UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

ISOLAMENTO SOCIAL PRECOCE E CONSUMO DE UMA DIETA HIPERLIPÍDICA: IMPLICAÇÕES NO COMPORTAMENTO DO TIPO DEPRESSIVO E EM ASPECTOS COGNITIVOS EM RATOS ADULTOS

Danusa Mar Arcego

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SUMÁRIO

APRESENTAÇÃO

A presente tese está organizada em: **Introdução; Objetivos; Materiais, Métodos e Resultados**: subdividos em *Capítulos 1 a 3* (referentes ao artigo publicado e aos manuscritos submetidos); **Discussão**; **Conclusões; Perspectivas**; e **Referências bibliográficas**.

Na **Introdução** encontra-se o embasamento teórico que fundamenta a proposta de estudo dessa tese. Os **Objetivos** (gerais e específicos) definem os propósitos centrais do trabalho, assim como de cada trabalho científico. A sessão **Materiais, Métodos e Resultados** está subdivida em Capítulos, sendo que cada Capítulo corresponde a um artigo científico específico referente aos trabalhos desenvolvidos durante o período do doutorado no Laboratório de Neurobiologia do Estresse (Departamento de Bioquímica, ICBS, UFRGS).

A sessão **Discussão** corresponde à discussão geral da tese, integrando os resultados dos três capítulos. Nas **Conclusões** estão os principais achados da presente tese e nas **Perspectivas** as possibilidades de futuros estudos a partir dos resultados obtidos.

As **Referências Bibliográficas** contêm as referências dos trabalhos citados nas sessões: **Introdução** e **Discussão**. No final de cada Capítulo está listada a bibliografia referente a cada artigo, formatada de acordo com as especificações de cada revista.

LISTA DE ABREVIATURAS

ACTH - hormônio adrenocorticotrópico

ADN- ácido desoxirribonucléico

BDNF - fator neurotrófico derivado do encéfalo

CB1- receptor canabinóide-1

CRH – hormônio liberador de corticotropina

D1- receptor dopaminérgico-1

GABA - ácido gama-aminobutírico

GAD65 - glutamato descarboxilase

GCs- glicocorticóides

GR- receptores de glicocorticóides

HFD – dieta hiperlipídica

HHA - hipotálamo-hipófise-adrenal

MR – receptor de mineralocorticóides

Nac – núcleo accumbens

NMDA - N-metil-D-aspartato

PI3K - fosfatidil-inositol-3-cinase

PLC – fosfolipase C

PSD – densidade pós-sináptica

SNC – sistema nervoso central

TrkB – receptor de tropomiosina cinase

VTA - área tegmentar ventral

RESUMO

Intervenções ambientais, como a exposição precoce a estressores ou a dietas ricas em calorias, podem alterar a trajetória da maturação neural e influenciar na susceptibilidade a certas patologias a longo-prazo. Neste contexto, o objetivo do presente estudo foi investigar os efeitos de uma exposição ao isolamento social durante o período pré-pubere associado ou não ao consumo precoce e crônico de uma dieta hiperlipídica (HFD) sobre aspectos cognitivos e emocionais, e possíveis mecanismos neuroquímicos associados a essas alterações, no hipocampo, no córtex pré-frontal e no núcleo accumbens de ratos machos na idade adulta. Os resultados mostraram que os dois fatores, estresse e dieta (separadamente), induziram um comportamento do tipo depressivo nos animais na idade adulta. Além disso, os animais isolados apresentaram um déficit cognitivo associado à memória de curta-duração e de trabalho, enquanto que animais com acesso à HFD demonstraram prejuízo somente na memória de curta-duração. Curiosamente, a interação entre os fatores (estresse e dieta) causou uma reversão dos déficits na memória de curta-duração. Em relação às avaliações do comportamento alimentar hedônico, observamos que o grupo com consumo crônico de HFD apresentou uma menor motivação para obter diferentes tipos de alimentos palatáveis doces. Essa redução motivacional não parece ser associada a uma menor palatabilidade e/ou a uma maior saciedade induzida pela HFD. Em relação aos marcadores de plasticidade analisados no córtex pré-frontal, observamos interações entre os fatores estresse e dieta na atividade da enzima $Na^{+}K^{+}$-ATPase, nos níveis de BNDF e no imunoconteúdo das proteínas AKT e MAPK/ERK, sendo que os fatores quando aplicados isolados diminuem os níveis dos parâmetros analisados, porém quando associados, os níveis retornam ou aumentam em relação aos valores do grupo controle. O hipocampo foi a estrutura mais afetada pelas intervenções ambientais neste trabalho. Observamos que tanto o estresse, como a dieta hiperlipídica (separadamente) causaram uma redução da plasticidade sináptica hipocampal, por meio de diferentes mecanismos: o acesso crônico à HFD afeta proteínas relacionadas ao funcionamento das sinapses, enquanto o isolamento social parece afetar mais particularmente a via de sinalização do BDNF. Com relação aos achados neuroquímicos no núcleo accumbens, observamos uma redução dos receptores dopaminérgico-1 (D1) e canabinóide-1 (CB1) com o consumo de HFD, enquanto que os animais isolados na pré-puberdade apresentaram uma redução do metabolismo dopaminérgico nesta estrutura. Em suma, esta tese demonstra que tanto o isolamento social e como o consumo contínuo de HFD causam comportamento do tipo depressivo e outras alterações comportamentais, e reduzem marcadores de plasticidade importantes no córtex prefrontal e hipocampo. O acesso à HFD também causa uma menor motivação para o comportamento alimentar hedônico. Assim, tais eventos precoces podem afetar a plasticidade neural levando a importantes alterações comportamentais, e assim predispor a diferentes patologias durante a vida.

Palavras-chave: neuroplasticidade; dieta rica em gordura; estresse; depressão.

ABSTRACT

Environmental interventions, such as early exposure to stressors or high calorie-diets, can alter the trajectory of neural maturation and influence the susceptibility to some pathologies throughout life. In this context, the aim of the present study was to investigate the effects of exposure to social isolation, during the pre-pubertal period, associated or not with early and chronic consumption of a hyperlipid diet (HFD) on cognitive and emotional aspects, and possible mechanisms associated with these changes, in the hippocampus, the prefrontal cortex and the nucleus accumbens of male rats in adulthood. The results showed that both pre-pubertal social isolation and chronic access to a hyperlipidic diet induced a depressive-like behavior in animals during adulthood. In addition, isolated animals had a cognitive deficit associated with short-term and working memory, whereas animals with access to HFD demonstrated impairment only in short-term memory. Interestingly, the interaction between the factors (stress and diet) caused a reversal in relation to short-term memory, remaining similar to the control group. Regarding the evaluations of the hedonic eating behavior, we observed that HFD group presented a lower motivation to obtain different sweet palatable foods. This impaired motivation does not appear to be associated with less palatability and/or satiety induced by the high-fat diet. Concerning plasticity markers in the prefrontal cortex, we observed interactions between stress and diet on Na^+ K^+-ATPase activity, BNDF levels and AKT and MAPK/ERK immunocontents. These interactions follow a similar profile: when applied alone, the levels of the analyzed parameters decrease, but when associated, the levels return or increase in relation to the values of the control group.The hippocampus was the structure most affected by the environmental interventions. Both stress and HFD caused a reduction of hippocampal synaptic plasticity through different mechanisms: chronic access to HFD affects proteins related to synaptic function, while social isolation affects the BDNF signaling pathway more significantly. Regarding the neurochemical findings in the nucleus accumbens, we observed a reduction in dopaminergic-1 (D1) and cannabinoid-1 (CB1) receptors with chronic HFD intake, whereas isolated animals had a reduction of dopaminergic metabolism in this same structure. In summary, this thesis shows that both social isolation and chronic consumption of HFD lead to depressive-like behavior and to other behavioral changes; they reduce plasticity markers in prefrontal cortex and hippocampus. HFD access also induced a lower motivation for hedonic feeding. Therefore, these early interventions may affect neural plasticity, leading to important behavioral changes, and thus, predispose to different pathologies later in life.

Key-words: neuroplasticity; high fat diet; stress; depression.

1. INTRODUÇÃO

1.1 Fatores ambientais no início da vida programam a fisiologia do organismo

As experiências vivenciadas ao longo da vida, principalmente durante os períodos iniciais da vida, podem ter significante impacto sobre a maturação do sistema nervoso central (SNC) (Andersen & Teicher, 2008). Sabe-se que o desenvolvimento é marcado por períodos sensíveis e críticos, os quais tornam o organismo mais susceptível a modificações, tanto por fatores extrínsecos como intrínsecos, podendo levar a mudanças permanentes fisiológicas, neuroquímicas e comportamentais (Fawcett & Frankenhuis, 2015, Stevens et al., 2016).

Dessa forma, os períodos iniciais da vida, incluindo a infância e a adolescência, são fases específicas onde processos dependentes da genética de cada indivíduo associados à exposição a fatores ambientais interagem para estabelecer as características funcionais (Crews *et al.*, 2007). Nesses períodos ocorre uma elevada maturação encefálica marcada pela organização funcional das redes neurais, podas sinápticas, proliferação neural, migração, diferenciação, além de gliogênese e mielinização (Rice & Barone, 2000, Ismail et al., 2016), fatores esses que são fundamentais para o estabelecimento dos circuitos neuroquímicos no SNC.

Diversos estudos em humanos vêm demonstrando a interação entre genes e o ambiente, mostrando que fatores genéticos individuais associados a condições ambientais adversas durante períodos precoces podem afetar mecanismos do desenvolvimento neurológico, aumentando a vulnerabilidade a certas patologias e transtornos (Giovanoli et al., 2013, Davis et al., 2016, Fraguas et al., 2017).

É importante ressaltar que, as experiências ocorridas no início da vida podem agir de formas distintas no desenvolvimento encefálico e seus desfechos dependerão das

características dessas experiências vivenciadas, de sua intensidade e da percepção de cada indivíduo (Horn et al., 2016, Lavoie et al., 2016). Assim, os fatores ambientais podem favorecer processos adaptativos, como aqueles associados à resiliência ao longo da vida, promovendo estratégias de enfrentamento e agindo de forma benéfica ao indivíduo (Lavoie *et al.*, 2016), ou então, exercer um papel crítico na predisposição a patologias futuras e/ou transtornos psicológicos/psiquiátricos (Homberg *et al.*, 2016).

Diversas evidências apontam para essa interação entre o ambiente no início da vida e alterações neuroquímicas e comportamentais ao longo da vida. Estudos em humanos mostram que exposição a traumas e estressores na infância como, por exemplo, abuso ou negligência, estão associados com o aumento do risco em desenvolver psicopatologias na idade adulta, incluindo transtornos depressivos (Heim & Nemeroff, 2001, Shea et al., 2005, Teicher et al., 2006), além de causar alterações neuroendócrinas e disfunções em algumas estruturas cerebrais (como hipocampo e córtex pré-frontal) (Bremner, 2003, Vermetten et al., 2003), levando a prejuízos na cognição (Brietzke *et al.*, 2012) e na resposta ao estresse na idade adulta (Pariante & Lightman, 2008).

Essas alterações a longo prazo induzidas pela exposição a fatores ambientais precoces também são observadas em estudos com modelos animais, como exposição a estressores, que alteram a programação neural, podendo levar a alterações cognitivas, emocionais e no funcionamento do eixo hipotálamo-hipófise-adrenal (HHA), afetando as respostas ao estresse na idade adulta (Isgor et al., 2004, McEwen, 2008, Juruena, 2014).

Além disso, a exposição a dietas com alto conteúdo calórico nesses períodos sensíveis do desenvolvimento pode levar a alterações neuronais e metabólicas

associadas a mudanças permanentes no comportamento alimentar, e associado a predisposição à obesidade na idade adulta (Frazier et al., 2008, Teegarden et al., 2009).

Dessa forma, a exposição a diferentes intervenções ambientais precoces pode levar a mudanças na programação metabólica e do SNC, podendo causar efeitos a longo-prazo, modificando a vulnerabilidade para psicopatologias e predisposição a doenças ao longo da vida.

1.2 Isolamento social no período pré-pubere

O período pré-pubere compreende uma fase importante do desenvolvimento que antecede a maturação do sistema reprodutivo. Em roedores, esse período compreende o final do desmame, por volta do 21º dia pós-natal, estendendo-se até os dias 30º a 35º pós-natal (McCormick et al., 2010, Eiland & Romeo, 2013). Em humanos esse período é correspondente ao final da infância, por volta de 8-10 anos de idade (Eiland & Romeo, 2013).

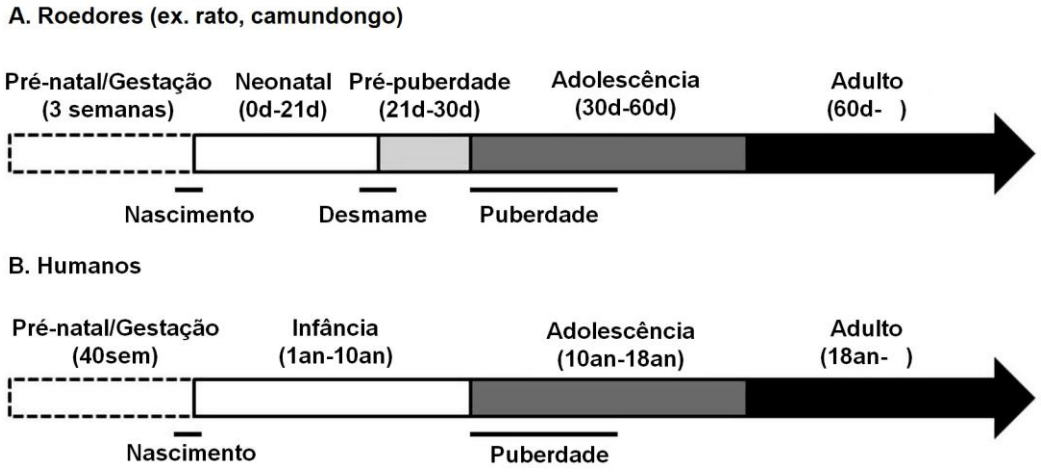

Figura 1. Linha do tempo do desenvolvimento em A. roedores e B. humanos. Fonte: adaptado de Eiland & Romeo (2013).

No período da pré-puberdade, o SNC passa por um intenso processo de transformação, em que ocorrem processos de maturação encefálica, sexual e organização dos circuitos neuroquímicos (Gogtay *et al.*, 2004). Essa maturação ocorre em diferentes estruturas encefálicas: no córtex prefrontal de roedores observa-se que tanto os espinhos dendríticos como o comprimento total das ramificações dendríticas aumentam entre o período de 20-35 dias pós-natal (Koss *et al.*, 2014); o hipocampo também apresenta um aumento na densidade de espinhos dendríticos e da neurogênese antes da puberdade (He & Crews, 2007, Yildirim et al., 2008), assim como o restante do sistema límbico, que possui uma maturação tardia dos seus sistemas neurotransmissores, como por exemplo, o sistema dopaminérgico (Spear, 2000).

Essa intensa plasticidade em diferentes estruturas encefálicas durante a pré-puberdade influencia o desenvolvimento dos comportamentos sociais e cognitivos, característicos desse período, que são demonstrados pelo aumento do comportamento exploratório e de comportamentos de brincadeiras (Sisk & Foster, 2004, McCormick et al., 2010). Dessa forma, as interações sociais durante essa fase são necessárias para um bom desenvolvimento sócio-emocional. Entretanto, quando ocorre uma restrição das influências sociais nesse período, podem ocorrer mudanças plásticas influenciando a estrutura e a função cerebral e assim, causar alterações e/ou disfunções que podem permanecer até a idade adulta (Einon & Morgan, 1977, Klein et al., 2010).

Estudos em humanos mostram que a privação social observada em orfanatos de crianças abandonadas (entre 8-10 anos de idade) leva a comprometimento cognitivo, déficit social e alterações em algumas estruturas encefálicas (Chugani et al., 2001, Eluvathingal et al., 2006, Nelson et al., 2007). Da mesma forma, o “bullying”, que é caracterizado como um comportamento agressivo, repetitivo e intencional (Olweus, 1994), faz com que a criança vítima desse tipo de comportamento acabe se isolando,

ocasionando prejuízos cognitivos e emocionais (Camodeca & Goossens, 2005, Medeiros et al., 2016).

Em modelos animais, o isolamento social é considerado um modelo de estresse por representar uma situação aversiva para ratos, uma vez que as interações sociais são recompensadoras para roedores e esses animais apresentam normalmente uma série de comportamentos sociais (Panksepp et al., 2007, Hong et al., 2012). O isolamento social após o desmame e aplicado cronicamente pode levar a alterações a longo-prazo, como aumento da atividade locomotora (Varty *et al.*, 2000) e da agressividade (Koike *et al.*, 2009), déficits cognitivos (Lu et al., 2003, Ibi et al., 2008), diminuição das interações sociais (Fone & Porkess, 2008, Koike et al., 2009), além de comportamento do tipo depressivo (Jahng *et al.*, 2012). Além disso, os animais isolados cronicamente apresentam alterações anatômicas e neuroquímicas, como diminuição dos espinhos dendríticos de neurônios piramidais no córtex prefrontal e hipocampo (Silva-Gomez *et al.*, 2003), diminuição de neurogênese e sinapses hipocampais (Varty et al., 1999, Ibi et al., 2008), além de alterações neuroquímicas, como aumento da liberação basal de dopamina e dessensibilização de receptores D2 no núcleo accumbens (Hall *et al.*, 1998).

Entretanto, a maioria dos estudos, incluindo os citados acima, avalia o isolamento social com exposição crônica, geralmente iniciando após o desmame e com duração até a idade adulta. Poucos estudos vêm analisando os efeitos do isolamento social antes da puberdade avaliando os seus efeitos comportamentais e neuroquímicos a longo prazo. É importante considerar que isolamentos por períodos mais curtos mimetizam de forma mais fidedigna a exposição a esse tipo de estressor em humanos, uma vez que são raros os casos de isolamento social por toda a vida.

1.3 A resposta ao estresse e alterações durante o desenvolvimento

Um estressor é definido como uma ameaça ou desafio ao indivíduo que pode potencialmente perturbar a homeostase do organismo (McEwen, 2007). A exposição a estressores físicos e/ou psicológicos gera diversas respostas do organismo, incluindo respostas neurovegetativas, imunológicas, comportamentais e neuroendócrinas (Tsigos & Chrousos, 2002) como uma forma de preparar o organismo para reagir e lidar com uma demanda adequada ao evento estressor (McEwen, 2007).

Classicamente, existem dois importantes sistemas que são ativados frente a um estressor: a) primeiramente, a ativação do sistema nervoso simpático que culmina na liberação de noradrenalina em diversos órgãos e de adrenalina pela glândula adrenal gerando respostas mais instantâneas frente a um estressor, descritas pelo paradigma das respostas de "luta ou fuga"; b) e a ativação do eixo HHA que inicia com a liberação do hormônio liberador de corticotropina (CRH) no hipotálamo, que por sua vez estimula a hipófise a secretar o hormônio adrenocorticotrópico (ACTH). O ACTH age no córtex da glândula adrenal estimulando a liberação de glicocorticóides na circulação (cortisol em humanos e corticosterona em roedores) (Tsigos & Chrousos, 2002, Lupien et al., 2005, McEwen, 2007, 2008). Os glicocorticóides (GCs) possuem várias ações no controle da homeostase corporal e das respostas ao estresse, como o aumento da disponibilidade de substratos energéticos no organismo e melhora do fluxo sanguíneo para órgãos-alvo. Além disso, os GCs também exercem ações no SNC, tais como alterações na plasticidade sináptica, na expressão de proteínas e de receptores de neurotransmissores, mudanças na neurogênese, indução de morte celular, entre outros (McEwen, 2007, McEwen & Gianaros, 2011).

Adicionalmente, os GCs exercem controle inibitório sobre o próprio eixo HHA, por meio de um sistema de retroalimentação negativa que ocorre via ligação dos GCs aos seus receptores localizados em estruturas do SNC envolvidas no controle desse eixo, dentre elas o hipotálamo, o hipocampo e o córtex cerebral (Tsigos & Chrousos, 2002). Existem dois tipos de receptores para os glicocorticóides com diferentes afinidades a seus ligantes: a) os receptores de mineralocorticóides (MR) possuem alta afinidade ao ligante e estão ativados com as concentrações basais de GCs; b) e os receptores de glicocorticóides (GR), com baixa afinidade ao ligante, são mais sensíveis às mudanças de concentrações dos GCs e assim, com as respostas ao estresse (Harris *et al.*, 2013). Esses receptores estão localizados no citosol das células e, quando ativados pelos GCs, migram para o núcleo, aonde irão se ligar a uma região específica responsiva aos GCs presente no ADN (ácido desoxirribonucléico) e assim, regular a transcrição de vários genes (Hayashi *et al.*, 2004).

A ativação dos sistemas relacionados ao estresse geralmente leva a uma adaptação do organismo para melhorar as habilidades e ajustar sua homeostase, facilitando a sobrevivência frente ao estressor (Tsigos & Chrousos, 2002). Entretanto, quando o estressor se torna prolongado e de forma crônica, a ativação constante desses sistemas e a liberação de glicocorticóides de forma prolongada pode ser uma condição indesejável, com alterações metabólicas e neuroquímicas prejudiciais para o organismo (McIntosh & Sapolsky, 1996). Dados da literatura demonstram que alterações biológicas no sistema de resposta ao estresse, que levam a uma desregulação da atividade do eixo HHA, podem estar relacionadas ao aparecimento de uma série de distúrbios/transtornos, como por exemplo, depressão, ansiedade, e predisposição à obesidade (de Kloet et al., 2005, Ridder et al., 2005, Pervanidou & Chrousos, 2012).

A exposição a estressores durante períodos de desenvolvimento, incluindo o período pré-pubere, pode afetar o desenvolvimento encefálico, uma vez afetando sua plasticidade contínua durante esses períodos, pode causar modificações funcionais e anatômicas nas respostas ao estresse. Vários estudos mostram que as consequências da exposição a estressores diferem entre adolescentes e adultos (McCormick et al., 2010): a exposição a estressores agudos em animais pré-puberes resulta em maiores respostas hormonais ao estresse, como aumento dos níveis de ACTH e corticosterona, quando comparados a animais adultos (Goldman et al., 1973, Romeo et al., 2004a, Romeo et al., 2004b, Romeo & McEwen, 2006, Lui et al., 2012). Além disso, quando os animais são repetidamente expostos ao mesmo estressor, os animais adultos demonstram uma maior habituação observada pela resposta ao estresse nos níveis de ACTH e corticosterona, enquanto que animais pré-puberes possuem uma resposta mais sensibilizada (Romeo & McEwen, 2006, Doremus-Fitzwater et al., 2009, Lui et al., 2012).

Além disso, essas alterações na resposta ao estresse podem influenciar também a maturação encefálica e certos circuitos neuroquímicos, como observado em um estudo em que a mesma dose de corticosterona aumentou a expressão de subunidades do receptor N-metil-D-aspartato (NMDA) hipocampal (ex. NR2A e NR2B) em animais pré-puberes quando comparados a animais adultos (Lee *et al.*, 2003). Adicionalmente, a exposição a estressores na pré-puberdade (27-29 PND) altera a expressão de moléculas de adesão celular neural no sistema límbico, envolvidas no desenvolvimento neural, na plasticidade sináptica e em processos de aprendizado (Tsoory et al., 2008). Dessa forma, esses estudos em modelos animais demonstram que a exposição a estressores durante a pré-puberdade e puberdade pode alterar a maturação de estruturas encefálicas importantes para funções cognitivas e para as respostas ao estresse.

1.4 Estresse e comportamento alimentar

A disponibilidade de nutrientes e a exposição a estressores podem afetar o comportamento alimentar de diferentes formas. O estresse pode influenciar o comportamento alimentar, intensificando ou atenuando o apetite, alterando as preferências alimentares, dependendo da intensidade e duração do agente estressor (Marti et al., 1994, Ortolani et al., 2011).

Dados da literatura mostram que o estresse possui um efeito sobre a escolha do tipo de alimento consumido: pessoas estressadas geralmente apresentam uma preferência maior por alimentos palatáveis, ricos em calorias provindos principalmente de açucares e gorduras (Wardle et al., 2000, Cartwright et al., 2003, Zellner et al., 2006, Tomiyama et al., 2011, Tryon et al., 2013). Da mesma forma, essa preferência também é observada em modelos animais (Pecoraro et al., 2004, Silveira et al., 2010, Oliveira et al., 2015).

Algumas linhas de pesquisa apontam para a hipótese de que o aumento do consumo de alimentos palatáveis induzido pelo estresse estaria relacionado a uma tentativa da diminuição das respostas do estresse, sendo que esses alimentos calóricos agiriam como "alimentos confortantes" (chamados "comfort foods") (Adam & Epel, 2007). Entretanto, existem algumas diferenças quanto às ações dessas dietas na atividade do eixo HHA, dependendo do tipo de dieta consumida: dietas ricas em açúcares estão mais associadas à redução das respostas ao estresse (Pecoraro *et al.*, 2004), enquanto dietas ricas em gorduras possuem dados controversos na literatura, mostrando que elas podem tanto realçar os níveis de GCs basais e induzidos pelo estresse, podendo agir como um tipo de estressor (Tannenbaum et al., 1997, Kamara et al., 1998, McNeilly et al., 2015), ou então da mesma forma que dietas ricas em

açúcares, causarem uma diminuição da atividade do eixo HHA (Auvinen *et al.*, 2012). Além disso, um estudo utilizando animais na pré-puberdade demonstrou que o consumo de uma dieta rica em gordura nesse período não foi capaz de alterar os níveis plasmáticos de corticostorena em machos (Boukouvalas *et al.*, 2008). Essas divergências podem estar relacionadas às diferentes composições de dietas que são utilizadas nos estudos e ao tempo de consumo, além do período em que é ofertado esse tipo de alimento (Auvinen *et al.*, 2011).

A ingestão alimentar é essencialmente regulada por duas vias complementares: (a) a via homeostática mais associada ao controle hipotalâmico, relacionada ao aumento da motivação para comer em função do balanço energético, sendo ativada quando ocorre depleção dos estoques energéticos, e (b) a via hedônica, ou regulação baseada na recompensa, ligada aos aspectos emocionais e recompensadores do alimento, geralmente relacionada ao consumo de alimentos palatáveis (Tulloch *et al.*, 2015) e associada ao sistema de recompensa, incluindo o núcleo accumbens. Muitas vezes, a via hedônica pode sobrepor-se à via homeostática durante períodos de relativa abundância de energia (Tulloch *et al.*, 2015). Assim, o controle alimentar envolve mecanismos complexos que sofrem influência não apenas das demandas energéticas, mas também do estado emocional do indivíduo.

É importante notar que há uma distinção nos fatores que levam à ativação dos mecanismos de recompensa do encéfalo. Pesquisas coordenadas por Kent Berridge levaram a um modelo que propõem dois componentes de resposta distintos envolvidos nessa regulação do comportamento alimentar hedônico (Berridge, 2009). Um deles, denominado “gostar” (“liking”), está relacionado ao prazer e à palatabilidade que envolve o consumo de determinado alimento. O segundo componente envolvido, caracterizado como “querer” (“wanting”), está mais associado com o valor motivacional

da recompensa ou saliência de incentivo, refere-se ao impulso direcionado para o estímulo alimentar alvo, sendo relacionado com o apetite (Berridge, 2009).

Essas duas respostas relacionadas ao comportamento alimentar são distintas; entretanto, ambas são necessárias para o processo de recompensa normal (Berridge, 2009). A modulação das respostas de "querer" (relacionadas à motivação em buscar o alimento) e de "gostar" (relacionadas à resposta de prazer no consumo da recompensa alimentar) possuem envolvimento das vias dopaminérgicas (receptores e transportadores dopaminérgicos, assim como todo processo de metabolização da dopamina) (Nicola, 2016), além do sistema canabinóide (receptor canabinóide 1, CB1) (Di Marzo *et al.*, 2009) e opióide (receptor μ-opióide) (Pecina & Berridge, 2000).

Um importante centro encefálico relacionado com as respostas motivacionais e com as características hedônicas alimentares é o núcleo accumbens (Nac), importante centro de projeções de neurônios dopaminérgicos com origem na área tegmentar ventral (VTA) (Haber & Knutson, 2010). O sistema dopaminérgico para o Nac parece ser importante na busca e na modulação do incentivo percebido pela recompensa (Wyvell & Berridge, 2000).

Algumas linhas de estudo mostram que a ingestão de dietas palatáveis ricas em gorduras pode alterar a função dos sistemas neurotransmissores encefálicos que participam da regulação do comportamento alimentar. Em animais expostos a uma dieta crônica hiperlipídica, foram observadas diferenças na metilação do ADN, com a redução na expressão de genes relacionados ao sistema dopaminérgico em várias estruturas cerebrais, incluindo o VTA (redução da expressão de tirosina-hidroxilase e do transportador de dopamina) e o Nac (redução da expressão de receptor D1) (Vucetic *et al.*, 2012). Esses resultados sugerem modificação epigenética do sistema dopaminérgico em função da dieta. Adicionalmente, uma exposição a esse tipo de dieta precocemente

também é capaz de alterar o sistema mesolímbico dopaminérgico e as respostas do eixo HHA frente a estressores na idade adulta, mostrando que animais que consomem esse tipo de dieta possuem menor sensibilização das respostas frente a um estressor crônico na idade adulta (Naef *et al.*, 2013). No mesmo sentindo, outros estudos também mostram alterações na neurotransmissão opióide (receptor μ-opióide) e canabinóide (receptor CB1) no sistema mesolímbico, associadas com o consumo de uma dieta rica em gordura, levando a alterações na regulação do comportamento alimentar (Deshmukh & Sharma, 2012, Pitman & Borgland, 2015).

Dessa forma, a exposição precoce e crônica a dietas hiperlipídicas, assim como a exposição a estressores no início da vida, podem causar alterações a longo-prazo na circuitaria envolvida no controle do comportamento alimentar e nas respostas hedônicas encefálicas, podendo levar a um aumento na predisposição ao ganho de peso e/ou obesidade ao longo da vida.

1.5 Estresse, dietas hiperlipídicas e alterações cognitivas dependentes de plasticidade

A plasticidade encefálica é a capacidade de remodelação do encéfalo frente às diferentes experiências do indivíduo, com reformulação de suas conexões em função das necessidades e dos fatores ambientais. Sendo essencialmente relacionada aos processos de desenvolvimento, aprendizado, resiliência, memória e de reparação (Pittenger, 2013).

A capacidade plástica é uma característica fundamental do SNC e prejuízos em processos de plasticidade sináptica, neurogênese, crescimento e remodelação celular

podem contribuir para déficits cognitivos e assim, podem induzir a uma variedade de doenças neuropsiquiátricas associadas (Pittenger, 2013).

É bem conhecido que a plasticidade neuronal mediada por alterações na morfologia e função sináptica está subjacente aos processos de aprendizado e memória (Lante *et al.*, 2006). Tais processos envolvem adaptações da circuitaria encefálica ao ambiente, ocorrendo ao longo de toda a vida. Define-se aprendizado como a aquisição de novas informações ou novos conhecimentos, enquanto que a memória é a retenção da informação aprendida (Korte & Schmitz, 2016, Kim & Kaang, 2017).

Existem diferentes tipos de memórias, sendo classificadas em: declarativas ou explícitas (memória para fatos e eventos) e não-declarativas ou implícitas (memória para habilidades, hábitos e comportamentos). As memórias também podem ser classificadas quanto a sua duração: memórias de longo-prazo são aquelas que podem ser evocadas por dias, meses ou anos após terem sido originalmente armazenadas; e as memórias de curto-prazo, que duram segundos a horas e são vulneráveis a perturbações (Korte & Schmitz, 2016). Um tipo de memória mantida por um curto período de tempo é a chamada memória de trabalho, que é uma forma temporária de armazenamento da informação, de capacidade limitada e exige certa repetição para ser conservada, mesmo que por período curto de tempo (por exemplo, armazenamento de um número de telefone por um tempo limitado) (Wang et al., 2017c).

Várias estruturas encefálicas fazem parte do processo de aquisição dos diferentes tipos de memórias. Classicamente, o envolvimento do hipocampo é bem discutido nos processos envolvendo memória e aprendizado (Squire, 1992), mas adicionalmente o córtex pré-frontal vem sendo reconhecido por mediar funções executivas, incluindo memórias de curta-duração, como a memória de trabalho (Levy & Goldman-Rakic, 1999, Tau & Peterson, 2010). Além disso, o córtex pré-frontal possui uma maturação

mais tardia, o que torna esta estrutura mais susceptível a intervenções ambientais durante o desenvolvimento (Gogtay *et al.*, 2004).

A exposição a fatores ambientais precoces, como estressores e as variações nutricionais, incluindo o consumo de dietas ricas em calorias, pode causar alterações em parâmetros biológicos relacionados à maturação neural e à plasticidade. Diversos estudos vêm demonstrando a associação entre o estresse precoce e alterações cognitivas ao longo da vida, induzidas por modificações na circuitaria encefálica. Estudos em roedores submetidos ao isolamento social crônico (durante o desenvolvimento até a idade adulta) relatam prejuízos, com mudanças comportamentais a longo-prazo, como hiperatividade em resposta à novidade (Fabricius et al., 2011, Watson et al., 2012) e prejuízo no aprendizado e na memória (Quan et al., 2010, Watson et al., 2012, McIntosh et al., 2013).

Da mesma forma, estudos em humanos demonstram que exposição a situações estressantes e/ou traumáticas durante certas fases do desenvolvimento, como a infância, afeta o desempenho em tarefas cognitivas envolvendo memória. Um estudo recente mostrou que indivíduos expostos a estressores precoces apresentaram menor velocidade de processamento e desempenho em tarefas envolvendo memória de trabalho, assim como menores volumes de várias áreas corticais (Saleh *et al.*, 2017), de acordo com estudos anteriores que mostraram essa associação entre estresse precoce e alterações volumétricas e funcionais em regiões encefálicas, observadas por estudos de neuroimagem (Cohen et al., 2006, van Harmelen et al., 2010, Dannlowski et al., 2012).

