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Ana Carolina de Moura

Análise comportamental, molecular e bioquímica de ratas lactantes com diferentes padrões de comportamento maternal.

Porto Alegre
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Tese submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor.

Orientadora: Profa. Dra. Márcia Giovenardi
Co-orientadora: Profa. Dra. Viviane Elsner

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Porto Alegre, ____ de __________ de_______.

BANCA EXAMINADORA

Profa. Dra. Lisiane Bizarro Araújo

Prof. Dr. Fernando Benetti

Profa. Dra. Ionara Rodrigues Siqueira

Profa. Dra. Renata Padilha Guedes

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RESUMO

INTRODUÇÃO: O cuidado materno é crucial para o desenvolvimento da prole, e os diferentes padrões de lamber os filhotes podem ser induzidos por variações nos níveis de moléculas neuromodulatórias e na expressão de seus receptores durante a gestação e pós-parto. Essas mudanças podem contribuir para o desenvolvimento e manejo do comportamento maternal. A frequência da lambida é usada para avaliar o cuidado materno, havendo mães com baixa (LL) e alta (HL) frequências de lamber. Funções encefálicas importantes, como a aprendizagem e a memória, podem ser influenciadas pelos níveis de estresse oxidativo, que também podem modular processos fisiopatológicos (por exemplo: depressão, ansiedade e outros transtornos psiquiátricos). **OBJETIVOS:** Analisar a expressão gênica dos receptores de dopamina (Drd1a), prolactina (Prlr), serotonina (Htr1a, Htr1b), estrogênio (Esr1, Esr2) e do fator neurotrófico derivado do cérebro (BDNF) no bulbo olfatório (OB), hipocampo (HP), córtex pré-frontal (PFC) e estriado (ST) de ratas Wistar de três grupos: LL, HL e fêmeas virgens em diestro. Verificar os níveis de acetilação da histona-H4 no OB de ratas LL e HL. Avaliar o estresse oxidativo no HP, OB e plasma de mães lactantes LL e HL. **MÉTODOS:** O comportamento materno foi estudado nos primeiros 7 dias pós-parto. Foi realizado qRT-PCR para avaliação da expressão gênica nas regiões encefálicas citadas acima. Os níveis de acetilação de histona-H4 foram determinados utilizando o Kit de Ensaio de Acetilação Global Histona-H4. O estresse oxidativo foi avaliado pela atividade da superóxido dismutase (SOD) e catalase (CAT); pelos danos ao DNA (ensaio cometa); e pelo ensaio da dihidrodiclorofluoresceína (DCF). **RESULTADOS E DISCUSSÃO:** Houve um aumento na expressão gênica de Drd1a, Prlr, Htr1a, Htr1b e Esr1 no OB do grupo HL, em comparação ao LL. No HP, apenas Drd1a, Prlr e Htr1a foram expressos de forma diferenciada, somente quando comparados os grupos HL ou LL, com o de ratas virgens. Mães HL tem níveis mais elevados de expressão gênica no OB, que é uma estrutura crucial para promover diferenças comportamentais. Ratas LL mostram uma diminuição na frequência de lamber, e um aumento da permanência afastadas dos filhotes. A análise epigenética revelou que ratas HL tem níveis de acetilação da histona-H4 maiores do que LL. A expressão aumentada dos receptores anteriormente citados no OB está relacionada, pelo menos em parte, ao estado de hiperacetilação da histona-H4 observado. Os resultados também demonstram que no HP de mães LL, a atividade das enzimas SOD e CAT estão aumentadas em relação às HL. No OB, a atividade de SOD e CAT também é maior em LL. O ensaio do cometa no HP mostrou que LL tem níveis mais elevados de danos basais e níveis aumentados de quebras de DNA, do que

HL. No OB, LL também possui maiores níveis de danos ao DNA. No plasma o ensaio de oxidação do DCF revelou que LL tem níveis mais elevados de produção de espécies reativas de oxigênio, do que HL. **CONCLUSÃO:** O aumento da expressão gênica observado em HL, provavelmente ocorre como consequência da acetilação das histonas, o que facilita a expressão dos genes. Além disso, as mães LL apresentaram evidência de aumento do estresse oxidativo quando comparadas a HL, sugerindo que as variações no comportamento materno podem estar relacionadas a esses parâmetros bioquímicos.

Palavras-chave: lambida/cuidado, hipocampo, bulbo olfatório, expressão gênica, acetilação das histonas, estresse oxidativo, dano ao DNA.

ABSTRACT

INTRODUCTION: Maternal care is crucial for offspring development and licking/grooming patterns can be induced by variations in the levels of neuromodulatory molecules, and in the expression of their receptors, during pregnancy and postpartum. These changes may contribute to the development and management of maternal behavior. The frequency of licking the pups is used to evaluate maternal care, having mothers with low licking (LL) and high licking (HL) frequencies. Important brain functions, such as learning and memory, can be influenced by oxidative stress, which can also modulate pathophysiological processes (e.g., depression, anxiety, and other psychiatric disorders). **OBJECTIVES:** Analyze the gene expression of the receptors for dopamine (Drd1a), prolactin (Prlr), serotonin (Htr1a, Htr1b), estrogen (Esr1, Esr2), and of the brain-derived neurotrophic factor (BDNF) in the olfactory bulb (OB), hippocampus (HP), prefrontal cortex, and striatum of Wistar rats from three groups: LL, HL, and virgin females in diestrus. Verify the acetylation levels of histone-H4 in the OB of LL and HL rats. Evaluate oxidative stress in the HP, OB, and plasma of LL and HL lactating mothers. **METHODS:** Maternal behavior was studied for the first 7 postpartum days. Brain areas were analyzed by qRT-PCR, for the gene expression. Acetylation levels of histone-H4 were determined using the Global Histone-H4 Acetylation Assay Kit. Oxidative stress was assessed by superoxide dismutase (SOD) and catalase (CAT) activities, DNA damage (comet assay), and dihydrodichlorofluorescein (DCF) oxidation assay. **RESULTS AND DISCUSSION:** There was an increase in gene expression of Drd1a, Prlr, Htr1a, Htr1b and Esr1 in the OB of HL, compared to LL. In the HP, Drd1a, Prlr and Htr1a were differently expressed only when comparing HL or LL to the virgin females. The main finding of the present study is that HL had higher gene expression levels in the OB, which is a crucial structure to promote behavioral differences. LL mothers also showed a decrease in the frequency of nursing, and an increase of remaining off the pups. Epigenetic analyses revealed that HL had increased histone-H4 acetylation levels than LL. The enhanced expression of the previously cited receptors in the OB could be related, at least in part, to the hyper acetylation status of histone-H4 observed. Results also demonstrate that in the HP of LL, the activity of SOD and CAT was increased compared to HL. In the OB, the activity of SOD and CAT was also increased in LL. The comet assay in the HP showed that LL had higher levels of basal damage and increased levels of DNA breaks than HL. In the OB, LL also had higher levels of DNA damage. DCF oxidation assay in the plasma revealed that LL had higher levels of reactive oxygen species production than HL. **CONCLUSION:** The increased gene expression observed in the HL group probably occurs as a consequence of the acetylation of histones, which facilitates the expression of such genes. Furthermore, LL mothers showed evidence of increased oxidative stress when compared to HL, suggesting that variations in maternal behavior might be related to these biochemical parameters.

Keywords: licking/grooming, hippocampus, olfactory bulb, gene expression, histone acetylation, oxidative stress, DNA damage.

LISTA DE ABREVIATURAS

BDNF	Brain-derived neurotrophic factor
BNST	Núcleo do leito da estria terminal
CAT	Catalase
SNC	Sistema nervoso central
DCF	Dihidrodiclorofluoresceína
DNA	Ácido Desoxirribonucleico
Drd1a	Receptor de dopamina 1a
E2	Estradiol
ER	Receptor de estrogênio
Esr1 ou ERα	Receptor de estrogênio 1 ou alfa
Esr2 ou ERβ	Receptor de estrogênio 2 ou beta
GR	Receptor de glicocorticoides
HL	Mães com alta frequência de lamber os filhotes (High licking)
HP	Hipocampo
Htr1a	Receptor de serotonina 1a
Htr1b	Receptor de serotonina 1b
LL	Mães com baixa frequência de lamber os filhotes (Low licking)
MeA	Amígdala medial
MPOA	Área pré-óptica medial
mRNA	Ácido ribonucleico mensageiro
OB	Bulbo olfatório
OT	Ocitocina
OTR	Receptor de ocitocina
PFC	Córtex pré-frontal
PRG	Progesterona
PRL	Prolactina
Prlr	Receptor de prolactina
qRT-PCR	Quantitative Reverse transcription – Polimerase chain reaction
RNS	Espécies reativas de nitrogênio
ROS	Espécies reativas de oxigênio
SOD	Superóxido dismutase
ST	Estriado

LISTA DE SÍMBOLOS

®	Marca registrada
cm	Centímetro
ml	Mililitro
ºC	Graus centígrados
α	Alfa
β	Beta

SUMÁRIO

1 REVISÃO DA LITERATURA

1.1 Comportamento Maternal

Em mamíferos, observa-se que no final da gestação e no início da lactação, o repertório comportamental da fêmea difere daquele exibido em outros períodos de seus ciclos reprodutivos. Elas sofrem importantes alterações hormonais e comportamentais que possibilitam o cuidado da prole. Além disso, a presença dos filhotes é essencial para o surgimento e a manutenção deste comportamento (Giovenardi *et al*, 2000; Ferreira *et al*, 2002; Numan, Insel, 2003; Giovenardi *et al*, 2005; Lonstein, Morrell, 2006).

As alterações comportamentais expressas durante a gestação e a lactação são resultantes de diversas modificações neuroendócrinas que ocorrem com a fêmea (Neumann, 2001). O período gestacional é caracterizado por uma expressiva elevação dos níveis de estradiol (E2) e progesterona (PRG). Já no período pós-parto, os hormônios folículo-estimulante, luteinizante e E2 se mantêm em níveis baixos (Escalada *et al*, 1997). Já a secreção de PRG aumenta progressivamente juntamente com a prolactina (PRL) e a ocitocina (OT) (Smith, Neill, 1977; Taya, Sesamoto, 1981; Taya, Greenwald, 1982). Outros hormônios como o do crescimento, os tireoidianos, os glicocorticoides, a insulina e o paratormônio, também são necessários para mediar diversas mudanças ocorridas neste período (Tucker, 1994).

A OT é o principal hormônio que induz a formação do vínculo entre os indivíduos, principalmente por possuir inúmeros receptores em áreas do sistema nervoso central (SNC) relacionadas aos comportamentos afetivos (Douglas, 2010). Esse hormônio também é fundamental para as interações com outros neurotransmissores, como a dopamina (Spielewoy *et al*, 2000; Champagne *et al*, 2004; Kavelaars *et al*, 2005). Estudos em animais e humanos sugerem que a expressão inicial do comportamento materno depende dos sistemas de OT e dopamina. No entanto, o mecanismo pelo qual esses sistemas afetam os comportamentos parentais e o momento em que ocorrem não são bem compreendidos (Tombeau Cost *et al*, 2016).

O estrogênio desempenha um papel importante na iniciação do comportamento materno em roedores, contribuindo tanto para a ansiedade reduzida, quanto para o aumento das abordagens aos filhotes, sendo ambos subjacentes ao início deste comportamento

(Murakami, 2016). A PRL também é fundamental para a expressão do comportamento maternal, sendo que em ratas virgens a sua administração acelera o início do cuidado materno (Salais-López *et al*, 2016).

Os sinais que conduzem o comportamento materno são complexos e envolvem várias áreas encefálicas, a maioria das quais são inervadas pela serotonina. A transmissão da serotonina influencia indiretamente os processos maternos através da liberação de hormônios relevantes para a mãe como a PRL, OT e vasopressina, mas também pode ter efeitos mais diretos na sobrevivência e na taxa de crescimento da prole, bem como no cuidado e agressão materna (Angoa-Pérez, Kuhn, 2015).

O comportamento maternal em roedores é caracterizado por ações que as mães exercem para garantir o sucesso no crescimento dos filhotes; estes nascem altriciais: são cegos, surdos e desprovidos de pelos, incapazes de se locomoverem, de excretarem e de manterem a temperatura corporal. Desta forma, os cuidados maternos são fundamentais para o desenvolvimento da prole (Mann, 2006). Outras ações praticadas pela mãe incluem: a construção e o recolhimento dos filhotes para o ninho, a estimulação à micção e à defecção por meio da lambida ano genital, o agrupamento dos filhotes no ninho e o posicionamento sobre eles provendo nutrição e calor (Stern, Johnson, 1990; Albert, Walsh, 1995; Mann, 2006).

O grau de intensidade do comportamento maternal apresenta variações naturais, principalmente na amamentação e na lambida dos filhotes (Champagne *et al*, 2007). A amamentação é um comportamento reflexo dependente da mãe. O ato de lamber expressa a motivação materna em relação à ninhada, ou seja, é um comportamento nato da mãe e independe da ação do filhote. A lambida melhora o crescimento somático e o desenvolvimento encefálico, provocando efeitos imediatos no sistema endócrino do recém-nascido (Schanberg, 1984; Levine, 1994; Stern, 1997). A ingestão da urina dos filhotes, por meio da lambida ano genital, auxilia a suprir as perdas maternas de água e eletrólitos (Gubernick, Alberts, 1983).

A avaliação usada como parâmetro para mensurar o comportamento maternal é realizada por meio da quantificação da frequência do ato de lamber os filhotes. Estes dados apresentam um padrão de distribuição normal quando verificada em uma grande população. Desta forma, as mães que apresentam elevada frequência (HL - *high licking*) e as que apresentam frequência reduzida (LL - *low licking*) deste comportamento, representam dois extremos de uma mesma população (Champagne *et al*, 2003).

1.2 Áreas Encefálicas Relacionadas ao Comportamento Maternal

Inúmeras regiões do SNC estão diretamente envolvidas no processamento das emoções e na exteriorização dos comportamentos (Antônio *et al*, 2008). Em relação à modulação do comportamento maternal em ratas destacamos duas regiões como sendo essenciais: a área pré-óptica medial (MPOA) e a parte ventral do núcleo do leito da estria terminal (BNST). Neurônios da MPOA e do BNST projetam-se direta ou indiretamente para várias regiões do encéfalo que estão envolvidas na regulação do comportamento das mães, como: o septo lateral, a habênula lateral, o núcleo acumbens, a área tegmental ventral, a substância cinzenta periaquedutal e o hipotálamo (Stack *et al*, 2002).

