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Influência do enriquecimento ambiental e do estresse imprevisível em camundongos pré-selecionados pelo perfil exploratório

PORTO ALEGRE, 2010.

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas- Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do título de doutor em Ciências Biológicas –Bioquímica.

Orientador: Prof. Dr. Diogo Rizzato Lara

PORTO ALEGRE, 2010.

Sem ambição, nada se começa. Sem esforço, nada se completa.
(Ralph Waldo Emerson)

Ao meu marido, Guilherme e à minha filha, Giulia
pelo carinho, amor e paciência.

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APRESENTAÇÃO

Esta tese está organizada em três partes:

Parte I: Resumo, Introdução e Objetivo Geral e Específico;

Parte II: Resultados que estão apresentados na forma de artigos científicos. Cada artigo representa um Capítulo e são subdivididos em: Introdução, Materiais e Métodos, Discussão e Referências Bibliográficas;

Parte III: Discussão, Conclusão, Perspectivas e Referências Bibliográficas citadas na Introdução da Parte I e na Discussão da Parte III.

Os trabalhos realizados durante esta tese foram desenvolvidos no Departamento de Bioquímica da UFRGS, laboratório 26, sob a orientação do Prof. Dr. Diogo Rizzato Lara.

SUMÁRIO

Parte I

RESUMO

O comportamento exploratório pode ser entendido a partir de uma perspectiva bidimensional envolvendo comportamentos inibitórios (evitação de dano) e comportamentos de ativação (busca de novidades). Estes comportamentos podem ser observados em animais e variam de acordo com suas diferenças individuais. Tais diferenças podem ser úteis para testar hipóteses sobre as bases biológicas do temperamento. O objetivo desta tese foi avaliar a efeito do estresse subcrônico imprevisível e do enriquecimento ambiental (EE) em camundongos da CF1, selecionados por seu comportamento exploratório na área central de um campo aberto: os menos exploradores (LE) e os mais exploradores (HE). Também foram investigados o número de astrócitos e a densidade óptica de GFAP e os níveis de S100B hipocampais destes camundongos HE e LE submetidos ao protocolo de EE. Além disso, estudamos se estes camundongos HE e LE se diferenciavam quanto à hiperlocomoção induzida pela anfetamina, dizocilpina, cafeína e apomorfina. Após o estresse subcrônico imprevisível tanto LE quanto HE apresentaram menor atividade exploratória, mas suas diferenças no comportamento exploratório permaneceram. Este protocolo de estresse por um curto período, não induziu alterações na ingestão de sacarose ou no tempo de imobilidade na tarefa de suspensão pela cauda.Os camundongos LE apresentaram menor desempenho na tarefa de reconhecimento do objetos (NOR) após o estresse. Os HE apresentaram níveis de corticosterona menores quando comparados com os LE em condições normais, sendo que os níveis de corticosterona aumentaram após o estresse apenas nos camundongos HE. BDNF hipocampal nos camundongos LE foi inferior ao dos HE, mas após o estresse, diminuiu apenas nos HE. Os níveis de S100B não foram diferentes entre os grupos. O protocolo de EE melhorou o comportamento exploratório, o desempenho nas tarefas de NOR e de esquiva inibitória, e os níveis de BDNF no hipocampo em ambos os camundongos LE e HE. Importante ressaltar que o perfil geral dos camundongos LE após dois meses de EE foi semelhante ao dos HE

mantidos sob condições normais. O protocolo de EE não alterou o número de astrócitos nem a densidade óptica de GFAP nas regiões CA1 e giro denteado do hipocampo. Os níveis de S100B diminuíram nos camundongos HE e LE após o EE. A atividade locomotora induzida por anfetamina, dizocilpina, cafeína e apomorfina foi semelhante para os dois grupos. Podemos ressaltar que os camundongos LE e HE apresentaram semelhanças e diferenças em parâmetros comportamentais e bioquímicos, quando submetidos ao estresse ou ao EE. Estes resultados indicam que as diferenças individuais devem ser levadas em consideração nos estudos de comportamento e podem ter implicações para a compreensão dos transtornos de humor.

ABSTRACT

Exploratory behavior can be understood from a bidimensional perspective involving inhibitory behaviors (harm avoidance) and activation behaviors (novelty seeking). These behaviors can be observed in animals and vary according to individual differences. Such differences can be useful for testing hypotheses on the biological basis of temperament. The purpose of this thesis was to evaluate the effect of unpredictable subchronic stress and enriched environment (EE) in two behavioral extremes of mice from the same strain (CF1) selected by their exploratory behavior of the central arena of an open field: the low exploratory (LE) and high exploratory (HE) mice. In addition, we investigate astrocytes number and GFAP optical density in the hippocampus and S100B levels of HE and LE mice exposed to EE. Also, we studied if they differed regarding hyperlocomotion induced by amphetamine, caffeine, dizocilpine, and apomorphine. After unpredictable subchronic stress both LE and HE stressed groups exhibited less exploratory activity, but their natural difference in exploratory behavior remained. This short stress protocol did not induce changes in sucrose intake or immobility in the tail suspension task. Also, LE mice exhibited impaired novel object recognition performance after stress. HE had lower corticosterone levels than LE mice, but corticosterone levels increased after stress only in HE mice. Hippocampal BDNF in LE was lower than in HE but decreased after stress only in HE mice, whereas S100B levels were not different between groups. EE protocol enhanced exploratory behavior, memory performance in the novel object and in the inhibitory avoidance tasks, and hippocampal BDNF levels in both LE and HE mice. Importantly, the general profile of LE mice after two months of EE was similar to HE mice housed in standard conditions. The EE protocol had no effect on number of astrocytes and on GFAP optical density in the CA1 and DG of hippocampus. The S100B levels decreased in both HE and LE mice after EE. LE and HE show similar locomotor activity induced by amphetamine, caffeine, dizocilpine, and

apomorphine. Therefore, HE and LE mice showed similarities and differences in behavior and biochemical parameters when subjected to stress or EE. These results point out that those individual differences should be taken into account in behavioral studies and may have implications for the understanding of mood disorders.

LISTA DE ABREVIATURAS

5-HT	Serotonina (5-hidroxitriptamina)
BAS	Sistema de ativação comportamental
BDNF	fator neurotrófico derivado do encéfalo
BIS	Sistema de Inibição comportamental
BN	Busca de novidades
D4	Receptor de Dopamina 4
D4KO	*Knock-out* de receptor de dopamina 4
EA	Enriquecimento Ambiental
ED	Evitação de dano
GCs	Glicocorticóides
GFAP	proteína glial fibrilar ácida
HE	High Exploratory
HPA	Hipotálamo-pituitária-adrenal
LE	Low Exploratory
S100A9	Proteína ligante de cálcio A9
S100B	Proteína ligante de cálcio S100B

1. Introdução

1.1 Temperamento

O temperamento é a predisposição a emoções básicas e reações comportamentais automáticas, em resposta a estímulos ambientais específicos (risco, novidade, recompensa) (Svrakic et al, 2002). Sua base neurobiológica é predominantemente hereditária e é relativamente estável durante toda a vida. O temperamento pode ser observado em animais, principalmente pelos comportamentos inibitórios e exploratórios (Cloninger et al, 1993).

1.1.1 Classificações dos tipos de temperamento

Vários modelos de classificação do temperamento já foram propostos por pesquisadores e estudiosos. Atualmente as classificações propostas por Gray (1982) e por Cloninger (1993) são as mais aceitas e utilizadas para o estudo do temperamento e seus transtornos.

Gray elaborou uma teoria de temperamento onde traços de personalidade podem ser determinados por dois principais sistemas:

- **Sistema de ativação comportamental** (**BAS**, do inglês *behavioral approach system*): sistema que organiza as reações a estímulos condicionados ligadas a um reforço, tanto positivo quanto negativo (não-punitivo) e que conduz a um comportamento de aproximação (realização do comportamento);
- **Sistema de inibição comportamental** (**BIS**, do inglês *behavioral inhibition system*): sistema que organiza as reações a estímulos desconhecidos ou que estão ligadas a uma punição (supressão de um reforço) e que conduz a uma inibição do comportamento (não realização do comportamento), a um aumento da tensão, da atenção e da excitação do sistema límbico.

Já Cloninger propôs que o temperamento depende de quatro dimensões, sendo as duas principais semelhantes aos sistemas de Gray. São elas:

• **Busca de novidades (BN):** caracteriza indivíduos com comportamentos ativos e exploratórios, impulsivos, extravagantes, impacientes, irritáveis, com grande curiosidade e busca por situações de recompensa, e que evitam situações de punição provável.

• **Evitação de dano (ED):** caracteriza indivíduos com inibição de comportamentos frente à possibilidade de frustração ou ameaça, conferindo natureza pessimista, evitativa, amena, passiva e tímida.

Cada um destes temperamentos é independente e expresso dimensionalmente, sendo importante avaliar quanto de cada uma dessas dimensões o indivíduo expressa.

1.2 Temperamento e transtornos de humor

Outro modelo bastante estudado atualmente foi proposto por Akiskal (1983), preconizando que os temperamentos podem ser entendidos como padrões predominantes de humor ou estilos afetivos. Estes padrões de humor poderiam também predispor para tipos específicos de transtornos de humor. Os tipos principais de temperamento segundo este modelo são:

• **Hipertímicos**: associado à busca por novidades, estímulos e sensações de prazer. São exploradores, impulsivos, otimistas, entusiasmados, extravagantes, curiosos, desorganizados, têm reações afetivas rápidas e intensas e se entediam facilmente.

• **Depressivos:** são pessimistas, quietos, tímidos, indecisos, preocupados, cautelosos, críticos e céticos. Apresentam ansiedade e inibição frente a situações de risco.

• **Ciclotímicos:** apresentam alternância entre fases de humor depressivo e hipertímico.

• **Irritáveis**: manifestam a irritabilidade como uma característica marcante e constante. Também são ameaçadores, desconfiados, combativos e destrutivos.

• **Ansiosos:** são apreensivos, sensíveis, inibidos, medrosos, preocupados e cautelosos.

1.2.1 Relação de BIS/ED e BAS/BN com temperamentos afetivos

Busca de novidades e evitação de dano propostas por Cloninger se correlacionam diretamente ao sistema de ativação e de inibição comportamental propostos por Gray, respectivamente (Mardaga e Hansenne, 2007). Por outro lado, evitação de dano e busca de novidades foram relacionadas com os tipos de temperamentos afetivos determinados por Akiskal (Akiskal et al, 2005, Maremanni et al, 2005), gerando o seguinte padrão:

- **Hipertímicos**: baixo BIS (ED), alto BAS (BN).
- **Depressivos:** baixo BAS (BN), alto BIS (ED).
- **Ciclotímicos:** BIS (ED) e BAS (BN) altos.
- **Irritáveis**: alto BAS (BN), médio BIS (ED).
- **Ansioso**: alto BIS (ED), médio BAS (BN).

Portanto, podemos dizer que os temperamentos afetivos, que são a base dos transtornos de humor (depressão e transtorno bipolar) estão intimamente relacionados a estas interações entre ativação e inibição emocional e comportamental.

1.3 Modelos animais para estudo dos transtornos de humor

Modelos animais são ferramentas importantes para o estudo e compreensão dos transtornos psiquiátricos, principalmente na busca de novos e melhores tratamentos. Os modelos têm sido delineados utilizando uma variedade de parâmetros farmacológicos, comportamentais e genéticos. Bons modelos animais nos possibilitam a oportunidade única de examinar profundamente os mecanismos neurobiológicos, genéticos e ambientais que predispõem aos transtornos de humor (Gosling e Samuel, 2001; Ray e Jansen, 2004). Ratos e camundongos são amplamente utilizados para investigações neurocomportamentais,

considerando que existem inúmeros testes para avaliar o perfil comportamental destes, tais como, campo aberto, labirinto em cruz elevado, esquiva ativa e inibitória, entre outros.

1.3.1 Modelos animais para estudo da depressão

Muitos modelos animais para estudo da depressão têm sido propostos baseados nos aspectos comportamentais, neurovegetativos e emocionais da depressão em humanos, tais como anedonia, distúrbios do sono, diminuição da atividade locomotora, do comportamento exploratório e social (Strekalova et al, 2004; Bhagya et al, 2010), sendo que estes fatores têm sido induzidos através de manipulações ou seleções genéticas, estresse crônico ambiental ou manipulação farmacológica. Uma estratégia bastante usada é a indução farmacológica de sintomas depressivos utilizando drogas como reserpina ou clonidina, que atuam inibindo a liberação de noradrenalina e reduzindo a atividade de neurônios noradrenérgicos no *locus coeruleus*, sendo este modelo baseado na hipótese da depressão estar relacionada à redução da atividade noradrenérgica cerebral (Malatynska e Knapp, 2005). Outro modelo com boa validade preditiva é o modelo de nado forçado proposto por Porsolt et al. (1978). Este modelo permite identificar drogas antidepressivas pela medida do tempo de imobilidade do animal, que é interpretada como "desespero". Com igual finalidade, também existe o teste de suspensão pela cauda. O protocolo de desamparo aprendido (choque inescapável) é um modelo de indução de estado depressivo crônico muito utilizado (Overmeir e Seligman, 1967; Chourbaji et al, 2005). Neste teste, os animais são submetidos a choques elétricos inescapáveis nas patas desenvolvendo déficits de esquiva quando expostos novamente, 24 horas depois, à nova situação na qual poderiam evitar ou escapar do estímulo aversivo. Estes déficits têm sido relacionados ao sentimento de desamparo que acomete pacientes deprimidos.

No entanto, estes modelos não identificam fármacos com ação antimaníaca ou estabilizadora do humor.

1.3.2 Modelos animais para estudo de mania e transtorno bipolar

A natureza da maioria dos sintomas de mania tais como euforia, expansividade, desinibição social, irritabilidade, impulsividade e diminuição da necessidade de sono, dificulta a elaboração de um modelo animal. Até o momento nos modelos propostos tem sido utilizada a indução farmacológica, o que limita a compreensão da neurobiologia deste transtorno. Um importante modelo farmacológico de mania é o de indução farmacológica por oubaína, um potente inibidor da bomba de sódio, produzindo hiperatividade locomotora (Herman et al, 2007). Visto que o mecanismo de ação desta droga mimetiza um parâmetro biológico de mania que é a desregulação iônica (indivíduos com mania aguda apresentam um aumento intracelular de cálcio e sódio, aumento da retenção de lítio e diminuição da atividade da bomba de sódio), este modelo é bastante aceito e utilizado para testar novas drogas para tratamento de mania (El-Mallakh et al, 2003). Sua validade preditiva foi estabelecida com o uso de lítio, um medicamento utilizado como estabilizador de humor para pacientes com transtorno bipolar. Outro modelo comumente utilizado para mania é a indução de hiperatividade pela administração de anfetamina, algumas vezes associada com clordiazepóxido, que serve para desenvolver sintomas semelhantes aos sintomas maníacos de euforia, agitação e distratibilidade (Cappeliez e Moore, 1989; Lamberty et al, 2001; Shaldivin et al, 2001; Arban et al, 2005). Outros modelos de mania têm sido propostos, como: privação do sono (Gessa et al, 1995); lesões cerebrais (Wilkinson et al, 1993); estimulação elétrica (Shaldivin et al, 2001) e modelos de manipulação genética (Niculescu et al, 2000; Maier et al, 2005).

O transtorno bipolar é caracterizado por alternância progressiva e espontânea de episódios de depressão e mania, e para a construção de um modelo ideal, este deveria incluir oscilações de comportamentos depressivos e maníacos. No entanto, a natureza progressiva e cíclica deste transtorno dificulta a tentativa de um modelo animal, portanto muitos modelos

tendem a focar em um só pólo desta doença (Einat et al, 2003). Em 1990, Cappeliez e Moore, sugeriram um modelo animal farmacológico para transtorno bipolar com base na administração e retirada de anfetamina para testar o efeito do lítio. A administração crônica de anfetamina induziria sintomas semelhantes aos estados maníacos (euforia, excitabilidade). Consequentemente, a retirada da anfetamina induziria sintomas depressivos (fadiga, diminuição da atividade locomotora). Não foi conferida validade preditiva para este modelo, pois o lítio não teve efeito. Os autores sugerem que lítio e anfetamina atuam em diferentes mecanismos relacionados à atividade locomotora.

Até o momento não existe um modelo experimental que não seja farmacológico para estudo de transtornos de humor bipolar ou de mania. Também os modelos utilizados para avaliar depressão e ansiedade não consideram fatores genéticos e de temperamento, o que limita a validade de face e de construção destes modelos.

Uma das estratégias possíveis para modelar transtornos psiquiátricos em animais é estudar as diferenças interindividuais em animais de laboratório baseado nas características que determinam o seu temperamento.

1.4 Modelos animais para estudo das diferenças comportamentais individuais

Os fatores individuais na neurociência comportamental são de fundamental importância, mas ainda muito negligenciados na área de investigação científica (Pawlak et al, 2008). Um dos problemas é a necessidade de um maior número de animais do que a abordagem comumente usada. Assim, as pesquisas que exigem muitas investigações (por exemplo, efeitos dose-resposta, diferentes tratamentos) raramente levam em conta as diferenças individuais. Nos últimos anos, no entanto, a necessidade de uma abordagem individual para pesquisa sobre as relações da neurobiologia e o comportamento tem se

tornado cada vez mais importante, e apesar dos progressos na pesquisa clínica, a neurociência comportamental em animais continua a ser indispensável.

Um dos principais desafios da investigação científica é compreender as causas das diferenças individuais e suas consequências em termos de aptidão, capacidade de adaptação, e vulnerabilidade individuais às patologias. Na literatura, a individualidade é definida como uma coleção de comportamentos ou características fisiológicas, tanto inatas quanto adquiridas, que distinguem os indivíduos de seus parentes próximos, com os quais compartilham componentes genéticos e até mesmo ambientais (Pawlak et al, 2008).

Existem modelos nos quais os animais são selecionados de acordo com um comportamento específico e sucessivamente cruzados entre si por várias gerações, e outros que se baseiam na seleção de características individuais em determinada tarefa comportamental realizada logo antes do estudo a ser feito. Diversas abordagens têm sido utilizadas para analisar ansiedade e atividade exploratória em roedores e estas se estendem desde seleções por diferenças comportamentais em animais selvagens (*wild-type* ou sem manipulação genética ou farmacológica) aos mais recentes modelos genéticos.

Fatores genéticos contribuem para a alta variabilidade interindividual quanto ao tipo e intensidade da resposta comportamental, neuroendócrina e fisiológica ao estresse observado em várias espécies, incluindo humanos e animais de laboratório. Este fato tem sido utilizado como base para desenvolver inúmeras linhagens bidirecionais (Maudsley, Roman, Syracuse entre outros) para o estudo da neurobiologia do estresse e/ou ansiedade. Em 1934, Hall propôs um modelo para ansiedade que consistia na seleção de ratos Wistar com alto e baixo número de defecações quando submetidos a um campo aberto. Desta maneira eles acessavam a emocionalidade dos animais baseados no conceito de que forte emoção de medo provoca, em humanos, maior freqüência de defecações e urina. Ratos inativos no campo aberto e com altos índices de defecações eram considerados com mais medo. Após oito gerações selecionadas,

diferenças entre ratos com alto e baixo padrão de defecações se tornaram consistentes e significantes (Broadhurst 1957,1960, 1975). A partir desta seleção, os ratos denominaram-se Maudsley reativos (alto padrão de defecações no campo aberto) e não-reativos (baixo padrão de defecações no campo aberto). Pesquisas subsequentes diferenciaram estes dois grupos do ponto de vista comportamental, farmacológico e neurofisiológico (Broadhurst, 1975; Eysenck, 1987). No entanto, não se conseguiu replicar a associação dos ratos Maudsley com parâmetros de ansiedade, pois o índice de defecações no campo aberto não correlacionou com outros testes que acessam ansiedade em ratos. Estas inconsistências podem ser devido às diferentes maneiras com as quais estes grupo foram mantidos durante os últimos 40 anos (Paterson et al, 2001). Atualmente não tem sido mais utilizado.

Muitos estudos baseiam-se na ideia de que diferenças individuais na resposta neural e hormonal a uma novidade contribuem para que se observem diferenças quanto ao comportamento buscador de novidades e suscetibilidade a psicopatologias (Zuckerman, 1990). Com o objetivo de acessar esta diferença na resposta à novidade, Piazza e colaboradores desenvolveram uma seleção de ratos quanto à resposta locomotora num ambiente novo (Piazza et al, 1989,1990). Este grupo classificou-os como ratos com Alta Resposta Locomotora e ratos com Baixa Resposta Locomotora. Observou-se que animais com alta resposta locomotora são mais suscetíveis às ações de psicoestimulantes. Através desta seleção, este grupo também conseguiu diferenciar a suscetibilidade a drogas de abuso entre estes dois grupos de animais utilizando abordagens neuroquímicas, como dosagem de dopamina e corticosterona (Kabbaj et al, 2000; Piazza et al,1993; Piazza e Le Moal, 1996). Levando em consideração os achados de Piazza e colaboradores, o grupo de Schwarting desenvolveu um método de triagem baseado na exploração vertical dos animais (*rearings*) no campo aberto. Esta seleção pelo comportamento emocional e exploratório dos animais tinha o objetivo de estudar as respostas dos sistemas dopaminérgicos, serotoninérgicos e

noradrenérgicos e também avaliar a suscetibilidade a drogas de abuso. Procuraram analisar se a atividade exploratória vertical (*rearing*) acessava os mesmos mecanismos da atividade exploratória horizontal (locomoção). Foi observado que os ratos que tem maior atividade exploratória (alto *rearing*) exploravam mais objetos novos (respondiam mais à novidade) e respondiam de maneira semelhante aos ratos com baixa atividade exploratória em relação aos psicoestimulantes. Portanto, esse método de triagem separa mais o componente novidade/reação a ambiente novo e não a suscetibilidade a drogas de abuso, como pôde ser visto no modelo do grupo de Piazza.