Além disso, o acesso a dietas altamente calóricas, especialmente as ricas em gorduras, também podem afetar a maturação e a plasticidade encefálica (Granholm *et al.*, 2008), alterando as respostas cognitivas, incluindo prejuízo no aprendizado e na memória, observado em roedores (Winocur & Greenwood, 2005, Granholm et al., 2008,

Valladolid-Acebes et al., 2011), assim como em humanos (Morris et al., 2004, Francis & Stevenson, 2011). Entretanto, mais estudos são necessários para identificar os mecanismos neurobiológicos que contribuem para essas modificações na plasticidade encefálica.

Alguns marcadores biológicos relacionados à plasticidade neuronal vêm sendo associados com as respostas de aprendizado e memória. Uma importante correlação entre o fator neurotrófico derivado do encéfalo (BDNF) e alterações em funções cognitivas vem sendo relatada (Kennedy et al., 2015, Azeredo et al., 2017). Essa neurotrofina está envolvida em processos de neurogênese e na modulação da plasticidade encefálica, exercendo seus efeitos biológicos através de sua ligação e ativação de receptores específicos de tropomiosina cinase (TrkB), que por sua vez desencadeiam a ativação de múltiplas cascatas de sinalização intracelulares, incluindo a ativação das vias fosfatidil-inositol-3-cinase (PI3K/AKT), fosfolipase C (PLC) e SHC/RAS/MAP cinase (Binder & Scharfman, 2004, Bennett & Lagopoulos, 2014) (ver Figura 2). A ativação dessas vias está envolvida na regulação de plasticidade dependente de síntese proteica e no transporte e tradução de proteínas sinápticas (Yoshii & Constantine-Paton, 2010). São relatadas alterações nos níveis de BDNF associadas ao consumo de uma dieta rica em gordura (Theriault et al., 2016, Wang et al., 2017a), assim como a exposições a estressores, como o isolamento social (Barrientos et al., 2003, Berry et al., 2012). Pelo fato do BDNF modular a comunicação e plasticidade sináptica, além de ter um envolvimento em sinapses glutamatérgicas e gabaérgicas (Manju et al., 2017), reduções nos níveis e/ou nas vias de sinalização dessa neurotrofina podem comprometer processos de aprendizado e memória.

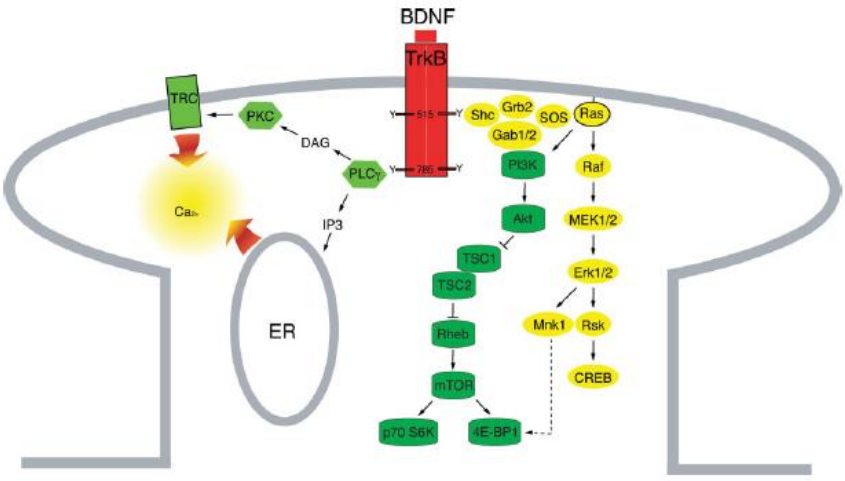

Figura 2. Vias de sinalização do BDNF (Fonte:Yoshii & Constantine-Paton, 2010).

Outro marcador relevante para processos de plasticidade e memória é a enzima Na^+,K^+-ATPase. Ela está envolvida com o transporte ativo de íons sódio e potássio, mantendo o gradiente eletroquímico necessário para excitabilidade e regulação do volume celular neuronal (Gloor, 1997). Adicionalmente, a Na^+,K^+-ATPase também exerce funções como transdutor de sinais da membrana plasmática para as organelas intracelulares (Xie & Askari, 2002). Sua modulação afeta diretamente a sinalização de neurotransmissores, a atividade neural bem como o comportamento. Além disso, a inibição da atividade dessa enzima está associada a prejuízos no aprendizado e memória (Zhan et al., 2004, Zhang et al., 2013). Estudos prévios mostram que a atividade da Na^+,K^+-ATPase pode ser influenciada por intervenções ambientais, como estressores no início da vida (Silveira et al., 2011, Noschang et al., 2012), assim como pela ingestão de dietas palatáveis durante a pré-puberdade (Krolow *et al.*, 2012).

Dessa forma, observa-se que variações ambientais em determinados períodos do desenvolvimento podem influenciar a plasticidade encefálica, modulando respostas cognitivas como, por exemplo, a memória. Entretanto, poucos estudos avaliaram a

interação a longo-prazo entre fatores como estresse e consumo de dietas hiperlipídicas durante a pré-puberdade.

1.6 Estresse e dietas hiperlipídicas associadas a alterações emocionais e ao comportamento do tipo depressivo.

A associação da vulnerabilidade genética com exposição a fatores ambientais específicos, especialmente em períodos importantes do desenvolvimento encefálico, pode estar relacionada a uma maior vulnerabilidade ao desenvolvimento de transtornos de humor, incluindo a depressão (Lopizzo *et al.*, 2015).

Embora os mecanismos patofisiológicos da depressão ainda não estejam completamente esclarecidos, vários conceitos tentam caracterizar as bases neurobiológicas desse transtorno, incluindo prejuízo da plasticidade neuronal e da neurogênese hipocampal, associado à redução de fatores neurotróficos, alterações no controle do eixo HHA, além da clássica teoria da deficiência monoaminérgica e/ou um desequilíbrio entre vários sistemas neurotransmissores (Nestler et al., 2002, Duman & Monteggia, 2006, Serafini et al., 2014).

Entre os fatores ambientais, a exposição a estressores, especialmente nas primeiras fases da vida, exerce um impacto importante no desenvolvimento encefálico levando a mudanças funcionais que podem contribuir para a depressão (Heim & Binder, 2012). Além disso, nesses períodos de alta plasticidade neural, regiões encefálicas envolvidas na regulação da emoção e na mediação das respostas ao estresse parecem estar particularmente mais sensíveis a exposição a eventos estressores. Nesse contexto, estudos epidemiológicos mostram que pacientes depressivos apresentam prejuízos no controle da atividade do eixo HHA, associado a uma redução de GR em estruturas

encefálicas envolvidas no controle desse eixo, além de aumento dos níveis de cortisol circulante (Pariante & Miller, 2001, Swaab et al., 2005, Vreeburg et al., 2009). Dessa forma, a plasticidade induzida pelo ambiente pode produzir alterações na circuitaria neural, além de respostas mal adaptativas ao ambiente, causando assim um aumento do risco para o desenvolvimento de transtornos depressivos (Hornung & Heim, 2014).

Numerosos estudos epidemiológicos e clínicos têm demonstrando evidências de uma forte associação entre variadas formas de estresse no início da vida e sintomas depressivos. Adversidades na infância, como abuso, negligência, maus tratos, perda de familiares, entre outros, estão associados com o aumento dos sintomas depressivos ao longo da vida (Agid et al., 1999, Edwards et al., 2003, Chapman et al., 2004, Heim & Binder, 2012). Entretanto, é importante salientar que, embora as experiências estressantes precoces tenham um impacto na vulnerabilidade a transtornos depressivos, elas não levam a transtornos psiquiátricos em todos os indivíduos expostos, uma vez que esse processo é dependente de um fundo genético individual, que por sua vez pode influenciar a forma de lidar com os estímulos estressantes (Mann & Currier, 2010).

Estudos em roedores também têm demonstrado que exposição a estressores precoces, como o isolamento social, provoca sintomas comportamentais semelhantes aos observados na depressão em humanos (Brenes Saenz et al., 2006, Vargas et al., 2016, Wang et al., 2017b). Entretanto, pouco se sabe sobre os efeitos de um isolamento social a curto-prazo, no início da vida, na predisposição de comportamentos do tipo depressivo na idade adulta.

Além disso, o consumo de dietas altamente calóricas também vem sendo associado com maior predisposição a transtornos psiquiátricos. Estudos em humanos (Akbaraly *et al.*, 2009) e em modelos animais (Abildgaard et al., 2011, Sharma & Fulton, 2013) mostram que o consumo de dietas ricas em calorias pode aumentar a

probabilidade de desenvolver depressão. É importante salientar que o acesso a dietas altamente calóricas vem aumentando consideravelmente em crianças, o que pode modificar a maturação encefálica e levar a mudanças em processos cognitivos e emocionais ao longo da vida (Sasaki et al., 2013, Boitard et al., 2015).

Mudanças na plasticidade neural causadas por exposição a fatores ambientais (como estresse e dietas hipercalóricas) durante o desenvolvimento podem estar associadas com o desenvolvimento de sintomas depressivos. Alterações estruturais encefálicas, como perda de sinapses e espinhos dendríticos, assim como, redução da ramificação dendrítica e mudanças em células gliais no hipocampo vem sendo relacionadas com a depressão (Serafini *et al.*, 2014). Por exemplo, o BDNF, envolvido na neurogênese e modulação da plasticidade neural, diminui após adversidades no início da vida (Choy et al., 2008, Roth & Sweatt, 2011) e sua redução tem sido correlacionada com a patofisiologia da depressão (Polyakova *et al.*, 2015). Além disso, a depressão pode estar associada a alterações em diversos sistemas neurotransmissores, tais como sistemas serotoninérgicos, glutamatérgicos e gabaérgicos (Yuksel & Ongur, 2010, Gao et al., 2013), sendo que a conectividade neural desses sistemas ocorre durante períodos específicos e a exposição a certos fatores ambientais afeta o seu desenvolvimento.

Dessa forma, considerando que a exposição a estressores induz aumento do consumo de alimentos palatáveis, e que o acesso a tais alimentos vem aumentando de forma marcante durante a infância, torna-se relevante entender como variações no ambiente, como o isolamento social, com a concomitante exposição a dietas altamente calóricas, podem propiciar, a longo prazo, o aparecimento de transtornos como a depressão, especialmente se aplicados durante períodos em que a maturação encefálica é maior.

Assim, tendo em vista que:

- o período pré-pubere é um período de grande maturação neural e assim, uma fase sensível no desenvolvimento da função do sistema nervoso;
- perturbações nesse período, como a exposição a fatores ambientais, podem afetar o desenvolvimento encefálico e o comportamento;
- a exposição a fatores ambientais adversos, tais como o isolamento social e o consumo de uma dieta rica em gordura, têm aumentado de modo marcante em períodos precoces;
- esses fatores em períodos sensíveis do desenvolvimento podem afetar a plasticidade neural, a conectividade dos circuitos neuroquímicos e o controle das respostas ao estresse, podendo dessa forma afetar permanentemente os processos cognitivos e emocionais;

o presente trabalho de Tese têm como **hipótese** que a exposição a um estressor durante o período pré-pubere pode perturbar a plasticidade neural, ocasionando mudanças significativas em processos cognitivos e emocionais na idade adulta e que o concomitante consumo precoce de uma dieta rica em gordura possa potencializar os efeitos do estresse, além de seus efeitos deletérios próprios no desenvolvimento encefálico e comportamental.

2. OBJETIVOS

2.1 Objetivo Geral

O objetivo da presente Tese foi investigar os efeitos da exposição a um estressor durante o desenvolvimento (período pré-pubere) associado a uma exposição crônica a uma dieta hiperlipídica sobre funções cognitivas e emocionais e estudar os possíveis mecanismos neuroquímicos responsáveis por essas alterações em ratos machos na idade adulta.

2.2 Objetivos Específicos

Analisar se a exposição ao isolamento social no período pré-pubere e o consumo precoce e crônico de uma dieta hiperlipídica causam:

a. Prejuízo da memória de curta-duração e alteram marcadores neuroquímicos envolvidos com a via de sinalização do BDNF, a enzima Na^+,K^+-ATPase e os receptores de glicocorticóides no córtex pré-frontal de ratos machos adultos (Capítulo I).

b. Comportamento do tipo depressivo e alterações em marcadores de plasticidade sináptica, assim como da atividade do eixo HHA no hipocampo de ratos machos na idade adulta (Capítulo II).

c. Modificações nas respostas hedônicas para o comportamento alimentar e alteram sistemas neurotransmissores envolvidos no sistema de recompensa alimentar,

como o dopaminérgico, o opióide e o canabinóide no núcleo accumbens de ratos machos na idade adulta (Capítulo III).

3. MATERIAIS, MÉTODOS E RESULTADOS

Os materiais, métodos e os resultados dessa Tese são apresentados a seguir, da seguinte forma:

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

- Capítulo II: Artigo submetido para publicação na revista *Molecular Neurobiology*;

- Capítulo III: Artigo a ser submetido para publicação na revista *European Journal of Nutrition.*

3.1 Capítulo I

Early life adversities or high fat diet intake reduce cognitive function and alter BDNF signaling in adult rats: Interplay of these factors changes these effects.

Artigo publicado na revista *International Journal of Developmental Neuroscience.*

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Early life adversities or high fat diet intake reduce cognitive function and alter BDNF signaling in adult rats: Interplay of these factors changes these effects

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ABSTRACT

Environmental factors, like early exposure to stressors or high caloric diets, can alter the early programming of central nervous system, leading to long-term effects on cognitive function, increased vulnerability to cognitive decline and development of psychopathologies later in life. The interaction between these factors and their combined effects on brain structure and function are still not completely understood. In this study, we evaluated long-term effects of social isolation in the prepubertal period, with or without chronic high fat diet access, on memory and on neurochemical markers in the prefrontal cortex of rats. We observed that early social isolation led to impairment in short-term and working memory in adulthood, and to reductions of Na^+,K^+-ATPase activity and the immunocontent of phospho-AKT, in prefrontal cortex. Chronic exposure to a high fat diet impaired short-term memory (object recognition), and decreased BDNF levels in that same brain area. Remarkably, the association of social isolation with chronic high fat diet rescued the memory impairment on the object recognition test, as well as the changes in BDNF levels, Na^+,K^+-ATPase activity, MAPK, AKT and phospho-AKT to levels similar to the control-chow group. In summary, these findings showed that a brief social isolation period and access to a high fat diet during a sensitive developmental period might cause memory deficits in adulthood. On the other hand, the interplay between isolation and high fat diet access caused a different brain programming, preventing some of the effects observed when these factors are separately applied.

1. Introduction

Early in life, the nervous system undergoes an intense process of maturation and adaptation, and the constant influence of environmental factors help to shape the cognitive and emotional aspects of the individual (Burggren and Mueller, 2015; Knudsen, 2004; Takesian and Hensch, 2013; Vickers, 2014). Early life events, like stressful events, can induce long-lasting changes in the brain that may result in alterations in several neuroendrocrinal and behavioral processes (Hoeijmakers et al., 2014; Marco et al., 2011; Naninck et al., 2015; Pervanidou and Chrousos, 2007). In this context, social behavior during developmental periods, especially during prepuberty and adolescence, has a significant impact on stress responses and behavior later in life (McCormick and Mathews, 2007; Weiss et al., 2004). In animals with social organization, like rodents, it has been observed that social isolation is aversive, as social interactions are necessary to ensure good cognitive and emotional development during growth (Hong et al., 2012). It was shown that long-term social isolation (during developmental periods until young adult age) disturbs brain maturation (Lapiz et al., 2003; Quan et al., 2010), and causes a series of long-lasting behavioral changes, like hyperactivity in response to novelty (Fabricius et al., 2011; Watson et al., 2012), and impaired learning and memory (McIntosh et al., 2013; Quan et al., 2010; Watson et al., 2012). Furthermore, acute social isolation (72 h) in rodents, increases plasma corticosterone (Kamal et al., 2014), indicating that a brief social isolation can be characterized as a potent stressor in these animals.

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The exposure to stressful events leads to increased release of glucocorticoids (GCs), due to activation of the hypothalamus-pituitary-adrenal (HPA) axis. Additionally, key signaling molecules, like glucocortidoid receptor (GR), can be affected by stress exposure, since this receptor mediate the negative feedback of GCs on the HPA axis following stress (Abildgaard et al., 2014; Buwalda et al., 2001; Groeneweg et al., 2012). One of the consequences of stress exposure is the change in food intake. It has been proposed a reward-based eating model ("comfort foods") which the high caloric food intake has been suggested as a means to reduce the stress response (Adam and Epel, 2007). In this sense, the association between stressful environment and access to high caloric diets early in life can be one of the reasons of the alarming higher prevalence of obesity and overweight in children and adolescents (Ogden et al., 2012). Furthermore, the access to high caloric diets in sensitive developmental periods could also modify brain maturation, with changes in cognitive processes (Winocur and Greenwood, 2005), as impaired learning and memory function, which has been observed in both humans (Francis and Stevenson, 2011; Morris et al., 2004) and rodents (Granholm et al., 2008; Valladolid-Acebes et al., 2011).

Is well known that neuronal plasticity mediated by changes in synaptic morphology and function underlies learning and memory processes (Lante et al., 2006). Early life exposure to factors such as stress and high caloric diet intake may cause alterations on biological parameters related to neural maturation and plasticity. Levels of brain-derived neurotrophic factor (BDNF), a neurotrophin involved in neurogenesis and modulation of brain plasticity, decreases after early-life adversities (Choy et al., 2008; Roth and Sweatt, 2011). This neurotrophin exerts many biological effects by binding and activating specific tropomyosin-related kinase (Trk) receptors, which in turn triggers multiple intracellular signaling cascades, including activation of phosphatidylinositol 3-kinase PI3 K/AKT, phospholipase Cγ (PLCγ), and SHC/RAS/MAPK pathways (Bennett and Lagopoulos, 2014). Alterations in BDNF have been associated with memory impairment induced by high fat diet intake (Cui et al., 2012; Granholm et al., 2008), or exposure to stressors early in life (Oomen et al., 2010; Wang et al., 2011). Also relevant to the development of central nervous system is the activity of Na^+,K^+-ATPase, an enzyme that maintains the neurochemical gradient essential for neuronal excitability, and which activity is influenced by environmental interventions, like stress in early life (Noschang et al., 2012; Silveira et al., 2011) and palatable diet intake (Krolow et al., 2012).

The prefrontal cortex is a brain structure involved in the orchestration of motor, cognitive, affective, and social behaviors (Kolb et al., 2012), mediating executive functions, including inhibitory control, attention and mental flexibility, reward sensitivity, and working memory (Blakemore and Choudhury, 2006; Crone, 2009; Tau and Peterson, 2010). In addition, the prefrontal cortex is also involved in stress responses (McEwen, 2008). This structure is susceptible to environmental interventions in early life, since its development and maturation occurs during childhood until early adulthood (Gogtay et al., 2004).

Here we studied a possible interplay between the effects of stress during the prepubertal period and the chronic access to a diet rich in calories on behavior, and on neurochemical aspects in prefrontal cortex in adulthood that could result from changes in early programming of central nervous system induced by early exposure to these factors. Thus, our aim was to evaluate whether the stress by social isolation in the prepubertal period and chronic high fat diet (HFD) intake induce memory impairments in male adult rats. Additionally, we analyzed neurochemical markers in the prefrontal cortex that could be related to the behavior alterations observed: BDNF levels and some markers of its signaling pathway (TrkB receptor, AKT, MAPK, phospho-AKT, and phospho-MAPK), Na^+,K^+-ATPase activity, and glucocorticoid receptors. Due to the effects of HFD on the stress response, our hypothesis was that these factors would interact, changing the outcome that is observed when they are applied alone.

2. Material and methods

2.1. Experimental subjects

All animal proceedings were performed in strict accordance to the recommendations of the Brazilian Society for Neurosciences (SBNeC) and Brazilian Law on the use of animals (Federal Law 11.794/2008), and were approved by the Institutional Ethical Committee (CEUA-UFRGS #25488). All efforts were made to minimize animal suffering, as well as to reduce the number of animals used. Animals were housed in home cages made of Plexiglas ($65 \times 25 \times 15$ cm) with the floor covered with sawdust, and maintained on a standard 12 h dark/light cycle (lights on between 7:00 h and 19:00 h), temperature of 22 ± 2 °C. On postnatal day (PND) 21, Wistar rats were weaned and only male pups were used in this study. Half of the animals were housed in groups of 4 per cage, the other half were subjected to stress by social isolation (one animal in a smaller home cage, $27 \times 17 \times 12$ cm) (Douglas et al., 2004). Isolation stress was applied during one week, between postnatal days (PND) 21–28. On PND 28, isolated animals were returned to the regular home cages ($65 \times 25 \times 15$ cm) in groups of four. Each group (control or stressed) was subdivided into 2 other groups, according to the diet: (1) receiving standard lab chow (50% carbohydrate, 22% protein and 4% fat) or (2) receiving *ad libitum* both standard chow plus HFD (25% carbohydrate, 28% protein and 42% fat). Therefore, animals from this group could choose from the two diets available. Animals were maintained on these diets until adulthood. Only one animal per litter was used per group. These procedures resulted in four experimental groups: controls receiving standard chow (control-chow); controls receiving standard chow plus high-fat diet (control-HFD); isolated animals receiving standard chow (stressed-chow); and isolated animals receiving standard chow plus HFD (stressed-HFD). The results were quantified by an investigator blind to the experimental group. Beginning on PND 60, the following behavioral tests were performed in different days: Exposure to the open field and novel object recognition task (same animals), and spontaneous alternation (others animals), (see timeline in Fig. 1A). One week after finishing the behavioral tests, each animal was euthanized by decapitation between 1:00PM to 3:00PM, and the brain was removed, dissected on ice, and a coronal slice of the prefrontal cortex was taken (Bregma +3.70 to +2.20), and punched bilaterally at L = 0.6 and V = 4.6 mm (Bakker et al., 2015; Calabrese et al., 2013; Paxinos, 1998). The punches were immediately frozen and stored at −80 °C for further biochemical evaluations.

We have demonstrated in a previous work (Arcego et al., 2014), the animals with both diets access, consumed more HFD compared to standard chow, but this is not reflected in a higher caloric intake (see supplementary material Fig. S1 in the online version at DOI:10.1016/j.ijdevneu.2016.03.001). However, animals with HFD had more abdominal fat, particularly in the stressed group with HFD, which is reflected in higher weight gain in these animals.

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.ijdevneu.2016.03.001.

2.2. High fat diet

The high fat diet (HFD) used in the study was enriched with fat (42%) from lard and soy oil. In addition, this diet contained vitamins and a salt mixture, purified soy protein, methionine, lysine and starch, in according with Arcego et al. (2014).

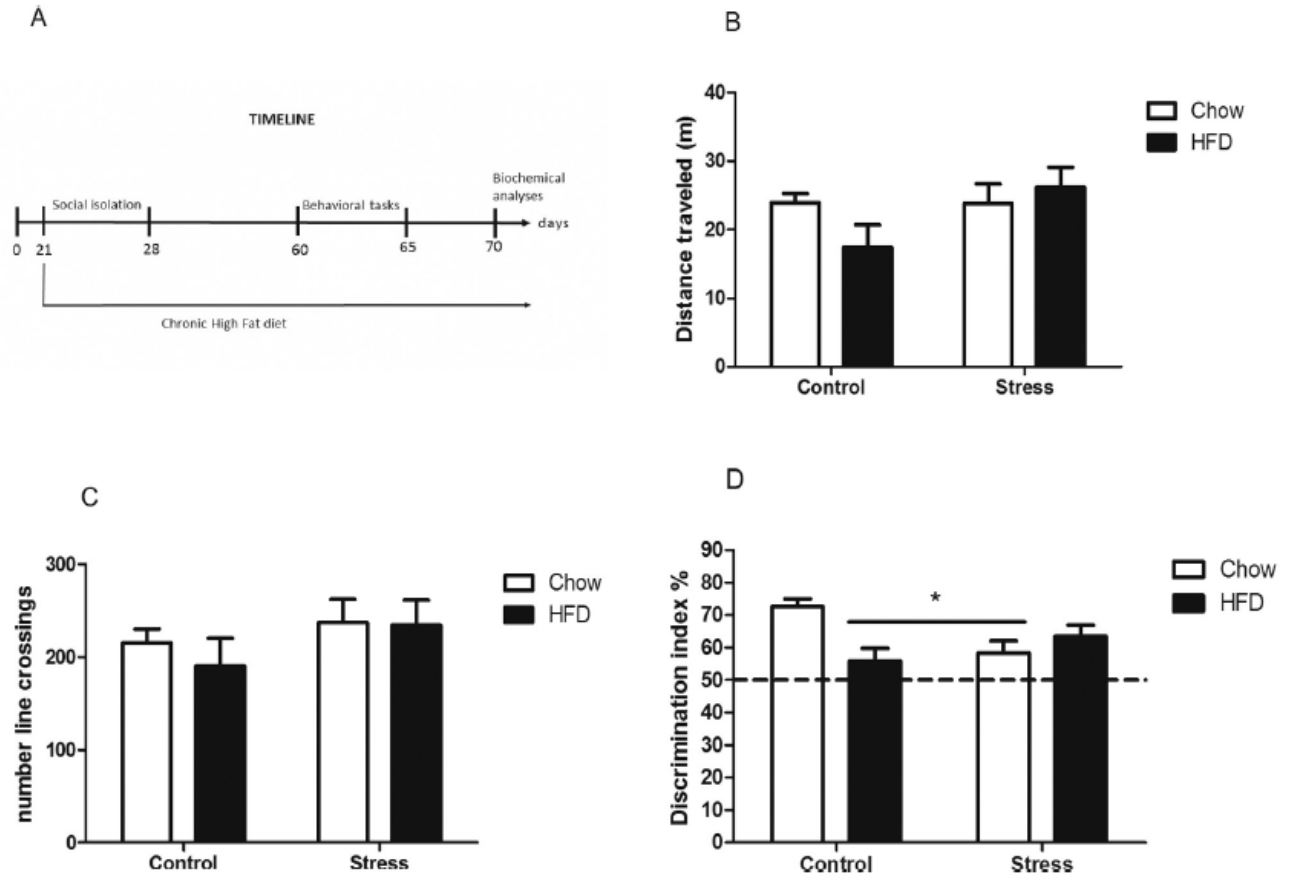

Fig. 1. Effects of social isolation in the prepubertal period, with or without chronic access to high fat diet (HFD), on the performance in an open field (first exposition), and on the discrimination index in novel object recognition in the adulthood (A) Timeline (B) Distance traveled (C) Crossings (D) Discrimination index in novel object recognition. Data are expressed as mean ± SEM. N = 8 for Control-Chow, Control-HFD and Stressed-HFD; N = 10 for Stressed-Chow. No significant differences were found between the groups for distance traveled and crossings ($P > 0.05$). Discrimination index represents the percentage of time spent exploring the novel object. A two-way ANOVA showed an interaction between stress and diet ($P = 0.003$). * different from Control-Chow group (Tukey post hoc test, $P < 0.05$).

2.3. Behavioral tests

Before each behavioral task, rats were placed in the test room (temperature 21 ± 2 °C) for one hour to allow habituation with the environment and researcher. The behavior in the open field, the novel object recognition task, and spontaneous alternation was recorded and analyzed (only open field) using the ANY-Maze video-tracking system (Stoelting, CO). Between each trial, apparatuses were cleaned with ethanol 70%.

2.3.1. Open field exposure

Open field exposure was used to evaluate locomotor activity in a new environment. A 50-cm high, 50 × 50-cm open field was used. The floor was subdivided with white lines into 16 equal 12.5- by 12.5-cm squares, and each animal was gently placed facing the left corner and allowed to explore the arena for 10 min, in two consecutive days. The performance was observed and the distance traveled (m) and crossings were evaluated during the two exposures. The same apparatus was used in the novel object recognition task, and was also considered as a habituation trial for that task.

2.3.2. Novel object recognition task

The novel object recognition task was based on Ennaceur and Delacour (1988) and adapted from the task previously described by Ouchi et al. (2013). This test was used to evaluate the rodents' ability to recognize a novel object in the environment. It consists of 3 phases: habituation, training and testing. In habituation (before training), all animals were exposed to an open-field arena for 10 min without objects during two days. In the training phase (after 24 h the last habituation session), the animal was placed in the open-field arena containing two identical objects, arranged separately (two balls or two towers of Lego® bricks), for 5 min. To prevent coercion to explore the objects, rodents were released against the center of the opposite wall with its back to the objects. Then, animals returned to a separate housing box and one of the objects was changed. After 15 min the animal was again placed in the same arena (test phase) with two objects, one equal to the previously explored objects and one novel object, and it was left to explore them for 5 min. After each trial, objects were cleaned in an attempt to remove any olfactory cues. To prevent preference for a particular object, the objects used as new or familiar ones changed between subjects. The exploration time of the familiar and the new objects was compared. Exploration time was defined as the time when the animal's nose was directed towards the object at a distance of no more than 2 cm and/or touching the object with the nose and paws (Hernandez-Rapp et al., 2015). If the animal climbed on top of the object, this period was not included in the exploration time. The total exploration time of both objects in both trials was calculated. The results are expressed as a % discrimination index, defined as the time spent exploring the novel object/total time exploring objects (familiar + new) × 100. It was taken as a memory index to the task an increase in the exploration time of the novel object compared to the familiar object (Ouchi et al., 2013).

2.3.3. Spontaneous alternation

The spontaneous alternation test in the Y-maze is widely used as a tool for assessing the sensory/attention and cognitive functions like, working memory, in rodents. This task is based on the natural tendency of rodents to alternate successive choices of the Y-maze arms (Hughes, 2004). The apparatus used have three sym-

metrical arms (each arm: 49 cm long × 13 cm wide × 33 cm high), placed in a room illuminated with 40 lux. The spontaneous alternation task was based on Kunisawa et al. (2015).The animal was placed in the middle of the Y-maze and allowed to freely explore the apparatus for 8 min in a single session. The Y-maze had any hint or reward. It is considered alternation when the animal moves to the other two arms without repetition (as ABC, ACB, BAC, CBA, BCA). It was considered an arm entry as the hind paws of the rats being completely within the arm. If the animal remained immobile for more than 2 min was excluded the test. The percentage alternation was calculated by: (number of alternations/total number of arm entries-2) × 100.

2.4. Biochemical analysis

2.4.1. Na^+,K^+-ATPase activity assay

For Na^+,K^+-ATPase activity determination, prefrontal cortex was homogenized in 10 vol (w:v) of 0.32 M sucrose solution containing 5 mM HEPES and 1 mM EDTA, pH 7.4. The homogenates were centrifuged at 1000*g* for 10 min and the supernatants were used. The reaction mixture contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, and 40 mM Tris–HCl buffer, pH 7.4, in a final volume of 200 μl. The reaction started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. Control was assayed under the same conditions with the addition of 1 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays (de Souza Wyse et al., 2000; Tsakiris and Deliconstantinos, 1984). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Enzyme specific activities were expressed as nmol Pi released per minute per milligram of protein [evaluated according to Bradford (1976)]. All assays were performed in duplicate, and the mean was used for statistical analysis.

2.4.2. Brain derived neurotrophic factor (BDNF) assay

Prefrontal cortex BDNF levels were measured by sandwich enzyme-linked immunosorbent assay, using a commercial kit (BDNF Emax® Immunoassay system, Promega, USA). Briefly, prefrontal cortex were homogenized 1:10 in a lysis buffer pH 7.9 containing 137 mM NaCl, 2.5 M KCl, 10 mM Hepes, 0.6 mm EDTA, 1% SDS, 10% glycerol and 1% protease inhibitor cocktail (PIC). Microtiter plates (96-well, flat-bottomed) were coated for 24 h with anti-BDNF, sample and standards were diluted in sample diluent, and ranged from 7.8 to 500 pg of BDNF. Sequential processing of the samples was performed according to manufacturer instructions, and the results were expressed as pg BDNF/mg protein [determined by the method described by Lowry et al. (1951)].

2.4.3. Western blot

Prefrontal cortex was homogenized in ice-cold lysis buffer pH 7.9: 2.5 M KCl, 10 mM Hepes, 0.6 mM EDTA, 0.1% NP 40 and 1% protease inhibitor cocktail (PIC). Equal protein concentrations (40 μg/lane of total protein, determined using a commercial kit BCA Protein Assay [Thermo Scientific, U.S.A]) were loaded onto NuPAGE® 4–12% Bis-Tris Gels. After electrophoresis, proteins were transferred (XCell SureLock® Mini-Cell, Invitrogen) to nitrocellulose membranes (1 h at 50 V in transfer buffer [48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS]) (Valentim et al., 2001). The blot was submitted to 2 h incubation in blocking solution (TBS plus 5% bovine serum albumin). After incubation, the blot was incubated overnight at 4 °C in blocking solution containing one of the following antibodies: anti-TrkB (1:1000, Milipore), anti-glucocorticoid receptor (GR, 1:1000; Sigma), anti-AKT (1:1000, Cell Signaling), anti-phospho-AKT (Ser473, 1:1000, Cell Signaling), anti-MAPK 1/2 (1:1000; Milipore), anti-phospho-p44/42 MAPK (Thr202/Tyr204, 1:1000, Cell Signaling), and anti-β-actin (1:2000, Milipore). The blot was then washed three times for 5 min with T-TBS and incubated for 2 h in solution containing peroxidase-conjugated anti-rabbit IgG (1:1000, Millipore). The blot was again washed four times for 5 min with T-TBS and then left in TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario). The chemiluminescence was detected using a digital imaging system (Image Quant LAS 4000, GE Healthcare Life Sciences) and analyzed using the Image J Software. Results were expressed as the ratio of intensity of the protein of interest to that of 1:2000 anti-β-actin (Milipore) from the same membrane.

2.5. Statistical analysis

Data are expressed as mean ± SEM of the mean, and analyzed using two-way ANOVA, with isolation stress and diet as factors. ANOVA tests were followed by the Tukey multiple range test, when indicated. All analyses were performed using IBM SPSS Statistic 22 software and significance level was set at $P < 0.05$ for all analyses.