O córtex cerebral é outra área relacionada com o comportamento afetivo, onde estão localizadas as funções cognitivas (Kandel *et al*, 1985). O córtex pré-frontal apesar de não fazer parte do circuito límbico tradicional, possui intensas conexões bidirecionais com o tálamo, a amígdala e outras estruturas subcorticais, explicando o importante papel que desempenha na gênese dos estados afetivos (Amaral, Oliveira, 2010). O córtex pré-frontal ventromedial e o estriado codificam o valor subjetivo de diferentes produtos ou ações durante a tomada de decisões, de uma forma que poderia guiar a escolha (Kable *et al*, 2009).

Por ser uma área integrada à tomada de decisões e considerada parte crucial dos sistemas neurobiológicos das emoções (Antônio *et al*, 2008), o hipocampo (HP) exerce importante função relacionada ao comportamento, à memória espacial e de reconhecimento, na conversão da memória de curto prazo em memória de longo prazo e na mediação da resposta ao estresse (Machado, 2006). Também está envolvido em algumas desordens psíquicas, como estresse crônico e depressão (Mao *et al*, 2017).

Outra região significativa na modulação do comportamento maternal é a amígdala medial (MeA), a qual recebe aferências do sistema olfatório principal e acessório (Numan, Insel, 2003). Ela está associada com circuitos neurais que regulam o medo, a reação defensiva e o comportamento de fuga (Ledoux, 1996). Ao receber um novo estímulo olfatório, o comportamento de medo é ativado, mediado pela MeA. Nas ratas lactantes, este estímulo é deprimido para que o comportamento maternal possa ocorrer (Numan, 2006). Acredita-se que as projeções da MeA para a MPOA estimulam o comportamento maternal, enquanto as projeções para área hipotalâmica anterior e núcleo ventromedial do hipotálamo são inibitórias (Numan, Insel, 2003).

O bulbo olfatório (OB) desempenha um papel fundamental na interação mãe – filhotes (Gonzalez, Poidron, 2002; Lévy *et al*, 2004; Fonseca *et al*, 2006). O sistema olfativo age como mediador destas interações comportamentais. Alterações profundas na regulação do olfato, moduladas por modificações fisiológicas que ocorrem ao final da gestação e no parto, levam a fêmea a responder de modo positivo ao estímulo oriundo do recém-nascido (Lévy *et al*, 2004). O OB, ou lóbulo olfativo, é a região encefálica em que se interpretam as aferências sensoriais das terminações nervosas dos receptores estimulados por odor (Gordon, 2003); ele codifica estas informações levando-as ao córtex cerebral por meio do trato olfatório, projetando-se para várias regiões encefálicas onde serão processadas (Broadwell, 1975).

O olfato é uma importante ferramenta reguladora do comportamento reprodutivo (Dias *et al*, 2008). Em fêmeas virgens, o odor oriundo dos filhotes ativa áreas encefálicas que geram medo e fuga. No final da gestação, eventos fisiológicos modificam o significado do odor dos filhotes de negativo para positivo. Esta alteração parece ser fundamental para o início do comportamento maternal (Numan, Insel, 2003).

Há evidências da atuação de mecanismos neuroendócrinos no comportamento maternal, por meio do trato olfatório, referindo-se à ativa participação da OT como principal hormônio facilitador deste processo (Fleming *et al*, 1992; Kolunie *et al*, 1994; Brouette-Lahlou, 1999). Mesmo não sendo fundamental para o desenvolvimento deste comportamento, sabe-se que danos periféricos de neurônios sensitivos olfatórios geram um déficit no recolhimento dos filhotes (aumento da latência) (Kolunie *et al*, 1994) e que o corte ou remoção dos nervos vomeronasais modifica a organização do comportamento de lambida ano genital (Brouette-Lahlou *et al*, 1999).

1.2.1 Regiões com Diferenças na Expressão Gênica

Em ratas, foi demonstrado que durante a gravidez, no parto e no pós-parto ocorrem alterações nos níveis dos receptores de ocitocina (OTR), especialmente no septo ventral (Landgraf *et al*, 1991), no núcleo supraóptico do hipotálamo (Caldwell *et al*, 1987; Mezey, Kiss, 1991; Landgraf *et al*, 1992) e na área pré-óptica medial do hipotálamo (Caldwell *et al*, 1987), bem como no BNST, no OB e na amígdala. Este aumento na expressão de OTR provavelmente ocorre para facilitar o aparecimento e manutenção do comportamento maternal (Leng *et al*, 2008). A elevação nos níveis de OTR no OB e HP de mães com cuidado maternal mais acentuado pode explicar as diferenças no comportamento maternal destas fêmeas (Ruthschilling *et al*, 2012).

Quando comparadas com mães LL, as HL possuem elevados níveis de receptores de estrogênio α (ERα) na MPOA (Champagne *et al*, 2003). Contudo, os principais responsáveis pela modulação da resposta materna são provavelmente os receptores de dopamina (D1 e D2), o D1 promovendo os atos de lamber e de recolher os filhotes, já o D2 prejudicando a amamentação (Miller, Lonstein, 2005). Lesões nesta área bloqueiam diversos comportamentos maternais, como o recolhimento, a construção de ninho e a amamentação (Wellman *et al*, 1997), não promovendo alterações de outros comportamentos como a atividade sexual ou locomotiva (Numan, 2006).

Outra região bastante importante para o comportamento maternal é o corpo do estriado, o qual é composto por três importantes subdivisões: o núcleo caudado, o putâmen e o estriado ventral, o qual inclui o núcleo accumbens – uma região envolvida com a emoção e a memória (Delong, 2000). Já foi demonstrado que mães HL possuem elevados níveis de receptores de dopamina (D1 e D3) no núcleo accumbens, quando comparadas à LL (Champagne *et al*, 2006).

1.3 Transmissão do Padrão de Comportamento Maternal

As relações entre genes, encéfalo e comportamento apresentam aspectos complexos em diferentes contextos, relacionados ao desenvolvimento dos organismos e à mecanismos fisiológicos. O comportamento não é diretamente especificado pelos genes, mas estes codificam produtos moleculares que constituem e modulam o funcionamento encefálico, por meio do qual o comportamento se expressa (Robinson *et al*, 2008).

O desenvolvimento e a atividade encefálica, juntamente com o comportamento, dependem de influências herdadas e de fatores ambientais. Desta forma, a informação social pode impactar a expressão gênica no encéfalo e o comportamento (Robinson *et al*, 2008). De modo geral, os comportamentos de mamíferos não dependem apenas de um pequeno número de genes, na verdade eles são o exemplo perfeito de traços multigênicos (Faff *et al*, 1997).

O genoma de um indivíduo é extremamente responsivo a uma variedade de estímulos, inclusive aqueles associados ao comportamento social, ao longo de toda sua vida. A informação social pode gerar modificações epigenéticas do genoma, que são definidas como modulações na expressão gênica, provocadas por alterações na estrutura da molécula, sem alterar a sequência de DNA, como exemplo o estado de acetilação das histonas e de metilação do DNA (Robinson *et al*, 2008).

A metilação do DNA é uma modificação epigenética que tem sido estudada extensivamente no contexto da regulação da transcrição, um papel observado pela primeira vez na década de 1980. A metilação do DNA que ocorre nos promotores gênicos está associada à repressão transcricional. No genoma, a metilação do DNA regula processos normais e anormais, sendo catalisada por enzimas chamadas DNA metil-transferases (DNMTs) (Lee, Bartolomei, 2013).

O nucleossomo consiste em 146 pares de bases de DNA envolvidos em torno de um octâmero de histonas, composto por duas cópias das proteínas histonas H2A, H2B, H3 e H4. Muitos de seus aminoácidos estão sujeitos a modificações químicas ou pós-translacionais. As modificações nas histonas são executadas, interpretadas e eliminadas por enzimas específicas; coletivamente, estas alterações são referidas como modificações pós-traducionais (Tarakhovsky, 2010; Musselman *et al*, 2012).

As modificações das histonas medeiam a transcrição influenciando o grau de condensação da cromatina. Especificamente, as caudas amino-terminais das histonas desempenham um papel importante na integração da cromatina em uma estrutura organizada, facilitando a estabilidade dos nucleossomos (Iwasaki *et al*, 2013). Essas modificações pós-traducionais podem induzir a transcrição - tornando a cromatina mais aberta; ou podem suprimir a transcrição - facilitando a condensação da cromatina (Dhall *et al*, 2014). Enquanto as modificações das histonas e a metilação do DNA são executadas por diferentes maquinarias celulares, os dois processos são dinamicamente ligados, havendo uma interação significativa entre esses mecanismos epigenéticos. As modificações das histonas têm demonstrado induzir a metilação do DNA, atuando para co-reprimir a transcrição (Paluch *et al*, 2016).

Os primeiros anos de vida representam um período de particular suscetibilidade à alterações epigenéticas, uma vez que mudanças ativas no DNA e nas histonas ocorrem em resposta a sinais ambientais, que incluem notavelmente a estimulação psicossocial e o comportamento maternal (Desplats, 2015). Sabe-se que a expressão de inúmeros genes, em ratos adultos, é modificada como consequência das diferenças do cuidado materno recebido por eles (McGowan *et al*, 2011).

A qualidade do cuidado maternal que o filhote recebe é um fator crucial no desenvolvimento do comportamento e da psicopatologia, sendo um componente comportamental indispensável para a sobrevivência e o sucesso reprodutivo em mamíferos

(Garfinkel *et al*, 2016). Portanto, uma hipótese de como o cuidado materno poderia ser programado e transmitido intergeracionalmente é por meio de alterações epigenéticas, ambientalmente induzidas, de genes importantes para o comportamento materno em regiões encefálicas relevantes (Nephew, Murgatroyd, 2013).

Sabe-se que ratas fêmeas, ao longo da vida, mantêm estável seu padrão comportamental de cuidado materno, independente do número de gestações ocorridas. O ato da lambida é um exemplo desta estabilidade da lactante, ou seja, a mãe repetirá sempre o padrão HL ou LL com todas as suas ninhadas. Ademais, o comportamento de lamber é mantido pelas fêmeas da prole num mecanismo de transmissão transgeracional não-genômico (Francis *et al*, 1999). Desta forma, as evidências para a transmissão intergeracional do comportamento parental são claras: a maternidade gera maternidade. Ambos os aspectos positivos e negativos da experiência inicial de cuidado maternal tendem a ser repetidos pela próxima geração (Fleming *et al*, 2002).

Outro aspecto interessante foi relatado por estudos prévios que demonstraram que filhas de ratas LL, quando criadas por HL, adotam o comportamento da mãe que as criaram, e vice-versa. Este fato provavelmente está associado com mudanças, a longo prazo, na expressão de alguns genes e como consequência, no comportamento da prole (Liu *et al*, 1997; Champagne *et al*, 2007).

Estudos epigenéticos em roedores realizados por Meaney (2010) e Szyf *et al* (2005) deixam claro que o ambiente de cuidado oferecido pela mãe, como exemplo, a quantidade de lambidas e a posição de amamentação com as costas arqueadas, pode alterar radicalmente os padrões epigenéticos e consequentemente a expressão gênica no SNC dos filhotes. Estas alterações ocorrem não apenas nos filhotes expostos diretamente aos cuidados materno, ou até mesmo privados destes cuidados, mas também estendem-se a descendência destes. Eles continuam sendo pais sensíveis ou insensíveis, dependendo das mudanças epigenéticas que herdaram dos seus pais (Mileva-Seitz *et al*, 2016).

1.3.1 Achados Epigenéticos no SNC

Estudos prévios (Weaver *et al*, 2004; Zhang, Meaney, 2010; Zhang *et al*, 2013) mostraram que a metilação alterada do gene do receptor de glicocorticoides (GR) parece induzir mudanças de longo prazo na resposta ao estresse, afetando a próxima geração. Em ratos, já se demonstrou que as variações no cuidado materno influenciam o estado epigenético e a atividade transcricional do gene GR no hipocampo, ou seja, o aumento da frequência de lambida materna promove o aumento da expressão do GR hipocampal de forma estável. Os mecanismos subjacentes a este efeito maternal ainda não foram definidos, incluindo a natureza das vias reguladoras de sinalização intracelular (Weaver *et al*, 2014).

As respostas aos recém-nascidos, dependentes de hormônios e não-hormonais, podem ser atribuídas à remodelação epigenética no hipotálamo que promove a ativação transcricional do ER. Esses caminhos respondem ao estrogênio, à experiência com filhotes e à experiência de desenvolvimento do cuidado materno, destacando o grau de plasticidade encefálica materna em resposta a amplos sinais ambientais (Stolzenberg, Champagne, 2016). Cabe destacar também que fêmeas adultas, filhas de mães HL, apresentam níveis mais elevados de ERα na MPOA, quando comparadas às filhas de LL (Champagne *et al*, 2008).

Beery *et al* (2015) demonstraram que a experiência de vida precoce, por meio da variação natural do cuidado materno durante a primeira semana de vida, está associada com diferenças de metilação do DNA no gene OTR no hipocampo, corpo estriado e hipotálamo. Blaze e Roth (2013) mostraram que as experiências dentro de um ambiente adverso de cuidado, como maus tratos, produzem padrões anormais de metilação do DNA no córtex medial pré-frontal de ratos adultos e em desenvolvimento.

A prole de HL apresenta um aumento da expressão gênica e de acetilação de histonas, e a redução da metilação de DNA, quando comparada com a prole de LL (McGowan *et al*, 2011). Estudos verificaram no HP de adultos descendentes de mães HL, em comparação com LL, níveis aumentados de mRNA e de proteínas, o que foi associado à diminuição na metilação do DNA e à níveis aumentados de acetilação e de metilação das histonas. Além disso, foi mostrado que a separação materna durante a infância também pode produzir alterações na acetilação das histonas e na metilação de DNA no HP de filhotes em desenvolvimento e em adultos (Bagot *et al*, 2012; Blaze, Roth, 2013).

1.4 Estresse Oxidativo

Relevantes pesquisas têm relacionado funções cognitivas e comportamentais com o estresse oxidativo (Patki *et al*, 2013). Estresse oxidativo é definido como uma perturbação no equilíbrio entre as defesas antioxidantes e a produção de radicais livres, especialmente de ROS (espécies reativas de oxigênio) e RNS (espécies reativas de nitrogênio) (Halliwell, Gutteridge, 1999). Este desequilíbrio pode resultar em danos celulares (perda de integridade e função da célula) e alteração de funções cerebrais essenciais, levando a diversas condições patológicas como doenças cardiovasculares, neurodegenerativas, depressão, distúrbios psiquiátricos, entre outros (Marcolin *et al*, 2012; Patki *et al*, 2013; Chiurchiu *et al*, 2016).

