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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:
BIOQUÍMICA

INVESTIGAÇÕES BIOQUÍMICAS E COMPORTAMENTAIS EM RATOS SUBMETIDOS AO ESTRESSE CRÔNICO VARIADO: PAPEL PROTETOR DAS VITAMINAS E & C

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica como requisito parcial para a obtenção do título de Doutor

Porto Alegre
Março de 2012

Dedico este trabalho a meus pais Sonia e Nilvar, incentivadores incondicionais de minha trajetória.

AGRADECIMENTOS

À minha amiga Profa. Angela Wyse, pela orientação que ultrapassa a tese, por ter me permitido sonhar, experimentar e realizar durante as minhas idas e vindas. Por compreender minhas inquietações e incentivar meu crescimento pessoal e profissional.

À Profa. Carla Dalmaz pelo carinho com que me recebeu em seu grupo de pesquisa, por me permitir a excursão pelo fascinante tema da depressão e pelas valorosas contribuições ao trabalho.

A todos os queridos colegas de laboratório 36 que conviveram comigo durante estes anos e que se tornaram grandes amigos. Agradeço pela solidariedade, pelo afeto, pelas longas conversas, pelas risadas, pelas discussões científicas, profissionais e sentimentais; obrigada por fazerem eu me sentir tão querida.

Aos colegas do laboratório 37 por dividirem comigo seu espaço de trabalho, e principalmente à Cristie pelo treinamento no modelo experimental.

Aos meus pais, que dedicaram a vida à educação dos filhos, obrigada por me ensinarem a beleza da ética, da dignidade e do trabalho. Obrigada pelo amor imensurável.

Ao Elias, pelo carinho, pela ternura, pela paz que me transmite, pelo silêncio que diz quase tudo, pela presença constante, pelo apoio e incentivo.

À Universidade Federal do Rio Grande do Sul, especialmente ao Departamento de Bioquímica, seus professores e funcionários pela formação de excelência que recebi desde a graduação e da qual tenho imenso orgulho.

Ao CNPq pelo apoio financeiro.

Àquele que ainda não descobri ao certo, mas sei que inquieta meu espírito, instiga meu coração e me leva à sempre buscar (Santo Agostinho).

RESUMO

A depressão é uma condição psiquiátrica relativamente comum, associada com significante morbidade e mortalidade. Embora essa patologia constitua um grande problema de saúde pública devido ao alto impacto psicossocial e socioeconômico a que está relacionada, seus determinantes psicológicos e neurobiológicos ainda não foram completamente elucidados. Considerando que eventos de vida estressantes são fatores de risco para o surgimento da depressão, o estresse crônico variado (ECV) tem sido amplamente utilizado como modelo animal de depressão. No presente trabalho, nós avaliamos o efeito do ECV sobre os seguintes parâmetros bioquímicos em cérebro e/ou sangue de ratos: níveis de citocinas pró-inflamatórias [interleucina-6 (IL-6), fator de necrose tumoral-α (TNF-α), óxido nítrico (NO) e proteína C reativa (PCR)]; atividades das enzimas acetilcolinesterase (AChE) e butirilcolinesterase (BuChe); parâmetros de estresse oxidativo [espécies reativas ao ácido tiobarbitúrico (TBARS), catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx)] e parâmetros de metabolismo energético [atividade dos complexos II (CII) e IV (CIV) da cadeia de transporte de elétrons e da enzima piruvato quinase]. Os níveis plasmáticos de homocisteína (HCY) e folato também foram determinados. Além disso, avaliamos possíveis alterações comportamentais induzidas pelo estresse. Resultados mostraram que houve uma indução do estresse oxidativo e diminuição na atividade da BuChE. Os níveis de HCY foram maiores nos animais estressados do que nos controles e os níveis de folato não foram alterados. O ECV não alterou os parâmetros do sistema imune avaliados em soro. Observamos um aumento nos níveis de citocinas pró-inflamatórias e da atividade da enzima AChE em hipocampo de ratos estressados. Utilizando o mesmo modelo de ECV, demonstramos que os complexos II e IV da cadeia transportadora de elétrons estão alterados em hipocampo e córtex pré-frontal de ratos, enquanto que a atividade da enzima piruvato quinase não foi alterada. Também demonstramos que a administração de vitaminas antioxidantes durante a indução do modelo experimental foi capaz de prevenir os danos observados na cadeia respiratória. Por fim, observamos que o ECV alterou de formas distintas as fases de aquisição e de retenção da memória e que os antioxidantes foram capazes de prevenir somente o dano induzido na fase de aquisição. O ECV também alterou a memória de trabalho e o prejuízo foi prevenido pelas vitaminas E e C. Com a finalidade de investigar os mecanismos responsáveis pelo prejuízo cognitivo, nós determinamos o imunoconteúdo de BDNF em hipocampos de ratos estressados. Os resultados não demonstraram nenhuma alteração em relação ao grupo controle. Nossos achados em conjunto, podem contribuir para a compreensão das bases neurológicas da depressão e para o desenvolvimento de novas terapias.

ABSTRACT

The major depressive disorder is a common psychiatric disorder; which is related to high morbidity and mortality. Although depression constitutes major public health problem due to the high socio-economic and psychosocial impact that is related, the psychological and neurobiological determinants have not been fully elucidated. Since life stressors contribute in some fashion to depression, chronic variable stress (CVS) has been used as an animal model of depression. In the present work, we evaluated the effect of CVS on the follow biochemical parameters in brain and/or blood of rats: levels of inflammatory markers [interleukin-6 (IL-6), tumor necrosis factorl-α (TNF-α), nitric oxide (NO) and C reactive protein (CRP)]; acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activities; oxidative stress parameters [thiobarbituric acid reactive species (TBARS), catalase (CAT), superoxide dismutase (SOD) and glutatione peroxidase (GPx)] and energy metabolism parameters [activities of complex II (CII) and IV (CIV) of respiratory chain and piruvate kinase activity] Plasmatic levels of homocysteine (HCY) and folate were also determined. Moreover, we evaluated the effect of CVS on behavior of animals in the water maze task. Results showed that there was na induction on oxidative stress and an inhibition of BuChE activity. The levels of HCY were higher in stressed animals than in controls and folate levels were not altered. CVS did not change the immune system parameters evaluated in serum. We observed an increase in the levels of cytokines and in the AChE activity in hippocampus of stressed rats. Using the same animal model, we demonstrated that the activities of CII and CIV of electron transport chain are altered in hippocampus and pre-frontal cortex, whereas the pituvate kinase activity was not changed. We also demonstrated that the administration of antioxidants during the period of stress induction was able in prevent the respiratory chain impairment. Lastly, we observed that CVS altered the acquisition and the retention phases of memory and that antioxidants have only been able to prevent the damage induced in the acquisition. Stress also changed the working memory and the damage was prevented by vitamins E and C. With the purpose of investigate the mechanisms involved in the cognitive impairment, we determined the immunocontent of BDNF in hippocampus of stressed rats. Our results showed no change in BDNF as compared with control group. Our findings together may contribute to understanding the neurological basis of depression and to development of new therapies.

LISTA DE ABREVIATURAS

ACh - acetilcolina

AChE - acetilcolinesterase

ACTH - hormônio adrenocorticotrófico

ADP - difosfato de adenosina

ATP - trifosfato de adenosina

AVP - arginina-vasopressina

BDNF - fator neurotrófico derivado de encéfalo

BuChE - butirilcolinesterase

CAT - catalase

COX - citocromo c oxidase

CPF - córtex pré-frontal

CRH - hormônio liberador de corticotrofina

DSM-IV - Diagnostic and Statistical Manual of Mental Disorders

ECV - estresse crônico variado

EO - estresse oxidativo

ER - espécies reativas

GABA - ácido gama-aminobutírico

GG - glicocorticóides

GPx - glutationa-peroxidase

GR - receptores glicocorticóides

GSH - glutationa

HCY - homocisteína

HPA- hipotálamo-pituitária-adrenal

IL - interleucina

INF- γ - interferon- γ

LCR - líquido cefalorraquidiano

LPS - lipopolissacarídeo

MCP-1 - quimiocina CCL2

MET - metionina

MR - receptores mineralocorticóides

NO - óxido nítrico

PCR - proteína C reativa

RL - radical livre

SI - sistema imune

SNC - sistema nervoso central

SOD - superóxido dismutase

TBARS - espécies reativas ao ácido tiobarbitúrico

TNF-α - fator de necrose tumoral-α

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1 INTRODUÇÃO

1.1 DEPRESSÃO

A depressão é uma condição comum, associada com significante morbidade e mortalidade e tem sido descrita como uma das condições médicas mais incapacitantes (CHARNEY and MANJI, 2004; SOLEIMANI et al., 2011). Este transtorno ocorre frequentemente em adultos jovens e pode apresentar um curso crônico, afetando negativamente o prognóstico de outras patologias como doenças cardiovasculares, diabetes e osteoporose (CHARNEY and MANJI, 2004). Dados da Organização Mundial de Saúde (World Health Organization – WHO) reportam que a depressão é a principal causa de anos vividos com incapacidade e a quarta causa de incapacidade produtiva, levando em conta a mortalidade prematura (WHO, 2001).

Estudos de prevalência em diferentes países mostram que a depressão é um transtorno frequente. A prevalência varia de 3,5% a 25,5% dependendo da região geográfica, dos métodos de diagnóstico e da coleta de dados (KESSLER et al., 2005; KOHN et al., 2005; VICENTE et al., 2006; LEIDERMAN et al., 2011; HIDAKA et al., 2012). A depressão também é um transtorno recorrente, aproximadamente 80% dos indivíduos que receberam tratamento para um episódio depressivo terão um segundo episódio, sendo quatro a mediana de episódios ao longo da vida (ANDERSON et al., 2000). A duração média de um episódio é entre 16 e 20 semanas e 12% dos pacientes têm um curso crônico sem remissão de sintomas (JUDD, 1997; SOUERY et al., 2007).

A depressão é considerada um grande problema de saúde pública devido ao alto impacto psicossocial e socioeconômico a que está relacionada, principalmente devido à redução da capacidade de trabalho e ao aumento dos gastos com saúde (WHO, 2006). Estima-se que os custos anuais associados a essa patologia cheguem a 44 bilhões de dólares, apenas nos Estados Unidos (GREENBERG et al., 1993).

De acordo com o Manual Diagnóstico e Estatístico de Transtornos Mentais (*Diagnostic and Statistical Manual of Mental Disorders, DSM-IV,* 2000), o diagnóstico de depressão requer que o paciente apresente pelo menos duas semanas de humor deprimido ou a perda de interesse ou prazer em praticamente todas as atividades, acompanhado de pelo menos quatro destes

sintomas: alterações de apetite, peso, sono (insônia ou hipersonia) ou de atividade psicomotora (retardo ou agitação); energia diminuída; sentimentos de inutilidade ou culpa; dificuldade de pensar, concentrar-se ou de tomar decisões; pensamentos recorrentes de morte, planos ou tentativas suicidas. Tais sintomas devem ter surgido recentemente ou ter piorado claramente em comparação ao estado prévio ao episódio da pessoa e devem persistir durante a maior parte do dia, em quase todos os dias, por pelo menos duas semanas, além de causar sofrimento ou prejuízo clinicamente significativo nas áreas social, ocupacional ou familiar.

No contexto das altas taxas de morbidade e mortalidade associadas aos transtornos depressivos, lamentavelmente os determinantes psicológicos e neurobiológicos da depressão ainda não foram completamente elucidados. Na maioria dos casos a depressão é idiopática, entretanto existem inúmeros fatores de risco como eventos de vida estressantes, anormalidades endócrinas (hipotireoidismo e hipercortisolismo), cânceres (pancreático, adenocarcinoma e câncer de mama) e efeitos colaterais de medicamentos (isotretinoína e interferon-α) (KRISHNAN and NESTLER, 2008; TRANCAS et al., 2010; WAGER-SMITH and MARKOU, 2011), além de fatores genéticos (LOHOFF, 2010).

1.2 EIXO HIPOTÁLAMO-PITUITÁRIA-ADRENAL

A hiperatividade do eixo hipotálamo-pituitária-adrenal (HPA) na depressão é um dos achados mais consistentes em psiquiatria (MELLO et al., 2007). Um significativo percentual de pacientes com depressão apresenta: concentrações aumentadas de cortisol (o glicocorticoide endógeno em humanos) no plasma, urina e líquido cefalorraquidiano (LCR); uma resposta exagerada do cortisol ao hormônio adrenocorticotrópico (ACTH); e um aumento das glândulas pituitária e adrenal (LEONARD, 2005; MELLO et al., 2007; ZUNSZAIN et al., 2011).

A atividade do eixo HPA é regulada pela secreção do hormônio liberador de corticotropina (CRH) e de arginina-vasopressina (AVP) pelo hipotálamo, que por sua vez ativam a liberação de ACTH pela pituitária (Figura 1). ACTH estimula a secreção de GCs (cortisol em humanos e corticosterona em roedores) pelo córtex adrenal e estes hormônios interagem com seus receptores em diferentes tecidos alvo, inclusive no eixo HPA, onde são responsáveis pelo *feedback* negativo na liberação de CRH e AVP pelo hipotálamo (ANTONI, 1993).