3. Results

3.1. Behavioral results

Fig. 1 shows the results from exposure to the open field. No significant differences in number of crossings and distance traveled were observed between the groups in the first (Fig. 1B and C, respectively, $P > 0.05$) and second day (see supplementary material Fig. S2 in the online version at DOI: 10.1016/j.ijdevneu.2016.03.001, $P > 0.05$) of exposure to open field, showing that these animals had no difference in locomotor activity.

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.ijdevneu.2016.03.001.

The novel object recognition task was carried out to assess short-term memory. In the training phase (two identical objects) there was no difference in the time exploring the two objects and there were no differences between groups (see Table 1, $P > 0.05$), consequently all groups explored the two objects by chance. In contrast, in the testing phase (familiar object × novel object), there was an interaction between stress and diet in the time in a new object ($P = 0.01$, $F[1,30] = 7.67$) and in the discrimination index ($P = 0.003$, $F[1,30] = 10.10$, followed by Tukey post-hoc). Groups exposed to stress only or to HFD only had decreased discrimination index, while the group receiving both treatments was not difference from the control-chow group (see Fig. 1D).

The spontaneous alternation in Y-maze was performed for evaluating working memory (Fig. 2). The stressed animals showed a reduced in percentage of spontaneous alternation ($P = 0.048$, $F[1,29] = 4.25$),(Fig. 2A), no interaction stress × HFD, and no HFD effect were observed ($P > 0.05$). There were no differences between the number of entries in the Y-maze arms during the 8-min task ($P > 0.05$ Fig. 2B).

3.2. Biochemical results

Changes in Na^+,K^+-ATPase activity and BDNF are important in learning and memory (Andero et al., 2014; Petzold et al., 2015; Sato et al., 2004). Therefore we evaluated the possible correlation of Na^+,K^+-ATPase activity and BDNF with behavioral findings. There was a significant interaction between stress and diet ($P = 0.001$, $F[1,24] = 13.04$, followed by Tukey post-hoc) on Na^+,K^+-ATPase activity, since the stress, during the prepubertal period reduced Na^+,K^+-ATPase activity in the adult prefrontal cortex, but in stressed animals with access to HFD, this enzyme activity increased (see Fig. 3). The BDNF levels in adult prefrontal cortex, also presented an interaction between stress and diet ($P = 0.048$, $F[1,15] = 4.63$, followed by Tukey post-hoc; see Fig. 4). Levels were reduced with

Table 1
Effects of social isolation in the prepubertal period, with or without chronic access to high fat diet (HFD) in novel object recognition in adult rats.

	Training		Test	
	Time in the object A	Time in the object B	Time in new object	Time in familiar object
Control-Chow	21.66 ± 2.39	23.85 ± 3.12	29.35 ± 3.18	12.12 ± 2.31
Control-HFD	19.47 ± 2.91	24.76 ± 6.40	14.73 ± 3.02	12.08 ± 3.40
Stressed-Chow	22.01 ± 3.64	28.48 ± 3.62	24.25 ± 5.71	16.53 ± 4.51
Stressed-HFD	21.68 ± 2.28	28.44 ± 3.75	36.35 ± 5.77	20.20 ± 3.48

Time in the objects was expressed in seconds. Data are expressed as mean ± SEM, N = 8 for Control-Chow, Control-HFD and Stressed-HFD; N = 10 for Stressed-Chow. There was an interaction between stress and diet in the time in new object in test phase (Two-way ANOVA, P = 0.01).

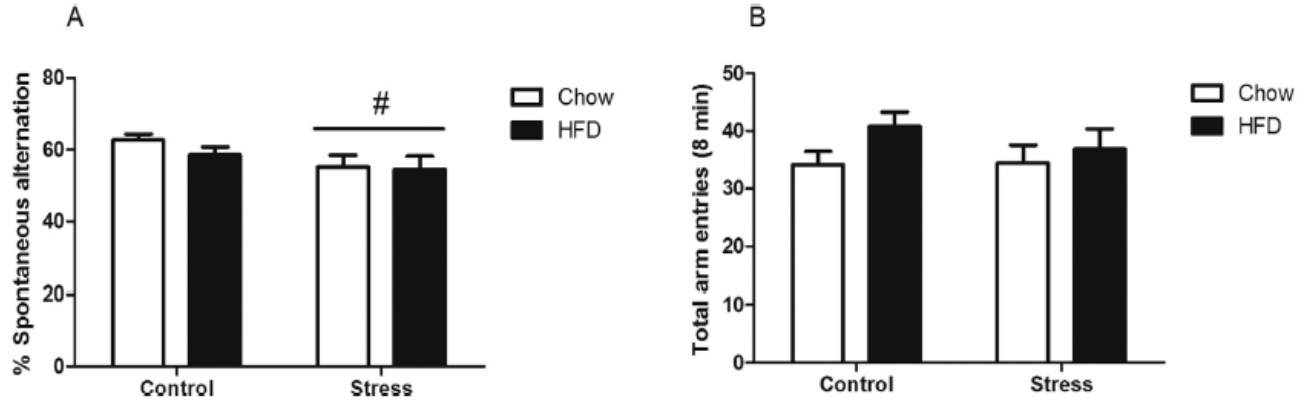

Fig. 2. Effects of social isolation in the prepubertal period, with or without chronic access to high fat diet (HFD), on the spontaneous alternation in adult rats. Data are expressed as mean ± SEM. N = 8 for Control-Chow, Control-HFD and Stressed-HFD; N = 9 for Stressed-Chow. (A) Percentage of spontaneous alternation (B) total number entries. A two-way ANOVA showed an effect of stress (P = 0.048) in percentage of spontaneous alternations and no significant differences were found in total number of entries in arms (P > 0.05). # stressed animals are significantly different from non-stressed animals (two-way ANOVA).

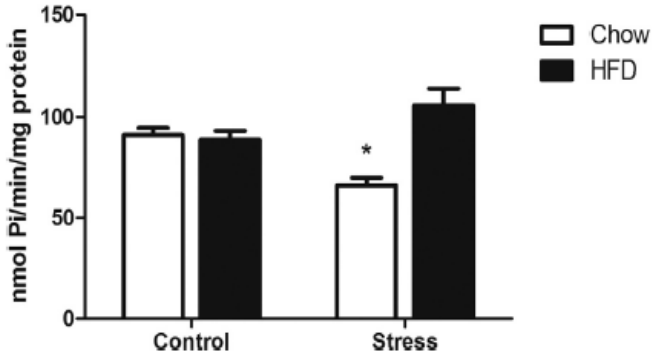

Fig. 3. Effects of social isolation in the prepubertal period, with or without chronic access to high fat diet (HFD), on Na+,K+ATPase activity in prefrontal cortex of adult rats. Data are expressed as mean ± SEM. N = 7 for Control-Chow and Stressed-Chow; N = 6 for Control-HFD; and N = 8 for Stressed-HFD. Two-way ANOVA showed an interaction between stress and diet (P = 0.001). *different from the other groups (Tukey post hoc test, P < 0.05).

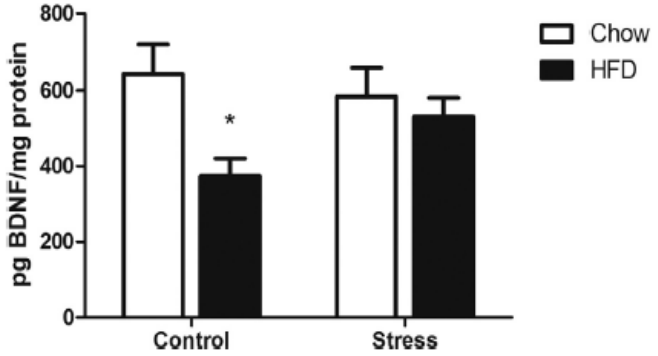

Fig. 4. Effects of social isolation in the prepubertal period, with or without chronic access to high fat diet (HFD), on BDNF levels in prefrontal cortex of adult rats. Data are expressed as mean ± SEM. N = 4 for Control-Chow and Stressed-Chow; N = 6 for Control-HFD; and N = 5 for Stressed-HFD. Two-way ANOVA showed an interaction between stress and diet (P = 0.048). *different from Control-Chow group (Tukey post hoc test, P < 0.05).

HFD; however, in the group exposed to stress and receiving HFD, BDNF levels were not different from the control-chow group.

The immunocontents of the BDNF receptor (TrkB) and the glucocorticoid receptor (GR) in the adult prefrontal cortex were similar for all groups (P > 0.05, see Fig. 5). Since BDNF levels showed an interaction between stress and diet, we evaluated the intracellular cascade, which is activated by the interaction of BDNF with its receptor TrkB. As is displayed in Fig. 6, interactions between stress and HFD were observed both in the immunocontent of the MAPK (P = 0.02, F[1,20] = 6.59, followed by Tukey post-hoc), AKT (P = 0.02, F[1,20] = 6.36), and phospho-AKT (P = 0.01, F[1,21] = 7.62, followed by Tukey post-hoc), since the effects of these factors when isolated were distinct from their effect when both factors were presented simultaneously. The immunocontents of phospho-MAPK, and the ratio phospho-MAPK/MAPK, phospho-AKT/AKT were similar for all groups (P > 0.05, see Fig. 6C, E, and F).

4. Discussion

Emotional and cognitive behaviors are influenced by experiences during sensitive periods of development, like infancy, prepuberty and adolescence. In this study, social isolation in the prepubertal period impaired short-term memory and working memory in adulthood. Animals taking high-fat diet presented impairment only in short-term memory in a novel object discrimination task. However, our results showed that stress and HFD when applied together caused a slight improvement on the performance of short-term memory. This interaction between stress and chronic consumption of HFD was also observed in several of the parameters measured involved in cellular function, such as Na^+,K^+-ATPase activity, or with neural plasticity, as BDNF levels and proteins related to its intracellular cascade. Interestingly, in all cases, the stressed group receiving HFD showed similar results to the control group.

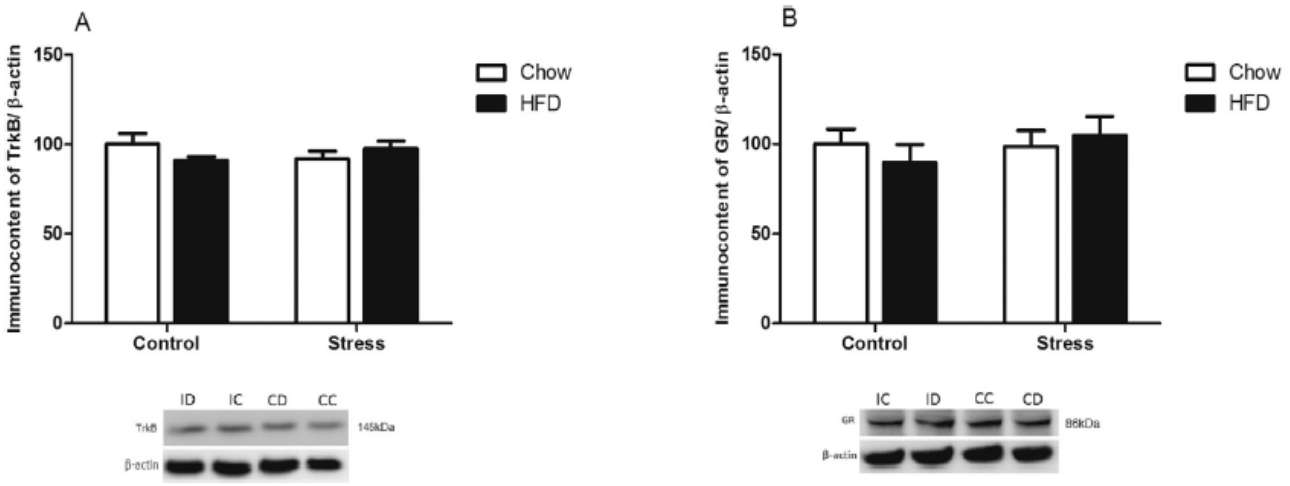

Fig. 5. Effects of social isolation in the prepubertal period, with or without chronic access to high fat diet (HFD), on TrkB and Glucocorticoid receptor evaluated by Western blot, in prefrontal cortex of adult rats. Data are expressed as mean ± SEM. (A) TrkB receptor; N = 6/group. (B) Glucocorticoid receptor; N = 5 for Control-Chow and Stressed-HFD; N = 4 for Control-HFD; and N = 6 for Stressed-Chow. No significant differences were found between the groups for TrkB and Glucocorticoid receptor ($P > 0.05$). CC: Control-Chow; CD: Control-HFD; IC: Stressed-Chow; ID: Stressed-HFD.

According to our findings, events early in life, such as the chronic high caloric diet intake beginning at weaning, or social isolation stress induced impairments in short-term memory in a novel object recognition task. This task is based on the natural tendency of rodents to explore new environments and/or new objects. The novel object recognition task is a non-spatial memory, and it requires a series of cognitive operations, such as perception, discrimination, identification and comparisons (Warburton and Brown, 2015). It is important to note that the behavioral effects observed are not related to altered locomotion, since no differences were observed in the open field exploration. Previous studies using high caloric diet have also reported that its intake may lead to a cognitive deficit and memory impairment, when offered either in a short (Kaczmarczyk et al., 2013) or long-term period (Kosari et al., 2012; Wang et al., 2015). This result could be related to the lower levels of BDNF, which was observed in the present study. BDNF is a neurotrophin involved in neuronal survival, differentiation and synaptic plasticity, which is important for learning and memory processes (Tapia-Arancibia et al., 2004). Other studies have also observed that high caloric diets may reduce BDNF in prefrontal cortex in rodents (Camer et al., 2015; Kanoski et al., 2007). Interestingly, animals receiving HFD only have deficit in novel object recognition, with no significant difference in working memory measured in the spontaneous alternation. It is known that the prefrontal cortex is involved in working memory, observed by differences in neuronal firing patterns recorded in the prefrontal cortex in rodents when perform in working memory task (Fujisawa and Buzsaki, 2011; Goldman-Rakic, 1995; Pfefferbaum et al., 2001). It is possible that these different interventions, such as stress or ingestion of HFD, during development, may affect differently the prefrontal cortex by changing functions involved in different memory process related to this structure. In addition, it is important to observe the two tasks evaluated in the present study use different brain circuits. The role of the rodent medial prefrontal cortex is well recognized in the working memory; however, in object recognition memory, its role has been intensively debated (Gonzalez et al., 2014; Pezze et al., 2015; Warburton and Brown, 2015). Recent studies have suggested that lesions of the medial prefrontal cortex do not produce such severe impairment in this particular task of recognition memory, and that this structure would have an important role in tasks in which integration of object and spatial location information is needed (Barker et al., 2007).

Long-term social stress also has been implicated in cognitive deficits and memory impairment (Gaskin et al., 2014; Jones et al., 2011; McIntosh et al., 2013; Watson et al., 2012). However, we are the first to demonstrate that an early social isolation (one week only) in the prepubertal period leads to a deficit in short-term and working memory in adulthood. It is important to note that this sensitive period of development requires higher energy, since the brain is in a constant process of organizing and of neural maturation. This is true especially for the prefrontal cortex, which has a characteristic late maturation and is sensitive to external factors such as diet and stress (Krolow et al., 2012; Sherman et al., 2014). The present study showed that the effects of stress alone were different in some responses when associated with a chronic HFD, indicating that different environmental interventions in high plasticity periods may program distinct outcomes in adulthood.

The association between stress and HFD also increased Na^+,K^+-ATPase activity in the prefrontal cortex. Na^+,K^+-ATPase activity is necessary to maintain the ionic gradient for neural excitability, plasticity, function and cell survival. Its activity is important for processes related with memory, and, consequently, the reduced activity of this enzyme is associated with memory impairment (Abdalla et al., 2014; Jaques et al., 2013; Wyse et al., 2004). In this study, the increased activity of this pump can be associated with better performance in novel object recognition, but not in spontaneous alternation. When we analyze the effect of social isolation without influence of HFD, we observed a decrease in Na^+,K^+-ATPase activity. This may be associated with a lower neuronal excitability that could be affecting the performance of these animals in the memory tasks evaluated, both of short duration. Since the stress was applied in early life and we observed changes in early adulthood, this finding suggests that stress in the prepubertal period may program the prefrontal cortex of these animals, leading to long-term changes. Moreover, in the groups receiving chronic HFD, both stressed or not, the activity of Na^+,K^+-ATPase was similar to the control group. It is known that modifications in the membrane fatty acid profile will translate into effects in Na^+,K^+-ATPase activity (Rodrigo et al., 2014). It is possible that a chronic administration of a HFD could be causing some alteration in membrane fluidity, changing the activity of this pump.

The most striking observation of the present results was that, when both factors (social isolation during the prepubertal period and access to a high fat diet) were applied together, interactions were observed in several of the parameters evaluated, both behavioral and neurochemical ones, and these interactions always showed that animals isolated in prepubertal period with access to HFD had results which were similar to the control group. This inter-

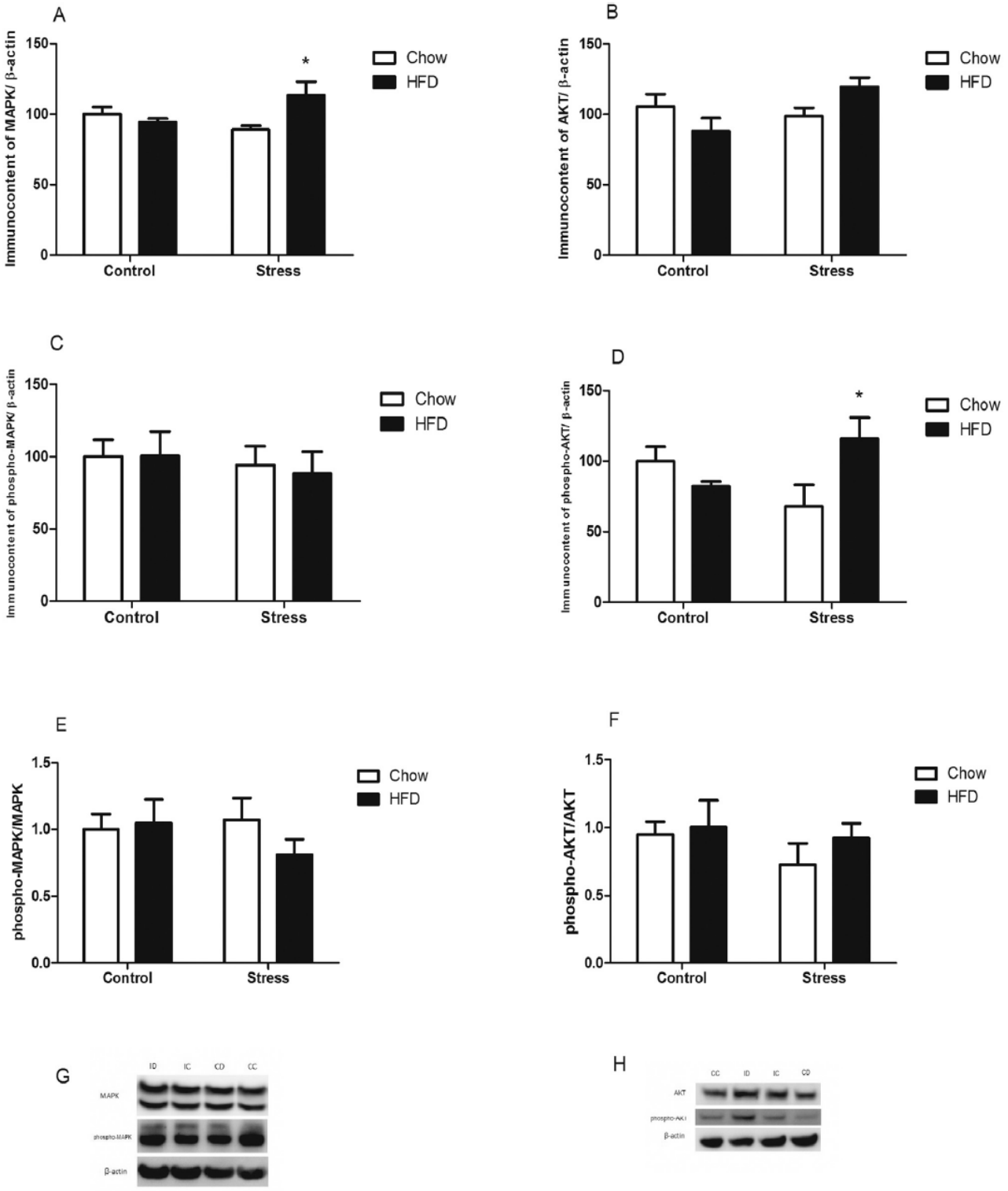

Fig. 6. Effects of social isolation in the prepubertal period, with or without chronic access to high fat diet (HFD), on AKT and MAPK immunocontents (total and phosphorylated forms), evaluated by Western blot, in prefrontal cortex of adult rats. Data are expressed as mean ± SEM. (A) MAPK; N = 6/group. (B) AKT; N = 6/group (C) Phospho-MAPK; N = 6/group (D) Phospho-AKT, N = 6 for Control-Chow; N = 5 for Control-HFD, Stressed-Chow and Stressed-HFD (E) Phospho-MAPK/MAPK, N = 6/group (F) Phospho-AKT/AKT N = 6/group. Two-way ANOVA showed an interaction between stress and diet for MAPK ($P = 0.02$), AKT ($P = 0.02$) and phospho-AKT immunocontents ($P = 0.01$). CC: Control-Chow; CD: Control-HFD; IC: Stressed-Chow; ID: Stressed-HFD. *different from the respective Stressed-Chow group (Tukey post hoc test, $P < 0.05$).

action was observed for Na^+,K^+-ATPase activity, for BDNF levels, as well as for intracellular signaling cascades activated by BDNF. In the case of the mitogen-activated protein/signal-regulated kinase extracellular (MAPK/ERK), an interaction was only observed in its total immunocontent, and not in its phosphorylated form; on the other hand, AKT, a serine/threonine kinase, involved in the signaling pathway of BDNF (Patapoutian and Reichardt, 2001), showed interactions both in its total immunocontent and in its phosphorylated form. Further studies are needed to evaluate whether this pathway is related to the behavioral effects observed.

Another possible explanation for the interaction between stress and access to HFD observed here could involve the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which may be a central mediator between stress and diet effects. Key signaling molecules of the HPA axis, as the glucocorticoid receptor (GR) and corticotrophin-releasing hormone (CRH) receptor, are susceptible

to environment influences. Some studies show that HFD intake alters some mediators of HPA axis, including GR expression, and post-stress plasma corticosterone levels in rats (Abildgaard et al., 2014; Auvinen et al., 2012; Laryea et al., 2015; McNeilly et al., 2015). In our study, however, no significant differences in glucocorticoid receptor (GR) immunocontent in the adult prefrontal cortex were found between groups. In relation to isolation stress, a previous study from our group (Arcego et al., 2014) showed that the adrenal weight was slightly increased in adult animals exposed to social isolation in the prepubertal period. In addition, acute social isolation (72 h) in rodents, increases plasma corticosterone (Kamal et al., 2014), indicating that these animals have an increased in HPA axis activation, thus releasing higher levels of glucocorticoids. In one previous study, we found increased plasma leptin levels in animals receiving HFD, while leptin was decreased in animals exposed to social isolation during the prepubertal period (Arcego et al., 2014). Many components of the HPA axis contain leptin receptors, and some studies have reported that leptin influences the stress responses. For example, leptin treatment blocks the increase in ACTH and corticosterone levels following stress (Heiman et al., 1997), decreases expression of mRNA CRH in the paraventricular hypothalamic nucleus (PVN) (Arvaniti et al., 2001; Huang et al., 1998; Oates et al., 2000), and CRH release from the hypothalamus (Heiman et al., 1997). Thus, leptin appears to act by reducing HPA-responses to stress. It is possible that increased leptin levels in animals receiving HFD could reduce the activation of the HPA axis, and consequently minimize the effects of social isolation. Thereby, this interplay between HFD and hormones related to feeding behavior and stress consequences could contribute to the positive effect observed in this study on short-term memory in isolated animals receiving HFD. This possibility is supported by the theory that highly palatable foods rich in sugar and fats, can act reducing the HPA axis responses, and thereby reducing stress responses (Adam and Epel, 2007).

The present findings show that adverse experiences early in life, such as stress and highly caloric diet intake, may have notably negative effects on cognitive behavior, which may arise during prepuberty and persist into adulthood. The signaling pathway related to BDNF in the prefrontal cortex is sensitive to the effects of early life stress and HFD. However, more studies are needed to elucidate possible mechanisms involved in interplay of these two factors (stress and diet) on brain function. In view of the importance of the prefrontal cortex to the maturation of cognitive and emotional functions, the study of the mechanisms underlying the cognitive changes may help to understand the pathophysiology of some psychiatric disorders.

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Supplementary Material

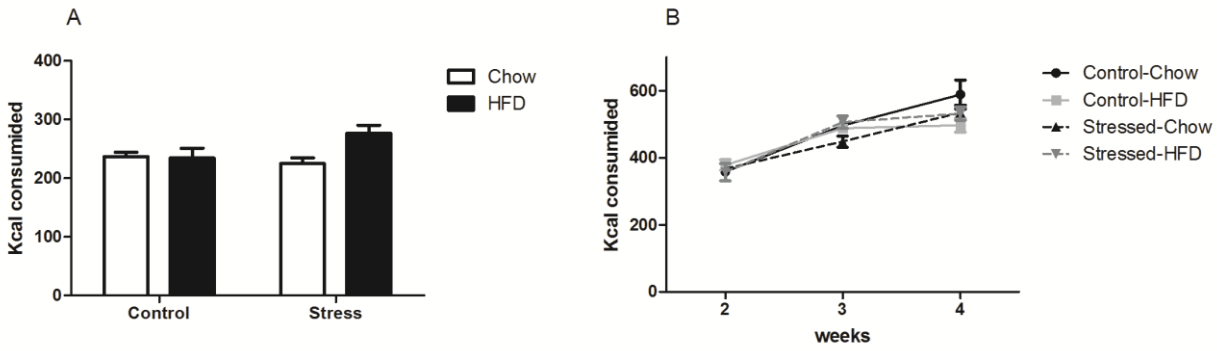

Fig. S1. Effects of social isolation in the prepubertal period, with or without chronic access to high fat diet (HFD), on caloric consumption. Data are expressed as mean ± SEM. (A) Caloric consumption during the period of social isolation (week 1), N = 8 for Control-Chow and Control-HFD and N = 20 for Stressed-Chow and Stressed-HFD. (B) Caloric consumption of diets during 2–4 weeks after the beginning of the treatment, N = 7 for Control-Chow and Control-HFD and N = 5 for Stressed-Chow and Stressed-HFD. No significant differences were found between the groups for caloric consumption ($P > 0.05$).

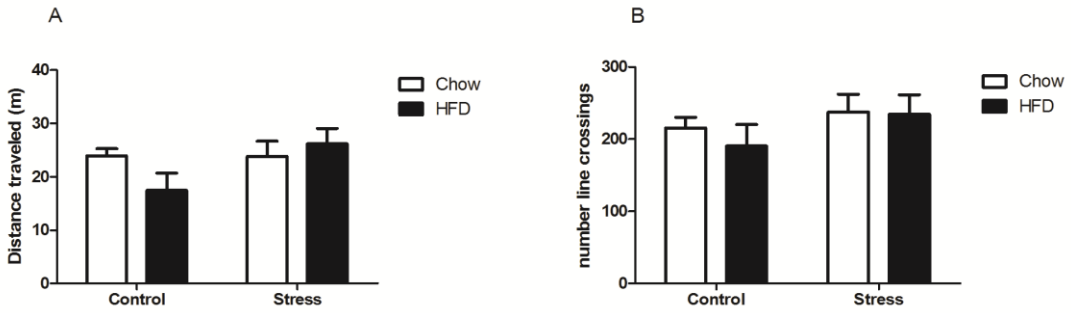

Fig. S2. Effects of social isolation in the prepubertal period, with or without chronic access to high fat diet (HFD), on the performance in an open field in the adulthood (second day). Data are expressed as mean ± SEM. N = 8 for Control-Chow, Control-HFD and Stressed-HFD; N = 10 for Stressed-Chow. (A) Distance traveled (B) Crossings. No significant differences were found between the groups for distance traveled and crossings ($P > 0.05$).

Table 1.

Effects of social isolation in the prepubertal period, with or without chronic access to high fat diet (HFD) in novel object recognition in adult rats.

	Training		Test	
	Time in the object A	Time in the object B	Time in new object	Time in familiar object
Control-Chow	21.66 ± 2.39	23.85 ± 3.12	29.35 ± 3.18	12.12 ± 2.31
Control-HFD	19.47 ± 2.91	24.76 ± 6.40	14.73 ± 3.02	12.08 ± 3.40
Stressed-Chow	22.01 ± 3.64	28.48 ± 3.62	24.25 ± 5.71	16.53 ± 4.51
Stressed-HFD	21.68 ± 2.28	28.44 ± 3.75	36.35 ± 5.77	20.20 ± 3.48

Time in the objects was expressed in seconds. Data are expressed as mean ± SEM, N = 8 for Control-Chow, Control-HFD and Stressed-HFD; N = 10 for Stressed-Chow. There was an interaction between stress and diet in the time in new object in test phase (Two-way ANOVA, P = 0.01).

3.2 Capítulo II

Impact of high-fat diet and early stress on depressive-like behavior and hippocampal plasticity in adult male rats.

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IMPACT OF HIGH-FAT DIET AND EARLY STRESS ON DEPRESSIVE-LIKE BEHAVIOR AND HIPPOCAMPAL PLASTICITY IN ADULT MALE RATS

--Manuscript Draft--

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Article Type:	Original Article
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Abstract:	During development, the brain goes through fundamental processes, including organization of neural networks and plasticity. Environmental interventions may alter initial brain programming, leading to long-lasting effects, and altering the susceptibility to psychopathologies, including depression disorder. It is known that depression is a psychiatric disorder with a high prevalence worldwide, including high rates among adolescents. In this study, we evaluated whether social isolation in the prepubertal period and chronic use of high-fat diet (HFD), may induce depressive-like behavior in male adult rats. We also investigated hippocampal plasticity markers and neurotransmitter systems. We found both social isolation and HFD induced a depressive-like behavior in the forced-swimming task. Moreover, chronic HFD increased anhedonia, as verified by the sucrose preference test, and impaired hippocampal plasticity, demonstrated by reductions in βIII-tubulin (neuronal marker), PSD-95, SNAP-25, and neurotrophin-3. The HFD group also presented decreased glutamatergic and GABAergic receptors subunits. On the other hand, stress affected hippocampal BDNF signaling pathways, and increased expression of subunit of the NMDA receptor (NR2A). Both factors (stress and diet) decreased GR in the hippocampus without affecting plasma corticosterone at basal levels. Interaction between early stress and HFD access was observed only in the TrkB receptor and synaptophysin. In summary, these findings showed that a brief social isolation and chronic HFD, during a sensitive developmental period, cause depressive-like behavior in adulthood. The mechanisms underlying these behavioral effects may reflect hippocampal synaptic activity alterations, but in different ways; HFD consumption appears to affect synaptic plasticity, while social isolation affected BDNF signaling more significantly.

IMPACT OF HIGH-FAT DIET AND EARLY STRESS ON DEPRESSIVE-LIKE BEHAVIOR AND HIPPOCAMPAL PLASTICITY IN ADULT MALE RATS

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ABSTRACT

During development, the brain goes through fundamental processes, including organization of neural networks and plasticity. Environmental interventions may alter initial brain programming, leading to long-lasting effects, and altering the susceptibility to psychopathologies, including depression disorder. It is known that depression is a psychiatric disorder with a high prevalence worldwide, including high rates among adolescents. In this study, we evaluated whether social isolation in the prepubertal period and chronic use of high-fat diet (HFD), may induce depressive-like behavior in male adult rats. We also investigated hippocampal plasticity markers and neurotransmitter systems. We found both social isolation and HFD induced a depressive-like behavior in the forced-swimming task. Moreover, chronic HFD increased anhedonia, as verified by the sucrose preference test, and impaired hippocampal plasticity, demonstrated by reductions in βIII-tubulin (neuronal marker), PSD-95, SNAP-25, and neurotrophin-3. The HFD group also presented decreased glutamatergic and GABAergic receptors subunits. On the other hand, stress affected hippocampal BDNF signaling pathways, and increased expression of subunit of the NMDA receptor (NR2A). Both factors (stress and diet) decreased GR in the hippocampus without affecting plasma corticosterone at basal levels. Interaction between early stress and HFD access was observed only in the TrkB receptor and synaptophysin. In summary, these findings showed that a brief social isolation and chronic HFD, during a sensitive developmental period, cause depressive-like behavior in adulthood. The mechanisms underlying these behavioral effects may reflect hippocampal synaptic activity alterations, but in different ways; HFD consumption appears to affect synaptic plasticity, while social isolation affected BDNF signaling more significantly.

Keywords: early life environment; prepubertal period; social isolation; emotional responses; anhedonia; BDNF.

1. Introduction

Major depressive is a multifactorial illness in which a variety of risk factors and causal pathways are likely to be involved. Depression is today one of the most common psychiatric disorders and epidemiological studies indicate that about half of the episodes of depression occur around adolescence (Kessler *et al.*, 2005). Hypotheses and evidence proposing roles for both vulnerability genes and environmental interventions, especially during developmental periods, in the progress of this disorder have been proposed (Lopizzo *et al.*, 2015). During the initial stages of an individual's life, environmental changes may influence the final process of maturation of the central nervous system's neurochemical circuit (Paus *et al.*, 2008).

Although the pathophysiological mechanisms of depression are not yet sufficiently clear, many theories have been proposed to explain this disorder; a well-characterized hypothesis regarding the neurobiological basis of major depression suggests roles for monoamine deficiency or imbalances between various neurotransmitter systems (Nestler *et al.*, 2002). However, new concepts have been associated with this disorder, including the impaired neuroplasticity and hippocampal neurogenesis associated with reduced neurotrophic factors and alterations in hypothalamus-pituitary-adrenal (HPA) axis control (Duman and Monteggia, 2006, Serafini et al., 2014) .