Níveis elevados de ROS podem desencadear danos nos lipídios, proteínas e DNA das células. Acredita-se que a proteção do DNA seja um elemento-chave na prevenção de certas patologias, bem como no adiamento do fenótipo relacionado ao envelhecimento. Quando o dano do DNA persiste no genoma, através de processos replicativos e/ou através de mutagênese associada à transcrição, torna-se permanente na forma de mutações e/ou ruptura cromossômica e instabilidade (Ribeiro *et al*, 2012).

A integridade do DNA é crítica para a função celular adequada e proliferação. O DNA não-danificado mantém uma estrutura organizada dentro do núcleo, mas torna-se interrompido quando a célula é prejudicada. Danos ao DNA podem ser analisados pelo ensaio cometa, que é um teste de genotoxicidade e pode detectar lesões pré-mutagênicas, tais como quebras (simples e duplas), locais de reparação incompletos e locais lábeis alcalinos. As quebras de fita simples podem surgir diretamente (por exemplo, a partir do ataque da desoxirribose por ROS) ou indiretamente por clivagem enzimática do esqueleto de fosfodiéster durante a reparação de DNA por excisão de base (Noschang *et al*, 2010; Portich *et al*, 2017).

O SNC é altamente sensível ao estresse oxidativo em consequência de seu alto consumo de oxigênio mesmo sob condições basais, da alta produção de ROS e RNS a partir de reações neuroquímicas específicas, do aumento da deposição de íons metálicos nos tecidos com o envelhecimento, além do abundante conteúdo lipídico (Marcolin *et al*, 2012; Patki *et al*, 2013; Chiurchiu *et al*, 2016). Algumas evidências já confirmam que o HP é a região encefálica mais sensível ao estresse, e que mudanças nessa área podem ter um papel na mediação da neurogênese e do comportamento (Ferreira *et al*, 2015).

Um estudo com ratos relatou que a indução farmacológica direta do estresse oxidativo promove importantes impactos comportamentais, afetando níveis de ansiedade, de aprendizado e a perda de memória. Porém, quando estes animais foram tratados com antioxidantes, esses comportamentos foram evitados, sugerindo um papel causal do estresse oxidativo nestes resultados comportamentais (Allam *et al*, 2013). Patki *et al* (2013) realizaram um experimento que provocava estresse psicológico, usando uma versão modificada do modelo residente-intruso, e mostraram que esta intervenção causa comportamentos depressivos e ansiosos, com um aumento concomitante do estresse oxidativo no HP.

Utilizando um modelo de isolamento social de roedores, demonstrou-se que este isolamento diminui a atividade das enzimas antioxidantes superóxido dismutase (SOD) e catalase (CAT) no HP. Essas alterações bioquímicas, induzidas pelo isolamento social, podem contribuir para as anormalidades comportamentais observadas nestes animais, incluindo um alto nível de ansiedade, déficit de interação social e memória de trabalho espacial comprometida (Shao *et al*, 2015).

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2 JUSTIFICATIVA

Há muitos anos já encontramos na literatura artigos que buscam elucidar os fatores que participam do desencadeamento e da manutenção do comportamento maternal de roedores. Há cerca de duas décadas, alguns pesquisadores se voltaram ao estudo das variações do cuidado materno que as ratas apresentam, onde destacamos dois grupos de mães, as chamadas muito cuidadoras e pouco cuidadoras. Contudo, ainda se tem pouco entendimento dos mecanismos moleculares e epigenéticos que participam neste contexto.

Desta forma, considerando a importância do cuidado materno no desenvolvimento cognitivo dos filhotes, este estudo buscou esclarecer alguns mecanismos que possam estar envolvidos nestas diferenças comportamentais, com o objetivo de avançar no conhecimento da expressão gênica, dos processos epigenéticos e do estresse oxidativo em áreas cruciais do SNC de mães pouco e muito cuidadoras.

Os resultados obtidos nesta tese irão avançar significativamente na compreensão da busca da causa desta diferença comportamental exibida por mães que vivem sob as mesmas circunstâncias e condições, e que ao mesmo tempo apresentam um comportamento totalmente oposto em relação aos filhotes.

3 OBJETIVOS

3.1 Objetivo geral

Analisar modificações na expressão gênica, além de alterações pós-traducionais e bioquímicas, em estruturas do SNC de ratas lactantes com diferentes padrões de comportamento maternal.

3.2 Objetivos específicos

- Investigar a expressão gênica, em nível transcricional, do BDNF, dos receptores de estrogênio Erα (Esr1) e Erβ (Esr2), dos receptores de serotonina (Htr1a e Htr1b), do receptor de dopamina (Drd1a) e do receptor de prolactina (Prlr), em diferentes áreas do SNC: bulbo olfatório (OB), hipocampo (HP), córtex pré-frontal e estriado, de ratas virgens e lactantes (Artigo 1).

- Mensurar os níveis de acetilação global de histona H4 no OB das ratas com aumento (HL) ou com redução (LL) do cuidado materno (Artigo 2).

- Analisar o estresse oxidativo no OB, HP e plasma, danos oxidativos ao DNA e a produção de espécies reativas de oxigênio em ratas HL e LL (Artigo 3).

4 ARTIGOS

4.1 Artigo 1: *Gene expression in the CNS of lactating rats with different patterns of maternal behavior*

Ana Carolina de Moura [a], Virgínia Meneghini Lazzari [a], Roberta Oriques Becker [a], Mirela Severo Gil [b], Carina Anicet Ruthschilling [a], Grasiela Agnes [a], Silvana Almeida [a], Ana Beatriz Gorini da Veiga [c], Aldo Bolten Lucion [d], Márcia Giovenardi [a]

[a] *Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil*
[b] *Biomédica, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil*
[c] *Programa de Pós-Graduação em Patologia, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil*
[d] *Programa de Pós-Graduação em Ciências Biológicas: Fisiologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*

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Gene expression in the CNS of lactating rats with different patterns of maternal behavior

Ana Carolina de Moura[a], Virgínia Meneghini Lazzari[a], Roberta Oriques Becker[a], Mirela Severo Gil[b], Carina Anicet Ruthschilling[a], Grasiela Agnes[a], Silvana Almeida[a], Ana Beatriz Gorini da Veiga[c], Aldo Bolten Lucion[d], Márcia Giovenardi[a,*]

[a] *Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil*
[b] *Biomédica, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil*
[c] *Programa de Pós-Graduação em Patologia, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil*
[d] *Programa de Pós-Graduação em Ciências Biológicas: Fisiologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*

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ABSTRACT

For most mammalian species, maternal behavior has an essential role in the development of the offspring. The frequency of licking/grooming (LG) the pups has been used as a parameter to evaluate maternal care, having mothers with high (HL) or low (LL) frequencies of LG. This study aimed to analyze the gene expression of the receptors for dopamine (Drd1a), prolactin (Prlr), serotonin (Htr1a, Htr1b), estrogen (Esr1, Esr2), and of Bdnf in the olfactory bulb (OB), hippocampus (HP), prefrontal cortex (PFC), and striatum (ST) of Wistar rats from three groups: LL ($n = 8$); HL ($n = 8$); virgin females in diestrus (D; $n = 6$). Maternal behavior was studied between the 1st and 7th postpartum days. Brain parts were analyzed by qRT-PCR. LL showed a decrease in the frequency of nursing, and an increase of remaining off the pups. There was an increase in gene expression of Drd1a, Prlr, Htr1a, Htr1b and Esr1 in the OB of HL, compared to LL. In the HP, Drd1a, Prlr and Htr1a were differently expressed when comparing HL, or LL, with D. The main finding is that HL had higher gene expression levels in the OB, which is a crucial structure to promote behavioral differences.

1. Introduction

For mammals, maternal care has an essential role in the development of the offspring. Rodents, such as rats, constitute ideal model systems to manipulate and investigate the maternal behavior, when mothers practice actions such as: crouching over the young for nursing and to keep them warm, nest building and retrieving the pups to the nest, body and genital licking and grooming the pups (Rosenblatt, 1975; Stern and Johnson, 1990; Numan and Insel, 2003). As well, lactating females, during this period, show an enhanced aggressive behavior toward intruders that approach the nest site, aiming to protect the offspring (Numan and Numan, 1994; Giovenardi et al., 1998; Souza et al., 2010).

Maternal behavior is expressed in different modalities, physiological conditions, and contexts. It is the result of a highly motivated brain that allows the female to flexibly adapt her caring activities to different situations and social demands (Olazábal et al., 2013). In rats, sensory stimuli from pups, such as sight, sound and odor contribute to contact-seeking, whereas physical contact from pups to dam elicit essential maternal behavioral reflexes involved in retrieval, licking, and the quiescent, upright nursing posture (Stern, 1997).

Lévy et al. (2004) suggest that olfaction has an essential role in the modulation of mother/pups relationship, referring to the influence of the neuroendocrine mechanism in the accessory and main olfactory system as a mediator of this behavior. A profound change in the regulation of olfaction, modulated by physiological changes that occur at the end of pregnancy and in the parturition, allows the female to respond positively to the stimulus coming from the newborn (Lévy et al., 2004).

Although olfaction is not necessary for the initiation of maternal behavior in parturient rats, once maternal responsiveness is established, olfaction is critical for the organization of some dam's specific behaviors. In fact, it was demonstrated that a specific olfactory cue regulates licking, a behavioral pattern crucial for pup survival (Lévy et al., 2004). Studies of bilateral bulbectomy

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* Corresponding author at: Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre, Sarmento Leite 245, sala 308, Porto Alegre, RS 90050-170, Brazil. Tel.: +55 51 33038751.
E-mail address: giovenardi.marcia@gmail.com (M. Giovenardi).

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performed before parturition interferes with the normal onset of maternal behavior, with profound disturbances of licking behavior and total time spent with pups (Lévy and Keller, 2009).

During the first postpartum week, lactating female rats engage in a high frequency of pup licking/grooming (LG). This behavior serves to stimulate pups, modify body and brain temperature, and allows the dam to reclaim salt and water to meet the physiological demands of lactation (Gubernick and Alberts, 1983). The LG frequency has been used as a parameter to evaluate maternal care (Liu et al., 1997; Caldji et al., 1998; Champagne et al., 2003; Parent and Meaney, 2008; Veenema and Neumann, 2009; Lenz and Sengelaub, 2009), and it has a normal distribution when assessed in a large sample (Champagne et al., 2003), where mothers with high frequencies of LG (HL) and low frequencies of LG (LL) represent the two extremes of the same population (Champagne, 2008).

HL and LL dams differ in mesolimbic dopaminergic activity associated with mother–pup interactions. Such differences may serve as neural substrates for individual differences in the motivational component of maternal behavior (Champagne et al., 2004). HL mothers spend more time taking care of the offspring, display a high frequency of biting intruders of the nest area (Ruthschilling et al., 2012), and their pups are less fearful; while LL have more sexually receptiveness, and pups are more able to react to stressful situations. Thus, HL uses strategies to obtain an offspring with more quality, whereas LL uses strategies to maximize the quantity of the offspring. Both strategies are important, depending on the environmental needs and conditions, and represent different forms of maintaining their own species (Cameron et al., 2008). These variations in maternal care are transmitted across generations, such that the lactating adult offspring of HL mothers are themselves high in pup LG, whereas the offspring of LL mothers exhibit decreased levels of pup LG (Francis et al., 1999; Champagne et al., 2001).

The different patterns of LG come from variations in the maternal care that involves sensorial, neuroendocrine and neural systems, in addition to genetic mechanisms. Several structures in the central nervous system (CNS), such as medial preoptic area (MPOA), medial (MA) and cortical nucleus of amygdale (CA), nucleus accumbens (NAC), paraventricular nucleus of hypothalamus (PVN), olfactory bulb (OB), hippocampus (HP), among others, are responsible for the development and maintenance of the maternal behavior (Consiglio and Lucion, 1996; Champagne et al., 2003; Teodorov et al., 2010; Ruthschilling et al., 2012). In addition to the neuroendocrine changes observed in lactating rats, important interactions among neurotransmitter systems occurs in the CNS, such as dopamine (DA), prolactin (PRL), serotonin (5-HT), estrogen, oxytocin (OT), norepinephrine, and gamma-aminobutiric-acid (GABA) (Spielewoy et al., 2000; Numan and Insel, 2003; Teodorov et al., 2010; Ruthschilling et al., 2012). Certain deficiencies in these substances or in their receptors show a modification in the maternal behavior, such as deficits in retrieval and decreased time in nest (Numan and Insel, 2003).

Molecular basis involved in postpartum behavioral changes and in the regulation of signaling pathways in the CNS are not fully elucidated. Understanding these mechanisms has been the basis of many behavioral studies in neuroscience. Such studies aim to address how a pattern of behavior is controlled by the expression of a candidate gene or group of genes. The present study aimed to analyze the gene expression, at transcriptional levels, of the receptors for dopamine (Drd1a), prolactin (Prlr), serotonin (Htr1a, Htr1b), estrogen (Esr1, Esr2), and of brain-derived neurotrophic factor (Bdnf) in different structures of the CNS, such as olfactory bulb (OB), hippocampus (HP), prefrontal cortex (PFC), and striatum (ST) of lactating females with different patterns of maternal behavior.

2. Materials and methods

2.1. Animals

Primiparous lactating Wistar rats ($n = 92$), approximately 90 days old, from the animal house of the Universidade Federal de Ciências da Saúde de Porto Alegre were used. During the last week of gestation, female rats were single-housed in 46 cm × 17 cm × 31 cm Plexiglas cages that allowed a clear view of all activities within the cage. Food and water were provided *ad libitum*. Animals were housed under controlled temperature ($21 \pm 1\,^{\circ}$C) and light (12:12 light-dark cycle with lights off at 5 pm) conditions. Litters were standardized with eight pups one day after the parturition. Virgin females ($n = 6$) showing three regular estrous cycles, in the diestrus phase, were used in the experiment (group D).

All procedures were performed in conformity with Conselho Nacional de Controle de Experimentação Animal (CONCEA) for the care and use of laboratory animals, and this study was approved by the Ethics Committee of the UFCSPA (protocol number 788/09).

2.2. Maternal behavior

The behavior of each mother was daily observed during observation periods of 75 min for the first 7 postpartum days (PPD). Observers were trained to ensure a high level of inter-rater reliability. Observations occurred at regular hours each day, with three periods during the light (10:00 am, 1:30 pm and 3:00 pm) and one period during the dark (5:30 pm) phases of the light/dark cycle. The distribution of these observation periods was based on findings that nursing in rats occurs more frequently during the light phase of the cycle. Within each 75 min observation period, the behavior of each mother was scored every 3 min (25 observations/period × 5 periods/day = 100 observations/mother per day) as previously described (Myers et al., 1989; Ruthschilling et al., 2012). The following behaviors were recorded: mother licking/grooming the pup; mother nursing the pups in an arched-back posture; a "blanket" 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 nurse; and the mother remaining off the pups. The behavioral categories were not mutually exclusive – a mother could be licking/grooming while nursing the pup.

