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EFEITOS SEXO-ESPECÍFICOS DA MANIPULAÇÃO NEONATAL EM RATOS: AVALIAÇÃO DE DIFERENTES TIPOS DE MEMÓRIAS, RELACIONADAS OU NÃO AO CONSUMO DE ALIMENTO PALATÁVEL, E DE PARÂMETROS NEUROQUÍMICOS

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

Orientadora: Profª Drª Carla Dalmaz

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somente até onde os outros já foram.

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SUMÁRIO

PARTE I

RESUMO

Consideráveis evidências demonstram que a qualidade do ambiente no início da vida influencia padrões de desenvolvimento que determinam a saúde e a produtividade do indivíduo ao longo da vida. Para estudar essa interação utilizamos o protocolo da manipulação neonatal que consiste em uma breve separação dos filhotes de sua mãe. Estudos demonstram que ratos submetidos a este procedimento apresentam quando adultos um aumento no consumo de alimento palatável, quando este é oferecido por um curto período de tempo, sem alterar o consumo de ração. Uma possibilidade para explicar este efeito seria que os ratos manipulados no período neonatal teriam alterações na memória relacionada ao consumo de alimento palatável. Desta forma, o objetivo desta tese foi avaliar os efeitos da manipulação neonatal sobre diferentes tipos de memória, relacionadas ou não ao alimento palatável, bem como parâmetros neuroquímicos que podem estar associados a alterações cognitivas, entre eles a atividade das enzimas Na^+/K^+-ATPase e acetilcolinesterase e os parâmetros do estresse oxidativo, considerando a possibilidade de diferenças sexo-específicas. Além disso, como o consumo aumentado de alimento palatável pelos animais manipulados no período neonatal é observado apenas após a puberdade, também avaliamos possíveis interações entre a manipulação neonatal e a remoção dos ovários antes da puberdade sobre a memória e sobre parâmetros neuroquímicos na idade adulta. Ninhadas de ratos Wistar foram manipuladas (10 min/dia, do 1º ao 10º dia após o nascimento) ou não. Na idade adulta foram avaliados os seguintes parâmetros: memória espacial, memória de hábito (associado ao alimento palatável) e aprendizado reverso (associado ou não ao alimento), assim como parâmetros neuroquímicos em machos e fêmeas. Ratas fêmeas manipuladas ou não no período neonatal também foram submetidas à cirurgia de remoção dos ovários ou apenas à cirurgia (sem remoção dos ovários) ou, ainda, não foram submetidas ao procedimento cirúrgico antes da puberdade e, quando adultas, foram avaliadas com relação à memória olfatória (associada ao alimento palatável) e à parâmetros neuroquímicos. Os mesmos parâmetros neuroquímicos também foram avaliados antes da puberdade em ratos jovens machos e fêmeas manipulados ou não no período neonatal. A manipulação neonatal (considerando ratos adultos) provocou um prejuízo na memória espacial em fêmeas mas não em machos, uma melhora na memória de hábito em fêmeas, um prejuízo no aprendizado reverso relacionado a este tipo de memória em machos e não alterou a memória olfatória em fêmeas. Além disso, no hipocampo de ratos adultos, aumentou o índice de quebras ao ADN em machos e em fêmeas reduziu a produção de óxido nítrico e aumentou a atividade da Na^+/K^+-ATPase. A manipulação neonatal também reduziu a relação superóxido dismutase/catalase no córtex pré-frontal e aumentou a atividade da Na^+/K^+-ATPase no bulbo olfatório de fêmeas adultas. Em ratos jovens, ela aumentou a atividade da Na^+/K^+-ATPase no bulbo olfatório e diminuiu a atividade da acetilcolinesterase no hipocampo de ratos machos. Nas fêmeas jovens, essa intervenção precoce diminuiu a atividade da glutationa peroxidase e também reduziu o conteúdo total de tióis no hipocampo. Conclui-se que a manipulação neonatal afetou os parâmetros avaliados nesta tese de forma sexo-específica, sendo a memória espacial prejudicada em fêmeas manipuladas e a memória de hábito facilitada; nos machos, por sua vez, a manipulação neonatal parece levar a uma menor flexibilidade comportamental. Os efeitos neuroquímicos dependem do período da vida e da estrutura cerebral avaliada. Esta tese reforça a importância de considerar o gênero quando se avaliam os efeitos da manipulação neonatal, e contribui para o melhor entendimento de como intervenções precoces afetam a memória e parâmetros neuroquímicos ao longo da vida.

ABSTRACT

Considerable evidences show that the quality of the early life environment influences patterns of development that determine the health and productivity of the individual throughout life. To study this interaction we used the neonatal handling protocol that consists of a brief separation of pups from their mother. Studies show that rats submitted to this procedure show when adults an increased consumption of palatable food, when this is offered during a brief period of time, without changing the consumption of chow. One possibility to explain this effect would be that neonatal handled rats would have memory changes related to the consumption of palatable food. Thus, the aim of this thesis was to evaluate the effects of neonatal handling on different types of memory, related or not to palatable food, as well as neurochemical parameters that may be related to cognitive changes, as Na^+/K^+-ATPase and acetylcholinesterase enzyme activities and oxidative stress parameters, considering the possibility of sex-specific differences. In addition, since the increased consumption of palatable food by neonatal handled animals is observed only after puberty, we also evaluated possible interactions between the neonatal handling and the ovaries removal before puberty on memory and on neurochemical parameters in adulthood. Wistar rats' litters were handled (10min/day, from the 1st to the 10th day after birth) or not. The following parameters were evaluated in adulthood: spatial memory, habit memory (related to palatable food) and reversal learning (related or not to food), as well as neurochemical parameters in males and females. Female rats handled or not in the neonatal period were also submitted to the ovaries removal surgery or only to the surgery (without removal of ovaries) or, still, were not submitted to the surgical procedure before puberty and, when adults, were evaluated in relation to the olfactory memory (related to palatable food) and to neurochemical parameters. The same neurochemical parameters were also evaluated before puberty in neonatal handled or not female and male juvenile rats. Neonatal handling (considering adult rats) led to impairment in females' spatial memory but not in males, improvement in habit memory in females, impairment in reversal learning related to this type of memory in males and did not change olfactory memory in females. In addition, in the hippocampus of adult rats, increased the DNA breaks index in males and in females reduced the nitric oxide production and increased the Na^+/K^+-ATPase activity. Neonatal handling also reduced the superoxide dismutase/catalase ratio in the prefrontal cortex and increased Na^+/K^+-ATPase activity in the olfactory bulb of adult females. In juvenile rats, it increased the Na^+/K^+-ATPase activity in the olfactory bulb and decreased the acetylcholinesterase activity in the hippocampus of male rats. In juvenile females, this early intervention decreased glutathione peroxidase activity and also decreased the total thiol content in the hippocampus. In conclusion, neonatal handling affected the parameters evaluated on this thesis in a sex-specific manner, with impairment in females' neonatal handled spatial memory and improvement in habit memory; on the other hand, in males, neonatal handling seems to lead to a lower behavioral flexibility. The neurochemical effects depend on the period of life and on the cerebral structure evaluated. This thesis reinforces the importance of considering the gender when evaluating neonatal handling effects, and contributes to a better understanding of how early interventions affect memory and neurochemical parameters throughout life.

LISTA DE ABREVIATURAS

ACTH - hormônio adrenocorticotrófico

ADN = ácido desoxirribonucleico

ATP = adenosina trifosfato

CAT = catalase

DOHaD = origens desenvolvimentistas da saúde e da doença

DOPAC = ácido 3,4-dihidroxifenilacético

ERO = espécies reativas do oxigênio

GABA = ácido gama-aminobutírico

GMPc = monofosfato de guanosina cíclico

GPx = glutationa peroxidase

5-HIAA = ácido 5-hidroxindolacético

HPA = hipotálamo-hipófise-adrenal

LTD = depressão de longa duração

LTP = potenciação de longa duração

NADPH = nicotinamida adenina dinucleotídeo fosfato reduzido

NMDA = N-metil-D-aspartato

SOD = superóxido dismutase

1. INTRODUÇÃO

1.1 Origens desenvolvimentistas da saúde e da doença

Atualmente existe um crescente interesse acerca de como o ambiente ou eventos precoces afetam o organismo na vida adulta. A hipótese do "fenótipo econômico", também conhecida como hipótese de Barker, propõe que o crescimento fetal reduzido está fortemente associado com vários processos crônicos ao longo da vida. Esta susceptibilidade aumentada resulta de adaptações feitas pelo feto em um ambiente com aporte limitado de nutrientes. Dentre tais processos crônicos, estão distúrbios cardíacos e metabólicos. Sugere-se que, em condições nutricionais pobres, o desenvolvimento do feto possa ser modificado, preparando-o para a sobrevivência em ambientes nos quais os recursos são escassos, resultando assim no fenótipo econômico (HALES e BARKER, 1992). Essas observações levaram à teoria de que a desnutrição durante a gestação é uma importante etiologia precoce de doenças metabólicas e cardíacas devido à programação fetal, a qual modula permanentemente a estrutura, a função e o metabolismo do corpo e contribui para a doença no indivíduo adulto. Essa teoria estimulou o interesse nas origens fetais das doenças em adultos, levando à formação da Sociedade Internacional para o Estudo das Origens Desenvolvimentistas da Saúde e da Doença (DOHaD, do inglês *Developmental Origins of Health and Disease*) (WADHWA ET AL., 2009).

Além do período fetal, existem outros períodos críticos no desenvolvimento, como por exemplo o período neonatal, a infância e a adolescência, os quais são caracterizados por alta plasticidade neuronal (CREWS, HE e HODGE, 2007). A exposição do indivíduo a um estímulo ou insulto nesses períodos pode levar a alterações persistentes no funcionamento do organismo (LUCAS, 1991). Períodos críticos são fases específicas durante o desenvolvimento, quando processos

dependentes da genética e ambientais interagem para estabelecer características funcionais (CREWS, HE e HODGE, 2007). Dessa forma, o desenvolvimento e a gravidade de diversas condições patológicas na vida adulta dependem da vulnerabilidade genética do indivíduo, da exposição a fatores ambientais adversos, e do período de ocorrência do insulto (CHARMANDARI ET AL., 2003).

1.2 Estresse neonatal

A resposta ao estresse leva a alterações comportamentais e metabólicas, num esforço de manter a homeostasia corporal e aumentar as chances de sobrevivência (CHROUSOS e GOLD, 1992; TSIGOS e CHROUSOS, 2002). Um dos principais mecanismos endócrinos é a ativação do eixo hipotalálamo-hipófise-adrenal, (AGUILERA, 1994), onde inicialmente ocorre a liberação do hormônio liberador de corticotropina do hipotálamo, que leva à liberação do hormônio adrenocorticotrópico (ACTH) da hipófise para a circulação sanguínea. O ACTH, por sua vez, estimula a liberação de glicocorticóides (cortisol em humanos e corticosterona em ratos) do córtex da adrenal (LUPIEN ET AL., 2005). Os glicocorticóides têm vários efeitos no organismo, como o aumento da disponibilidade de substrato energético em diferentes partes do corpo e a adaptação às alterações do ambiente (LUPIEN ET AL., 2005).

Intervenções feitas na infância influenciam a relação da mãe com os filhotes, pois a mãe pode assumir determinados comportamentos que afetam o desenvolvimento do sistema nervoso dos seus filhotes. Estudos sobre o estresse neonatal têm mostrado a importância de um ambiente adequado para um desenvolvimento saudável, pois os recém-nascidos são mais vulneráveis neste período. Assim, experimentos nesta área são de grande importância para futuramente

relacionarmos quais eventos adversos na infância podem ser responsáveis pelo aparecimento de algumas patologias.

A manipulação neonatal em ratos tem sido usada como um modelo para avaliar como as intervenções no período neonatal influenciam na vida adulta. Ela consiste tipicamente na "manipulação" dos filhotes (onde eles são separados de sua mãe) por alguns minutos, em geral durante as duas primeiras semanas de vida. Esse processo modula o desenvolvimento do eixo hipotálamo-hipófise-adrenal (HPA) e está associado na idade adulta com a diminuição da resposta ao estresse (LEVINE ET AL., 1967). O eixo HPA é moderadamente responsivo ao estresse no momento do nascimento, mas a resposta ao hormônio adrenocorticotrópico diminui durante o período neonatal (YOSHIMURA ET AL., 2003). Nos ratos, o último estágio fetal, durante o qual muita corticosterona é secretada, é seguido por um período de menor secreção até o final da segunda semana pós-natal, o qual é conhecido como período hiporresponsivo ao estresse (YOSHIMURA ET AL., 2003). Durante essa fase, a resposta do eixo HPA a estímulos nocivos é reduzida (HALTMEYER ET AL., 1966; BARTOVA, 1968), pois há uma exacerbação do mecanismo de retroalimentação negativa dos glicocorticóides na hipófise e diminuição da sensibilidade da adrenal ao ACTH (YOSHIMURA ET AL., 2003). Nesse período, mesmo que a concentração total da corticosterona plasmática seja baixa, a concentração de corticosterona biologicamente ativa (isto é, não ligada a proteínas no plasma) é relativamente alta, o que é suficiente para que o hormônio exerça suas ações biológicas e, possivelmente, atue programando o sistema nervoso central de forma persistente. Por outro lado, o período hiporresponsivo ao estresse parece desempenhar um efeito protetor, prevenindo que altos níveis de glicocorticóides alcancem o cérebro durante o período neonatal.

Estudos demonstram que as mães de filhotes manipulados lambem mais a sua prole do que mães de filhotes não-manipulados. Esse comportamento da mãe em relação ao filhote afeta o desenvolvimento do sistema nervoso deste (LEVINE, 1994). Acredita-se que a perturbação dessa relação é o que induziria o padrão comportamental e endócrino observado na vida adulta do rato manipulado no período neonatal.

Os ratos manipulados no período neonatal apresentam (quando adultos) uma menor liberação de glicocorticóides em resposta ao estresse (ADER e GROTA, 1969). Ainda, a manipulação neonatal leva a alterações comportamentais na idade adulta como, por exemplo, o aumento na exploração de um ambiente diferente da caixa moradia (LEVINE ET AL., 1967), bem como uma redução do medo a ambientes novos (MADRUGA ET AL., 2006). Estudos anteriores do nosso laboratório demonstraram que ratos (machos e fêmeas) submetidos à manipulação neonatal apresentaram na idade adulta, um maior consumo de alimento palatável, quando este foi oferecido por um curto período de tempo, sem que houvesse alteração no consumo de ração padrão. Isso nos leva a acreditar que essa mudança no comportamento motivado esteja relacionada a razões hedônicas mais que metabólicas (isto é, não seria resultado de maior necessidade calórica e sim de motivação pelo alimento palatável). Além disso, esse aumento de ingestão de alimento palatável não estava relacionado com a ansiedade, pois não foi revertido pela administração de diazepam e as avaliações comportamentais de ansiedade através do labirinto em cruz elevado e do teste claro-escuro confirmaram a ausência do comportamento do tipo ansioso em ratos manipulados no período neonatal (SILVEIRA ET AL., 2005).

É interessante observar que a avaliação do comportamento alimentar antes da puberdade em ratos machos manipulados no período neonatal mostrou uma redução

(embora não significativa, P = 0,063) na ingestão de alimento palatável quando este foi oferecido por um curto período de tempo (SILVEIRA ET AL., 2006). Além disso, ratas fêmeas manipuladas no período neonatal não apresentaram diferença com relação ao consumo desse tipo de alimento antes da puberdade quando comparadas a controles não manipuladas (Noschang C, resultados não publicados). Tais observações exemplificam o fato de que, especialmente com relação ao comportamento motivado, as alterações resultantes da manipulação neonatal são observadas apenas após a puberdade, sugerindo que esse evento precoce (no período neonatal) deixe uma marca que levará a alterações comportamentais posteriormente, talvez pela interação com eventos que ocorrem durante a puberdade.

1.3 Manipulação neonatal e comportamento motivado: possíveis fatores relacionados

Um aumento dos estímulos motivacionais relacionados aos efeitos comportamentais citados acima poderiam resultar de diferentes fatores, algumas possibilidades sendo (1) uma alteração no comportamento de busca e/ou na percepção do valor hedônico da recompensa, mas também poderia estar relacionado (2) a alterações na memória, envolvida nas lembranças positivas ou negativas relacionadas ao consumo de determinado alimento e processadas por um conjunto de estruturas encefálicas que inclui o hipocampo.

1.3.1 O “querer” e o “gostar”

Pesquisas neuropsicológicas vêm auxiliando a compreensão dos substratos encefálicos que medeiam a recompensa alimentar. Por exemplo, pesquisas na Universidade de Michigan coordenadas por Kent Berridge levaram a um modelo que propõem a existência de dois componentes distintos envolvidos. O primeiro é um componente hedônico denominado "gostar", que é o resultado de um processo central incorporando não apenas propriedades sensoriais, mas também o estado fisiológico do indivíduo. No "gostar" estão envolvidos o prazer e o quão palatável o alimento é. O segundo componente é a motivação ou a saliência de incentivo, classificada como "querer", e refere-se ao processo que direciona ao objetivo e pode ser visto como o impulso direcionado ou a demanda para o estímulo alimentar alvo, sendo relacionado com o apetite. Em termos operacionais, isso reflete o processo neural que modula a alteração no comportamento, desde a busca ativa pelo objeto até o comportamento de ignorá-lo (FINLAYSON, KING e BLUNDELL, 2007). "Querer e gostar" apresentam substratos neuronais separados. A modulação do "gostar" está relacionada com a recompensa alimentar e envolve sistemas neurotransmissores como os opióides e o sistema GABA/benzodiazepínico, bem como estruturas anatômicas como o pálido ventral e o tronco encefálico. Já a modulação do "querer" envolve os sistemas dopaminérgicos mesotelencefálicos, bem como o núcleo accumbens e a amígdala (BERRIDGE, 1996). Uma possível explicação para o aumento no consumo de alimento palatável por ratos manipulados no período neonatal seria por alterações no "querer" e/ou no "gostar". Conforme será abordado a seguir, alguns efeitos da manipulação neonatal no consumo de alimento palatável parecem estar associados ao "querer" enquanto outras sugerem uma relação com o "gostar".

O aumento do consumo de alimento palatável por ratos manipulados no período neonatal pode estar associado a diferentes mecanismos. Uma possibilidade seria uma menor percepção do sabor doce e dos efeitos relacionados ao prazer e à recompensa resultante do consumo desse tipo de alimento. Sabe-se que esses ratos apresentam na idade adulta uma redução na atividade da enzima 5'-nucleotidase em membranas sinápticas no núcleo accumbens (SILVEIRA ET AL., 2006), sendo esta enzima responsável pela formação de adenosina a partir do ATP (CUNHA e RIBEIRO, 2000). Dessa forma, acredita-se que esses animais apresentem uma diminuição nos níveis extracelulares de adenosina no accumbens quando adultos (SILVEIRA ET AL., 2006), e isto poderia estar relacionado a uma menor percepção do sabor doce, uma vez que a adenosina está envolvida nesta percepção (SCHIFFMAN, GILL e DIAZ, 1985). Baseado neste achado parece que a manipulação neonatal altera o "gostar". Também foi demonstrado que ratos manipulados no período neonatal apresentam uma diminuição nas reações hedônicas positivas frente a uma solução de sacarose, sugerindo que o impacto hedônico do alimento doce seja menos proeminente nestes ratos (SILVEIRA ET AL., 2010).

Estudos demonstram que a interação adenosina/dopamina no accumbens é necessária para induzir o efeito de reforço da recompensa (AROLFO ET AL., 2004), de modo que uma redução no tônus dopaminérgico no núcleo accumbens também pode estar relacionada ao consumo aumentado de alimento palatável, como uma tentativa de alcançar uma maior ativação deste circuito (SILVEIRA ET AL., 2006). De acordo com tal possibilidade, a manipulação neonatal leva a uma diminuição no metabolismo dopaminérgico no núcleo accumbens de ratos adultos, havendo um aumento nos níveis de dopamina e uma redução nos níveis de ácido 3,4-dihidroxifenilacético (DOPAC) e na razão DOPAC/dopamina (SILVEIRA ET AL.,

2010). Por outro lado, estes ratos apresentam um menor condicionamento no teste de preferência de local condicionado, o que também ocorre em ratos tratados com haloperidol (bloqueador do receptor D_2 de dopamina) (SPYRAKI, FIBIGER e PHILLIPS, 1982). Além disso, eles apresentam uma maior saliência de incentivo no teste do corredor, tendo apresentado uma menor latência para alcançar o alimento palatável (SILVEIRA ET AL., 2010). Desta forma, considerando este último efeito, a manipulação neonatal parece afetar o "querer". Experimentos do nosso grupo também demonstraram que o metilfenidato, o qual inibe a recaptação de dopamina, não tem impacto no consumo de alimento palatável quando os ratos manipulados no período neonatal estão alimentados (isto é, animais manipulados continuam comendo mais alimento palatável em relação aos controles), o que parece ser explicado pelo fato de, neste estado, os níveis de insulina no plasma estarem aumentados, o que estimula a recaptação da dopamina (FIGLEWICZ ET AL., 1994). Já no estado de jejum, os animais não manipulados no período neonatal que receberam metilfenidato na idade adulta mostraram um aumento no consumo de alimento palatável, enquanto que os manipulados não apresentaram efeito (SILVEIRA ET AL., 2010).

Com relação ao sistema serotoninérgico, estudos recentes sugerem o envolvimento deste sistema no consumo aumentado de alimento palatável por animais manipulados no período neonatal (PORTELLA ET AL., 2010). Foi demonstrado que esses animais apresentam, na idade adulta, uma diminuição no metabolismo serotoninérgico no núcleo accumbens, apresentando níveis aumentados de serotonina e diminuídos de ácido 5-hidroxindolacético (5-HIAA), bem como na razão 5-HIAA/serotonina. Além disso, o tratamento crônico com imiprimina foi capaz de prevenir o efeito da manipulação neonatal sobre o consumo de alimento palatável (PORTELLA ET AL., 2010). A imipramina é um antidepressico tricíclico que atua

inibindo a recaptação de serotonina e noradrenalina, agindo também na neurotransmissão dopaminérgica e acetilcolinérgica (SARKO, 2000).

1.3.2 O comportamento alimentar e a memória

Na primeira vez que experimentamos um alimento, sensações são produzidas as quais podem ser positivas ou negativas. Cria-se então uma memória referente àquela experiência, que pode ser no sentido de gostar ou não do alimento ingerido. Isso influenciará futuras decisões com relação ao consumo ou não do alimento em questão. Além disso, a habilidade de procurar e achar comida é crucial para a sobrevivência. Após achar o alimento e ingerí-lo, é importante lembrar onde ele pode ser encontrado e, talvez mais importante do que isso, ser capaz de reter a estratégia usada para achá-lo. Desta forma, uma memória e um aprendizado efetivo parecem ser críticos para a sobrevivência em períodos com deficiência alimentar (OLSZEWSKI, SCHIOTH e LEVINE, 2008).

O hipocampo é uma estrutura conhecida por seu papel no aprendizado e na memória. Estudos encorajam a hipótese que mecanismos da memória e do aprendizado dependentes do hipocampo podem contribuir diretamente para o controle do consumo alimentar (DAVIDSON ET AL., 2007). Por outro lado, a habilidade de hormônios modularem o comportamento apetitivo pode também ser dependente de seus efeitos nos processos de memória e aprendizado dependente do hipocampo. Esta estrutura contém alta densidade de receptores de insulina e leptina (LATHE, 2001) e a administração destes hormônios aumenta tanto a memória espacial dependente do hipocampo quanto a potenciação de longa duração hipocampal (LTP, do inglês *long term potentiation*) (FARR, BANKS e MORLEY,

2006; ZHAO ET AL., 2004), reportada como base celular para o aprendizado e a memória. Existem também evidências de que o controle inibitório do consumo alimentar dependa da integridade funcional do hipocampo. Por exemplo, após o consumo de uma refeição completa, pacientes com acentuada amnésia (com dano hipocampal) comeram uma segunda refeição completa oferecida apenas alguns minutos depois (DAVIDSON ET AL., 2007). Também foi demonstrado que, em humanos neurologicamente sadios, a memória de uma refeição prévia ajuda a inibir o consumo subsequente (HIGGS, 2005). Outro hormônio relacionado ao comportamento alimentar e que atua também sobre a memória é a grelina, tendo sido demonstrado que ela é capaz de atuar no hipocampo ligando-se a neurônios onde gera a potenciação de longa duração (DIANO ET AL., 2006). Além disso, animais *knockout* para a grelina apresentaram prejuízo em tarefas de memória, sendo este prejuízo revertido pela administração de grelina (OLSZEWSKI, SCHIOTH e LEVINE, 2008).

É importante lembrar que respostas sensoriais ao sabor, como o odor e a textura, ajudam a determinar a preferência alimentar (DREWNOWSKI, 1997). Nos humanos cerca de 2/3 dos receptores olfatórios não são funcionais, possivelmente como uma consequência da redução da importância da função olfatória para os humanos comparados com ratos, por exemplo (BEAUCHAMP e MENNELLA, 2009). Desta forma, em ratos, o componente olfatório torna-se bastante relevante e consequentemente a memória olfatória relacionado ao alimento também.

1.4 Memória

1.4.1 Memória: tipos e estruturas encefálicas relacionadas

O aprendizado e a memória são adaptações da circuitaria cerebral ao ambiente que ocorrem ao longo de toda a vida. Eles nos permitem responder de modo adequado a situações que experimentamos anteriormente. Define-se aprendizado como a aquisição de novas informações ou novos conhecimentos, enquanto que a memória é a retenção da informação aprendida. Sabe-se que diferentes partes do encéfalo participam em diferentes tipos de memórias (BEAR, CONNORS e PARADISO, 2008). Segundo Squire (1987), a memória apresenta dois sistemas principais, os quais têm sido reconhecidos ao longo dos anos: o sistema da memória declarativa, o qual está sob o controle do hipocampo e estruturas do lobo temporal relacionadas, e o sistema da memória de procedimentos ou hábitos, o qual está sob o controle do estriado e suas conexões. Mais especificamente, a memória pode ser declarativa (relacionada a fatos e eventos) ou não-declarativa (por exemplo, condicionamento simples, bem como a aquisição de habilidades motoras, perceptuais e cognitivas) (DAUM e ACKERMANN, 1997). Diferentes tarefas comportamentais foram desenvolvidas para avaliar memórias em modelos animais; exemplos relacionados a este trabalho de tese são as tarefas do Labirinto Aquático de Morris (memória declarativa, espacial), a memória de reconhecimento (também declarativa) e a formação de hábitos (não-declarativa) (ANDERSEN e TUFIK, 2010).

A memória espacial está tipicamente associada ao hipocampo (VANN e ALBASSER, 2011). Células hipocampais apresentam várias propriedades eletrofisiológicas consistente com seu papel na formação de representações alocêntricas do espaço (DERDIKMAN e MOSER, 2010). Estudos demonstram que essas propriedades eletrofisiológicas se desenvolvem em ratos muito jovens, desde quando eles exploram ambientes fora do ninho pela primeira vez (LANGSTON ET

AL., 2010). O Labirinto Aquático de Morris é um teste amplamente utilizado para avaliar a memória espacial em ratos (MORRIS, GARRUD e RAWLINS, 1982). Na versão que usa dicas, os ratos aprendem a nadar a menor distância possível entre a borda do tanque contendo água até a plataforma submersa ligeiramente abaixo da superfície. Nesta versão, eles aprendem a utilizar as figuras fixadas nas paredes, bem como outras dicas visuais externas ao aparato para encontrar a plataforma. A principal estrutura cerebral relacionada com este teste é o hipocampo. No entanto, não podemos esquecer que existe também um aprendizado de procedimentos, ou seja, nadar para escapar do labirinto, o qual é rapidamente adquirido como um hábito (IZQUIERDO ET AL., 2006). Dessa forma, neste teste existe tanto um componente declarativo quanto a formação de hábito.

Existe uma versão não-espacial do Labirinto Aquático de Morris, no qual não existem dicas espaciais externas. Neste caso, o animal pode ver a plataforma, a qual fica ligeiramente acima do nível da água, e aprende o caminho até ela pela exploração do tanque. Esta versão é puramente baseada na memória de procedimentos e depende do estriado (TEATHER ET AL., 2005), mas estudos sugerem que ambas versões podem ser moduladas pela amígdala basolateral (PACKARD, CAHILL e MCGAUGH, 1994).

