Universidade Federal do Rio Grande do Sul
Instituto de Ciências Básicas da Saúde
Programa de Pós-Graduação em Neurociências

Mecanismos Subjacentes ao Efeito da Manipulação Neonatal Sobre o Vínculo Mãe/filhote

Adolfo Rodrigues Reis

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Tese apresentada ao Curso de Pós-Graduação em Neurociências, da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do Titulo de Doutor em Ciências Biológicas: Neurociências.

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- ✓ *5-HIAA – METABOLITO DA SEROTONINA (Ácido 5-Hidroxi-indolacetico)*
- ✓ *ACTH – HORMÔNIO ADRENOCORTICOTRÓPICO*
- ✓ *ADN – ÁCIDO DESOXIRRIBONUCLEICO*
- ✓ *ANOVA – ANÁLISE DE VARIÂNCIA*
- ✓ *AVP – VASOPRESSINA*
- ✓ *BO – BULBO OLFATÓRIO*
- ✓ *BDNF – FATOR NEUROTRÓFICO DERIVADO DO ENCÉFALO*
- ✓ BNSTv – Núcleo da estria terminal ventral
- ✓ *CBP – PROTEÍNA LIGANTE AO CREB*
- ✓ *CRE – ELEMENTO RESPONSIVO AO AMPc*
- ✓ *CREB – PROTEÍNA DE LIGAÇÃO AO ELEMENTO RESPONSIVO AO AMPc*
- ✓ *CRH – HORMÔNIO LIBERADOR DA CORTICOTROPINA*
- ✓ *DPN – DIA PÓS-NATAL*
- ✓ *DPP – DIA PÓS-PARTO*
- ✓ *EPM – ERRO PADRÃO DA MÉDIA*
- ✓ *HPA – EIXO HIPOTÁLAMO-PITUITÁRIA-ADRENAL*
- ✓ *LC – LOCUS COERULEUS*
- ✓ *MHPG – METABOLITO DA NORADRENALINA (3-metoxy-4-hidroxifeniletilenoglicol)*
- ✓ MPO – Área Pré-optica Medial

- ✓ *NA – NORADRENALINA*
- ✓ NAs-Concha do Núcleo Accumbens
- ✓ *pro-BDNF – PROTEÍNA PRECURSORA DO BDNF*
- ✓ *pCREB – PROTEÍNA DE LIGAÇAO AO ELEMENTO RESPONSIVO AO AMPc FOSFORILADA*
- ✓ PAGc – Substancia Cinzenta Periaquedutal
- ✓ *PKA – PROTEÍNA CINASE A*
- ✓ *PKC – PROTEÍNA CINASE C*
- ✓ *PVN – NÚCLEO PARAVENTRICULAR*
- ✓ *SNC – SISTEMA NERVOSO CENTRAL*
- ✓ VP – Pálido Ventral
- ✓ VTA – Área Tegmentar Ventral

Resumo

Reis, Adolfo Rodrigues. ***Mecanismos Subjacentes ao Efeito da Manipulação Neonatal Sobre o Vínculo Mãe/filhote.*** Tese (Doutorado) – Programa de Pós-Graduação em Ciências Biológicas: Neurociências, Universidade Federal do Rio Grande do Sul, 2014.

Ao nascerem, os mamíferos não estão com o sistema nervoso plenamente desenvolvido e os primeiros dias de vida representam uma fase crítica para o desenvolvimento desse sistema. De fato, nesta fase o encéfalo está passando por diversos processos fundamentais como organização funcional das redes neurais, proliferação neuronal, migração, diferenciação, além de gliogênese e mielinização.

Em ratos, um procedimento simples, como "manipular" os filhotes por alguns minutos durante a primeira semana de vida, pode marcar decisivamente o desenvolvimento do indivíduo. Assim, a manipulação neonatal tem sido muito utilizada para se examinar os mecanismos pelos quais variações ambientais podem afetar o desenvolvimento do filhote.

A manipulação neonatal promove uma série de alterações comportamentais e neuroendócrinas que se caracterizam basicamente por uma diminuição do medo e da resposta ao estresse no adulto. Embora muitos autores até caracterizem a manipulação como uma intervenção positiva ela também pode provocar graves déficits em comportamentos sociais e reprodutivos para a prole aparecendo desde o inicio do desenvolvimento e persistindo até a vida adulta. Além de seu efeito sobre os filhotes, estudos tem demostrado que intervenções na prole no período neonatal também afetam de forma duradoura a resposta ao estresse das genitoras, mas este tema ainda é muito pouco explorado pela literatura.

Portanto, na primeira parte desta tese, iremos estudar os efeitos da manipulação neonatal sobre a formação do vínculo mãe-filhote, tentando associar mudanças no comportamento da mãe ao longo dos 10 primeiros dias pós-parto com a preferencia pelo odor do ninho em filhotes testados no labirinto em Y. Na segunda parte desta tese iremos abordar os efeitos da manipulação sobre a resposta ao estresse agudo e crônico em ratas que tiveram seus filhotes manipulados no período neonatal, para isso submetemos genitoras dos grupos controle e manipulado após o desmame a um dos dois protocolos descritos a seguir: com estresse (estresse por contenção de movimentos 1h/dia por 7 dias) ou sem estresse (nenhuma intervenção após o desmame) e testamos os animais através do teste de nado forçado para observar mudanças na resposta emocional. Também medimos os níveis de BDNF e corticosterona no plasma após o teste e medimos o peso das adrenais para verificar o efeito da manipulação na resposta ao estresse das genitoras

Os resultados dessa tese mostram que a manipulação neonatal afeta a estrutura do comportamento maternal, mudando a sequencia e a sincronia do comportamento da mãe com o filhote, o que poderia ser em parte a causa da alteração no comportamento de preferencia pelo odor do ninho observado em animais manipulados, principalmente nas fêmeas. Além disso, observamos que a manipulação afeta de forma duradoura a resposta ao estresse (agudo e crônico) das genitoras, podendo alterar a resposta emocional desses animais e predispor a sintomas do tipo depressivo em resposta ao estresse agudo.

Esses resultados reforçam a ideia de que o estudo dos efeitos duradouros da manipulação não só nos filhotes, mas também no organismo materno, podem servir como uma importante ferramenta para elaboração de projetos clínicos, visando a exploração da existência de comportamentos similares em humanos. Isso ajudará na elaboração de politicas de saúde publica que visem minimizar os efeitos de eventos adversos acontecidos no inicio da vida sobre a saúde física e mental tanto da mãe quanto da criança.

Abstract

Mammals are not born with fully developed nervous system, and the first days of life represent a critical stage in the development of this system. In fact, at this stage, the brain is undergoing many fundamental processes such as functional organization of neural networks, neuronal proliferation, migration, differentiation, gliogenesis and myelination.

In rats, a simple procedure such as "handling" the pups for a few minutes during the first week of life can decisively mark the development of the individual. Thus, neonatal handling has been widely used to examine the mechanisms by which environmental adversity can affect the development of the pups. Neonatal handling promotes a series of behavioral and neuroendocrine changes that are characterized primarily by a decrease of fear and stress responses in the adult. Although many authors characterize the handling procedure as a positive intervention, it is also associated with severe deficits in social and reproductive behaviors of the offspring that appear early during development and persist into adulthood. Apart from its effect on the pups, studies have shown that interventions in the offspring during the neonatal period can also induce long lasting effects in the maternal stress response, but this subject is still little explored in the literature.

Therefore, in the first part of this thesis, we will study the effects of neonatal handling on the mother-pups’ bond formation, trying to associate changes in maternal behavior over the first 10 days postpartum with the preference for the odor of the nest in pups tested the Y maze. The second part of this thesis will address the effects of neonatal handling on the acute and chronic stress response in dams that had their pups handled. For this purpose, we submitted mothers of control and manipulated groups after weaning to: stress (restraint 1h/day for 7 days) or no stress (no intervention after weaning) and tested the animals using the forced swim test to observe changes in emotional response. We also measured plasma BDNF and corticosterone levels after the test and the adrenals’ weight to verify the effect of handling on the dam’s stress response.

The results of this thesis show that neonatal handling affects the structure of maternal behavior, changing the behavioral sequence and synchrony of the mother with her pups, which could be in part the cause of the altered social behaviors observed in handled pups, especially in females. Moreover, we observed that handling affects the dam´s response to stress (acute and chronic), and may alter the emotional response of the dams increasing the susceptibility to developing psychiatric disorders such as depression at least in response to acute stress.

These results reinforce the idea that investigating the long lasting effects of handling not only in the young, but also in the dam’s physiology becomes an important tool for the development of clinical studies, aiming at exploring the existence of similar effects in humans. The final goal will be the elaboration of public health policy to minimize the effects of early life adverse events on physical and mental health of both mothers and their children.

Key Words – Neonatal Intervention, Maternal Behavior, Attachment, Nest odor Preference, Depression, Forced Swimming Test, Corticosterone.

1 INTRODUÇÃO

1.1 Período Neonatal

Em mamíferos o impacto de sair do ambiente uterino protegido e enfrentar estímulos ambientais desconhecidos depende da atuação de um cuidador. Esta é uma fase crítica para o desenvolvimento do sistema nervoso, quando o cérebro está sob um processo intenso de organização funcional, proliferação neuronal, migração e diferenciação, gliogênese e mielinização (Rice & Barone 2000). Durante esse período, a mãe é uma interface entre o mamífero recém-nascido e o ambiente (Korosi & Baram 2009). A mãe e o filhote estabelecem um relacionamento muito próximo, tornando-se difícil separar se estímulos ambientais podem afetar os filhotes diretamente ou através de mudanças no comportamento maternal (Korosi & Baram 2009). Em ratos, tanto os filhotes como as mães apresentam adaptações durante o período neonatal, que ajudam a proteger o desenvolvimento da prole.

1.1.1 Adaptações dos Filhotes no Período Neonatal

Durante as duas primeiras semanas de vida, em ratos, a concentração de corticosterona plasmática é baixa e permanece assim até aproximadamente o 14º dia de vida. Além disso, as concentrações hipofisárias de ACTH, e hipotalâmicas de CRH, também são diminuídas neste período. Assim, estímulos que normalmente induziriam o aumento de ACTH em adultos são incapazes de fazê-lo em animais neonatos, durante esta fase. Este período é chamado de período hiporresponsivo ao estresse (Levine 2001, Sapolsky & Meaney 1986).

Embora haja uma redução da resposta do eixo HPA à maior parte dos estressores no período hiporresponsivo, esta resposta é muito variável sendo alterada por fatores como, por exemplo, a idade do animal o tempo e o tipo de estressor ao qual ele é submetido (De Kloet et al 1998).

A resposta do Eixo Hipotálamo-Pituitária-Adrenal dos filhotes ao estresse sofre grande influência do cuidado materno. Filhotes submetidos à privação materna apresentarão uma resposta mais acentuada de ACTH e corticosterona, e essa influência pode persistir até a vida adulta do animal (Knuth & Etgen 2007, Levine 2001). Foi demonstrado que o eixo HPA de ratos neonatos responde a estímulos ambientais de uma maneira peculiar, diferente de um animal adulto (Dent et al 2000).

Em ratos, a administração de altas doses de glicocorticóides causa um decréscimo na mitose, na mielinização, na migração e também é capaz de alterar a neuromorfogênese (Hadoke et al 2006). Além disso, pode aumentar o risco ao desenvolvimento de doenças cardiovasculares nos adultos (Hadoke et al 2009). Sendo assim, a manutenção de uma baixa concentração de corticosterona durante este período da vida do rato é necessária para um desenvolvimento normal do animal (Levine 2001).

O período hiporresponsivo ao estresse é, então, um importante mecanismo para proteger o filhote de uma secreção aumentada de glicocorticóides, durante este período crítico de desenvolvimento cerebral (Sapolsky & Meaney 1986). Devido a este fator, o neonato necessita do cuidado maternal adequado nesta fase, para diminuir sua exposição a estressores (Sapolsky & Meaney 1986).

1.1.2 Adaptações das Mães no Período Neonatal

Em ratos, no período em torno do parto, ocorrem profundas alterações comportamentais e neuroendócrinas, sendo um pré-requisito para um desenvolvimento embrionário seguro, para o processo do parto, para o desenvolvimento do comportamento materno e da nutrição do recém-nascido de forma a garantir a sua sobrevivência (Broad et al 2006, Kojima & Alberts 2009, Sanchez-Andrade & Kendrick 2009, Sullivan 2001, Sullivan 2005, Sullivan & Wilson 2003, Wilson & Sullivan 1994). Neste período pode ocorrer por um lado, a ativação de sistemas que são necessários para processos reprodutivos como a lactação e o comportamento materno que envolvem principalmente a ocitocina, prolactina e opióides endógenos e por outro lado a desativação de sistemas não relevantes, como o do hipotálamo-pituitária-adrenal (HPA). Existe ainda a possibilidade destes mecanismos adaptativos neurobiológicos também serem necessários para a proteção do organismo materno contra as dramáticas mudanças hormonais que ocorrem nesse período e que de outra forma poderiam resultar em distúrbios emocionais, como a depressão pós-parto ou psicose pós-parto (Hillerer et al 2011).

Apesar de tanto a mãe como o filhote apresentarem adaptações que os protegem durante o período neonatal, eventos estressantes durante o período pós-natal poderiam predispor a prole ao desenvolvimento de diversos tipos de transtornos psiquiátricos na vida adulta (Heim & Nemeroff 2001). Além disso, os efeitos da exposição ao estresse no período neonatal no organismo materno são pouco conhecidos, mesmo que, em seres humanos, este período pareça ser especialmente vulnerável a perturbações externas (Llewellyn et al 1997, Steiner 1979). Cerca de 20-30% das mulheres desenvolvem um breve “Baby Blues” no período pós-parto, e a prevalência de depressão maior não-psicótica é cerca de 10% nos primeiros meses após o parto (Mastorakos & Ilias 2000).

1.2 Estresse no Período Neonatal

O estresse neonatal seria capaz de induzir mudanças neurobiológicas e comportamentais definitivas no fenótipo dos adultos (Cui et al 2004, Levine 2001, Vazquez et al 2005, Walker et al 2004, Walker et al 2003).

Uma das interfaces mais estudadas é a relação de alterações do comportamento da mãe em resposta as condições ambientais e suas implicações para o desenvolvimento dos filhos. Em humanos, negligência e alterações na qualidade do cuidado materno são um desafio para a saúde pública com efeitos graves em longo prazo para a saúde e desenvolvimento da criança (Strathearn 2011), desde déficits cognitivos no caso de falhas em prover alimento, roupas, abrigo, cuidados médicos e educação, até déficits afetivos no caso de falhas em prover atenção, afeto, contato e segurança emocional (Strathearn 2011) (Figura 1).

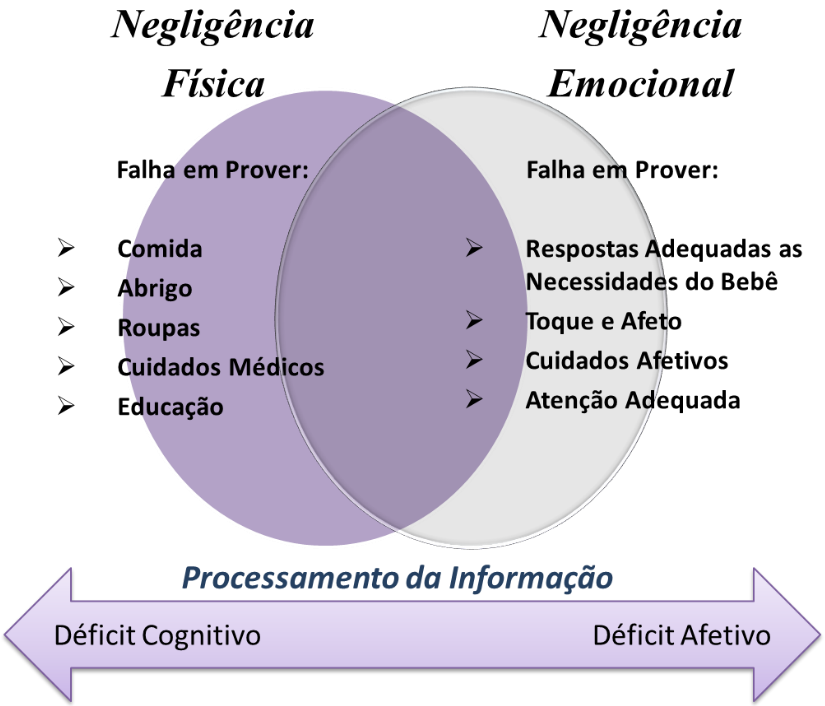


Figura 1 – Associação entre o cuidado maternal e as principais consequências para a criança em caso de negligencia (Figura adaptada de L. Strathearn, 2011).

O impacto de intervenções no ambiente neonatal também tem sido estudado utilizando vários modelos animais em que mudanças no comportamento da mãe parecem ter um papel chave neste processo (Meaney et al 1988, Poeggel et al 2003, van Oers et al 1998, Weaver et al 2004, Weaver et al 2002)

Mais de 50 anos de estudo têm explorado as implicações das mudanças no comportamento maternal sobre a programação neonatal e as consequências persistentes desta alteração em parâmetros comportamentais e neuroquímicos da prole na vida adulta (Avishai-Eliner et al 2001, Baram et al 2012, Barnett & Burn 1967, Fenoglio et al 2006, Korosi & Baram 2009, Korosi et al 2011, Levine & Lewis 1959, Meaney et al 1985a, Villescas et al 1977, Weaver et al 2002).

1.3 Manipulação Neonatal

A manipulação neonatal é um procedimento experimental envolvendo breves períodos de separação materna e estimulação tátil dos filhotes sem dor ou jejum (Noschang et al 2012, Raineki et al 2013, Reis et al 2014, Veenema 2012, Zhang et al 2012). Primeiro, a caixa moradia com a mãe e toda ninhada é levada do nosso biotério setorial para uma sala anexa com o mesmo período de luz e temperatura. Em seguida, a mãe é removida e colocada gentilmente em outra caixa. O experimentador manipula toda ninhada de uma vez com cuidado, utilizando as duas mãos cobertas com luvas de látex por 1 min. Após o protocolo de manipulação, todos os filhotes são colocados novamente em sua caixa moradia, a mãe é colocada de volta e a caixa moradia é devolvida para o seu lugar dentro do biotério onde permanecerá sem ser perturbada até o mesmo horário do dia seguinte. As ninhadas são manipuladas do primeiro ao décimo dia pós-parto (DPP 1 ao 10), durante o período de luz do ciclo de fotoperíodo diário (10:00-12:00) e o tempo total da separação das genitoras do filhotes é de cerca de 1 min e 30 s.

Esta intervenção repetida na relação mãe-filhote afeta a prole adulta de muitas maneiras, como através da redução do medo (Meerlo et al 1999, Padoin et al 2001, Severino et al 2004) e da resposta da corticosterona a uma variedade de estressores (Liu et al 2000, Plotsky & Meaney 1993). Esta intervenção também pode diminuir o comportamento sexual e fertilidade em ratos machos e fêmeas (Gomes et al 2006a, Gomes et al 2006b, Gomes et al 1999, Raineki et al 2008). Além das mudanças comportamentais e neuroendócrinas, a manipulação neonatal pode também alterar a plasticidade central através da sinalização neurotrófica, produzindo mudanças estruturais duradouras no encéfalo (Bodnoff et al 1987, Camozzato et al 2009, Lucion et al 2003, Todeschin et al 2009, Winkelmann-Duarte et al 2007).

1.3.1 Manipulação Neonatal e Comportamento Maternal

Assim como acontece em humanos, mudanças no comportamento maternal poderiam estar causalmente relacionadas aos desfechos observados nos filhotes. (Liu et al 1997).

Sabe-se que esse comportamento da mãe em relação ao filhote pode alterar o desenvolvimento do SNC (Francis et al 1999, Levine 2001, Liu et al 2000, Weaver et al 2004, Weaver et al 2002). Variações naturais no cuidado maternal também alteram permanentemente o comportamento e neuroquímica dos filhotes (Champagne et al 2003, Francis et al 1999, Weaver et al 2004, Weaver et al 2002). Animais filhos de mães muito lambedoras apresentam diminuição da resposta ao estresse e menor ansiedade, de forma persistente, assim como nos filhotes manipulados (Liu et al 2000, Liu et al 1997, Plotsky &

Meaney 1993). Liu *et al.* (1997) mostraram que filhotes cujas mães permanecem mais tempo no ninho e apresentam aumento na frequência do comportamento de lambida, quando adultos desenvolvem um aumento da concentração de receptores para glicocorticóides no hipocampo e uma menor secreção de ACTH e corticosterona em resposta ao estresse. Esses dados também são similares aos dos animais que foram manipulados no período neonatal (Levine 1994, Meaney et al 1993, Plotsky & Meaney 1993).

Animais cujas mães são “muito lambedoras” apresentam um aumento na taxa de sinaptogênese ou aumento na sobrevivência das sinapses no hipocampo comparados a animais cujas mães têm um baixo cuidado com a sua prole (Liu et al 2000). Sendo assim, a variação no cuidado maternal pode ser considerada o diferencial para as experiências sensoriais no desenvolvimento dos filhotes.

1.4 Controle do Comportamento Maternal

Os comportamentos maternos que envolvem movimentos mais ativos, como lamber e construção do ninho, e comportamentos que são quiescentes como as posturas de amamentação, são controlados por diferentes regiões do sistema nervoso central que têm interações opostas com o sistema dopaminérgico (Cummings et al 2010) (Figura 2). Comportamentos maternais ativos são estimulados por receptores dopaminérgicos na concha do núcleo accumbens (NAs) (Cummings et al 2010). Em contraste, comportamentos quiescentes são inibidos pelos receptores dopaminérgicos, sugerindo que o início de um comportamento quiescente requer uma diminuição no tônus dopaminérgico no NAs (Keer & Stern 1999).

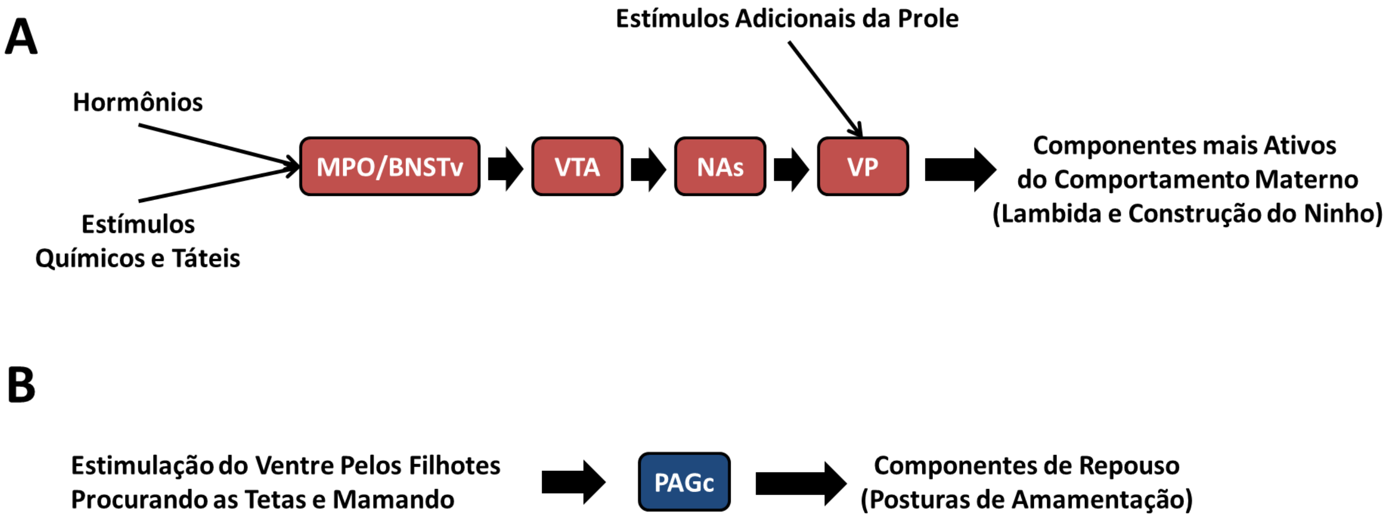


Figura 2 – Mecanismos de controle do comportamento maternal (Figura adaptada de Cummings et al 2010). MPO – Área Pré-optica Medial, BNSTv – Núcleo da estria terminal ventral, VTA – Área Tegmentar Ventral, NAs – Concha do Núcleo Accumbens, VP – Pálido Ventral, PAGc – Substancia Cinzenta Periaquedutal.