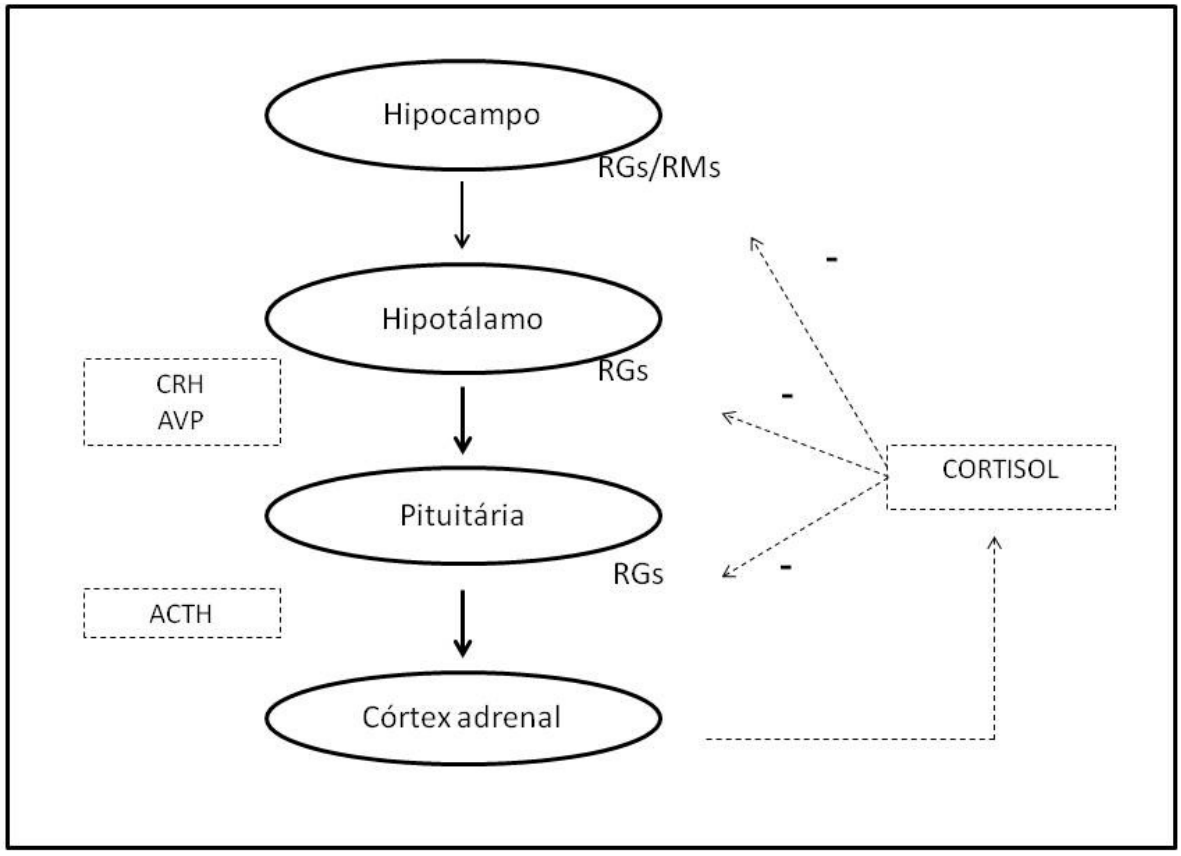

Figura 1. Diagrama esquemático do eixo hipotálamo-pituitária-adrenal, descrevendo a regulação e o feedback negativo (-) do cortisol via receptores de glicocorticoides (RG) e de mineralocorticoides (RM). ACTH: hormônio corticotrópico; CRH: hormônio liberador de corticotropina; AVP: arginina-vasopressina. (Adaptado de McQUADE and YOUNG, 2000)

1.2.1 Glicorticoides e estresse

Os GCs regulam funções corporais periféricas como metabolismo e imunidade, e representam a resposta endócrina clássica ao estresse. Por atuarem como um componente essencial da homeostase, seus níveis plasmáticos e teciduais precisam permanecer dentro de intervalos considerados ótimos. Quando uma estimulação constante e prolongada altera os padrões de concentração dos GCs podem ocorrer sequelas psicopatológicas, já que esses hormônios atuam na regulação da sobrevivência neuronal, neurogênese, aquisição de novas memórias, avaliação emocional de eventos e na resposta imune ao estresse (LEONARD, 2005; HERBERT et al., 2006; ZUNSZAIN et al., 2011).

O eixo HPA atua de formas diferentes diante de uma ativação causada por estresse agudo ou por estresse crônico. A secreção de glicocorticóides pela glândula adrenal é estimulada em resposta a uma variedade de estressores. Quando os níveis de glicocorticóides aumentam de forma moderada por um período de poucas horas, eles podem melhorar a cognição mediante a indução da plasticidade sináptica no hipocampo (MCEWEN and SAPOLSKY, 1995). Como nem todos os eventos que ocorrem no ambiente são consolidados em memórias, o aumento da plasticidade sináptica, induzido pelos GC, é um mecanismo adaptativo pelo qual o organismo foca em eventos emocionalmente importantes. Esses efeitos benéficos são mediados pelos receptores de corticóides de alta afinidade ou receptores mineralocorticoides (MR) encontrados em altas concentrações no hipocampo. Por outro lado, quando os níveis de GC aumentam em razão de estressores ambientais mais fortes e mais prolongados, estes esteróides produzem efeitos deletérios ao hipocampo. Tais efeitos são mediados por outro grupo de receptores, os receptores glicocorticoides (GR) que possuem baixa afinidade (LEE et al., 2002). A exposição excessiva aos GC pode ser diretamente tóxica aos neurônios ou pode potencializar o dano causado por outros insultos ao hipocampo como hipóxia/isquemia, convulsões ou hipoglicemia (SAPOLSKI, 1996).

O hipocampo é a estrutura cerebral que atua na intersecção dos circuitos límbicos, cognitivos/executivos e neuroendócrinos, incluindo o eixo HPA, desta forma, esta estrutura é particularmente vulnerável à depressão e consequentemente é a estrutura mais amplamente estudada. Entretanto, outras regiões cerebrais também parecem estar morfológica e funcionalmente alteradas. O córtex pré-frontal (CPF), a amígdala e o hipocampo funcionam como um circuito integrado e atuam tanto para regulação do humor quanto no aprendizado e na memória contextual. Dentro do CPF, a porção ventro-medial medeia dor, agressividade, comportamento sexual e apetite, enquanto a porção latero-orbital avalia riscos e modula comportamentos afetivos mal-adaptativos e de preservação. Muitos dos sintomas da depressão envolvem o mau funcionamento desta estrutura cerebral. Além disso, a região é rica em receptores de GC, o que a torna sensível à ação neurotóxica do excesso de GC (MCEWEN, 2005). Estudos de imagem demonstram que ocorre uma redução no volume de massa cinzenta no CPF em pacientes deprimidos quando comparados com controles saudáveis (DREVETS, 2000). O metabolismo e fluxo sanguíneo cerebral também estão alterados no CPF e são correlacionados com a gravidade dos sintomas depressivos (DEVRETS, 2000; 2001).

1.2.2 Estresse e depressão

Perturbações no sistema de estresse podem desencadear distúrbios cognitivos e emocionais que se assemelham a alguns sintomas observados nos pacientes com depressão. Entre as evidências que podem comprovar esta associação estão:

• a hiperatividade dos neurônios hipotalâmicos liberadores de CRH e AVP e a hiper-reatividade do eixo HPA resultantes do estresse crônico também ocorrem na depressão;

• os sinais neuroendócrinos da depressão podem ser distinguidos de outros transtornos psiquiátricos induzidos por estresse como o transtorno do estresse pós-traumático, que é caracterizado por hiperatividade de CRH/VPA e hipocortisolemia;

• a secreção aumentada de cortisol pode prejudicar a regulação da ansiedade e agressividade, e produzir déficit cognitivo que é associado com um fenótipo similar ao observado na depressão;

• a hipercortisolemia também prejudica o sistema monoaminérgico de uma maneira semelhante à depressão;

• o aumento nos níveis de cortisol causa redução no volume de estruturas do sistema límbico, que também é observada na depressão;

• a atividade do eixo HPA é um fator preditivo para a recaída ou remissão dos sintomas depressivos;

• administração intracerebrovascular de CRH induz fenótipos semelhantes à depressão e ansiedade;

• mutagênese dos GR e dos receptores CRH1 modula ansiedade, agressividade e desempenho cognitivo em camundongos;

• antidepressivos aumentam a expressão dos MR e GR no sistema límbico, ao mesmo tempo em que normalizam a atividade do eixo HPA;

• antagonistas dos receptores CRH1 melhoram os sinais e sintomas de depressão;

• antagonistas dos GR acentuam sintomas psicóticos;

• antagonistas dos MR inibem a ação de antidepressivos;

Estas evidências indicam que uma hiperatividade prolongada do eixo HPA e um desequilíbrio MR/GR desencadeados por estresse geram um fenótipo vulnerável à depressão (DE KLOET et al., 2005).

1.2.3 Modelo animal de depressão

Transtornos afetivos como ansiedade e depressão envolvem interações mal-adaptativas entre processos cognitivos (tomada de decisões, atribuição e recuperação de memórias) e fatores emocionais (condicionamento, motivação e reforço); esses transtornos muitas vezes são precipitados por circunstâncias estressantes. Além das experiências condicionantes e da exposição ao estresse, a definição de depressão também precisa levar em conta as diferenças individuais resultantes da predisposição genética para produzir

estes estados patológicos de comportamento (MINEKA and OEHLBERG, 2008).

Embora muitos progressos estejam sendo feitos, ainda estamos longe de determinar os antecedentes genéticos e moleculares subjacentes à depressão. Desta forma, os modelos animais utilizados para o estudo da depressão devem focar em sinais e sintomas (FERNADO and ROBBINS, 2011). A maioria dos modelos é baseada na exposição do animal a diferentes estressores, desde que como evidenciamos anteriormente, a depressão é fortemente influenciada por eventos estressantes e traumáticos ao longo da vida, sugerindo que os pacientes possam ter prejuízos em suas estratégias para lidar com situações aversivas (DE KLOET et al., 2005).

O modelo de estresse crônico variado (ECV) apresenta três importantes características que o diferenciam dos demais modelos: as condições para indução são relativamente realistas, o foco do modelo é um sintoma central da depressão, anedonia, e o curso prolongado é apropriado para investigar os efeitos de tratamentos crônicos (WILNER, 2005). Em 1981, Katz observou que, quando expostos a uma variedade de estressores relativamente graves por um período prolongado de tempo, os ratos reduziam a ingestão de líquidos doces, o que foi sugerido como reflexo de uma diminuição na reatividade hedônica. O modelo de Katz foi modificado, primeiro por razões éticas e segundo para mimetizar de forma mais realista os estressores da vida diária, assim, a intensidade dos estressores foi substancialmente diminuída. O modelo de ECV utilizado neste trabalho foi adaptado por Manoli e colaboradores (2000) e os estressores utilizados foram a) 24h de privação de água, b) 1 h a 3 h de imobilização (colocando o animal em um tubo plástico de 25x7cm, ajustado para impedir que o rato se mova), c)1,5 h a 2 h de imobilização a 4ºC, d) luz piscante durante 120 min a 210 min, e) isolamento durante 2 a 3 dias, f) inclinação das caixas moradia em um ângulo de 45º por 4-6h, g) maravalha suja (300 mL de água na caixa por 1,5-2h), por um período total de 40 dias. A exposição diária a estressores moderados ao invés da exposição a eventos traumáticos parece simular melhor a condição humana. Além disso, os animais apresentam sintomas do tipo depressivo similares aos observados em humanos e estes sintomas são revertidos por antidepressivos clássicos

(MANOLI et al., 2000). Desta forma, considera-se que o modelo de ECV tem bom valor preditivo (as alterações comportamentais são revertidas por antidepressivos), validade de face (muitos sintomas da depressão são observados no modelo) e validade de conteúdo ou constructo (ECV causa uma diminuição na reatividade à recompensa ou anedonia que é o principal sintoma da depressão) (WILNER, 1997).

