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

MANIPULAÇÃO NEONATAL EM RATOS: AVALIAÇÃO DE PARÂMETROS COMPORTAMENTAIS E NEUROQUÍMICOS RELACIONADOS À ATENÇÃO E À IMPULSIVIDADE NA IDADE ADULTA

CAMILLA LAZZARETTI

Porto Alegre
2016

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

MANIPULAÇÃO NEONATAL EM RATOS: AVALIAÇÃO DE PARÂMETROS COMPORTAMENTAIS E NEUROQUÍMICOS RELACIONADOS À ATENÇÃO E À IMPULSIVIDADE NA IDADE ADULTA.

CAMILLA LAZZARETTI

Orientadora: Profª. Dra. Carla Dalmaz
Co-orientador: Prof. Dr. Tadeu Mello e Souza

Tese apresentada ao Programa de Pós-Graduação em Neurociências da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do grau de Doutora em Neurociências.

Porto Alegre
2016

AGRADECIMENTOS

Agradeço ao programa de pós-graduação em neurociências da UFRGS, representado por todos seus professores, secretaria e alunos colegas de pós-graduação, que contribuíram de forma direta ou indireta para minha formação.

Ao CNPq pela bolsa de estudos, que foi imprescindível para a continuidade dos meus estudos.

Agradeço às pessoas que cruzaram meu caminho e enriqueceram minha jornada. Primeiramente à prof.ª Carla Dalmaz por ter me acolhido como doutoranda em 2011 no laboratório de neurobiologia do estresse. Ela foi e sempre será, um grande exemplo a ser seguido por mim, como orientadora, mãe científica, profissional pesquisadora e professora, e acima de tudo amiga, nos momentos de felicidade e descontração, bem como nos puxões de orelha.

Ao meu co-orientador, Tadeu Mello e Souza, por continuar comigo nesta caminhada, desde o mestrado, por seus ensinamentos, e auxílios que sempre me ajudaram a evoluir.

À professora Cecília Scorza, que abriu as portas do instituto Clemente Estable em Montevidéu, para que eu pudesse realizar experimentos, com a ajuda do Gustavo e Martín.

À Rachel, ou Rac, que me trouxe pela mão ao laboratório com um sorrisão no rosto, e de cara eu já sabia que seria uma grande amiga. Me ensinou não só o Western blotting, proteínas, estresse oxidativo, mas sim me mostrou como ser uma pós-graduanda de qualidade e humilde, e que inteligência rima sim com amizade, risadas, e conselhos. Minha grande professora e amiga do lab 37.

À Grasi, que chegou de mansinho, me ensinou tarefas comportamentais que seriam chatas se não fosse a companhia dela foi uma grande amiga e parceirona. Me ajudou a ser uma doutoranda, a trabalhar, pedir ajuda, conversar, orientar os ICs, e acima de tudo ser uma grande amiga.

Ao Pablo, que junto da Grasi e eu, formaram um time dos comportamentos. Que dedicou seu tempo ao meu auxílio.

À Danusa e a Ana, que foram especiais. Sempre disponíveis para o que der e vier, me ajudaram sempre, meninas brilhantes de iniciativa e energia para experimentos até altas horas, e ainda com disposição para rir e tomar um café no Jesus para descontrair.

À meninas do lab 37, Nati, Carine, Marina, Letícia, e aos meninos André, e Eduardo, que foram colegas importantes para minha formação.

Ao Fares pelo auxílio nos artigos, foi uma ajuda imprescindível.

Aos ICs que foram do lab 37 e sempre me auxiliaram. A Raquelzinha menina de ouro sempre disponível e prestativa. Ao Dieguinho um doce de guri e sempre no meu auxílio. A Ane, Carol, Vivian, Isabella, sempre ajudaram em meus experimentos.

Ao pessoal do lab 35, à Simone, Eduardo, Fabrício, que nos socorreram nos materiais faltantes, no cafezinho e na amizade.

Aos professores, coordenadores, alunos e colegas da FACOS, que acreditaram no meu potencial, e puderam fazer um sonho realidade, ser professora. Que me fizeram compreender que o conhecimento científico que adquirimos na graduação e pós-graduação só tem valia se chegar às pessoas, que talvez não tiveram tanto acesso a ele, sendo compartilhando e facilitado por nós, sem egos inflados arrogância.

Às minhas colegas de moradia e amigonas da Miguel Tostes, Mile e Beta, sempre estiveram presentes nos momentos de angústias, dúvidas e felicidade. Viram minha jornada de perto, e foram muito importantes em mostrar-me algo mais da vida, a leveza e a importância das grandes amizades. A Nicole que chegou depois, e participou desta linda república.

Aos amigos de Caxias, que mesmo distantes estão presentes no meu coração, dando aquela força.

À Márcia, que no meio do doutorado, eu cheia de aflições, ela me mostrou como pensar, e ouvir as vozes que vem de dentro, me ajudando a sanar dores que nos fazem cair.

À minha família, pai, mãe, Sil, que sempre me apoiaram e incentivaram a buscar aquilo que quero, me mostrando que é preciso lutar por aquilo que ser na vida, mesmo que obstáculos apareçam no meio do caminho. Amo vocês.

Ao meu companheiro, amigo e amor, Anderson, que esteve sempre presente, mesmo não entendendo o Blotting que tanto eu falava, sempre ouvindo minhas angústias e lamúrias, e me proporcionando momentos inesquecíveis ao seu lado. Te amo meu amor. E a minha segunda família, minha sogra Inês e meu sogro Juarez, que me acolheram como uma filha, sempre preocupados com meu bem estar e sempre prontos a me ajudar.

Obrigada a todos, vocês tem parte neste trabalho!

Camilla

“Numa sociedade com base no conhecimento,
por definição, é necessário que você seja estudante a vida toda".
Tom Peters

SUMÁRIO

APRESENTAÇÃO **7**
LISTA DE ABREVIATURAS **8**
LISTA DE FIGURAS **10**
RESUMO **12**
ABSTRACT **14**
1. INTRODUÇÃO **16**
1.1Janela de vulnerabilidade e manipulação neonatal 16
1.2 O eixo hipotálamo-pituitária-adrenal (HPA) e a manipulação durante o período neonatal 17
1.3 Efeitos comportamentais da manipulação neonatal 20
1.4 Inibição comportamental, manipulação neonatal e comportamentos correlatos.... 21
1.5 Manipulação neonatal, atenção e flexibilidade cognitiva 23
1.6 Neurobiologia dos processos executivos 26
1.7 O desenvolvimento do córtex pré-frontal e marcadores de plasticidade: Sinaptofisina e BDNF..... 29
1.8 Manipulação neonatal e aminas biogênicas 31
1.9 Na+/K+ -ATPase e manipulação neonatal..... 33
2. OBJETIVOS **34**
2.1 Objetivo Geral 34
2.2 Objetivos específicos 34
3. MATERIAL, MÉTODOS E RESULTADOS **36**
3.1 Capítulo I..... 36
3.2 Capítulo II 52
3.3 Capítulo III - Resultados Adicionais..... 91
4. DISCUSSÃO **94**
5. CONCLUSÕES **103**
6. PERSPECTIVAS **103**
7. REFERÊNCIAS BIBLIOGRÁFICAS **105**
8. ANEXOS **114**
8.1 Carta de aprovação do comitê de ética em pesquisa..... 114

APRESENTAÇÃO

A presente tese está delimitada nos seguintes tópicos dispostos abaixo:

1. Introdução: estabelece a fundamentação teórica para a compreensão dos objetivos apresentados.

2. Objetivos (geral e específicos): definem os propósitos centrais do trabalho estruturados em cada capítulo.

3. Capítulos: cada capítulo corresponde a um artigo específico, e/ou resultados adicionais, ordenados de acordo com os objetivos propostos.

3.1 Capítulo I: compreende o primeiro artigo da presente tese intitulado: ("*Neonatal handling causes impulsive behavior and decreased pharmacological response to methylphenidate in male adult Wistar rats*.") **Publicado na revista "Journal of Integrative Neuroscience", volume. 15, Nº. 1 (2016) 81–95.**

3.2 Capítulo II: compreende o segundo artigo da presente tese intitulado: (*"Neonatal handing alters attentional set shifting task performance and medial pré-frontal córtex biochemical parameters in adult rats".)* **A ser submetido para publicação.**

4. Capítulo III: (compreende resultados adicionais da presente tese.)

5. Discussão: compreende a discussão geral da tese, envolvendo os dois artigos submetidos, e os resultados ainda não publicados.

6. Conclusões: abordam as conclusões gerais da tese.

7. Perspectivas: propõem possibilidades de continuidade para complementação do presente trabalho.

8. Referências bibliográficas: lista as referências bibliográficas citadas nas seções *Introdução* e *Discussão.* No final de cada capítulo estão listadas suas referências para cada respectivo artigo condizente e formatadas de acordo com as especificações das revistas submetidas.

9. Anexos: contêm a carta de aprovação do Comitê de Ética na Utilização de Animais (CEUA – UFRGS) referente aos experimentos realizados na presente tese.

LISTA DE ABREVIATURAS

5-HT	Serotonina
5-HIAA	Ácido 5-hidroxiindoleacético
ACTH	Hormônio Adrenocorticotrópico
ADHD	Transtorno do Déficit de Atenção com Hiperatividade
ASST	Tarefa do Deslocamento da Atenção
ASST maze	Tarefa de Mudança de Estratégia
ASSTpot	Tarefa do Deslocamento da Atenção
BDNF	Fator Neurotrófico Derivado do Encéfalo
CA	Cenário Atencional
CD	Discriminação Composta
CEUA	Comitê de ética para o uso de animais
CORT	Corticosterona
CPF	Córtex Pré-Frontal
CPFm	Córtex Pré-Frontal Medial
CPFvm	Córtex Pré-Frontal Medial Ventro-medial
CRH	Hormônio Liberador de Corticotropina
D2	Receptor D2 de Dopamina
D3	Receptor D3 de Dopamina
DA	Dopamina
DOPAC	Ácido 3,4-Di-hidroxifenilacético
ED	Discriminação Extradimensional
EDTA	Ácido Etilenodiamino Tetra-acético
GC	Glicocorticoides
GluA3	Receptor AMPA 3 de Glutamato
GR	Receptores de Glicocorticoides
HEPES	Ácido 4,2-Hidroxietil 1-Piperazina Etano Sulfônico
HPA	Hipófise-Pituitária-Adrenal
HPLC-ED	Cromatografia Líquida de Alta pressão com Detector Eletroquímico
HUGO	Organização do Genoma Humano
HVA	Ácido Homovanílico
IC	Inibição Comportamental
ID	Discriminação Intradimensional
LC	Locus Coeruleous

L-DOPA	Levodopa
MN	Manipulação Neonatal
mPFC	Córtex Pré-frontal Medial
MR	Receptores de Mineralocorticoides
MPH	Metilfenidato
NE	Noradrenalina
NAcc	Núcleo Accunbens
NH	Manipulação Neonatal
NR	Núcleo da Rafe
OFC	Córtex Órbito-frontal
PHE	Período Hiporrespossivo ao Estresse
Pi	Fosfato Inorgânico
PIC	Coquetel Inibidor de Protease
PND	Dia Pós-natal
PRS	Estresse pré-natal de restrição
REST	R1 silencing transcription factor
REV	Discriminação Reversa
SD	Discriminação Simples
SDS	Sódio Dodecil-sulfato
SHR	Ratos Espontaneamente Hipertensivos
SYP	Sinaptofisina
SNC	Sistema Nervoso Central
TBS	Tampão Tris-salina
TBS-T	Tampão Tris-salina com Tween
TDAH	Transtorno do Déficit de Atenção e Hiperatividade
TAR	Tolerância ao Atraso da Recompensa
TDA	Tarefa do Deslocamento da Atenção
TH	Tirosina Hidroxilase
THf	Tirosina Hidroxilase Fosforilada
pTH	Tirosina Hidroxilase Fosforilada
TME	Teste de Mudança de Estratégia
TW	Task Switching
VAMP1	Sinaptobrevina 1
VAMP2	Sinaptobrevina 2
VTA	Área tegmentar ventral

LISTA DE FIGURAS

Introdução

Figura1a. Fotografia exemplificando o procedimento de manipulação neonatal.....17
Figura1b. Filhotes na incubadora durante o procedimento de manipulação neonatal.....17
Figura 2. Eixo hipotálamo-pituitária-adrenal ativado pelo estresse e causa liberações hormonais subsequentes.....18
Figura3. Labirinto em "T" utilizado no teste TAR.....23
Figura 4. Labirinto de quatro braços fechados utilizado no teste de mudança de estratégia.....25
Figura 5a. Ilustração do teste de TDA.....26
Figura 5b. Imagem do rato avaliando os potes no teste de TDA.....26
Figura 6. Esquema determinando as partes do córtex pré-frontal.....27
Figura 7. Ilustração do córtex pré-frontal medial 28
Figura 8. Vias modulatórias aferentes do CPFm em encéfalo de rato.....29
Figura 9. Linha do tempo do desenvolvimento encefálico em humanos e ratos.....30
Figura 10. Funções da Sinaptofisina.....31
Figura 11. Via de síntese das catecolaminas.....32

Capítulo I

Figura 1. A) Linha do tempo e gráficos relativos aos experimentos comportamentais em animais manipulados e controles na tarefa tolerância ao atraso da recompensa mostrados nas fases B) pré-treino, C) treino e D) teste.....41
Tabela 1. Níveis de dopamina (DA), serotonina (5-HT), e seus metabólitos (ácido 3,4-dihidroxiphenilacético (DOPAC), ácido homovanílico (HVA) e ácido 5-hidroxi-indoleacético (5-HIAA), bem como noradrenalina (NE) em CPFm de animais manipulados machos e fêmeas e comparados aos controles.....45

Capítulo II.

Figura 1. A) Linha do tempo e B) gráficos relativos aos experimentos em animais manipulados e controles tarefa comportamental de mudança de estratégia.....77
Figura 2. Gráfico relativo aos experimentos em animais manipulados e controles na tarefa comportamental de deslocamento da atenção.....78

Figura 3. Gráficos relativos as dosagens das proteínas A) Imunoconteúdo de tirosina hidroxilase; B) Imunoconteúdo de tirosina hidroxilase em sua forma fosforilada; C) Níveis de BDNF; D) Imunoconteúdo de sinaptofisina em córtex pré-frontal medial ventro-medial.....79
Figura 4. Gráfico representando a atividade enzimática da proteína Na+/K+-ATPase em córtex pré-frontal medial ventro-medial.....80
Figura 5. Rede "*in silico*".....81
Tabela 1. Materiais utilizados em todas as discriminações na tarefa comportamental de deslocamento da atenção.....82
Tabela 2. Exemplo da sequência de discriminações realizadas no teste de deslocamento da atenção.....82
Tabela 3. Identificadores de proteínas dos genes que pertencem ao "Na+/K+-ATPase" no modelo de "*rede in silico*."".....83
Tabela 4. Escores de confiança sobre as ligações da rede "*in silico*" Na+/K+-ATPase.....84

Capítulo III.

Figura 1. Linha do tempo relativa aos experimentos em animais manipulados e controles.....91
Figura 2. Gráficos relativos aos experimentos em animais manipulados e controles durante a tarefa comportamental de mudança de estratégia.....92
Figura 3. Gráfico relativo aos experimentos em animais manipulados e controles na tarefa comportamental de deslocamento da atenção.....93
Figura 4. Gráfico relativo à atividade da enzima Na+/K+-ATPase na estrutura encefálica do CPFvm.....93

RESUMO

O período neonatal é considerado uma janela de vulnerabilidade a alterações fisiológicas e comportamentais no decorrer da ontogenia. Modificações ambientais nessa fase podem predispor o indivíduo a transtornos, como o déficit de atenção com hiperatividade (TDAH), abusos de substâncias psicoativas e transtornos alimentares. Modelos animais que mimetizem intervenções nessa fase são relevantes cientificamente. A manipulação neonatal (MN) é um desses modelos, em que breves separações entre a mãe e a ninhada são realizadas durante as primeiras duas semanas de vida (1º-10º dia de vida) por 10 minutos diários. No decurso do período neonatal, o eixo hipotálamo-pituitária-adrenal (HPA) trabalha de maneira distinta da fase embrionária, levando ao assim denominado período hiporresponsivo ao estresse, com menores liberações de corticosterona e hormônio adrenocorticotrópico (ACTH). Estudos apontam que a MN induz uma modulação a longo prazo sobre o eixo HPA, diminuindo a reatividade a eventos estressores e modificando alguns comportamentos na vida adulta. O objetivo central da presente tese foi avaliar comportamentos executivos e possíveis alterações neuroquímicas no córtex pré-frontal medial vento-medial de ratos manipulados no período neonatal. Os resultados mostraram-se sexo-específicos, pois animais machos manipulados no período neonatal mostraram-se mais impulsivos na vida adulta, apresentando também alterações na atenção e na flexibilidade cognitiva. Além disso, a responssividade ao fármaco metilfenidato (Ritalina®) nesse grupo foi encontrada diminuída. Com referência ao controle inibitório comportamental, mediante a tarefa de tolerância ao atraso da recompensa, a impulsividade foi observada apenas em animais machos e manipulados no período neonatal, e esta alteração não foi revertida pelo fármaco metilfenidato. Contudo ao analisar os níveis das aminas biogênicas, dopamina e serotonina e seus metabólitos 3,4-ácido dihidroxifenilacético (DOPAC), ácido homovanílico (HVA) e 5-ácido 5-hidroxiindolacético (5-HIAA) e noradrenalina, não se verificou modificação em relação aos grupos experimentais, entretanto, fêmeas tiveram concentrações maiores de DOPAC que os machos. Em relação à atenção

e à flexibilidade cognitiva em machos, verificou-se no teste de deslocamento da atenção (TDA) um pior desempenho dos animais manipulados nas associações simples e na mudança intradimensional; entretanto, com melhora na extradimensional. A respeito das análises neuroquímicas no CPFvm, observou-se diminuição da atividade da enzima Na+/K+-ATPase e do imunoconteúdo da proteína sinaptofisina. Não houve alterações, contudo, nos imunoconteúdos de tirosina hidroxilase (TH) e sua forma fosforilada (THf), e nos níveis do fator neurotrófico derivado do encéfalo (BDNF). Ao observar a flexibilidade cognitiva e a atenção, os resultados indicaram pior desempenho na tarefa do deslocamento da atenção apenas em fêmeas do grupo MN, na discriminação de reversão. Juntamente com esses parâmetros, a atividade da enzima Na+/K+-ATPase foi encontrada diminuída em CPFvm no grupo MN. Tendo em vista os resultados desta tese, percebe-se que a manipulação neonatal em ratos altera alguns comportamentos cognitivos de forma distinta entre os sexos: provoca a diminuição do controle inibitório e resposta ao metilfenidato em machos; e modifica a atenção e flexibilidade cognitiva em ambos os sexos, com maior volume de alterações em machos. Coube a esta tese considerar que, breves separações entre a mãe e os filhotes no período neonatal provocam diferentes consequências cognitivo-comportamentais na vida adulta da prole, e isto é dependente do sexo. Com isso, pôde-se concluir que, a fase neonatal é um momento vulnerável a influências ambientais, e estas produzem múltiplos desfechos comportamentais ao longo da vida.

ABSTRACT

The neonatal period is considered a vulnerability window to physiological and behavioral alterations during ontogeny. Enviromental modifications in this period may predispose the individual to disorders as attention defficit hyperactivity disorder (ADHD), psicoative substance abuse, and eating disorder. Animal models which mimic interventions in this phase are scientifically relevant. Neonatal handling (NH) is one of these models: brief separations between the mother and the litters are made during the first two weeks of life (1^{st}-10^{th} day of life) for 10 minutes daily. During the neonatal period, the hypothalamus-pituitary-adrenal (HPA) axis works differently from the embrionary phase, resulting in a stress hiporresponsive period, with a less release of corticosterone and adrenocorticotropic hormone (ACTH). Studies show that NH induces a long-term modulation on HPA axis, decreasing its reactivity to stressors in adult life. The main objective of this thesis was to evaluate executive behaviors and possible neurochemical alterations in the medial ventro-medial prefrontal cortex of rats handled in the neonatal period. The results were sex-specific, because neonatal handled male animals showed more impulsive behavior in adult life, also presenting modifications in attention and cognitive fexibility. Furthermore, the responsiveness to methilphenidate (Ritalina®) in this group was decreased. In relation to behavioral inhibitory control by tolerance to delay of reward task, the impulsivity was observed only in male handled animals, and it was not reversed by methilphenidate. However, when biogenic amines (dopamine and serotonin and their metabolites 3,4-dihidroxidphenilacetic acid (DOPAC), homovanilic acid (HVA), 5-hidroxy-indol-acetic acid (5-HIAA), and noradrenaline) were measured, their levels were not modified, though females showed increased concentrations of DOPAC compared to males. In relation to attention and cognitive flexibility in males, we verified a reduced performance of the neonatal handled group in the attentional set shifting task, both in simple associations and intradimensional shifts, despite an improvement in extradimensional shift.

The neurochemical analysis in PFCvm showed a decrease in the activity of Na+/K+-ATPase, and in the immunocontent of synaptophysin. There were no alterations in the immunocontent of tyrosine hydroxylase (TH) or its phospholitated form (pTH), and no difference was observed in the levels of brain derived neurotrophic factor (BDNF). When studying cognitive flexibility and attention in attentional set shifting task in females, the results indicated a decreased performance in reverse discrimination in the NH group. Additionally, the activity of Na+/K+-ATPase enzyme was found decreased in PFCvm in NH group. Thus, the results of this thesis show that neonatal handling in rats alter, in a distinct form, cognitive behaviors between sexes: Provoke a decrease of inhibitory behavior and together the response to methilphenidate in males; and addictionally modifies attention and cognitive flexibility in both sexes, with more alterations in males. Therefore, this thesis considered that briefly separations between dam and pups in the neonatal period provoke differents behavioral cognitive consequences in adult life of litters, and this subject depend on sex. Thus, we can conclude that neonatal period is a vulnerable moment to enviromental influences, and these features produce multiple behavior outcomes along life.

1.1 Janela de vulnerabilidade e manipulação neonatal

O conjunto de experiências, aversivas ou não, vivenciadas pelos indivíduos durante toda a vida propicia a construção da personalidade e o desenvolvimento de comportamentos em resposta a distintos estímulos ambientais contextuais (Obradovic and Boyce, 2009, Raineki *et al*., 2014). Juntamente com esses fatores, ações simultâneas entre heranças genéticas e epigenéticas moldam sistemas comportamentais (Weaver, 2014).

Durante todo o desenvolvimento, existem períodos considerados "*janelas de vulnerabilidade"* para o desencadeamento de transtornos psicológicos e/ou psiquiátricos (Raineki *et al*., 2014). Fases como os períodos intraútero, neonatal e a puberdade são exemplos de janelas de vulnerabilidade (para uma revisão ver: Raineki *et al*., 2014). No decorrer dessas fases, situações negativas, como negligência parental, abusos de conteúdo violento, conflitos familiares, e relações distantes entre pais e filhos (Fleitlich and Goodman, 2001, Pires Tde *et al*., 2012, O'Donnell *et al*., 2014) podem estimular a susceptibilidade a transtornos psicobiológicos, *e.g.* déficit de atenção com hiperatividade (TDAH) (Froehlich *et al*., 2011), distúrbios alimentares (Skowron *et al*., 2014), abuso de substâncias psicoativas (Kosten *et al*., 2000), entre outros. Experiências de cunho neutro e/ou benéficas, contudo, como o cuidado parental adequado, proporcionam melhores respostas contextuais a eventos estressores ou adversidades (Obradovic and Boyce, 2009, O'Donnell *et al*., 2014). Dessa forma, há uma interação de experiências precoces com o desenvolvimento intrínseco do indivíduo permitindo um direcionamento positivo ou negativo no decorrer da vida (O'Donnell *et al*., 2014, Raineki *et al*., 2014).

O período pós-natal é um momento crítico para o desencadeamento de alterações de longo prazo no funcionamento do encéfalo, que podem levar futuramente a distúrbios mentais ou à aquisição de resiliência (Raineki *et al*., 2014). Nessa fase, importantes modificações fisiológicas e comportamentais são verificadas, e há uma plasticidade exacerbada em estruturas do sistema

nervoso central (SNC) (Raineki *et al*., 2014). Modelos animais utilizando intervenções durante o período neonatal demonstram as implicações dessa fase ao longo da vida. A manipulação neonatal em ratos (MN) (Figura 1) é um desses modelos, tendo sido descrita inicialmente na década de 1950, por Levine e colaboradores (1957). Nesse modelo, os filhotes são brevemente separados da mãe, que permanece na mesma sala por 3 a 15 minutos por dia, nas primeiras semanas de vida (dias 1-21 pós-natal), podendo haver variações de acordo com cada protocolo de pesquisa (Noschang *et al*., 2012b).

Figura 1.a.(esquerda). Fotografia que exemplifica o procedimento de manipulação neonatal. Durante sua realização, a caixa moradia (esquerda) com a mãe permanece na sala durante todo o procedimento (10 min). **b.** (direita). Os filhotes estão na incubadora. Papel toalha permanece no assoalho em contato com os filhotes, e luvas diferentes são utilizadas para a manipulação de cada ninhada.

1.2 O eixo hipotálamo-pituitária-adrenal (HPA) e a manipulação durante o período neonatal

Respostas neuroendócrinas ao estresse se dão por ativação de neurônios parvocelulares do núcleo paraventricular do hipotálamo e consequente liberação do hormônio liberador de corticotropina (CRH). Este último, por sua vez, atua na pituitária (hipófise) anterior proporcionando a liberação de hormônio adrenocorticotrópico (ACTH) (Ulrich-Lai and Herman, 2009, Silberman *et al*., 2016). Nessas condições, o ACTH liberado de forma endócrina é transportado pela corrente sanguínea e, por sua vez, atua no córtex da glândula adrenal provocando a liberação de glicocorticoides (GC) (cortisol em humanos e corticosterona (CORT) em roedores) (Ulrich-Lai and

Herman, 2009) (Figura 2). Altos níveis de glicocorticoides realizam o controle do eixo, com o estabelecimento da retroalimentação negativa. Receptores de glicocorticoides (GR) e mineralocorticoides (MR) são expressos em regiões como hipotálamo e hipocampo, promovendo a diminuição de níveis de cortisol pela ativação de circuitos inibitórios direcionadas ao hipotálamo (Lupien *et al*., 2009).

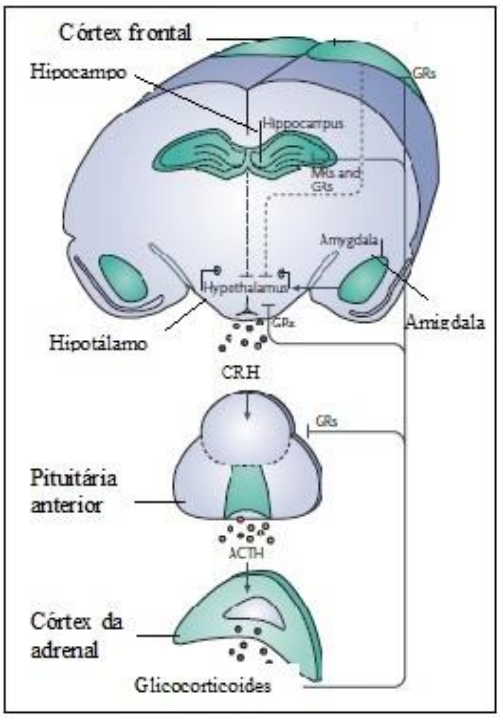

Figura 2. Esquema **e**xemplificado em encéfalo de rato, o eixo hipotálamo-pituitária- adrenal ativado pelo estresse e causa liberações hormonais subsequentes. Retirada e adaptada de Lupien *et al*., (2009).

Inicialmente, na década de 50, estudos acerca da manipulação neonatal em roedores mostraram efeitos hormonais permanentes ao longo da vida, resultados esses que foram reproduzidos em muitos trabalhos: verificou-se que animais manipulados possuem uma menor reatividade a eventos estressores na vida adulta (Levine, 1957, Meerlo *et al*., 1999), isto é, menores níveis de ACTH e corticosterona liberados, com rápido retorno a níveis basais (Levine, 1957, Meaney *et al*., 1993, Raineki *et al*., 2014).

O eixo HPA dos roedores desenvolve-se no período gestacional, e observa-se elevada secreção de glicocorticoides, hormônios primordiais na maturação de órgãos como encéfalo, pulmões e rins (Sapolsky and Meaney, 1986, Constantinof *et al*., 2015). No momento do nascimento, o eixo HPA modula respostas moderadas ao estresse. Em torno da segunda semana de

vida pós-natal, observa-se uma diminuição da CORT, sendo esta fase denominada "Período Hiporresponsivo ao Estresse"(PHE) (Yoshimura *et al*., 2003, Lupien *et al*., 2009, Raineki *et al*., 2014). Eventos estressores nesse momento, como cirurgias, manipulações e alterações térmicas, geram fracas respostas fisiológicas (Sapolsky and Meaney, 1986, Raineki *et al*., 2014). A causa biológica do PHE está relacionada com a imaturidade dos secretagogos adrenocorticais, que possuem diminuída sensibilidade ao ACTH, e um aumento exacerbado da retroalimentação negativa dos glicocorticoides no hipotálamo e hipófise (Halasz *et al*., 1997, Yoshimura *et al*., 2003). Por essas razões, há, nessa fase, uma diminuição da resposta do eixo HPA a estímulos nocivos. No entanto, mesmo com a concentração total de corticosterona plasmática baixa, a fração biologicamente ativa é relativamente alta, determinando suas ações hormonais, sistêmicas e, principalmente, encefálicas (Haltmeyer *et al*., 1966, Bartova, 1968). Gradualmente, em seguida ao PHE, há um aumento da responssividade adrenocortical ao ACTH devido à maturação fisiológica do rato, o que ocorre, no caso deste sistema, em torno dos 16-21 dias de vida (Arai and Widmaier, 1993).

Comportamentos maternos diante da ninhada, como diversos tipos de contato e estimulações tácteis, podem alterar níveis de glicocorticoides no PHE (Raineki *et al*., 2014). Após o procedimento de MN, com o retorno dos filhotes ao contato com a mãe, esta assume comportamentos diante da ninhada que podem alterar o desenvolvimento encefálico nesse importante período (Champagne and Meaney, 2001, Cirulli *et al*., 2003, Raineki *et al*., 2014). Isso porque, quando afastados de suas progenitoras, roedores filhotes emitem vocalizações ultrassônicas, as quais são sons afetivos percebidos pelas mães, que servem como sinalização para a assistência parental (Hahn and Lavooy, 2005).

1.3 Efeitos comportamentais da manipulação neonatal

Roedores, como os ratos (*Rattus novergicus*) utilizados em experimentação animal, possuem um comportamento inato neofóbico quando adentram locais desconhecidos. Esses animais apresentam comportamentos defensivos e, normalmente, possuem preferência por zonas não iluminadas, de paredes fechadas (comportamento de tigmotaxia: deslocamento em ambiente por meio do toque sensorial em paredes), o que evita a exposição a possíveis predadores (Barnett, 1963, Carobrez and Bertoglio, 2005).

Mudanças comportamentais provocadas pela MN são observadas ao longo da ontogenia dos animais. Como citado anteriormente, animais manipulados no período neonatal possuem reatividade ao estresse diminuída (Raineki *et al*., 2014). Parâmetros que avaliam a emocionalidade estão igualmente reduzidos: (i) a exploração em ambientes novos (campo aberto) é aumentada mesmo em presença de predador (Padoin *et al*., 2001), visto que exibem menor medo e menor tigmotaxia (Madruga *et al*., 2006); (ii) em labirinto em cruz elevado, o comportamento do tipo ansioso mostra-se atenuado, pois há menor permanência nos braços fechados (Meerlo *et al*., 1999, Caldji *et al*., 2000).