O grupo de Landgraf e colaboradores selecionaram ratos Wistar quanto ao desempenho no teste de labirinto em cruz elevado, que se baseia na criação de um conflito entre o desejo natural de exploração e o medo de áreas abertas/expostas, separando dois grupos: ratos com Alta ou Baixa Ansiedade (Henniger et al, 2000, Liebsch et al, 1998a,1998b). A partir desta seleção, analisaram outros parâmetros comportamentais e neuroendócrinos na tentativa de compreender a neurobiologia da ansiedade. Observaram que os ratos com baixa ansiedade são mais ativos, expressam maior agressividade e exploram mais a área central do campo aberto. Em relação a parâmetros neuroquímicos, os ratos com alta ansiedade possuem maior concentração plasmática de corticosterona e redução da transmissão serotoninérgica hipocampal em relação aos ratos com baixa ansiedade (Landgraf et al, 1999, Murgatroyd et al., 2004). A partir de animais selecionados com alto e baixo desempenho na tarefa de evitação na esquiva ativa (grupos Roman e Syracuse), chegou-se a diferenças de comportamento que servem como um modelo animal para analisar as variações genéticas que predispõe à ansiedade (Brush, 2003; Escorihuela et al, 1999; Piras et al, 2010). Uma série de estudos demonstrou que os ratos selecionados pelo alto desempenho neste teste de triagem (esquiva ativa), em relação aos de baixo desempenho, são menos ansiosos, mais buscadores de novidades, mais impulsivos frente a estímulos aversivos e com menor resposta

do eixo hipófise pituitária adrenal (HPA). Os ratos Roman com alto desempenho na esquiva ainda se mostraram mais suscetíveis à sensibilização por anfetamina, apresentando maior resposta dopaminérgica. Ramos e colaboradores fizeram um intercruzamento de três grupos de ratos (Wistar, Hooded e Lewis) e criaram os ratos Floripa que diferem quanto à locomoção na área central do campo aberto, denominados Floripa H (high - alta locomoção) e Floripa L (Low - baixa locomoção). Este grupo tem buscado a caracterização genética destes animais que se diferenciam em inúmeros testes comportamentais e tem como objetivo utilizar este modelo animal para estudo de ansiedade e tendência ao consumo abusivo de álcool (Ramos et al, 2003; Izıdio & Ramos, 2007; Hinojosa et al., 2006; Ramos e Mormede, 1998).

Uma outra maneira de caracterizar diferenças interindividuais entre animais é através da análise do controle territorial e agressividade. Camundongos selecionados geneticamente pela sua latência de ataque formam um modelo para estudo dos mecanismos responsáveis pela variação da suscetibilidade ao estresse. Animais menos agressivos reagem de maneira passiva ou inativa a mudanças ambientais, além de ter secreção aumentada e prolongada de corticosterona após situação de estresse, comparado aos mais agressivos (Sluyter et al, 1996; Veenema et al, 2002).

De um modo geral, todos os estudos citados anteriormente levam em consideração diferenças individuais dos animais a fim de obter um modelo animal como ferramenta para o estudo de psicopatologias.

1.5 Modelo de Alta e Baixa Atividade Exploratória no Campo Aberto desenvolvido em nosso laboratório

O nosso modelo foi desenvolvido com a finalidade de traçar o perfil temperamental de cada animal baseando-se na avaliação individual do comportamento no teste de campo aberto com um objeto central para estimular a exploração. Este modelo permite a observação do

perfil exploratório destes animais, que decorre da combinação de características de temperamento evitador de dano (inibitório) e buscador de novidades (ativador) (Kazlauckas et al, 2005). Os resultados obtidos neste estudo demonstraram que podemos identificar diferenças interindividuais comportamentais em camundongos e que estas influenciam em outras tarefas comportamentais. O teste de campo aberto utilizado para essa seleção é reproduzível e estável no decorrer do tempo (por pelo menos 8 meses), como esperado quando se está avaliando tais características. Os camundongos mais exploradores (HE – *high exploratory mice*) mostraram-se mais agressivos frente ao intruso, evitam melhor a punição condicionada (choque elétrico na esquiva inibitória) e tiveram melhor desempenho no labirinto de Lashley com estímulo apetitivo (comida). O componente medo ou evitação de dano foi analisado através dos testes de claro-escuro e labirinto em cruz elevado, que são específicos para medir ansiedade. Foi identificado um menor índice de ansiedade nos animais mais exploradores enquanto que os camundongos menos exploradores (LE – *low exploratory mice*) se mostraram bastantes ansiosos em ambos os testes. Assim, é possível estudar os substratos biológicos das diferenças interindividuais em animais de laboratório sem a necessidade de grupos selecionados geneticamente.

1.6 Estresse

Diariamente estamos submetidos a situações de estresse que podem desencadear alterações em nosso organismo. A exposição ao estresse é capaz de alterar a homeostasia fisiológica e psicológica de um indivíduo induzida pela ativação do eixo hipotálamo-pituitária-adrenal (HPA). Os glicocorticóides (GCs) (cortisol em humanos e corticosterona em roedores) são os efetores finais do eixo HPA e participam do controle da homeostase corporal e da resposta do organismo ao estresse, exercendo ações adaptativas e essenciais para a sobrevivência imediata quando em resposta a estímulos agudos (Tsigos e Chrousos, 2002;

Chrousos e Gold, 1992). O fato de um evento emocional como o estresse afetar o organismo se deve ao íntimo relacionamento entre o sistema imunológico (defesa), sistema nervoso (controle) e sistema endócrino (hormonal) (Tsigos e Chrousos, 2002).

O estresse pode alterar o aprendizado e a memória (Oitzl et al., 2001), sendo aceito de forma geral que eventos estressantes são muito bem lembrados (Olff, Angeland e Gersons, 2005). Estudos com animais demonstraram que o estresse facilita e pode até ser indispensável para o aprendizado e a memória (Oitzl et al., 2001). Um aumento nos níveis dos hormônios do estresse, principalmente os corticosteróides, dentro do contexto da situação de aprendizado, ajuda a lembrar um evento em particular. Por outro lado, o estresse crônico também tem sido associado com um prejuízo no desempenho cognitivo em certas situações (Joels et al, 2006).

O encéfalo determina o que será suficiente para desencadear uma situação estressante e também controla as respostas comportamentais e fisiológicas ao evento estressor (Figura 1).

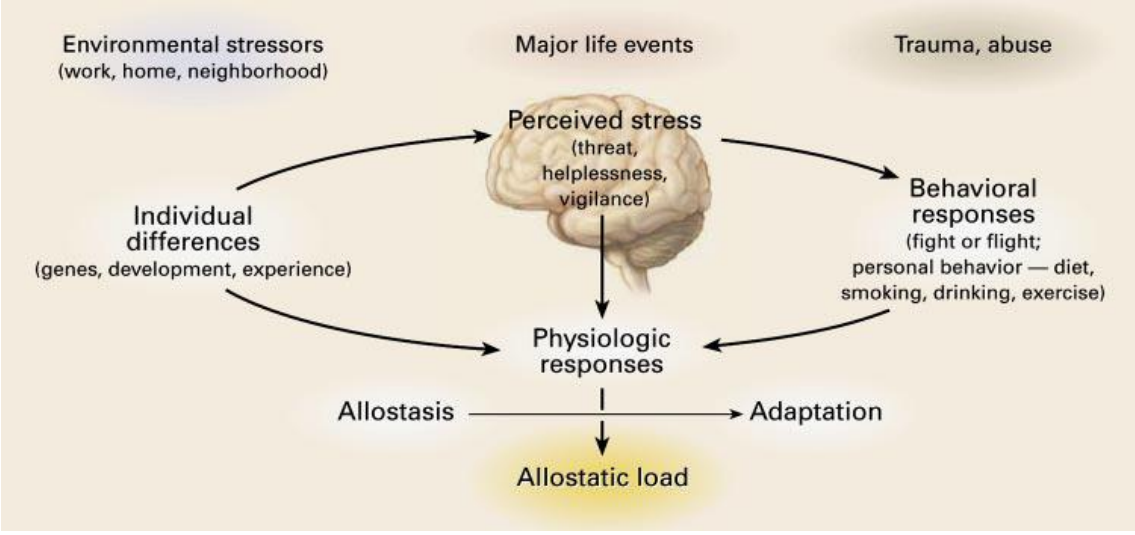

Figura 1 – Vias de controle encefálico na alostase e nas respostas comportamentais e fisiológicas em resposta a agentes estressores (McEwen BS. Protective and damaging effects of stress mediators. *N Engl J Med* 338: 171–179, 1998.211).

O hipocampo tem sido amplamente estudado no que diz respeito às ações do estresse e depressão, e os neurônios do hipocampo podem ser danificados pela exposição crônica ao estresse ou a ativação do eixo HPA, e o consequente aumento dos níveis de glicocorticóides. Disfunção do hipocampo pode resultar em prejuízos cognitivos e déficits de memória (de Vasconcelos et al., 2006; Smith, 1996).

Os fatores neurotróficos também podem ser alterados pelo estresse, sendo principalmente estudado o fator neurotrófico derivado do encéfalo (BDNF), que atua fornecendo suporte para sobrevivência, crescimento e diferenciação sobre os neurônios do sistema nervoso central e periférico. Ratos expostos a protocolos de estresse tiveram uma diminuição nos níveis de BDNF hipocampal, indicando que o BDNF também está envolvido na fisiopatologia do estresse relacionada aos transtornos de humor (Larsen et al, 2010, Gronli et al, 2006; Shirayama et al, 2002). A proteína S100B também já foi estudada em situação de estresse, tendo sido demonstrado um aumento de S100B sérico independente de GCs em ratos estressados por contenção (Scaccianoce et al, 2004).

Muitos protocolos de estresse têm sido desenvolvidos e utilizados em laboratórios buscando elucidar os mecanismos de resposta aos agentes estressores e suas consequências ao organismo, sendo o modelo de estresse crônico o mais utilizado para o estudo da neurobiologia do estresse e da depressão (Larsen et al, 2010; Detanico BC, 2009; Willner et al, 2005). O protocolo de estresse imprevisível tem sido amplamente utilizado para acessar alterações comportamentais em animais, principalmente por apresentar estresse social que representa um tipo de estresse encontrado no ambiente natural dos animais (Ducottet et al., 2004). É importante ainda salientar que o componente de imprevisibilidade também contribui para que este modelo mimetize o estresse diário em humanos, não necessariamente desencadeando um quadro depressivo, como é o caso do protocolo de estresse crônico.

1.7 Enriquecimento ambiental

Enriquecimento ambiental (EA) consiste na combinação de interação social, exercício físico e exposição a diferentes objetos que contribuem para o bem-estar dos animais. Existem diversos modelos de EA para roedores, com variações no tempo de enriquecimento, idade inicial de exposição ao EA, entre outras (Nithianantharajah e Hannan, 2006; Mohammed et al, 2002; van Praag et al, 2000).

Uma das características do EA é proporcionar aos animais um aumento da interação social, dos estímulos visuais, além de possibilitar a realização de atividade física, estimulando assim diversas regiões encefálicas. A troca dos objetos com os quais os animais interagem e a troca de posição destes objetos dentro do ambiente são de fundamental importância para o funcionamento do modelo, com o intuito de promover um aumento na formação de mapas espaciais e na plasticidade hipocampal dos animais submetidos a este protocolo. (vide Figura 2; Nithianantharajah e Hannan, 2006).

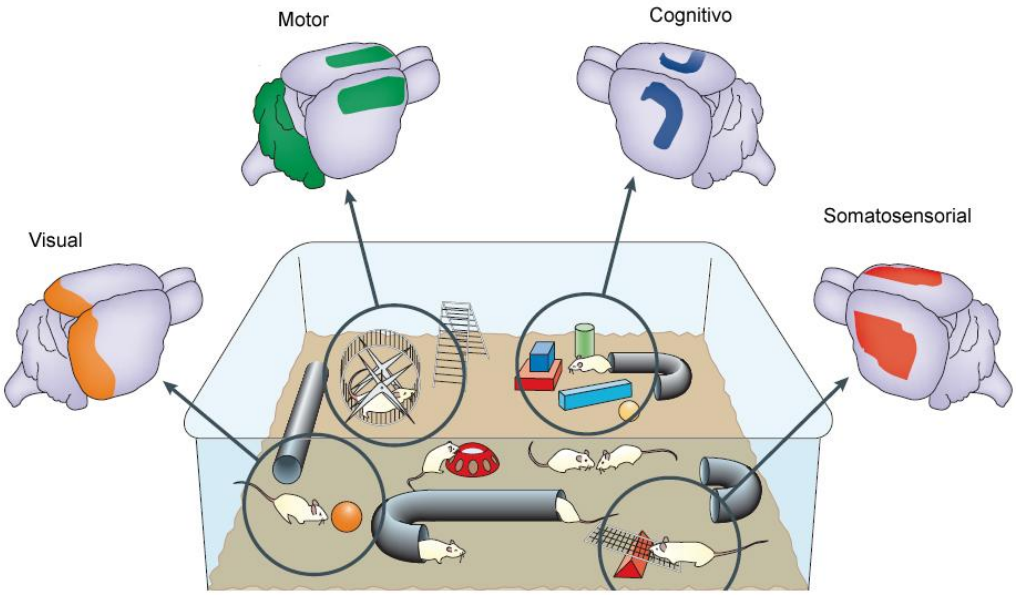

Figura 2: Enriquecimento Ambiental e os efeitos da estimulação motora, sensorial e cognitiva em diversas áreas cerebrais (Nithianantharajah e Hannan, 2006).

Mudanças na capacidade de armazenar e acessar novas informações tornam o EA um modelo interessante para avaliar desempenhos comportamentais (Tang et al., 2001, Viola et al., 2010; Amaral et al., 2008). Outro fator que faz com que o EA seja um interessante modelo de estudo para análises de tarefas comportamentais é a diferença de resposta entre as espécies, e até mesmo de linhagens de roedores empregadas neste modelo nas distintas tarefas (Nithianantharajah e Hannan, 2006). Nos últimos anos a discussão sobre interação gene-ambiente vem merecendo grande destaque em trabalhos envolvendo o EA (Abramov et al., 2008).

É importante salientar que o EA propicia uma melhora no desempenho das funções cognitivas avaliadas em animais testados em diferentes tarefas comportamentais (Rampon et al., 2000, Tang et al., 2001), além de reduzir o déficit de memória decorrente do envelhecimento (Bennett al., 2006). Entretanto, em relação aos trabalhos que avaliam a atividade exploratória, a literatura apresenta resultados discrepantes (Chapillon et al., 1999; Amaral et al., 2008), o que corrobora com a ideia de que o EA exacerbe as características comportamentais inerentes a cada espécie animal e suas diferentes linhagens.

Estudos demonstram que o enriquecimento ambiental induz mudanças bioquímicas, morfológicas e funcionais que levam a um melhor desempenho em tarefas comportamentais e cognitivas (Roy et al, 2001; Pamplona et al, 2009; Amaral et al, 2008, Viola et al, 2010, van Praag et al, 2000). Também é importante ressaltar que ocorre um aumento nos níveis de BDNF após um período de enriquecimento ambiental, assim como uma melhora na memória espacial e na atividade exploratória de roedores (Rossi et al, 2006; Gobbo et al, 2005; Ickes et al, 2000).

1.8 Resposta farmacológica com base em diferenças individuais

Muitos autores têm proposto que diferenças individuais servem de ferramenta para predizer a resposta a tarefas comportamentais (Kazlauckas et al, 2005; Thiel et al, 1999; Dellu

et al, 1996), eventos estressores, e vulnerabilidade individual a administração de diferentes tratamentos farmacológicos (Redolat et al, 2009). Grande parte dos estudos em relação à resposta farmacológica consiste numa seleção prévia através de um teste comportamental específico como descrito anteriormente (seção 1.4 Modelos animais para estudo das diferenças comportamentais individuais) ou utilizando camundongos *knock-out* para alguma proteína específica (Barbier et al, 2007; Helms et al, 2008). Nos últimos anos, têm sido desenvolvidas inúmeras pesquisas para elucidar estas diferenças. Estudos sobre respostas individuais já utilizaram a cafeína (antagonista adenosinérgico não seletivo), a anfetamina (agonistas catecolaminérgico indireto), a apomorfina (agonista direto não-seletivo de dopamina), entre outros (Barbelivien et al, 2008; Barbier et al, 2007; Piazza et al, 1989).

A atividade dopaminérgica mesolímbica está relacionada com a resposta a novidade e a drogas estimulantes (Bardo et al, 2006; Pentz et al, 2006). Dados recentemente publicados indicam que camundongos *knock-out* para receptor D4 (D4KO) são mais sensíveis à novidade do que os camundongos sem a mutação, quando expostos a um campo aberto (Helms et al, 2008). Outros dados também sugerem a implicação de serotonina (5-HT), especialmente os receptores 5-HT6 e 5-HT7, em comportamentos de busca de novidades e respostas associadas ao abuso de drogas (Ballaz et al, 2007). Todos estes estudos citados buscam investigar os sistemas neurotransmissores, e uma das ferramentas para se realizar este tipo de estudo é o uso de tratamento farmacológico com psicoestimulantes que atuam em receptores específicos (dopamina, serotonina, noradrenalina), levando a uma determinada resposta comportamental.

Grande parte dos trabalhos utiliza o protocolo de sensibilização, que consiste na administração repetida e espaçada da substância (Dietz et al, 2008; Giménez-Llort et al, 2005; Adriani e Laviola, 2002). Entretanto, a administração aguda destas substâncias também pode permitir o estudo das diferenças neurobiológicas entre determinados grupos de animais.

2. Objetivo

2.1 Objetivo Geral

Avaliar a resposta comportamental e bioquímica dos camundongos selecionados no campo aberto como mais (HE) e menos (LE) exploradores submetidos ao protocolo de enriquecimento ambiental e ao protocolo de estresse imprevisível, bem como avaliar o efeito da administração aguda de fármacos psicoestimulantes na locomoção destes animais sob condições normais, ou seja, sem serem submetidos a nenhum dos protocolos.

2.2 Objetivos Específicos

2.2.1 Avaliar os efeitos do protocolo de estresse subcrônico imprevisível nos camundongos HE e LE em relação a sua atividade exploratória e locomotora na tarefa de campo aberto, na memória através da tarefa de reconhecimento de objetos, e sobre os parâmetros bioquímicos de corticosterona, BDNF e S100B.

2.2.2 Investigar a influência do enriquecimento ambiental durante a vida adulta em camundongos HE e LE sobre a atividade exploratória na tarefa de campo aberto, a memória através das tarefas de reconhecimento de objetos e esquiva inibitória, e também avaliar o imunoconteúdo de BDNF hipocampal.

2.2.3 Analisar a resposta locomotora dos camundongos HE e LE após administração aguda de fármacos psicoestimulantes.

2.2.4. Analisar as modificações no número e na densidade astrocitária hipocampal e nos níveis de S100B hipocampal de camundongos HE e LE submetidos ao protocolo de enriquecimento ambiental.

Parte II

CAPÍTULO I

Distinctive effects of unpredictable subchronic stress on memory, serum corticosterone and hippocampal BDNF levels in high and low exploratory mice

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Behavioural Brain Research

Dear Pharmacist Vanessa Kazlauckas,

I have received the revised version of the manuscript referenced above. The revised version adequately addresses the issues raised by the reviewers, and therefore, I am pleased to inform you that it is now acceptable for publication in Behavioural Brain Research. The paper will be forwarded directly to the publisher, who will be in contact with you regarding the publication schedule in due course.

Thank you for submitting your work to this journal.

With kind regards,

Stephen Maren, PhD
Editor-in-Chief
Behavioural Brain Research

Distinctive effects of unpredictable subchronic stress on memory, serum corticosterone and hippocampal BDNF levels in high and low exploratory mice

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Abstract

Stress affects learning and memory processes and sensitivity to stress greatly varies between individuals. We studied behavioral and neurobiological effects of unpredictable subchronic stress (USCS) in two behavioral extremes of mice from the same strain (CF1) selected by their exploratory behavior of the central arena of an open field. The top and bottom 25% explorers were classified as low exploratory (LE) and high exploratory (HE) mice, respectively. The open field task, the novel object recognition task (NOR), sucrose intake and tail suspension task were evaluated in LE and HE groups exposed to USCS for two weeks or control conditions. Also serum corticosterone and hippocampal BDNF and S100B levels were analyzed. Both stressed groups exhibited less exploratory activity when submitted to USCS, but their difference in exploratory behavior remained. This short stress protocol did not induce changes in sucrose intake or immobility in the tail suspension task. Also, LE mice exhibited impaired NOR performance after USCS, whereas HE mice changed their pattern of exploration towards less exploration of the familiar object. HE had lower corticosterone levels than LE mice, but corticosterone levels increased after stress only in HE mice. Hippocampal BDNF in LE was lower than in HE but decreased after USCS only in HE mice, whereas S100B levels were not different between groups and did not change with USCS. In conclusion, our results suggest that individual differences in exploratory behavior in rodents from the same strain influence cognitive and biochemical response to stress.

Keywords: exploratory behavior; unpredictable subchronic stress; corticosterone; BDNF, memory, open field.

List of abbreviations: GCs: glucocorticoids; HE: high exploratory; LE: low exploratory; OF: open field; USCS: Unpredictable Subchronic Stress.