Por outro lado, a flexibilidade comportamental, a capacidade de ajustar as respostas de acordo com as alterações nas estratégias, regras ou estímulos, é mediada pelo córtex pré-frontal. Esta região cerebral tem sido identificada como uma estrutura-chave para o aprendizado reverso em várias espécies, incluindo humanos, macacos e roedores (CLARKE, ROBBINS e ROBERTS, 2008; HORNAK ET AL., 2004; MCALONAN e BROWN, 2003). No entanto, tem sido sugerido que a flexibilidade comportamental não está baseada exclusivamente no córtex pré-frontal:

outra estrutura que interagiria com o córtex para mediar a flexibilidade cognitiva seria o estriado (KOLB, 1977).

Outro tipo de memória é a memória olfatória, que podemos classificar como memória de reconhecimento, e que tem um importante papel na organização do comportamento de mamíferos, sendo relevante no reconhecimento de parentes, do parceiro e dos recém-nascidos. Por exemplo, os recém-nascidos rapidamente aprendem a identificar e preferir o odor materno naturalmente dentro do ninho (LEON, 1992), uma vez que a identificação da mãe é essencial para sua sobrevivência e desenvolvimento. Além disso, estudos sugerem que, como não há especificidade funcional do sistema olfatório principal e acessório no desenvolvimento do comportamento materno entre as espécies, apenas o sistema olfatório principal estaria envolvido quando a discriminação olfatória individual do filhote é necessária (LÉVY, KELLER e POINDRON, 2004). Dessa forma, a memória olfatória esta intimamente relacionada com o bulbo olfatório. É importante lembrar que a memória olfatória também exerce um papel no reconhecimento de quais alimentos serão consumidos e quais territórios são dominados por predadores (KEVERNE e BRENNAN, 1996).

1.4.2 Memória e manipulação neonatal

Diferentes efeitos da manipulação neonatal têm sido observados sobre a memória. Um estudo clássico dos efeitos da manipulação neonatal sobre a memória em ratos machos foi conduzido por Meaney e colaboradores (1988). Neste estudo os animais foram avaliados na tarefa do Labirinto Aquático de Morris em diferente idades (6, 12 e 24 meses). Foi observado o aparecimento de déficit na memória

espacial de ratos controles com a idade, no entanto não houve surgimento de déficit nos animais manipulados no período neonatal durante o envelhecimento, sugerindo que a manipulação neonatal é um fator protetor para o hipocampo, por reduzir o declínio cognitivo. Além disso, neste estudo também foi demonstrado que a manipulação neonatal promove uma redução na perda neuronal hipocampal com a idade. Kosten e colaboradores (2007) mostraram que esta intervenção precoce prejudica o aprendizado na esquiva inibitória, mas não observaram efeito no aprendizado no labirinto circular, ao passo que houve melhora na memória relacionada ao reconhecimento de objetos em ratos machos e fêmeas. Esses resultados sugerem um efeito facilitatório da manipulação neonatal para memórias relacionadas ao reconhecimento de objetos (embora outros tipos de memória de reconhecimento não tenham sido testados), enquanto há um prejuízo em memórias aversivas (a esquiva inibitória é uma tarefa com componentes declarativos e não-declarativos) (ANDERSEN e TUFIK, 2010).

Com relação à memória e ao aprendizado olfatório, foi demonstrado que a manipulação neonatal reduz o comportamento de aproximação em direção a um odor familiar (odor materno) em fêmeas, mas não em machos, no período inicial da vida (RAINEKI ET AL., 2009). Por outro lado, diferenças sexo-específicas com relação à manipulação neonatal também foram observadas na tarefa de reconhecimento social em adultos, onde apenas os ratos machos manipulados no período neonatal apresentaram uma redução no comportamento social (TODESCHIN ET AL., 2009).

É importante salientar que o envolvimento da memória nos efeitos da manipulação neonatal pode estar presente nos estudos conduzidos por Silveira e colaboradores (2004), onde foi demonstrado que os animais manipulados no período neonatal apresentam, quando adultos, um consumo aumentado de alimento palatável.

Isso porque no primeiro dia da tarefa não há diferença entre animais manipulados e controles, sendo que a diferença só aparece em sessões posteriores. Assim, embora ambos os grupos apresentem um aumento do consumo com o tempo (mostrando aprendizado), o grupo manipulado apresenta um aumento maior e uma possibilidade seria diferenças na memória do valor positivo da recompensa ou uma habituação mais intensa à tarefa.

1.5 Estresse oxidativo

O sistema nervoso central é especialmente vulnerável aos danos causados por radicais livres, devido ao alto consumo de oxigênio pelo cérebro, o conteúdo abundante de lipídios e a insuficiência relativa de enzimas antioxidantes em comparação com outros tecidos (HALLIWELL e GUTTERIDGE, 1985). As espécies reativas do oxigênio (ERO) são produzidas em baixos níveis nas células dos mamíferos por vários processos metabólicos, como a cadeia respiratória na mitocôndria, a atividade da NADPH oxidase e o metabolismo oxidativo do ácido araquidônico. ERO são espécies muito oxidantes, capazes de afetar a estrutura e a função de distintos componentes celulares. O organismo por sua vez apresenta sistemas de defesas para combater essas espécies, os quais podem ser antioxidantes enzimáticos e não-enzimáticos (HALLIWELL e CROSS, 1994). O sistema antioxidante enzimático é composto por enzimas como a superóxido dismutase (SOD), que converte radical superóxido em peróxido de hidrogênio, a catalase (CAT), que promove a degradação do peróxido de hidrogênio, e a glutationa peroxidase (GPx), que promove a degradação de peróxidos, especialmente os derivados da oxidação dos fosfolipídios de membrana (KEHRER, 2000). O funcionamento

inadequado dessas enzimas, uma produção aumentada de espécies reativas que não é acompanhada do aumento proporcional na atividade dessas enzimas, e/ou uma diminuição de agentes redutores podem gerar um excesso de espécies reativas, as quais podem danificar os lipídios de membranas, as proteínas e o ADN celular (KOVÁCS ET AL., 1996; COCHRANE, 1991). Como resultado, podemos ter um declínio das funções fisiológicas e possível morte celular. Um exemplo de funcionamento inadequado ocorre quando existe um aumento na atividade da SOD (para combater um produção exarcebada de ânion superóxido e consequentemente aumentando a produção de peróxido de hidrogênio), o qual não é acompanhado por uma aumento na atividade da GPx e/ou da CAT. Neste caso, teremos um acúmulo de peróxido de hidrogênio, que pode reagir com o ânion superóxido, através da reação de Haber Weiss, formando o altamente reativo radical hidroxila (HABER e WEISS, 1934). A produção do radical hidroxila pode ocorrer também pela reação do peróxido de hidrogênio com o ferro, na reação de Fenton (HALLIWELL, 2001). Além do sistema antioxidante enzimático, a célula também conta com antioxidantes não-enzimáticos, como colocado acima, para a proteção contra o ataque da radicais livres aos seus componentes. Esses antioxidantes são compostos com baixa massa molecular, como a glutationa reduzida, a qual neutraliza vários radicais livres diretamente ou por ser substrato para a enzima GPx. Outro antioxidante não-enzimático é o α-tocoferol, o qual é o mais importante capturador de radicais livres nas membranas (HALLIWELL e CROSS, 1994).

1.5.1 Estresse oxidativo e manipulação neonatal

Até o inicio do desenvolvimento desta tese, nenhum trabalho que seja do meu conhecimento havia abordado os efeitos da manipulação neonatal sobre parâmetros do estresse oxidativo. Dessa forma, esta tese apresenta os primeiros trabalhos neste sentido. Além disso, durante o desenvolvimento desta, outros dois trabalhos foram publicados pelo meu grupo de pesquisa onde eu sou co-autora abordando este tema (ARCEGO ET AL., 2011; MARCOLIN ET AL., 2012). Estes estudos mostraram que a manipulação neonatal afetou parâmetros do estresse oxidativo no hipocampo e no córtex pré-frontal de ratos machos aos 29 dias de idade causando um desequilíbrio oxidativo (aumentou a atividade da SOD e a relação SOD/GPx nestas duas estruturas e diminuiu o conteúdo total de tióis apenas no córtex pré-frontal) (MARCOLIN ET AL., 2012) enquanto que não alterou parâmetros do estresse oxidativo (atividade das enzimas SOD, GPx e CAT, produção de espécies reativas e conteúdo total de tióis) no cérebro de fêmeas adultas (ARCEGO ET AL., 2011). Ainda, um terceiro trabalho foi publicado por Rodriguez e colaboradores (2011) mostrando que as atividades da SOD e da CAT diminuem em ratos manipulados no início da vida quando avaliadas aos 10 dias de idade em relação a atividade destas enzimas avaliadas em períodos precoces também em ratos manipulados.

1.5.2 Estresse oxidativo, óxido nítrico e memória

Como relatado acima, um desequilíbrio oxidativo pode afetar as proteínas celulares. Estudos demonstraram que as enzimas Na^+/K^+-ATPase e acetilcolinesterase são suscetíveis ao ataque por radicais livres (PETRUSHANKO ET AL., 2006; TSAKIRIS ET AL., 2000). Além disso, ambas tem sido relacionadas com cognição (PETRUSHANKO ET AL., 2006; STANCAMPIANO ET AL., 1999). Por outro lado,

radicais livres não são exclusivamente nocivos. A prova disso é o radical livre óxido nítrico, o qual atua como um neurotransmissor atípico em diferentes tecidos, inclusive no tecido nervoso (PAUL e EKAMBARAM, 2011). Estudos sugerem que o óxido nítrico esteja envolvido no processo de aprendizado e memória, por potencializar ou facilitar principalmente o processo de aquisição (QUIAN ET AL., 1997). Foi demonstrado em ratos que inibidores da óxido nítrico sintase (enzima responsável pela formação de óxido nítrico) levam a um leve prejuízo da memória, enquanto que doadores de óxido nítrico facilitam a memória no teste do Labirinto Aquático de Morris (DEMIRGOREN e POGUN, 1995).

1.6 Na^+/K^+-ATPase, acetilcolinesterase e memória

A Na^+/K^+-ATPase é um enzima muito importante para o funcionamento do tecido nervoso. É responsável pelo transporte ativo de íons sódio e potássio, mantendo e restabelecendo após cada despolarização o gradiente eletroquímico necessário para a excitabilidade neuronal e a regulação do volume celular neuronal. Está presente em altas concentrações nas membranas celulares cerebrais, consumindo cerca de 40-50% do ATP gerado neste tecido (GLOOR, 1997). Além disso, a Na^+/K^+-ATPase também funciona como um transdutor de sinal para liberar mensagens da membrana plasmática para as organelas intracelulares (XIE e ASKARI, 2002).

A modulação da atividade da $Na^{+/}K^+$-ATPase afeta diretamente a sinalização de neurotransmissores, a atividade neural bem como o comportamento animal. A inibição desta enzima diminui a captação de noradrenalina, dopamina e serotonina, além de aumentar a liberação de acetilcolina (VIZI e OBERFRANK, 1992; VATTA

ET AL., 2004). Sabe-se que a administração de inibidores da Na^+/K^+-ATPase prejudica o aprendizado espacial, bem como outras formas de aprendizado e a memória (SATO ET AL., 2004; ZHAN ET AL., 2004). Por exemplo, foi demonstrado que a administração de ouabaína (um inibidor dessa enzima) prejudica a memória de camundongos no teste da esquiva inibitória (SATO ET AL., 2004).

A acetilcolinesterase é a enzima envolvida na hidrólise do neurotransmissor acetilcolina. Dessa forma, um aumento na atividade desta enzima pode causar uma diminuição nos níveis de acetilcolina na fenda sináptica e, consequentemente, uma diminuição na atividade colinérgica (MONTEIRO ET AL., 2005). As funções neuromodulatórias não-enzimáticas da acetilcolinesterase afetam o crescimento de neuritos e a sinaptogênese (ZIMMERMAN e SOREQ, 2006). Estudos demonstram que a ativação colinérgica no hipocampo é necessária para a formação da memória espacial (HERRERA-MORALES ET AL., 2007), e que os níveis extracelulares de acetilcolina aumentam nesta estrutura durante uma tarefa de memória espacial (STANCAMPIANO ET AL., 1999). Além disso, a administração de um inibidor da acetilcolinesterase em ratos melhora o desempenho destes no aprendizado reverso (CHEN ET AL., 2009).

1.7 Na^+/K^+-ATPase, acetilcolinesterase e manipulação neonatal

Estudos prévios mostram que ratos machos manipulados no período neonatal apresentaram quando adultos uma diminuição na atividade da enzima Na^+/K^+-ATPase no hipocampo e um aumento na atividade desta na amígdala (SILVEIRA ET AL., 2011). Por outro lado, fêmeas manipuladas no período neonatal não tiveram alteração

na atividade da Na^+/K^+-ATPase nestas estruturas com relação as controles na idade adulta (DA S BENETTI ET AL., 2010).

Com relação ao sistema colinérgico e à manipulação neonatal, foi demonstrado que ratos machos manipulados no período neonatal e que foram submetidos a um desafio neurotóxico com 11 meses de idade [administração unilateral de N-metil-D-aspartato (NMDA) no núcleo basal magnocelular] apresentaram no hemisfério lesionado uma perda aumentada (em relação aos controles não-manipulados) de fibras imunorreativas para colina-acetiltransferase e de fibras positivas para acetilcolinesterase no córtex somato-sensorial. Por outro lado, não houve diferença nestes aspectos quando foi avaliado o lado do cérebro não lesionado (HORVATH ET AL., 2004). Outro estudo avaliou a influência de eventos precoces, como o cuidado materno, na função colinérgica e mostrou que a prole adulta que foi exposta a um cuidado materno aumentado no início da vida apresentou uma atividade aumentada da colina-acetiltransferase e da marcação de acetilcolinesterase hipocampal, além de um aumento na acetilcolina nesta estrutura (LIU ET AL., 2000). Como é sabido (ver acima) que a manipulação induz maior grau de cuidados maternos, esses resultados sugerem uma maior atividade colinérgica no hipocampo desses animais; por outro lado também mostram maior sensibilidade à neurodegeneração colinérgica no córtex após insultos.

Assim, tendo em vista o exposto acima, nossa hipótese para este trabalho de tese inicialmente era que o comportamento de maior consumo de alimento palatável apresentado por animais manipulados no período neonatal quando adultos poderia estar relacionado a efeitos sobre a memória de tarefas apetitivas e que esse efeito poderia ser sexo-específico. Além disso, como algumas alterações comportamentais

só ocorrem após a adolescência nesses animais, hipotetizamos que tais alterações poderiam ser dependentes da liberação de hormônios gonadais durante a adolescência, embora mudanças neuroquímicas possivelmente já se apresentem como marcas resultantes da manipulação ainda antes da adolescência.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar os efeitos da manipulação neonatal sobre diferentes tipos de memória, relacionadas ou não ao alimento palatável, bem como parâmetros neuroquímicos que podem estar associados a alterações cognitivas, como a atividade das enzimas Na^+/K^+-ATPase e acetilcolinesterase e os parâmetros relacionados ao estresse oxidativo, considerando possíveis diferenças sexo-específicas em ratos jovens e adultos.

2.2 Objetivos específicos

- Avaliar os efeitos da manipulação neonatal sobre a memória espacial, a produção de óxido nítrico, a atividade de enzimas antioxidantes e sobre o índice de quebras ao ADN no hipocampo (estrutura envolvida com a memória espacial) de ratos machos e fêmeas adultos.

- Avaliar as diferenças sexo-específicas no aprendizado reverso em ratos adultos manipulados no período neonatal bem como parâmetros do estresse oxidativo no estriado e no córtex pré-frontal (estruturas relacionadas com o aprendizado reverso) destes animais.

- Verificar os efeitos e as possíveis interações da manipulação neonatal e da remoção dos ovários antes da puberdade sobre a memória olfatória associada ao alimento palatável, bem como sobre parâmetros do estresse oxidativo e

sobre a atividade da enzima Na^+/K^+-ATPase no hipocampo e no bulbo olfatório de fêmeas adultas.

- Investigar os efeitos da manipulação neonatal em parâmetros do estresse oxidativo bem como na atividade das enzimas Na^+/K^+-ATPase e acetilcolinesterase no hipocampo e no bulbo olfatório de ratos machos e fêmeas jovens.

PARTE II

1. MATERIAIS E MÉTODOS E RESULTADOS

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

- Capítulo 1: Artigo publicado na revista *Neurochemical Research*

- Capítulo 2: Artigo publicado na revista *International Journal of Developmental Neuroscience*

- Capítulo 3: Artigo aceito para publicação na revista *Neurochemical Research*

- Capítulo 4: Artigo submetido para publicação na revista *Brain & Development*

1.1 Capítulo 1

Neonatal handling impairs spatial memory and leads to altered nitric oxide production and DNA breaks in a sex specific manner

Artigo publicado na revista *Neurochemical Research*

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ORIGINAL PAPER

Neonatal Handling Impairs Spatial Memory and Leads to Altered Nitric Oxide Production and DNA Breaks in A Sex Specific Manner

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Abstract Early life events lead to behavioral and neurochemical changes in adulthood. The aim of this study is to verify the effects of neonatal handling on spatial memory, nitric oxide (NO) production, antioxidant enzymatic activities and DNA breaks in the hippocampus of male and female adult rats. Litters of rats were non-handled or handled (10 min/day, days 1–10 after birth). In adulthood they were subjected to a Morris water maze or used for biochemical evaluations. Female handled rats showed impairment in spatial learning. They also showed decreased NO production, while no effects were observed in these parameters in male rats. No effects were observed on the number of hippocampal NADPH diaphorase positive cells. In the Comet Assay, male handled rats showed increased DNA breaks index when compared to non-handled ones. We conclude that neonatal handling impairs learning performance in a sex-specific manner, what may be related to NO decreased levels.

Keywords Neonatal handling · Spatial learning · Nitric oxide · Oxidative stress · DNA break · Sex-specific effects

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Introduction

Several studies have documented the impact of early life events on neurochemical and behavioral status in adulthood [1–3]. Some experimental approaches have been developed to study this interaction; one of them is neonatal handling, a brief, repeated, and apparently innocuous separation of rat pups from their mother. As adults, handled rats exhibit attenuated fearfulness (decreased freezing, increased exploration) in novel environments, increased ingestion of palatable food [4] and a less pronounced increase in the secretion of adrenal glucocorticoids in response to a variety of stressors [5]. In addition, they show a permanent increase in glucocorticoid receptors in the hippocampus, a critical region in the negative feedback inhibition of adrenocortical activity. This increased receptor concentration leads to greater hippocampal sensitivity to glucocorticoids and enhanced negative-feedback efficacy in the handled rats [6].

Glucocorticoid receptors influence spatial learning (a process in which the hippocampus is critical) and are involved in the consolidation of learned information [7]. Glucocorticoid levels also play a role in the ability for learning and memory processes [8]. Some studies have investigated the effects of early environmental experiences on learning and memory processes in adult rats and different results have been found depending on the task evaluated [6, 9–11]. Various outcomes concerning neonatal

handling and memory are possibly dependent also on age and sex [9, 11].

The hippocampus, as well as the whole brain, is especially vulnerable to free radical-induced damage because of its high oxygen consumption, abundant lipid content and relative paucity of antioxidant enzymes [12, 13], and lower hippocampal oxidative damage has been related to better spatial learning ability [14, 15]. Oxidative stress happens when there is an imbalance between antioxidant defenses and oxidative species. In this case, the antioxidant defenses, such as the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), are not able to neutralize the reactive species efficiently [12]. As consequences, cellular proteins, lipids and DNA may be damaged [16].

The nitric oxide (NO) is an example of reactive species. It is interesting to notice that this molecule can have paradoxical effects [17, 18]. It can react with superoxide anion leading to the production of peroxynitrite, a potent oxidant specie, which in excess can contribute to cell death [17]. On the other hand, NO modulates blood flow, neural activity [17] and is thought to be involved in synaptic plasticity, contributing to learning and memory in several brain areas including the hippocampus [19]. It has been suggested that neonatal handling is a protective factor to the hippocampus as it reduces aging-related hippocampal neuronal loss and cognitive decline [6]. However, as mentioned above, these animals show reduced hypothalamus–pituitary–adrenal (HPA) axis response, and this response is known to be important for memory storage in several tasks (e.g., [8]). Besides, to our knowledge, the effects of neonatal handling on oxidative stress in male and female hippocampi in relation to cognitive ability have not been reported. Therefore, the aim of this study is to verify the effects of neonatal handling on NO production, on the antioxidant enzymatic activities and on DNA breaks in the hippocampus of male and female adult rats. In addition, this study wants to contribute to elucidate the effects of neonatal handling on spatial memory, verifying if possible effects on memory can be mediated by changes in NO.

Experimental Procedure

Subjects

All animal proceedings were approved by the Institutional Ethical Committee and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS), and of the Federation of Brazilian Societies for Experimental Biology. All efforts were done to minimize animal suffering as well as to reduce the number of animals.

Twenty-eight pregnant Wistar rats bred at our own animal facility were randomly selected. They were housed alone in home cages made of Plexiglas (65 × 25 × 15 cm) with the floor covered with sawdust and were maintained in a controlled environment: lights on between 07:00 and 19:00 h, temperature of 22 ± 2°C, cage cleaning twice a week, food and water provided ad libitum. The day of birth was considered as day 0. All litters were culled within 24 h of birth to eight pups and were maintained undisturbed except for handling procedures, which were carried out between 10:00 and 15:00 h. Several litters were submitted to the handling procedures in the same day. The researcher changed gloves between the handling procedures of each litter to avoid any kind of odor to be spread from nest to nest. Litters were weaned and separated by sex on postnatal day 21. When adults, no more than two animals per litter were used in the behavioral experiment and only one animal per litter was used for biochemical measurements. Experiments using males and females were performed separately, at different times. Rats had free access to food (standard lab rat chow) and water, except during the period when the behavioral task was applied (task was performed between 11:00 and 16:00 h).

Neonatal Handling [20]

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

Handled group: The dam was gently pulled to one side of the cage and the pups were removed from their home cage and were placed into a clean cage lined with clean paper towel. This cage was placed into an incubator set to maintain an ambient temperature at 30–32°C. After 10 min, pups were returned to their dams which were in the same room. This procedure was performed from day 1–10 following birth, and then pups were left undisturbed until the 21st day of life (weaning). It was also stated on the cage that these animals should not be touched, not even for cage cleaning. The same procedure of non-handled group was done to change dirty sawdust.

Spatial Memory Evaluation

The Morris water maze [21] is a behavioral test in which animals are required to find a submerged (1 cm) platform located at the center of a quadrant of the tank (a black circular pool with 200 cm in diameter and 100 cm high), using only distal, spatial cues available within the testing room. Rats are proficient, but reluctant swimmers and readily use the platform to escape the water. When animals

were 3 months of age, they were submitted to daily sessions of four trials per day during 7 days (in the case of male rats) or 10 days (female rats) to find the submerged platform. We observed that all animals of each group were able to swim in a normal manner during all trials. On each trial, the rat was placed in the water, facing the edge of the tank, at one of the four standard start locations (N, S, W and E). The order of the start locations varied in each sequence so that, for each block of four trials, any given sequence was not repeated on consecutive days. The rat was then allowed 60 s to search for the platform. Latency to find the platform (escape latency) was measured in each trial. Once the rat located the platform, it was allowed to remain on it for 15 s. If the rat did not find the platform within this time, it was guided to it and allowed to remain on it for 15 s. After each trial, the rats were removed, dried with a towel and put back in their home cages. The interval between trials was around 15 min [22].

Preparation of the Samples for Biochemical Measurements

When they were around 4 months old, animals were killed by decapitation and the hippocampus was quickly dissected out. For the comet assay, this structure was immediately used as described below. For determination of antioxidant enzymes activities and for NO evaluation the hippocampus was stored at −70°C until analysis when it was homogenized in 10 vol (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA. The homogenate was centrifuged (at 3,000 rpm for the enzymes activities and 4,000 rpm for NO) for 10 min at 4°C and the supernatant was used.

Nitric Oxide Production

NO production was determined by measuring its metabolites nitrate (NO^{3-}) and nitrite (NO^{2-}) according to Miranda et al. [23]. The principle of this assay is the reduction of nitrate by vanadium (III) combined with detection by the acidic Griess reaction. The resulting pink-stained pigment was determined in a spectrophotometer at 540 nm. A calibration curve was performed using sodium nitrite (0.2–4.0 nmol). NO production values were calculated as nmol nitrite/mg protein.

Superoxide Dismutase Activity

SOD activity was determined using a RANSOD kit (Randox Labs., USA) which is based on the procedure described by Delmas-Beauvieux et al. [24]. This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a formazan dye that is assayed spectrophotometrically at 492 nm at 37°C. The inhibition in production of the chromogen is proportional to the activity of SOD present in the sample.

Glutathione Peroxidase Activity

GPx activity was determined according to Wendel [25], with modifications. The reaction was carried out at 37°C in 200 µl of solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione and 0.4 U glutathione reductase. The activity of GPx was measured taking tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as nmol NADPH oxidized per minute per mg protein.

Catalase Activity

CAT is an enzyme that degrades hydrogen peroxide (H_2O_2), and its activity assessment is based upon establishing the rate of H_2O_2 degradation spectrophotometrically at 240 nm at 25°C [26]. CAT activity was calculated in terms of micromol of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$.

Protein Assay

The total protein concentrations were determined using the method described by Lowry et al. [27] with bovine serum albumin as the standard.

Single Cell Gel Electrophoresis: Comet assay

A standard protocol for comet assay preparation and analysis was adopted [28]. The hippocampus was gently homogenized in phosphate-buffered saline solution (PBS) pH 7.4. The slides were prepared by mixing 20 µl of hippocampus homogenate (in cold PBS), with 80 µl of low melting point agarose (0.75%). The mixture (cells-agarose) was added to a microscope slide coated with a layer of 500 µl of normal melting agarose (1%). After solidification, the cover slip was gently removed and the slides were placed in a lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.5, with freshly added 1% Triton X-100 and 10% DMSO) for 1 day. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM

NaOH and 1 mM EDTA, pH > 13) for 5 min. The DNA was electrophoresed during 20 min at 25 V (0.90 V/cm) and 300 mA. Afterwards, the slides were neutralized with Tris buffer (0.4 M; pH 7.5). Finally, the DNA was stained with SYBR Safe. The stained nuclei were blindly analyzed by fluorescence microscopy with visual inspection (200×). Cells were scored from zero (no breaks observed) to 4 (maximal break index), according to the tail intensity (size and shape), resulting in a single DNA break score for each cell, and, consequently, for each group. The DNA break index was calculated by multiplying the number of cells by its respective index score and than summing it up. Therefore, group index could range from zero (all cells with no tail, 100 cells × 0) to 400 (all cells with maximally long tails, 100 cells × 4) [29] (Fig. 3a).

NADPH Diaphorase (NADPH-d) Histochemistry

Other animals (both handled and non-handled) were used for histochemistry. NADPH-d staining was used to evaluate NOS expression. This technique provides a specific histochemical marker for neurons producing NO [30]. The animals were anesthetized with an overdose of chloral hydrate and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4. Brains were removed, postfixed overnight in 4% paraformaldehyde, and cryoprotected with sucrose.

The brains were cut into 40 μm serial coronal sections with a cryostat and stored at −20°C in a solution containing glycerol and phosphate-buffered saline until analysis. By that day, free-floating sections were incubated in 0.1 M phosphate buffer containing 0.1% Triton X-100, 0.05% β-NADPH and 0.02% nitro blue tetrazolium at 37°C for 90 min [31]. All sections were then mounted onto gelatin-coated glass slides, air-dried, quickly colored with hematoxylin, cleared with xylene and cover slipped.

The number of NADPH-d-positive cells was counted in three hippocampal sections (between −3.14 and −3.3 mm from bregma) [32]. Using a 40× objective. Cells were counted in all extention of Amon's horn and dentate gyrus. Hippocampal area of each section was measured using a computerized image analysis system Image J. The results are expressed as the mean number of NADPH-d positive cells/mm^2. To avoid bias during counting the slides were coded.

Statistical Analysis

Data were expressed as means ± standard error of the mean, and were analyzed using Repeated Measures ANOVA in the case of the spatial memory test and Student's t test in the others cases. Significance level was accepted as different when the P value was less than 0.05.