Quesitos mnemônicos já foram bastante estudados: (i) na avaliação da memória espacial em labirinto aquático de Morris realizada por nosso laboratório, apenas fêmeas manipuladas mostraram prejuízo na tarefa (Noschang *et al*., 2010), enquanto que outros estudos encontraram melhora em machos (Kosten *et al*., 2012); (ii) Noschang e colaboradores (2012a) avaliaram a memória olfativa, que se mostrou apropriada, o que também expõe boas condições olfativas a alimentos palatáveis no grupo experimental (Noschang *et al*., 2012a). (iii) Em tarefas com componente aversivo, como o medo condicionado, nota-se uma diminuição de respostas corporais (congelamento) ou aumento da extinção dessas memórias (Kosten *et al*., 2012); (iv) entretanto, em esquiva inibitória e sobressalto potencializado por medo, existem prejuízos relatados (Kosten *et al*.,

2012); (v) observando-se a memória de trabalho em labirinto radial, verifica-se melhor desempenho em animais idosos machos (Vallee *et al*., 1999, Kosten *et al*., 2012). Outros comportamentos serão citados nos próximos tópicos.

1.4 Inibição comportamental, manipulação neonatal e comportamentos correlatos

Inibição comportamental (IC) é um componente cognitivo motor da tomada de decisão, pertencente às funções executivas dos indivíduos, juntamente com memória de trabalho, planejamento de ações, resolução de problemas, atenção e flexibilidade cognitiva (Elliott, 2003, Bari and Robbins, 2013). A inibição de comportamentos não apropriados é, muitas vezes, voluntária e consciente, essencial para a sobrevivência de um indivíduo em um dado contexto, uma vez que esses comportamentos podem ser reajustados para um melhor desempenho no ambiente no qual o indivíduo está inserido (Bari and Robbins, 2013).

A falha do processo inibitório comportamental se denomina impulsividade e, nessa situação, há prejuízo das funções executivas (Bari and Robbins, 2013). Traços impulsivos implicam, geralmente, em consequências negativas e manifestam-se pelos seguintes sintomas:

- Imediatismo temporal diante de recompensas (Bari and Robbins, 2013);
- Atos realizados sem reflexão prévia ou sem informações disponíveis sobre o contexto (Bari and Robbins, 2013);
- Interrupções em conversações para responder questionamentos rapidamente (Bari and Robbins, 2013);
- Alimentação excessiva de alimentos palatáveis (Yeomans and Brace, 2015) ;
- Comportamentos de risco (como drogadição e comportamento sexual de risco) (Wilson and Vassileva, 2016);
- Necessidade de novidade (Lukkes *et al*., 2015).

Muitos quadros clínicos psiquiátricos trazem consigo a impulsividade como um de seus sintomas: ADHD, esquizofrenia, doença de Parkinson, uso abusivo de drogas, indivíduos portadores do quadro obsessivo-compulsivo, entre outras (Bari and Robbins, 2013). Implicações negativas futuras estão interligadas à impulsividade: início precoce da delinquência juvenil (John *et al*., 1994), indivíduos antissociais (Luengo *et al*., 1994) e comportamento suicida (Dumais *et al*., 2011). Entretanto, alguns estudos apontam indícios adaptativos, como mentes criativas e decisões rápidas para problemas fáceis, que são exemplos de pontos benéficos de personalidades impulsivas (Bari and Robbins, 2013).

Estudos analisando animais manipulados sugerem características impulsivas, especialmente no comportamento alimentar. Primeiramente, Silveira e colaboradores (2004) evidenciaram uma maior ingestão de alimentos palatáveis doces (Froot loops®) bem como salgados (Cheetos®) em curtos espaços de tempo, sem alteração em ração padrão. Este resultado mostrou-se reprodutível em outros estudos (Silveira et al., 2008, Portella et al., 2010, Silveira et al., 2010). Quando se observou o parâmetro da saciedade desses animais, fez-se a administração repetida de cereal doce, e novamente houve ingestão de ampla quantidade em um curto tempo, ocorrendo sua saciedade antes dos controles, que alimentaram-se mais devagar e espaçadamente (Silveira et al., 2006b). Com isso, a MN induz a abrupta alimentação palatável, o que condiz com um fenótipo impulsivo (Garza et al., 2016).

Testes em humanos e animais mensuram o comportamento impulsivo. Em pessoas, são utilizados tanto instrumentos operantes como a tarefa do atraso descontado (*delay disconcouting test*) (Odum, 2011), como questionários respondidos pelo próprio indivíduo (Bari and Robbins, 2013). Em animais, tarefas comportamentais diversificadas aferem esse comportamento. Nesta tese foi realizada a tarefa "*Tolerância ao atraso da recompensa*" (TAR) para medir o índice de impulsividade dos animais. A tarefa foi realizada em um labirinto em T e constitui-se de três fases (pré-treino, treino e fase de teste), baseando-se na escolha entre uma recompensa menor e imediata, ou outra, maior e com tempo de espera antes de atingí-la (Bizot et al., 2007) (Figura 3).

A metodologia detalhada está descrita no capítulo I.

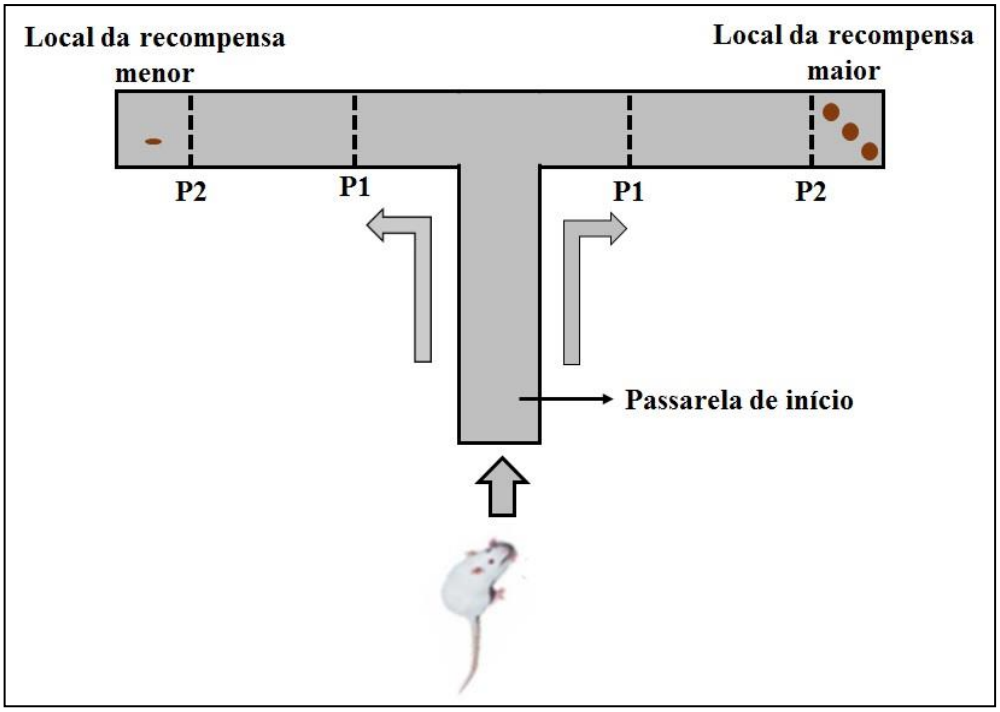

Figura 3. Labirinto em "T" utilizado no teste TAR. Há uma passarela de início na qual o rato é colocado para o começo da tarefa. Tanto nas laterais direita e esquerda encontram-se 2 portas: P1, porta 1; P2, porta 2. Entre P1 e P2, o rato pode se acomodar com as portas fechadas. Após a P2, encontra-se o local de disposição das recompensas, tanto maior como a menor dispostas aleatoriamente para cada animal.

1.5 Manipulação neonatal, atenção e flexibilidade cognitiva

Atenção é a capacidade de um indivíduo focalizar-se em subsídios relevantes de seu contexto e ignorar estímulos irrelevantes (Callahan and Terry, 2015). Existem subdivisões dentro do parâmetro da atenção:

- Atenção sustentada: dentro de um período de tempo, há a focalização em um estímulo;
- Atenção seletiva: manutenção da atenção em um dado estímulo, e simultaneamente na presença de outros irrelevantes;
- Atenção orientada: atenção direcionada espacialmente a um estímulo;

- Atenção dividida: atenção à realização de múltiplas tarefas ao mesmo tempo (Callahan and Terry, 2015).

Um aspecto da atenção é a flexibilidade cognitiva, habilidade esta que desenvolve respostas em um ambiente em modificação (Dajani and Uddin, 2015). Trata-se de uma capacidade cognitiva, a qual permite o deslocamento atencional de um estímulo, previamente relevante em um dado contexto, para outro, antes considerado irrelevante, gerando respostas comportamentais apropriadas (Dajani and Uddin, 2015). Ambas as capacidades citadas são essenciais para a sobrevivência animal, e estão associadas com desfechos positivos, relacionados com maior resiliência a eventos negativos e ao estresse ao longo da vida (Dajani and Uddin, 2015). Deficiências nestas habilidades são observadas em indivíduos portadores de ADHD, doença de Parkinson, doença de Alzheimer, esquizofrenia e depressão, entre outras (Callahan and Terry, 2015).

Podem-se testar essas capacidades tanto em humanos quanto em animais. Em humanos, o "*Teste de Classificação de Cartas de Wiscosin*" foi desenvolvido inicialmente na Universidade de Wiscosin em 1948, e utiliza cartas de baralho como instrumento de teste. Por meio de sequências de cores, formas e números, o sujeito submetido ao teste, deve classificar as cartas, e ocasionalmente o experimentador modifica as regras de sorteio das mesmas, o que permite que a flexibilidade do indivíduo seja testada através de erros e acertos resultantes (Grant and Berg, 1948, Tait et al., 2014). Em roedores diversos testes comportamentais mensuram atenção e a flexibilidade cognitiva, tais como:

- Teste das cinco escolhas (Patel et al., 2006, Semenova et al., 2007);
- Aprendizado reverso em labirinto aquático de Morris, e Labirinto em Y (Noschang et al., 2012b);
- Teste de mudança de estratégia (TME, do inglês *Task switching)*, executado em um labirinto de quatro braços fechados (Floresco et al., 2006a);

- A tarefa do deslocamento da atenção (TDA) ou do inglês *"Attentional set shifting task"*, que utiliza potes de cerâmica com distintas características sensoriais (Birrell and Brown, 2000).

A tarefa de TME foi realizada na presente tese. Esta tarefa (Figura 4) baseia-se no protocolo de Floresco et al. (2006a), com adaptações de Pandolfo et al. (2013), e utiliza um labirinto de quatro braços fechados, onde os animais devem procurar recompensas alimentares (pedaços de ração Royal Canin junior ®) seguindo dicas impostas ou sensório-visuais, como na figura abaixo.

A metodologia detalhada está descrita no capítulo II.

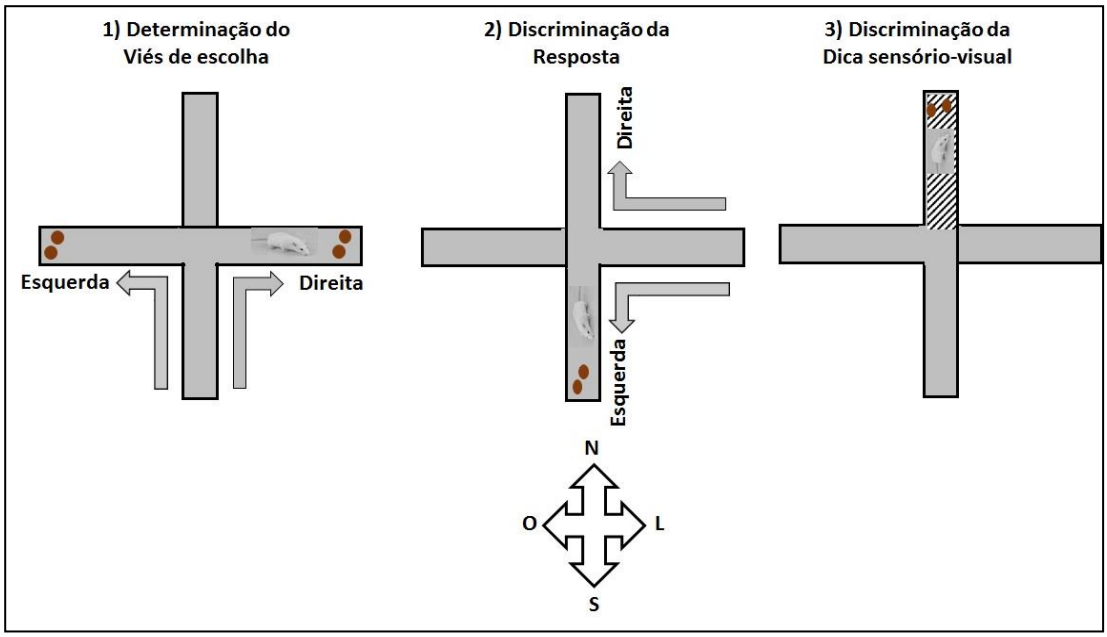

Figura 4. Labirinto de quatro braços fechados utilizado no teste de TME. O animal deve buscar os pedações de recompensa nos braços do aparelho durante as fases da tarefa.

A tarefa de TDA, também realizada na presente tese (Figura 5), se baseia no protocolo de Birrel e Brown (2000), o qual utiliza recipientes de cerâmica contendo uma recompensa palatável (Froot Loops®). Os potes diferem em características sensoriais (odor, textura e meio de escavação). O objetivo da tarefa é o encontro da recompensa escondida, enterrada no meio interno de um pote, do par apresentado ao animal.

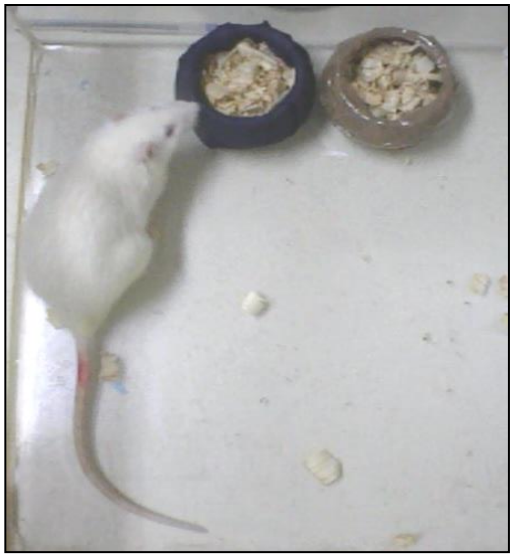

Figura 5a. Ilustração do teste de TDA. **Figura 5b.** Imagem do rato avaliando os potes no teste de TDA. Par de potes apresentado ao animal, que contém características sensoriais diferenciadas, e apenas um possui a recompensa palatável oculta.

1.6 Neurobiologia dos processos executivos

Evidências indicam que o córtex pré-frontal (CPF) controla funções cognitivas, com modulações pelo estriado e tálamo (Rossi et al., 2009, Puig et al., 2015). Um caso ocorrido no ano de 1848, no estado de Vermont, nos Estados Unidos, onde operários dinamitavam rochas, chamou a atenção para esta estrutura: um operário chamado *Phineas Gage* sofreu um grave acidente (Damasio et al., 1994). Uma barra de ferro atravessou seu crânio destruindo-lhe o olho esquerdo, e provocando lesões sérias em sua massa encefálica frontal (Damasio et al., 1994, Garcia-Molina, 2012). Felizmente, Phineas sobreviveu ao acidente. Entretanto, inúmeras alterações de cunho neurobiológico foram desencadeadas: além de crises epilépticas e instabilidade de humor, comportamentos executivos foram afetados, como a ausência de planos futuros, impulsividade e impaciência (Damasio et al., 1994, Thiebaut de Schotten et al., 2015). No final década de 1960, Alexander Luria, um neuropsicólogo soviético, descreveu que lesões localizadas no córtex frontal em humanos provocavam alterações comportamentais nas funções executivas (Luria, 1969).

Estudos em primatas não humanos e roedores (ratos) indicam que lesões localizadas no CPF causam prejuízos em tarefas que avaliam a flexibilidade comportamental, atenção (Birrell and

Brown, 2000) e o controle inibitório (impulsividade) (Donnelly et al., 2014) (Rossi et al., 2009). Obviamente o CPF não age isoladamente na modulação destes comportamentos, entretanto o intuito deste trabalho é verificar a importância desta região em relação à MN.

Anatomicamente o córtex pré- frontal divide-se em sub-regiões: medial, ventral medial, lateral e ventral (Dalley et al., 2004, Kesner and Churchwell, 2011). A presente tese deteve-se em estudar o córtex pré-frontal medial (CPFm), e mais especificamente sua parte ventral (CPFvm) nos comportamentos executivos já citados. O CPF é, por sua vez, subdividido em distintas regiões: médio-dorsal, ventro-medial, lateral e ventral. A primeira região contém o córtex pré-central e cingulado anterior, já a segunda possui os córtices pré-límbico e infralímbico, e a terceira, medial orbital, e a terceira é respectivamente composta pelas regiões, córtices dorsal, ventral agranular insular e lateral orbital, e finalmente a quarta possui as regiões ventral orbital e ventrolateral orbital (Dalley et al., 2004, Kesner and Churchwell, 2011). Para melhor visualização e entendimento, as figuras 6 e 7 mostram esquematicamente as regiões estudadas.

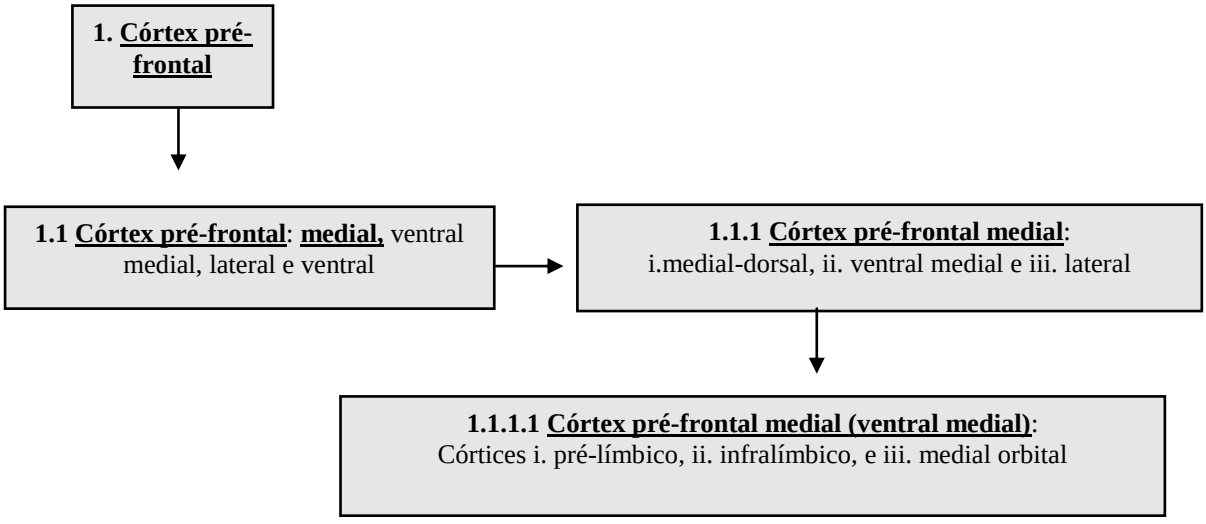

Figura 6. Esquema determinando as partes do córtex pré-frontal.

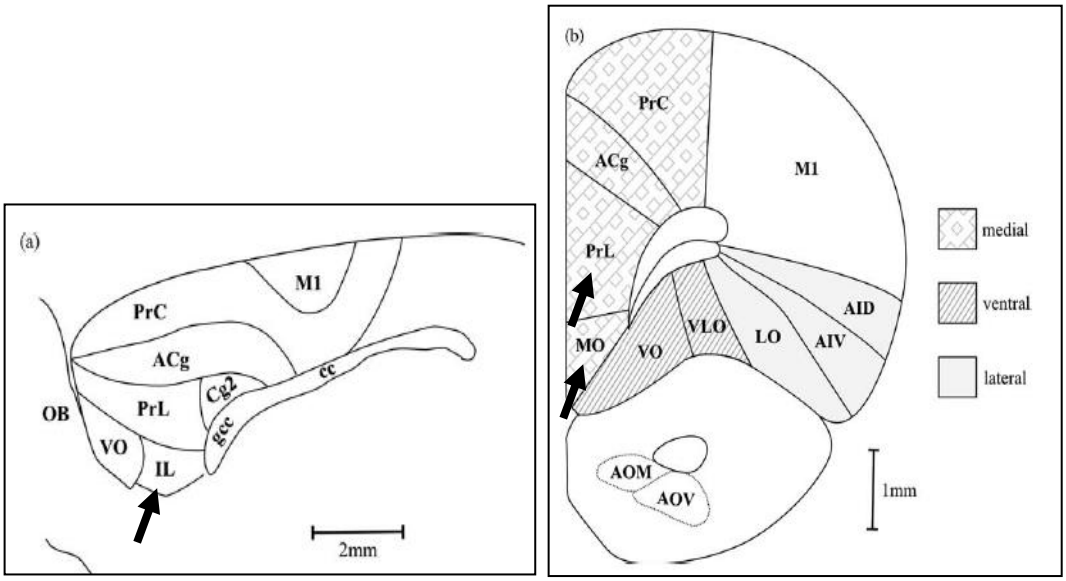

Figura 7. Ilustrações do córtex pré-frontal medial. As setas indicam as regiões utilizadas neste trabalho. a) Vista lateral. b) Vista coronal. Retirada e adaptada de Dalley et al. (2004). PrC: pré-central, ACg: cingulado anterior, PrL: pré-límbico, MO: medial orbital, VO: ventral orbital, VLO: ventral lateral orbital, LO: lateral orbital, AIV: agranular insular ventral e AID: agranular insular dorsal.

As conexões aferentes do CPFvm vêm de núcleos talâmicos (médio dorsal, núcleos da linha média), sistema límbico e associações (córtex perirrinal, entorrinal e hipocampo), núcleo basal da amígdala, prosencéfalo basal medial, mesencéfalo e tronco encefálico (Logue and Gould, 2014). Suas eferências se dão por conta do caudado-putâmen e a parte central (*core*) do núcleo *accumbens* (pré-límbico), sendo que a cápsula (*shell*) deste relaciona-se mais com a parte do infralímbico e orbital medial (Dalley et al., 2004, Kesner and Churchwell, 2011). Inervações para o CPFm, como um todo, são realizadas pela área tegmentar ventral (VTA), locus coeruleus (LC) e núcleos dorsais da rafe, que utilizam as aminas biogênicas dopamina, noradrenalina e serotonina, respectivamente (Logue and Gould, 2014). A Figura 8 mostra as vias citadas acima de modulação neuroquímica do CPFm. Esses neurotransmissores são moduladores de comportamentos executivos, como a atenção, a flexibilidade comportamental e o controle inibitório. Assim, em humanos, os tratamentos farmacológicos para distúrbios relacionados a essas funções executivas utilizam fármacos como o metilfenidato (que inibe a recaptação de catecolaminas), que é utilizado em indivíduos com TDAH

e impulsividade (Mehta et al., 2004), e a fluoxetina (que inibe a recaptação de serotonina) (Carlisi et al., 2016).

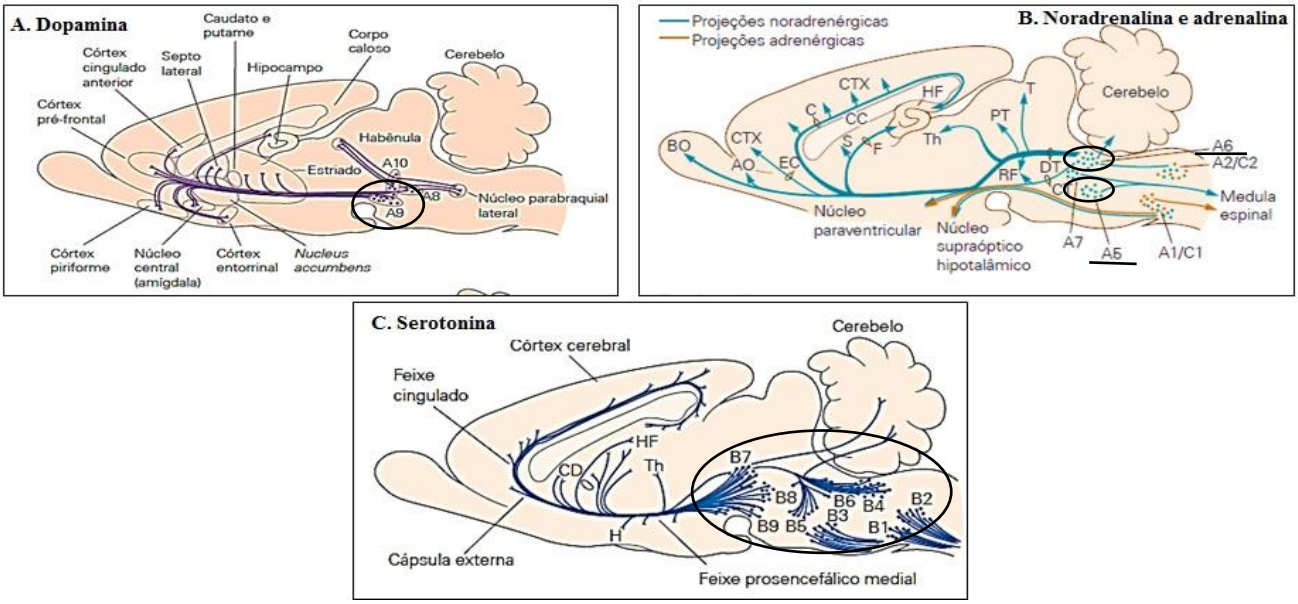

Figura 8. Vias modulatórias aferentes do CPFm em encéfalo de rato. **A.** Via dopaminérgica em A9, a partir da área tegmentar ventral (VTA). **B.** Via noradrenérgica em A5 e A6, a partir do locus coeruleus (LC). **C.** Via serotoninérgica a partir dos núcleos dorsais da rafe a partir de B (NDR). Retirado de Kandel (2015).

1.7 O desenvolvimento do córtex pré-frontal e marcadores de plasticidade: Sinaptofisina e BDNF

O córtex pré frontal medial participa da retroalimentação negativa do eixo HPA, e este controle torna-se mais efetivo por meio do aumento de receptores de glicocorticoides na estrutura (Raineki et al., 2014). A MN causa um aumento do número dos receptores GR no CPFm, bem como no hipocampo (Meaney et al., 1985, Myers et al., 2012).

O CPFm possui um desenvolvimento considerado tardio frente a outras estruturas encefálicas (Kolb et al., 2012). Na Figura 9 observa-se a linha do tempo do desenvolvimento encefálico, e percebe-se que esse processo é finalizado aproximadamente na quadragésima década em humanos, ou ao redor dos 200 dias de vida em ratos (Kolb et al., 2012). No período neonatal, há exacerbada produção de sinapses, que sofrerão a poda sináptica natural a partir da infância,

processo este que ocorre mais vagarosamente no CPF, alongando-se até a vida adulta (Petanjek et al., 2011, Kolb et al., 2012). Desse modo, diferentes experiências vivenciadas ao longo do desenvolvimento, bem como alterações metabólicas e hormonais nos períodos pré e neonatal, provocam modificações comportamentais e neuronais nesta estrutura (Kolb et al., 2012).

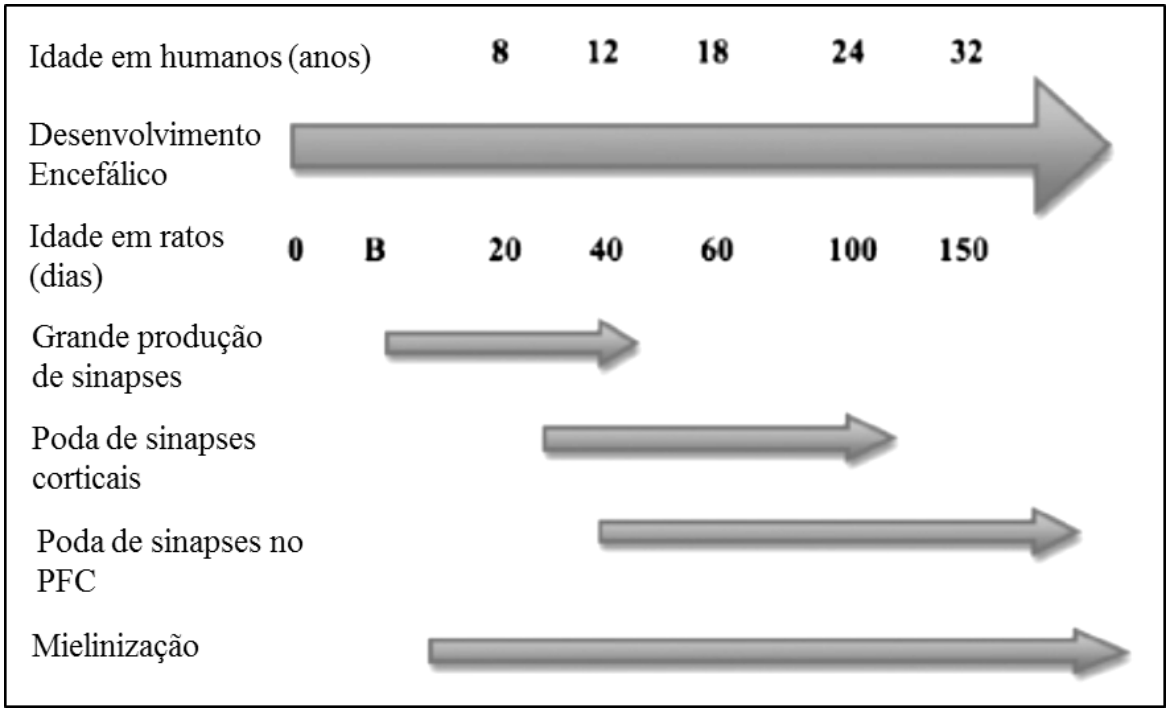

Figura 9. Linha do tempo do desenvolvimento encefálico em humanos e ratos. Observa-se que a maturação completa do sistema nervoso se dá na vida adulta de ambas espécies. Retirado e adaptado de Kolb et al., (2012).

Inúmeros fatores influenciam a quantidade de sinapses e espinhos dendríticos por exemplo. Estimulações táteis/motoras, estresse pré-natal, privação materna e/ou paterna, comportamentos sociais e uso de drogas ou fármacos em diferentes idades são fatores que incrementam ou diminuem estruturas sinápticas (Petanjek et al., 2011).

A sinaptofisina, uma proteína encontrada em vesículas sinápticas, é um indicativo de integridade sináptica e participa da neurotransmissão química (Valtorta et al., 2004, Glantz et al., 2007). Seu pico de aparecimento encefálico ocorre na primeira infância e seu decaimento na puberdade (Glantz et al., 2007). Sua função é de auxiliar a fusão e liberação de neurotransmissores na fenda sináptica (Valtorta et al., 2004). Sua ausência, em animais alterados geneticamente,

promove alterações comportamentais como locomoção exacerbada (Schmitt et al., 2009) (Figura 10).