1. Introduction

It is widely known that stressful events can affect learning and memory [4, 8]. Moreover, stress affects differently the pathways involved in learning and memory, such as encoding, consolidation and retrieval [16, 19]. The effects of stress on memory depend critically on the timing and frequency of exposure to the stressors as well as the environment or context in which they occur [36]. Furthermore, sensitivity to stress varies greatly between individuals [26].

Glucocorticoids (GCs) are hormones that coordinate the stress system and the ability of an organism to cope with stress [8]. These hormones easily cross the blood brain barrier and subsequently interact with their specific intracellular receptors, i.e. glucocorticoids and mineralocorticoids receptors, in different brain areas related to cognitive processes [26, 30]. It is still controversial whether the effect of these hormones is positive or negative on memory and learning processes [16, 36]. In a study in rodents, exogenous GCs facilitated memory consolidation [8], whereas retrieval processes were impaired after high levels of GCs administration [26]. Moreover, a study by Scaccianoce et al. (2003) showed that animals with high levels of corticosterone presented superior performance in a learning paradigm [31]. However, a study from Luine et al. (1993) showed that some rats submitted to corticosterone treatment had their learning performance impaired, whereas other corticosterone-treated rats showed no change on radial maze task performance [22]. These examples illustrate individual differences in vulnerability to stress hormones and subsequent effects on cognitive processes [12, 24, 38].

Neurotrophins, as brain-derived neurotrophic factor (BDNF) and S100B, are important molecules that regulate development, maintenance, and function of nervous system [15, 23, 31]. BDNF plays a pivotal role in the structure and function of hippocampal neurons and are

important mediators of corticosterone actions in the hippocampus [14, 33]. Stress-induced elevation of GCs is accompanied by reduced expression of BDNF, structural changes and neuronal damage in the hippocampus subfields [39]. The calcium-binding protein S100B, an astroglial-specific neurotrophic factor produced primarily by glial cells, has been largely used as a parameter of glial activation and/or death in several conditions of brain injury [20, 41]. S100B has also been related to memory processes, as shown by Donato et al (2003) [11]. Interestingly, S100B-deficient mice exhibit enhanced spatial and fear-associated memories. In addition, S100B serum levels were found increased in rats subjected to stress [11]. Restraint stress has been shown to increase serum S100B levels in control and in adrenalectomized rats but not in corticosterone-injected rats, indicating a relationship between stress and S100B that is independent of glucocorticoids [32]. Also, Schulte-Herbrüggen et al. (2008) found that hippocampal BDNF was significantly increased (+53%) in S100B KO mice compared to wild-type mice, suggesting their interaction to maintain the neurotrophic tone in the hippocampus [35].

In a previous study, our group has characterized two behavioral extremes of mice according to their exploratory behavior in the open field (OF) task: low exploratory (LE) and high exploratory (HE) mice [18]. HE mice show less anxiety, more aggressive behavior against intruders, higher avoidance to conditioned punishment (electric footshock) and better performance in a maze with positive reinforcement (food) when compared to their LE counterparts [18]. Since these LE and HE mice phenotypes have different behavioral characteristics, they might also respond differently to stress conditions. Unpredictable subchronic stress (USCS) is an experimental model that simulates human daily stress by applying various kinds of stressors at different times of the day, producing significant changes in behavior and cognitive processes [40].

Individual differences in behavioral and biological responses to stress have been poorly studied in animal research. In particular, little is known about the early effects of stress on cognitive tasks and neurobiological parameters without the development of depressive state. If a depressive state is fully developed, it is not possible to differentiate if neurobiological or behavioral changes reflect a stress response or are secondary to this depressive state. Therefore, the aim of the present study was to evaluate the effect of a relatively short course of USCS (two weeks) in LE and HE mice on: i) exploratory and locomotor behavior in the open field task, ii) on the novel object recognition task that deals with their natural ability to recognize novelties in a familiar environment, iii) serum corticosterone levels and adrenal gland weight as stress biochemical parameters and iv) hippocampal BDNF and S100B levels to evaluate neurotrophic tone.

2. Material and Methods

2.1. Animals

Eighty male albino CF1 mice (60 days old), weighting approximately 35–40 g, were obtained from State Foundation for Health Science Research (FEPPS, Porto Alegre, RS, Brasil). They were housed in groups of six to eight in standard conditions of temperature and humidity with a 12h light/dark cycle (lights on at 7:00 a.m.) and access to food and water *ad libitum*. All experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals and Brazilian Society for Neuroscience and Behavior (SBNeC). Recommendations for animal care were followed throughout all the experiments; in accordance to the project approved by the ethical committee from Universidade Federal do Rio Grande do Sul. All efforts were made to minimize the number of animals employed in the present study and their suffering.

The animals were weighed the day before the USCS starting and in the sample collection day. All the behavioral tasks were performed between 10:00 A.M and 4:00 P.M.

2.2. LE and HE mice selection

Eighty mice were selected into low (LE) and high exploratory (HE) mice, according to their exploratory behavior in the central area of the open field (OF), as previously described [18]. Briefly, this test was used to separate the two mice phenotypes depending on the animal's exploration in a new environment. For this, the animal was placed in an open field (50 cm × 50 cm × 50 cm) with an object (a white cylinder of 1.5 cm radius and 5 cm high) placed in the center of the arena to stimulate exploration. Exploratory behavior was video recorded for 5 min and the time spent by the animal in and out of an imaginary center square of 30 cm × 30 cm was analyzed with ANYmaze software (Stoelting, Woods Dale). From the eighty mice screened in the OF, the bottom and top 20 explorers were selected to compose the LE and HE exploratory groups, respectively. Then, LE and HE mice were randomly subdivided in two subgroups: LE and HE mice submitted to unpredictable subchronic stress (USCS), LE_{USCS} and HE_{USCS} respectively, and LE and HE mice maintained under control/ standard conditions, $LE_{control}$ and $HE_{control}$, respectively. Within LE and HE, no significant difference in baseline open-field parameters was found between those ascribed to stress or control protocols ($P > 0.10$). All mice remained in their home cages appropriately identified without changing housemates until the end of behavioral testing.

2.3. Unpredictable subchronic stress experimental design

Unpredictable subchronic stress (USCS) was designed according to other models of variable stress with some modifications [7, 13, 40]. The following stressors were used: (a) inclination of the home cages at a 45º angle for 14 h, (b) 2h of tube restraint (c), forced

swimming for 3 minutes at 15°C (d) social isolation (3 days), (e) overnight illumination, (f) overnight wet sawdust, (g) intermittent 0,6mA foot-shock during 3 minutes, (h) food and water deprivation followed by access to an empty water bottle during 2hs, (i) pairing with another stressed mouse. Animals were exposed to only one stressor every day and in order to minimize its predictability, the protocol was carried out at different times each day. They were exposed to stressful situations for 15 days (see Table 1 for the sequence of stressors applied during all the experimental procedures). The control groups were not manipulated or stressed, other than the food and water deprivation that preceded the sucrose preference task.

At the end of the USCS protocol, LE (control and USCS) and HE (control and USCS) mice were tested according to the following schedules: Sucrose Intake on day 1; OF task followed by Tail suspension on day 2 and NOR task on days 3 and 4.

2.4. Behavioral studies

2.4.1. Open field after USCS

The 5-min open field task with a central object was performed again as described above (Materials and Methods, 2.2 LE and HE Selection).

2.4.2. Novel object recognition task

Novel object recognition (NOR) task was performed in an apparatus consisting of a small black wood chamber (25 cm×25 cm×40 cm). Before the experimental sessions, animals were habituated to the experimental room for 60 min in dim light conditions. A light bulb was switched on during the experimental sessions, with uniform light intensity in the different parts of the apparatus. The objects were placed equidistant from two corners, 12 cm apart from the wall. Mice were placed individually in the chamber. In the habituation sessions, mice explored the apparatus during 10 min, in the absence of objects. In the training session, performed 24 h later, 2 similar objects unfamiliar to mice were used. In the test session,

performed 1.5 h later, one familiar and one unfamiliar object were presented. The objects employed were 2 kinds of soda bottles with different shape and color (white and amber) presenting the same texture and size. These objects do not have ethological significance for mice. Objects were cleaned between sessions with 70 % ethanol solution. Exploration was defined by directing the nose to the object at a distance less than 2 cm and/or touching the object with the nose or forepaws. Exploration time was measured with the use of chronometers by observers blind to the treatment groups. Animals that presented less than 3 seconds of exploration time were excluded from the experiment. Following parameters were quantified: (a) the discrimination ratio that was expressed by the ratio of the total time spent exploring the novel object by the sum of the time exploring the novel and familiar objects (discrimination ratio = TN/(TN+TF), where TN = time spent exploring the novel object; TF = time spent exploring familiar object), the ratio for the training session was calculated by the ratio between the time spent on one of the objects randomly chosen and the total time of exploration in the training session and (b) time spent exploring the familiar object during the training and test sessions in seconds (according to Bevins & Besheer [3]).

2.4.3. Tail suspension

Mice were suspended by the tail using adhesive tape and an observer unaware of the group being tested recorded immobility time for 10 min (adapted from [37]). The immobility time was defined as the total duration that animal showed no movement and is considered a measure of helplessness.

2.4.4. Sucrose preference task

Mice were first habituated to consume a palatable 2% sucrose solution over a 2-day period after the selection and 4 days before the USCS protocol. Two bottles, one with water and another with sucrose solution were available during 24 hours. The sucrose preference test

was carried out in the day after USCS protocol ended. After a 12-h food and water deprivation, mice were tested singly housed with both sucrose and tap water bottles and allowed to consume water and 2% sucrose solution for 2hs. The bottles were weighed before and after the test. The sucrose intake changes and water intake changes were defined as the differences between the final and the initial volume values (adapted from [7]).

2.5. Samples collection

One day after the end of the behavioral experiments, all animals were decapitated and trunk blood was collected within two hours between 10:00 A.M and 12:00 P.M. Samples were collected among the four groups (LE and HE control and USCS groups) to avoid any substantial time lag in samples collection. The left adrenal gland of each mouse was removed and subsequently weighed. Mice brains were rapidly removed on ice and dissected for hippocampus isolation and homogenized in phosphate buffer solution (PBS) consisting of 100 mM Tris / HCl, pH 7, containing 2% bovine serum albumin (BSA), 1 M NaCl, 4 mM EDTA.Na2, 2% Triton X-100, 0.1% sodium azide and protease inhibitors (Sigma). Samples were stored at -20°C until analysis.

2.6. Determination of serum corticosterone

Total serum corticosterone concentration was determined by double antibody radioimmunoassay (Immuchem 125I Corticosterone Kit; ICN Biomedicals, Inc., Costa Mesa, CA) according to the manufacturer's instructions. The sensitivity of the measurement was 7.7ng/mL. The intra- and inter- assay coefficients of variation were 7.1% and 9.5%, respectively. The results are presented as ng/mL.

2.7. Determination of hippocampal BDNF levels

BDNF levels in the hippocampus were measured by anti-BDNF sandwich-ELISA, according to the datasheet from Millipore kit (Chemicon, USA). Microtiter plates (96-well flat-bottom) were coated overnight with samples (diluted 1: 2 in diluent buffer) and reference curve standards (ranging from 7.8 to 500 pg/ml BNDF). The plates were then washed four times and a diluted biotinylated mouse anti-BDNF monoclonal 1:1000) was added to each well and incubated for 3 h at room temperature. After this time, the plates were washed four times and then a diluted streptavidin-HRP conjugate solution was added to each well and incubated at room temperature for 1 h. Wells were washed four times before adding substrate and maintained at room temperature for 15 minutes before the addition of stop solution. The amount of BDNF was determined at 450 nm and expressed as pmol/mg wet tissue protein.

2.8. Determination of hippocampal S100B levels

S100B concentrations were measured using an enzyme linked immunosorbent assay (Diasorin® S100 ELISA Kit) in a Spectra Max M5 molecular Devices (USA). Calibrators and hippocampal samples (100 µL of each) were incubated in a plate already coated with anti-S100B antibody. The S100 ELISA was a two-site, one-step, enzyme linked immunosorbent assay. In the assay calibrators, controls and unknown samples react simultaneously with 2 solid phase capture antibodies and a detector antibody conjugated with horseradish peroxidase (HRP) during the incubation in the microtiter wells for 2 hours. After a washing step a TMB chromogen (Tetramethylbenzidine) was added and the reaction was allowed to proceed for 15 minutes. The enzyme reaction was stopped by adding stop solution and the absorbance was measured at 450 nm. S100B concentrations were derived by comparison with the calibration curve based on the total absorbance for each given calibrator provided with the assay. All determinations were carried out within the same experiment. The S100B calibration curve is cubic spline up to 5 µg/L, and the CVs for duplicates across the entire concentration range for

the calibrators and samples were 5%. The detection limit of the assay is 0.03 μg/L. The results are expressed as pg/mg wet tissue protein.

2.9. Statistical analysis

All results are presented as mean ± S.E.M. Differences between LE and HE in the selection by their exploratory profile in the open field were analyzed using Student´s t-test. After the USCS protocol, open field, tail suspension test, sucrose preference, corticosterone, adrenal gland weight, BDNF and S100B levels were analyzed using a two-way ANOVA with group (LE/HE) and treatment (control/USCS) as independent variables. Bonferroni post-hoc comparisons were conducted when appropriate. In the novel object recognition test we used three-way ANOVA with groups, treatment and trials as independent variables. The Graphpad Prism 5 and SPSS 17.0 softwares were used and significant differences were considered when $P < 0.05$.

3. Results

3.1. LE and HE mice phenotypes selection

LE and HE mice were selected in the open field task according to their exploratory behavior. The 20 mice that spent less time in the central area of the arena (16.27 ± 2.04 %) were denominated LE group and the top 20 explorers (40.81 ± 1.39 %, $P < 0.001$ compared to LE mice), the HE group (Fig. 1A). Locomotor activity did not differ between LE and HE groups (Fig. 1B).

3.2. Effect of USCS in the OF task

The time spent in the central area was reduced after USCS in both groups (two-way ANOVA for treatment [F (1, 36) = 6.80; $P < 0.05$], but HE mice exhibited greater exploratory behavior compared to LE mice for both conditions (two-way ANOVA for groups [F (1, 36) = 14.43; $P < 0.001$]), as shown in Figure 2A. Locomotor activity was not affected by exposure to USCS (Fig. 2B).

3.3. Novel object recognition task

As a normal behavior, rodents usually spent less time exploring the familiar object during the test session, which implies that they recognized the object presented previously.

Analysis of the discrimination ratio in the novel object recognition test revealed a significant main effect of trials [F (1, 24) =18.88, $P < 0.001$] and also a significant effect of groups [F (3, 24) = 3.91, $P < 0.05$]. Except for LE_{USCS} mice, post hoc analysis showed that all groups of mice recognized the novel object as shown by the difference in the discrimination ratio between training and test sessions: $LE_{control}$ (0.49 ± 0.03 *versus* 0.61 ± 0.03, $P < 0.05$), $HE_{control}$ (0.51 ± 0.01 *versus* 0.62 ± 0.02, $P < 0.05$) and HE_{USCS} (0.51 ± 0.01 *versus* 0.66 ± 0.03, $P < 0.05$). Thus, LE mice were able to recognize the novel object similarly to HE mice, but USCS protocol impaired novel object recognition only in the LE mice (Fig. 3A).

The time spent exploring the familiar object was evaluated in the figure 4B and three way ANOVA revealed a significant main effect of trials ($F(1,24) = 42.44$; $P < 0.001$). Thus, except for LE_{USCS} mice, all groups spent less time on the familiar object during the test session. Also, the post hoc analysis revealed that the time spent exploring the familiar object was particularly low for HE_{USCS} mice during the test session when compared to its high and low exploratory counterparts ($P < 0.001$) (Fig. 3B).

3.4. Sucrose preference task

There was no difference between groups in the sucrose intake ($LE_{control}$: 2.98 ml ± 0.64; LE_{USCS}: 3.26 ml ± 0.19; $HE_{control}$: 2.41 ml ± 0.17 and HE_{USCS}: 2.71 ml ± 0.36; N.S.).

3.5. Tail suspension

There was no difference between groups in the tail suspension test ($LE_{control}$: 124.70 s ± 12.28; LE_{USCS}: 98.81 s ± 13.12; $HE_{control}$: 109.50 s ± 18.17 and HE_{USCS}: 111.60 s ± 14.93; N.S.).

3.6. Serum corticosterone levels and adrenal gland weight

Corticosterone serum levels were lower in $HE_{control}$ when compared to $LE_{control}$ group (Fig. 4A (a), $P < 0.05$). Also, two-way ANOVA indicated a significant interaction between group and treatment [F (1, 28) = 10.92; $P < 0.001$] which revealed that USCS had a different effect between HE and LE groups. Also, Bonferroni post hoc showed that corticosterone serum levels significantly increased only in the HE_{USCS} group (Fig. 4A (b), $P < 0.001$). Adrenal gland weight values were normalized to body weight. HE_{USCS} mice had significantly higher adrenal gland weight (normalized to body weight) when compared to the $HE_{control}$ mice [F (1, 28) = 9.70; (b), $P < 0.01$] (Fig. 4B). Total body weight was not different between groups ($LE_{control}$: 44.69g ± 1.08; LE USCS: 42.65g ± 1.40; $HE_{control}$: 44.85g ± 1.19 and HE_{USCS}: 41.91g ± 1.28; N.S). As observed, USCS led to increased corticosterone and adrenal gland weight only in the HE mice.

3.7. Hippocampal BDNF and S100B levels

Hippocampal BDNF levels were analyzed by two-way ANOVA that revealed an interaction [F (1, 35) = 5.94; $P < 0.05$] and groups effects [F (1, 35) = 8.69; $P < 0.05$] indicating that USCS had a different effect on the BDNF levels between HE and LE groups. Post hoc analysis showed that BDNF levels were significantly increased in $HE_{control}$ mice

when compared to $LE_{control}$ and also we observed that HE_{USCS} had diminished BDNF levels when compared to the $HE_{control}$ group (Fig. 5A).

There was no difference between groups and treatments in S100B levels (Figure 5B).

4. Discussion

Low exploratory (LE) and high exploratory (HE) mice are two distinct phenotypes selected according to their exploratory behavioral patterns in the OF with a central object to stimulate exploration. In the same OF task, both LE and HE groups submitted to USCS exhibited less exploratory activity when compared to the LE and HE control groups, respectively. However, their differences in the natural exploratory behavior remained, since the HE_{USCS} showed higher exploratory activity when compared to LE_{USCS} mice. Moreover, locomotor activity was not different between the LE and HE control and USCS groups, showing that stress specifically affects the exploratory behavior of the central arena or thygmotaxis. In accordance to the results presented here, Dalm et al. (2009) demonstrated that stressed mice exhibited a different pattern of exploratory behavior in a circular hole board and also decreased exploratory behavior in their home cages without changes in locomotor activity [6].

The NOR task is widely employed to evaluate recognition memory since it deals with the animal's natural proclivity to recognize novelty in a familiar context [2, 3, 5]. Our experiments revealed that LE mice subjected to USCS had impaired recognition memory since this group did not discriminate the novel object, whereas HE_{USCS} learned the task. HE_{USCS} showed predominantly a decrease in time of exploration of the familiar object. These findings suggest that HE mice subjected to USCS presented diminished interest for the familiar object in the test session. It is interesting to note that in our previous study

characterizing the phenotypes, LE and HE mice presented large differences in the inhibitory avoidance task performance, a task that has an aversive stimulus, and in the Lashley maze using appetitive reward. In the present study, LE and HE control groups had no differences in the NOR task, possibly because this task did not include reward or punishment. Nevertheless, our findings showed that stress differently affected LE and HE groups response to novelty (represented by the novel object in the NOR task), which corroborates the characterization of these two extreme behavioral phenotypes.

Individual characteristics in cellular and hormonal responses to stress may contribute to differences in behavior and vulnerability to psychopathology [9, 12, 13, 17]. In the present study, biochemical analysis revealed that the HE control group showed lower corticosterone serum levels than the LE control group. In the CF1 mice strain used in our study, the basal levels of corticosterone were described to range from 90 to 100 ng/mL [10]. In our study, corticosterone levels were 165 ng/mL in $LE_{control}$ and 60 ng/mL in $HE_{control}$. Therefore, we hypothesize that high baseline corticosterone levels in LE mice may preclude detecting any rise in its levels following exposure to stress, which would explain the lack of change in corticosterone levels after USCS. In contrast, the HE group exhibited low levels of corticosterone under control conditions but were very responsive to stress, showing a significant increase in corticosterone levels when submitted to USCS. Similar results were described in rats exhibiting high or low rates of locomotor activity when exposed to a novel environment [28]. The high responder (HR) group exhibited a significant increase in stress-induced secretion of serum corticosterone after exposure to a novel environment relative to their low responders (LR) group counterparts, but differently from our study, stress was acute [27, 29]. These HR rats not only were more active when exposed to a novel environment but also preferred novel and aversive situations compared to LR rats. From the neuroendocrine standpoint, it was suggested that HR rats engaged in these behaviors not necessarily because

they find them less stressful, but as a response to the activation of limbic–hypothalamo–pituitary adrenal axis (HPA) [9, 17, 28]. In addition, some studies showed that individuals exposed to chronic stress can either habituate or respond with a sustained overactivation of the HPA axis in pathological conditions [12, 17, 25].