Results

There were no statistical differences between non-handled and handled male rats in spatial memory evaluated by Morris water maze test ($P > 0.05$). Repeated Measures ANOVA showed that male animals learned the task and showed an adequate memory since their time to find the platform decreased along the days; mean time to find the platform on the seventh day of training was 55% of the mean time spent on the first day for the non-handled group and 53% for the handled group [$F(1,17) = 19.24$, $P < 0.001$] (Fig. 1a). Swimming speed was also evaluated: handled males showed higher speed (non-handled: 1.9 cm/s; handled: 2.5 cm/s; t (16) = 2.60, $P < 0.05$). On the other hand, when evaluating female rats, Repeated Measures ANOVA demonstrated that neonatal handled rats spent more time looking for the platform than non-handled ones; for example, in the seventh day of training, mean

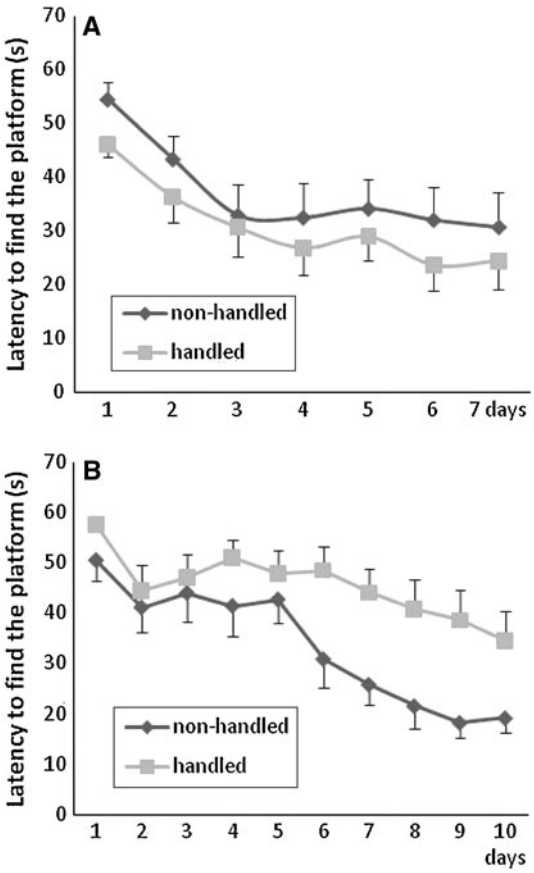

Fig. 1 Latencies to find the platform in the water maze. Data are expressed as mean ± SEM. **a** Males, $N = 9$–10/group. Repeated Measure ANOVA showed no effect between groups and an effect of the task sessions in both groups ($P < 0.001$). **b** Females, $N = 7$/group. Repeated Measure ANOVA showed an increased latency to find the platform in the handled group compared to non-handled animals ($P < 0.05$), and a general effect of the task sessions ($P < 0.001$)

Table 1 Nitric oxide production and NADPH-diaphorase (NADPH-d) positive cells in hippocampus

	Males		Females	
	Non-handled	Handled	Non-handled	Handled
Nitrites (nmol/mg protein)	22.5 ± 4.4	20.3 ± 3.6	26.7 ± 1.1	17.4 ± 1.5*
NADPH-d positive cells (mean number/mm^2)	5.24 ± 0.42	4.50 ± 0.87	6.04 ± 0.32	5.20 ± 0.73

Data are expressed as mean ± SEM. Nitrites: $N = 5$–6/group. NADPH diaphorase staining: $N = 4$–5/group

* Significantly different compared to non-handled group (Student's t test, $P < 0.001$)

time to find the platform was 51% of the mean time spent on the first day for the non-handled group of females, and 77% for the handled group [$F(1,12) = 8.68$, $P < 0.05$]. There was also a reduction in the time to find the platform along the days in both female groups [$F(1,12) = 34.24$, $P < 0.001$] (Fig. 1b), and no effect was observed on swimming speed (non-handled: 2.4 cm/s; handled: 2.4 cm/s; t (12) = 0.162, $P > 0.05$).

When evaluating the biochemical parameters, female handled rats showed a decrease of 35% on NO production when compared to non-handled females [t (8) = 5.07, $P < 0.001$], while no effect was observed on male rats ($P > 0.05$; Table 1). We investigated the effects of neonatal handling on NO-producing cells in the hippocampus using nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry. In the hippocampus, NADPHd-positive cells were counted in the pyramidal layer of Ammon's horn and the dentate gyrus totally. The staining pattern of NADPH-d positive cells was consistent with previous reports from the literature [33, 34]. Results were expressed as mean number of cells/mm^2 and are shown in Table 1. No significant differences were observed (Student's t test, $P > 0.05$), neither in males nor in females. Similarly, no differences where found when CA area and dentate gyrus were analyzed separately. Representative photomicrographs are shown in Fig. 2.

Additionally, no differences between groups (non-handled and handled) were observed on the antioxidant enzymes activities (SOD, GPx and CAT) neither in males nor in females ($P > 0.05$; Table 2). In the Comet Assay, male handled rats showed a 160% increase in DNA breaks index when compared to non-handled ones [t (7) = 5.01, $P < 0.01$]. No effect was observed in females in this index ($P > 0.05$; Fig. 3b, c).

Discussion

The major findings in this work are that neonatal handling effects on spatial memory, NO production and on DNA breaks in adult rats are sex specific. Handling procedures reduced female performance in the water maze task, as well as NO production in hippocampus. In males, only DNA breaks were affected by this procedure.

The effects of neonatal handling on spatial memory in rats differ between studies [6, 9, 35]. It has been observed that postnatal handling has no effect on the performance of adult male animals in a spatial memory task [36]. That result agrees with ours, since we did not observe any differences either. In addition, we verified that male handled animals had a higher swimming speed in the water maze when compared to non-handled males, which may explain the lower latencies (although not significant) for the former to find the platform. Different results from ours seem to be related to the animals age and condition (stressed or not), as it was shown that the handling procedure improved spatial learning in male rats following stress exposure [37], and also attenuated memory impairments in both males and females animals in later life [38].

Female handled rats had a lower performance in water maze and a decreased NO production when compared to non-handled females. Additionally, no differences were observed between male handled and non-handled animals on NO production. NO is synthesized from L-arginine, in the presence of NADPH and O_2, by a series of isoenzymes of the family of NO synthases (NOS) [39]. It seems to be involved in learning and memory formation by potentiating or facilitating mainly the acquisition process [40]. In rats, it has been verified that NOS inhibition leads to a slight impairment of memory, while a NO donor facilitates memory in the Morris Water Maze; furthermore, a correlation was observed between performance during the probe trial and hippocampal cGMP levels, suggesting the involvement of NO in spatial memory [41]. Male rats prefer to use spatial cues and NO has a supportive role in this behavioral strategy, which requires memory relating to spatial cues [42]. These considerations agree with our findings, since male rats had a better performance in the water maze than females from both groups, and handled females, which had a decrease in NO production, had a worse performance in the water maze. It was suggested that NOS inhibition is involved in the impairment of earlier phases of acquisition of learning in the water maze and that the effect is more pronounced in females than males [42].

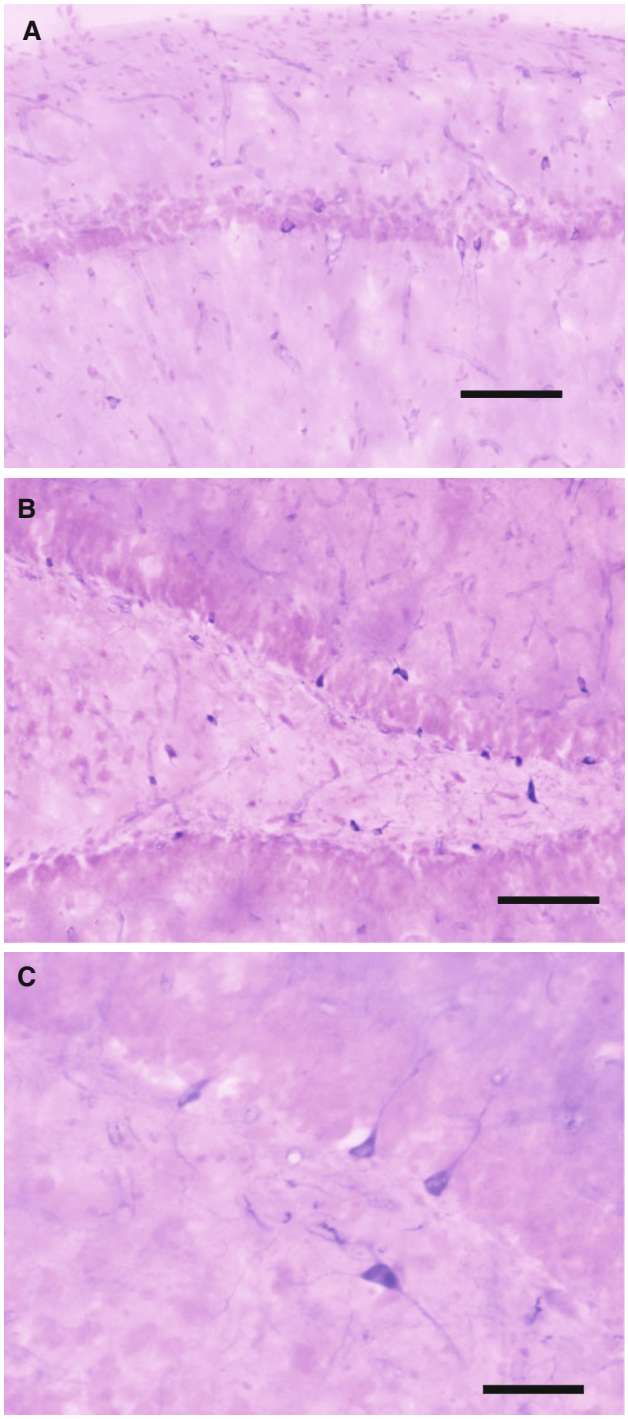

Fig. 2 Representative photomicrograph of NADPH-d positive cells in sections of CA field (**a**) and dentate gyrus (**b**) of hippocampus; **c** cells from dentate gyrus. No differences were observed between groups. *Scale bar* = 100 μm (**a** and **b**); 50 μm (**c**)

Our results go in the same direction (at least for handled females).

On the other hand, although hippocampal NO production was markedly decreased in neonatally handled female rats, the number of NOS-positive cells in hippocampus showed no changes. Noteworthy, the relation between NOS activity and nNOS-positive neurons is not necessarily positive: For example, Stepanichev et al. [43] reported changes in NOS activity in hippocampus without changes in NOS-positive neurons in this structure; other study [44] demonstrates decreased number of NO-producing cells in the rat hippocampus following 2,4-diamino-6-hydroxypyrimidine administration, and no changes in NO production. It is possible that NO is being less produced in these NOS-positive cells from handled females due to some mechanism such as a reduced intracellular Ca^{2+} concentration. A decreased production of NO may be due to decreased activation of nNOS. This enzyme activity is calcium-dependent, and an increase in its activity may be mediated, for example, by activation of N-methyl-D-aspartate (NMDA) receptors and subsequent Ca^{2+} entry into neurons; the activation of this type of receptors in the hippocampus is known to be essential to the process of memory storage (e.g. [45]). Further studies are necessary to clarify the regulation of NO production in the hippocampal NOS-positive cells of neonatally handled female rats.

Neonatal handling has been reported to counteract deleterious effects of prenatal stress, such as the reduced hippocampal cell proliferation [46]. It also appears to attenuate spontaneous aging-related neurodegeneration in rats [6, 38]. Based on these studies, it seems that this early intervention has a protective effect in brain. Therefore, we verified the neonatal handling effect on the antioxidant enzymes activities once, as far as we know, there are no studies evaluating these parameters in rats submitted to this procedure. However, no differences were found between non-handled and handled groups, neither in males nor in females. The lack of differences observed could be due to the age of the animals and it can not be excluded the possibility of differences appearing later in life.

Regarding the comet assay, this test is frequently used to measure DNA damage in individual cells [47]. Under alkaline conditions (pH $>$ 13), this assay can detect single and double-stranded breaks, incomplete repair sites, alkali labile sites, and also possibly both DNA–protein and DNA–DNA cross-links in eukaryotic cells [48]. Single-strand breaks can arise either directly (e.g. from attack of deoxyribose by reactive oxygen species) or indirectly via enzymatic cleavage of the phosphodiester backbone during DNA base excision repair [49]. We observed that DNA breaks index is substantially different in male and female non-handled rats. To our knowledge, this observation has not been reported previously, and it is possible that gonadal hormones are involved in this result: 17β-estradiol can increase the rate of DNA damage [50–52]. One mechanism for this action would be the formation of estradiol metabolites that react with DNA to form depurinating estrogen-DNA adducts [51]. Considering the effect of neonatal

Table 2 Evaluation of the antioxidant enzymes activities in hippocampus of neonatally handled and non-handled rats

Antioxidant enzyme	Males		Females	
	Non-handled	Handled	Non-handled	Handled
SOD	6.00 ± 1.66	6.68 ± 0.95	11.46 ± 1.73	11.61 ± 1.52
GPx	22.25 ± 0.62	21.30 ± 1.21	33.85 ± 0.95	36.49 ± 1.53
CAT	1.10 ± 0.12	1.12 ± 0.22	2.26 ± 0.18	2.19 ± 0.11

Data are expressed as mean ± SEM of SOD (U/mg protein), GPx (nmol NADPH oxidized/min/mg protein), and CAT (μmol H2O2 transformed/min/mg protein) activities. Males, $N = 6$/group. Females, $N = 4$–9/group

Student's t test showed no differences between groups neither in males nor in females

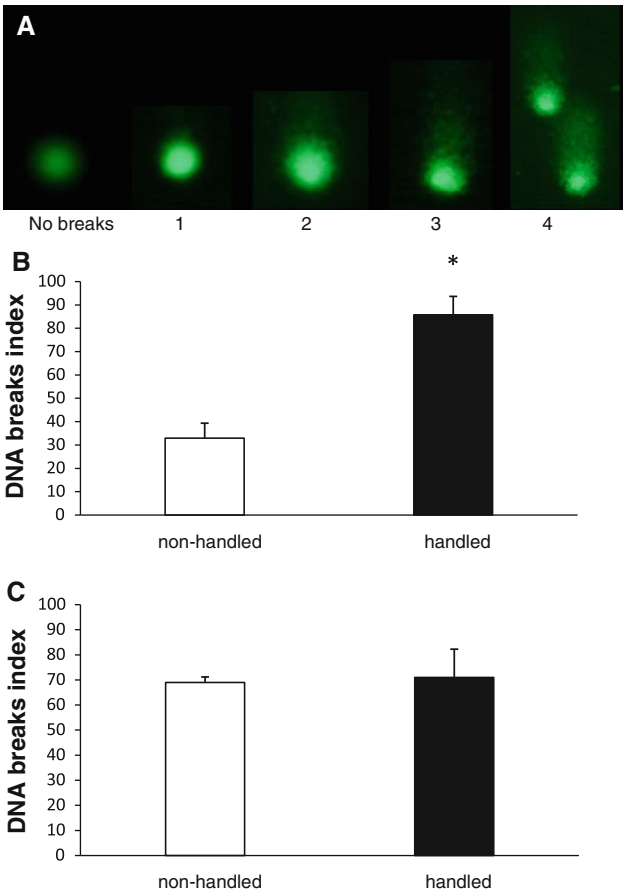

Fig. 3 **a** Some typical examples of Comet assay. Evaluation of DNA breaks using Syber Safe (200×). The cells are assessed visually and received scores from 0 (no breaks observed) to 4 (maximal breaks index), according to the size and shape of the tail. **b**, **c** DNA breaks in hippocampus evaluated by the Comet Assay. **b** Males, **c** Females. Data are expressed as mean ± SEM. $N = 4$–5/group. * Significantly different compared to non-handled group (Student's t test; $P < 0.01$)

manipulation, we observed an increase in DNA breaks in male animals submitted to neonatal handling. Since we did not observe changes in the antioxidant enzymes activities, it is possible that this result may be related to the repair process. However, we cannot rule out the possibility of occurrence of oxidative stress, and other studies evaluating damage indexes and free radicals production would help to clarify this question. In the last few years, it has been proposed that early experiences alter behavior and physiology through sustained alterations in gene expression in specific brain regions, such as differences in the DNA methylation pattern [53]. These effects could increase DNA accessibility and, consequently, its attack by several substances and the need to activate repair systems. However, further studies are necessary to confirm this hypothesis.

In conclusion, this study supports the idea that male rats have a better spatial memory than females and that the neonatal handling impairs learning performance only in females which may be related to NO decreased levels. The sex specific differences are also observed in the evaluation of the DNA breaks, with neonatal handling affecting only males. This study contributes to the understanding of how early life events can influence behavior and neurochemistry in adult life considering sex specific differences.

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1.2 Capítulo 2

Neonatal handling affects learning, reversal learning and antioxidant enzymes activities in a sex-specific manner in rats

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Neonatal handling affects learning, reversal learning and antioxidant enzymes activities in a sex-specific manner in rats

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ABSTRACT

Early life experiences have profound influences on behavior and neurochemical parameters in adult life. The aim of this study is to verify neonatal handling-induced sex specific differences on learning and reversal learning as well as oxidative stress parameters in the prefrontal cortex and striatum of adult rats. Litters of rats were non-handled or handled (10 min/day, days 1–10 after birth). In adulthood, learning and reversal learning were evaluated using a *Y* maze associated with palatable food in male and female rats. Morris water maze reversal learning was verified in males. Oxidative stress parameters were evaluated in both genders. Male neonatal handled animals had a worse performance in the *Y* maze reversal learning compared to non-handled ones and no difference was observed in the water maze reversal learning task. Regarding females, neonatal handled rats had a better performance during the *Y* maze learning phase compared to non-handled ones. In addition, neonatal handled female animals showed a decreased SOD/CAT ratio in the PFC compared to non-handled females. We conclude that neonatal handling effects on learning and memory in adult rats are sex and task specific. The sex specific differences are also observed in the evaluation of antioxidant enzymes activities with neonatal handling affecting only females.

1. Introduction

Several studies have documented the impact of early life events on neuroendocrine and behavioral status in adulthood (Levine, 1957; Meaney et al., 1996; Padoin et al., 2001). A suitable environment during the neonatal period is essential for a healthy development of neonates, since they are more vulnerable during this period. The hypothalamic–pituitary–adrenocortical (HPA) axis is one of the most important neuroendocrine systems activated in response to actual or presumed environmental challenges. In rats, it has been demonstrated that both prenatal and postnatal factors may influence the development of the HPA axis (Liu et al., 1997; Maccari et al., 2003; Plotsky and Meaney, 1992; Weinstock, 1997). Rats who are briefly and repeatedly separated from their mothers at the beginning of their lives, by a procedure called neonatal handling, show when adults a less pronounced increase in the secretion of adrenal glucocorticoids in response to a variety of stressors (Meaney et al., 1991). They also have attenuated fearfulness (decreased freezing, increased exploration) in novel environments and an increased ingestion of palatable food (Silveira et al., 2004). In addition, we have demonstrated in a previous study that adult female neonatal handled rats have impairment in spatial learning (a sex-specific effect) in the water maze task (Noschang et al., 2010). However, many outcomes concerning neonatal handling and learning and memory are possible dependent on age, sex and task evaluated (Kosten et al., 2007; Meaney et al., 1988; Vallee et al., 1999; Weinberg and Levine, 1977).

Different types of learning involve distinct cerebral structures. The hippocampus and related structures support learning in spatial tasks like water maze (Morris et al., 1982). On the other hand, behavioral flexibility, the ability to adjust responses according to changes in strategies, rules or stimulus-reward contingencies, is mediated by the prefrontal cortex (PFC). This cortical region has been identified as a key structure for reversal learning in many species, including humans, monkeys and rodents (Clarke et al., 2008; Hornak et al., 2004; McAlonan and Brown, 2003). Additionally, it has been suggested that flexible behavior is not supported uniquely by PFC. One brain structure that may interact with PFC to mediate cognitive flexibility is the striatum (Kolb, 1977).

It has been demonstrated that oxidative stress contributes to age-related impairments in learning and memory (Liu et al., 2003). The whole brain is vulnerable to free radicals-induced damage

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because of its high oxygen consumption, abundant lipid content and relative paucity of antioxidant enzymes (Halliwell and Gutteridge, 2007; Olanow, 1992). Oxidative stress happens when there is an imbalance between antioxidant defenses and oxidative species. In this case, the antioxidant defenses, such as the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), are not able to neutralize the reactive species efficiently (Halliwell and Gutteridge, 2007). As consequences, oxidative stress may affect structure and function of different proteins and enzymes; it may also affect membranes, lipids and DNA (Cochrane, 1991). Therefore, function and plasticity of neurons could be changed leading to altered memory processes.

Once we have already observed sex specific differences in spatial learning in adult neonatal handled rats when compared to non-handled ones, we wonder how this early intervention would affect reversal learning associated to food reward. In addition, striatum and PFC are involved in reversal learning, and no study was found evaluating oxidative stress parameters in these structures in adult neonatal handled rats considering sex differences. Therefore, the aim of this study is to evaluate the sex specific differences in reversal learning in adult neonatal handled rats as well as in oxidative stress parameters in the striatum and PFC of these animals.

2. Materials and methods

2.1. Subjects

All animal proceedings were approved by the Institutional Ethical Committee and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS), and of the Federation of Brazilian Societies for Experimental Biology. All efforts were done to minimize animal suffering as well as to reduce the number of animals.

Pregnant Wistar rats bred at our own animal facility were randomly selected. They were housed alone from gestational day 18th in home cages made of Plexiglas (65 cm × 25 cm × 15 cm) with the floor covered with sawdust and were maintained in a controlled environment: lights on between 07:00 and 19:00 h, temperature of 22 ± 2 °C, cage cleaning twice a week, food and water provided ad libitum. The day of birth was considered as day 0. All litters were culled within 24 h of birth to eight pups and were maintained undisturbed except for handling procedures which were carried out between 10:00 and 15:00 h. Several litters were submitted to the handling procedures in the same day. The researcher changed gloves between the handling procedures of each litter to avoid any kind of odor to be spread from nest to nest. Litters were weaned and separated by sex on postnatal day 21. Animals were maintained in groups from 3 to 5 rats per cage. Experiments were performed when adults, and each experimental group had no more than two animals per litter (around three months old) in the behavioral experiments and only one animal per litter (around four months old) for biochemical measurements. Experiments using males and females were performed separately at different times. Rats had free access to food (standard lab rat chow) and water, except during the period when the behavioral task was applied (task was performed between 9:00 and 14:00 h).

2.2. Neonatal handling (Silveira et al., 2006)

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

Handled group: The dam was gently pulled to one side of the cage and the pups were removed from their home cage and were placed into a clean cage lined with clean paper towel. This cage was placed into an incubator set to maintain an ambient temperature at 30–32 °C. After 10 min, pups were returned to their dams. This procedure was performed from day 1 to 10 following birth, and then pups were left undisturbed until the 21st day of life (weaning). It was also stated on the cage that these animals should not be touched, not even for cage cleaning. The same procedure of non-handled group was performed to change dirty sawdust.

2.3. Learning and reversal learning evaluation

A *Y* shaped maze with three wood arms (each one measuring 30 cm high × 51 cm long × 12 cm wide) with a 120° angle from each other was used and the behavior was performed under red light. On the first day of training, animals were allowed to freely explore the maze for 5 min. On the following days, they were introduced in the maze facing the wall in the extremity of the arm that was closer to the evaluator. Animals could choose to enter one of the two other arms of the maze, but only one arm had five units of sweet palatable food (Froot Loops®). Once in the maze, animals had 60 s to choose one of the arms. If they did not do it they were returned to their cages. On the other hand, once they chose one of the arms, the other entrance was closed with a door and they could stay in the maze for additional 60 s. This procedure was repeated four times a day for each rat, using a inter-trial interval of around 20 min. Animals were trained in the learning phase to a criterion of four trials with a maximum of one error/day (error was defined by entrance in the arm with no food). After reaching that, the palatable food was switched to the other arm, requiring the subject to reverse what was previously learned (reversal phase). Successful reversal performance was defined as four trials with a maximum of one error/day. The animals were food restricted during this task (receiving about 80% of habitual ingestion of standard lab chow). Results show the number of correct choices (entrance in the arm with food) as well as the number of pellets consumed (corrected by the number of correct choices) in both learning and reversal phases.

2.4. Morris water maze reversal learning

Another set of neonatal handled and non-handled male rats with three months of age were used in this task. The Morris water maze (Morris et al., 1982) acquisition phase was performed as described in detail in Noschang et al. (2010). After seven days of acquisition, reversal learning was performed. Briefly, animals were trained to find the hidden platform now located in a different position from the acquisition phase, during four days (four trials per day). Latency to find the platform was determined in each trial.

2.5. Preparation of the samples for biochemical measurements

Animals were killed by decapitation when they were four months old, and the PFC and striatum were quickly dissected out. The brain structures were stored at −70 °C until analysis, when they were homogenized in 10 vol. (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA. The homogenate was centrifuged (at 960 × *g*) for 10 min at 4 °C and the supernatant was used for the evaluation of reactive species production by the chemical oxidation of dichlorodihydrofluorescein (DCFH), the determination of total thiol content and the evaluation of antioxidant enzymes activity.

2.5.1. Superoxide dismutase activity

SOD activity was determined using a RANSOD kit (Randox Labs., USA), which is based on the procedure described by Delmas-Beauvieux et al. (1995). SOD activity is expressed as U/mg of protein. One unit of SOD causes a 50% inhibition of the rate of reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride under the conditions of the assay.

2.5.2. Glutathione peroxidase activity

GPx activity was determined according to Wendel (1981), with modifications (Noschang et al., 2010). The reaction was carried out at 37 °C, and the activity of GPx was measured taking tert-butylhydroperoxide as the substrate at 340 nm. GPx activity was expressed as nmol NADPH oxidized per minute per mg of protein.

2.5.3. Catalase activity

CAT is an enzyme that degrades hydrogen peroxide (H_2O_2) and its activity assessment is based upon establishing the rate of H_2O_2 degradation spectrophotometrically at 240 nm at 25 °C (Aebi, 1984). CAT activity was calculated in terms of micromol of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

2.5.4. Evaluation of reactive species production by the chemical oxidation of DCFH (Lebel et al., 1992)

The samples were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA 100 μM) at 37 °C for 30 min. DCFH-DA is cleaved by cellular esterases and the DCFH formed is eventually oxidized by reactive oxygen species (ROS) or reactive nitrogen species (RNS) present in the samples. The formation of the oxidized fluorescent derivative dichlorofluorescein (DCF) was monitored using excitation and emission wavelength of 488 and 525 nm, respectively, using a spectrophotometer. The amount of reactive oxygen/nitrogen species was quantified using a DCF standard curve and results were expressed as nmoles of DCF formed per mg of protein.

2.5.5. Determination of total thiol (SH) content

This assay is based on the reduction of 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) by SH groups, which becomes oxidized (disulfide), yielding a yellow compound (TNB) whose absorption is measured spectrophotometrically at 412 nm (Riddles et al., 1983).

2.5.6. Protein assay

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

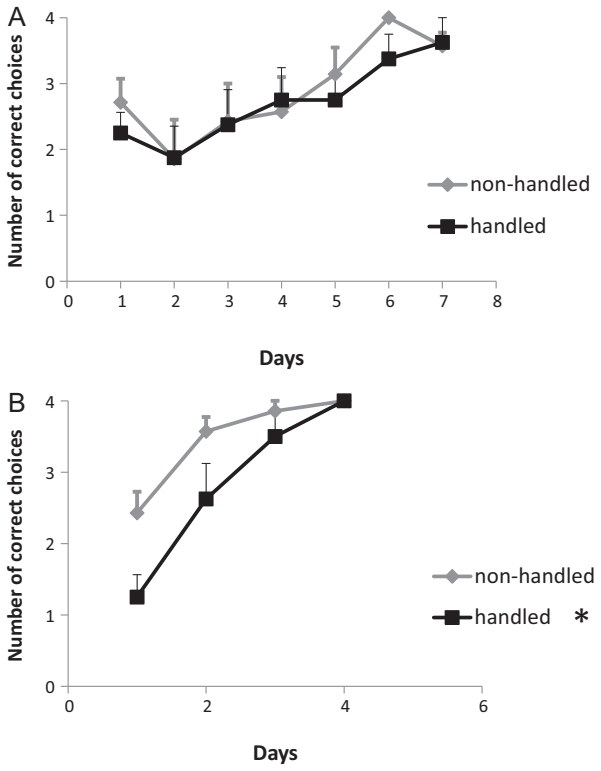

Fig. 1. Effect of neonatal handling on the number of correct choices (entrance in the arm with food) in learning (A) and reversal phases (B) in male rats. Data are expressed as mean + SEM. $N = 7–8$/group. Repeated Measures ANOVA showed a day effect on learning phase of the task ($P < 0.001$). On reversal learning, male neonatal handled animals had a worse performance than non-handled ones ($P = 0.05$). Also on reversal learning, there was day effect ($P < 0.001$).

