PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
ESCOLA DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM PEDIATRIA E SAÚDE DA CRIANÇA
DOUTORADO EM SAÚDE DA CRIANÇA

LUIS EDUARDO WEARICK DA SILVA

IMPACTO DE UM MODELO EXPERIMENTAL DE ESTRESSE PRECOCE NA COGNIÇÃO, MOTRICIDADE E CORRELATOS NEUROBIOLÓGICOS DURANTE A ADOLESCÊNCIA

Porto Alegre
2018

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PROF. DR. RODRIGO GRASSI-OLIVEIRA

Orientador

PORTO ALEGRE
2018

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APROVADA EM:_____ DE_____DE_____.

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Suplente

PORTO ALEGRE
2018

Dedico esta tese ao amor da minha vida,

Nina Schmal Wearick

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Primeiramente, gostaria de agradecer à Pontifícia Universidade Católica do Rio Grande do Sul por me acolher durante toda a minha trajetória profissional. Ao programa de Pós-Graduação em Pediatria e Saúde da Criança por me dar a oportunidade de realizar o Mestrado e o Doutorado em um programa de excelência. Da mesma forma, agradeço à CAPES pela bolsa de estudos do Doutorado e ao CNPq pela bolsa de estudos via programa Ciências sem Fronteiras, que me possibilitou passar preciosos 12 meses no exterior aprimorando meus conhecimentos.

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RESUMO

Introdução: A exposição ao estresse no início da vida está associado a uma diminuição na qualidade de vida e é considerada como fator de risco para diversas patologias. Ainda que o impacto do estresse precoce no desenvolvimento cerebral e cognitivo, bem como no sistema motor, esteja bem documentado na literatura, pouco se sabe sobre os mecanismos neurobiológicos mediadores destes efeitos.

Objetivos: Este trabalho tem como objetivo investigar o impacto de um modelo experimental de estresse precoce no funcionamento cognitivo e na adaptabilidade da marcha na adolescência, avaliando a expressão gênica de alvos relacionados à memória e aprendizagem em diferentes regiões do cérebro.

Métodos: Camundongos machos da linhagem C57BL/6 foram expostos ao modelo de *Limited Bedding* do P2 ao P9 e testados no Labirinto Radial de 8 braços, Labirinto Y, Esquiva Inibitória e Escada Horizontal no final da adolescência. RT-qPCR foi realizado para investigar a expressão gênica do exon IV do BDNF, Drd1 e Drd2 nas regiões do mPFC, Córtex Motor e Cerebelo.

Resultados: Camundongos expostos ao modelo de *Limited Bedding* na infância cometeram menos erros perseverativos quando comparados ao grupo controle. Este efeito foi seguido por um aumento na expressão do exon IV do BDNF no mPFC, embora nenhuma diferença entre Drd1 e Drd2 tenha sido observada.

Ao observar a adaptabilidade da marcha, encontramos dois subgrupos distintos de animais que apresentaram desempenho superior (SP) quando comparados aos controles ou desempenho inferior (IP). Observamos uma expressão exarcebada de Drd1 no mPFC de animais com performance inferior e aumento na expressão de Drd1 no cerebelo de animais com performance superior, sem diferenças em relação à expressão de Drd2 no mPFC, córtex motor e cerebelo. Observamos que ambos os grupos aumentaram a expressão do BDNF no mPFC, juntamente com uma diferença significativa entre os grupos SP e IP na expressão do BDNF no córtex motor. Encontramos uma forte correlação negativa entre a expressão do exon IV do BDNF no córtex motor e a adaptabilidade da marcha. Não foram observadas diferenças entre os grupos em relação à expressão de TrkB nas regiões do cérebro investigadas, embora haja uma correlação positiva entre a expressão de TrkB no mPFC e uma melhor capacidade de adaptação da marcha.

Conclusão: Nosso estudo demonstrou que camundongos expostos ao *Limited Bedding* apresentaram menos comportamentos perseverativos e aumento da expressão do exon IV do BDNF na adolescência. Além disso, nossos dados sugerem que a exposição ao *Limited Bedding* pode levar a fenótipos distintos na tarefa de adaptabilidade da marcha, seguido de uma expressão diferenciada de Drd1 e BDNF em regiões cerebrais envolvidas na adaptabilidade da marcha.
Palavras-Chave: Estresse Precoce, Adolescência, cognição, adaptabilidade da marcha, dopamina, BDNF;

ABSTRACT

Introduction: Early life stress exposure is a global issue and is associated with decreased quality of life and it is considered a risk factor for several diseases. There are several evidences in the literature suggesting that early life stress impact brain development, as well as cognition and motricity. The neurobiological mechanisms behind these effects are poorly understood.

Aim: This study aims to investigate the impact of an experimental model of early life stress on cognitive abilities and walk adaptability during adolescence, looking at the gene expression of targets related to learning and memory in different brain regions.

Methods: Briefly, we exposed male C56BL/6 mice to the limited bedding protocol post-natal day (PND)2 to PND9 and then tested animals in the radial 8-arm maze, Y-maze and Step-Down avoidance task and Ladder Rung Walking Test at the end of adolescence. RT-qPCR was used to investigate BDNF exon IV, Drd1 and Drd2 gene expression in the mPFC, Motor Cortex and Cerebellum 2h after the task.

Results: Mice raised in *Limited Bedding* conditions presented fewer perseverative errors compared to our reference group. This effect was followed by an increased BDNF exon IV expression in the mPFC with no differences in Drd1 and Drd2. When looking at the ability of adapt walking of mice, we found two distinct subgroups of animals that presented a superior performance (SP) when compared to controls or an inferior performance (IP). We observed that Drd1 expression is increased in the mPFC of IP animals and in the cerebellum of SP with no differences regarding Drd2 expression on mPFC, motor cortex and cerebellum. We observed that both SP and IP groups increased BDNF expression in the mPFC together with a significant difference between SP and IP groups in BDNF expression on motor cortex. We found a strong negative correlation between BDNF exon IV expression in the motor cortex and walking adaptability. No differences between groups regarding TrkB mRNA expression in any brain region investigated were observed although there is a positive correlation between TrkB expression in the mPFC and a better ability to adapt walking.

Conclusions: Our study showed that mice exposed to *Limited Bedding* showed fewer perseveration and increased BDNF exon IV expression in the mPFC during adolescence. Also, our data suggest that exposure to *Limited Bedding* early in life can lead to distinct phenotypes followed by differential expression in Drd1 and BDNF in brain regions involved in the regulation of walking adaptability.

Keywords: Early-Life Stress, Adolescence, Cognition, Gait Adaptability, Dopamine, BDNF;

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ANOVA – Análise de variância

BDNF – Fator Neurotrófico Derivado do Cérebro

cDNA – DNA complementar

CEMBE – Centro de Modelos Biológicos e Experimentais

DRD1 – Receptor de Dopamina D1

DRD2 – Receptor de Dopamina D2

EPM – Erro Padrão da Média

HIV – Vírus da Imunodeficiência Humana

IBGE – Instituto Brasileiro de Geografia e Estatística

mPFC – Córtex pré-frontal medial

P – Dia pós-natal

RNA – ácido ribonucleico

RT-qPCR – Reação em cadeira de polimerase quantitativa em tempo real

SUMÁRIO

1. INTRODUÇÃO

Esta tese de doutorado é composta por dois artigos empíricos que buscaram investigar o impacto de um modelo de estresse precoce na infância no funcionamento cognitivo e na adaptabilidade da marcha no final da adolescência. O primeiro artigo, portanto, teve como objetivo avaliar a aprendizagem e a memória de trabalho em uma tarefa de memória espacial em camundongos criados em um ambiente empobrecido usando o modelo de *Limited Bedding*. Além disso, busco investigar a expressão gênica de alvos relacionados a memória, aprendizagem e neuroplasticidade nas regiões do cérebro envolvidas na tarefa. O segundo artigo buscou explorar o impacto do modelo de *Limited Bedding* na adaptabilidade da marcha no final da adolescência, buscando avaliar possíveis correlatos neurobiológicos associados em regiões do cérebro importantes do sistema motor.

1.1 REVISÃO DA LITERATURA

1.1.1 O IMPACTO DA ESTRESSE NO DESENVOLVIMENTO COGNITIVO E MOTOR

Dados epidemiológicos evidenciam que o estresse gera consequências negativas em processos cognitivos durante a vida adulta, (7). Mani et al (2013) demonstrou experimentalmente que indivíduos de famílias expostas ao estresse apresentaram um desempenho inferior em tarefas cognitivas (8). Dados de estudos com neuroimagem mostram diversos fatores que estariam associados a alterações estruturais em regiões cerebrais importantes para o processamento cognitivo. Dentre eles, a educação parental estaria relacionada com volume e espessura cortical entre 4-18 anos de idade(9, 10). Outros fator importante associado com alterações estruturais é a renda e a educação materna, que estaria relacionada com o crescimento e alterações volumétricas em regiões frontais e parietais do cérebro(11).

Além do impacto que o estresse exerce sobre o desenvolvimento cognitivo, outro fator importante afetado pelo estresse é o desenvolvimento motor. O desenvolvimento das habilidades motoras durante a infância aparece como um importante fator mediador da saúde, tendo em vista que crianças que apresentam maior proficiência em tarefas motoras são mais propensas a engajarem-se em atividade física durante a própria infância, adolescência e vida adulta(13). Morley et al (2015) demonstrou que crianças expostas a situações adversas no início da vida demonstraram prejuízos em tarefas de motricidade fina e ampla(14). Além disso, outros estudos também compararam crianças de diferentes condições e observaram um efeito negativo do estresse em habilidades motoras gerais, equilíbrio e destreza manual(15, 16). Baseado nisso, pode-se hipotetizar que o estresse possui um efeito não somente na cognição mas também no desenvolvimento do sistema motor.

Crianças criadas em situação estressantes estariam sendo expostas cronicamente a diversos fatores estressantes como confusão e desordem, conflitos e violência intrafamiliar e barulho excessivo(18). Com isso, o efeito estresse parece estar relacionado com a ação fisiológica que os hormônios relacionados a resposta ao estresse exercem em processos adjacentes ao desenvolvimento.

1.1.2 ESTRESSE PRECOCE E NEURODESENVOLVIMENTO

Ainda que o efeito do estresse precoce no desenvolvimento cognitivo e motor desperte o interesse de pesquisadores durante décadas, uma pergunta que ainda não foi completamente respondida é: Quais os efeitos do estresse precoce no neurodesenvolvimento?

O neurodesenvolvimento é um processo dinâmico que é regulado por fatores genéticos e ambientais(20-22). Ainda que alguns processos maturacionais estejam completos no momento do nascimento, o cérebro no período pós-natal ainda apresenta diversos processos maturacionais como crescimento dendrítico, neurogênese e poda sináptica(23-25). Trata-se de um período crítico para o desenvolvimento e influências ambientais podem promover alterações duradouras ou até mesmo irreversíveis no cérebro(26, 27). O estresse precoce parece estar associado à vulnerabilidade para transtornos de humor, distúrbios emocionais e dependência química(28-30). Evidências clínicas sugerem que a exposição a maus-tratos no início da vida tem sido associado a alteração na morfologia e na funcionalidade de diversas regiões cerebrais (31). Um estudo demonstrou que crianças entre 2-4 anos que foram criadas em um orfanato apresentaram severos prejuízos cognitivos e funcionamento social(32). Edmiston et al (2011) demonstrou que adolescentes que auto reportaram histórico de maus-tratos na infância demonstraram diminuição no volume de massa cinzenta em regiões como o mPFC,

amigdala, estriado e cerebelo(33). Além disso, Andersen et al (2008) demonstrou que mulheres que sofriam repetidos episódios de abuso sexual na infância e adolescência apresentaram redução no volume do hipocampo, volume de massa cinzenta do mPFC e diminuição do corpo caloso(34).

Para a investigação dos mecanismos moleculares relacionados à exposição a eventos adversos no início da vida, diversos modelos de estresse precoce surgiram, como a separação materna e o *Limited Bedding.* Muitos dos modelos de estresse precoce buscaram impactar a relação entre a mãe e a ninhada, tendo em vista que o cuidado e a interação materna são os principais fatores associados aos efeitos do estresse ao longo da vida [para revisão, Molet et al (2014)(35)].O cuidado materno vai muito além dos aspectos nutricionais e de fornecer um ninho. As mães são essências para fornecer sinais sensoriais e pistas ambientais para seus filhotes(36, 37). O comportamento materno consiste em comportamentos de lamber, arrumar e amamentar(38). A fragmentação deste cuidado, através da remoção da mãe por períodos intermitentes, ou da privação de recursos básicos para a construção do ninho tem sido utilizados como modelos naturalísticos de estresse precoce(35).

Mais recentemente, Naninck et al (2015) demonstrou que o estresse crônico causado pelo modelo de moradia empobrecida aumentou a neurogênese durante o desenvolvimento, assim como o funcionamento cognitivo dos animais(39). Além disso, Bath et al (2016) sugere que o *Limited Bedding* promove uma aceleração na chegada de marcadores de maturidade sináptica e o desenvolvimento precoce da aprendizagem emocional em camundongos(40). Baseado nisso, uma das hipóteses desta tese é que o estresse pode induzir uma maturação precoce de regiões cerebrais envolvidas em processos cognitivos, apesar dos mecanismos moleculares por trás desta alteração não terem sido completamente elucidados.