1.3 SISTEMA IMUNITÁRIO E DEPRESSÃO

Trabalhos de pesquisa provenientes do campo da neuroimunomodulação vêm tornando explícitas as intrincadas relações existentes entre o sistema nervoso central (SNC) e o sistema imunitário (SI). Esses trabalhos suportam a existência de comunicações diretas e bidirecionais entre os dois sistemas (MAES et al., 2009; MILLER et al., 2009; ZUNSZAIN et al., 2011). O primeiro estudo a sugerir esta relação foi o trabalho do cientista Hans Seyle no ano de 1936, que cunhou o termo “estresse”. Neste artigo, republicado em 1998, Seyle descreveu o desenvolvimento de uma síndrome decorrente da exposição de um animal a um conjunto diversificado de estímulos nocivos; os achados de necropsia característicos dessa síndrome incluíam hipertrofia das glândulas adrenais, aparecimento de úlceras gástricas e, curiosamente para a época, atrofia de órgãos linfóides, como timo, baço e linfonodos. Como esses achados eram independentes do estímulo empregado, Seyle concluiu que os mesmos representavam uma resposta orgânica à injúria, denominando-os coletivamente de ‘síndrome de adaptação geral’, posteriormente chamada de estresse (Seyle, 1998). Mais recentemente, o cérebro deixou de ser considerado um órgão privilegiado do ponto de vista imune, inteiramente separado das células imunes circulantes pela barreira cérebro-sangue. Sabe-se que diversos estímulos provenientes do SNC são capazes de modular a resposta imune e que citocinas produzidas perifericamente podem atuar dentro do SNC (SCHIEPERS et al., 2005).

Estudos têm demonstrado que parâmetros pró-inflamatórios como interleucinas (IL-1, IL-2, IL-6, IL-8 e IL-12), interferon- γ (INF- γ) e fator de necrose tumoral-α (TNF-α) estão aumentados em pacientes com depressão

(SCHIEPERS et al., 2005). Por outro lado, a injeção de lipopolissacarídeo (LPS) (central ou periférica) em ratos e camundongos induz aumento na produção de citocinas pró-inflamatórias e neuroinflamação, o que pode resultar na chamada síndrome do comportamento doentio, que inclui sintomas específicos como anorexia, redução da atividade locomotora e exploratória, anedonia e distúrbios cognitivos muito similares aos observados na depressão (DANTZER, 2006; QIN et al., 2007; SONG and WANG, 2011).

O sistema endócrino – e, em especial, o eixo HPA – é um dos responsáveis pela ligação entre o SI e o SNC (LICINIO and FROST, 2000). A ativação do eixo HPA e a consequente produção dos glicocorticóides durante o estresse são um dos principais mecanismos responsáveis pelas alterações da resposta imune encontradas no decorrer deste processo. Os GC estão entre os hormônios anti-inflamatórios mais potentes do corpo humano (VINSON, 2009). No entanto, enquanto a administração de dexametasona suprime a proliferação linfocitária e a produção de IL-1β em indivíduos saudáveis, pacientes deprimidos não têm a mesma resposta supressora (MAES et al., 1991). Crianças com doenças alérgicas cutâneas ou asma apresentam níveis plasmáticos elevados de cortisol em reposta ao estresse e são mais suscetíveis à depressão (STERNBERG et al., 1989). Além disso, o estresse e a depressão têm sido associados no ser humano a reduções de imunidade e progressão dos sintomas da infecção por HIV (ADER, 2000). Em animais, a administração subcrônica de IL-1β causou aumento na concentração de corticosterona em soro de ratos (SONG et al., 2006). As alterações na função imune observadas na depressão sugerem o desenvolvimento de uma resistência imune aos GC (MAES et al., 1994; ALVES and PALERMO-NETO, 2007). Entretanto, o exato mecanismo envolvido ainda não foi elucidado.

1.4 SISTEMA COLINÉRGICO E DEPRESSÃO

A acetilcolina (ACh) é um neurotransmissor que está envolvido nos processos de cognição, atenção e excitabilidade neuronal. A atividade do neurotransmissor é finalizada pela ação hidrolítica de colinesterases (DARVESH et al., 2003). A acetilcolinesterase (AChE) e a butirilcolinesterase

(BuChE) são constituintes ubíquos do sistema colinérgico e diferem basicamente quanto à distribuição tecidual, propriedades cinéticas, especificidade por substratos e por inibidores (MASSOULIÉ et al., 1993). A AChE está presente em maior concentração no SNC, na junção neuromuscular e na membrana de eritrócitos, hidrolisando preferencialmente a acetilcolina (MASSOULIÉ et al., 1993). A BuChE é mais abundante no soro e embora não se conheça sua exata função fisiológica, a enzima tem a capacidade de hidrolisar rapidamente acetilcolina e de substituir a AChE na manutenção da integridade estrutural e funcional das vias colinérgicas centrais (MESULAM et al., 2002).

A integridade da transmissão colinérgica é fundamental para os processos de cognição e memória (MICHEAU and MARIGHETTO, 2011). Estudos farmacológicos em humanos demonstram que o bloqueio dos receptores muscarínicos por drogas como escopolamina prejudica a codificação de novas memórias, mas não a recuperação de memórias previamente armazenadas (ATRI et al., 2004; HASSELMO and McGAUGHY, 2004) e prejudica a memória de trabalho em algumas tarefas específicas (GREEN et al., 2005). Por outro lado, drogas que ativam os receptores nicotínicos melhoram a codificação de novas informações (BUCCAFUSCO et al., 2005; LEVIN et al., 2006).

Além de seu papel clássico na transmissão colinérgica, recentes evidências sugerem que a ACh está envolvida na chamada “via anti-inflamatória colinérgica”, onde atua inibindo a produção ou liberação de TNF-α, IL-1β, IL-6 e a migração de macrófagos (BOROVIKOVA et al., 2000). Por sua vez, a liberação de ACh (estimulada farmacológica ou eletricamente) não altera a síntese e secreção de citocinas anti-inflamatórias (por exemplo, IL-10) (BOROVIKOVA et al., 2000). Considerando que as atividades da AChE e da BuChE estão aumentadas em plasma e eritrócitos em várias condições inflamatórias sistêmicas (DAS, 2007) e que ACh é uma molécula anti-inflamatória, o aumento na atividade destas enzimas pode resultar em um estado pró-inflamatório. É importante ressaltar, que a depressão não afeta apenas o SNC, e que alterações periféricas como ativação da resposta imune, ativação do sistema renina-angitensina-aldosterona e alterações no sistema

colinérgico também podem ser importantes contribuintes para a patofisiologia deste transtorno.

Estudos demonstram que o estresse é capaz de induzir uma hiperexcitação colinérgica (SAPOLSKY, 1996; TRACEY, 2002) e têm-se demonstrado que há uma relação entre a atividade da BuChE e risco de doença arterial coronária (ALCANTARA et al., 2002), que é uma comorbidade comum à depressão (JOHNSON and GRIPPO, 2006). Em ratos, distúrbios no sistema colinérgico têm sido apontados como uma das causas das alterações comportamentais induzidas por estresse (SUNANDA et al., 2000; MIZOGUCHI et al., 2001; SRIKUMAR et al., 2006). Rada e colaboradores (2006) demonstraram que os níveis de Ach estão aumentados em animais submetidos ao nado forçado e que esta alteração é compensada pela ativação da AChE. Baseados nestes resultados, acredita-se que a capacidade de hidrolisar ACh possa estar envolvida nos prejuízos da resposta imune e da cognição observados em pacientes com depressão.

1.5 ESTRESSE OXIDATIVO E DEPRESSÃO

Um radical livre (RL) é uma estrutura química que possui um elétron desemparelhado, ou seja, ocupando um orbital atômico ou molecular sozinho. Isto o torna muito instável, extraordinariamente reativo e com enorme capacidade para combinar-se inespecificamente com as diversas moléculas integrantes da estrutura celular e derivados de cada uma delas (HALLIWELL and GUTTERIDGE, 2007).

Espécies reativas (ER) é o termo utilizado para designar radicais (por exemplo, ânion superóxido, radical hidroxila, óxido nítrico) e alguns não radicais derivados do oxigênio ou nitrogênio (peroxinitrito, peróxido de hidrogênio, oxigênio singleto). As ER podem ser produzidas por fontes endógenas ou exógenas, a principal fonte endógena é a redução do oxigênio molecular até água na mitocôndria durante a respiração celular, cerca de 2 a 5% do oxigênio consumido pode não receber todos os elétrons gerando ER de oxigênio. Alterações na cadeia de transporte de elétrons podem elevar ainda mais os níveis de radicais livres dentro da mitocôndria. Além disso, outras

fontes intracelulares de radicais livres são os peroxissomos, o retículo endoplasmático (contém o citocromo P-450) e os processos de fagocitose (SALVADOR and HENRIQUES, 2004); o processo inflamatório também é acompanhado pela produção de radicais livres (MAES et al., 2009). Os radicais livres possuem funções fisiológicas importantes, como sinalização celular, resposta imune e mitose, entretanto, por serem espécies muito instáveis têm o potencial de danificar proteínas, lipídeos, carboidratos e ácidos nucléicos (HALLIWELL, 2006). Esse dano pode resultar em apoptose ou necrose (AKSENOVA et al., 2005; HOVATTA et al., 2010).

O dano induzido pelas ER é normalmente controlado por sistemas antioxidantes naturais enzimáticos (superóxido dismutase [SOD], glutationa-peroxidase [GPx] e catalase [CAT]) e não enzimáticos (glutationa [GSH], vitaminas A, E e C, selênio e outros) (Figura 2). Quando ocorre um desequilíbrio entre a produção e a remoção das ER ocorre o que chamamos de estresse oxidativo (EO), que pode ser decorrente do aumento na produção de ER e/ou da diminuição das defesas antioxidantes.

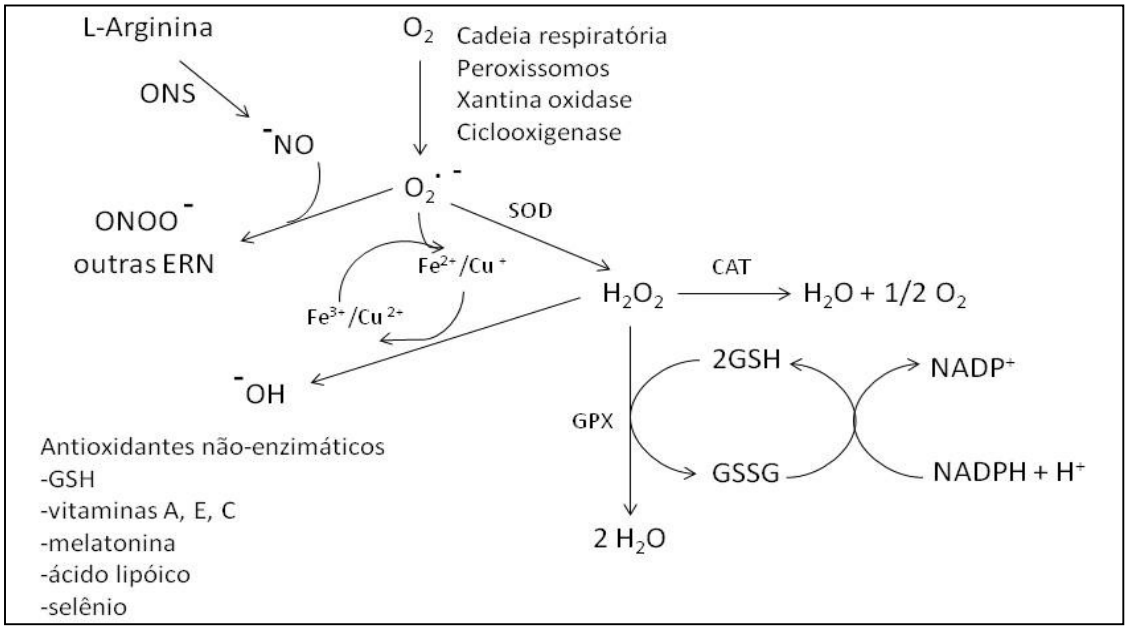

Figura 2. Principais vias de produção de espécies reativas e defesas antioxidantes. CAT: catalase; GPx: glutationa peroxidase; GSSG/GSH: glutationa oxidada/reduzida; $NADP^+$/NADPH: nicotinamida adenina dinucleotídeo fosfato; ONS: óxido nítrico sintase; ERN: espécies reativas de nitrogênio; SOD: superóxido dismutase. (Adaptado de HOVATTA et al., 2010)

O papel do EO está bem estabelecido na patogênese de diversas doenças como doença cardiovascular, isquemia/reperfusão, câncer, diabetes, e envelhecimento (HALLIWELL, 2006; NG et al., 2008). O cérebro é um órgão altamente suscetível ao EO por apresentar elevado consumo de oxigênio, grande concentração de neurotransmissores auto-oxidáveis, membranas neurais ricas em ácidos graxos poli-insaturados, alto nível de ferro e modesta defesa antioxidante (HALLIWELL, 2006). Dessa forma, também se tem cogitado um possível envolvimento do EO em doenças neurológicas (HALLIWELL, 2006; REYNOLDS et al., 2007) e neuropsiquiátricas (BOUAYED et al., 2009; HOVATTA et al., 2010), incluindo a depressão (CUMURCU et al., 2009; MAES et al., 2011).