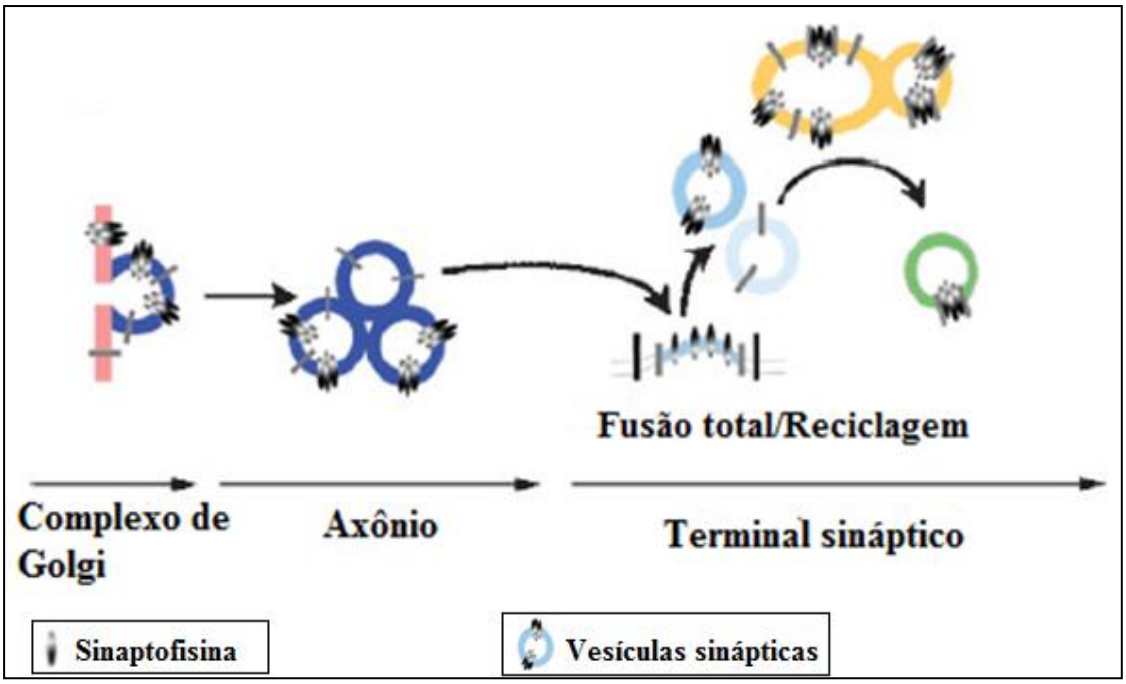

Figura 10. Funções da Sinaptofisina. Sua atuação se dá desde o tráfego vesicular até a liberação do neurotransmissor e reciclagem de vesículas sinápticas.

Outra importante biomolécula relacionada com o desenvolvimento e a plasticidade encefálica é o fator neurotrófico derivado do encéfalo (BDNF) (Sakata et al., 2013). Sabe-se que suas concentrações são influenciadas por modificações ambientais e experiências precoces. Estudos avaliando BDNF em animais submetidos à MN demonstram um efeito sexo-dependente em bulbo olfatório minutos após a manipulação (Reis et al., 2014). Em CA4 hipocampal, foi observado que a MN aumenta os níveis deste fator neurotrófico na idade adulta, melhorando também a memória espacial (Garoflos et al., 2005a, Garoflos et al., 2005b).

1.8 Manipulação neonatal e aminas biogênicas

Estudos com animais adultos submetidos no período neonatal à manipulação mostram que este procedimento altera as concentrações de certos neurotransmissores em distintas regiões encefálicas. No núcleo accumbens, dopamina e serotonina apresentam níveis aumentados, com

diminuição de seus metabólitos, ácido 3,4-diidroxifenilacético (DOPAC), ácido homovanílico (HVA), e ácido 5-hidroxiindolacético (5-HIAA), sem alteração em noradrenalina; esses efeitos foram relacionados com um aumento do apetite para alimento doce (Portella et al., 2010, Silveira et al., 2010). Na região da VTA e na parte compacta da substância negra não se observam modificações na enzima tirosina-hidroxilase, a qual é o passo limitante na síntese de DOPA (diidroxifenilalanina), precursor das catecolaminas (Madruga et al., 2006) (Figura 10). Na região da amígdala, há diminuição dos neurotransmissores NE, DA e 5-HT (Caldji et al., 2000, Arborelius and Eklund, 2007).

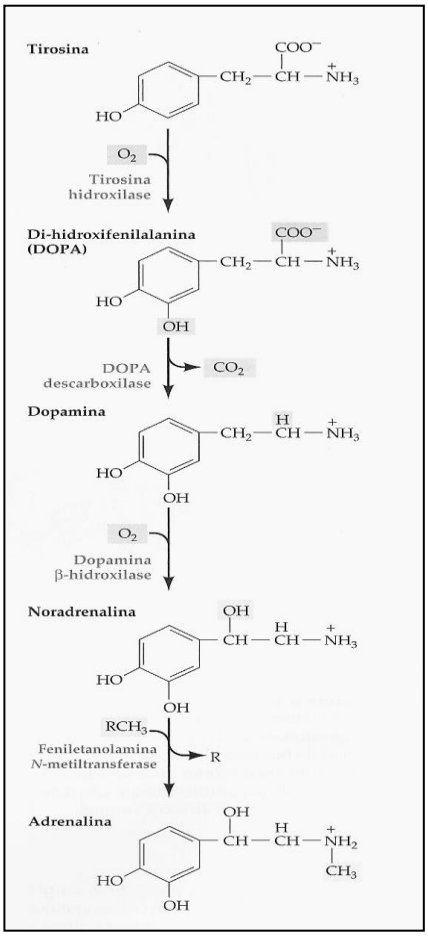

Figura 11. Via de síntese das catecolaminas. A partir do aminoácido tirosina, por ação da enzima tirosina-hidroxilase, ocorre a síntese de DOPA, precursor de dopamina, noradrenalina e adrenalina. Retirado de Goodman & Gilman (2010).

1.9 Na+/K+ -ATPase e manipulação neonatal

A enzima Na+/K+-ATPase atua fisiologicamente na manutenção do potencial de membrana neuronal, realizando o co-transporte ativo de íons Na+ para fora da célula e de íons K+ para o ambiente interno, permitindo a formação do gradiente iônico (Therien and Blostein, 2000). Esta proteína é fundamental para o estabelecimento do potencial de repouso nos neurônios e para a ocorrência do potencial de ação, na transmissão da informação nervosa (Therien and Blostein, 2000).

O procedimento de MN altera significativamente a atividade da Na+/K+-ATPase no encéfalo. Estudos realizados em nosso laboratório mostram aumento da atividade da enzima no hipocampo e no bulbo olfatório em ratas fêmeas manipuladas (Noschang et al., 2012a). Em um estudo avaliando a resiliência adquirida pelos animais manipulados a um quadro do tipo depressivo após estresse crônico e variado, observou-se que a exposição ao estresse crônico causou diminuição na atividade da enzima em todas as estruturas estudadas (hipocampo, amígdala e córtex parietal), e este efeito ocorreu principalmente nos animais não manipulados, sugerindo que os animais manipulados apresentariam menor susceptibilidade aos efeitos do estresse crônico na vida adulta (Silveira et al., 2011). Finalmente, um estudo realizado por Benetti e colaboradores (2010) mostrou que a atividade desta enzima, que diminui em hipocampo e amígdala após exposição crônica a uma dieta com chocolate, não sofre o mesmo tipo de alteração em fêmeas manipuladas no período neonatal.

Conforme o exposto acima, tendo em conta as alterações comportamentais e neuroquímicas já descritas na literatura, levantamos a hipótese, de que a manipulação neonatal provoca modificações nos comportamentos executivos: (i) atenção, (ii) flexibilidade cognitiva e (iii) impulsividade. Da mesma forma estas possíveis modificações comportamentais podem resultar de

alterações neuroquímicas no córtex pré-frontal medial ventro-medial, local de influência importante destes comportamentos.

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar comportamentos executivos em animais manipulados no período neonatal, e alterações neuroquímicas em córtex pré-frontal medial ventro-medial em ratos machos e fêmeas na idade adulta.

2.2 Objetivos específicos

Capítulo I

1. Avaliar o comportamento impulsivo na vida adulta, em animais manipulados no período neonatal, machos e fêmeas, por meio da tarefa tolerância ao atraso da recompensa, com e sem administração do fármaco metilfenidato.

2. Mensurar em CPFvm, os níveis de dopamina, serotonina, e seus metabólitos, DOPAC, ácido homovanílico, e 5-HIIAA, bem como noradrenalina, na vida adulta de animais manipulados no período neonatal, machos e fêmeas.

Capítulo II

3. Avaliar o comportamento atencional e a flexibilidade cognitiva, na vida adulta, em ratos machos manipulados no período neonatal, por meio do teste de mudança de estratégia e da tarefa do deslocamento da atenção.

4. Verificar, em ratos machos adultos manipulados no período neonatal, na estrutura encefálica CPFvm, o imunoconteúdo da enzima tirosina hidroxilase em sua forma fosforilada ou não, da proteína sinaptofisina, e também os níveis do fator neurotrófico derivado do encéfalo (BDNF), bem como a atividade da enzima Na+/K+-ATPase,.

5. Gerar uma rede computacional de bioinformática para procurar pontos de interligação entre as proteínas de interesse: tirosina hidroxilase, sinaptofisina, fator neurotrófico derivado do encéfalo e Na+/K+-ATPase.

6. Capítulo III

7. Avaliar o comportamento atencional e a flexibilidade cognitiva, na vida adulta, em fêmeas manipuladas no período neonatal, por meio dos testes de mudança de estratégia e de deslocamento da atenção.

8. Verificar na estrutura encefálica CPFvm, a atividade da enzima Na+/K+-ATPase, na idade adulta de fêmeas manipuladas no período neonatal.

3. MATERIAL, MÉTODOS E RESULTADOS

3.1 Capítulo I

Journal of Integrative Neuroscience, Vol. 15, No. 1 (2016) 81–95
© Imperial College Press
DOI: 10.1142/S0219635216500047

Imperial College Press
www.icpress.co.uk

Neonatal handling causes impulsive behavior and decreased pharmacological response to methylphenidate in male adult wistar rats

Camilla Lazzaretti*,††, Grasielle Clotildes Kincheski†, Pablo Pandolfo‡, Rachel Krolow§,¶, Ana Paula Toniazzo§, Danusa Mar Arcego§, Natividade de Sá Couto-Pereira§, Fares Zeidán-Chuliá§, Martin Galvalisi‖, Gustavo Costa**, Cecilia Scorza‖, Tadeu Mello e Souza*,§ and Carla Dalmaz*,§

*Programa de Pós-Graduação em Neurociências
Instituto de Ciências Básicas da Saúde (ICBS)
Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

†Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil

‡Universidade Federal Fluminense (UFF), Rio de Janeiro, RJ, Brazil

§Programa de Pós-Graduação em Ciências Biológicas: Bioquímica
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde (ICBS)
Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

¶Programa de Pós-Graduação em Saúde e Comportamento
Universidade Católica de Pelotas (UCPel), Pelotas, RS, Brazil

‖Departamento de Neurofarmacología Experimental
Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay

**Departamento de Neuroquímica
Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay
††camillinhalazzaretti@yahoo.com.br

[Received 19 April 2015; Accepted 5 October 2015; Published 27 November 2015]

Neonatal handling has an impact on adult behavior of experimental animals and is associated with rapid and increased palatable food ingestion, impaired behavioral flexibility, and fearless behavior to novel environments. These symptoms are characteristic features of impulsive trait, being controlled by the medial prefrontal cortex (mPFC). Impulsive behavior is a key component of many psychiatric disorders such as attention deficit hyperactivity disorder (ADHD), manic behavior, and schizophrenia. Others have reported a methylphenidate (MPH)-induced enhancement of mPFC functioning and improvements in behavioral core symptoms of ADHD patients. The aims of the present study were: (i) to find *in vivo* evidence for an association between neonatal handling and the development of impulsive behavior in adult Wistar rats and (ii) to test whether neonatal handling could have an impact on monoamine levels in the mPFC and the pharmacological response to MPH *in vivo*. Therefore, experimental animals (litters) were classified as: "non-handled" and "handled" (10 min/day, postnatal days 1–10). After puberty, they were exposed to either a larger and delayed or smaller and immediate reward (tolerance to delay of reward task). Acute MPH (3 mg/Kg. i.p.) was used to suppress and/or regulate impulsive behavior. Our results show that only neonatally handled male adult Wistar rats exhibit impulsive behavior with no significant differences in monoamine levels in

the medial prefrontal cortex, together with a decreased response to MPH. On this basis, we postulate that early life interventions may have long-term effects on inhibitory control mechanisms and affect the later response to pharmacological agents during adulthood.

Keywords: Neonatal handling; impulsivity; prefrontal cortex; psychiatric disorder; manic behavior; attention deficit hyperactivity disorder; ADHD; monoamines.

1. Introduction

Early life individual experiences acting simultaneously with genetic inheritance and epigenetic factors mold both personality and behavior (Roth, 2013; Reichl *et al.*, 2014). During neonatal age, the immature brain is under intense neural plasticity and interfering environmental factors can modify the response to stressors and its resilience (Raineki *et al.*, 2014).

Familiar experiences, such as maternal care in early life, have impact on behavior and neuroendocrine physiology in adulthood, as observed *in vivo* in studies where litters separated from their mothers displayed distinct stress responsiveness in the future (Levine *et al.*, 1957; Meaney *et al.*, 1996; Padoin *et al.*, 2001). Maltreatment, traumatic experiences, or parental neglect, and other childhood adversities can disrupt the control from inhibitory pathways in adult life (Elton *et al.*, 2014; Skowron *et al.*, 2014). Consequently, the neonatal period is considered as a high emotional and physiological window of vulnerability when environmental factors may alter the susceptibility to a number of psychiatric disorders (McIntosh *et al.*, 1999).

Neonatal handling is an established animal model to study the effects of interventions during the neonatal period. This model consists of briefly separating the litters (for 3–15 min) from the mother during the first two weeks of life, approximately (Noschang *et al.*, 2012). It is known that handling modulates the hypothalamic-pituitary-adrenocortical (HPA) axis, which is one of the most important neuroendocrine axes responsive to stress. In adult life, handled animals show persistent hormonal changes such as decreased adrenocorticotropic hormone (ACTH) and corticosterone in response to stress, fearless behavior to new environments, and increased ingestion of palatable food (Meaney *et al.*, 1991; Padoin *et al.*, 2001; Silveira *et al.*, 2004). In addition, a recent study from our own group showed that handling impairs reversal learning in the Y-maze in male rats (Noschang *et al.*, 2012). Reversal learning has been linked by others to behavioral inhibition in preclinical and clinical studies and seems to mainly require the activation of the prefrontal cortex (Bertoux *et al.*, 2012; Bari & Robbins, 2013).

The medial prefrontal cortex (mPFC) is an important brain structure related to inhibitory control of actions together with the orbitofrontal cortex (OFC), and lesions in these two regions can trigger impulsive phenotypes (Cardinal *et al.*, 2004). Adequate serotonin (5-HT), dopamine (DA), and norepinephrine (NE) neurotransmission is required to prevent impulsive response and inappropriate actions, and then select and shift to a new cognitive/behavioral set (Winstanley *et al.*, 2006; Bari & Robbins, 2013; Pardey *et al.*, 2013). Interventions during early life, such as neonatal

handling, can increase the levels of DA and decrease the levels of 5-HT in nucleus accumbens (NAcc), a brain area connected with the mPFC (Portella *et al.*, 2010; Silveira *et al.*, 2010). In addition, early interventions in life (e.g., handling, maternal separation) can alter the response of psychostimulants drugs (Meaney *et al.*, 2002; Silveira *et al.*, 2010).

Methylphenidate (MPH) is a well-known psycho-stimulant drug utilized as a therapeutic agent for the treatment of attention deficit hyperactivity disorder (ADHD). This drug helps to improve characteristic symptoms of the disorder: inattention, impulsivity, hyperactivity, and cooperates in brain performance function (Cooper *et al.*, 2005; Hermens *et al.*, 2007; Kulkarni, 2014). In rodents, MPH is largely utilized in animal models of ADHD as spontaneously hypertensive rat (SHR) or common rat laboratory lineages such as Wistar and Wistar Kyoto (Bizot *et al.*, 2007).

Sex differences regarding the effects of early interventions, such as maternal separation, handling, and hypoxia-ischemia, are frequently observed. Sex-specificity in outcomes from events that occur during the neonatal period have been reported in studies evaluating anxiety (Weinberg *et al.*, 1978; Raineki *et al.*, 2014), spatial learning and memory (Noschang *et al.*, 2010), aversive memory (Kosten *et al.*, 2005), and mitochondrial dysfunction (Renolleau *et al.*, 2008; Weis *et al.*, 2012). Additionally, social stress during development induces long-lasting morphological alterations that include deficits in prefrontal cortex myelination that may potentially be sex-specific (Leussis & Andersen, 2008). In this context, alterations associated to early life events, such as neonatal handling, may contribute to changes in brain functionality and behavioral responses of the offspring.

On this basis, the aim of the present study was (i) to find *in vivo* evidence between early interventions during the neonatal period and impulsive behavior in the adulthood, main characteristic symptom of ADHD and other psychiatric conditions, by using the well-characterized neonatal handling model, (ii) to analyze the monoamine levels in the prefrontal cortex of neonatally handled adult Wistar rats when compared to control animals, (iii) to test *in vivo* the pharmacological response to MPH in these animals since this drug is considered a conventional therapeutic agent for ADHD, and finally (iii) to analyze potential sex-dependent behavioral and pharmacological effects.

2. Material and Methods

2.1. *Animal subjects*

The proceedings were conducted in agreement with the UFRGS – CEUA (Ethics Committee on Animal Use), protocol number 21460, and the Brazilian Law on the use of animals (Federal Law 11.794/2008) and Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003). Female ($N = 22$) primiparous pregnant Wistar rats bred at our own animal facility were randomly selected. They were housed alone from around gestational day

18 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: 12 hours light/12 hours dark, temperature of 22 ± 2°C, cage cleaning twice a week, food and water provided *ad libitum*. All litters were culled to eight pups within 24 hours from birth. The day of birth was considered postnatal day (PND) 0. Weaning occurred on PND 21. When evaluations began, no more than two animals per sex from each litter were used for behavioral experiments and one animal/sex from each litter was used for biochemical measurements for avoiding potential litter bias that could confuse the results, as it was previously observed by Noschang and collaborators (2012). For the experimental groups, the number of animals that were submitted to the behavioral task was as follows: females, non-handled, $N = 8$, and handled, $N = 10$; and males, non-handled, $N = 12$, and handled, $N = 13$.

2.2. *Neonatal handling model*

The neonatal handling *in vivo* model was performed as previously described by Noschang *et al.* (2012). Briefly (Fig. 1(a)), in the non-handled group, pups were left undisturbed with the dam until weaning. Dirty sawdust was carefully removed from one side of the cage, without disturbing the mother and the nest, and then replaced with clean sawdust at that side by the main researcher. Same procedure was performed for changing dirty sawdust in the handled group. In the handled group, the dam was gently pulled to one side of the cage and the pups were carefully removed from their home cage and placed into a clean cage lined with clean paper towel. This cage was placed into an incubator set at 32°C. After 10 min, pups were returned to their dams which were in the same room. The researcher changed gloves between the neonatal handling procedures of each litter to avoid any kind of odor being spread from one nest to the other. These procedures were performed between 10:00 a.m. and 4:00 p.m., and from PND 1 to 10, and then pups were left undisturbed until PND 21, when litters were weaned and separated based on sex.

2.3. *Tolerance to delay of reward*

Animals were food-restricted when reached 45 days old in order to achieve 80% of the original weight. At PND 50, five palatable pellets (see below) were given to the rats to get the animals used to this new food. The behavioral experiment began at PND 52. A wood-made black color T-maze was used to measure the tolerance to delay of reward. With 50-cm high walls, it consists of a starting runaway 50-cm long together with two 50-cm long and 15-cm wide arms. Four removable guillotine wood doors were vertically inserted at the entry and 10-cm from the end of each arm. The between-doors space in each arm was enough for placing a rat. The task was divided into four different phases: habituation, pre-training, training, and testing, adapted from a previous work of Bizot *et al.* (2007). This task was performed between 10:00 and 16:30 hours.

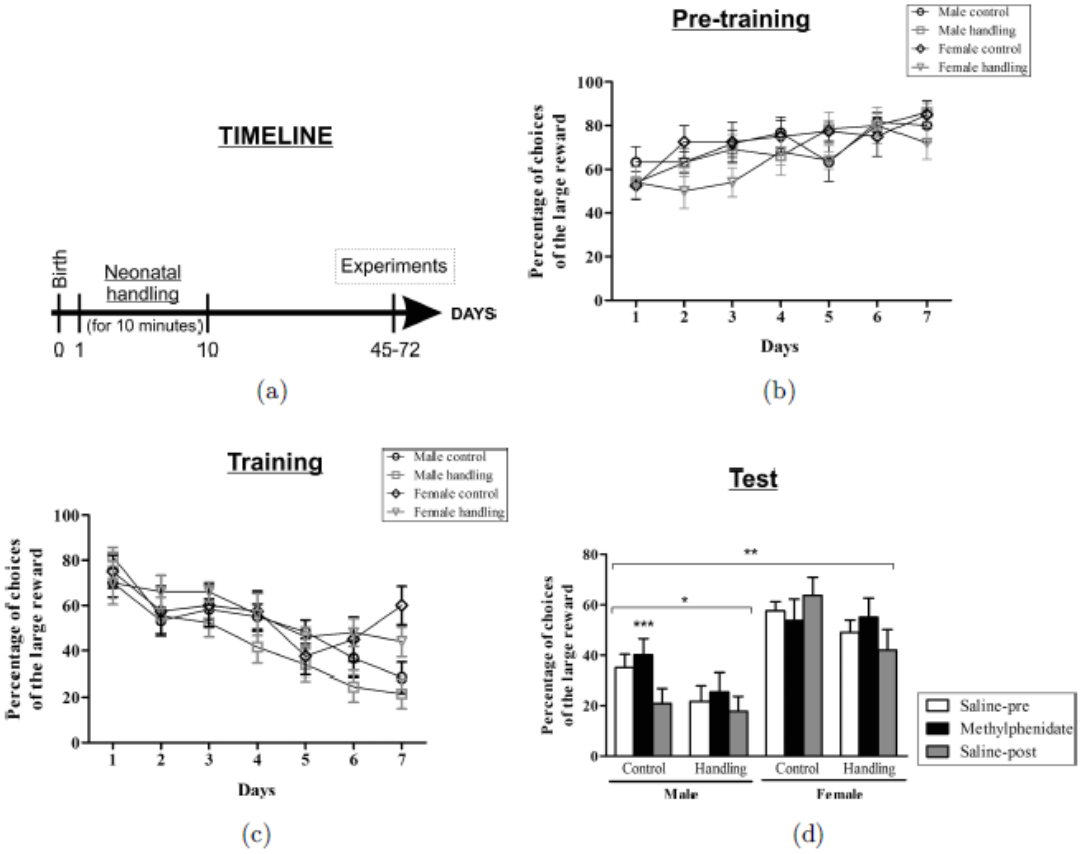

Fig. 1. Neonatally handled male adult Wistar rats exhibit impulsive behavior and decreased response to MPH. (a) Timeline for the experimental design. (b) Percentage (mean ± S.E.M.) of choices for the large reward (pre-training). There was a main effect of session ($p < 0.001$) (Repeated measures ANOVA; $p < 0.001$), but no significant effect of handling was observed. (c) Percentage (mean ± S.E.M.) of choices for the large reward with a 15-s delay (training) in early handled animals vs. controls. Male animals showed a decrease in choices for the large reward side (Repeated measures ANOVA; $p = 0.050$). There was a main effect of session ($p < 0.001$) (Repeated measures ANOVA; $p < 0.001$), but no significant effect of handling was observed. (d) Percentage (mean ± SEM) of choices for a large reward presented after a 30-s delay (testing) 30 min after challenging both groups with either saline or MPH.

Note: *Interaction between group and sex, handling decreasing the percentage of choices of large reward only in males (Repeated measures ANOVA; $p < 0.020$). **Significant difference between sexes; female animals, both control and handling ones, exhibited more choices for the large reward side (Repeated measures ANOVA; $p < 0.001$). ***Significant difference between sessions carried after MPH when compared to saline administration for control male animals (Student's *t*-test, $p = 0.040$). $N = 8$, $N = 10$ (Non-handled and handled females, respectively), $N = 12$, $N = 13$ (Non-handled and handled males, respectively) *per* group.

2.3.1. *Habituation*

Animals were individually placed on the starting runaway and allowed to freely explore the apparatus. Each arm had 3 pellets (Royal Canin Junior®), including the starting runaway, in a total of 9 pellets. After 5 min of exposure to the task, the number of pellets ingested was verified. If the animal had not eaten all of them, it was then subjected to a new habituation section in the next day.

2.3.2. *Pre-training*

In the pre-training phase, each rat was placed on the starting runaway, and it could choose between a small and a large reward (0.5 and 3 pellets, respectively), which were placed in the two arms. The arm where the large reward was placed into was randomly selected for each rat but it was always in the same side throughout the whole experiment for each rat. Animals had equal access to both arms, independent of the amount of reward. Both doors in the chosen arm were opened as soon as the animal turned to its direction. As soon as it crossed the first door, it was closed in order to prevent the animal to escape from the chosen arm. The second door from that arm remained open to allow the animal to eat its reward. After eating the reward, another trial was carried out. Each daily section had five trials. After the fifth daily trial, the animal was returned to its home cage. The criteria to finish the pre-training phase were choosing the large reward at least 4 times within a single section. Otherwise, further trials were carried out in the following day.

2.3.3. *Training*

In the training phase, a delay of 15 s was introduced before the animal had access to the large reward; for instance, after choosing the arm with the large reward, both doors were closed right after the animal crossed the first one, letting the animal between doors during this period. No delay was imposed after entering the small reward arm. This phase ended when the animal chose the large reward at most twice a day, from five trials.

2.3.4. *Testing phase*

The task protocol of tolerance to delay of reward described by Bizot and colleagues (2007) was used in the present study, and uses 3 mg/kg of MPH to test and suppress and/or regulate impulsive behavior showed in choices for large reward with a delay (30 s) (Bizot *et al.*, 2007). The testing phase was carried during six 5-trial daily sessions. About 30 min before each session, animals were received an injection of either saline or MPH (Ritalina – Novartis®, 3 mg/kg, i.p.) as following: saline in sessions 1, 2, 5, and 6, and MPH in sessions 3 and 4. The MPH dose was chosen according to the study by Bizot *et al.* (2007) that obtained a suppression of impulsive behavior in the test phase using Wistar, Wistar Kyoto, and SHR rats at the same age. This phase was similar to training, except for the delay period, which was 30 s according to the protocol of Bizot *et al.* (2007).

3. Neurochemical Analysis

After behavioral evaluations (three days after in order to avoid possible behavioral stress interference), animals were killed by decapitation and the brains were quickly removed and chilled in ice cold saline. A coronal slice of mPFC was taken (Bregma

+3.70 to +2.20), and punched bilaterally at $L = 0.6$ and $V = 4.6$ mm (Paxinos & Watson, 1998; Calabrese *et al.*, 2013; Bakker *et al.*, 2015), and the punches were immediately frozen and stored at −80°C until use. On the day of the assay, tissue samples were weighed and suspended in 0.1 M $HClO_4$ (1:30, w:v), sonicated for 5 s and centrifuged at 15,000 rpm for 15 min at 4°C (Meikle *et al.*, 2013). The pellet was discarded and NE, DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxy-indoleacetic acid (5-HIAA) tissue concentrations was analyzed in the supernatants (50 μL) using high pressure liquid chromatography coupled with electrochemical detection (HPLC-ED, BAS, USA). The sensitivity of the amperometric detector was 5 and 20 nA and oxidation potential was fixed at 0.75 V (Jacobsson *et al.*, 1980; Claustre *et al.*, 1986). Chromatographic separations were performed using a C18 reverse phase column (150 mm 4.6 mm, Phenomenex, USA) packed on microparticulate (5 mm). The mobile phase consisted of 0.15 M citric acid, 0.06 mM sodium octyl sulfate, 4% acetonitrile (v:v); 1.6% tetrahydrofurane (v:v), in double-distilled water, pH 3.0. The mobile phase was filtered through a 0.2 mm filter, degassed under vacuum and delivered at a flow rate of 1.2 mL/min. The position and height of the peaks in tissue homogenates were measured and compared to 50 μL samples of an external calibrating standard solution containing 5 ng of each NE, DA, 5-HT, DOPAC, HVA, and 5-HIAA; and concentrations of these substances in the samples were calculated and expressed as ng/g of wet tissue. DA and 5-HT turnover values were calculated as DOPAC/DA, HVA/DA, and 5-HIAA/5-HT ratio, respectively.

4. Statistical Analysis

Data are expressed as mean ± S.E.M., and were analyzed by a repeated-measure ANOVA. Greenhouse-Greisser correction was applied considering violation of the sphericity assumption, indicated by the Mauchly test (pre-training, training, and testing phase). The efficacy of MPH was analyzed by the Student's *t*-test for related samples. The monoamine levels were analyzed by Two-Way ANOVA. Results were considered significant when the $p < 0.05$. Kolmogorov–Smirnov normality test was run to all phases of tolerance to delay of reward and monoamine measurements and $p > 0.05$ were observed for all these cases, which is consistent with the parametrical statistics.

5. Results

5.1. *Body weight*

During the task, rats were maintained food-restricted, receiving just 60% in the amount of food that animals of the same age would ingest when feeding *ad libitum*. The body weight was 183.0 ± 11.1 g (mean ± standard error of the mean [S.E.M.]) at the end of experiment (PND 72).

5.2. *Effect of neonatal handling on tolerance to delay of reward*

During the pre-training phase, animals learnt to choose the large reward until they reached a criterion of at least 80% of correct choices *per* daily session. We observed a significant effect of session [$F(6, 234) = 7.892; p < 0.001$], since the performance improved with repetition. However, no effect of neonatal handling [$F(1, 39) = 1.436$; $p = 0.238$] and no interactions were observed [$F(6, 234) = 1.587; p = 0.152$] (Fig. 1(b)). Sex had no significant effect in this phase.

During the training phase, a delay of 15 s was imposed for the large reward side and animals showed a decrease in the number of times this side was chosen. In addition, there was a significant effect of session [$F(4.468, 174.262) = 16.497$; $p < 0.001$, correction for Greenhouse-Geisser]. No main effect of neonatal handling [$F(1, 39) = 0.439; p = 0.511$] was observed. A possible relation between training sessions and sex [$F(4.468, 174.262) = 2.342; p = 0.050$, correction for Greenhouse-Geisser] was observed (Fig. 1(c)) since male animals showed a significant decrease in the percentage of choices for the large reward side during the training days. In particular, in the last day of training (day 7), males had significantly lower number of higher reward choice when compared to females [$F(1, 39) = 14.415; p = 0.001$].

During the testing phase, sex [$F(1, 39) = 20.800; p < 0.001$], session [$F(5, 195) = 2.756$; $p = 0.020$], and interaction between factors (sex and group) [$F(5, 195) = 2.746; p = 0.020$] seemed to have an influence, since females exhibited a higher percentage of choices for the large reward and the handling-induced decrease in the percentage of choices for large reward was exclusively observed in male rats.

Regarding the effect of acute drug treatment with MPH, non-handled male animals showed an increase in the percentage of choices for the larger reward when challenged with MPH [Student's paired t-test, $t(11) = 2.323; p = 0.040$], while the other groups (handled male animals and both groups of females) showed no difference between their performance with MPH and drug-free conditions [handled, $t(12) = 1.522$; $p = 0.154$; non-handled females, $t(7) = 0.482; p = 0.644$; and handled females, $t(9) = 0.919; p = 0.382$] (Fig. 1(d)).

5.3. *Effect of neonatal handling on monoamine levels in the prefrontal cortex (mPFC)*

No effect of neonatal handling was observed on DA [$F(1, 24) = 2.351; p = 0.138$] and 5-HT levels [$F(1, 25) = 3.355$; $p = 0.079$] and its metabolites [DOPAC, [$F(1, 26) = 0.518; p = 0.478$]; HVA, [$F(1, 24) = 0.352; p = 0.558$]; 5HIAA, [$F(1, 26) = 2.238$; $p = 0.147$] (Table 1). No significant differences were observed in DOPAC/DA [$F(1, 24) = 1.676; p = 0.208$], HVA/DA [$F(1, 23) = 0.983; p = 0.332$], and 5HIAA/5HT ratios, representing the DA and 5-HT turnover, respectively [$F(1, 25) = 1.102; p = 0.304$]. No difference in NA levels were observed when handled and control groups [$F(1, 18) = 0.385; p = 0.543$] were compared. In contrast, female rats showed increase in DOPAC levels when compared to male animals [$F(1, 26) = 0.033; p = 0.035$]. No further interactions were observed.