1.1.3 NEUROBIOLOGIA DO ESTRESSE PRECOCE

Uma molécula que desempenha um papel fundamental em mecanismos de neuroplasticidade é o BDNF (Brain-Derived Neurotrophic Factor, da sigla em inglês). O BDNF é uma proteína importante para funções celulares como neurogênese, crescimento e maturação sináptica e neuronal, além de ser componente chave nos mecanismos de aprendizagem e memória(41). O gene *bdnf* é composto de 9 exons, controlados por 8 regiões promotoras diferentes. Os exons I-VIII irão se juntar ao exon IX após o mecanismo de *splicing* antes de serem traduzidos para a proteína. Análises por bioinformática sugerem que o BDNF pode possuir até 22 isoformas diferentes(42). Tamanha complexidade permite uma regulação fina no seu padrão transcripcional dinâmico em diferentes tipos celulares e estímulos neuronais. Apesar disso, o exon IV, por possuir propriedades de regulação e ser o principal exon responsivo aos estímulos do ambiente, também é o mais investigado(43). Seo et al (2016) demonstrou que ratos expostos a um estresse precoce por separação materna apresentaram diminuição na expressão do exon IV do BDNF no hipocampo mais tarde na vida(44). Calabrese et al também observou uma diminuição do exon IV do BDNF na porção ventral do hipocampo e na porção ventromedial do Córtex Pré-Frontal quando os animais expostos a separação materna atingiram a adultez(45). Coletivamente, estes dados sugerem que o estresse precoce promove alterações prejudiciais duradouras na expressão do BDNF ao longo da vida. Entretanto, em um estudo recente publicado por Suri et al (2013), animais expostos ao estresse precoce, quando testados durante o início da vida adulta, apresentavam uma neurogênese aumentada seguida de uma melhora na performance no Labirinto Aquático de Morris. O mais interessante é que, quando os animais foram testados durante a meia idade, o efeito foi o oposto: diminuição na expressão do exon IV do BDNF e prejuízos

cognitivos(46). Claramente o BDNF está sendo afetado pela exposição ao estresse precoce, apesar do efeito ainda não ser completamente elucidado. Entretanto, consideradas as propriedades que esta molécula apresenta, torna-se um alvo potencial para se estudar os efeitos do *Limited Bedding* na cognição e neuroplasticidade durante a adolescência.

Outra molécula envolvida em processos de memória e aprendizagem é a dopamina [para revisão, ver Puig et al (2014)(47)]. Andersen et al (2000) demonstrou que, em um cérebro de camundongo que se desenvolve normalmente, o pico de densidade dos receptores do tipo 1 (Drd1) e do tipo 2 (drd2) acontece aos 40 dias de idade no mPFC, idade correspondente à adolescência(48). Estudos iniciais que investigaram o papel da dopamina na memória e aprendizagem propuseram um princípio no qual a dopamina no córtex pré-frontal, via ativação de Drd1, tem um efeito em forma de “U invertido”, onde bloqueando a ativação de Drd1 ou uma sinalização exacerbada acabaria trazendo prejuízos cognitivos(49-51). Novos estudos, entretanto, propuseram um modelo mais complexo considerando os diferentes receptores de dopamina a diferentes tarefas cognitivas(52). A estimulação farmacológica de Drd1 pode melhorar a performance em uma tarefa de memória de trabalho quando tempos de retenção mais longos são necessários para executar a tarefa. Por outro lado, administração do agonista de Drd1 em uma tarefa de curta-duração prejudicou a memória de trabalho. No que diz respeito a ativação de Drd2 no mPFC, a modulação da memória de trabalho parece ter uma função mais linear, quando comparada a função do Drd1, com baixos níveis de ativação associados a melhor performance e altos níveis de ativação associados a piora na performance(53). Mais recentemente, Brenhouse et al (2013) demonstrou que ratos expostos a separação materna apresentam uma expressão elevada de Drd1 durante a

adolescência e uma atenuada expressão de Drd2 no córtex pré-frontal(54). Além do papel da dopamina nos processos cognitivos, pesquisadores demonstraram que a atividade dopaminérgica no córtex motor desempenha um papel fundamental na regulação da aprendizagem motora e na manutenção da plasticidade do córtex motor(55). Bloquear a sinalização dopaminérgica no córtex motor através da administração do antagonista causou a diminuição na sinalização do potencial de longa duração, um importante mecanismo de aprendizagem(56). Além disso, outra região cerebral envolvida no controle motor é o cerebelo, que já foi demonstrado possuir inervações para a gânglia basal e fazer parte do sistema de controle motor(57).

2. OBJETIVOS

2.1. OBJETIVO GERAL

Este trabalho tem como objetivo investigar o impacto de um modelo experimental de estresse precoce no funcionamento cognitivo e na adaptabilidade da marcha na adolescência, avaliando a expressão gênica de alvos relacionados à memória e aprendizagem em diferentes regiões do cérebro.

2.2. OBJETIVOS ESPECÍFICOS

- Investigar o impacto do *Limited Bedding* na memória e aprendizagem no labirinto radial de 8 braços;
- Investigar o impacto do *Limited Bedding* na memória de trabalho no labirinto em Y;
- Investigar o impacto do *Limited Bedding* na memória de medo na tarefa de Esquiva Inibitória;
- Investigar o impacto do *Limited Bedding* na adaptabilidade da marcha;
- Investigar o efeito do *Limited Bedding* na expressão gênica de BDNF exon IV*, TrkB, Drd1 e Drd2* no mPFC, Córtex Motor e Cerebelo;
- Correlacionar os níveis de expressão gênica com os desfechos comportamentais anteriormente descritos;

3. MÉTODOS

3.1 CONSIDERAÇÕES ÉTICAS E MANEJO DOS ANIMAIS

Este projeto foi submetido e aprovado pelo CEUA desta universidade sob os números de aprovação 15/00472 e 16/0040. Além disso, todos os procedimentos foram conduzidos seguindo os princípios dos “três Rs” e conforme o Guide for the Care and Use of Laboratory Animals do National e a Lei Arouca (nº11.794/08). Os animais eram mantidos no vivário do CEMBE em um ambiente com temperatura, umidade e iluminação controlados automaticamente em um ciclo 12h/12h (12h claro e 12h escuro). Os animais foram alimentados com ração peletizada para pequenos roedores em um regime *ad libitum*. Os animais que foram treinados no labirinto em radial de 8 braços foram mantidos em um regime de restrição hídrica ao longo do treinamento. Os demais animais foram mantidos em um regime de hidratação *ad libitum*. Após o desmame, os animais eram separados por sexo e agrupados em caixa com animais da mesma ninhada em grupos de 2-4 animais por caixa. Após o término dos experimentos, os animais foram eutanasiados através de deslocamento cervical por membros da equipe altamente treinados.

3.2 AMOSTRA E DELINEAMENTO EXPERIMENTAL

Este projeto foi realizado usando camundongos da linhagem C57BL/6, que foram divididos entre os dois estudos. Fêmeas adultas eram adquiridas do CEMBE/PUCRS e após um período de aclimatação (~1 semana) eram acasaladas em um regime 2:1 (2 fêmeas e um macho) por um período de 7 dias. Após esse período, o macho era retirado e as fêmeas permaneciam em duplas até 1-2 dias antes da provável data do parto. Após separadas, as fêmeas eram observadas diariamente para verificar o nascimento das ninhadas. Confirmado o nascimento, as fêmeas eram então designadas, aleatoriamente, para os grupos experimentais de *Limited Bedding* ou controle, descritos detalhadamente a seguir. Os filhotes ficavam com as mães até

o momento do desmame, onde eram separados por sexo e agrupados em 2-4 animais por caixa até o dia dos testes. Neste estudo, apenas os filhotes machos foram utilizados. As fêmeas foram designadas para outro projeto em andamento no laboratório. Para o estudo 1, foram necessárias duas coortes independentes de animais (A e B, conforme ilustrado na Figura 1). Para o estudo 2, apenas uma coorte foi utilizada (C). Com isso, foram utilizadas um total de 60 fêmeas adultas (32 controle e 30 ambiente empobrecido). Para evitar um efeito de ninhada, cada família contribuiu com, no máximo, 3 animais em cada grupo. O número de filhotes machos utilizados foi:

- Coorte A – Controles: 29
- Coorte A – Empobrecimento Ambiental: 28
- Coorte B – Controles:22
- Coorte B – Empobrecimento Ambiental: 27
- Coorte C – Controles: 13
- Coorte C – Empobrecimento Ambiental: 19

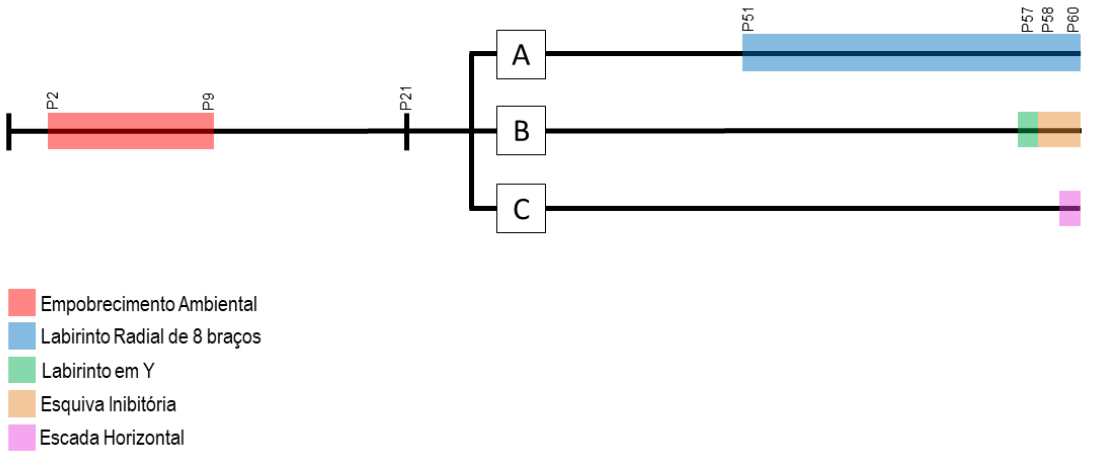

Figura 1 – Delineamento Experimental: A) Coorte de animais que foram treinados no labirinto em radial de 8 braços e foram incluídos no estudo 1; B) Coorte de animais que foram treinados no labirinto em Y e na tarefa de Esquiva Inibitória e inclusos no estudo 1; C) Coorte de animais que foram treinados na tarefa da Escada Horizontal e fizeram parte do estudo 2;

3.3 MODELO DE EMPOBRECIMENTO AMBIENTAL

O protocolo do *Limited Bedding* foi realizado conforme descrito anteriormente (58, 59). O fundo da caixa residência foi coberto com maravalha autoclavada para absorver fezes e urina e uma malha de alumínio separava a ninhada e os filhotes da maravalha. Acima da malha, 1g de algodão era fornecido para a mãe construir o seu ninho. Não era possível para a mãe recolher a maravalha do fundo da caixa para incorporar ao seu ninho. Para as ninhadas do grupo controle eram fornecidos 4g de algodão e quantidade padrão de maravalha (~150g) para construção do ninho. No P2, as ninhadas eram alocadas aleatoriamente para os grupos controle ou *Limited Bedding*. Resumidamente, as mães eram removidas primeiro da caixa residência, os filhotes então removidos, um por um, e colocados na nova caixa. A mãe era então colocada na nova caixa contendo a malha de alumínio ou a maravalha e não foram manipuladas até o P9, quando eram removidas da condição de *Limited Bedding* e retornavam para condições de moradia igual ao grupo controle (~150g de maravalha e 4g de algodão). Os animais recebiam uma nova troca de caixa no P16. Mães do grupo controle foram deixadas sem manipulação, exceto para limpezas da caixa no P2, P9 e P16.

3.4 LABIRINTO RADIAL DE 8 BRAÇOS

Para investigar os efeitos do *Limited Bedding* em uma memória de memória e aprendizagem, nós utilizamos uma versão com recompensa em 4 braços, conforme publicado anteriormente(60). Primeiramente, os animais eram colocados em um aparato contendo 8 braços, numerados de 1 a 8, de 30cm de comprimento por 5cm de largura, irradiando de um compartimento central de 25cm². Dois dias antes de iniciar o protocolo os animais foram habituados a recompensa (10% leite condensado, em água). Uma tampa contendo 2mL da solução recompensa foi colocado na caixa

residência por um período de 48h para habituação à solução e para reduzir a neofobia. Para aumentar a motivação à recompensa para coletar a recompensa, foi restringido o acesso a agua por 15h, diariamente. As garrafas de água eram retiradas da caixa residência as 6pm e os animais treinados no outro dia pela manhã. Animais foram mantidos neste regime de restrição hídrica durante todo o protocolo. Após completar os dois trials, os animais tinham acesso a agua na sua caixa residência. O protocolo de treino foi composto de 3 fases. Em todas elas os animais realizavam 2 trials consecutivos e para evitar pistas olfatórias, o aparato era limpo com álcool isopropílico entre os trials e entre os animais. Na primeira delas, chamada pré-treino, os animais eram expostos ao aparato com recompensas no final dos oito braços e os animais exploravam livremente o aparato até encontrar as 8 recompensas ou 300s decorridos. No próximo dia, a segunda fase do protocolo iniciava-se, chamada de aquisição com duração de 7 dias, onde os animais eram expostos ao aparato e apenas 4 dos 8 braços continham recompensa (braços pares ou ímpares, randomizados entre os animais). Os braços contendo a recompensa permaneceram os mesmo durante toda a fase de aquisição. Animais exploravam o labirinto até que as 4 recompensas fossem coletadas ou 300s decorressem. Dois dias após o último trial de aquisição, os animais retornavam para o labirinto para um teste de retenção idêntico à fase de aquisição. Na versão do labirinto com recompensas em apenas 4 braços, os animais precisam aprender a ignorar os braços que não contém a recompensa e explorar apenas os braços contendo a recompensa. Além disso, precisam da memória de trabalho para lembrar qual braço já foi visitado dentro do mesmo trial. Para analisar a aprendizagem e a memória dos animais através dos dias, a média entre os trials foi calculado usando as variáveis dependentes descritas na tabela 1.