Estudos clínicos já demonstraram que os níveis de vitaminas E e C estão reduzidos em soro de pacientes deprimidos (MAES et al., 2000; KHANZODE et al., 2003; OWEN et al., 2005). Além disso, drogas antidepressivas demonstram propriedades antioxidantes (BILICI et al., 2001; KHANZODE et al., 2003; HERKEN et al., 2007) e alguns antioxidantes têm efeitos antidepressivos (FERREIRA et al., 2008, BINFARÉ et al., 2009). Estudos pré-clínicos demonstraram que animais submetidos ao modelo de ECV também apresentaram alterações em alguns parâmetros de EO como níveis de espécies reativas ao ácido tiobarbitúrico (TBARS) e na atividade das enzimas SOD e GPx em diferentes regiões do cérebro (VASCONCELLOS et al., 2006). Também foi demonstrado que tanto a depleção de serotonina (CIOBICA et al., 2010) quanto a administração de corticosterona (SATO et al., 2010) promovem EO e consequentemente causam prejuízos na memória de ratos.

Embora o entendimento sobre a etiologia/patofisiologia da depressão tenha aumentado significativamente nos últimos anos, este aumento não se refletiu em importantes avanços em relação ao tratamento. Menos de dois terços dos pacientes atingem remissão com os tratamentos farmacológicos baseados no aumento dos níveis de serotonina, os medicamentos apresentam efeitos colaterais persistentes e um há um período longo entre o início do tratamento e a resposta clínica (NEMEROFF and OWENS, 2002; MAES et al., 2009; BARTOVA et al., 2010; RABL et al., 2010). Assim, novas alternativas de

tratamento precisam ser estudadas. Considerando os trabalhos sobre EO e depressão citados acima, a suplementação com antioxidantes passa a ser considerada como possível adjuvante no tratamento.

1.6 METABOLISMO ENERGÉTICO E DEPRESSÃO

Mitocôndrias são organelas celulares localizadas no citoplasma, cuja função fundamental é a produção de trifosfato de adenosia (ATP) na cadeia respiratória. A síntese de ATP é um processo que requer a ação de vários complexos enzimáticos localizados na membrana mitocondrial interna (Figura 3). Na maioria dos organismos a cadeia respiratória mitocondrial é formada por quatro complexos. A cadeia de elétrons é acoplada com a translocação de prótons da matriz mitocondrial para o espaço intermem branas, e o gradiente de prótons gerado é utilizado pela enzima ATP sintase para catalisar a formação de ATP a partir da fosforilação de difosfato de adenosina (ADP) (GARDNER and BOLES, 2011).

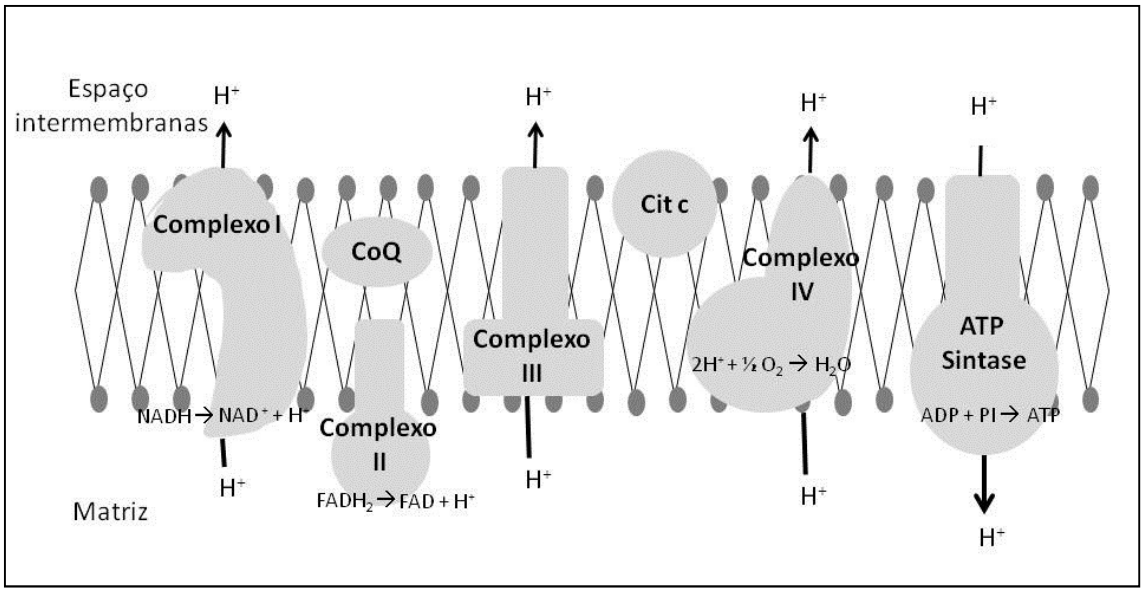

Figura 3. Complexos da cadeia transportadora de elétrons inseridos na membrana mitocondrial interna. CoQ: co-enzima Q; Cit c: citocromo c; ATP: trifosfato de adenosina; ADP: difosfato de adenosina; Pi: fosfato inorgânico. (Adaptado de GARDNER and BOLES, 2011).

Disfunções mitocondriais podem se originar, entre outras causas, de mutações (tanto no DNA nuclear dentro dos cromossomos, quanto no DNA mitocondrial), de deficiências nos cofatores necessários para a transferência de elétrons ou de alterações na fluidez da membrana mitocondrial (GARDNER and BOLES, 2011). Órgãos e tecidos que são altamente dependentes da produção de ATP, principalmente cérebro e músculos, são os primeiros afetados por disfunções mitocondriais e reduções no metabolismo oxidativo (SMEITINK, 2003). Alterações na função mitocondrial têm sido implicadas na patogênese de várias doenças que afetam o cérebro, como demência, isquemia cerebral, doença de Alzheimer e doença de Parkinson (HEALES et al., 1999; BLASS, 2001; SCHURR, 2002; MONSALVE et al., 2007; MOREIRA et al., 2007).

Por outro lado, pacientes com doenças mitocondriais hereditárias apresentam déficits de memória e alterações de atenção e das funções executivas e psicomotoras (TURCONI et al., 1999; BOSBACH et al., 2003). Muitos destes sintomas podem ser episódicos, e tipicamente ocorrem em situações onde há alta demanda energética associada com estressores fisiológicos como doenças, jejum, exercício prolongado e temperaturas ambientes extremas. Estressores psicológicos também podem desencadear a sintomatologia, inclusive episódios graves de depressão, possivelmente devido a uma alta demanda energética do cérebro que a produção de ATP não consegue suprir (GARDNER et al., 2003). Em geral, disfunções mitocondriais contribuem para neurodegeneração tanto por indução de apoptose quanto por geração de ER (McKENZIE et al., 2004).

A comorbidade entre as doenças mitocondriais e os transtornos psiquiátricos aponta na direção de um possível papel das disfunções mitocondriais na patofisiologia da depressão. Estudos *pos-mortem* relataram alterações na transcrição de genes ligados à função mitocondrial nos córtices frontal, pré-frontal e visual de indivíduos deprimidos (WHATLEY et al., 1996; KARRY et al., 2004). Os níveis da subunidade NDUFS7 do complexo I da cadeia respiratória também estão diminuídos em córtex pré-frontal, assim como a atividade do complexo I (ANDREAZZA et al., 2010). Alterações mitocondriais e a consequente morte celular também têm sido relacionadas com a atrofia do

hipocampo e do córtex pré-frontal observada em pacientes deprimidos (LEE et al., 2002; GARDNER and BOLES, 2011). Zhang e colaboradores (2006) sugerem que níveis elevados de cortisol induzem alterações no potencial de membrana mitocondrial que resultam em liberação de citocromo c da mitocôndria para o citoplasma, onde promove a ação de caspases que levam à apoptose.

Estudos em modelos animais também têm indicado uma associação entre metabolismo energético e depressão. Madrigal e colaboradores (2001) demonstraram que animais submetidos ao estresse por imobilização apresentaram uma inibição da atividade dos complexos I-III e II-III da cadeia respiratória em cérebro. Em concordância, Kanarik e colaboradores (2008) relataram que a denervação serotonérgica e sua combinação com estresse crônico por 3 semanas alteraram os níveis de citocromo c oxidase (COX) em diferentes regiões do cérebro de ratos.

Além da cadeia mitocondrial de transporte de elétrons, outros aspectos do metabolismo energético celular também podem estar associados com a fisiopatologia da depressão. A piruvato quinase é uma importante enzima tiólica da via glicolítica, fundamental para o provimento de energia para o cérebro e para a transmissão sináptica. Evidências sugerem que ATP sintetizado pela piruvato quinase associada às vesículas sinápticas é aproveitado para o transporte de glutamato, GABA, dopamina e serotonina para dentro das vesículas. É possível que o ATP gerado localmente pela glicólise forneça a energia necessária para o acúmulo vesicular dos neurotransmissores (ISHIDA et al., 2009) e alterações no fluxo dos neurotransmissores são relacionadas com depressão (FOLEY et al., 2006; TORDERA et al., 2007).

1.7 MEMÓRIA E DEPRESSÃO

O aprendizado e a memória são funções distintas do SNC, mas que estão intimamente ligadas, já que aquilo que aprendemos precisa ser conservado. Define-se aprendizagem como aquisição ou mudança relativamente estável de comportamentos ou processos mentais, devido a uma interação com o meio, experiência ou exercício. Por outro lado, a memória é

um processo cognitivo que consiste na capacidade de reter e recuperar informação. O processo mnésico divide-se basicamente em três estágios: a) aquisição, que é a entrada de conteúdos ou informações nos sistemas neurais ligados à memória; b) retenção ou consolidação, que consiste no armazenamento destes conteúdos por longos períodos; e c) evocação, processo pelo qual a informação armazenada pode ser requisitada para uso na cognição, emoção e/ou expressão de um comportamento. (IZQUIERDO, 2002; SQUIRE and KANDEL, 2003).

Recentes trabalhos teóricos e empíricos sugerem que a organização da memória no cérebro de mamíferos e os sistemas neurais que medeiam os processos cognitivos exercem um papel fundamental na estrutura dos pensamentos, emoções, escolhas, ações e até mesmo na personalidade. Estes circuitos neurais não contêm apenas resquícios do nosso passado, mas também exercem enormes influências em nosso comportamento futuro. Assim, os sistemas neuronais, em grande medida, determinam quem são os indivíduos e como estes se comportam em determinadas situações, exercendo um papel central nas manifestações normais e anormais do comportamento (McDONALD et al., 2004).

Muitos fatores contribuem para definir a forma como os sistemas de aprendizado e memória interagem um com o outro e com o resto do cérebro para afetar o comportamento. Entre estes fatores estão genética, eventos pré e pós-natais e as experiências acumuladas ao longo da vida. Disfunções nos sistemas neurais envolvidos na memória e aprendizagem contribuem para o desenvolvimento de distúrbios neuropsicológicos, incluindo esquizofrenia (HANLON and SUTHERLAND, 2000; LIPSKA and WEINBERGER, 2002); ansiedade (HARIRI et al., 2002) e depressão (McEWEN et al., 2002; SANTARELLI et al., 2003; SHELINE et al., 2003) e de outras condições como abuso de drogas (EVERITT et al., 2001; WHITE, 2002) e envelhecimento (McDONALD, 2002).

O hipocampo é uma estrutura tradicionalmente relacionada a processos cognitivos como aprendizado e memória (RIEDEL and MICHEAU, 2001) e como descrito anteriormente, é rico em receptores de GC, que respondem de formas distintas ao estresse agudo e crônico via regulação da plasticidade

sináptica. A exposição a GC por períodos prolongados leva a alterações neuroquímicas, morfológicas, de excitabilidade e inclusive à morte celular (McEWEN and MAGARINOS, 2001; SAPOLSKY, 2003; JOËLS et al., 2004; BREMNER, 2006; CONRAD, 2006; LUCASSEN et al., 2006). Assim, estudos têm demonstrado que pacientes deprimidos apresentam prejuízos em diferentes domínios cognitivos, incluindo memória (BEARDEN et al., 2006; VASIC et al., 2008), atenção (ZIHL et al., 1998) e funções executivas (STORDAL et al., 2004). Em modelos animais, o estresse crônico tem sido associado com déficits cognitivos observados em diferentes tarefas, como labirinto aquático de Morris (VASCONCELLOS et al., 2003), labirinto em cruz (SRIKUMAR et al., 2007) e labirinto em Y (ORSETTI et al., 2007). Entretanto, os mecanismos envolvidos nas alterações cognitivas observadas ainda não estão completamente elucidados.

A plasticidade neuronal, mediada por modificações na morfologia e funções sinápticas, parece ser um processo envolvido tanto na formação de memórias quanto nos processos de adaptação ao estresse (FUCHIKAMI et al., 2010). Há um número crescente de evidências pré-clínicas demonstrando que o estresse agudo diminui a expressão do fator neurotrófico derivado de encéfalo (BDNF) em estruturas límbicas que controlam o humor (BARRIENTOS et al., 2003; PIZARRO et al., 2004; ROCERI et al., 2004; FUCHIKAMI et al., 2010), e que o tratamento com antioxidantes é capaz de reverter ou bloquear os efeitos do estresse (DUMAN and MONTEGGIA, 2006), entretanto, poucos estudos tem demonstrado uma relação entre o estresse crônico e expressão de BDNF.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Com o objetivo de melhor compreender os mecanismos envolvidos nas alterações neurológicas e periféricas presentes na depressão e em outras doenças relacionadas ao estresse, o objetivo geral deste trabalho foi investigar o efeito do ECV sobre alguns parâmetros bioquímicos (níveis de citocinas pró-inflamatórias, atividade da AChE e da BuChe, parâmetros de estresse oxidativo e metabolismo energético) em cérebro e/ou sangue de ratos, bem como avaliar possíveis alterações comportamentais induzidas pelo estresse. Considerando que um dos maiores desafios em psiquiatria é o desenvolvimento de tratamentos mais eficazes para a depressão, também avaliamos o possível papel neuroprotetor dos antioxidantes vitaminas E e C sobre alguns destes parâmetros.