Several studies have been performed to evaluate the effect of stress on brain BDNF levels [21, 24, 33, 34]. Severe stress protocols, such as daily immobilization stress, showed a noticeable decrease of BDNF levels in the hippocampus [21, 25]. However, it should be considered that these protocols of severe stress have limited validity with regard to the human daily situation. In contrast, another study demonstrated that chronic unpredictable stress increased BDNF mRNA expression in the hippocampus, possibly reflecting an adaptive protective response to the unpredictable stressors [21]. In the present study we demonstrated that $HE_{control}$ mice have higher levels of hippocampal BDNF when compared to the $LE_{control}$ mice. However, USCS reduced hippocampal BDNF levels in HE but not in LE mice. One possible explanation for the lack of reduction in BDNF levels in LE mice after stress is a "floor effect", but additional studies with more prolonged or severe stress protocols are necessary to clarify this issue.

In agreement with our study, Schaaf et al. [33] described that stress induced elevation of GCs was accompanied by reduced expression of BDNF. Also, stress was able to decrease BDNF in the dentate gyrus in the absence of glucocorticoids, suggesting that corticosterone feedback is not necessarily matched with the observed decrease on BDNF expression caused by stress. Thus, changes in corticosterone and BDNF observed here may not be correlated with behavior since even HE submitted to USCS had good performance in a memory task despite presenting diminished levels of BDNF. The effect of GCs enhancing memory consolidation [1] may explain the adequate cognitive performance of HE even after USCS. Finally, LE showed impaired NOR performance despite no observable changes in serum

corticosterone or neurotrophins, suggesting other neurobiological correlates for this behavioral change.

The chronic unpredictable stress can also be used to model depression. To evaluate the possible interference of depressive behavior as helplessness or anhedonia in our relatively short protocol we performed the tail suspension and sucrose intake experiments. The results showed that LE and HE mice subjected to USCS did not exhibit substantial depressive behavior as measured with these classical parameters, so the OF and NOR task results are due to USCS without a clear mood impairment. These data also suggest that stress can reduce brain BDNF levels and affect cognition before the development of changes in helplessness and anhedonia.

The brain levels of the glial neurotrophin S100B were not different under normal or stressful conditions in the LE and HE mice. Moreover, restraint stress increased serum S100B levels in control and in adrenalectomized but not in corticosterone-injected rats, indicating a GCs-independent relationship between stress and S100B [32]. It is likely that the differences observed in the literature and in the results described here in S100B levels are a consequence of the nature of the stress protocol employed, the strain and age of the animals and also which kind of samples were analyzed.

In conclusion, these results suggest that individual differences in exploratory behavior in rodents from the same strain influence cognitive and biochemical response to stress. We also found that exploratory behavior is associated with higher hippocampal BDNF levels and those changes in cognition and neurotrophic levels seem to precede the development of a clear depressive state in the USCS model. Therefore, such individual differences should be taken into account in order to improve our understanding of the neurobiology of behavior under normal and pathological conditions.

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Legends

Fig. 1: Selection of low (LE) and high (HE) exploratory mice behavioral pattern. Animals (n = 80) were subjected to the open field task with a central object, and time spent in the central area and locomotor activity were recorded during five minutes. LE (n=20) and HE (n=20) mice were evaluated for time spent in the central area (A) and locomotion (cm) (B). Results are presented as mean ± S.E.M. Statistical analysis was performed using Student´s *t* test. *** $P < 0.001$.

Fig. 2: Exploratory and locomotor activities in the open field task after USCS. LE and HE control and USCS mice were subjected to the open field task with a central object, and time spent in the central area (A) and locomotor activity (B) were recorded during five minutes. White bars represent the LE and HE control groups and the gray bars represent LE and HE USCS groups. Results are presented as mean ± S.E.M. Statistical analysis was performed by Two-way ANOVA followed by Bonferroni post hoc test. * $P < 0.05$; ** $P < 0.001$. $LE_{control}$ (n = 11); LE_{USCS} (n = 9); $HE_{control}$ (n = 10); HE_{USCS} (n = 10).

Fig. 3: Novel object recognition task. LE and HE control and USCS mice were evaluated for: (A) discrimination ratio for the training and test sessions and (B) total time spent exploring the familiar object during training and test sessions. Results are presented as mean ± S.E.M. Statistical analysis was performed by Three-way ANOVA with differences between groups, treatment and trials (as independent variables). * $P < 0.05$ and ** $P < 0.001$ differences between training and test session and # $P < 0.05$ difference between HE_{USCS} and other groups in the test session. $LE_{control}$ (n = 8); LE_{USCS} (n = 6); $HE_{control}$ (n = 8); HE_{USCS} (n = 6).

Fig. 4: Serum corticosterone levels (A) and normalized adrenal gland weight (B) for LE and HE control and USCS mice. Results are presented as mean ± S.E.M. Statistical analysis was

performed by Two-way ANOVA followed by Bonferroni post hoc test. ** $P < 0.001$ between LE and HE groups and (a) $P < 0.001$ between the LE and HE control groups (figure A), (b) $P < 0.001$ between the control and USCS HE groups (figure A and B). $LE_{control}$ (n = 10); LE_{USCS} (n = 7); $HE_{control}$ (n = 8); HE_{USCS} (n = 7).

Fig. 5: Hippocampal BDNF (A) and S100B (B) levels for LE and HE control and USCS mice. White bars represent the LE and HE control groups and the gray bars represent LE and HE USCS groups. Results are presented as mean ± S.E.M. Statistical analysis was performed by Two-way ANOVA followed by Bonferroni post hoc test. ** $P < 0.001$ between LE and HE groups and (a) $P < 0.05$ between LE and HE control groups and (b) $P < 0.05$ between $HE_{control}$ and HE_{USCS}. $LE_{control}$ (n = 10); LE_{USCS} (n = 10); $HE_{control}$ (n = 10); HE_{USCS} (n = 9).

Table legend

Table 1: Unpredictable Subchronic Stress (USCS). LE and HE mice were exposed to only one stressor every day, during 15 days, starting at different time periods each day.

Table 1:

Day	*STRESSOR ACTION*	*Time*
1	Inclination of home cages	10:30 am – 3:30 pm
2	Tube restraint	2:30 pm – 4:30 pm
3	Cold swimming during 3 minutes	3:00 pm (starting time)
3	Food and water deprivation	6:00 pm – 9:00 am
4	Access to the empty water bottle	9:00 am – 11:00 am
5	Overnight illumination	10:00 pm – 10:00 am
6	Intermittent 0,6mA foot-shock during 3 minutes	9:00 am (starting time)
7	Exposition to the foot-shock cage	2:00 (starting time)
8	Social isolation	48 h (from 10:00am to 10:00am)
9	Social isolation	48 h (from 10:00 am to 10:00am)
10	Return to the home cage	10:00 am
11	Wet sawdust	10:30 pm– 10:30 am
12	Inclination of home cage	12:00 pm – 2:00 pm
13	Home cage came back to the normal angle	2:00 pm
14	Tube restraint	4:00 am – 6:00 pm
15	Animal stressed paired	11:00 am (starting time)

Figure 1

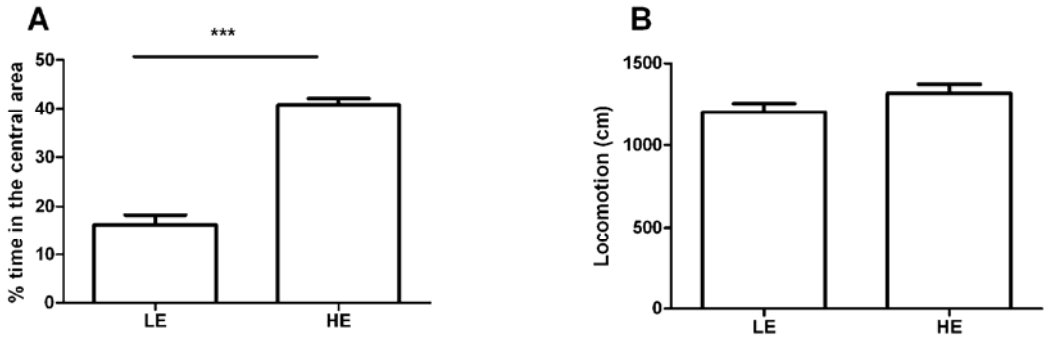

A
***
% time in the central area
50
40
30
20
10
0
LE
HE
B
Locomotion (cm)
1500
1000
500
0
LE
HE

Figure 2

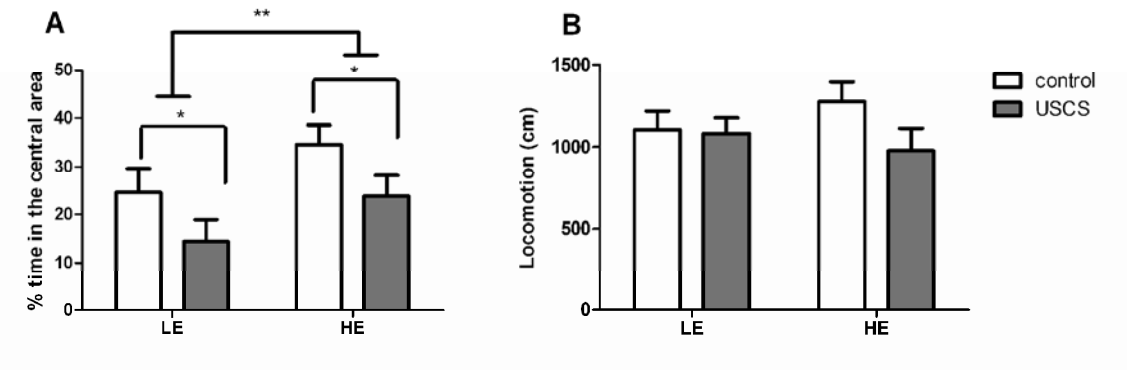

A
B
**
*
*
% time in the central area
50
40
30
20
10
0
LE
HE
Locomotion (cm)
1500
1000
500
0
LE
HE
control
USCS

Figure 3

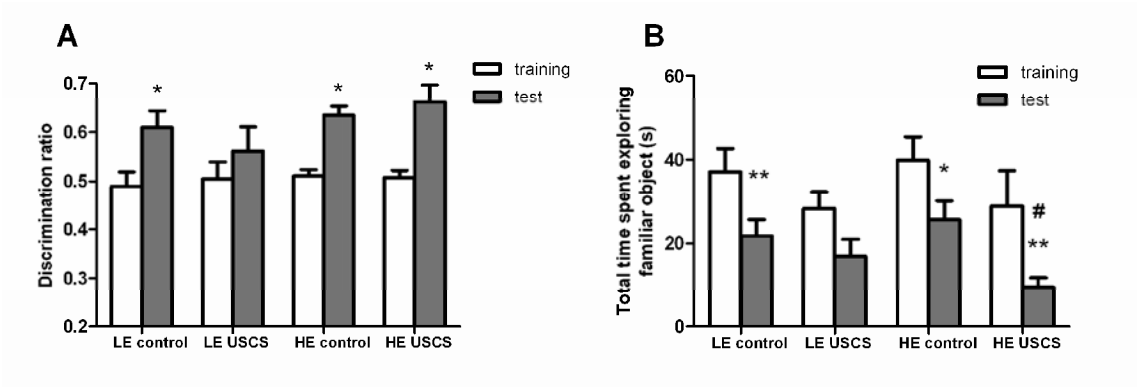

A
Discrimination ratio
0.7
0.6
0.5
0.4
0.3
0.2
*
*
*
training
test
LE control
LE USCS
HE control
HE USCS
B
Total time spent exploring familiar object (s)
60
40
20
0
**
*
#
**
training
test
LE control
LE USCS
HE control
HE USCS

Figure 4

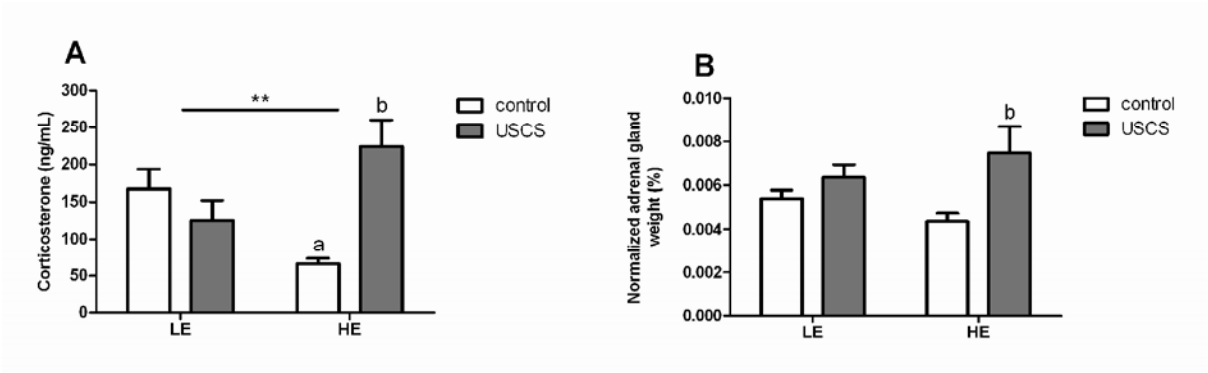
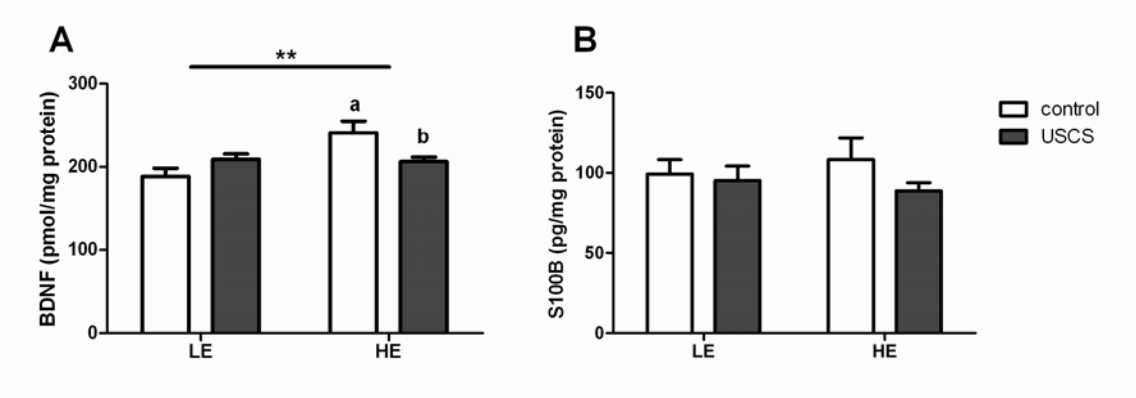

A
Corticosterone (ng/mL)
300
250
200
150
100
50
0
**
a
b
LE
HE
control
USCS
B
Normalized adrenal gland weight (%)
0.010
0.008
0.006
0.004
0.002
0.000
b
LE
HE
control
USCS

Figure 5


A
**
a
b
BDNF (pmol/mg protein)
300
200
100
0
LE
HE
B
S100B (pg/mg protein)
150
100
50
0
LE
HE
control
USCS

CAPÍTULO II

Enriched environment effects on behavior, memory and BDNF in Low and High Exploratory mice.

RUNNING TITLE: Environmental effects on mice traits

Vanessa Kazlauckas [a], Natalia Pagnussat [b], Sabrina Mioranzza [a], Eduardo Kalinine [a], Fernanda Nunes [a], Leticia Pettenuzzo [a], Diogo O.Souza [a], Luis V. Portela [a], Lisiane O. Porciúncula [a], Diogo R. Lara [b].

Artigo publicado no periódico Physiology & Behavior

Carta de Aceite

Ms. Ref. No.: PHB-D-10-00570R1

Title: Enriched environment effects on behavior, memory and BDNF in Low and High Exploratory mice.

Physiology & Behavior

Dear Mrs. Vanessa Kazlauckas,

I am pleased to confirm that your paper "Enriched environment effects on behavior, memory and BDNF in Low and High Exploratory mice." has been accepted for publication in Physiology & Behavior.

Thank you for submitting your work to this journal.

With kind regards,

Anton Scheurink

Editor-in-Chief

Physiology & Behavior

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Number of pages: 22

Number of figures: 5

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Abstract

Environmental enrichment (EE) has been largely used to investigate behavioral modifications and neuroplasticity in the adult brain both in normal and pathological conditions. The interaction between individual behavioral traits with EE responsiveness has not been investigated within the same strain. By using two extremes of CF1 mice that differ by their exploratory behavior in the Open Field (OF) task (Kazlauckas V, 2005), denominated as Low (LE) and High (HE) Exploratory Mice, the present study evaluated if EE during adulthood could modify the putative differences between LE and HE mice on exploratory behavior, memory performance and hippocampal BDNF levels. To this end, we investigated the effect of adult LE and HE mice after 2 months of enriched or standard housing conditions on the open field, on novel object recognition, on the inhibitory avoidance task and on hippocampal BDNF immunocontent. LE showed low exploratory behavior, less retention in the inhibitory avoidance and lower hippocampal BDNF levels. EE enhanced exploratory behavior, memory performance and hippocampal BDNF levels both in LE and HE mice. Importantly, the general profile of LE mice submitted to EE was similar to HE mice housed in standard conditions. These results show that internalized behavior of LE mice can be significantly modified by exposure to an enriched environment even during adulthood. These observations may contribute to investigate biological mechanisms and therapeutical interventions for individuals with internalized psychiatric disorders.

Keywords: Environmental enrichment; low exploratory mice, high exploratory mice, Open-field, BDNF, novel object recognition, inhibitory avoidance.

1. Introduction

Enriched housing is an environmental condition that provides enhanced possibilities of complex inanimate and social stimulation as compared to standard laboratory conditions [1] This protocol has been largely used to investigate behavioral modifications and neuroplasticity by increasing physical activity, learning experiences, visuals inputs, and social interactions [2, 3, 4, 5]. It has been observed that rodents submitted to an enriched environment even during adulthood present biochemical, morphological and functional changes in the adult brain both in normal and pathological conditions [5, 6].

The exposure to environmental enrichment (EE) induces plastic changes particularly at the level of the hippocampus and cerebral cortex [5, 7]. In rodents, hippocampal expression of BDNF and NGF neurotrophins increases after taking part in spatial learning tasks or performing physical exercise [8, 9]. In addition, these neurotrophins are correlated to an improved performance in learning and memory tasks [8, 10, 11,12, 13].

Previous studies using the anxious BALB/c strain as a possible model of neophobia and the nonanxious C57BL/6 strain revealed that BALB/c was more affected by EE than C57BL/6 [14; 15], suggesting that EE could have a strain specific effect on rodents behavior. EE also improved Spontaneously Hypertensive Rats (SHR) performance in open field habituation, water maze spatial reference, social and object recognition tasks, whereas non-cognitive traits, such as nociception and hypertension, were not affected by EE [16, 17].

A study with Roman high- and low-avoidance rats (RHA/Verh and RLA/Verh), which represent low emotional/anxious and high novelty seeker vs. high emotional/anxious and low novelty seeker profiles, respectively, showed that early-life EE increased head-dipping behavior in both rat lines, without affecting locomotor activity. They reported that these genetically divergent novelty seeking patterns can be enduringly modified to the point of

considerably reducing the between-line differences by early life rearing in an enriched environment [18].

Within the same strain, the interaction between individual behavioral traits and EE responsiveness during adulthood has not been investigated. Our group has characterized two extremes of mice that differ by their exploratory behavior in the Open Field (OF) task [19], denominated as Low (LE) and High (HE) Exploratory Mice. HE mice present less anxiety-like behavior, more aggression against intruders, higher avoidance of conditioned punishment (electric footshock), and better performance in a maze with positive reinforcement (food) compared to LE mice [19]. Thus, LE and HE mice may represent a model for internalized and externalized behaviors and disorders in humans. Internalized disorders, such as generalized anxiety, major depression and phobias show a common trait called neuroticism, with high fear, sensitivity and distress, whereas externalized disorders, such as antisocial personality disorder and drug abuse are characterized by impulsivity and aggression [20].

The purpose of the present study was to investigate if the influence of EE during adulthood could modify the putative differences between LE and HE mice on exploratory behavior, memory performance and hippocampal BDNF levels, or at least significantly affect LE behavior towards a less internalized profile. To this end, we evaluated adult LE and HE mice after 2 months of standard (ST) or enriched (EE) housing conditions with the open field for exploratory behavior, the novel object recognition and the inhibitory avoidance tasks for memory. After behavioral analysis, the hippocampal immunocontent of BDNF was determined.

2. Materials and Methods

2.1 Animals

Male albino CF1 mice (2 months), weighing approximately 35–40 g, were obtained from State Foundation for Health Science Research (FEPPS, Porto Alegre, RS, Brasil). They were housed in groups of six to eight in standard conditions of temperature and humidity, in a 12h light/dark cycle (lights on at 7:00 am), with access to food and water *ad libitum*. Sawdust was changed 2 times a week. All experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals and Brazilian Society for Neuroscience and Behavior (SBNeC). Recommendations for animal care were followed throughout all the experiments in accordance to the project approved by the ethical committee from Universidade Federal do Rio Grande do Sul. All efforts were made to minimize the number of animals employed in the present study and their suffering.

2.2 LE and HE mice selection

Two batches of eighty mice each were selected into low (LE) and high exploratory (HE) mice, according to their exploratory behavior in the central area of the open field (OF), as described in our previous research [19]. This test was used to separate the two different mice populations depending on the animal's response to a novel object in a new environment. Briefly, the animal was placed in an open-field (50 cm × 50 cm × 50 cm) with an object (a white cylinder of 1.5 cm radius and 5 cm high) in the center of the arena to stimulate exploration. Exploratory behavior was video recorded for 5 min, and the time spent by the animal in and out of an imaginary center square of 30 cm × 30 cm was analyzed using the ANYmaze software (Stoelting, Woods Dale). From 160 mice screened, the bottom and top 25% explorers of the central area of the arena composed the LE and HE exploratory groups, respectively. All mice were kept within their same housing groups and were randomly allocated to enriched environment (LE-EE, n = 18 and HE-EE, n = 23) or standard housing conditions (LE-ST, n = 22 and HE-ST, n = 17).