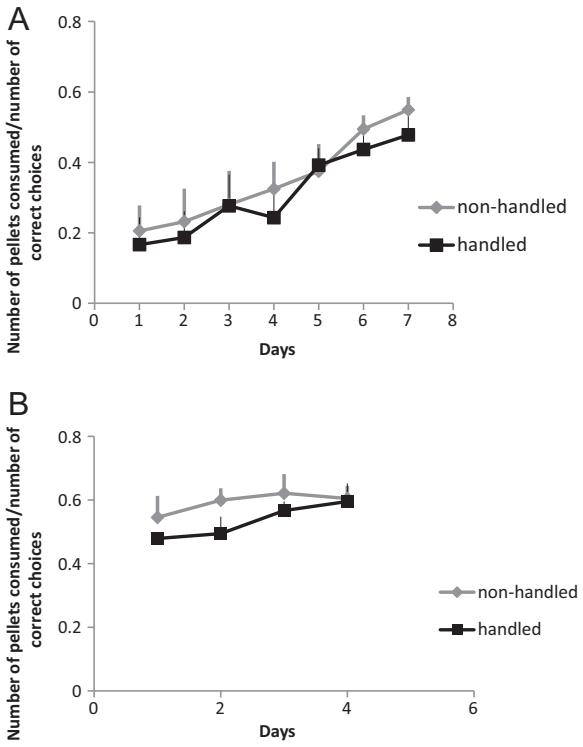

Fig. 2. Effect of neonatal handling on the number of pellets consumed (corrected by the number of correct choices) in learning (A) and reversal phases (B) in male rats. Data are expressed as mean + SEM. $N = 7–8$/group. Repeated Measures ANOVA showed a day effect on the learning phase of the task ($P < 0.001$).

2.6. Statistical analysis

Data were expressed as means ± standard error of the mean, and were analyzed using Repeated Measures ANOVA for learning and reversal learning evaluation and Student's *t* test in the other cases. Significance level was accepted as different when the *P* value was less than 0.05. Regarding Repeated Measures ANOVA, Greenhouse-Greisser correction was applied considering violation of the sphericity assumption as shown by the Mauchly test.

3. Results

3.1. Learning and reversal learning evaluation

Considering male animals, during the learning phase of the task there was a day effect on the number of correct choices, since animals increased that number as the days passed by showing adequate learning [$F(3.29, 42.78) = 8.789$ $P < 0.001$, correction for Greenhouse-Geisser] (Fig. 1A). A day effect was also observed regarding the number of pellets consumed during that phase of the task as all animals increased pellet consumption within the days [$F(2.61, 23.48) = 10.31$, $P < 0.001$, correction for Greenhouse-Geisser] (Fig. 2A). According to that, the latency to start consuming the food decreased throughout the days ($P < 0.05$, data not showed). In addition, no difference between groups was observed during the learning phase ($P > 0.05$). On the reversal learning, male neonatal handled animals had a worse performance than non-handled ones [$F(1,13) = 4.619$, $P = 0.05$]. There was also a day effect [$F(3,39) = 30.24$, $P < 0.001$], since the animals increased the number of correct choices throughout the period (Fig. 1B). According to that, the latency to start consuming the food was higher in neonatal handled animals compared to non-handled ones and this measure also decreased within the days in both groups ($P < 0.05$, data not showed). However, no difference between groups neither a day effect was observed in pellet consumption during reversal learning for male animals ($P > 0.05$) (Fig. 2B). On the other hand, female neonatal handled animals had a better performance in the learning phase in relation to non-handled ones regarding the number of correct choices [$F(1,16) = 12.921$, $P < 0.01$] (Fig. 3A). Moreover, a day effect was observed in both learning and reversal learning phases since both groups increased the number of correct choices as days went by [$F(3.66, 58.58) = 14.273$, $P < 0.001$, correction for Greenhouse-Geisser for learning phase, and $F(2.53, 40.49) = 18.75$, $P < 0.001$, correction for Greenhouse-Geisser, for reversal learning] (Fig. 3A and B). Regarding the latency to start consuming the food, it was lower in neonatal handled rats compared to non-handled ones during the learning phase ($P < 0.05$, data not showed) and it decreased within the days for both groups during learning and reversal learning phases ($P < 0.05$, data not showed). A day effect was also observed regarding pellet consumption during learning [$F(2.86, 37.18) = 21.802$, $P < 0.001$] and reversal learning phases [$F(2.62, 41.88) = 4.322$, $P = 0.013$] (correction for Greenhouse-Geisser in both cases), since both groups increased the consumption with the days, although the increase observed during reversal learning was subtler and seemed to have reached a ceiling effect (Fig. 4A and B). No difference between groups was observed in reversal learning ($P > 0.05$).

3.2. Morris water maze reversal learning

This task was performed in order to evaluate if the deficit in reversal learning observed in neonatal handled male rats could also be observed in reversal learning of a different type of task, to verify if male rats would behave in the same way in a reversal learning task that did not involve reward. Only male animals were used because neonatal handled male rats had a worse performance than non-handled ones on the *Y* maze reversal learning while there

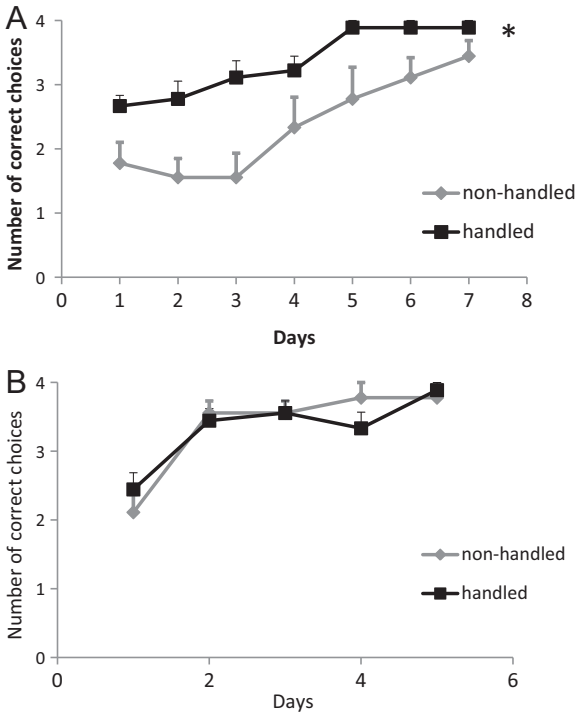

Fig. 3. Effect of neonatal handling on the number of correct choices (entrance in the arm with food) in learning (A) and reversal phases (B) in female rats. Data are expressed as mean + SEM. $N = 9$/group. Repeated Measures ANOVA showed that female neonatal handled animals had a better performance in the learning phase in relation to non-handled ones ($P < 0.01$). A day effect was observed in learning ($P < 0.001$) and reversal learning ($P < 0.001$) phases.

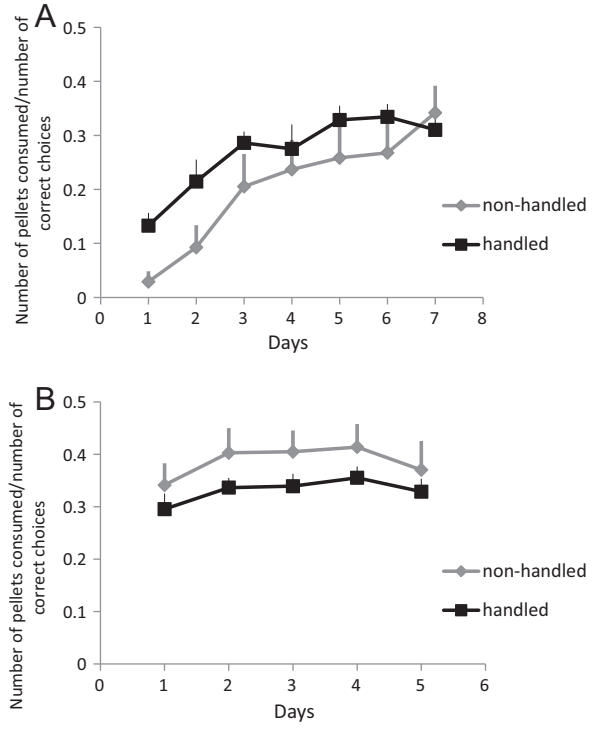

Fig. 4. Effect of neonatal handling on the number of pellets consumed (corrected by the number of correct choices) in learning (A) and reversal phases (B) in female rats. Data are expressed as mean + SEM. $N = 9$/group. Repeated Measure ANOVA showed a day effect in learning ($P < 0.001$) and reversal learning ($P = 0.0013$) phases.

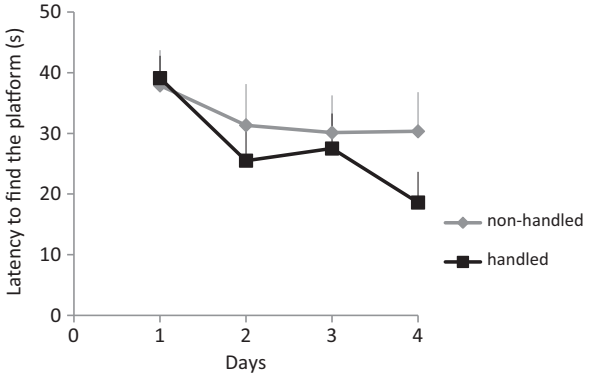

Fig. 5. Effect of neonatal handling on the latency to find the platform on the Morris water maze reversal learning task in male rats. Data are expresses as mean + SEM. $N = 9$–10/group. Repeated Measure ANOVA showed a day effect ($P < 0.01$).

was no difference between females groups, as described above. No difference was observed between neonatal non-handled and handled animals ($P > 0.05$) on the Morris water maze reversal learning task. There was only a day effect since both groups decreased the latency to find the platform within the days [$F(3,51) = 5.733$, $P < 0.01$] (Fig. 5).

3.3. Biochemical measurements

No difference between groups (non-handled and handled) was observed on the antioxidant enzymes activities (SOD, GPx and CAT) in the striatum and in the PFC, neither in males nor in females ($P > 0.05$). However, female neonatal handled rats showed a lower SOD/CAT ratio in the pre-frontal cortex [$t(10) = 3.178$, $P = 0.01$]. In addition, no difference between groups (for both genders) was observed in the total thiol content and in the reactive species production (DCFH oxidation) in the striatum and in the PFC ($P > 0.05$) (Tables 1 and 2).

4. Discussion

The main findings on this paper are that male neonatal handled animals had a worse performance in the *Y* maze reversal learning compared to non-handled ones and no difference occurred in the water maze reversal learning task. Regarding females, neonatal handled rats had a better performance during the *Y* maze learning phase compared to non-handled ones. In addition, neonatal handled female animals showed a decreased SOD/CAT ratio in the PFC compared to non-handled females.

Sex-specific differences were observed on learning and reversal learning evaluation. It is interesting that during the *Y* maze reversal learning, although neonatal handled male rats showed a higher latency to start consuming the food, there was no difference in the amount of food consumed. On the other hand, in the *Y* maze learning phase, female neonatal handled rats had a decreased latency to start consuming the food and there was no difference in the amount of food consumed. However, the important point for the present study is that both handled and non-handled groups showed similar consumption, and therefore both groups were motivated for the task, suggesting that the differences in learning are not related to motivation.

Neonatal handling alters learning in adult male and female rats in a task-specific manner. It was showed that neonatal handling impairs inhibitory avoidance learning, has no effect on circular maze learning, and enhances object recognition memory in rats of both genders (Kosten et al., 2007). These results suggest that neonatal handling has a different effect on different types of memory. In this study, we observed impairment of reversal learning in

Table 1
Evaluation of oxidative stress parameters in the striatum of neonatal handled and non-handled rats.

	Males		Females	
	Non-handled	Handled	Non-handled	Handled
DCF content	6.26 ± 0.32	6.60 ± 0.61	3.95 ± 0.30	4.44 ± 0.33
SOD	14.45 ± 2.07	11.87 ± 1.99	14.88 ± 2.14	16.96 ± 2.20
GPx	36.59 ± 6.11	39.52 ± 7.50	47.03 ± 1.79	46.82 ± 1.20
CAT	1.24 ± 0.30	1.21 ± 0.25	2.30 ± 0.33	2.34 ± 0.14
SOD/CAT ratio	16.13 ± 5.35	15.19 ± 5.81	5.63 ± 1.65	5.69 ± 1.07
SOD/GPx ratio	0.39 ± 0.04	0.37 ± 0.05	0.22 ± 0.05	0.24 ± 0.05
Total thiol content	57.14 ± 2.35	61.10 ± 1.18	42.57 ± 3.89	45.81 ± 3.39

Data are expressed as mean ± S.E.M. of SOD (U/mg protein), GPx (nmol NADPH oxidized/min/mg protein), and CAT (μmol H_2O_2 transformed/min/mg protein) activities. For DCF and thiol content data are expressed as nmoles of DCF formed per mg of protein and nmoles of SH per mg of protein. Males, $N = 4–7$/group. Females, $N = 4–9$/group. Student's t test showed no differences between groups neither in male nor in females in all parameters evaluated.

adult male rats which had been handled during the neonatal period, indicating reduced cognitive flexibility in a task related to reaching a reward, while no impairment was detected on reversal learning of a spatial task. Reversal learning is important to direct behavior according to changes in the environment. It is interesting to point out that interventions on a particular neurotransmitter (CB1) system in the PFC have lead to reversal learning impairment in male rats in the attentional set shift test, a task that is related to food reward (Klugmann et al., 2011). On that study as well as on ours, deficits were only observed when the rats were required to change the behavioral strategy previously learned, suggesting that the impairment does not involve general mechanisms of memory acquisition and storage. It is possible that neonatal handled males have a lower ability to extinguish learned behaviors, particularly related to food location, while the capacity to learn new spatial information is intact.

Different outcomes concerning neonatal handling and learning and memory are possibly dependent on sex. Earlier studies using latent inhibition (a behavioral paradigm in which repeated exposure to stimuli not followed by meaningful consequences renders these stimuli ineffective for subsequent learning), showed that latent inhibition was obtained in neonatal handled and non-handled female animals, while neonatal handled males showed that effect, but non-handled ones failed to develop it (Weiner et al., 1985, 1987). We also observed sex-specific differences regarding the handling procedure and learning and memory. We showed that neonatal handled male and female animals behave differently during learning and reversal learning phases using the *Y* maze task (neonatal handled male rats had a worse performance in reversal learning compared to non-handled ones, while neonatal handled female rats had a better performance in the learning phase compared to non-handled ones). In addition, our group (Noschang et al., 2010) showed sex-specific differences regarding neonatal handling in the acquisition phase of the water maze. We observed that there was no difference between neonatal handled and non-handled male ones on that parameter, however neonatal handled female rats had a worse performance than non-handled ones. These results suggest that neonatal handled female rats improve their performance regarding learning if a motivational factor is involved, in our case a sweet palatable food, however this does not apply to male rats.

Another interesting possibility is the fact that neonatal handling could interact with sex-specific behaviors during development. Since our animals were reared after weaning separated by sex in groups of 3–5 per cage, males and females had different rearing conditions once male rats are known to present intermale aggression, which is dependent of testosterone and also on experience (Albert et al., 1992). In the present study, we did not evaluate aggressive behavior. It would be possible that stress during development induced by different levels of aggressive behavior could help to explain sex-specific differences, however, we must consider that no signs of increased aggressive behavior were observed while rearing the animals.

Between the possible mechanisms that could explain the neural plasticity induced by neonatal handling are epigenetic modifications. The molecular mechanisms involved in the epigenetics of the genome are numerous and complex including RNA interference, chromatin remodeling, histone modification and DNA methylation (Turner, 2001). It has been proposed that the quality of the parent–infant interactions may induce a molecular change in the offspring, which alters the patterns of gene expression present in specific brain regions (emerging in infancy and sustained into adulthood). These epigenetic effects indicate that the quality of the early-life environment can change the activity of genes, thus illustrating the dynamic interplay between genes and environmental experiences in shaping development (Gudsnuk and Champagne, 2011). On the other hand, the sex-specific difference on learning ability presented by neonatal handling animals in adulthood could be explained by the influence of gonadal hormones that can be observed on cellular morphology, on physiological responses,

Table 2
Evaluation of oxidative stress parameters in the PFC of neonatal handled and non-handled rats.

	Males		Females	
	Non-handled	Handled	Non-handled	Handled
DCF content	7.88 ± 0.56	8.36 ± 0.64	3.49 ± 0.54	4.36 ± 0.29
SOD	7.86 ± 2.00	7.36 ± 1.23	11.99 ± 1.80	10.51 ± 1.21
GPx	34.40 ± 4.27	30.89 ± 3.06	32.14 ± 3.39	32.08 ± 2.87
CAT	3.73 ± 0.78	3.01 ± 0.73	2.24 ± 0.08	2.64 ± 0.20
SOD/CAT ratio	5.62 ± 0.66	7.33 ± 1.39	5.75 ± 0.59	3.58 ± 0.35*
SOD/GPx ratio	0.22 ± 0.05	0.33 ± 0.06	0.39 ± 0.03	0.33 ± 0.03
Total thiol content	65.54 ± 1.70	62.99 ± 1.15	43.04 ± 3.91	46.01 ± 3.72

Data are expressed as mean ± S.E.M. of SOD (U/mg protein), GPx (nmol NADPH oxidized/min/mg protein), and CAT (μmol H_2O_2 transformed/min/mg protein) activities. For DCF and thiol content data are expressed as nmoles of DCF formed per mg of protein and nmoles of SH per mg of protein. Males, $N = 4–11$/group. Females, $N = 6–9$/group.
* Significantly different compared to non-handled group (Student's t test, $P = 0.01$).

and on neurobiological and behavioral outcomes (Shughrue and Merchenthaler, 2000). Within the brain, estrogen can act either directly to modify neural activity or indirectly through cascades of changes in gene expression (Boonyaratanakornkit and Edwards, 2004). Genomic effects of estrogen are achieved through activation of nuclear estrogen receptors (ERa and ERb). Disruption to the early-life environment has been demonstrated to alter gene expression and estrogen receptor levels in females (Curley et al., 2011).

In this paper, oxidative stress parameters were evaluated in two brain structures related to reversal learning: PFC and striatum. Concerning PFC, the equivalence of dorsolateral PFC in primates and others species has been discussed (Uylings et al., 2003; Preuss, 1995). Although it is not possible to make a complete analogy between primates and rodents PFC, recently functional and structural characteristics have been used and it has been concluded that rats have a PFC, which includes some features of the primate dorsolateral PFC (Uylings et al., 2003).

We observed a decrease in the SOD/CAT ratio in PFC of female neonatal handled rats compared to non-handled ones. SOD is the enzyme responsible for converting superoxide radicals into hydrogen peroxide that needs to be further scavenged by CAT and GPx (Halliwell, 2001). A decreased SOD/CAT ratio may lead to increased superoxide radicals and alteration of membrane integrity, DNA instability and protein damage (Cochrane, 1991), when it is due to a decreased SOD activity. However, in this study, SOD activity showed no difference between groups. Additionally, we did not observe any difference regarding the production of reactive species, neither in the total thiol content that could be affected by protein damage. Based on that, we suggest that the SOD/CAT decreased ratio is not enough to produce high levels of superoxide radical that would lead to protein damage. Nonetheless, we cannot rule out the possibility of oxidative stress, since the technique we used to measure the production of reactive species may be influenced by the availability of reducible substrates and might have not been sensible enough to catch an increased production of reactive species. Other structures like lipids and DNA (that are not the focus of this paper) might have been affected. More studies are needed to understand that. Even so, if there is oxidative stress, it is clear that it is not impairing learning processes in the *Y* maze, considering female neonatal handled rats.

In conclusion, neonatal handling effects on learning and memory in adult rats are sex and task specific. The sex specific differences are also observed in the evaluation of antioxidant enzymes activities with neonatal handling affecting only females. This study contributes to the understanding of how early life events can influence behavior and neurochemistry in adult life considering sex specific differences.

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1.3 Capítulo 3

The influence of early life interventions on olfactory memory related to palatable food, and on oxidative stress parameters and Na^+/K^+-ATPase activity in the hippocampus and olfactory bulb of female adult rats

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The influence of early life interventions on olfactory memory related to palatable food, and on oxidative stress parameters and Na+/K+-ATPase activity in the hippocampus and olfactory bulb of female adult rats

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Abstract

The effects of neonatal handling and the absence of ovarian hormones on the olfactory memory related to a palatable food in adulthood were investigated. Oxidative stress parameters and Na+/K+-ATPase activity in the hippocampus and olfactory bulb of adult pre-puberty ovariectomized female rats handled or not in the neonatal period were also evaluated. Litters were non-handled or handled (10min/day, days 1-10 after birth). Females from each litter were divided into: OVX (subjected to ovariectomy), sham, and intact. When adults, olfactory memory related to a palatable food (chocolate) was evaluate using the hole-board olfactory task. Additionally, oxidative stress parameters and Na+/K+-ATPase activity were measured in the hippocampus and olfactory bulb. No difference between groups was observed considering olfactory memory evaluation. Neonatal handled rats presented an increase in Na+/K+-ATPase activity in the hippocampus and in the olfactory bulb, compared to non-handled ones. Considering the surgical procedure, there was a decrease in Na+/K+-ATPase and catalase activities in sham and OVX groups, compared to intact animals in the olfactory bulb. We concluded that olfactory memory related to a palatable food in adulthood was not affected by neonatal handling or by pre-puberty surgery, with or without removal of ovaries. The difference observed between groups in catalase and Na+/K+-ATPase activity does not seem to be related to the olfactory memory. Additionally, the increase in Na+/K+-ATPase activity (an enzyme that maintains the neurochemical gradient necessary for neuronal excitability) induced by neonatal handling may be related to neuroplastic changes in the hippocampus and olfactory bulb.

Keywords: neonatal handling, ovariectomy, oxidative stress, Na+/K+-ATPase, olfactory memory

Introduction

Early life environmental events can induce long-lasting alterations in behavior and neurochemical parameters [1]. During the neonatal period, a stable mother-infant interaction seems to be critical for normal growth and behavioral development in rodents [2, 3] and humans [2, 4]. Infant rats rapidly learn to identify, approach and prefer the maternal odor naturally within the nest [5], since the identification of the mother is essential for survival and development of mammals. To study the impact of early life events in adulthood, some approaches have been used [6-8], and one of these is neonatal handling, consisting of a brief, repeated, and apparently innocuous separation of pups from the mother during the neonatal period.

Neonatal handling modulates the development of the hypothalamic-pituitary-adrenal (HPA) axis, and is associated with decreased stress reactivity [9] and an increased ingestion of palatable food in adulthood [10]. It has been shown that the neonatal handling procedure reduces the approach behavior towards a familiar odor (maternal odor preference) in female pups, but not in males [11]. This gender effect has been attributed to differences in sex hormones, although it has also been suggested to be related to increased maternal behavior, usually directed to males when compared to female pups [11, 12].

The classic view of sexual differentiation is that the male brain develops under the influence of testicular secretions, whereas the female brain develops in the absence of any hormonal simulation. However, several studies have suggested a possible role of estradiol in female neural development [13, 14], and it has been suggested that, in mice, estrogens are required for the development of the main and accessory olfactory systems [14]. In addition, the olfactory bulb is necessary for long-

term retention of olfactory memories, and it is possible that estrogen promotes the preservation of these memories by acting in the olfactory bulb [15].

With regard to the postnatal development of the olfactory bulb of rats, most neurons (75-80%) originate during the first three weeks of life; however all interneuronal populations, including accessory bulb granule cells, show some neurogenesis beyond postnatal day 20 (up to postnatal day 60 in the accessory bulb and up to postnatal day 180 in the main bulb) [16]. Furthermore, it has been shown that the volume of the main olfactory bulb increases by over seven-fold by postnatal day 30 and remains unchanged thereafter [17].

Interventions during the neonatal period, such as neonatal handling, may have long term effects on some neurochemical parameters related to oxidative stress, such as DNA breaks and nitric oxide production [18], which may be related to oxidative stress. Additionally, the presence of gonadal hormones during development may affect oxidative stress, since estradiol has been reported to have neuroprotective properties [19, 20]. It is becoming increasingly clear that estrogens are neuroprotective hormones acting via estrogen receptor-dependent pathways, which binds to specific sites in the nuclear DNA, and neuroprotective steroidal structures acting independently of the activation of specific estrogen receptors. In addition, one striking activity of the estradiol molecule is its intrinsic antioxidant activity, which makes it a potential chemical shield for neurons [20]. Moreover, data from the literature suggest that estradiol may protect against a wide range of toxic insults, including free radical generators [21].

Oxidative stress happens when there is an imbalance between high levels of reactive species in relation to antioxidant defenses (for example antioxidant enzymes), resulting in several dysfunctions in the cell metabolism, including alterations in

protein structure. One enzyme that is known to be affected is the Na+/K+-ATPase [22, 23], an ion pump that is responsible for maintaining the ionic gradient necessary for neuronal excitability and neurotransmission; it also functions as a signal transducer to relay messages from the plasma membrane to the intracellular organelles [24].

Considering the exposed above, the aim of this study was to verify the effects and possible interactions of neonatal handling and the pre-puberty ovariectomy on the olfactory memory related to a palatable food in adulthood. Our initial hypothesis was that neonatal handling would facilitate memory, as it has already been demonstrated in a previous study from our group using a *Y* maze task [25]. Additionally, we believed that the absence of ovarian hormones would influence the development of neonatal handling effects later in life. Since an olfacto-hippocampal network is dynamically involved in odor-discrimination learning [26], functional impairments in the nervous system have been associated with oxidative stress [27, 28] and Na+/K+-ATPase is especially sensitive to oxidative stress [29, 30], we evaluated oxidative stress parameters and Na+/K+-ATPase activity in the hippocampus and olfactory bulb of adult pre-puberty ovariectomized female rats, handled or not, in the neonatal period.

Materials and Methods

Subjects

All animal proceedings were approved by the Institutional Ethical Committee and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS), and of the Federation of Brazilian Societies for

Experimental Biology. All efforts were made to minimize animal suffering as well as to reduce the number of animals.

Fifty pregnant *Wistar* rats bred at our own animal facility were randomly selected. Animals were housed alone from gestational day 18th in home cages made of Plexiglas (65 X 25 X 15 cm) with the floor covered with sawdust and were maintained in a controlled environment: lights on between 07:00 and 19:00 h, temperature of 22 ± 2°C, cage cleaning twice a week, food and water provided ad libitum. The day of birth was considered as day 0. All litters were culled within 24 h of birth to eight pups and were maintained undisturbed except for neonatal handling procedures, which were carried out between 10:00 and 15:00 h. Several litters were submitted to the neonatal handling procedures on the same day. The researcher changed gloves between the neonatal handling procedures of each litter to avoid any kind of odor being spread from nest to nest. Litters were weaned and separated by sex on postnatal day 21. Between 24-26 post-natal days (PND), females from each litter were divided into the following groups: OVX (subjected to ovariectomy), sham, and intact (no surgery). Experiments were performed when adults and each experimental group had no more than two animals per litter (around 90 day-old) in the behavioral experiments and only one animal per litter (around 90 day-old) for biochemical measurements. Different sets of rats were used in behavior and biochemical measures. Seventy animals were used for the behavioral procedures and 79 animals were used for the biochemical measures. Rats had free access to food (standard lab rat chow) and water, except during the period when the behavioral task was applied.