Poderíamos inferir que a manipulação dos filhotes induz um aumento da atividade dopaminérgica no núcleo accumbens em função do aumento do comportamento de lambida já relatado em trabalhos anteriores de nosso laboratório (Azevedo et al 2010). Levando em conta a combinação deste aumento de atividade com uma possível redução da atividade dos filhotes após a manipulação esperamos encontrar um aumento no comportamento de lambida por parte da mãe logo após o protocolo de manipulação assim como em outros trabalhos (Azevedo et al 2010, Smotherman et al 1977), seguido por uma redução no comportamento de amamentação. A associação destes dois fatores pode ser a causa das alterações em comportamentos sociais causadas pela manipulação neonatal (Raineki et al 2013, Todeschin et al 2009).

1.4.1 Comportamento Maternal e o Aprendizado Olfatório

Durante o inicio da vida, a mãe e a criança estabelecem uma relação muito próxima. Sistemas sensoriais, auditivo e visual, estão fortemente implicados neste processo, mas durante o período neonatal, o aprendizado olfativo é um fator chave para o estabelecimento de vínculo, especialmente em mamíferos de cérebro pequeno, tais como roedores (Broad et al 2006, Kojima & Alberts 2009, Okabe et al 2012, Sullivan 2005, Sullivan & Wilson 2003, Wilson & Sullivan 1994).

Em ratos, os filhotes aprendem a identificar a mãe através de um processo semelhante ao paradigma de condicionamento clássico, envolvendo estimulação tátil promovida pela mãe (estímulo incondicionado) e o odor da mãe (estímulo condicionado) (Kojima & Alberts 2009, Moriceau & Sullivan 2004, Sullivan 2001, Sullivan & Wilson 2003, Wilson & Sullivan 1994). O bulbo olfatório (BO) e lócus coeruleus (LC) são estruturas importantes no mecanismo de aprendizado olfativo (Chiaramello et al 2007, Gascon et al 2007, Imamura & Greer 2009, Tran et al 2013). O processo de aprendizado olfatório produz mudanças metabólicas e anatômicas no bulbo olfatório que são mantidas ao longo da vida do animal (Sullivan 2005), e este processo está intimamente relacionado com a atividade noradrenérgica do LC (Moriceau & Sullivan 2004).

A noradrenalina do LC tem um papel fundamental no desenvolvimento e modificação do sistema sensorial de mamíferos (Sullivan et al 1994). Como mostra a figura 3, uma das áreas que recebe densa projeção noradrenérgica do LC é o bulbo olfatório (BO), área essa que apresenta receptores β-adrenérgicos funcionais durante as primeiras semanas de vida dos ratos (McLean & Shipley 1991, Wilson & Leon 1988).

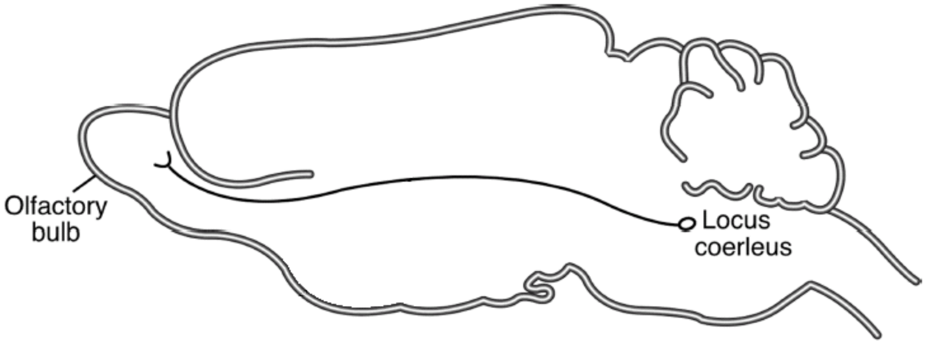


Figura 3 – Representação esquemática da via noradrenérgica do LC para o BO. Para o filhote aprender a ter preferência por um odor é necessário o pareamento deste odor com a liberação de NA proveniente do LC (Modificado de Sullivan 2003).

Rangel and Leon (1995) demonstraram que a estimulação tátil com pincel é capaz de promover um aumento no conteúdo de NA no BO. De fato, a estimulação do LC com acetilcolina, pareado com a apresentação de um novo odor, induz a preferência por esse odor no filhote (Sullivan et al 2000). No entanto, lesão bilateral do LC em filhotes, com 6-OHDA, reduz o conteúdo de NA no BO impedindo a formação do aprendizado olfatório nesses animais (Sullivan et al 1994).

Portanto, logo após o nascimento, quando a mãe lambe o filhote (estimulação tátil) ela promove liberação de NA pelo LC que age no BO e promove o aprendizado olfatório, fazendo com que o filhote tenha preferência pelo cheiro da mãe.

A ligação da NA aos receptores β-adrenérgicos do BO (Langdon et al 1997, Sullivan et al 2000, Sullivan et al 1989) promove um aumento da concentração de AMPc (Adenosina monofosfato cíclico) que é um segundo mensageiro intracelular, que por sua vez, ativa uma proteína cinase dependente de AMPc (PKA). A PKA atua fosforilando inúmeros substratos intracelulares, entre eles o fator de transcrição CREB (proteína ligante ao elemento responsivo ao AMPc), cuja fosforilação ocorre na serina 133, passo fundamental para a ativação desse fator de transcrição (Lamprecht 1999, Silva et al 1998).

O CREB pertence a uma família de fatores de transcrição denominada CREB/ATF e participa de eventos de sinalização intracelular que regulam uma grande variedade de funções biológicas, incluindo a proliferação celular na pituitária (Struthers et al 1991), diferenciação sexual do SNC (Auger et al 2001), espermatogênese (Don & Stelzer 2002, Scobey et al 2001) e formação de memória (Cammarota et al 2000, Silva et al 1998).

A ativação transcricional mediada por CREB depende da presença de uma proteína nuclear denominada CBP (proteína ligante ao CREB), a qual se liga especificamente à forma fosforilada do CREB. Após, o complexo CREB/CBP liga-se a regiões especificas do ADN, pois as proteínas pertencentes à família de fatores de transcrição CREB/ATF apresentam, em sua estrutura, domínios para a ativação da transcrição e regiões que permitem a dimerização e a ligação ao ADN, os quais são responsáveis pela ligação à sequência CRE (elemento responsivo ao AMPc), permitindo o início da transcrição de genes que apresentam uma sequência que responde ao estímulo por CREB nos seus promotores (Lamprecht 1999, Silva et al 1998).

Embora não existam relatos sobre o efeito da manipulação neonatal sobre a morfologia do bulbo olfatório, estudos mostram que a manipulação repetida até o sétimo dia pós-parto promove mudanças importantes no sistema monoaminérgico dos filhotes no dia 7, além de serem encontradas também diferenças nos níveis de CREB no bulbo olfatório [(Raineki et al 2009) e Reis, A.R. 2010, Dissertação de Mestrado].

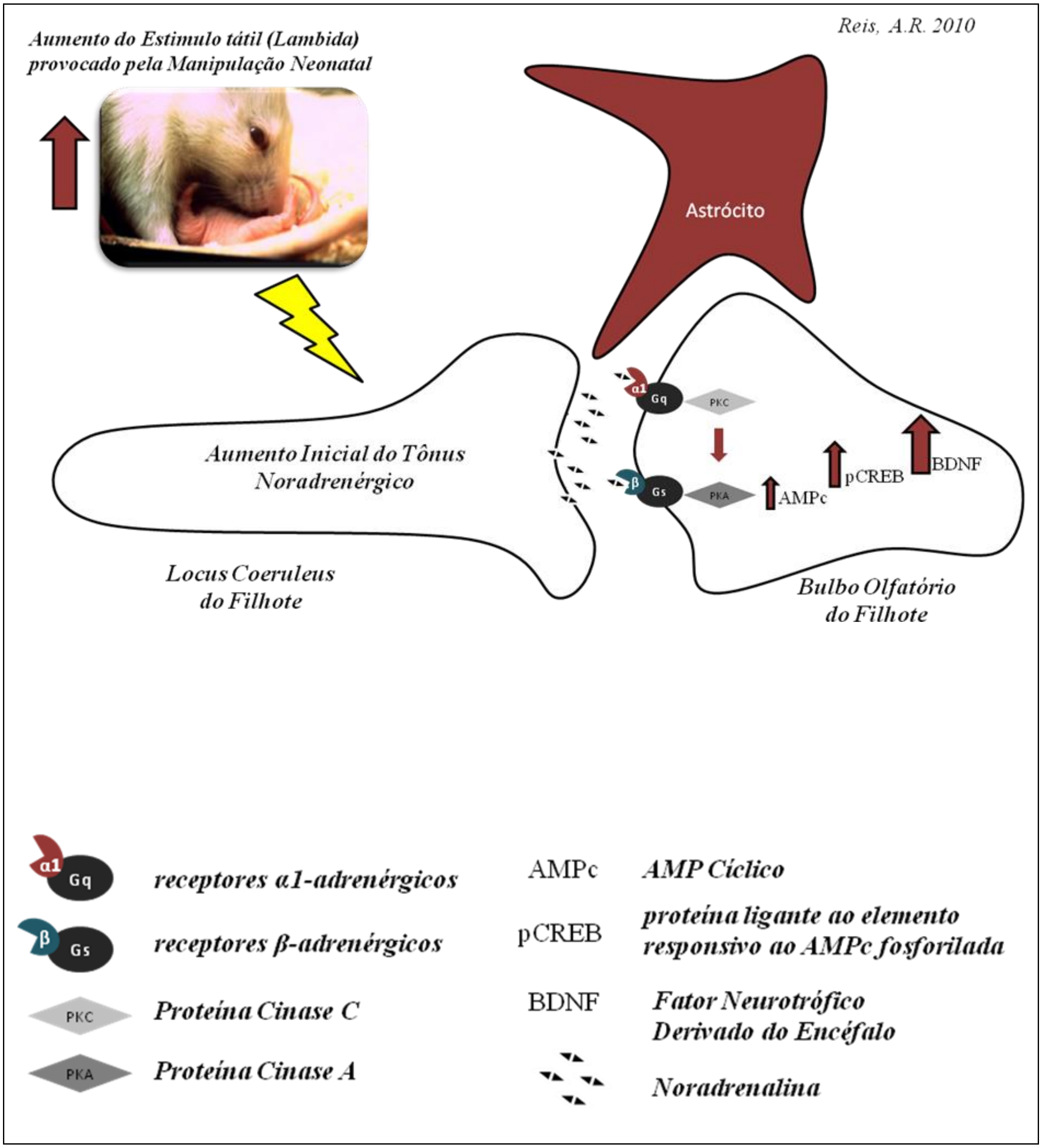
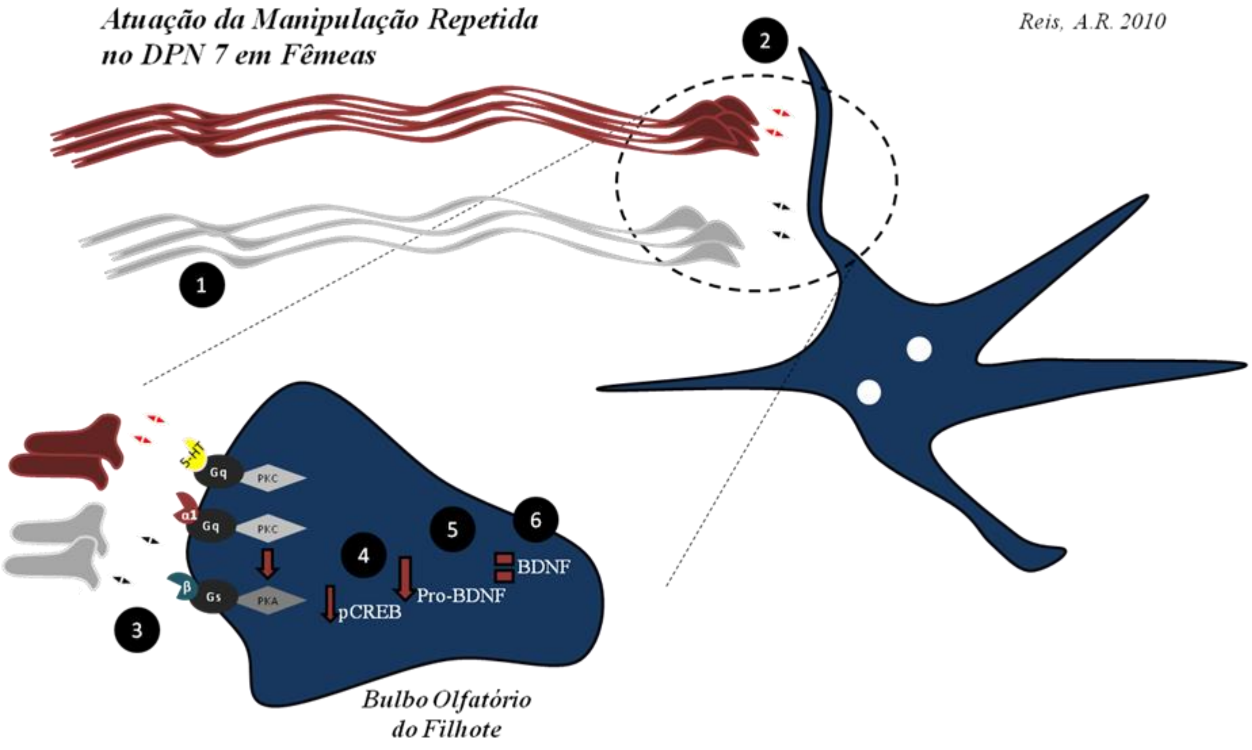


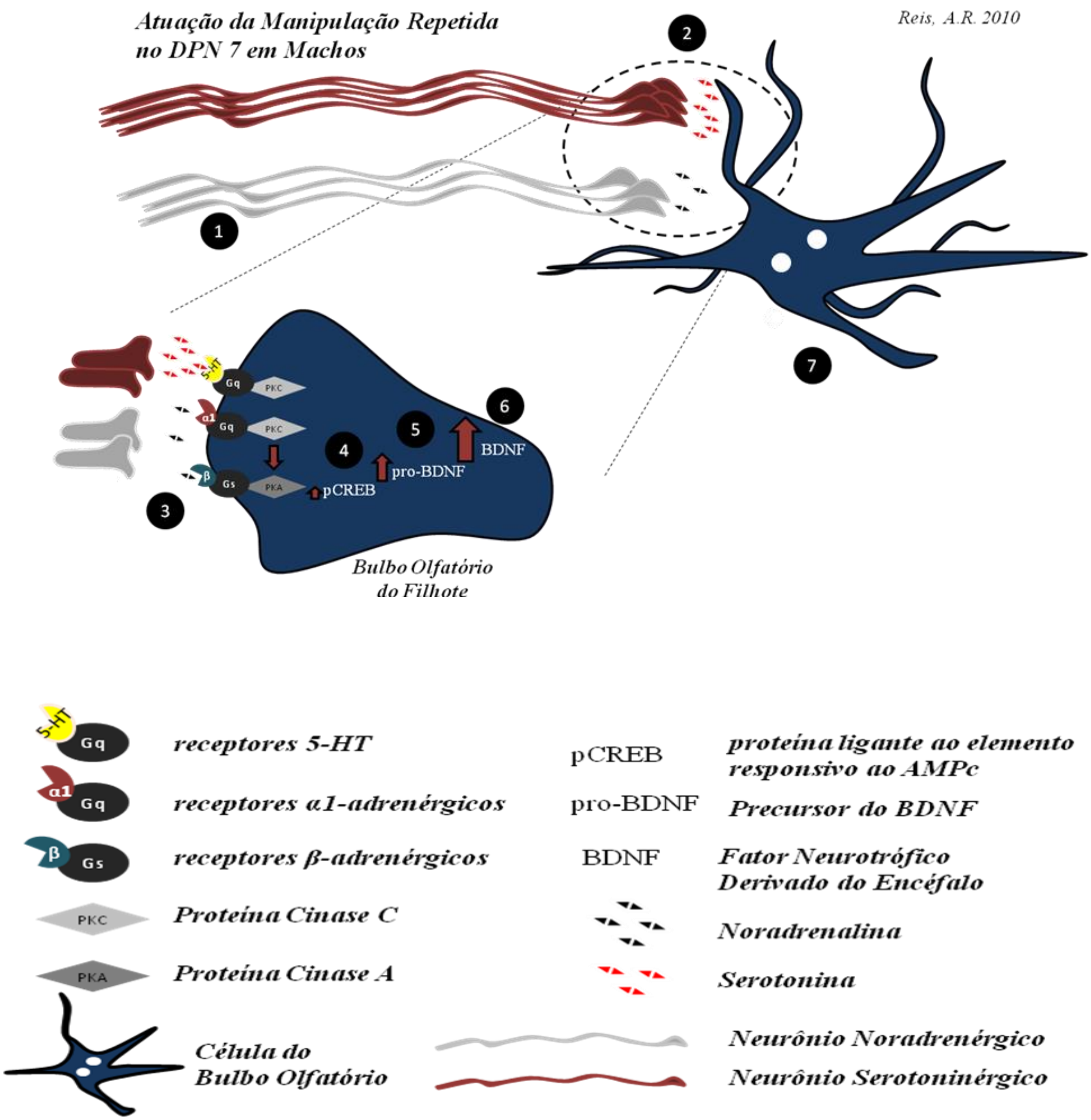
Figura 4 - Representação esquemática da via noradrenérgica do LC para o BO mostrando a possível atuação do comportamento da mãe no funcionamento da via e na produção de BDNF no bulbo olfatório dos filhotes após o protocolo de manipulação.

Essas alterações no sistema monoaminérgico e na fosforilação do CREB induzidas pela manipulação neonatal e sua atuação no comportamento da mãe, poderiam alterar o sinal de BDNF e promover alterações na estrutura do bulbo olfatório, como acontece em outras áreas do cérebro (Figura 4).

Reis, A.R. 2010, fez medidas em tempos diferentes e em pontos diferentes da cascata de produção do BDNF os dados se apresentam sólidos e demonstram uma clara diferença no efeito da manipulação neonatal em machos e em fêmeas no período neonatal (Figura 5). Embora ainda não se saiba ao certo qual o motivo deste dimorfismo sexual na resposta ao protocolo de manipulação neonatal, acreditamos que a resposta possa estar em parte no comportamento da mãe. Com esse intuito, neste trabalho também iremos avaliar o comportamento de preferência pelo odor do ninho nos filhotes, na tentativa de associá-lo com mudanças no comportamento de suas respectivas genitoras.

Figura 5 – Mecanismo proposto para as modificações encontradas no sinal de BDNF nas fêmeas e nos filhotes machos promovidas pela manipulação repetida até o DPN 7. Os resultados estão detalhados em Reis, A.R. 2010 (Dissertação de Mestrado).




Atuação da Manipulação Repetida
no DPN 7 em Machos
Reis, A.R. 2010
1
2
3
4
5
6
7
5-HT
Gq
PKC
α1
Gq
PKC
β
Gs
PKA
pCREB
pro-BDNF
BDNF
Bulbo Olfatório
do Filhote
5-HT Gq
receptores 5-HT
α1 Gq
receptores α1-adrenérgicos
β Gs
receptores β-adrenérgicos
PKC
Proteína Cinase C
PKA
Proteína Cinase A
Célula do
Bulbo Olfatório
pCREB
proteína ligante ao elemento
responsivo ao AMPc
pro-BDNF
Precursor do BDNF
BDNF
Fator Neurotrófico
Derivado do Encéfalo
Noradrenalina
Serotonina
Neurônio Noradrenérgico
Neurônio Serotoninérgico

2 JUSTIFICATIVA

A manipulação neonatal pode alterar várias respostas comportamentais induzindo a redução de comportamentos sociais e comprometer o desenvolvimento normal de diversas estruturas cerebrais nos filhotes. Em humanos, alterações como as que ocorrem nos modelos animais poderiam estar relacionadas com o aparecimento de diversos distúrbios psiquiátricos e neurológicos na vida adulta. A utilização de modelos animais como a manipulação que simulam eventos adversos no inicio da vida podem ajudar a desvendar os mecanismos pelos quais essa predisposição ocorre no adulto. Assim o estudo da influência do cuidado materno e do ambiente neonatal no desenvolvimento do sistema nervoso é de vital importância para proporcionar uma melhor compreensão da relação do ambiente com o desenvolvimento de distúrbios psiquiátricos.

Além do ponto de vista da prole, a utilização de modelos animais para verificar os efeitos do estresse neonatal no organismo materno também é de grande importância uma vez que em seres humanos, este período parece ser especialmente vulnerável a perturbações externas. O estudo do efeito de eventos adversos nas respostas emocionais das mães neste período irá levar a uma melhor compreensão dos mecanismos pelos quais as genitoras desenvolvem susceptibilidade a transtornos psiquiátricos como a depressão.

Investigar os mecanismos pelos quais ocorrem essas alterações associadas a mudanças do comportamento da mãe pode servir como um importante subsídio para elaboração de estudos clínicos, visando à exploração da existência de comportamentos similares em humanos com objetivo final de elaborar politicas de saúde publica que visem minimizar os efeitos de eventos adversos acontecidos no inicio da vida sobre a saúde física e mental tanto da mãe quanto da criança.

3 OBJETIVOS

3.1 Objetivo Geral

Esta tese irá tratar do efeito de uma breve separação com manipulação da ninhada no período neonatal sobre a formação do vínculo mãe-filhote em ratos e suas implicações para o comportamento social dos filhotes nos primeiros de 10 dias de vida.

Além disso, iremos discutir o efeito do estresse neonatal no organismo materno, desde seu efeito a curto prazo, sobre o comportamento maternal, até seu efeito a longo prazo, sobre a resposta ao estresse (agudo e crônico) das genitoras após o desmame.

3.2 Objetivos Específicos

Experimento 1 - Verificar se a manipulação neonatal altera o padrão diário dos componentes do comportamento materno não limitado a mudanças no comportamento de lambida. Finalmente, com a expectativa de associar as mudanças no comportamento maternal com mudanças no comportamento social dos filhotes, analisamos o comportamento de preferência pelo odor do ninho no DPP 11.

I. Verificar se a manipulação neonatal altera o comportamento maternal nos 10 primeiros dias de vida dos filhotes por meio de 4 sessões diárias de observação (Antes da manipulação, logo após a manipulação, 3 horas após a manipulação e 5 horas após a manipulação) sendo 50 registros por observação, 200 registros por dia.

II. Verificar se o protocolo de manipulação altera a preferência natural dos filhotes pelo odor do ninho no DPP 11 por meio de um labirinto em Y onde de um lado será colocado maravalha do ninho e do outro maravalha limpa. Foram feitas 5 sessões de teste com cada animal.

