as diferentes intervenções neonatais podem ter programado os mecanismos hipocampais envolvidos na reconsolidação de uma memória aversiva não foi diretamente estudado nesta tese, mas os dados coletados no período neonatal e a avaliação da secreção de corticosterona em resposta à exposição ao contexto aversivo, juntamente com trabalhos prévios de outros autores, permitem formular algumas hipóteses. Um modelo de estudo do impacto do comportamento materno sobre diferentes desfechos na vida adulta da prole tem permitido muitas descobertas interessantes na última década: neste modelo ("*artificial rearing*", em inglês), os filhotes são isolados da mãe e são alimentados através de sondas implantadas no estômago; diferentes componentes do cuidado materno (estimulação tátil, social e olfativa) são administrados isoladamente e a sua importância para o desenvolvimento é avaliada em função da prevenção do desfecho (Lomanowska & Melo 2016). Assim, verificou-se que animais sem estímulos maternos e animais controles não diferem em tarefas cognitivas, mas têm comportamento tipo-ansioso exacerbado em diversos paradigmas, que são parcialmente prevenidas pela estimulação tátil artificial; o mesmo modelo permitiu identificar que os benefícios da estimulação tátil têm um efeito teto

(Lomanowska & Melo 2016). Emboras as conclusões destes estudos tenham limitações, estes parecem indicar que a estimulação tátil pela mãe tem um papel importante na programação da resposta ao estresse, o que está de acordo com os estudos que sugerem que o comportamento materno medeia os efeitos da manipulação (Liu et al. 1997) e com um estudo em humanos que mostrou que a frequência de toque materno avaliada em uma sessão previu a conectividade funcional no mPFC, em crianças pequenas (Brauer et al. 2016); por outro lado, o efeito teto observado em relação à estimulação tátil, juntamente com a nossa observação que o aumento do cuidado materno nas mães das ninhadas separadas, incluindo o aumento do comportamento de lamber a ninhada, vem junto com um aumento global da inconsistência comportamental, podem ajudar a explicar a ausência da mesma programação benéfica do eixo HHA nos animais separados (Lajud et al. 2012) que os animais manipulados apresentam (Meaney et al. 1985). Outro aspecto interessante é que as mães roedoras lambem mais os filhotes machos do que as fêmeas (Moore & Morelli 1979); assim, o aumento deste comportamento nas mães das ninhadas separadas pode ter sido dirigido principalmente à prole do sexo masculino, e pode em parte explicar os efeitos opostos na secreção de corticosterona face ao contexto aversivo, quando comparados com os respectivos controles.

Obviamente, a própria intervenção e ausência da mãe impacta diretamente os filhotes. A ausência de alimentação pelo período relativamente longo usado neste modelo pode programar alguns dos aspectos do desenvolvimento da prole, no entanto, estudos em que se mostrou um impacto importante da ausência de alimentação foram realizados no modelo de privação materna por 24 horas, no qual se mostrou que a ausência de alimentação é responsável pela elevação da secreção de corticosterona durante a intervenção, enquanto a ausência de estimulação tátil modulou o controle central desta resposta endócrina (Suchecki, Rosenfeld & Levine 1993), de acordo com os estudos citados acima. No entanto, o aumento

da secreção de corticosterona em resposta à ausência da mãe acontece apenas nos primeiros dias do protocolo (Daskalakis et al. 2011), e a separação materna pelo período de 3 horas, como usado nesta tese, não modificou os níveis basais de corticosterona a partir do terceiro dia de intervenção (Lajud et al. 2012), o que é consistente com a hiporresponsividade do eixo HHA neste período. A separação materna interfere com a neurogênese do hipocampo conforme avaliado ainda no período neonatal (Lajud et al. 2012) e com a flutuação da densidade sináptica nesta estrutura e na amígdala, ao longo do desenvolvimento de machos e fêmeas separados (Andersen & Teicher 2004). Assim, uma interação entre os efeitos da ausência da mãe e o seu comportamento materno alterado parece explicar os efeitos comportamentais e as alterações nas regiões hipocampais observadas em animais separados, neste estudo. Em relação aos animais manipulados, o seu comportamento de congelamento diminuído frente a um contexto aversivo e ausência de generalização do medo coincidem com o comportamento materno aumentado.

CONCLUSÕES

Em suma, as intervenções neonatais usadas neste trabalho configuram experiências precoces diferentes em vários níveis, com consequências distintas para a prole. Em adultos, quando expostos a um evento aversivo, os animais que foram separados da mãe no início da vida exibiram alterações na secreção de corticosterona, que foram opostas em machos e fêmeas, e uma resposta sugestiva de um cenário de vulnerabilidade, devido à sua resistência à interferência no processo de reconsolidação, generalização do medo a contextos novos e alterações na sinalização intracelular mediada por espécies reativas, no HcV. Os ratos machos que foram manipulados no período neonatal apresentam um comportamento de enfrentamento de situações aversivas distinto e, apesar de também se mostrarem resistentes a interferências na reconsolidação no protocolo utilizado, exibiram uma resposta coincidente com o conceito de resiliência, com baixo congelamento quando re-expostos ao contexto condicionado e ao contexto novo.

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Lista de Figuras

Anexo – Carta de aprovação do projeto pela Comissão de Ética no Uso de Animais da Universidade Federal do Rio Grande do Sul

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 23844
Título: Reconsolidação da memória em um modelo de medo condicionado em ratos adultos: efeitos da separação materna e da manipulação na fase neonatal

Pesquisadores:

Equipe UFRGS:

CARLA DALMAZ - coordenador desde 01/11/2012
GRASIELLE CLOTILDES KINCHESKI - pesquisador desde 01/11/2012
Camilla Lazzaretti - Aluno de Doutorado desde 01/11/2012
Natividade de Sá Couto Pereira - Aluno de Doutorado desde 01/11/2012

Comissão De Ética No Uso De Animais aprovou o mesmo em seus aspectos éticos e metolodológicos, para a utilização de 131 ratas, Wistar,prenhas e 628 ratos, Wistar, machos e fêmeas, de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Terça-Feira, 13 de Novembro de 2012

STELA MARIS KUZE RATES
Coordenador da comissão de ética