Neonatal Handling [31]

The neonatal handling procedure consists of a brief, repeated and apparently innocuous separation from the mother during the first days of life [32]. During the

first two weeks of life, rat pups show markedly reduced adrenocortical response to stress (this period has been termed the “stress hyporesponsive period” [33]), and it has been demonstrated that neonatal handling leads to behavioral and neurochemical changes in the adult offspring [34, 35].

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

Handled group: The dam was gently pulled to one side of the cage and the pups were removed from their home cage and placed in a clean cage lined with clean paper towel. This cage was placed into an incubator set to maintain an ambient temperature at 30–32°C. After 10 min, pups were returned to their dams. This procedure was performed from day 1–10 following birth and pups were then left undisturbed until the 21st day of life (weaning). It was also stated on the cage that these animals should not be touched, not even for cage cleaning. The same procedure for the non-handled group was performed to change dirty sawdust.

Surgery

Ovariectomy (OVX) was performed between 24 and 26 PND. Rats were anesthetized with 120 mg/kg ketamine HCl (Dopalen: Agribrands, Campinas, SP, Brazil) and 16 mg/kg xylazine (Anasedan: Agribrands, Campinas, SP, Brazil) and bilateral ovariectomy was performed using a single abdominal incision. The abdominal skin was then cut, the peritoneum was opened, both ovarian arteries were linked, and both ovaries were removed. The muscle and the skin were sutured. Sham animals were subjected to surgery, but the ovaries were not removed.

Exposure to the open field

A 50 cm high, 40×60 cm open field made of brown plywood with a frontal glass wall was used [36]. The floor was subdivided with white lines into 12 equal 13.3 by 15.0 cm rectangles. Measurements were taken in a brightly lit room, set up so that uniform light was applied on the floor of the open field. The animals were gently placed facing the left corner and allowed to explore the arena for 5 min. The line crossings (ambulation) were counted.

Hole board olfactory task

A wood box with 42 cm high x 67 cm long and 46 cm wide was used. The bottom of the box had nine holes, measuring 4 cm in diameter and 1 cm deep covered with sawdust. The task consisted of a habituation trial during which the animals could explore the box for three minutes. After habituation, animals were returned to their home cages and received a small piece of chocolate (1.5g/rat). On the next day, overnight food restricted (receiving about 80% of habitual ingestion of standard lab chow) rats received another small piece of chocolate (1.5g/rat) before the test. Two holes were selected to contain chocolate; more specifically in each hole one piece of chocolate (1.5g) was hidden under the sawdust; the remaining holes were also filled with sawdust. Animals were placed in the box and the following parameters were measured during three minutes: latency to find the chocolates, latency to start eating the first and second piece of chocolate and the total amount of chocolate consumed. When analyzing the data regarding the latency to start consuming the chocolates and the amount of chocolate consumed, only the animals that found the chocolate were considered (from a total of 70 animals 61 found the chocolate; the animals that did not find it were distributed between the groups). Both habituation and test phases were performed under red light.

Preparation of the Samples for Biochemical Measurements

Animals were killed by decapitation; the olfactory bulb and hippocampus were quickly dissected out and stored at -70°C until analysis. These brain structures were homogenized in 10 vol (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. The homogenate was centrifuged (at 960 g) for 10 min at 4°C and the supernatant was used for the evaluation of reactive species production by the chemical oxidation of dichlorodihydrofluorescein (DCFH), the determination of total thiol content and antioxidant enzyme activities. For the determination of Na+/K+-ATPase activity, the cerebral structures were homogenized in 10 volumes (w:v) of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. The homogenate was centrifuged (at 960 g) for 10 min and the supernatant was used.

Superoxide Dismutase (SOD) Activity

SOD activity was determined using a RANSOD kit (Randox Labs., USA), which is based on the procedure described by Delmas-Beauvieux et al. [37]. SOD activity is expressed as U/mg of protein. One unit of SOD causes a 50% inhibition of the rate of reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride under the conditions of the assay.

Glutathione Peroxidase (GPx) Activity

Glutathione peroxidase activity was determined according to Wendel [38], with modifications. The reaction was carried out at 37°C in 200 μL of solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione and 0.4 U glutathione reductase. The activity of GPx was measured, taking tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted

from the overall reaction ratio. GPx activity was expressed as nmol NADPH oxidized per minute per mg protein.

Catalase (CAT) Activity

CAT is an enzyme that degrades hydrogen peroxide (H_2O_2) and its activity assessment is based upon establishing the rate of H_2O_2 degradation spectrophotometrically at 240 nm at 25°C [39]. CAT activity was calculated in terms of micromol of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$.

Evaluation of reactive species production by the chemical oxidation of DCFH [40]

The samples were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA 100μM) at 37° C for 30 minutes. DCFH-DA is cleaved by cellular esterases and the DCFH formed is eventually oxidized by reactive oxygen species (ROS) or reactive nitrogen species (RNS) present in the samples. The formation of the oxidized fluorescent derivative dichlorofluorescein (DCF) was monitored using excitation and emission wavelength of 488 and 525nm, respectively, using a spectrophotometer. The amount of reactive oxygen/nitrogen species was quantified using a DCF standard curve and results were expressed as nmoles of DCF formed per mg of protein.

Determination of total thiol (SH) content

This assay measures protein thiol and non-protein thiol content, the latest mainly represented by the reduced form of glutathione. The method is based on the reduction of 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) by SH groups, which becomes oxidized (disulfide), yielding a yellow compound (TNB) whose absorption is measured spectrophotometrically at 412 nm [41].

Na+/K+-ATPase Activity

The reaction mixture for Na+/K+-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl and 40 mM Tris–HCl, pH 7.4. After 10 min of sample pre-incubation at 37ºC, the reaction was initiated by addition of ATP to a final concentration of 3 mM and was incubated for 20 min. Controls were carried out under the same conditions with the addition of 1 mM ouabain. Na+/K+-ATPase activity was calculated by the difference between the two assays according to de Souza Wyse et al. [42]. Released inorganic phosphate (P_i) was measured by the method of Chan et al. [43]. Specific activity of the enzyme was expressed as nmol P_i released/min/mg of protein.

Protein Assay

The total protein concentration was determined using the method described by Lowry et al. [44], with bovine serum albumin as standard. For the determination of Na+/K+-ATPase activity, total protein concentration was measured by Bradford [45].

Statistical Analysis

Data were expressed as mean ± standard error of the mean, and were analyzed using repeated measures or two-way ANOVA (factors were neonatal handling and surgery) followed by the Duncan multiple range test, when indicated. The significance level was accepted as different when the P value was equal or less than 0.05.

Results

Exposure to the open field

No difference in the number of crossings was observed between the groups (data not shown; two-way ANOVA $P>0.05$).

Hole board olfactory task

No difference between groups was observed in the latency to find the two pieces of chocolate (two-way ANOVA $P>0.05$) (Figure 1A). With regard to the latency to start eating the chocolates, there was a tendency of neonatal handled animals to start consuming the first piece of chocolate faster than non-handled ones [$F(1,55) = 3.104$, $P=0.084$, two-way ANOVA] (Figure 1B). The same pattern of behavior was observed in the latency to start eating the second piece of chocolate, however in this case there was a significant difference [$F(1,55) = 5.029$, $P<0.05$, two-way ANOVA] (Figure 1C). Repeated measures ANOVA was run to verify possible differences when comparing the time elapsed from finding and ingesting the food reward and no significant effects were observed ($P>0.05$). Considering the total amount of chocolate consumed there was no difference between groups (two-way ANOVA $P>0.05$) (Figure 1D).

Biochemical Measurements

No difference was observed between groups (two-way ANOVA $P>0.05$) in the hippocampus for the following parameters: SOD, GPx, CAT activities, reactive species production and total thiol content (Table 1). On the other hand, neonatal handled animals had a higher Na+/K+-ATPase activity compared to non-handled ones [$F(1,21) = 14.211$, $P<0.05$, two-way ANOVA] (Figure 2A).

With regard to the olfactory bulb, there was an effect of surgery on CAT activity [$F(2,24) = 10.669$, $P<0.05$, two-way ANOVA] with Duncan post-hoc analysis showing that CAT was lower in both sham and OVX animals, compared to the intact group; in addition, CAT activity in the sham group was lower than that of OVX animals (Table 2). No difference between groups was observed in the SOD and GPx activities, as well as in the reactive species production and in the total thiol content

(two-way ANOVA P>0.05) (Table 2). Neonatal handled animals presented higher values of Na+/K+-ATPase activity, compared to non-handled ones [$F(1,18) = 7.107$, $P<0.05$, two-way ANOVA], and there was also an effect of surgery [$F(2,18) = 11.854$, $P<0.01$, two-way ANOVA], with sham and OVX groups presenting lower activity than the intact group (Duncan post-hoc analysis) (Figure 2B).

Discussion

The main findings of this study were that neonatal handled animals showed a lower latency to start eating chocolates in the hole board olfactory task and presented an increase in the Na+/K+-ATPase activity in the hippocampus and in the olfactory bulb, compared to non-handled animals. With regard to the surgical procedure, the main effects were observed in the olfactory bulb where there was a decrease in Na+/K+-ATPase activity in the sham and OVX groups, compared to intact animals, and a lower CAT activity in sham and OVX animals compared to the intact group. In addition, CAT activity in the sham group was lower than in the OVX animals. Our hypothesis that neonatal handling could have beneficial effects on olfactory memory was not confirmed, since no difference was found between handled and non-handled groups. Additionally, according to the aim of this study, it was expected that the main effects related to the surgical procedure would be due to the pre-puberty OVX; however we observed that surgery per se led to important effects, as discussed below. In addition, ovariectomy was not able to influence the effects of neonatal handling, and none of the early interventions used were able to influence olfactory memory related to a palatable food.

Several lines of evidence point to sex-specific effects of neonatal interventions on behavior and cognition. Considering olfactory abilities, it has been shown that the behavior of rat pups in response to maternal odor and biochemical parameters related to the olfactory learning mechanism (observed in 7-day-old pups handled from PND 1 to 7) is gender dependent. The neonatal handling procedure has been shown to clearly reduce the approach behavior towards a familiar odor in female pups, but not in males [11]. It should be pointed out that, in this previous study, the neonatal handling procedure used was different and the total time of the mother–infant separation was approximately 1 min and 30 s per day instead of ten minutes, as we used in the current study. In addition, serotonin activity increased in the olfactory bulb of male pups, but not in females [11]. These effects of neonatal handling on the olfactory memory of infant females prompted us to study olfactory memory in neonatal handled adult females and the involvement of gonadal hormones. Moreover, sex-specific effects of neonatal handling on learning in rats have been suggested for different tasks [18, 25]. In adult neonatal handled rats, females showed impairment in spatial learning compared to non-handled ones, while no difference was observed in males [18]. Additionally, in a *Y* maze memory task female neonatal handled animals had a better performance in the learning phase, in relation to non-handled females, while male neonatal handled animals had a worse performance in the reversal learning, compared to non-handled ones [25]. In the present study, we did not find any difference in the latency to find the chocolates in the hole board olfactory task. The effects of neonatal handling on the memory of females appear to be dependent on the task used, the age as well as the type of memory evaluated. It is interesting to notice that, using a *Y* maze task, neonatal handled female rats showed a lower latency to start consuming the palatable food in the learning phase, compared to non-handled

females, and no differences in the amount of food consumed were observed [25]. These data support our results for the latency to start eating and the amount of chocolate consumed. On the other hand, other studies have observed that neonatal handling increases the ingestion of palatable food [10]; in this context, we must consider that the incentive value of the chocolate may have been increased in neonatal handled animals, what could explain the reduced latency to eat. It should be pointed out that the animals did not ingest the total amount of food retrieved; therefore there was not a ceiling effect on this measurement. Additionally, no difference was observed in the motor activity and this parameter did not affect the results observed.

Sex-specific effects have also been observed with regard to early life interventions and social recognition, which is related to olfactory abilities, in adulthood [46]. In the study by Todeschin et al. [46] only maternally separated adult males showed a reduction in social behavior, which was also related to alterations in brain areas involved in social bonding. This effect due to an early intervention appears in adulthood, suggesting that some sex-specific influence during development is responsible for such alterations. It is possible that both early interventions and gonadal hormones may interact during development to establish behaviors related to social interactions.

Considering the influence of sexual hormones on olfactory tasks, estradiol may be crucial for female recognition of males at 120 min after a brief encounter [47]. We did not observe any difference in the latency to find the chocolate in the hole board task as a result of the ovariectomy procedure. Rats submitted to this procedure have very low plasma gonadal hormones levels [19]; since no difference was found between the OVX group, compared to the other groups, it is possible that these hormones do not influence this behavior. It is important to point that the hole board

task performed here depends on two factors: memory and olfactory perception. Since we did not find any difference between groups in the latency to find the chocolates, we concluded that none of these factors were affected by the interventions evaluated.

With regard to Na+/K+-ATPase activity, interventions during development have been reported to alter the activity of this enzyme. We observed an increase in Na+/K+-ATPase activity in the hippocampus and in the olfactory bulb of adult female neonatal handled animals, compared to non-handled ones. Using preparations of hippocampal synaptic membranes, previous studies have shown that adult male neonatal handled rats had decreased Na+/K+-ATPase activity [48], while adult female neonatal handled animals presented no difference when compared to non-handled females [49]. This discrepancy with the present study could be explained by the fact that different preparations were used; while we evaluated the enzyme activity in the total membrane fraction (including different cellular types), the previous study used the synaptic plasma membrane [49]. We chose to study this fraction since modulation of Na+/K+-ATPase activity by some neurotransmitters appears to also affect the glial fraction [50].

Na+/K+-ATPase activity in the brain seems to be modulated by serotonin and norepinephrine activity [51, 52]. It has been shown that adult female neonatal handled animals have the same basal tissue levels of serotonin, norepinephrine and their metabolites in the hippocampus [53], when compared to non-handled ones; however, these animals were aged females, and the neonatal handling procedure was different from ours. Further studies are needed to verify whether neurotransmitters, such as those pointed above, could be involved in the increased Na+/K+-ATPase activity in the hippocampus and olfactory bulb of the adult female neonatal handled rats. In

addition, the increased Na+/K+-ATPase activity induced by neonatal handling may be related to neuroplastic changes in the hippocampus and olfactory bulb.

Sexual hormones and pre-puberty surgery do not seem to influence oxidative stress parameters and Na+/K+-ATPase activity in the hippocampus of adult females whether neonatal handled or not. However, we observed an effect of surgery, on CAT activity in the olfactory bulb, especially in sham animals, which presented the lowest activity. These results agree with earlier studies from our group that showed a significant effect of pre-puberty surgery, causing decreased CAT activity in the cerebral cortex of adult female rats [54]. CAT is an enzyme that degrades hydrogen peroxide. If hydrogen peroxide is not eliminated properly it can lead to the production of the highly reactive hydroxyl radical [55, 56]. As a consequence, cellular proteins, lipids and DNA can be damaged [57]. Accordingly, the decreased CAT activity, as observed in this study, may lead to an increased hydrogen peroxide production and cellular damage. Although we did not find any difference in the total thiol content (a measurement related to protein damage), this does not exclude the chance of cellular damage, since lipids and DNA may have been affected. It is interesting to note that CAT activity was lower in the sham group, compared to OVX; this may be related to the fact that the surgery was performed during the pre-puberty period, when neuronal rearrangements occur, resulting in refined connectivity and functionality of brain regions in adulthood [58, 59]. We believe that the absence of sexual hormones from a critical period of development, such as pre-puberty until adulthood may have led to brain changes that possibly explain the difference in CAT activity between sham and OVX groups. With regard to the decreased Na+/K+-ATPase activity in the olfactory bulb presented by animals submitted to surgery (sham and OVX) compared to intact animals, findings agree with previous studies showing that reduced antioxidants or

antioxidant enzyme activities are related to reduced Na+/K+-ATPase activity [22, 23]. Given that surgery reduced catalase and Na+/K+-ATPase activities in the olfactory bulb, one may suggest that the anesthetics used could be responsible for this alteration. However, we believe this is unlikely, since these effects were not observed in the hippocampus and since about two months were elapsed between the surgery and these enzymes evaluation. In addition, it has been shown that male rats exposed to isolation stress in the pre-puberty period showed a decreased Na+/K+-ATPase activity in the prefrontal cortex during adulthood [60], also in accordance with our findings, pointing to the fact that different stressors (isolation and surgery) during pre-puberty may reduce Na+/K+-ATPase activity in brain structures.

In conclusion, olfactory memory related to a palatable food in adulthood was not affected by neonatal handling or by pre-puberty surgery, with or without the removal of ovaries. The difference observed in CAT and Na+/K+-ATPase activity between groups does not seem to be related to the olfactory memory. Additionally, the increase in Na+/K+-ATPase activity, induced by neonatal handling, may be related to neuroplastic changes in the hippocampus and olfactory bulb. This study contributes to the understanding of how early events (neonatal handling and pre-puberty surgery) affect neurochemical parameters in the adult brain.

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

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Table 1
Effects of neonatal handling and pre-puberty ovariectomy procedures in oxidative stress parameters in the hippocampus of adult rats

Groups		DCF content	SOD	GPx	CAT	Total thiol content
Non-handled	Intact	8.70±1.46 (7)	5.41±0.87 (9)	22.40±0.99 (9)	1.64±0.12 (8)	59.50±4.40 (9)
	Sham	6.59±1.99 (6)	8.73±1.35 (8)	21.67±0.48 (8)	1.32±0.16 (7)	62.89±4.76 (8)
	OVX	8.83±2.17 (5)	7.91±1.50 (6)	22.61±1.77 (6)	1.38±0.15 (4)	66.26±4.63 (6)
Handled	Intact	6.33±1.17 (10)	6.77±0.59 (11)	22.03±0.99 (11)	1.48±0.17 (7)	58.48±3.22 (11)
	Sham	8.15±2.19 (6)	7.56±1.26 (9)	21.94±1.32 (9)	1.57±0.20 (9)	63.35±4.46 (9)
	OVX	5.40±0.91 (8)	7.20±0.45 (9)	20.53±0.48 (9)	1.24±0.10 (8)	59.84±3.43 (9)

Data are expressed as mean ± S.E.M. of SOD (U/mg protein), GPx (nmol NADPH oxidized/min/mg protein), and CAT (μmol H2O2 transformed/min/mg protein) activities. For DCF and thiol content data are expressed as nmoles of DCF formed per mg of protein and nmoles of SH per mg of protein. N= 4-11/group (specified between parentheses).

Two-way ANOVA showed no difference between groups in all parameters evaluated.

Table 2
Effects of neonatal handling and pre-puberty ovariectomy procedures in oxidative stress parameters in the olfactory bulb of adult rats

Groups		DCF content	SOD	GPx	CAT	Total thiol content
Non-handled	Intact	15.47±2.59 (5)	10.99±2.03 (5)	33.95±3.04 (5)	2.45±0.15 (5)	58.20±0.86 (5)
	Sham	15.90±0.90 (6)	10.84±0.72 (6)	30.74±1.87 (6)	1.49±0.09 (6)*#	49.31±1.84 (6)
	OVX	15.74±1.43 (6)	11.51±1.24 (6)	33.86±2.11 (6)	1.69±0.22 (6)*	52.54±3.29 (6)
Handled	Intact	17.09±3.92 (5)	8.50±0.89 (5)	30.38±3.16 (5)	2.66±0.07 (4)	54.90±3.84 (5)
	Sham	14.55±2.26 (4)	10.57±2.62 (4)	36.28±6.56 (4)	1.25±0.41 (4)*#	60.36±7.93 (4)
	OVX	16.80±1.50 (5)	12.00±1.89 (5)	36.39±3.77 (5)	2.41±0.40 (5)*	58.63±2.02 (4)

Data are expressed as mean ± S.E.M. of SOD (U/mg protein), GPx (nmol NADPH oxidized/min/mg protein), and CAT (μmol H2O2 transformed/min/mg protein) activities. For DCF and thiol content data are expressed as nmoles of DCF formed per mg of protein and nmoles of SH per mg of protein. N= 4-6/group (specified between parentheses).

Two-way ANOVA showed a surgery effect on CAT activity ($P<0.05$). No difference between groups was observed in the other parameters ($P>0.05$).

* Sham and OVX groups were different compared to intact groups (Duncan post-hoc, $P<0.05$).
# Sham groups were different compared to OVX groups (Duncan post-hoc, $P<0.05$).

Legends to Figures

Fig. 1 Effects of neonatal handling and pre-puberty ovariectomy procedures on the latency to find two pieces of chocolate (A) the latency to start eating the first (B) and the second (C) piece of chocolate and on the total amount of chocolate consumed (D) in the hole board task

Data are expressed as mean + S.E.M.

N = 10-14/group for the latency to find two pieces of chocolate

N = 8-12/group for the latency to start eating the first and the second piece of chocolate as well as for the total amount of chocolate consumed

Two-way ANOVA showed that neonatal handled rats start eating the second piece of chocolate faster than non-handled ($P< 0.05$).

* Neonatal handled groups were different compared to non-handled groups.

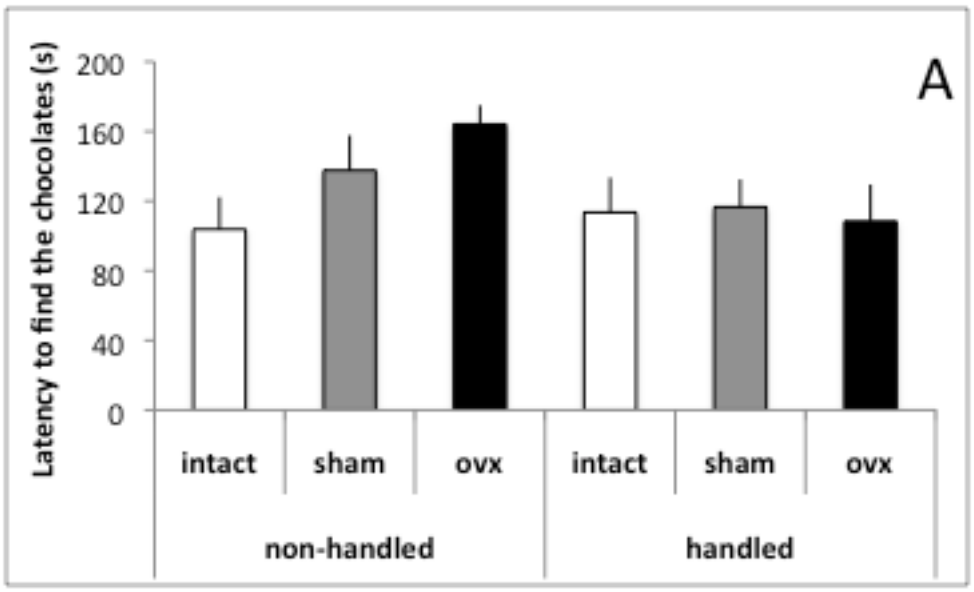
Fig. 2 Effects of neonatal handling and pre-puberty ovariectomy procedures on Na+/K+-ATPase activity in the hippocampus (A) and olfactory bulb (B) of adult rats

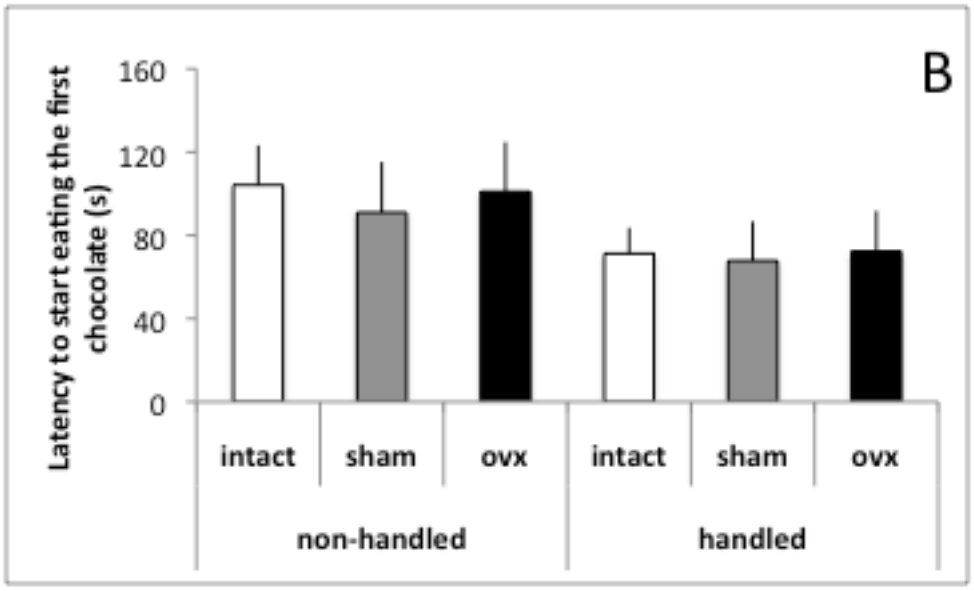
Data are expressed as mean + S.E.M. N = 4-6/group. In the hippocampus, two-way ANOVA showed that neonatal handled animals had a higher enzyme activity compared to non-handled animals ($P<0.05$). In the olfactory bulb, two-way ANOVA showed that neonatal handled increased Na+/K+-ATPase activity, compared to non-handled animals ($P<0.05$), and an effect of surgery ($P<0.01$), with sham and OVX groups presenting lower activities than the intact groups (Duncan post-hoc test, $P<0.05$).

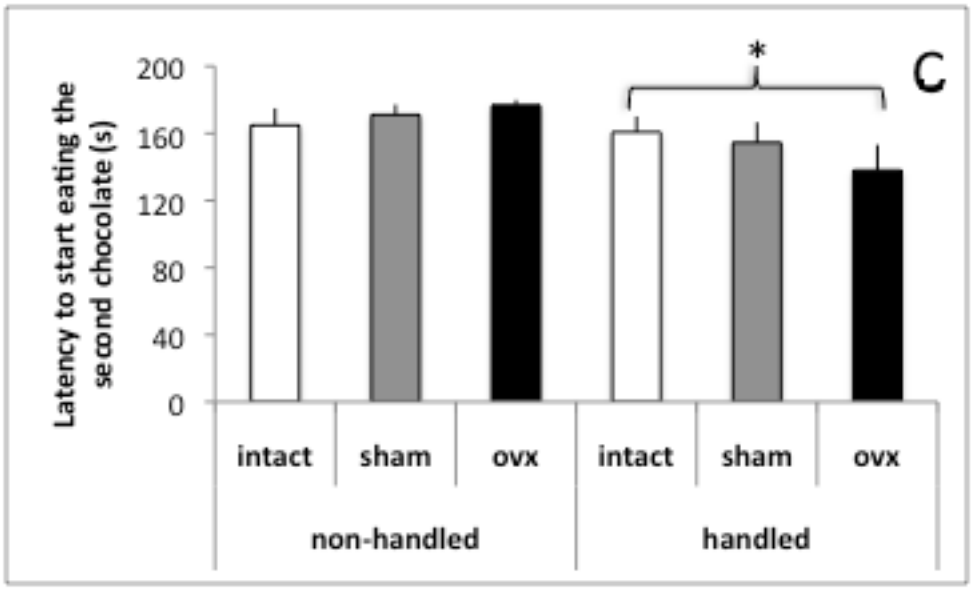
* Neonatal handled groups were different compared to non-handled groups.

Sham and OVX groups were different compared to intact groups.