Epidemiological studies show that most depressed patients have a hyperactive HPA axis, associated with an impairment in HPA axis negative feedback, which is regulated mainly by the glucocorticoid receptor (GR) (Pariante and Miller, 2001). Exposure to stressful events leads to increased release of glucocorticoids (GCs), due to the activation of the hypothalamus-pituitary-adrenal (HPA) axis. Hippocampal GRs mediate the negative feedback of GCs on this axis (Groeneweg et al., 2012, Abildgaard et al., 2014), and stress exposure during the early stages of development, especially stressful psychological events, may induce persistent changes in the HPA's ability to respond to stress during adulthood (Juruena, 2014). This mechanism may lead to an increased susceptibility to depression throughout life (Pariante and Miller, 2001, Juruena, 2014).

Furthermore, in animal models, social stress evokes symptoms that resemble those found in depressed patients (Fuchs and Flugge, 2004). In rodents, social interactions are necessary to ensure good emotional development during growth, and disruption of social interactions between conspecifics is aversive (Hong *et al.*, 2012). Post-weaning long-term social isolation induces a variety of behavioral abnormalities, including depressive-like behaviors (Berry et al., 2012, Haj-Mirzaian et al., 2015). However, less is known about the effects of short-term social isolation, early on in life, on the predisposition to depressive-like behavior during adulthood.

Activity of the HPA axis can also be affected by palatable diet exposure (Buwalda et al., 2001, Abildgaard et al., 2014). Some studies have shown that high fat diet (HFD) intake alters some mediators of the HPA axis, including GR expression, and post-stress plasma corticosterone levels in rats (Abildgaard et al., 2014, Laryea et al., 2015, McNeilly et al., 2015). It should be noted that access to highly caloric diets during sensitive developmental periods may also modify brain maturation, with changes in cognitive and emotional processes during life (Sasaki et al., 2013, Boitard et al., 2015). Therefore, the consumption of these high-calorie diets may increase the probability of developing

depression, as observed in both human (Akbaraly *et al.*, 2009) and animal studies (Abildgaard et al., 2011, Sharma and Fulton, 2013).

Brain structural changes in plasticity, such as loss of synapses and dendritic spines, as well as reduced dendritic branching and reduction of glial cells in the hippocampus, have been associated with depressive disorder (Serafini *et al.*, 2014). These changes may be caused by exposure to environmental factors (such as stress and high caloric diet intake) early on during development. For example, brain-derived neurotrophic factor (BDNF), a neurotrophin involved in neurogenesis and modulation of brain plasticity, decreases after early-life adversities (Choy et al., 2008, Roth and Sweatt, 2011) and has been correlated with the pathophysiology of depression (Polyakova *et al.*, 2015). Additionally, neurotransmitter systems related to synaptic plasticity, such as the GABAergic and glutamatergic systems, can also be altered in mood disorders (Yuksel and Ongur, 2010, Gao et al., 2013). These changes may be associated with early life adversities, since maturation of their connectivity occurs during these important periods (Shikanai *et al.*, 2013).

Since early life interventions during sensitive developmental periods may change the programming of the central nervous system and modify its vulnerability to the development of psychopathologies later in life, our aim in this study was to evaluate whether social isolation stress in the prepubertal period and chronic access to a high-fat diet (HFD) would induce depressive-like behavior in adult male rats. Additionally, we analyzed synaptic markers of plasticity in the hippocampus that may be involved in the behavioral alterations observed. Our hypothesis was that both these factors (HFD and social stress), when applied during sensitive periods of early life, would induce changes in neuronal development, increasing susceptibility to the development of diseases, such as depression, in adulthood, and alter plasticity and neurotransmission in the hippocampus.

2. Material and Methods

2. 1 Experimental subjects

All animal procedures were performed in strict accordance with the recommendations of the Brazilian Society for Neurosciences (SBNeC) and Brazilian Laws on the use of animals (Federal Law 11.794/2008), and were approved by the Institutional Ethical Committee (CEUA-UFRGS #25488). All efforts were made to minimize animal suffering, as well as to reduce the number of animals used. Animals were housed in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust, and maintained on a standard 12h dark/light cycle (lights on between 7:00h and 19:00h), temperature of 22 ± 2°C. On postnatal day (PND) 21, Wistar rats were weaned and only male pups were used in this study. Half of the animals were housed in groups of 4 per cage, the other half were subjected to stress by social isolation (one animal in a smaller home cage, 27x17x12 cm) (Douglas *et al.*, 2004). Isolation stress was applied during one week, between postnatal days (PND) 21-28. On PND 28, isolated animals were returned to the regular home cages (65 x 25 x 15 cm) in groups of four. Each group (control or stressed) was subdivided into 2 other groups, according to their diet: (1) receiving standard lab chow (50% carbohydrate, 22% protein and 4% fat) or (2) receiving both standard chow plus HFD *ad libitum* (25% carbohydrate, 28% protein and 42% fat). Therefore, animals from this group could choose from the

two diets available. Animals were maintained on these diets until adulthood. Only one animal per litter was used per group. These procedures resulted in four experimental groups: controls receiving standard chow (control-chow); controls receiving standard chow plus high-fat diet (control-HFD); isolated animals receiving standard chow (stressed-chow); and isolated animals receiving standard chow plus HFD (stressed-HFD). From PND 60, the following behavioral tests were performed on different days: exposure to the Porsolt forced-swimming test and sucrose preference test (different animals were used for each behavioral task, see timeline in Figure 1A). One week after finishing the behavioral tests, each animal was euthanized by decapitation between 8:00 pm to 10:00 pm, the trunk blood was collected into tubes with heparin to assess basal corticosterone levels, and the brain was removed, dissected on ice, and hippocampi were immediately frozen and stored at -80°C for further biochemical evaluations.

In a previous study (Arcego *et al.*, 2014), we demonstrated that animals that had access to both diets consume more HFD, compared to standard chow, but that this is not reflected in a higher caloric intake. However, animals with access to HFD had more abdominal fat, particularly in the stressed group with HFD, which showed the greatest weight gain.

2.2 High Fat Diet

The high fat diet (HFD) used in this study was enriched with fat (42%) from lard and soy oil. In addition, this diet contained vitamins and a salt mixture, purified soy protein, methionine, lysine and starch, as previously described by Arcego et al. (2014).

2.3 Behavioral tests

Before each behavioral task, rats were placed in the test room (temperature 21±2°C) for one hour to allow habituation in the environment and with the researcher.

2.3.1 Sucrose preference test: This test aims to assess anhedonia-related behavior in animals. On the first day, animals were pre-habituated to a 1% sucrose solution (w/v), with free access to two similar bottles for 24 hours in home cages. On the 2nd day, the animals were deprived of food and water for 7 hours before performing the test. The sucrose preference test was then performed, for which rats were housed in individual cages with free access to two bottles containing (1) 100 ml sucrose solution (1%, w/v) and (2) 100 ml water. The consumption from each bottle was measured after 4 hours of exposure. On the 3rd day, after water and food deprivation for 7 hours, the same procedure was carried out, and the animals received free access to two bottles (1% sucrose and water) for 4 hours. The position of the bottles was changed to avoid a preferential side. Sucrose preference was calculated by the following formula: sucrose consumption ratio for days 2 and 3 = (sucrose consumption, day 2 + day 3)/(total consumption, day 2 + day 3) (adapted from Lee *et al.*, 2013).

2.3.2 Porsolt forced swimming test: This is a test that is widely used to evaluate depressive-like behavior in rodents (Porsolt *et al.*, 1979). The animals were placed individually in Plexiglas cylinders (height of 40 cm, diameter of 18 cm) filled with water at 25±1°C. Two swimming sessions were carried out; one exposure for 15 minutes on the first day (day 1), followed 24h later by a 5-minute exposure (day

2). Following each swimming session, the rats were towel dried and returned to their home cages. The immobility time and the time during which the animal tried to escape from the apparatus were evaluated on both days. The time of immobility was determined when no additional activity was observed, other than the movements necessary to keep the rat's head above the water (Porsolt *et al.*, 1979). Swimming was measured only when the rat showed active swimming movements, e.g., moving around in the cylinder.

2.4 Biochemical analysis

2.4.1 Western Blotting: Hippocampi were homogenized in ice-cold lysis buffer (pH 7.9): 10mM KCl, 10mM Hepes, 0.6mM EDTA, 1% NP 40 and 1% protease inhibitor cocktail and centrifuged at 1000g for 10 min. Equal protein concentrations (40 µg/lane of total protein, determined using a commercial kit BCA Protein Assay [Thermo Scientific, U.S.A]) were loaded onto NuPAGE® 4-12% Bis-Tris Gels. After electrophoresis, proteins were transferred (XCell SureLock® Mini-Cell, Invitrogen) to nitrocellulose membranes (1 h at 50V in transfer buffer [48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% sodium dodecyl sulfate]) (Arcego *et al.*, 2016). The blot was submitted to 2-h incubation in blocking solution (5% bovine serum albumin in Tris-buffered-saline -TBS). After the incubation, the blot was incubated overnight at 4°C in blocking solution containing one of the following antibodies: anti-BDNF (1:1000, Abcam, Cat#ab6201, RRID:AB_305367), anti-TrkB (1:1000, Millipore, Cat#07-225, RRID:AB_310445), anti-glucocorticoid receptor (GR, 1:200; Santa Cruz, Cat#sc-1004, RRID:AB_2155786), anti-AKT (1:1000, Cell Signaling, Cat#4685, RRID:AB_2225340), anti-phospho-AKT (Ser473, 1:1000, Cell Signaling, Cat#4060, RRID:AB_2315049), anti-synaptophysin (SYP, 1:500; Millipore Cat#AB9272, RRID:AB_570874), anti-neurotrophin-3 (NT-3, 1:1000, Abcam, Cat#ab101753, RRID:AB_10710856), anti-synaptosomal-associated protein 25 (SNAP-25, 1:1000, Sigma-Aldrich, Cat#S9684, RRID:AB_261576), anti-spinophilin/neurabin-II (1:1000, Millipore, Cat#06-852, RRID:AB_310266), anti-postsynaptic density protein 95 (PSD-95, 1:000, Cell Signaling Technology, Cat#2507S, RRID:AB_10695259), anti-βIII-tubulin (1:1000, Millipore Cat# MAB1637 RRID:AB_2210524), anti-glial fibrillary acidic protein (GFAP, 1:1000, Thermo Fisher Scientific Cat#MA5-12023, RRID:AB_10984338), Anti-$GABA_A$ receptor (1:500, Santa Cruz, Cat#sc-376282, RRID:AB_10988210), anti-GAD65 (1:1000, Abcam, Cat#ab26113, RRID:AB_448989), anti-AMPA receptor (subunit GlutA2, 1:1000, Millipore, Cat#AB1768, RRID:AB_2313802), anti-NR2A (N-methyl-D-aspartate receptor subunit 2A, 1:1000, Sigma-Aldrich, Cat#M264, RRID:AB_260485), anti-NR2B (N-methyl-D-aspartate receptor subunit 2B, 1:2000, Millipore, Cat#06-600, RRID:AB_310193), anti-phospho-NR2B (Tyr1472, 1:1000, Sigma-Aldrich, Cat#M2442, RRID:AB_262150), or anti-β-actin (1:3000, Millipore, Cat#04-1040, RRID:AB_11214202). The blot was then washed with Tween-TBS and incubated in solution containing peroxidase-conjugated anti-rabbit IgG (1:1000, Millipore, Cat#AP307P, RRID:AB_11212848) or anti-mouse IgG (1:1000, Millipore, Cat#402335-2ML, RRID:AB_10679589), before developing with a chemiluminescence ECL kit (Amersham, Oakville, Ontario). The chemiluminescence was detected using a digital imaging system (Image Quant LAS 4000, GE Healthcare Life Sciences) and analyzed using the Image Studio Lite Software 5.2

(https://www.licor.com/bio/products/software/image_studio_lite, RRID:SCR_014211). Results were expressed as the ratio of the intensity of the protein of interest to that of 1:3000 anti-β-actin (Millipore, Cat#04-1040, RRID:AB_11214202) on the same membrane.

2.4.2 Flow Cytometry: Astrocytes were analyzed by flow cytometry in the hippocampus. For analysis of GFAP markers, hippocampal tissue was dissociated in PBS containing 1% collagenase, 1% DNase, and 0.1% trypsin, and filtered through a 40 μm membrane (Millipore). Fixed cells were re-suspended in PBS containing 2% BSA, 0.1% Triton X-100 and rabbit anti-GFAP antibody (1:250; Thermo Fisher Scientific, Cat#MA5-12023, RRID:AB_10984338), for 30 min at room temperature. Cells were subsequently centrifuged and the resultant pellet washed with cold PBS (pH 7.4). Secondary anti-rabbit Alexa Fluor 488 antibody (1:500, Thermo Fisher Scientific, Cat#A-11008, RRID:AB_143165) was then added and, after incubating for 60 min, fluorescence intensity was determined by flow cytometry. Data acquisition was made using a FACS Calibur cytometry system and Cell Quest software (BD CellQuest Pro, http://www.bdbiosciences.com, RRID:SCR_014489). Data obtained was analyzed with FCS Express 4 Software (FlowJo, http://www.flowjo.com, RRID:SCR_008520).

2.4.3 Corticosterone assay: plasma was obtained as described in 2.1 section. Corticosterone was extracted with ethyl acetate (Couto-Pereira *et al.*, 2016) and analyzed with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Co., USA), following the manufacturer's instructions. Results are expressed as ng corticosterone/ml.

2.5 Statistical Analysis

Data are expressed as means ± SEM of the mean, and analyzed using two-way ANOVA, with isolation stress and diet as factors. ANOVA tests, followed by the Tukey multiple range test, were used when indicated. All analyses were performed using IBM SPSS Statistic 22 software (http://www-01.ibm.com/software/uk/analytics/spss/, RRID:SCR_002865) and the significance level was set at $P < 0.05$ for all analyses.

3. Results

3.1. Early chronic access to HFD reduced preference for sucrose intake

For evaluating behavior related to anhedonia, the sucrose preference test was performed (Figure 1B). The animals that had chronic access to HFD showed a reduced preference for sucrose intake, when compared to controls [$P=0.046$, $F(1,29)=4.33$], on days 2 and 3.

3.2 Early chronic access to HFD and social isolation in prepubertal period induces depressive-like behavior

The Porsolt forced swimming test was performed to assess the emotional responses of animals and evaluate depressive-like behavior (results are shown in Figure 2). When evaluating the total immobility time (during 15 minutes), we observed an increased immobility time upon the first exposition

(day 1) for both stress [P=0.01, F(1,38)=6.69] and chronic HFD exposure [P=0.02, F(1,38)=5.93] groups, with no difference between groups in swimming time (P>0.05). Upon the second exposure (day 2), we observed an increased immobility time [P=0.02, F[1,38]=6.16) and reduced swimming time [P=0.03, F[1,38]=5.34] only in the stressed groups.

3.3 Hippocampal plasticity markers alters after early social isolation and chronic HFD

Since depression has been related to impaired hippocampal neuroplasticity, in association with reduced neurotrophic factors (Pariante and Miller, 2001, Duman and Monteggia, 2006, Serafini et al., 2014), we then evaluated proteins related to neuroplasticity, in the hippocampus, using Western blotting analyses. Hippocampal levels of βIII-tubulin [P=0.003, F(1,17) = 12.05], PSD-95 [P=0.02, F(1,28) = 6.21], SNAP-25 [P=0.007, F(1,31)=8.26], as well as neurotrophin-3 [P=0.02, F(1,20)=6.57], were reduced after chronic HFD access (see Figures 3 and 4D). Additionally, the group stressed during the prepubertal period presented reductions in the immunocontent of phospho-AKT [P=0.03, F(1,21)=5.39] during adulthood (as depicted in Figure 5B).

Significant interactions were observed between stress and HFD and synaptophysin levels [P=0.004, F(1,19)=10.47, followed by Tukey post-hoc], and the TrkB receptor protein [P<0.001, F(1,16)=43.44, followed by Tukey post-hoc], where stress during the prepubertal period and HFD intake caused reductions in these proteins. However, when these factors were associated (stress and diet) increased levels were observed that were similar to those of the control-chow group (Figures 3F and 4C, respectively).

In the other hand, there was no significant difference between groups with regard to the immunocontents of neurabin II, GFAP, BDNF, pro-BDNF, AKT, phospo-AKT/AKT ratio (P>0.05 for all, see Figures 3, 4 and 5). In addition, analyses of the astrocytes by flow cytometry using an anti-GFAP antibody did not indicate any changes between the groups (P>0.05, see Figure 6).

3.4 Hippocampal immunocontents of GR reduced after early social isolation and chronic HFD

Knowing the relationship between depression and activity of the HPA axis, we investigated some related markers. The glucocorticoid receptors immunocontent (GR) decreased in social isolated animals [P=0.025, F(1,19)=5.90] and with chronic access to HFD [P=0.02, F(1,19)=6.46] (see Figure 7A). However, no differences between the groups were observed in plasma corticosterone basal levels (P>0,05, see Figure 7B).

3.5 Hippocampal immunocontents of glutamatergic and GABAergic markers alters after early social isolation and chronic HFD

Reductions in synaptic proteins, such as SNAP-25, and PSD-95, suggested a decreased synaptic function, and could lead to altered levels of receptors. After chronic HFD access, we observed a reduction in the enzyme responsible for GABA synthesis, GAD-65 [P=0.02, F(1,33)=5.74], as well as, in the GlutA2 subunit of the AMPA receptor [P=0.008, F(1,21)=8.74], and the phospho-NR2B subunit of the NMDA receptor [P=0.02, F(1,18)=6.53] (see Figures 8 and 9). The group that was stressed during the prepubertal period presented increased of NR2A subunit of NMDA receptor [P=0.04, F(1,21)=4.64]

during adulthood (see Figure 9B). Further, there was no significant difference between groups in the $GABA_A$ receptors, and NR2B subunits of NMDA receptor immunocontents ($P>0.05$, see Figures 8A and 9C, respectively).

4. Discussion

Maturation and plasticity of specific brain regions are influenced by experiences during sensitive periods of development, such as infancy, prepuberty and adolescence, and these early interventions may be related to susceptibility to the development of psychiatric disorders, such as depression, later on in life. In this study, both social isolation stress in the prepubertal period and exposure to a chronic high-fat diet induced depressive-like behaviors in adult male rats. Additionally, alterations in synaptic markers and in some neurotransmission systems, such as the glutamatergic and GABAergic systems, were found in the hippocampus of adult rats in response to these factors.

We herein used the forced swimming task to assess signs of depressive-like behavior (Bogdanova *et al.*, 2013). Exposure to both stress in the prepubertal period and to chronic HFD intake led to increased immobility times in the forced swimming task (FST). This behavioral response is considered to be an indicator of despair and depressive-like behavior (Bogdanova *et al.*, 2013). Additionally, these effects cannot be attributed to locomotor activity changes, since a previous study from our group reported no differences in locomotion (as evaluated by exposure to the open field) between the experimental groups, when using the same experimental protocol (Arcego *et al.*, 2016). It is important to note that animals with access to HFD displayed an increase in immobility time only during their first exposure to forced swimming, while the stressed animals presented increased floating behavior for two days. In cases where rats have undergone prior manipulations (as used during the exposure to stress and a high-calorie diet, employed herein), other authors have recommended that behavior be estimated during the first two sessions (Slattery and Cryan, 2012, Bogdanova et al., 2013), as exposure to these environmental factors could potentially alter their baseline activity (Slattery and Cryan, 2012). Alternatively, it has been proposed that increased immobility during the second FST session may be interpreted as a learning process based on the animal's previous experience in the same environment, and could be a coping strategy for successful behavior that employs energy conservation (Borsini and Meli, 1988, West, 1990). Considering the reasons listed above, we believe that the immobility time during the first exposure to forced swimming test is a better parameter for evaluating depressive-like behavior in these animals. Previous reports have suggested that exposure to chronic social isolation during different periods of development leads to depressive-like behavior (Hong et al., 2015, Ieraci et al., 2016); accordingly, the present study showed that social isolation, applied during the prepubertal period for just one week, also affected animals' emotional behavior during adulthood.

With regard to HFD consumption from the prepubertal period, the depressive-like behavior presented by these animals is supported by findings from the sucrose preference test. In this task, these animals showed increased anhedonistic behavior. It is known that one of the notable symptoms in the majority of depressed patients is the inability to feel pleasure from positive incentives (Grillo, 2016, Rizvi et al., 2016). In addition, these symptoms of anhedonia are also found in animal models of depression

(Willner, 1997). Literature reports have suggested that the overconsumption of caloric-dense foods contributes to overweight and obesity, in association with mood disorders (Sharma and Fulton, 2013), which corroborates with our findings.

An important role for hippocampal formation in the development of major depression has been well documented (Sheline et al., 1996, Kempermann, 2002). Studies in humans have shown a decrease in hippocampal volume in depressive patients (Sheline *et al.*, 1996) that is also observed in rodents (Veena *et al.*, 2011). This hippocampal atrophy that is associated with depression may lead to a reduction in brain plasticity and a decrease in neurotrophic factors (Duman and Monteggia, 2006). A variety of studies have associated decreased levels of an important neurotrophin, brain-derived neurotrophic factor (BDNF), with a higher incidence of depression, considering BDNF as a possible marker for this condition (Polyakova *et al.*, 2015). BDNF is essential for neurite outgrowth, cell survival and synaptic strengthening (Lu *et al.*, 2005). In this study, no significant differences were found between the groups for the hippocampal imunnocontents of pro and mature forms of BDNF. However, a decrease in the BDNF receptor was observed in stressed animals and in animals with chronic access to HFD. Decreased TrkB levels may be associated with a lower effect of BDNF in these animals. In contrast, when the two factors are applied together (diet and stress), an increase in the levels of this receptor was observed compared to stressed-chow and control-HFD groups. This increase, however, does not imply an increase in its activation, since these animals had decreased levels of phosphorylated Akt, suggesting a reduction in the BDNF signaling cascades, in this case, the phosphatidylinositol 3-kinase PI3K/AKT pathway. Thus, a decrease in both BDNF receptor immunocontent (TrkB) and one of its activation pathways (phosphorylated AKT) could be related to the depressive-like behavior observed, particularly in stressed animals. In contrast, a decrease in neurotrophin-3 (NT-3), another important brain neurotrophin, was observed in animals with chronic access to HFD. This neurotrophin belongs to the same family as BDNF, and also has an impact on adult synaptic plasticity, including neurite development, maintenance and establishment of synaptic connections (Gomez-Palacio-Schjetnan and Escobar, 2013). As such, the depressive-like behavior observed in the animals with access to HFD may be more related to a decrease in NT-3 immunocontent than in BDNF activation pathways.

There is preclinical evidence that stress and glucocorticoids negatively affect hippocampal neuroplasticity, and neuronal and glial survival (Czeh and Lucassen, 2007, Pittenger and Duman, 2008), and may contribute to the onset of psychiatric disorders, including depression. Moreover, one major finding in depression is a dysregulation of the HPA axis, since the majority of depressed patients exhibit HPA axis hyperactivity (Pariante and Miller, 2001), often associated with abnormal GR expression and function (Pariante, 2009, Anacker et al., 2011). Here, we found decreased GR immunocontent in the hippocampus of both the stressed and HFD intake groups (separate effects), however the basal levels of plasma corticosterone were not affected. The absence of alterations on basal corticosterone does not necessarily imply absence of effects of these factors on HPA axis functioning, since these measurements were performed in a basal situation, and the results may be different in response to stress exposure: the rats may have an altered response to acute stress, with plasma corticosterone levels returning to baseline differently from controls after an acute insult, with a less efficient glucocorticoid negative feedback due to the decreased levels of glucocorticoid receptors we observed in the hippocampus. In addition, other

types of depressive disorder do not have hyperactive HPA axis, such as atypical depression (related to lethargy, fatigue and sense of disconnectedness), that is more associated to hypoactive HPA axis (Gold and Chrousos, 2002, O'Keane et al., 2012).

Although the pathophysiological mechanisms of depression are not completely understood, this disorder has also been associated with impaired neuroplasticity and hippocampal neurogenesis (Kempermann and Kronenberg, 2003, Serafini et al., 2014). An important synaptic marker is synaptophysin, a synaptic vesicle-associated protein, commonly used as an estimate of the number of functional synapses involved in neurotransmission (Valtorta *et al.*, 2004). We found a decrease in the immunocontent of this marker in stressed animals and in animals with access to HFD. However, when the two factors were associated, synaptophysin immunocontent remained similar to that of the control group. Although the isolated plus HFD group appears to be more protected against synaptophysin depletion, the depressive-like behavior was not reversed in these animals.

The HFD intake group presented alterations in several synaptic plasticity markers in the hippocampus. This group showed a decreased immunocontent of βIII-tubulin (a microtubule element expressed exclusively in neurons), and appears to present a reduction in synaptic function, as observed by reduced SNAP-25 (protein associated with synaptic vesicles that participates in the regulation of synaptic vesicle exocytosis) and PSD-95 (post-synaptic density marker). On the other hand, no changes in a dendritic spines marker (Neurabin II) and astrocytes (GFAP) were observed. Alterations in synaptic activity lead to modifications in the post synaptic density (PSD) composition, including changes in the levels of receptors, scaffolding and signaling proteins (Keith and El-Husseini, 2008). In excitatory glutamatergic synapses, the major scaffolding molecule localized at the PSD is the PSD-95. Alterations in the amount of PSD-95 influence the recruitment of AMPA receptors at the synapse, thereby modulating synaptic strength (El-Husseini et al., 2000, Schnell et al., 2002). Therefore, the modulation of AMPA receptor retention at the synapse by PSD-95 may also regulate synaptic plasticity (Keith and El-Husseini, 2008). Stimulations that induce long-term depression (LTD) lead to both PSD-95 reduction and a net loss of AMPA receptors from the postsynaptic membrane (Bredt and Nicoll, 2003). Accordingly, we found decreased AMPA (subunit GluA2) immunocontent in animals with access to HFD, possibly due to a reduction in PSD-95. Additionally, these animals demonstrate a decrease in the phosphorylation of NDMA receptor subunits (phospho-NR2B), suggesting that glutamatergic neurotransmission may be impaired in this group. This impairment in glutamatergic neurotransmission in the hippocampus may be associated with the reduction in NT-3 found in those animals receiving chronic HFD, as NT-3 appears to be involved in increased glutamatergic currents *in vitro* (Paul *et al.*, 2001). In addition, activation of the NT-3 receptor (TrkC) has been shown to regulate long-term potentiation, through its activation pathways (PI 3-kinase and PLC-γ pathways), which culminate in the activation of the synthesis of some proteins, therefore regulating synaptic connections and modulating plasticity (Je et al., 2005, Je et al., 2006), in turn contributing to the synaptic protein disruptions found in this group.

In contrast, the group subjected to social isolation during the prepubertal period showed an increase in the NR2A subunit of the NMDA receptor. In agreement with these findings, studies in both humans (Frye et al., 2007, Yuksel and Ongur, 2010) and animals (Padovan and Guimaraes, 2004) have suggested an association between glutamatergic system changes and the pathophysiology of depression.

The strongest support for this hypothesis came from pharmacological studies with NMDA receptor blockers, which lead to antidepressant-like effects in animal models of depression (Przegalinski et al., 1997, Padovan and Guimaraes, 2004). Moreover, the changes observed in the composition of glutamatergic receptors, caused by exposure to stress and chronic diet, may lead to differences in hippocampal synaptic plasticity (Erreger *et al.*, 2005).

Among the theories of mechanisms of depression, GABAergic dysfunction has been proposed to play a role in this pathophysiology; where a reduction in GABAergic function has been reported in mood disorder patients (Karolewicz et al., 2010, Gao et al., 2013), and animal models of depression (Holm et al., 2011, Guidotti et al., 2012). In animals with access to HFD, we found a decrease in the immunocontent of glutamic acid decarboxylase (GAD65), the enzyme responsible for the synthesis of GABA, and no change in the $GABA_A$ receptor in the hippocampus. It could be assumed that a decrease in GAD65 immunocontent in these animals would lead to a reduction in GABA levels, and thus to a decrease in inhibitory neurotransmission provided by GABA. The hypothesis of reduced GABAergic activity in mood disorders may complement the monoaminergic theories, indicating that the balance between multiple neurotransmitter systems may be altered in these disorders.

The present findings show that early experiences, such as stress and highly caloric diet intake, may augment the predisposition to depression development and change neurochemical markers in the hippocampus. These changes may arise during pre-puberty and persist into adulthood. These two factors appear to increase susceptibility to depression through different mechanisms, and they may also interact in this regard. The signaling pathway related to BDNF in the hippocampus is more sensitive to the effects of early life stress; furthermore, an increase in glutamatergic neurotransmission appears to increase in this group. In contrast, chronic HFD intake decreased important synaptic markers, suggesting that a HFD leads to reduced glutamatergic and GABAergic neurotransmission. Additionally, both factors (stress and diet) may affect the HPA axis response to stress (although not affecting basal corticosterone levels) (see Figure 10 for summary of mainly results). Despite the differences in the altered neurochemical markers observed in the hippocampus of animals exposed to the different environmental factors studied here, our results further support the hypothesis that interventions that occur early in life, continuous or not, can permanently alter the brain of individuals. Additionally, the hippocampus is susceptible to environmental changes during development. The study of the mechanisms underlying the changes caused by early experiences may help us to understand the pathophysiology of some psychiatric disorders, such as depression.