These four groups were tested after 2 months of environmental enrichment or standard conditions. Mice were appropriately identified and remained in their respective home cages without changing housemates until the end of behavioral testing.

2.3 Housing conditions

Standard housing conditions consisted of a 27cm x 16cm x 12cm acrylic box with sawdust containing groups of 6-8 mice. Enriched housing conditions consisted of 38cm x 32cm x 16cm acrylic box with sawdust containing 8 mice. The apparatus contained one running wheel and a variety of objects, including wood and plastic objects, tunnels, hiding places and nesting materials where the animals could be out of luminosity. The objects were changed 2 times a week.

2.4 Behavioral tasks

The behavioral tasks were conducted in two independent cohorts of LE and HE mice. One group was tested in the open field and novel object recognition task, and the other group was tested in the open field, in the novel object recognition task and in the inhibitory avoidance task.

2.4.1 Open field after EE

The open field task was performed as previously described above in 2.2 LE and HE mice selection.

2.4.2 Novel object recognition task

Novel object recognition task (NOR) was performed in an apparatus consisting of a small black wood chamber (25 cm×25 cm×40 cm). Before the experimental sessions, animals

were habituated to the experimental room for 60 min in dim light conditions. A light bulb was switched on during the experimental sessions, with uniform light intensity in the different parts of the apparatus. The objects were placed equidistant from two corners, 12 cm apart from the wall. Two observers blind to the housing conditions performed the behavioral evaluation. Mice had been acclimated in the apparatus during ten minutes twenty-four hours before sample session. The sample session consisted of placing a mouse in the apparatus containing two identical objects, and allowed it to explore for 10 minutes. Each mouse was always placed in the apparatus facing the wall. In the discrimination sessions, performed 1.5 h and 24 h later, one familiar (used in the sample session) and a novel object were presented. The objects employed were 2 kinds of small bottles with different shape and color (white and amber) presenting the same texture and size. The objects do not have ethological significance for mice. Objects were cleaned between sessions with 70% ethanol solution. Exploration was defined as directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose or forepaws. The time of exploration was manually recorded. Animals presenting less than 3 seconds of exploration time were excluded from the experiment. The following parameters were analyzed: (a) the discrimination ratio, analyzed and expressed by the ratio of total time spent exploring the novel object by the total time spent in both objects and (b) time spent exploring both objects during the sample and discrimination sessions in seconds. The discrimination for sample session was calculated by the ratio between the time spent on one of the objects randomly chosen and the total time of exploration for both objects in the sample session [21].

2.4.3 Inhibitory avoidance

The inhibitory avoidance task was assessed in an acrylic box (50 × 25 × 25 cm) with parallel stainless-steel bars (1 mm diameter) spaced 1 cm apart as the floor. A platform (2 cm

high and 4 cm × 6 cm wide) was placed in the center of the box. In the training session, mice were placed on the platform and the latency to step-down onto the floor with the four paws was recorded; immediately after stepping-down mice received a 0.4 mA, 2 s footshock and were placed in their home cage. The test session was performed 1.5 hours after training (short-term memory) or 24 hours after training (long-term memory). No footshock was given in the test session, and step-down latencies (180 s ceiling) were taken as a measure of retention.

2.5 Immunoblotting

After behavioral analysis mice were sacrificed by decapitation and the whole hippocampus was dissected out immediately after the end of the experiments. Hippocampi were homogenized in 5% SDS solution containing A protease inhibitor cocktail (Sigma, São Paulo/Brazil) and kept at −70 °C. Protein content was further determined by using bicinchoninic acid assay using bovine serum albumin (BSA) as standard (Pierce, São Paulo/Brazil). Hippocampal extracts were diluted to a final protein concentration 2 μg/μl in SDS-PAGE buffer and 85 μg of the samples and dual-color prestained molecular weight standards (Bio-Rad, Porto Alegre, Brazil) were separated by SDS-PAGE (16% with 4% concentrating gel). After electro-transfer, the membranes were incubated overnight with Tris–buffered saline 0.1% Tween-20 (TBS-T) containing 3% BSA. After blocking, the membranes were incubated for 24 h at 4 °C with mouse anti-BDNF antibody (1:500, Sigma, São Paulo, Brazil) or with mouse anti-β-tubulin antibody (1:1000; Sigma, São Paulo, Brazil). After primary antibodies incubation, membranes were washed and incubated with horseradish peroxidase conjugated secondary antibodies for 2 h at room temperature and developed with ECL (Amersham, São Paulo/Brazil). The autoradiographic films were scanned, and densitometric analyses were performed using Image J software. As an additional control of

the protein loading, membranes were stained with Ponceau S. The results were presented by BDNF/ β-tubulin density.

2.6 Statistical analysis

Differences in exploratory profile between LE and HE in the open field task were analyzed using Student´s t-test. For Open field exploration after EE and BDNF immunocontent determination, differences were analyzed using two-way ANOVA with groups (LE / HE) and housing conditions (ST / EE) as independent variables, followed by Bonferroni to compare each EE to their ST group. In the novel object recognition test, we used Three-way ANOVA with differences between groups, housing conditions and trials and also used the test of within subjects contrast. Separate analyses were performed in order to test for specific differences between groups. In the inhibitory avoidance test, step-down latencies are expressed as medians (interquartile ranges). Differences between training and test session were analyzed by Wilcoxon and difference between groups were analyzed by Kruskal Wallis followed by Dunns multiple comparison tests. Graphpad Prism 5 and SPSS16.0 softwares were used, and significant differences were considered when $P < 0.05$. Except for inhibitory avoidance, results are expressed as mean + S.E.M.

3. Results

3.1 LE and HE phenotypes selection

LE and HE mice were selected in the open field task according to their exploratory behavior (n = 40 in each group). LE mice spent 14.8 ± 1.4 % of the time in the central area of the arena compared to 41.6 ± 1.1 % for HE mice ($P < 0.001$, Fig.1A). Locomotor activity did not differ between LE and HE groups (Fig. 1B).

3.2 Effects of Environmental Enrichment in the Open field task

EE conditions significantly increased exploration of the central area in both LE and HE groups [F (1, 72) = 19.44; $P < 0.001$]. After two months under standard or enriched housing conditions, the exploratory behavior of HE groups remained higher than their respective LE groups [F (1, 72) = 32.35; $P < 0.001$], but LE-EE was not different from HE-ST ($P > 0.1$) (Fig. 2A). Locomotor activity did not change for any group after environmental enrichment exposure (Fig. 2B).

3.3 Novel object recognition task

The performance of both groups was improved by enriched environment as shown in Figure 3A. All groups had an increase in the discrimination ratio across trials [F (2, 94) =1 06.47, $P < 0.05$] and there was a significant interaction [F (2, 94) =11.29, $P < 0.05$]. The test of within subjects contrast revealed a quadratic relation between trials *versus* housing conditions (F (1, 47) =13.9, $P < 0.05$), showing that enriched groups reached the most prominent performance in this task in the second trial, whereas standards groups reached the best performance only in the third trial.

LE groups showed lower exploration of both objects when compared to HE [F (1, 47) = 26.65, $P < 0.05$]. Also, EE in both LE and HE groups decreased object exploration compared to standard [F (1, 47) = 16.66, $P < 0.05$]. We observed a trend for interaction ($P = 0.051$) indicating that this effect of EE is more prominent in HE groups (Figure 3B).

3.4 Inhibitory avoidance task

In the inhibitory avoidance task, latency to step-down in the training session was similar for all groups (data not shown). All four groups significantly increased latency to step-down at 1.5 h and 24 h after training session (Figure 4, $*P < 0.05$ and $**P < 0.001$). Under

standard housing conditions, HE showed higher retention than LE mice at 24 h ($P < 0.001$) but not at the 1.5 h after training. LE-EE had significantly higher step-down latency compared to LE-ST both 1.5 h and 24 h after training ($P < 0.001$, Figure 4A and 4B). The performance of HE under standard and enriched conditions was not different as revealed by similar latencies to step-down in the test session. Thus, these results indicate that environmental enrichment promotes an improvement in performance of inhibitory avoidance task especially in LE mice.

3.5 Hippocampal BDNF immunocontent:

BDNF levels increased in LE and HE groups after environmental enrichment [$F(1, 18) = 5.56$; $P < 0.05$], and also both HE groups presented higher levels of BDNF when compared to LE groups [$F(1, 18) = 17.80$; $P < 0.001$] (Figure 5). Hippocampal BDNF levels were not significantly different between LE-EE and HE-ST groups ($P > 0.10$).

4. Discussion

This study showed that environmental enrichment enhanced exploratory behavior, memory performance and hippocampal BDNF levels both in LE and HE mice. Trait differences in exploratory behavior and hippocampal BDNF levels remained within the same housing conditions. Importantly, the general profile of LE mice submitted to environmental enrichment was similar to HE mice housed in standard conditions. These results show that the more internalized behavior, inferior cognitive performance and neurotrophic tone of LE mice can be significantly modified by exposure to an enriched environment even during adulthood. However, overall these results do not support that individual trait differences between HE and LE mice can be substantially modified by environmental interventions.

Postweaning rats exposed to enriched environment explored more the central area of the open field when compared to rats housed in isolation or standard conditions [22]. Furthermore, rats exposed to an environmental enrichment presented reduced latency to explore the novel open field and faster exploration of novel objects in the NOR task [23]. These studies corroborate our findings of increased OF central area exploration in adult mice exposed to EE, which occurred in both LE and HE groups. However, in the NOR task, EE lead to reduced time exploring objects. This was particularly evident in HE mice, which explored both objects extensively in standard conditions, in agreement with these mice natural behavioral patterns [19]. Thus, depending on the parameter analyzed and time of exposure to novelty, EE may have different effects on exploratory activity. In general, EE seems to change mice exploratory behavior towards an immediate start of exploration, but for shorter periods of time. Thus, this faster habituation induced by an enriched environment may lead to higher exploration in a short protocol (5’ min in the OF) and lower exploration in a longer protocol (10' in the NOR task).

Enrichment protocol at postweaning and during adulthood also decreases locomotion in rodents probably by increasing habituation [3, 17, 24, 25]. However, Fernandez-Teruel et al. (2002) reported that EE did not produce changes in the locomotor activity in their Roman rats [18] which corroborates our study,that locomotor activity in the OF task did not change after environmental enrichment, but our protocol was shorter (5’ compared to 10’ in other studies) and the OF had an object in the central area, which may have changed the locomotor pattern.

Improved learning and memory by environmental enrichment is one of the most consistent findings in the literature [5, 10, 13, 26, 27]. Therefore, to further understand the effect of enriched conditions in LE and HE mice, we analyzed their behavioral performance in the novel object recognition task, which accesses memory based on the natural motivation of

animals to explore novelty in a familiar context. This task has been widely used to evaluate the effects of pharmacological, genetic or environmental interventions on memory processes [28]. The hippocampus seems to play a central role in this task, both in memory processes and in environmental interactions [29, 30]. A previous study, using CF1 male mice, showed that mice in EE correctly discriminate objects using less time exploring objects [2]. Our results showed that LE-ST and HE-ST mice presented similar memory performance in the NOR task. Furthermore, LE and HE submitted to EE clearly discriminated the novel object in the 1.5 h test whereas the standard groups showed this performance only in the 24 hours test after training. These results suggest that EE can especially improve recognition memory.

Another task that deals with the animal's natural exploratory behavior is the inhibitory avoidance task which consists in the animal's ability to avoid a conditioned punishment repressing their tendency to explore beyond the safe areas [31]. The trait difference was clearly evident when long-term memory was assessed (24 h after training), since the retention for LE-ST was lower than HE-ST, confirming our previous observation [19]. Thus, this trait difference was reflected for long- but not for short-term memory. Short and long-term memories present different molecular mechanisms, which includes protein synthesis for long-term memory. Recently, studies in rodents using the inhibitory avoidance task showed that BDNF induces memory persistence by transforming a nonlasting long-term memory trace into a persistent memory trace, revealing that BDNF is essential for long-term memory persistence [32]. Thus, the lower density of BDNF presented for LE mice compared to HE housed in the same conditions could help to explain the lower performance for long-term memory in the inhibitory avoidance task.

Interestingly, the environmental enrichment significantly improved the performance in the inhibitory avoidance task only for LE mice. Probably, the performance for HE mice was not modified due to a ceiling effect. Considering that the consolidation of memory is a

process that lasts few hours through which memories are transformed from a labile into a more stable state, probably interventions could be more effective for consolidation than acquisition. Previous studies have demonstrated significant alterations in the BDNF protein levels in several brain regions as a result of an enriched environment, providing a possible biochemical basis for its behavioral and morphological alterations [11, 33]. Given that LE presented lower density of BDNF than HE mice housed in the same conditions, the environmental enrichment promoted an increase on BNDF immunocontent in both groups. Once more the increase on BDNF immunocontent caused by EE may be involved in the environmental enrichment benefits on memory presented mainly for LE-EE mice when compared with their LE counterparts. Thus, memory and BDNF levels depend on both trait and environmental conditions.

In conclusion, trait behavior, memory and neurobiological markers can be substantially modified by environmental interventions in adult mice. More specifically, internalized traits, as in LE mice and in patients with internalized psychiatric disorders, may be attenuated by exposure to an enriched environment even during adulthood.

Acknowledgments

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Legends

Fig. 1: Selection of low (LE) and high (HE) exploratory mice behavioral pattern. Animals were subjected to the open field task with a central object, and time spent in the central area (A) and locomotor activity (B) were recorded during five minutes. LE (n = 40) and HE (n = 40) mice were evaluated for time spent in the central area (A) and locomotion (cm) (B). Results are presented as mean + S.E.M. Statistical analysis was performed using Student´s *t* test. *** $P<0.001$.

Fig. 2: Exploratory and locomotor activities in the open field task after environmental enrichment. LE and HE standard (ST) and enriched (EE) mice were subjected to the open field task with a central object, and time spent in the central area (A) and locomotor activity (B) were recorded during five minutes. White bars represent the LE and HE ST groups and gray bars represent LE and HE EE groups. Results are presented as mean + S.E.M. Statistical analysis was performed by Two-way ANOVA followed by Bonferroni post hoc test. ** $P<$ 0.001. LE-ST (n = 20); LE-EE (n = 19); HE-ST (n = 19); HE-EE (n = 19).

Fig. 3: Novel object recognition task. LE and HE standard (ST) and enriched (EE) mice were evaluated for: (A) discrimination ratio for the sample and discrimination sessions and (B) total time spent exploring both objects during the sample and discrimination sessions. Results are presented as mean + S.E.M. Statistical analysis was performed by Three-way ANOVA with differences between groups, housing conditions and trials. * $P< 0.05$ indicates difference between sample and discrimination sessions within groups. # $P<0.05$ indicates difference between sample and discrimination sessions within groups and differences between

discrimination sessions within groups [LE (ST and EE)]; [HE (ST and EE)]. LE-ST (n = 12); LE-EE (n = 13); HE-ST (n = 11); HE-EE (n = 15).

Fig. 4: Effect of environmental enrichment in the inhibitory avoidance task in mice tested 1.5 h (A) and 24 h (B) after the training session. Results are presented as dots and dash represents median (interquartile range) values. Differences between groups were analyzed by Kruskal Wallis followed by Dunns multiple comparison tests. LE-ST (n = 8); LE-EE (n = 8); HE-ST (n = 10); HE-EE (n = 9). ** $P<0.001$ between groups. (ST): standard and (EE): enriched.

Fig. 5: Hippocampal BDNF immunocontent for LE and HE standard (ST) and enriched (EE) mice. White bars represent the LE and HE ST groups and gray bars represent LE and HE EE groups. Results are presented as mean + S.E.M. Statistical analysis was performed by Two-way ANOVA followed by Bonferroni post hoc test. *** $P< 0.001$ between LE and HE groups and * $P< 0.05$ between ST and EE groups. LE-ST (n = 6); LE-EE (n = 5); HE-ST (n = 5); HE-EE (n = 6).

Figure 1

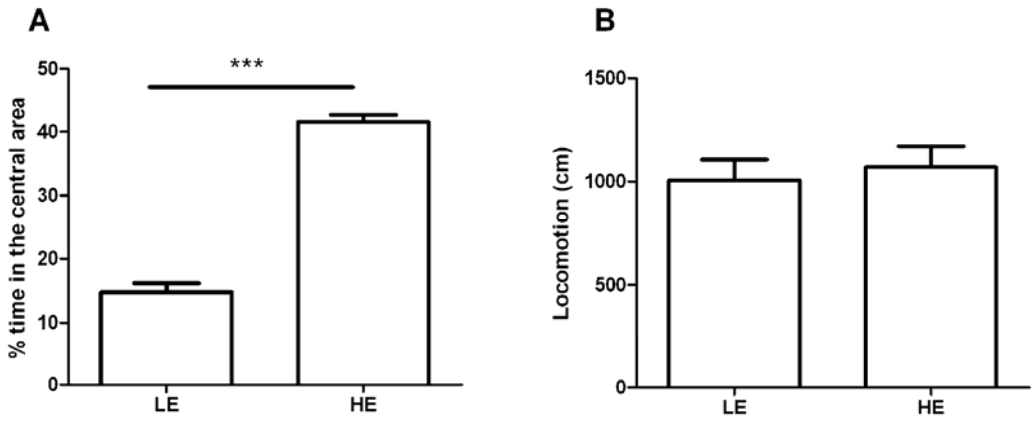

A
***
% time in the central area
50
40
30
20
10
0
LE
HE
B
Locomotion (cm)
1500
1000
500
0
LE
HE

Figure 2

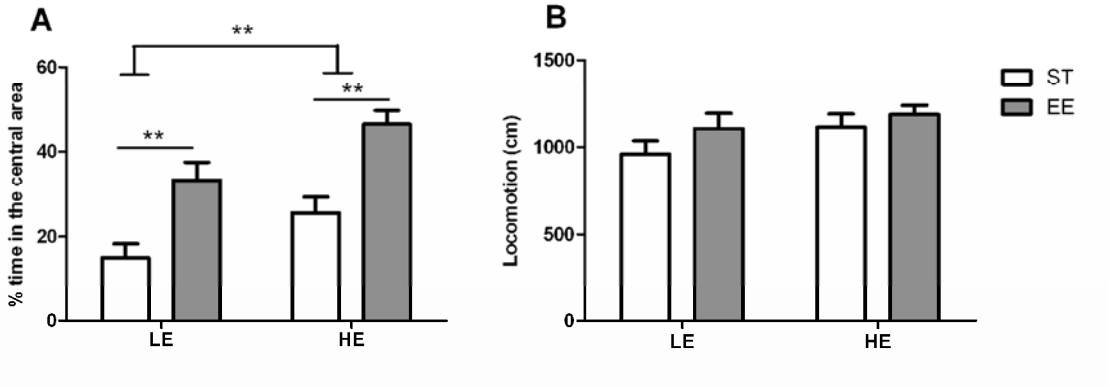

A
B
**
**
**
% time in the central area
60
40
20
0
LE
HE
Locomotion (cm)
1500
1000
500
0
LE
HE
ST
EE

Figure 3

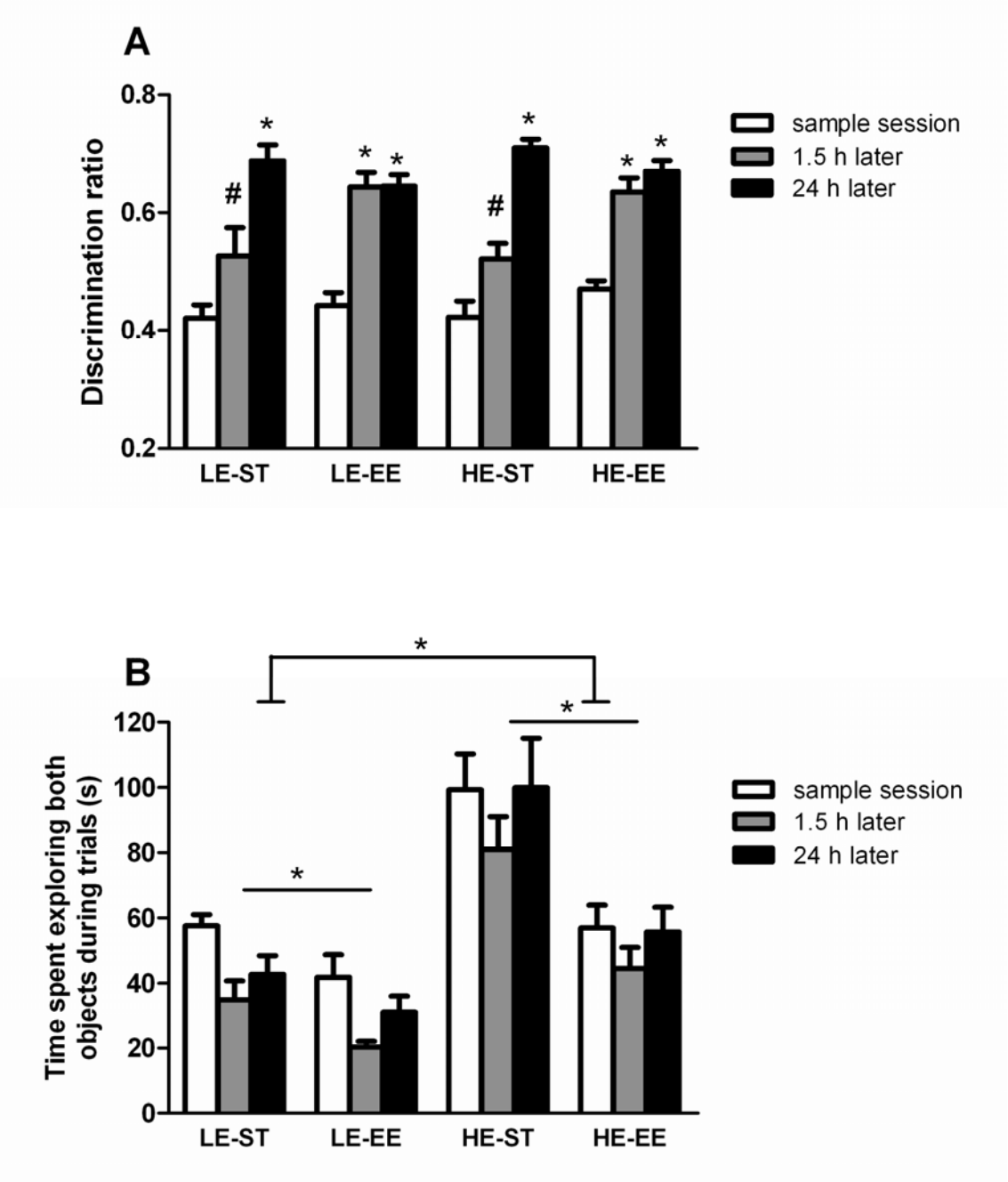

A
Discrimination ratio
0.8
0.6
0.4
0.2
#
*
LE-ST
LE-EE
HE-ST
HE-EE
sample session
1.5 h later
24 h later
B
Time spent exploring both objects during trials (s)
120
100
80
60
40
20
0
*
LE-ST
LE-EE
HE-ST
HE-EE
sample session
1.5 h later
24 h later