2.2 OBJETIVOS ESPECÍFICOS

2.1.1 Objetivo específico 1

Avaliar alguns parâmetros de estresse oxidativo (TBARS, CAT, SOD e GPx), assim como a atividade da enzima BuChE em sangue de ratos submetidos ao modelo de ECV. Determinar os níveis de homocisteína (HCY), folato e de marcadores de resposta inflamatória [IL-6, TNF-α, óxido nítrico (NO) e proteína C reativa (PCR)].

2.1.2 Objetivo específico 2

Investigar o efeito do ECV em alguns parâmetros do sistema imunitário, incluindo níveis de citocinas (IL-1 β, IL-6 e TNF- α) e da quimiocina CCL2 (MCP-1) em hipocampo de ratos. Determinar a atividade da enzima AChE na mesma estrutura cerebral.

2.1.3 Objetivo específico 3

Determinar as atividades das enzimas piruvato quinase e dos complexos II e IV da cadeia respiratória em hipocampo e córtex pré-frontal de ratos submetidos ao ECV e avaliar o papel neuroprotetor das vitaminas E e C sobre as alterações observadas.

2.1.4 Objetivo específico 4

Investigar o efeito do tratamento crônico com vitaminas E e C sobre os prejuízos causados pelo ECV na performance de ratos na tarefa do labirinto aquático de Morris e determinar o imunoconteúdo de BDNF no hipocampo destes animais.

3 MATERIAIS E MÉTODOS

Todos os experimentos bioquímicos e comportamentais apresentados nesta tese foram realizados em ratos adultos (60 dias) machos, submetidos ao modelo de ECV. O protocolo de estresse foi adaptado de Manoli e colaboradores (2000). Os animais foram submetidos a um estressor por dia, em diferentes horários, durante 40 dias. Os estressores utilizados foram: 24h de privação de água, 1-3h de contenção, 1,5-2h de contenção a 4ºC, luz piscante durante 120-210 min, isolamento (2-3 dias), inclinação das caixas moradia em um ângulo de 45º por 4-6h, maravalha suja (300 mL de água na caixa por 1,5-2h), de acordo com a tabela 1.

Os materiais e métodos utilizados nos experimentos estão descritos com detalhes em cada um dos artigos científicos.

Tabela 1 – Estressores utilizados

Dia	Estressor	Dia	Estressor
1	Contenção (4ºC)	21	Isolamento
2	Inclinação das caixas	22	Isolamento
3	Luz piscante	23	Contenção (4ºC)
4	Contenção	24	Privação de água
5	Isolamento	25	Inclinação das caixas
6	Isolamento	26	Contenção
7	Isolamento	27	Luz piscante
8	Maravalha suja	28	Contenção
9	Inclinação das caixas	29	Maravalha suja
10	Nenhum estressor	30	Nenhum estressor
11	Luz piscante	31	Privação de água
12	Privação de água	32	Inclinação das caixas
13	Contenção	33	Luz piscante
14	Maravalha suja	34	Contenção (4ºC)
15	Inclinação das caixas	35	Isolamento
16	Contenção (4ºC)	36	Isolamento
17	Luz piscante	37	Isolamento
18	Contenção	38	Luz piscante
19	Maravalha suja	39	Maravalha suja
20	Isolamento	40	Contenção

4 RESULTADOS

Os resultados desta tese geraram quatro publicações científicas de acordo com os objetivos propostos.

4.1 Artigo 1: Chronic variable stress induces oxidative stress and decreases butyrylcholinesterase activity in blood of rats

Publicado em: Journal of Neural Transmission (2010) 117:1067–1076

J Neural Transm (2010) 117:1067–1076
DOI 10.1007/s00702-010-0445-0

BASIC NEUROSCIENCES, GENETICS AND IMMUNOLOGY - ORIGINAL ARTICLE

Chronic variable stress induces oxidative stress and decreases butyrylcholinesterase activity in blood of rats

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Received: 6 April 2010 / Accepted: 9 July 2010 / Published online: 5 August 2010

Abstract Depressive disorders, including major depression, are serious and disabling, whose mechanisms are not clearly understood. Since life stressors contribute in some fashion to depression, chronic variable stress (CVS) has been used as an animal model of depression. In the present study we evaluated some parameters of oxidative stress [thiobarbituric acid reactive substances (TBARS), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx)], and inflammatory markers (interleukin 6, C reactive protein, tumor necrosis factor-alpha and nitrites), as well as the activity of butyrylcholinesterase in blood of rats subjected to chronic stress. Homocysteine and folate levels also were measured. Stressed animals were submitted to different mild stressors for 40 days. After CVS, a reduction in weight gain was observed in the stressed group, as well as an increase in immobility time in the forced swimming test as compared with controls. Stressed animals presented a significant increase on TBARS and SOD/CAT ratio, but stress did not alter GPx activity and any inflammatory parameters studied. CVS caused a significant inhibition on serum butyrylcholinesterase activity. Stressed rats had higher plasmatic levels of homocysteine without differences in folate levels. Although it is difficult to extrapolate our findings to the human condition, the alterations observed in this work may be useful to help to understand, at least in part, the pathophysiology of depressive disorders.

Keywords Depression · Oxidative stress · Inflammation · Butyrylcholinesterase · Homocysteine

Introduction

Major depression is a common, severe, chronic, and often life-threatening illness. There is a growing appreciation that, far from being a disease with purely psychological

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manifestations, major depression is a systemic disease with deleterious effects on multiple organ systems (Charney and Manji 2004). On the other hand, stressful life events have a substantial causal association with depression, and there is now compelling evidence that even early life stress constitutes a major risk factor for the subsequent development of depression (Charney and Manji 2004). The chronic variable stress (CVS) model of depression has high validity, since a large number of recent publications have confirmed that CVS causes behavioral changes in rodents that parallel symptoms of depression (Gamaro et al. 2008; Ni et al. 2008; Wilner 2005; Katz and Hersh 1981).

Despite extensive research, the current theories on serotonergic dysfunctions do not provide sufficient explanations for the nature of depression (Maes et al. 2009). In this context, both oxidative stress and inflammatory mediators have been suggested to contribute to the neuropathology of depression (Lucinio and Wong 1999; Cumurcu et al. 2009). Oxidative stress, characterized by the imbalance between production of free radicals and the antioxidant capacity of organism, has been implied in the pathogenesis of several psychiatric disorders such as schizophrenia, bipolar disorder, and depression (Ng et al. 2008). Free radicals are molecules that play physiological roles in cellular signaling, immunological responses, and mitosis. However, being highly unstable molecules with unpaired electrons, they have differential oxidative strengths and hence the potential to damage cellular proteins, lipids, carbohydrates and nucleic acids (Halliwell 2006). Chronic stress has been shown to cause oxidative damage in the central nervous system (CNS) (Lucca et al. 2009; Madrigal et al. 2001; Olivenza et al. 2000), but there is a lack of works investigating peripheral effects of CVS.

New developments in psychiatric research have led to the hypothesis that inflammatory processes and neural-immune interactions are involved in the pathogenesis of major depression (Maes et al. 2009). Studies have demonstrated that proinflammatory parameters such as interleukins (IL-1, IL-2, IL-6, IL-8 and IL-12), interferon-γ (IFNγ), and tumor necrosis factor-α (TNFα) are increased in patients with depression (Schiepers et al. 2005). However, other studies have failed to find an association between the immune system and depression (Haack et al. 1999; Steptoe et al. 2003), indicating the need for more investigations in order to confirm the activation of immune system as cause of depressive symptoms.

Acetylcholine (ACh) is the principal vagus neurotransmitter and its action is finished by hydrolysis catalyzed by acetylcholinesterase (AChE) (E.C.3.1.1.7) and butyrylcholinesterase (BuChE) (E.C.3.1.1.8) (Darvesh et al. 2003). In humans, AChE is more abundant in the CNS, end plate of skeletal muscle, and erythrocytes membranes, while BuChE is more abundant in serum (Massoulié et al. 1993). Although the exact physiological function of BuChE is unclear, it has been shown that it can promptly hydrolyze acetylcholine and to substitute AChE in maintaining the structural and functional integrity of central cholinergic pathways (Mesulam et al. 2002). In addition, reports from the literature suggest a relationship between BuChE activity and risk factors for coronary artery disease (Alcantara et al. 2002) and that heart disease and depression are highly co-morbid (Johnson and Grippo 2006). In relation to stress, Rada et al. (2006) demonstrated that ACh levels are elevated in animals subjected to forced swimming and that this alteration is compensated by AChE activation. Otherwise, stress insults induce hyperexcitation of cholinergic circuits (Tracey 2002; Sapolsky 1996).

Another alteration that has been related to depressed patients concerns Hcy metabolism (Jendricko et al. 2009; Levine et al. 2008; Tolmunen et al. 2004). Homocysteine (Hcy) is a sulfurated amino acid derived from ingested methionine. It is directly toxic to neurons and blood vessels and can induce DNA strand breakage, oxidative stress and apoptosis (Mattson and Shea 2003; Lipton et al. 1997). On the other hand, the methionine–homocysteine metabolic pathway produces methyl groups required for the synthesis of catecholamines and DNA. This is accomplished by remethylating homocysteine—using B12 and folate as cofactors—back to methionine. A recent study demonstrated that serum homocysteine levels correlate positively with cortisol levels (Cascalheira et al. 2008). Furthermore, there are evidences supporting an association of depression with high blood homocysteine in humans (Bottiglieri et al. 2000; Tolmunen et al. 2004; Folstein et al. 2007) though the results are still controversial.

Thus, in line of the foregoing considerations, in the present study, we evaluated some parameters of oxidative stress [thiobarbituric acid reactive substances (TBARS), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx)], and inflammatory markers [interleukin 6 (IL-6), C reactive protein (CRP), tumor necrosis factor-alpha (TNF-α) and nitrite (NO)], as well as the activity of butyrylcholinesterase in blood of rats subjected to chronic stress. Homocysteine and folate levels were also measured.

Materials and methods

Animals and reagents

Fifty-two (20 for oxidative stress measurements; 20 for inflammatory markers and BuChE; 12 for Hcy and folate assay), male Wistar rats (60 days old; 200–270 g weight) were obtained from the Central Animal House of the Department of Biochemistry of the Federal University of

Rio Grande do Sul, Porto Alegre, Brazil. The experimentally naive animals were housed in groups of 4–5 in home cages made of Plexiglas material (65 × 25 × 15 cm) with the floor covered with sawdust. They were maintained under a standard dark–light cycle (lights on between 7:00 and 19:00 h) at a room temperature of 22 ± 2°C. The rats had free access to food (standard rat chow) and water, except for the stressed group during the period when the stressor applied required no water. After being randomized to assure all groups presented similar body weights, the animals were divided into two groups: control and stressed. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

Stress model

The CVS protocol was applied as described by Gamaro et al. (2003) with some modifications in the stressors applied, such as inclination of the home cages instead of food deprivation and damp bedding instead of forced swimming. Control animals were handled daily. A variable-stressor paradigm was used for the animals in the stressed group. This protocol differs from other chronic stress protocols that use only one stressor in that the different stressors used diminish adaptation to stress (Marin et al. 2007). Animals were subjected to one stressor per day, at different times each day, in order to minimize predictability. The following stressors were used: (a) 24 h of water deprivation, (b) 1–3 h of restraint, as described below, (c) 1.5–2 h of restraint at 4°C, (d) flashing light during 120–210 min, (e) isolation (2–3 days), (f) inclination of the home cages at a 45° angle for 4–6 h, (g) damp bedding (300 mL water spilled onto bedding during 1.5–2 h). Restraint was carried out by placing the animal in a 25 × 7 cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1-cm hole at the far end for breathing. Exposure to flashing light was made by placing the animal in a 50-cm high, 40 × 60 cm open field made of brown polywood with a frontal glass wall. A 40-W lamp, flashing at a frequency of 60 flashes per minute, was used. Rats were submitted to chronic variate stress for 40 days as described in Table 1.