Table 1. Levels of DA, serotonin (5-HT), their metabolites (3,4-dihydroxyphenylacetic acid [DOPAC], homovanilic acid [HVA], and 5-hydroxy-indoleacetic acid [5-HIAA]), as well as NE in the mPFC of early handled male and female animals, when compared to controls.

	Males		Females	
	Non-Handled	Handled	Non-Handled	Handled
DA	3.94 ± 0.56	5.98 ± 1.69	9.68 ± 2.49	8.51 ± 2.88
5-HT	39.15 ± 8.47	32.25 ± 4.27	50.06 ± 7.60	25.16 ± 10.20
NE	38.58 ± 6.29	40.02 ± 4.87	46.07 ± 5.70	39.03 ± 8.71
DOPAC	17.45 ± 3.56	15.24 ± 2.73	27.26 ± 3.68*	23.58 ± 4.51*
HVA	16.99 ± 5.58	11.33 ± 2.34	16.55 ± 3.66	17.14 ± 4.48
5-HIAA	890.28 ± 130.57	1050.68 ± 104.31	936.10 ± 104.13	1120.97 ± 127.54
DOPAC/DA	4.40 ± 0.64	3.11 ± 0.50	6.69 ± 1.51	3.75 ± 1.74
HVA/DA	4.38 ± 1.43	2.58 ± 0.96	3.40 ± 1.06	2.85 ± 1.23
5-HIAA/5-HT	33.46 ± 9.48	39.10 ± 10.33	32.35 ± 8.66	47.48 ± 11.62

Note: (data presented as mean ± S.E.M. of ng/g tissue). DOPAC/DA, HVA/DA, and 5-HIAA/5-HT ratios are also shown. $N = 5–8$/group (Two-Way ANOVA, $p = 0.035$ for sex effect*).

6. Discussion

In the present study, we observed (i) sex-dependent differences in the pharmacological response to MPH in regard to impulsive behavior of experimental animals and that (ii) only control male animals exhibited differences in impulsivity after MPH administration, which suggests potential long-lasting interactions of early postnatal stress interventions and the behavior of adult animals.

The task used for evaluating the tolerance to delay of reward requires the animals to learn in which side the larger reward is placed and also, the time of delay during the training and testing phases (Bizot *et al.*, 2007). Problems related to either learning and/or memory of this task could interfere in the observed behavioral effects in handled animals. With all, it has already been reported that handling does not affect learning in training phases of behavioral tasks that require several days, such as Morris water maze (Noschang *et al.*, 2010) or Y-maze (Noschang *et al.*, 2012). In contrast, animals show some deficits in reversal learning (Noschang *et al.*, 2012).

To date, a limited number of studies have analyzed parameters related to impulsivity in handled animals (3–15 min) in neonatal life. Studying maternal separated (MS) and neonatally handled rats, Colorado *et al.* (2006), using the velocity of ambulation in open field, observed an increased velocity in MS animals (but not in handled ones) during pre-puberty (PND 28). However, some of the effects of early handling only appeared after puberty and opposite effects may be observed before puberty and in the adult life (Silveira *et al.*, 2006a).

Impulsivity is an executive process characterized by increased risk behavior and it is associated with drug addiction, schizophrenia with violent personality, suicidal behavior (Dumais *et al.*, 2011), and obsessive compulsive patients that have less ability to inhibit non-required thoughts and actions as binge eating (Bari & Robbins, 2013). The increased risk behavior is normally seen in behavioral animal tasks as

more ambulation in open field center zone than thigmotaxic activities (Clement *et al.*, 1995; Colorado *et al.*, 2006), more time in the light part of a light-dark box (Colorado *et al.*, 2006), and more time in the open arms in a plus maze, pointing to less defensive behavior that could protect the animal against possible predators in nature (Carobrez & Bertoglio, 2005; Radhakrishnan *et al.*, 2015). Handled animals have increased risk assessment behavior in the presence of predator odor (Siviy & Harrison, 2008) or the predator itself (Padoin *et al.*, 2001). In addition, handled rats show less anxiety and consequently increased risk behavior than non-handled groups (Fujimoto *et al.*, 2014) and also demonstrate attenuate stressful responses in novel environments such as less defecation (Fujimoto *et al.*, 2014). Impulsive traits are also involved in the development of eating disorders, specially overeating and binge eating (Schag *et al.*, 2013; Meule *et al.*, 2014; Velazquez-Sanchez *et al.*, 2014). Neonatally handled animals eat more sweet food (Froot Loops®) (Silveira *et al.*, 2004) and present a lower latency to reach palatable food (McIntosh *et al.*, 1999; Silveira *et al.*, 2004), eating more quantities of a palatable food in a small period of time (Silveira *et al.*, 2006b).

Our results evidence that male Wistar rats tend to exhibit a greater impulsive action than those seen in females, which is consistent with previously published reports by others in animals (Weafer & de Wit, 2014). On the contrary, in humans, sex differences on measures of impulsive action depend on tasks as well as the characteristics of the subjects (for example, drug users or control subjects) (Weafer & de Wit, 2014; Mitchell & Potenza, 2015). The absence of MPH-derived effects in female rats could be expected if one considers these animals exhibited a significant number of higher reward choices, even in the basal state. Of note, in adolescent humans with ADHD, no interactions between sex and MPH efficacy have been observed, even though both males and females were largely equivalent in impairment (Mikami *et al.*, 2009). Our strategy of alternating MPH and saline administration when testing MPH effect has been used by different authors (Cooper *et al.*, 2005; Bizot *et al.*, 2007; Hermens *et al.*, 2007) to prevent effects related to repetition of the task, which would be independent of drug effect.

The absence of effects on monoamine levels (with the exception of DOPAC) in the mPFC in male handled rats suggests other factors/routes possibly explaining these differences in impulsive behavior. For instance, (i) the NAcc ascending pathway to mPFC may participate (Donnelly *et al.*, 2014), (ii) an altered D_3/D_2 DA internal circuitry in NAcc has additionally been reported in impulsive subjects (Jupp *et al.*, 2013), and finally (iii) others have reported that the D_2 antagonists can cause impairment in delayed choices in the OFC (Pardey *et al.*, 2013). We cannot discard, however, potential synaptic differences in smaller brain regions than those analyzed in the present study, and/or technique limitations (e.g., sensitivity). Only DOPAC levels were higher in mPFC of female animals suggesting a higher metabolism of DA in this structure. With further investigation would be required to better comprehend these differences.

Our present findings are consistent with the concept linking early life stressful events as an interfering agent possibly modifying the later drug response during the adulthood (Meaney *et al.*, 2002). The absence of MPH response (3 mg/kg) in neonatally handled rats is in agreement with Silveira *et al.* (2010) that reported no increase in food consumption in response to MPH in contrast to control animals. In addition, it has been demonstrated (Brake *et al.*, 2004) that the addictive profile of psychostimulant drugs is also influenced by neonatal rearing conditions. In agreement with the considerations above, it has been described in the literature that female individuals show less impulsive behavior than males in childhood (Cote *et al.*, 2002), and that women are less impulsive than men at the peak of fertility, which has led to a consideration of the differing selection pressures placed on males and females (Hosseini-Kamkar & Morton, 2014). A well-known parental theory described by Bjorklund and Kipp (1996) postulates that females are less impulsive than males in fertile periods, involving the choosing for a reproductive partner, with the purpose of protecting a possible bad choice in reproductive moments. In our study, although the estrous cycle was not controlled, the testing phase lasted for a longer time period than the entire estrous cycle (6 days) and indicates that the estrous cycle may have not affected these results.

In conclusion, our study reports evidence for a cause-effect link between neonatal handling of and the induction of impulsive behavior in adult male experimental animals, together with differences in the pharmacological response to MPH, a common drug utilized for ADHD patients.

Acknowledgments

CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). The proceedings were conducted in agreement with the UFRGS — CEUA (Ethics Committee on Animal Use), protocol number 21460, and with the Brazilian Law on the use of animals (Federal Law 11.794/2008) and Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003). Fares Zeidán-Chuliá holds a PNPD postdoctoral position (Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Universidade Federal do Rio Grande do Sul) funded by CAPES.

Conflict of Interest

The authors declare that they have no conflict of interest.

REFERENCES

Bakker, R., Tiesinga, P. & Kotter, R. (2015) The scalable brain atlas: Instant web-based access to public brain atlases and related content. *Neuroinformatics*, **13**, 353–366.

Bari, A. & Robbins, T.W. (2013) Inhibition and impulsivity: Behavioral and neural basis of response control. *Prog. Neurobiol.*, **108**, 44–79.

Bertoux, M., Volle, E., Funkiewiez, A., de Souza, L.C., Leclercq, D. & Dubois, B. (2012) Social cognition and emotional assessment (SEA) is a marker of medial and orbital frontal functions: A voxel-based morphometry study in behavioral variant of frontotemporal degeneration. *J. Int. Neuropsychol. Soc.*, **18**, 972–985.

Bizot, J.C., Chenault, N., Houze, B., Herpin, A., David, S., Pothion, S. & Trovero, F. (2007) Methylphenidate reduces impulsive behaviour in juvenile Wistar rats, but not in adult Wistar, SHR and WKY rats. *Psychopharmacology (Berl)*, **193**, 215–223.

Bjorklund, D.F. & Kipp, K. (1996) Parental investment theory and gender differences in the evolution of inhibition mechanisms. *Psychol. Bull.*, **120**, 163–188.

Brake, W.G., Zhang, T.Y., Diorio, J., Meaney, M.J. & Gratton, A. (2004) Influence of early postnatal rearing conditions on mesocorticolimbic dopamine and behavioural responses to psychostimulants and stressors in adult rats. *Eur. J. Neurosci.*, **19**, 1863–1874.

Calabrese, E., Badea, A., Watson, C. & Johnson, G.A. (2013) A quantitative magnetic resonance histology atlas of postnatal rat brain development with regional estimates of growth and variability. *Neuroimage*, **71**, 196–206.

Cardinal, R.N., Winstanley, C.A., Robbins, T.W. & Everitt, B.J. (2004) Limbic corticostriatal systems and delayed reinforcement. *Ann. N Y Acad. Sci.*, **1021**, 33–50.

Carobrez, A.P. & Bertoglio, L.J. (2005) Ethological and temporal analyses of anxiety-like behavior: The elevated plus-maze model 20 years on. *Neurosci. Biobehav. Rev.*, **29**, 1193–1205.

Claustre, Y., Rivy, J.P., Dennis, T. & Scatton, B. (1986) Pharmacological studies on stress-induced increase in frontal cortical dopamine metabolism in the rat. *J. Pharmacol. Exp. Ther.*, **238**, 693–700.

Clement, Y., Martin, B., Venault, P. & Chapouthier, G. (1995) Involvement of regions of the 4th and 7th chromosomes in the open-field activity of mice. *Behav. Brain Res.*, **70**, 51–57.

Colorado, R.A., Shumake, J., Conejo, N.M., Gonzalez-Pardo, H. & Gonzalez-Lima, F. (2006) Effects of maternal separation, early handling, and standard facility rearing on orienting and impulsive behavior of adolescent rats. *Behav. Process.*, **71**, 51–58.

Cooper, N.J., Keage, H., Hermens, D., Williams, L.M., Debrota, D., Clark, C.R. & Gordon, E. (2005) The dose-dependent effect of methylphenidate on performance, cognition and psychophysiology. *J. Integr. Neurosci.*, **4**, 123–144.

Cote, S., Tremblay, R.E., Nagin, D., Zoccolillo, M. & Vitaro, F. (2002) The development of impulsivity, fearfulness, and helpfulness during childhood: Patterns of consistency and change in the trajectories of boys and girls. *J. Child Psychol. Psychiatry*, **43**, 609–618.

Donnelly, N.A., Holtzman, T., Rich, P.D., Nevado-Holgado, A.J., Fernando, A.B., Van Dijck, G., Holzhammer, T., Paul, O., Ruther, P., Paulsen, O., Robbins, T.W. & Dalley, J. W. (2014) Oscillatory activity in the medial prefrontal cortex and nucleus accumbens correlates with impulsivity and reward outcome. *PLoS One*, **9**, e111300.

Dumais, A., Potvin, S., Joyal, C., Allaire, J.F., Stip, E., Lesage, A., Gobbi, G. & Cote, G. (2011) Schizophrenia and serious violence: A clinical-profile analysis incorporating impulsivity and substance-use disorders. *Schizophr. Res.*, **130**, 234–237.

Elton, A., Tripathi, S.P., Mletzko, T., Young, J., Cisler, J.M., James, G.A. & Kilts, C.D. (2014) Childhood maltreatment is associated with a sex-dependent functional reorganization of a brain inhibitory control network. *Human Brain Mapping*, **35**, 1654–1667.

Fujimoto, T., Kubo, K., Nishikawa, Y. & Aou, S. (2014) Brief neonatal handling alters sexually dimorphic behaviors in adult rats. *J. Integr. Neurosci.*, **13**, 61–70.

Hermens, D.F., Cooper, N.J., Clark, C.R., Debrota, D., Clarke, S.D. & Williams, L.M. (2007) An integrative approach to determine the best behavioral and biological markers of methylphenidate. *J. Integr. Neurosci.*, **6**, 105–140.

Hosseini-Kamkar, N. & Morton, J.B. (2014) Sex differences in self-regulation: An evolutionary perspective. *Front. Neurosci.*, **8**, 233.

Jacobsson, S.E., Jonsson, S., Lindberg, C. & Svensson, L.A. (1980) Determination of terbutaline in plasma by gas chromatography chemical ionization mass spectrometry. *Biomed. Mass Spectrometry.*, **7**, 265–268.

Jupp, B., Caprioli, D., Saigal, N., Reverte, I., Shrestha, S., Cumming, P., Everitt, B.J., Robbins, T.W. & Dalley, J.W. (2013) Dopaminergic and GABA-ergic markers of impulsivity in rats: Evidence for anatomical localisation in ventral striatum and prefrontal cortex. *Eur. J. Neurosci.*, **37**, 1519–1528.

Kosten, T.A., Miserendino, M.J., Bombace, J.C., Lee, H.J. & Kim, J.J. (2005) Sex-selective effects of neonatal isolation on fear conditioning and foot shock sensitivity. *Behav. Brain Res.*, **157**, 235–244.

Kulkarni, M. (2014) Attention deficit hyperactivity disorder. *Ind. J. Pediatr.*, **82**, 267–271.

Leussis, M.P. & Andersen, S.L. (2008) Is adolescence a sensitive period for depression? Behavioral and neuroanatomical findings from a social stress model. *Synapse*, **62**, 22–30.

Levine, S., Alpert, M. & Lewis, G.W. (1957) Infantile experience and the maturation of the pituitary adrenal axis. *Science*, **126**, 1347.

McIntosh, J., Anisman, H. & Merali, Z. (1999) Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: Gender-dependent effects. *Brain Res. Dev. Brain Res.*, 113, 97–106.

Meaney, M.J., Brake, W. & Gratton, A. (2002) Environmental regulation of the development of mesolimbic dopamine systems: A neurobiological mechanism for vulnerability to drug abuse? *Psychoneuroendocrinology*, **27**, 127–138.

Meaney, M.J., Diorio, J., Francis, D., Widdowson, J., LaPlante, P., Caldji, C., Sharma, S., Seckl, J.R. & Plotsky, P.M. (1996) Early environmental regulation of forebrain glucocorticoid receptor gene expression: Implications for adrenocortical responses to stress. *Dev. Neurosci.*, **18**, 49–72.

Meaney, M.J., Mitchell, J.B., Aitken, D.H., Bhatnagar, S., Bodnoff, S.R., Iny, L.J. & Sarrieau, A. (1991) The effects of neonatal handling on the development of the adrenocortical response to stress: Implications for neuropathology and cognitive deficits in later life. *Psychoneuroendocrinology*, **16**, 85–103.

Meikle, M.N., Prieto, J.P., Urbanavicius, J., Lopez, X., Abin-Carriquiry, J.A., Prunell, G. & Scorza, M.C. (2013) Anti-aggressive effect elicited by coca-paste in isolation-induced aggression of male rats: Influence of accumbal dopamine and cortical serotonin. *Pharmacol. Biochem. Behav.*, **110**, 216–223.

Meule, A., Lutz, A.P., Vogele, C. & Kubler, A. (2014) Impulsive reactions to food-cues predict subsequent food craving. *Eat Behav.*, **15**, 99–105.

Mikami, A.Y., Cox, D.J., Davis, M.T., Wilson, H.K., Merkel, R.L. & Burket, R. (2009) Sex differences in effectiveness of extended-release stimulant medication among adolescents with attention-deficit/hyperactivity disorder. *J. Clin. Psychol. Med Settings*, **16**, 233–242.

Mitchell, M.R. & Potenza, M.N. (2015) Importance of sex differences in impulse control and addictions. *Front. Psychiatry*, **6**, 24.

Noschang, C., Krolow, R., Arcego, D.M., Toniazzo, A.P., Huffell, A.P. & Dalmaz, C. (2012) Neonatal handling affects learning, reversal learning and antioxidant enzymes activities in a sex-specific manner in rats. *Int. J. Dev. Neurosci.*, **30**, 285–291.

Noschang, C.G., Krolow, R., Fontella, F.U., Arcego, D.M., Diehl, L.A., Weis, S.N., Arteni, N.S. & Dalmaz, C. (2010) Neonatal handling impairs spatial memory and leads to altered nitric oxide production and DNA breaks in a sex specific manner. *Neurochem. Res.*, **35**, 1083–1091.

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

Pardey, M.C., Kumar, N.N., Goodchild, A.K. & Cornish, J.L. (2013) Catecholamine receptors differentially mediate impulsive choice in the medial prefrontal and orbitofrontal cortex. *J. Psychopharmacol.*, **27**, 203–212.

Paxinos, G. & Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates*. San Diego: Academic Press.

Portella, A.K., Silveira, P.P., Diehl, L.A., Crema, L.M., Clemente, Z., Peres, W., Costa, G., Scorza, C., Quillfeldt, J.A. & Dalmaz, C. (2010) Early life handling decreases serotonin turnover in the nucleus accumbens and affects feeding behavior of adult rats. *Dev. Psychobiol.*, **52**, 190–196.

Radhakrishnan, A., Aswathy, B.S., Kumar, V.M. & Gulia, K.K. (2015) Sleep deprivation during late pregnancy produces hyperactivity and increased risk-taking behavior in offspring. *Brain Res.*, **1596**, 88–98.

Raineki, C., Lucion, A.B. & Weinberg, J. (2014) Neonatal handling: An overview of the positive and negative effects. *Dev. Psychobiol.*, **56**, 1613–1625.

Reichl, C., Kaess, M., Resch, F. & Brunner, R. (2014) The role of genotype in the intergenerational transmission of experiences of childhood adversity. *Z. Kinder Jugendpsychiatr. Psychother.*, **42**, 349–359.

Renolleau, S., Fau, S. & Charriaut-Marlangue, C. (2008) Gender-related differences in apoptotic pathways after neonatal cerebral ischemia. *Neuroscientist*, **14**, 46–52.

Roth, T.L. (2013) Epigenetic mechanisms in the development of behavior: Advances, challenges, and future promises of a new field. *Dev. Psychopathol.*, **25**, 1279–1291.

Schag, K., Teufel, M., Junne, F., Preissl, H., Hautzinger, M., Zipfel, S. & Giel, K.E. (2013) Impulsivity in binge eating disorder: Food cues elicit increased reward responses and disinhibition. *PLoS One*, **8**, e76542.

Silveira, P.P., Cognato, G., Crema, L.M., Pederiva, F.Q., Bonan, C.D., Sarkis, J.J., Lucion, A.B. & Dalmaz, C. (2006a) Neonatal handling, sweet food ingestion and ectonucleotidase activities in nucleus accumbens at different ages. *Neurochem. Res.*, **31**, 693–698.

Silveira, P.P., da Silva Benetti, C., Ayres, C., Pederiva, F.Q., Portella, A.K., Lucion, A.B. & Dalmaz, C. (2006b) Satiety assessment in neonatally handled rats. *Behav. Brain Res.*, **173**, 205–210.

Silveira, P.P., Portella, A.K., Assis, S.A., Nieto, F.B., Diehl, L.A., Crema, L.M., Peres, W., Costa, G., Scorza, C., Quillfeldt, J.A., Lucion, A.B. & Dalmaz, C. (2010) Early life experience alters behavioral responses to sweet food and accumbal dopamine metabolism. *Int. J. Dev. Neurosci.*, **28**, 111–118.

Silveira, P.P., Portella, A.K., Clemente, Z., Bassani, E., Tabajara, A.S., Gamaro, G.D., Dantas, G., Torres, I.L., Lucion, A.B. & Dalmaz, C. (2004) Neonatal handling alters feeding behavior of adult rats. *Physiol. Behav.*, **80**, 739–745.

Siviy, S.M. & Harrison, K.A. (2008) Effects of neonatal handling on play behavior and fear towards a predator odor in juvenile rats (Rattus norvegicus). *J. Comp. Psychol.*, **122**, 1–8.

Skowron, E.A., Cipriano-Essel, E., Gatzke-Kopp, L.M., Teti, D.M. & Ammerman, R.T. (2014) Early adversity, RSA, and inhibitory control: Evidence of children's neurobiological sensitivity to social context. *Dev. Psychobiol.*, **56**, 964–978.

Velazquez-Sanchez, C., Ferragud, A., Moore, C.F., Everitt, B.J., Sabino, V. & Cottone, P. (2014) High trait impulsivity predicts food addiction-like behavior in the rat. *Neuropsychopharmacology*, **39**, 2463–2472.

Weafer, J. & de Wit, H. (2014) Sex differences in impulsive action and impulsive choice. *Addict. Behav.*, **39**, 1573–1579.

Weinberg, J., Krahn, E.A. & Levine, S. (1978) Differential effects of handling on exploration in male and female rats. *Dev. Psychobiol.*, **11**, 251–259.

Weis, S.N., Pettenuzzo, L.F., Krolow, R., Valentim, L.M., Mota, C.S., Dalmaz, C., Wyse, A.T. & Netto, C.A. (2012) Neonatal hypoxia-ischemia induces sex-related changes in rat brain mitochondria. *Mitochondrion*, **12**, 271–279.

Winstanley, C.A., Theobald, D.E., Dalley, J.W., Cardinal, R.N. & Robbins, T.W. (2006) Double dissociation between serotonergic and dopaminergic modulation of medial prefrontal and orbitofrontal cortex during a test of impulsive choice. *Cereb. Cortex*, **16**, 106–114.

3.2 Capítulo II

NEONATAL HANDLING ALTERS ATTENTIONAL SET SHIFTING TASK PERFORMANCE AND MEDIAL PREFRONTAL CORTEX BIOCHEMICAL PARAMETERS IN ADULT RATS

Camilla Lazzaretti [a*], Grasielle Clotildes Kincheski [b], Pablo Pandolfo [c], Rachel Krolow [d,e], Ana Paula Toniazzo [d], Danusa Mar Arcego [d], Natividade de Sá Couto-Pereira [d], Fares Zeidán-Chuliá [d], Ben-Hur Neves de Oliveira [d], Andréa Kurek Ferreira [d], Janaína Kolling [d], Cassiana Siebert [d], Angela Teresinha Wyse [d,f], Tadeu Mello e Souza [a,f], Carla Dalmaz[a,d,f]

[a] *Programa de Pós-Graduação em Neurociências, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.*

[b] *Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil.*

[c] *Universidade Federal Fluminense (UFF), Rio de Janeiro, RJ, Brazil.*

[d] *Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.*

[e] *Programa de Pós-Graduação em Saúde e Comportamento, Universidade Católica de Pelotas (UCPel), Pelotas, RS, Brazil.*

[f] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.*

Running title: Neonatal Handling and Attention

***Corresponding address:** Camilla Lazzaretti (Corresponding author); Departamento de Bioquímica, ICBS, UFRGS; Rua Ramiro Barcelos, 2600 (Anexo), Lab. 37; 90035-003 Porto Alegre, RS, Brasil. Phone: 55 51- 3308-5570. Fax: 55 51- 3316-5535.

E-mail address: camillinhalazzaretti@yahoo.com.br

HIGHLIGHTS

- Neonatal handling alters attentional set shifting task performance in adult rats;
- Synaptophysin is decreased in the medial prefrontal cortex from neonatally handled rats;
- Na^+/K^+-ATPase activity is decreased in the medial prefrontal cortex from neonatally handled rats.

ABSTRACT

Early post-natal environmental factors are able to influence different aspects of physiology and behavior during adulthood. Cognitive impairment represents a core pathological hallmark of different neuropsychiatric disorders and parallels with dysfunction of the prefrontal cortex (PFC). Neonatal handling (NH) is an experimental animal model for evaluating the effect of neonatal disruptive environments on adult mental health, and induces changes in anxiety, task-dependent deficits in reversal learning, and higher locomotion in new environments. The aim of this study was to observe the effects, in adult rats, of NH (i) on the performance in two different attentional set shifting tasks; (ii) on the immunocontent of proteins such as the brain-derived neurotrophic factor (BDNF), tyrosine hydroxylase (TH and phosphorylated TH), and synaptophysin (SYP), as well as on Na+/K+-ATPase activity in the medial PFC; and, finally, (iii) we intended to study the molecular landscape of interactions of these proteins by developing an *in silico* network model with the STRING 10 search tool to better comprehend any potential difference. Our results showed that neonatally handled animals, when adults, exhibit impairments in simple and intradimensional discrimination as well as facilitated extradimensional discrimination. These effects were accompanied by a significant decrease in SYP immunocontent and Na^+/K^+-ATPase activity in the mPFC. Tyrosine hydroxylase (TH and pTH) immunocontent and BDNF levels remained unchanged. Finally, we developed an *in silico* network model that shows the landscape of interactions between Na^+/K^+-ATPase, SYP, TH, and BDNF. Based on our results, we conclude that frequent neonatal brief separations alters

performance in an attentional set shifting task probably because it increases attention to irrelevant and/or impairs associative learning, and this is accompanied by a decrease in a synaptic vesicle protein (SYP) and in the activity of Na^+/K^+-ATPase in the medial prefrontal cortex.

Keywords: Neonatal handling; Attentional set shifting tasks; Prefrontal Cortex; Synaptophysin; Na+/K+-ATPase activity; NaK-ATPase network.

Abbreviations:

NH, neonatal handling; mPFC, medial prefrontal cortex; BDNF, brain-derived neurotrophic factor; TH, tyrosine hydroxylase; pTH tyrosine hydroxylase phosphorilate isoform; SYP, synaptophysin; PND, postnatal day; ASST, attentional set shifting task; ADHD, attention deficit hyperactivity disorder**.**

1. INTRODUCTION

Infancy and early childhood are periods of life when numerous brain modifications take place, such as synapse production, neuron migration, high brain plasticity, and maturation of cerebral areas (Kolb et al., 2012). Environmental factors interacting with an immature brain system during these periods can direct the organism to either mental health or disease and may increase the vulnerability to psychiatric disorders such as addiction (Kosten et al., 2000), eating disorders, impulsivity (Elton et al., 2014, Skowron et al., 2014) , and attention deficit hyperactivity disorder (ADHD) (Latimer et al., 2012). In the case of ADHD, for instance, it has been observed that early traumatic events with violent content can potentiate its development (Pires Tde et al., 2012, Thapar et al., 2013), indicating evidence for a cause-effect link between disruptive early life environments and some of ADHD symptoms.

Since modifications in the early post-natal environment are capable of influencing different aspects of physiology and behavior during lifetime (Raineki et al., 2014), animal models of neonatal intervention have been studied in an effort to better comprehend how environmental factors during early life may influence behavior in adulthood (Raineki et al., 2014). Neonatal handling (NH) is a form of early-life stress induced in pups by brief and frequent separations from their dam, which may lead to some behavioral phenotypes, such as an increased locomotion in new environments (Padoin et al., 2001) and a task- and gender-dependent reversal learning impairment (Noschang et al., 2012b). Therefore, the cognitive impairments observed in the NH model (Raineki et al., 2014) could be assigned to changes in attentional processes (Noschang et al., 2012a, Noschang et al., 2012b, Raineki et al., 2014).

The ability to allocate attention to particular features of the environment is critical for animal survival. However, changes within the environment not only requires the proper skills to disconnect a previously relevant stimulus but also to be able to respond to other group of stimuli that have previously been experienced as irrelevant (Birrell and Brown, 2000, Dajani and Uddin, 2015). In other words, attention and their shifts provide the ability to the organism for readjusting its actions, thus increasing its chances for survival. The attentional set shifting tasks (ASST) evaluate the ability to make associations between stimuli and rewards, to make sets of these associations (Birrell and Brown, 2000), and to shift attention. Two different ASST were used in the present study: i) The first task uses a four-arm maze, and the animal must change from an egocentric spatial to a visual-cued strategy (Floresco et al., 2006a); ii) and the second one, described by Birrel and Brown (2000), uses different features (sets) of stimuli (called here as dimensions), related to odor, digging medium or texture of a food pot, and the significance of these dimensions to find the reward changes while performing the task. In rodents, attention and cognitive flexibility depends on the neuronal integrity/functionality of the medial prefrontal cortex (mPFC) (Floresco et al., 2006b, Logue and Gould, 2014).

Interventions during early life, such as NH, can modify the levels of neurotransmitters in nucleus accumbens and olfatory bulb, brain areas connected with the mPFC (Raineki et al., 2009, Portella et al., 2010, Silveira et al., 2010). Some proteins involved in neuronal function seem to be especially relevant in this context, such as (i) the brain-derived neurotrophic factor (BDNF), which is involved in synaptic plasticity and known to be influenced by different environmental changes (Sakata et al., 2013); (ii) tyrosine hydroxylase

(TH) and its phosphorylated (at serine 40) form (pTH), which is responsible for the production of L-DOPA, a precursor of dopamine and noradrenaline, which are essential neurotransmitters to an adequate performance in ASST (Floresco et al., 2006b, McGaughy et al., 2008, Winter et al., 2009, Kehagia et al., 2010); (iii) synaptophysin (SYP), which is a synaptic vesicle protein that participates in the synaptic transmission (Schmitt et al., 2009) and represents a reliable indicator of synaptic integrity and has previously been demonstrated to correlate well *in vivo* with the loss of cognitive function in animal models of disease (Zhang et al., 2014); and finally (iv) the Na^+/K^+-ATPase, which is an enzyme that maintains the necessary membrane gradient for neuronal function (Therien and Blostein, 2000, Noschang et al., 2012a). Thus, the aim of the present study was (i) to examine potential differences in performance of two different attentional set shifting tasks in animals submitted to NH when compared to control (non-handled) rats, (ii) to find potential changes in neuronal molecules (BDNF, TH and pTH, SYP and Na^+/K^+-ATPase) in the mPFC, and finally, to (iii) to develop an *in silico* network model of interactions between the studied proteins to better comprehend the mechanisms underlying the observed effects.

2. EXPERIMENTAL PROCEDURES

2.1 Subjects

The proceedings were approved by the UFRGS ethics committee (protocol number 21,460), and followed the recommendations of the Brazilian Federal Law 11.794/2008 on the use of animals and of the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research. Female primiparous pregnant Wistar rats, bred at our own animal facility, were randomly

selected (N=23). From days 16^{th}-18^{th} of gestation, animals were housed alone in home cages made of Plexiglas (65 cm x 25 cm x 15 cm) with the floor covered with sawdust and maintained in a controlled environment: 12h light/12h dark, temperature of 22±2°C, cage cleaned twice a week, and food and water provided *ad libitum*. The day of the birth was considered postnatal day (PND) 0, and the litters were standardized in eight pups for cage 24h later. After birth and until weaning, 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. Litters were weaned and separated by sex on PND 21. No more than two males from each litter were used in the behavioral and biochemical procedures.