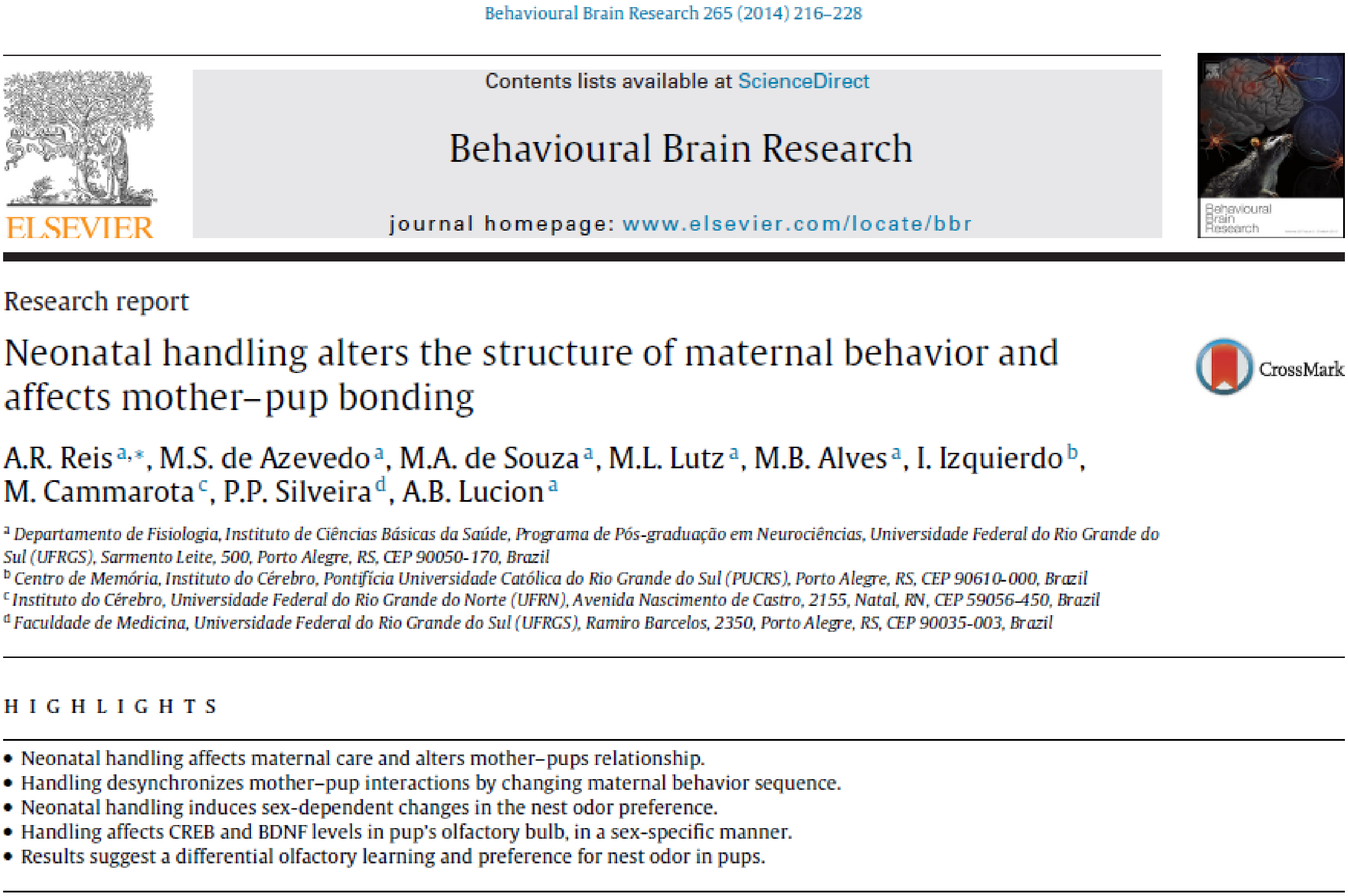
Experimento 2 - Verificar se a manipulação neonatal altera a resposta ao estresse (agudo e crônico) e sensibiliza as mães dos animais que foram manipulados ao desenvolvimento de transtornos psiquiátricos como depressão após o desmame como detalhado nos tópicos abaixo:

I. Verificar se o protocolo de manipulação promove alterações duradouras na resposta emocional de ratas que tiverem os filhotes manipulados, induzindo o aparecimento de comportamentos do tipo depressivo no teste de nado forçado. Os animais foram testados nos DPP 29 e 30 (7 dias após o desmame).

II. Verificar se a manipulação dos filhotes no período neonatal afeta de forma duradoura a resposta ao estresse agudo e crônico das genitoras após o desmame dos filhotes. Para este fim medimos os níveis basais de BDNF e corticosterona no plasma, além disso, verificamos o peso das adrenais 1 dia após o teste de nado forçado (DPP 31).

4 COLETANEA DE ARTIGOS

4.1 Capítulo 1 – Resultados do Experimento 1



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Research report

Neonatal handling alters the structure of maternal behavior and affects mother–pup bonding

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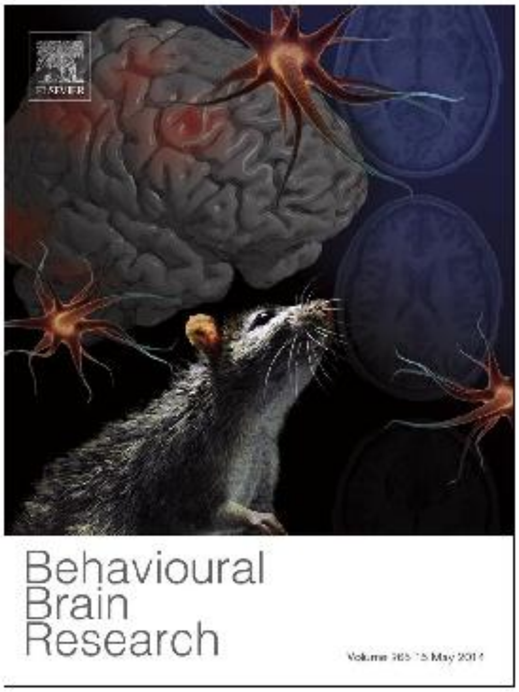
HIGHLIGHTS

- Neonatal handling affects maternal care and alters mother–pups relationship.
- Handling desynchronizes mother–pup interactions by changing maternal behavior sequence.
- Neonatal handling induces sex-dependent changes in the nest odor preference.
- Handling affects CREB and BDNF levels in pup's olfactory bulb, in a sex-specific manner.
- Results suggest a differential olfactory learning and preference for nest odor in pups.



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Brain
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Neonatal Handling Alters the Structure of Maternal Behavior and Affects Mother-Pup Bonding

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ABSTRACT

During early life, a mother and her pups establish a very close relationship, and the olfactory learning of the nest odor is very important for the bond formation. The olfactory bulb (OB) is a structure that plays a fundamental role in the olfactory learning (OL) mechanism that also involves maternal behavior (licking and contact). We hypothesized that handling the pups would alter the structure of the maternal behavior, affect OL, and alter mother-pup relationships. Moreover, changes in the cyclic AMP-response element binding protein phosphorylation (CREB) and neurotrophic factors could be a part of the mechanism of these changes. This study aimed to analyze the effects of neonatal handling, 1 minute per day from postpartum day 1 to 10 (PPD 1 to PPD 10), on the maternal behavior and pups’ preference for the nest odor in a Y maze (PPD 11). We also tested CREB´s phosphorylation and BDNF signaling in the OB of the pups (PPD 7) by Western Blot analysis. The results showed that handling alters mother-pups interaction by decreasing mother-pups contact and changing the temporal pattern of all components of the maternal behavior especially the daily licking and nest-building. We found sex-dependent changes in the nest odor preference, CREB and BDNF levels in pups OB. Male pups were more affected by alterations in the licking pattern, and female pups were more affected by changes in the mother-pup contact (the time spent outside the nest and nursing).

Key Words – Neonatal Intervention, Neurotrophic Signaling, CREB, Attachment, Olfactory Learning

1-INTRODUCTION

In mammals, the impact of leaving the safe uterine environment and facing many unfamiliar environmental stimuli and risks requires the protection of a caregiver. During the neonatal period, the mother is an interface between the newly born mammal and the environment and can powerfully shape infant development (Korosi & Baram 2009). Early life is a critical phase for the nervous system, when the brain undergoes functional organization, neuronal proliferation, migration and differentiation, gliogenesis and myelination (Rice & Barone 2000). More than 50 years of study have explored the implications of changes in maternal behavior on neonatal programming and its persistent consequences on behavioral and neurochemical outcomes later in life (Avishai-Eliner et al 2001, Baram et al 2012, Barnett & Burn 1967, Claessens et al 2012, Fenoglio et al 2006, Korosi et al 2011, Levine & Lewis 1959, Miranda et al 2011, Villescas et al 1977, Young et al 1965).

During early life, mother and infant establish a very close relationship. Auditory and visual sensory systems are heavily implicated in this process but during the neonatal period, olfactory learning is a key factor for the attachment establishment, especially in small-brain mammals such as rodents (Broad et al 2006, Kojima & Alberts 2009, Okabe et al 2012, Sanchez-Andrade & Kendrick 2009, Sullivan 2005, Sullivan & Wilson 2003, Wilson & Sullivan 1994). In rats, the pups learn how to identify the mother through a process similar to the classic paradigm of conditioning, involving tactile stimulation from the dam (unconditioned stimulus) and the dam’s odor (conditioned stimulus) (Kojima & Alberts 2009, Moriceau & Sullivan 2004, Sullivan 2001, Sullivan 2005, Wilson & Sullivan 1994). The olfactory bulb (OB) and the locus coeruleus (LC) are important structures in the olfactory learning mechanism (Moriceau et al 2010, Moriceau et al 2009, Rangel & Leon

1995, Sullivan & Wilson 2003), enabling the rat pup, born deaf and blind, to direct its behavior toward the mother (Moriceau et al 2009). Tactile stimulation can activate pups' LC, which increases Noradrenaline (NA) in the olfactory bulb mitral cells (Rangel & Leon 1995). This association activates a chain of events in the mitral neurons of the pup's OB increasing the phosphorylation of CREB (cyclic AMP-response element binding protein), which is responsible for the transcription of a variety of factors (including brain derived neurotrophic factor [BDNF]) that lead to biochemical and morphological changes in memory formation (McLean & Harley 2004, McLean et al 1999, Moriceau & Sullivan 2005, Raineki et al 2010, Sullivan & Wilson 2003, Yuan et al 2003). BDNF appears to be a key factor in olfactory association learning (Zimmerberg et al 2009). BDNF gene expression increases in response to several stimuli, including neurotransmitters signaling and CREB phosphorylation (Tao et al 1998); for a review, see (Binder & Scharfman 2004) and is critical in the OB morphologic development (Cao et al 2007, Chiaramello et al 2007, Gascon et al 2007, Imamura & Greer 2009, Matsutani & Yamamoto 2004, Tran et al 2008).

Neonatal handling is an experimental procedure that involves brief maternal separation and tactile stimulation, which is extensively used to investigate the effects of early life interventions on behavioral and endocrine alterations. This repeated disruption in the mother-pup relationship reduces fear (Padoin et al 2001), alters HPA axis (Hypothalamic-Pituitary-Adrenal) response to a variety of stressors (Liu et al 2000, Meaney et al 1985a, Meaney et al 1985b, Meaney et al 2007, Plotsky & Meaney 1993) and may also affect social behaviors and fertility in both male and female rats (Gomes et al 1999, Raineki et al 2009, Raineki et al 2013, Raineki et al 2008). In addition to the behavioral and neuroendocrine aspects of these changes, neonatal handling alters the brain plasticity and neurotrophic signaling, thus producing long-lasting structural changes (Lucion et al 2003, Todeschin et al 2009, Winkelmann-Duarte et al 2011).

Previous studies from our laboratory have shown that the handling procedure reduces the pup’s preference for the nest odor in a sex depend way (Raineki et al 2009, Raineki et al 2013). This lack of preference may be due to an alteration in the olfactory learning mechanisms; changes in the NA activity and CREB´s phosphorylation in the OB of 7-day-old rat pups suggest that this hypothesis could be right (Raineki et al 2009). BDNF is the perfect candidate to test this hypothesis since it is implicated in plasticity, dendritic branching, neuronal survival, migration and differentiation and axonal competition in this area during the neonatal period (Cao et al 2007, Chiaramello et al 2007, Gascon et al 2007, Imamura & Greer 2009, Matsutani & Yamamoto 2004, Tran et al 2008).

Other studies using early handling show that neurotrophic factors like BDNF play a key role in the establishment of these changes and also point out sex dependent changes (Garoflos et al 2005b, Garoflos et al 2007, Garoflos et al 2008) . However, is still not clear if the handling procedure could affect the neurotrophic signaling in the olfactory bulb of rat pups and if these changes could also present a sex dependent pattern like in other brain areas.

Therefore, our hypothesis is that neonatal handling alters the daily pattern of maternal behavior components beyond the licking component, and that these changes are part of the mechanism that alters BDNF gene expression through sex-dependent modifications in CREB phosphorylation and production in the pup's OB. For that, we analyzed the effects of handling on CREB phosphorylation and BDNF levels in the olfactory bulb of 7-day-old rat pups to verify whether the alterations in CREB phosphorylation are transient or are translated into protein alterations (changes in BDNF levels), which would indicate a more prolonged effect. Finally, expecting to associate the changes in maternal behavior with the pups' social behavior, we also analyzed the pups' social behavior with the nest odor preference test on PPD 11 to confirm whether the biochemical alterations in the olfactory bulb would affect social behavior already in early life.

2-EXPERIMENTAL PROCEDURES

2.1-Animals

Pregnant female Wistar rats were brought from the colony of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil) to the animal room in our laboratory. Approximately 7 days before delivery, the females were housed individually, and the presence of the pups was checked twice daily. Birth was considered to be day 0, and on postpartum day 1 (PPD 1), the number of pups was culled to 8 per dam by randomly removing a few pups while ensuring minimal contact with the remaining rats, the sex of the pups were not considered in this procedure. All of the animals were maintained on a 12-h light/dark cycle with the lights on at 6 a.m. The room temperature was 22 ± 1 °C, and water and food (Rodent chow, Nutrilab, Colombo, Brazil) were available at all times. Cage bedding was not changed from PPD 0 to 10. The experiments were performed in accordance with the National Institutes of Health (NIH) and Colégio Brasileiro de Experimentação Animal (COBEA) guidelines. These guidelines were designed to minimize the discomfort of animals and were approved by the Ethics in Research Committee of Federal University of Rio Grande do Sul (Process CEP/UFRGS nº 2007937 and nº 19759) and followed Brazilian legislation.

2.2-Neonatal Handling

Pups were handled for 1 min per day from PPD 1 to PPD 10 for behavioural studies while for western blot analysis this procedure lasted until PPD 7. First, the home cage containing the mother and the litter was moved to a quiet room next to the animal facility and were given the same light period and temperature as described above. Then, the mother was removed from the home cage and placed into another cage. The experimenter gently handled all of the pups at the same time using both hands, covered with fine latex gloves, for 60 s. No apparent harm was inflicted to the pups; they were simply touched. After handling, all of the pups were taken to the nest at the same time, and the mother was placed back inside the home cage. The home cage was then returned to the animal facility room and left undisturbed until the same time the next day. The pups were handled during the light period of the daily photoperiod cycle (10:00-12:00) at a distance of approximately 100 cm from the mother. The total time of the mother–infant separation was approximately 90 s (Gomes et al 1999, Padoin et al 2001, Raineki et al 2009, Raineki et al 2013, Raineki et al 2008, Todeschin et al 2009).

2.3-Experiments and Groups

In the first experiment, we analyzed the effect of neonatal handling on maternal behavior. Litters were divided into 2 groups based on the handling procedure: the non-handled group or control group (NH, n=9), in which the pups were left undisturbed with their mother during the first 10 postnatal days, and the repeatedly handled group (RH, n=9), in which the pups were handled as described above from PPD 1 to 10.

In the second experiment, the litters that originated from the experiment one (NH, n=9; RH, n=9) were used for the odor of the nest preference test to analyze the social behavior of the pups on the PPD 11.

In the third experiment, the molecular mechanism in the OB related to maternal behavior was analyzed on PPD 7. Previous studies showed changes in the monoaminergic system after the handling procedure on that day (Raineki et al 2009). A total of 48 pups (24 males and 24 females) from the two experimental groups described above (NH; n=12 from each sex and RH; n=12 from each sex) were divided into 4 subgroups based on the time of tissue collection for western blot analysis (n=6 of each sex in all groups). Two samples of tissue were obtained in both groups, 30 and 120 minutes after the handling procedure.

2.4-Experiment 1: Maternal Behavior

From the 1st to the 10th PPD, the maternal behavior was scored daily using a procedure adapted from previous studies (Caldji et al 1998, Champagne et al 2003, Francis et al 1999, Liu et al 1997, Uriarte et al 2007). Four 72-minute recording sessions were conducted. The recording sessions occurred at regular times with 3 periods during the light phase (9:00, 12:00 and 15:00) and 1 period during the dark phase (18:00) of the light-dark cycle. Within each observation session, the behavior of the mother at that specific moment was scored every 1 minute and 30 seconds. Thus, we had 50 observations per session in 4 periods per day, rendering a total of 200 observations per mother per day. Usually, only one behavior was recorded in each observation sample; however, 2 simultaneous behaviors (for example, nursing and licking) were also recorded. The data were expressed as the number of observations in which the pups received the target maternal behavior during the 10 postpartum days (the total number of events was 2000).

The target maternal behaviors were as follows: (1) licking any pup (the body surface and/or anogenital region), (2) nest building, (3) going outside of the nest, (4) carrying pups (retrieving the pups and placing them in the nest), and (5) nursing pups (in either a high arched-back posture or a low arched-back posture, in which the mother lays over the pups, or a passive posture, in which the mother is lying either on her back or side while the pups suckle). A detailed description of the behaviors is provided in Myers et al (1989) and Champagne et al (2003).

2.5-Experiment 2: Nest Odor Preference Test

The social behavior of the pups was tested in the 11th PPD with a protocol adapted from (Raineki et al 2009, Raineki et al 2013). Briefly, the maternal odor preference test was a two-odor choice between areas with the nest or fresh bedding. A Y-maze (neutral arm 15x10 cm, test arms 20x10 cm) was used to perform this test. In each area, 300 ml of fresh or nest bedding were placed in adjacent arms. At PPD 11, a pup was removed from the nest and placed in the neutral arm of the Y-maze. All of the sessions were videotaped and further analyzed by a researcher who was blind to the experimental groups. During the 1-min trial, the amount of time that the pup (whole body) spent over each of the two arms was recorded. We also recorded the time that was spent in reaching the nest area. Animals were tested for 5 trials, with an inter-trial interval of 2 min, during which the maze was cleaned with 70% ethanol to remove trace odors. We used one male and one female pup from each litter, and the order of the test was counterbalanced across litters to prevent any effect from the testing order. In each trial, the nest or fresh bedding was switched between the different arms of the Y-maze. The video recordings were later analyzed using the Noldus Observer software (Noldus Information Technology, Wageningen, Netherlands).We analyzed the time in each area in each trial, the percentage of total time over the areas and the latency to reach the nest area. The total time in each area was obtained by calculating the sum of the five trials, and the data were expressed as the percentage of time that the animal spent over the bedding areas (fresh or the nest). The latency to reach the nest area was obtained by calculating the mean of the five trials, and the data were expressed in seconds.

2.6-Experiment 3: Western Blot Analysis

The levels of BDNF, CREB and pCREB were analyzed on PND 7 (postnatal day 7), which is the optimal day for expressing odor preference learning for the maternal odor (Moriceau & Sullivan 2005, Raineki et al 2010, Sullivan & Holman 2010) by western blot analysis, as described by Raineki et al (2009). Briefly, pups were rapidly decapitated immediately after taking them from the home cage. The OBs were removed and immediately frozen in a container with isopentane in dry ice and stored at -70 °C. They were homogenized using 500 μL of homogenization buffer (20 mM Tris–HCl, pH 7.4 containing 1 mM sodium orthovandate, 1 mM EDTA, 1 mM EGTA, 50 mM NaF and 1 mM PMSF). Protein content was determined using the Bradford (1976) method. Equivalent amounts of protein (20 μg for each sample) were loaded into each lane. Proteins were separated on the basis of gel mobility using SDS-PAGE; they were then electrotransferred to polyvinylidene difluoride membrane (PVDF membrane; Immobilon-P, Millipore, Billerica, USA). The membranes were immersed in Ponceau S solution to determine whether equal amounts of protein were loaded; they were then washed with water. The membranes were incubated in Tween–Tris buffer saline (TTBS; 100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween 20) that contained 5% albumin for 2 h at room temperature to block nonspecific binding. Afterward, the membranes were rinsed four times for 15 min in TTBS, followed by incubation overnight on a shaker at 4 °C in the presence of primary antibodies that recognize the following antigens: BDNF (1:5000, Santa Cruz Biotechnology, Santa Cruz, USA), CREB (1:5000, New England BioLabs, USA) and pCREB (1:4000, Sigma-Aldrich, USA). The blots were rinsed four times for 15 min in

TTBS, and they were then incubated for 2 h with the secondary antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology), at a 1:80.000 (BDNF) or 1:50.000 (CREB and pCREB) dilution in TTBS. The membranes were then rinsed four times for 15 min in TTBS. Immunoreactivity was detected using the West-Pico enhanced chemiluminescence kit (Pierce, IL, USA). Densitometric analysis was conducted using an ImageQuant RT-ECL system (GE, Piscataway, NJ, USA).

2.7-Statistical Analysis

We used time series decomposition to access trends and seasonal patterns in the components of the maternal behavior of the two groups (NH and H) in all observation sessions in the first 10 PPDs. To access trend in the components of the maternal behavior we used Dickey-Fuller Test and were considered significant when $p>0.05$. To access seasonal patterns we used Fisher G Test and were considered significant when $p<0.05$ (Fig. 1A, B, C and D).

The number of events of each component of the maternal behavior was expressed as the mean (± SEM).We analyzed the maternal behavior across the 4 daily observation sessions (sum of the 10 days in each daily observation sessions, with 500 observations per session) for each component of the maternal behavior in the first 10 postpartum days. We used a Generalized Estimating Equations analysis (GEE) to compare the maternal behavior in the two groups (NH and H) across the 4 daily observation sessions. To assess differences between the groups for each observation point, we used Bonferroni multiple comparisons test when appropriate. In all cases, differences were considered significant when $p<0.05$ (Fig. 2A, B, C and D).

We analyzed the components of the nursing behavior (Active Nursing - High and Low Arched-back Nursing and Passive Nursing) across the 4 daily observation sessions using a Generalized Estimating Equations analysis (GEE) comparing the two groups (NH and H) across the 4 daily observation sessions. To assess differences between the groups for each observation point, we used Bonferroni multiple comparisons test when appropriate. In all cases, differences were considered significant when $p<0.05$ (Fig. 3A, B and C).

To analyze the performance in the Nest Odor Preference Test, we compared the percentage of time spent in the nest bedding area and in the fresh bedding area using an analysis of variance (ANOVA) for handling procedure, sex and bedding area (Fresh or Nest). To assess differences between the groups for each point, we used Bonferroni multiple comparisons test when appropriate (Fig. 4).

To analyze the performance in each trial of the Nest Odor Preference Test, we compared the time (seconds) spent in the bedding areas (Fresh and Nest) using used a Generalized Estimating Equations analysis (GEE) for time (time in each of the 5 trials of the Nest Odor Preference Test), handling procedure, sex and bedding area (Fresh or Nest). To assess differences between the groups for each point, we used Bonferroni multiple comparisons test when appropriate (Fig. 5A, B, C and D).

To analyze the latency to reach the nest bedding area and the total of movement during the odor preference test, we used an analysis of variance (ANOVA) for handling procedure and sex. To access differences between the groups, we used Bonferroni multiple comparisons test when appropriate. In all of the cases, differences were considered to be significant when $p<0.05$ (Fig. 6).

The levels of CREB and pCREB in the OB were analyzed using Student *t*-test, and the BDNF levels were analyzed using a two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test (factors treatment X time of tissue collection). In all cases, differences were considered to be significant when $p<0.05$ (Fig. 7A, B, C and Fig. 8A, B, C).