Table 1 Schedule of stressor agents

Day of treatment	Stressor applied
1	Cold restraint (1.5 h)
2	Inclination of home cages (4 h)
3	Flashing light (2 h)
4	Restraint (2 h)
5	Isolation
6	Isolation
7	Isolation
8	Damp bedding (2 h)
9	Inclination of home cages (6 h)
10	No stressor applied
11	Flashing light (2 h)
12	Water deprivation (24 h)
13	Restraint (3 h)
14	Damp bedding (3 h)
15	Inclination of home cages (4 h)
16	Cold restraint (2 h)
17	Flashing light (3 h)
18	Restraint (2.5 h)
19	Damp bedding (3 h)
20	Isolation
21	Isolation
22	Isolation
23	Cold restraint (1.5 h)
24	Water deprivation (24 h)
25	Inclination of home cages (4 h)
26	Restraint (3 h)
27	Flashing light (3 h)
28	Restraint (1 h)
29	Damp bedding (2 h)
30	No stressor applied
31	Water deprivation (24 h)
32	Inclination of home cages (6 h)
33	Flashing light (2 h)
34	Cold restraint (2 h)
35	Isolation
36	Isolation
37	Isolation
38	Flashing light (3 h)
39	Damp bedding (2 h)
40	Restraint (3 h)

After 40 days of stress, forced swimming test was performed according to Porsolt et al. (1977), in order to confirm the ability of this stress to increase the immobility time, an indicative of depressive behavior. The test involves two individual exposures to a cylindrical tank with water in which rats cannot touch the bottom of the tank or escape. The tank is made of clear Plexiglas, 50 cm tall, 30 cm in diameter, and filled with water (22–23°C) to a depth of 30 cm. Water in the tank was changed after each rat swimming test section. For the first exposure, rats were placed in the water for 15 min (pre-test session). Twenty-four hours later, rats were placed in the water again for a 5-min session (test session), and the immobility time of rats was recorded in seconds.

Body weight was measured at different times during treatment, since several works reported that chronic stress-induced significant reduction in body weight gain (Konarska et al. 1990; Harro et al. 2001).

Erythrocyte and plasma preparation

Erythrocytes and plasma were prepared from whole blood samples obtained from rats (controls and stressed rats) after decapitation.

Whole blood was collected and transferred to heparinized tubes for erythrocyte separation. Blood samples were centrifuged at 1,000×*g*, plasma was removed by aspiration and frozen at −80°C until determination of TBARS, HCy, and folate levels. Erythrocytes were washed three times with cold saline solution (0.153 mol/L sodium chloride). Lysates were prepared by the addition of 1 mL of distilled water to 100 μL of washed erythrocytes and frozen at −80°C until determination of the antioxidant enzyme activities.

For antioxidant enzyme activity determination, erythrocytes were frozen and thawed three times, and centrifuged at 13,500×*g* for 10 min. The supernatant was diluted in order to contain approximately 0.5 mg/mL of protein.

Thiobarbituric acid reactive substances

Usually, lipid peroxidation is quantified by measuring malondialdehyde (MDA), which is formed by the degradation products of polyunsaturated fatty acid hydroperoxides (Halliwell and Gutteridge 2006). The main source of MDA in biological samples is the peroxidation of polyunsaturated fatty acids. TBARS is a widely adopted method for measuring lipid oxidation (Ferreira et al. 2010; Kunz et al. 2008; Del Rio et al. 2005; Draper and Hadley 1990); however, the TBARS assay is not specific for MDA. This way, we expressed the results in terms of the amount of thiobarbituric acid reactive substances formed per unit of time instead of the amount of malondialdehyde produced. TBARS was determined according to the method described by Ohkawa et al. (1979) for in vivo studies. TBARS measures malondialdehyde (MDA), a product of lipoperoxidation caused mainly by hydroxyls free radicals. Plasma diluted in 1.15% KCl was mixed with 20% trichloroacetic acid and 0.8% thiobarbituric acid and heated in a boiling water bath for 60 min. TBARS were determined by the absorbance at 535 nm. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as that of the plasmas. TBARS was calculated as nanomoles of malondialdehyde formed per milligram of protein.

Catalase assay

CAT activity was assayed by the method of Aebi (1984). H_2O_2 disappearance was continuously monitored with a spectrophotometer at 240 nm for 90 s. One unit of the enzyme is defined as 1 mmol of hydrogen peroxide consumed per minute and the specific activity is reported as units per mg protein.

Superoxide dismutase assay

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on $O_2^{-\cdot}$, which is a substrate for SOD (Marklund 1985). The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results were reported as units/mg protein.

Glutathione peroxidase

GSH-Px activity was measured by the method of Wendel (1981), except for the concentration of NADPH, which was adjusted to 0.1 mM after previous tests performed in our laboratory. Tert-butylhydroperoxide was used as substrate. NADPH disappearance was continuously monitored with a spectrophotometer at 340 nm for 4 min. One GSH-Px unit is defined as 1 mmol of NADPH consumed per minute and specific activity is reported as units per mg protein.

Serum preparation

After decapitation, the blood was collected and centrifuged for 10 min at 1,000×*g*. The serum was used for the inflammatory marker assays and enzymatic (BuChE) analyses.

Cytokines (TNF-α and IL-6) assay

TNF-α and IL-6 levels in serum were quantified by rat high-sensitivity enzyme-linked immunoabsorbent assays (ELISA) with commercially available kits (Biosource®, Camarillo, CA).

Nitrite assay (NO)

Nitrite levels were measured using the Griess reaction; 100 μL of supernatant of hippocampus and cerebral cortex was mixed with 100 μL Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1%

naphthylethylenediamine dihydrochloride in water) and incubated in 96-well plates for 10 min at room temperature. The absorbance was measured on a microplate reader at a wavelength of 543 nm. Nitrite concentration was calculated using sodium nitrite standards (Green et al. 1982).

Acute-phase protein assay (CRP)

CRP levels in serum were determined by a colorimetric assay with commercially available kits (BioSystems® and Bioclin®, Brazil).

Butyrylcholinesterase activity assay

BuChE activity was determined by the method of Ellman et al. (1961) with some modifications. Hydrolysis rate was measured at acetylthiocholine concentration of 0.8 mM in 1 mL assay solutions with 100 mM potassium phosphate buffer pH 7.5 and 1.0 mM 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). Fifty microliters of rat diluted serum was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2 min (intervals of 30 s) at 25°C. All samples were run in duplicate. Specific enzyme activity was expressed as mmol acetylthiocholine hydrolyzed per hour per milligram of protein.

Homocysteine level determination

Hcy levels in plasma were determined as described by Magera et al. (1999), using liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS). After reduction and deproteinization of samples, Hcy concentration was detected through the transition from the precursor to the product ion (*m/z* 136 to *m/z* 90). Homocysteine-d was added as internal standard.

Folate levels determination

For folate determination, heparinized blood was collected and plasma was separated. Plasma folate concentration was measured by an automated chemiluminescence system (ACS: 180, Siemens). The method is based on a competitive immunoassay with acridinium ester-labeled folate in solid phase.

Protein determination

Protein was measured according to Bradford (1976) for butyrylcholinesterase assay and according to Lowry et al. (1951) for all others techniques. Serum bovine albumin was used as standard.

Statistical analysis

Data were analyzed by unpaired Student's *t* test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 15.0 in a PC-compatible computer. The results were expressed as mean ± SEM and differences were considered statistically significant if $P < 0.05$.

Results

The body weight of animals was evaluated before and after CVS. We verified that the control group gained more weight after 40 days than the stressed group [$t(18) = 2.763$; $P < 0.01$]. Chronic stress also increased immobility time in the forced swimming test when compared with controls [$t(18) = 3.066$; $P < 0.01$].

The effect of chronic stress upon the levels of TBARS in plasma was measured. As can be observed in Fig. 1, chronic stress increased TBARS levels [$t(14) = 5.100$; $P < 0.001$]. Figure 2a shows a significant increase in SOD/CAT ratio in the stressed group [$t(18) = 3.363$; $P < 0.05$]. To verify whether other antioxidant enzyme was compensating the imbalance verified between SOD and CAT, we determined glutathione peroxidase activity. As shown in Fig. 2b, stress did not alter GPx activity [$t(18) = 0.644$; $P > 0.05$].

We also investigated some inflammatory parameters in serum of rats subjected to CVS. Table 2 shows that these inflammatory parameters studied were not affected in stressed group when compared with controls [IL-6: $t(17) = 1.433$, $P > 0.05$; TNF: $t(18) = 1.644$, $P > 0.05$; NO: $t(18) = 3.363$; $P > 0.05$; PCR: $t(16) = 1.490$; $P > 0.05$].

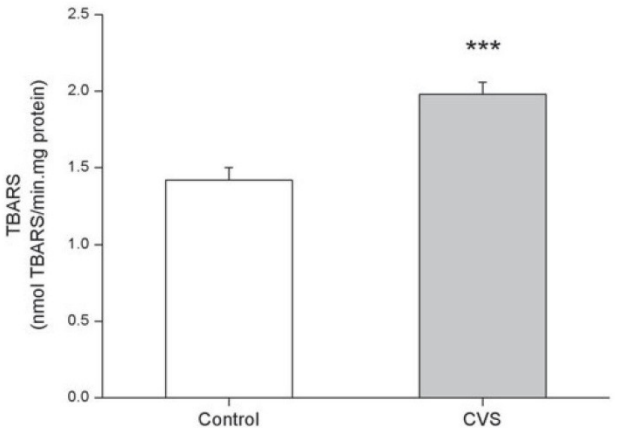

Fig. 1 Effect of chronic variable stress (CVS) on thiobarbituric acid reactive substances (TBARS) in plasma of rats. TBARS is expressed as nmol of thiobarbituric acid reactive substances per mg protein. Results are expressed as mean ± SEM for eight independent experiments performed in duplicate. ***$P < 0.001$ compared with control group (Student's *t* test)

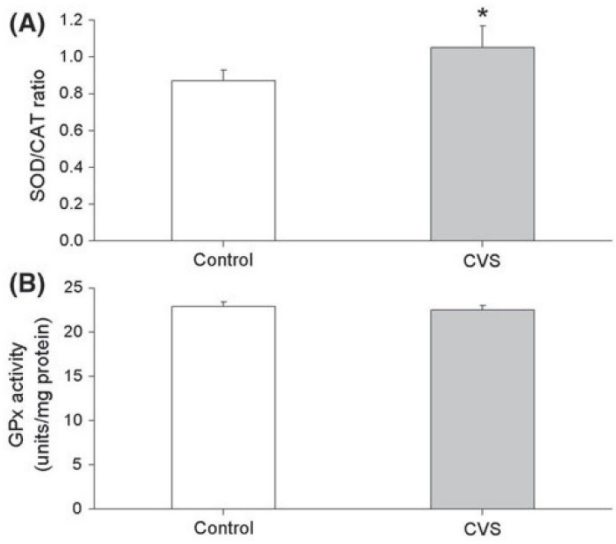

Fig. 2 Effect of chronic variable stress (CVS) on superoxide dismutase/catalase ratio (**a**) and on glutathione peroxidase activity (**b**). Results are expressed as mean ± SEM for ten independent experiments performed in duplicate. *$P < 0.05$ compared with control group (Student's *t* test). *SOD* superoxide dismutase, *CAT* catalase, *GPx* glutathione peroxidase

Table 2 Effect of chronic variable stress (CVS) on proinflammatory cytokines levels in serum of rats

Parameters	Control	CVS
IL-6 (pg/mL)	6.4 ± 0.95	6.1 ± 1.10
TNF (pg/mL)	1.5 ± 0.09	1.7 ± 0.11
NO (μM)	0.50 ± 0.08	0.59 ± 0.06
CRP (mg/L)	0.30 ± 0.06	0.17 ± 0.06

Results are mean ± SEM for 8–10 independent experiments (animals), $P > 0.05$

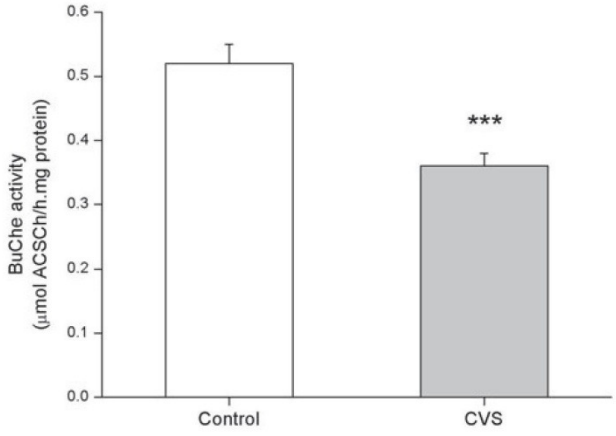

Fig. 3 Effect of chronic variable stress (CVS) on butyrylcholinesterase activity in serum of rats. Results are mean ± SEM for eight independent experiments performed in duplicate. ***$P < 0.001$ compared with control (Student's *t* test)

The effect of CVS on the activity of BuChE in serum of rats was also studied. Figure 3 shows that this enzyme was significantly inhibited in the stressed group [$t(14) = 4.793$; $P < 0.001$] as compared with the control group.

Plasma levels of Hcy and folate in animals submitted to CVS are demonstrated in Table 3. Results showed that the stressed group had higher plasma Hcy than the control group [$t(10) = 5.433$; $P < 0.001$], while no significant difference in folate concentration was detected between groups [$t(9) = 1.078$; $P > 0.05$].