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Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1 Effects of social isolation during the prepubertal period, with or without chronic access to high fat diet (HFD), on the performance in the sucrose preference test in adulthood. Data are expressed as mean + SEM. N= 8-9/group. **(a)** Timeline **(b)** Ratio of sucrose consumption on days 2 and 3. A two-way ANOVA showed an effect of HFD intake on sucrose consumption ratio (P=0.05). ** HFD groups are significantly different from chow groups (two-way ANOVA)

Figure 2 Effects of social isolation during the prepubertal period, with or without chronic access to a high fat diet (HFD), on the performance in the forced-swimming test in adulthood. Data are expressed as mean + SEM. N= 10-12/group. **(a)** Immobility time on day 1 **(b)** Swimming time on day 1 **(c)** Immobility time on day 2 **(d)** Swimming time on day 2. A two-way ANOVA showed an effect of HFD on immobility time on day 1 (P=0.02) and an effect of stress on immobility time on day 1 (P=0.01), and day 2 (P=0.02), and on swimming time on day 2 (P=0.03). ** HFD groups are significantly different from chow groups (two-way ANOVA). # stressed animals are significantly different from non-stressed animals (two-way ANOVA)

Figure 3 Effects of social isolation during the prepubertal period, with or without chronic access to high fat diet (HFD), on synaptic and neural cell markers: βIII-tubulin, GFAP, Neurabin-II, PSD-95, SNAP-25 and Synaptophysin (evaluated by Western blot) in the hippocampus of adult rats. Data are expressed as mean + SEM. **(a)** βIII-tubulin (N=5-6/group) **(b)** GFAP (N=5-9/group) **(c)** Neurabin-II (N=5-7/group) **(d)** PSD-95 (6-10/group) **(e)** SNAP-25 (N=7-10/group) **(f)** Synaptophysin (N=5-6/group). CC: Control-Chow; CD: Control-HFD; IC: Stressed-Chow; ID: Stressed-HFD. A two-way ANOVA showed an interaction between stress and diet (P=0.004) in synaptophysin; and an effect of diet on βIII-tubulin (P<0.01), PSD-95 (P=0.02) and SNAP-25 (P<0.01). * Significantly different from control-HFD and stressed-chow groups (Tukey post hoc test). ** HFD groups are significantly different from chow groups (two-way ANOVA)

Figure 4 Effects of social isolation during the prepubertal period, with or without chronic access to high fat diet (HFD), on BDNF, proBDNF, TrkB receptor, and neurotrophin-3 (NT-3) immunocontent (evaluated by Western blot) in the hippocampus of adult rats. Data are expressed as mean + SEM. **(a)** BDNF (N=5-7/group) **(b)** proBDNF (N=5-7/group) **(c)** TrkB receptor (N=5/group) **(d)** NT-3 (N=5-7/group). CC: Control-Chow; CD: Control-HFD; IC: Stressed-Chow; ID: Stressed-HFD. A two-way ANOVA showed an interaction between stress and diet (P<0.001) on the immunocontent of TrkB receptor, and an effect of diet on NT-3 immunocontent (P<0.05). * Significantly different from control-HFD and stressed-chow groups (Tukey post hoc test). ** HFD groups are significantly different from chow groups (two-way ANOVA)

Figure 5 Effects of social isolation during the prepubertal period, with or without chronic access to high fat diet (HFD), on AKT and phospho-AKT, evaluated by Western blot, in the hippocampus of

adult rats. Data are expressed as mean + SEM. **(a)** AKT (N=5-7/group) **(b)** phospho-AKT (Ser473; N=5-7/group) **(c)** phospho-AKT/AKT ratio (N=5-7/group). CC: Control-Chow; CD: Control-HFD; IC: Stressed-Chow; ID: Stressed-HFD. A two-way ANOVA showed an effect of stress on phospho-AKT immunocontent (P=0.03). # stressed animals are significantly different from non-stressed animals (two-way ANOVA)

Figure 6 Effects of social isolation during the prepubertal period, with or without chronic access to high fat diet (HFD), on GFAP (evaluated by Flow Cytometry) in the hippocampus of adult rats. **(a)** Representative dot plot of hippocampal cell subpopulations based on FSC vs SSC. **(b)** Mean fluorescence intensity (MFI) histogram of GFAP-immunolabeled cells. **(c)** Relative fluorescence of GFAP. Data are expressed as mean ± SEM. N=6/group. No significant differences were found between the groups (P>0.05)

Figure 7 Effects of social isolation during the prepubertal period, with or without chronic access to high fat diet (HFD), on glucocorticoid receptor (GR) (evaluated by Western blot) expression in the hippocampus and on plasma corticosterone levels of adult rats. Data are expressed as mean + SEM. **(a)** GR (N=5-7/group). **(b)** plasma corticosterone levels (ng/ml; N=5-7/group). CC: Control-Chow; CD: Control-HFD; IC: Stressed-Chow; ID: Stressed-HFD. A two-way ANOVA showed an effect of diet (P=0.02) and an effect of stress (P=0.02) on GR immunocontent. ** HFD groups are significantly different from chow groups (two-way ANOVA) # stressed animals are significantly different from non-stressed animals (two-way ANOVA)

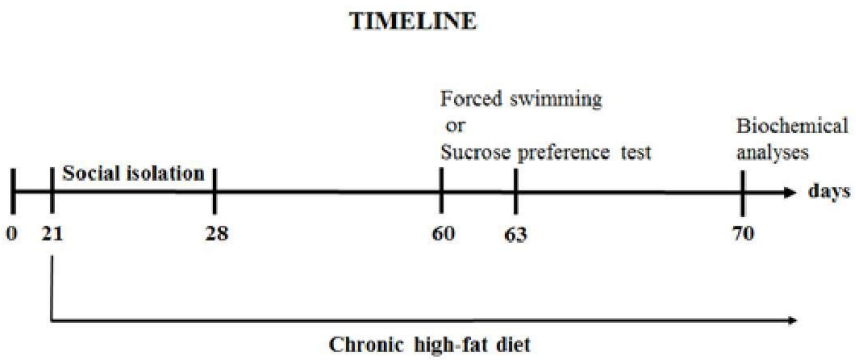
Figure 8 Effects of social isolation during the prepubertal period, with or without chronic access to high fat diet (HFD), on $GABA_A$ receptor and GAD65 (evaluated by Western blot) expression in the hippocampus of adult rats. Data are expressed as mean + SEM. **(a)** $GABA_A$ receptor (N=5-6/group) **(b)** GAD65 (N=8-11/ group). CC: Control-Chow; CD: Control-HFD; IC: Stressed-Chow; ID: Stressed-HFD. A two-way ANOVA showed an effect of diet on GR (P=0.02) and GAD65 (P=0.02); and an effect of stress on GR (P=0.02). ** HFD groups are significantly different from chow groups (two-way ANOVA)

Figure 9 Effects of social isolation during the prepubertal period, with or without chronic access to high fat diet (HFD), on glutamatergic system markers (evaluated by Western blot) in the hippocampus of adult rats. Data are expressed as mean ± SEM. **(a)** AMPA (subunit GlutA2) receptor (N=5-8/group) **(b)** NR2A subunit of NMDA receptor (N=5-7/group) **(c)** NR2B subunit of NMDA receptor (N=5-7/group) **(d)** phospho-NR2B subunit of NMDA receptor (N=5-7/group) **(e)** phospho-NR2B/NR2B ratio (5-7/group). CC: Control-Chow; CD: Control-HFD; IC: Stressed-Chow; ID: Stressed-HFD. A two-way ANOVA showed an effect of diet on AMPA (subunit GlutA2) receptor (P<0.01) and on phospho-NR2B subunit of NMDA receptor (P=0.02); in addition, an effect of stress was shown on the NR2A subunit of the NMDA receptor (P=0.04). ** HFD groups are significantly different from chow groups (two-way ANOVA). # stressed animals are significantly different from non-stressed animals (two-way ANOVA)

Figure 10 Illustrative figure showing the main effects of social isolation during the prepubertal period and chronic HFD access on the hippocampus of adult rats. **(a)** Chronic HFD intake reduced glutamatergic receptors (GlutA2 subunit of AMPA receptor and phospho-NR2B subunit of NMDA receptor) and the GABA synthesizing enzyme (GAD65), possibly leading to decreased glutamatergic and GABAergic neurotransmission. In addition, reductions in other plasticity markers are found in these animals, including proteins associated with synaptic vesicles [SNAP-25 and synaptophysin (however, see interaction HFD x stress)], neurotrophin-3 (possibly also associated with the decrease in the glutamatergic system), PSD-95 and βIII-tubulin. Moreover, these animals showed a reduction in glucocorticoid receptors (GR). **(b)** The effects of social isolation during the prepubertal period; increased expression of the NR2A subunit of the NMDA receptor may be associated with increased glutamatergic neurotransmission. The BDNF pathways appears to be less activated in these animals, as indicated by a reduction in TrkB receptor protein (however, see interaction stress x HFD) and phospho-AKT, which suggests impaired plasticity and cellular growth. Accordingly, decreased synaptophysin was also observed in these animals (however, see interaction stress x HFD), as well as a decline in GR. This figure was created using the images from the Servier Medical Art bank (www.servier.com)

Figure 1

a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Sucrose preference test
Biochemical
analyses
Social isolation
days
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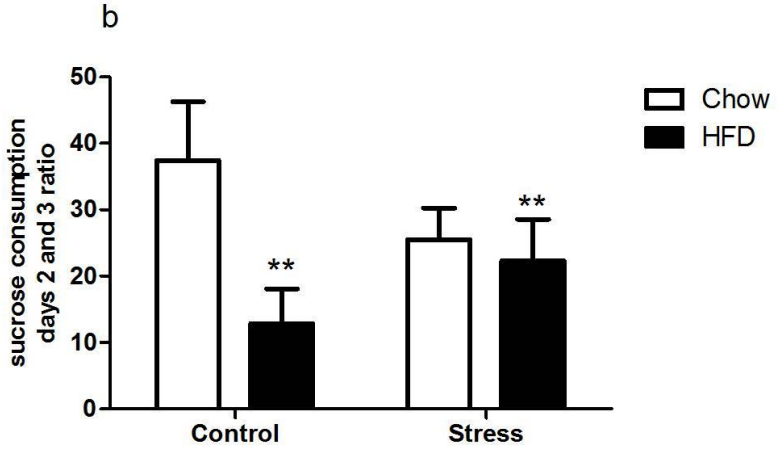
Chronic high-fat diet

b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Stress
Chow
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Figure 2

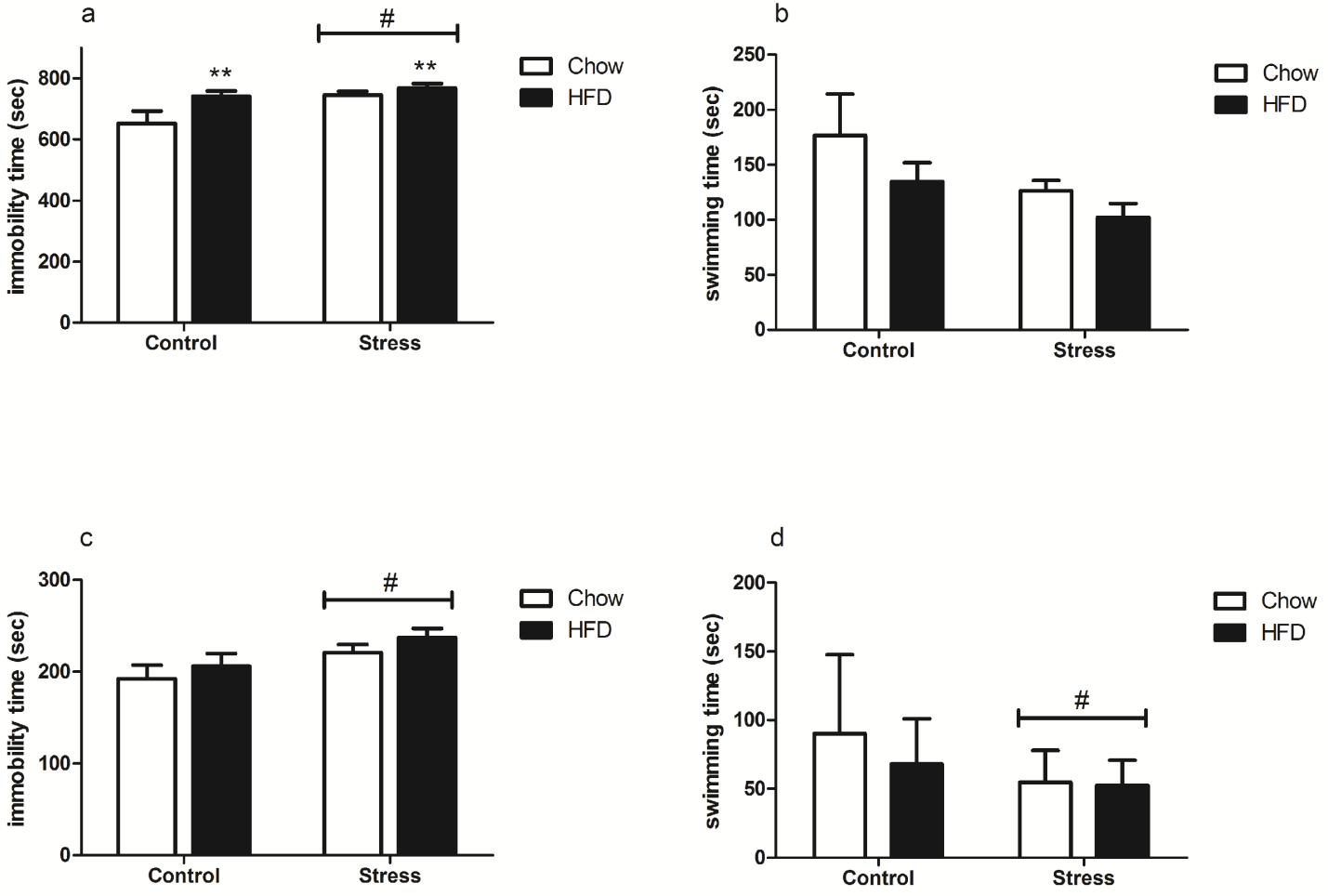
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Figure 3

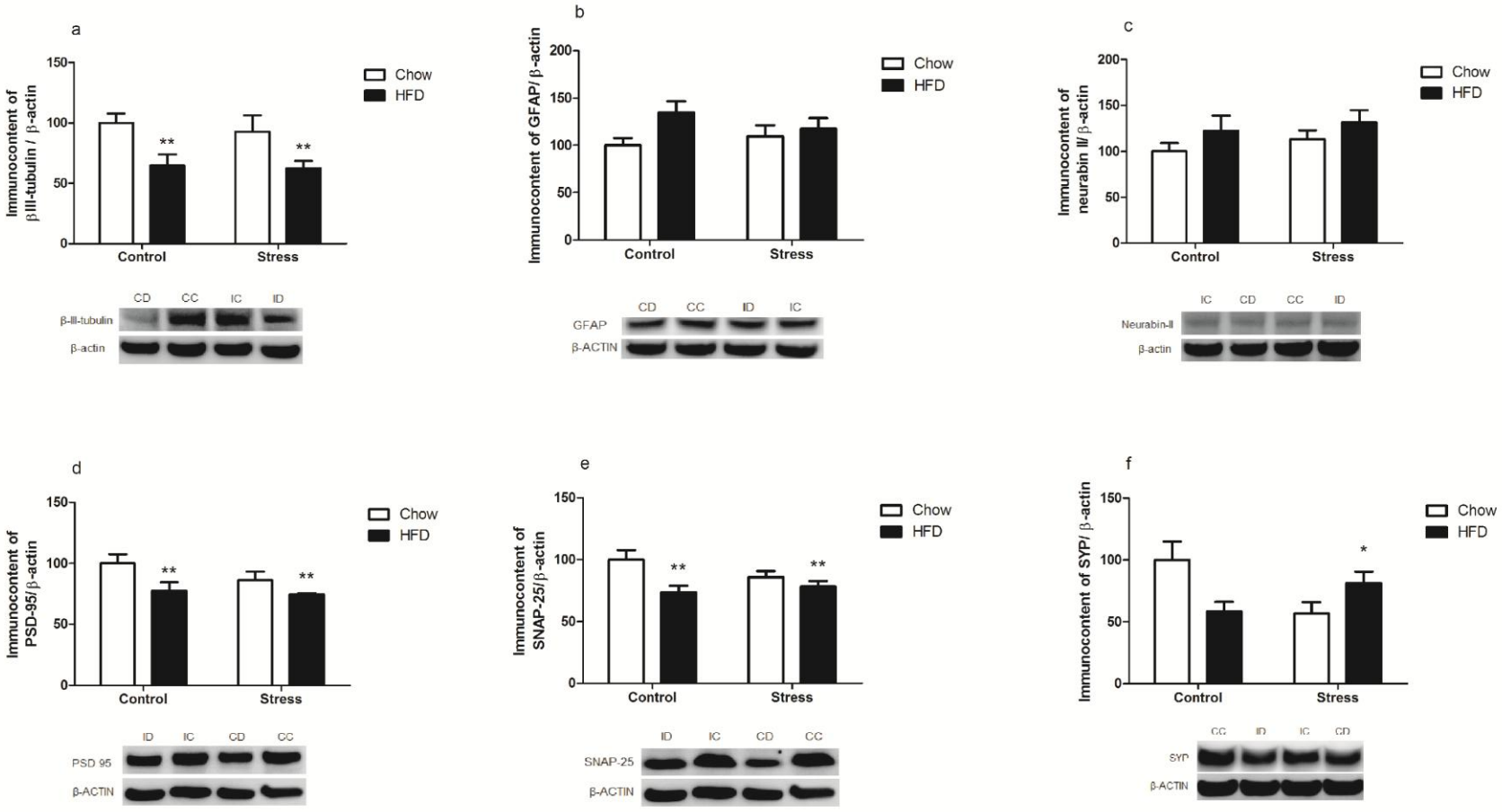
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Figure 4

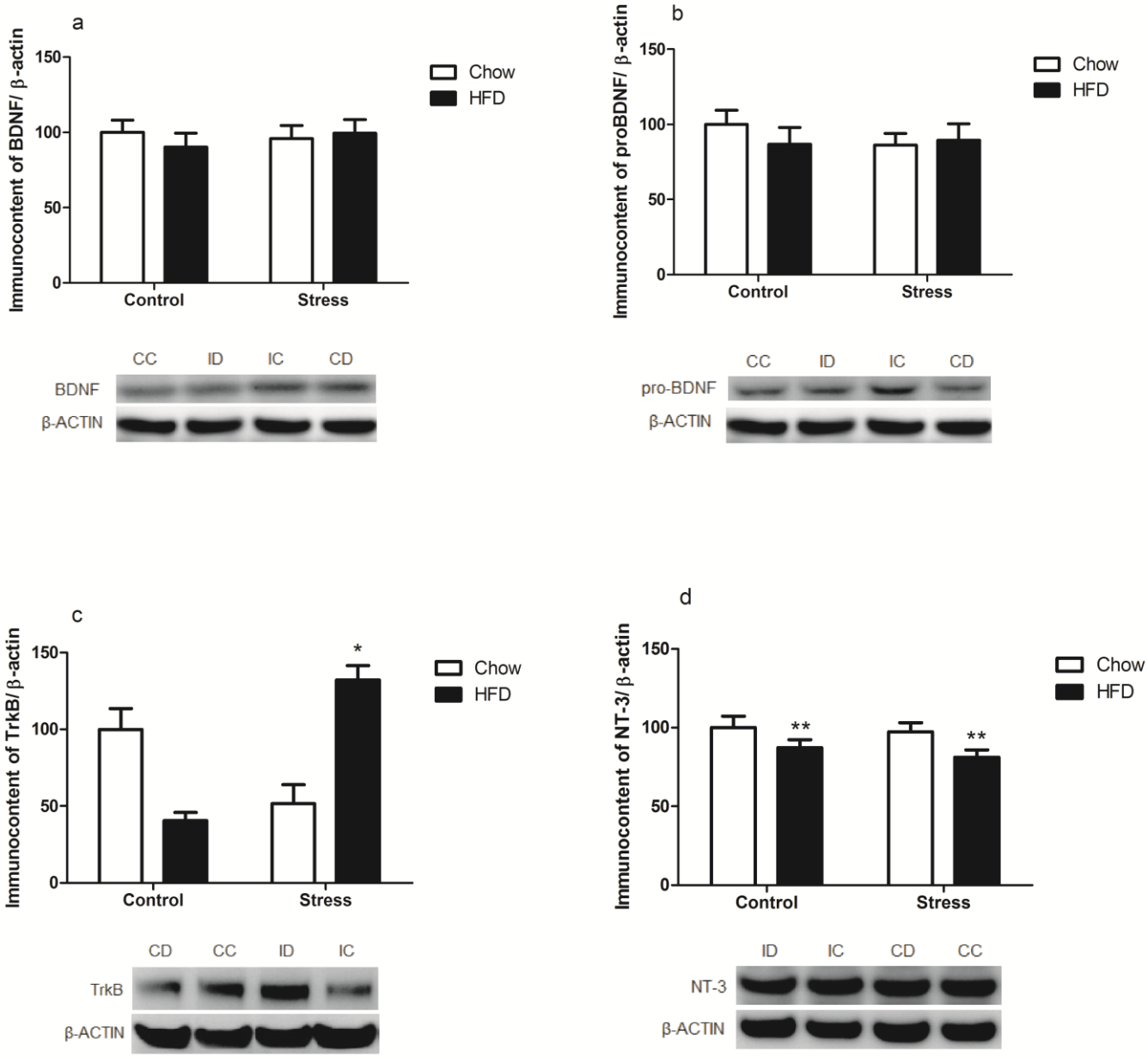
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Figure 5

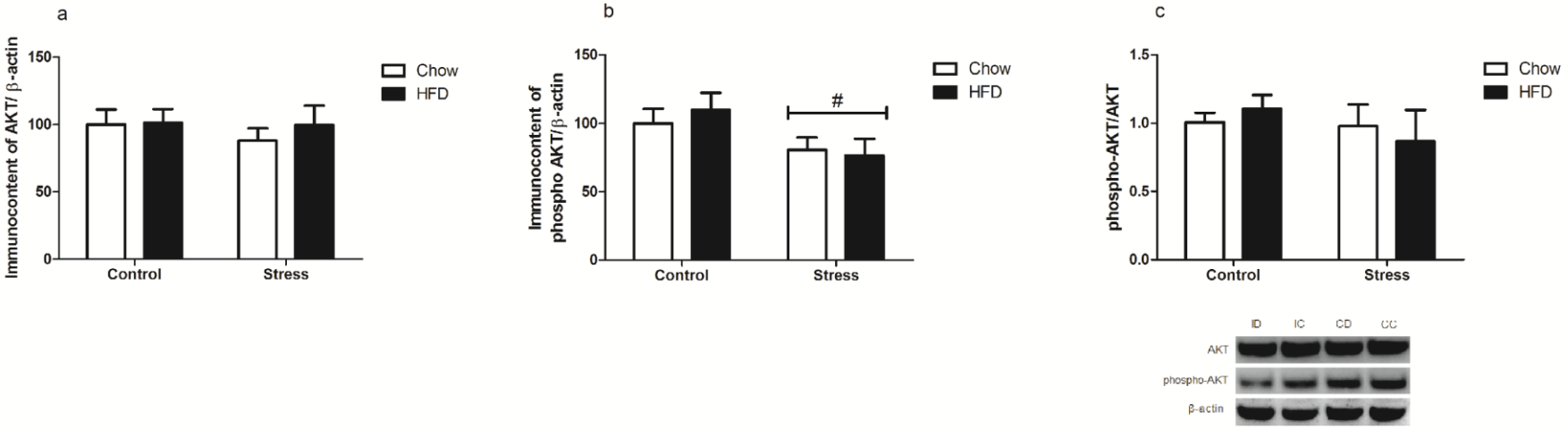
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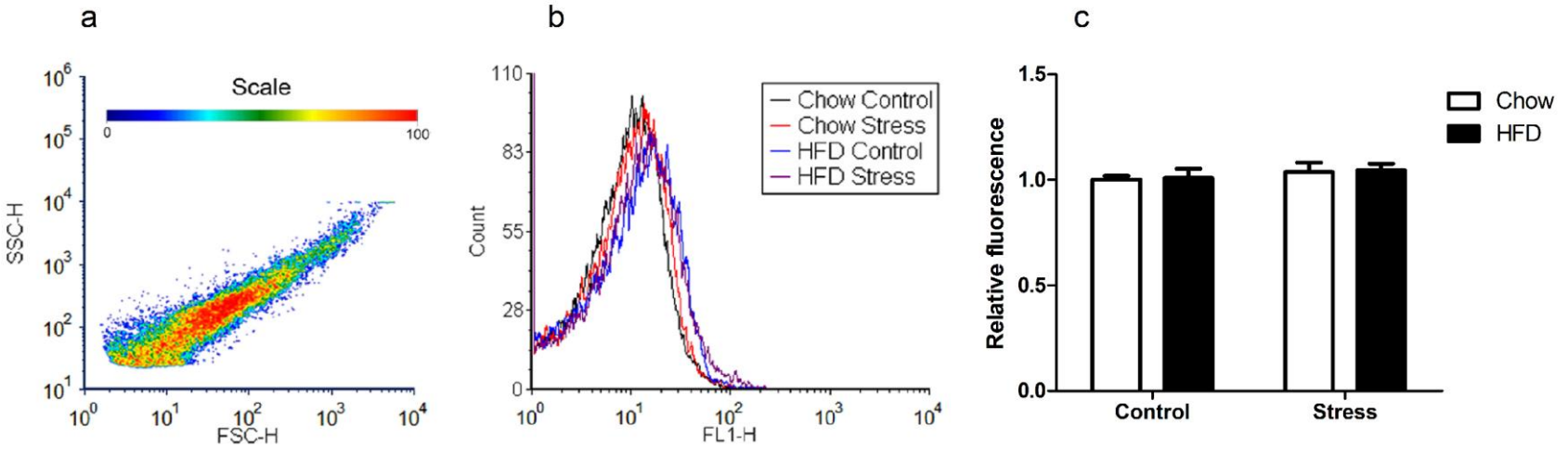
Figure 6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Stress

Figure 7

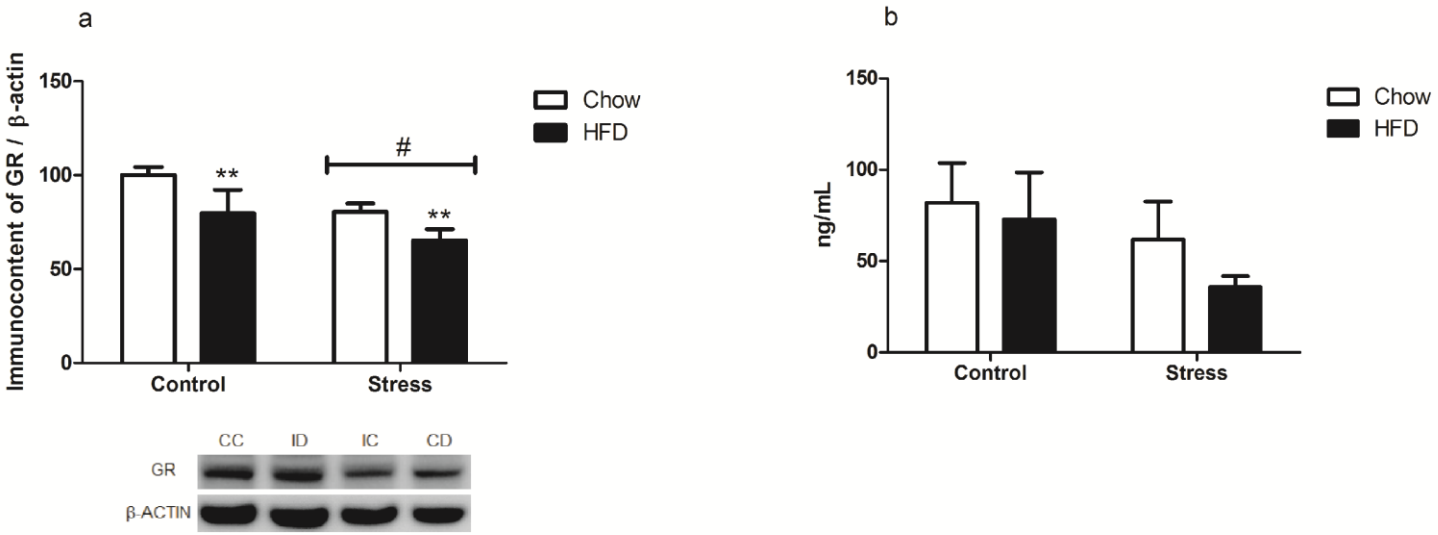
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Figure 8

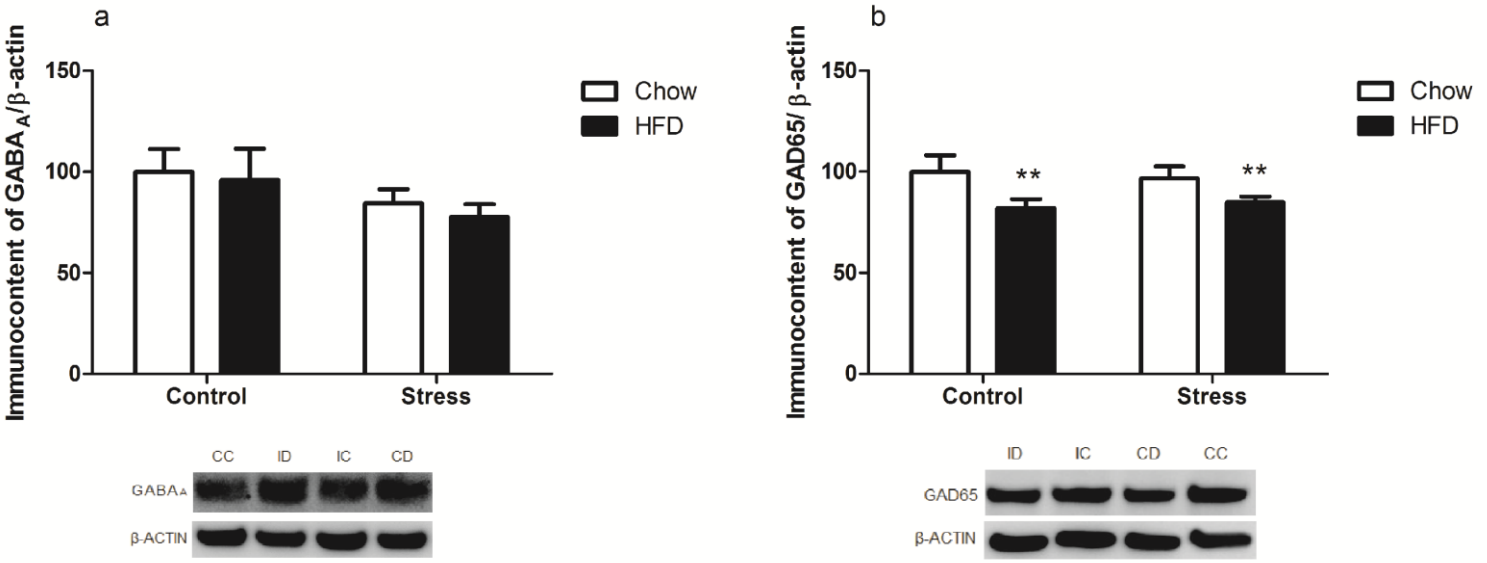
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Figure 9

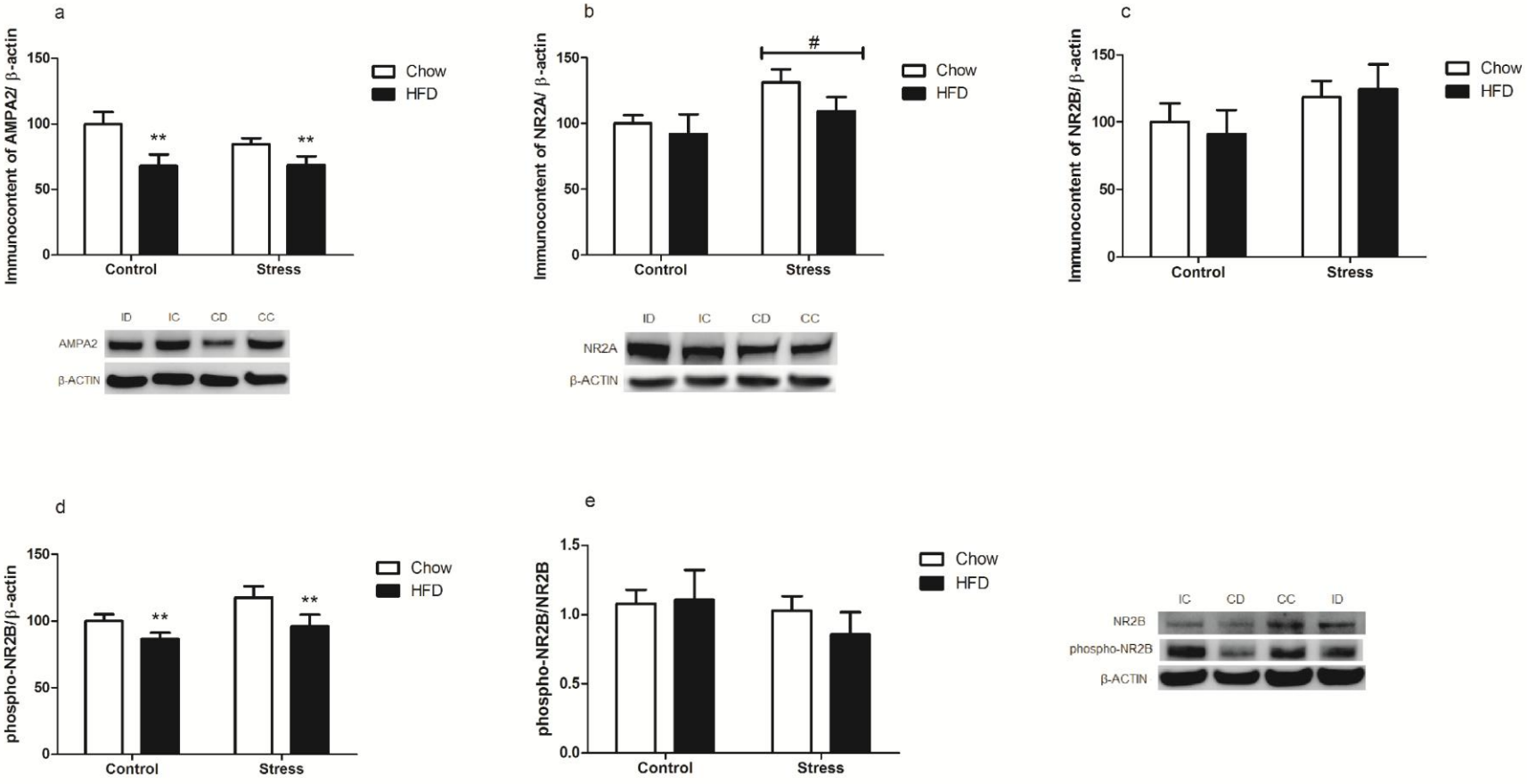
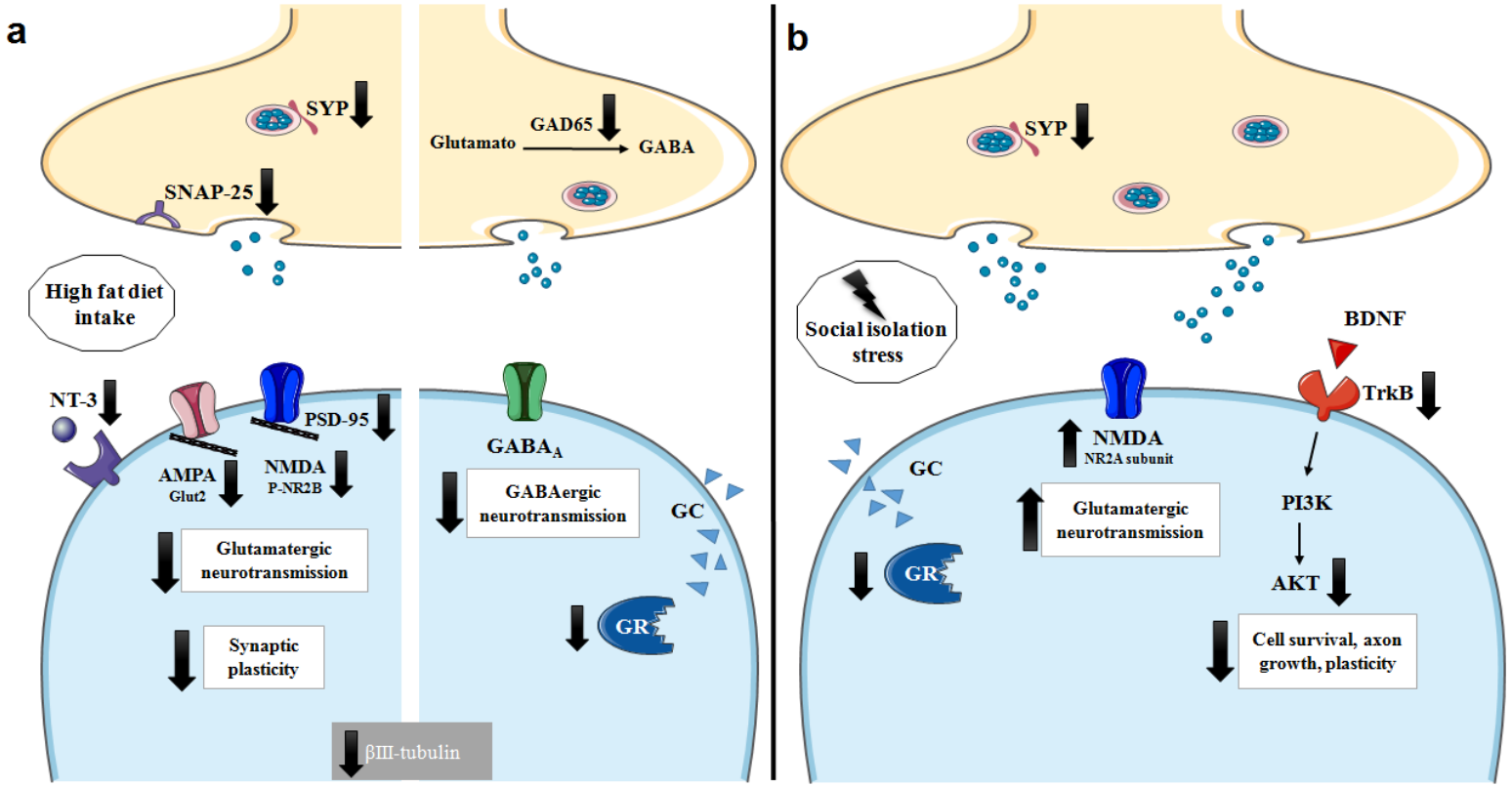

a
Immunocontent of AMPA2/ β-actin
150
100
50
0
**
**
Control
Stress
Chow
HFD
ID
IC
CD
CC
AMPA2
β-ACTIN
b
Immunocontent of NR2A/ β-actin
#
Control
Stress
Chow
HFD
ID
IC
CD
CC
NR2A
β-ACTIN
c
Immunocontent of NR2B/ β-actin
Control
Stress
Chow
HFD
d
Immunocontent of phospho-NR2B/β-actin
**
**
Control
Stress
Chow
HFD
e
phospho-NR2B/NR2B
1.5
1.0
0.5
0.0
Control
Stress
Chow
HFD
IC
CD
CC
ID
NR2B
phospho-NR2B
β-ACTIN

Figure 10


a
SYP
SNAP-25
High fat diet intake
NT-3
PSD-95
AMPA
Glut2
NMDA
P-NR2B
Glutamatergic neurotransmission
Synaptic plasticity
Glutamato
GAD65
GABA
GABA$_A$
GABAergic neurotransmission
GC
GR
βIII-tubulin
b
SYP
Social isolation stress
BDNF
TrkB
NMDA
NR2A subunit
GC
GR
Glutamatergic neurotransmission
PI3K
AKT
Cell survival, axon growth, plasticity

3.3 Capítulo III

Early high-fat diet exposure reduces food-seeking behavior for different palatable foods during adulthood

Artigo a ser submetido para publicação na revista *European Journal of Nutrition.*

EARLY HIGH-FAT DIET EXPOSURE REDUCES FOOD-SEEKING BEHAVIOR FOR DIFFERENT PALATABLE FOODS DURING ADULTHOOD

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ABSTRACT

Purpose

Exposure to early environmental events can alter early brain programming leading to changes in feeding behavior and predisposing to the development of obesity/overweight throughout life. Here, we investigated the long-term effects of social isolation stress and chronic high-fat diet (HFD) access, on hedonic feeding responses of adult Wistar rats.