For the cohorts ($n = 92$) used in the present study, the mean ± SEM percentage of licking across the first 7 PPD was 7.99 ± 2.49. Thus, HL ($n = 8$) were defined as females whose frequency scores for LG were greater than 1 SD above the mean (10.48), and LL ($n = 8$) were defined as females whose frequency scores for LG were greater than 1 SD below the mean (5.50), as previously described (Champagne et al., 2003; Cameron et al., 2008; Ruthschilling et al., 2012).

2.3. Brain tissue samples

At the 11th PPD the LL and HL rats, also the virgin females (D), were decapitated and brains were quickly removed. The OB, HP, ST and PFC (*only the front portion*) from the left hemisphere were manually dissected as described below, *with the use of a stereomicroscope, on ice, using sterile materials.* Samples were always collected in the morning, during the light cycle and in a noiseless room.

The structures dissection was performed as previously described and illustrated by Chiu et al. (2007), following the coordinates stated in the maps and guides to dissection published by Palkovits (1988) and Swanson (2004) to separate each specific brain area of interest for the present study. After removing the brain, RNA-later (Ambion) was dropped on the tissue. The brain was cut in the sagittal plane, separating the right hemisphere from the left hemisphere. The first collected structure was the left olfactory bulb; soon after the left prefrontal cortex was removed with the aid of a scalpel lamina (number 20, Solidor). Then, the left striatum was collected with the help of two tip-curved forceps (number 12, Duflex). To collect the hippocampus, the ventral side of the brain was placed upwards and the midbrain was removed to expose the hippocampus, which was dissected from the cortex using two tweezers (number 12, Duflex).

Immediately after dissection, samples were placed in tubes containing RNA-later (1:1, v/v) for 24 h at 4 °C. After, RNA-later was removed and the tissue stored at −80 °C until the RNA extraction.

2.4. Molecular analyses

2.4.1. RNA extraction

Total RNA was extracted from samples using Trizol (Invitrogen), according to manufacturer's guidelines. Briefly, each brain structure was homogenized in the presence of Trizol; chloroform was added (1:5, v/v); and the aqueous phase was obtained after centrifugation (12,000 × g, 15 min). RNA was precipitated with isopropanol for 15 min, at room temperature; followed by centrifugation at 12,000 × g for 10 min. Pellets were resuspended in 0.1% DEPC-treated water. The concentration of total RNA was determined by measuring the optical density at 260 nm and the RNA purity was assessed based on the 260 nm/280 nm ratio and agarose gel electrophoresis (Langnaese et al., 2008).

2.4.2. cDNA synthesis

Total RNA (1 μg) was used as a template to synthesize cDNA. RNA was first incubated with 1 μl oligo (dT) (0.5 μg/μl, Invitrogen), 1 μl 10 mM dNTPs and DEPC-water to a final volume of 12 μl, for 5 min at 65 °C and then 1 min in ice. The following reagents were then added to reach a final volume of 19 μl: 4 μl RT buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 2 μl 0.1 M DTT, and 1 μl RNaseOUT (40 U/μl, Invitrogen). After a 2-min incubation at 37 °C, 1 μl M-MLV-RT (200 U/μl, Invitrogen) was added and cDNA synthesis was performed at 50 °C for 1 h; the reaction was inactivated by incubation at 70 °C for 15 min.

2.4.3. Real time PCR (qPCR)

To analyze the expression of the receptors for dopamine (Drd1a), prolactin (Prlr), serotonin (Htr1a, Htr1b), estrogen (Esr1, Esr2), and also of brain-derived neurotrophic factor (Bdnf), qPCR was performed. Housekeeping genes that have been shown to be stable in each brain area were used as control–ActB in HP and ST, UbC in OB and CypA in PFC (Moura et al., 2014). Table 1 shows all primer sequences used in this study, which were designed using the software Primer-3 (Rozen and Skaletsky, 2000), based on rat mRNA sequences in the GenBank database (Benson et al., 2013). The specificity of the primers was checked using BLAST search against nucleotide collection (nr) of the NCBI database. All primers were from Invitrogen (São Paulo, Brazil).

Amplification was carried out using 7.5 μL of SYBR Green PCR Master Mix (Applied Biosystems, São Paulo, Brazil), 0.5 μL of forward and reverse primers (0.33 μM each), 100 ng of cDNA and nuclease-free water, in a total volume of 15 μL. Reactions were performed in an optical 96-well plate, using a StepOnePlus™ thermocycler (Applied Biosystems, Foster City, CA, USA). After an initial denaturation step at 95 °C for 10 min, amplification was performed in 50 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 40 s and extension at 72 °C for 40 s. Amplification was followed by a melting curve analysis to confirm PCR product specificity. No signals were detected in no-template controls. The experimental Ct (cycle threshold) was calculated using the algorithm enhancements provided by the equipment. All samples were run in duplicate and the mean value of each duplicate was used for all further calculations (Langnaese et al., 2008; Cook et al., 2010; Nelissen et al., 2010). The Ct value of each reaction was used to calculate the level of mRNA expression of that specific gene, after normalizing it in relation to the expression of the control HKG gene analyzed in parallel in the same reaction plate.

2.5. Statistical analyses

The frequencies of mother nursing the pups (considering the sum of all nursing positions) and of mother remaining off the pups (mean ± SEM) were compared by Student's *t*-test. Data of molecular analyses (mean ± SEM) were compared by the Kruskal–Wallis test. When significant differences were found, Dunn's multiple comparison test was used to compare the three groups. In all cases $p < 0.05$ was considered significant.

3. Results

In analyses of maternal behaviors, we found that the frequency of nursing in HL group was 30.75 ± 5.55 and in LL group was 16.95 ± 3.41, the difference between the groups was almost significant ($p = 0.05$). In contrast, the frequency of mother remaining off the pups was significantly higher ($p < 0.05$) in LL group than in HL group (HL, 24.68 ± 2.93; LL, 33.74 ± 2.62).

Fig. 1 shows the results for the gene Drd1a, where a significant difference was observed in the OB, with HL expressing higher levels than LL (Fig. 1A); and also HL expressing more receptors than D in the HP (Fig. 1B). For this receptor no significant results were found in the PFC or ST (Fig. 1C and D).

For Prlr we found that HL expresses significantly more receptors than LL in the OB (Fig. 2A) and less receptors than D in the HP (Fig. 2B). However, in the PFC and ST no significance was found (Fig. 2C and D).

Fig. 3A shows a significant increase in mRNA expression levels of Htr1a in the OB, in HL females when compared to LL and D. It is also observed a significant decrease in mRNA levels in the HP of D, when compared to HL and LL (Fig. 3B). On the other hand, no significant difference in gene expression of this receptor was found in the PFC and ST, in all studied groups (Fig. 3C and D).

Our results also showed that HL females have a significant increase in Htr1b mRNA expression in the OB, when compared to LL females (Fig. 4A). But no significant difference in gene expression of this receptor was found in the HP, PFC or ST, among the groups (Fig. 4B–D).

Regarding Esr1, we only found significant difference in the OB, where HL expressed a higher amount of Esr1 than LL and D (Fig. 5A). In the other structures, HP, PFC and ST (Fig. 5B–D), no differences were found.

For the genes Esr2 and Bdnf, results showed that there were no significant differences in mRNA expression levels in the studied groups and areas [Esr2: OB ($p = 0.10$), HP ($p = 0.78$), PFC ($p = 0.95$) and ST ($p = 0.63$); Bdnf: OB ($p = 0.38$), HP ($p = 0.83$), PFC ($p = 0.88$) and ST ($p = 0.96$)].

4. Discussion

Studies that identify genes involved in maternal behavior are fundamentally important because these findings can lead to molecular pathways that are crucial to brain circuitry modulating behavior (Teodorov et al., 2010). Our main finding showed that HL has higher expression levels of the receptors Drd1a, Prlr, Htr1a, Htr1b, Esr1 in the OB when compared with LL. The olfactory system is modulated of hormonal changes that occur during pregnancy, delivery and lactation (Lévy et al., 2004), allows the

Table 1
Primer sequences used in the study.

Gene[a]	ID[b]	Primer F	Primer R
Drd1a	24316	5′CATTCTGAACCTCTGCGTGATC3′	5′GTTGTCATCCTCGGTGTCCTC3′
Prlr	24684	5′CTGGAGAAGGGCAAGTCTGA3′	5′GTCGGGATCTAGGTGTGTGG3′
Htr1a	24473	5′CTGTTGCTCATGCTGGTTCTC3′	5′CAGAGGAAGGTGCTCTTTGG3′
Htr1b	25075	5′GTGCTGGACTGCTTTGTGAAC3′	5′TAATGGAGGTGACCGAGGAC3′
Esr1	24890	5′GATGGTCAGTGCCTTATTGGATGC3′	5′GCAGGTTCATCATGCGGAATCGA3′
Esr2	25149	5′CAATCATCGCTCCTCTATGC3′	5′GGCCTTACATCCTTCACATGA3′
Bdnf	24225	5′GATGAGGACCAGAAGGTTCG3′	5′GATTGGGTAGTTCGGCATTG3′
ActB	81822	5′TATGCCAACACAGTGCTGTCTGG3′	5′TACTCCTGCTTGCTGATCCACAT3′
CypA	25518	5′TATCTGCACTGCCAAGACTGAGTG3′	5′CTTCTTGCTGGTCTTGCCATTCC3′
UbC	50522	5′TTTCCATAGACAATGCAGATCTTT3′	5′AGGGTGGACTCCTTCTGGAT3′

[a] Abbreviations: Drd1a, dopamine receptor 1A; Prlr, prolactin receptor; Htr1a, serotonin receptor 1A; Htr1b, serotonin receptor 1B; Esr1, estrogen receptor alpha; Esr2, estrogen receptor beta; Bdnf, brain-derived neurotrophic factor; ActB, beta-actin; CypA, cyclophilin A; UbC, ubiquitin C.
[b] Gene Identification Number, available at: http://www.ncbi.nlm.nih.gov/gene.

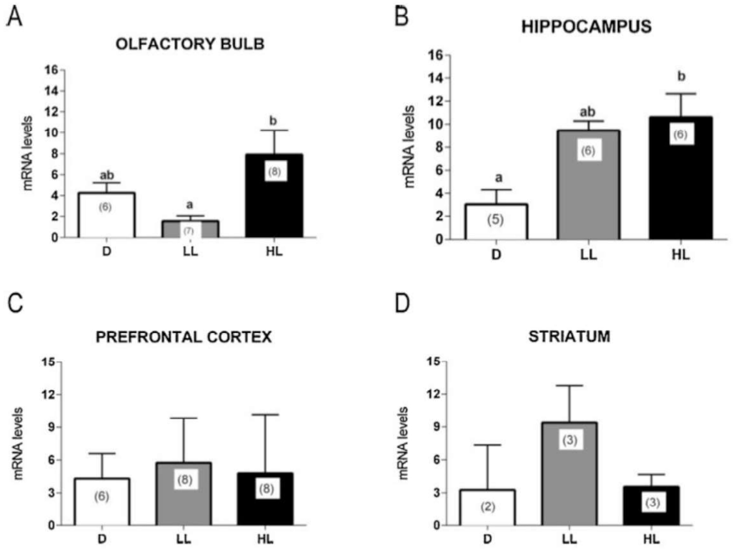

Fig. 1. Expression of Drd1a in the CNS of LL (low licking) and HL (high licking) lactating females, and of virgin females in the diestrus period (D). Data expressed as mean [±SEM]. Kruskal–Wallis test (Dunn's multiple comparison test); $p < 0.05$. [a,b] Indicates significant difference between the groups. The number of animals (*n*) is given between parentheses.

mother to respond positively to the stimulus coming from the influencing mother/pup binding and recognition (Sullivan et al., 1989). Once maternal responsiveness is established, olfaction is critical for the organization of maternal behavior (Lévy et al., 2004). A previous study (Meddle et al., 2007) showed that the oxytocin receptor (OXTR) expression in the OB is increased in the parturition period when compared with pre-parturition and in the postpartum period. OT within the OB has been known to play crucial roles in induction of maternal behavior (Yu et al., 1996; Kendrick et al., 1997), olfactory recognition of the offspring (Lévy et al., 1995) and preservation of social recognition (Dluzen et al., 2000). Thus our main finding allows us to infer that there is an association between increased gene expression in OB and behavioral pattern of HL. Furthermore, the genes studied in this work, decode receptors where neurotransmitters and neuropeptides will bind, have an important role in neurochemistry of maternal behavior.

Until now, literature reports few studies linking gene expression and differences in behavioral patterns observed between HL and LL. A previous study (Francis et al., 2000) demonstrated that HL mothers have higher levels of OXTR in the central nucleus of the amygdale, in the ventral part of the bed nucleus of the stria terminalis (BNST), in the MPOA and ventral region of the septum when compared to LL. Ruthschilling et al. (2012) showed that HL has lower expression of OXTR in the OB and greater expression of OXTR in the HP when compared to HL. The levels of Esr1 in the MPOA in HL are higher than LL (Champagne et al., 2003). In addition, the gene expression of OXTR is significantly altered in the HP of adult rats as a repercussion of the maternal care received during lactation (Meaney, 2001).

The brain dopamine (DA) system is involved in the control of the motivational aspects of maternal care. Thus, lesions in DA-rich brain areas and administration of DA receptor antagonists interfere with maternal motivation by affecting most forms of active behaviors (retrieval and grouping of the pups at the nest site, pup licking, and nest building) that precede and promote nurturance (Numan and Insel, 2003). The DA activity is increased in the NAC, what may occur throughout the reproductive cycle to prepare the female for maternal behavior. The NAC is an important area for maternal behavior, as well as for the processing of pup-related stimuli likely to be involved in motivation. Changes of DA in the MPOA during

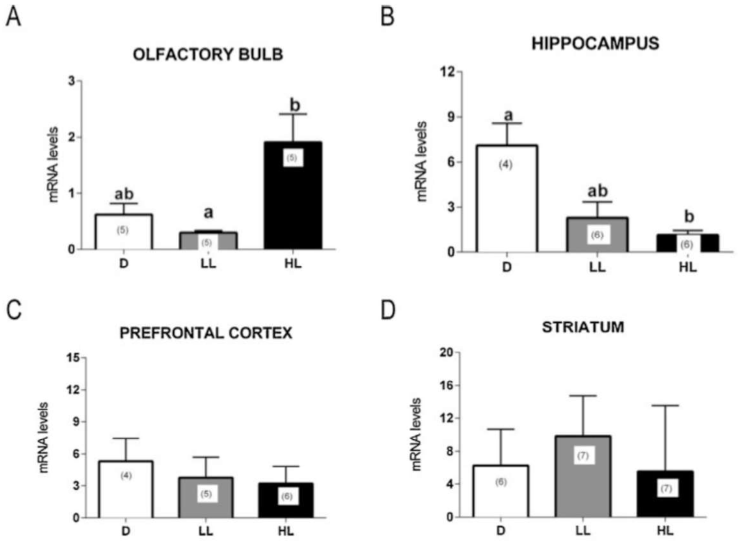

Fig. 2. Expression of Prlr in the CNS of LL (low licking) and HL (high licking) lactating females, and of virgin females in the diestrus period (D). Data expressed as mean [±SEM]. Kruskal–Wallis test (Dunn's multiple comparison test); $p < 0.05$. ^{a,b} Indicates significant difference between the groups. The number of animals (n) is given between parentheses.

pregnancy and lactation can influence the behavior of the mother toward the pup (Teodorov et al., 2010). However, we did not find any difference in the ST, among all groups, for the studied genes. It is also important to point that our study has a methodological difference from the previous cited, because the NAC was not separated from the ST. Thus, we did not actually quantify the dopaminergic content in the NAC.