Tabela 1: Descrição das variáveis dependentes no Labirinto Radial de 8 braços

Variável	Descrição
Tempo	Tempo gasto para coletar todas as recompensas.
Erro de Referência	Entrada em um braço sem recompensa.
Erro de Memória de Trabalho	Re-entrada, no mesmo trial, em um braço com recompensa.
Erro Perseverativo	Re-entrada, no mesmo trial, em um braço sem recompensa.

3.5 LABIRINTO EM Y

Para avaliar a memória de trabalho no Labirinto em Y, a alternância espontânea foi investigada nos animais conforme descrito anteriormente(61). Este teste é baseado na tendência natural que os camundongos apresentam para explorar um novo ambiente. Quando colocados no aparato, o animal vai explorar um braço e depois tenderia a alternar entre os braços e visitar o menos recentemente visitado. Para fazer isso, os camundongos precisam usar a memória de trabalho para manter um registro contínuo do braço previamente visitado. Os animais eram levados para a sala de experimentação 30min antes do teste. Todos os testes foram realizados de manhã (aprox. 2-3h após o início do ciclo claro) e a luminosidade da sala era ~100lux. Animais eram expostos ao labirinto em Y, que consistiu de uma sessão única de 5min, onde os animais puderam explorar livremente os três braços do labirinto. O braço inicial foi randomizado entre os animais para evitar um possível viés de colocação. Alternações foram definidas como entradas consecutivas em três braços, em tercetos sobrepostos (por exemplo, CBA, BCA, ACB, etc.). O escore de alternância espontânea foi calculado dividindo o número de tercetos pela alternância possível (entradas totais –

2) x 100. O retorno alternado ao mesmo braço ou retorno ao mesmo braço foi calculado para investigar erros na alternância espontânea(62).

3.6 ESQUIVA INIBITÓRIA

Animais que realizaram o labirinto em Y foram treinados e testados na tarefa de Esquiva Inibitória, baseado em estudos anteriormente publicados(63, 64). A Esquiva Inibitória é uma tarefa clássica de condicionamento de medo, dependente do hipocampo, onde os animais aprendem que descer de uma plataforma desencadeia um estímulo aversivo (choque). O aparato consiste em uma caixa de acrílico de 50x25x25cm com o chão formado por barras de aço inoxidável espaçados em 1cm contendo uma plataforma no canto da caixa de 5cm². Na sessão de treino, animais eram colocados na plataforma, de frente para o canto, e a latência para descer da plataforma era cronometrado. Imediatamente após descer da plataforma, animais recebiam três choques leves (0.5mA, separados por 2s) e depois retornavam para a caixa residência. O teste de retenção foi realizado 48h depois do treino e foi realizado de forma idêntica ao treino, mas nenhum choque foi apresentado. A latência para descer da plataforma no teste foi usada como uma medida de retenção.

3.7 ESCADA HORIZONTAL

Para avaliar a adaptabilidade da marcha dos camundongos na adolescência, uma versão adaptada para camundongos da escada horizontal foi utilizada conforme descrito anteriormente(65). Os camundongos eram incentivados a atravessar três vezes uma escada horizontal de 1m de comprimento com degraus aleatoriamente espaçados, situada 30cm acima do chão. Durante o teste, os camundongos foram gravados cruzando a escada utilizando uma GoPro Hero 4 com uma taxa de aquisição de 240 quadros por segundo e posterior análise quadro-a-quadro. O primeiro trial foi considerado habituação e, portanto, não considerado para a análise. Desta forma, a

média de desempenho dos trials 2 e 3 foram calculados. O desempenho foi verificado calculando o número de erros dos membros anteriores e membros posteriores conforme anteriormente descritos (66). O número de erros foi normalizado em relação ao grupo controle utilizando a seguinte fórmula: (Número de errors/Número de erros do grupo controle)*100. Deste modo, um escore de 100% indica desempenho similar ao grupo controle, ao passo que um escore inferior à 100% indica que o animal cometeu menos erros em comparação ao grupo controle. Um escore superior a 100% indica que os animais cometeram mais erros que o grupo controle e, portanto, possuem um desempenho inferior na tarefa.

3.8 COLETA DE MATERIAL BIOLÓGICO

Os animais foram eutanasiados 2h após o último trial do labirinto radial de 8 braços para o estudo 1 e 2h após o teste da escada horizontal no estudo 2. O cérebro era dissecado em gelo para a extração do mPFC, Córtex Motor e Cerebelo. Após a dissecção, o tecido era imediatamente congelado em gelo seco e armazenados em freezer -80ºC até o momento das análises.

3.9 ANÁLISE DE EXPRESSÃO GÊNICA

Para a expressão gênica, o tecido era primeiramente descongelado e então o RNA extraído usando Trizol® (Qiagen - Hilden, Germany) de acordo com as instruções do fabricante. A concentração do RNA foi mensurada usando o NanoDrop (Thermo Fisher Scientific – Waltham, EUA) e um total de 500ng de RNA de cada amostra foi utilizado para a conversão para cDNA usando o miScript II RT kit (Qiagen - Hilden, Germany). O cDNA foi utilizado em cada reação de RT-qPCR no RotorGene (Qiagen - Hilden, Germany) usando o miScript SYBR green kit (Qiagen - Hilden, Germany).

Os primers utilizados para a análise de expressão gênica e o tamanho do produto de RT-qPCR são descritos na Tabela 2. PCR em gradiente foi utilizado para verificação da temperatura ótima de anelamento e gels de DNA foram corridos para verificação da especificidade. Cada reação de RT-qPCR foi corrida em duplicata e repetidas uma vez. O cálculo para análise de expressão gênica relativa foi realizado usando o método ΔΔCt com o grupo controle como referência e os valores de Ct do GAPDH como controle endógeno.

Tabela 2 – Primers utilizados para análise de expressão gênica:

Gene	Forward	Reverse	bp
GAPDH	TCATATTTCTCGTGGTTCACACC	CTGAGTATGTCGTGGAGTCTACTGG	149
BDNF exon IV	GCAGCTGCCTTGATGTTTAC	GCATGGCATAGTAGTTGTAGTGG	147
TrkB	CTCGGTAGCTGGAAGCACAT	GGACTCTTTGGGTCGCAGAA	155
Drd1	ATGGCTCCTAACACTTCTACCA	GGGTATTCCCTAAGAGAGTGGAC	124
Drd2	ACCTGTCCTGGTACGATGATG	GCATGGCATAGTAGTTGTAGTGG	105

3.10 ANÁLISE ESTATÍSTICA

Todas as análises estatísticas foram realizadas no SPSS 20.0 (IBM – New York, EUA) e os gráficos foram plotados usando o Prism GraphPad 6.0 (La Jolla, EUA). A normalidade das variáveis foi verificada utilizando o teste de Shapiro-Wilk. Para investigar o efeito do ambiente empobrecido nas variáveis dependentes, teste t-student ou ANOVA de uma via por utilizada com comparação múltipla de Tukey, quando cabível. A correlação entre os desfechos moleculares e o escore nos testes comportamentais foi analisada através do teste de correlação de Pearson. Uma ANOVA de medidas repetidas foi conduzida para determinar se os animais reduziam o tempo e o número de erros ao longo dos dias durante a fase de aquisição. Em todas

as análises, os dados foram expressos como média±EPM e o nível de significância adotado foi de 5%.

4. DISCUSSÃO E CONCLUSÕES

4.1. Camundongos machos expostos ao Limited Bedding apresentam menor perseveração no erro

O estudo 1 desta tese teve como objetivo investigar o impacto do *Limited Bedding* na memória de trabalho e aprendizagem espacial durante a adolescência, assim como correlatos neurobiológicos no mPFC. Interessantemente, camundongos expostos ao modelo de *Limited Bedding* apresentaram menos erros perseverativos quando comparados com o grupo controle, seguido por aumento da expressão do exon IV do BDNF e nenhuma diferença significativa nos receptores de dopamina Drd1 e Drd2.

Neste estudo, utilizamos a versão com recompensas em quatro dos oito braços do labirinto radial. Esta abordagem permite uma investigação da memória de trabalho (caracterizado por re-entradas nos braços previamente visitados em um mesmo trial) e também a aprendizagem espacial através dos erros de referência (entradas em braços que nunca possuíram recompensa). Yoon et al (2008) demonstrou que uma inativação seletiva do mPFC com muscimol levou a prejuízos de memória de trabalho. Por outro lado, a inativação do hipocampo aumentou o número de referência (67). Além disso, inativação do mPFC de roedores diminuiu a flexibilidade cognitiva, ou seja, a capacidade de inibir uma estratégia utilizada anteriormente para desenvolver uma nova (68, 69). Nesta tarefa, o animal precisa manter a o registro em tempo real dos braços já visitados no mesmo trial, além de lembrar-se de ignorar, ao longo do período de treino, os braços que nunca possuíram recompensa. Neste caso, o animal precisa de um funcionamento adequado tanto do mPFC quando do hipocampo para desempenhar a tarefa com o menor número de erros possível. Podemos observar que os animais expostos ao protocolo de *Limited Bedding* apresentaram uma diminuição no número de erros perseverativos quando comparados ao grupo controle e outra

coorte independente de animais criados em *Limited Bedding* também teve um número de erros de memória de trabalho inferior ao grupo controle. Esta observação contrasta com dados publicados anteriormente, que mostraram que camundongos expostos ao protocolo de Limited Bedding apresentam prejuízos em uma tarefa de localização de objetos e também na tarefa de aprendizagem espacial do labirinto aquático de Morris (58, 70). No que diz respeito aos erros de referência e o desempenho da tarefa de esquiva inibitória (ambas tarefas dependentes do hipocampo), não foram observadas diferenças significativas entre os grupos. Nossa hipótese inicial era que os animais criados em *Limited Bedding* iriam apresentar prejuízo no desempenho destas tarefas, baseado em dados descritos na literatura que mostraram que este modelo reduziu a potenciação de longa duração, mecanismo importante para a aprendizagem e memória, além do número de espinhas dentríticas na área CA3 do hipocampo (71). Além disso, foi reportado que a plasticidade sináptica do hipocampo nas áreas CA1 e CA3 do hipocampo estaria reduzida após o modelo de *Limited Bedding* (72). Ainda que estes estudos tenham testado os animais em diferentes fases da vida comparado com o nosso experimento, está poderia ser uma das possíveis explicações para a discrepância nos dados. Entretanto, nosso estudo contribui para a literatura com uma observação dos efeitos do *Limited Bedding* em tarefas dependentes de hipocampo durante a adolescência.

Conforme mencionado anteriormente, o período da vida que os animais são testados após o modelo de estresse variou da adolescência até o animal envelhecido, o que pode ser um fator de confusão para se fazer uma conclusão. A adolescência é um período crítico do desenvolvimento com o início da puberdade, marcado por alterações substanciais no desenvolvimento cerebral (73). A maioria das demandas emergentes na adolescência exigem uma alta flexibilidade cognitiva, como a alteração

na relação com os pares, transição para o ensino superior e carreira profissional. Quando o adolescente falha em ajustar-se a estas demandas do ambiente pode apresentar diversas consequências negativas como abandono escolar, exclusão social e transtornos psiquiátricos (74). Em roedores, por exemplo, Koss et al demonstrou que adolescentes cometem mais erros perseverativos que adultos em uma tarefa de alternância (dependente de mPFC). Portanto, nós podemos racionalizar que adolescentes irão apresentar comportamentos perseverativos normal para esta fase do desenvolvimento, considerando que o cérebro ainda está em desenvolvimento e maturação. Seguindo na linha das evidências que sugerem que o estresse precoce poderia acelerar processos neurobiológicos de maturação(40, 75), nossa hipótese é que os animais expostos ao *Limited Bedding* na infância, ao apresentarem menos comportamentos perseverativos que controles, poderiam ser considerados como tendo comportamento semelhantes aos adultos. Entretanto, deve-se ter cuidado ao reivindicar isto, tendo em vista que mais experimentos são necessários para confirmar esta hipótese.

Outro achado importante que poderia contribuir com esta hipótese é o aumento da expressão do exon IV do BDNF em uma região importante para a regulação da memória de trabalho e aprendizagem guiada por recompensa (76). Este dado é interessante tendo em vista que estes mesmos animais apresentam diminuição na perseveração, como mencionado anteriormente. O exon IV do gene do BDNF é um gene que é expresso de forma dependente da atividade cerebral e tem um papel fundamental em funções celulares relacionadas a memória e aprendizagem e neurogênese(77). Alguns estudos sugerem que o estresse crônico no início da vida pode reduzir a expressão do BDNF ao longo do desenvolvimento (78, 79). Estas evidências contrastam com as observações do nosso estudo que mostrou um

aumento da expressão deste gene após o protocolo de estresse. A diferença entre os modelos empregados nos diferentes estudos pode ser um fator que contribui para a discrepância dos resultados. Aparentemente, diferentes modelos de estresse precoce promovem distintas alterações neurobiológicas. Uma hipótese alternativa para a explicação desta variabilidade é a intensidade da exposição ao estresse durante o desenvolvimento, ou seja, a exposição ao estresse no início da vida poderia levar a fenótipos de vulnerabilidade ou resiliência mais tarde na vida. Mesmo que não tenhamos explorado esta hipótese, comparando diferentes modelos de estresse e os níveis de estresse após a exposição, entender como os modelos de estresse podem levar a fenótipos distintos pode ser um tópico interessante para estudos futuros(80).