2.2 Neonatal Handling (NH) model

The neonatal handling (NH) model was previously described elsewhere by Noschang and colleagues (2012b) in our own lab. Briefly, animals were divided into two groups, as follows: (1) the handled group, in which 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 30-32ºC. After 10 min, pups were returned to their dams, which were in the same room. This procedure was performed from PND 1-10 following birth, and then pups were left undisturbed until PND 21 (weaning); and, (2) the control group (non-handled), in which pups were left completely undisturbed (not touched) with the dam since birth until weaning. About half of the litters were submitted to

NH starting on PND 1 and always handled by the same person, who changed gloves between litters in order to avoid potential odor interference. Figure. 1A represents the timeline of experiments.

2.3 Attentional set shifting tasks (ASST)

On PND 60, the animals were housed individually in cages and were weighted and submitted for 7 days to a restricted diet composed of twelve to fifteen grams of standard chow *per* rat per day in order to achieve 80% of the original weight. A different cohort of animals was used for each attentional set shifting task (ASST): ASST maze, which requires the animal to change from a spatial egocentric to a cued-version strategy (Floresco et al., 2006a, Pandolfo et al., 2013); and ASST pot, in which rats have to make simple or complex (varying) discriminations by using three different perceptual features: a pot texture, digging meddium, or odor (Birrell and Brown, 2000). Group sizes were 8 and 10 for ASST maze and ASST pot, respectively. All behavioral tests were performed between 9:30 and 16:30 h. Three days before the beginning of the tasks, the animals were habituated to reward food pellets, Royal Canin (Purina®) Junior for ASST maze, and Froot Loops (Kellogg's ®) for ASST pot. Rats were habituated in the test room for 1 hour before the tests started in order to minimize the influence of stress. After each animal, test arenas and objects were carefully cleaned.

2.3.1 ASST maze

ASST maze has already been described by others (Floresco et al., 2006a, Pandolfo et al., 2013). Briefly, it is composed of three phases described below and was carried out by using a maze with four closed arms, with 40x10x20 cm^3 of dimension.

- *Familiarization:* each rat was allowed to freely explore the maze and eat twelve pellets (three per arm) in 15 minutes (dog food, Royal Canin Junior®). If the animal did not eat all pellets, a second or even a third day of familiarization was carried out until the criterion was reached. The maze was configured in a “T” shape to the following phases.
- *Discrimination phase:* it was determined the turning bias (right or left) of each animal in four consecutive turns. After that, it was imposed that the rat turned to the opposite side of the bias to obtain the reward pellet, independently of the output arm (south, east, or west), until reaching a criterion of 10 correct consecutive trials (egocentric strategy). Then, a final probe trial was carried out, in which the animal started from the north arm, until then never used as a start arm.
- *Sensory/visual cue phase.* The animal had to find the reward by turning to the arm containing a plastic placed on the floor independent of the starting arm (west, south or east). The criterion to finish was also ten consecutive correct trials. Three kinds of errors were recorded: (i) perseverative (when a rat made the same egocentric response as required for the response discrimination phase three times or more); (ii) regressive (once a rat made less than three perseverative errors); and (iii) never-reinforced (when a rat entered the incorrect arm because of its turning bias). Regressive and never-reinforced errors provide an index of the ability to maintain an attentional set and perseverative errors represent an index for behavioral flexibility.

2.3.2 ASST pot

ASST pot is described elsewhere (Birrell and Brown, 2000). Briefly, it consists of three phases described below and is carried out in a 50-cm high, 60-cm × 40-cm rectangular arena with a frontal glass, with the floor subdivided with grey lines into 12 rectangles (13.3 cm by 15.0 cm). Ceramic pots with the following dimensions were used: 4-cm high, 6.5 cm of diameter, and 3.5 cm of internal depth.

- *Habituation:* each rat was allowed to explore the arena for 5 min, and crossings through the lines on the floor were recorded. Thereafter, a ceramic pot was placed in the arena so that the animals could explore it. Then, one pellet (Froot Loops Kellogg's®) was placed into the pot and the animal was allowed to eat it, and the baited pot was offered three or four times. Afterwards, sawdust was introduced into the pot, covering the pellet, and the animals were allowed to dig the sawdust and eat the pellet three or four times.
- *Training session:* after habituation, the rats were trained to choose only one of two pots, which contained a reward, in a series of three simple discriminations (SDs) in the following order: first, texture (crepe paper *vs.* adhesive tape); second, digging medium (tnt fabric *vs.* paper chips); and third, odor (cumin *vs.* sweet paprika). They had to achieve six consecutive correct trials in each SD. The rewarding pot was randomly placed in either side so that spatial localization could not be used as a cue. After the first choice, both pots were removed and changed by new ones some seconds later.

- *Testing phase: a* trial was initiated by giving the rat access to the two digging pots, only one of which was baited. The first four trials were discovery trials: the rat was permitted to dig in both of the pots, but only one was baited. An error was recorded if the rat dug first in the unbaited pot. On subsequent trials, if the rat started to dig in the unbaited pot, an error was recorded, and the trial was terminated. Testing continued until the rat reached a criterion level of performance of six consecutive correct trials.

Materials used (Table. 1) and each step in this task (Table. 2) are provided. In SD, the pair of pots differed in odor, texture or medium, just one type of feature, which is from now considered a dimension. For compound discrimination (CD), a second dimension was introduced, but the correct pot remained the same. In Reversals (Rev1, Rev2, Rev 3), the rat needed to learn that, in the same dimension, the previously correct stimulus was now incorrect and the correct stimulus is one that was previously wrong. For intradimensional discriminations (ID), a new pair of relevant stimuli in the same dimension was introduced. Finally, in the extradimensional discrimination (ED), a new dimension was introduced and considered to be the relevant to be associated with the reward; for instance, if the relevant stimuli until then were odors, now medium is considered relevant. There were six possible patterns of shift (odor to texture or odor to medium, medium to odor or texture, texture to medium or odor). No rat of the same group used the same combination of materials.

2.4 Neurochemical analysis

Three days after finishing the behavioral procedures, all animals were sacrificed by decapitation and brains were quickly removed and chilled in ice

cold saline. A coronal slice of mPFC was taken (Bregma, from +3.70 to +2.20 mm), and punched bilaterally at coordinates L=0.6 and V=4.6 mm (Paxinos and Watson, 1998). Punches were immediately frozen and stored at -80º C until use.

2.4.1 BDNF levels

BDNF levels in the mPFC were measured by sandwich enzyme-linked immunosorbent assay (ELISA), using a commercial kit (BDNF Emax® Immunoassay system, Promega, USA). Briefly, brain samples were homogenized 1:10 (w:v) in a lysis buffer containing 10mM Hepes, 10mM KCl, 0.6mM EDTA, 127 mM NaCl, 0.4% of SDS, 10% glycerol and 1% PIC, pH 7.9. Microplates (96-well, flat-bottomed) were coated for 24 h (overnight 4°C) with Anti-BDNF 1:1000; the standard curve ranged from 7.8 to 500 pg of BDNF/mL. Sequential processing of the samples was performed according to the manufacturer's instructions. Sample protein concentrations were determined by using the Lowry method (Lowry et al., 1951), with bovine serum albumin as the standard.

2.4.2 Tyrosine hydroxylase, phosphorilated tyrosine hydroxylase (pSer40) and synaptophysin proteins immunocontent

Punches from medial mPFC were homogenized in ice-cold lysis buffer pH 7.9: 2.5M KCl, 10mM HEPES, 0.6mM EDTA, and 1% protease inhibitor cocktail (PIC). Homogenized samples were centrifuged at 1.000g/10min/4°C and the supernatant was used. Protein was measured by using the Lowry method (Lowry et al., 1951), with bovine serum albumin as the standard. Equal protein concentrations (50 µg/lane of total protein) were mixed with LDS Sample Buffer (Invitrogen) and mercaptoethanol, and were loaded onto 10% polyacrylamide

gels, analyzed by SDS-PAGE and transferred (MiniVE Electrophoresis System Amersham Biosciences) to nitrocellulose membranes (1 h at 50V in transfer buffer containing 48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS) (Valentim et al., 2001). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution with 5% powdered milk or bovine albumin (depending on the protein analised) in Tris-buffered saline plus 0.1% Tween-20. After blocking, membranes were incubated overnight at 4°C in Tris-buffered saline plus 0.1% Tween-20 (TBS-T) solution containing one of the following antibodies: Tyrosine hydroxylase (TH, 1:2000; Millipore, AB152), phosphorilated Tyrosine hydroxylase (at Ser40) (pTH,1:1000; Invitrogen,36-8600), Synaptophysin (SYP, 1:1000; Millipore, MAB368). The membranes were then washed three times for 5 min with TBS-T and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-rabbit IgG (1:1000, Millipore) at room temperature. Anti-β-actin antibody was used as control. (1:1000, Cell Signaling Technology, 4967S). Membranes were developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario). The chemiluminescence was detected using X-ray films that were scanned and analyzed by using the ImageJ Software.

2.4.3 Na^+/K^+-ATPase activity

The reaction mixture for Na^+/K^+-ATPase assay contained 5 mM MgCl2, 80 mM NaCl, 20 mM KCl, and 40 mM Tris-HCl (pH 7.4). After 10 min of sample pre-incubation at 37º C, the reaction was initiated by adding ATP to a final concentration of 3 mM, followed by 20 min incubation. 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 Wise et al. (2000). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Specific activity of the enzyme was expressed as nmol Pi released/min/mg of protein.

2.5. *In silico* network development of Na+/K+ transporting human ATPases, brain-derived neurotrophic factor (BDNF), tyrosine hydroxylase (TH), and synaptophysin (SYP) protein-protein interactions.

The “NaK-ATPase” network model was developed by using the STRING 10 database (http://string-db.org/) (Szklarczyk et al., 2011) with ‘‘Experiments’’ and ‘‘Databases’’ as input options and a confidence score of 0.300. STRING is a search tool for the retrieval of interacting genes/proteins extracted from different curate and public databases with information on direct (physical) and indirect (functional) associations/interactions derived from different sources: i) genomic context, ii) high-throughput experiments, iii) conserved co-expression, and also iv) previous knowledge (i.e., Pubmed). As starting point, we selected the Na+/K+ transporting human ATPases-related genes/proteins (Table. 3) together with BDNF, TH, and SYP as preliminary members (nodes) of interest for checking potential protein-protein interactions in the database. Under these input options, STRING 10 returned a 17 genes/proteins in total interconnecting within a network, which description is fully provided in Table 3. These genes/proteins integrating the “NaK-ATPase” *in silico* network model were identified by using the Human Genome Organization (HUGO) Gene Symbol (Wain et al., 2004) and Ensembl protein ID (Birney et al., 2006) (Table. 4). Detailed information about the confidence score network links within the “NaK-ATPase” model can be found in table 4.

2.6. Statistical analysis

Shapiro-Wilk normality test analyzed all data in order to choose the appropriate statistical test for each case. Therefore, data are expressed as mean ± S.E.M for ASST maze, BDNF levels, protein immunocontent, and Na^+/K^+-ATPase activity. These measurements were analyzed by Student's t test. For ASST pot, data are expressed as median (interquartile range) and were analyzed by Mann-Withney U test and the Wilcoxon test for between-group and within-subject analyses, respectively. Significance (*) level was considered when $P<0.05$.

3. RESULTS

3.1 Effect of neonatal handling on the attentional set shifting (ASST) tasks

a) ASST maze

No group difference was found in response to discrimination [Student's t test for independent samples, t(14)=0.732; *P*=0.476] or in the sensory/visual cue phase [Student's t test for independent samples, t(14)= 0.523; *P*=0.609] (Figure. 1B). No differences in perseverative [Student's t test for independent samples, t(14)= 0.205; *P*=0.841], regressive [Student's t test for independent samples, t(14)= 0.530; *P*=0.604], and never-reinforced [Student's t test for independent samples, t(14)= 0.145; *P*=0.887] errors were found (Figure. 1C).

b) ASST pot

No differences between groups were observed in crossings during the habituation session *(data not shown)*. During training, there were no differences between groups (NH and controls) independently of the dimension employed - texture, digging medium and odor (Mann-Whitney U=44.00, *P*=0.466, Z=-0.730;

U=46.00, *P*=0.626, Z=-0.487; U=40.00, *P*=0.146, Z=-1,453, respectively) *(data not shown*). However, in the testing phase, we observed impairments in the neonatally handled group in two discriminations, with increased trials to achieve the criterion: in the simple (SD; Mann-Whitney U=30.00, *P*=0.030, Z=2.164) and in the intradimensional (ID; Mann-Whitney U=30.00, *P*=0.031, Z=2.163). Interestingly, in the extradimensional discrimination (ED), neonatally handled animals used less trials to achieve the criterion, indicating a facilitation for this discrimination (Mann-Whitney U= 24.500, *P*=0.034, Z=2.123), as is seen in Figure 2. No differences were found in CD, REV1, REV2, and REV3 (Mann-Whitney U=33.00, *P*=0.129, Z=1.51; U=49.00, *P*=0.936, Z=0.080; U=49.00, *P*=0.936, Z=0.080; U=42.50, *P*=0.559, Z=0.585; respectively) (Figure 2). In controls, there was a difference in the following between-discrimination comparisons using the Wilcoxon test: SD-REV1 (Z=2.226, *P*=0.026), SD-REV2 (Z=2.226, *P*=0.026), SD-ED (Z=2.388, *P*=0.017), SD-REV3 (Z=2.724, *P*=0.006), CD-ED (Z=2.058, *P*=0.040), CD-REV3 (Z=2.372, *P*=0.018), REV1-ID (Z=2.226, *P*=0.026), ID-REV2 (Z=2.226, *P*=0.026), ID-ED (Z=2.388, *P*=0.017), ID-REV3 (Z=2.724, *P*=0.017). In NH animals, between-discrimination comparisons were different between ED and REV3 (Z=2.254, *P*=0.024) and a trend towards a difference in ID-REV3 (Z=1.849, *P*=0.064). No differences were observed in the total time to perform the task (data not shown).

3.2 Effects of neonatal handling on BDNF levels and on the immunocontents of total and phosphorylated tyrosine hydroxylase, and of synaptophysin in the mPFC.

No significant differences between groups were observed in the mPFC on TH and pTH immunocontent [Student's t test for independent samples, t(11)=1.028;

P=0.326; t (10)=0.109; *P*=0.915, respectively] (Figure. 3A and B), and on BDNF levels [Student's t test for independent samples, t(9)=0.028; *P*=0.978] (Figure. 3C). However, NH showed a significant decrease on synapthophysin levels in the mPFC [Student's t test for independent samples, t(12)=2.438; *P*=0.031] (Figure. 3D).

3.3 Effect of neonatal handling on Na^+/K^+-ATPase activity in the mPFC.

Handling of neonatal animals significantly decreased Na^+/K^+-ATPase activity in mPFC when compared to control animals [Student's t test for independent samples, t(11)=2.966; *P*=0.013] (Figure. 4).

3.4 *In silico* characterization of Na+/K+ transporting human ATPases, BDNF, TH, and SYP protein-protein network interactions.

The newly developed *in silico* network model "NaK-ATPase" (Figure. 5) generated by using ''Databases'' and ''Experiments'' as input options, is composed by 17 interacting nodes and represents the protein-protein landscape of interactions between Na^+/K^+-ATPase, BDNF, TH, and SYP. No direct interconnection between Na^+/K^+-ATPase, BDNF, TH, and SYP was detected by the resource search tool STRING 10. Of note, our model revealed that SYP protein is able to interact with the rest of the *in silico* model exclusively by either the interaction with synaptobrevin-1 (VAMP1) or synaptobrevin-2 (VAMP2) network nodes; and that SYP interaction with the Na^+/K^+-ATPase-related cluster of proteins would only require the additional interaction with either UBC or YWHAZ and MAPK1. By clicking the ''Actions view'' option of STRING 10, a representation between the network nodes by either ''activation,'' ''inhibition,'' ''binding,'' ''catalysis,'' and ''reaction'' was developed and shown within the network in different colors.

4. DISCUSSION

To the best of our knowledge, this is the first study showing evidence for an influence of neonatal handling on the performance of an ASST pot in adult Wistar rats, with impairment in simple and intradimensional discrimination but improvement in extradimensional discrimination. However, NH does not modify the performance in ASST maze in which animals have to change from an egocentric spatial to a cued strategy. These behavioral changes are accompanied by a decrease in SYP immunocontent and lower activity of the Na^+/K^+-ATPase in the mPFC.

The ASST pot is the rodent version (Birrell and Brown, 2000, Floresco et al., 2006a) of the Wisconsin Card Sorting Task (Nyhus and Barcelo, 2009, Bissonette et al., 2013), which is used to evaluate attentional skills in humans. Attention, associative learning, and/or cognitive flexibility are intertwined processes. The putative involvement of each of them in the effects of NH on the ASST pot performance is discussed below.

For simple and intradimensional discrimination in the ASST pot, new associative learning occurs and the animal does not need to change a previous rewarding behavior. Therefore, associative learning, attention, or both, but possibly not cognitive flexibility, were impaired in NH animals to promote SD and ID deficits in the ASST pot (Wright et al., 2015).

Over successive CD and ID trials, the animal must attend selectively to the features that remain the best predictors of reward, and learn to successively ignore a non-rewarding dimension: *i.e.*, there is set formation, which is a higher-order learning process, since rules or categories but not concrete stimuli are the

subject to be selected. In ED, relevant contingencies change, such that a previously relevant dimension is not rewarded, and attention must shift to a previously irrelevant dimension, which is now rewarded. So, the need of more trials for ED than for ID is an indication of set formation (Robbins and Roberts, 2007, Tait et al., 2014). In the present study, ED showed to be more difficult than ID for controls. In NH animals, however, there was no ED-ID difference. Therefore, it is very likely that there was no set formation in these animals. Curiously, NH animals showed a difference in the number o trials between the last reversal and ED, which did not occurred with controls. One possible explanation of these results is that the neonatal handling procedure induced impaired SD, ID, and attentional set formation because animals had disrupted capacity for attending to relevant stimuli by increasing attention to irrelevant domains and, then, had difficulties in associative learning. This hypothesis was also formulated for explaining very similar results that occurred after anterior thalamic lesions (Wright et al., 2015): the difference from that study and ours was that reversals were not clearly impaired after neonatal handling.

Another explanation of our results is that associative learning per se, for a stimulus or a set, was impaired. This is reinforced by the fact that every step that involved the introduction of a new stimulus (SD, ID, and ED but neither CD nor reversals) was clearly impaired in NH animals. However, this possibility is less likely if one consider that there are no behavioral impairments while analyzing olfactory short-term memory (Noschang et al., 2012a), or novel object recognition long-term memory in NH animals (Kosten et al., 2007). In addition, there is a lack of effect of NH on cathecolaminergic parameters (observed in this and in a previous work; Lazzaretti et al., (2015), and the role of

cathecolamines in the prefrontal cortex is very known in associative learning for concrete stimuli or set formation (Robbins and Roberts, 2007).

The ability to respond to a changing environment is essential for reversals and the extradimensional shift, since the cue that was previously associated with the reward is still present. Therefore, we may not rule out a putative involvement of cognitive flexibility deficits for explaining the results in ED. However, this possibility is unlikely for the following reasons: (1) it requires that a set formation might have been formed in the NH group, but these animals did not show behavioral evidence of this formation, as mentioned before; (2) no effect of neonatal handling was observed in the ASST maze, in which the animal must change from an egocentric strategy to another that uses a novel visual, tactile cue on the maze floor, which is presented to the animal in all further phases; and (3) the first and second reversals were not changed in the ASST pot. Interestingly, reversal learning deficits may be found in a Y- task but not in a Morris water-maze task in male NH rats (Noschang et al., 2012b). However, an effect in reversal learning might not be completely ruled out in the ASST pot, since a few NH rats showed exceptional high number of trials during reversals. Whatever the reason for the behavioral results found in the ASST pot, they indicate that neonatal handling may be either beneficial or detrimental, depending on the demands of the environment: when only SD is required, NH may decrease animal performance; in case ED is required, NH might increase its performance.

In rodents, attention and cognitive flexibility are mediated by mPFC (Floresco et al., 2006b, Logue and Gould, 2014). We thus selected this cortical region to make our biochemical analyses from adult animal samples.

Our results showed that neonatal handling decreases SYP immunocontent. SYP is a synaptic protein that has been linked to ADHD (Brookes et al., 2005, Brookes et al., 2006, Guan et al., 2009, Schmitt et al., 2009, Liu et al., 2013). Behavioral alterations and learning deficits have previously been reported in knock-out mice lacking *SYP* (Schmitt et al., 2009). In rodents, frontal regions decline their growth in the transition from childhood to adolescence, which are characterized by synaptic overproduction and synaptic pruning, respectively (Glantz et al., 2007, Kolb et al., 2012). This transition occurs in parallel with an improvement in executive functions (Fuster, 2002) and a decrease in SYP levels in the PFC (Glantz et al., 2007). Therefore, our results indicate that NH is able to decrease SYP content even further. Reduced levels of SYP and PSD95 (postsynaptic marker) were found in frontal cortex neurons, in puberty animals subjected to maternal deprivation on PND 9 during 24h, establishing that maternal contact modulates the synapses protein content (Marco et al., 2013). Addictionally, synaptophysin transcription are controled by R1 silencing transcription factor (REST) (Hohl and Thiel, 2005), and REST are modulated by neonatal interventions models, such as neonatal handling increasing its levels (Korosi et al., 2010) and maternal separation deacresing them in mPFC (Uchida et al., 2010). Therefore, early life experiences can modify the brain basic circuitry to be later pruned during adolescence and consequently affect behavior in cognitive tasks during adulthood (Kolb et al., 2012, Noschang et al., 2012b). However, distinct neonatal interventions may lead to different outcomes. For example, maternal separation, which is a procedure that involves separating the dam from the pups during several hours in the

neonatal period, is known to increase anxiety (Neumann et al., 2005), an effect opposite to that induced by neonatal handling, which causes less defensive behavior (Padoin et al., 2001, Macri et al., 2004, Madruga et al., 2006). Some behavioral characteristics of neonatally handled rats, such as increased exploratory behavior (Padoin et al., 2001) and impaired spatial memory (Noschang et al., 2010) are also known in knock-out animals for *SYP* (Schmitt et al., 2009). Additionally, NH is able to downregulate GluA3 receptors in prelimbic and infralimbic regions of the mPFC (Katsouli et al., 2014), and this reduction in GluA3 receptors might be related to reduced levels of SYP.

Our results also highlight a decrease in the Na^+/K^+-ATPase activity in the mPFC from adult animals that underwent neonatal handling. It has been described that early handling decreases the Na^+/K^+-ATPase activity in the hippocampus during adulthood (Silveira et al., 2011) and increases it in the olfactory bulb and amygdala (Silveira et al., 2011, Noschang et al., 2012a). It is worth to mention that behavioral alterations are found after infusion of the specific Na^+/K^+-ATPase inhibitor ouabain into the frontal cortex, such as hyperactivity (Kim et al., 2013), and that Na^+/K^+-ATPase activity is decreased in different animal models of psychiatric disorders (Silveira et al., 2011, Sun et al., 2015). We can speculate that the lower Na^+/K^+-ATPase activity induced by neonatal handling in the mPFC is related to the decrease in SYP immunocontent, since both could be the result of a decrease in the number of synapses in this brain region, even though Na^+/K^+-ATPase is found not only in the synapse, but throughout the membrane. Very interestingly, SYP interacts with the rest of the *in silico* model exclusively by either the interaction with

synaptobrevin-1 (VAMP1) or synaptobrevin-2 (VAMP2) network nodes. Abnormalities of glutamate release have recently been associated with large reductions in the levels of synaptic vesicle-related proteins, such as VAMP, syntaxin-1, and SYP among other in the ventral hippocampus of prenatally restraint stressed (PRS) rats (Marrocco et al., 2012). To our knowledge, there are no reports regarding the effect of neonatal handling on the levels of VAMP1 and/or VAMP2. Considering Marrocco et al. (2012) results in the PRS model and our own *in silico* evidence, these proteins may worth further investigation.

There was a lack of effect of neonatal handling on both total and phosphorylated (pSer40) TH immunocontent. In a previous study, our laboratory showed that NH animals do not present alterations in the levels of dopamine and noradrenaline in the mPFC Lazzaretti et al. (2015). In addition, Bobrovskaya and colleagues (2013) showed no differences in TH and pTH (pSer40) in adrenal glands from handled animals at adult age. Therefore, no gross alterations due to early life stress are observed in the mPFC on the catecholaminergic systems. On the other hand, changes in the dopaminergic system have been observed in neonatally handled animals in the accumbens (Silveira et al., 2010), and we cannot rule out the possibility of some alteration on these systems in mPFC.

In summary, the present study reports that a brief neonatal intervention (NH) induces changes in attention and/or learning, impairing performance in simple and intradimensional discrimination but facilitating extradimensional discrimination. Additionally, SYP content and Na^+/K^+-ATPase activity were decreased in the mPFC. Therefore, this study provides further evidence about

how early life events can program behavior and direct the organism to either mental health or increased vulnerability to disorders.

Conflict of interest

All authors disclose no conflict of interest including any financial, personal or other relationships with other people or organizations.

Acknowledgements

CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), PROFIX (CNPq), and FINEP (‘‘Rede Instituto Brasileiro de Neurociências” - IBN-Net, No 01.06.0842-00). The proceedings were conducted in agreement with the UFRGS - CEUA (Ethics Committee on Animal Use), protocol number 21460, and with the Brazilian Law on the use of animals (Federal Law 11.794/2008) and Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003). F. Zeidán-Chuliá (UFRGS) and G.C. Kincheski (UFRJ) hold postdoctoral positions, supported by CAPES.

Figures:

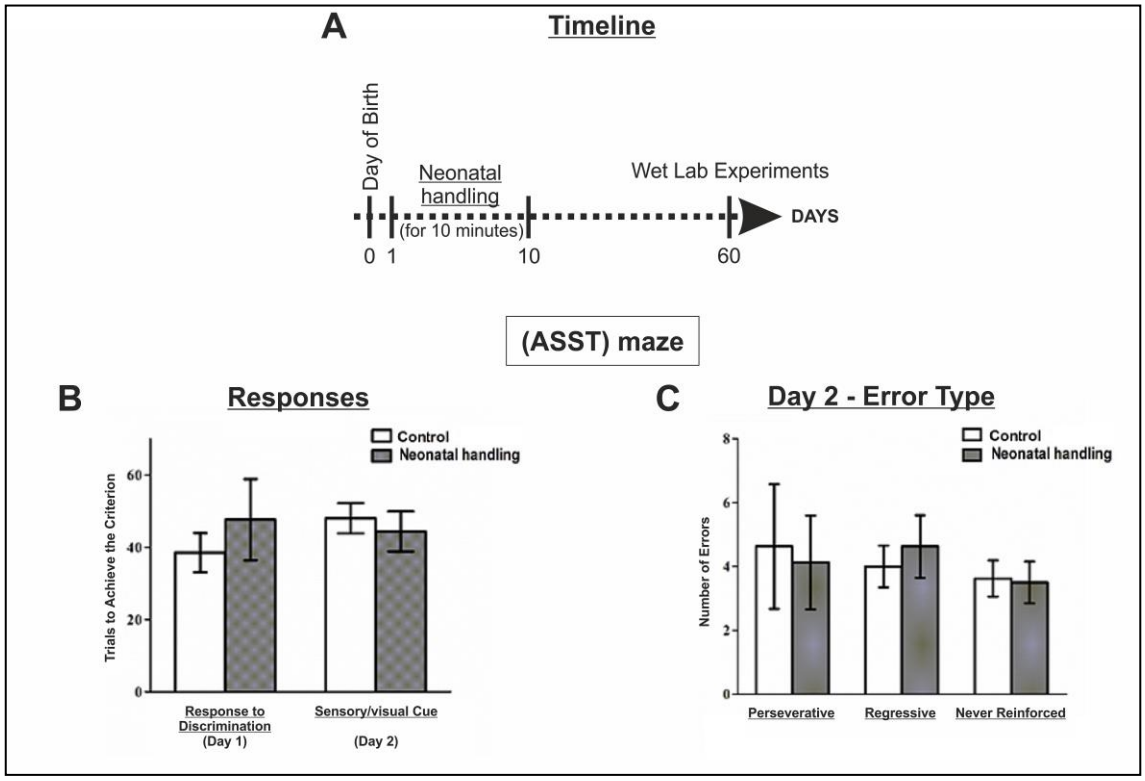

Figure 1. Effect of neonatal handling (NH) on attentional set shifting maze task (Floresco et al., 2006a, Pandolfo et al., 2013). (A) Timeline for the experimental design. (B) Trials to achieve the criterion in the responses to discrimination phase and visual/sensory cue phase. Student's t test shows *P*=0.476 (day 1), *P*=0.609 (day 2). (C) Number and types of errors. Student's t test, perseverative *P*=0.841, regressive *P*=0.604, and never reinforced *P*=0.887. No significant effect in any phases or number of errors; non-handled (N=8), NH (N=8).

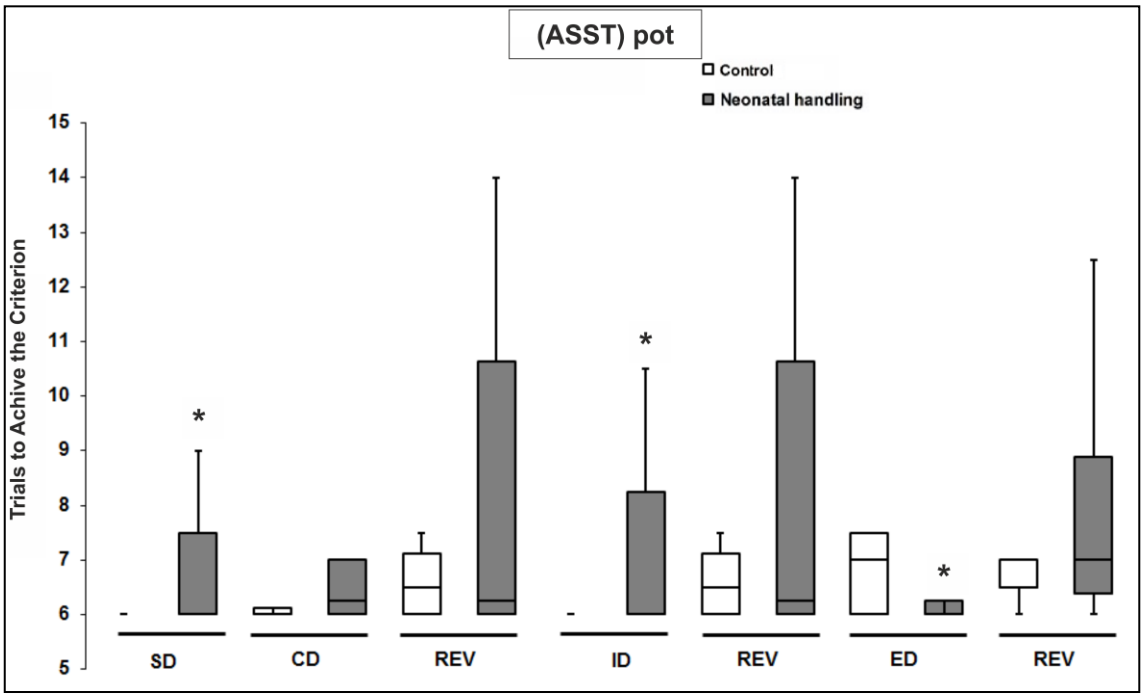

Figure 2. Effect of neonatal handling (NH) on the total number of trials to achieve six consecutive correct ones in attentional set shifting pot task (Brown and Birrel, 2000). Data within the box plot: upper horizontal line in the box, 75th percentile; lower horizontal line in the box, 25th percentile; horizontal bar within box, median; lower and higher horizontal bars outside the box represent minimum and maximum values, respectively. *Significant effect of NH on trials to achieve the criterion when compared to controls: increase in simple and intradimensional discriminations (Mann-Whitney test: SD, *P*=0.030; ID, *P*=0.031) and decrease in extradimensional discrimination (Mann-Whitney test: ED, *P*=0.034. Non-handled (N=10), NH (N=10).