The serotonergic system participates in the modulation of several behaviors, such as aggression, anxiety, hyperactivity, exploratory and emotional behaviors (Clark and Neumaier, 2001).

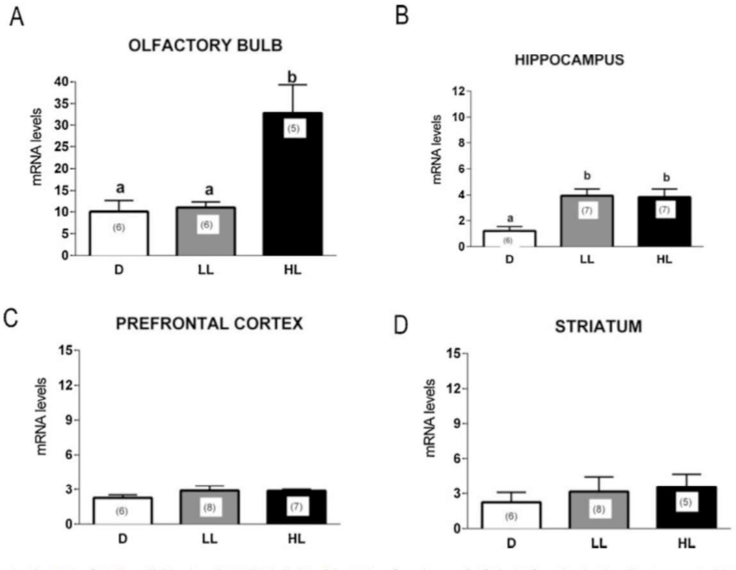

Fig. 3. Expression of Htr1a in the CNS of LL (low licking) and HL (high licking) lactating females, and of virgin females in the diestrus period (D). Data expressed as mean [±SEM]. Kruskal–Wallis test (Dunn's multiple comparison test); $p < 0.05$. ^{a,b} Indicates significant difference between the groups. The number of animals (n) is given between parentheses.

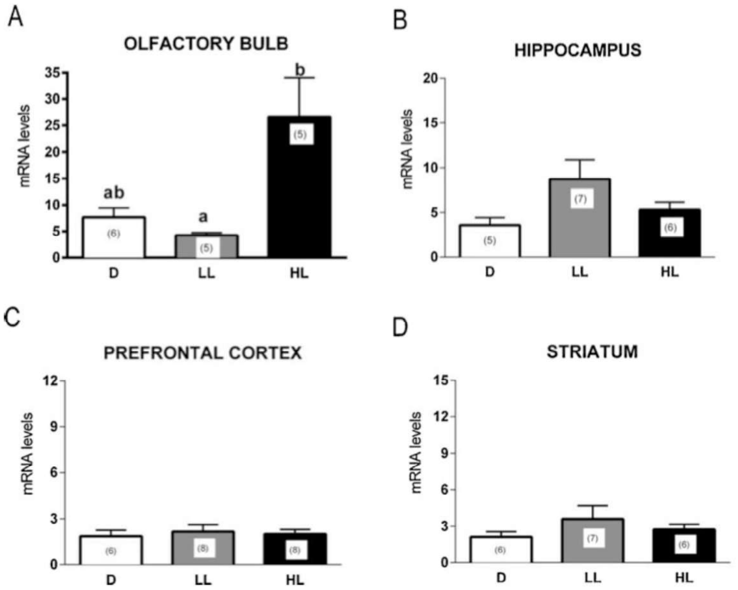

Fig. 4. Expression of Htr1b in the CNS of LL (low licking) and HL (high licking) lactating females, and of virgin females in the diestrus period (D). Data expressed as mean [±SEM]. Kruskal–Wallis test (Dunn's multiple comparison test); $p < 0.05$. [a,b] Indicates significant difference between the groups. The number of animals (n) is given between parentheses.

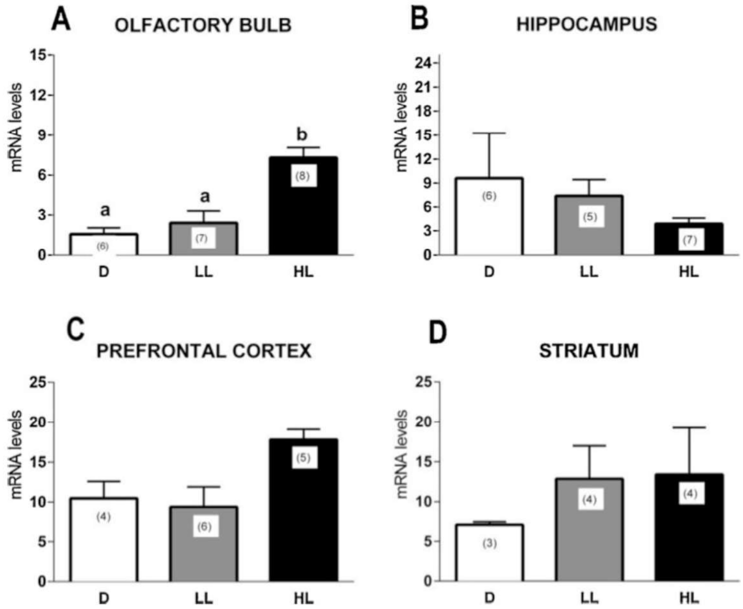

Fig. 5. Expression of Esr1 in the CNS of LL (low licking) and HL (high licking) lactating females, and of virgin females in the diestrus period (D). Data expressed as mean [±SEM]. Kruskal–Wallis test (Dunn's multiple comparison test); $p < 0.05$. [a,b] Indicates significant difference between the groups. The number of animals (n) is given between parentheses.

Lesions in the median raphe nucleus caused short-term disruption of all aspects of maternal behavior except the prolactin response to the suckling stimulus (Teodorov et al., 2010). Our results show that HL mothers have increased levels of Htr1a and Htr1b than LL in the OB, but not in the other studied areas.

Other important factor related to maternal behavior is that during the reproductive period some alterations in sensitivity of estrogen receptors occur in relevant brain areas, which might affect this behavior. The LG suffer influence of the estrogen–oxytocin interaction, even during the postpartum period (Teodorov et al., 2010). Previous study (Ogawa et al., 1998) with knockout mice for Esr1 demonstrated a deficit in maternal behavior and some females exhibit infanticide. However, knockout mouse for Esr2 do not shows deficit in maternal care (Ogawa et al., 1999).

In a study correlating gene expression and maternal care, Champagne et al. (2003) showed that HL has higher levels of Esr1 in the MPOA than LL. Corroborating these findings, we found more expression of ESR1 in the OB only in the females with higher maternal care, however no difference was found in the other studied areas.

Another point to be highlighted in this study is the results of the diestrus group (D). The females of the D expressed less Htr1a and Esr1 in the OB when compared to HL; and in the HP, they expressed less Drd1a than HL, and less Htr1a than lactating females, but more Prlr than HL mothers. An alternative explanation for the differences in behavior between diestrus and lactating females could rely on the reproductive experience of the latter.

The diestrus period is characterized by low concentrations of circulating estrogens and progesterone, and the lactating period is similar in this aspect, but with several other characteristics, like high plasma concentrations of PRL, OT, glucocorticoids, insulin, growth and thyroid hormones (Tucker, 1994). Females in diestrus and lactating females are in different states and have different reproductive experience. Wartella et al. (2003) demonstrated that the experience of pregnancy, parturition and lactation reduces anxiety-like behavior and attenuated neural activation in brain areas associated with emotionality.

Our data point to a pattern of similarity in gene expression observes in D and LL, which is different from HL. Cameron et al. (2008) discusses that HL are more motivated to strive for quality rather than quantity; while LL (have low maternal behavior) invest the minimum necessary for the survival of offspring and has no maternal motivation. This behavioral pattern is similar to the D group, where virgin females have no maternal behavior signs.

To study social behavior, several approaches are used, including gene expression assays. This way, several studies (Nephew et al., 2009; Zhang et al., 2010; Chiavegatto et al., 2012; Bagot et al., 2012; Driessen et al., 2014) have been using real time PCR, through mRNA relative quantification, to analyze gene expression in the CNS. However, an inherent limitation of our study is that we evaluate the expression of mRNA of receptors, and not the protein translated.

Current studies have shown that maternal behavior is related to epigenetic mechanisms of gene expression in the genes' control regions of hormones and neurotransmitters (Meaney, 2001; Champagne et al., 2003, 2004, 2008; Weaver et al., 2004; Cummings et al., 2010). Another aspect to be highlighted is the need of further analyzes, including epigenetic studies in the OB, that are required for a better understanding of the molecular basis of maternal behavior.

5. Conclusions

The OB is a key component of the neural circuit that mediates maternal behavior. Our results allow us to infer that this area have a decisive role in the behavioral differences observed in HL and LL rats.

The main finding in this study is that HL mothers show higher expression levels of Drd1a, Prlr, Htr1a, Htr1b and Esr1 in the OB when compared to LL. Furthermore, differences were found in the expression levels of Drd1a, Prlr, Htr1a and Esr1 between lactating and virgin females.

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4.2 Artigo 2: *Global Histone H4 Acetylation in the Olfactory Bulb of Lactating Rats with Different Patterns of Maternal Behavior*

Ana Carolina de Moura[1], Ivy Reichert Vital da Silva[2], Gustavo Reinaldo[3], Caroline Dani[2], Viviane Rostirola Elsner[2], Márcia Giovenardi[1]

[1] *Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil.*

[2] *Programa de Pós Graduação em Biociências e Reabilitação do Centro Universitário Metodista do IPA, Porto Alegre, RS, Brazil.*

[3] *Curso de Fisioterapia do Centro Universitário Metodista do IPA, Porto Alegre, RS, Brazil.*

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BRIEF COMMUNICATION

Global Histone H4 Acetylation in the Olfactory Bulb of Lactating Rats with Different Patterns of Maternal Behavior

Ana Carolina de Moura[1] · **Ivy Reichert Vital da Silva**[2] · **Gustavo Reinaldo**[3] ·
Caroline Dani[2] · **Viviane Rostirola Elsner**[2] · **Márcia Giovenardi**[1]

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Abstract In rats, variations in the levels of neuromodulatory molecules and in the expression of their receptors are observed during pregnancy and postpartum. These changes may contribute to the development and management of maternal behavior. The frequency of licking the pups is used to evaluate maternal care, having mothers with low licking (LL) and high licking (HL) frequencies. Previously, we found that HL had increased levels of transcriptional expression of the receptors for serotonin (HTR1a, HTR1b), estrogen (Erα), dopamine (D1a), and prolactin (Prlr) than LL in the olfactory bulb (OB); however, the molecular mechanisms behind this phenomenon are unknown. Since evidences pointed out that epigenetic marks, which may alter gene expression, are modulated by environmental factors such as exercise, diet, maternal care, and xenobiotic exposure, our objective was to verify the acetylation levels of histone-H4 in the OB of LL and HL rats. Maternal behavior was studied for the first 7 postpartum days. LL ($n = 4$) and HL ($n = 5$) mothers were selected according to the behavior of licking their pups. Acetylation levels of histone-H4 were determined using the Global Histone-H4 Acetylation Assay Kit and expressed as ng/mg protein (mean ± SD). Analysis revealed that HL (278.36 ± 68.95) had increased H4 acetylation levels than LL (183.24 ± 73.05; $p = 0.045$). The enhanced expression of the previously studied receptors in the OB could be related, at least in part, to the hyperacetylation status of histone-H4 here observed. Afterward, the modulation of histone acetylation levels could exert a pivotal role through molecular mechanisms involved in the different patterns of maternal behavior.

Keywords Epigenetics · Histone H4 acetylation · Gene expression · Maternal behavior · Licking/grooming · High/low licking mothers

Introduction

During the first 2 weeks postpartum, lactating female rats engage in a high frequency of licking/grooming (LG) the pups. The LG frequency has been used as a parameter to evaluate maternal care (Liu et al. 1997; Caldji et al. 1998; Parent and Meaney 2008; Lenz and Sengelaub 2009; Veenema and Neumann 2009), and it has a normal distribution when assessed in a large sample (Champagne et al. 2003), where mothers with high frequencies of LG (HL) and low frequencies of LG (LL) represent the two extremes of the same population (Champagne 2008).

In the perinatal period, the brain is particularly sensitive to remodeling by environmental factors, such as maternal LG, which might influence stress responsiveness, the levels of anxiety and depressive-like behaviors, cognitive performance, and social/reproductive behavior in the offspring (Champagne et al. 2008; Maccari et al. 2014; Peña et al. 2014). Additionally, some studies reported that these

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✉ Ana Carolina de Moura
anacarol.demoura@gmail.com

Márcia Giovenardi
giovenardi.marcia@gmail.com

[1] Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre, Sarmento Leite 245, Sala 308, Porto Alegre, RS 90050-170, Brazil

[2] Programa de Pós Graduação em Biociências e Reabilitação do Centro Universitário Metodista do IPA, Porto Alegre, RS, Brazil

[3] Curso de Fisioterapia do Centro Universitário Metodista do IPA, Porto Alegre, RS, Brazil

variations in maternal care are transmitted across generations, such that the lactating adult offspring of HL mothers are themselves high in pup LG, whereas the offspring of LL mothers exhibit decreased levels of pup LG (Francis et al. 1999; Champagne et al. 2001).