4.2 O modelo de Limited Bedding e a identificação de subgrupos distintos baseado na capacidade de adaptação da marcha

No estudo 2, investigamos o impacto do *Limited Bedding* durante a infância na adaptabilidade da marcha no teste da escada horizontal. Conforme esperado, nós observamos que nem todos os animais responderam de forma similar aos efeitos do ambiente empobrecido. Alguns animais demonstraram uma performance superior no teste enquanto outro subgrupo de animais apresentou uma performance superior. Este resultado é interessante considerando que nem todos os indivíduos expostos à eventos estressores no início da vida irão manifestar as consequências negativas desta exposição. Alguns indivíduos possuem a capacidade de manter a resposta fisiológica e comportamental em níveis normais. Apesar de alguns efeitos do estresse no início da vida (através da separação materna ou do *Limited Bedding*) estarem documentados, pouco se sabe sobre quais os mecanismos biológicos que levam a estes fenótipos distintos (81). Nosso estudo se propôs a investigar esta variabilidade entre os grupos subdividindo os animais expostos ao estresse de acordo com a

performance na tarefa. Além disso, encontramos alguns relatos neurobiológicos em diferentes regiões do cérebro que podem servir como base para futuros estudos que busquem investigar os efeitos do estresse na adaptabilidade da marcha.

A respeito da sinalização dos receptores de Dopamina, observamos que o Drd1 estaria diferentemente expresso no mPFC de camundongos machos que foram expostos ao *Limited Bedding*. Além disso, animais expostos ao *Limited Bedding*, independente da performance na tarefa, apresentaram níveis elevados do exon IV do BDNF no mPFC. Nós hipotetizamos que o mPFC está envolvido nesta tarefa pelo fato de, quando expostos ao aparato, o animal precisa cruzar a plataforma contendo degraus aleatoriamente espaçados, o que exige precisão na colocação das patas e no movimento de agarrar. Este teste parece ser suficientemente desafiador a ponto de detectar deficiências motoras que exigem controle cortical (66). Além disso, em humanos foi demonstrado uma maior ativação do mPFC e do córtex sensório-motor primário em uma tarefa de adaptabilidade da marcha na esteira (82). Nós observamos que a expressão de Drd1 estaria aumentada no mPFC e Drd2 não estaria diferentemente expresso. Estudos anteriores mostraram que o aumento leve da dopamina no cérebro parece ter um efeito positivo na cognição, ao passo que uma ativação prolongada e exacerbada ou insuficiente (este efeito geralmente sendo descrito como um “U” invertido) traria prejuízos em tarefas cognitivas (83). Animais do grupo que teve prejuízo na adaptabilidade da marcha demonstrou um aumento significativo de Drd1 no mPFC, o que poderia corroborar com esta hipótese. Entretanto, para este experimento, todos os animais foram eutanasiados no mesmo período. Estudos futuros poderiam investigar se, após a tarefa, a sinalização de Drd1 permanece exacerbada, o que poderia fornecer ainda mais evidências de uma

possível desregulação da sinalização de Drd1 no mPFC de animais com performance inferior na tarefa.

Outro resultado interessante do nosso estudo foi a correlação positiva entre a expressão do exon IV do BDNF no córtex motor e a capacidade de adaptabilidade da marcha. Em outras palavras, quanto maior a expressão deste exon no córtex motor, melhor a capacidade de adaptar a caminhada. Apesar de estudos futuros serem necessários para afirmar que a expressão do exon IV do BDNF no córtex motor prediz a adaptabilidade da marcha, com base no papel mediador que o BDNF exerce em vias de sinalização celular importantes para a aprendizagem, como o potencial de longo duração e ativação do TrkB, nós hipotetizamos que esta molécula possui propriedades que o tornam um candidato importante para esta função.

4.3 Conclusões e Perspectivas Futuras

Esta tese teve como objetivo principal investigar os impactos de um modelo de estresse precoce em tarefas cognitivas e de adaptabilidade da marcha, assim como a expressão de BDNF e dos receptores Drd1 e Drd2 de dopamina. Observamos que os animais expostos ao *Limited Bedding* apresentam menor quantidade de erros perseverativos e consequente aumento da expressão de BDNF no mPFC durante a adolescência. Hipotetizamos que os animais expostos ao *Limited Bedding* na infância, apresentando menos comportamentos perseverativos que os animais do grupo controles, poderiam ser considerados como tendo comportamento semelhantes aos adultos, que poderia ser explicado através de uma maturação precoce de regiões cerebrais envolvidas neste comportamento. Como perspectiva futura para a investigação desta hipótese, do ponto de vista comportamental, poderíamos investigar a performance dos animais no labirinto em radial de 8 braços na fase adulta (~120 dias). Os resultados deste experimento podem demonstrar que, de fato, os animais

adultos cometem menos erros perseverativos que os animais adolescentes (e consequentemente similar aos adolescentes expostos ao ambiente empobrecido). A nível molecular, poderíamos investigar a sinalização de marcadores de maturação neural, como a parvalbumina, ou marcadores de proliferação celular como a Ki-67. Para isso, os animais expostos ao *Limited Bedding* deveriam apresentar uma maior expressão destes marcadores no mPFC.

Além disso, no que diz respeito à capacidade de adaptabilidade da marcha, buscamos investigar os distintos fenótipos associados ao *Limited Bedding*. Identificamos dois subgrupos distintos baseado na performance e observamos que os grupos possuem alteração nos padrões de expressão gênica específicas. Ambos os grupos, independente da performance na tarefa, apresentaram aumento da expressão de BDNF no mPFC, o que poderia corroborar com a hipótese de aceleração da maturação descrita no estudo 1. Além disso, o aumento exacerbado de Drd1 no mPFC dos animais que apresentaram uma performance inferior poderia ser um indicador do envolvimento desta via de sinalização com a tarefa. Para investigar isso, estudos futuros que busquem bloquear farmacologicamente esta via, especificamente nesta região, através da administração do antagonista de Drd1, poderiam fornecer evidências do envolvimento desta via na regulação da capacidade dos animais de adaptar a marcha. Além disso, uma abordagem similar poderia ser empregada para investigar a forte correlação entre o exon IV do BDNF no córtex motor com o desempenho na tarefa. Se a sinalização do BDNF no córtex motor prediz a adaptabilidade da marcha, um bloqueio farmacológico desta via, especificamente nesta região, traria prejuízos na performance na escada horizontal.

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6. ANEXOS E APÊNDICES

6.1 CARTA DE APROVAÇÃO DO CEUA

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Oficio 111/2015 - CEUA

Porto Alegre, 18 de dezembro de 2015.

Prezado Sr(a). Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 15/00472 intitulado **"Efeitos do exercício físico e suplementação probiótica na microbiota intestinal: Marcadores inflamatórios e resposta ao estresse em camundongos expostos à separação materna".**

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está **autorizada** a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Nº de Animais	Espécie	Duração do Projeto
260	Mus musculus	12/2015 – 09/2019

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

Ilmo. Sr.
Prof. Dr. Rodrigo Grassi de Oliveira
IPB
Nesta Universidade

PUCRS
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SIPESQ

Sistema de Pesquisas da PUCRS

Código SIPESQ: 7665 Porto Alegre,21 de dezembro de 2016

Prezado(a) Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou o Projeto de Pesquisa "IMPACTO DA EXPOSIÇÃO AO ESTRESSE PRECOCE SOBRE O SISTEMA NEUROENDÓCRINO E O DESENVOLVIMENTO SENSÓRIO-MOTOR DA MARCHA EM UM MODELO MURINO" coordenado por REGIS GEMERASCA MESTRINER.

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está autorizada a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Duração do Projeto: 21/12/2016 - 21/08/2017

Nº de Animais	Espécie
64	Mus Musculus
16	Mus Musculus
8	Mus Musculus
Total de Animais: 88	

Atenciosamente,

Comissão de Ética no Uso de Animais(CEUA)

6.2 ESTUDO 1

Em preparação para submissão para *Developmental Psychobiology*

Limited Bedding affects cognition and BDNF expression during adolescence with no differences in Dopamine Receptors D1 and D2

Wearick-Silva, L.E. [1,2]; Viola, T.W.[1,2]; Centeno-Silva, A.[2]; Creutzberg, K.C. [2]; Orso, R. [2]; de Freitas, B.C.[3]; Schroder, N.[4]; Marshall, P.[5]; Li, X. [5]; Bredy, T.W. [5]; Grassi-Oliveira, R. [1,2];

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Financial support: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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ABSTRACT

Introduction: Early life stress is a global issue and essentially associated with a decreased quality of life and is a risk factor for different neuropsychiatric diseases. Evidences points to the deleterious effects that early life has on cognitive outcomes, which is even more critical during childhood. The neurobiology behind the effect that early life stress exerts on cognition is not completely understood. The aim of this study is investigate the impact of a model of early life stress during infancy in cognitive outcomes on adolescence, followed by the investigation of BDNF, Drd1 and Drd2 in the mPFC. **Methods:** Briefly, we exposed male C56BL/6 mice to the limited bedding protocol post-natal day (PND)2 to PND9 and then tested animals in the radial 8-arm maze, Y-maze and Step-Down avoidance task at the end of adolescence. RT-qPCR was used to investigate BDNF exon IV, Drd1 and Drd2 gene expression in the mPFC 2h after the task. **Results:** Mice raised in LB conditions presented fewer perseverative errors compared to our reference group. This effect was followed by an increased BDNF exon IV expression in the mPFC with no differences in Drd1 and Drd2. **Conclusions:** Taken together, our study showed that mice exposed to Limited Bedding showed fewer perseveration and increased BDNF exon IV expression in the mPFC during adolescence. Our study provide new evidences on the impact that poverty has on cognition during adolescence, which could help to understand the consequences that being raised under poverty-like conditions could have on adolescent cognition

KEYWORDS: Early Life Stress; Poverty, Adolescence; Learning and Memory, BDNF; Dopamine.

INTRODUCTION

Early-life stress is a global issue and is essentially associated with a decreased quality of life, increased mortality and vulnerability to a wide range of diseases such as neurological diseases(1), diabetes (2) and psychiatric disorders(3).

Experimental and epidemiological evidences shows the negative consequences of early life stress in cognitive outcomes (8). The deleterious influences of early life stress in cognition is even more critical during childhood, given the importance of a supportive environment on the development of emotional, cognitive and language processes in the newborns(58). This way, early-life stress caused by low-socioeconomic status is a predictor of inferior IQ, as well as increased risk of learning disorders and decreased years of formal education in children and adolescents(59).

Although the impact of early-life stress on cognition has caught attention of researchers from almost a century, little experimental data addressing this relationship exists, making the neurobiological consequences of such exposure throughout development is still poorly understood. More recently, Naninck et al (2015) showed that chronic early life stress caused by limited bedding altered developmental and adult neurogenesis, as well as cognitive function in mice(39). In addition, Bath et al (2016) suggests that limited bedding promoted accelerated arrival of markers of synaptic maturity and precocious development of emotional learning in mice(40). Taken together, we hypothesize that ELS induces an early maturation of brain regions involved in cognitive processes, although the mechanism behind this effect are not completely understood.

One molecule that plays a role in neuroplasticity mechanisms is the Brain-Derived Neurotrophic factor (BDNF). The BDNF is a protein important for functions like neurogenesis, synaptic and neuronal growth and is a key regulator of learning and memory processes, especially in brain regions such as mPFC and hippocampus. Several previous studies showed that BDNF exon IV is released in an activity-dependent manner(43) and is sensitive to stress early in life(60). Seo et al (2016) showed that rats exposed to maternal separation stress showed decreased expression of BDNF exon IV in the

hippocampus later in life(44). Calabrese et al also found decreased expression of BDNF exon IV in ventral hippocampus and ventromedial pre-frontal cortex when rats exposed to maternal separation reached adulthood(45). Collectively, these data suggests that ELS promotes long-lasting effect in the BDNF signaling that could be harmful to developmental processes in the brain. Corroborating with the hypothesis that ELS induces an early maturation, Suri et al (2013) found that when animals exposed to ELS were tested during young adulthood they showed enhanced neurogenesis followed by increased performance in the Morris Water Maze. But when animals were tested during middle-age, the effect was the opposite: decreased expression of BDNF exon IV and cognitive impairments(46). Clearly, BDNF exon IV is playing a role in ELS-induced changes throughout development, making him a candidate to study the impact of Limited Bedding on cognition and neuroplasticity during adolescence.

Another molecule involved in learning and memory is dopamine [DA; for review, see (47). Andersen et al (2000) showed that, in a normal developing mouse brain, Drd1 and Drd2 density peaked in rodents with 40 days of age in the mPFC, period that correspond to adolescence(48). Early studies investigating the role of DA in learning and memory proposed a principle that PFC DA, acting via activation of Drd1, has an "inverted-U shape" effect, where either blocking DA in the mPFC or excessive Drd1 signaling would have an deleterious effect on cognition(49-51). Novel studies, however, proposed a more complex model taking into account the different DA receptors and cognitive operations(52). Pharmacological stimulation of Drd1 can improve working memory performance when longer delays are required to perform the task. In the other hand, administration of Drd1 agonist impaired working memory in a short-delayed working memory task. Regarding Drd2 activation in the mPFC, modulation of working memory might have a more linear function compared to Drd1, with lower levels of Drd2 activation associated with better performance and higher levels of activation associated with poorer performance(53). More recently, Brenhouse et al (2013) showed that rats exposed to maternal separation have exacerbated Drd1 expression during adolescent and a blunted Drd2 expression(54).

Although we have evidences to support the hypothesis that early life stress impairs learning and memory during adolescence, the specific consequences of Limited Bedding are not clear. Thus, we firstly sought to investigate how Limited Bedding could affect cognitive performance in tasks dependent of correct mPFC functioning, looking at BDNF and Dopamine receptors D1 and D2 expression as neurobiological correlates. Secondly, we looked at a hippocampal-dependent task to verify if Limited Bedding could impact this brain region as well or if the effect is specifically related to working memory.