Figure 4

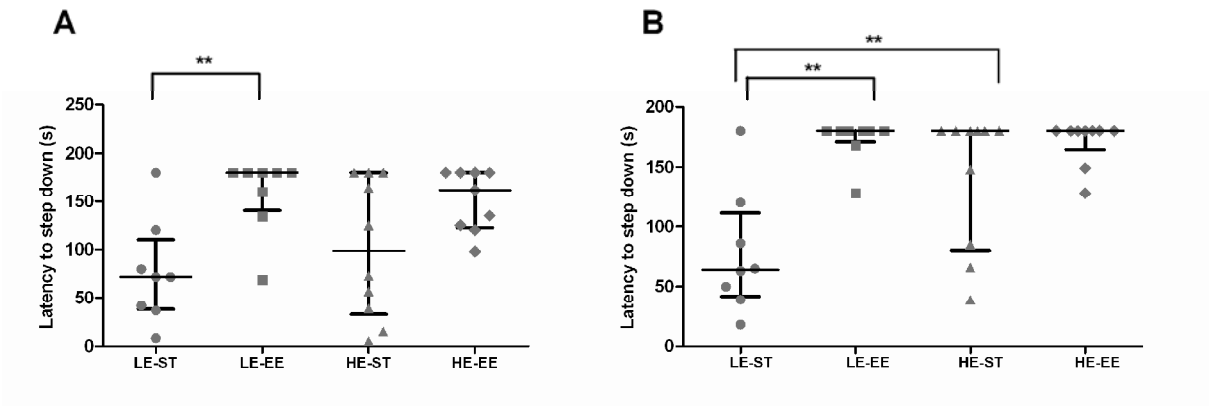
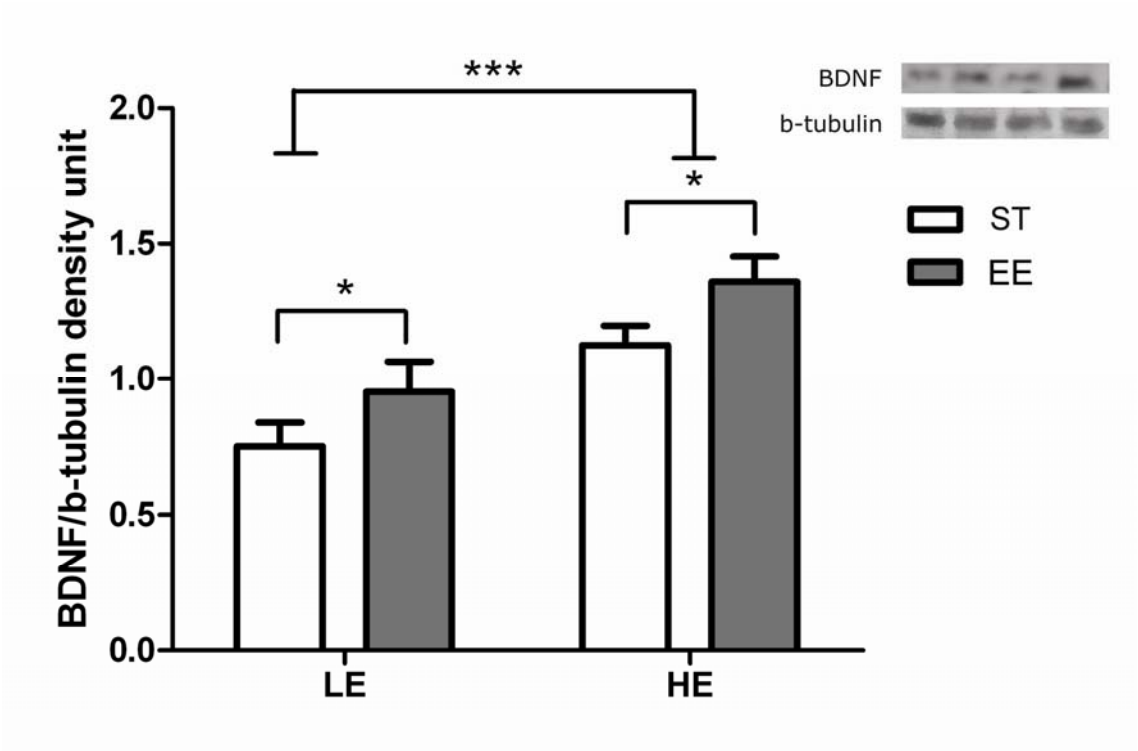

A
**
Latency to step down (s)
250
200
150
100
50
0
LE-ST
LE-EE
HE-ST
HE-EE
B
**
**
Latency to step down (s)
200
150
100
50
0
LE-ST
LE-EE
HE-ST
HE-EE

Figure 5


***
*
*
BDNF
b-tubulin
ST
EE
BDNF/b-tubulin density unit
2.0
1.5
1.0
0.5
0.0
LE
HE

CAPÍTULO III

Environmental enrichment decreases S100B levels in low and high exploratory mice.

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Artigo em preparação

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Abstract

Environmental enrichment (EE) is an experimental model to study neuroplasticity. Rodents submitted to an enriched environment even during adulthood present biochemical, morphological and functional changes in the adult brain both in normal and pathological conditions. Increases in astrocytic volume and number have been reported after EE in cortical region. By using two extremes of CF1 mice that differ by their exploratory behavior in the Open Field (OF) task (Kazlauckas V, 2005), denominated as Low (LE) and High (HE) Exploratory Mice, the present study evaluated if EE during adulthood could modify the astrocytes number and GFAP optical density in the CA1 and DG of hippocampus and S100B hippocampal levels. The present study showed that enriched environments had no effects on the GFAP optical density on the CA1 and on the DG of the hippocampus in both groups. Also, EE has significant effects on exploratory behavior and specifically reduces hippocampal S100B levels in both LE and HE mice. Thus, this is the first report of decrease of hippocampal S100B levels in response to EE.

Key-words: astrocytes; exploratory behavior; S100B; GFAP; open field

1. Introduction

Environmental enrichment (EE) is an experimental model that allows the study of neuroplasticity by providing increases in physical activity, learning experiences, somatosensorial and visual inputs and social interaction among animals in their home environment (van Praag et al., 2000; Mohammed et al., 2002). Rodents submitted to an enriched environment even during adulthood present biochemical, morphological and functional changes in the adult brain both in normal and pathological conditions (Viola et al, 2009; Amaral et al, van Praag et al., 2000; Will et al., 2004).

Studies of environmental effects on brain plasticity have focused on altered neuronal morphology; however, substantial morphological changes have also been shown to occur in glial cells (Sirevaag & Greenough, 1991; Komitova et al., 2002), and the first report to describe glial multiplication associated with enriched environment was published in 1964 using autoradiography (Altman & Das, 1964).

Increases in astrocytic volume and number have been reported after EE in cortical regions (Szeligo and Leblond, 1977; Sirevaag and Greenough, 1991). However, even though astrocytic plasticity is probably involved in the effects of EE in the hippocampus, there is little data on whether EE can induce changes in hippocampal astrocytic networks (Sirevaag et al., 1991; Briones et al., 2006).

Our group has characterized two behavioral extremes of mice that differ by their exploratory behavior in the Open Field (OF) task (Kazlauckas et al, 2005), denominated as Low (LE) and High (HE) Exploratory Mice. HE mice present less anxiety-like behavior, more aggression against intruders, higher avoidance of conditioned punishment (electric footshock), and better performance in a maze with positive reinforcement (food) compared to LE mice (Kazlauckas et al, 2005). Thus, LE and HE mice may represent a model for internalized and externalized behaviors disorders in humans.

The purpose of the present study was to investigate astrocytes number and GFAP optical density in the CA1 and dentate gyrus (DG) of hippocampus and S100B hippocampal levels of HE and LE mice exposed to EE during adulthood. Exploratory behavior was also analyzed.

2. Materials and Methods

2.1 Animals

Male albino CF1 mice (2 months), weighing approximately 35–40 g, were obtained from State Foundation for Health Science Research (FEPPS, Porto Alegre, RS, Brasil). They were housed in groups of six to eight in standard conditions of temperature and humidity, in a 12h light/dark cycle (lights on at 7:00 am), with access to food and water *ad libitum*. All experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals and Brazilian Society for Neuroscience and Behavior (SBNeC). Recommendations for animal care were followed throughout all the experiments in accordance to the project approved by the ethical committee from Universidade Federal do Rio Grande do Sul. All efforts were made to minimize the number of animals employed in the present study and their suffering.

2.2 LE and HE mice selection

Eighty mice were selected into low (LE) and high exploratory (HE) mice, according to their exploratory behavior in the central area of the open field (OF), as described in our previous research (Kazlauckas et al, 2005). This test was used to separate the two different mice populations depending on the animal's response to a novel object in a new environment. Briefly, the animal was placed in an open-field (50 cm × 50 cm × 50 cm) with an object (a white cylinder of 1.5 cm radius and 5 cm high) in the center of the arena to stimulate exploration. Exploratory behavior was video recorded for 5 min, and the time spent by the

animal in and out of an imaginary center square of 30 cm × 30 cm was analyzed using the ANYmaze software (Stoelting, Woods Dale). The bottom and top 25% explorers of the central area of the arena composed the LE and HE exploratory groups, respectively. All mice were kept within their same housing groups and were randomly allocated to enriched environment (LE-EE, n = 9 and HE-EE, n = 10) or standard housing conditions (LE-ST, n = 11 and HE-ST, n = 10).

These four groups were tested after 2 months of environmental enrichment or standard conditions. Mice were appropriately identified and remained in their respective home cages without changing housemates until the end of behavioral testing.

2.3 Housing conditions

Standard housing conditions consisted of a 27cm x 16cm x 12cm acrylic box with sawdust containing groups of 6-8 mice. Enriched housing conditions consisted of 38cm x 32cm x 16cm acrylic box with sawdust containing 8 mice. The apparatus contained one running wheel and a variety of objects, including wood and plastic objects, tunnels, hiding places and nesting materials where the animals could be out of luminosity. The objects and sawdust were changed 2 times a week.

2.4 Immunohistochemistry

For the immunohistochemical study, animals were deeply anesthetized with ketamine (100 mg/kg) and xylasine (10 mg/kg). They were then transcardially perfused through the left cardiac ventricle using 60 mL of saline solution followed by 60 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4; PB). The brains were removed and post-fixed in the same fixative solution at room temperature for 4 h, then cryoprotected in a 30% sucrose solution in PB at 4 °C until they sank. Coronal sections (30 μm) were obtained using a vibratome (Leica,

Germany). Sections were collected in phosphate buffer saline solution (PBS, pH=7.4) and processed for immunohistochemical detection for GFAP as a marker of astrocytes (Pekny and Nilsson, 2005). We carried out in the sections with all fields of the hippocampus. The sections were first rinsed for 5 min with PBS (140mM NaCl, 3mM KCl, 20mM Na2HPO4, 1.5mM KH2PO4) and then three times for 5 min with Trizma base buffer (TBS: 0.05M containing 150mM NaCl, pH 7.2) at room temperature. The sections were blocked with TBS containing 0.2% Triton X-100 and 10% goat serum for 45 min, then incubated in the presence of the rabbit anti-GFAP antibody (1:500 dilution in TBS containing 0.2% Triton X-100 and 10% goat serum) for 48h at -4 ^{o}C. After incubation, sections were rinsed three times for 10 min in TBS and subsequently incubated with goat antirabbit secondary antibody conjugated with a fluorophore (Alexa Fluor 594; Invitrogen, São Paulo, Brazil; 1:200 dilution in 0.1M TBS containing 0.2 % Triton X-100 and 10% goat serum) overnight at -4 ^{o}C. After rinsing three times for 10 min in TBS, the sections were mounted on slides, using Vectashield mounting medium (Vector Laboratories, São Paulo, Brazil). All sections were examined under fluorescence Nikon (Nikon Eclipse E600) fluorescence microscope with Nikon ACT 1C software.

2.5 Estimation of GFAP density and astrocyte number

The intensity of GFAP immunoreactivity was measured by semi-quantitative densitometric analysis (Ferraz et al., 2003; Xavier et al., 2005; Martinez et al., 2006) using a Nikon Eclipse E-600 microscope coupled to a Nikon DXM 1200C CCD camera and analyzed by Image J software. All lighting conditions and magnifications were kept constant during the capture process. Picture elements (pixels) employed to measure optical density were obtained from region of interest (ROI) squares with 157500 μm^2 with background correction. Six images were analyzed per subfield from a total of four-six animals per group. The number of

GFAP-immunoreactive astrocytes per mm^2 in the CA1 and DG located inside the square ROI or intersected by the lower and/or right edges of the square were counted using the Nikon ACT 1C software.. Obvious blood vessels and other artifacts were avoided. These images corresponding to an area of the hippocampus extending from bregma -1.58 mm to -2.06 mm (Franklin and Paxinos, 1997).

2.6 Determination of hippocampal S100B levels

S100B concentrations were measured using an enzyme linked immunosorbent assay (Diasorin® S100 ELISA Kit) in a Spectra Max M5 molecular Devices (USA). Calibrators and hippocampal samples (100 µL of each) were incubated in a plate already coated with anti-S100B antibody. The S100 ELISA was a two-site, one-step, enzyme linked immunosorbent assay. In the assay calibrators, controls and unknown samples react simultaneously with 2 solid phase capture antibodies and a detector antibody conjugated with horseradish peroxidase (HRP) during the incubation in the microtiter wells for 2 hours. After a washing step a TMB chromogen (Tetramethylbenzidine) was added and the reaction was allowed to proceed for 15 minutes. The enzyme reaction was stopped by adding stop solution and the absorbance was measured at 450 nm. S100B concentrations were derived by comparison with the calibration curve based on the total absorbance for each given calibrator provided with the assay. All determinations were carried out within the same experiment. The S100B calibration curve is cubic spline up to 5 µg/L, and the CVs for duplicates across the entire concentration range for the calibrators and samples were 5%. The detection limit of the assay is 0.03 µg/L. The results are expressed as pg/mg wet tissue protein

2.7 Statistical analysis

Differences between LE and HE in the selection by their exploratory profile in the

open field task were analyzed using Student´s t-test. For Open field exploration after EE and GFAP density and astrocyte number, differences were analyzed using two-way ANOVA with groups (LE / HE) and housing conditions (ST / EE) as independent variables, followed by Bonferroni to compare each EE to their ST group.

3. Results

3.1 LE and HE phenotypes selection

LE and HE mice were selected in the open field task according to their exploratory behavior (n = 20 in each group). LE mice spent 10.9 ± 1.5 % of the time in the central area of the arena compared to 36.8 ± 1.1 % for HE mice ($P < 0.001$, Fig.1A). Locomotor activity did not differ between LE and HE groups (Fig. 1B).

3.2 Effects of Environmental Enrichment in the Open field task

EE conditions significantly increased exploration of the central area in both LE and HE groups [$F(1, 36) = 10.53$; $P < 0.001$]. After two months under standard or enriched housing conditions, exploratory behavior of HE groups remained higher than their respective LE groups [$F(1, 36) = 28.86$; $P < 0.001$], but LE-EE was not different from HE-ST ($P > 0.1$) (Fig. 2A). Locomotor activity did not change for any group after environmental enrichment exposure (Fig. 2B).

3.3 GFAP optical density

Quantitative results for GFAP OD in the CA1 region and GFAP OD in the DG are shown in Figures 3A and B, respectively. There was no significant difference between the EE and ST groups.

3.4 Astrocytes number

Number of astrocytes in the CA1 region had a trend level reduction in the HE-EE group [$F(1,18) = 3.51$, $P= 0.06$] Figure 4 A. There was no significant difference in astrocyte number in the DG region (Figure 4B).

3.5 S100B levels

S100B levels were analyzed by two-way ANOVA revealed a treatment effect [$F (1, 17) = 4.98$; $P< 0.05$]. No significant difference between HE and LE groups was found. This result revealed that the S100B levels decreased in both groups of mice under enriched conditions (Figure 5).

4. Discussion

The present study showed that EE has significant effects on behavior and specifically reduces hippocampal S100B levels in both LE and HE mice. Interestingly, EE exposure alters the expression of genes involved in signaling and neural plasticity in different hippocampal areas in adult mice (Rampon et al, 2000) and S100A9 gene expression is down-regulated after EE treatment in Presenilin Knockout mice as a model for Alzheimer disease (Dong et al., 2007).

Hippocampal BDNF significantly increases in S100B KO mice compared to wild-type mice (Schulte-Herbrüggen et al, 2008). EE increases brain BDNF levels, mainly in the hippocampus, and such increase may contribute to the biochemical basis for behavioral and brain morphological alterations after EE (Rossi et al, 2006; Ickes et al, 2000). Thus, we could interpret that the decrease in the S100B levels after adulthood EE is in line with these observation. On the other hand, S100B is reduced in the raphe region and hippocampus of BDNF KO mice with unchanged number of astrocytes (Djalali et al, 2005).

Environmental enrichment and other factors are known to influence adult neurogenesis and gliogenesis, driving the brain plasticity, including astrocyte morphology and biochemistry (Steiner et al, 2004). Astrocyte morphology has long been known to change in response to postweaning EE, with the changes depending both on the duration of EE exposure and on the cortical layer in which the astrocytes reside (Nilsson and Pekny, 2007). In this context, we observed that astrocyte number tends to reduce in CA1 region of hippocampus in the HE-EE group. Increases in astrocytic volume and number have been reported after postweaning EE in cortical regions (Szeligo and Leblond, 1977; Sirevaag and Greenough, 1991). However, another study reported that old rats under enriched conditions have decreased astrocyte number and size in the frontal cortex, corpus callosum and mainly in the hippocampus (Soffié et al, 1999). Also, Viola et al. (2009) observed no significant difference in hippocampal number of astrocytes and in GFAP optical density between their enriched and control mice postweaning enriched (Viola et al, 2009). In the present study, enriched environments had no effects on the GFAP optical density on the CA1 and on the DG of the hippocampus in both groups.

Moreover, EE can induce pronounced biochemical, morphological and functional changes in the brain, influencing the brain in many ways that in due course modulate its function (van Praag et al., 2000). Also, EE enhances neurogenesis in DG of hippocampus increasing levels of certain compounds that have important roles in neural signalling and cellular plasticity (Segovia et al, 2009), so further studies regarding neurogenesis and astrocytes morphology in our HE and LE mice would lead to better evidence on the changes caused by EE.

In conclusion, this is the first report of decrease of hippocampal S100B levels in response to EE. This change takes place without histological differences in astrocytes.

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Legends:

Fig. 1: Selection of low (LE) and high (HE) exploratory mice behavioral pattern. Animals were subjected to the open field task with a central object, and time spent in the central area (A) and locomotor activity (B) were recorded during five minutes. LE (n = 40) and HE (n = 40) mice were evaluated for time spent in the central area (A) and locomotion (cm) (B). Results are presented as mean + S.E.M. Statistical analysis was performed using Student´s *t* test. *** $P<0.001$.

Fig. 2: Exploratory and locomotor activities in the open field task after environmental enrichment. LE and HE standard (ST) and enriched (EE) mice were subjected to the open field task with a central object, and time spent in the central area (A) and locomotor activity (B) were recorded during five minutes. White bars represent the LE and HE ST groups and gray bars represent LE and HE EE groups. Results are presented as mean + S.E.M. Statistical analysis was performed by Two-way ANOVA followed by Bonferroni post hoc test. ** $P<$ 0.001. LE-ST (n = 20); LE-EE (n = 19); HE-ST (n = 19); HE-EE (n = 19).

Fig. 3. Effects of environmental enrichment on GFAP optical density measurements in the CA1 (A) and DG (B) regions from standard (white bar) and EE (gray bar) or LE and HE groups. Expressed as mean ± S.E.M (n= 5–6). A.U., arbitrary units.