3-RESULTS

3.1-Time Series Analysis of Maternal Behavior Across the Frist 10 Postpartum Days

3.1.1-Nursing Behavior

We observed a trend in the nursing behavior in the non-handled group (Dickey-Fuller= -1.56, Lag order=3, p=0.74) and also in the handled group (Dickey-Fuller = -2.51, Lag order=3, p = 0.36). Dams showed a natural reduction in the pattern of the nursing behavior across the first 10 PPDs, but this reduction was more pronounced in the dams from the handled group (Fig. 1C) compared to the non-handled group (Fig. 1A). There was also a seasonal pattern across the 4 daily observation sessions in both groups (fisher g test, NH=p<0.01 and H=p<0.01) but the handled group (Fig. 1C) exhibits a different daily pattern compared to non-handled group (Fig. 1A).

3.1.2-Mother Off the Nest

We observed a trend in the time that the mother spent outside the nest in the non-handled group (Dickey-Fuller= -2.07, Lag order=3, p=0.54) and also in the handled group (Dickey-Fuller = -1.92, Lag order=3, p = 0.60). Dams showed a natural increase in the time spent outside the nest across the first 10 PPDs, but this increase was more pronounced in the dams from the handled group (data not show) compared to the non-handled group (data not show). There was also a seasonal pattern across the 4 daily observation sessions in both groups (fisher g test, NH=p<0.05 and H=p<0.01) but the handled group (data not show) exhibits a different daily pattern compared to non-handled group (data not show).

3.1.3-Licking Pup (Body and Anogenital)

We observed a trend in the licking behavior in the non-handled group (Dickey-Fuller= -2.29, Lag order=3, p=0.45) and also in the handled group (Dickey-Fuller = -2.33, Lag order=3, p = 0.44). Although there was observed a trend in both groups, the dams of the non-handled group (Fig. 1B) show more complex trend (polynomial) them the handled group that exhibits a linear reduction of licking across the first 10 PPDs (Fig. 1D). There was no seasonal pattern detected across the 4 daily observation sessions in the non-handled group (Fig. 1B) there is no significant change across the day (fisher g test, p>0.05) but the handled group (Fig. 1D) exhibits a different daily pattern with an abrupt increase in licking in the session immediately after handling group (fisher g test, p<0.01)

3.1.4-Nest-building Behavior

We observed a trend in the time nest-building behavior in the non-handled group (Dickey-Fuller= -3.29, Lag order=3, p=0.08) and also in the handled group (Dickey-Fuller = -3.03, Lag order=3, p = 0.16). Dams showed a natural decrease in the time spent building the nest across the first 10 PPDs, but this decrease was less pronounced in the dams from the handled group (data not show) compared to the non-handled group (data not show). There was also a seasonal pattern across the 4 daily observation sessions in both groups (fisher g test, NH=p<0.01 and H=p<0.001) but the handled group (data not show) exhibits a different daily pattern compared to non-handled group with an abrupt increase in the session immediately after handling (data not show).

3.2-Maternal Behavior Across 4 Daily Observation Sessions

3.2.1-Nursing Behavior

Dams showed a natural reduction in the pattern of the nursing behavior across the 4 daily observation sessions (main effect observation session Wald chi-square $_{(3,14)}$ = 132.05

$p<0.001$), but this reduction was more pronounced in the dams from the handled group (main effect handling Wald chi-square $_{(1,16)}$ = 6.81, $p<0.01$) especially in the observation sessions immediately after (Bonferroni multiple comparisons test $p<0.05$) and 3 hours after the handling procedure (Bonferroni multiple comparisons test $p<0.01$) (Interaction handling X observation session Wald chi-square $_{(3,14)}$ = 2.68 $p<0.05$) (Fig. 2A).

3.2.2-Mother Off the Nest

Dams showed a natural increase in the time spent outside the nest across the 4 daily observation sessions (main effect observation session Wald chi-square $_{(3,14)}$ = 230.99, $p<0.001$), but this increase was more pronounced in the dams from the handled group (main effect handling Wald chi-square $_{(1,16)}$ = 7.53, $p<0.01$). The results showed significant main effects for handling and observation sessions, and there was an interaction between the two factors (Interaction handling X observation session Wald chi-square $_{(3,14)}$ = 23.39, $p<0.001$) that showed that the increase in the time outside the nest was different between the groups before the handling procedure (Bonferroni multiple comparisons test, $p<0.05$) and 3 hours after the handling procedure (Bonferroni multiple comparisons test, $p<0.001$) (Fig. 2B).

3.2.3-Licking Pup (Body and Anogenital)

When the distribution of the licking behavior was analyzed across the four daily observation sessions, the results showed no significant main effect for the treatment and (main effect handling Wald chi-square $_{(1,16)}$ = 0.73, $p>0.05$). Dams of handled pups showed an increase in licking behavior (main effect observation session, Wald chi-square $_{(3,14)}$ = 11.96, $p<0.05$ and interaction handling X observation session Wald chi-square $_{(3,14)}$ = 24.09, $p<0.001$), but this increase was limited to the recording session immediately after the handling procedure (Bonferroni multiple comparisons test, $p<0.01$) (Fig. 2C).

3.2.4-Nest-building Behavior

Dams showed a natural increase in nest-building behavior across the 4 daily observation sessions (main effect observation sessions, Wald chi-square $_{(3,14)}$ = 32.88 $p<0.001$). The results showed no main effect of the treatment alone (main effect handling Wald chi-square $_{(1,16)}$ = 0.07, $p>0.05$), but there was a significant interaction between the two factors (Interaction handling X observation session Wald chi-square $_{(3,14)}$ = 23.89 $p<0.001$), in which dams of the handled pups showed an increase in nest-building behavior that was limited to the recording session immediately after the handling procedure, which is similar to what was found for the licking behavior (Bonferroni multiple comparisons test, $p<0.05$) (Fig. 2D).

3.3- Active and Passive Nursing Behavior Across 4 Daily Observation Sessions

3.3.1-Active Nursing - High Arched-back Nursing

Dams showed a natural reduction in the pattern of the nursing behavior across the 4 daily observation sessions (main effect observation session Wald chi-square $_{(3,14)}$ = 67.66 $p<0.001$) but there were no differences between the groups (main effect handling Wald chi-square $_{(1,16)}$ = 1.32, $p>0.05$) and no interaction between the two factors (Interaction handling X observation session Wald chi-square $_{(3,14)}$ = 2.79 $p>0.05$) (Fig. 3A).

3.3.2-Passive Nursing

There were no differences in the passive nursing across the 4 daily observation sessions (main effect observation session Wald chi-square $_{(3,14)}$ = 2,18 $p>0.05$), no differences between the groups (main effect handling Wald chi-square $_{(1,16)}$ = 3.17, $p>0.05$) and no interaction between the two factors (Interaction handling X observation session Wald chi-square $_{(3,14)}$ = 3.35 $p>0.05$) (Fig. 3B).

3.3.3-Active Nursing - Low Arched-back Nursing

Dams showed a natural reduction in the pattern of the low arched-back nursing across the 4 daily observation sessions (main effect observation session Wald chi-square $_{(3,14)} = 101.26$ $p<0.001$), but this reduction was more pronounced in the dams from the handled group (main effect handling Wald chi-square $_{(1,16)} = 6.01$, $p<0.05$) especially in the observation sessions immediately after (Bonferroni multiple comparisons test $p<0.05$) and 3 hours after the handling procedure (Bonferroni multiple comparisons test $p<0.01$) (Interaction handling X observation session Wald chi-square $_{(3,14)} = 7.96$ $p<0.05$) (Fig. 3C).

3.4.4-Nest odor preference test

3.4.1-Percentage of Time Spent Over the Areas

There were no main differences between the pups of both groups in the nest odor preference test when the percentage of the total time (sum of the 5 trials) was analyzed. Male and female pups of both groups appeared to show preference for the side of the nest bedding (Main effect handling $F_{(1,34)} = 0.01$ $p>0.05$; main effect sex $F_{(1,34)} = 3.02$ $p>0.05$; main effect area – Fresh or Nest Bedding Area – $F_{(1,34)} = 45.25$ $p<0.0001$).

There was a difference between male and female pups in the time spent over the area (Interaction sex X area $F_{(1,34)} = 6.18$ $p<0.05$). Male pups spent more time in the nest bedding area than did the female pups (Bonferroni multiple comparisons test, $p<0.05$) (Fig. 4).

There were no interactions between handling X sex ($F_{(1,34)} = 0.33$, $p>0.05$) and handling X sex X area ($F_{(1,34)} = 0.02$, $p>0.05$).

3.4.2- Time Spent Over the Areas in each trial of the Odor Preference Test

There were no main differences between the pups of both sex and groups in the nest odor preference test when the time in each trial was analyzed (Main effect handling Wald chi-square $_{(1,34)}$ = 0.02 $p>0.05$; main effect sex Wald chi-square $_{(1,34)}$ = 2.19 $p>0.05$) but there were differences between the time spent in the bedding areas (main effect area – Fresh or Nest Bedding Area Wald chi-square $_{(1,34)}$ = 41.58, $p<0.001$) and in the performance in each trials (main effect trial Wald chi-square $_{(1,31)}$ = 43.59, $p<0.001$).

The trial that the pups had the worst performance was the second (Interaction area X trial Wald chi-square $_{(1,31)}$ = 16.71, $p<0.01$). There was a difference between male and female pups in the time spent over the area (Interaction sex X area Wald chi-square $_{(1,34)}$ = 7.79 $p<0.01$). Male pups spent more time in the nest bedding area than did the female pups (Bonferroni multiple comparisons test, $p<0.05$), and this result was clearly caused by the lack of preference for the nest bedding area observed in the female pups that were handled in the neonatal period (handling X sex X area X trial Wald chi-square $_{(1,31)}$ = 10.59, $p<0.05$). Male pups from both groups preferred the nest area in the trial 3 (Bonferroni multiple comparisons test,NH $p<0.05$ and H $p<0.001$), 4(Bonferroni multiple comparisons test, NH $p<0.001$ and H $p<0.05$) and 5(Bonferroni multiple comparisons test, NH and H $p<0.001$), female pups the non-handled group preferred the nest area in the trials 1 (Bonferroni multiple comparisons test, $p<0.05$) and 4 (Bonferroni multiple comparisons test, $p<0.001$), female pups of the handled group did not preferred any side of the maze in the 5 trials of the Odor Preference Test.

There were no interactions between handling X sex Wald chi-square $_{(1,34)}$ = 1.72, $p>0.05$), handling X area Wald chi-square $_{(1,34)}$ = 0.03, $p>0.05$), handling X trial Wald chi-square $_{(1,34)}$ = 2.60, $p>0.05$), sex X trial Wald chi-square $_{(1,31)}$ = 0.98, $p>0.05$), handling X sex X area Wald chi-square $_{(1,34)}$ = 0.11, $p>0.05$), handling X sex X trial Wald chi-square $_{(1,31)}$ = 5.22, $p>0.05$), handling X area X trial Wald chi-square $_{(1,31)}$ = 7.41, $p>0.05$).

3.4.3-Time to Reach the Nest Bedding Area

The results showed that handled pups spend more time reaching the nest bedding area than the pups of the non-handled group (main effect handling $F_{(1,28)} = 7.23$, $p<0.05$; main effect sex $F_{(1,28)} = 0.31$ $p>0.05$; interaction handling X sex $F_{(1,28)} = 0.00$ $p>0.05$) (Fig. 6).

3.4.4- Total time of movement in the Odor Preference Test

There were no differences between the groups in the total amount of movement during the 5 trials of the Odor Preference Test (main effect handling $F_{(1,28)} = 2.09$, $p>0.05$; main effect sex $F_{(1,28)} = 0.23$ $p>0.05$; interaction handling X sex $F_{(1,28)} = 3.33$ $p>0.05$) (data not show).

3.4.5- Percentage of Time Spent Over the Areas, Time to Reach the Nest Bedding Area and the Maternal Behavior

Alterations in the maternal behavior could disrupt the olfactory learning, and our results showed that the effect could be different for male and female pups. Although male and female pups of the handled group used more time to reach the nest bedding area, this alteration was correlated with the maternal licking pattern for male pups of the handled group (an increase in licking in the session immediately after handling $r = 0.568$, $p<0.05$). For the female pups, there was a negative correlation between the time that each pup spent to reach the nest bedding area and the maternal nursing pattern ($r = -0.5$, $p<0.05$) and a correlation tendency between the time spent by the pup to reach the nest bedding area and the maternal time spent outside the nest ($r = 0.49$, $p=0.054$).

An association was also present between the nest odor preference of female pups and the maternal behavior. There was a correlation between the percentage of time that the female pups spend in the nest area and the nursing behavior ($r= 0.62$, $p<0.01$) and a negative correlation with the maternal time spent outside the nest ($r = -0.57$, $p<0.05$).

3.5-Western Blot Analysis

3.5.1-CREB and BDNF levels in the OB of 7-day-old males

There were no differences in the CREB level 30 minutes after handling between the group submitted to repeated handling (PND1 to PND7) compared to the non-handled group in male pups ($t_{10}=0.722$, $p>0.05$) (Fig. 7A). There were also no differences in the pCREB level between the groups ($t_9=1.219$, $p=0.25$) (Fig. 7B).

The BDNF level was increased in the RH group at 30 (Interaction handling X time $F_{(1,10)} =16.71$ $p<0.01$) and 120 minutes (Interaction handling X time $F_{(1,10)} =7.43$, $p<0.05$) after handling (main effect handling $F_{(1,10)} =56.99$, $p<0.0001$). There was no effect of the time alone (main effect time of tissue collection $F_{(1,10)}=0.023$, $p>0.05$) (Fig. 7C).

3.5.2-CREB and BDNF levels in the OB of 7-day-old females

There were no differences in the CREB level 30 minutes after handling between the groups ($t_{10}=0.536$, $p>0.05$) (Fig. 8A). There was a decrease in the pCREB level in the female pups of the RH group ($t_{10}=4.298$, $p<0.01$) (Fig. 8B).

The BDNF level did not differ in any of the groups at 30 and 120 minutes after handling (main effect handling $F_{(1,10)} =0.474$, $p>0.05$; main effect time of tissue collection $F_{(1,10)} =0.001$, $p>0.05$; Interaction handling X time $F_{(1,10)} =0.556$ and $F_{(1,10)} =0.166$, $p>0.05$) (Fig. 8C).

4-DISCUSSION

The results showed that handling the pups during the neonatal period induces complex changes on maternal behavior that are not limited to an increase or decrease in licking behavior after the intervention. Moreover, we demonstrated that the handling intervention alters CREB phosphorylation and BDNF levels in a sex dependent way in the pups' OB, which could be associated with differential olfactory learning and the odor of the nest preference in the pups.

4.1- Neonatal Handling and Maternal Behavior

This study shows, for the first time (to the best of our knowledge) that handling alters the trend along the first 10 postpartum days and the seasonal daily pattern of licking, nest building, nursing and time spent outside the nest.

Studies that had interventions in the postpartum period using slightly different handling protocols, such as brief (15 min) daily separation from the pups, showed an enhancement in mother-pup interactions by provoking bursts of maternal sensory stimulation of the pups immediately after their return to the home cage (Brown et al 1977, Champagne et al 2003, Claessens et al 2012, Fenoglio et al 2006, Garoflos et al 2005b, Garoflos et al 2007, Garoflos et al 2008, Stamatakis et al 2006).

In this study, we expected to find the same increase in maternal care, especially in the licking behavior. Indeed, our results showed an increase in licking behavior immediately after the handling procedure, as we had previously demonstrated (Azevedo et al 2010), but this increase was limited to that specific period, which is in agreement with the results described by Claessens et al (2012). Increased licking behavior could represent a maternal response to the environmental intervention because mothers of non-handled pups showed an expected stable daily pattern in this maternal behavior. Changes in the stability of the licking pattern that were induced by handling could be perceived by the offspring as an environmental threat, which could lead to persistent alterations in their development.

One important suggestion made by Pryce et al (2001) is that the rat strain could affect the result of the handling procedure in the maternal behavior. Using Wistar rather than Long Evans rats (used in other studies) (Claessens et al 2012, Levine & Lewis 1959), they did not find an increase in licking after the handling procedure [51]. We also used Wistar rats, but we found an increase in licking after the handling procedure in agreement with other the studies that also use this rat strain (Garoflos et al 2005b, Garoflos et al 2007, Garoflos et al 2008, Stamatakis et al 2006). There are two important differences in the protocol of the maternal behavior observation that could explain this discrepancy. Pryce et al (2001) measured the effect of handling on maternal care during the dark period, when rats are more active, and the mothers usually spend less time in contact with their pups in comparison to the light period in which we performed our observations. Moreover, we started recording maternal behavior immediately after the handling procedure and their observations began 15 minutes after the intervention.

We also observed a reactive increase in the nest-building behavior in the session immediately after handling, which was also expected because the handling procedure alters the nest area and introduces external unfamiliar odors that can trigger the motor active components of the maternal behavior (Stern 1989, Stern & Johnson 1989).

In addition to differences in the frequency of licking, rather complex changes in other components of the maternal behavior were found, which were more persistent than the licking burst. The total frequency of nursing decreased in dams whose pups were handled compared to the non-handled pups especially in the sessions immediately and 3 hour after handling procedure. Moreover, dams that had theirs pups handled increased the time spent outside the nest, and this change was not observed after the procedure, as would be expected; instead, a persistent effect on this behavior was observed before the handling and only after 3 hours after the intervention, this difference may be due to the increase in licking in the session immediately after handling, decreasing the time outside the nest in this period.

Maternal behaviors that involve more active movements (such as licking and nest building) and the maternal behaviors that are more quiescent (such as nursing) are be controlled by different CNS regions with opposite relations to the dopaminergic system (Cummings et al 2010). Maternal active behaviors are stimulated by dopaminergic receptors in the shell of the Nucleus Accumbens (NCc) (Cummings et al 2010). In contrast, quiescent behaviors are inhibited by dopaminergic receptors, so that the onset of quiescent behavior demands a decrease in the dopaminergic tonus within the NCc (Keer & Stern 1999). These mechanisms could be involved in the disparity licking/nursing behavioral sequence in handled nests as described in our study.

Handling per se can affect directly the pups (Villescas et al 1977), although the role of the offspring in early social dynamic is difficult to analyze, as it is dominated by the behavior of the caregiver (Cromwell 2011). However, pups' behavior may play an important role in the effect of handling controlling in part the maternal behavior (Fuertes et al 2006, Smotherman et al 1977, Thoman & Levine 1970). The increase in licking could be a reaction of the dam to an increase in ultrasonic vocalization of the pups. The behavior of the pups could also explain the trend of decreasing the licking across the postpartum days.

Neonatal handling alters the functioning of the HPA axis (Hypothalamic-Pituitary-Adrenal) in response to a variety of stressors (Liu et al 2000, Meaney et al 1985a, Meaney et al 2007, Plotsky & Meaney 1993) and may also change the morphology and function of brain structures (Lucion et al 2003, Todeschin et al 2009, Winkelmann-Duarte et al 2011). If these alterations occur already in the first days of the pups' life (Lucion et al 2003, Meaney et al 2007, Todeschin et al 2009, Weaver et al 2004, Winkelmann-Duarte et al

2007) they may affect the pups' early behavioral response to the handling protocol and lead to a differential stimulation of the maternal behavior across the postpartum days. Other possibility is that the mother could adapt to the repeated handling and reduce the reactivity to the protocol across the 10 postpartum days. Future measures of ultrasonic vocalization of the pups will add to the study in terms of being able to evaluate the pup's level of motivation and affective state change.

The results are intriguing and leave open the possibility that pup behavior could be playing a major role in the changes in maternal care. Recent work has emphasized the role that pup motivation plays in triggering and maintaining maternal care in rodents (Cromwell 2011), in fact active nursing behavior that is persistently altered by the handling procedure, is directly affected by the pups behavior (Pryce et al 2001, Stern 1997, Stern & Lonstein 1996, Stern & Lonstein 2001, Villescas et al 1977).

Based on the natural sequence of the maternal behavior (Stern 1989, Stern & Johnson 1989), we expected to find an increase in the nursing behavior and a decrease in the time spent outside the nest after the increase in the motor active components of the maternal behavior, but we found the opposite. These results suggest that handling does not only alter maternal behavior by increasing or decreasing its behavioral components, but also desynchronizes the mother-pup interactions by changing the behavioral sequence.

Stability of the mother-infant relationship is important for the development of emotional and cognitive functions; for a review, see (Baram et al 2012). Indeed, synchrony in mother-infant interactions appears to be a crucial variable in human development (Feldman et al 2011) and additionally for rats (Reeb-Sutherland & Tang 2012, Tang et al 2011). We infer that these alterations in the behavioral sequence of the maternal care associated with changes in the mother-pup interaction synchrony lead to a reduction in the mother-pup contact (increased time spent outside the nest and decreased nursing) and alters the stability of the maternal behavior throughout the day (especially licking and nest building).

Besides the changes in the seasonal daily pattern of nursing and time spent outside the nest, handling also affected the trend of both components of the maternal behavior. Dams that had theirs pups handled showed a reduction in the contact with the pups and increased trend to be outside the nest across the first 10 postpartum days. Dams of the non-handled group also show a trend of increasing the time outside the nest, but the handling procedure seems to accelerate the separation between the mother and the pups, as dams of the handled group show the same amount of time outside the nest in the 4 PPD than dams of the non-handled group in PPD 8. The same pattern occurs with the nursing behavior but in the opposite direction, in which dams of handled group show practically the same score of nursing in 4 PPD than dams of the non-handled group in PPD 8. These changes could affect the olfactory learning process during a critical period for the bonding formation and could be a key cause of long-lasting effects of this intervention on social behaviors (Raineki et al 2013, Todeschin et al 2009).

4.2-Maternal Behavior and Olfactory Learning of the Pups

Infant rats rapidly and naturally learn to identify, orient, approach and prefer the nest odor (McLean et al 1999, Moriceau & Sullivan 2005, Rangel & Leon 1995). The nest odor preference depends on the maternal behavior, especially the tactile stimulation of licking and mother-pup contact (Kojima & Alberts 2009, Nakamura et al 1987, Sullivan 2001, Sullivan & Wilson 2003), Considering that the handling procedure increases the licking behavior and the natural tactile stimulation of the pups by the mother, activates the LC (Nakamura et al 1987), increases noradrenalin (NA) levels in the OB of the pups

(Rangel & Leon 1995, Sullivan & Wilson 2003) and induces the phosphorylation of the cyclic AMP response element binding protein (CREB) (McLean et al 1999, Sullivan & Wilson 2003, Yuan et al 2000, Yuan et al 2003), we expected to find an increase in the levels of CREB phosphorylation in the pups’ olfactory bulbs; however, the results showed no such increase. In fact, in handled female pups, CREB phosphorylation was even reduced, while in males, we observed no significant effect on PPD 7. One possible explanation could be an adaptation process to the repeated increased licking, which, after some days of the handling, could cause a reduction in the pup´s response to this increase. We have described a reduction in NA in the OB on PPD 7 after the handling procedure (Raineki et al 2009), and this reduction could lead to a lack of increase in CREB phosphorylation after the handling on PPD 7.

The fact that only the handled females show a decrease in CREB phosphorylation could indicate that, in a yet unknown way, females are more sensitive to the decrease in mother-pup contact than males. Indeed, the delay in the time spent in reaching the nest area was correlated to the nursing behavior only in the female pups. The results of the nest odor preference test also agree with this hypothesis because female pups that had less contact with theirs dams (decreased nursing and increased time outside the nest) demonstrated less time in the nest area. In fact, when we analyzed the trials of the nest odor preference test, female pups of the handled group did not show any sign of learning across the 5 trails in contrast with the male pups of the same group and the female pups of the non-handled group. As there was an overall difference between male and female pups, it is possible that the task was experienced differently between sexes, regardless of the experimental group.

Differences in the maternal licking distribution among the pups of different sexes can also contribute to this effect. Studies have shown that, in rats, mothers lick the male more than they do the female pups due to their different urine odors (Hao et al 2011, Moore 1985, Moore et al 1996, Oomen et al 2009), and we demonstrated that the licking pattern was correlated to the social behavior changes in the male pups. Alternatively, it is possible that early life interventions can induce sex-changes according to the variable measured later in life.