METHODS

Animals and Experimental Design

The present study was performed was conducted in accordance with the guidelines of the Brazilian Association for Laboratory Animal Sciences and all the procedures described above were approved by the local Ethics Committee for Animal Research under the #15/00472. In this study, female C57BL/6 acquired from CEMBE/PUCRS were breed with a male for 48h and the day of birth was considered PND (post-natal day) 1. From PND2 to PND9, mice were exposed to either Limited Bedding or control condition (described in details below) and weaned at PND21, where they were placed with same-sex littermates in 2-3 per cage. After PND21, male animals were randomly assigned to two independent cohort. Animals from the first cohort were designed to perform the radial maze protocol starting at PND42 (corresponding to adolescence). The second cohort of male mice performed the Y-Maze at PND48 and the Step-Down Avoidance between PND49 and PND60. All animals were euthanized at PND60 for tissue collection and molecular analysis.

A total of 48 litters were used (25x23) and to avoid any potential litter effects, no more than 3 animals per litter were used. Additional animals from the same litter were assigned to different cohorts or additional ongoing projects in the laboratory to maintain the criteria of no more than 3 animals/litter/group.

Animal were maintained in automatically controlled room (temperature 23ºC+-1ºC and 12h light/12h dark cycle) with mouse chow *ad libitum* throughout the entire study. Animals that performed the radial maze training were maintained in a regime of *ad libitum* water until they started the training.

Limited Bedding Protocol

The Limited Bedding was performed based on previously described (61, 62). The cage floor was covered with bedding to absorb feces and urine and a stainless steel wire floor was placed 2cm above the floor. The wire has 10mm squared openings and 0,6mm diameter. Dams were not able to retrieve bedding from the floor to build the nest. In addition to the wire floor, 1g of autoclaved cotton was provided to the dams to build the nest. Control cages were provided with normal bedding amount (~150g) and an additional 4g of cotton to incorporate in their nests. On the PND2, dams were assigned to either Limited Bedding or control conditions. Briefly, dams were firstly removed from home cage. Then, pups were removed, one-by-one by hand from the home cage, weighted and placed in the new cage. The dam and the litter were left undisturbed until PND9, when they were removed from the Limited Bedding condition and returned to a normal cage, with access to bedding and 4g of cotton. They received an additional cage cleaning at P16 where the bedding was replaced with another 4g of cotton as well. Dams from control conditions were left undisturbed, except for regular clean cage at PND2, PND9 and PND16.

Radial 8-arm Maze

To investigate the effects of Limited Bedding on learning and memory, we used the four-arm baited version of the radial 8-arm maze adapting the protocol described by Valladolid-Acebes et al (2011)(63). Firstly, animals were exposed to the apparatus containing eight arms, numbered 1 to 8, of 30cm long for 5cm wide, and radiating from a central compartment of 25cm². Two days before starting the protocol, animals were nd habituated to the reward solution (10% condensed milk in water). A falcon tub cap containing 2mL of reward solution was placed in the homecage for 48h for habituation for the solution and to reduce neophobia. To enhance the motivation to search the reward, animals were water restricted for 15h daily. Water bottles were removed from the homecage at 6pm and animals were trained in the next morning. Animals were kept under this water restriction regime until the end of the protocol. After completing the trials of the day, animals were allowed to freely drink water in their home cage. The training protocol consisted of three phases. In all phases, animals performed two consecutive trials. To

avoid olfactory cues, the apparatus were cleaned with Isopropyl Alcohol between trials and between animals. Extra-maze cues were placed in the room and were kept constant during the entire protocol. One experimenter held the training and positioned himself in the same place in the room, serving as one of the extra-maze cues. In the first phase, animals performed two pre-training trials where animals were exposed to the apparatus and all the eight arms were baited and the animal was allowed to explore the maze until collecting all the rewards or 300s elapsed. Twenty-four hours later, the acquisition phase began, where animals were exposed to the apparatus and only four arms were baited. Rewards were placed in even arms (#2, #4, #6 and #8) or odds arms (#1, #3, #5 and #7), randomized between animals. The baited arms remained the same throughout the entire acquisition phase. Animals were allowed to explore the maze until four rewards were collected or 300s elapsed. Two days after the last acquisition trial, animals returned to the apparatus for two retention trials identical to the acquisition phase. In the four-arm baited version of the eight-arm maze, animals should learn to ignore the non-baited arms and explore only the baited arms. Also, they need the working memory to keep track of which arm was already visited within a trial. To analyze the learning and memory of animals throughout the days, the average between trials were calculated for the dependent variables described in Table 1.

Table 1: Description of the dependent variables analyzed in the Radial 8-arm maze.

Variable	Description
Time	Time taken to animal collect all the rewards.
Reference Error	Entry into a non-baited arm.
Working Memory Error	Re-entry, within the same trial, in a baited arm.
Perseverative Error	Re-entry, within the same trial, in a non-baited arm.

Y-Maze

Spontaneous alternation test were conducted in the Y-Maze as previously showed by Wietrzych et al (2005)(64). This test in based on the natural trend that mice have to explore a new environment. When placed in an arm maze, mice will explore one arm and then tend to alternate between arms and

will explore the least recently visited arm. To do so, mice need to use working memory in order to maintain an ongoing record of the previously visited arm.

Briefly, animals were placed in the experimental room for 30min prior testing. All the tests were held in the morning (aprox. 2-3 hour after the light phase) and the luminosity in the room was ~100lux. Animal were then exposed to the Y-maze, which consisted of a single 5-min trial which the animals were allowed to freely explore the three arms of the maze. The starting arm were randomized between animals to avoid placement bias. Alternations were defined by consecutive entries in three arms in an overlapping triplets (i.e., CBA, BAC, ACB, …). The Spontaneous Alternation Score (SAP) was calculated by dividing the number of triplets by possible alternation (total arm entries – 2) x 100. The alternated arm returns (AAR) and same arm returns (SAR) were calculated to investigate errors in spontaneous alternation(65).

Step-Down Avoidance

Animal that performed the Y-maze were trained and tested in the Step-Down Avoidance based on previously published (66, 67). This single-trial Step-Down Avoidance is a classical fear-related, hippocampal-dependent task, where animals learn that stepping down the platform will trigger an aversive stimulus (foot-shock). The apparatus consisted of acrylic 50x25x25cm box with a stainless steel bars floor spaced 1cm apart containing a small platform (5cm²) in a corner of the box. In the training session, animals were placed on the platform facing the corner of the box and latency to step-down with all four paws was measured manually. Immediately after stepping down, animals received three 1s 0.5mA footshock (2s apart) and then were returned to the home cage. Retention test were carried out 48h after training and was procedurally identical to training, but no foot shock was presented. Latency to step-down was used as dependent variable as a measure of retention.

Gene expression analysis

Mice brains were collected 2h after the last retention trial in the Radial Maze test and rapidly dissected on ice for medial Pre-Frontal Cortex (mPFC) isolation. After dissection, brain regions were snap frozen in dry ice and maintained at -80ºC until analysis. Considering that animals from Y-Maze and Step-

Down avoidance cohort were performed to provide additional behavioral data from different tasks, the tissue from these animals were not collected. Total RNA were isolated from tissue using Trizol® (Qiagen - Hilden, Germany) reagent following manufacturer's protocol and reconstituted in 20uL of RNase-free water. The concentration of RNA was measured using NanoDrop (Thermo Fisher Scientific – Waltham, EUA) and a total of 500ng of RNA from each sample was reverse transcribed using miScript II RT Kit (Qiagen - Hilden, Germany) and cDNA used in each real-time quantitative PCR (RT-qPCR) reaction in the RotorGene (Qiagen - Hilden, Germany) machine using the miScript SYBR green kit (Qiagen - Hilden, Germany).

The primers following primers, purchased from IDT, were used: *BDNF exon IV*: Forward: GCAGCTGCCTTGATGTTTAC; Reverse: CCGTGGACGTTTACTT; *Drd1:* Forward: ATGGCTCCTAACACTTCTACCA; Reverse: GGGTATTCCCTAAGAGAGTGGAC; *Drd2:* Forward: ACCTGTCCTGGTACGATGATG; Reverse: GCATGGCATAGTAGTTGTAGTGG; *Pgk:* Forward: TGCACGCTTCAAAAGCGCACG; Reverse: AAGTCCACCCTCATCACGACCC. Gradient PCR and DNA gels were ran for specificity analysis, as well as melting curve analysis. Each RT-qPCR were run in duplicates for each sample and repeated once. The fold-change relative expression calculation with ΔΔCt method was used with the control group as reference and the Pgk Ct values as endogenous controls for mRNA analysis.

Statistical Analysis

All statistical analysis were performed using the SPSS 20.0 (IBM – New York, EUA) and the graphs were constructed using the Prism GraphPad 6.0 (La Jolla, EUA). Normality of the data distribution were analyzed using Shapiro-Wilk's test. A repeated-measures ANOVA with the between subjects factors "rearing" (control or Limited Bedding) and the within subjects "session" to analyze time to complete the task and number of total errors was performed. Bonferroni *Post* Hoc analyses were ran after repeated measures to determine whether the differences were significant between groups during acquisition phase. In order to compare the number of errors in the retention test, as well as gene expression, a student's t-

test was performed to each dependent variable that were normally distribute. Dependent variables that were not normally distributed were analyzed by Mann-Whitney's U test. To evaluate mice performance on Step-Down Avoidance, a one-way ANOVA was used with latency to step down as dependent variable with Tukey multiple comparisons. In all analysis, data are expressed as mean ± SEM and the level of statistical significance was set as 5%.

RESULTS

Male Mice raised in the Limited Bedding committed fewer perseverative errors in a radial-arm maze

The first cohort of animals was trained in the eight-arm radial maze in order to evaluate the spatial learning and memory. As expected, statistical analysis revealed a significant "session" effect, indicating that regardless the experimental conditions, both groups decrease the time to complete the task throughout the acquisition phase [Figure1A: $F(5.27, 290.19)=3.443$, $p = 0.004$]. In addition, animals from both group decreased the number of total errors during the acquisition phase, suggesting that both controls and LB animals proficiently learn the task during acquisition phase [Figure 1B: $F(5.00,275.31) = 5.546$, $p < 0.001$].

After completing the acquisition phase, a retention test was conducted 48h after the last trial. Regarding the reference errors, student's t-test showed no significant differences between groups (Figure 1C: $t(55)=0.770$, $p = 0.44$). Also, when looking at the working memory errors, no differences were observed between groups as well (Figure 1D: $U = 191$, $p = 0.06$). However, interestingly, there is a statistically significant difference between groups in Perseverative Errors, which is a re-entry in a previously visited non-baited arm within that trial, with LB animals committing fewer errors than controls (Figure 1E: $U = 163$, $p = 0.01$). Differently than expected, these data suggests that controls and LB animals presented a similar learning pattern but LB animals showed less perseverative errors compared to controls.

Limited Bedding affects Working Memory in the Y-Maze

To further investigate this effect, an independent set of male animals were trained in a different working memory task using the Y-Maze. No group differences were observed in locomotor activity (measured as number of arm entries; Figure 2A: $t(35)=0.881$, $p = 0.38$). Also, no differences in Spontaneous Alternation Performance nor Alternated Arm Return (Figure 2B: $t(35)=1.000$, $p = 0.32$ and Figure 2C: $t(35)=0,415$, $p = 0.68$, respectively) were present when comparing both groups. However, corroborating with the eight-arm radial maze, animals raised in Limited Bedding presented fewer same arm return errors when compared to controls [Figure 2D: $t(33)=2.703$, $p = 0.01$].

Retention of a fear-related memory is preserved in Limited Bedding-raised mice

We also tested animals in a fear-memory, hippocampal-dependent task to investigate if the working memory effect we observed in the retention trials were due to a better retention capability. Thus, looking to the latency to step-down in the training session, animals from both group quickly explored the apparatus [Figure 3: Controls mean: 22.15s±6.5; IE mean: 19.82s±3.6; $F(3.93) = 24.15$, $p <0.001$]. When comparing the latency to step-down in the test session, multiple comparisons showed that animals increased the latency to step-down ($p <0.05$) suggesting that both groups learned the task. However, no group difference was observed ($p > 0.05$) suggesting that regardless of rearing conditions, both groups took equivalent time to step down in the test session. Thus, the observation that LB animals committed fewer perseverative errors are not due to a better retention capability, suggesting that the effects observed are specific to working memory processes.

BDNF exon IV is differentially expressed in the mPFC with no differences in Dopamine Receptors D1 and D2 mRNA levels

To investigate possible neurobiological correlates in the LB animals, we looked at the BDNF exon IV mRNA levels and Dopamine Receptors D1 and D2. Mann-Whitney's U test showed a statistically significant difference between controls and LB animals regarding BDNF exon IV, with LB showing an increased expression in the mPFC compared to controls [Figure 4A; ($U = 6.00$, $p = 0.03$)]. No differences

in D1 and D2 mRNA levels were observed between groups [Figure 4B: U = 14.00, p = 0.55) and Figure 4C: t(10)=0.048, p = 0.96, respectively).

DISCUSSION

This study aimed to investigate the impact of Limited Bedding on working memory and spatial learning during adolescence, as well as differentially expressed neurobiological correlates in the mPFC. Interestingly and different than expected, male mice raised in LB conditions presented fewer perseverative errors compared to our reference group. This effect was followed by an increased BDNF exon IV expression in the mPFC with no differences in Drd1 and Drd2.

In our study, we used four-arm baited version of the eight-arm radial maze, which allows us the investigation of the impact of Limited Bedding in both working memory and spatial reference in mice. More recently, Yoon et al (2008) showed that selective inactivation of mPFC with muscimol leads to impaired working memory. In the other hand, inactivation of dorsal hippocampus increased the number of reference errors in that given task(68). Moreover, inactivation of the mPFC of rodents impaired the behavioral flexibility, capability to inhibit an old strategy and utilize a new one(69, 70). Interestingly, mice exposed to Limited Bedding performed better than controls in both independently conducted cohorts of animals. This is contrasting with the findings of previous studies that used the limited bedding model of Limited Bedding. Rice et al (2008) found that mice raised under limited bedding conditions had impaired performance in the Morris Water maze later in life(61). Kanatsou et al (2017) showed that mice exposed to the same limited bedding model showed impaired memory formation in an object-location task(71).