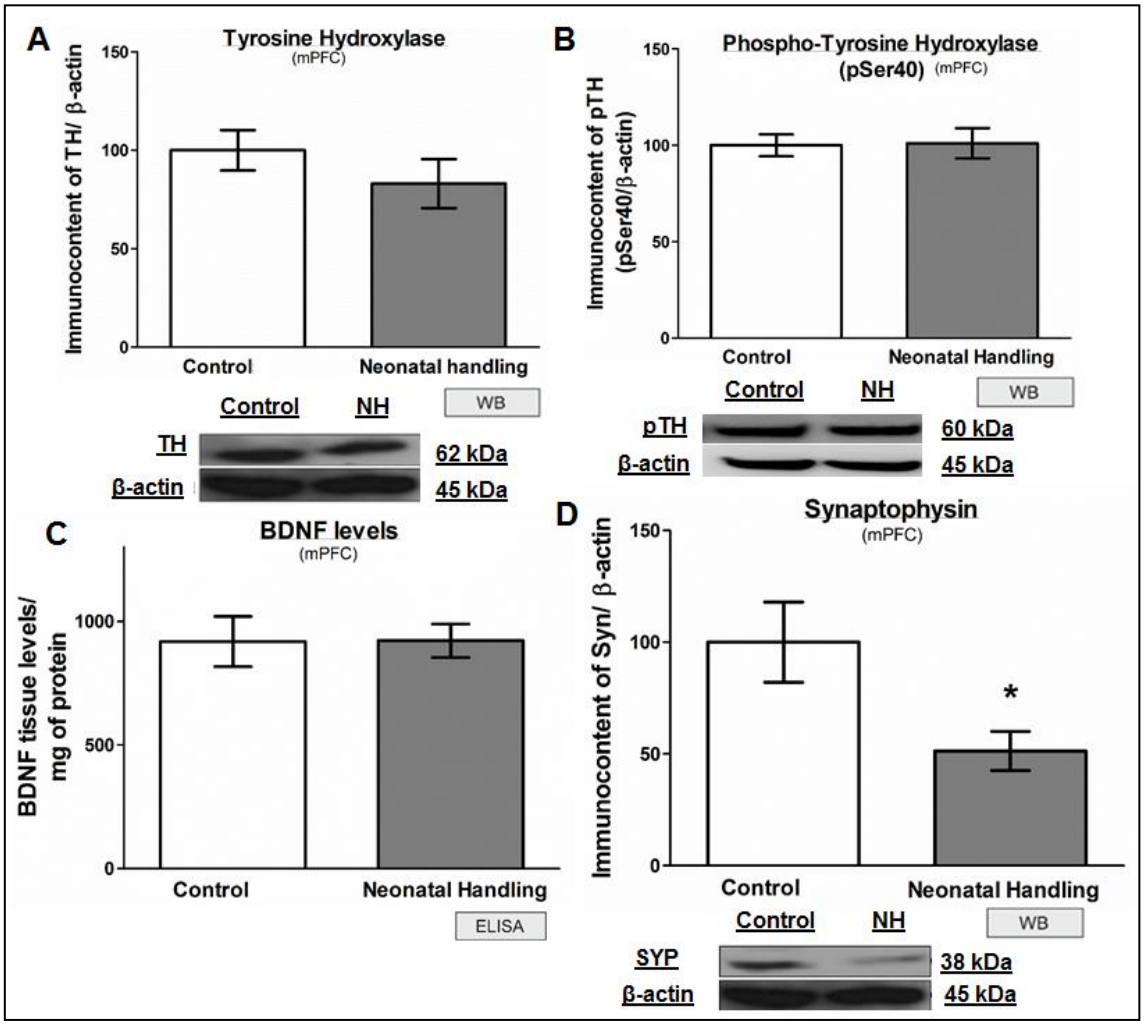

Figure 3. Levels of TH, Phospho-Tyrosine Hydroxilase (pSer40), BDNF and SYP in the medial prefrontal cortex (mPFC) of control *vs.* early neonatally handled animals. Data are expressed as mean ± S.E.M. (A) Quantification of tyrosine hydroxylase (TH) immunocontent shows no significant differences between control (N=6) and neonatally handled (NH) adult Wistar rats (N=7) (*P*=0.326). Bars illustrate TH content normalized to that of β-actin. (B) Quantification of phosphorylated tyrosine hydroxylase (pSer40) immunocontent shows no significant differences between controls (N=6) and NH adult Wistar rats (N=6) (*P*=0.915). Bars illustrate pTH content normalized to that of β-actin. (C) No significant differences in BDNF levels analyzed by ELISA assay were observed (controls, N=5; NH, N=6; *P*=0.978). (D) Quantification of

synaptophysin (SYP) immunocontent shows significant differences between controls (N=7) and NH adult Wistar rats (N=7; *Student's *t* test, *P*=0.031). Bars illustrate SYP content normalized to that of β-actin.

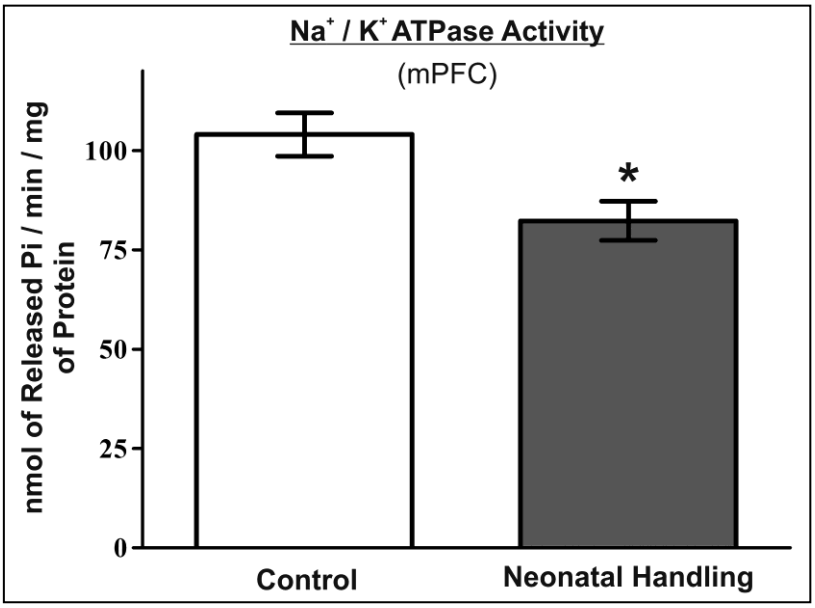

Figure 4. Effects of neonatal handling on Na^+/K^+-ATPase activity in the medial prefrontal cortex (mPFC). Data are expressed as mean ± S.E.M. Na^+/K^+-ATPase activity was significantly decreased in neonatally handled (NH, N=7) adult Wistar rats when compared to controls (N=6, *Student's t test, *P*=0.013).

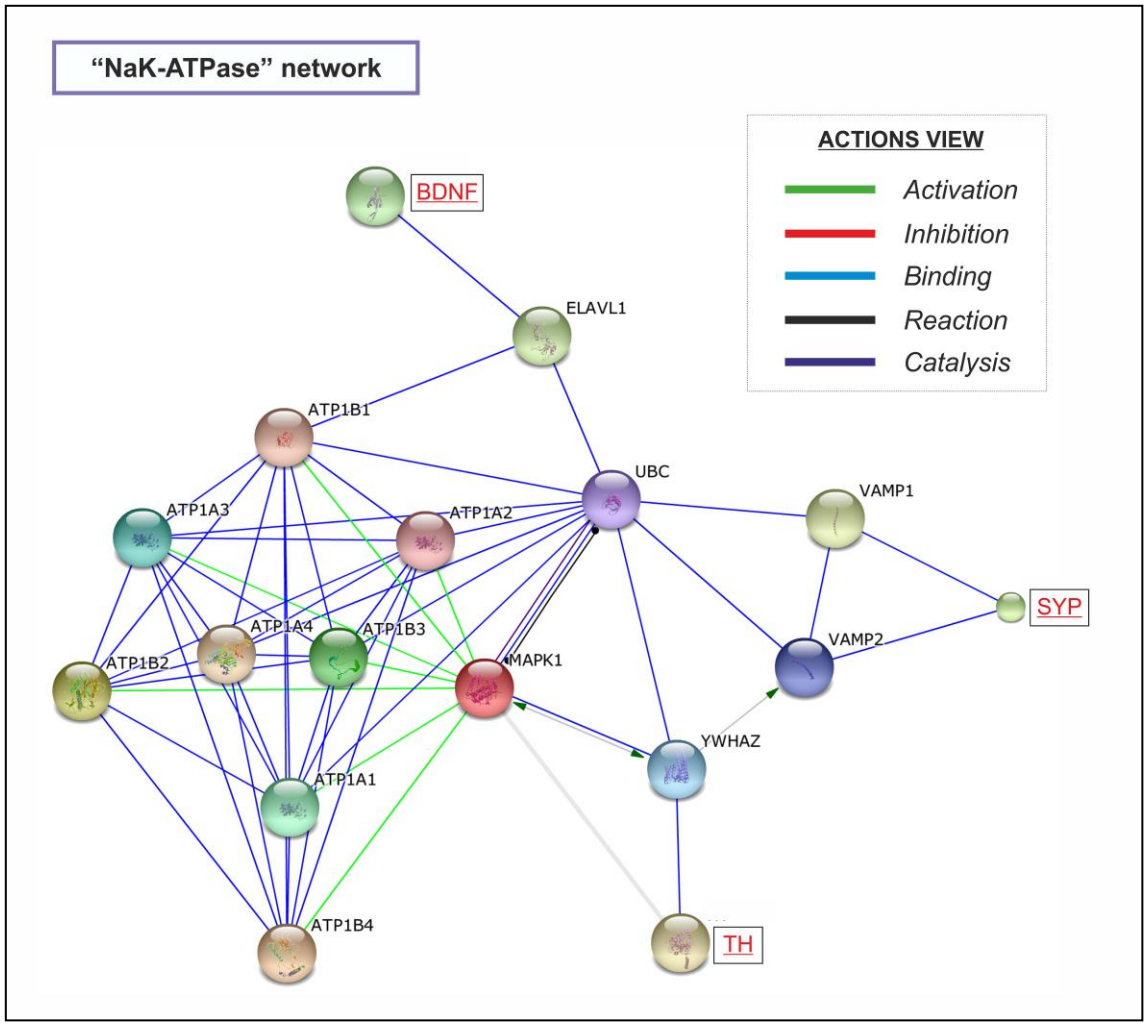

Figure 5. *In silico* network model of the interactions between BDNF, synaptophysin (SYP), tyrosine hydroxylase (TH), and Na+/K+ transporting human ATPases ("NaK-ATPase" model) by using the STRING 10 resource with "experiments" and "databases" as input options and a confidence score of 0.300. "Actions view" representation between the network nodes by either "activation", "inhibition", "binding", "reaction", and "catalysis" are shown within the network in different colors and specified in the inset.

Table 1. Materials utilized in pairs for all discriminations, mixing textures, medium, and odor.

	PAIR 1		PAIR 2		PAIR 3	
Textures	Velvet	Newspaper	Fine Sandpaper	Coarse Sandpaper	Rubber	Cardboard Cover
Medium	Buttons	Chopped Cardboard	Clips	Ball Beads	Ferrule	Needle
Odor	Cinnamon	Nutmeg	Strawberry	Vanilla	Thyme	Oregano

Table 2. Example of a putative sequence of discriminations carried out in the ASST maze. In this case, the dimensions used were odor (O) and medium (M) (texture was used in other combinations). (+) and (–) indicate reward and no-reward, respectively. Numbers indicate possible materials listed in table 1. According to Birrel and Brown.,(2000).

	Dimensions		Exemplar combinations	
	Relevant	Irrelevant	+	-
Simple (SD)	Odor		O1	O2
Compound (CD)	Odor	Medium	O1/M1 O1/M2	O2/M2 O2/M1
Reversal (REV1)	Odor	Medium	O2/M1 O2/M2	O1/M2 O1/M2
Intradimensional shift (ID)	Odor	Medium	O3/M3 O3/M4	O4/M4 O4/M3
Reversal (Rev2)	Odor	Medium	O4/M3 O4/M4	O3/M4 O3/M3
Extradimensional shift (ED)	Medium	Odor	M5/O5 M5/O6	M6/O6 M6/O5
Reversal (Rev3)	Medium	Odor	M6/O5 M6/O6	M5/O6 M5/O5

Table 3. Ensembl protein identifiers of the genes belonging to the "NaK-ATPase" *in silico* network model.

Gene Symbol	**Ensembl ID** (ENSP)	**Description**
VAMP1	ENSP00000379602	Vesicle-associated membrane protein 1 (synaptobrevin 1)
BDNF	ENSP00000414303	Brain-derived neurotrophic factor
ATP1B1	ENSP00000356789	ATPase, Na+/K+ transporting, beta 1 polypeptide
VAMP2	ENSP00000314214	Vesicle-associated membrane protein 2 (synaptobrevin 2)
SYP	ENSP00000263233	Synaptophysin
UBC	ENSP00000344818	Ubiquitin C
TH	ENSP00000370571	Tyrosine hydroxylase
ATP1A3	ENSP00000302397	ATPase, Na+/K+ transporting, alpha 3 polypeptide
ATP1B3	ENSP00000286371	ATPase, Na+/K+ transporting, beta 3 polypeptide
ATP1A2	ENSP00000354490	ATPase, Na+/K+ transporting, alpha 2 polypeptide
ELAVL1	ENSP00000385269	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)
ATP1A1	ENSP00000295598	ATPase, Na+/K+ transporting, alpha 1 polypeptide
ATP1B4	ENSP00000218008	ATPase, Na+/K+ transporting, beta 4 polypeptide
ATP1B2	ENSP00000250111	ATPase, Na+/K+ transporting, beta 2 polypeptide
MAPK1	ENSP00000215832	Mitogen-activated protein kinase 1
ATP1A4	ENSP00000357060	ATPase, Na+/K+ transporting, alpha 4 polypeptide
YWHAZ	ENSP00000309503	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

Table 4. Information about the confidence score network links within the "NaK-ATPase" *in silico* network model.

Node 1	Node 2	Node 1 External ID	Node 2 External ID	Experimental	Knowledge	Combined Score
ATP1B2	*ATP1B4*	ENSP00000250111	ENSP00000218008	0	0.36	0.36
VAMP2	*SYP*	ENSP00000314214	ENSP00000263233	0.391	0	0.391
VAMP1	*UBC*	ENSP00000379602	ENSP00000344818	0.938	0	0.938
ATP1A1	*ATP1B2*	ENSP00000295598	ENSP00000250111	0.326	0.9	0.929
ATP1B3	*MAPK1*	ENSP00000286371	ENSP00000215832	0	0.8	0.8
ATP1A4	*ATP1A1*	ENSP00000357060	ENSP00000295598	0	0.9	0.899
ATP1A3	*ATP1B3*	ENSP00000302397	ENSP00000286371	0.326	0.9	0.929
ATP1A2	*ATP1B4*	ENSP00000354490	ENSP00000218008	0.326	0.36	0.55
ATP1B3	*ATP1B4*	ENSP00000286371	ENSP00000218008	0	0.36	0.36
ATP1B1	*UBC*	ENSP00000356789	ENSP00000344818	0.898	0	0.898
VAMP2	*YWHAZ*	ENSP00000314214	ENSP00000309503	0	0.9	0.899
ATP1A3	*ATP1B2*	ENSP00000302397	ENSP00000250111	0.326	0.9	0.929
ELAVL1	*ATP1B1*	ENSP00000385269	ENSP00000356789	0.321	0	0.321
ATP1A3	*ATP1A1*	ENSP00000302397	ENSP00000295598	0	0.9	0.899
ATP1A2	*UBC*	ENSP00000354490	ENSP00000344818	0.95	0	0.95
VAMP1	*SYP*	ENSP00000379602	ENSP00000263233	0.301	0	0.3
ATP1A4	*ATP1B3*	ENSP00000357060	ENSP00000286371	0.326	0.9	0.929
ATP1B1	*ATP1B3*	ENSP00000356789	ENSP00000286371	0	0.9	0.899
ATP1A1	*ATP1B4*	ENSP00000295598	ENSP00000218008	0.326	0.36	0.55
ATP1A4	*ATP1B1*	ENSP00000357060	ENSP00000356789	0.632	0.9	0.961
TH	*YWHAZ*	ENSP00000370571	ENSP00000309503	0.917	0	0.917
ATP1A1	*ATP1B3*	ENSP00000295598	ENSP00000286371	0.326	0.9	0.929
ATP1A4	*ATP1B4*	ENSP00000357060	ENSP00000218008	0.326	0.36	0.55
ATP1A4	*UBC*	ENSP00000357060	ENSP00000344818	0.87	0	0.87
ATP1B1	*ATP1A2*	ENSP00000356789	ENSP00000354490	0.632	0.9	0.961
UBC	*ATP1A1*	ENSP00000344818	ENSP00000295598	0.99	0	0.99
UBC	*ATP1B3*	ENSP00000344818	ENSP00000286371	0.943	0	0.943
TH	*MAPK1*	ENSP00000370571	ENSP00000215832	0.812	0	0.812
ATP1A2	*ATP1B2*	ENSP00000354490	ENSP00000250111	0.326	0.9	0.929
ATP1A2	*ATP1A3*	ENSP00000354490	ENSP00000302397	0	0.9	0.899
ATP1A3	*MAPK1*	ENSP00000302397	ENSP00000215832	0	0.8	0.8
ATP1B3	*ATP1B2*	ENSP00000286371	ENSP00000250111	0	0.9	0.899
ATP1A3	*ATP1B4*	ENSP00000302397	ENSP00000218008	0.326	0.36	0.55
ATP1B1	*ATP1A3*	ENSP00000356789	ENSP00000302397	0.665	0.9	0.965
UBC	*YWHAZ*	ENSP00000344818	ENSP00000309503	0.992	0	0.992
ATP1B4	*MAPK1*	ENSP00000218008	ENSP00000215832	0	0.8	0.8
ATP1A2	*MAPK1*	ENSP00000354490	ENSP00000215832	0	0.8	0.8
BDNF	*ELAVL1*	ENSP00000414303	ENSP00000385269	0.321	0	0.321
ELAVL1	*UBC*	ENSP00000385269	ENSP00000344818	0.998	0	0.998
ATP1A2	*ATP1B3*	ENSP00000354490	ENSP00000286371	0.326	0.9	0.929
ATP1B2	*MAPK1*	ENSP00000250111	ENSP00000215832	0	0.8	0.8
ATP1A2	*ATP1A1*	ENSP00000354490	ENSP00000295598	0	0.9	0.899
YWHAZ	*MAPK1*	ENSP00000309503	ENSP00000215832	0.089	0.9	0.904
ATP1B1	*MAPK1*	ENSP00000356789	ENSP00000215832	0	0.8	0.8
ATP1B1	*ATP1B2*	ENSP00000356789	ENSP00000250111	0	0.9	0.899
ATP1A4	*ATP1B2*	ENSP00000357060	ENSP00000250111	0.326	0.9	0.929
UBC	*MAPK1*	ENSP00000344818	ENSP00000215832	0.969	0	0.969
UBC	*ATP1A3*	ENSP00000344818	ENSP00000302397	0.95	0	0.95
ATP1B1	*ATP1A1*	ENSP00000356789	ENSP00000295598	0.665	0.9	0.965
ATP1B1	*ATP1B4*	ENSP00000356789	ENSP00000218008	0	0.36	0.36
VAMP1	*VAMP2*	ENSP00000379602	ENSP00000314214	0	0.54	0.54

ATP1A1	*MAPK1*	ENSP00000295598	ENSP00000215832	0.576	0.8	0.911
ATP1A4	*ATP1A3*	ENSP00000357060	ENSP00000302397	0	0.9	0.899
UBC	*VAMP2*	ENSP00000344818	ENSP00000314214	0.967	0	0.967
ATP1A4	*ATP1A2*	ENSP00000357060	ENSP00000354490	0	0.9	0.899

References

Birney E, Andrews D, Caccamo M, Chen Y, Clarke L, Coates G, Cox T, Cunningham F, Curwen V, Cutts T, Down T, Durbin R, Fernandez-Suarez XM, Flicek P, Graf S, Hammond M, Herrero J, Howe K, Iyer V, Jekosch K, Kahari A, Kasprzyk A, Keefe D, Kokocinski F, Kulesha E, London D, Longden I, Melsopp C, Meidl P, Overduin B, Parker A, Proctor G, Prlic A, Rae M, Rios D, Redmond S, Schuster M, Sealy I, Searle S, Severin J, Slater G, Smedley D, Smith J, Stabenau A, Stalker J, Trevanion S, Ureta-Vidal A, Vogel J, White S, Woodwark C, Hubbard TJ (2006) Ensembl 2006. Nucleic Acids Res 34:D556-561.

Birrell JM, Brown VJ (2000) Medial frontal cortex mediates perceptual attentional set shifting in the rat. J Neurosci 20:4320-4324.

Bissonette GB, Powell EM, Roesch MR (2013) Neural structures underlying set-shifting: roles of medial prefrontal cortex and anterior cingulate cortex. Behav Brain Res 250:91-101.

Bobrovskaya L, Maniam J, Ong LK, Dunkley PR, Morris MJ (2013) Early life stress and post-weaning high fat diet alter tyrosine hydroxylase regulation and AT1 receptor expression in the adrenal gland in a sex dependent manner. Neurochem Res 38:826-833.

Brookes K, Xu X, Chen W, Zhou K, Neale B, Lowe N, Anney R, Franke B, Gill M, Ebstein R, Buitelaar J, Sham P, Campbell D, Knight J, Andreou P, Altink M, Arnold R, Boer F, Buschgens C, Butler L, Christiansen H, Feldman L, Fleischman K, Fliers E, Howe-Forbes R, Goldfarb A, Heise A, Gabriels I, Korn-Lubetzki I, Johansson L, Marco R, Medad S, Minderaa R, Mulas F, Muller U, Mulligan A, Rabin K, Rommelse N, Sethna V, Sorohan J, Uebel H, Psychogiou L, Weeks A, Barrett R, Craig I, Banaschewski T, Sonuga-Barke E, Eisenberg J, Kuntsi J, Manor I, McGuffin P, Miranda A, Oades RD, Plomin R, Roeyers H, Rothenberger A, Sergeant J, Steinhausen HC, Taylor E, Thompson M, Faraone SV, Asherson P (2006) The analysis of 51 genes in DSM-IV combined type attention deficit hyperactivity disorder: association signals in DRD4, DAT1 and 16 other genes. Mol Psychiatry 11:934-953.

Brookes KJ, Knight J, Xu X, Asherson P (2005) DNA pooling analysis of ADHD and genes regulating vesicle release of neurotransmitters. Am J Med Genet B Neuropsychiatr Genet 139B:33-37.

Chan KM, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca2+ -stimulated ATPase activity. Anal Biochem 157:375-380.

Dajani DR, Uddin LQ (2015) Demystifying cognitive flexibility: Implications for clinical and developmental neuroscience. Trends Neurosci 38:571-578.

de Souza Wyse AT, Streck EL, Worm P, Wajner A, Ritter F, Netto CA (2000) Preconditioning prevents the inhibition of Na+,K+-ATPase activity after brain ischemia. Neurochem Res 25:971-975.

Elton A, Tripathi SP, Mletzko T, Young J, Cisler JM, James GA, Kilts CD (2014) Childhood maltreatment is associated with a sex-dependent functional reorganization of a brain inhibitory control network. Hum Brain Mapp 35:1654-1667.

Floresco SB, Ghods-Sharifi S, Vexelman C, Magyar O (2006a) Dissociable roles for the nucleus accumbens core and shell in regulating set shifting. J Neurosci 26:2449-2457.

Floresco SB, Magyar O, Ghods-Sharifi S, Vexelman C, Tse MT (2006b) Multiple dopamine receptor subtypes in the medial prefrontal cortex of the rat regulate set-shifting. Neuropsychopharmacology 31:297-309.
Fuster JM (2002) Frontal lobe and cognitive development. J Neurocytol 31:373-385.
Glantz LA, Gilmore JH, Hamer RM, Lieberman JA, Jarskog LF (2007) Synaptophysin and postsynaptic density protein 95 in the human prefrontal cortex from mid-gestation into early adulthood. Neuroscience 149:582-591.
Guan L, Wang B, Chen Y, Yang L, Li J, Qian Q, Wang Z, Faraone SV, Wang Y (2009) A high-density single-nucleotide polymorphism screen of 23 candidate genes in attention deficit hyperactivity disorder: suggesting multiple susceptibility genes among Chinese Han population. Mol Psychiatry 14:546-554.
Katsouli S, Stamatakis A, Giompres P, Kouvelas ED, Stylianopoulou F, Mitsacos A (2014) Sexually dimorphic long-term effects of an early life experience on AMPA receptor subunit expression in rat brain. Neuroscience 257:49-64.
Kehagia AA, Murray GK, Robbins TW (2010) Learning and cognitive flexibility: frontostriatal function and monoaminergic modulation. Curr Opin Neurobiol 20:199-204.
Kim SH, Yu HS, Park HG, Ha K, Kim YS, Shin SY, Ahn YM (2013) Intracerebroventricular administration of ouabain, a Na/K-ATPase inhibitor, activates mTOR signal pathways and protein translation in the rat frontal cortex. Prog Neuropsychopharmacol Biol Psychiatry 45:73-82.
Kolb B, Mychasiuk R, Muhammad A, Li Y, Frost DO, Gibb R (2012) Experience and the developing prefrontal cortex. Proc Natl Acad Sci U S A 109 Suppl 2:17186-17193.
Kosten TA, Karanian DA, Yeh J, Haile CN, Kim JJ, Kehoe P, Bahr BA (2007) Memory impairments and hippocampal modifications in adult rats with neonatal isolation stress experience. Neurobiol Learn Mem 88:167-176.
Kosten TA, Miserendino MJ, Kehoe P (2000) Enhanced acquisition of cocaine self-administration in adult rats with neonatal isolation stress experience. Brain Res 875:44-50.
Latimer K, Wilson P, Kemp J, Thompson L, Sim F, Gillberg C, Puckering C, Minnis H (2012) Disruptive behaviour disorders: a systematic review of environmental antenatal and early years risk factors. Child Care Health Dev 38:611-628.
Lazzaretti C, Kincheski GC, Pandolfo P, Krolow R, Toniazzo AP, Arcego DM, Couto-Pereira NS, Zeidan-Chulia F, Galvalisi M, Costa G, Scorza C, Souza TM, Dalmaz C (2015) Neonatal handling causes impulsive behavior and decreased pharmacological response to methylphenidate in male adult wistar rats. J Integr Neurosci 1650004.
Liu L, Chen Y, Li H, Qian Q, Yang L, Glatt SJ, Faraone SV, Wang Y (2013) Association between SYP with attention-deficit/hyperactivity disorder in Chinese Han subjects: differences among subtypes and genders. Psychiatry Res 210:308-314.
Logue SF, Gould TJ (2014) The neural and genetic basis of executive function: attention, cognitive flexibility, and response inhibition. Pharmacol Biochem Behav 123:45-54.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275.

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

Madruga C, Xavier LL, Achaval M, Sanvitto GL, Lucion AB (2006) Early handling, but not maternal separation, decreases emotional responses in two paradigms of fear without changes in mesolimbic dopamine. Behav Brain Res 166:241-246.

Marco EM, Valero M, de la Serna O, Aisa B, Borcel E, Ramirez MJ, Viveros MP (2013) Maternal deprivation effects on brain plasticity and recognition memory in adolescent male and female rats. Neuropharmacology 68:223-231.

Marrocco J, Mairesse J, Ngomba RT, Silletti V, Van Camp G, Bouwalerh H, Summa M, Pittaluga A, Nicoletti F, Maccari S, Morley-Fletcher S (2012) Anxiety-like behavior of prenatally stressed rats is associated with a selective reduction of glutamate release in the ventral hippocampus. J Neurosci 32:17143-17154.

McGaughy J, Ross RS, Eichenbaum H (2008) Noradrenergic, but not cholinergic, deafferentation of prefrontal cortex impairs attentional set-shifting. Neuroscience 153:63-71.

Neumann ID, Wigger A, Kromer S, Frank E, Landgraf R, Bosch OJ (2005) Differential effects of periodic maternal separation on adult stress coping in a rat model of extremes in trait anxiety. Neuroscience 132:867-877.

Noschang C, Krolow R, Arcego DM, Laureano D, Fitarelli LD, Huffell AP, Ferreira AG, da Cunha AA, Machado FR, Wyse AT, Dalmaz C (2012a) 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. Neurochem Res 37:1801-1810.

Noschang C, Krolow R, Arcego DM, Toniazzo AP, Huffell AP, Dalmaz C (2012b) Neonatal handling affects learning, reversal learning and antioxidant enzymes activities in a sex-specific manner in rats. Int J Dev Neurosci 30:285-291.

Noschang CG, Krolow R, Fontella FU, Arcego DM, Diehl LA, Weis SN, Arteni NS, Dalmaz C (2010) Neonatal handling impairs spatial memory and leads to altered nitric oxide production and DNA breaks in a sex specific manner. Neurochem Res 35:1083-1091.

Nyhus E, Barcelo F (2009) The Wisconsin Card Sorting Test and the cognitive assessment of prefrontal executive functions: a critical update. Brain Cogn 71:437-451.

Padoin MJ, Cadore LP, Gomes CM, Barros HM, Lucion AB (2001) Long-lasting effects of neonatal stimulation on the behavior of rats. Behav Neurosci 115:1332-1340.

Pandolfo P, Machado NJ, Kofalvi A, Takahashi RN, Cunha RA (2013) Caffeine regulates frontocorticostriatal dopamine transporter density and improves attention and cognitive deficits in an animal model of attention deficit hyperactivity disorder. Eur Neuropsychopharmacol 23:317-328.

Paxinos G, Watson C (1998) The Rat Brain in Stereotaxic Coordinates. San Diego: Academic Press.