Methods

Half of rats were subjected to social isolation between 21-28 postnatal day. Additionally, rats were fed from weaning to a control diet or HFD, for 10 weeks. In adulthood, hedonic feeding behavior were evaluated and related to the dopaminergic, cannabinoid and opioid systems evaluations in nucleus accumbens (Nac).

Results

Animals with chronic HFD intake were less motivated to seek different sweet palatable foods. This reduced motivation did not appear to be associated with less pleasure upon tasting sweet food, as no effect in reactivity to sweet taste was observed. Interestingly, the animals receiving HFD presented decreased immunocontents of the D1 and CB1 receptors, while the stressed group displayed a reduction in dopamine turnover.

Conclusions

Results suggest that the long-term HFD intake causes a significant motivational impairment in animals when searching for different palatable foods; these changes may be associated with decreased dopaminergic and cannabinoid neurotransmission to the NAc. In contrast, a brief social isolation during the prepubertal period was unable to alter the behavioral parameters studied, but caused a decreased dopaminergic turnover in the NAc of adult rats. These findings highlight the importance of early environmental events on programming hedonic feeding behavior and related neurochemical systems during adulthood.

Keywords: social isolation stress, prepubertal period, hedonic responses, motivation, dopaminergic system, cannabinoid system.

1. Introduction

The prevalence of obesity and overweight has become a major global public health problem and is increasingly evident in young children and adolescents (Ogden et al., 2014, Koletzko, 2016). Dietary patterns and lifestyles have changed drastically, with constant exposure to an obesogenic environment (Finkelstein *et al.*, 2005). In fact, current food patterns are increasingly based on the consumption of highly energetic and palatable foods that are rich in sugars and fat (Shang *et al.*, 2012). These diets are widely used, including during sensitive periods of developmental when feeding behavior and dietary preferences are shaped (Mennella and Beauchamp, 2002, Teegarden et al., 2009). The relationship between the consumption early in life of these caloric foods with long-term dietary changes, including predisposition to obesity/overweight, has been demonstrated in both animal models (Frazier et al., 2008, Teegarden et al., 2009) and humans (Carnell *et al.*, 2012). During these early periods of life, the brain undergoes several fundamental processes, such as functional organization of neural networks (Rice and Barone, 2000), which will influence on the programming of eating behaviors throughout life (Crespi and Unkefer, 2014). Therefore, changes in nutritional status during development may predispose to childhood obesity that can persist into adulthood, making these children more vulnerable to the development of a non-healthy life style and its associated pathologies (Park *et al.*, 2012).

Moreover, a growing body of literature suggests that exposure to stress contributes to the development and maintenance of obesity in youth (Wilson and Sato, 2014). Stress is a factor which may influence emotional eating behavior and food preferences, and it is usually associated with food intake increase, especially of highly palatable foods that are rich in sugars and fat (Adam and Epel, 2007). The consumption of calorie-dense foods that is induced by stress has been suggested to function as a means to reduce the stress response, as proposed by the reward-based eating model ("comfort foods") (Adam and Epel, 2007). Accordingly, reports indicate that early life stress, usually induced by psychosocial stressors, is associated with increased obesity/overweight prevalence during developmental periods and throughout life (Burke et al., 2011, Gundersen et al., 2011, Ogden et al., 2014). Therefore, additional studies regarding the stimulation of the intake of high-energy-dense foods by early stressful experiences are needed to identify the mechanisms involved in this association.

Feeding behavior involves complex mechanisms that respond to the external environment, as well as body storage depots and metabolism, and also depends on individual behavioral and psychological

profiles (Pandit et al., 2012, Tulloch et al., 2015). Two complementary pathways regulate motivated eating behaviors: (a) Homeostatic, associated with increased motivation to eat in relation to energy balance, and (b) hedonic, linked to the reward and emotional aspects of eating. This latter pathway is usually associated with an increased search for highly palatable foods, which can, in some cases, even override the homeostatic regulation of eating behavior (Tulloch *et al.*, 2015). The hedonic aspects of eating are much less understood than homeostatic mechanisms (Tulloch *et al.*, 2015).

Importantly, the activation of the brain reward circuitry demonstrates distinct "liking" and "wanting" responses. Berrigde (2009) proposed "liking" to be a hedonic reaction related to the pleasure of the reward, while "wanting" is associated more with the motivational value of reward (or motivation process of incentive salience). These two responses are different and are mediated through distinct pathways; however both are necessary for the normal reward (Berridge, 2009). Additionally, some neurotransmitter systems are strongly associated with these responses, such as mesolimbic dopamine neurotransmission, as well as cannabinoid and opioid neurotransmission (Berridge, 2009, Nicola, 2016), especially in the nucleus accumbens (NAc), an important brain center related to the motivation and hedonic characteristics of food intake (Colantuoni et al., 2001, Levine and Billington, 2004). Alterations in neurotransmitter systems in this brain structure can affect the incentive value and pleasure associated with food reward (Berridge, 2009, McNeilly et al., 2016).

Although some studies have shown the effects of stress and high-caloric food intake on hedonic responses to food, little is known about how exposure to these environmental factors during important developmental periods affects these responses, and possible interactions between them. Thus, the main objective of the present study was to test the hypothesis that early environmental factors, such as stress and access to high-fat diet, can alter the neurotransmission systems involved in the reward system and change long-term hedonic responses to food. With this aim, we analyzed the effects of social isolation during the prepubertal period, in association with chronic high-fat diet exposure, on "wanting" and "liking" responses to sweet food later in life; we also determined whether these conditions alter some markers related to dopaminergic, opioid and cannabinoid neurotransmission in the NAc of adult animals.

2. Material and Methods

2. 1 Experimental subjects

Animals were housed in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust, and maintained in a controlled environment: standard 12h dark/light cycle (lights on between 7:00h and 19:00h) and, temperature of 22 ± 2°C. Wistar rats were weaned on postnatal day (PND) 21, and only male pups were used in this study. At PND 21, half of the animals were housed in groups of 4 per cage, and the other half were subjected to stress by social isolation (one animal in a smaller home cage, 27x17x12 cm) (Douglas *et al.*, 2004). Isolation stress was applied during one week, between PND 21-28. On PND 28, isolated animals were returned to the regular home cages (65 x 25 x 15 cm) in groups of four. Each group (control or stressed) was subdivided into 2 other groups, according to their diet: (1) receiving *ad libitum* standard lab chow (50% carbohydrate, 22% protein and 4% fat) or (2) receiving *ad libitum* both standard chow plus high-fat diet (HFD) (25% carbohydrate, 28% protein and 42% fat). Therefore, animals from this group could choose from the two diets available. Animals were maintained on these diets until adulthood. Only one animal per litter was used per group. These procedures resulted in four experimental groups: controls receiving standard chow (control-chow; CC); controls receiving standard chow plus high-fat diet (control-HFD, CD); isolated animals receiving standard chow (stressed-chow, IC); and isolated animals receiving standard chow plus HFD (stressed-HFD, ID). On PND 60, behavioral tests were performed on different days (see timeline in Figure 1). After the behavioral tests, on approximately PND 75, each animal was killed by decapitation between 1:00 pm to 3:00 pm, and the brain was removed, NAc was dissected on ice, and immediately frozen and stored at -80°C for further biochemical evaluations.

The animal proceedings were conducted in strict accordance with the recommendations of the Brazilian Society for Neurosciences (SBNeC) and Brazilian Law on the use of animals (Federal Law 11.794/2008), and were approved by the Institutional Ethical Committee (CEUA-UFRGS #25488). All efforts were made to minimize animal suffering, as well as to reduce the number of animals used.

2.2 High-Fat Diet

The high-fat diet (HFD) used in the study was enriched with fat (42%) from lard and soy oil. In addition, this diet contained vitamins and a salt mixture, purified soy protein, methionine, lysine and starch, as previously described by Arcego et al. (2014). The calorie content of the HFD was 588 cal/100g, compared to that of standard chow, which was 301.2 kcal/100g. Previous data (Arcego *et al.*, 2014) showed that the animals with access to both diets consumed more HFD compared to standard chow, but

this was not reflected in a higher caloric intake. However, animals on the HFD had more abdominal fat, particularly in the stressed group with HFD, which was reflected in a higher weight gain in these animals.

2.3 Behavioral experiments

The behavioral tests began on PND 60 (different animals were used for each behavioral test, since previous exposure to the test could compromise the performance for the next test, except for novelty suppressed feeding and reactivity to sweet taste tasks in which the same animals were used). Prior to each behavioral task, rats were placed in the test room (temperature 21±2°C) for one hour to allow habituation to the environment and researcher.

2.3.1 Experiment 1: Motivation assessment for seeking out different types of palatable foods

2.3.1.1 Palatable food ingestion

To assess motivation for seeking out different types of palatable food, Froot Loops® (Kellogg's® - pellets of wheat, corn starch and sucrose), or chocolate tablets (Nestle Classic®), were offered to different animals. Animals were placed in a lightened rectangular box (40x15x20 cm) with floor and side walls made of wood and a glass ceiling. The palatable food (ten Froot Loops® or chocolate tablets) was placed in one extremity of the box. The animals were habituated to this environment during 5 days, 3 min each day, under food restriction (80% of habitual ingestion). After the last habituation session, the animals were fed *ad libitum* and were exposed to a 3-min test session, 24 hours later. Time spent to reach the food and until initiation of eating, as well the total number of ingested palatable foods were measured in each trial and in test session (Lampert *et al.*, 2013).

2.3.1.2 Novelty suppressed feeding

For this task, the animals had all their food removed 24h prior to the test. The testing apparatus consisted of a Plexiglas box (50x50x50cm) with the room illuminated approximately 150 lux. A small piece of standard chow was placed in the center of the arena on a white circular filter paper (9.5 cm in diameter). Each animal was placed in the corner of the testing arena, and the time until the first feeding episode was measured. A maximum time (12 minutes) was stipulated for starting pellet consumption. Immediately after the rat began to eat the chow, the animal being tested was placed alone in its home cage

with a weighed piece of chow for 5 min. The amount of food consumed in 5 minutes in the home cage was controlled to discard a general disturbance in food intake. At the end of this period, the amount of food consumed was determined by weighing the piece of chow. After all the animals from a single cage had been tested, the rats were returned to their home cage with food and water provided *ad libitum*. This procedure elicits a conflict between the drive to eat the food and the fear of risk in the center of the apparatus (Mineur *et al.*, 2007).

2.3.2 Experiment 2: Motivation assessment to seeking out palatable food without the influence of consumption of HFD

This experiment tested the possibility that chronically HFD feeding had some interference in palatable food ingestion, as observed in experiment 1. Therefore, the palatable food ingestion task using Froot Loops® was repeated in different animals that had the HFD removed one week prior to the behavioral task, receiving only standard chow to avoid any interference by HFD-induced satiety.

2.3.3 Experiment 3: Evaluation of hedonic taste reactivity responses (liking)

The hedonic responses to a sweet solution was evaluated, in order to test the possibility of any change in response to the "liking" of sweetness might interfere in the findings from experiments 1 and 2.

2.3.3.1 Reactivity to sweet taste

In this test, patterns of facial responses to a sweet taste were recorded on video and analyzed. Initially, the animals were habituated to oral administration of water (100µl) using a plastic pipette during 5 days. On the sixth day, sucrose solutions were administered at concentrations of 0.1M and 1M (with an interval of 1 hour between the administrations). Affective facial reactions were recorded with a digital camera for later analysis. The reactions were evaluated by video analysis (frame by frame – 30 frames/s) using KMPlayer® Software, expressed as the frequency and duration (number of frames) of tongue protrusions held for 60 seconds (adapted of Berridge, 2000, and Laureano et al., 2016). Frontal and lateral rhythmic tongue protrusions were considered positive hedonic responses ("like") (Berridge, 2000).

2.4 Neurochemical analysis

2.4.1 Western Blot

Nucleus accumbens was homogenized in ice-cold lysis buffer (10mM KCl, 10mM Hepes, 0.6mM EDTA, 1% NP 40 and 1% protease inhibitor cocktail) pH 7.9, and centrifuged at 1000g for 10 min. Equal protein concentrations (40 µg/lane of total protein, determined using a commercial kit BCA Protein Assay [Thermo Scientific, U.S.A]) were loaded onto NuPAGE® 4-12% Bis-Tris Gels. After electrophoresis, proteins were transferred (XCell SureLock® Mini-Cell, Invitrogen) to nitrocellulose membranes (1 h at 50V in transfer buffer [48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS]) (Arcego *et al.*, 2016). The blot was incubated for 2 h in blocking solution [tris-buffered-saline (TBS) plus 5% bovine serum albumin]. Afterwards, the blot remained overnight at 4 °C in blocking solution containing one of the following antibodies: anti-dopamine transporter (DAT, 1:500, Millipore), anti-dopamine D1a receptor (1:500, Millipore), anti-dopamine D2 receptor (1:1000, Millipore), anti-tyrosine hydroxylase (TH, 1:2000, Millipore), anti-phospho-tyrosine hydroxylase (pSer40, 1:1000, Invitrogen), anti-cannabinoid receptor 1 (CB1, 1:200, Abcam), anti-µ-opioid receptor (1:500, Millipore) and anti-β-actin (1:2000, Millipore). The blot was then washed with Tween-TBS and incubated for 2 h in solution containing peroxidase-conjugated anti-rabbit IgG (1:1000, Millipore) or anti-mouse IgG (1:1000, Millipore). The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario). Chemiluminescence was detected using a digital imaging system (Image Quant LAS 4000, GE Healthcare Life Sciences) and analyzed using the Image J Software. Results were expressed as the ratio of intensity of the protein of interest to that of anti-β-actin from the same membrane.

2.4.2 Monoamines measurement

On the day of the assay, NAc samples were weighed and suspended in 0.1 M $HClO_4$ (1:30, w:v), sonicated for 5s and centrifuged at 15000 rpm for 15 min at 4 ºC (Meikle *et al.*, 2013). The pellet was discarded and dopamine (DA) and dihydroxyphenylacetic acid (DOPAC) tissue concentrations were analyzed in the supernatants (50 µl) using High Pressure Liquid Chromatography coupled with Electrochemical Detection (HPLC-ED, BAS, USA). The oxidation potential of the amperometric detector was set at 0.75 V versus an Ag/AgCl reference electrode (Jacobsson et al., 1980, Claustre et al., 1986). Chromatographic separations were performed using a C18 reverse phase column (150 mm 4.6 mm,

Phenomenex, USA) packed on microparticulate (5 mm). The mobile phase flow rate was 1.2 ml/min and its composition was 0.15 M citric acid, 0.06 mM sodium octyl sulfate, 4 % acetonitrile (v:v); 1.6 % tetrahydrofurane (v:v), in double-distilled water, pH 3.0. The positions and heights of the peaks in tissue homogenates were measured and compared to 50-µl samples of an external standard solution containing 5 ng of each DA and DOPAC, and concentrations of these substances were calculated in each sample and expressed as ng/g of wet tissue (Scorza et al., 1997, Costa et al., 2001). DA turnover values were calculated as DOPAC/DA ratio.

2.5 Statistical Analysis

Data are expressed as mean ± SEM of the mean, and analyzed using two-way ANOVA (isolation stress and diet as factors), and repeated measures ANOVA for habituation sessions in the palatable food ingestion task. ANOVA tests were followed by the Tukey multiple range test, when indicated. All analyses were performed using IBM SPSS Statistic 22 software and the significance level was set at $P < 0.05$ for all analyses.

3. Results

3.1 Experiment 1- effects of early social isolation and chronic HFD intake on motivation to seek out different types of palatable food

3.1.1 Palatable food ingestion when chocolate was offered: During the habituation sessions, no significant differences were found between groups in the latency to reach the food when animals were kept under food restriction ($P>0.05$; Fig.2A); however, the animals that received chronic HFD showed an increased latency to start eating chocolate [$F(1,30)=8.65$; $P=0.006$; Fig.2B], and ate smaller amounts [$F(1,30)=29.72$, $P<0.001$; Fig.2C]. For the test, when animals had been fed *ad libitum* for the last 24 hours, similarly to the habituation session, no differences in latency to reach the food were found ($P> 0.05$; Fig.2D), but the HFD group presented a greater latency to start eating [$F(1,30)=9.97$, $P=0.004$; Fig.2E] and ate less chocolate [$F(1,30)=17.15$, $P<0.001$; Fig.2F]. In addition, a significant interaction between stress and diet was observed for the latency to eat during the test [$F(1,30)=5.49$, $P=0.03$, followed by Tukey post hoc test; Fig.2E]; animals on the HFD in association with stress presented a

lower latency, compared to non-stressed animals receiving HFD. A trend towards the same interaction between factors was found in the amount of chocolate consumed [F(1,30)=3.14; P=0.086; Fig.2F].

3.1.2 Palatable food ingestion when Froot Loops® were offered: The results found in this task were similar to those of the task performed using chocolate. During habituation, the animals did not exhibit differences in latency to reach the food (P> 0.05; Fig.3A), however increased latency to eat [F(1,34)=7.11; P=0.01; Fig.3B], and decreased consumption of Froot Loops® [F(1,34)=31.78, P<0.001; Fig.3C] were observed for the HFD group. On the sixth day, when the test session was performed, we observed an interaction between stress and diet on the latency to reach the food [F(1,34)=7.76; P=0.009, followed by Tukey post hoc test; Fig.3D], as the HFD group presented increased latency, which was not observed in the HFD stressed group. When evaluating latency to eat the sweet cereal, the group on HFD took longer [F(1,34)=4.16; P=0.05; Fig.3E], and ate less of this type of palatable food [F(1,34)=6.37; P=0.02; Fig.3F].

3.1.3 Novelty suppressed feeding. When we evaluated the latency to seek food in an aversive environment, such as the illuminated center of the apparatus, we observed that the groups with access to chronic HFD had higher latencies to eat the chow pellet positioned in the center, when compared to other groups [F(1,47)=20.97; P<0.001, see Figure 4]. Additionally, no significant effects were found between the groups in the total amount consumed during 5 minutes in the familiar home cage, showing no major effect on rats' appetites (P>0.05, data not shown).

3.2 Experiment 2 - effects of early social isolation and chronic HFD intake on the motivation to seek out palatable food without the influence of consumption of HFD

3.2.1 Palatable food ingestion when Froot Loops® were offered several days after HFD withdrawal: To assess whether the behavioral effects found in the groups that received chronic HFD (high calorie diet) were due to the fact that they were still receiving HFD, or were related to a long-term effect induced by this diet, we observed the performance of the animals when HFD was removed one week before the task. Similarly to the effects observed in Experiment 1, we did not observe significant differences in the latency to reach the food during the habituation sessions (P>0.05; Fig. 5A), however there was an increased latency to eat [F(1,48)=5.75; P=0.02; Fig. 5B), and a decrease in the amount of sweet cereal consumed [F(1,48)=11.15; P=0.002; Fig. 5C] in the groups that had previously received the HFD. For the

test, animals were fed *ad libitum* with only standard chow for 24 hours before the test and did not demonstrate any differences in the latency to reach (P>0.05; Fig.5D), and eat the food (P>0.05; Fig.5E); however, the HFD groups still consumed less sweet food [F(1,52)=4.50; P=0.04; Fig.5F].

3.3 Experiment 3 – effects of early social isolation and chronic HFD intake on hedonic taste reactivity responses (liking)

3.3.1 Reactivity to sweet taste: No significant differences were observed between the groups tested, for either the number of frames or the frequency of tongue protrusions in response to a sweet taste, for concentrations of both 0.1M and 1M (P>0.05 in every case, see Figure 6).

3.4 Neurochemical Analyses

3.4.1 Immunocontent of proteins related to dopaminergic, cannabinoid and opioid systems. The results of Western blot analyses are shown in Figures 7 and 8. Chronic HFD groups showed a reduction in the immunocontents of D1 receptor [F(1,20)=6.65; P=0.02; Fig. 7A], as well as CB1 receptors [F(1,22)=4.46; P=0.05; Fig. 7D] in the NAc. No significant differences between the groups were observed for the immunocontents of the D2 receptor (see Fig. 7B), μ-opioid receptor (Fig. 7C), or tyrosine hydroxylase (total and phosphorylated forms; Fig. 8A and 8B, respectively) (P>0.05). The group that had been stressed during the prepubertal period showed an increase in dopamine transporter (DAT) immunocontent [F(1,17)=8.26; P=0.01; Fig. 8C].

3.4.2 Dopamine and DOPAC measurements. We observed increased accumbal dopamine levels [F(1,23)=4.11; P=0.05] in the stressed groups. DOPAC levels were not significantly different between groups (P>0.05); however we found a reduction in DOPAC/Dopamine ratio [F(1,23)=6.76; P=0.02] in NAc (Figure 9).

4. Discussion

Eating patterns can be influenced by early experiences during sensitive periods of development. In the present study, rats receiving chronic HFD, from prepuberty to adulthood, showed a reduced

motivation to obtain other forms of palatable foods. These findings may be associated with a decrease in cannabinoid (CB1) and dopaminergic (D1) receptors found in the Nac of these animals. In addition, the animals that had been socially isolated during the prepubertal period showed a reduction in dopamine turnover in adulthood in the same brain structure, although they did not present any changes in the behaviors assessed.

We herein evaluated incentive salience ("wanting") by monitoring behaviors involving the search for different palatable foods. The animals that had chronically consumed a highly caloric diet (that was rich in fat) took longer to eat and, therefore, ate less palatable food, regardless of whether the food was rich in sugar (Froot loops®), or both sugar and fat (Classic® chocolate), showing a reduced motivation to obtain different palatable foods. Moreover, these findings were not due to an influence of the higher caloric ingestion obtained from the HFD, since these animals also presented the behavior during the caloric restriction that was imposed during the habituation period. Additionally, one of the tasks was repeated with the prior removal of this diet, and results suggest that the increased latency to eat and the reduced ingestion of other palatable foods may be related to a long-term effect that is induced by the access to this diet during development. Interestingly, HFD animals had a free choice between standard chow and HFD in their cages, and demonstrated preference for the HFD compared to chow (previously published data in Arcego *et al.*, 2014). Therefore, we may conclude that the group with access to HFD does not appear to be less motivated to eat the familiar diet that had been received post weaning in their home cages; however, when exposed to new palatable foods that were rich in sugar, they were less motivated to obtain these types of palatable food.

In the novelty suppressed feeding task, the HFD groups showed similar results, with greater latency to seek the food in the center of the open field apparatus. This task involves a conflict that evokes competing motivations, the drive to eat versus the fear to enter the center of a brightly box. Although this test is usually associated with anxiety assessment and depression-like behavior, it also involves an additional motivational component, since food deprivation should induce the ability to decide between the innate avoidance to novel open areas and the incentive to consume food (Savalli *et al.*, 2015). In addition, the anxiety-like behavior assessment that was previously performed with the same experimental design, in the open field and plus-maze tasks, demonstrated that animals with chronic HFD intake had no differences in these classical tasks for anxiety analyses in rodents (Arcego *et al.*, 2013).

Although there are controversial results in the literature, some studies in animals fed on diets rich in fat support our findings, demonstrating that chronic administration of HFD reduces motivated behaviors, as shown by a decrease in operant food self-administration rate (Davis et al., 2008, Finger et al., 2012, Ibias et al., 2016, McNeilly et al., 2016), in addition to failure to acquire place preference (CPP) for different rewards, such as sucrose pellets (Tracy *et al.*, 2015), cocaine (Morales *et al.*, 2012), amphetamine (Davis *et al.*, 2008), and nicotine (Blendy *et al.*, 2005). Taken together, data show that continuous access to a HFD appears to decrease the reward value of different palatable foods, or even drugs. Interestingly, a study evaluating food preference in mice acutely exposed to a HFD, early on in life, showed a significant preference for a fat-rich diet in adulthood, compared to controls, showing that this early exposure may alter the programming of reward pathways leading to changes in the adult dietary preferences (Teegarden *et al.*, 2009). In addition, the same study also found that animals also exposed to a diet rich in carbohydrates early in life showed no difference in preferences during adulthood, suggesting that this preference is not due to familiarity to the diet (Teegarden *et al.*, 2009).

The activation of reward-associated responses in relation to dopaminergic signaling has been consistently related in the literature (Baik, 2013). The dopaminergic projections from the ventral tegmental area (VTA) to the Nac are involved in driving behavior goals, as motivation to obtain food rewards (Berridge, 2004). In this study, a decrease in D1 receptors immunocontent was observed in the NAc of animals with chronic access to HFD. Although there are no differences in dopamine levels and other dopaminergic markers in this group, a reduction of this receptor may represent a lower activation of dopaminergic neurotransmission in this structure. Moreover, the function of D1 receptors appears to be more related to the activation of responses oriented to reward, while D2 receptors play a greater role in the evaluation of the reward (D'Aquila, 2010, Galistu and D'Aquila, 2012). Therefore, a decrease in D1 receptor activation may be related to the behavioral findings of this study, reducing the motivation to get food rewards; when little effort is necessary to obtain food, the animals normally consume that food (in the case of HFD provided in the home cages).

The endocannabinoid system also contributes to incentive processes and to the hedonic evaluation of food stimuli via effects in NAc. (Kirkham, 2009). Stimulation of the cannabinoid receptor 1 (CB1) facilitates activity in incentive pathways, promoting orientation to food reward and increases the motivation to eat (Cavuoto and Wittert, 2009, Kirkham, 2009). Conversely, CB1 antagonists reduce the effort to obtain food, and CB1 knockout mice exhibit lower levels of response to sweet food (Solinas and

Goldberg, 2005, Kirkham, 2009). In addition, blockade of CB1 receptors in the NAc attenuates the conditioned place preference for reward stimuli, such as morphine (Zhang *et al.*, 2016). In this study, we found decreased CB1 immunocontent in NAc in HFD groups. This finding may be related to lower cannabinoid system activation and, thus, supports a poor behavioral performance of these animals in food-seeking and eating initiation. In addition, CB1 knockout mice in neurons expressing D1 reportedly show a reduced preference for sweet solutions, when compared to a wild-type control group (Terzian *et al.*, 2011), demonstrating that there may be an interaction between CB1 and D1 receptors in modulating motivated behaviors, and that the interplay between these receptors could be altered in animals with chronic HFD consumption during development.

Furthermore, opioid neurotransmission is strongly associated with the hedonic impact of sensory pleasure ("liking") (Berridge, 2009). Opioids regulate the palatability or the hedonic evaluation of food (Berridge, 1996), and opioid peptides and their receptors are broadly expressed throughout the limbic system (Le Merrer *et al.*, 2009). In this study, we did not observe significant differences between the groups in relation to the immunocontent of μ-opioid receptors. This finding corroborates the behavioral results of reactivity to the sweet taste task, which assesses the hedonic responses to a sweet solution, in which no difference between the groups was found in relation to "liking" the sweet taste. This result also reinforces the finding that the reduced search to obtain sweet palatable food is related more to motivation (and associated with the dopaminergic and cannabinoid systems).

In relation to the neurochemical effects found with the socially isolated group in the prepubertal period, we observed an increase in dopamine levels in the NAc, while DOPAC/DA ratio was decreased. This reduced ratio suggests a lower dopamine metabolism, which means less dopamine being released in the synaptic cleft, and more stored in vesicles. In addition, the dopamine transporter (DAT) immunocontent was increased in this group, contributing to a lower availability of dopamine in the synaptic clefts of these animals.

Studies using chronic social isolation are controversial in relation to dopaminergic activity; some suggest an increase in dopaminergic neuron phasic bursting activity in the VTA (Fabricius *et al.*, 2010), with increased dopamine levels in the NAc (Miura *et al.*, 2002), and dopamine turnover in the amigdala (Heidbreder *et al.*, 2000), while others report no differences in dopamine levels in the VTA and NAc (Wang *et al.*, 2012). Our present results suggest that a brief exposure to social isolation after weaning for only one week could lead to a reduced dopamine turnover, in contrast to the effects of prolonged

isolation. Since our animals went through a shorter isolation period and analyzes were performed long after this stress exposure, these results could represent a negative compensatory regulation of the dopaminergic system response. This suggestion is based on studies that observed a reduction of 50% in tonic dopaminergic activity after using a stressor or withdrawal of amphetamine (responsible for tonic increase in dopaminergic firings) (Chang and Grace, 2013, Belujon et al., 2016, Grace, 2016). In our animals, however, the decreased dopaminergic turnover is possibly due to a lower activation of dopaminergic tonic inputs from the VTA, since the measurements were performed at baseline. However, the phasic dopaminergic response (in response to stimulus) does not appear to be affected in these animals, since when exposed to a stimulus, such as the search for a palatable food, this behavior is not impaired. In contrast, the animals exposed to both factors, stress and HFD, presented some significant interactions in the behaviors tests (latency to reach the Froot Loops® and latency to start eating chocolate in the tests) suggesting an interaction that increased reward motivation.

In conclusion, our study reports that the chronic early consumption of a high-fat diet alters the motivation to eat distinct palatable foods, and that these effects may be related to the disruption of the accumbal dopaminergic and cannabinoid systems. Despite the fact that social isolation stress in the prepubertal period did not consistently affect the assessed behaviors, we found long-term changes in dopaminergic circuitry in the same brain structure in this group (see Figure 10 for summary of mainly results). Our data indicate an altered neural programming of hedonic behavior and preference for palatable foods in the Nac after early environmental interventions. More studies are needed to elucidate the possible mechanisms and activation pathways involved in food-seeking behavior ("wanting") that may be altered in animals subjected to these environmental factors. Understanding how previous life events occuring during development affect long-term brain and ingestive behavior may have implications for understanding individuals' vulnerability to eating disorders and obesity.

Conflict of interest

The authors declare no conflict of interest.