Interestingly, there is evidence that maternal care also modulates the expression of several genes related to brain function (Patisaul et al. 2003; Champagne et al. 2006; Weaver et al. 2006; Ruthschilling et al. 2012). Previously, we showed that HL had increased levels of transcriptional expression of the receptors for serotonin (HTR1a, HTR1b), estrogen (Erα), dopamine (D1a), and prolactin (Prlr) than LL in the olfactory bulb (OB) of Wistar rats, using the same sample for the present study (de Moura et al. 2015).

Despite this finding, the molecular mechanisms behind this phenomenon are unknown. Emerging studies suggest that it is related, at least in part, to epigenetic pathways (Weaver 2007; Champagne and Curley 2008; McGowan et al. 2011; Mileva-Seitz et al. 2015). Epigenetics involves modifications in the structure of chromatin, DNA, and histones, which can modulate the transcriptional machinery and allow for long-lasting modifications in the genome (Kouzarides 2007).

The reports concerning maternal care and epigenetic modulation are restricted to the DNA methylation parameters (Beery et al. 2015; Blaze and Roth 2013) and histone-H3 acetylation levels (Weaver et al. 2004; Weaver 2007; McGowan et al. 2011), while less attention has been paid to histone-H4 acetylation modulation. It has been demonstrated that histones show a distinct specificity, which may be stimulus-dependent (Bredy et al. 2007; Elsner et al. 2011; Lalonde et al. 2014), reflecting the need to investigate the impact of maternal care on this marker. Also, several studies associate histone modifications to disorders like schizophrenia, depression, and addiction (Kumar et al. 2005; Sharma 2005; Tsankova et al. 2006), linking the epigenetic mechanisms to different patterns of behavior.

Most findings regarding epigenetic modulations on gene expression in response to maternal care are focused in different brain areas such as medial preoptic area (MPOA), hippocampus, striatum, and hypothalamus (Champagne et al. 2006; Beery et al. 2015; Blaze and Roth 2013). To our knowledge, there are no investigations in the OB, which is a key component of the neural circuit that mediates maternal behavior and where we previously found significant differences in the expression of key genes (de Moura et al. 2015). Olfaction has an essential role in the modulation of mother/pups relationship, as physiological changes that occur during pregnancy and parturition allow the female to respond positively to the stimulus coming from the newborn. Furthermore, olfaction is critical for the organization of some dam's specific behaviors, when specific olfactory cue regulates licking, a behavioral pattern crucial for pup survival (Lévy et al. 2004).

Therefore, the aim of the present study was to verify the acetylation levels of histone-H4 in the OBs of LL and HL Wistar rats.

Materials and Methods

Maternal Behavior

Maternal behavior was studied in primiparous lactating Wistar rats of approximately 90 days old (animals were housed under controlled conditions). The behavior of each mother was daily observed for the first 7 postpartum days (PPD) as previously described (Myers et al. 1989). For the used cohorts ($n = 92$), the mean ± SEM percentage of licking across the first 7 PPD was 7.99 ± 2.49. Thus, HL ($n = 5$) were defined as females whose frequency scores for LG were greater than 1 SD above the mean (10.48), and LL ($n = 4$) were defined as females whose frequency scores for LG were greater than 1 SD below the mean (5.50) (for details, see Ruthschilling et al. 2012).

Tissue Preparation

Rats were decapitated and their whole brains were quickly removed. The OBs were manually dissected on ice using sterile materials, as previously described by Chiu et al. (2007). Samples were collected during the morning, in the light cycle and in a noiseless room. After dissection, the OBs were immediately frozen in liquid nitrogen and stored at −80 °C until the day of the assay.

Determination of Global Histone-H4 Acetylation

Acetylation levels of histone-H4 were determined in the OB using the Global Histone-H4 Acetylation Assay Kit (Colorimetric Detection, catalog number P-4009, EpiQuik/USA) according to the manufacturer's instructions by following these steps: tissue disaggregation (cell lysis), histone extraction, histone coating onto assay wells, capture antibody addition, detection antibody addition, and developing solution (for developing color and measuring absorbance) addition. Finally, samples were incubated with the Stop Solution and their absorbance was measured on a microplate reader (450 nm). The ratio or amount of acetylated H4 can be quantified through HRP (horseradish peroxidase)-conjugated secondary antibody-color development system, which is proportional to the intensity of color development, and was expressed as ng/mg protein. The protein concentration of each sample was measured by the Coomassie Blue method using bovine serum albumin as standard (Bradford 1976).

Statistical Analyses

Global histone-H4 acetylation levels were expressed as mean ± SD and analyzed by Student's *t* test ($p < 0.05$ indicates significant difference).

Results

The levels of global histone-H4 acetylation in the OB of HL and LL are depicted in Fig. 1. *t* test revealed that HL had a significant increase in this parameter compared to LL (278.36 ± 68.95; 183.24 ± 73.05, respectively; $p = 0.045$).

Discussion

In the present study, we showed that HL mothers have increased levels of global histone-H4 acetylation than LL mothers in the OB. Therefore, we may suggest that the enhanced gene expression previously reported by our group in these HL rats (de Moura et al. 2015) can be associated, at least in part, with histone-H4 hyperacetylation in this area.

In accordance, McGowan et al. (2011) showed that HL offspring had increased gene expression of the NR3C1 glucocorticoid receptor (GR) in the hippocampus, which was associated to enhanced histone acetylation levels of H3, and DNA methylation reduction when compared to LL offspring. Maternal separation during infancy has also been shown to produce changes in histone acetylation and methylation within the developing and adult hippocampus (Blaze and Roth 2013). These studies in the pups are in accordance with our results in the mothers, since high frequencies of maternal care and epigenetic patterns are transmitted transgenerationally.

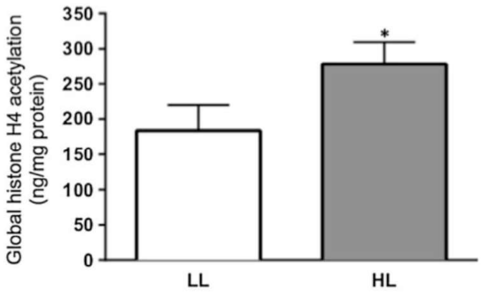

Fig. 1 Global histone-H4 acetylation in the Olfactory Bulb of low licking (LL) and high licking (HL) mothers (*$p < 0.05$)

Histone acetylation is catalyzed by histone acetyltransferases (HATs), producing a more opened chromatin structure, a characteristic that allows transcriptional activation. Inversely, histone deacetylases (HDACs) exert the opposite effect (Kouzarides 2007). Interestingly, it was also shown that H3-hypoacetylation status was reversed by the HDAC inhibitor trichostatin-A (Weaver et al. 2004). Moreover, Stolzenberg et al. (2012) demonstrated that treatment with sodium butyrate (SB), another HDAC inhibitor, amplified the effects of maternal experience, both on maternal responsiveness and on gene expression in the MPOA. Furthermore, Weaver (2007) showed increased association of acetylated histone-H3 with the GR promoter in the offspring of HL mothers.

As we can observe, the findings regarding histone acetylation and maternal behavior were focused in histone-H3. However, several studies had also analyzed the histone-H4 acetylation levels related to other parameters, such as in the long-term memory for extinction of conditioned fear in the hippocampus and prefrontal cortex (Bredy et al. 2007); in the storage of hippocampus-dependent latent inhibition memory (Levenson et al. 2004); in the abuse of certain substances—excessive alcohol drinking and its effects in the nucleus accumbens (Warnault et al. 2013), and of morphine-induced reward sensitization in the rat central nucleus of the amygdala (Bie et al. 2012); and also the use of HDAC inhibitor (Yamawaki et al. 2012). These studies thus developed our interest in analyzing the relation between histone-H4 and maternal care, and our results indeed expressed this relationship.

In conclusion, it seems that the enhanced expression of the receptors HTR1a, HTR1b, Erα, D1a, and Prlr in the OB of HL mothers can be related, at least in part, to the hyperacetylation status of histone-H4 here observed. Therefore, we suggest that the modulation of epigenetic parameters, specifically acetylation levels, could exert a pivotal role through molecular mechanisms involved in the different patterns of maternal behavior. However, it is still necessary to evaluate other epigenetic parameters, such as HAT and HDAC enzymes activities, DNA methylation, and other histone modifications, for a better understanding of the relationship between gene expression and maternal behavior in this brain area.

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Compliance with Ethical Standards

Conflict of interest The authors have no financial conflicts of interest.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of CONCEA (Conselho Nacional de Controle de Experimentação Animal) and were approved by the Ethics Committee of UFCSPA (Protocol Number 788/09).

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4.3 Artigo 3: *Low maternal care is associated with increased oxidative stress in the brain of lactating rats*

Ana Carolina de Moura[1], Verônica Bidinotto Brito[1], Marilene Porawski[2], Jenifer Saffi[1,2], Márcia Giovenardi[1,2]

[1] Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, Brazil
[2] Programa de Pós-Graduação em Biociências, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, Brazil

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Research report

Low maternal care is associated with increased oxidative stress in the brain of lactating rats

Ana Carolina De Moura[a], Verônica Bidinotto Brito[a], Marilene Porawski[b], Jenifer Saffi[a,b], Márcia Giovenardi[a,b,*]

[a] *Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, Brazil*
[b] *Programa de Pós-Graduação em Biociências, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, Brazil*

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ABSTRACT

Maternal care is crucial for offspring development and licking/grooming patterns can be induced by sensorial, neuroendocrine, and metabolic variations in the CNS. Important brain functions, such as learning and memory, can be influenced by oxidative stress, which can also modulate pathophysiological processes (e.g., depression, anxiety, and other psychiatric disorders). This study evaluated oxidative stress in the hippocampus (HP), olfactory bulb (OB), and plasma in Low-Licking (LL) and High-Licking (HL) lactating rats through superoxide dismutase (SOD) and catalase (CAT) activities, DNA damage (comet assay), and dihydrodichlorofluorescein (DCF) oxidation assay. Results demonstrate that in the HP of LL, the activities of SOD and CAT were increased compared to HL. In the OB, the activities of SOD and CAT were also increased in LL. The comet assay in the HP showed that LL had higher levels of basal damage and increased levels of DNA breaks than HL. In the OB, LL also had higher levels of DNA damage. In the plasma, no difference was observed in either SOD or CAT activities, but the DCF oxidation assay revealed that LL had higher levels of ROS production than HL. In conclusion, we observed that LL mothers showed evidence of increased oxidative stress when compared to HL, suggesting that variations in maternal behavior might be related to these biochemical parameters.

1. Introduction

The development of mammalian offspring is highly influenced by maternal care. In rodents, maternal behavior is expressed by the mothers' actions, such as crouching over the young for nursing and keeping them warm, building the nest and retrieving the pups back to it, and the act of licking the body and genital area of newborns (Rosenblatt, 1975; Stern and Johnson, 1990; Numan and Insel, 2003).

Maternal behavior results from a highly motivated brain, which enables the female to adapt their caring activities to different situations and social demands (Olazabal et al., 2013). During the first 2 weeks postpartum, lactating rats develop increasingly frequent licking and grooming behaviors that stimulate the pups, modifies their body and brain temperature, and allows the mother to reclaim salt and water to meet the physiological demands of lactation (Gubernick and Alberts, 1983). The frequency of this behavior is commonly used as a measure for the evaluation of maternal care (Liu et al., 1997; Caldji et al., 1998; Champagne et al., 2003; Parent and Meaney, 2008; Lenz and Sengelaub, 2009; Veenema and Neumann, 2009). This behavior has a normal distribution when assessed in a large sample (Champagne et al., 2003), suggesting that mothers with a low frequency (LL) and a high frequency (HL) of licking constitute the two extremes of the same population (Champagne, 2008).

Several structures of the central nervous system (CNS), such as the medial preoptic area (MPOA), medial and cortical nucleus of the amygdale, nucleus accumbens, paraventricular nucleus of the hypothalamus (PVN), hippocampus (HP), and the olfactory bulb (OB) are involved in the development and maintenance of maternal care (Consiglio and Lucion, 1996; Champagne et al., 2003; Teodorov et al., 2010; Ruthschilling et al., 2012; De Moura et al., 2015).

The olfactory system is modulated by hormonal changes that occur during pregnancy, parturition, and lactation (Lévy et al., 2004), which enable the female to respond positively to the stimulus coming from their offspring, thus influencing the mother–pup bonding and recognition (Sullivan et al., 1989). Olfaction is critical for the organization of certain behaviors after maternal responsiveness has been established (Lévy et al., 2004). Moreover, results suggest that the OB has a decisive role in the behavioral differences observed in lactating rats, because the genes as the dopamine, serotonin, prolactin, and estrogen receptors were downregulated in the OB of LL mothers when compared to HL

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* Correspondence to: Federal University of Health Science, Division of Physiology, Rua Sarmento Leite, 245/308, Porto Alegre, RS 90050-170, Brazil.
E-mail address: giovenardi.marcia@gmail.com (M. Giovenardi).

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(De Moura et al., 2015).

Essential brain functions, such as learning and memory, can be influenced by oxidative stress, which can also modulate pathophysiology processes, such as depression, anxiety, and other psychiatric disorders (Patki et al., 2013). Evidence indicates that the HP is the most stress-sensitive brain region and that changes in this area might play a role in mediating neurogenesis and behavior (Ferreira et al., 2015).

As mentioned before, maternal care is crucial in the development of the offspring and the distinct licking patterns are influenced by variations in the sensorial, neuroendocrine, and metabolic systems, as well as for differences in gene expression in key structures of the CNS. Therefore, the present study aimed to determine whether the maternal behavior could be related to oxidative stress and DNA damage in the HP, OB, and plasma of LL and HL mothers.

2. Results

Our results demonstrate that the activity of the antioxidant enzyme superoxide dismutase (SOD) in the HP was significantly increased (t(8) =3.679, p=0.006) in LL, when compared to HL, and that the activity of the enzyme catalase (CAT) was also increased (t(8)=11.27, p < 0.0001) in LL (Fig. 1).

Moreover, in the OB, the activity of SOD (t(8)=3.310, p=0.011) and CAT (t(8)=3.209, p=0.012) were also increased in low licking dams (Fig. 2).

DNA damage was analyzed with Endonuclease III (Endo III) and Formamidopyrimidine [fapy]-DNA glycosylase (FPG) enzymes, which recognize oxidative base damaged and convert it into single-strand breaks. The comet assay in the HP (Fig. 3-A) demonstrated that LL had higher levels of DNA damage than HL mothers, as shown by the basal DNA breaks (t(8)=3.993, p=0.004) and increased level of DNA breaks with the use of Endo III, which detects pyrimidine oxidized bases (t(8) =3.799, p=0.005) and FPG, which detects purine oxidized bases (t(8) =14.60, p < 0.0001). Fig. 3-B shows the results for the comet assay in the OB, where LL also had higher levels of DNA damage than HL (basal: t(8)=3.667, p=0.006) (Endo III: t(8)=4.299, p=0.003) (FPG: t(8)=3.936, p=0.004).