These correlational results suggest relationships between the care behaviors and pup learning but it is difficult to tease apart the effects of handling versus maternal care on the neurochemical changes. Studies using an artificial model of maternal behavior are able to disentangle these effects and are very useful to evaluate the degree of importance of each variable in this matter (de Medeiros et al 2009, Gonzalez & Fleming 2002, Lovic & Fleming 2004).

The biochemical alterations found in the repeatedly handled pups are most likely associated with changes in the monoaminergic system (Papaioannou et al 2002, Raineki et al 2009, Stamatakis et al 2006, Vicentic et al 2006). Raineki et al (2009) showed that male and female repeatedly handled pups (PPD 1 - PPD 7) demonstrate a decrease in the noradrenergic tonus in the OB after handling on PPD 7, but only males have an increase in serotonin activity, which could act as a compensatory mechanism in males. However, it is still poorly understood why male and female rat pups on PPD 7 show these differences in the pCREB levels in the OB.

It is possible that, already at this early age, male and female pups have different responses to environmental or adverse stimuli (McCormick et al 1995, Shanks et al 1994, Viveros et al 2009). Stamatakis et al (2006) showed that a protocol of neonatal handling induces sexually dimorphic changes in the levels of hippocampal 5-HT1A receptors in adult animals, with males having an increase and females a decrease. In addition, repeated neonatal handling increases BDNF levels in the CA4 area in the hippocampus of male rat pups, while females show no alteration (Garoflos et al 2005a, Garoflos et al 2005b). Our results in the OB are in line with that study. Handled male pups did not demonstrate differences in CREB phosphorylation, but they had increased levels of BDNF at 30 and 120 minutes after the intervention compared to control groups. Because there was no change in the CREB phosphorylation, it is possible that the increase in BDNF levels at 30 and 120 minutes after the handling procedure could be caused by a cumulative effect of the increased licking over the previous days. Thus, the increased BDNF signaling in the OB in male pups could characterize a persistent effect of the handling procedure. An increase in hippocampal BDNF is associated with an increase in spatial memory in adult rats that were handled in the neonatal period (Garoflos et al 2005a, Garoflos et al 2005b), and therefore it is possible that the same effect exists for the olfactory learning.

In the OB, no significant change in the BDNF levels was detected in the repeatedly handled female pups; however, this result does not necessarily mean that there were no changes in the BDNF levels in different regions of that structure. We used a Western blot protocol with the homogenization of the entire OB, but this structure has several layers with different populations of neurons. The use of morphological analysis that could allow differentiation of the layers in the OB would be a good experimental approach to detecting more specific and precise effects of neonatal handling on the functioning and morphology of the OB.

5-CONCLUSIONS

In conclusion, we demonstrated that the handling procedure induces complex changes in critical components of maternal care. This study shows, for the first time (to the best of our knowledge), that handling can change the mother-pup interaction synchrony, thus altering the behavioral sequence of maternal care, reducing the mother-pups contact during the first 10 PPDs and changing the daily pattern of nursing, licking behavior and nest building. Handling induces a burst in licking behavior immediately after the intervention and causes biochemical changes on the pup's brain in a sex-specific way. These maternal behavioral changes could explain the increase in BDNF in the olfactory bulb of male pups and the decrease in CREB phosphorylation in female pups because differences in the behavior of the pups were correlated with differences in the maternal behavior components (also in a sex-specific way). These biochemical changes in the nervous system of the pups could be the cause of the sexual differences shown in the nest odor preference test.

Although the female pups appear to be more affected by the handling procedure, males also have alterations in the time to reach the nest area, and the increased levels of BDNF in the olfactory bulb could contribute to the long-lasting effects of early life environmental intervention on the development of the behavioral strategies in males.

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7-FIGURE AND LEGENDS

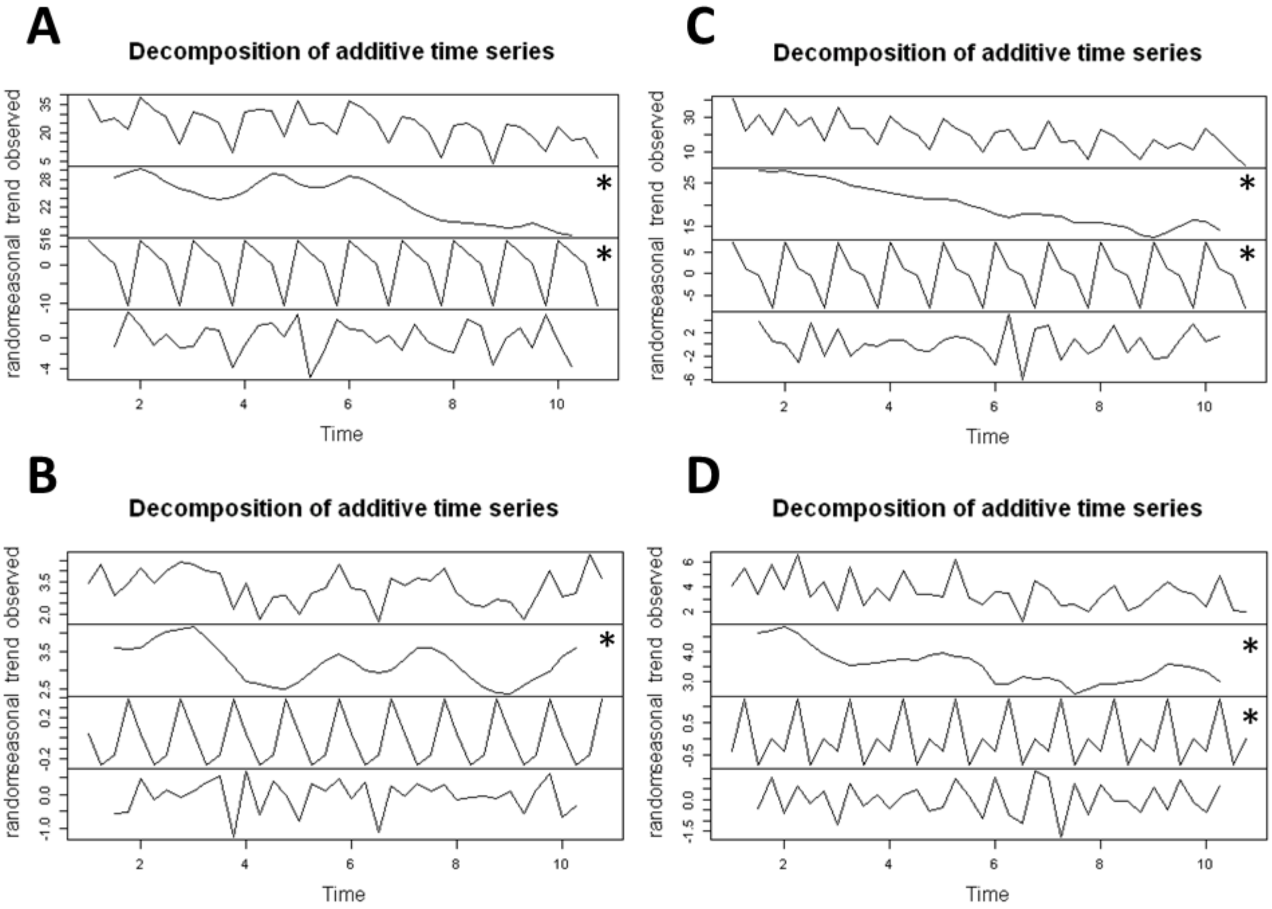

FIGURE 1. Time series decomposition analysis of the Nursing Behavior of non-handled (A) and handled dams (C) and Licking (Body and Anogenital) of non-handled (B) and handled dams (D) in the first 10 PPDs. Data were analyzed using decomposition of additive time series, verifying trend and seasonal patterns in the first 10 days postpartum across the 4 daily observation sessions (before, after, 3 hours after and 5 hours after handling). * represents presence of trend or seasonal pattern, n=9 in all groups.

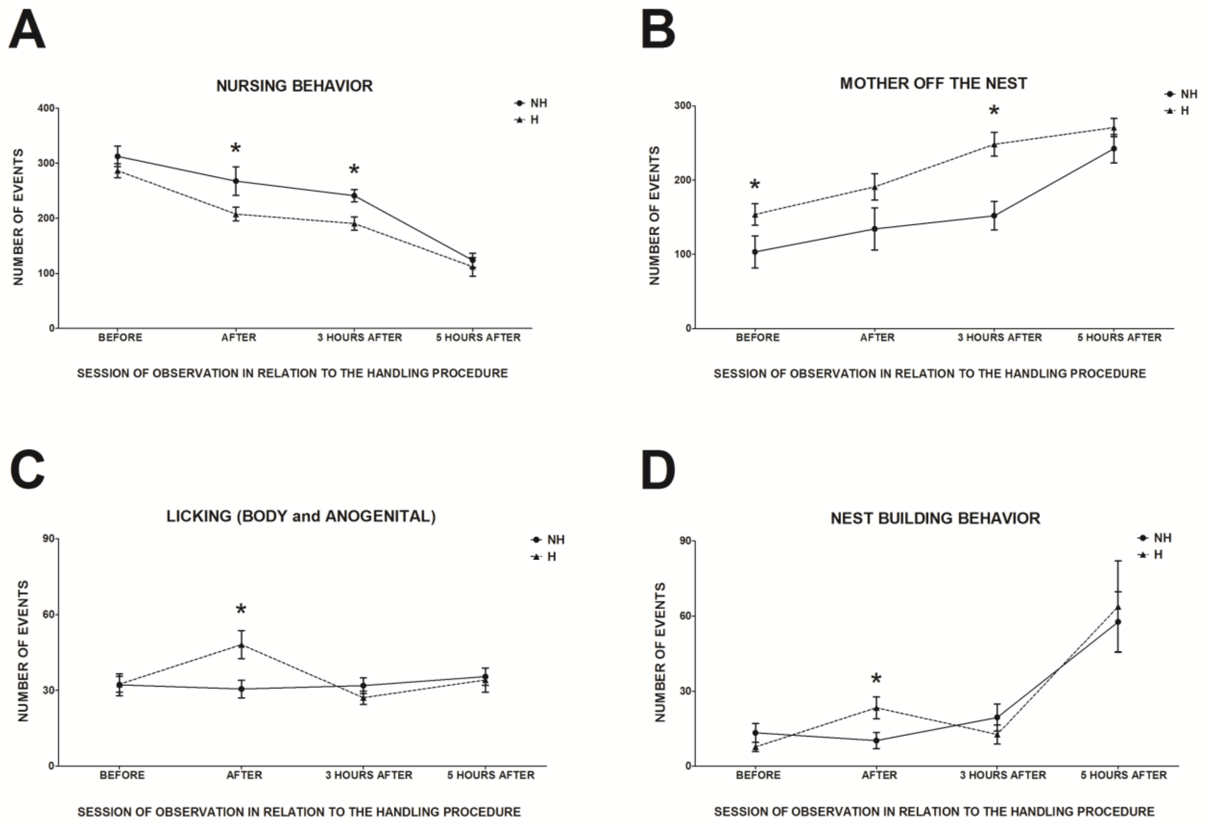

FIGURE 2. Components of the maternal behavior in the 4 daily observation sessions: Nursing Behavior (A), Mother off the Nest (B), Licking (Body and Anogenital) (C) and Mother Building Nest (D). Data were expressed mean (± SEM) and analyzed using GEE for handling procedure and 4 daily observation sessions (before, after, 3 hours after and 5 hours after handling) for each component of maternal behavior (nursing, off the nest, licking and building nest) followed by Bonferroni´s multiple comparisons test to access differences in each point. * represents a significant difference ($p<0.05$) for interaction handling X observation session in the all graphics, n=9 in all groups.

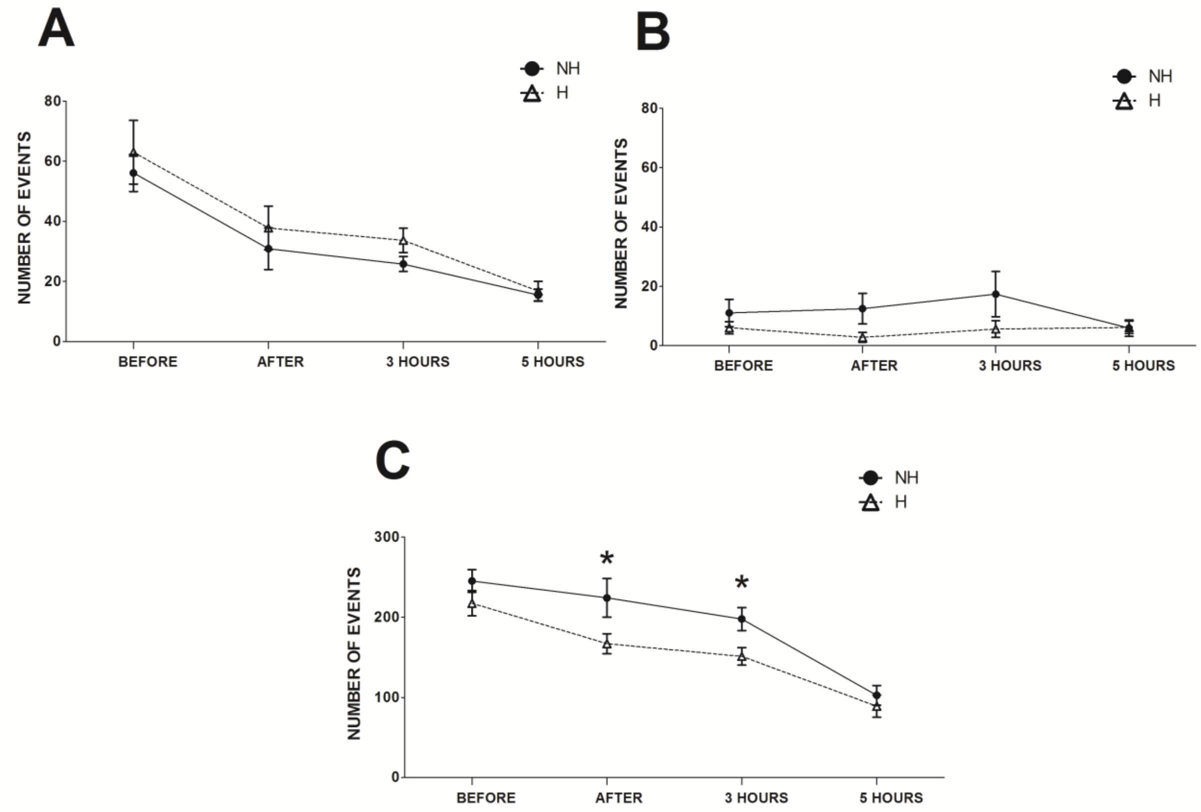

FIGURE 3. Components of the nursing behavior in the 4 daily observation sessions: Active Nursing - High Arched-back Nursing (A), Passive Nursing (B) and Active Nursing - Low Arched-back Nursing (C). Data were expressed mean (± SEM) and each component of the nursing behavior was analyzed using GEE for handling procedure and 4 daily observation sessions (before, after, 3 hours after and 5 hours after handling) followed by Bonferroni multiple comparisons tests to access differences in each point. * represents a significant difference ($p<0.05$) for interaction handling X observation session, n=9 in all groups.

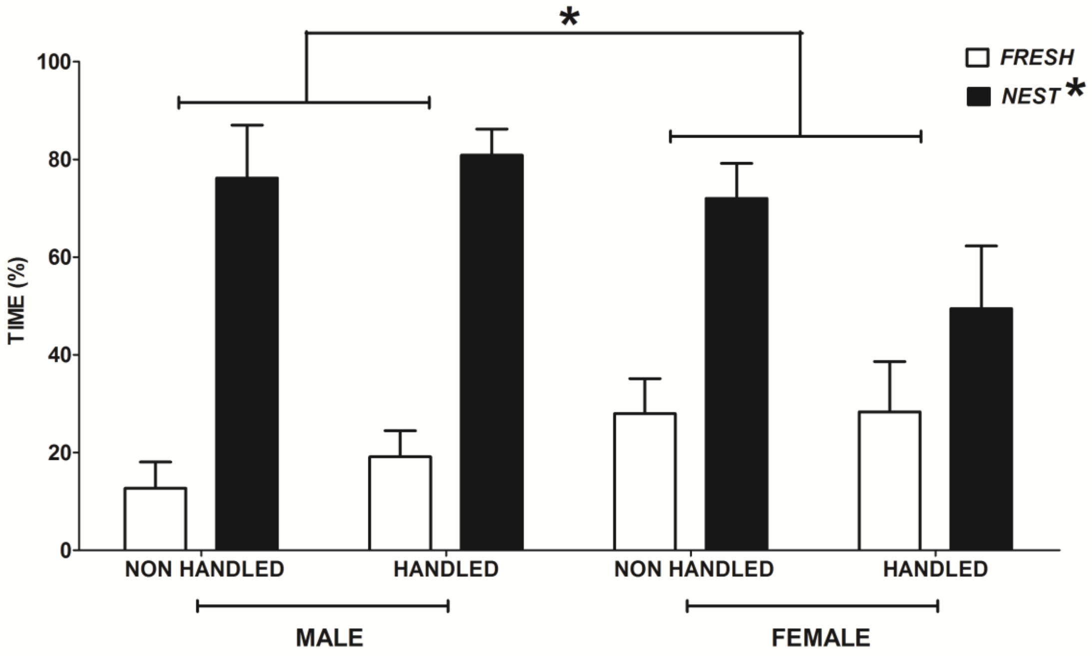

FIGURE 4. Percentage of time spent in the bedding areas (Fresh and Nest) in the odor preference test on PPD 11. Data were expressed mean (± SEM) and analyzed using ANOVA for handling procedure, sex and bedding area (Fresh and Nest) followed by Bonferroni multiple comparisons tests to access differences in each point. * represents significant difference ($p<0.05$) for the main effect handling and for main effect sex, n=9 in all groups.

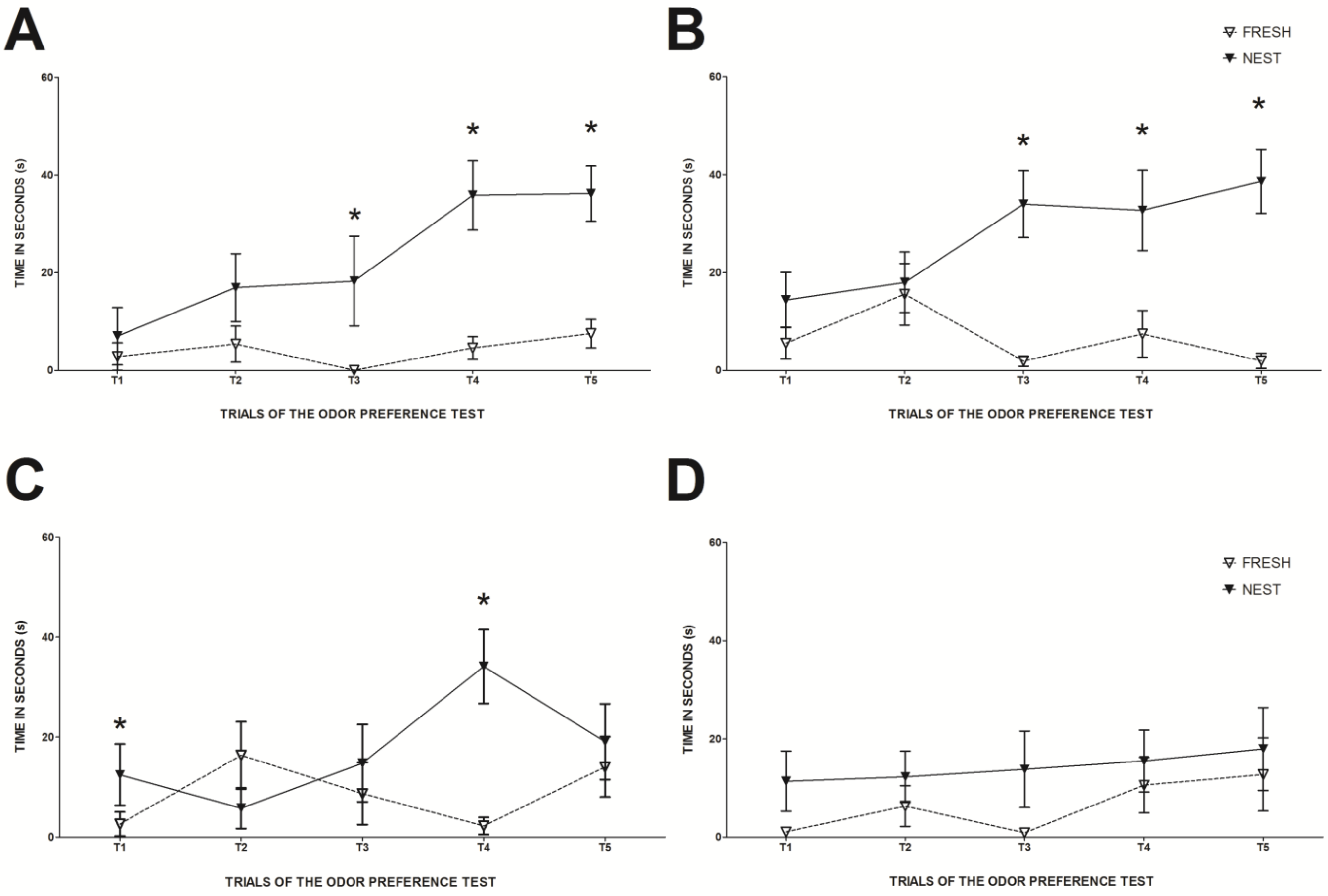

FIGURE 5. Time in seconds spent in the bedding areas (Fresh and Nest) in each session of the odor preference test on PPD 11 for male pups of the nonhladed (A) and handled group (B) and for female pups of the nonhdled (C) and handledgroup (D). Data were expressed mean (± SEM) and analyzed using GEE for time (sessions of the odor preference test), handling procedure, sex and bedding area (Fresh and Nest) followed by Bonferroni multiple comparisons tests to access differences in each point. * represents significant difference ($p<0.05$) for the interaction time X handling X sex X bedding area, n=9 in all groups.

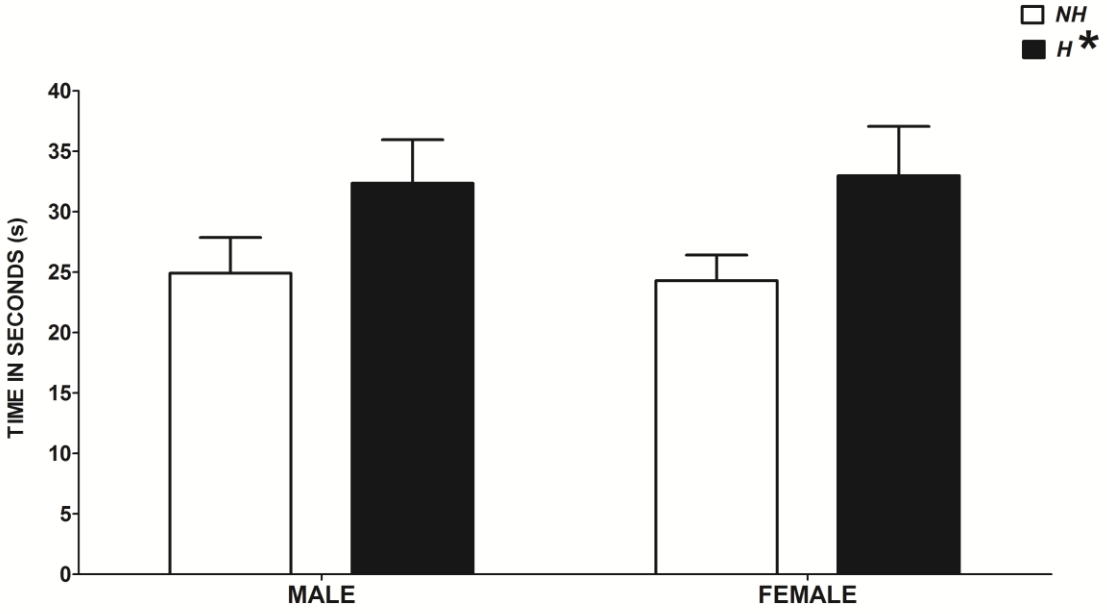

FIGURE 6. Time spend by the pups to reach the nest bedding area in the nest odor preference test on PPD 11. * represents significant difference ($p<0.05$) for the main effect handling, n=8 in all groups.