Pires Tde O, Silva CM, Assis SG (2012) [Family environment and attention-deficit hyperactivity disorder]. Rev Saude Publica 46:624-632.
Portella AK, Silveira PP, Diehl LA, Crema LM, Clemente Z, Peres W, Costa G, Scorza C, Quillfeldt JA, Dalmaz C (2010) Early life handling decreases serotonin turnover in the nucleus accumbens and affects feeding behavior of adult rats. Dev Psychobiol 52:190-196.
Raineki C, De Souza MA, Szawka RE, Lutz ML, De Vasconcellos LF, Sanvitto GL, Izquierdo I, Bevilaqua LR, Cammarota M, Lucion AB (2009) Neonatal handling and the maternal odor preference in rat pups: involvement of monoamines and cyclic AMP response element-binding protein pathway in the olfactory bulb. Neuroscience 159:31-38.
Raineki C, Lucion AB, Weinberg J (2014) Neonatal handling: an overview of the positive and negative effects. Dev Psychobiol 56:1613-1625.
Robbins TW, Roberts AC (2007) Differential regulation of fronto-executive function by the monoamines and acetylcholine. Cereb Cortex 17 Suppl 1:i151-160.
Sakata K, Martinowich K, Woo NH, Schloesser RJ, Jimenez DV, Ji Y, Shen L, Lu B (2013) Role of activity-dependent BDNF expression in hippocampal-prefrontal cortical regulation of behavioral perseverance. Proc Natl Acad Sci U S A 110:15103-15108.
Schmitt U, Tanimoto N, Seeliger M, Schaeffel F, Leube RE (2009) Detection of behavioral alterations and learning deficits in mice lacking synaptophysin. Neuroscience 162:234-243.
Silveira PP, Portella AK, Assis SA, Nieto FB, Diehl LA, Crema LM, Peres W, Costa G, Scorza C, Quillfeldt JA, Lucion AB, Dalmaz C (2010) Early life experience alters behavioral responses to sweet food and accumbal dopamine metabolism. Int J Dev Neurosci 28:111-118.
Silveira PP, Portella AK, Benetti Cda S, Zugno AI, Scherer EB, Mattos CB, Wyse AT, Lucion AB, Dalmaz C (2011) Association between Na(+),K(+)-ATPase activity and the vulnerability/resilience to mood disorders induced by early life experience. Neurochem Res 36:2075-2082.
Skowron EA, Cipriano-Essel E, Gatzke-Kopp LM, Teti DM, Ammerman RT (2014) Early adversity, RSA, and inhibitory control: evidence of children's neurobiological sensitivity to social context. Dev Psychobiol 56:964-978.
Sun MK, Nelson TJ, Alkon DL (2015) Towards universal therapeutics for memory disorders. Trends Pharmacol Sci 36:384-394.
Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguez P, Doerks T, Stark M, Muller J, Bork P, Jensen LJ, von Mering C (2011) The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. Nucleic Acids Res 39:D561-568.
Tait DS, Chase EA, Brown VJ (2014) Attentional set-shifting in rodents: a review of behavioural methods and pharmacological results. Curr Pharm Des 20:5046-5059.
Thapar A, Cooper M, Eyre O, Langley K (2013) What have we learnt about the causes of ADHD? J Child Psychol Psychiatry 54:3-16.
Therien AG, Blostein R (2000) Mechanisms of sodium pump regulation. Am J Physiol Cell Physiol 279:C541-566.
Valentim LM, Geyer AB, Tavares A, Cimarosti H, Worm PV, Rodnight R, Netto CA, Salbego CG (2001) Effects of global cerebral ischemia and

preconditioning on heat shock protein 27 immunocontent and phosphorylation in rat hippocampus. Neuroscience 107:43-49.
Wain HM, Lush MJ, Ducluzeau F, Khodiyar VK, Povey S (2004) Genew: the Human Gene Nomenclature Database, 2004 updates. Nucleic Acids Res 32:D255-257.
Winter S, Dieckmann M, Schwabe K (2009) Dopamine in the prefrontal cortex regulates rats behavioral flexibility to changing reward value. Behav Brain Res 198:206-213.
Wright NF, Vann SD, Aggleton JP, Nelson AJ (2015) A critical role for the anterior thalamus in directing attention to task-relevant stimuli. J Neurosci 35:5480-5488.
Zhang W, Wang GM, Wang PJ, Zhang Q, Sha SH (2014) Effects of neural stem cells on synaptic proteins and memory in a mouse model of Alzheimer's disease. J Neurosci Res 92:185-194.

3.3 Capítulo III - Resultados Adicionais

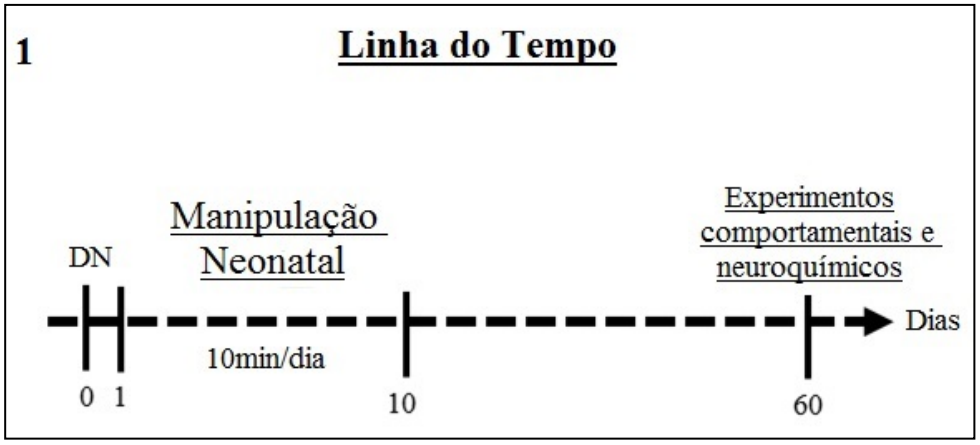

Figura 1. Linha do tempo dos experimentos. DN: Dia do nascimento dos animais, considerado o dia zero.

Teste de mudança de estratégia

Foi realizado o teste de mudança de estratégia, em animais fêmeas manipuladas no período neonatal, como visto na linha do tempo acima (Figura 1), para a sequência temporal de experimentos. Demonstrou-se ausência de diferenças estatísticas significantes tanto no primeiro dia (Figura 2A), nas discriminações das respostas (teste t de Student para amostras independentes, $t(13)= 0,338$; $P=0,741$), quanto no segundo dia (Fig. 2B), perante a dica sensório-visual (teste t de Student para amostras independentes, $t(13)= 0,855$; $P=0,408$). Da mesma maneira, não foram encontradas diferenças nos erros observados na figura 2B, (perseverativos, teste t de Student para amostras independentes, $t(13)= 0,459$; 0,653; regressivos $t(13)= 0,338$; $P=0,741$; nunca reforçados $t(13)= 1,933$; $P=0,075$); neste último, entretanto, observou-se uma tendência.

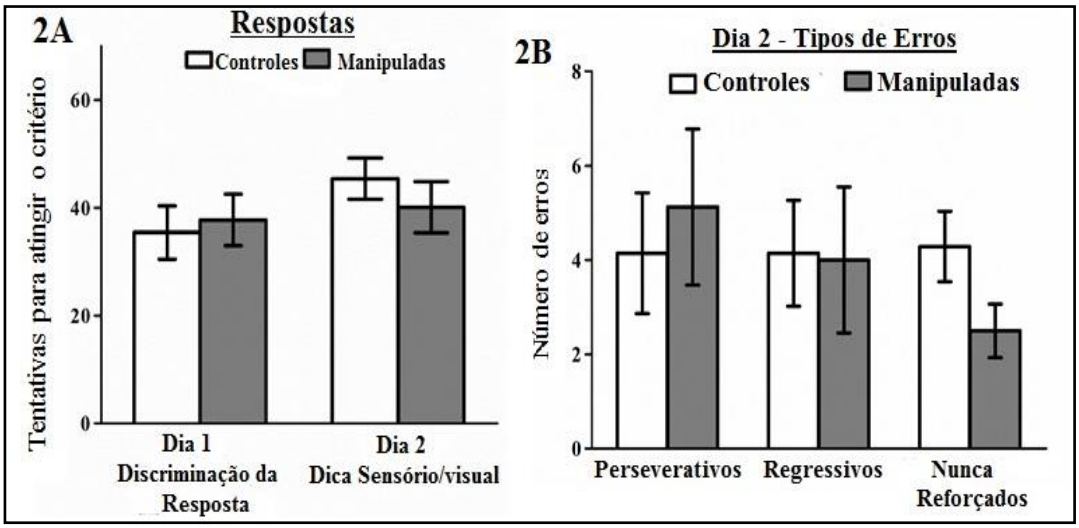

Figura 2. Efeito da manipulação neonatal no teste de mudança de estratégia. A) Tentativas para atingir o critério de nas respostas as fases de discriminação da resposta e com uso de dica sensório-visual, teste *t* de Student mostra *P*= 0,741 , e *P*= 0,408, respectivamente. B) Quantidade e tipos de erros, teste *t* de Student aponta, *P*=0,653 erros perseverativos, *P*=0,943, regressivos, *P*=0,075 e nunca reforçados. Nenhuma diferença foi encontrada nas fases do teste, Controles (N=7), Manipuladas (N=8).

Teste do deslocamento da atenção

No teste do deslocamento da atenção, foi observada diferença estatística significante apenas na segunda discriminação reversa (Mann-Whitney U=19,500, *P*=0,030, Z=-2,176), e uma tendência significativa na intradimensional (Mann-Whitney U=26,000, *P*=0,078, Z=-1,761). Entretanto as demais discriminações não mostraram significância (simples, Mann-Whitney U=45,000, *P*=1,000, Z=0,000; composta, Mann-Whitney U=37,500, *P*=0,457, Z=0,743; reverso 1, Mann-Whitney U=25,000, *P*=0,096, Z=-1,666; extradimensional, Mann-Whitney U=32,500, *P*=0,268, Z=-1,107; e a última fase de reverso, Mann-Whitney U=36,000, *P*=0,452, Z=-0,752) (Figura 3).

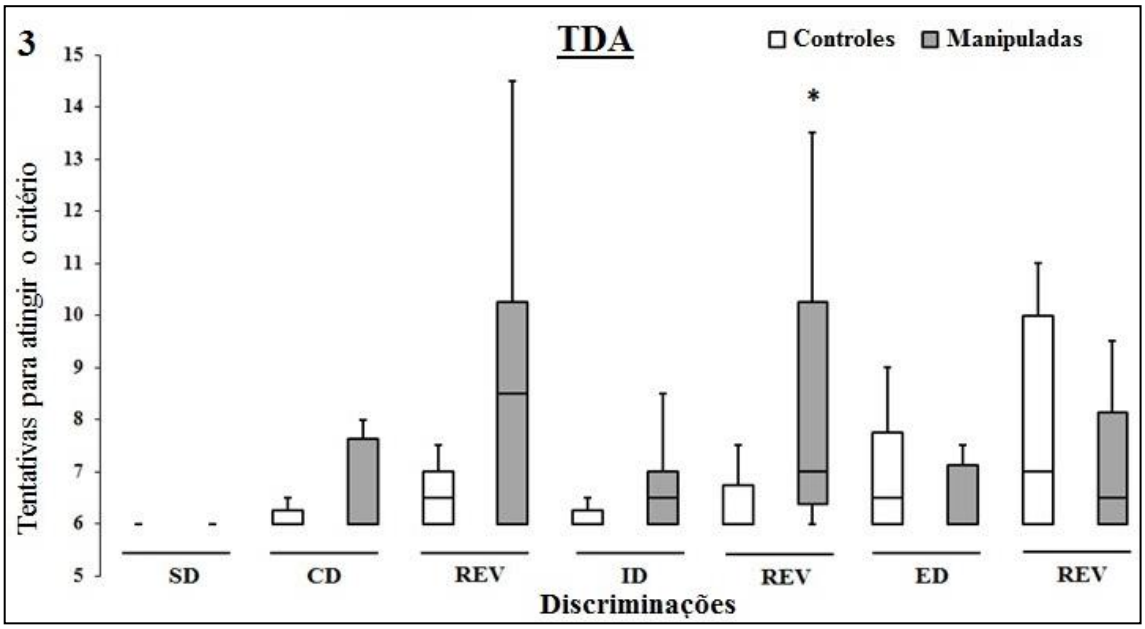

Figura 3. Efeito da manipulação neonatal no teste do deslocamento da atenção. Tentativas para atingir o critério de 6 consecutivas corretas. Houve diferença estatística significante na segunda discriminação reversa* (Mann-Whitney U=19,500, *P*=0,030, Z=-2,176). Controles (N=9), Manipuladas (N=10).

Atividade da enzima Na+/K+-ATPase

Realizou-se a atividade da enzima Na+/K+-ATPase na estrutura encefálica do CPFvm. Verificou-se diminuição desta atividade, no grupo manipulado no período neonatal comparado com o controle, (teste *t* de Student para amostras independentes, t(10)=3,557;P=0,05) (Figura 4).

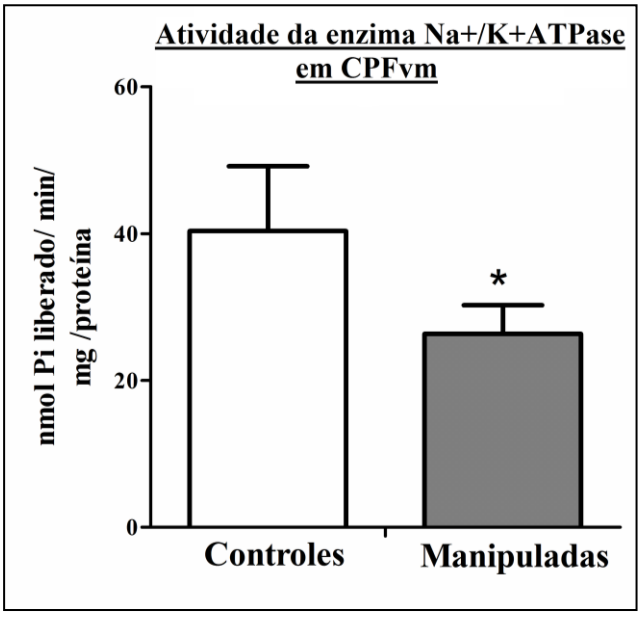

Figura 4. Atividade da enzima Na+/K+-ATPase em CPFvm. A manipulação neonatal diminuiu esta atividade, teste *t* de Student para amostras independentes P=0,05. Controles (N=6), Manipuladas

Esta tese teve como hipótese fundamental a possível influência da intervenção breve e precoce, realizada no início da vida, representada pelo procedimento de manipulação neonatal, em comportamentos executivos como a atenção, flexibilidade comportamental e a impulsividade.

São consideradas claras e evidentes as alterações comportamentais e neuroquímicas relacionadas à manipulação neonatal na literatura corrente. De autoria pioneira na descrição dos efeitos comportamentais deste modelo, Seymor Levine e colaboradores, na década de 50 (Levine, 1956, Levine et al., 1956), já apontavam que intervenções nesta fase da vida produziam fenótipos comportamentais diferenciados. Este autor demonstrou inicialmente que animais manipulados na idade neonatal tiveram melhor aprendizado e menor congelamento e defecação em condicionamento aversivo (que utilizara choque nas patas), propondo uma maior resiliência emocional a eventos estressores. Manipulações tardias breves (Levine, 1956), feitas aos 50 dias de vida, não produzem os mesmos efeitos, e se assemelham aos grupos controles. Esta diferença pode se dar pelo fato de que, no período neonatal, os ratos apresentam um período hiporrespossivo ao estresse (Yoshimura et al., 2003, Lupien et al., 2009, Raineki et al., 2014), e intervenções nesta fase deixam marcas duradouras no encéfalo desses animais (Raineki et al., 2014).

Após os trabalhos de Levine, vários autores publicaram relatos de alterações causadas pela MN. Uma das consequências preponderantes da MN na literatura, é a diminuição da emocionalidade na vida adulta, representada por diminuições do comportamento do tipo ansioso (Kosten et al., 2012), das respostas ao estresse (Meerlo et al., 1999) e do medo (Padoin et al., 2001). O comportamento de risco é aumentado

nos animais MN, isto é, expõem-se com maior frequência a ambientes abertos e claros em testes comportamentais como o campo aberto, com maior permanência no centro (Caldji et al., 2000), e o labirinto em cruz elevado, com maior permanência nos braços abertos desse aparato (Meerlo et al., 1999, Kiosterakis et al., 2009). Da mesma maneira, em presença de odores de predador (Padoin et al., 2001) ou na apresentação de um predador (Siviy and Harrison, 2008), os animais manipulados no período neonatal mostram redução no comportamento de avaliação de risco. Observa-se maior comportamento de risco em indivíduos impulsivos, uma característica comportamental que frequentemente é observada em usuários abusivos de drogas, esquizofrênicos, e pessoas com personalidade violenta ou suicida (Dumais et al., 2011). Os resultados acerca do comportamento inibitório, na presente tese, por meio do teste "tolerância ao atraso da recompensa", mostram maior impulsividade no grupo de animais machos e manipulados, com aumento da escolha da recompensa imediata (sem tempo de espera), embora em quantidade menor. Sabe-se que a impulsividade está envolvida em distúrbios alimentares envolvendo alta ingestão e na compulsão alimentar (Schag et al., 2013a, Schag et al., 2013b). Com base nisso, nota-se que a MN provoca características notáveis neste ponto: (i) aumento do consumo (Silveira et al., 2004), (ii) e em grandes quantidades de alimento doce (Froot Loops®), bem como (iii) diminuição da latência para obtê-lo (McIntosh et al., 1999, Silveira et al., 2004). Um estudo de Colorado et al. (2006) mediu a impulsividade na pré-puberdade por meio da velocidade de ambulação em campo aberto, entretanto a MN não demonstrou este comportamento. Todavia, percebe-se que os efeitos da MN citados acima (relacionados ao consumo de alimento) costumeiramente aparecem somente após a puberdade, na vida adulta (Silveira et al., 2006a).

A MN não altera o aprendizado e a memória em tarefas comportamentais que necessitem de vários dias de realização, como já relatado por Noschang acerca do labirinto aquático de Morris (Noschang et al., 2010) e em labirinto em Y (Noschang et al., 2012b).

Notavelmente, os resultados acerca da impulsividade em fêmeas indicam uma certa proteção relacionada ao sexo, mesmo quando a característica da manipulação neonatal está presente. Na literatura, nota-se que animais machos Wistar são mais impulsivos (Weafer and de Wit, 2014). Na literatura, o sexo feminino é menos impulsivo na infância (Cote et al., 2002), bem como na vida adulta, pois nos picos de fertilidade mulheres costumam ser menos impulsivas que homens, e até mesmo na escolha do parceiro reprodutivo, um modo de designar parceiros melhores evolutivamente (Hosseini-Kamkar and Morton, 2014). Este parâmetro pode ser associado à maior escolha da recompensa maior no estado basal e sob efeito do MPH. Este fármaco mostrou-se sem a ação esperada na fase do teste da tarefa TAR, de modo que o resultado parece ter atingido um efeito teto na tarefa. Em indivíduos humanos portadores de ADHD, distintos efeitos do MPH em relação ao sexo não são comuns (Mikami et al., 2009). Em nosso estudo, verificamos apenas em fêmeas níveis maiores de DOPAC no CPFvm, indicando um maior metabolismo de dopamina na estrutura, contudo este dado ainda necessita maiores investigações.

A ausência de efeito relacionado ao MPH na dose de 3mg/Kg em machos manipulados mostra que o estresse precoce pode alterar a resposta farmacológica a drogas e diminui transportadores de dopamina, como apresentado por Meaney (2002) usando separação neonatal, um modelo distinto daquele usado no presente trabalho. Nossos resultados estão de acordo com o estudo de Silveira et al. (2010), que também observaram falta de efeito do MPH em animais manipulados, que não mostram

diferenças no consumo de alimento após a administração da droga, enquanto os controles aumentam a ingestão de alimento. Esses dados corroboram o estudo de Brake (2004), que sugere que intervenções precoces podem alterar o padrão aditivo a psicoestimulantes.

Ainda a respeito dos resultados encontrados em fêmeas, nesta tese, observamos que aquelas que foram manipuladas mostraram um pior desempenho na tarefa de deslocamento da atenção, especificamente na discriminação reversa. Um estudo realizado por Lovic e Fleming (2004) corrobora com nossos resultados, e ainda demonstra outros efeitos nessa tarefa. Naquele estudo, fêmeas manipuladas por estimulação táctil de 60 segundos diários necessitam maior número de tentativas para atingir o critério em todas as discriminações reversas, na intradimensional, e na extradimensional. Nossos resultados na segunda discriminação reversa indicam uma perseveração nos erros, o que concorda com o trabalho descrito acima; este parâmetro, entretanto, não foi confirmado na tarefa de mudança de estratégia, que em seu protocolo também avalia esse tipo de erro.

Consideramos interessante aprofundar os estudos em fêmeas em relação à flexibilidade cognitiva e atenção: em estudos não relatados neste trabalho de tese, em que observou-se fêmeas MN que se tornaram mães, verificou-se que seu comportamento em relação à prole é desatento e negligente, e há distração durante o comportamento reprodutivo (Gonzalez et al., 2001), o que pode alterar a escolha do parceiro. Por outro lado, não observamos aumento da impulsividade em nosso trabalho.

Nesta mesma tarefa de TDA, os animais pertencentes ao grupo MN machos mostraram resultados distintos dos das fêmeas, com a maior quantidade de tentativas para atingir o critério de seis escolhas consecutivas corretas na discriminação simples e na intradimensional. Entretanto, na extradimensional, surpreendentemente a MN

determinou um melhor desempenho em relação aos controles. Este teste comportamental possui tanto a versão para roedores utilizada nesta tese (Birrell and Brown, 2000), quanto uma versão para humanos, determinada pela tarefa de sorteio de cartas de Wiscosin (Nyhus and Barcelo, 2009). Aos nos determos na avaliação das discriminações simples e mudanças intradimensionais, sabe-se que um novo aprendizado associativo ocorre, e o animal deve modificar seu comportamento em relação a um aprendizado anterior realizado por intermédio de recompensa. Desse modo o aprendizado associativo, a atenção ou ambos estão prejudicados no grupo MN de machos, fatores que promoveram pior desempenho nestas duas descriminações SD e ID, que não é relatado estar associado com flexibilidade cognitiva (Wright et al., 2015).

A formação de um "*attentional set*" ou cenário atencional (CA) na tarefa de TDA se dá a partir da importância que o animal dá às características de um objeto que estejam associadas a uma recompensa, e da irrelevância que este direciona a dimensões não recompensadas. Para o alcance do CA na TDA, há a necessidade do seguimento de uma categorização e/ou regras pelo animal, ao invés de um estímulo concreto. Na discriminação extradimensional, as contingências mudam, uma vez que a dimensão prévia relevante não mais é recompensada, e a atenção deve se deslocar à dimensão que previamente se mostrava irrelevante, e que agora passa a ser premiada. Logo, há necessidade de maior número de tentativas para a ED do que a ID, o que indica a formação de um cenário atencional (Robbins and Roberts, 2007, Tait et al., 2014).

No presente estudo, a ED mostrou ser mais difícil que a ID para os controles. Em animais MN, entretanto, não existiu diferença entre ED-ID, indicando que, não houve formação do cenário atencional nesses animais. Curiosamente, o grupo MN mostrou diferença no número de tentativas entre o último reverso e a ED, que não ocorreu nos controles. Uma possível explicação dos resultados é que o procedimento de

manipulação neonatal induz prejuízos em SD e ID, e na formação do cenário atencional, pois estes animais diminuem sua atenção ao estímulo relevante, talvez por aumento da atenção a características irrelevantes, possuindo dificuldades no aprendizado associativo. Essa hipótese também foi levantada em trabalho com resultados semelhantes, encontrados com lesões talâmicas (Wright et al., 2015).

Outra hipótese seria o prejuízo *per se* no aprendizado associativo para um estímulo ou um cenário. Cada discriminação na tarefa de TDA em que há a inserção de novos estímulos (SD, ID e ED, mas não CD e reversos) foi prejudicada nos animais. Essa possibilidade, contudo, não é corroborada por estudos da avaliação da memória olfatória (Noschang et al., 2012a) ou da memória de longo prazo no reconhecimento de objetos novos (Kosten et al., 2007). Há também uma ausência de efeitos da MN em parâmetros catecolaminérgicos, como já mostramos com dosagens de neurotransmissores e do imunoconteúdo da enzima tirosina hidroxilase e sua forma fosforilada (pSer40), sendo estes parâmetros essenciais para a formação do cenário atencional e o aprendizado associativo (Robbins and Roberts, 2007).

Nos resultados mostrados em ED, não se pode descartar alteração na flexibilidade cognitiva, entretanto, esta possibilidade pode não ser concreta, pois (i) não houve formação do cenário atencional no grupo MN; (ii) nenhum efeito foi relatado no teste de mudança de estratégia, em que o animal deve mudar sua estratégia egocêntrica para outra que usa dicas intralabirinto; (iii) as discriminações reversas não foram modificadas na TDA. É interessante observar que desempenhos prejudicados no aprendizado reverso já foram encontrados em MN na tarefa com labirinto em Y, porém não em labirinto aquático de Morris (Noschang et al., 2012b).

Em roedores, a atenção, flexibilidade cognitiva e impulsividade são mediadas pelo PFCvm (Cardinal et al., 2004, Floresco et al., 2006b). Nossos resultados em

machos demonstram diminuição nesta estrutura do imunoconteúdo de SYP, proteína que tem sido associada a TDAH em humanos (Brookes et al., 2005, Schmitt et al., 2009). Durante o desenvolvimento, há uma grande produção de sinapses, seguindo-se um período de poda destas (Glantz et al., 2007). Deste modo, fisiologicamente, há diminuição de SYP, paralelamente ao aprimoramento de comportamentos executivos (Glantz et al., 2007, Kolb et al., 2012). Nossos resultados indicam que a MN diminui ainda mais o imunoconteúdo de SYP. Níveis reduzidos desta proteína também foram descritos na puberdade no modelo de privação maternal durante 24h no dia pós-natal 9 (Marco et al., 2013). Adicionalmente, a transcrição da SYP é regulada pelo fator de transcrição silenciador R1 (REST) que é um silenciador neuronal (Hohl and Thiel, 2005), e este pode ser modulado pela manipulação neonatal, aumentando seus níveis no hipotálamo (Korosi et al., 2010) e por meio da separação materna de 3h diárias (PND 2-9), diminuindo-os em mCPF (Uchida et al., 2010), deste modo pode-se expecular que a diminuição de sinaptofisina neste trabalho pode estar relacionada com o fator REST, que é um silenciador neuronal. Tem sido sugerido que intervenções precoces podem determinar uma eliminação tardia de sinapses na adolescência, alterando o comportamento na vida adulta (Kolb et al., 2012). Animais deletados geneticamente (*knockout*) para a proteína SYP, possuem características comportamentais compartilhadas com a manipulação neonatal, como o aumento da exploração de ambientes novos e diminuição do medo (Padoin et al., 2001), e alterações em memória espacial (Schmitt et al., 2009, Noschang et al., 2010). Também observou-se que a MN altera a expressão de subunidade de receptores AMPA (Katsouli et al., 2014), o que podem estar relacionados aos níveis de SYP.

A manipulação por si só, independente de interação com o sexo, deixa marcas neuroquímicas importantes. Na atividade da enzima Na+/K+-ATPase, tanto machos

como fêmeas do grupo MN apresentaram diminuição da atividade desta enzima no CPFvm. Tem sido relatado que a MN altera a atividade da Na+/K+-ATPase de modo distinto quando consideramos diferentes estruturas encefálicas: em hipocampo, no estudo de Silveira et al. (2011), verificou-se diminuição dessa atividade, enquanto que na região da amígdala e do bulbo olfatório (Silveira et al., 2011, Noschang et al., 2012a), a atividade dessa enzima encontrou-se aumentada. Há indícios de que a inibição dessa enzima no córtex frontal resulte em alterações comportamentais semelhantes à mania (Kim et al., 2013). Podemos especular que a redução da atividade desta enzima se deu pela possível diminuição no número de sinapses, apesar de esta enzima ser encontrada ao longo de toda membrana neuronal.

No modelo "*in silico*", interações da SYP foram realizadas com sinaptobrevina-1 (VAMP1) e sinaptobrevina-2 (VAMP-2) nos pontos da via. Em ratos estressados no modelo de restrição de movimentos no período pré-natal, houve reduções de glutamato associadas a diminuição de proteínas vesiculares neuronais, como VAMP, sintaxina-1, e SYP na parte ventral do hipocampo (Marrocco et al., 2012). Fica a perspectiva de avaliar tais proteínas no modelo de MN.

A respeito da ausência de efeitos da MN no conteúdo da forma fosforilada da enzima tirosina hidroxilase (pTH), Bobrovskaya (2013) e colaboradores também não obtiveram diferenças neste parâmetro e na TH em glândulas adrenais de animais manipulados. Além disso, não observamos alterações neuroquímicas em catecolaminas em nosso trabalho em CPFvm. A MN, contudo, causa alteração nos níveis de dopamina e de seu metabolismo em núcleo accunbens (Silveira et al., 2010), de modo que não podemos descartar possíveis alterações desse neurotransmissor em outras regiões do CPF.

As causas que levam a estas consequências comportamentais na MN, não são totalmente explicadas, alguns estudos mostram que o comportamento da mãe frente a prole nesta fase é modificado. Estudos apontam ainda resultados contraditórios a esse respeito na literatura, Reis e colaboradores (2014) indicaram uma diminuição do comportamento maternal nesta fase, o que afeta animais MN de forma distinta em relação ao sexos, sendo os machos afetados por alterações em lambidas maternas e fêmeas pelo tempo que a mãe passa fora da ninhada. Entretanto o estudo de Stamatakis et al., (2015) mostra o contrário, um aumento do comportamento com diminuição da ansiedade materna. Com isso, verifica-se a importância de buscar uma correlação futura do comportamento materno diante da prole no modelo de MN.

Em resumo, as observações feitas nesta tese mostraram que as alterações dos comportamentos executivos causadas pela MN são sexo- e tarefa-específicas. Há prejuízo no controle inibitório em machos, bem como alterações em discriminações atencionais. Já nas fêmeas, vimos uma certa ação protetora da MN em termos comportamentais, pois o único parâmetro prejudicado encontrado foi a discriminação reversa. As alterações neuroquímicas merecem destaque pela interação do modelo de MN independente de sexo na enzima Na+/K+-ATPase, e estudos posteriores devem ser realizados acerca da proteína sinaptofisina em fêmeas. Acreditamos que mais estudos deverão ser realizados para a obtenção de um perfil comportamental completo relacionado a MN e cognição e suas possíveis modificações em outras estruturas encefálicas.

Deste modo, a pesquisa básica acerca da MN nos mostra a importância das interações entre mães e filhos durante o período neonatal, uma fase rica em modificações fisiológicas que provocam distintas programações encefálicas na vida futura.

5. CONCLUSÕES

Concluiu-se nesta tese que o modelo de manipulação neonatal altera cognitivamente os animais na vida adulta de forma distinta entre os sexos, sendo os machos mais acometidos por modificações em comportamentos executivos (diminuição da resposta ao MPH), prejuízo no aprendizado associativo e ausência do cenário atencional. Entretanto nas fêmeas a flexibilidade cognitiva mostrou-se alterada. Da mesma maneira, este procedimento deixa marcas neuroquímicas importantes no encéfalo dos animais. Entretanto, não se pode afirmar com certeza que estes prejuízos atuem de forma maléfica nestes animais, acredita-se que respostas contextuais a diferentes ambientes, *e.g.* ambiente enriquecido, possam nortear os resultados obtidos.

6. PERSPECTIVAS

Temos como perspectivas deste trabalho alguns tópicos relevantes, que irão elucidar o padrão comportamental por inteiro em relação à MN e devem futuramente ser analisados.

1. Avaliar a compulsão alimentar, relacionada à manipulação neonatal, uma vez que existem comprovadamente alterações alimentares nestes animais, bem como a impulsividade em machos;
2. Avaliar estruturas encefálicas que estão intimamente envolvidas com os parâmetros comportamentais analisados nesta tese, estriado, córtex órbito-frontal, e núcleo accumbens;
3. Nas estruturas encefálicas relacionadas no item anterior, buscar alterações em de aminas biogênicas, na proteína sinaptofisina e receptores de dopamina;

4. Realizar a avaliação do imunoconteúdo de SYP, TH e pTH em fêmeas;
5. Procurar correlacionar possíveis alterações no comportamento maternal neonatal com os resultados nesta tese obtidos;
6. Mensurar o fator de transcrição REST em animais manipulados e controles.
7. Mensurar as proteínas VAMP 1 e VAMP 2 nas estruturas encefálicas citadas.

Arai M, Widmaier EP (1993) Steroidogenesis in isolated adrenocortical cells during development in rats. Mol Cell Endocrinol 92:91-97.

Arborelius L, Eklund MB (2007) Both long and brief maternal separation produces persistent changes in tissue levels of brain monoamines in middle-aged female rats. Neuroscience 145:738-750.

Bari A, Robbins TW (2013) Inhibition and impulsivity: behavioral and neural basis of response control. Prog Neurobiol 108:44-79.

Barnett SA (1963) The Rat: A Study in Behavior.

Bartova A (1968) Functioning of the hypothalamo-pituitary-adrenal system during postnatal development in rats. Gen Comp Endocrinol 10:235-239.

Birney E, Andrews D, Caccamo M, Chen Y, Clarke L, Coates G, Cox T, Cunningham F, Curwen V, Cutts T, Down T, Durbin R, Fernandez-Suarez XM, Flicek P, Graf S, Hammond M, Herrero J, Howe K, Iyer V, Jekosch K, Kahari A, Kasprzyk A, Keefe D, Kokocinski F, Kulesha E, London D, Longden I, Melsopp C, Meidl P, Overduin B, Parker A, Proctor G, Prlic A, Rae M, Rios D, Redmond S, Schuster M, Sealy I, Searle S, Severin J, Slater G, Smedley D, Smith J, Stabenau A, Stalker J, Trevanion S, Ureta-Vidal A, Vogel J, White S, Woodwark C, Hubbard TJ (2006) Ensembl 2006. Nucleic Acids Res 34:D556-561.