Discussion

Major depressive disorder has traditionally been considered to have a neurochemical basis, but despite the devastating impact of the illness, little is known about its etiology or pathophysiology. However, several studies have demonstrated that although the monoaminergic neurotransmitter systems may be involved in this disease, they are limited in elucidating the pathogenesis of depression. It has been demonstrated that depression arises from the complex interaction of multiple susceptible (and likely protective) genes and environmental factors, and disease phenotypes include not only episodic and often profound mood disturbances, but also a range of cognitive, motoric, autonomic, endocrine, and sleep/wake abnormalities (Manji et al. 2001). These observations have led to the appreciation that although dysfunction within the monoaminergic neurotransmitter systems is likely to play important roles in mediating some facets of the pathophysiology of depression, there are other possible mechanisms involved (Maletic et al. 2007; Belmaker and Agam 2008).

The HPA axis and its final effector system, glucocorticoids, are essential components of an individual's capacity to cope with stress, and in fact, a hyperactivity of the HPA axis is observed in the majority of patients with depression (Gillespie and Nemeroff 2005; Bao et al. 2008; Swaab et al. 2005). Considering that life stressors contribute in some fashion to depression and are an extension of what occurs normally, chronic stress has been used as an animal model of depression (Gamaro et al. 2008; Ni et al. 2008) since animals displayed typical changes in hedonic status.

Table 3 Homocysteine (Hcy) and folate levels in plasma of rats submitted to chronic variable stress (CVS)

Parameters	Control	CVS
Homocysteine (μmol/L)	5.93 ± 0.5	9.93 ± 0.5***
Folate (μg/mL)	47.33 ± 4.0	52.5 ± 2.2

Results are mean ± SEM for 5–6 independent experiments (animals)
*** $P < 0.001$ compared with control (Student's *t* test)

Using this model, in the present study we evaluated some parameters of oxidative stress in plasma and erythrocytes of rats. We observed a significant increase in TBARS, a method that evaluates the oxidative stress assayed for malondialdehyde, the last product of lipid breakdown caused by oxidative stress (Halliwell and Gutteridge 2006). Beyond generating pathways, in this study, we pay attention to consuming pathways of free radicals, namely SOD, CAT, and GPx, the major enzymatic system responsible for protecting cells against free radical attacks (Halliwell and Gutteridge 2006). Animals exposed to stress presented an imbalance between SOD and CAT, expressed by increased SOD/CAT ratio. When a cell has decreased activity of CAT, a large amount of H_2O_2 (the product of SOD action) becomes available to react with transition metals and generates the radical hydroxyl, which is the most harmful radical (Kelner et al. 1995; Matés et al. 1999). On the other hand, stress did not alter the activity of GPx; this result reinforces the view that oxidative stress responses do not always involve a coordinated regulation of all antioxidant enzymes and that their activities are regulated by different mechanisms (Röhrdanz et al. 2000; Wilson and Johnson 2000).

These data are consistent with evidences that indicate that oxidative stress is a major pathological mechanism in the maladaptation to chronic stress in rats (Lucca et al. 2009; Madrigal et al. 2001; Olivenza et al. 2000). In this line, clinical studies also demonstrate an induction of oxidative stress in serum of depressed patients (Cumurcu et al. 2009; Khanzode et al. 2003; Bilici et al. 2001). Oxidative stress induction caused by chronic stress could be explained by several pathways, for example, through over-stimulation of glucocorticoids receptors (You et al. 2009; Zafir and Banu 2009), inhibition of mitochondrial electron transport chain complexes (Tagliari et al. 2010), and alterations on homocysteine metabolism (de Souza et al. 2006).

Recent studies have demonstrated that inflammatory and neurodegenerative processes play an important role in depression and that enhanced neurodegeneration in depression may—at least partly—be caused by inflammatory processes (Maes et al. 2009; Miller et al. 2009; Dantzer 2006; Schiepers et al. 2005). Based on these studies, in the present study, we evaluated the effects of stress on some inflammatory markers such as IL-6, TNF-α, NO, and PCR. Results showed that chronic stress did not alter any of the inflammatory parameters studied. Although clinical studies showed an increase of cytokines levels in blood of depressed patients, animal models using stress as model of depression have inconsistent results. In this context, Kubera et al. (1996) demonstrated increased blood levels of IL-1 and IL-2 after 8 weeks of mild stress. On the other hand, mice exposed to a 3-week chronic mild stress had decreased expression of peripheral IL-1beta and IL-6 and an increased expression of brain IL-6 (Mormède et al. 2003). In addition, another study has also reported elevated cytokine levels in brain of mice subjected to chronic mild stress for 5 weeks (Goshen et al. 2008). These inconsistent results concerning blood interleukins may be due to different stress protocols or different periods of stress exposure.

We also measured the activity BuChE in serum of animals submitted to chronic stress. Results showed that this enzyme was inhibited in stressed animals as compared with the control group. Moreover, since the results of the present study show an imbalance between CAT and SOD activities, what could result in increased levels of H_2O_2, our results are in agreement with other studies demonstrating that hydrogen peroxide can inhibit serum cholinesterase (Schallreuter and Elwary 2007).

Since there are data from literature showing that Hcy metabolism can be altered in depression and/or stressed patients (Jendricko et al. 2009; Levine et al. 2008; Tolmunen et al. 2004) and that this amino acid induces oxidative stress (Matté et al. 2009; Faraci and Lentz 2004; Wyse et al. 2002), we investigate the plasma levels of homocysteine and folate in control and stressed rats. Our results showed an increase in homocysteine levels in the stressed group; however, there were no differences in folate levels between control and stressed groups. Previous studies regarding Hcy metabolism in depression have provided contradictory results. Several works suggest that Hcy levels are increased in depressed patients (Wilhelm et al. 2010; Alexopoulos et al. 2010; Resler et al. 2008; Tolmunen et al. 2004; Reif et al. 2003; Bottiglieri et al. 2000); however, the lack of correlation between Hcy and depression has been demonstrated by Kelly et al. (2004). In most cases, however, increased levels of Hcy were observed in depressed patients with vitamin B12 or folate deficiency (Kim et al. 2008; Bottiglieri et al. 2000; Refsum et al. 2006). Our results are in agreement with studies that demonstrated increased levels of Hcy in the absence of folate deficiency after restraint stress in rats (de Souza et al. 2006). In addition, Triantafyllou et al. (2008) showed that multiple sclerosis patients that present elevated Hcy levels are particularly prone to develop depressive symptomatology. Previous studies from our laboratory have shown that BuChE can be inhibited by Hcy (Scherer et al. 2007; Matté et al. 2006), and this inhibition is mediated by the generation of free radicals (Stefanello et al. 2005). Therefore, the reduced BuChE activity could be related to the altered Hcy observed in the present study. Besides, elevated levels of Hcy increase oxidative stress (Matté et al. 2009; Wyse et al. 2002).

Besides augmenting the probability of oxidative damage, increased Hcy levels in stressed animal may ultimately

be related to an imbalance at the monoamine or neurotransmitter level, since the rise in Hcy levels could be ascribed to failure of methylation of Hcy to methionine. Methionine, in turn, is the precursor of *S*-adenosylmethionine, the methyl donor in a host of methylation reactions in the CNS involving monoamines and various neurotransmitters, amongst other cellular constituents.

In conclusion, we found in the present study that the CVS model of depression provokes an increase in oxidative stress, an inhibition of BuChE, and an increase in Hcy levels in blood of rats. Since the pathophysiology of depression still is poorly understood, if confirmed in humans, our results could be useful to explain some symptoms observed in patients.

Acknowledgments We thank Lucas P. Mocelin for his technical assistance. This work was supported in part by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) and by the FINEP Research Grant "Rede Instituto Brasileiro de Neurociência (IBN-Net), # 01.06.0842-00".

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4.2 Artigo 2: Chronic variable stress alters inflammatory and cholinergic parameters in hippocampus of rats

Publicado em: Neurochemical Research (2011) 36:487–493

Neurochem Res (2011) 36:487–493
DOI 10.1007/s11064-010-0367-0

ORIGINAL PAPER

Chronic Variable Stress Alters Inflammatory and Cholinergic Parameters in Hippocampus of Rats

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Accepted: 5 December 2010 / Published online: 24 December 2010

Abstract In the present study we investigated the effect of chronic variable stress (CVS) on some parameters of the immune system, including levels of cytokines [interleukin 1β (IL-1 β), interleukin 6 (IL-6), tumor necrosis factor α (TNF- α)] and chemokine CCL2 (MCP-1) in the hippocampus of rats. Acetylcholinesterase activity was also evaluated. Sixty-day old Wistar rats were submitted to different mild stressors for 40 days. After the last stress section, the cytokines and MCP-1 were determined by immunoassay and acetylcholinesterase activity by colorimetric method. Results showed that chronic stress significantly increased the levels of IL-1β, IL-6 and TNF-α, but did not alter the levels of MCP-1. In addition, acetylcholinesterase activity was increased in the hippocampus of rats subjected to CVS. These findings suggest that inflammation and cholinergic dysfunction may be, at least in part, important contributors to the neurological dysfunction observed in some depressed patients.

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Keywords Chronic variable stress · Depression · Neuroinflammation · Acetylcholinesterase

Introduction

Stress is defined as an integrated bodily response that is produced to deal with extraordinary circumstances. While appropriate stress responses help in facing up to diseases and are beneficial, severe and prolonged stress can be harmful [1]. Thus, the stress response and major depression share many features, both associated with a decline of cognitive and affective flexibility, alterations in arousal, and perturbations in neuroendocrine and autonomic function [2].

The stress response is associated with activation of the hypothalamo-pituitary-adrenal (HPA) axis and an increase in the level of glucocorticoids (GC) [3]. These hormones are considered to be anti-inflammatory, immunosuppressive and immunomodulatory under normal conditions. However, studies in the central nervous system (CNS) have demonstrated that glucocorticoids can augment aspects of inflammation, like cell extravasation and migration, microglia proliferation, inflammatory messengers and transcription factors [4, 5].

Recent evidence supports the hypothesis that inflammatory responses have an important role in the pathophysiology of depression. In this context, studies have shown that patients with major depression can present activation of inflammatory pathways [6–9]. However, there are still controversies about the so-called "cytokine hypothesis of depression", since studies exist that do not find any association between immune activation and depression [10, 11] and some positive studies have failed to find a correlation between inflammation and depressive severity [7, 12]. On the other hand, few animal studies have

been performed to investigate the role of inflammation on depression and other stress-related states.

Acetylcholine (ACh) is a neurotransmitter that is involved in cognitive processing, arousal, and attention in the brain. Activity of Ach in the brain is terminated by the hydrolytic action of cholinesterases, mainly acetylcholinesterase (AChE) [13] (E.C.3.1.1.7) [14]. Recent evidence suggests that AChE may have important roles beyond the 'classical' co-regulation of ACh-mediated neurotransmission, including the "cholinergic anti-inflammatory pathway", where acetylcholine acts by inhibiting the production of TNF-α (tumor necrosis factor alpha), IL-1β (interleukine-1 beta) and macrophage migration inhibitory factor [15]. On the other hand, cholinergic dysfunction is identified as the underlying cause of the behavioral deficits following stress [16–18]. Based on this knowledge, we predicted that variations in the capacity for hydrolyzing ACh might be causally involved in both the immune response and neurotransmission deficits observed in depressed patients.

Considering that chronic variable stress (CVS) causes behavioural changes in rodents that parallel symptoms of depression, the objectives of this study were to investigate the levels of some proinflammatory cytokines named TNF-α, IL-1β, IL-6 (interleukine 6) and chemokine CCL2 [MCP-1 (monocyte chemoattractant protein-1)] and AchE activity in the hippocampus of adult rats submitted to this animal model. Hippocampus was used because of its rich concentration of receptors for glucocorticoids and because depressed patients present morphological alterations in this structure [19, 20] as well as impairment in learning and memory processes [17].

Experimental Procedure

Animals and Reagents

Adult, male Wistar rats (60 days old; 200 g weight) were used. The experimentally-naive animals were housed in groups of 4–5 in home cages made of Plexiglas material (65 × 25 × 15 cm) with the floor covered with sawdust. Animals were maintained in a controlled environment: standard dark/light cycle (lights on between 07:00 h and 19:00 h), temperature of 22 ± 2°C. The rats had free access to food (standard rat chow) and water, except for the stressed group during the period when the stressor applied required no water. After being randomized to assure all groups presented similar body weights, the animals were divided into two groups: control and stressed. Animal care followed the "EC Directive 86/609/EEC for animal experiments" and was approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul, Brazil (project number 2008102). All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

Stress Model

CVS protocol was applied, as described by Gamaro et al. [21], with some modifications in the types of stressors applied, such as inclination of the home cages instead of food deprivation and damp bedding instead of forced swimming. Control animals were handled daily. A variable stressor paradigm was used for the animals in the stressed group. This protocol differs from other chronic stress protocols that use only one stressor in that the different stressors used diminished adaptation to stress [22]. Animals received one stressor per day, at different times each day, in order to minimize predictability. The following stressors were used: (a) 24 h of water deprivation, (b) 1–3 h of restraint, as described below, (c) 1.5–2 h of restraint at 4°C, (d) flashing light during 120–210 min, (e) isolation (2–3 days), (f) inclination of the home cages at a 45° angle for 4–6 h, (g) damp bedding (300 ml water spilled onto bedding for 1.5–2 h). Restraint was carried out by placing the animal in a 25 × 7 cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. Exposure to flashing light was carried out by placing the animal in a 50 cm-high, 40 × 60 cm open field made of brown plywood with a frontal glass wall. A 40 W lamp, flashing at a frequency of 60 flashes per minute, was used. Rats were submitted to CVS for 40 days, as described in Table 1.