Fig.4. Quantitative analysis of GFAP-stained astrocytes from CA1 and DG of the hippocampus from LE and HE mice under ST or EE conditions. Results are means + S.E.M of the number of GFAP-stained astrocytes/mm2 (n = 4/ 6 animals per group).

Fig. 5: Hippocampal S100B levels for LE and HE ST and EE mice. White bars represent the LE and HE control groups and the gray bars represent LE and HE USCS groups. Results are

presented as mean ± S.E.M. Statistical analysis was performed by Two-way ANOVA with * $P < 0.05$ between LE-ST and LE-EE and between HE-ST and HE-EE.

Figure 1

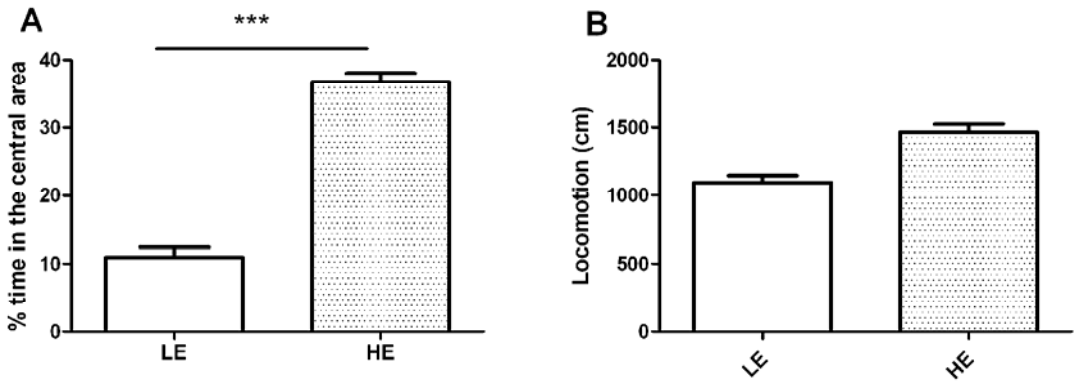
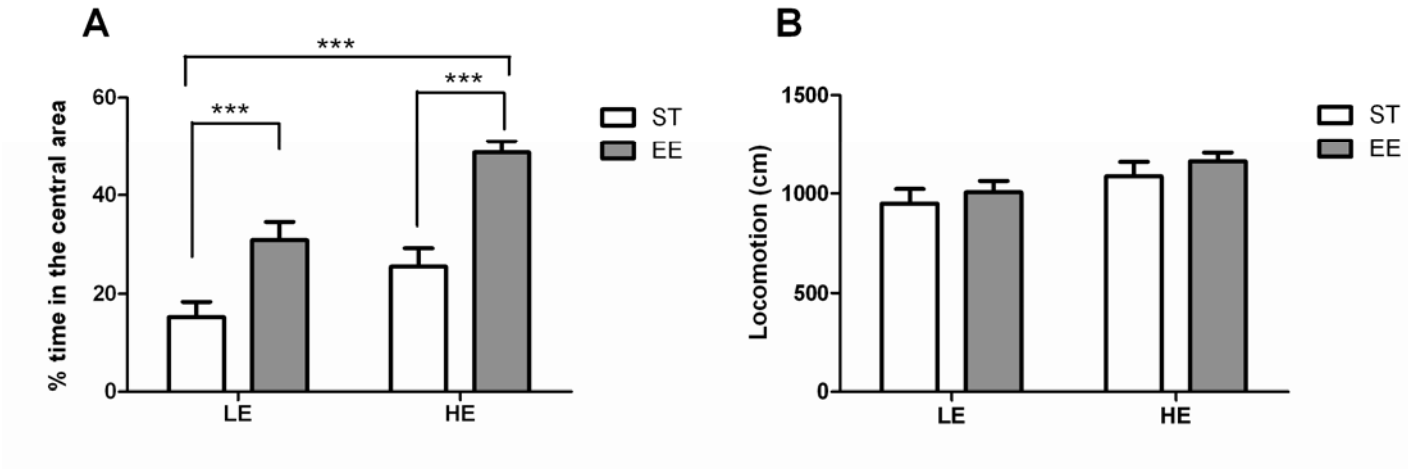

A
***
% time in the central area
40
30
20
10
0
LE
HE
B
Locomotion (cm)
2000
1500
1000
500
0
LE
HE

Figure 2


A
***
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% time in the central area
60
40
20
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LE
HE
ST
EE
B
Locomotion (cm)
1500
1000
500
0
LE
HE
ST
EE

Figure 3

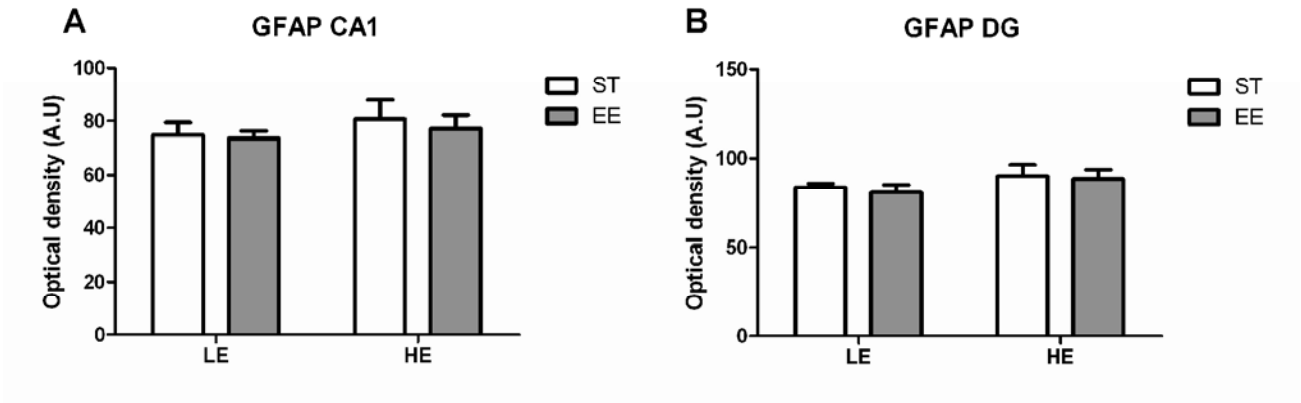

A
GFAP CA1
Optical density (A.U)
100
80
60
40
20
0
LE
HE
ST
EE
B
GFAP DG
Optical density (A.U)
150
100
50
0
LE
HE
ST
EE

Figure 4

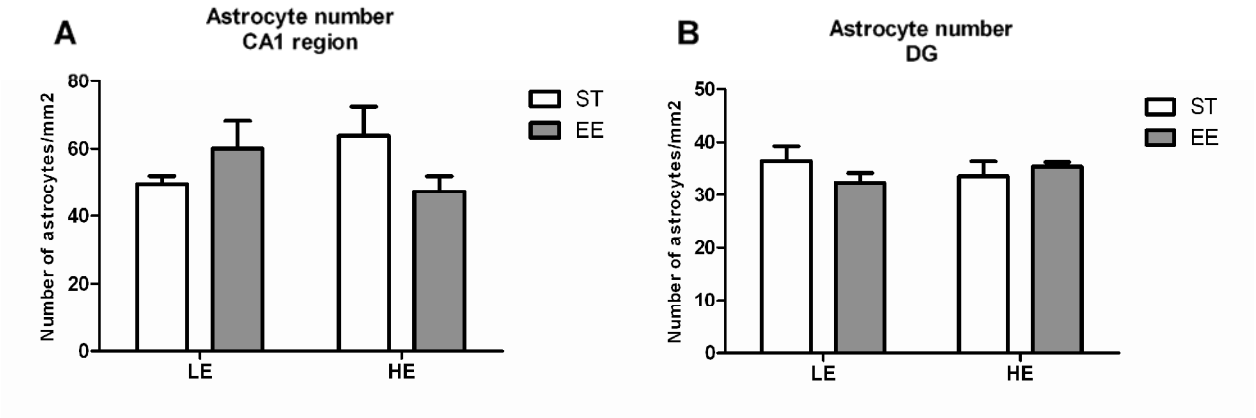
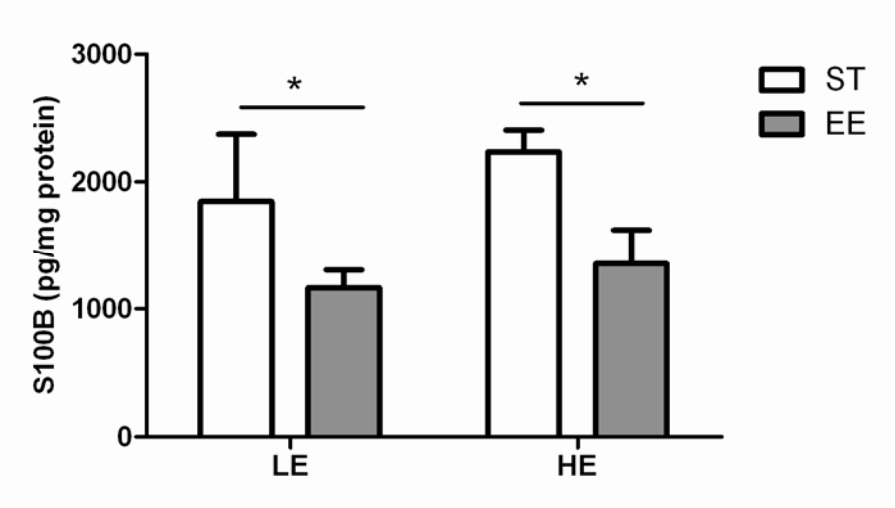

A
Astrocyte number
CA1 region
Number of astrocytes/mm2
80
60
40
20
0
LE
HE
ST
EE
B
Astrocyte number
DG
Number of astrocytes/mm2
50
40
30
20
10
0
LE
HE
ST
EE

Figure 5



CAPÍTULO IV

Locomotor response to psychostimulant drugs in low and high exploratory mice.

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Artigo em preparação

Locomotor response to psychostimulant drugs in low and high exploratory mice.

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Number of figures: 2

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Abstract

Exploratory behavior can be understood from a bidimensional perspective involving protective inhibitory behaviors and novelty seeking as the activation system. Because these behaviors can also be observed in animals, their analysis can be useful for testing hypothesis on the biological basis mood and temperament, with putative implications for the understanding of human mood disorders. The purpose of this study was to evaluate if mice with low and high exploratory activity (LE and HE) differ regarding to hyperlocomotion induced by amphetamine, caffeine, dizocilpine, and apomorphine. Significant differences between LE and HE groups concerning habituation were seen in 4 out of 6 experiments, confirming that LE and HE mice differ in exploratory behavior. However no significant differences were observed in the response of LE and HE mice in regard to hyperlocomotion induced by any of the drugs. These equivalent responses indicate that the sensitivity of these systems in LE and HE mice is not clearly distinct. Further studies with other psychoactive drugs or behavioral endpoints should be performed to better study the neurobiological differences between LE and HE mice.

Keywords: temperament, individual differences, open-field, amphetamine, caffeine, dizocilpine, apomorphine.

1. Introduction

The search for psychological models that could more adequately explain human behavior and aspects of human personality has been present through in science. Recently, Lara et al (2006 a,b) proposed a theoretical model based on Gray's and Cloninger's models (Pickering and Gray, 1999; Cloninger et al. 1993) in which the behavioral and mood spectrum are organized in a bidimensional model involving the interaction between behavioral activation and inhibition. In this model, inhibition or harm avoidance (HA) is responsible for inhibiting behaviors, acting as a "brake" and activation or novelty seeking (NS) would be responsible for initiating behaviors. Since activation and inhibition are two interdependent axes, any combination between them is possible, representing a wide spectrum of mood and behavior as well as related psychopathologies.

The combination of low inhibition and high activation is associated with externalizing disorders and symptoms, whereas low activation and high inhibition relate to internalized disorders. Since behavioral inhibition can be observed in animals, the utilization of tasks to analyze parameters related with these two traits can be useful for testing hypothesis on biological basis of temperament, and its relation to human mood disorders. Accordingly, our previous study (Kazlauckas et al. 2005) showed that the exploratory activity in an open-field with a central object is an adequate strategy to differentiate mice according to behavioral patterns that represent temperament types: animals with a higher object exploration rate – represented as the time spent in the central area of the open-field – showed more behavioral traits of NS, less HA and higher aggressiveness, whereas animals with low exploratory activity rates shown the opposite behavior. Therefore, mice with high and low exploratory activity can be classified as externalized and internalized phenotypes, respectively.

Pharmacological challenges can help clarify the neurobiological differences in these behavioral phenotypes. Thus, the purpose of this study was to search for differential locomotor effects induced by amphetamine (an indirect dopamine/noradrenaline agonist), caffeine (an A1-A2A adenosine receptor antagonist), dizolcipine (a non-competitive NMDA receptor antagonist), and apomorphine (a direct dopamine receptor agonist) in low and high exploratory mice.

2. Materials and methods

2.1 Animals

Male albino CF1 mice (60 days old), weighing approximately 35–40 g, were obtained from State Foundation for Health Science Research (FEPPS, Porto Alegre, RS, Brasil). They were housed in groups of six to eight in standard conditions of temperature and humidity, in a 12h light/dark cycle (lights on at 7:00 am), with access to food and water *ad libitum*. All experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals and Brazilian Society for Neuroscience and Behavior (SBNeC). Recommendations for animal care were followed throughout all the experiments in accordance to the project approved by the ethical committee from Universidade Federal do Rio Grande do Sul. All efforts were made to minimize the number of animals employed in the present study and their suffering.

2.2 LE and HE mice selection

Eighty mice were selected into low (LE) and high exploratory (HE) mice, according to their exploratory behavior in the central area of the open field (OF), as described in our previous research (Kazlauckas et al, 2005). This test was used to separate the two different mice phenotypes populations depending on the animal's response to a novel object in a new

environment. For this, the animal was placed in an open-field (50 cm × 50 cm × 50 cm) with an object (a white cylinder of 1.5 cm radius and 5 cm high) in the center of the arena to stimulate exploration. Exploratory behavior was video recorded for 5 min, and the time spent by the animal in and out of an imaginary center square of 30 cm × 30 cm was analyzed using the ANYmaze software (Stoelting, Woods Dale). 12 animals were selected from each extreme of exploratory behavior (the most and the least explorers) to compose the HE and LE groups, respectively. Mice were appropriately identified and remained in their respective home cages without changing housemates until the end of behavioral testing.

2.3 Pharmacological treatment and locomotor activity measurement

Mice were placed individually in the same open-field arena used before without object and the locomotor activity of four mice were analyzed simultaneously during 2 hours. The distance traveled by each animal was calculated by ANYMAZE software. Initially, mice were allowed to acclimatize to the new environment during 60 min, followed by an i.p injection (10ml/kg) of saline (0.9% NaCl; n=12); D-amphetamine (1.0 mg/kg n=12 and 3.0 mg/kg; n=12); dizolcipine (0.25 mg/kg; n=10), apomorphine (2mg/kg; n=10) and caffeine (30 mg/kg; n=10). The animals had a period of drug withdrawal of one week between treatments.

2.4 Statistical Analysis

Differences between LE and HE in the selection by their exploratory profile in the open field task were analyzed using Student´s t-test. Locomotor activity between groups across time was evaluated by Two-way ANOVA with repeated measures, with time as a repeated measure and group (LE / HE) as independent variable. We evaluated only the difference between groups in each drug trial. Graphpad Prism 5 and SPSS 16.0 softwares

were used, and significant differences were considered when $P < 0.05$. Results are expressed as mean ± S.E.M.

3. Results

3.1 LE and HE phenotypes selection

The mean time of the whole group (n = 80) in the center of the open-field was 37.92 ± 0.99 % (mean + S.E.M.) and the higher and lower cutoff values for these 12 mice were > 47% and < 29.33%. The mean time of the LE group (n = 12) in the center of the open-field was 23.94 ± 6% whereas the HE group was 50.11 ± 2.56% (mean + S.E.M.) ($t = 13.37$, $P < 0.001$) (Fig.1A). Locomotor activity did not differ between high and low groups (Fig 1B). The remaining 56 animals (between 29.33% and 47% time in the center of the open-field) were kept to maintain the home groups of high and low exploratory mice.

3.2 Effect of treatment with psychoactive drugs on the locomotor activity of adult male mice (Figure 2).

During the habituation period (first 60 minutes prior the administration of the drug) a statistical difference was found between HE and LE groups in saline [$F(1,110)= 4.47$; $P = 0.0461$], amphetamine 3 mg/kg [$F(1,110)=5.77$; $P = 0.0252$], amphetamine 1mg/kg [$F(1,110)=4.42$; $P = 0.0472$] and apomorphine [$F(1,90)=5.46$; $P = 0.0312$] but not in dizolcipine [$F(1,90)= 1.76$; $P = 0.2014$] and caffeine [$F(1,90)= 3.59$; $P = 0.0742$]. There was no statistical difference between HE and LE groups in all treatments as evidenced by the drug-time interaction in the ANOVA with repeated measures: saline $F(1,176) = 1.42$; $P = 0.24$ (Fig. 2A); amphetamine 3mg/kg $F(1,176) = 1.52$ (Fig. 2B); $P = 0.23$; amphetamine 1m/kg $F(1,176) = 0.02$; $P = 0.90$ (Fig 2C); dizolcipine (MK-801) $F(1,144) = 0.35$; $P = 0.20$

(Fig 2D); apomorphine F (1,144) = 0.21; $P = 0.03$ (Fig. 2E) and caffeine F (1,144) = 1.39; $P = 0.07$ (Fig. 2F).

Discussion

In this study we found no significant differences between high and low exploratory groups in locomotor response to the psychoactive drugs used. Taken together, these observations indicate that dopamine, noradrenaline, adenosine and NMDA receptor responses are not particularly different between these behavioral phenotypes.

Similar studies in rats seem to vary: Piazza et al (1989, 1993) showed that in rats separated as high or low responders to novel environment (HR and LR respectively), locomotor activity induced by d-amphetamine was higher in HR rats in relation to LR. Of note, in this study, high and low phenotypes were selected based on locomotor response to novelty, which is different from our approach. Nevertheless, Gingras and Cools (1997) found no differences between HR and LR in the locomotor activity induced by d-amphetamine in a wide range of doses, similarly to Antoniou et al (2004), who found only differences in other parameters such as sniffing, hearing and scratching. The involvement of dopamine neurotransmission in behavioral responses to novelty is suggested by reports that reward is related to increased dopamine activity and that dopamine modulates exploratory behavior in animals (Dulawa et al, 1999). Five distinct dopamine (DA) receptors, named D1-D5, are expressed in the central nervous system, where they control motor function, emotional states, and endocrine physiology. D4 receptors have been shown to be activated by all three catecholamine neurotransmitters: dopamine, epinephrine, and norepinephrine (Hartman et al., 1997) and this receptor appear to be critical for the behavioral expression of novelty seeking (Dulawa et al, 1999). In humans, polymorphisms of D4R have been associated with novelty-

seeking traits in general and attention deficit-hyperactivity disorder in particular. Similarly, D4R (-/-) mice exhibit less novel object exploration than D4R (+/+) mice (Hartman et al., 1997; Dulawa et al., 1999).

A clinical study revealed that novelty seeking correlated positively with dopamine transporter density, which is in line with Cloninger's theory concerning personality and character (Laine et al, 2001). Furthermore, the association between temperamental personality dimensions measured with the Temperament and Character Inventory (TCI) and polymorphisms of the dopamine (DAT), norepinephrine (NET) and serotonin (5-HTT) transporter genes were studied in humans. There were no significant differences between means of TCI temperamental dimensions (novelty seeking, reward dependence, persistence and harm avoidance) and the transporter genes. Also, the NET transporter gene polymorphism showed no significant association with any of the temperamental TCI subdimensions (Samochowiec J, 2001).

Despite the clear differences in the exploratory behavior of mice there was no association of those differences in the effects response to psychoactive drugs used in the present study. This suggests that the dopaminergic, noradrenergic, adenosinergic and glutamatergic systems are not directly associated with these traits differences. However, other important systems, such as serotonergic, opiod and cholinergic systems were not investigated. Given their interaction with the dopaminergic systems in particular, they may differently modulate this system in rodents with high and low exploratory traits. One example that the opiod system may be involved is those rats that prefer novel environment show higher morphine consumption in a free choice paradigm and higher place preference induced by morphine (Pelloux et al, 2006). Also, the HPA system may be involved, since HE and LE show differences in basal and stress-induced levels of corticosterone (Kazlauckas et al, in press). Further studies will be needed to clarify these issues.

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Legends

Figure 1: Performance of low and high exploratory mice in the open-field with a central object. LE: white bars and HE gray bars). ***P <0.001 (Student's t-test).

Figure 2: Locomotor activity of LE and HE mice acutely treated with (A) saline, (B) amphetamine (3 mg/kg), (C) amphetamine (1mg/kg), (D) dizolcipine (0.25 mg/kg), apomorphine (2 mg/kg) and (E) caffeine (30 mg/kg) after 60 min of habituation period in a novel environment. Data shown mean ± S.E.M. *P <0.05 (One-way ANOVA with repeated measures).