Birrell JM, Brown VJ (2000) Medial frontal cortex mediates perceptual attentional set shifting in the rat. J Neurosci 20:4320-4324.

Bissonette GB, Powell EM, Roesch MR (2013) Neural structures underlying set-shifting: roles of medial prefrontal cortex and anterior cingulate cortex. Behav Brain Res 250:91-101.

Bizot JC, Chenault N, Houze B, Herpin A, David S, Pothion S, Trovero F (2007) Methylphenidate reduces impulsive behaviour in juvenile Wistar rats, but not in adult Wistar, SHR and WKY rats. Psychopharmacology (Berl) 193:215-223.

Bobrovskaya L, Maniam J, Ong LK, Dunkley PR, Morris MJ (2013) Early life stress and post-weaning high fat diet alter tyrosine hydroxylase regulation and AT1 receptor expression in the adrenal gland in a sex dependent manner. Neurochem Res 38:826-833.

Brake WG, Zhang TY, Diorio J, Meaney MJ, Gratton A (2004) Influence of early postnatal rearing conditions on mesocorticolimbic dopamine and behavioural responses to psychostimulants and stressors in adult rats. Eur J Neurosci 19:1863-1874.

Brookes K, Xu X, Chen W, Zhou K, Neale B, Lowe N, Anney R, Franke B, Gill M, Ebstein R, Buitelaar J, Sham P, Campbell D, Knight J, Andreou P, Altink M, Arnold R, Boer F, Buschgens C, Butler L, Christiansen H, Feldman L, Fleischman K, Fliers E, Howe-Forbes R, Goldfarb A, Heise A, Gabriels I, Korn-Lubetzki I, Johansson L, Marco R, Medad S, Minderaa R, Mulas F, Muller U, Mulligan A, Rabin K, Rommelse N, Sethna V, Sorohan J, Uebel H, Psychogiou L, Weeks A, Barrett R, Craig I, Banaschewski T, Sonuga-Barke E, Eisenberg J, Kuntsi J, Manor I, McGuffin P, Miranda A, Oades RD, Plomin R, Roeyers H, Rothenberger A, Sergeant J, Steinhausen HC, Taylor E, Thompson M, Faraone SV, Asherson P (2006) The analysis of 51 genes in DSM-IV combined type attention deficit hyperactivity disorder: association signals in DRD4, DAT1 and 16 other genes. Mol Psychiatry 11:934-953.

Brookes KJ, Knight J, Xu X, Asherson P (2005) DNA pooling analysis of ADHD and genes regulating vesicle release of neurotransmitters. Am J Med Genet B Neuropsychiatr Genet 139B:33-37.

Caldji C, Francis D, Sharma S, Plotsky PM, Meaney MJ (2000) The effects of early rearing environment on the development of GABAA and central benzodiazepine receptor levels and novelty-induced fearfulness in the rat. Neuropsychopharmacology 22:219-229.

Callahan PM, Terry AV, Jr. (2015) Attention. Handb Exp Pharmacol 228:161-189.

Cardinal RN, Winstanley CA, Robbins TW, Everitt BJ (2004) Limbic corticostriatal systems and delayed reinforcement. Ann N Y Acad Sci 1021:33-50.

Carlisi CO, Chantiluke K, Norman L, Christakou A, Barrett N, Giampietro V, Brammer M, Simmons A, Rubia K (2016) The effects of acute fluoxetine administration on temporal discounting in youth with ADHD. Psychol Med 46:1197-1209.

Carobrez AP, Bertoglio LJ (2005) Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. Neurosci Biobehav Rev 29:1193-1205.

Champagne F, Meaney MJ (2001) Like mother, like daughter: evidence for non-genomic transmission of parental behavior and stress responsivity. Prog Brain Res 133:287-302.

Chan KM, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca2+ -stimulated ATPase activity. Anal Biochem 157:375-380.

Cirulli F, Berry A, Alleva E (2003) Early disruption of the mother-infant relationship: effects on brain plasticity and implications for psychopathology. Neurosci Biobehav Rev 27:73-82.

Colorado RA, Shumake J, Conejo NM, Gonzalez-Pardo H, Gonzalez-Lima F (2006) Effects of maternal separation, early handling, and standard facility rearing on orienting and impulsive behavior of adolescent rats. Behav Processes 71:51-58.

Constantinof A, Moisiadis VG, Matthews SG (2015) Programming of stress pathways: A transgenerational perspective. J Steroid Biochem Mol Biol.

Cote S, Tremblay RE, Nagin D, Zoccolillo M, Vitaro F (2002) The development of impulsivity, fearfulness, and helpfulness during childhood: patterns of consistency and change in the trajectories of boys and girls. J Child Psychol Psychiatry 43:609-618.

da SBC, Silveira PP, Matte C, Stefanello FM, Leite MC, Goncalves CA, Wyse AT, Dalmaz C, Goldani MZ (2010) Effects of a chronic exposure to a highly palatable diet and its withdrawal, in adulthood, on cerebral Na+,K+-ATPase and plasma S100B in neonatally handled rats. Int J Dev Neurosci 28:153-159.

Dajani DR, Uddin LQ (2015) Demystifying cognitive flexibility: Implications for clinical and developmental neuroscience. Trends Neurosci 38:571-578.

Dalley JW, Cardinal RN, Robbins TW (2004) Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates. Neurosci Biobehav Rev 28:771-784.

Damasio H, Grabowski T, Frank R, Galaburda AM, Damasio AR (1994) The return of Phineas Gage: clues about the brain from the skull of a famous patient. Science 264:1102-1105.

de Souza Wyse AT, Streck EL, Worm P, Wajner A, Ritter F, Netto CA (2000) Preconditioning prevents the inhibition of Na+,K+-ATPase activity after brain ischemia. Neurochem Res 25:971-975.

Donnelly NA, Holtzman T, Rich PD, Nevado-Holgado AJ, Fernando AB, Van Dijck G, Holzhammer T, Paul O, Ruther P, Paulsen O, Robbins TW, Dalley JW (2014)

Oscillatory activity in the medial prefrontal cortex and nucleus accumbens correlates with impulsivity and reward outcome. PLoS One 9:e111300.
Dumais A, Potvin S, Joyal C, Allaire JF, Stip E, Lesage A, Gobbi G, Cote G (2011) Schizophrenia and serious violence: a clinical-profile analysis incorporating impulsivity and substance-use disorders. Schizophr Res 130:234-237.
Elliott R (2003) Executive functions and their disorders. Br Med Bull 65:49-59.
Elton A, Tripathi SP, Mletzko T, Young J, Cisler JM, James GA, Kilts CD (2014) Childhood maltreatment is associated with a sex-dependent functional reorganization of a brain inhibitory control network. Hum Brain Mapp 35:1654-1667.
Fleitlich B, Goodman R (2001) Social factors associated with child mental health problems in Brazil: cross sectional survey. BMJ 323:599-600.
Floresco SB, Ghods-Sharifi S, Vexelman C, Magyar O (2006a) Dissociable roles for the nucleus accumbens core and shell in regulating set shifting. J Neurosci 26:2449-2457.
Floresco SB, Magyar O, Ghods-Sharifi S, Vexelman C, Tse MT (2006b) Multiple dopamine receptor subtypes in the medial prefrontal cortex of the rat regulate set-shifting. Neuropsychopharmacology 31:297-309.
Froehlich TE, Anixt JS, Loe IM, Chirdkiatgumchai V, Kuan L, Gilman RC (2011) Update on environmental risk factors for attention-deficit/hyperactivity disorder. Curr Psychiatry Rep 13:333-344.
Fuster JM (2002) Frontal lobe and cognitive development. J Neurocytol 31:373-385.
Garcia-Molina A (2012) [Phineas Gage and the enigma of the prefrontal cortex]. Neurologia 27:370-375.
Garoflos E, Panagiotaropoulos T, Pondiki S, Stamatakis A, Philippidis E, Stylianopoulou F (2005a) Cellular mechanisms underlying the effects of an early experience on cognitive abilities and affective states. Ann Gen Psychiatry 4:8.
Garoflos E, Stamatakis A, Mantelas A, Philippidis H, Stylianopoulou F (2005b) Cellular mechanisms underlying an effect of "early handling" on pCREB and BDNF in the neonatal rat hippocampus. Brain Res 1052:187-195.
Garza KB, Ding M, Owensby JK, Zizza CA (2016) Impulsivity and Fast-Food Consumption: A Cross-Sectional Study among Working Adults. J Acad Nutr Diet 116:61-68.
Gilman. G (2010) Goodman & Gilman. As Bases Farmacológicas da Terapêutica.
Glantz LA, Gilmore JH, Hamer RM, Lieberman JA, Jarskog LF (2007) Synaptophysin and postsynaptic density protein 95 in the human prefrontal cortex from mid-gestation into early adulthood. Neuroscience 149:582-591.
Gonzalez A, Lovic V, Ward GR, Wainwright PE, Fleming AS (2001) Intergenerational effects of complete maternal deprivation and replacement stimulation on maternal behavior and emotionality in female rats. Dev Psychobiol 38:11-32.
Grant DA, Berg EA (1948) A behavioral analysis of degree of reinforcement and ease of shifting to new responses in a Weigl-type card-sorting problem. J Exp Psychol 38:404-411.
Guan L, Wang B, Chen Y, Yang L, Li J, Qian Q, Wang Z, Faraone SV, Wang Y (2009) A high-density single-nucleotide polymorphism screen of 23 candidate genes in attention deficit hyperactivity disorder: suggesting multiple susceptibility genes among Chinese Han population. Mol Psychiatry 14:546-554.
Hahn ME, Lavooy MJ (2005) A review of the methods of studies on infant ultrasound production and maternal retrieval in small rodents. Behav Genet 35:31-52.

Halasz I, Rittenhouse PA, Zorrilla EP, Redei E (1997) Sexually dimorphic effects of maternal adrenalectomy on hypothalamic corticotrophin-releasing factor, glucocorticoid receptor and anterior pituitary POMC mRNA levels in rat neonates. Brain Res Dev Brain Res 100:198-204.

Haltmeyer GC, Denenberg VH, Thatcher J, Zarrow MX (1966) Response of the adrenal cortex of the neonatal rat after subjection to stress. Nature 212:1371-1373.

Hohl M, Thiel G (2005) Cell type-specific regulation of RE-1 silencing transcription factor (REST) target genes. Eur J Neurosci 22:2216-2230.

Hosseini-Kamkar N, Morton JB (2014) Sex differences in self-regulation: an evolutionary perspective. Front Neurosci 8:233.

John OP, Caspi A, Robins RW, Moffitt TE, Stouthamer-Loeber M (1994) The "little five": exploring the nomological network of the five-factor model of personality in adolescent boys. Child Dev 65:160-178.

Kandel E (2015) Princípios de Neurociências.: Mc Gill.

Katsouli S, Stamatakis A, Giompres P, Kouvelas ED, Stylianopoulou F, Mitsacos A (2014) Sexually dimorphic long-term effects of an early life experience on AMPA receptor subunit expression in rat brain. Neuroscience 257:49-64.

Kehagia AA, Murray GK, Robbins TW (2010) Learning and cognitive flexibility: frontostriatal function and monoaminergic modulation. Curr Opin Neurobiol 20:199-204.

Kesner RP, Churchwell JC (2011) An analysis of rat prefrontal cortex in mediating executive function. Neurobiol Learn Mem 96:417-431.

Kim SH, Yu HS, Park HG, Ha K, Kim YS, Shin SY, Ahn YM (2013) Intracerebroventricular administration of ouabain, a Na/K-ATPase inhibitor, activates mTOR signal pathways and protein translation in the rat frontal cortex. Prog Neuropsychopharmacol Biol Psychiatry 45:73-82.

Kiosterakis G, Stamatakis A, Diamantopoulou A, Fameli M, Stylianopoulou F (2009) Long-term effects of neonatal handling on mu-opioid receptor levels in the brain of the offspring. Dev Psychobiol 51:439-449.

Kolb B, Mychasiuk R, Muhammad A, Li Y, Frost DO, Gibb R (2012) Experience and the developing prefrontal cortex. Proc Natl Acad Sci U S A 109 Suppl 2:17186-17193.

Korosi A, Shanabrough M, McClelland S, Liu ZW, Borok E, Gao XB, Horvath TL, Baram TZ (2010) Early-life experience reduces excitation to stress-responsive hypothalamic neurons and reprograms the expression of corticotropin-releasing hormone. J Neurosci 30:703-713.

Kosten TA, Karanian DA, Yeh J, Haile CN, Kim JJ, Kehoe P, Bahr BA (2007) Memory impairments and hippocampal modifications in adult rats with neonatal isolation stress experience. Neurobiol Learn Mem 88:167-176.

Kosten TA, Kim JJ, Lee HJ (2012) Early life manipulations alter learning and memory in rats. Neurosci Biobehav Rev 36:1985-2006.

Kosten TA, Miserendino MJ, Kehoe P (2000) Enhanced acquisition of cocaine self-administration in adult rats with neonatal isolation stress experience. Brain Res 875:44-50.

Latimer K, Wilson P, Kemp J, Thompson L, Sim F, Gillberg C, Puckering C, Minnis H (2012) Disruptive behaviour disorders: a systematic review of environmental antenatal and early years risk factors. Child Care Health Dev 38:611-628.

Lazzaretti C, Kincheski GC, Pandolfo P, Krolow R, Toniazzo AP, Arcego DM, Couto-Pereira NS, Zeidan-Chulia F, Galvalisi M, Costa G, Scorza C, Souza TM, Dalmaz C (2015) Neonatal handling causes impulsive behavior and decreased

pharmacological response to methylphenidate in male adult wistar rats. J Integr Neurosci 1650004.
Levine S (1956) A further study of infantile handling and adult avoidance learning. J Pers 25:70-80.
Levine S (1957) Infantile experience and resistance to physiological stress. Science 126:405.
Levine S, Alpert M, Lewis GW (1957) Infantile experience and the maturation of the pituitary adrenal axis. Science 126:1347.
Levine S, Chevalier JA, Korchin SJ (1956) The effects of early shock and handling on later avoidance learning. J Pers 24:475-493.
Liu L, Chen Y, Li H, Qian Q, Yang L, Glatt SJ, Faraone SV, Wang Y (2013) Association between SYP with attention-deficit/hyperactivity disorder in Chinese Han subjects: differences among subtypes and genders. Psychiatry Res 210:308-314.
Logue SF, Gould TJ (2014) The neural and genetic basis of executive function: attention, cognitive flexibility, and response inhibition. Pharmacol Biochem Behav 123:45-54.
Lovic V, Fleming AS (2004) Artificially-reared female rats show reduced prepulse inhibition and deficits in the attentional set shifting task--reversal of effects with maternal-like licking stimulation. Behav Brain Res 148:209-219.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275.
Luengo MA, Carrillo-de-la-Pena MT, Otero JM, Romero E (1994) A short-term longitudinal study of impulsivity and antisocial behavior. J Pers Soc Psychol 66:542-548.
Lukkes JL, Thompson BS, Freund N, Andersen SL (2015) The developmental inter-relationships between activity, novelty preferences, and delay discounting in male and female rats. Dev Psychobiol.
Lupien SJ, McEwen BS, Gunnar MR, Heim C (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci 10:434-445.
Luria A (1969) Frontal lobe syndromes. New York: Elsevier.
Macri S, Mason GJ, Wurbel H (2004) Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats. Eur J Neurosci 20:1017-1024.
Madruga C, Xavier LL, Achaval M, Sanvitto GL, Lucion AB (2006) Early handling, but not maternal separation, decreases emotional responses in two paradigms of fear without changes in mesolimbic dopamine. Behav Brain Res 166:241-246.
Marco EM, Valero M, de la Serna O, Aisa B, Borcel E, Ramirez MJ, Viveros MP (2013) Maternal deprivation effects on brain plasticity and recognition memory in adolescent male and female rats. Neuropharmacology 68:223-231.
Marrocco J, Mairesse J, Ngomba RT, Silletti V, Van Camp G, Bouwalerh H, Summa M, Pittaluga A, Nicoletti F, Maccari S, Morley-Fletcher S (2012) Anxiety-like behavior of prenatally stressed rats is associated with a selective reduction of glutamate release in the ventral hippocampus. J Neurosci 32:17143-17154.
McGaughy J, Ross RS, Eichenbaum H (2008) Noradrenergic, but not cholinergic, deafferentation of prefrontal cortex impairs attentional set-shifting. Neuroscience 153:63-71.
McIntosh J, Anisman H, Merali Z (1999) Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: gender-dependent effects. Brain Res Dev Brain Res 113:97-106.

Meaney MJ, Aitken DH, Bodnoff SR, Iny LJ, Tatarewicz JE, Sapolsky RM (1985) Early postnatal handling alters glucocorticoid receptor concentrations in selected brain regions. Behav Neurosci 99:765-770.

Meaney MJ, Bhatnagar S, Larocque S, McCormick C, Shanks N, Sharma S, Smythe J, Viau V, Plotsky PM (1993) Individual differences in the hypothalamic-pituitary-adrenal stress response and the hypothalamic CRF system. Ann N Y Acad Sci 697:70-85.

Meaney MJ, Brake W, Gratton A (2002) Environmental regulation of the development of mesolimbic dopamine systems: a neurobiological mechanism for vulnerability to drug abuse? Psychoneuroendocrinology 27:127-138.

Meerlo P, Horvath KM, Nagy GM, Bohus B, Koolhaas JM (1999) The influence of postnatal handling on adult neuroendocrine and behavioural stress reactivity. J Neuroendocrinol 11:925-933.

Mehta MA, Goodyer IM, Sahakian BJ (2004) Methylphenidate improves working memory and set-shifting in AD/HD: relationships to baseline memory capacity. J Child Psychol Psychiatry 45:293-305.

Mikami AY, Cox DJ, Davis MT, Wilson HK, Merkel RL, Burket R (2009) Sex differences in effectiveness of extended-release stimulant medication among adolescents with attention-deficit/hyperactivity disorder. J Clin Psychol Med Settings 16:233-242.

Myers B, McKlveen JM, Herman JP (2012) Neural Regulation of the Stress Response: The Many Faces of Feedback. Cell Mol Neurobiol.

Neumann ID, Wigger A, Kromer S, Frank E, Landgraf R, Bosch OJ (2005) Differential effects of periodic maternal separation on adult stress coping in a rat model of extremes in trait anxiety. Neuroscience 132:867-877.

Noschang C, Krolow R, Arcego DM, Laureano D, Fitarelli LD, Huffell AP, Ferreira AG, da Cunha AA, Machado FR, Wyse AT, Dalmaz C (2012a) 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. Neurochem Res 37:1801-1810.

Noschang C, Krolow R, Arcego DM, Toniazzo AP, Huffell AP, Dalmaz C (2012b) Neonatal handling affects learning, reversal learning and antioxidant enzymes activities in a sex-specific manner in rats. Int J Dev Neurosci 30:285-291.

Noschang CG, Krolow R, Fontella FU, Arcego DM, Diehl LA, Weis SN, Arteni NS, Dalmaz C (2010) Neonatal handling impairs spatial memory and leads to altered nitric oxide production and DNA breaks in a sex specific manner. Neurochem Res 35:1083-1091.

Nyhus E, Barcelo F (2009) The Wisconsin Card Sorting Test and the cognitive assessment of prefrontal executive functions: a critical update. Brain Cogn 71:437-451.

O'Donnell KA, Gaudreau H, Colalillo S, Steiner M, Atkinson L, Moss E, Goldberg S, Karama S, Matthews SG, Lydon JE, Silveira PP, Wazana AD, Levitan RD, Sokolowski MB, Kennedy JL, Fleming A, Meaney MJ, Team MR (2014) The maternal adversity, vulnerability and neurodevelopment project: theory and methodology. Can J Psychiatry 59:497-508.

Obradovic J, Boyce WT (2009) Individual differences in behavioral, physiological, and genetic sensitivities to contexts: implications for development and adaptation. Dev Neurosci 31:300-308.

Odum AL (2011) Delay discounting: I'm a k, you're a k. J Exp Anal Behav 96:427-439.

Padoin MJ, Cadore LP, Gomes CM, Barros HM, Lucion AB (2001) Long-lasting effects of neonatal stimulation on the behavior of rats. Behav Neurosci 115:1332-1340.

Pandolfo P, Machado NJ, Kofalvi A, Takahashi RN, Cunha RA (2013) Caffeine regulates frontocorticostriatal dopamine transporter density and improves attention and cognitive deficits in an animal model of attention deficit hyperactivity disorder. Eur Neuropsychopharmacol 23:317-328.

Patel S, Stolerman IP, Asherson P, Sluyter F (2006) Attentional performance of C57BL/6 and DBA/2 mice in the 5-choice serial reaction time task. Behav Brain Res 170:197-203.

Paxinos G, Watson C (1998) The Rat Brain in Stereotaxic Coordinates. San Diego: Academic Press.

Petanjek Z, Judas M, Simic G, Rasin MR, Uylings HB, Rakic P, Kostovic I (2011) Extraordinary neoteny of synaptic spines in the human prefrontal cortex. Proc Natl Acad Sci U S A 108:13281-13286.

Pires Tde O, Silva CM, Assis SG (2012) [Family environment and attention-deficit hyperactivity disorder]. Rev Saude Publica 46:624-632.

Portella AK, Silveira PP, Diehl LA, Crema LM, Clemente Z, Peres W, Costa G, Scorza C, Quillfeldt JA, Dalmaz C (2010) Early life handling decreases serotonin turnover in the nucleus accumbens and affects feeding behavior of adult rats. Dev Psychobiol 52:190-196.

Puig MV, Gulledge AT, Lambe EK, Gonzalez-Burgos G (2015) Editorial: Neuromodulation of executive circuits. Front Neural Circuits 9:58.

Raineki C, De Souza MA, Szawka RE, Lutz ML, De Vasconcellos LF, Sanvitto GL, Izquierdo I, Bevilaqua LR, Cammarota M, Lucion AB (2009) Neonatal handling and the maternal odor preference in rat pups: involvement of monoamines and cyclic AMP response element-binding protein pathway in the olfactory bulb. Neuroscience 159:31-38.

Raineki C, Lucion AB, Weinberg J (2014) Neonatal handling: an overview of the positive and negative effects. Dev Psychobiol 56:1613-1625.

Reis AR, de Azevedo MS, de Souza MA, Lutz ML, Alves MB, Izquierdo I, Cammarota M, Silveira PP, Lucion AB (2014) Neonatal handling alters the structure of maternal behavior and affects mother-pup bonding. Behav Brain Res 265:216-228.

Robbins TW, Roberts AC (2007) Differential regulation of fronto-executive function by the monoamines and acetylcholine. Cereb Cortex 17 Suppl 1:i151-160.

Rossi AF, Pessoa L, Desimone R, Ungerleider LG (2009) The prefrontal cortex and the executive control of attention. Exp Brain Res 192:489-497.

Sakata K, Martinowich K, Woo NH, Schloesser RJ, Jimenez DV, Ji Y, Shen L, Lu B (2013) Role of activity-dependent BDNF expression in hippocampal-prefrontal cortical regulation of behavioral perseverance. Proc Natl Acad Sci U S A 110:15103-15108.

Sapolsky RM, Meaney MJ (1986) Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. Brain Res 396:64-76.

Schag K, Schonleber J, Teufel M, Zipfel S, Giel KE (2013a) Food-related impulsivity in obesity and binge eating disorder--a systematic review. Obes Rev 14:477-495.

Schag K, Teufel M, Junne F, Preissl H, Hautzinger M, Zipfel S, Giel KE (2013b) Impulsivity in binge eating disorder: food cues elicit increased reward responses and disinhibition. PLoS One 8:e76542.

Schmitt U, Tanimoto N, Seeliger M, Schaeffel F, Leube RE (2009) Detection of behavioral alterations and learning deficits in mice lacking synaptophysin. Neuroscience 162:234-243.

Semenova S, Stolerman IP, Markou A (2007) Chronic nicotine administration improves attention while nicotine withdrawal induces performance deficits in the 5-choice serial reaction time task in rats. Pharmacol Biochem Behav 87:360-368.

Silberman DM, Acosta GB, Zorrilla Zubilete MA (2016) Long-term effects of early life stress exposure: Role of epigenetic mechanisms. Pharmacol Res.

Silveira PP, Cognato G, Crema LM, Pederiva FQ, Bonan CD, Sarkis JJ, Lucion AB, Dalmaz C (2006a) Neonatal handling, sweet food ingestion and ectonucleotidase activities in nucleus accumbens at different ages. Neurochem Res 31:693-698.

Silveira PP, da Silva Benetti C, Ayres C, Pederiva FQ, Portella AK, Lucion AB, Dalmaz C (2006b) Satiety assessment in neonatally handled rats. Behav Brain Res 173:205-210.

Silveira PP, Portella AK, Assis SA, Nieto FB, Diehl LA, Crema LM, Peres W, Costa G, Scorza C, Quillfeldt JA, Lucion AB, Dalmaz C (2010) Early life experience alters behavioral responses to sweet food and accumbal dopamine metabolism. Int J Dev Neurosci 28:111-118.

Silveira PP, Portella AK, Benetti Cda S, Zugno AI, Scherer EB, Mattos CB, Wyse AT, Lucion AB, Dalmaz C (2011) Association between Na(+),K(+)-ATPase activity and the vulnerability/resilience to mood disorders induced by early life experience. Neurochem Res 36:2075-2082.

Silveira PP, Portella AK, Clemente Z, Bassani E, Tabajara AS, Gamaro GD, Dantas G, Torres IL, Lucion AB, Dalmaz C (2004) Neonatal handling alters feeding behavior of adult rats. Physiol Behav 80:739-745.

Silveira PP, Portella AK, Crema L, Correa M, Nieto FB, Diehl L, Lucion AB, Dalmaz C (2008) Both infantile stimulation and exposure to sweet food lead to an increased sweet food ingestion in adult life. Physiol Behav 93:877-882.

Siviy SM, Harrison KA (2008) Effects of neonatal handling on play behavior and fear towards a predator odor in juvenile rats (Rattus norvegicus). J Comp Psychol 122:1-8.

Skowron EA, Cipriano-Essel E, Gatzke-Kopp LM, Teti DM, Ammerman RT (2014) Early adversity, RSA, and inhibitory control: evidence of children's neurobiological sensitivity to social context. Dev Psychobiol 56:964-978.

Stamatakis A, Kalpachidou T, Raftogianni A, Zografou E, Tzanou A, Pondiki S, Stylianopoulou F (2015) Rat dams exposed repeatedly to a daily brief separation from the pups exhibit increased maternal behavior, decreased anxiety and altered levels of receptors for estrogens (ERalpha, ERbeta), oxytocin and serotonin (5-HT1A) in their brain. Psychoneuroendocrinology 52:212-228.

Sun MK, Nelson TJ, Alkon DL (2015) Towards universal therapeutics for memory disorders. Trends Pharmacol Sci 36:384-394.

Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguez P, Doerks T, Stark M, Muller J, Bork P, Jensen LJ, von Mering C (2011) The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. Nucleic Acids Res 39:D561-568.

Tait DS, Chase EA, Brown VJ (2014) Attentional set-shifting in rodents: a review of behavioural methods and pharmacological results. Curr Pharm Des 20:5046-5059.

Thapar A, Cooper M, Eyre O, Langley K (2013) What have we learnt about the causes of ADHD? J Child Psychol Psychiatry 54:3-16.

Therien AG, Blostein R (2000) Mechanisms of sodium pump regulation. Am J Physiol Cell Physiol 279:C541-566.

Thiebaut de Schotten M, Dell'Acqua F, Ratiu P, Leslie A, Howells H, Cabanis E, Iba-Zizen MT, Plaisant O, Simmons A, Dronkers NF, Corkin S, Catani M (2015) From Phineas Gage and Monsieur Leborgne to H.M.: Revisiting Disconnection Syndromes. Cereb Cortex 25:4812-4827.

Uchida S, Hara K, Kobayashi A, Funato H, Hobara T, Otsuki K, Yamagata H, McEwen BS, Watanabe Y (2010) Early life stress enhances behavioral vulnerability to stress through the activation of REST4-mediated gene transcription in the medial prefrontal cortex of rodents. J Neurosci 30:15007-15018.

Ulrich-Lai YM, Herman JP (2009) Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci 10:397-409.

Valentim LM, Geyer AB, Tavares A, Cimarosti H, Worm PV, Rodnight R, Netto CA, Salbego CG (2001) Effects of global cerebral ischemia and preconditioning on heat shock protein 27 immunocontent and phosphorylation in rat hippocampus. Neuroscience 107:43-49.

Vallee M, MacCari S, Dellu F, Simon H, Le Moal M, Mayo W (1999) Long-term effects of prenatal stress and postnatal handling on age-related glucocorticoid secretion and cognitive performance: a longitudinal study in the rat. Eur J Neurosci 11:2906-2916.

Valtorta F, Pennuto M, Bonanomi D, Benfenati F (2004) Synaptophysin: leading actor or walk-on role in synaptic vesicle exocytosis? Bioessays 26:445-453.

Wain HM, Lush MJ, Ducluzeau F, Khodiyar VK, Povey S (2004) Genew: the Human Gene Nomenclature Database, 2004 updates. Nucleic Acids Res 32:D255-257.

Weafer J, de Wit H (2014) Sex differences in impulsive action and impulsive choice. Addict Behav 39:1573-1579.

Weaver IC (2014) Integrating early life experience, gene expression, brain development, and emergent phenotypes: unraveling the thread of nature via nurture. Adv Genet 86:277-307.

Wilson MJ, Vassileva J (2016) Neurocognitive and psychiatric dimensions of hot, but not cool, impulsivity predict HIV sexual risk behaviors among drug users in protracted abstinence. Am J Drug Alcohol Abuse 1-11.

Winter S, Dieckmann M, Schwabe K (2009) Dopamine in the prefrontal cortex regulates rats behavioral flexibility to changing reward value. Behav Brain Res 198:206-213.

Wright NF, Vann SD, Aggleton JP, Nelson AJ (2015) A critical role for the anterior thalamus in directing attention to task-relevant stimuli. J Neurosci 35:5480-5488.

Yeomans MR, Brace A (2015) Cued to Act on Impulse: More Impulsive Choice and Risky Decision Making by Women Susceptible to Overeating after Exposure to Food Stimuli. PLoS One 10:e0137626.

Yoshimura S, Sakamoto S, Kudo H, Sassa S, Kumai A, Okamoto R (2003) Sex-differences in adrenocortical responsiveness during development in rats. Steroids 68:439-445.

Zhang W, Wang GM, Wang PJ, Zhang Q, Sha SH (2014) Effects of neural stem cells on synaptic proteins and memory in a mouse model of Alzheimer's disease. J Neurosci Res 92:185-194.

8.1 Carta de aprovação do comitê de ética em pesquisa

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

PROPESQ

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 21460
Título: Manipulação neonatal: avaliação de parâmetros comportamentais e neuroquímicos relacionados à atenção na idade adulta

Pesquisadores:

Equipe UFRGS:

CARLA DALMAZ - coordenador desde 01/09/2011
MARINA DE LIMA MARCOLIN - Aluno de Graduação desde 01/09/2011
Rachel Krolow Santos Silva - Aluno de Doutorado desde 01/09/2011
Camilla Lazzaretti - Aluno de Doutorado desde 01/09/2011

Equipe Externa:

Grasielle Clotildes Kincheski - pesquisador desde 01/09/2011

Comissão De Ética No Uso De Animais aprovou o mesmo, em reunião realizada em 12/09/2011 - Sala de Reuniões do 2º andar da Reitoria, Campus Central, em seus aspectos éticos e metolodológicos de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Sexta-Feira, 23 de Setembro de 2011

FLAVIC ANTONIO PACHECO DE ARAUJO
Coordenador da comissão de ética

1