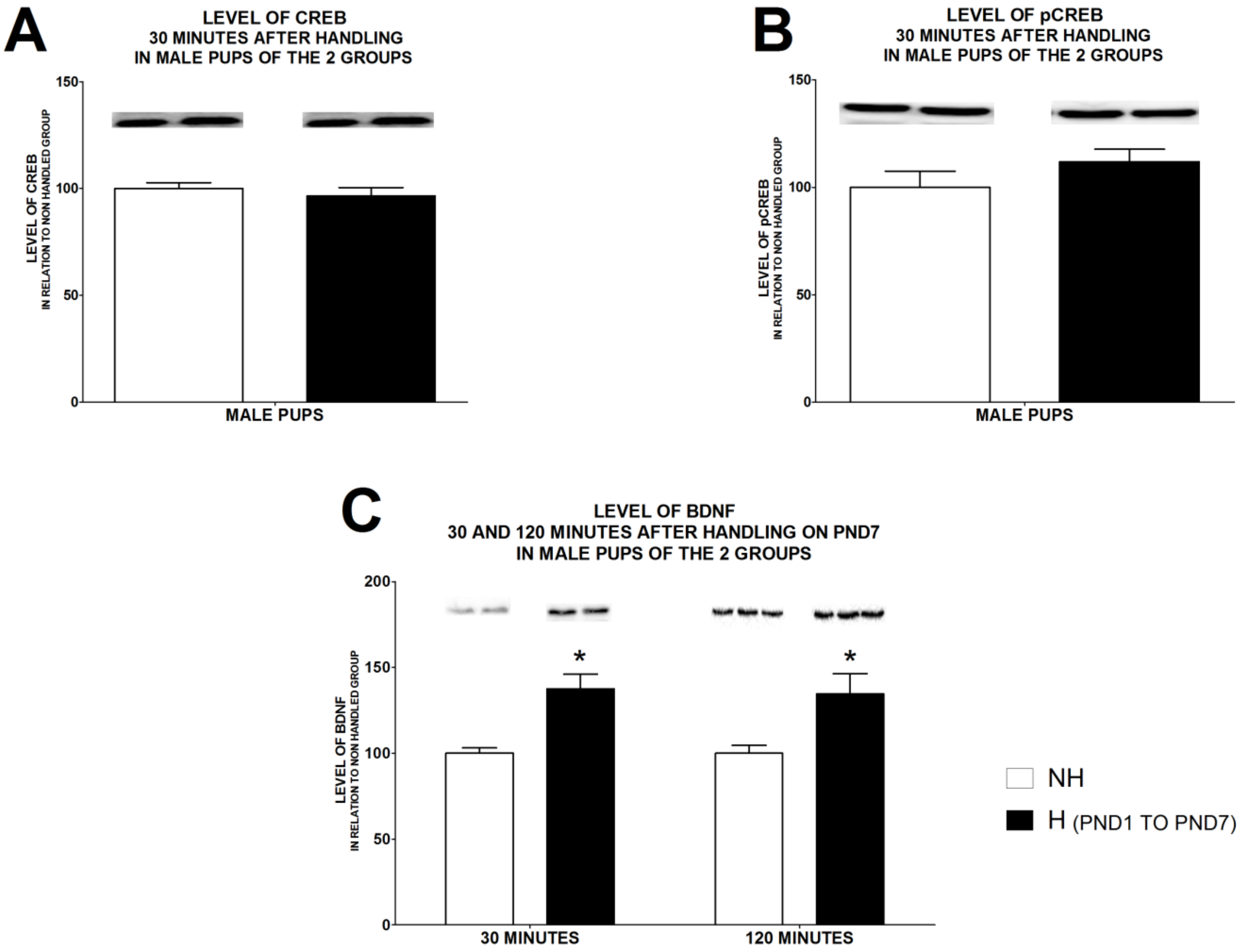

FIGURE 7. Western blot analysis of male pups olfactory bulb after the handling procedure on PPD 7. CREB (A), pCREB (B), and BDNF levels (C) in the olfactory bulb of male pups were expressed as mean±S.E.M. and analyzed using Student *t* test (A and B) or Two-Way ANOVA followed by the Bonferroni Post-tests (C). * represents significant difference ($p<0.05$) compared to control group (non-handled); n=6 in all groups, except for pCREB levels in the handled group (n=5).

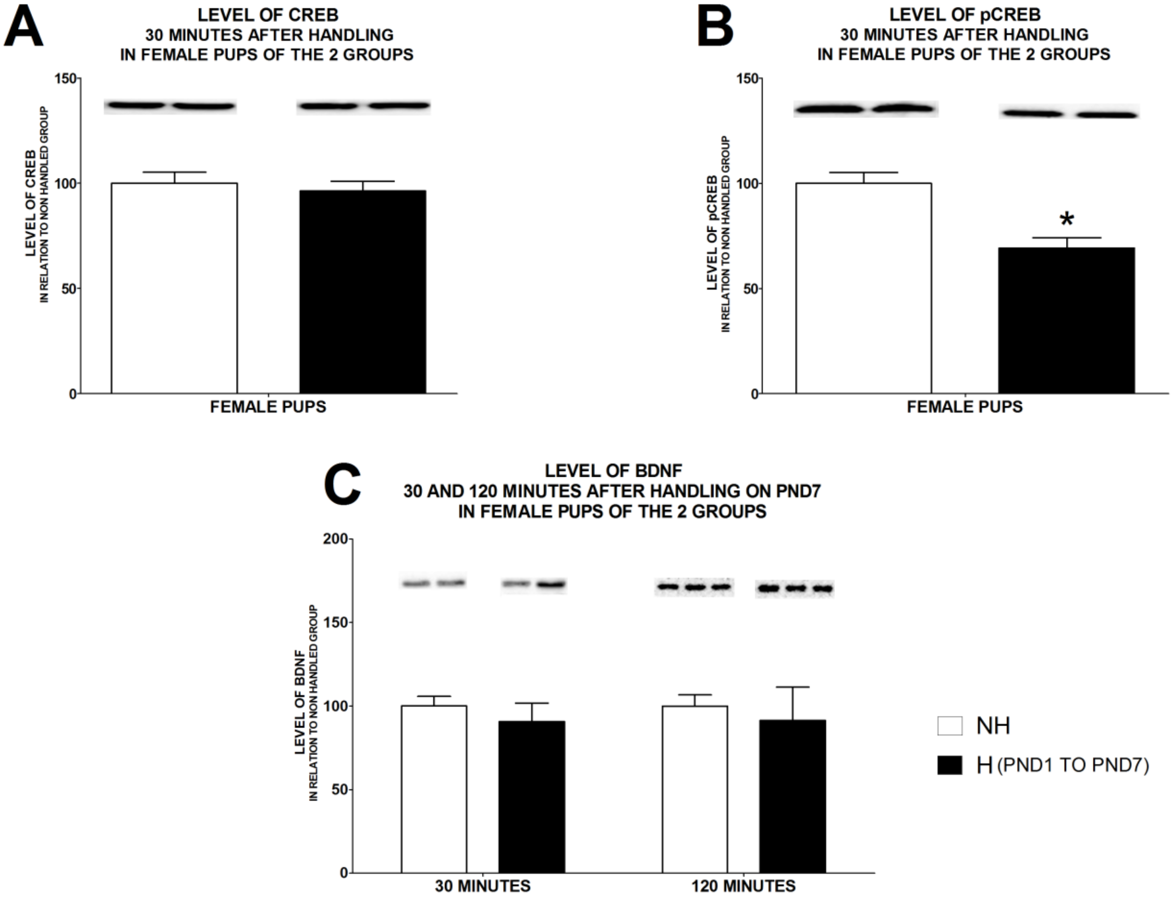

FIGURE 8. Western blot analysis of female pups olfactory bulb after the handling procedure on post-natal day7. CREB (A), pCREB (B), and BDNF levels (C) in the olfactory bulb of female pups were expressed as mean±S.E.M. and analyzed using Student *t* test (A and B) or Two-Way ANOVA followed by the Bonferroni Post-tests (C). * represents significant difference ($p<0.05$) compared to control group (non-handled); n=6 in all groups.

4.2 Capítulo 2 – Resultados do Experimento 2

Neonatal Handling Alters Maternal Emotional Response

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ABSTRACT

Stressful events during early life period may exert major consequences on health disorders later in life. Neonatal handling is an experimental procedure that has been used to analyze long-lasting effects of environmental intervention during early postpartum days (PPD). However, long-lasting effects of repeated stress exposure in the neonatal period on the maternal organism are poorly studied. The aim of this study was to see if neonatal handling induces lasting effects on maternal stress responses increasing the risk for depression. Dams were divided in 2 groups (NH-Non handled and H-Handled) based on the handling procedure (Pups were handled for 1 min per day from PPD 1 to PPD 10) and them subdivided in 4 groups (NH, NH+S, H and H+S) base on the exposure or not to restraint stress after weaning (1 hour/per day for 7 days, PPD22-PPD28). We analyzed forced swimming test behavior (FST PPD29 and PPD30), plasma basal corticosterone and BDNF levels, as well as adrenal weight of the dams (PPD31). The results show that handling procedure alters dam´s emotional response to acute and to chronic stress, in which dams of the H group had increased immobility in the first day of FST similarly to NH+S, but there were no differences between the groups on the second day. Dams of the H and H+S groups show decreased levels of corticosterone when compared to NH and NH+S groups. The H+S also shows increased adrenal weight suggesting an increased sensibility of the maternal organism to chronic stress applied after weaning.

We show that handling may present a long lasting effect in maternal stress response increasing the response to acute and chronic stress. These changes in the dam´s stress response may increase the susceptibility of the development of psychiatric disorders like depression at least in response to acute stress.

Key Words – Neonatal Intervention, Stress Response, Depression, Corticosterone, Adrenal Weight.

1-INTRODUCTION

Stressful events during early life period may exert major consequences on health disorders later in life. Neonatal handling is an experimental procedure involving brief maternal separation and tactile stimulation, extensively used to investigate the effects of early life interventions on behavioral and endocrine alterations. This repeated disruption in the mother–pup relationship reduces fear (Padoin et al 2001), corticosterone response to a variety of stressors (Liu et al 2000, Plotsky & Meaney 1993) and may also affect social behaviors and fertility in male and female rats (Gomes et al 1999, Raineki et al 2009, Raineki et al 2012, Raineki et al 2008, Reis et al 2014). Besides the behavioral and neuroendocrine changes, neonatal handling also alters the brain plasticity and neurotrophic signaling, producing long lasting structural changes (Lucion et al 2003, Todeschin et al 2009, Winkelmann-Duarte et al 2011).

The most common target of interest of researches is the long lasting effects of these interventions on the pups, however, the effects of repeated stress exposure in the neonatal period on the maternal organism are poorly studied, even though, in humans, this period seems to be especially vulnerable to external disturbances (Llewellyn et al 1997, Steiner 1979). In postpartum women, about 20–30% develop a short-lived ‘postpartum blues’, and the prevalence of non-psychotic major depression is about 10% in the first months after delivery (Mastorakos & Ilias 2000).

In rats, in the period around parturition, severe behavioral and neuroendocrine alterations occur and they are reflected at almost all brain levels and are a prerequisite for protected embryonal development, successful delivery process, maternal behavior, and nurturance of the newborn ensuring their survival ((Douglas 2005, Douglas et al 2003, Neumann et al 1998, Neumann et al 2000, Pearlman 1983) and for review see (Neumann 2003)). These neurobiological adaptive mechanisms are most likely to be also necessary for the protection of the maternal organism against dramatic hormonal changes occurring at this time that may otherwise result in emotional disturbances, postpartum depression or postpartum psychosis (Neumann 2003). In this period may occur on one hand, activation of systems that are necessary for reproductive processes like labour, lactation, and maternal behavior which mainly involve oxytocin, prolactin, and endogenous opioids and on the other hand deactivation of non relevant systems like the hypothalamo-pituitaryadrenal (HPA) axis (Neumann 2003).

Neonatal handling persistently affects different components of the maternal behavior, decreasing nursing and mother-pups contact (Reis et al 2014), which suggests a disturbance between the activation of reproductive behaviors and deactivation of the HPA response. Nursing is highly influenced by oxytocin via stimulation of dopaminergic neurons in the ventral tegmental area projecting to the nucleus accumbens (Shahrokh et al 2010), therefore, this intervention could also lead to permanent changes in the emotional response of the dams. Indeed, we have previously shown that brief separation from the pups during the postpartum period induces dam’s behavioral sensitization to psychostimulants and increased corticosterone secretion in response to acute stress (Silveira et al 2013). However, the dam’s response to chronic stress following neonatal handling is not known.

Therefore, our hypothesis is that neonatal handling induces long lasting effects on the maternal responses to repeated stress, increasing their risk for depression. As such, we hypothesized that dams that had theirs pups handled in the neonatal period and were exposure to mild chronic stress after weaning demonstrate depressive-like behavior in the forced swimming test and changes in biochemical parameters related to the stress response.

The knowledge of the mechanism by which these changes take place will be very important for a better understanding and treatment of psychiatric disorders like postpartum depression.

2-EXPERIMENTAL PROCEDURES

2.1-Animals

Pregnant female Wistar rats were brought from the colony of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil) to the animal room in our laboratory. Approximately 7 days before delivery, the females were housed individually, and the presence of the pups was checked twice daily. Birth was considered to be day 0, and on postpartum day 1 (PPD 1), the number of pups was culled to 8 per dam by randomly removing a few pups while ensuring minimal contact with the remaining rats. All of the animals were maintained on a 12-h light/dark cycle with the lights on at 6 a.m. The room temperature was 22 ± 1 °C, and water and food (Rodent chow, Nutrilab, Colombo, Brazil) were available at all times. Cage bedding was not changed from PPD 0 to 10. The experiments were performed in accordance with the National Institutes of Health (NIH) and Colégio Brasileiro de Experimentação Animal (COBEA) guidelines. These guidelines were designed to minimize the discomfort of animals and were approved by the Ethics in Research Committee of Federal University of Rio Grande do Sul (Process CEP/UFRGS nº 19759) and followed Brazilian legislation.

2.2-Neonatal Handling

Pups were handled for 1 min per day from PPD 1 to PPD 10. First, the home cage containing the mother and pups was transferred to a quiet room next to the animal facility providing the same light period and temperature as described above. Then, the mother was removed from the home cage and placed into another cage. The experimenter gently handled all of the pups at the same time using both hands, covered with fine latex gloves, for 60 s. No apparent harm was inflicted to the pups; they were simply touched. After handling, all of the pups were taken to the nest at the same time, and the mother was placed back inside the home cage. The home cage was then returned to the animal facility room and left undisturbed until the same time the next day. The pups were handled during the light period of the daily photoperiod cycle (10:00-12:00) at a distance of approximately 100 cm from the mother. The total time of the mother–infant separation was approximately 90 s (Gomes et al 1999, Padoin et al 2001, Raineki et al 2009, Raineki et al 2012, Raineki et al 2008, Todeschin et al 2009).

2.3-Experiments and Groups

A total of 40 pregnant female Wistar rats were first divided in two experimental groups based in the protocol of neonatal handling described above (Non handled; n=21 and Handled; n=19), them the dams were divided into 4 subgroups based on the presence or absence of restraint stress (1 hour per day in a Plexiglas restraint apparatus 24 X 9 X 5 cm, during 7 days after weaning): NH-non handled, n=12; NH+S-non handled plus restraint stress, n=9; H-handled, n=10 and H+S-handled plus restraint stress, n=9.

2.4-Forced Swimming Test (FST)

The detailed procedures of FST have been described elsewhere [19]. Briefly, each rat was placed individually into a vertical rigid plastic cylinder (50 cm tall X 20 cm in diameter) containing 25°C water (35 cm depth). The pretest session consisted in placing the rats in the cylinder during 15 min. At the end of this pretest phase, the rat was removed from the water, dried with a towel and placed in a cage. Twenty-four hours later, in a 5-min test session; the rats were placed in the cylinder again.

Immobility, i.e., when the rats remained floating in the water, making only the necessary movements to keep their heads above water (Cervo & Samanin 1988); and the active behaviors: swimming, i.e., when the rats made active swimming motions; and climbing, i.e., when they made vigorous movements with their forepaws in and out of the water, usually directed against the walls (Detke & Lucki 1996) were scored. All swimming sessions were video-taped and scored by observers unaware of experimental groups.

2.5-Adrenal Weight/Body Weight

Immediately following decapitation, the adrenals were dissected, cleaned, weighed on a precision balance. The adrenal weight was analyzed in relation to the dams´ body weight to avoid natural anatomical differences. The body weight was measured in the second day of forced swimming test.

2.6-Plasma Corticosterone and BDNF Levels

Animals were decapitated on postpartum day 31. Trunk blood was collected into heparinized tubes for the determination of corticosterone levels. Tubes were centrifuged at 4°C and plasma was separated and frozen until the day of the analysis. Hormone levels were measured using commercially available ELISA kit for corticosterone (ENZO Chemical Co., USA; intra-assay coefficient of variation: 7.5%; inter-assay coefficient of variation: 14.6%). BDNF levels were measured using commercially available ELISA kit (PROMEGA Corporation, BDNF Emax® ImmunoAssay System – G7611).

2.7-Statistical Analysis

All results were expressed as mean (± SEM). The percentage of time spent climbing, swimming and in immobility during the forced swimming test was analyzed with a Generalized Estimating Equations analysis (GEE) to compare the percentage of time of each behavior in the four groups (NH, NH+S, H and H+S) across the 2 days of test (habituation and test). To assess differences between the groups for each time point, we used Bonferroni multiple comparisons test when appropriate. (Fig. 1A, B and C).

To analyze the basaline plasma levels of corticosterone and BDNF one day after the FST we used an analysis of variance (ANOVA) using handling and stress exposure as variables. To assess differences between the groups we used Bonferroni multiple comparisons test when appropriate (Fig. 2 and 4).

We used an analysis of variance (ANOVA) for analyzing the adrenal weight dissected one day after the forced swimming test, adjusting it for the dams’ body weight; to assess differences between the groups we used Bonferroni multiple comparisons test when appropriate (Fig. 3). In all cases, differences were considered significant when $p<0.05$.

3-RESULTS

3.1-Forced Swimming Test

The behavior of the dams in the forced swimming test was different depending by the treatment (NH, NH+S, H and H+S), the type of behavior analyzed (Immobility, Swimming and Climbing) and the day of test (Interaction treatment X behavior X time Wald chi-square $_{(6,46)} = 24.71$, $p<0.001$), the pos hoc analysis of these interactions will be described in the next four sections (Fig. 1A, B and C).

3.1.1-Immobility

NH+S and H dams demonstrate increased immobility time than NH dams on the first day of the FST (habituation, Bonferroni multiple comparisons test $p<0.01$ and $p<0.001$)). H+S dams spent more time in immobility than NH dams, but less time than the other 2 groups although no significant statistic differences were observed. There were also no differences between the 4 groups in the immobility time on the second day of the FST (Fig. 1A).

Dams from the NH+S and H groups have a decreased immobility time from the first day to the second day of FST (Bonferroni multiple comparisons test $p<0.05$ and $p<0.01$). There were no differences in the percentage of time spent in immobility in the two days of test in the NH and H+S groups.

3.1.2-Climbing

H+S dams spent less time climbing in the first day of FST (habituation) when compared to NH and H groups (Bonferroni multiple comparisons test $p<0.001$ and $p<0.05$) and also a tendency when compared to NH+S group (Bonferroni multiple comparisons test $p=0.05$). There was no significant difference between the 4 groups in the time spent climbing on the second day of the FST (Fig. 1B).

Dams from all the four groups show an increase in the time spent climbing from the first day to the second day of FST (Bonferroni multiple comparisons test, NH $p<0.001$, NH+S $p<0.01$, H $p<0.001$ and H+S $p<0.001$)

3.1.3-Swimming

Dams from the NH+S and H groups demonstrate less swimming time than dams of the NH group on the first day of the FST (habituation) (Bonferroni multiple comparisons test $p<0.001$ and $p<0.05$). Dams of the H+S group spent less time swimming than the dams of the NH group and more time than the other 2 groups but there was no significant difference. There was no statistic difference between the 4 groups in the percentage of time spent swimming on the second day of the FST (Fig. 1C).

Dams from the NH and H+S groups had decreased percentage of time spent swimming from the first to the second day of FST (Bonferroni multiple comparisons test $p<0.001$ and $p<0.05$). There were no differences in the percentage of time spent swimming in the 2 days of test in the NH+S and H groups.

3.2- Corticosterone Levels

Dams of the groups that had theirs pups handled in the neonatal period (H and H+S) have lower levels of plasma corticosterone than the other 2 groups (NH and NH+S) (Main effect handling $F_{(1,27)} = 6.22$, $p>0.05$).

There was also a tendency to increased corticosterone in the groups exposed to restraint stress (NH+S and H+S) compared to non-exposed (NH and H) (Main effect restraint stress $F_{(1,27)} = 3.96$, $p=0.058$) (Fig. 2). This effect is visually resultant from an increase in NH+S only, although there was no interaction between handling X restraint stress exposure($F_{(1,27)} = 2.25$, $p>0.05$).

3.3- Adrenal Weight/Body Weight

There was no effect of handling alone on the adrenal weight (Main effect handling $F_{(1,32)} = 0.95$, $p>0.05$) but there was an effect of restraint stress (Main effect restraint stress $F_{(1,32)} = 6.85$, $p<0.05$) and also an interaction between the 2 factors (handling X restraint stress; $F_{(1,32)} =4.82$, $p<0.05$). H+S dams show a higher adrenal mass compared to the other 3 groups (Bonferroni multiple comparisons test, NH $p<0.01$, NH+S $p<0.05$, H $p<0.01$) (Fig. 3).

3.4-BDNF Levels

There was no effect of handling on the plasma BDNF levels (Main effect handling $F_{(1,27)} = 0.23$ $p>0.05$) and only a tendency to increase BDNF levels in the groups without restraint stress (NH and H) compared and with restraint stress (NH+S and H+S) (Main effect restraint stress $F_{(1,27)} = 4.08$ $p=0.055$) (Fig. 4). This tendency is a result of an increase specifically in the H, although there was no interaction between handling X restraint stress ($F_{(1,27)} = 1.44$, $p>0.05$).

4-DISCUSSION

The results show that environmental adversity during the neonatal period can have a long term impact on the dam’s behavior, emotional responses and increase the depressive-like behaviors at least in response to acute stress. Exposure to various stressors during pregnancy or the postpartum period are often linked to the development of mood disorders (Beck 2001, Hillerer et al 2012, Hillerer et al 2011, Robertson et al 2004), and in humans, chronic exposure to psychosocial stressors, such as social conflict, is one of the strongest predictors of postpartum depression (Westdahl et al 2007). Repeated handling could be interpreted by the dams as a social defeat paradigm, considering that they cannot avoid the daily separation from their pups, which most likely works as an intense psychological stressor and therefore may predisposes to depressive-like behaviors as we demonstrated.

Dams that had theirs pups handled from PPD1 to 10 show altered stress and emotional responses, but the results were different depending on the exposure to repeated stress after weaning. Dams of the H group have a depressive like behavior on the first day of the FST, similarly to what we observed in the NH+S. It is possible that the habituation to the FST works as an acute stress exposure, causing an increased immobility time in these groups. This result would be in agreement with another study indicating that mothers that had theirs pups handled show increase response to acute stress (Silveira et al 2013). Dams of the H+S group demonstrate a slight different behavior: although they show an increase in the immobility time in the first day of FST, there were no differences compared to the other groups, suggesting that there is an interaction between the handling procedure and the chronic stress applied after weaning. Based on the behavioral analysis we may infer that handling attenuate the response to chronic stress, but the increase in adrenal weight observed only in this group suggests otherwise.