On the other hand, no differences were observed in the activities of SOD or CAT in the plasma (Fig. 4) between the groups. However, the dihydrodichlorofluorescein (DCF) oxidation assay revealed significantly higher levels of ROS production in LL than in HL dams (t(14) =2.396, p=0.031).

3. Discussion

Previous studies have demonstrated that the brains of low and high licking dams show significant differences. LL mothers have lower transcriptional expression levels of important genes (i.e., receptors for dopamine, serotonin, prolactin, and estrogen) related to maternal care in the OB, when compared to HL (De Moura et al., 2015). The variations found in these two specific areas raise questions about the role of oxidative stress in diversified behavioral patterns. Our results demonstrated this relationship with an elevated pattern of oxidative stress in LL, when compared to HL, with the rise in the activity of the antioxidant enzymes SOD and CAT, in both HP and OB, indicating greater elimination pathway of ROS. Also in these areas, LL had more extensive DNA damage than HL. However, when the plasma was analyzed in LL, the ROS amount was increased, but not the SOD and CAT activities. This peripheral ROS may have been derived from a source outside the tissues studied and not as a direct result of variations in maternal behavior.

Furthermore, a study with rats reported that direct pharmacological induction of oxidative stress promotes behavioral change, causing anxiety-like behaviors, learning, and memory impairment; however, when the animals were treated with antioxidants, these behaviors were prevented, suggesting a causal role of oxidative stress in this behavioral outcomes (Allam et al., 2013). A study of social defeat-mediated stress, involving psychological stress induction using a modified version of the resident-intruder model for social stress, showed that this intervention causes depression-like and anxiety-like behaviors with a concomitant rise in oxidative stress in the HP (Patki et al., 2013). Increased oxidative stress is a result of a reduced antioxidant response, which most likely occurs due to diminished SOD protein expression. A failing antioxidant response may lead to further ROS production, thus leading to inflammation and cytotoxicity. Therefore, a reduction in anxiety-like behaviors corresponds with less oxidative stress, and higher levels of oxidative stress correspond with greater memory impairment (Patki et al., 2013).

Marcolin et al. (2012) suggests that the brain is especially vulnerable to free radicals and numerous studies have demonstrated their effects in the HP and pre-frontal cortex (PFC). The HP seems more susceptible to oxidative stress-induced damage and it plays a role in regulating the antioxidant pathway (Patki et al., 2013). Additionally, Zlatkovic et al. (2014) examined stress-induced changes in SOD activity in the HP and PFC of male Wistar rats exposed to acute and chronic stress, observing significant decreases of SOD following chronic social isolation, compared to controls.

Social isolation decreases the activity of SOD and CAT in the HP. These social isolation-induced neurochemical and biochemical changes contribute to behavioral abnormalities including high levels of anxiety, social interaction deficits, and impaired spatial working memory (Shao et al., 2015). The HP also seems to be sensitive to early interventions, when animals stressed by isolation in childhood show an imbalance in the antioxidant enzyme system. Evidence points to a relationship between anxiety-like behavior and oxidative stress, when psychosocial stress can lead to alterations in some cellular processes, which may cause oxidative stress (Marcolin et al., 2012).

The above-cited studies corroborate our results, showing that the HP is a brain region sensitive to oxidative stress in studies of behavioral models. Besides the HP, we also analyzed the OB (which is a region essential to maternal behavior) showing results related to oxidative

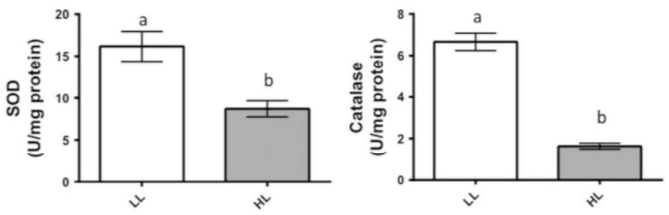

Fig. 1. Superoxide dismutase (SOD) and Catalase (CAT) activities in the Hippocampus (HP) of low (LL; n=5) and high (HL; n=5) licking mothers. Data expressed as Mean ± SEM, compared by the Student's *t*-test. ^{a,b} indicate significant difference between the groups.

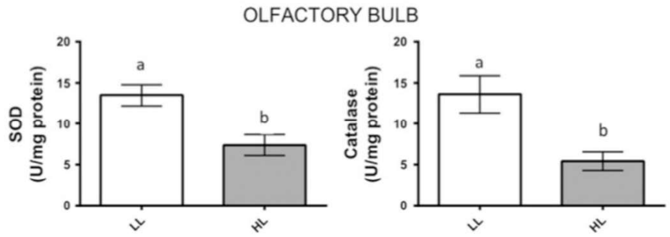

Fig. 2. Superoxide dismutase (SOD) and Catalase (CAT) activities in the Olfactory Bulb (OB) of low (LL; n=5) and high (HL; n=5) licking mothers. Data expressed as Mean ± SEM, compared by the Student's *t*-test. ^{a,b} indicate significant difference between the groups.

stress, demonstrating that the oxidative parameters of LL exceed those of HL. Olfaction also has an important role in mother–pup interactions, suggesting that neuroendocrine mechanisms in the olfactory system are a mediator of maternal care (Lévy et al., 2004).

Another important finding of our study is that low licking dams showed higher levels of DNA damage than HL, in both HP and OB, as shown in the comet assay. These results, corroborated with the data in Figs. 1 and 2, indicate greater antioxidant enzyme activity in the same structures of LL, than in HL. When DNA damage persists in the genome, through replicative processes and/or transcription-associated mutagenesis, it becomes permanent in the form of mutations and/or chromosomal breakage and instability (Ribeiro et al., 2012). Noschang et al. (2010) reported that male rats handled during the neonatal period, displayed higher levels of DNA breaks in the HP as adults, possibly due to an oxidative imbalance. It was also demonstrated that the HP of adult male rats, exposed to neonatal stress, exhibit an increased DNA break index, nitric oxide production impairment, and altered antioxidant enzymatic activity levels.

Unfortunately, with a paucity of studies comparing oxidative stress and maternal behavior, we cannot affirm what is cause or consequence of this relationship. In other words, it is not possible to confirm if oxidative stress could modify the mothers' behavior, or if the differences in maternal care can alter the oxidative stress parameters. However, the results found in this study show a connection between oxidative stress in the HP and OB with the variations in maternal behavior, and it is noteworthy that all the rats (LL and HL) lived under the same laboratory conditions, had standardized the same number of pups, and did not suffer any intervention.

Mothers who take less care of the offspring (low licking) have decrease levels of gene expression of the receptors for dopamine, serotonin, prolactin, and estrogen in the HP and OB (De Moura et al., 2015). In addition, LL showed increased DNA damage, in the HP and OB, as well as a higher activity of antioxidant enzymes in these areas, however this rise in antioxidant defenses was not able to prevent oxidative damage. Thus, we can speculate that there is a relationship between enlarged oxidative DNA damage and reduced maternal care. On the other hand, high licking dams seem to have developed a mechanism that preserves oxidative damage to the DNA in important areas related to maternal care. Further studies, therefore, should try to answer why LL and HL develop this different intensity of licking and grooming.

4. Experimental procedure

4.1. Animals

Animals used for the behavioral analyses were approximately 90 d old primiparous Wistar rats (*n*=92). They were housed under controlled temperature and lighting conditions, while food and water were provided ad libitum. In the last week of gestation, females were single-housed in Plexiglas cages (46 cm×17 cm×31 cm), remaining in the same cages for the behavioral observations. In the first postpartum day (PPD) each litter was randomly standardized at 8 pups.

All procedures were performed in conformity with the Brazilian Society of Neuroscience and Behavior Guidelines for the care and use of laboratory animals, and this study was approved by the Ethics Committee of the Universidade Federal de Ciências da Saúde de Porto Alegre (Protocol Number 788/09).

4.2. Maternal behavior

The licking behavior was observed daily for 75 min for the first 7 PPD. Observations occurred at regular hours each day, with three periods during the light (10:00 am, 1:30 pm, and 3:00 pm) and one period during the dark (5:30 pm) cycle. The observation in the dark was illuminated by a red light. Within each 75 min observation period, the behavior of each mother was scored every 3 min (25 observations/period×5 periods/day=100 observations/mother per day) as previously described by Myers et al. (1989).

The frequency of licking shows a normal distribution, so HL and LL

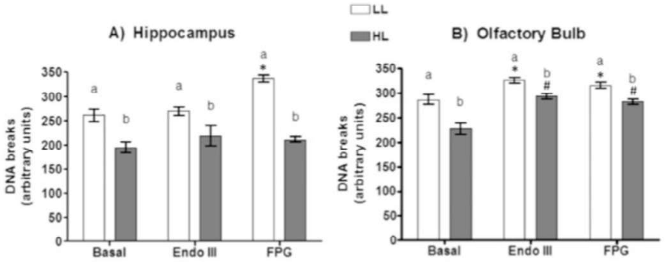

Fig. 3. DNA damage by comet assay in the Hippocampus (panel A) and Olfactory Bulb (panel B) of low (LL; n=5) and high (HL; n=5) licking mothers. DNA damage was analyzed with EndoIII and FPG enzymes, which recognize oxidative base damaged and convert it into single-strand breaks. ^{a,b} indicate significant difference between the LL and HL groups. $^{*, \#}$ indicate that oxidative base damage recognized by Endo III or FPG have a $p < 0.05$ from basal damage. Abbreviations: Formamidopyrimidine [fapy]-DNA glycosylase=FPG; Endonuclease III=EndoIII.

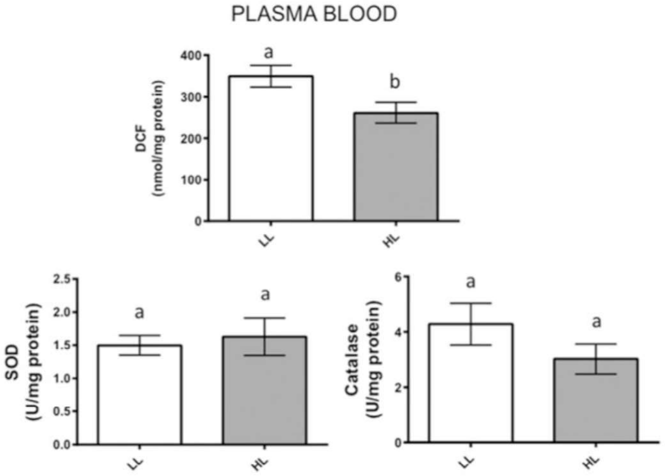

Fig. 4. The 2′,7′-dihydrodichlorofluorescein (DCF) oxidation assay; Superoxide dismutase (SOD) and Catalase (CAT) activities in the Hippocampus (HP) of low (LL; n=5) and high (HL; n=5) licking mothers. Data expressed as Mean ± SEM, compared by the Student's *t*-test. ^{a,b} indicate significant difference between the groups.

mothers represent opposites ends of a population (Ruthschilling et al., 2012). To define the sample for this study, the behavior of 92 mothers was observed. The mean ± standard deviation (SD) of licking behavior was 7.99 ± 2.49, and HL mothers (n=8) were defined as females whose frequency scores for licking were greater than 1 SD above the mean (10.48) and LL mothers (n=8) were defined as females whose frequency scores for licking were greater than 1 SD below the mean (5.50).

4.3. Tissue preparation

After behavioral tests were performed, females were decapitated without the use of anesthetics. Following blood was collected, and the brain rapidly removed. The HP and OB were dissected in a Petri dish placed on ice and stored at −80 °C. The dissection of the structures was performed as previously described in De Moura et al. (2015). These tissues were homogenized in 19 volumes (1:20, w/v) of 20 mM sodium phosphate buffer, pH 7.4 for the oxidative stress parameters, and in 19 volumes (1:20, w/v) 20 mM sodium phosphate buffer, pH 7.4, containing 20 mM EDTA and 10% DMSO for the DNA damage analysis. Homogenates were centrifuged at 1500*g* for 10 min at 4 °C. The pellet was discarded and the supernatant was retained for measurement.

Blood was separated in heparinized tubes. Whole blood samples were then centrifuged at 5000*g* for 10 min at 5 °C to yield a plasma fraction, which was used for subsequent biochemical analysis.

4.4. Superoxide dismutase (SOD) activity

SOD activity was evaluated by quantifying the inhibition of superoxide-dependent autoxidation of epinephrine, verifying the absorbance of samples at 480 nm (Misra and Fridovich, 1972). Briefly, to 20 µl of each sample were added 170 µl of a mixture containing 50 mM glycine buffer, pH 10.2, and 10 mM catalase. After that, 10 µl of 60 mM epinephrine were added and the absorbance was recorded immediately, each 36 s for 10 min, at 480 nm in a SpectraMax M2e Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California). The inhibition of autoxidation of epinephrine occurs in the presence of SOD, whose activity can indirectly be assayed spectrophotometrically. One SOD unit is defined as the amount of SOD necessary to inhibit 50% of epinephrine autoxidation and the specific activity is reported as SOD Units/mg protein.

4.5. Catalase (CAT) activity

CAT activity was assayed according to the method described by Aebi (1984), based on the disappearance of H_2O_2 at 240 nm. Briefly, 10 µl of each sample were added to 180 µl of 20 mM potassium phosphate buffer, pH 7.2. Subsequently, 10 µl of 10 mM H_2O_2 were added and the absorbance was recorded immediately, each 36 s for 5 min, at 240 nm using a SpectraMax M2e Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California). One CAT unit is defined as one µmol of hydrogen peroxide consumed per minute and the specific activity is calculated as CAT Units/mg protein.

4.6. Plasma dihydrodichlorofluorescein diacetate (H2DCF-DA) oxidation assay

The H2DCF-DA assay was used to estimate the intracellular generation of reactive species in a cell-free assay according to Karlsson et al. (2010) and Yin et al. (2013). Briefly, a 2′,7′-dihydrodichlorofluorescein diacetate (H2DCF-DA) stock solution was prepared and stored at −20 °C. Prior each experiment, 1 mL of 0.1 M NaOH was added to 250 µl of stock H2DCF-DA and incubated at room temperature for 30 min. Reaction was stopped by neutralizing the solution with phosphate buffered saline (PBS), which converts H2DCF-DA to dihydrodichlorofluorescein (H2DCF). H2DCF oxidation by reactive species from the plasma sample generates DCF. The DCF fluorescence intensity is correlated with the amount of reactive species present in the sample. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. All experiments were performed in a dark room to prevent the oxidation of DCFH. A calibration curve was prepared with DCF standard and the levels of reactive species were calculated from the curve and expressed as nmol DCF formed/mg protein.