Figure 1


A
Latency to find the chocolates (s)
200
160
120
80
40
0
intact
sham
ovx
intact
sham
ovx
non-handled
handled


B
Latency to start eating the first chocolate (s)
160
120
80
40
0
intact
sham
ovx
intact
sham
ovx
non-handled
handled


C
*
Latency to start eating the second chocolate (s)
200
160
120
80
40
0
intact
sham
ovx
intact
sham
ovx
non-handled
handled

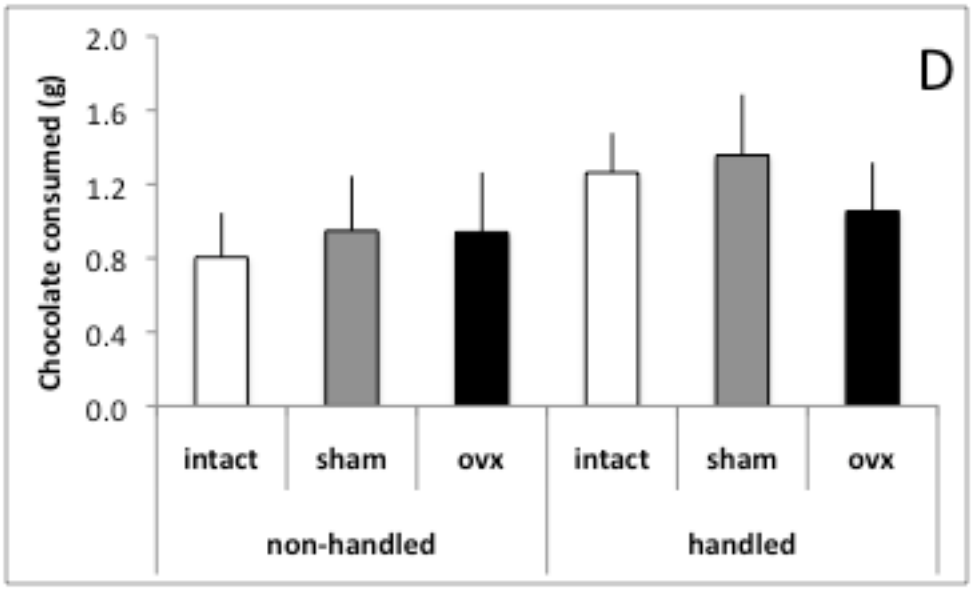
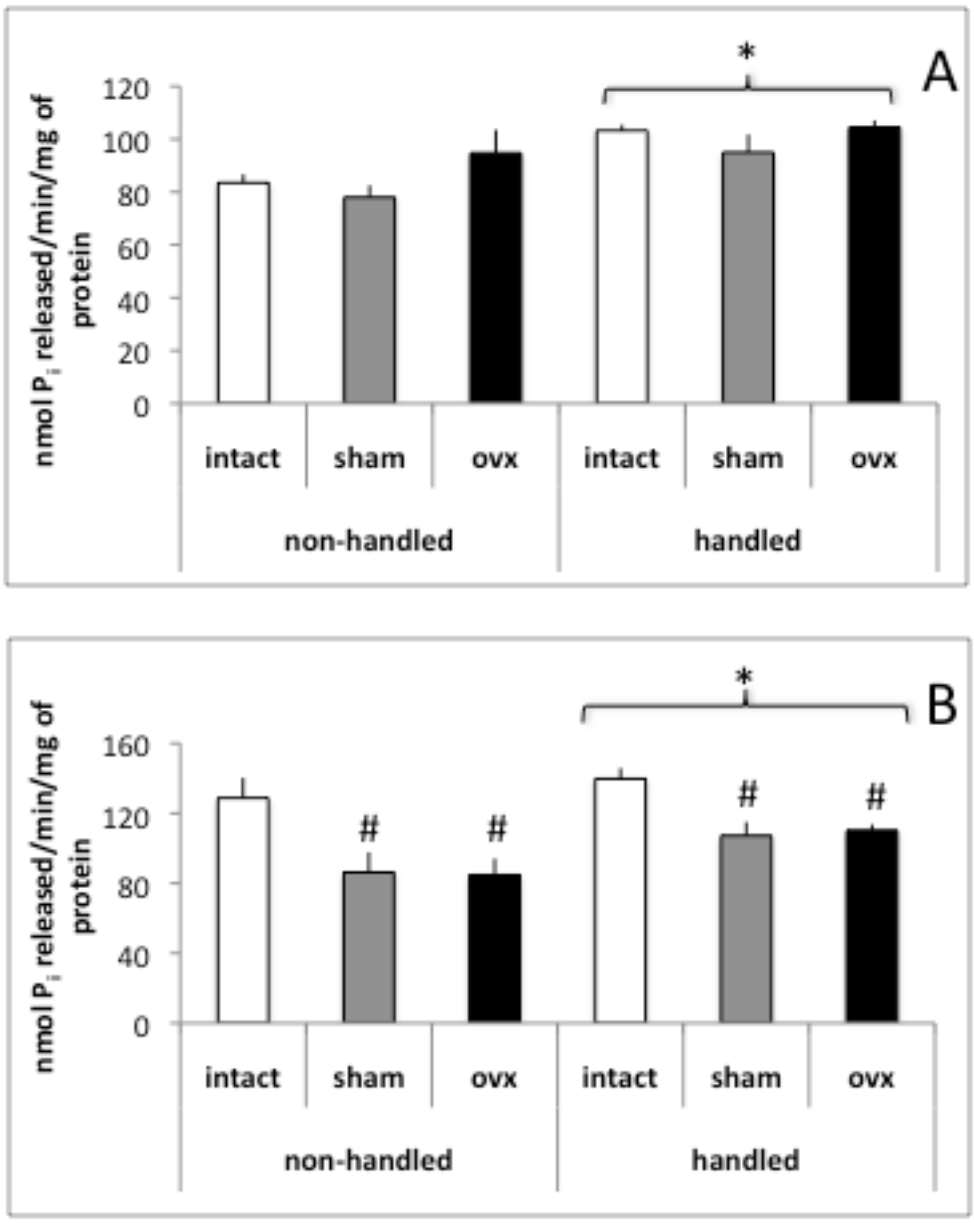

D
Chocolate consumed (g)
2.0
1.6
1.2
0.8
0.4
0.0
intact
sham
ovx
intact
sham
ovx
non-handled
handled

Figure 2


A
*
nmol P_i released/min/mg of protein
120
100
80
60
40
20
0
intact
sham
ovx
intact
sham
ovx
non-handled
handled
B
*
#
#
#
#
nmol P_i released/min/mg of protein
160
120
80
40
0
intact
sham
ovx
intact
sham
ovx
non-handled
handled

1.4 Capítulo 4

Neonatal handling affects oxidative stress, Na^+/K^+-ATPase and acetylcholinesterase in juvenile rats

Artigo submetido para publicação na revista *Brain & Development*

Neonatal handling affects oxidative stress, Na^+/K^+-ATPase and acetylcholinesterase in juvenile rats

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19 text pages , 2 figures and 2 tables

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Abstract

Intoduction: Considerable evidences demonstrate that the quality of the early environment influences patterns of development. Neonatal handling leads to altered behavior in adulthood, which may be related to neurochemical changes during development. The aim of this study was to investigate the effects of neonatal handling on oxidative stress parameters as well as on Na^+/K^+-ATPase and acetylcholinesterase activities in the hippocampus and olfactory bulb of male and female juvenile rats. Materials and Methods: Litters of rats were non-handled or handled (10 min/day, days 1–10 after birth). On postnatal day 21, biochemical parameters in the hippocampus and olfactory bulb were evaluated. Results: Juvenile male neonatal handled rats showed an increase in Na^+/K^+-ATPase activity in the olfactory bulb and a decrease in acetylcholinesterase activity in the hippocampus compared to non-handled ones. Female juvenile neonatal handled animals showed a decrease in glutathione peroxidase activity and in the total thiol content in the hippocampus compared to non-handled females, suggesting a higher susceptibility to oxidative insults. Conclusions: neonatal handling affects oxidative stress parameters as well as Na^+/K^+-ATPase and acetylcholinesterase activities differently in male and female juvenile rats. The effects are also dependent on the brain structure evaluated. This study reinforces the importance of considering the gender when evaluating neonatal handling effects.

Keywords

Neonatal handling; Oxidative stress; Na^+/K^+-ATPase; Acetylcholinesterase; Juvenile rats

1. Introduction

Considerable evidences demonstrate that the quality of the early environment influences patterns of development that, in turn, determine the health and productivity of the individual throughout their life span [1]. Some experimental approaches have been developed to study this interaction; one of these is neonatal handling, consisting of a brief, repeated, and apparently innocuous separation of pups from the mother during the neonatal period.

Besides the neonatal period, there are other critical stages during development. For example, in the olfactory bulb of rats, most neurons (75-80%) originate during the first three weeks of life [2]. Additionally, the peripuberal period is also a time of transition, and enhanced brain architecture [3]. During this period neuronal rearrangements occur, resulting in refined connectivity and functionality of brain regions in adulthood [4]. The hippocampus is one brain structure where maturational changes are evidenced during the peripuberal period [5].

Considering the effects of neonatal handling in the hippocampus and olfactory bulb of rats, earlier studies from our group have shown that neonatal handling increases the DNA break index in the hippocampus of adult male rats, an index that can be affected by an increased production of free radicals [6]. These reactive species can damage proteins, DNA and lipids, affecting cellular functions. In order to neutralize the effects of free radicals, the cell uses antioxidant defences, for example, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [7]. The brain is especially vulnerable to free radical-induced damage because of its high oxygen consumption, abundant lipid content and relative paucity of antioxidant enzymes [7].

Between the enzymes that are susceptible to free radicals attack are Na+/K+-ATPase and acetylcholinesterase (AChE) [8,9] and both have been related to cognition [8,10]. Although behavioral effects of neonatal handling have been reported mainly in adulthood [6,11], it is possible that neurochemical alterations might precede these effects, and it is not known if changes in Na^+/K^+-ATPase and AChE activities are present before puberty in neonatal handled rats.

Na^+/K^+-ATPase is a crucial enzyme, responsible for the generation of membrane potential through the active transport of sodium and potassium ions [12]. It is necessary to maintain the ionic gradient for neuronal excitability [12], consuming about 40–50% of the ATP generated in brain cells.

Acetylcholinesterase (AChE) is involved in the hydrolysis of the neurotransmitter acetylcholine and it has been shown that its stimulation could cause a decrease in acetylcholine in the synaptic cleft and, consequently, a decrease in cholinergic activity [13]. On the other hand, a significant correlation has been suggested between acetylcholinesterase inhibition and cognitive improvement [10].

Considering the exposed above, the aim of this study was to investigate the effects of neonatal handling on oxidative stress parameters as well as on Na^+/K^+-ATPase and AChE activities in the hippocampus and olfactory bulb of male and female juvenile rats. Since this period is a time of transition and enhanced brain architecture, we believed that neonatal handling would change oxidative stress parameters probably leading to an oxidative imbalance and this could affect the activity of the enzymes Na^+/K^+-ATPase and AChE.

2. Materials and Methods

2.1 Subjects

All animal procedures were approved by the Institutional Ethical Committee and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS), and of the Federation of Brazilian Societies for Experimental Biology. All efforts were made to minimize animal suffering as well as to reduce the number of animals.

Thirty-six pregnant Wistar rats bred at our own animal facility were randomly selected. Animals were housed alone from gestational day 18th in home cages made of Plexiglas (65 X 25 X 15 cm) with the floor covered with sawdust and were maintained in a controlled environment: lights on between 07:00 and 19:00 h, temperature of 22 ± 2°C, cage cleaning twice a week, food and water provided *ad libitum*. The day of birth was considered as day 0. All litters were culled within 24 h of birth to eight pups and were maintained undisturbed except for handling procedures which were carried out between 10:00 and 15:00 h. Several litters were submitted to this procedure on the same day. The researcher changed gloves between the handling procedures of each litter to avoid any kind of odor being spread from nest to nest. On postnatal day 21, one male and one female rat were killed per litter for biochemical measurements (the remaining animals were used in different studies when adults). Different animals were used for the evaluation of oxidative stress parameters, Na+/K+-ATPase and acetylcholinesterase activities.

2.2 Neonatal Handling [6]

Non-handled group: Pups were left undisturbed with the dam. It was stated on the cage that these animals should not be touched, not even for cage cleaning. Dirty sawdust was carefully removed from one side of the cage, without disturbing the

mother and the nest, and replaced by clean sawdust at that side by the main researcher.

Handled group: The dam was gently pulled to one side of the cage and the pups were removed from their home cage and placed in a clean cage lined with clean paper towel. This cage was placed into an incubator set to maintain an ambient temperature at 30–32°C. After 10 min, pups were returned to their dams. This procedure was performed from day 1–10 following birth and pups were then left undisturbed until postnatal day 21. It was also stated on the cage that these animals should not be touched, not even for cage cleaning. The same procedure for the non-handled group was performed to change dirty sawdust.

2.3 Preparation of the Samples for Biochemical Measurements

Animals were killed by decapitation on postnatal day 21; the olfactory bulb and hippocampus were quickly dissected out and stored at -70°C until analysis. These brain structures were homogenized in 10 vol (w:v) of ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. The homogenate was centrifuged (at 960 g) for 10 min at 4°C and the supernatant was used for the evaluation of reactive species production by the chemical oxidation of dichlorodihydrofluorescein (DCFH), the determination of total thiol content and antioxidant enzyme activities. For the determination of Na^+/K^+-ATPase activity, the cerebral structures were homogenized in 10 volumes (w:v) of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. The homogenate was centrifuged (at 960 g) for 10 min and the supernatant was used. In the case of acetylcholinesterase, the hippocampus and olfactory bulb were homogenized in 10 volumes of 0.1 mM potassium phosphate buffer, pH 7.5, centrifuged for 10 min at 1000 *g* and the supernatant was used.

2.3.1 Superoxide Dismutase (SOD) Activity

SOD activity was determined using a RANSOD kit (Randox Labs., USA) and it was expressed as U/mg of protein. One unit of SOD causes a 50% inhibition of the rate of reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride under the conditions of the assay.

2.3.2 Glutathione Peroxidase (GPx) Activity

GPx activity was determined according to Wendel [14], with modifications [6]. The reaction was carried out at 37°C and the activity of GPx was measured taking tert-butylhydroperoxide as the substrate at 340 nm. GPx activity was expressed as nmol NADPH oxidized per minute per mg protein.

2.3.3 Catalase (CAT) Activity

CAT is an enzyme that degrades hydrogen peroxide (H_2O_2) and its activity assessment is based upon establishing the rate of H_2O_2 degradation spectrophotometrically at 240 nm at 25°C [15]. CAT activity was calculated in terms of micromol of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$.

2.3.4 Evaluation of reactive species production by the chemical oxidation of DCFH [16]

The samples were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA 100μM) at 37° C for 30 minutes. DCFH-DA is cleaved by cellular esterases and the DCFH formed is eventually oxidized by reactive oxygen species (ROS) or reactive nitrogen species (RNS) present in the samples. The formation of the oxidized fluorescent derivative dichlorofluorescein (DCF) was monitored using excitation and emission wavelength of 488 and 525nm, respectively, using a spectrophotometer. The amount of reactive oxygen/nitrogen species was quantified

using a DCF standard curve and results were expressed as nmoles of DCF formed per mg of protein.

2.3.5 Determination of total thiol (SH) content

This assay measures protein thiol and non-protein thiol content, the latest mainly represented by the reduced form of glutathione. The method is based on the reduction of 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) by SH groups, which becomes oxidized (disulfide), yielding a yellow compound (TNB) whose absorption is measured spectrophotometrically at 412 nm [17].

2.3.6 Na^+/K^+-ATPase Activity

The reaction mixture for Na^+/K^+-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl and 40 mM Tris–HCl, pH 7.4. After 10 min of sample pre-incubation at 37ºC, the reaction was initiated by the addition of ATP to a final concentration of 3 mM and was incubated for 20 min. Controls were carried out under the same conditions with the addition of 1 mM ouabain. Na^+/K^+-ATPase activity was calculated by the difference between the two assays according to de Souza Wyse et al. [18]. Released inorganic phosphate (P_i) was measured by the method of Chan et al. [19]. Specific activity of the enzyme was expressed as nmol P_i released/min/mg of protein.

2.3.7 Acetylcholinesterase Activity (AChE)

AChE activity was verified, according to Ellman and colleagues [20], with some modifications. Hydrolysis rates were measured at acetylthiocholine (ASCh) concentration of 0.8 mM in 300 µL assay solution with 30 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB at 25 ºC. 15 µL of supernatant was added to the reaction mixture and pre-incubated for 3 min. The hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2-3 min (intervals of 30 s) and specific

enzyme activity was determined as μmol ASCh per hour per milligram of protein. All samples were run in triplicate.

2.3.8 Protein Assay

The total protein concentration was determined using the method described by Lowry et al. [21], with bovine serum albumin as standard, unless for the determination of Na^+/K^+-ATPase activity where total protein concentration was measured by Bradford [22].

2.4 Statistical Analysis

Data were expressed as mean ± standard error of the mean, and were analyzed using Student's *t* test for independent samples, controlling for homogeneity of variances using the Levine test. The significance level was accepted as different when the P value was equal or less than 0.05. The sex was not considered as a factor for the statistical analysis since the evaluation of the biochemical parameters in male and females were done in different moments.

3. Results

Male neonatal handled animals showed a decrease in AChE activity in the hippocampus [$t(8)= 2.638$, $P=0.03$] (Figure 1A), as well as an increase in Na^+/K^+-ATPase activity in the olfactory bulb [$t(7)=2.62$, $P=0.03$] (Figure 2B) compared to non-handled males. No difference was observed between groups in male rats regarding the total thiol content, the reactive species production (DCFH oxidation) and the antioxidant enzyme activities ($P>0.05$) in the hippocampus (Table 1) and in the olfactory bulb (Table 2). Additionally, no difference was observed between groups in the AChE activity in the olfactory bulb (Figure 1B) as well as in the

Na^+/K^+-ATPase activity in the hippocampus (Figure 2A) considering male rats ($P>0.05$).

Female neonatal handled animals showed a decrease in GPx activity [$t(9)=2.827$, $P=0.02$] as well as in total thiol content [$t(7)=2.952$, $P=0.02$] in the hippocampus (Table 1) compared to non-handled females. No difference between groups was observed in the SOD, CAT, Na^+/K^+-ATPase and AChE activities as well as in the reactive species production (DCFH oxidation) in the hippocampus of female animals (Table 1 for SOD, CAT and DCFH oxidation; Figure 2A for Na^+/K^+-ATPase; Figure 1A for AChE) ($P>0.05$). Moreover, there was no difference between groups in all biochemical parameters evaluated in this study when considering the olfactory bulb of female rats ($P>0.05$) (Table 2, Figure 1B and Figure 2B).

4. Discussion

The main findings of this study were that juvenile male neonatal handled animals had an increased Na^+/K^+-ATPase activity in the olfactory bulb and a decreased AChE activity in the hippocampus when compared to male non-handled animals. Additionally, juvenile female neonatal handled rats had a decreased GPx activity and total thiol content compared to non-handled females in the hippocampus. The results regarding females' hippocampus support our hypothesis that the neonatal handling would change oxidative stress parameters. On the other hand, Na^+/K^+-ATPase and AChE activities were not modified in female neonatal handled rats counteracting the idea that an oxidative imbalance would affect the activity of these enzymes. With regard to males' findings, the results do not support our hypothesis suggesting different possibilities as we discuss below.

Neonatal handling affected the biochemical parameters evaluated on this paper differently in male and female animals. Our group and others have already demonstrated that neonatal handling leads to sex-specific differences in adult and adolescent rats on behavioral and biochemical parameters [6,11,23]. Considering oxidative stress evaluation during adulthood, female neonatal handled rats presented alterations in the antioxidant enzyme activities when compared to non-handled females while no difference was observed in males [6,11]. It is important to notice that the effects during adulthood are related to the brain structure analysed [6,11] as we also demonstrated here in juvenile rats. The mechanisms to explain the differences regarding the gender are still unknown, however previous studies have indicated that rodent dams spend more time rearing male pups than females [24] and this may contribute to the different effects observed on the present study in juvenile male and female handled rats as well as other studies considering sex-difference in adult neonatal handled rats.

In this study, we observed that the effects on female rats were restricted to the hippocampus, with a lower GPx activity and total thiol content in female neonatal handled animals compared to non-handled animals. GPx is an antioxidant enzyme that is involved in the degradation of H_2O_2. A decrease in its activity can lead to an increase in H_2O_2 levels, which can interact with superoxide anion leading to the formation of the highly reactive hydroxyl radical, and this can also happen when H_2O_2 is in the presence of iron [25]. Hydroxyl radical, in turn, can damage lipids, protein and DNA. Once the total thiol content assay measures protein thiol and non-protein thiol amount, the latter mainly represented by the reduced form of glutathione, and large portion of biological properties and functions involving protein structure, enzyme catalysis, and redox signaling pathways depends on the redox properties of

the thiol group present both in protein and in low-molecular-weight molecules [26], an alteration in total thiol content can badly affect the cell functionality. With regard to the oxidative stress scenario presented here, a relevant point is the fact that there was no difference in the chemical oxidation of DCFH, however this assay does not detect peroxides very well [7] and this may explain the absence of effect observed on the reactive species production. Additionally, although there was a significant statistical effect on GPx activity and on total thiol content in female neonatal handled rats compared to non-handled ones, the difference between groups was not very high and one could question the biological significance of these effects; however it is possible that these animals have a predisposition to suffer oxidative stress damage and thus, facing adverse situations, the differences observed here would increase.

The observation that an adverse effect of neonatal handling occurred only in females regarding oxidative stress is interesting considering that an impairment on water maze learning, a task which is dependent on the hippocampal function, was observed in adult female rats that were handled in the neonatal period [6]. Additionally, sex-specific differences towards the stress response have been shown in neonatal handled animals during puberty, with females being more susceptible to stress effects during this period [23]: neonatal handling reduced restraint stress-induced hormone levels in adolescent males and increased plasma hormone concentrations in handled females [23]. Since it has been shown that high corticosterone levels induce reactive oxygen species production leading to oxidative damage in the hippocampus [27], the higher susceptibility of neonatal handled female hippocampus to oxidative stress may be related to their higher response to stress, and to the spatial memory impairment observed later in life [6].

The effects regarding juvenile male neonatal handled animals are related to Na^+/K^+-ATPase and AChE activities and are dependent on the brain structure. Although both enzymes' functionality can be affected by free radicals [8,9], it does not appear to be the case in the present study, since none of the oxidative stress parameters evaluated was affected in the hippocampus and olfactory bulb of juvenile male neonatal handled rats. Additionally, an inhibition of Na^+/K^+-ATPase activity has been associated with free radicals [8], and we observed an increased activity of this enzyme in the olfactory bulb of juvenile male neonatal handled animals.

Na^+/K^+-ATPase is very important to brain function. It is responsible for the active transport of sodium and potassium ions, maintaining and re-establishing, after each depolarization, the electrochemical gradient necessary for neuronal excitability and regulation of neuronal cell volume [12]. It was shown that Na^+/K^+-ATPase activity increased in brain after incubation with serotonin, suggesting that this neurotransmitter modulates brain Na^+/K^+-ATPase activity through a specific receptor located in target neurons or glial cells [28]. We observed an increase in Na^+/K^+-ATPase activity in the olfactory bulb of juvenile male neonatal handled rats compared to non-handled animals. Additionally, an earlier study showed that neonatal handling increased serotonin activity in the olfactory bulb of 7 days old male rats [29]. Although the age of our animals differs from that previous study and the neonatal handling procedure used was somehow different, we believe that serotonin might be involved in the neonatal handling effects regarding Na^+/K^+-ATPase activity in the olfactory bulb of juvenile male rats. More studies are needed to confirm this hypothesis.

It has been proposed that Na^+/K^+-ATPase not only pumps Na^+ and K^+ across cell membranes, but also relays the extracellular ouabain signal to intracellular

compartments via activation of different protein kinases, resulting in the assembly and activation of different pathways [30]. Therefore, increased ouabain signaling in the olfactory bulb of juvenile male neonatal handled animals would be a possibility and further studies are required. In addition, the increased Na^+/K^+-ATPase activity induced by neonatal handling may be related to neuroplastic changes in the olfactory bulb of juvenile male rats.

AChE catalyses the hydrolysis of acetylcholine, thereby interrupting cholinergic activity in the synapse. It has been associated with brain development, learning and memory [31]. It was shown that cholinergic activation in the hippocampus is necessary for spatial memory formation [32] and that extracellular acetylcholine levels in the hippocampus increase during a spatial memory task [10]. In addition, several "nonclassical" AChE activities have been described, as neurite growth and synaptic development and maintenance [31]. In normal rats, the number of cholinergic fibers that reach the hippocampal formation increases between embryonic day 20 and postnatal day 3, and the adult pattern of cholinergic innervation is reached between postnatal day 14 and 21 [33].

Considering the influence of early life events (maternal care variation) on cholinergic function, it was observed an increased hippocampal choline acetyltransferase activity and acetylcholinesterase staining as well as increased hippocampal acetylcholine release in the adult offspring of mothers that show high levels of pup licking and grooming and arched-back nursing. Additionally, an enhanced spatial learning and memory was observed [34]. We observed a decrease in AChE activity in the hippocampus of juvenile male neonatal handled rats when compared to non-handled animals, and this decreased activity might be related to increased levels of acetylcholine. Therefore, it would be interesting to see if juvenile

male neonatal handled rats have memory improvement; however this was not the focus of this study and future researches may address this point. This could also help to elucidate the question about the biological significance of the effect observed here since the decrease in AChE activity presented by juvenile male neonatal handled rats was of 11% compared to non-handled ones.

In conclusion, neonatal handling affects oxidative stress parameters as well as Na^+/K^+-ATPase and AChE activities differently in juvenile male and female rats, with females being more susceptible to oxidative stress modifications, while males are more vulnerable to changes on Na^+/K^+-ATPase and AChE activities. The effects are also dependent on the brain structure evaluated. This study suggests that hippocampal oxidative stress in juvenile females may be related to impairments later in life and reinforces the importance of considering the gender when evaluating neonatal handling effects.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Evaluation of oxidative stress parameters in the hippocampus of juvenile neonatal handled and non-handled rats.

	Males		Females	
	Non-handled	Handled	Non-handled	Handled
DCF content	6.12 ± 0.38 (4)	6.81 ± 1.23 (4)	7.22 ± 0.43 (5)	7.05 ± 0.45 (4)
SOD	6.45 ± 1.02 (6)	5.59 ± 0.60 (6)	3.46 ± 0.23 (6)	4.14 ± 0.43 (6)
GPx	14.90 ± 0.75 (5)	14.33 ± 0.54 (5)	14.79 ± 0.41 (6)	12.93 ± 0.53* (5)
CAT	2.66 ± 0.30 (4)	2.45 ± 0.36 (6)	2.73 ± 0.30 (5)	2.32 ± 0.11 (4)
Total thiol content	49.31 ± 2.39 (4)	48.60 ± 1.18 (5)	50.57 ± 0.81 (5)	47.47 ± 0.58* (4)

Data are expressed as mean ± S.E.M. of SOD (U/mg protein), GPx (nmol NADPH oxidized/min/mg protein), and CAT (μmol H_2O_2 transformed/min/mg protein) activities. For DCF and thiol content data are expressed as nmoles of DCF formed per mg of protein and nmoles of SH per mg of protein. Males, N=4-6/group. Females, N=4-6/group. The exact number of animals used is specified between parentheses.

* Significantly different compared to non-handled female group (Student's t test, $P < 0.05$)

Table 2. Evaluation of oxidative stress parameters in the olfactory bulb of juvenile neonatal handled and non-handled rats.

	Males		Females	
	Non-handled	Handled	Non-handled	Handled
DCF content	6.62 ± 1.08 (5)	4.89 ± 0.21 (5)	7.09 ± 1.23 (5)	7.77 ± 1.47 (4)
SOD	5.43 ± 0.73 (4)	4.80 ± 0.81 (5)	6.41 ± 1.81 (6)	9.07 ± 1.94 (5)
GPx	20.68 ± 3.09 (4)	23.16 ± 1.25 (5)	22.82 ± 2.46 (5)	24.25 ± 1.87 (4)
CAT	2.67 ± 0.24 (4)	3.05 ± 0.25 (5)	3.20 ± 0.21 (5)	2.90 ± 0.39 (4)
Total thiol content	34.25 ± 1.62 (4)	34.24 ± 3.05 (4)	32.79 ± 3.93 (4)	37.10 ± 2.23 (4)

Data are expressed as mean ± S.E.M. of SOD (U/mg protein), GPx (nmol NADPH oxidized/min/mg protein), and CAT (μmol H_2O_2 transformed/min/mg protein) activities. For DCF and thiol content data are expressed as nmoles of DCF formed per mg of protein and nmoles of SH per mg of protein. Males, N=4-5/group. Females, N=4-6/group. The exact number of animals used is specified between parentheses.

Student's t test showed no difference between groups neither in male nor in females in all parameters evaluated.