One important factor is the timing of test. The age of mice when tested ranged from adolescence to aged animal, which might by a confound factor to make a conclusion. More studies are required at specific timings to make a more reliable conclusion. Adolescence is a critical period of development with the onset of puberty, marked by fundamental alterations in the brain(72). A lot of demands that rises during adolescence requires high cognitive flexibility, like peer-relationship changes(73), as well as transition to higher education and professional career. Failure to adjust to this new environmental

requirements could cause several negative consequences like dropout from school, social exclusion and psychiatric disorders(74).. In rodents, for example, Koss et al (2011) showed that adolescent rats committed more perseverative errors than adults rats in a delayed alternation task, mPFC-dependent(75). Taken together, we can rationalize that adolescents will present perseveration during adolescence given that the brain is still under development. However, following the rationale that early-life stress could accelerates the maturation of affected brain regions(40, 76), we hypothesize that ELS adolescents presenting fewer perseverative behaviors than controls could be considered as having adult-like behaviors. However, caution should be taken to claim this, as further experiments are needed to confirm this hypothesis.

Regarding reference errors in the eight-arm maze and fear consolidation, both hippocampal-dependent tasks, no differences were observed among our experimental groups. We firstly hypothesize that limiting the bedding and nesting material from the dams would impact these tasks based on previous studies showing that this model reduced rat long-term potentiation and number of dendritic spines in CA3 area of hippocampus(77). Also, synaptic plasticity within the CA1 and CA3 areas of hippocampus are reduced after limited bedding conditions(78). Even that these studies tested animals in different periods of life than ours study, our study contribute to the literature another evidence of the impact of Limited Bedding in cognitive processes during adolescence.

Our study explored BDNF exon IV in an important brain region that regulates both working memory and reward-guided learning(79). We found that BDNF exon IV expression is up-regulated in Limited Bedding -raised animals. This finding is interesting since animals raised under same conditions also showed enhanced working memory performance in a task that is mPFC-dependent. This exon is expressed in an activity-dependent manner and plays a role in cellular functions related to learning and memory (41). There are evidences in the literature that early life promotes a down-regulation in the expression pattern of BDNF during developing. This contrast with our study could be explain by the differences in the early life stress models employed, since the majority of classical studies investigating

this signaling pathway used maternal separation as a stress protocol. One hypothesis is that, depending on the stress intensity during development, early stress exposure could lead to a resilience or vulnerability later on life. Even though we did not compared stress levels and models in our study to claim that conclusion, exploring more the differences in stress during development to promote resilience could be an interesting topic for future research.

Regarding dopamine receptors D1 and D2 expression, we did not observed any statistically significant difference between groups in the mPFC. It is important to state that we looked at the gene expression layer and some consideration could be made based on this approach before generalizing or jump to conclusions. First, mRNA levels do not always correlate with protein levels, mainly because there are many post-transcriptional mechanisms of regulation between mRNA transcription and protein translation(80). However, profiling the transcriptome as a screening for the discovery of new potential targets has been used for decades with the advances of technology(81, 82). Thus, looking at the mRNA levels could be a useful screening tool but careful should be made to major conclusions based solely on this observation.

This study has some limitations that needed to be clarified before generalizing the findings. First, this study was conducted just with males. There is a sex difference response to stress regulates behaviors dependent on the mPFC dopaminergic signaling(48, 83, 84). Although this was not the aim of this work, future studies addressing how early-life stress modulates dopamine in both sexes could provide a better framework of the neurobiology of early life stress. Second, as mentioned before, we investigated molecular targets looking at the gene expression layer. Although looking at the transcription levels of receptor and its ligand could provide useful screening information, maybe looking at the protein levels could provide a more consistent story. Also, manipulating these targets using specific antagonist or agonists could help to stablish a cause-effect relationship. Finally, the hands-free dissection technique used for brain region isolation is appropriated and relatively easy to conduct but does not allow for sub region specificity or cell-type specificity, which was already been showed that dopamine acts as a

neuromodulator in a manner that depends on the functional state of the target neurons in different brain regions(85).

CONCLUSION

In this study, we focused on the cognitive performance of mice that were raised under Limited Bedding and how BDNF exon IV and Dopamine receptors D1 and D2 would be expressed during adolescence. Taken together, our study showed that mice exposed to Limited Bedding showed fewer perseveration and increased BDNF exon IV expression in the mPFC during adolescence. Our study provide new evidences on the impact that poverty has on cognition during adolescence, which could help to understand the consequences that being raised under poverty-like conditions could have on adolescent cognition.

FIGURES

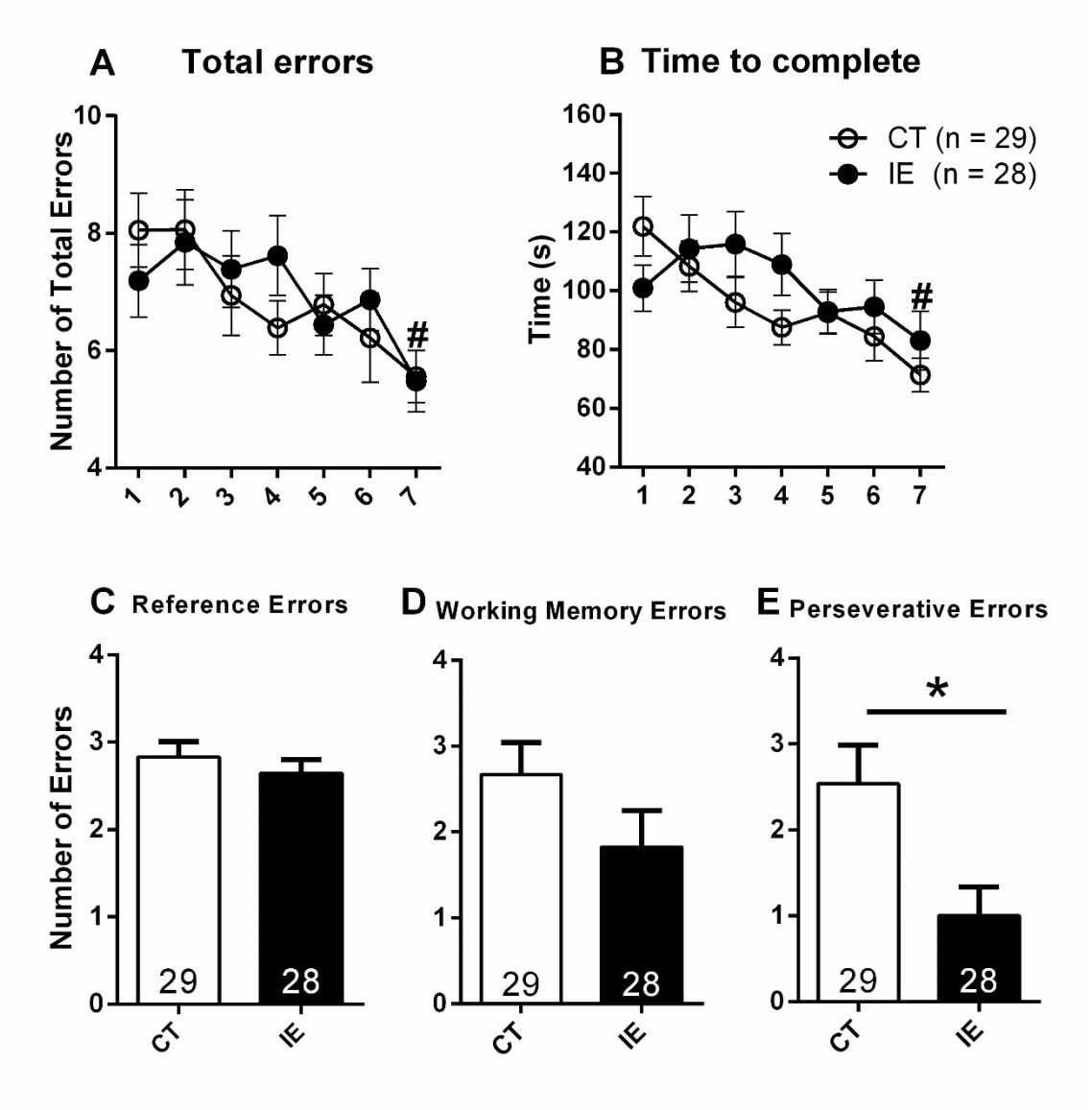

Figure 1 – Radial 8-arm Maze performance: A) Number of total errors during the acquisition phase of the radial 8-arm training; B) Latency to complete the task throughout the acquisition phase; C) Number of reference errors in the retention test; D) Number of working memory errors errors during the retention test; E) Number of Perseverative Errors during the retention test. Number inside the bars represent the sample size; Data are presented as mean± SEM; * represent $p < 0.05$; # represent $p < 0.05$ compared to the first day of the acquisition phase.

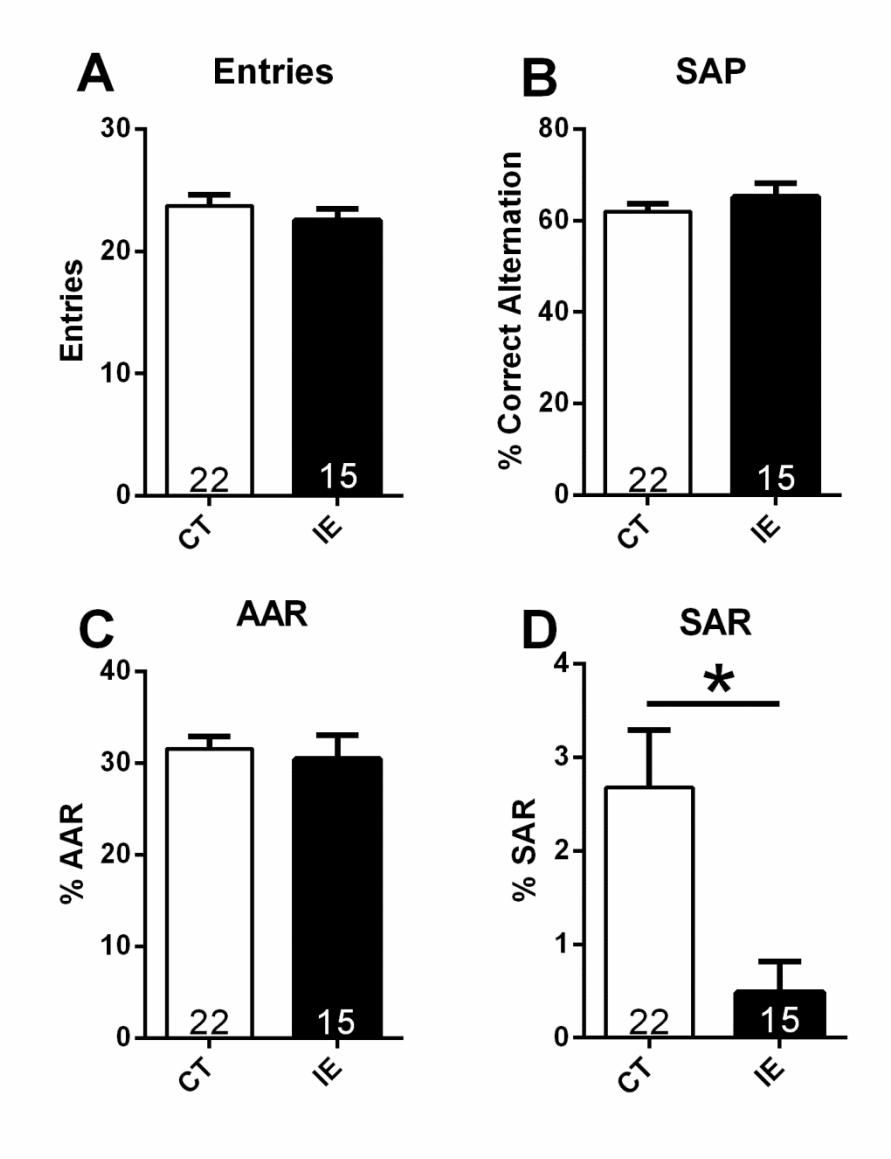

Figure 2 – Y-Maze Spontaneous Alternation Performance: A) Number of total arm entries; B) % of correct alternation, Spontaneous Alternation Performance (SAP); C) % Alternated Arm Return (AAR); D) % Same arm return (SAR); Number inside the bars represent the sample size; Data are presented as mean± SEM; * represent $p < 0.05$.

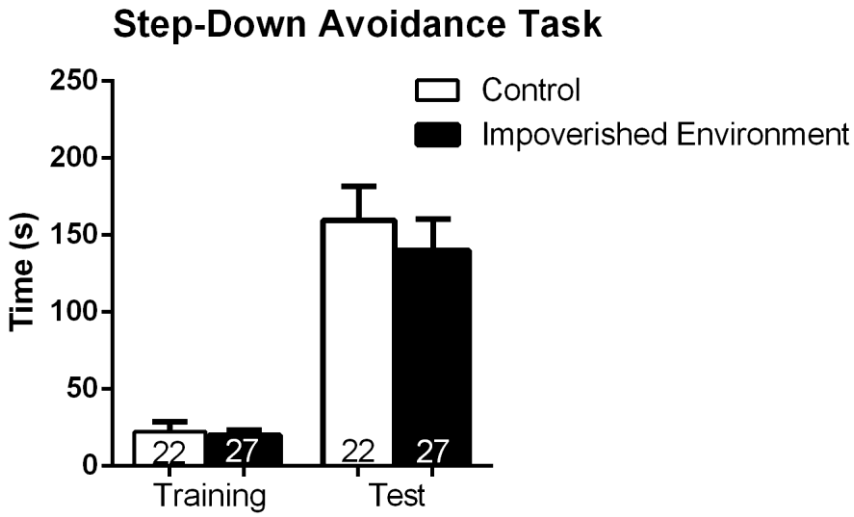

Figure 3 - Step-Down Avoidance Task: Latencies to step-down from the platform during training and test session. Data are presented as mean± SEM. Number inside bars represent sample size.