4.7. DNA damage by comet assay

The viability of cells in the frozen tissues (HP and OB) was measured by a Trypan Blue exclusion test, as previously described by Robichová and Slameňová (2002). Tissues were gently homogenized in ice and with cold PBS (10% w/v), in a dark room. After centrifugation (500*g*, 5 °C, 5 min) 10 µl of this cellular suspension was stained with TB (0.4%) and the number of viable and dead cells was counted in an automated cell counter (CountessTM, Thermo Fisher Scientific).

The alkaline comet assay, used to measure single and double DNA strand breaks, was performed as described by Singh et al. (1988) in accordance with general guidelines for use of the comet assay (Tice et al., 2000). Aliquots of 20 µl of homogenized tissues were mixed with 90 µl low melting point agarose and added to 1.5% agarose pre-coated microscope slides. Slides were placed in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10.0–10.5 with 10% DMSO and 1% Triton X-100) to remove cell proteins, leaving DNA as "nucleoids". In the modified comet assay, slides were removed from the lysis solution, washed three times in an enzyme buffer (40 mM Hepes, 100 mM KCl, 0.5 mM Na_2EDTA, 0.2 mg/mL BSA, pH 8.0), and incubated with 100 µl of Endonuclease III (Endo III) (300 mU per gel; 45 min 37 °C), or Formamidopyrimidine [fapy]-DNA glycosylase (FPG) (300 mU per gel; 45 min 37 °C). After the lysis procedure and enzyme incubation, the slides were placed on a horizontal electrophoresis unit and were covered with fresh buffer (300 mM NaOH and 1 mM EDTA, pH 13.0) for 15 min to allow DNA unwinding. Electrophoresis was subsequently performed for 15 min (300 mA, 25 V). All the above steps were performed under yellow light or in the dark in order to prevent additional DNA damage. Slides were neutralized (0.4 M Tris, pH 7.5) and stained using silver nitrate staining protocol (Nadin et al., 2001).

After staining, gels were left to dry at room temperature overnight and subject to analysis the following day. For DNA damage evaluation, 100 cells per sample were analyzed by optical microscopy. The cells were visually scored by measuring the DNA migration length and classifying the amount of DNA in the tail into five classes. Furthermore, a damage index (DI) value was calculated for each sample. These five classes are described as follows: Class 0: undamaged, without a tail; Class 1: with a tail shorter than the diameter of the head (nucleus); Class 2: with a tail length one to two times the diameter of the head; Class 3: with a tail two times longer than the diameter of the head and; Class 4: comets with no head. DI ranged from 0 (completely undamaged: 100 cells×0) to 400 (maximum damage: 100 cells×4). International guidelines and recommendations for the comet assay consider visual scoring of comets to be a well-validated evaluation method (Collins et al., 2008).

4.8. Protein measurement

The protein content of samples was quantified for data normalization according to Lowry et al. (1951) with bovine serum albumin as a standard.

4.9. Statistical analyses

Data distribution was determinate by a D′agostino-Pearson omnibus test. Data was expressed (mean ± SEM) and compared using the software Prism™ by the Student's *t*-test. In all cases, $p < 0.05$ was considered statistically significant.

Acknowledgements

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5 CONCLUSÕES E CONSIDERAÇÕES FINAIS

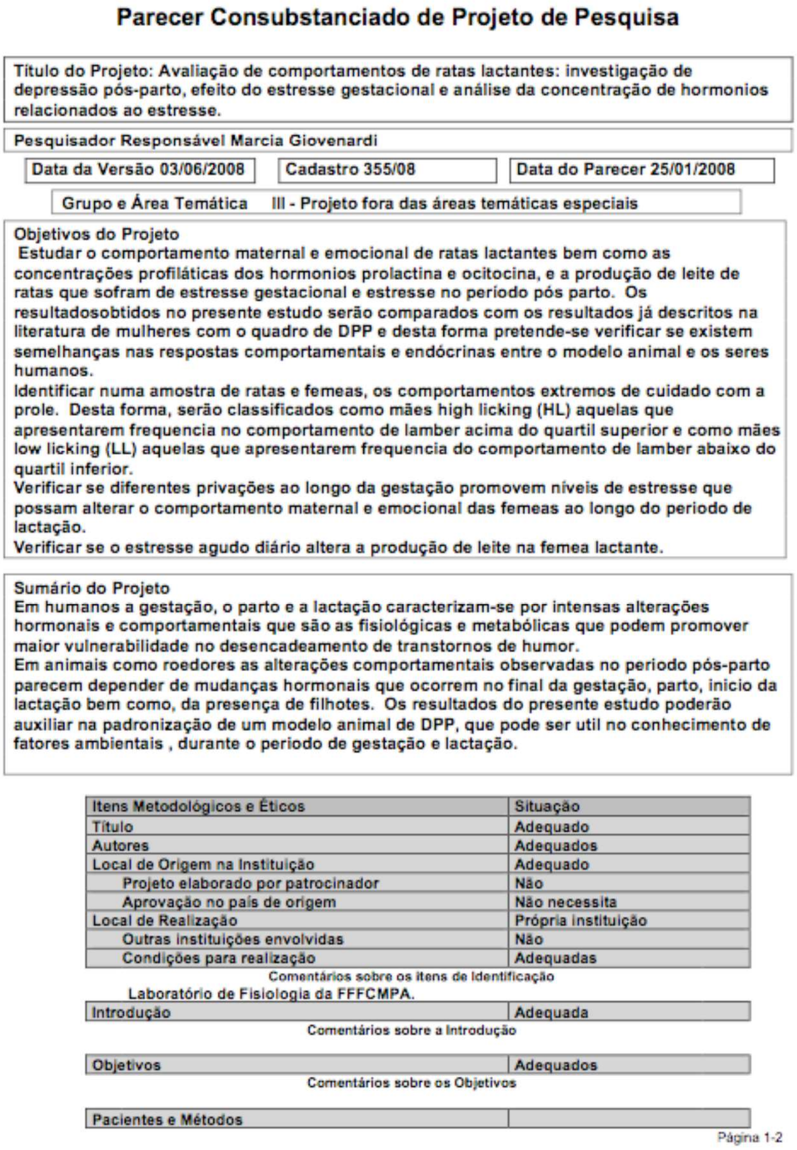
- Mães HL apresentam aumento na expressão gênica (Drd1a, Prlr, Htr1a, Htr1b e Esr1) no OB quando comparadas as LL.
- Observamos o aumento no nível de acetilação da histona H4 no OB do grupo HL.
- Mães HL apresentam menor atividade das enzimas antioxidantes (SOD e CAT) e menor dano oxidativo ao DNA no OB e HP, além de menor produção de ROS no plasma, do que mães LL.

Com os resultados obtidos nesta tese podemos inferir que o aumento da expressão gênica descrita no OB das mães mais cuidadoras ocorreu pelo aumento da acetilação da histona H4. Porém, há necessidade de realizar mais testes epigenéticos que visem confirmar ou ampliar este resultado.

Em relação aos resultados do estudo de estresse oxidativo, não podemos afirmar se este modificou o comportamento das mães ou se as diferenças nos cuidados maternos alteraram os parâmetros do estresse oxidativo. Podemos inferir que mães HL parecem ter desenvolvido um mecanismo que preserva o dano oxidativo ao DNA em áreas importantes relacionadas ao cuidado materno.

Os resultados encontrados nesta tese ampliam as respostas na busca do entendimento das diferenças comportamentais das lactantes, contudo, mais estudos serão necessários para desvendar os mecanismos que geram estes padrões opostos de cuidado materno.

ANEXO A - PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA



Parecer Consubstanciado de Projeto de Pesquisa

Título do Projeto: Avaliação de comportamentos de ratas lactantes: investigação de depressão pós-parto, efeito do estresse gestacional e análise da concentração de hormonios relacionados ao estresse.

Pesquisador Responsável Marcia Giovenardi

Data da Versão 03/06/2008

Cadastro 355/08

Data do Parecer 25/01/2008

Grupo e Área Temática III - Projeto fora das áreas temáticas especiais

Objetivos do Projeto

Estudar o comportamento maternal e emocional de ratas lactantes bem como as concentrações profiláticas dos hormonios prolactina e ocitocina, e a produção de leite de ratas que sofram de estresse gestacional e estresse no período pós parto. Os resultadosobtidos no presente estudo serão comparados com os resultados já descritos na literatura de mulheres com o quadro de DPP e desta forma pretende-se verificar se existem semelhanças nas respostas comportamentais e endócrinas entre o modelo animal e os seres humanos.

Identificar numa amostra de ratas e femeas, os comportamentos extremos de cuidado com a prole. Desta forma, serão classificados como mães high licking (HL) aquelas que apresentarem frequencia no comportamento de lamber acima do quartil superior e como mães low licking (LL) aquelas que apresentarem frequencia do comportamento de lamber abaixo do quartil inferior.

Verificar se diferentes privações ao longo da gestação promovem níveis de estresse que possam alterar o comportamento maternal e emocional das femeas ao longo do periodo de lactação.

Verificar se o estresse agudo diário altera a produção de leite na femea lactante.

Sumário do Projeto

Em humanos a gestação, o parto e a lactação caracterizam-se por intensas alterações hormonais e comportamentais que são as fisiológicas e metabólicas que podem promover maior vulnerabilidade no desencadeamento de transtornos de humor.

Em animais como roedores as alterações comportamentais observadas no periodo pós-parto parecem depender de mudanças hormonais que ocorrem no final da gestação, parto, inicio da lactação bem como, da presença de filhotes. Os resultados do presente estudo poderão auxiliar na padronização de um modelo animal de DPP, que pode ser util no conhecimento de fatores ambientais , durante o periodo de gestação e lactação.

Itens Metodológicos e Éticos	Situação
Título	Adequado
Autores	Adequados
Local de Origem na Instituição	Adequado
Projeto elaborado por patrocinador	Não
Aprovação no país de origem	Não necessita
Local de Realização	Própria instituição
Outras instituições envolvidas	Não
Condições para realização	Adequadas

Comentários sobre os itens de Identificação

Laboratório de Fisiologia da FFFCMPA.

Introdução	Adequada

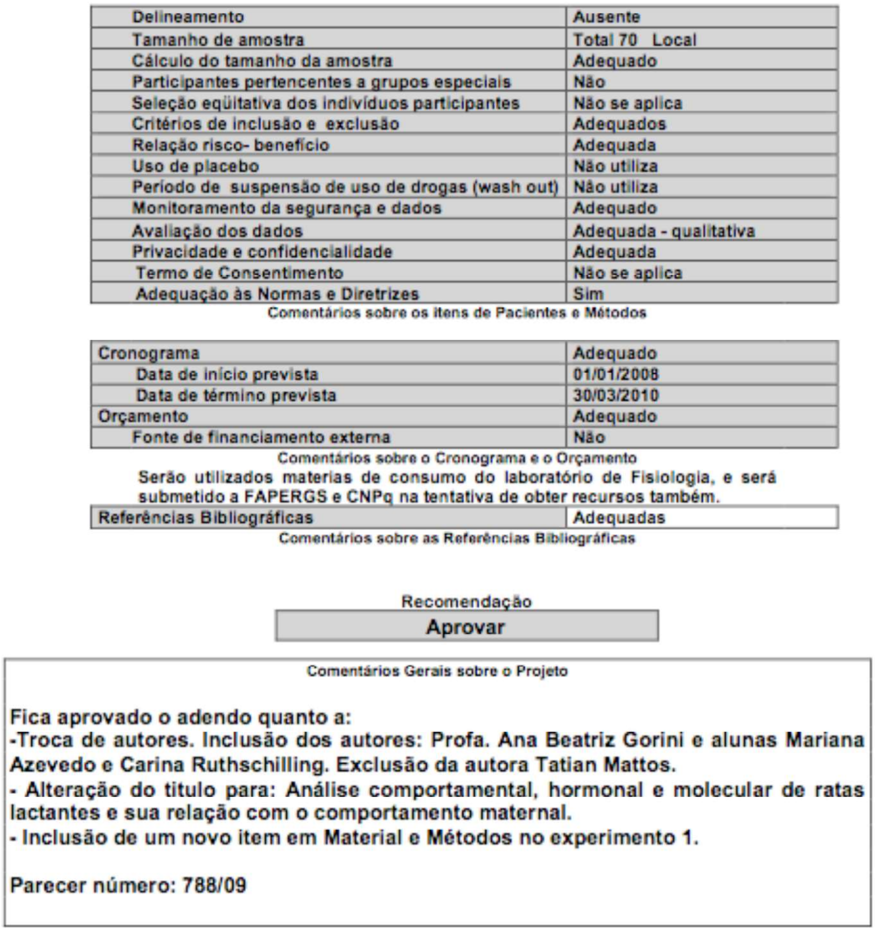
Comentários sobre a Introdução

Objetivos	Adequados

Comentários sobre os Objetivos

Pacientes e Métodos	

Página 1-2



Delineamento	Ausente
Tamanho de amostra	Total 70 Local
Cálculo do tamanho da amostra	Adequado
Participantes pertencentes a grupos especiais	Não
Seleção eqüitativa dos indivíduos participantes	Não se aplica
Critérios de inclusão e exclusão	Adequados
Relação risco- benefício	Adequada
Uso de placebo	Não utiliza
Período de suspensão de uso de drogas (wash out)	Não utiliza
Monitoramento da segurança e dados	Adequado
Avaliação dos dados	Adequada - qualitativa
Privacidade e confidencialidade	Adequada
Termo de Consentimento	Não se aplica
Adequação às Normas e Diretrizes	Sim

Comentários sobre os itens de Pacientes e Métodos

Cronograma	Adequado
Data de início prevista	01/01/2008
Data de término prevista	30/03/2010
Orçamento	Adequado
Fonte de financiamento externa	Não

Comentários sobre o Cronograma e o Orçamento

Serão utilizados materias de consumo do laboratório de Fisiologia, e será submetido a FAPERGS e CNPq na tentativa de obter recursos também.

Referências Bibliográficas	Adequadas

Comentários sobre as Referências Bibliográficas

Recomendação

Aprovar

Comentários Gerais sobre o Projeto

Fica aprovado o adendo quanto a:
-Troca de autores. Inclusão dos autores: Profa. Ana Beatriz Gorini e alunas Mariana Azevedo e Carina Ruthschilling. Exclusão da autora Tatian Mattos.
- Alteração do titulo para: Análise comportamental, hormonal e molecular de ratas lactantes e sua relação com o comportamento maternal.
- Inclusão de um novo item em Material e Métodos no experimento 1.

Parecer número: 788/09