Legends to Figures

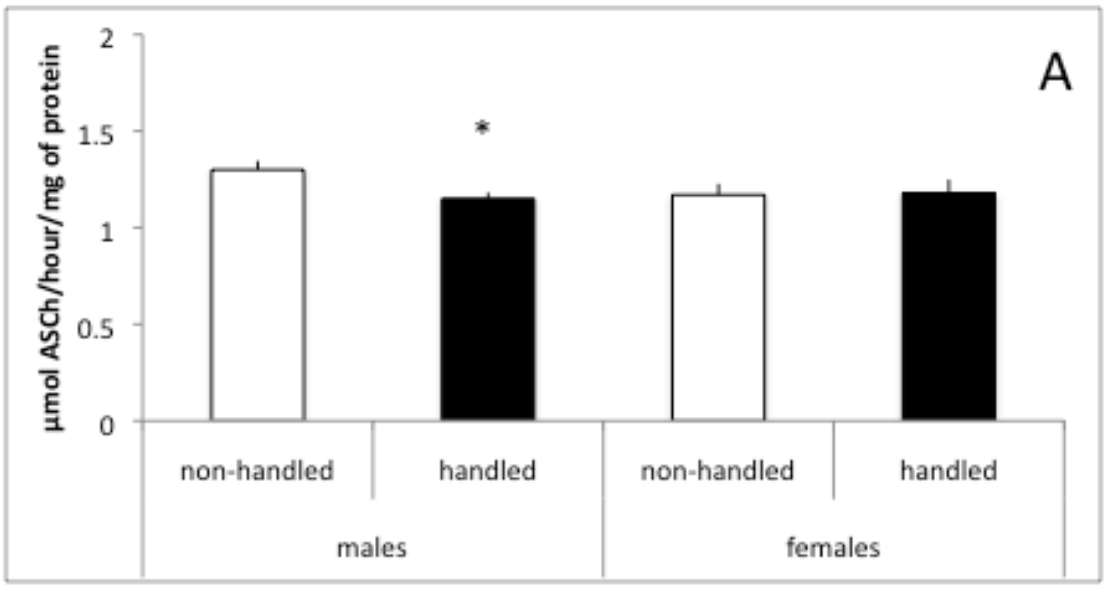
Fig. 1 Effects of neonatal handling on AChE activity in the hippocampus (A) and olfactory bulb (B) of male and female juvenile rats. Data are expressed as mean + S.E.M. N = 5/group. Student's t test showed a decrease in AChE activity in the hippocampus of male neonatal handled animals compared to non-handled ones ($P<0.05$).

* Different compared to non-handled males.

Fig. 2 Effects of neonatal handling on Na^+/K^+-ATPase activity in the hippocampus (A) and olfactory bulb (B) of male and female juvenile rats. Data are expressed as mean + S.E.M. N = 4-7/group. Student's t test showed an increase in Na^+/K^+-ATPase activity in the olfactory bulb of male neonatal handled animals compared to non-handled ones ($P<0.05$).

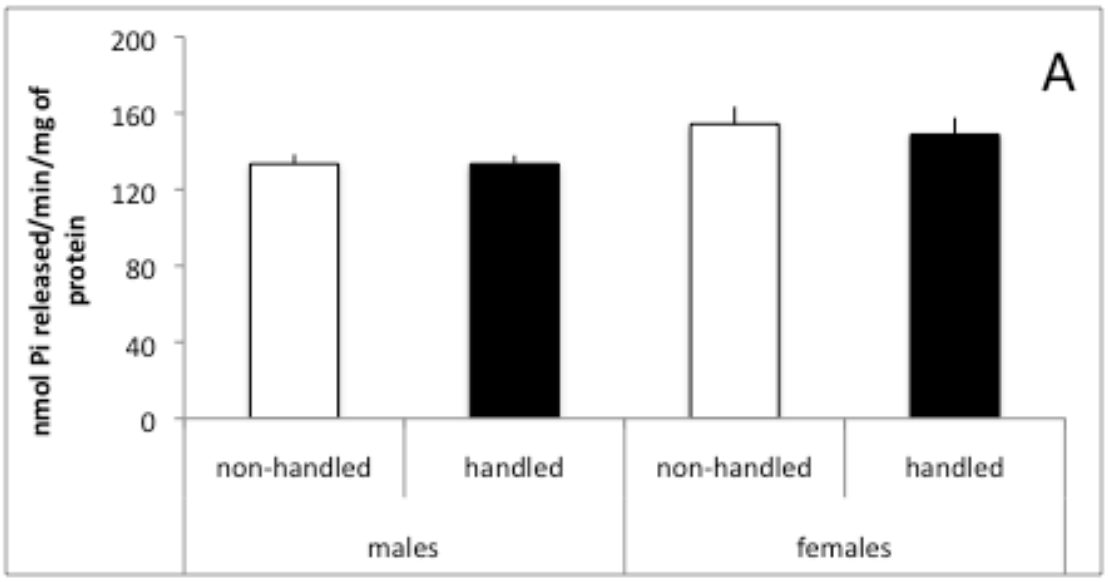
* Different compared to non-handled males.

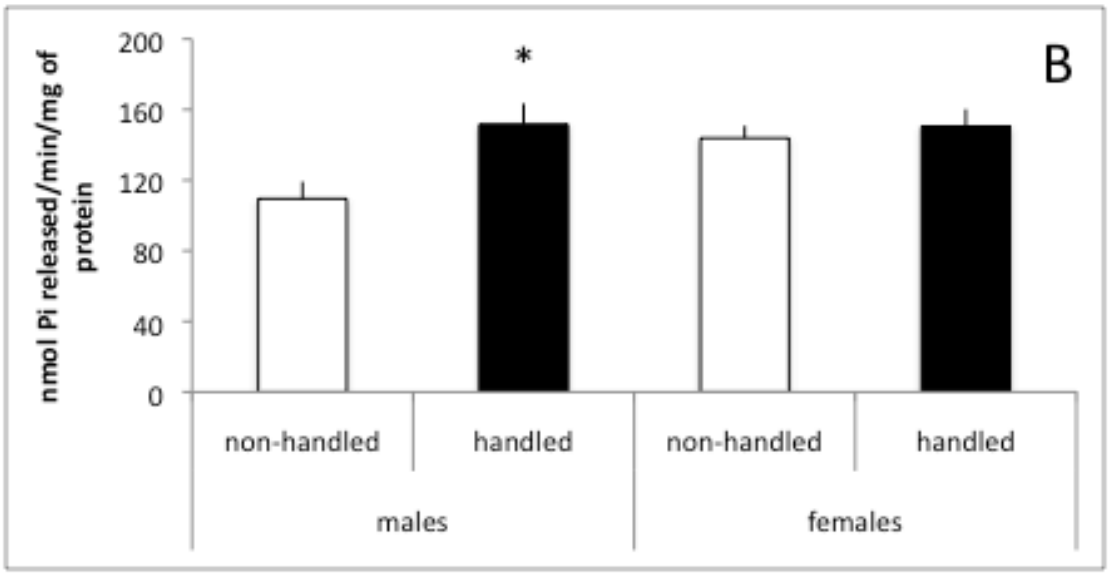
Figure 1


A
µmol ASCh/hour/mg of protein
2
1.5
1
0.5
0
*
non-handled
handled
non-handled
handled
males
females


B
µmol ASCh/hour/mg of protein
1.5
1
0.5
0
non-handled
handled
non-handled
handled
males
females

Figure 2


A
nmol Pi released/min/mg of protein
200
160
120
80
40
0
non-handled
handled
non-handled
handled
males
females


B
*
nmol Pi released/min/mg of protein
200
160
120
80
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non-handled
handled
non-handled
handled
males
females

PARTE III

1. DISCUSSÃO

Esta tese mostra interessantes resultados com relação aos efeitos da manipulação neonatal, um procedimento aparentemente inócuo no início da vida. Este procedimento foi capaz de alterar o aprendizado e a memória de ratos na vida adulta, bem como parâmetros neuroquímicos tanto no período antes da puberdade quanto na idade adulta. Mais especificamente, este trabalho mostrou que a manipulação neonatal provocou um prejuízo na memória espacial em fêmeas mas não em machos, uma melhora na memória de hábito em fêmeas e um prejuízo no aprendizado reverso relacionado a este tipo de memória em machos. Além disso, não alterou a memória olfatória em fêmeas. Com relação aos parâmetros neuroquímicos, a manipulação neonatal aumentou o índice de quebras ao ADN em machos, reduziu a produção de óxido nítrico e aumentou a atividade da enzima Na^+/K^+-ATPase em fêmeas. Esses efeitos foram observados no hipocampo de ratos adultos. A manipulação neonatal também reduziu a relação SOD/CAT no córtex pré-frontal e aumentou a atividade da enzima Na^+/K^+-ATPase no bulbo olfatório, ambos efeitos observados em fêmeas adultas. Considerando o período antes da puberdade, a manipulação neonatal provocou um aumento na atividade da enzima Na^+/K^+-ATPase no bulbo olfatório e uma diminuição na atividade da enzima acetilcolinesterase no hipocampo de ratos machos. Nas fêmeas, essa intervenção precoce diminuiu a atividade da enzima GPx e também reduziu o conteúdo total de tióis no hipocampo. Com relação ao exposto acima fica evidente que a manipulação neonatal afetou os parâmetros avaliados nesta tese de forma sexo-específica e que os efeitos dependem do período da vida e da estrutura cerebral avaliada.

Acerca dos efeitos da manipulação neonatal na memória, diferentes resultados têm sido demonstrados entre os estudos abordando este tema, como será discutido a seguir. Estudos considerando a memória espacial mostraram que ratos machos

manipulados no período neonatal apresentaram um menor declínio cognitivo associado à idade com relação aos ratos controles, efeito este observado na tarefa do Labirinto Aquático de Morris (MEANEY ET AL., 1988), uma tarefa bem adaptada para o estudo da memória espacial utilizando fatores visuo-espaciais (HODGES, 1996; ANDERSEN e TUFIK, 2010). Utilizando essa mesma tarefa, também foi demonstrado que a manipulação neonatal não afetou o aprendizado e a memória espacial em machos aos 4, 6 e 7 meses de idade (VALLÉE ET AL., 1997; MEANEY ET AL., 1988; VALLÉE ET AL., 1999). Em outra tarefa avaliando a memória espacial, onde um labirinto em Y foi utilizado, ratos machos manipulados no período neonatal também não diferiram dos controles aos 6 meses de idade (VALLÉE ET AL., 1999). Além disso, esses animais não apresentaram diferença no aprendizado reverso com relação aos controles (VALLÉE ET AL., 1997). Esses dados concordam com os resultados desta tese no Labirinto Aquático de Morris com relação aos ratos machos. Ainda em relação a este sexo, a avaliação da memória espacial (usando o Labirinto Aquático de Morris) em ratos submetidos previamente a 30 minutos de estresse agudo por contenção mostrou que os animais manipulados no período neonatal têm um melhor desempenho na tarefa em questão (GAROFLOS ET AL., 2005). Este dado, juntamente com a constatação de que a manipulação neonatal diminui o declínio cognitivo associado com a idade (MEANEY ET AL., 1988), conforme comentado acima, mostram que os efeitos desta intervenção precoce sobre a memória espacial em ratos machos dependem da idade do animal bem como da sua condição (estressado ou não). No conjunto, poderíamos sugerir que situações que possam prejudicar a memória (envelhecimento ou estresse) apresentam menor impacto em ratos machos manipulados. É interessante ressaltar que esta tese mostrou que ratos jovens machos manipulados no período neonatal apresentaram uma redução

na atividade da enzima acetilcolinesterase no hipocampo. Esta redução não parece estar associada a um desequilíbrio oxidativo, contrariando a hipótese levantada no capítulo 4. Por outro lado, conforme descrito na introdução, esta enzima hidrolisa o neurotransmissor acetilcolina (MONTEIRO ET AL., 2005) e os níveis extracelulares deste neurotransmissor aumentam durante uma tarefa de memória espacial (STANCAMPIANO ET AL., 1999). Desta forma, conforme discutido no capítulo 4, uma redução na atividade da acetilcolinesterase pode estar relacionada a um aumento nos níveis extracelulares de acetilcolina, e seria interessante averiguar se ratos machos jovens manipulados no período neonatal apresentam um melhor desempenho na tarefa do Labirinto Aquático de Morris. Além disso, seria também interessante verificar a atividade desta enzima em ratos machos senis, uma vez que, conforme descrito acima, ratos machos manipulados no período neonatal apresentam um menor declínio cognitivo associado à idade com relação aos controles não-manipulados (MEANEY ET AL., 1988).

Considerando os efeitos da manipulação neonatal sobre diferentes tipos de memórias em ratos machos e fêmeas, Kosten e colaboradores (2007) demonstraram que este procedimento não tem efeito no labirinto circular e melhora a memória relacionada ao reconhecimento de objetos em ambos os sexos. O teste do labirinto circular avalia a memória espacial, assim como o Labirinto Aquático de Morris, sendo que a diferença entre eles é que, no primeiro, o escape é da luz para um compartimento escuro, enquanto no segundo o escape consiste em alcançar uma plataforma para deixar de nadar (BARNES, 1979). Já a tarefa de reconhecimento de objetos pode ser dependente do hipocampo (BAKER e KIM, 2002; HAIJIMA e ICHITANI, 2012) ou não, dependendo das estratégias utilizadas na tarefa (BARKER e WARBURTON, 2011), e pode basear-se em uma memória não-espacial. Outro

estudo também não encontrou efeito da manipulação neonatal sobre a memória espacial considerando ratos machos e fêmeas adultos (3 a 6 meses de idade). Neste caso, a tarefa utilizada para avaliar a memória foi o Labirinto Aquático de Morris (PRYCE ET AL., 2003). Com relação a esses resultados, existe uma concordância com os achados desta tese com relação aos ratos machos, mas não com relação às fêmeas. É importante ressaltar que, tanto no estudo conduzido por Kosten e colaboradores (2007) quanto nos conduzidos por Pryce e colaboradores (2003), o procedimento de separação dos filhotes das suas mães foi diferente do executado nesta tese. No caso deles, a separação foi feita do dia pós-natal 1 ao 21, por 15 minutos, com os filhotes irmãos ficando separados uns dos outros durante o procedimento, enquanto no presente trabalho foi utilizada a separação do dia pós-natal 1 ao 10, por 10 minutos, com os filhotes irmãos permanecendo juntos. Se a diferença de protocolo usado para a manipulação neonatal justifica diferentes efeitos observados na memória espacial de fêmeas, surge por outro lado a pergunta: por que não modifica os resultados encontrados com relação aos machos? Conforme demonstramos nos capítulos 1, 2 e 4, a manipulação neonatal afeta de modo diferente machos e fêmeas em vários parâmetros; desta forma, pode ser que variações no protocolo da breve separação dos filhotes das suas mães no início da vida afetem as fêmeas e não os machos na vida adulta com relação à memória espacial.

Concordando com os achados no comportamento, estão os resultados bioquímicos com relação à produção de óxido nítrico. Ou seja, a manipulação neonatal prejudicou a memória espacial e reduziu a produção de óxido nítrico (determinado medindo os metabólitos do óxido nítrico, nitratos e nitritos) no hipocampo em ratas adultas, enquanto que nos machos ambos não foram alterados. Tem sido sugerido o envolvimento do óxido nítrico nos processos de aprendizado e

memória conforme descrito na introdução deste trabalho (QUIAN ET AL., 1997; DEMIRGOREN e POGUN, 1995). Muitos efeitos do óxido nítrico são mediados através do seu receptor canônico, a guanilato-ciclase solúvel e do segundo mensageiro monofosfato de guanosina cíclico (GMPc) (FEIL e KLEPPISCH, 2008). O óxido nítrico estimula a guanilato-ciclase solúvel para formar o segundo mensageiro GMPc nas células alvo. A via óxido nítrico – GMPc tem sido implicada na indução hipocampal da LTP e da LTD (depressão de longa duração, do inglês *long term depression*), as quais são referidas como os mecanismos predominantes dos processos de aprendizado e memória (PAUL e EKAMBARAM, 2011). Além disso, a LTP é dependente do influxo de cálcio através do receptor NMDA pós-sináptico. De acordo com a hipótese de que o óxido nítrico age como um mensageiro retrógrado, sugere-se que o cálcio se ligue a calmodulina e ative a óxido nítrico sintase, a qual converte arginina em citrulina levando à formação do óxido nítrico, que então se difunde para o terminal pré-sináptico, possibilitando um aumento na liberação do neurotransmissor glutamato (HUANG, 1997). Estudos em ratos demonstraram que a inibição da óxido nítrico sintase leva a um ligeiro prejuízo da memória, enquanto que doadores de óxido nítrico facilitam a memória no Labirinto Aquático de Morris (DEMIRGOREN e POGUN, 1995). Assim, o prejuízo na memória espacial associado a diminuição na produção de óxido nítrico em ratas manipuladas no período neonatal estão de acordo com os relatos da literatura em relação a memória versus óxido nítrico. No entanto, mais estudos são necessários para entender porque a manipulação neonatal reduz a produção de óxido nítrico em fêmeas adultas. Deve-se observar que, em nosso estudo, essa alteração foi observada no estado basal, e não se pode afirmar qual seria o status da produção de óxido nítrico após uma tarefa comportamental, por exemplo. Por outro lado, vale também ressaltar que uma outra possibilidade considerando o óxido

nítrico, seria que a redução nos níveis de nitratos e nitritos observada no capítulo 1 nas fêmeas manipuladas no período neonatal estararia associada à um aumento nos níveis de peroxinitrito e não significaria necessariamente uma redução na produção de óxido nítrico. O peroxinitrito, formado pela reação do ânion superóxido com o óxido nítrico, pode reagir com diferentes biomoléculas incluindo proteínas levando a alterações na estrutura e na função destas (ALVAREZ e RADI, 2003; AUGUSTO, 2006; RUBBO, TROSTCHANSKY e O'DONNELL, 2009) e isto poderia estar relacionado com o prejuízo observado na memória.

Ainda com relação às alterações bioquímicas provocadas no hipocampo de fêmeas adultas pela manipulação neonatal, um aumento na atividade da enzima Na^+/K^+-ATPase foi observado. Sabe-se que a inibição da atividade desta enzima esta relacionada com um prejuízo no aprendizado espacial (ZHAN ET AL., 2004). Estes resultados contrariam o déficit de memória espacial observado nas fêmeas manipuladas. No entanto, a atividade aumentada da Na^+/K^+-ATPase no hipocampo pode estar relacionada a outros processos que não a memória, uma vez que conforme descrito na discussão do capítulo 3, a atividade desta enzima parece ser modulada por neurotransmissores como a serotonina e a noradrenalina (PEÑA-RANGEL, MERCADO e HERNÁNDEZ-RODRIGUEZ, 1999; HERNÁNDEZ-R, 1992).

Alterações bioquímicas sugerindo um desequilíbrio oxidativo foram observadas no hipocampo de fêmeas jovens manipuladas no período neonatal. A redução da atividade da GPx, associada à diminuição no conteúdo total de tióis, pode afetar gravemente o funcionamento celular conforme detalhado no capítulo 4. Poderíamos pensar que alterações no funcionamento da célula neste período (pré-púbere) estejam relacionadas com o prejuízo na memória espacial observado em fêmeas adultas manipuladas no período neonatal. Por outro lado, o desequilíbrio

oxidativo observado foi restrito ao período pré-púbere, não havendo alterações nos parâmetros de estresse oxidativo avaliados no hipocampo na idade adulta nas fêmeas manipuladas, conforme relatado nos capítulos 1 e 3. É importante ressaltar que o desequilíbrio oxidativo foi observado no período antes da puberdade. Estudos sugerem que a presença de hormônios gonadais durante o desenvolvimento possa afetar o estresse oxidativo, uma vez que propriedades neuroprotetoras têm sido associadas ao estradiol (PREDIGER ET AL., 2004; BEHL ET AL., 2000). No entanto, o capítulo 3 desta tese mostra que a remoção dos ovários antes da puberdade não alterou os parâmetros do estresse oxidativo avaliados no hipocampo de ratas adultas manipuladas ou não no período neonatal. Ainda com relação ao desequilíbrio oxidativo observado no período pré-púbere, sabe-se que fêmeas manipuladas no período neonatal são mais suscetíveis aos efeitos do estresse durante a puberdade, apresentando níveis aumentados de corticosterona (PARK ET AL., 2003). Uma vez que tem sido demonstrado que altos níveis de corticosterona induzem a produção de ERO levando ao dano oxidativo no hipocampo (SATO ET AL., 2010), a suscetibilidade aumentada ao estresse oxidativo apresentada nesta estrutura por fêmeas jovens manipuladas no período neonatal pode estar relacionada a sua maior resposta ao estresse. No entanto, no estudo feito por Park e colaboradores (2003), os animais apresentavam 40 dias de idade. Seria interessante verificar como os ratos manipulados de 21 dias se comportam frente ao estresse, e como este afeta os níveis de corticosterona e os parâmetros relacionados ao estresse oxidativo no período pré-púbere. Outro ponto importante é o fato de, embora haver significância estatística na atividade da GPx e no conteúdo total de tióis avaliados em fêmeas jovens manipuladas no período neonatal com relação as controles, a diferença entre os grupos foi pequena, o que levanta a questão sobre a significância biológica destes

achados. Além disso, o desequilíbrio oxidativo observado não afetou a atividade das enzimas Na^+/K^+-ATPase e acetilcolinestrease, contrariando a hipótese levantada no capítulo 4.

Esta tese avaliou o efeito da manipulação neonatal sobre outros tipos de memória além da memória espacial em ratos adultos. Concordando com os efeitos sexo-específicos da manipulação neonatal sobre a memória aqui apresentados estão os trabalhos mencionados na discussão do capítulo 2 desta tese (WEINER ET AL., 1985; WEINER, FELDON e ZIV-HARRIS, 1987). Utilizando o labirinto em Y, avaliamos a memória de formação de hábito para a localização do braço contendo o alimento, e observamos os efeitos descritos a seguir. Ratos machos manipulados no período neonatal apresentaram um pior desempenho com relação aos controles no aprendizado reverso enquanto que as fêmeas manipuladas no período neonatal apresentaram um melhor desempenho na fase de aprendizado quando comparadas às controles. A tarefa do labirinto em Y usada nesta tese foi feita sob luz vermelha, desta forma os ratos não eram capazes de usar dicas espaciais e dependiam de dicas egocêntricas, relacionadas à resposta, para determinar em qual braço do labirinto entrariam. O aprendizado reverso foi realizado no sentido de avaliar a rigidez comportamental apresentada por esses ratos. Curiosamente os machos manipulados no período neonatal persistiram mais do que os controles na entrada no braço antigo quando o alimento foi colocado no novo braço, no entanto essa rigidez comportamental não foi observada na tarefa do aprendizado reverso do Labirinto Aquático de Morris. Parece que o déficit no aprendizado reverso apresentado pelos machos manipulado no período neonatal está associado à memória de formação de hábito associada ao alimento palatável e não a memória espacial. Por outro lado, um fator relevante e diferente entre as duas tarefas (labirinto em Y e Labirinto Aquático

de Morris) é o fato de o labirinto em Y usar o alimento palatável e o labirinto aquático não. Seria interessante ver como os ratos machos manipulados no período neonatal executariam a tarefa do Y associada ao alimento palatável em outra versão onde eles pudessem utilizar dicas espaciais. O aprendizado reverso é importante para direcionar o comportamento de acordo com as alterações no ambiente. Conforme discutido no capítulo 2 foi demonstrado que animais machos super-expressando receptores CB1 no córtex pré-frontal também apresentaram um déficit no aprendizado reverso em uma tarefa utilizando alimento palatável (KLUGMANN ET AL., 2011), sugerindo desta forma o envolvimento do sistema endocanabinóide neste processo. Assim, tanto naquele estudo quanto no nosso, os déficits foram observados apenas quando os animais tinham que alterar a estratégia aprendida e, desta forma, sugere que os mecanismos de armazenamento da memória não foram afetados.

Estudos também mostraram que a administração sistêmica de um inibidor da acetilcolinesterase (donepezila) melhorou o desempenho de ratos no aprendizado reverso de uma tarefa associada ao alimento (CHEN ET AL., 2009), sugerindo que alterações no sistema colinérgico podem estar envolvidas com a rigidez comportamental. Quanto à possibilidade de um desequilíbrio oxidativo estar relacionado com alterações na memória de hábito em machos, os resultados desta tese sugerem não ser o caso uma vez que não houve alterações nos parâmetros de estresse oxidativo avaliados no estriado e córtex pré-frontal nos animais deste sexo. Com relação às fêmeas manipuladas no período neonatal, o que aconteceu na fase de aprendizado dos 2 testes em questão foi de certa forma o contrário do que vimos nos machos no aprendizado reverso. Fêmeas manipuladas no período neonatal tiveram um prejuízo no aprendizado espacial, enquanto que no aprendizado na tarefa do labirinto em Y foram melhores do que as controles. Sabe-se que o estriado é uma estrutura

chave na memória de hábito (KREITZER, 2009). Sendo assim e visto que, como comentado acima, o óxido nítrico está envolvido com a memória, e que fêmeas manipuladas no período neonatal apresentaram níveis reduzidos de óxido nítrico no hipocampo e um prejuízo na memória espacial, poderíamos pensar que fêmeas manipuladas no período neonatal que apresentam um melhor desempenho no labirinto em Y tenham níveis aumentados de óxido nítrico no estriado. No entanto isso não ocorreu, e os níveis de óxido nítrico nesta estrutura não diferiram entre fêmeas manipuladas ou não (Noschang C. resultados não publicados).

Outra possibilidade para os diferentes efeitos da manipulação neonatal sobre as memórias avaliadas em fêmeas seria em função da alteração de receptores de estrógeno no hipocampo. Sabe-se que alterações no ambiente neonatal alteram a expressão gênica e os níveis de receptores de estrógeno em fêmeas (CURLEY ET AL., 2011). Estudos demonstram que o estradiol age através dos receptores de estrógeno α e β em várias sub-regiões hipocampais, levando a alterações que contribuem para a influência deste hormônio na plasticidade sináptica hipocampal (SPENCER-SEGAL ET AL., 2012). Desta forma, se a manipulação neonatal for capaz de reduzir os receptores de estrógenos hipocampais, podemos ter um prejuízo na memória associado a esta estrutura. Por outro lado, estudos sugerem que ratos com altos níveis de estrógeno usam estratégias alocêntricas de forma eficiente, superando ratos privados de hormônios em tarefas que requerem o uso de dicas extra-labirinto, enquanto que ratos com baixos níveis de estrógeno tendem a usar estratégias egocêntricas nas tarefas em que a troca de direção (por exemplo, esquerda ou direita) é necessária (KOROL, 2004). Assim, é possível que as alterações observadas aqui estejam relacionadas a uma diminuição dos níveis de estrógeno em fêmeas adultas manipuladas no período neonatal (GOMES ET AL., 2005). Com relação à associação

entre um desequilíbrio oxidativo e alterações na memória de hábito nas fêmeas, os resultados desta tese não vão nesta direção. Foi observada uma redução na relação SOD/CAT no córtex pré-frontal de fêmeas manipuladas adultas, o que poderia estar relacionado com uma alteração no aprendizado reverso, uma vez que esta estrutura está relacionada com a flexibilidade comportamental (CLARKE, ROBBINS e ROBERTS, 2008; HORNAK ET AL., 2004; MCALONAN e BROWN, 2003), no entanto não houve diferença entre os grupos neste parâmetro. Por outro lado, uma redução na relação SOD/CAT pode levar a um aumento do ânion superóxido afetando lipídios, proteínas, bem como o ADN (COCHRANE, 1991) e, consequentemente outras funções podem estar comprometidas. Além disso, é importante ressaltar que a redução desta relação tem como consequência uma redução nos níveis de peróxido de hidrogênio, o qual tem sido proposto como uma molécula mensageria capaz de difundir-se e modular a atividade de proteínas fosfatases resultando na modulação da plasticidade neuronal (KAMSIER e SEGAL, 2004).