In animal models, studies show that depressive states generated are associated with decreased brain BDNF levels, and that central BDNF administration improves the depressed state (Angelucci et al 2004, Reus et al 2011). Although the correlation between central and peripheral levels of BDNF it still matter of debate (Elfving et al 2010) , we expected to find a decrease in plasma BDNF in those groups showing increased immobility time. This was not the case, supposedly, this could be explained by a compensatory mechanism in response to the acute stress of the FST paradigm.

Neonatal handling persistently affects different components of the maternal behavior, increasing licking and time outside the nest and decreasing nursing and mother-pups contact (Reis et al 2014). Nursing is highly influenced by oxytocin via stimulation of the dopaminergic neurons in the ventral tegmental area projecting to the nucleus accumbens (Shahrokh et al 2010). Oxytocin levels and milk ejection are also associated with the contact and behavior of the pups hooting and sucking (Freund-Mercier & Richard 1984, Lambert et al 1993a, Lambert et al 1993b). The increase in licking followed by a decrease in time spent nursing and in contact with the pups could indicate a decrease in the levels of the dam’s oxytocin. Oxytocin plays an inhibitory role on the hypothalamic stress response during the peripartum period (Neumann et al 2000), therefore a decrease in central levels of oxytocin may be followed by an increase in the dam’s stress reactivity after neonatal handling. We have previously shown that dams present a sensitization to psychostimulants and increased corticosterone secretion in response to acute stress (Silveira et al 2013).

Dams that had theirs pups handled from PPD1 to 10 (H and H+S) have lower basal levels of corticosterone 1 day after the FST compared to the groups without handling (NH and NH+S) regardless the stress protocol after weaning. This shows that handling alters the stress response of the mothers, possibly due to an impairment in the neuroendocrine adaptations that occur during lactation, which include hypercorticism (Lightman et al 2001) and elevated levels of corticosterone binding globulin (Douglas et al 2003, Pearlman 1983). One hypothesis is that handling the pups repeatedly during the neonatal period affects the onset of these adaptations in the mothers including the increase in the basal levels of corticosterone and corticosterone binding globulin. Preventing the adaptive increase in the basal levels of this hormone and increasing the availability of free corticosterone may lead to more responsiveness to stress and a consequent higher liberation and production of corticosterone in response to a stressor.

The increase in the adrenal weight in the H+S group is in accordance with this hypothesis. It is very intriguing that such change in the anatomy occurs in response to only 7 days of stress exposure. One possibility is that there was an increased in the hormonal production in the gland and that may be the explanation for the increased structure weight. Unfortunately we did not perform histological analyses to verify if this increase results from a higher number of cells and in which layer of the adrenal was accounting for this increase.

Thus, prevention of the peripartum-associated elevations in basal corticosterone and oxytocin system activity by pregnancy stress reveal two alterations that may increase the risk of postpartum psychiatric disorders (Hillerer et al 2011). Here we demonstrated that a similar process may occur in our animal model in response to a neonatal intervention that may increase the risk of pospartum depression by changing the stress response of the dams following acute and chronic stress.

5 – CONCLUSION

Dams that had theirs pups handled from PPD1 to 10 present altered stress response showing increased adrenal weight and also depressive-like behavior during the first day of FST but these result was affect by the exposure to stress after weaning. On the second day of the FST, both non-handled and handled dams that were submitted to restraint stress after weaning didn’t show increased immobility time but handled dams present increase adrenal weight and a reduced basal corticosterone. The behavioral results suggest that neonatal handling of the pups predisposes the mothers to depressive-like behaviors but only in response to acute stress; meanwhile, the biochemical and anatomical analysis suggest that there is also an alteration in the stress response to chronic stress. We show that the handling procedure may have a long lasting effect in the dam’s emotional response increasing the stress response to acute and chronic stress and these alterations may predispose the dams to psychiatric disorders like postpartum depression.

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Conflict of interest: The authors declare that they have no conflict of interest.

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7-FIGURE AND LEGENDS

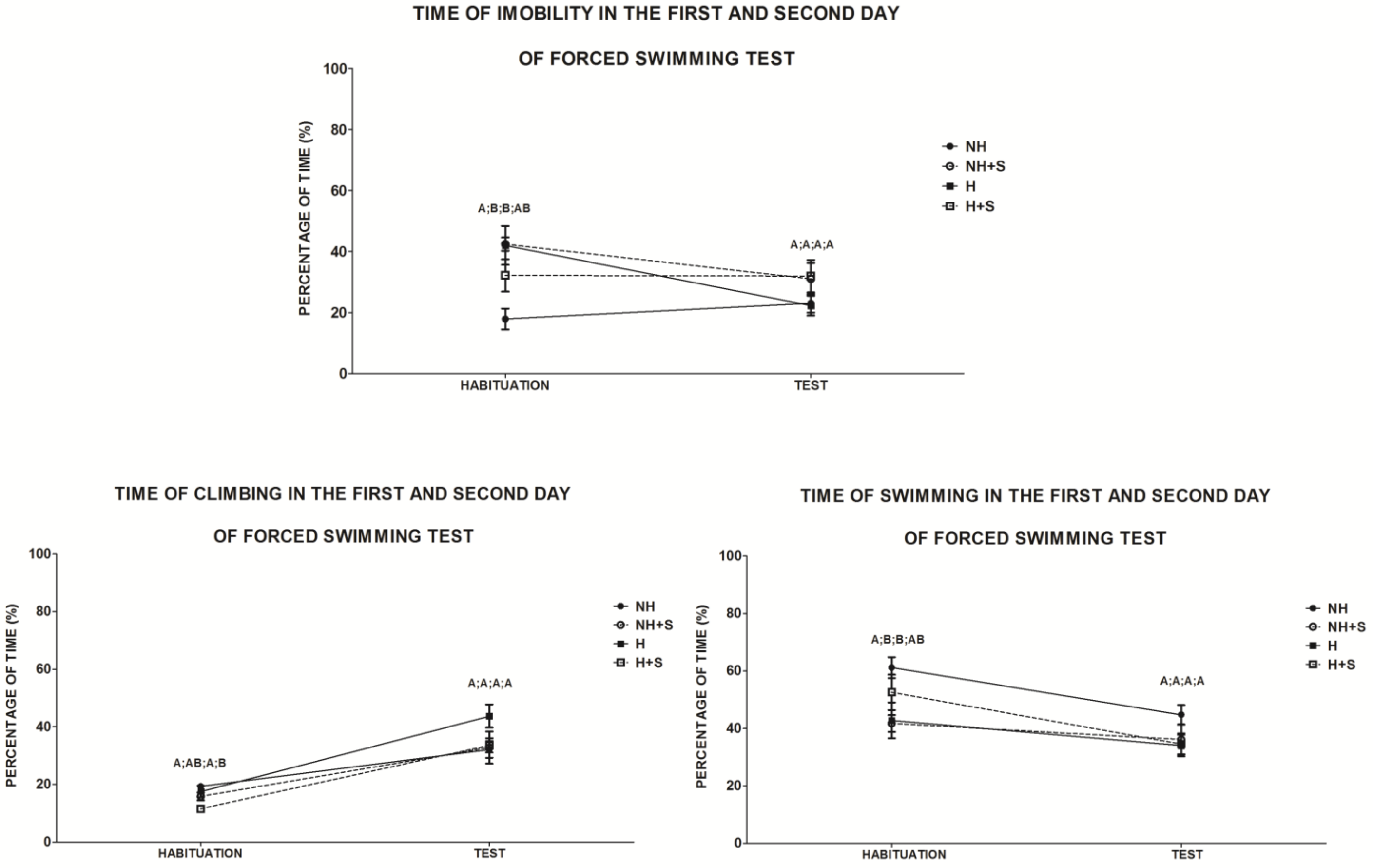

FIGURE 1. Percentage of time that the dams spent in each behavior during the 2 days of FST: Immobility (A), Climbing (B) and Swimming (C). Data were expressed mean (± SEM) and analyzed using GEE for handling procedure, restraint stress and time (habituation and test of FST) followed by Bonferroni´s multiple comparisons test to access differences in each point. Different letters represents significant difference between the groups ($p<0.05$) for interaction handling X stress X time in all graphics, n=12(NH)-9(NH+S)-10(H)-9(H+S).

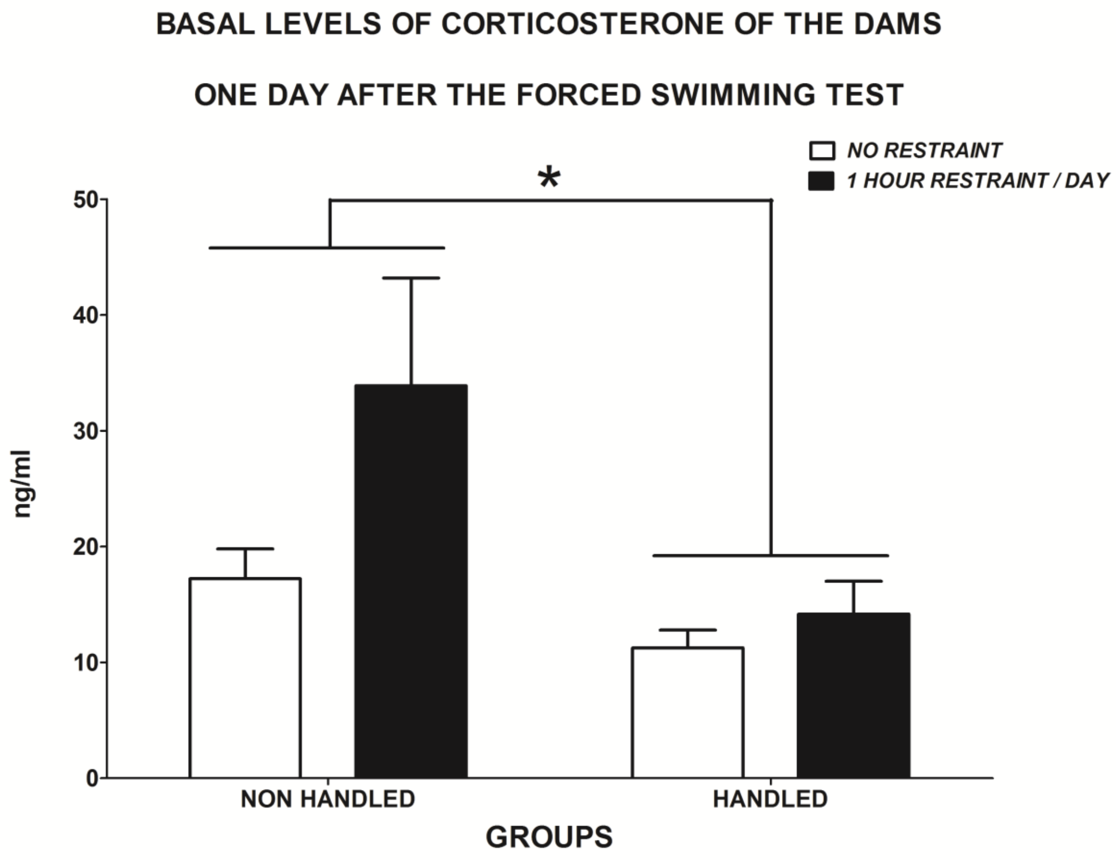

FIGURE 2. Plasm levels of corticosterone 1 day after the FST. Data were expressed mean (± SEM) and analyzed using ANOVA for handling procedure and restraint stress followed by Bonferroni multiple comparisons tests to access differences in each point. * represents significant difference ($p<0.05$) for the main effect handling, n=9(NH)-7(NH+S)-6(H)-6(H+S).

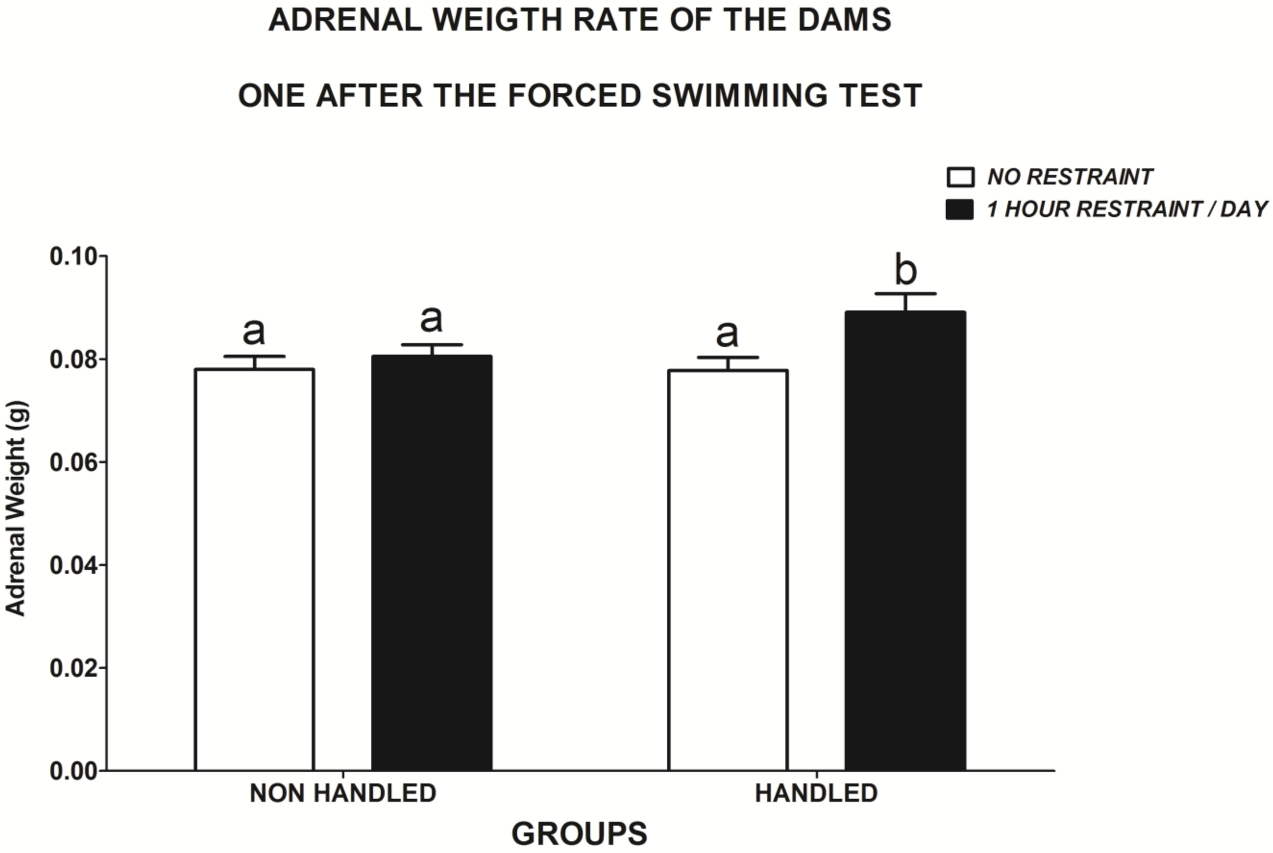

FIGURE 3. Dams adrenal weight/body wight 1 day after the FST. Data were expressed mean (± SEM) and analyzed using ANOVA for handling procedure and restraint stress followed by Bonferroni multiple comparisons tests to access differences in each point, the dams body weight was used as a covariant.to avoid individual differences. Different letters represents significant difference ($p<0.05$) for interaction handling X stress, n=12(NH)-9(NH+S)-10(H)-9(H+S).

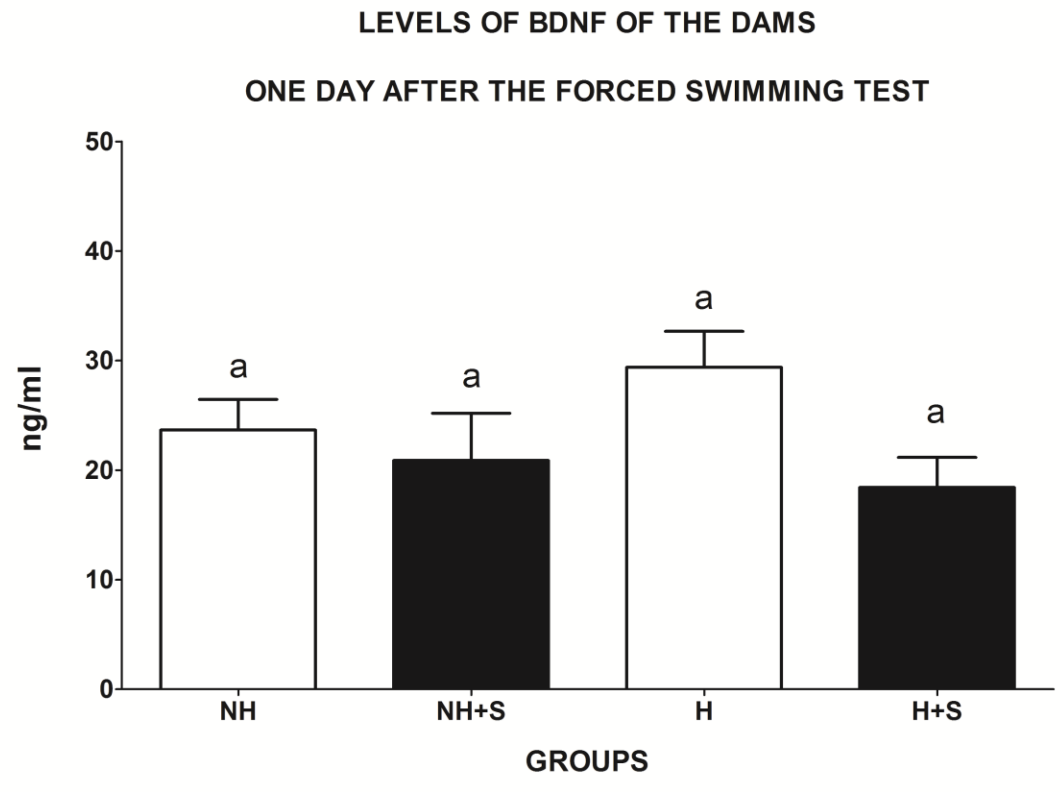

FIGURE 4. Plasm levels of Brain Derived Neurotrophic Factor (BDNF) 1 day after the FST. Data were expressed mean (± SEM) and analyzed using ANOVA for handling procedure and restraint stress followed by Bonferroni multiple comparisons tests to access differences in each point, n=9(NH)-7(NH+S)-6(H)-6(H+S).

5 DISCUSSÃO GERAL

5.1 Manipulação Neonatal e o Comportamento Maternal

Nesta tese mostramos que a manipulação altera a estrutura do comportamento maternal ao longo dos primeiros 10 dias pós-parto e afeta também o padrão diário dos comportamentos de lamber os filhotes, a construção do ninho, amamentação e tempo de permanência fora do ninho.

Estudos que utilizaram intervenções no período pós-parto ligeiramente diferentes de nosso protocolo de manipulação, como 15 minutos de separação diária dos filhotes, mostraram um aumento na interação mãe-filhote, provocando estimulação sensorial materna dos filhotes imediatamente após o seu regresso à caixa moradia (Brown et al 1977, Champagne et al 2003, Claessens et al 2012, Fenoglio et al 2006, Garoflos et al 2005b, Garoflos et al 2008, Stamatakis et al 2006). Esperávamos encontrar o mesmo aumento no cuidado maternal, especialmente no comportamento lamber os filhotes. Observamos um aumento do comportamento de lamber os filhotes imediatamente após o procedimento de manipulação, como já havíamos mostrado em outro estudo (Azevedo et al 2010). No entanto, este aumento foi limitado a esse período específico, o que está de acordo com os resultados descritos por Claessens et al (2012). Esse aumento no comportamento lambida poderia representar uma resposta materna à intervenção ambiental, pois as mães de filhotes não-tratados mostraram um padrão diário estável para este comportamento. As alterações na estabilidade do comportamento de lamber induzido pela manipulação poderiam ser percebidas como uma ameaça ambiental pela prole, o que poderia levar a alterações persistentes no seu desenvolvimento.

Uma sugestão importante feita por Pryce et al (2001) é que a linhagem de ratos utilizados poderia afetar o resultado da manipulação neonatal sobre o comportamento maternal. Usando Wistar em vez de ratos Long Evans (como utilizados em outros estudos) (Claessens et al 2012, Levine & Lewis 1959), eles não encontraram um aumento no comportamento de lambida após o procedimento de manipulação (Winkelmann-Duarte et al 2011). Também utilizamos ratos Wistar, mas encontramos um aumento no comportamento de lambida após a manipulação, o que está de acordo com outros estudos que também usam esta linhagem de ratos (Garoflos et al 2005b, Garoflos et al 2008, Stamatakis et al 2006). Há duas diferenças importantes no protocolo de observação de comportamento maternal que poderiam explicar esta discrepância. Pryce et al (2001) mediram o efeito da manipulação sobre os cuidados maternos durante o período de escuro, quando os ratos são mais ativos , e as mães costumam passar menos tempo em contato com seus filhotes em comparação com o período de luz, durante o qual realizamos as nossas observações. Além disso, começamos a observar o comportamento materno imediatamente após a manipulação dos filhotes, diferente deles, cujas observações começaram apenas 15 minutos após a intervenção.

Observamos também um aumento reativo no comportamento de construção de ninho na sessão imediatamente após a manipulação, o que também poderia se considerar como esperado, uma vez que o procedimento de manipulação altera a área do ninho e introduz odores externos. Isso poderia desencadear a ativação de respostas motores ativas do comportamento materno como a construção de ninho e a lambida (Stern 1989, Stern & Johnson 1989).

Além de diferenças na frequência do comportamento de lamber, observamos mudanças complexas em outros componentes do comportamento materno, que se apresentaram de forma mais persistente do que o aumento reativo no comportamento de lambida. A frequência total de amamentação diminuiu nas genitoras cujos filhotes foram manipulados em comparação com as não-manipuladas, especialmente nas sessões imediatamente e três horas após a manipulação. Além disso, as genitoras do grupo manipulado tiveram um aumento no tempo gasto fora do ninho, e essa mudança não foi observada logo após o procedimento, como seria de esperar; em vez disso, um efeito persistente sobre este comportamento foi observado antes do tratamento e apenas 3 horas após a intervenção, esta diferença pode ser atribuída ao aumento do comportamento de lambida na sessão imediatamente após a manipulação, diminuindo o tempo fora do ninho neste período.

5.2 Mecanismo de Atuação da Manipulação Neonatal sobre o Controle do Comportamento Maternal

Comportamentos maternos que envolvem movimentos mais ativos (como lamber e construção do ninho) e os comportamentos maternos que são mais de repouso (tais como amamentação) são controlados por diferentes regiões do sistema nervoso central com relações opostas ao sistema dopaminérgico (Cummings et al 2010). Comportamentos mais ativos são estimulados por receptores dopaminérgicos na concha do núcleo accumbens (NAs) (Cummings et al 2010). Em contraste, os comportamentos quiescentes, que são mais de repouso, são inibidos pelos receptores dopaminérgicos, de modo que o início de um comportamento quiescente exige uma diminuição no tônus dopaminérgico no NAs (Keer & Stern 1999). Estes mecanismos podem estar envolvidos na mudança da sequência comportamental do cuidado materno, levando a disparidade lamber/amamentar em ninhadas manipuladas conforme descrito nos resultados do experimento 1.

A manipulação por si só poderia afetar diretamente os filhotes (Villescas et al 1977), embora seja difícil de analisar o papel dos filhotes na dinâmica social no inicio da vida, uma vez que seu comportamento está intimamente ligado ao comportamento do cuidador (Cromwell 2011). No entanto, o comportamento da prole pode desempenhar um papel importante no efeito da manipulação sobre o comportamento maternal (Fuertes et al 2006, Smotherman et al 1977, Thoman & Levine 1970). O aumento na lambida poderia ser uma reação da mãe a um aumento na vocalização ultrassônica dos filhotes. O comportamento dos filhotes também poderia explicar a tendência de diminuir a lamber através dos dias pós-parto.