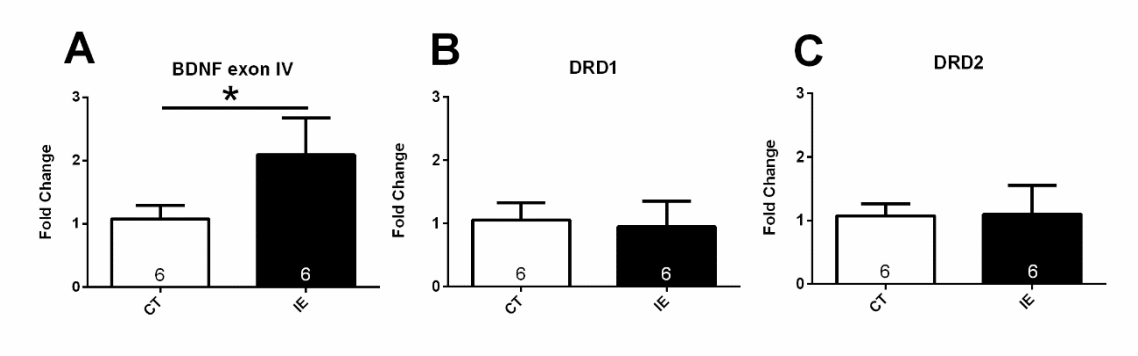

Figure 4 – Gene expression Analysis: A) Expression of BDNF exon IV mRNA levels in the mPFC of animals that underwent radial 8-arm maze training. B) Expression of Drd1 in the mPFC; C) Expression of Drd2 in the mPFC; The fold-change relative expression calculation with ΔΔCt method was used with the control group as reference and the Pgk Ct values as endogenous controls for mRNA analysis; Data are expressed as mean ± SEM; * represent $p < 0.05$.

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6.3 ESTUDO 2

Em preparação para submissão para B*ehavioural Brain Research*

Impoverished environment impairs walking adaptability and changes BDNF/TrkB and Drd1/Drd2 expression in different mouse brain regions

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ABSTRACT

Introduction: Evidence suggests poverty impairs development, quality of life and increases vulnerability to disease and death. One important aspect of poverty is its impact on cognitive-motor development, since children raised in families with low socioeconomic status (SES) have impaired motor skills when compared to children with high SES. This study aimed to investigate how impoverished environment affects walking adaptability of mice, while investigating BDNF/TrkB and Drd1/Drd2 expression in different brain regions. **Methods:** Briefly, we exposed male C56BL/6 to the limited bedding protocol (LB) from post-natal day (PND) 2 to PND9 and then tested animals in the ladder walking task at PND60. RT-qPCR was used to investigate gene expression in the mPFC, Motor Cortex and Cerebellum 2h after the task. **Results:** When analyzing the walking adaptability of the mice, we found two distinct subgroups of animals within the LB group: a) superior performance (SP); and b) inferior performance (IP), compared to controls. Additionally, Drd1 expression was increased in the mPFC of IP animals and in the cerebellum of SP animals, while Drd2 expression was unchanged. BDNF expression in the mPFC and motor cortex was increased in both the IP and SP subgroups. No inter-group differences were found in TrkB mRNA expression in the analyzed brain regions. A strong negative correlation was found between walking adaptability performance and BDNF exon IV expression in the motor cortex. Conversely, a positive correlation was found between walking adaptability performance and TrkB expression in the mPFC. **Conclusions:** Overall, our findings suggest exposure to impoverished environment in early life leads to distinct walking adaptability phenotypes, which may be related to differential BDNF and Drd1 expression in brain regions that influence walking adaptability.

Keywords: Early Life Stress, Walking Adaptability, Dopamine, BDNF.

1. Introduction

The impact of poverty on neural development has been studied for many years, since poverty decreases quality of life and increases mortality and vulnerability to a wide range of diseases (1, 2).

Deprivation of basic resources such as food and shelter has critical consequences during childhood. Premature birth and poverty in the first three years of life increases the likelihood of growth, intelligence and behavior-related problems (3). Also, children from low-socioeconomic status (SES) families tend to experience inadequate neurodevelopment (4). Poverty would also seem to have a negative impact on motor development, a key factor to promote health and physical activity. For example, children who present higher motor skill proficiency are more likely to engage in physical activity (5, 6). Morley et al (2015) showed children from high-SES families outperformed children from low-SES families in gross and fine-motor skill tasks (7). Comparisons among children based on their SES and/or ethnicity have demonstrated both factors affect balance, object control and overall motor skill proficiency (8, 9). In addition, being raised in poverty also exposes children to a very stressful environment, with a wide range of negative factors that affect development (10).

Whilst vulnerability is linked with stress exposure, not all individuals that experience stressful life events, trauma or chronic adversity will present this vulnerability-like phenotype (11). An early study led by Bradley in 1994 found children living in poverty showed early signs of resilience, functioning in the normal range for cognitive, health and growth parameters at age three (12). However, the resilience-related mechanisms that explain these findings remain unclear.

Although clinical studies show low-SES may influence motor performance in children, few experiments have attempted to shed light on the neurobiological mechanisms involved. For instance, chronic pharmacological activation of glucocorticoid and mineralocorticoid receptors (GR and MR, respectively) led to impairments in a skilled-reaching motor task in rats (13). More recently, Kokubo et al (2017) showed early-life stress exposure resulted in motor coordination dysfunction in adult mice (14). Moreover, the dopaminergic connection between the cerebellum and basal ganglia constitutes an

important pathway within the motor control system (15). Additionally, dopamine activity in the motor cortex has been shown to play a major role in regulating motor learning and motor-cortex plasticity (16). Using antagonists to block dopamine in the motor cortex induced a decrease in long-term potentiation, which is an important mechanism in learning (16,17).

Synergistically with dopaminergic neurotransmission, BDNF plays a role in memory and learning, as well as in synaptic plasticity and neurogenesis (18). For instance, Fritsch and cols. have shown activity-induced BDNF and TrkB activation in the primary motor cortex are required for the acquisition of new motor skills. Indeed, the loss of BDNF has been linked to neurodegenerative diseases such as Parkinson's Disease, Huntington's Disease and Amyotrophic Lateral Sclerosis (19). BDNF levels and its receptor TrkB are crucial for motor development (17,19) and may provide insights into the relationship between impoverished environment, stress and walking adaptability. Thus, a better understanding of the mechanisms behind early life stress exposure could contribute towards reducing the negative effects such stress has on motor control.

However, to the best of our knowledge, no previous studies have investigated the impact of early life stress (impoverished environment) on walking adaptability in the adulthood. In the same way, whether dopaminergic and BDNF expression in the medial pre-frontal cortex, motor cortex and cerebellum influence walking adaptability is still unknown. Therefore, the present exploratory study was designed to address these issues.

2. Material and methods

The present study was conducted in accordance with the guidelines of the Brazilian Association for Laboratory Animal Sciences and all the procedures described above were approved by the local Ethics Committee for Animal Research under the #15/00475. In this study, adult female C57BL/6 acquired from CEMBE/PUCRS were mated with a male for 48h. The day of birth of the resulting litters was considered post-natal day (PND) 1. From PND2 to PND9, the pups were exposed to either impoverished environment

or control condition (described in detail below) and weaned at PND21, when they were placed with same-sex littermates in 2-3 per cage. At P60, animals were assessed using the ladder walking task (described in detail below). A total of 20 litters were used (10x10) and to avoid any potential litter effects, no more than 2 animals per litter were used. Animals from the same litter were assigned to additional ongoing research projects. The animals were maintained in automatically controlled room (temperature 23ºC+-1ºC and 12h light/12h dark cycle) with mouse chow and water *ad libitum* throughout the entire study.

The impoverished environment was performed based on a previously described protocol (20, 21). The cage floor was covered with bedding to absorb feces and urine and a stainless steel wire floor was placed 2cm above the floor. The 0.6mm gauge wire floor has 10mm squared openings. Dams were unable to retrieve bedding from the floor to build nests, however 1g of autoclaved cotton was provided for this purpose. In the control group cages, there was no wire floor and the normal amount of bedding (~150g) was provided in addition to 4g of cotton for nest building. On PND2, dams were assigned to either impoverished environment or control conditions. Briefly, dams were firstly removed from the home cage. Then, pups were removed, one-by-one by hand from the home cage, weighed and placed in a new cage. The dam and the litter were left undisturbed until PND9, when they were removed from the impoverished environment condition and returned to a cage similar to that of controls. In addition, the cages were cleaned on a PND16 when the bedding was replaced. Dams from control conditions were left undisturbed, except for regular cage cleaning on PND2, PND9 and PND16.

To assess the ability to adapt walking of the mice, we used the adapted version of the ladder walking task, as previously described (22, 23). Briefly, mice were encouraged to cross a 1m long horizontal ladder with variably spaced rungs situated 30cm above the floor. During the task session, mice were recorded crossing the ladder three times using a GoPro Hero 4 with 12mp and an acquisition rate of 240 FPS. The first trial was considered habituation and was discounted from the analysis. Thus, the performance score was calculated using the average number of total errors (forelimb errors + hindlimb errors) committed on trials 2 and 3. The numbers of errors were normalized to the control group using the

following formula: (Number of total errors/Mean of total errors from control group) * 100. Hence, a percentage (%) lower than 100% means that animals committed fewer errors than controls, thus performing better. A percentage higher than 100% means that animals committed more errors than controls, thus performing worse.

Mice brains were collected 2h after the last trial in the ladder walking task and rapidly hand-free dissected on ice to access the medial Pre-Frontal Cortex (mPFC), Motor Cortex and Cerebellum. After dissection, brain regions were snap frozen in dry ice and maintained at -80ºC until analysis. Total RNA were isolated from tissue using Trizol® (Qiagen - Hilden, Germany) according to the manufacturer's protocol and reconstituted in 20uL of RNase-free water. The concentration of RNA was measured using NanoDrop (Thermo Fisher – Waltham, USA) and a total of 500ng of RNA from each sample was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems - Foster City, USA). The cDNA used in each real-time quantitative PCR (RT-qPCR) reaction in the RotorGene (Qiagen - Hilden, Germany) machine was processed with the miScript SYBR green kit (Qiagen - Hilden, Germany).

The primers used for RT-qPCR analysis are described in Table 1. Gradient PCR and DNA gels were checked for specificity analysis, as well as melting curve analysis. Each RT-qPCR was run in duplicate for each sample and repeated once. The fold-change relative expression was calculated using the ΔΔCt method (with the control group as reference) and the GAPDH Ct values as endogenous controls for mRNA analysis.

---------- Table 1 here ----------

All statistical analyses were performed using the SPSS 20.0 (IBM – New York, USA) and the graphs were constructed using the Prism GraphPad 6.0 (La Jolla, USA). Data normality distribution was analyzed using the Shapiro-Wilk's test. In order to analyze the effects of impoverished environment on walking adaptability and gene expression, one-way ANOVA was performed followed by Tukey's multiple comparison adjustments. To investigate whether the behavior correlated with gene expression, Pearson's

correlation was used. In all the analyses, data are expressed as mean±SEM and the level of statistical significance was set at 5%.

3. Results

In the ladder walking task, the average percentage of errors in the control group was 100.1±13.13 (n = 13). The animals exposed to impoverished environment showed distinct patterns of error in this task, and were therefore divided into two subgroups: a) superior performance (SP) - mice showing at least one standard deviation below the mean of the control group (n=6, showed fewer errors during the task) and b) inferior performance (IP) - mice exhibiting at least one standard deviation above mean of the control group (n=7, showing more errors during the task). Another six animals exposed to impoverished environment performed similarly to the controls (performance score between 86.97-113.23) and therefore were not included in the analysis. These selection criteria were adapted from a previous study led by Albuquerque Filho, et. al. (2017) (24).

One-way ANOVA showed a significant difference among groups when comparing the performance in the ladder walking task [Figure 1; $F(2,23) = 28.99$, $p < 0.0001$), as expected. Subsequent analyses using Tukey's post hoc test confirmed that animals in the SP group had fewer errors relative to the control group ($p < 0,001$). Animals in the IP group committed more errors relative to controls ($p < 0,01$).

---------- Figure 1 here ----------

To investigate the connection between the expression of dopaminergic receptors and walking adaptability, we first assessed Drd1 expression in the mPFC, motor cortex and cerebellum. Results related to Drd1 expression in those brain regions are shown in Figure 2A. We observed Drd1 expression was increased in the mPFC of the IP animals [$F(2,16) = 9.89$, $p = 0.0016$)]. We also showed Drd1 increased in the cerebellum of the SP group compared to controls [$F(2,15) = 6.97$, $p = 0.007$)]. Secondly, regarding the Drd2 expression there were no between-group differences in the mPFC, motor cortex and

cerebellum [Figure 2B; $F(2,17) = 0.03$, $p = 0.96$; $F(2,15) = 0.02$, $p = 0.97$ and $F(2,16) = 1.35$, $p = 0.28$, respectively]. Interestingly, we found a negative correlation between the expression of Drd1 in the cerebellum and errors in the ladder waking task, which suggests lower cerebellar Drd1 expression may be associated with poor performance in adaptive walking (Figure 3A: Pearson's correlation, $R = -0.63$, $R^2 = 0.39$, $p = 0,005$).