Os efeitos da manipulação neonatal foram abordados sobre um terceiro tipo de memória nesta tese: a memória olfatória. Considerando que estudos anteriores mostraram que a manipulação neonatal reduziu o comportamento de aproximação em direção a um odor familiar (odor materno) em fêmeas, mas não em machos no período inicial da vida (RAINEKI ET AL., 2009), nesta tese abordamos a memória olfatória em fêmeas. Além disso, conforme abordado na introdução desta tese, os efeitos da manipulação neonatal aumentando o consumo de alimento palatável foram observados apenas após a puberdade. Desta forma, conforme descrito no capítulo 3, avaliamos também a influência da remoção dos hormônios gonadais antes da puberdade e possíveis interações entre ambas intervenções sobre a memória olfatória relacionada a um alimento palatável (chocolate) na idade adulta. Como diferentes

desfechos são obtidos com a manipulação neonatal conforme o período da vida que se estuda, achávamos que, apesar de no início da vida fêmeas manipuladas demonstrarem um prejuízo na memória olfatória, na idade adulta, quando esse tipo de memória fosse avaliada associada a um alimento palatável (cujo consumo é aumentado por esses animais), o resultado seria uma melhora com relação aos controles, até mesmo porque já havíamos observado um melhor desempenho das fêmeas adultas manipuladas no período neonatal na tarefa do labirinto em Y associado ao alimento palatável (capítulo 2). Não observamos diferenças com relação a este parâmetro, no entanto. Da mesma forma, a influência dos hormônios gonadais neste processo não foi confirmada. Seria interessante avaliar os efeitos da manipulação neonatal sobre outras tarefas olfatórias e também em ratos machos, uma vez que, conforme comentado na introdução e no capítulo 3, diferenças sexo-específicas com relação à manipulação neonatal foram observadas na tarefa de reconhecimento social em adultos, onde apenas os ratos machos manipulados no período neonatal apresentaram uma redução no comportamento social (TODESCHIN ET AL., 2009).

Considerando os efeitos da manipulação neonatal relatados nesta tese sobre os diferentes tipos de memória bem como os dados da literatura, fica evidente que os efeitos desta intervenção precoce sobre o aprendizado e a memória parecem estar relacionados com a idade dos animais e o tipo de memória avaliada. Com relação à nossa hipótese de que o consumo aumentado de alimento palatável na idade adulta em animais manipulados no período neonatal estaria relacionado com a memória, ela parece ser válida para fêmeas, se considerarmos os resultados na fase de aprendizado da tarefa do labirinto em Y (a manipulação neonatal melhorou a memória). Por outro lado, o mesmo não ocorre nos machos. Poderia ser argumentado que, como os

machos apresentam deficiência no aprendizado reverso desta tarefa, isto poderia sugerir um aprendizado de hábito mais marcante, o que também poderia estar envolvido no comportamento alimentar. Assim, podemos dizer que, se existem componentes de memória envolvidos na alteração do comportamento alimentar, estes componente são diferentes em machos e em fêmeas.

No capítulo 3, parâmetros bioquímicos foram avaliados na tentativa de fazer uma correlação com as possíveis alterações na memória. Como já foi descrito acima, não houve diferença entre os grupos com relação à memória olfatória e as alterações bioquímicas observadas devem estar relacionadas a outros processos. Mais detalhadamente, observamos um aumento na atividade da enzima Na^+/K^+-ATPase no bulbo olfatório de ratas manipuladas no período neonatal. Da mesma forma como foi comentado anteriormente com relação ao hipocampo, este aumento pode estar relacionado a alterações nos níveis de neurotransmissores como serotonina e noradrenalina, os quais parecem modular a atividade daquela enzima (PEÑA-RANGEL, MERCADO e HERNÁNDEZ-RODRIGUEZ, 1999; HERNÁNDEZ-R, 1992). Chama a atenção o fato de ratos machos manipulados no período neonatal apresentarem um aumento na atividade da Na^+/K^+-ATPase aos 21 dias de idade também no bulbo olfatório, enquanto que não houve diferença entre as fêmeas. Uma possibilidade para o aumento na atividade dessa enzima apenas na idade adulta nas fêmeas manipuladas poderia ser em função dos hormônios gonadais. Pensando assim, a remoção dos ovários antes da puberdade levaria, na idade adulta, aos mesmos efeitos observados no período pré-púbere. No entanto, o que vimos foi que, tanto a cirurgia de remoção dos ovários quanto o procedimento cirúrgico por si só (grupo *sham*, sem a remoção dos ovários) reduziram a atividade da enzima nas fêmeas adultas manipuladas ou não no período neonatal. O fato da cirurgia ter sido feita antes

da puberdade, em um período crítico do desenvolvimento, pode justificar as alterações encontradas. Seria interessante verificar como a remoção dos ovários na idade adulta afetaria a atividade da Na^+/K^+-ATPase nas fêmeas manipuladas no período neonatal e nas controles. Por outro lado, sabe-se que esta enzima é suscetível ao ataque por radicais livres (PETRUSHANKO ET AL., 2006), de modo que uma redução na atividade da Na^+/K^+-ATPase poderia estar associada a um desequilíbrio oxidativo. Conforme comentado no capítulo 3, vimos que tanto os animais ovariectomizados quanto os *sham* apresentaram uma menor atividade da enzima CAT com relação aos intactos. Além disso, a atividade foi ainda menor nos *sham* com relação aos ovariectomizados. Estes resultados concordam com estudos que mostram que a redução de antioxidantes ou da atividade de enzimas antioxidantes está relacionada com uma redução na atividade da enzima Na^+/K^+-ATPase (STRECK ET AL., 2001; PETRUSHANKO ET AL., 2006). Uma possível explicação para as diferenças entre os animais *sham* e ovariectomizados com relação à atividade da CAT poderia ser que a ausência de hormônios sexuais desde antes da puberdade até a idade adulta tenha levado a alterações cerebrais que explicariam esses resultados.

Um ponto interessante com relação às tarefas que avaliaram a memória utilizando alimento palatável nesta tese é o fato de que as fêmeas manipuladas no período neonatal mostraram uma menor latência para consumir o alimento palatável, sem alterar a quantidade consumida na tarefa do labirinto em Y na fase de aprendizado e também na tarefa da tábua com buracos com relação as controles. Conforme abordado na introdução, sabe-se que animais adultos manipulados no período neonatal apresentam um consumo aumentado de alimento palatável quando expostos a este por um curto período de tempo (SILVEIRA ET AL., 2004). Uma possibilidade para explicar porque, mesmo com uma menor latência para começar a

comer, o consumo de alimento palatável não tenha sido alterado, pode ser o fato de que a tarefa usada por Silveira e colaboradores (2004) é mais simples do que as utilizadas nesta tese. Naquela tarefa, o rato apenas deve ir até o final de um corredor, o qual não é muito longo. Por outro lado, na tarefa da tábua com buracos, o animal perde mais tempo procurando o alimento para só então começar a comer. Assim, é possível que, em função do menor tempo de exposição ao alimento, não tenham sido observadas diferenças no consumo no nosso caso. O mesmo vale para a tarefa em Y, onde o animal fica apenas 1 minuto em contato com o alimento para cada escolha do braço correto. No entanto, partindo-se dessa suposição, este parece ser outro efeito sexo-específico, pois os machos manipulados não diferiram dos controles com relação à latência para consumir o alimento palatável na fase do aprendizado na tarefa do labirinto em Y. Ainda, o fato de os machos manipulados no período neonatal apresentarem um aumento na latência para consumir o alimento palatável na fase de aprendizado reverso concorda com os resultados do prejuízo da memória apresentado por eles nesta fase, o que já foi discutido anteriormente.

Não podemos deixar de abordar nesta discussão que as alterações causadas pela manipulação neonatal podem estar relacionadas a alterações epigenéticas. Nesta tese, estes tipos de alterações não foram estudadas, mas com certeza uma avaliação neste sentido ajudaria a esclarecer os efeitos descritos neste trabalho. O estudo epigenético tornou-se importante uma vez que, após o sequenciamento do genoma humano, ficou claro que as informações genéticas sozinhas não eram suficientes para o entendimento das manifestações fenotípicas, e que a interação com fatores ambientes era fundamental (MARTÍN-SUBERO, 2011). É nessa interface que a ciência da epigenética exerce seu papel crucial. Mecanismos epigenéticos como a metilação do ADN e a modificação de histonas são essenciais para que vários

processos fisiológicos e fatores ambientais como, por exemplo, o cuidado materno, sejam capazes de induzir alterações epigenéticas (MARTÍN-SUBERO, 2011; TURNER, 2001). No capítulo 1 desta tese foi demonstrado que ratos machos manipulados no período neonatal apresentam quando adultos um aumento no índice de quebra ao ADN. É possível que a manipulação, por meio do aumento no cuidado materno dos filhotes, provoque alterações nos padrões de metilação do ADN em estruturas cerebrais como o hipocampo, tornando-o mais acessível ao ataque por várias substâncias.

Nesta tese observamos diferenças sexo-específicas em vários parâmetros avaliados. Uma possível explicação para isso seria o fato de que as mães, em uma ninhada mista, cuidam mais dos filhotes machos do que das fêmeas (MOORE e MORELLI, 1979) e como colocado acima, variações no cuidado materno podem induzir alterações epigenéticas que poderiam explicar essas diferenças (MARTÍN-SUBERO, 2011). A influência do estradiol também seria uma possibilidade. Apesar de não termos encontrado efeito da remoção dos ovários antes da puberdade sobre a memória olfatória em fêmeas adultas manipuladas no período neonatal, mais estudos são necessários para verificar a influência do estradiol nesta e em outras tarefas, bem como em parâmetros bioquímicos.

Mas afinal, a manipulação neonatal é protetora ou prejudicial? Esta tese contribuiu para mostrar que essa intervenção precoce aparentemente inócua é capaz de gerar efeitos protetores e também efeitos prejudiciais em ratos machos e fêmeas. Possivelmente, tais alterações, em especial as alterações comportamentais, sejam adaptativas ou mal-adaptativas, dependendo do tipo de ambiente em que esses animais viverão. Além disso, este trabalho ressalta a importância do estudo de intervenções no início do desenvolvimento com relação aos aspectos cognitivos e

neuroquímicos ao longo da vida. Um melhor entendimento destes processos pode levar ao desenvolvimento de estratégias para evitar o aparecimento de patologias na vida adulta associadas a interferências no período inicial do desenvolvimento.

2. CONCLUSÕES

- ✓ A manipulação neonatal prejudicou a memória e o aprendizado espacial apenas em fêmeas adultas, o que parece estar relacionado com uma redução nos níveis de óxido nítrico no hipocampo. As diferenças sexo-específicas também foram observadas na avaliação do índice de quebras ao ADN, com a manipulação neonatal afetando apenas os machos.

- ✓ Os efeitos da manipulação neonatal sobre o aprendizado reverso em ratos adultos foram dependentes da tarefa usada e do sexo. A manipulação neonatal prejudicou o aprendizado reverso no labirinto em Y e não teve efeito sobre o aprendizado reverso no Labirinto Aquático de Morris considerando os machos. Os efeitos sexo-específicos também foram observados na avaliação dos parâmetros do estresse oxidativo onde a manipulação neonatal reduziu a relação SOD/CAT apenas no córtex pré-frontal de fêmeas.

- ✓ A memória olfatória relacionada ao alimento palatável avaliada em fêmeas na idade adulta não foi afetada pela manipulação neonatal nem pela cirurgia de remoção dos ovários ou pelo procedimento cirúrgico por si só (sem remoção dos ovários = *sham*) realizado antes da puberdade. A redução na atividade da CAT e da Na^+/K^+-ATPase no bulbo olfatório de fêmeas adultas que foram submetidas a cirurgia antes da puberdade não parece estar relacionada com a memória olfatória. Além disso, a atividade aumentada da enzima Na^+/K^+-ATPase no hipocampo e no bulbo olfatório induzida pela manipulação neonatal pode estar relacionada com alterações neuroplásticas.

- ✓ A manipulação neonatal afetou parâmetros do estresse oxidativo, bem como a atividade das enzimas Na^+/K^+-ATPase e acetilcolinesterase de modo diferente em ratos machos e fêmeas jovens, com as fêmeas manipuladas sendo mais suscetíveis a alterações nos parâmetros relacionados ao estresse oxidativo (redução na atividade da GPX e no conteúdo total de tióis no hipocampo) e os machos manipulados sendo mais vulneráveis a alterações na atividade das enzimas Na^+/K^+-ATPase (aumentada no bulbo olfatório) e acetilcolinesterase (reduzida no hipocampo).

- Como conclusão geral, esta tese mostrou que a manipulação neonatal afetou a memória e o aprendizado bem como parâmetros neuroquímicos de modo sexo-específico, possivelmente por direcionar a diferentes estratégias de aprendizado nos dois sexos. Além disso, as alterações observadas nos parâmetros neuroquímicos dependem da estrutura estudada bem como da idade dos animais. Ainda, as alterações nas atividades enzimáticas observadas em machos não parecem estar relacionadas ao estresse oxidativo enquanto que as fêmeas parecem ser mais suscetíveis a este. Esta tese reforça a importância de considerar o gênero quando avaliando os efeitos da manipulação neonatal e contribui para o melhor entendimento de como intervenções precoces afetam a memória e parâmetros neuroquímicos ao longo da vida.

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ANEXO

Neste anexo apresento os resultados referentes a minha participação no Programa de Doutorado no País com Estágio no Exterior (PDEE) financiado pela Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES) o qual foi realizado na Universidade de Cambridge na Inglaterra sob a supervisão do Professor Dr. Trevor W. Robbins no período de Setembro de 2010 a Julho de 2011. Os resultados estão apresentados na forma de pôsteres, os quais foram apresentados nos congressos indicados na página que antecede cada um deles. Durante o PDEE, trabalhei no desenvolvimento de um novo modelo para o estudo do transtorno obsessivo compulsivo em ratos, baseado na indução de "respostas de observação" ou "comportamento de conferência" pela injeção intraperitoneal de quinpirole (agonista do receptor de dopamina D_2) conforme descrito nos pôsteres a seguir. Este estágio visou o aprendizado de técnicas relacionadas ao comportamento compulsivo com o objetivo de realizar futuros estudos no grupo de pesquisa verificando a possibilidade de tal comportamento estar envolvido no comportamento alimentar de ratos adultos manipulados no período neonatal.

The observing response test for rats as a model of the checking symptoms of obsessive-compulsive disorder: parametric manipulations of quinpirole-induced checking behaviour

Trabalho apresentado no Congresso da Associação Britânica de Neurociência (*British Neuroscience Association*).

The Observing Response Test for rats as a model of the checking symptoms of obsessive-compulsive disorder: parametric manipulations of quinpirole-induced checking behaviour

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Introduction

Obsessive-compulsive disorder (OCD) is a psychiatric affliction classified as an anxiety disorder by DSM-IV[1]. OCD checking involves the performance of actions supposedly related to security, orderliness or accuracy, and is characterized by the repeated and excessive re-doing of such rituals[2].

Compulsive checking may be linked with intolerance of uncertainty, or with unpredictability in the environment.

Although existing animals models of compulsive checking (e.g., quinpirole-induced "home-base" checking[3]) help us understand the neuropharmacology underpinning OCD symptoms, they tell us little about how such behaviours evolve, or to what extent they interfere with normal function.

We present a novel model of compulsive checking: the Observing Response Test (which allow us to examine compulsive-like behaviour in great detail). We verified how an increase in the work requirement and/or temporal uncertainly affect checking behaviour in rats.

Hypothesis

Quinpirole is a dopamine D2/D3 receptor agonist that can induce long-lasting compulsive checking in rats following chronic treatment.

If work investment or temporal uncertainty are increased, checking behaviour for information about the environment could be an effective strategy for maintaining performance. We predict that quinpirole-treated rats may be more sensitive to these parametric task manipulations.

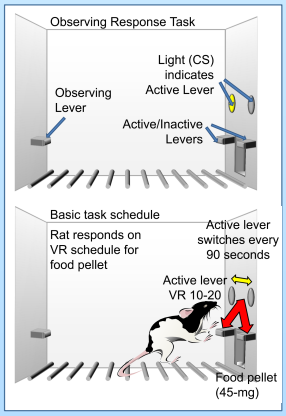
Methods

24 male Lister-Hooded rats were trained on the Observing Response Test.

Following training, median-split groups of High and Low Checkers were selected based on their Observing lever responses, selected from 2 days of testing before the original pre-drug session.

Rats were treated for 10 consecutive days with quinpirole (0.5 mg/kg i.p.) or vehicle (6 High and 6 Low Checkers per drug/vehicle group).

Data are presented for parametric manipulations within the post-quinpirole treatment period: days17-79.



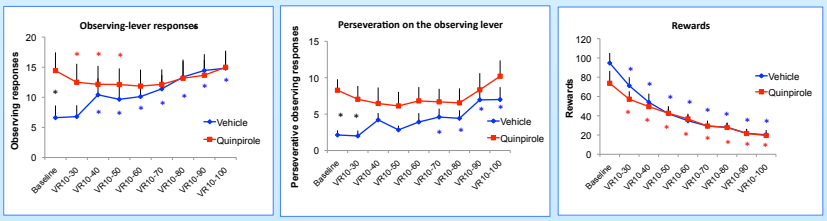
Two manipulations of the task were made that aimed to increase checking behaviour.

1. Investment increase: VR 10-20 was increased in steps of 10 up to VR10-100. Active/Inactive levers switched sides on a fixed interval (FI) 90s schedule.

2. Temporal Uncertainty + Investment Increase: Temporal uncertainty was introduced by changing the Active/Inactive lever switch schedule from FI 90s to variable interval (VI) 20-120s. Then, the investment increase under the temporal uncertainty was verified using a VR 10-50 and VR 10-70.

Results

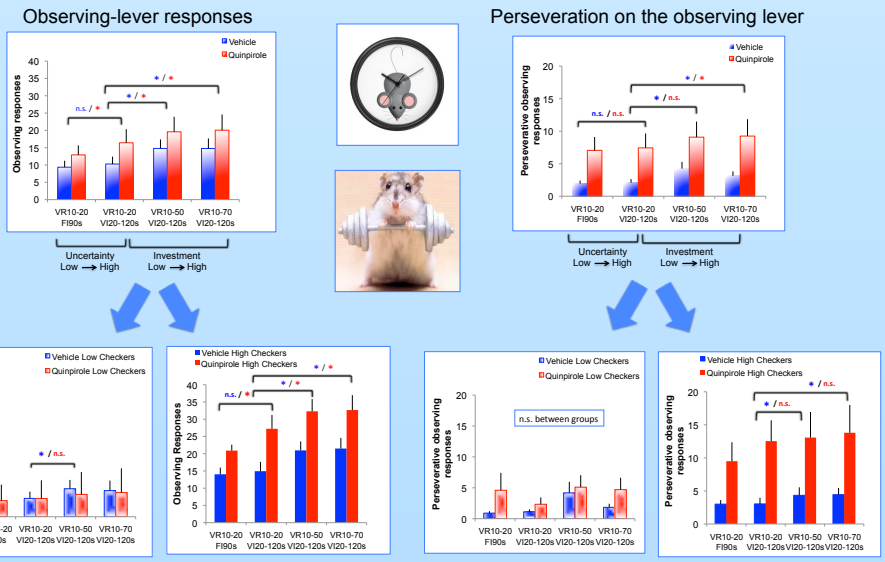
Task Manipulation: Investment Increase



Increasing investment /workload (increased VR) increased both observing-lever responses and perseveration on the observing lever in the vehicle-treated rats but not the quinpirole-treated rats. Both groups decreased the amount of reward obtained in the task as the VR increased.

* = P<0.05 for quinpirole (manipulation vs baseline)
* = P<0.05 for vehicle (manipulation vs baseline)
• = P<0.05 for quinpirole vs vehicle

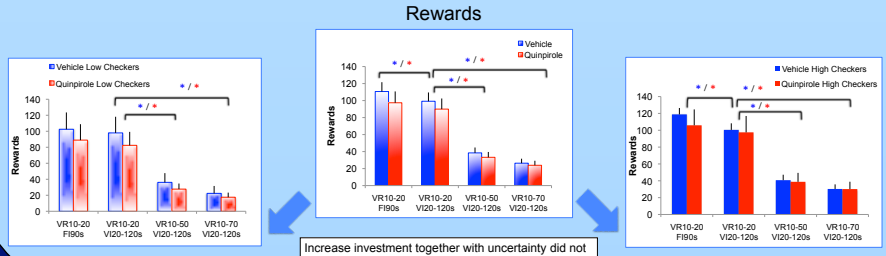
Task Manipulation: Temporal Uncertainty plus Investment Increase



Temporal uncertainty increased observing-lever responses by quinpirole-treated rats, but not vehicle-treated rats. This effect was significant in the High-Checker group of quinpirole-treated rats. However, Temporal uncertainty had little effect on perseverative observing-lever responses.

Increased investment/workload plus temporal uncertainty increased both observing-lever responses and perseverative observing-lever responses in quinpirole- and vehicle-treated rats. The effects were significant for the High-Checker rats, and more marked for the vehicle-treated rats.

Rewards



Increase investment together with uncertainty did not affect the amount of reward obtained by quinpirole compared to vehicle rats.

Conclusions

Rats that had previously been treated with chronic quinpirole increased checking when Active lever position was more uncertain. However, their checking and perseverative checking was less affected by changes in investment/workload on the Active lever.

In contrast, vehicle-treated rats increased checking and perseveration when Active lever investment/workload was increased, but were less affected when the Active lever position was more uncertain.

This selectivity increases the validity of quinpirole-induced checking in rats as a model for checking symptoms of OCD, as quinpirole-induced checking appears sensitive to changes in environmental uncertainty.

Acknowledgments & References

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2. Szechtman H et al 2001 BMC Neuroscience 2:4
3. Szechtman et al 1998 Behav Neurosci 112: 1475-85

The observing response test for rats as a model of checking symptoms of obsessive-compulsive disorder: chronic quinpirole treatment increases checking-related behaviours.

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The Observing Response Test for rats as a model of checking symptoms of obsessive-compulsive disorder: chronic quinpirole treatment increases checking-related behaviours.

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Introduction

Obsessive-compulsive disorder (OCD) is a common, debilitating psychiatric disorder (prevalence 1-3%[1,2]).

The most commonly-treated symptom of OCD is **compulsive checking**[2,3]: "*I have to go and check, but then I'm not convinced about the checking. So I have to go and check that I checked properly.*"

Compulsive-checking routines may extend to several hours per day, at the expense of normal function, and are linked with anxiety and intolerance of uncertainty[4].

Animal models of compulsive checking help us to understand the neuropharmacology underpinning OCD. For example, chronic **quinpirole** (dopamine D2/D3 receptor agonist) has been established as a potential pharmacological model of compulsive checking (of a 'home-base' site in an open field)[2].

However, it is unclear to what extent the quinpirole-induced behavioural profile of existing animal models is unnecessary, or if it interferes with normal function.

We present a novel model of 'checking' behaviour in the rat: the **Observing Response Test**. This model can be used to investigate repetitive, compulsive-like behaviour in detail, e.g., how compulsive checking may evolve from a more 'normal' behavioural repertoire, and how this relates to behavioural flexibility and tolerance of uncertainty.

Methods

24 male Lister-Hooded rats were trained on the Observing Response Test.

Following training, median-split groups of **High and Low Checkers** (observing lever press (OLP) responses) were selected from 2 days of testing before the pre-drug session.

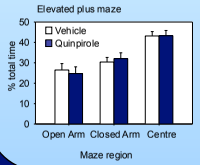
Rats were treated for 10 consecutive days with quinpirole (0.5 mg/kg i.p.) or vehicle (6 High and 6 Low Checkers per drug/vehicle group). Drugs were administered 20 minutes before behavioural testing (except on days 1-3: 1 hour before behavioural testing. Quinpirole-induced behavioural sensitization requires rats to perform on task as soon as possible after drug administration. However, early treatments induced behavioural suppression and a longer pre-test period was required on days 1-3).

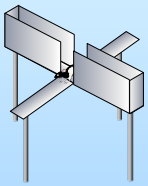
Rats were tested in elevated plus maze on day 92 post-quinpirole treatment (5-minute test-room habituation, 5-minute test session).

Are quinpirole-treated rats more anxious?

Elevated plus maze

Reduced time in open arms is an indicator of increased anxiety (open arm use is increased by anxiolytic drugs)[5]



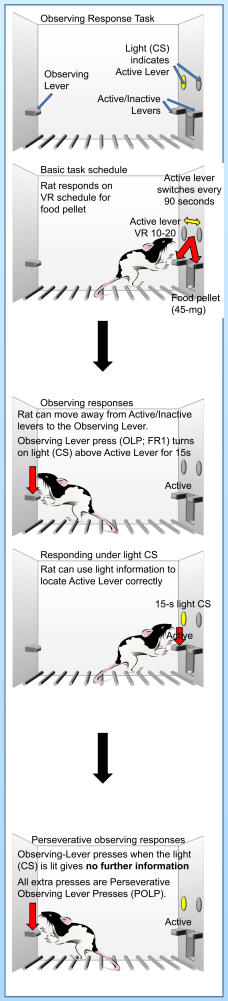


Quinpirole treatment

There were no differences in elevated plus maze performance between quinpirole- and vehicle-treated rats.

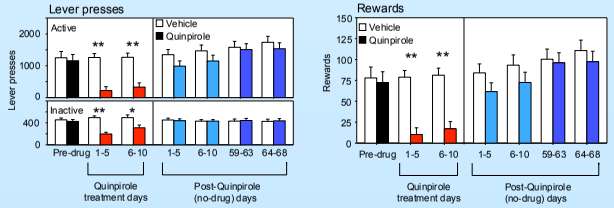
Results

Task Design



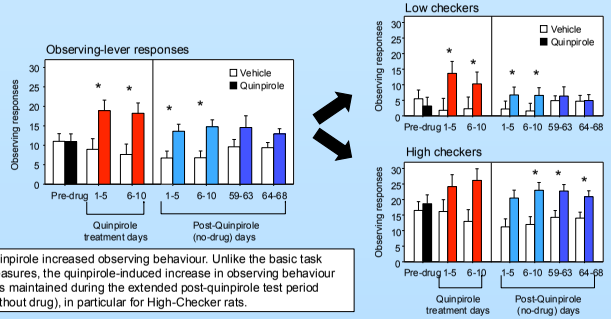
Basic task – Active/inactive levers and reward

*/** denotes significant difference from vehicle at p<0.05/p<0.01



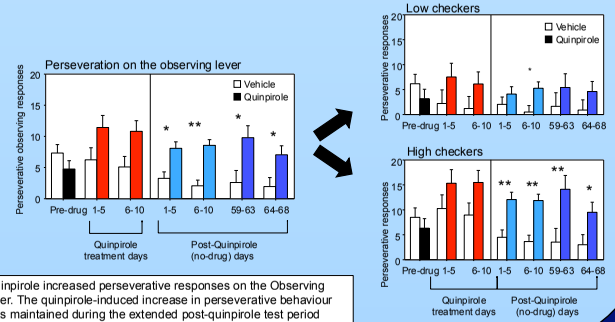
Quinpirole reduced both Active and Inactive lever presses and rewards but only during the 10-day drug treatment period.

Observing responses – 'checking' gives information about Active lever



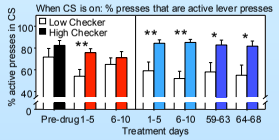
Quinpirole increased observing behaviour. Unlike the basic task measures, the quinpirole-induced increase in observing behaviour was maintained during the extended post-quinpirole test period (without drug), in particular for High-Checker rats.

Perseveration – 'checking' gives no further information



Quinpirole increased perseverative responses on the Observing lever. The quinpirole-induced increase in perseverative behaviour was maintained during the extended post-quinpirole test period (without drug), in particular for High-Checker rats.

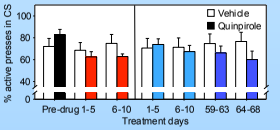
Do rats use the Observing Lever for information?



An Observing-Lever press turns on the light CS above the Active lever. This reduces uncertainty about which lever is active.

When the Active lever light is on, High-Checker rats make relatively more presses on the Active lever than Low-Checker rats.

Quinpirole had no significant effect on the ability to use the light to select Active lever responses



Conclusions

Quinpirole selectively increased 'checking' (responding on the Observing Lever to turn on the light CS), but did not increase Active or Inactive lever presses.

Quinpirole selectively increased perseverative Observing Lever presses (OLP when the light is already on), which had no function and gave no further information. These responses may model excessive checking in OCD.

Checking-related quinpirole-induced behavioural changes were long-lasting and were still present more than 60 days after quinpirole treatment.

Both OLP and perseverative OLP increases were more marked in rats that were High Checkers. High-Checker rats used the Observing Lever to give information about the location of the Active lever. These rats may be less tolerant of uncertainty.

Rats that show high levels of checking to reduce uncertainty show marked increases in checking and perseverative checking following quinpirole treatment. This further validates chronic quinpirole treatment as a model of OCD-like checking.

References & Acknowledgements

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