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Legends to Figures

Fig. 1 Timeline for the experimental design

Fig 2 Evaluation of palatable food ingestion, when chocolate tablets were offered to adult male rats receiving standard lab chow or lab chow + a high fat diet, and subjected (or not) to isolation during the prepubertal period. Data are expressed as mean ± SEM. N= 8-9/group. **(a)** Latency to reach the food during habituation sessions **(b)** Latency to start eating this food during habituation sessions **(c)** Amount consumed in the habituation sessions **(d)** Latency to reach the food on test day (animals fed *ad libitum*) **(e)** Latency to start eating in the test **(f)** Amount consumed in the test. Repeated measures ANOVA showed an effect of diet on the latency to start eating ($P=0.006$) and on the amount consumed ($P<0.001$) during habituation sessions. Two-way ANOVA showed an interaction between stress and diet on latency to start eating ($P=0.03$, Tukey post hoc test) and an effect of diet on the amount consumed ($P<0.001$) in the test. * Control-HFD group is significantly different from control-chow group (two-way ANOVA followed by Tukey post hoc test). ** HFD groups are significantly different from chow groups (Repeated measures and two-way ANOVA)

Fig. 3 Evaluation of palatable food ingestion using Froot Loops® in adult male rats receiving standard lab chow or lab chow + a high fat diet, and subjected (or not) to isolation during the prepubertal period. Data are expressed as mean ± SEM. N= 8-10/group. **(a)** Latency to reach the food during the habituation sessions **(b)** Latency to start eating during the habituation sessions **(c)** Amount consumed in the habituation sessions **(d)** Latency to reach the food in the test **(e)** Latency to start eating in the test **(f)** Amount consumed in the test. Repeated measures ANOVA showed an effect of diet on the latency to start eating ($P=0.01$) and on the amount consumed ($P<0.001$) during habituation sessions. In the test, a two-way ANOVA showed an interaction between stress and diet in the latency to reach the food ($P=0.009$), as well as an effect of diet on the latency to start eating food ($P=0.05$), and on the amount consumed ($P=0.02$). *Control-HFD group is significantly different from control-chow and stress-HFD groups (two-way ANOVA, followed by Tukey post hoc test). ** HFD groups are significantly different from chow groups (Repeated measures and two-way ANOVA)

Fig. 4 Latency to start eating a chow pellet in the Novelty Supressed Feeding test in adult male rats receiving standard lab chow or lab chow + a high fat diet, and subjected (or not) to isolation during the prepubertal period. Data are expressed as mean ± SEM. N= 11-14/group. Two-way ANOVA showed an effect of diet ($P<0.001$). ** HFD groups are significantly different from chow groups (two-way ANOVA)

Fig. 5 Evaluation of palatable food ingestion using Froot Loops® after previous withdrawal of HFD for one week before the behavioral testing in adult male rats receiving standard lab chow or lab chow + a high fat diet since weaning, and subjected (or not) to social isolation during the prepubertal period. Data are expressed as mean ± SEM. N= 12-15/group. **(a)** Latency to reach the food in habituation sessions **(b)** Latency to start eating food in habituation sessions **(c)** Amount consumed during habituation sessions **(d)** Latency to reach the food in the test session **(e)** Latency to start eating food in the test **(f)** Amount consumed during the test. Repeated measures ANOVA showed an effect of diet on the latency to start eating food ($P=0.02$) and the amount consumed ($P=0.002$) during habituation sessions. In the test, a two-way ANOVA showed an effect of diet in the amount consumed ($P=0.04$). ** HFD intake groups are significantly different from chow intake groups (Repeated measures and two-way ANOVA)

Fig. 6 Patterns of facial responses during the reactivity to sweet taste evaluation in adult male rats receiving standard lab chow or lab chow + a high fat diet, and subjected (or not) to isolation during the prepubertal period. Data are expressed as mean ± SEM. N= 10-12/group. **(a)** Number of frames of tongue protrusions with 0.1M sucrose solution. **(b)** Frequency of tongue protrusions with 0.1M sucrose solution. **(c)** Number of frames of tongue protrusions with 1M sucrose solution **(d)** Frequency of tongue protrusions with 1M sucrose solution. No significant differences were found between the groups (Two-way ANOVA, $P>0.05$)

Fig. 7 Immunocontent of D1 and D2 dopaminergic, canabinoid CB1 and opioid μ receptors, as evaluated by Western blot, in the nucleus accumbens of adult male rats receiving standard lab chow or lab chow + a high fat diet, and subjected (or not) to isolation during the prepubertal period. Data are expressed as mean ± SEM. **(a)** D1 receptor (N= 6-7/group) **(b)** D2 receptor (N= 6-7/group) **(c)** μ-opioid receptor (N= 5-7/group) **(d)** CB1 receptor (N= 6-7/group). CC: Control-Chow; CD: Control-HFD; IC: Stress-Chow; ID:

Stress-HFD. Two-way ANOVA showed an effect of diet in D1 (P=0.02) and CB1 receptors (P=0.05). ** HFD intake groups are significantly different from chow intake groups (two-way ANOVA)

Fig. 8 Immunocontent of tyrosine hydroxylase and dopamine transporter (DAT), as evaluated by Western blot, in the nucleus accumbens of adult male rats receiving standard lab chow or lab chow + a high fat diet, and subjected (or not) to isolation during the prepubertal period. Data are expressed as mean ± SEM. **(a)** Tyrosine hydroxylase (N= 5-7/group) **(b)** Tyrosine hydroxylase phosphorylated at Ser 40 (N= 5-7/group) **(c)** Dopamine transporter (DAT) (N= 4-6/group). CC: Control-Chow; CD: Control-HFD; IC: Stress-Chow; ID: Stress-HFD. Two-way ANOVA showed an effect of stress on DAT (P=0.01). # stressed animals are significantly different from non-stressed animals (two-way ANOVA)

Fig. 9 Levels of dopamine and its metabolite, DOPAC, as evaluated by HPLC, in the nucleus accumbens of adult male rats receiving standard lab chow or lab chow + a high fat diet, and subjected (or not) to isolation during the prepubertal period. Data are expressed as mean ± SEM; N= 6-7/group. **(a)** Dopamine **(b)** DOPAC **(c)** DOPAC/DA ratio. Two-way ANOVA showed an effect of stress on dopamine levels (P=0.05) and DOPAC/DA ratio (P=0.02). # stressed animals are significantly different from non-stressed animals (two-way ANOVA)

Fig. 10 Illustrative figure showing the main effects of social isolation during the prepubertal period and chronic HFD access on the nucleus accumbens of adult rats. This figure was created using the images from the Servier Medical Art bank (www.servier.com)

Figure 1

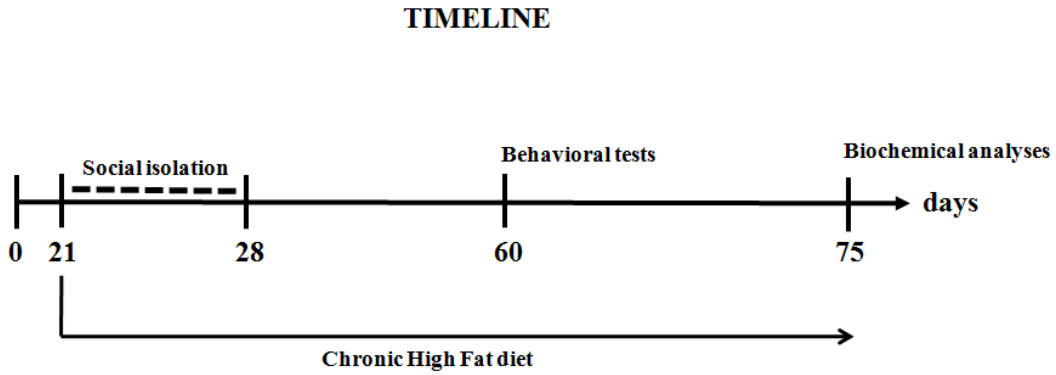

TIMELINE
Social isolation
Behavioral tests
Biochemical analyses
days
0
21
28
60
75
Chronic High Fat diet

Figure 2

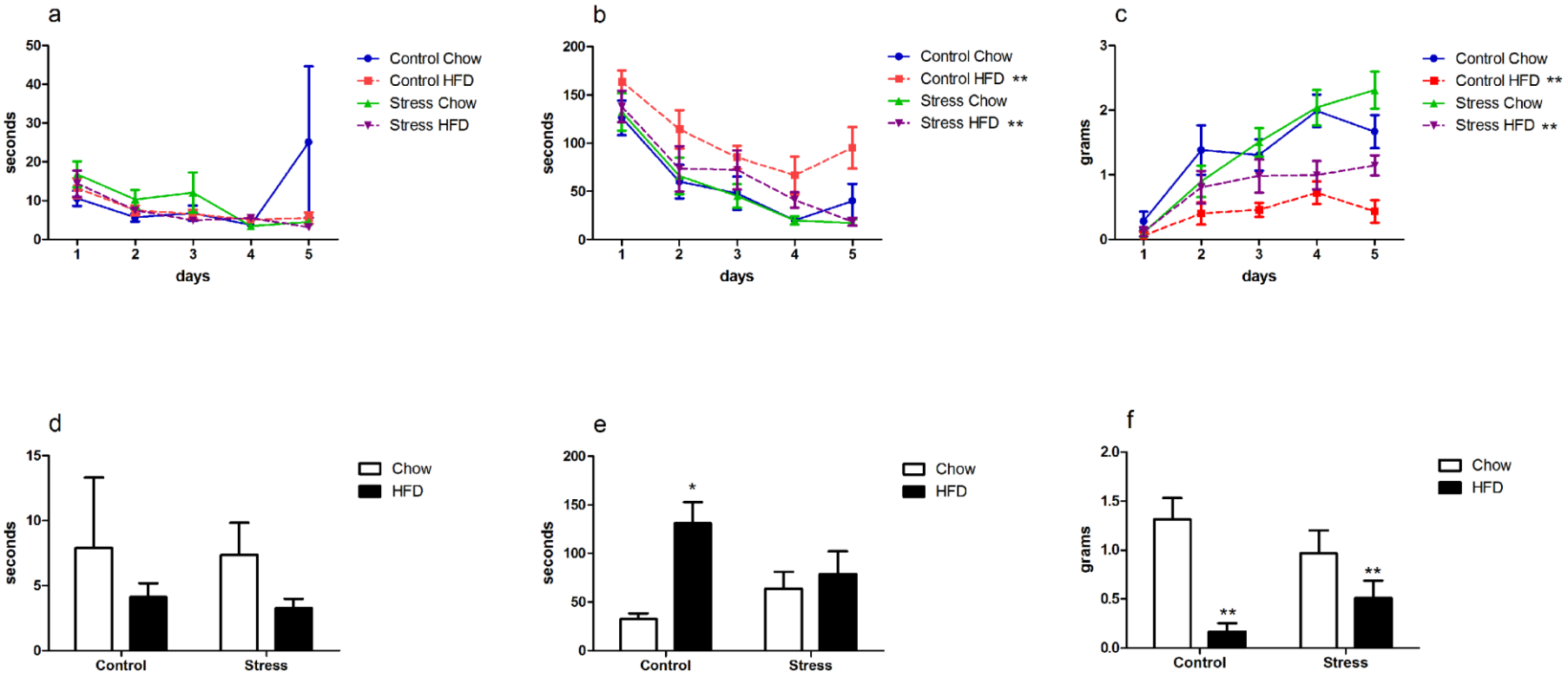

a
seconds
days
Control Chow
Control HFD
Stress Chow
Stress HFD
b
seconds
days
Control Chow
Control HFD **
Stress Chow
Stress HFD **
c
grams
days
Control Chow
Control HFD **
Stress Chow
Stress HFD **
d
seconds
Chow
HFD
Control
Stress
e
seconds
*
Chow
HFD
Control
Stress
f
grams
**
**
Chow
HFD
Control
Stress

Figure 3

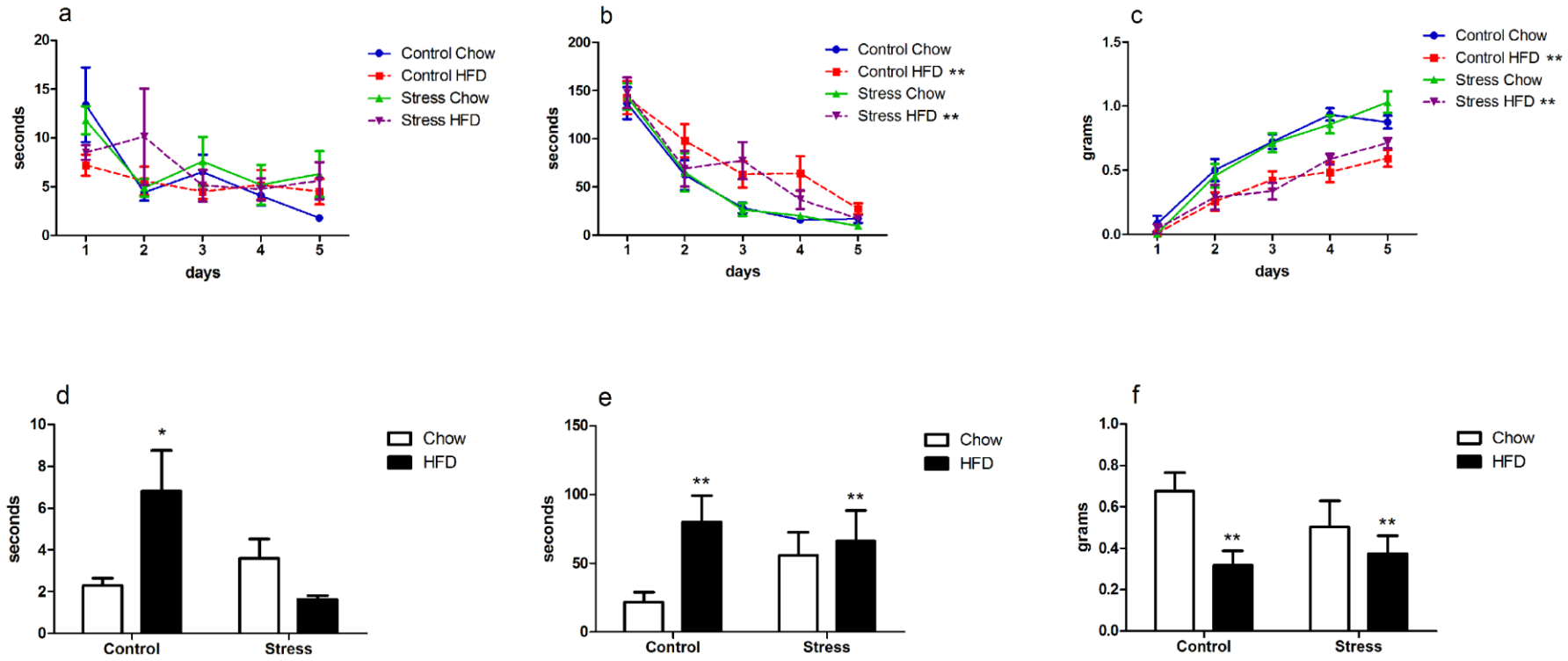

a
seconds
days
Control Chow
Control HFD
Stress Chow
Stress HFD
b
seconds
days
Control Chow
Control HFD **
Stress Chow
Stress HFD **
c
grams
days
Control Chow
Control HFD **
Stress Chow
Stress HFD **
d
seconds
Chow
HFD
Control
Stress
e
seconds
Chow
HFD
Control
Stress
f
grams
Chow
HFD
Control
Stress

Figure 4

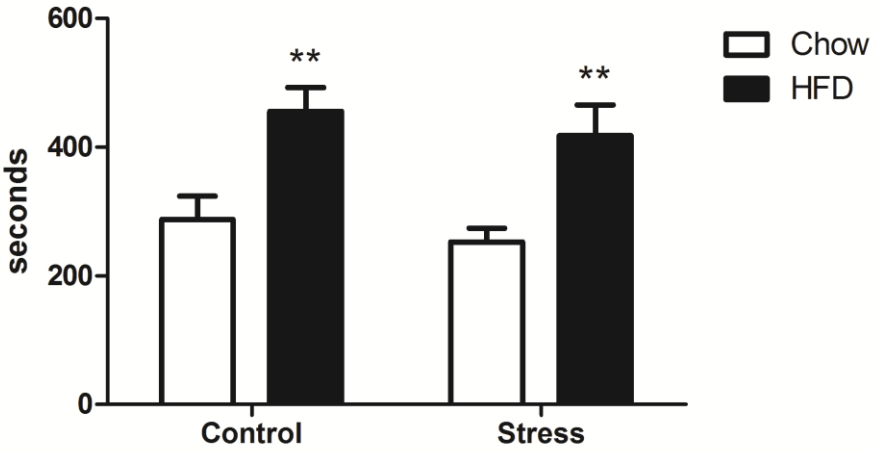


Figure 5

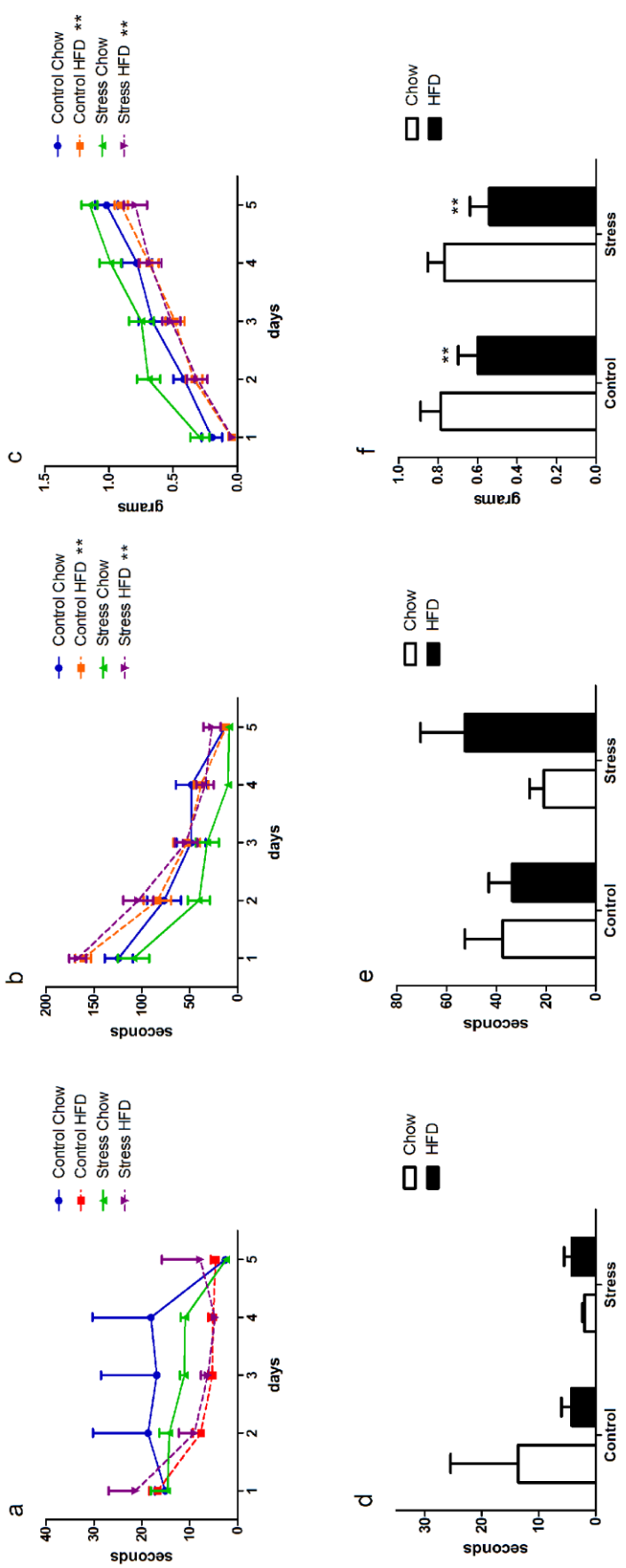

a
b
c
d
e
f
seconds
grams
days
Control Chow
Control HFD
Stress Chow
Stress HFD
Control HFD **
Stress HFD **
Chow
HFD
Control
Stress
**

Figure 6

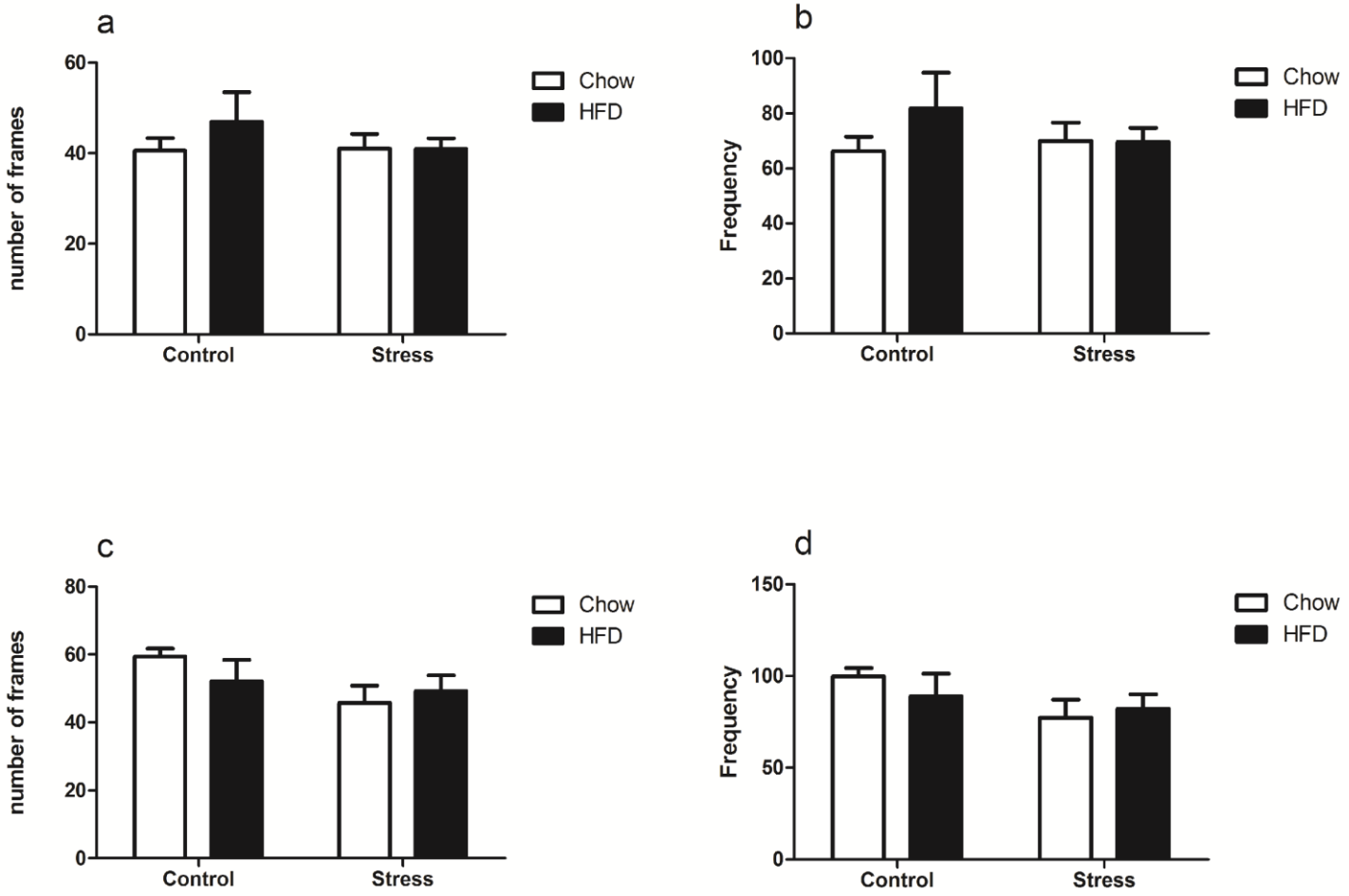

a
number of frames
60
40
20
0
Control
Stress
Chow
HFD
b
Frequency
100
80
60
40
20
0
Control
Stress
Chow
HFD
c
number of frames
80
60
40
20
0
Control
Stress
Chow
HFD
d
Frequency
150
100
50
0
Control
Stress
Chow
HFD

Figure 7

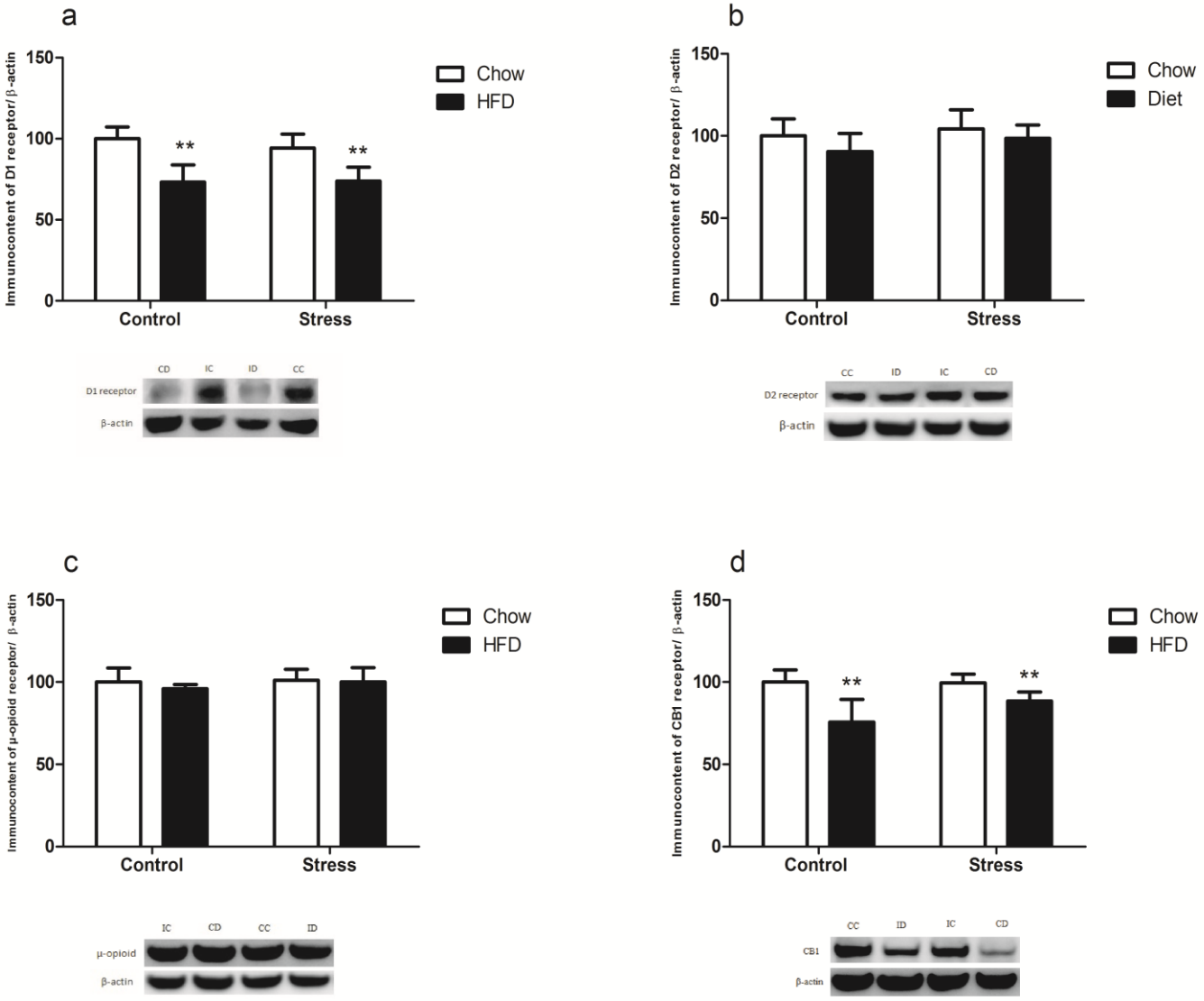

a
Immunocontent of D1 receptor/ β-actin
150
100
50
0
**
**
Control
Stress
Chow
HFD
CD
IC
ID
CC
D1 receptor
β-actin
b
Immunocontent of D2 receptor/ β-actin
150
100
50
0
Control
Stress
Chow
Diet
CC
ID
IC
CD
D2 receptor
β-actin
c
Immunocontent of μ-opioid receptor/ β-actin
150
100
50
0
Control
Stress
Chow
HFD
IC
CD
CC
ID
μ-opioid
β-actin
d
Immunocontent of CB1 receptor/ β-actin
150
100
50
0
**
**
Control
Stress
Chow
HFD
CC
ID
IC
CD
CB1
β-actin

Figure 8

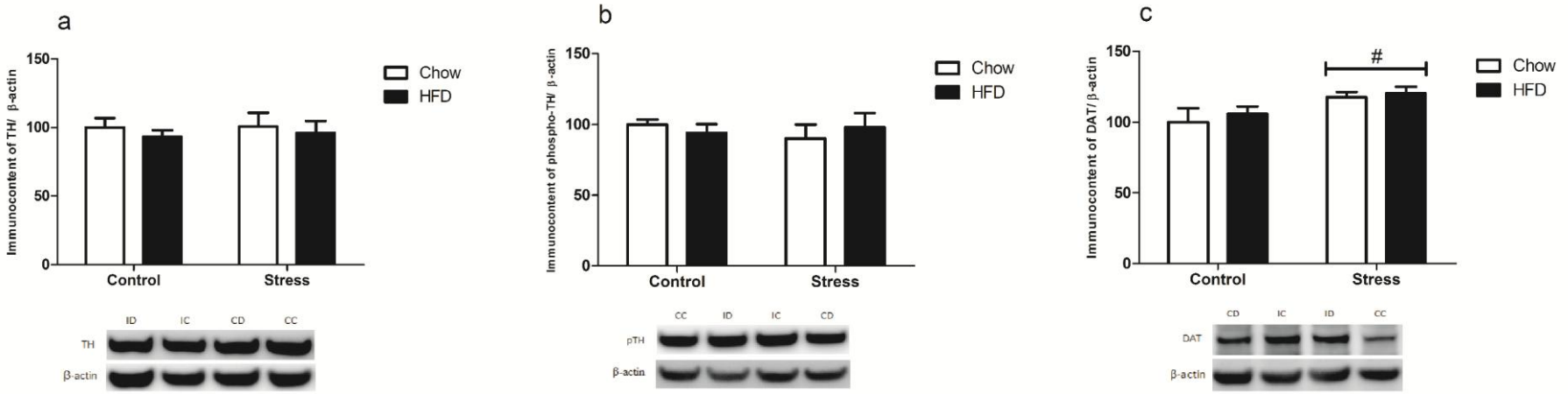

a
Immunocontent of TH/ β-actin
150
100
50
0
Control
Stress
Chow
HFD
ID
IC
CD
CC
TH
β-actin
b
Immunocontent of phospho-TH/ β-actin
150
100
50
0
Control
Stress
Chow
HFD
CC
ID
IC
CD
pTH
β-actin
c
Immunocontent of DAT/β-actin
150
100
50
0
#
Control
Stress
Chow
HFD
CD
IC
ID
CC
DAT
β-actin

Figure 9

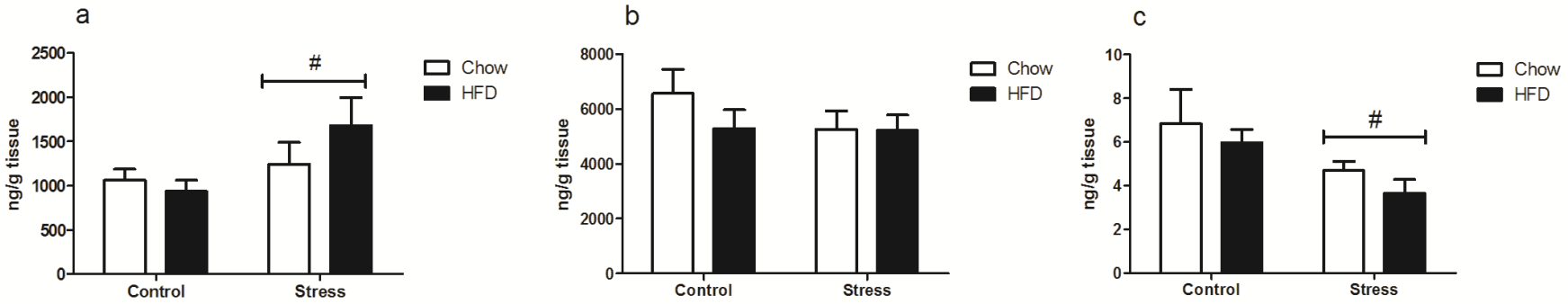
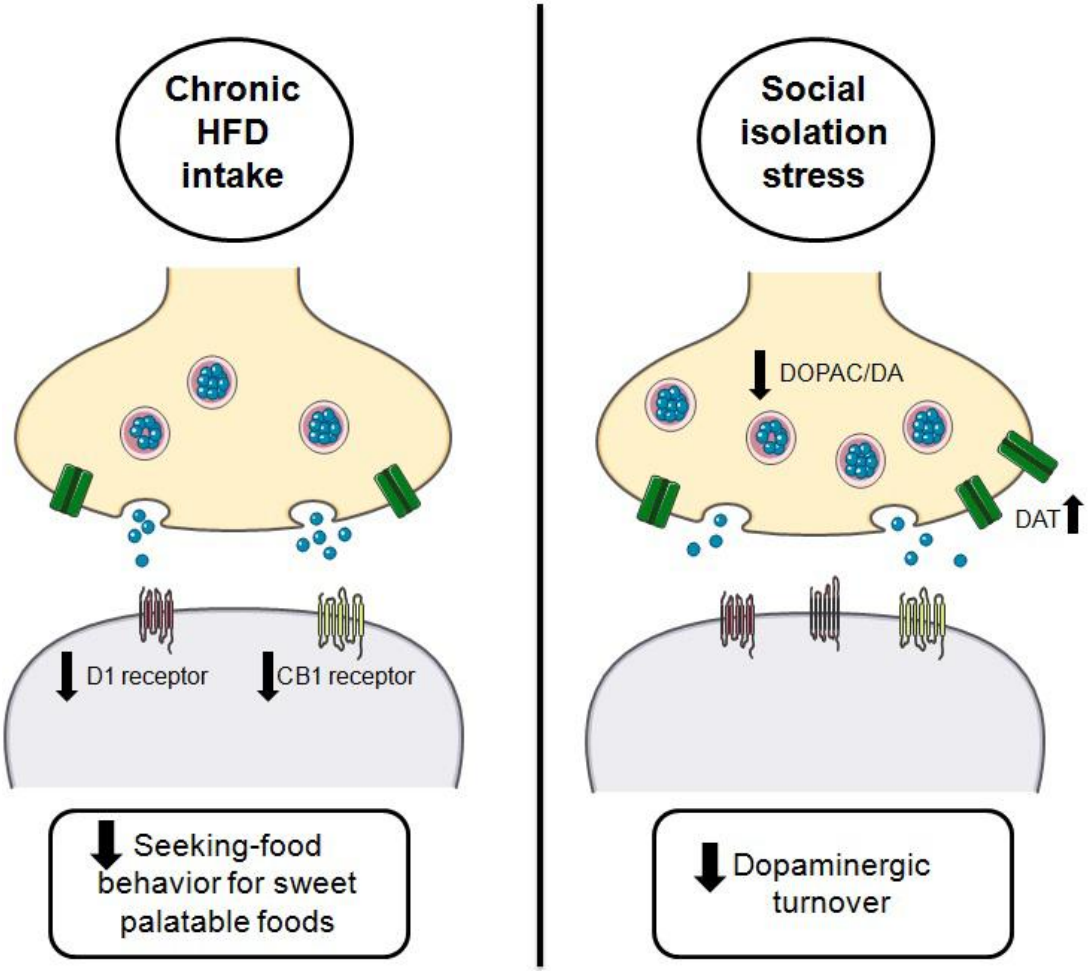

a
2500
2000
1500
1000
500
0
ng/g tissue
#
Chow
HFD
Control
Stress
b
8000
6000
4000
2000
0
ng/g tissue
Chow
HFD
Control
Stress
c
10
8
6
4
2
0
ng/g tissue
#
Chow
HFD
Control
Stress

Figure 10


Chronic HFD intake
D1 receptor
CB1 receptor
Seeking-food behavior for sweet palatable foods
Social isolation stress
DOPAC/DA
DAT
Dopaminergic turnover

4. DISCUSSÃO

As tabelas 1 e 2 abaixo resumem os principais resultados encontrados no presente trabalho de Tese.