---------- Figure 2 here ----------

One-way ANOVA showed a significant difference in mPFC expression between groups [$F(2,16) = 7.18$, $p = 0.005$)]. Tukey's post-hoc analyses revealed both the SP and IP groups increased the expression within the mPFC when compared to controls ($p = < 0.05$ and $p = 0,01$, respectively). BDNF exon IV expression in the motor cortex was significantly different between the SP and IP groups [$F(2,15) = 7.86$, $p = 0.004$); Tukey's post-hoc $p < 0.01$]. No between-group differences for this gene were observed in the cerebellum [$F(2,15) = 1.68$, $p = 0.21$)]. Figure 2C shows the BDNF exon IV expression. A strong negative correlation was found between BDNF exon IV expression in the motor cortex and walking adaptability (Figure 3B). This finding suggests lower expression of this transcript in the motor cortex is also associated with poor performance in adaptive walking (Pearson's correlation, $R = -0.71$, $R^2 = 0.51$, $p < 0.001$).

Finally, we measured the expression of TrkB, a BDNF receptor, in the same brain regions. A one-way ANOVA revealed no differences between groups [Figure 2D; $F(2,17) = 1.34$, $p = 0.28$; $F(2,16) = 0.33$, $p = 0.71$ and $F(2,13) = 1.13$, $p = 0.35$, respectively]. However, a positive correlation between TrkB expression in the mPFC and the ladder walking task performance was found (Figure 3C), which suggests the higher expression of TrkB expression in this brain region is necessary to properly adapt walking (Pearson's correlation, $R = 0.52$, $R^2 = 0.27$, $p = 0.02$). No additional correlations were revealed.

---------- Figure 3 here ----------

4. Discussion

In this study, we have used a mouse model to investigate: a) the impact of an impoverished environment early in life on walking adaptability; and b) whether the impoverished environment-related changes in dopaminergic and BDNF expression in the medial pre-frontal cortex, motor cortex and cerebellum influence walking adaptability. As expected, animals responded differently to the effects of impoverished environment - some animals showed a superior performance in the ladder walking task, while other animals showed an inferior performance. This finding suggests that some individuals exposed to stressful conditions are more resilient and maintain normal physiological and behavioral responses. Whilst several reports have described the effects of early life stress on animal physiology and behavior, little is known about the stress response of individuals (25). Moreover, the biological mechanisms underlying the different responses after suffering threats are still unclear. Our study has started to fill this gap in the literature by subdividing the animals based on their performance in the ladder walking task and trying to establish some neurobiological correlations to guide this research field.

The mPFC establishes stress-related behaviors (26) and plays a role in adaptive waking (27). We observed the IP group increases Drd1 expression in mPFC, whereas impoverished environment increased BDNF exon IV expression in both the IP and SP groups. No differences were found for Drd2 expression. On the one hand, dopamine has both positive and negative effects on cognition, exhibiting an inverted U-shaped dose-response curve (for review, see Savitz et al, 2006 (27)). Gene expression changes in the dopaminergic signaling pathway are linked with the resilient/vulnerability profile, which is in agreement with our data (28). In addition, we also found a negative correlation between cerebellar Drd1 gene expression and walking adaptability performance in mice. The literature shows dysfunction in dopaminergic signaling impairs gait automatism by modifying cerebellar processing (29), which is aligned with the current results. On the other hand, stress triggers an activity-dependent BDNF upregulation, as seen in the mPFC results, probably acting as a compensatory mechanism to recover and reestablish neuronal function and prevent neuronal loss (30-32). However, the performance in the ladder waking task

was uncorrelated with the expression of both Drd1 and BDNF exon IV genes in the mPFC. Hence, studies assessing other genes are needed to clarify how the mPFC acts to control walking adaptability.

To the best of our knowledge, this is the first study to show a positive correlation between BDNF exon IV expression in the motor cortex and performance in adaptive walking. In other words, higher levels of BDNF exon IV in the motor cortex may induce more precise walking. This hypothesis is supported by previous studies, since BDNF mediates important downstream signaling pathways involved in learning and memory, such as the long-term potentiation (33) and TrkB activation (34). Thus, walking adaptability might involve BDNF exon IV as a signaling mediator in the motor cortex, which is indeed a matter for further investigation.

Whilst the TrkB expression remained the same for all studied groups, we found a positive correlation between TrkB mRNA expression in the mPFC and errors in adaptive walking. This finding suggests TrkB signaling in the mPFC may play a role in linking higher brain functions (such as working memory and attention) to the process of adjusting interlimb coordination to the environmental context. BDNF and other neurotrophic factors such as NT-3 and NT-4 (involved in learning and brain plasticity) bind with high affinity to TrkB receptors (35). Additionally, Choi et al 2012 has shown inhibiting both BDNF and TrkB in the mPFC impairs the formation of memory (36), which may be required to adjust and adapt walking. Notwithstanding, further studies blocking TrkB receptors in the mPFC (using techniques such as lentiviral-mediated knockdown or antagonist administration directly in the mPFC) are needed to establish a causal relationship between TrkB activation and adaptive walking.

This study has some limitations. Firstly, only male mice were used to avoid hormonal cycling influences. Therefore, the connection between impoverished environment and walking adaptability in female mice is unknown. Secondly, we investigated molecular targets looking at the gene expression layer. Although useful as an exploratory screening, further studies addressing the proteins at cell-type level are needed to confirm our findings. Furthermore, using specific receptor antagonists or agonists

could help to establish a cause-effect relationship. Finally, the free-hand technique of brain dissection prevents sub region analyses, which should be considered when generalizing the molecular results.

5. Conclusion

Together, our findings suggest an impoverished environment early in life can lead to distinct phenotypes (superior or inferior) of walking adaptability in adolescent mice. In addition, cerebellar Drd1, motor cortex BDNF exon IV and mPFC TrkB expressions were correlated with walking adaptability performance and may play a role in its neural control. Further studies addressing the questions raised here should provide more conclusive evidence relating early life stress and walking adaptability.

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FIGURES

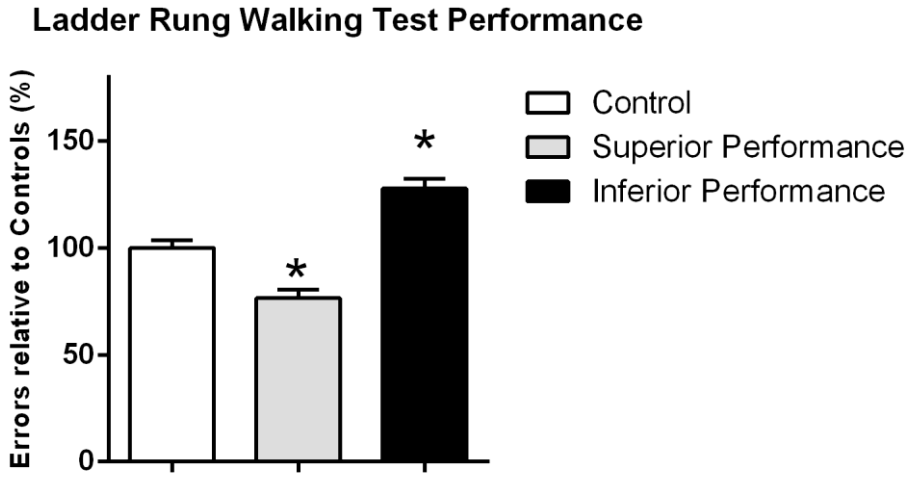

Figure 1. Walking adaptability of mice exposed to impoverished environment. Performance in the task is presented as percentage of errors compared to the control group. The average (%) of errors from the control group was 100,1±13.13 (n = 13); Superior Performance n = 6; Inferior Performance n = 7. Data are expressed as mean ± SEM; * represent $p < 0.05$ compared to the control group.

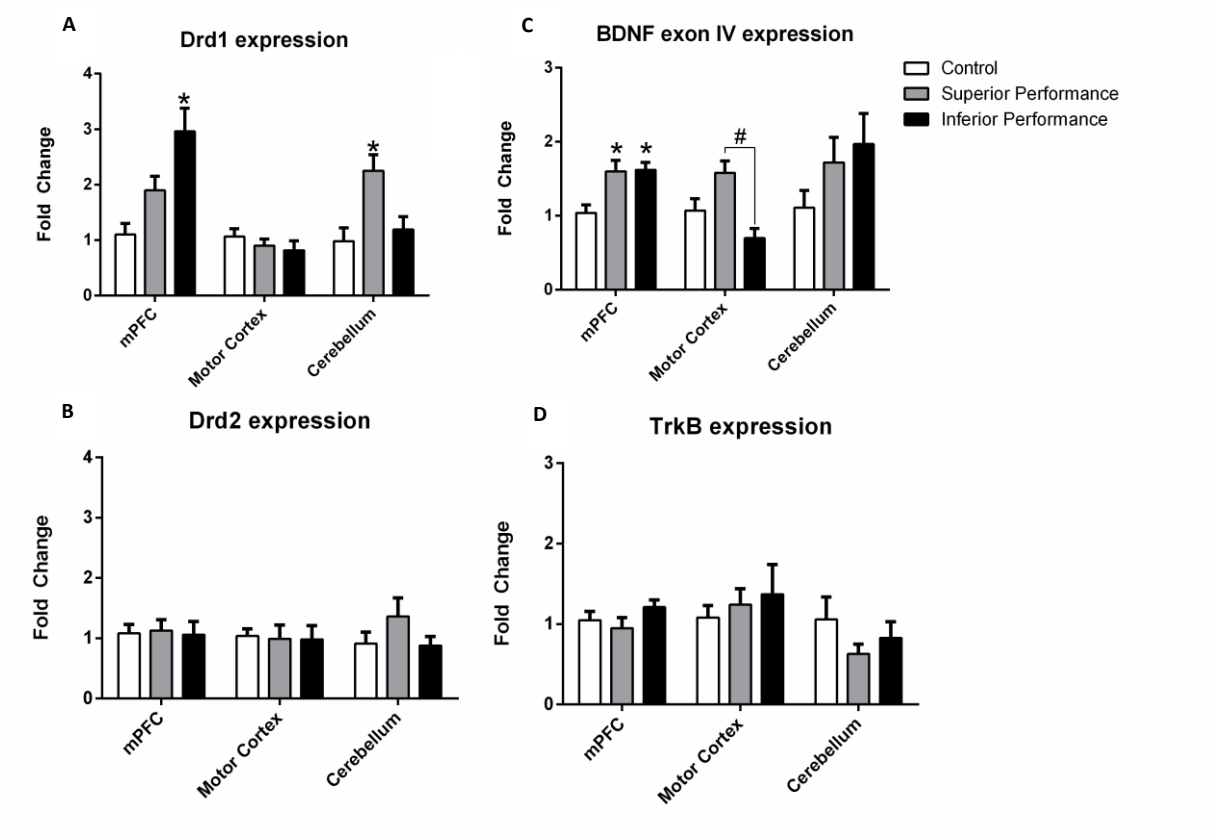

Figure 2. Drd1, Drd2, BDNF exon IV and TrkB levels. A) Expression of Drd1 mRNA levels in different brain regions of animal 2h after the ladder walking task. B) Expression of Drd2 mRNA levels in different brain regions; C) Expression of BDNF exon IV levels in different brain regions of animal 2h after the ladder walking task; D) Expression of TrkB mRNA levels in different brain regions. The fold-change relative expression calculation with ΔΔCt method was used with the control group as reference and the Pgk Ct values as endogenous controls for mRNA analysis; Control group n = 7; Superior Performance n = 6; Inferior Performance n = 7 Data are expressed as mean ± SEM; *p < 0.05 compared to the control group. # p < 0.05 between SP and IF groups.

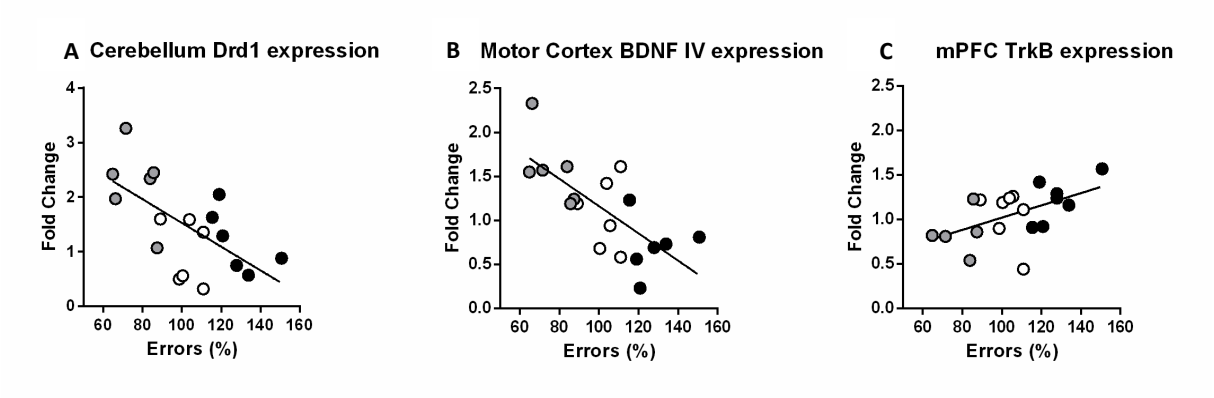

Figure 3. Correlation between walking adaptability and gene expression. A) Pearson's correlation between percentage of errors in the ladder walking task and Cerebellum Drd1 mRNA expression ($R = -0.63$, $R^2 = 0.39$, $p = 0.005$). B) Pearson's correlation between percentage of errors in the ladder walking task and motor cortex BDNF exon IV mRNA expression ($R = -0.71$, $R^2 = 0.51$, $p < 0.001$). C) Pearson's correlation between percentage of errors in the ladder walking task and mPFC TrkB mRNA expression ($R = 0.52$, $R^2 = 0.27$, $p = 0.02$).

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