A manipulação neonatal altera o funcionamento do eixo HPA (hipotálamo-hipófise-adrenal), em resposta a uma variedade de estressores (Liu et al 2000, Meaney et al 1985a, Meaney et al 2007, Plotsky & Meaney 1993) e também pode alterar a morfologia e função de estruturas cerebrais (Lucion et al 2003, Todeschin et al 2009, Winkelmann-Duarte et al 2011). Se estas alterações já ocorrem nos primeiros dias de vida dos filhotes (Lucion et al 2003, Meaney et al 2007, Todeschin et al 2009, Weaver et al 2002), isso poderia afetar a resposta comportamental dos filhotes à manipulação, levando a uma estimulação diferencial do comportamento materno ao longo dos 10 primeiros dias pós-parto. Outra possibilidade é que a mãe pode adaptar-se à manipulação repetida, reduzindo a reatividade ao protocolo ao longo dos 10 dias pós-parto.

Os resultados são intrigantes e deixam em aberto a possibilidade de que o comportamento dos filhotes poderia desempenhar um papel importante na mudança do cuidado materno. Um trabalho recente enfatiza o papel que desempenha o filhote na motivação e manutenção do cuidado materno em roedores (Cromwell 2011). Na verdade, o comportamento de amamentação ativo, que é alterado de forma persistente pela manipulação neonatal, é diretamente afetado pelo comportamento filhotes (Pryce et al 2001, Stern 1989, Stern & Johnson 1989, Stern & Lonstein 2001, Villescas et al 1977).

Com base na sequência natural do comportamento maternal (Stern 1989, Stern & Johnson 1989), esperávamos encontrar um aumento nas posturas de amamentação e uma diminuição do tempo fora do ninho logo após o aumento dos componentes mais ativos do comportamento maternal, mas nós encontrado o oposto. Estes resultados sugerem que a manipulação não só altera o comportamento maternal, aumentando ou diminuindo a prevalência de seus componentes comportamentais, mas também dessincroniza a interação mãe-filhotes alterando a sequência comportamental natural do cuidado materno.

A estabilidade da relação mãe-filhote é importante para o desenvolvimento de funções emocionais e cognitivas da prole; para uma revisão, ver Baram et al (2012). De fato, a sincronia nas interações mãe-bebê parece ser uma variável crucial no desenvolvimento humano (Feldman et al 2011) e de ratos (Reeb-Sutherland & Tang 2012, Tang et al 2011). Poderíamos inferir que alterações na sequência comportamental do cuidado materno associadas a alterações na sincronia da interação mãe-filhote levam a uma redução do contato mãe-filhote (aumentando o tempo fora do ninho e diminuindo o tempo amamentando) e alteram a estabilidade do comportamento maternal ao longo do dia (especialmente por meio de alterações no comportamento de lambida e construção do ninho).

Baseado em resultados descritos nesta tese e dados não publicados de nosso laboratório, propomos o seguinte mecanismo de atuação da manipulação neonatal sobre o comportamento maternal (Figura 6).

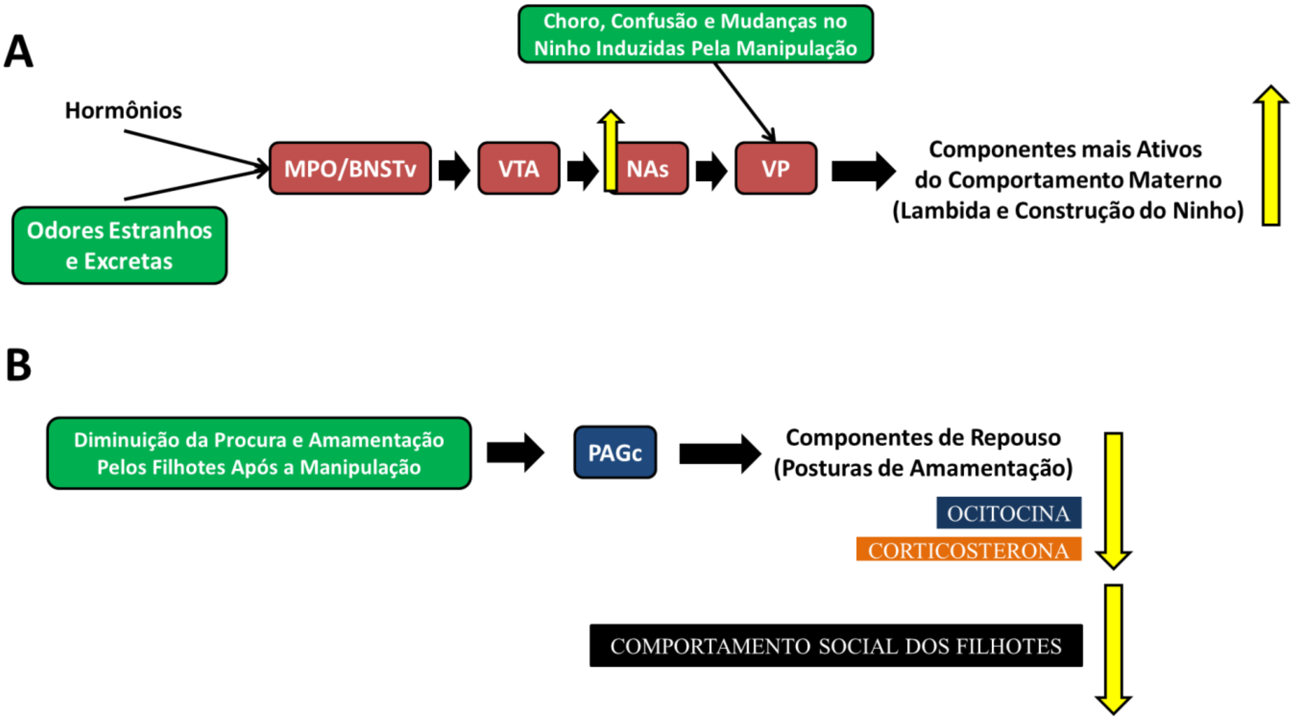

Figura 6 – Mecanismo sugerido de atuação da manipulação neonatal sobre o controle do comportamento maternal e suas implicações para a formação do vínculo Mãe-filhote (**quadros verdes e setas amarelas**). (Figura adaptada de Cummings et al 2010). MPO – Área Pré-optica Medial, BNSTv – Núcleo da estria terminal, VTA – Área Tegmentar Ventral, Concha do Núcleo Accumbens, VP – Pálido Ventral, PAGc – Substancia Cinzenta Periaquedutal.

A manipulação atuaria ativando a VTA pela atuação da ocitocina e do choro dos filhotes durante o processo de manipulação (vocalizações ultrassônicas), e também as áreas MPO, BNSTv que por sua vez seriam estimuladas pela a introdução de odores estranhos na área do ninho devido ao toque dos filhotes pelo experimentador e pelo cheiro das excretas dos filhotes que são muitas vezes estimuladas durante o processo de manipulação. Esse conjunto de fatores levaria a um aumento da atividade dopaminérgica no NAs, desencadeando um aumento dos comportamentos mais ativos do cuidado materno como o de lamber e de construção do ninho. Uma parte importante deste controle é que as áreas final do controle do comportamento os NAs e a VT também podem ser afetas pelo comportamento dos filhotes o que regula de forma mais precisa o comportamento da mãe (Cummings et al 2010).

Pela sequencia natural do cuidado materno, esperaríamos uma redução dos comportamentos mais ativos sendo substituídos aos poucos por comportamentos quiescentes como as posturas de amamentação, mas isso não acontece. A manipulação além de estimular uma ativação mais robusta do NAs, também poderia interferir no comportamento dos filhotes, diminuindo a procura e estimulação da mãe para amamentar (talvez por ficarem confusos e estressados durante o processo de manipulação) levando a uma redução da prevalência deste comportamento (para revisão sobre o controle do comportamento maternal, ver Cummings et al 2010). A diminuição das posturas de amamentação reduziria o tempo de contato entre a prole e a mãe, diminuindo os níveis de ocitocina (da mãe e do filhote) e aumentando os níveis de corticosterona nos filhotes uma vez que o contato e a amamentação atuam reduzindo a resposta ao estresse da prole (Levine 2002). Essas alterações poderiam afetar a formação do vínculo mãe-filhote no inicio do período pós-parto e serem fatores chaves para o surgimento de alterações em comportamentos sociais observados na prole de animais manipulados no período neonatal (Raineki et al 2013, Todeschin et al 2009).

5.3 Comportamento Maternal e o Aprendizado Olfatório nos Filhotes

Ratos filhotes aprendem rapidamente e naturalmente a identificar e preferir o odor ninho (Moriceau & Sullivan 2005, Rangel & Leon 1995). A preferência pelo odor ninho depende do comportamento materno, especialmente da estimulação tátil de lamber os filhotes e do contato mãe-filhote (Kojima & Alberts 2009, Sullivan 2001, Sullivan & Wilson 2003), considerando que a manipulação aumenta o comportamento de lamber os filhotes ativando o LC (Nakamura et al 1987), aumentando os níveis de noradrenalina no BO dos filhotes (Rangel & Leon 1995, Sullivan & Wilson 2003) e induzindo a fosforilação do CREB (McLean et al 1999, Sullivan & Wilson 2003, Yuan et al 2000, Yuan et al 2003) (Figura 4). Em um trabalho anterior mostramos que ocorre uma modificação dependente do sexo nos níveis de CREB e de BDNF no bulbo olfatório de ratos em resposta a manipulação (Reis, A R. 2010 Dissertação de Mestrado) (Figura 5). Uma possível explicação poderia estar em diferenças no cuidado materno direcionado a cada sexo (Moore 1985, Moore & Chadwick-Dias 1986, Oomen et al 2009), os filhotes machos poderiam ser mais afetados pelo comportamento de lambida, uma vez que eles são privilegiados com esse tipo de estimulação, já as fêmeas poderiam sofrer mais com a falta de estimulo e diminuição do contato mãe-filhote em função da diminuição do comportamento de amamentação e o aumento do tempo fora do ninho. De fato, os animais que foram manipulados levam mais tempo para encontrar a área da maravalha do ninho no teste de preferência pelo odor do ninho, no entanto, esse resultado apresentou uma correlação com o comportamento de amamentação apenas para as fêmeas. Os resultados do tempo gasto na área das maravalhas (limpa e do ninho) no teste de preferência pelo odor ninho também parecem suportar essa hipótese, uma vez que as fêmeas que tiveram menos contato com as mães (diminuição do comportamento de amamentação e o aumento do tempo fora do ninho) foram as que apresentaram menos tempo na área da maravalha do ninho. Fêmeas do grupo manipulado não mostram qualquer sinal de aprendizagem ou preferência pelo lado do ninho nas 5 sessões do teste, ao contrário do que acontece com os filhotes machos de ambos os grupos e com os filhotes do sexo feminino do grupo controle. Como observamos um efeito do sexo nos resultados do tempo de permanência na área das maravalhas é possível que a tarefa seja mais difícil para fêmeas do que para machos, independentemente do grupo experimental.

Diferenças na distribuição das lambidas entre os filhotes de sexos diferentes também pode contribuir para este efeito. Estudos têm demonstrado que, em ratos, a mãe lambe mais filhotes machos do que fêmeas, devido a diferenças sexuais nos odores de urina (Moore 1985, Moore & Chadwick-Dias 1986, Oomen et al 2009), nós demonstramos que o padrão de lambida apresenta uma correlação com as mudanças de comportamento social apenas dos filhotes machos. Estes resultados sugerem uma relação entre o comportamento da mãe e a aprendizagem olfatória do filhote, mas é difícil separar os efeitos da manipulação *per se* de alterações do cuidado materno sobre os filhotes.

É possível que já nessa idade filhotes machos e fêmeas apresentem diferentes respostas a estímulos ambientais (McCormick et al 1995, Shanks et al 1994, Viveros et al 2009). Stamatakis et al (2006) mostrou que um protocolo de manipulação neonatal induz mudanças dependentes do sexo nos níveis de receptores 5-HT1A do hipocampo de animais adultos, com os machos tendo um aumento e fêmeas uma diminuição. Além disso, a manipulação neonatal repetida aumenta os níveis de BDNF na área de CA4 do hipocampo de filhotes machos, enquanto que as fêmeas não apresentam esta alteração (Garoflos et al 2005b).

5.4 Manipulação Neonatal e a Resposta Emocional das Genitoras

Os resultados mostram que a manipulação dos filhotes durante o período neonatal pode ter um impacto a longo prazo sobre o comportamento das mães, alterar respostas emocionais e pode predispor o organismo materno à depressão. A exposição ao estresse durante a gravidez ou no período pós-parto está muitas vezes associado ao desenvolvimento de transtornos do humor (Beck 2001, Hillerer et al 2012, Hillerer et al 2011, Robertson et al 2004), em humanos, a exposição crônica a estressores psicossociais, como o conflito social, é um dos mais fortes preditores de depressão pós-parto (Westdahl et al 2007). O procedimento de manipulação repetido de forma crônica dos primeiros 10 dias pós-parto pode ser interpretado pelas genitoras como paradigma de derrota social, considerando que elas não podem evitar a separação diária de seus filhotes. Isso provavelmente funcionaria como um intenso estressor psicológico e, portanto, poderia predispor a comportamentos do tipo depressivo como demonstramos nesta tese.

Genitoras que tiveram seus filhotes manipulados no período neonatal apresentam respostas emocionais alteradas após o desmame, mas os resultados foram diferentes dependendo da exposição ao estresse por contenção após o desmame. Mães do grupo manipulado (H) apresentam um comportamento do tipo depressivo no primeiro dia do teste de nado forçado (FST) assim como o que foi observado no grupo não-manipulado estressado após o desmame (NH+S). É possível que a própria habituação ao FST funcione como uma exposição ao estresse agudo, evidenciando o efeito da manipulação (no grupo H) e do estresse após o desmame (no grupo NH+S) sobre essa resposta, causando um aumento do tempo de imobilidade nestes grupos. Este resultado estaria de acordo com outro estudo que indica que as mães que tiveram seus filhotes manipulados mostram aumento de resposta ao estresse agudo (Silveira et al 2013). Genitoras do grupo manipulado e estressado após o desmame (H + S) apresentaram um comportamento um pouco diferente, embora mostrem um aumento do tempo de imobilidade no primeiro dia do FST, não houve diferença significativa quando comparado com os outros grupos, o que sugere que existe uma interação entre o procedimento de manipulação e o estresse crônico aplicado após o desmame. Com base na análise comportamental poderia se inferir que o tratamento atenua a resposta ao estresse crônico, mas o aumento do peso adrenal observado apenas no grupo H+S sugere o contrário.

Genitoras dos grupos que passaram pela manipulação neonatal (H e H + S) têm níveis basais mais baixos de corticosterona um dia depois do FST em comparação com os grupos sem manipulação (NH e NH + S), independentemente do protocolo de estresse após o desmame. Isso mostra que a manipulação afeta de forma duradoura a resposta ao estresse das mães, possivelmente devido a uma alteração das adaptações neuroendócrinas que ocorrem durante a lactação, como o hipercorticalismo crônico (Hillerer et al 2011, Lightman et al 2001, Stern et al 1973, Windle et al 1997) e os níveis elevados de globulina de ligação a corticosterona (Douglas et al 2003, Pearlman 1983).

É possível que a manipulação dos filhotes repetidamente durante o período neonatal interfira na instalação destas adaptações, evitando o aumento adaptativo dos níveis basais de corticosterona mantendo uma maior disponibilidade de corticosterona livre o que pode conduzir a uma maior responsividade ao estresse. Este resultado estaria de acordo com outro estudo que indica que as mães que tiveram seus filhotes manipulados mostram aumento da liberação de corticosterona em resposta ao estresse agudo (Silveira et al 2013).

O aumento do peso das adrenais no grupo H + S está de acordo com esta hipótese. É intrigante que uma mudança na anatomia ocorre em resposta a apenas 7 dias de exposição ao estresse. Podemos inferir que houve aumento da produção e na demanda hormonal da glândula e talvez isso seja a explicação para o aumento de peso da estrutura. Infelizmente não realizamos análises histológicas para verificar se este aumento resulta de um maior número de células e qual camada das adrenais foi responsável por este aumento.

Levando em consideração as alterações encontradas no experimento 1 desta tese, os dados do experimento 2 e dados que ainda estão sendo preparados para publicação, propomos o seguinte mecanismo para a alteração induzida pela manipulação neonatal na resposta ao estresse na genitoras (Figura 7).

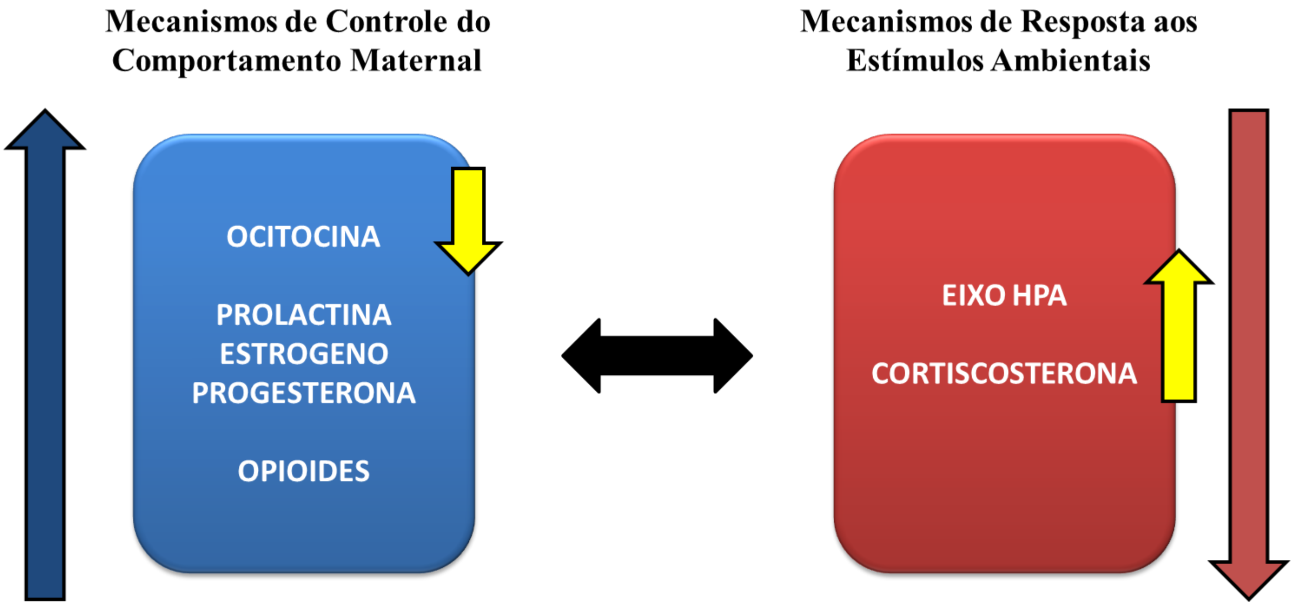

Figura 7 – Mecanismos adaptativos da resposta ao estresse e do controle do comportamento das mães que atuam durante o período perinatal (Figura baseada em Neumann, I.D. 2003). As setas amarelas representam os possíveis mecanismos pelos quais a manipulação neonatal atua alterando a implantação destas adaptações e comprometendo de forma duradoura as respostas emocionais das genitoras.

A manipulação neonatal afeta de forma persistente diferentes componentes do comportamento materno, aumentando o tempo de permanência fora do ninho, diminuindo as posturas de amamentação e o contato mãe-filhotes (Reis et al 2014). O controle do comportamento de amamentação é altamente influenciado pela ocitocina que atua em neurônios dopaminérgicos na área tegmental ventral e suas projeções para o núcleo accumbens (Shahrokh et al 2010). Por sua vez, os níveis de ocitocina da mãe e o controle da ejeção do leite também estão associados com o contato da mãe com os filhotes e com o comportamento dos mesmos estimulando o ventre da mãe e sugando o leite (Freund-Mercier & Richard 1984, Lambert et al 1993a, Lambert et al 1993b). O aumento no comportamento de lambida seguido por um decréscimo de amamentação e contato com os filhotes poderia indicar uma diminuição nos níveis de ocitocina das mães.

A ocitocina também desempenha um papel na inibição da resposta ao estresse no hipotálamo durante o período de perinatal (Neumann et al 2000), portanto, uma diminuição dos níveis centrais de ocitocina poderia ser seguida por um aumento da reatividade do organismo materno ao estresse após o período de manipulação neonatal.

O impedimento da instalação de adaptações da resposta ao estresse no organismo da mãe, como o aumento dos níveis basais de corticosterona e dos níveis de globulina de ligação a corticosterona e o aumento da atividade do ocitocinérgica revelam duas alterações que poderiam aumentar o risco de transtornos psiquiátricos pós-parto (Hillerer et al 2011). Aqui demonstramos que um processo semelhante pode ocorrer em resposta a uma intervenção neonatal e isso pode aumentar o risco de aparecimento de sintomas depressivos no período pós-parto ou até em eventos futuros através de alterações da resposta ao estresse das genitoras.

A compreensão dos mecanismos pelos quais ocorrem essas alterações associadas a mudanças do comportamento da mãe pode servir como uma importante ferramenta para elaboração de estudos clínicos. A observação da existência de comportamentos similares em humanos ajudaria na elaboração de politicas de saúde publica que visem minimizar os efeitos de eventos adversos acontecidos no inicio da vida sobre a saúde física e mental tanto da mãe quanto da criança.

6 CONCLUSÕES

A manipulação neonatal induz mudanças complexas em componentes críticos de cuidados maternos. Este estudo mostra que a manipulação pode alterar a sincronia da interação mãe - filhote, afetando a sequência comportamental do cuidado materno o que leva a uma redução do contato mãe - filhotes nos primeiros 10 DPP, muda o padrão diário do comportamento de amamentação, de lamber os filhotes e construir o ninho. A manipulação induz um aumento reativo no comportamento lamber os filhotes imediatamente após a intervenção e provoca alterações comportamentais nos filhotes de uma forma sexo-específica. Estas modificações no comportamento maternal poderiam explicar as diferenças no comportamento dos filhotes no labirinto em Y uma vez que o resultado do teste de preferência pelo odor do ninho se correlacionou com diferentes componentes do comportamento maternal (também de uma forma sexo-específica). Embora as fêmeas pareçam ser mais afetadas pelo processo de manipulação, os filhotes machos também apresentam um aumento do tempo necessário para chegar à área do ninho, o que sugere que eles também sejam afetados pelo procedimento.

Além dos efeitos para os filhotes, mostramos nesta tese que genitoras que tiveram filhotes manipulados (DPP1-10) mostram um aumento na resposta ao estresse, aumento do peso adrenal e também um comportamento do tipo depressivo durante o primeiro dia do FST, mas estes resultados foram afetados pela exposição ao estresse após o desmame. No segundo dia do FST, não houve diferença entre os grupos, no entanto, mães que tiveram seus filhotes manipulados e que foram submetidos ao estresse de contenção após o desmame apresentaram um aumento de peso adrenal e diminuição dos níveis basais de corticosterona. Os resultados comportamentais sugerem que a manipulação neonatal dos filhotes predispõe as mães a comportamentos do tipo depressivo, mas apenas em resposta ao estresse agudo. Enquanto isso, as análises bioquímicas e anatômicas realizadas nas genitoras sugerem que exista também um efeito da manipulação na resposta ao estresse crônico. Mostramos que manipulação neonatal pode ter um efeito duradouro na resposta emocional das mães, aumentando a resposta ao estresse (agudo e crônico) e predispondo as genitoras ao desenvolvimento de comportamentos do tipo depressivo, principalmente em resposta ao estresse agudo.

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