Tabela 1. Resumo dos principais resultados neuroquímicos

Córtex pré-frontal	Na+,K+-ATPase: interação estresse*dieta BDNF: interação estresse*dieta TrkB: sem efeito GR: sem efeito ERK/MAPK total: interação estresse*dieta AKT fosforilada: interação estresse*dieta
Hipocampo	BDNF e pró-BDNF: sem efeito Receptor TrkB: interação estresse*dieta AKT fosforilada: estresse↓ β-III-tubulina: dieta ↓ Sinaptofisina: interação estresse*dieta Neurotrofina-3: dieta ↓ SNAP-25: dieta ↓ PSD-95: dieta ↓ GR: estresse↓ e dieta ↓ Receptor GABAa: sem efeito GAD-65: dieta ↓ Subunidade GluA2 receptor AMPA: dieta ↓ Subunidade NR2B fosforilada receptor NMDA: dieta ↓ Subunidade NR2A receptor NMDA: estresse ↑
Núcleo Accumbens	Receptor D1: dieta ↓ Receptor D2: sem efeito Tirosina hidroxilase (total e fosforilada): sem efeito DAT: estresse ↑ Dopamina: estresse ↑ DOPAC/DA: estresse↓ Receptor CB1: dieta ↓ Receptor μ-opióide: sem efeito

Tabela 2. Resumo dos principais resultados comportamentais

Memória de reconhecimento de objetos	Interação estresse*dieta
Memória de trabalho	estresse↓
Teste de preferência a sacarose	dieta ↓ preferência a sacarose
Nado Forçado	estresse↓ e dieta ↓ tempo de imobilidade
Consumo alimento palatável – Froot Loops®	Habituação: dieta ↑ latência para comer e ↓ quantidade consumida. Teste: interação estresse*dieta na latência para chegar; dieta ↑ latência para comer e ↓ quantidade consumida.
Consumo alimento palatável - Chocolate	Habituação: dieta ↑ latência para comer e ↓ quantidade consumida. Teste: interação estresse*dieta na latência para comer e dieta↓ quantidade consumida.
Consumo alimento palatável – Froot Loops® (com retirada da HFD)	Habituação: dieta ↑ latência para comer e ↓ quantidade consumida. Teste: dieta↓ quantidade consumida.
Teste de alimentação suprimida pela novidade	Dieta ↑ latência para começar a comer pellet de ração.

A hipótese da presente tese foi de que o isolamento social no período pré-pubere, associado ao consumo precoce e contínuo de uma dieta hiperlipídica, levaria a alterações da plasticidade neural, modificando sistemas neuroquímicos e, através dessas modificações, ocasionando mudanças nos processos cognitivos e emocionais na idade adulta. Alguns de nossos achados mostraram que a exposição a esses fatores ambientais precocemente pode alterar a programação do SNC de forma prejudicial na idade adulta, causando importantes mudanças comportamentais.

São observadas diversas alterações comportamentais na literatura em modelos animais que utilizam o isolamento social como um estressor. Entretanto, tais estudos utilizam métodos drásticos, como o isolamento social contínuo do desmame até a idade adulta, e poucos estudos avaliam os efeitos de um isolamento social breve no período antes da puberdade e seus efeitos a longo-prazo, na idade adulta. Neste trabalho, encontramos que uma exposição a esse tipo de estresse na pré-puberdade foi capaz de alterar comportamentos como do tipo depressivo e prejudicar a memória de curta-duração e de trabalho na idade adulta de ratos machos. É importante considerar que neste trabalho de tese foram apenas estudados ratos machos e que, na experiência de nosso grupo, intervenções precoces, mesmo antes da puberdade, muitas vezes resultam em efeitos sexo-específicos. Assim, não é possível generalizar nossos resultados e estudos com fêmeas seriam de grande importância.

Na avaliação da memória de curta-duração, utilizamos a tarefa de reconhecimento de objetos, que se baseia na tendência natural dos roedores para explorar ambientes novos/objetos novos. Esta tarefa pode envolver avaliações tanto de memória de curta, como de longa-duração, dependendo do tempo de intervalo entre o treino (dois objetos apresentados) e o teste (troca de um dos objetos). Neste trabalho avaliamos somente a memória de curta-duração, com um intervalo de 15 minutos entre as exposições. Vários estudos têm demonstrado que o isolamento social crônico (período do desmame até a idade adulta) causa prejuízo neste tipo de tarefa, geralmente associado a uma memória de longa-duração (Bianchi et al., 2006, Fone & Porkess, 2008, Gaskin et al., 2014), e que a capacidade de discriminação nesse modelo pode diminuir somente após 1 hora de intervalo (Bianchi *et al.*, 2006), inclusive não sendo encontrado déficit nesta tarefa com intervalo de 1 minuto entre as sessões (Lapiz *et al.*, 2000). No entanto, os resultados observados neste trabalho de tese demonstraram que a

exposição ao isolamento social precoce durante uma semana já foi capaz de causar prejuízo seletivo na discriminação de objetos com um intervalo curto (15 minutos) entre as exposições. Essa redução na memória de reconhecimento de objetos nos animais isolados deve-se provavelmente ao comprometimento cognitivo, uma vez que não houve diferença significativa na atividade locomotora desses animais, assim como no tempo gasto explorando ambos os objetos no treino. Poderíamos especular que esse prejuízo na memória de reconhecimento de objetos se devesse a uma inflexibilidade cognitiva, impedindo a atenção a um novo estímulo, entretanto outras tarefas comportamentais deveriam ser realizadas para confirmar essa hipótese.

Além disso, neste trabalho demonstramos que a memória de trabalho, avaliada no labirinto em Y, também foi prejudicada nos animais isolados. Um estudo realizado por Shao e colaboradores (2015) com animais isolados por oito semanas, a partir do desmame, mostrou um desempenho prejudicado neste tipo de tarefa. Contudo, este é o primeiro estudo demonstrando nos efeitos na idade adulta de um isolamento precoce nesse tipo de memória.

Uma das consequências mais notáveis do isolamento social crônico na literatura é o aparecimento do comportamento do tipo depressivo (Hong et al., 2015, Wang et al., 2017b). Neste trabalho, mostramos que o isolamento social aplicado somente no período pré-pubere já foi capaz de induzir um comportamento do tipo depressivo na idade adulta. Nossos achados corroboram com os de Takatsu-Coleman e colaboradores (2013), que demonstraram que uma exposição aguda ao isolamento por 12 horas causa comportamento do tipo depressivo em camundongos. Dessa forma, a partir dos resultados analisados, observamos que a exposição a um estressor social precocemente pode causar alterações persistentes na idade adulta, aumentando os sintomas depressivos, além de causar prejuízos em tarefas cognitivas envolvendo a memória.

Essas alterações comportamentais podem estar relacionadas, uma vez que uma das características de transtornos depressivos inclui prejuízos cognitivos (Austin *et al.*, 2001).

Da mesma forma, observamos que o consumo crônico de uma dieta hiperlipídica (HFD) desde o desmame até a idade adulta também ocasionou um prejuízo na memória de curta-duração (sem alteração na memória de trabalho) e levou a um comportamento do tipo depressivo, juntamente com aumento da anedonia (no teste de preferência a sacarose). Além disso, o consumo desse tipo de dieta foi capaz de alterar o comportamento alimentar hedônico nesses animais, mostrando que eles possuem menor motivação para obter diferentes alimentos palatáveis, sendo que esse fator não é associado a uma alteração na palatabilidade.

Cabe ressaltar que foi observado em um estudo prévio que os animais com acesso a ambas dietas simultaneamente consomem mais dieta hiperlipídica comparada a ração padrão, mas isso não é refletido em maior ganho calórico (Arcego et al., 2014). Entretanto, esses animais possuem maior ganho de gordura abdominal, particularmente no grupo com associação entre estresse e HFD, observado pelo maior ganho de peso nesses animais.

Alguns estudos já demonstram a associação entre obesidade e sintomas depressivos, inclusive em crianças (Muhlig et al., 2016, Schwartz et al., 2016). Sabendo que a obesidade está intrinsecamente relacionada ao consumo aumentado de alimentos ricos em calorias, é possível que uma dieta rica em gordura possa estar envolvida nos mecanismos que predispõem a depressão. Essa relação entre obesidade e depressão muitas vezes é associada a fatores psicológicos e culturais. Entretanto, a partir dos resultados encontrados nesta tese, observamos que tanto mecanismos fisiológicos, como moleculares estão envolvidos nessa associação. Além disso, estudos em humanos vêm

sugerindo que a redução do consumo calórico provindo de gorduras e alimentos processados possa estar associada com uma melhora no humor e uma redução do risco de desenvolver depressão (Akbaraly et al., 2009, Brinkworth et al., 2009).

Estudos prévios utilizando modelos animais sugerem que o consumo exagerado de alimentos altamente calóricos contribui para o ganho de peso e obesidade em associação com sintomas depressivos (Abildgaard et al., 2011, Sharma & Fulton, 2013), o que está de acordo com nossos achados. De modo interessante, as alterações comportamentais observadas neste trabalho, como prejuízo cognitivo e motivacional, nos animais com acesso crônico a uma dieta hiperlipídica talvez possam estar correlacionadas ao comportamento do tipo depressivo. Estudos considerando transtornos de humor vêm sendo associados com prejuízos cognitivos, como em processos de memória e aprendizado, além de diminuição da motivação e da capacidade de sentir prazer em resposta a estímulos positivos (anedonia) (Austin et al., 2001, Grillo, 2016, Rizvi et al., 2016). Adicionalmente, estudos em roedores relacionam o fenótipo de depressão causada por uma dieta hiperlipídica com prejuízos cognitivos em diferentes tarefas relacionadas à memória (Winocur & Greenwood, 2005, Pathan et al., 2008), incluindo a memória de reconhecimento de objetos (Abildgaard *et al*., 2011).

A diminuição da motivação para buscar alimentos palatáveis observada nos animais com consumo crônico de uma dieta hiperlipídica, aqui observada nas tarefas realizadas com alimentos doces (Froot Loops® e chocolate), bem como a menor preferência por sacarose, interpretada como anedonia, pode estar relacionada com as características da depressão que normalmente são observadas em pacientes (Grillo, 2016, Rizvi et al., 2016). Adicionalmente, as vias relacionadas ao sistema de recompensa e o comportamento alimentar hedônico vêm sendo cada vez mais associadas com a sintomatologia da depressão, tanto em humanos (Tremblay et al.,

2005, Epstein et al., 2006, Nauczyciel et al., 2013), como em modelos animais (Chaudhury *et al.*, 2013).

A partir desse panorama comportamental observado, foi realizado o teste de reatividade ao sabor doce para avaliar se esses efeitos ligados à menor motivação e à anedonia poderiam ser devidos a um menor prazer desses animais em resposta ao sabor doce. Não foram encontradas, no entanto, diferenças significativas entre os grupos, mostrando que esses efeitos não são mediados pelas características de "gostar".

Um questionamento adicional que levantamos em relação a esses achados foi tomando em consideração a possível saciedade provinda da dieta hiperlipídica, uma vez que essa dieta possui maior conteúdo calórico quando comparado à dieta padrão, mesmo tendo em vista que os testes de comportamento alimentar foram realizados sob restrição alimentar. Para isso, repetimos a mesma tarefa com alimento palatável (Froot Loops®), porém com a retirada total da dieta hiperlipídica uma semana antes da realização da avaliação comportamental, sendo que esses animais foram mantidos somente com ração padrão durante toda a análise. Os resultados foram semelhantes aos obtidos anteriormente, confirmando que a menor motivação encontrada nesses animais não se deve a uma maior saciedade provocada pela dieta hiperlipídica e nem a uma menor palatabilidade ao sabor doce. Além disso, foi excluída a possibilidade de deficiência locomotora nesses animais, uma vez que na tarefa do Campo Aberto não houve diferenças significativas entre os grupos na atividade locomotora.

Outro indicativo que sustenta nossa hipótese relacionada à sintomatologia da depressão nesses animas foram os resultados obtidos no teste de "alimentação suprimida pela novidade". Essa tarefa, apesar de ser usualmente utilizada para avaliar efeitos de ansiedade, também pode ser relacionada ao comportamento do tipo-depressivo (Savalli *et al.*, 2015). No nosso caso, a diminuição da motivação para buscar o alimento no

centro do aparato no grupo de animais com a dieta hiperlipídica pode estar relacionado ao fenótipo depressivo, uma vez que, em estudos anteriores, já foi observado que esse mesmo grupo de animais, quando testados nas tarefas do campo aberto e labirinto em cruz elevado, que são tarefas clássica para avaliar comportamento do tipo ansioso, não apresentaram diferenças significativas em relação a esse parâmetro (Arcego *et al.*, 2013).

Nossos resultados estão de acordo com evidências na literatura, que demonstram que o consumo crônico de uma dieta hiperlipidica pode estar associado a uma redução de comportamentos motivados, já observados por uma diminuição da auto-administração de alimentos palatáveis em tarefas de condicionamento operante (Ibias et al., 2016, McNeilly et al., 2016) e prejuízos na escolha de prefência de lugar (tarefa preferência condicionada de lugar) para diferentes tipos de recompensas, incluindo sacarose (Tracy *et al.*, 2015) e drogas de abuso (Blendy et al., 2005, Morales et al., 2012). Esses dados em conjunto demonstram que o acesso contínuo a HFD parece diminuir o valor de estímulos recompensadores, tanto associados a drogas de abuso como a alimentos palatáveis; entrentanto, os mecanismos subjacentes a esses comportamentos ainda não foram esclarecidos.

Surpreendentemente, observarmos nesse trabalho que a associação dos fatores isolamento social e consumo de HFD reverte o prejuízo observado no desempenho na tarefa de reconhecimento de objetos em relação aos fatores isolados, sendo que o índice de discriminação entre o objeto novo/familiar apresenta-se em níveis similares dos controles. Da mesma forma, foram observadas algumas interações entre os fatores na avaliação do comportamento alimentar hedônico, no sentido de aumentar a motivação para a recompensa alimentar (latência para chegar ao alimento com Froot Loops® e

latência para comer o chocolate, quando os animais foram testados sem restrição alimentar).

Relacionando essas interações das tarefas comportamentais com os achados neuroquímicos realizados neste trabalho de tese, observamos que semelhantes interações entres os fatores (estresse e dieta) ocorreram na atividade da enzima $Na^{+}K^{+}$-ATPase, no imunoconteúdo de BDNF, MAPK/ERK e AKT total e fosforilada no córtex pré-frontal, assim como no imunoconteúdo do receptor do BDNF (TrkB) e sinaptofisina no hipocampo de ratos machos na idade adulta. Todas as interações seguem um perfil semelhante, os fatores quando aplicados isolados diminuem os níveis do parâmetro analisado, porém quando associados, os níveis retornam ou aumentam em relação aos valores do grupo controle. Assim, podemos observar que o isolamento social associado ao consumo de uma dieta hiperlipídica parece restaurar marcadores importantes para a plasticidade sináptica, tanto no córtex pré-frontal como no hipocampo, e isso poderia estar relacionado aos achados comportamentais, em que não ocorrem prejuízos na memória de reconhecimento de objetos quando os dois fatores estão associados. Entretanto, em relação à memória de trabalho e ao comportamento do tipo depressivo, essas interações não foram suficientes para melhorar a performance desse grupo nas tarefas avaliadas.

Uma possível explicação para essas interações seria o envolvimento dos fatores (estresse e dieta) na atividade do eixo HHA. No Capítulo I da presente tese, colocou-se a hipótese das ações do hormônio leptina no eixo HHA, diminuindo as respostas ao estresse (Heiman et al., 1997, Huang et al., 1998, Oates et al., 2000, Arvaniti et al., 2001). Uma vez que em resultados anteriores com o mesmo grupo experimental foi observado aumento da leptina em animais com HFD e diminuição em animais isolados no período pré-pubere (Arcego *et al.*, 2014), poderíamos esperar que quando os dois

fatores estivessem associados, o aumento da leptina pela HFD poderia minimizar os efeitos do isolamento social, e assim contribuir para os efeitos positivos observados na memória de curta-duração.

Outra possibilidade para explicar essas interações baseia-se na teoria de que os alimentos palatáveis, ricos em açúcares e gorduras, reduzem a ativação do eixo HHA e, dessa forma, diminuem as respostas ao estresse (Adam & Epel, 2007). Entretanto, para dietas ricas em gorduras, ainda não há um consenso sobre os seus efeitos no eixo HHA, como discutido na Introdução desta tese.

A partir disso, nós investigamos alguns marcadores relacionados à atividade do eixo HHA nesses grupos experimentais. Em relação ao imunoconteúdo do receptor de glicocorticóides (GR), não encontramos diferença significativa entre os grupos no córtex pré-frontal, porém no hipocampo esse receptor se encontra diminuído tanto no grupo isolado como no grupo com acesso à HFD, sem interação entre os fatores. Entretanto, os níveis plasmáticos de corticosterona basal não alteraram entre os grupos.

Para melhor investigarmos a hipótese da influência da ativação diferenciada do eixo HHA nas interações observadas, outras medidas neuroquímicas devem ser realizadas. Cabe ressaltar também que a ausência de alteração nos níveis basais de corticosterona não significa necessariamente a ausência dos efeitos desses fatores no funcionamento do eixo HHA, uma vez que essas medidas foram realizadas em uma situação basal e podem ser diferentes frente a uma exposição a estressores.

O que podemos concluir com os resultados encontrados é que quando os fatores como estresse e dieta são associados, ocorre uma programação encefálica diferente daquela que resultaria da aplicação desses fatores isoladamente. Além disso, apesar de parecer ocorrer um possível efeito positivo na memória de curta-duração com a interação de estresse e dieta hiperlipídica, muitos efeitos deletérios observados no grupo

com acesso à HFD não foram revertidos pela associação com o estresse (e da mesma forma com o grupo estresse isoladamente).

Acerca dos resultados neuroquímicos, relacionados ao consumo crônico de uma dieta rica em gordura isoladamente, eles mostraram que em geral houve uma diminuição de marcadores de plasticidade e de neurotransmissão no córtex pré-frontal, hipocampo e núcleo accumbens na idade adulta. Destas, a estrutura mais afetada foi o hipocampo.

No córtex pré-frontal observamos que a dieta hiperlipídica causou uma diminuição nos níveis de BDNF (mais marcante no grupo não associado ao estresse), enquanto que no hipocampo não houve alteração no imunoconteúdo dessa neurotrofina. Apesar disso, no hipocampo, houve uma interação entre os fatores isolados (estresse ou dieta) em relação ao receptor desta neurotrofina (receptor TrkB), cujos níveis encontraram-se diminuídos, porém retornam aos níveis do grupo controle quando os fatores estão associados. Por sua vez, no córtex pré-frontal não houve diferença entre os grupos nos níveis deste receptor. Esses resultados sugerem que a sinalização do BDNF possa ser reduzida pelos fatores estudados nessas duas estruturas através de mecanismos distintos. Além disso, diversos marcadores de plasticidade sináptica no hipocampo apresentaram-se diminuídos, tais como a neurotrofina-3, a β-III-tubulina (considerada um marcador neuronal), a SNAP-25 (proteína associada a vesículas sinápticas, envolvida no processo de exocitose), a PSD-95 (marcador de densidade pós-sináptica) e a sinaptofisina (proteína sináptica associada a vesículas; não diminui no grupo dieta associado ao estresse).

A diminuição significativa de importantes marcadores sinápticos com a dieta hiperlipídica reflete um prejuízo na neuroplasticidade desses animais na idade adulta. Como demonstrado no Capítulo II, essas alterações na plasticidade sináptica podem

estar relacionadas ao comportamento do tipo depressivo apresentado por esse grupo. Assim, esses achados estão de acordo com a hipótese dos mecanismos patofisiológicos da depressão, uma vez que prejuízos de plasticidade no hipocampo vêm sendo atribuídos a etiologia desse transtorno (Serafini *et al.*, 2014). Além disso, a formação hipocampal parece ter um papel especial no desenvolvimento da depressão, uma vez que pacientes depressivos muitas vezes apresentam uma redução do volume dessa estrutura encefálica (Sheline *et al.*, 1996), o que também é observado em modelos de depressão em roedores (Veena *et al.*, 2011), podendo esse fato estar relacionado com a redução da plasticidade e diminuição dos fatores neurotróficos.

Adicionalmente, uma redução da plasticidade sináptica também pode mediar processos envolvendo memória (Lante *et al.*, 2006). Dessa forma, o déficit cognitivo observado nos animais com acesso à HFD na tarefa de reconhecimento de objetos pode estar associado a uma diminuição da plasticidade neural hipocampal, além da diminuição do BDNF encontrada nesses animais no córtex pré-frontal, uma vez que essa tarefa possui também um envolvimento hipocampal, apesar da avaliação realizada nesta tese ser uma memória não-espacial de curta-duração (Gonzalez et al., 2014, Warburton & Brown, 2015).

Em relação ao imunoconteúdo de alguns marcadores de sistemas neurotransmissores, como o glutamatérgico e gabaérgico, foram observadas alterações no hipocampo de ratos com acesso à dieta hiperlipídica, tais como redução da subunidade GluA2 do receptor AMPA e da subunidade NR2B fosforilada do receptor NMDA, além de diminuição da enzima glutamato descarboxilase (GAD65), responsável pela síntese de GABA (ácido gama-aminobutírico), um importante neurotransmissor inibitório do SNC.

A diminuição das subunidades dos receptores AMPA e NMDA pode sugerir que esses animais possuem um prejuízo na neurotransmissão glutamatérgica no hipocampo, entretanto, mais estudos necessitam ser realizados para compreender melhor essa modificação. Alterações na atividade sináptica levam a mudanças na composição da densidade pós-sináptica (PSD), incluindo alteração em níveis de receptores (Keith & El-Husseini, 2008), sendo que nas sinapses excitatórias glutamatérgicas a principal proteína envolvida no ancoramento dos receptores é a PSD-95. Uma vez que alterações na quantidade de PSD-95 podem influenciar no recrutamento de receptores AMPA na sinapse, é possível que a diminuição observada da PSD-95 possa estar correlacionada a uma redução do imunoconteúdo da subunidade do receptor AMPA encontrada nos animais com acesso à HFD e, portanto, à regulação da plasticidade sináptica (Keith & El-Husseini, 2008). Adicionalmente, o provável prejuízo na neurotransmissão glutamatérgica desses animais pode também ser associado aos níveis reduzidos da neurotrofina-3. Essa neurotrofina está envolvida com aumento de correntes glutamatérgicas *in vitro* (Paul *et al.*, 2001), além de ativar a síntese de algumas proteínas envolvidas na modulação de conexões sinápticas e na plasticidade relacionada à potenciação de longa-duração (Je et al., 2005, Je et al., 2006).

Além disso, é possível que esses animais com acesso à HFD tenham uma diminuição na neurotransmissão inibitória no hipocampo, pois foi observada uma diminuição do imunoconteúdo da enzima GAD65, o que pode significar uma redução nos níveis de GABA, uma vez que ela é a enzima responsável por sua síntese. Associado a isso, alguns estudos têm reportado uma redução na função gabaérgica em pacientes com transtornos de humor (Karolewicz *et al.*, 2010), e também em modelos animais para depressão (Holm *et al.*, 2011), o que corrobora com os nossos achados comportamentais para esse grupo.

A dieta hiperlipídica também afetou o sistema dopaminérgico e canabinóide no núcleo accumbens, observado pela redução do imunoconteúdo dos receptores dopaminérgico-1 (D1) e canabinóide-1 (CB1). Apesar de não haver diferença nos níveis de dopamina e seus metabólitos nessa estrutura, é possível que a redução desses receptores possa se refletir na menor motivação hedônica apresentada pelos animais que consomem esse tipo de dieta. Além disso, a função do receptor D1 parece estar mais envolvida na ativação das respostas orientadas para a recompensa, enquanto que o receptor D2 possui um papel na avaliação da recompensa (D'Aquila, 2010, Galistu & D'Aquila, 2012). Como a dieta hiperlipídica não alterou o imunoconteúdo dos receptores D2, é possível que a menor ativação de receptores D1 possa estar relacionada aos achados comportamentais de redução da saliência de incentivo (na busca do alimento palatável), diminuindo assim a motivação. Entretanto, para afirmar isso, mais parâmetros necessitam ser analisados, incluindo avaliações *in vivo* da neurotransmissão dopaminérgica e canabinóide frente a um alimento palatável, além de um possível mecanismo sinérgico entre os receptores D1 e CB1 no comportamento hedônico desses animais.

Alguns estudos mostram que os receptores dopaminérgicos (D1 e D2) e CB1 interagem aumentando o disparo de potenciais de ação em neurônios no Nac (Seif et al., 2011). Além disso, camundongos nocaute para receptor CB1 em neurônios que expressam receptor D1 apresentam uma menor preferência para soluções doces comparados ao grupo controle (Terzian et al., 2011), mostrando que a interação entre o sistema dopaminérgico e canabinóide pode influenciar na modulação de comportamentos alimentares motivados. Uma possível diminuição nos receptores D1 e CB1 encontrada nos animais com acesso a HFD, também pode estar relacionada ao comportamento do tipo depressivo observado no Capítulo II nesse animais, uma vez que

muitos estudos vêm demonstrando que a perda de motivação e a anedonia encontradas em pacientes com depressão possam estar ligadas a uma disfunção no sistema de recompensa e motivacional, principalmente relacionada ao sistema mesolímbico dopaminérgico (Tremblay et al., 2005, Epstein et al., 2006, Nauczyciel et al., 2013). Além disso, o sistema canabinóide também pode influenciar nesse tipo de resposta, já que possui uma influência na neurotransmissão do sistema monoaminérgico, como serotoninérgico e dopaminérgico (Esteban & Garcia-Sevilla, 2012), assim como pode causar alterações na liberação de neurotransmissores importantes para a plasticidade encefálica, como o GABA e glutamato (Alger, 2002, Dow-Edwards & Silva, 2017).

Com relação à sinalização opióide no núcleo accumbens, não encontramos diferenças significativas entre os grupos para o imunoconteúdo do receptor μ-opióide. Sabendo que o sistema opióide influencia as respostas hedônicas relacionadas à palatabilidade (Berridge, 2009), esse resultado corrobora com os achados comportamentais observados na tarefa de reatividade ao sabor doce (que avaliou as respostas hedônicas relacionadas ao "gostar" em relação a uma solução doce), em que não houve diferença nessa característica nos grupos experimentais analisados.

A respeito dos efeitos neuroquímicos analisados com o isolamento social, observamos também um prejuízo em marcadores de plasticidade hipocampal, na idade adulta. Entretanto, parece que seus efeitos foram mais relacionados à via de sinalização PI3K/AKT ligada ao BDNF, observada pela redução do imunoconteúdo de TrkB (sem interação com a HFD) e da proteína AKT na forma fosforilada. Essa via de ativação está relacionada com o transporte e com a tradução de proteínas sinápticas (Yoshii & Constantine-Paton, 2010). Como relatado anteriormente, quando os fatores são associados (estresse e dieta) não ocorre alteração nos níveis de TrkB no hipocampo, que se mantêm semelhantes aos níveis do grupo controle. Apesar desse grupo apresentar-se

aparentemente protegido quanto a essa redução nos níveis de TrkB, esses animais possuem igualmente uma diminuição da ativação da via PI3K/AKT, demonstrada pela redução do imunoconteúdo da proteína AKT fosforilada.

Associado a isso, os animais isolados também apresentaram diminuição do imunoconteúdo de sinaptofisina e aumento da subunidade NR2A do receptor NMDA no hipocampo. Já no córtex pré-frontal, observamos uma diminuição nos níveis de $Na^{+}K^{+}$-ATPase somente nos animais isolados com acesso à ração padrão, e o mesmo padrão de interação foi observado no imunoconteúdo de MAPK/ERK e de AKT fosforilada. Todos esses fatores podem estar associados a diferenças na plasticidade sináptica, no hipocampo e no córtex pré-frontal. A redução dos níveis da enzima $Na^{+}K^{+}$-ATPase nos animais estressados pode estar relacionada a uma baixa excitabilidade neuronal, o que pode em parte explicar o desempenho prejudicado desses animais nas tarefas de memória avaliadas.

Notavelmente, os resultados acerca dos marcadores de plasticidade hipocampal nos animais isolados indicam um prejuízo nessa estrutura encefálica, mesmo que em menor proporção que o grupo com acesso a HFD. Da mesma forma, esses achados podem ser relacionados ao fenótipo depressivo encontrado nesses animais.

Além disso, no núcleo accumbens dos animais isolados no período pré-pubere, observamos uma diminuição do metabolismo dopaminérgico. Apesar de ser observado um aumento nos níveis de dopamina nesta estrutura, ocorre uma diminuição da razão dopamina/DOPAC, sendo o DOPAC um metabólito da dopamina. Essa razão diminuída significa que menor quantidade de dopamina é liberada na fenda sináptica e posteriormente metabolizada, ficando dessa forma, estocada nas vesículas sinápticas. Além disso, o imunoconteúdo do transportador de dopamina (DAT) encontra-se

aumentado neste grupo, sugerindo uma mais ágil recaptação da dopamina a partir da fenda sináptica.

Apesar dessa diminuição no metabolismo dopaminérgico no núcleo accumbens de ratos isolados, isso não se refletiu nos achados comportamentais relacionados à busca de alimento palatável. Sabendo que o isolamento social foi aplicado no início da vida e as análises comportamentais e neuroquímicas realizadas na idade adulta, é possível que esses resultados demonstrem uma regulação negativa compensatória da resposta do sistema dopaminérgico. Esta hipótese é baseada em estudos que observaram uma redução de 50% na atividade tônica dopaminérgica após exposição a um estressor ou na retirada da anfetamina (um fármaco responsável por aumentar os disparos neuronais tônicos no sistema dopaminérgico) (Chang & Grace, 2013, Belujon et al., 2016). Possivelmente, a diminuição do metabolismo dopaminérgico é resultado de uma menor ativação das projeções dopaminérgicas tônicas da área tegmentar ventral (VTA), uma vez que as medidas foram realizadas no estado basal. Entretanto, a resposta dopaminérgica fásica (ativada em resposta a um estímulo, como a exposição a um alimento palatável), não parece ter sido afetada nesses animais, pois não houve prejuízo na resposta à exposição a um estímulo (alimento palatável) na tarefa de comportamento alimentar hedônico.

Em suma, os resultados apresentados na presente tese demonstraram que o período correspondente à pré-puberdade se mostra uma fase sensível a fatores ambientais, como exposição a estresse e a uma dieta rica em gordura, levando a alterações a longo-prazo e predispondo ao aparecimento de psicopatologia como a depressão e alterações comportamentais associadas. Essas experiências precoces deixam marcas na programação neural, principalmente relacionadas a fatores neurotróficos e à plasticidade sináptica, incluindo modificações em alguns sistemas neurotransmissores

encefálicos, o que leva a alterações comportamentais cognitivas e emocionais relacionadas à memória, à depressão e ao comportamento alimentar na idade adulta. Das estruturas encefálicas avaliadas neste trabalho, o hipocampo apresentou-se bastante susceptível aos fatores ambientais precoces utilizados. Dessa forma, uma melhor compreensão dos mecanismos envolvidos nessas alterações pode contribuir para o desenvolvimento de estratégias de prevenção e/ou terapêuticas, diminuindo a vulnerabilidade ao aparecimento de psicopatologias na idade adulta causadas por adversidades ambientais precoces.

5. CONCLUSÕES

- O acesso precoce e crônico a uma dieta hiperlipídica levou a um prejuízo na memória de curta-duração, induziu comportamento do tipo depressivo, com aumento de anedonia, e alterou o comportamento alimentar hedônico, causando uma menor motivação para a busca de outros tipos de alimentos palatáveis na idade adulta. Também foram observadas diminuições em marcadores de plasticidade sináptica no hipocampo e no córtex pré-frontal. Além disso, a dieta hiperlipídica afetou marcadores da neurotransmissão glutamatérgica e gabaérgica, sugerindo uma diminuição da neurotransmissão hipocampal desses dois sistemas neurotransmissores. No núcleo accumbens, houve uma redução do imunoconteúdo de receptores D1 e CB1, nos animais com acesso a esse tipo de dieta.

- A exposição precoce ao isolamento social durante o período pré-pubere causou prejuízos cognitivos relacionados à memória de curta-duração e de trabalho, bem como induziu comportamento do tipo depressivo na idade adulta. Além disso, foram observadas alterações em marcadores de plasticidade sináptica no hipocampo e córtex pré-frontal, bem como uma sugestiva mudança na composição de receptores NMDA no hipocampo. No núcleo accumbens, o isolamento social diminuiu o metabolismo dopaminérgico na idade adulta.

- A interação entre estresse e dieta hiperlipídica reverteu o déficit cognitivo na memória de reconhecimento de objetos observado pelos fatores isolados. Da mesma forma, a interação preveniu a longo-prazo os efeitos deletérios dos fatores isolados na atividade da enzima $Na^{+}K^{+}$-ATPase, nos níveis de BDNF, imunoconteúdo de MAPK/ERK e AKT fosforilada no córtex pré-frontal, assim como no imunoconteúdo de sinaptofisina e no receptor TrkB no hipocampo.

6. PERSPECTIVAS

- Estudar a ontogenia das alterações no eixo hipotálamo-hipófise-adrenal;
- Avaliar diferentes aspectos das capacidades cognitivas nesses animais;
- Realizar um estudo epigenético referente às alterações encontradas;
- Avaliar diferenças sexo-específicas quanto à influência das intervenções ambientais utilizadas nesta Tese;
- Investigar possíveis fatores capazes de reverter o comportamento do tipo depressivo e as alterações neuroquímicas que observamos no modelo experimental utilizado nesta Tese.

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