Figure 1

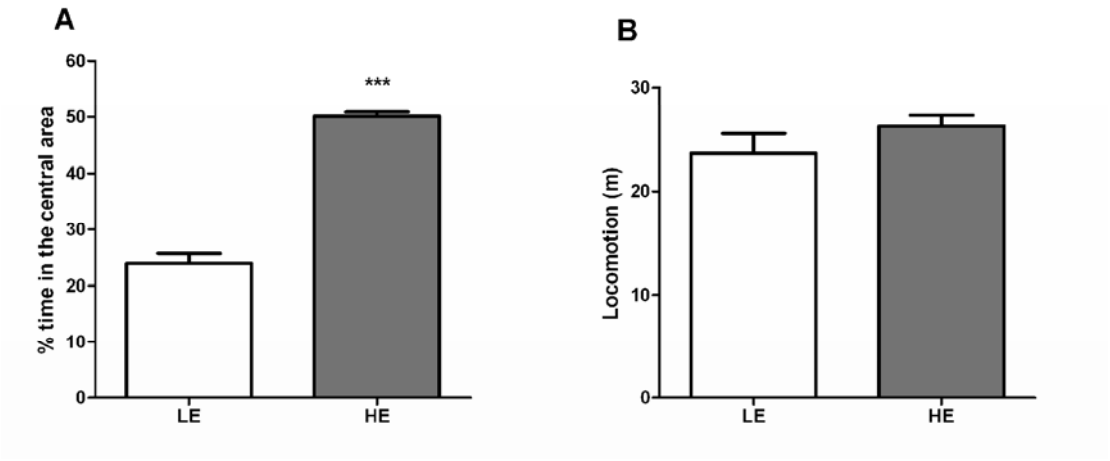
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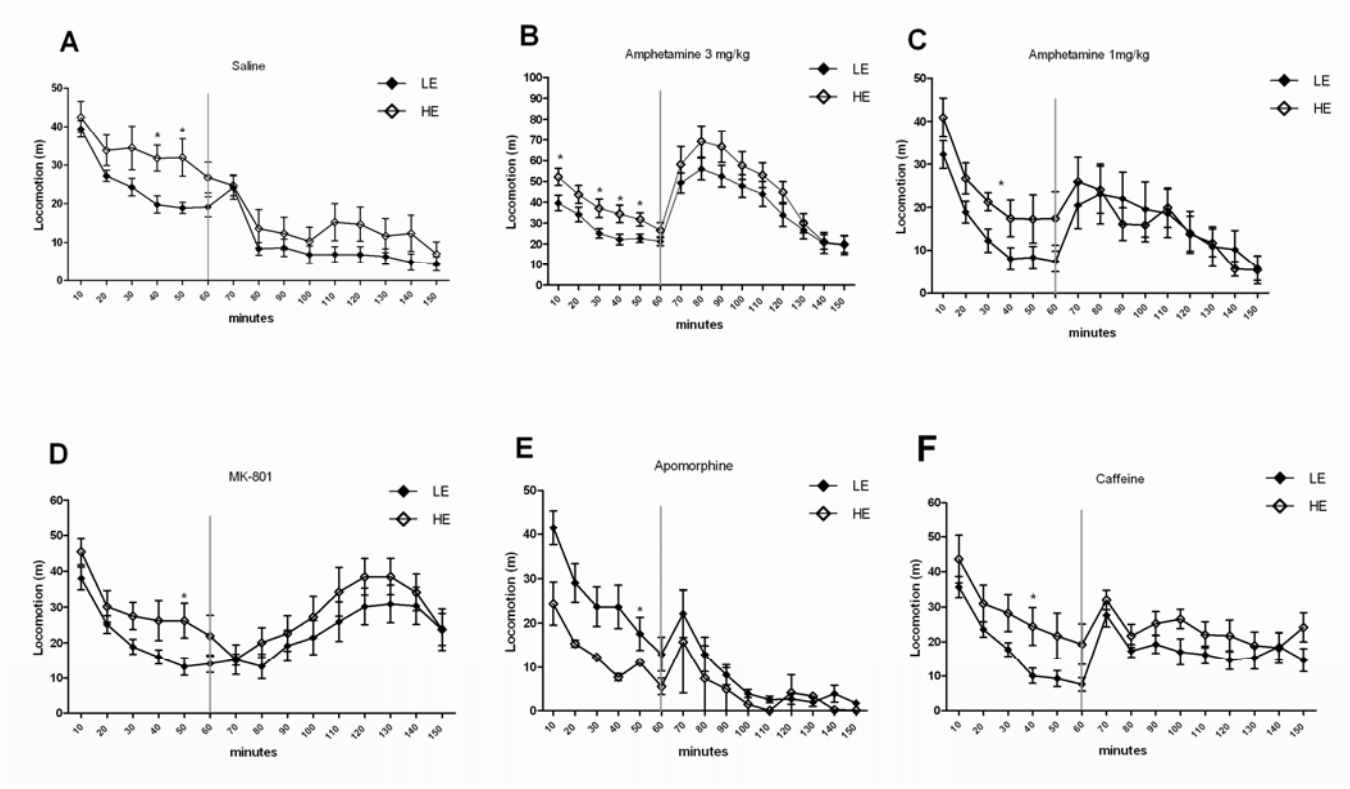
Figure 2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Saline
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Locomotion (m)
minutes
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LE
HE
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minutes
C
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LE
HE
Locomotion (m)
minutes
D
MK-801
LE
HE
Locomotion (m)
minutes
E
Apomorphine
LE
HE
Locomotion (m)
minutes
F
Caffeine
LE
HE
Locomotion (m)
minutes

Parte III

3. Discussão

A elaboração de um modelo animal para estudo do temperamento é de fundamental importância para se entender a influência do temperamento em respostas comportamentais e neurobiológicas. Modelos animais podem auxiliar no estudo detalhado do substrato neurobiológico dos diversos tipos de temperamento assim como propiciar a avaliação das intervenções que possam alterá-lo.

Fatores genéticos contribuem para a alta variabilidade interindividual quanto ao tipo e intensidade da resposta comportamental, neuroendócrina e fisiológica ao estresse observado em várias espécies, incluindo humanos e ratos de laboratório. Este fato tem sido utilizado como base para desenvolver inúmeras linhagens bidirecionais (Maudsley, Roman, Syracuse, Floripa entre outros) para o estudo da neurobiologia dos transtornos emocionais.

O modelo desenvolvido por nosso grupo buscou traçar o perfil temperamental individual de cada animal baseando-se na avaliação individual do comportamento explorador no teste de campo aberto com objeto central (Kazlauckas et al, 2005). Os resultados obtidos neste estudo confirmaram que podemos identificar diferenças interindividuais comportamentais em camundongos e que estas influenciam outras tarefas comportamentais.

Ainda há muito para se estudar em relação a diferenças comportamentais e neurobiológicas individuais em resposta a situações de estresse, principalmente em relação ao estresse imprevisível. Muito pouco se sabe em relação aos efeitos iniciais do estresse em tarefas cognitivas e parâmetros biológicos sem que tenha sido desenvolvido um estado depressivo, portanto avaliamos o efeito de um protocolo de estresse imprevisível relativamente curto (15 dias) em camundongos selecionados, HE e LE na exploração e locomoção no campo aberto, na tarefa de reconhecimento de objetos para avaliação de memória de reconhecimento e sobre os níveis de BDNF e S100B.

Nossos resultados demonstraram que na tarefa de campo aberto os camundongos HE e LE submetidos ao protocolo de estresse imprevisível tiveram menor atividade exploratória quando comparados aos seus grupos controle. Entretanto, suas diferenças na atividade exploratória permaneceram distintas visto que os camundongos HE, mesmo após serem submetidos ao protocolo de estresse, mostraram-se mais exploradores quando comparados aos LE também submetidos ao estresse imprevisível. Como a atividade locomotora não foi alterada após o estresse podemos dizer que esse protocolo teve efeito especifico na atividade exploratória (ou tigmotaxia) destes animais. Esse resultado já havia sido representado de maneira semelhante num estudo de Dalm e colaboradores, no qual foi observado que ratos estressados tinham tido sua atividade exploratória diminuída sem alteração na atividade locomotora (Dalm et al, 2009).

A avaliação do efeito do estresse sob a memória destes animais foi feita pela realização da tarefa de reconhecimento de objetos visto que este protocolo avalia o desempenho de animais através da sua propensão natural de reconhecer a novidade em um contexto familiar. Sendo assim, nossos resultados revelaram que os camundongos LE submetidos ao protocolo de estresse tiveram seu desempenho prejudicado nesta tarefa enquanto que os camundongos HE mesmo tendo sido submetidos ao estresse, e apresentando baixa exploração dos objetos, aprenderam a tarefa assim como os HE e LE controles. É importante ressaltar que em nosso estudo anterior (Kazlauckas et al, 2005) os HE e LE apresentaram diferença nos testes de memória de esquiva inibitória e no labirinto de Lashley e no presente estudo esta diferença entre os HE e LE controles não foi observada quando analisados na tarefa de reconhecimento de objetos. Uma explicação plausível para a ausência de diferença entre os grupos controle no teste de reconhecimento pode ser devido à ausência de estímulos positivos (comida como recompensa no teste do labirinto de Lashley) e/ou negativos (choque como punição no teste de esquiva).

No presente estudo (capítulo I) também pudemos observar que os camundongos HE controles apresentaram baixo nível de corticosterona basal (60 ng/mL) quando comparados com os LE controles (165 ng/mL). Como já foi descrito em estudo recente utilizando os mesmos camundongos CF1 utilizados em nosso trabalho, os níveis basais de corticosterona variam de 90 a 100 ng/mL (Detânico et al, 2009). Sendo assim, podemos hipotetizar que o nível já alto de corticosterona nos camundongos LE pode estar relacionado à falta de um aumento nestes níveis nos LE submetidos ao protocolo de estresse por um mecanismo de dessensibilização. Em contraste, os camundongos HE se mostraram hiper-responsivos ao estresse, visto que seus níveis de corticosterona foram bastante elevados após a submissão ao protocolo de estresse imprevisível.

Um estudo de Piazza e colaboradores (1993) utilizando ratos selecionados pela atividade locomotora denominados mais e menos respondedores também demonstrou que os ratos mais respondedores exibiram altos níveis de corticosterona após exposição a um ambiente novo. É importante ressaltar que estes ratos mais respondedores apresentam alguma semelhança aos nossos camundongos HE, visto que são mais ativos em um ambiente novo, tem preferência por novidade e situações aversivas quando comparados com os menos respondedores (Piazza et al., 1989 e 1993). Outros estudos demonstraram que indivíduos expostos a estresse crônico podem se habituar a esta situação e até mesmo responder com uma hiper-ativação do sistema HPA (Driscoll et al, 1998; Kabbaj et al, 2000; McEwen et al, 2007). Este padrão de resposta auxilia na compreensão dos nossos resultados sobre os níveis de corticosterona, construindo a hipótese de que os nossos camundongos HE, por apresentarem características de buscadores de novidade/BAS podem, quando submetidos em situações deste tipo, explorar e responder melhor quando testados.

Estudos sobre os efeitos do estresse nos níveis de BDNF têm sido amplamente desenvolvidos com o objetivo de relacionar o papel deste fator neurotrófico na adaptação do

organismo frente às situações de estresse (Larsen et al, 2010; McEwen et al, 2007; Schaaf et al, 1998 e 2000). Os resultados obtidos no nosso estudo demonstraram que camundongos HE apresentam alto nível de BDNF hipocampal quando comparados com os LE em condições controles. Entretanto, sob condições de estresse, os níveis de BDNF diminuem nos camundongos HE, mas não se alteram nos LE. Um estudo de Schaaf e colaboradores (1998) demonstrou que o estresse induziu elevações nos níveis de glicocorticóides acompanhado de uma redução na expressão de BDNF, sendo que o mesmo ocorreu em nossos camundongos HE. Sabe-se que a corticosterona suprime a expressão de BDNF hipocampal (Schaaf et al, 2000), mas em algumas situações, como pós-treino no labirinto aquático de Morris, o aumento na concentração plasmática de corticosterona não é acompanhado de diminuição nos níveis de BDNF. Esta resistência pode ter alta relevância na formação da memória visto que a alta expressão de BDNF em regiões específicas do hipocampo parece estar relacionada com o desempenho do animal em determinadas tarefas. Assim, a corticosterona e o BDNF podem não estar diretamente relacionados quanto aos seus efeitos sobre a formação da memória. No presente estudo, as mudanças nos níveis de corticosterona e BDNF podem não estar relacionadas com o comportamento observado, considerando que mesmo os camundongos HE submetidos ao protocolo de estresse tiveram bom desempenho na tarefa de memória mesmo tendo apresentado uma diminuição nos níveis de BDNF hipocampal. É importante salientar que os efeitos dos glicocorticóides na melhora da consolidação da memória relatados por Barsegyan et al. (2010) podem explicar o adequado desempenho dos nossos HE mesmo após terem sido submetidos ao protocolo de estresse.

Os camundongos LE não tiveram alterações em seus níveis de BDNF e corticosterona, sugerindo que outros parâmetros neurobiológicos possam estar relacionados às suas mudanças comportamentais.

Outro parâmetro bioquímico analisado em nosso trabalho foi o nível hipocampal de S100B. Já foi relatado um aumento de S100B em ratos estressados por contenção (Scaccianoce S, 2004); mas não observamos diferença em nossos grupos controles e estressados. Este resultado pode ser devido à diferença de protocolos de estresse (mais intenso no caso da contenção), da linhagem dos animais e até mesmo das técnicas de dosagem.

As diferenças comportamentais e bioquímicas encontradas entre camundongos HE e LE com ou sem estresse imprevisível reforçam a importância dos traços de comportamento/temperamento nas respostas ao estresse. Nossos resultados também demonstram que alterações comportamentais e nos níveis de BDNF precedem o desenvolvimento de um estado depressivo. Salientamos também que os camundongos LE foram mais sensíveis ao estresse imprevisível, podendo representar a observação clínica de maior sensibilidade ao estresse em pacientes com quadros de ansiedade e depressão (Kunugi et al, 2010).

Tendo visto a resposta de nossos camundongos HE e LE quando confrontados com uma situação de efeito negativo como o estresse imprevisível, o segundo estudo desta tese (capítulo II) teve por objetivo investigar a influência do enriquecimento ambiental como um estímulo positivo durante a vida adulta destes camundongos HE e LE.

O enriquecimento ambiental tem sido amplamente utilizado para o estudo de modificações comportamentais e de neuroplasticidade, e a interação entre traços individuais comportamentais com a resposta ao enriquecimento durante a vida adulta não havia sido estudada entre roedores dentro de um mesmo grupo até o presente momento. Neste estudo utilizamos camundongos CF1 selecionados pelo seu padrão exploratório na tarefa de campo aberto para avaliar se a exposição ao ambiente enriquecido durante dois meses poderia modificar as diferenças entre os camundongos HE e LE sobre a atividade exploratória na

tarefa de campo aberto, sobre a memória através das tarefas de reconhecimento de objetos e esquiva inibitória e sobre os níveis de BDNF no hipocampo.

Nossos resultados demonstram que as diferenças entre os traços comportamentais nos camundongos HE e LE permanecem após dois meses de enriquecimento ambiental, mesmo observando um aumento na exploração da área central do campo aberto em ambos os grupos. É importante salientar que outros estudos demonstram que o enriquecimento ambiental provoca uma diminuição na locomoção de roedores provavelmente por aumentar a habituação (Falkenberg et al, 1992; Amaral et al, 2008, Pamplona et al, 2009), mas no nosso estudo essa alteração não foi observada, talvez pela diferença de protocolo pelo campo aberto ter sido realizado durante cinco minutos e com a presença de um objeto central, o que pode ter contribuído para um diferente padrão locomotor nestes camundongos após o enriquecimento ambiental.

Para analisar o efeito do enriquecimento na memória de nossos camundongos HE e LE, utilizamos a tarefa de reconhecimento de objetos, que se baseia na motivação natural dos animais de explorar uma novidade num contexto familiar e também a tarefa de esquiva inibitória, que leva em consideração a habilidade dos animais de evitar punição e reprimir sua natureza exploratória. Ambos os testes utilizam a exploração de um ambiente/aparato para avaliar memória, portanto são bastante interessantes para serem utilizados em nossos camundongos considerando que estes se diferenciam justamente pela característica exploratória.

Nossos resultados demonstraram que o enriquecimento facilitou o aprendizado dos HE e LE já no primeiro teste de reconhecimento, 1,5 h após o treino enquanto que os HE e LE que foram mantidos sob condições padrões só tiveram um desempenho comparável 24 horas após o treino, demonstrando que o enriquecimento beneficia o aprendizado em ambos os grupos. Já no teste de esquiva inibitória, pode-se observar que o enriquecimento beneficiou

mais o grupo dos LE, considerando que esse grupo teve um desempenho melhor do que o grupo LE sob condições padrões. Talvez o grupo HE não tenha sido diferente quando sob condições de enriquecimento, pois já sob condições padrões havia atingido o teto no teste, ou seja, não poderia melhorar ainda mais seu desempenho. No teste 24 horas após o treino os grupos LE e HE apresentaram o mesmo padrão de desempenho observado no nosso estudo anterior (Kazlauckas V, 2005), ou seja, o grupo LE apresentou um índice de retenção menor que o grupo HE.

Inúmeros estudos relataram que o enriquecimento ambiental provoca alterações nos níveis de BDNF em regiões cerebrais, principalmente no hipocampo (Rossi et al, 2006; Ickes et al, 2000). Os nossos camundongos LE apresentaram baixos níveis de BDNF hipocampal quando comparados aos grupos HE e o enriquecimento fez com que ocorresse um aumento nesses níveis em ambos os grupos. Esse aumento nos níveis de BDNF após o enriquecimento é compatível com a melhora no desempenho dos camundongos LE na tarefa de esquiva inibitória, em concordância com os dados da literatura que demonstram os benefícios do enriquecimento na memória devido ao aumento dos níveis de BDNF.

Esse segundo estudo da presente tese demonstrou que o desempenho em tarefas cognitivas depende dos traços de temperamento que levam os animais a apresentarem diferenças comportamentais individuais, e estas diferenças podem ser modificadas por intervenções ambientais. Mesmo durante a vida adulta, o enriquecimento ambiental pôde atenuar traços de comportamento internalizado dos camundongos LE.

O terceiro capítulo desta tese tratou de avaliar a resposta locomotora dos camundongos HE e LE com a administração aguda de psicoestimulantes (anfetamina, apomorfina, cafeína e dizocilpina), buscando caracterizar qual ou quais os sistemas neurotransmissores estariam envolvidos na diferença comportamental destes animais.

Nenhum dos psicoestimulantes utilizado no presente estudo foi capaz de induzir alteração diferenciada na locomoção destes animais com as quais poderíamos indicar um possível mecanismo neuroquímico para essas diferenças comportamentais entre nossos camundongos. Mais estudos, com outros fármacos psicoestimulantes ou utilizando outros parâmetros comportamentais, são necessários para elucidar as diferenças neurobiológicas e bioquímicas destes animais. Uma possibilidade é de que as diferenças estejam em mecanismos que regulam indiretamente a atividade dopaminérgica desses animais, como sistema opióide, serotonérgico ou o próprio sistema HPA.

Em relação à investigação imunohistoquímica dos camundongos HE e LE após um período de enriquecimento ambiental (capítulo IV), não encontramos um aumento na densidade óptica de GFAP nas regiões CA1 e giro denteado do hipocampo. No entanto, observamos uma diminuição consistente de S100B hipocampal em ambos os grupos HE e LE submetidos ao enriquecimento ambiental. É interessante notar que a exposição ao EA altera a expressão de genes envolvidos na plasticidade e sinalização neural em diferentes áreas do hipocampo (Rampon et al, 2000) e também já foi observada uma diminuição do gene S100A9 após enriquecimento (Dong et al., 2007). Baseado nestes estudos e considerando que até o momento não existem estudos relacionando S100B e EA, podemos concluir que o EA leva a uma diminuição dos níveis de S100B hipocampais.

4. Conclusão

Nesta tese um modelo de seleção comportamental em camundongos se mostrou consistente e foi utilizado para o estudo da influência dos traços de temperamento em roedores em tarefas comportamentais e parâmetros bioquímicos. Um achado consistente sem a intervenção ambiental foi de que os camundongos mais exploradores apresentam níveis mais altos de BDNF no hipocampo.

As diferenças comportamentais e bioquímicas encontradas entre camundongos HE e LE antes e depois do estresse imprevisível reforçam a importância dos traços de comportamento/temperamento nas respostas ao estresse, particularmente no eixo HPA e na queda do BDNF especificamente nos animais mais exploradores.

Em relação ao protocolo de enriquecimento ambiental, ambos os grupos apresentaram benefícios, mas foram mais robustos e significativos nos camundongos LE, fazendo com que estes desenvolvessem resposta semelhante a dos camundongos HE. Já uma diminuição dos níveis de S100B hipocampais após enriquecimento ambiental foi observada de modo similar nos dois grupos.

Portanto, características individuais devem ser levadas em consideração quando se realizam estudos com modelos animais. Essa estratégia pode ser útil para entender a neurobiologia dos diferentes temperamentos associados à depressão e ao transtorno bipolar. Por outro lado, diferenças comportamentais individuais podem ser modificadas tanto positiva como negativamente por intervenções ambientais.

5. Perspectivas

Para dar continuidade aos estudos aqui apresentados, temos como perspectiva:

- Submeter nova leva de camundongos HE e LE ao protocolo de enriquecimento ambiental com a finalidade de avaliar o efeito sobre parâmetros de ansiedade, bem como investigar a expressão de receptores dopaminérgicos D2 e D4 por técnica de PCR em tempo real;
- Investigar a neurogênese assim como as alterações morfológicas de astrócitos e neurônios dos camundongos HE e LE após o enriquecimento ambiental;
- Verificar se um protocolo de estresse crônico mais prolongado ou intenso afeta diferentemente os animais HE e LE no desencadeamento de um estado depressivo;
- Avaliar o efeito de outros fármacos psicoestimulantes na atividade locomotora destes animais e também avaliar o efeito destes em diferentes testes comportamentais;
- Avaliar diferenças de expressão gênica com a técnica de microarrays em camundongos HE e LE.

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