Forced Swimming Test

After 40 days of stress, the forced swimming test was performed according to Porsolt et al. [23], in order to confirm the effectiveness of the CVS procedure to increase the immobility time, an indicative of depressive behavior. The test involves two individual exposures to a cylindrical tank with water in which rats cannot touch the bottom of the tank or escape. The tank is made of clear Plexiglas, 50 cm tall, 30 cm in diameter, and filled with water (22–23°C) to a depth of 30 cm. Water in the tank was changed after each rat swimming test session. For the first exposure, rats were placed in the tank for 15 min (pre-test session). Twenty-four hours later, rats were placed in the tank again for a 5 min session (test session), and the immobility time was recorded in seconds.

Adrenal Gland and Body Weight

Body weight was measured at different times during treatment, since several studies have reported that chronic stress induced significant reduction in body weight gain [24, 25]. Animals were sacrificed 24 h after the last stress session. After

Table 1 Schedule of stressor agents

Day of treatment	Stressor applied
1	Cold restraint (1.5 h)
2	Inclination of home cages (4 h)
3	Flashing light (2 h)
4	Restraint (2 h)
5	Isolation
6	Isolation
7	Isolation
8	Damp bedding (2 h)
9	Inclination of home cages (6 h)
10	No stressor applied
11	Flashing light (2 h)
12	Water deprivation (24 h)
13	Restraint (3 h)
14	Damp bedding (3 h)
15	Inclination of home cages (4 h)
16	Cold restraint (2 h)
17	Flashing light (3 h)
18	Restraint (2.5 h)
19	Damp bedding (3 h)
20	Isolation
21	Isolation
22	Isolation
23	Cold restraint (1.5 h)
24	Water deprivation (24 h)
25	Inclination of home cages (4 h)
26	Restraint (3 h)
27	Flashing light (3 h)
28	Restraint (1 h)
29	Damp bedding (2 h)
30	No stressor applied
31	Water deprivation (24 h)
32	Inclination of home cages (6 h)
33	Flashing light (2 h)
34	Cold restraint (2 h)
35	Isolation
36	Isolation
37	Isolation
38	Flashing light (3 h)
39	Damp bedding (2 h)
40	Restraint (3 h)

killing the animals, the adrenal gland weight was evaluated as an indirect parameter of HPA axis activation [26].

Tissue Preparation

After decapitation, the brain was quickly removed and hippocampi were dissected and homogenized 1:5 (w/v) in saline solution (0.9% NaCl). The homogenate was centrifuged at 800 × g for 10 min at 4°C and the supernatant was used in cytokines assays. For the AChE assay, hippocampi were homogenized 1:10 (w/v) in 0.5 M potassium phosphate buffer pH 7.5 and centrifuged for 10 min at 1000×g.

Cytokines (TNF-α, IL-1β, IL-6) and Chemokine CCL2 (MCP-1) Assay

TNF-α, IL-1β, IL-6 and MCP-1 levels in hippocampus were quantified by Multiplexed Immunoassay with a commercially available kit, and analyzed on a Luminex 200® ™.

Acetylcholinesterase

AChE activity was determined according to Ellman et al. [27], with some modifications. Hydrolysis rates were measured at an ACh concentration of 0.8 mM in 1 mL assay solutions with 30 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB at 25°C. About 50 μL of rat hippocampus supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). All samples were run in duplicate.

Protein Determination

Protein was measured according to Bradford [28] for the AChE assay and according to Lowry et al. [29] for all other techniques. Serum bovine albumin was used as standard.

Statistical Analysis

Data are mean ± SD for five animals in each group and were analyzed by Student's t test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software version 15.0 in a PC compatible computer. A $P < 0.05$ was considered significant.

Results

Body weight, before and after chronic mild stress, was measured. As can be observed in Table 2, the control group gained more weight after 40 days than the stressed group in the same period [$t(18) = 2.91$; $P < 0.01$]. Adrenal gland weight was also measured as an index of chronic stress. Results showed that this gland weight was significantly increased, when compared to that of the control group [$t(18) = 4.33$; $P < 0.001$]. The effect of chronic stress

Table 2 Effect of chronic variable stress (CVS) on body weight and adrenal gland weight of rats

	Control	CVS
Variantion of body weigth (g)	76.5 ± 16.3	56.5 ± 14.3**
Adrenal gland weigth (mg)	20.6 ± 2.1	26.1 ± 3.4***

Results are mean ± SD for 10 independent experiments (animals)
** $P < 0.01$; *** $P < 0.001$

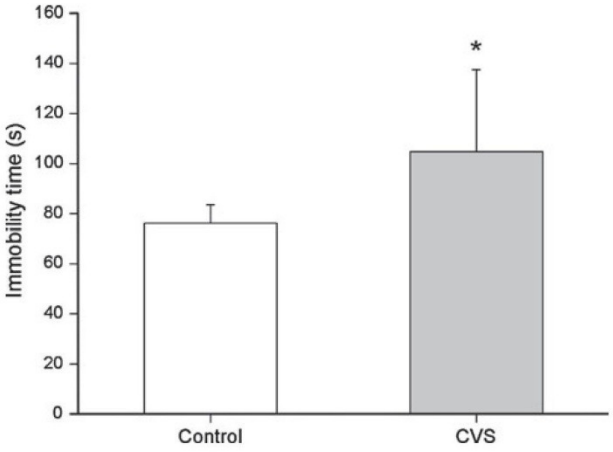

Fig. 1 Effect of chronic variable stress on time of immobility in forced swimming test. Results are expressed as mean ± SD for ten animals in each group. Different from control, *$P < 0.05$ (Student's t test). *CVS* chronic variable stress

upon the immobility time in the forced swimming test in rats was also evaluated and is shown in Fig. 1. We verified that stressed animals presented an increase in immobility time [$t(18) = 2.2$; $P < 0.05$].

The levels of cytokines (IL-1β, IL-6 and TNF-α) and of chemokine MCP-1 in the hippocampus of rats subjected to CVS were determined. Figure 2 shows that CVS significantly increased the levels of IL-1β (A) [$t(8) = 3.41$; $P < 0.01$]; IL-6 (B) [$t(8) = 2.54$; $P < 0.05$] and TNF-α (C) [$t(8) = 2.44$; $P < 0.05$]. The stressed group did not present any alteration in the levels of MCP-1, when compared with the control group (D) [$t(8) = 1.31$; $P > 0.05$].

Finally, we determined the activity of AChE. As can be observed in Fig. 3, chronic stress provoked an increase in the AChE activity in the hippocampus of rats [$t(8) = 2.91$; $P < 0.05$].

Discussion

Although major depression is a serious and recurrent disorder, affecting 17–20% of the population of the world and may result in premature death and in major social and economic consequences [30], its psychological and neurobiological determinants have not been precisely defined. As such, we used an animal model of depression to investigate the possible involvement of immune and cholinergic systems in the pathophisiology of depression.

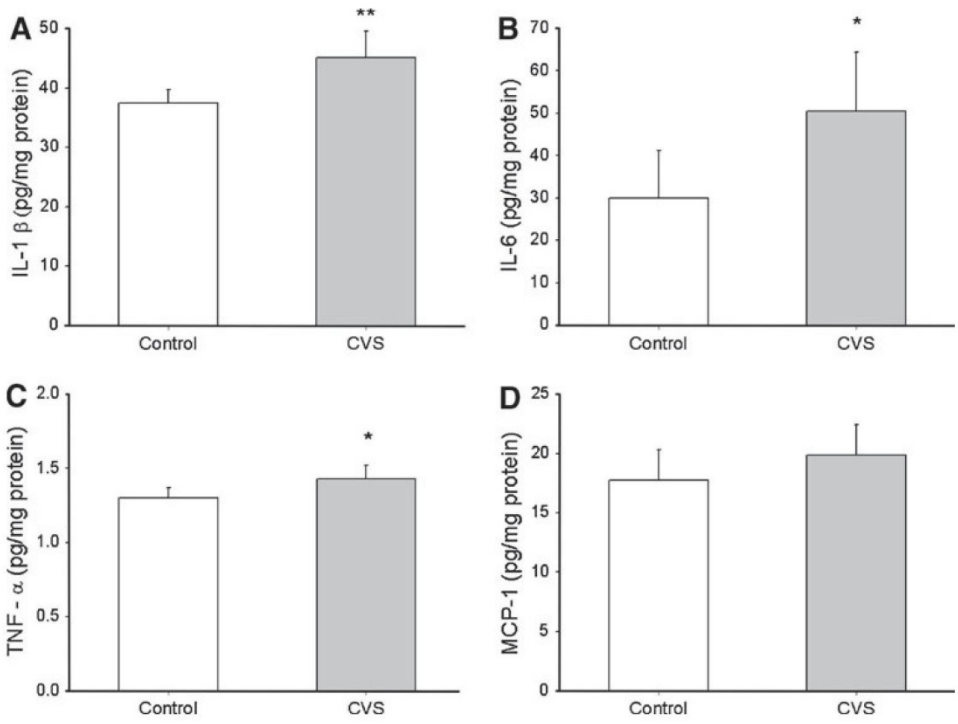

Fig. 2 Effect of chronic variable stress on cytokines IL-1β (**A**), IL-6 (**B**), TNF-α (**C**) and chemokine CCL2 (MCP-1) (**D**) levels in the hippocampus of rats. Results are expressed as mean ± SD for five animals in each group. Different from control, *$P < 0.05$; **$P < 0.01$ (Student's t test). *CVS* chronic variable stress, *IL-1 β* interleukin-1 beta, *IL-6* interleukin-6, *TNF-α* tumor necrosis factor alpha, *MCP-1* monocyte chemoattractant protein-1

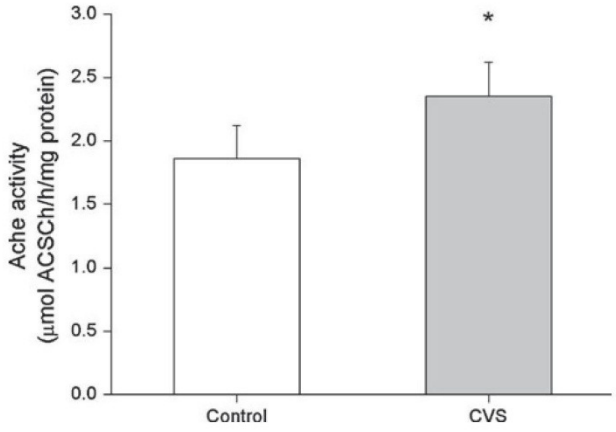

Fig. 3 Effect of chronic variable stress (CVS) on acetylcholinesterase activity in hippocampus of rats. Results are mean ± SD for five animals in each group. Different from control, $^*P < 0.05$ (Student's *t* test). *CVS* chronic variable stress

The CVS procedure was developed as an animal model of depression that presents three important characteristics: the inducing conditions are relatively realistic, the focus of the model is a core symptom of depression, anhedonia, and the protracted time course of the model is suitable for investigating the effects of chronic drug treatments [31]. A series of publications have reported that the CVS procedure causes decreased intake of sucrose solutions (or pellets) [32–35], impairments in other measures of hedonic reactivity such as place preference conditioning [36, 37] and brain stimulation reward [38, 39], decreased sexual and aggressive behaviors [40, 41] and self-care [42–44], in addition to changes in sleep architecture [39, 45]. Moreover, these behavioral changes are reversed by chronic treatment with all classes of clinically-effective antidepressant drugs, but not by drugs known to be ineffective as antidepressants.

Our results demonstrate that animals submitted to chronic stress presented changes in depressive-like behavior, characterized by a decrease in body weight gain and increased immobility time in the forced swimming test. Furthermore, there was an increase in adrenal gland weight, a common feature of chronic stress because glucocorticoid hormones are released by the adrenals in response to physical and psychological stressors [26].

It is known that depression is related to changes in the functional activity of the immune system, however there are still controversies about whether cytokines play a causal role in depressive illness or are the consequence of brain injury provoked by other causes. Thus, we decided to investigate the levels of important proinflammatory cytokines in the hippocampus of rats submitted to CVS. We observed that chronic stress provoked an increase in the levels of IL-1β, IL-6 and TNF-α, but did not alter the level of chemokine MCP-1. These results are in agreement with those of other investigators who demonstrated an increase in inflammatory markers in the hippocampus of rats submitted to different stress regimens [4, 46–48].

Considering that a previous study by our group showed that the levels of these cytokines are not increased in the serum of rats submitted to chronic stress [40], our hypothesis is that the increase in hippocampus cytokines is secondary to brain injury and not caused by passage of cytokines produced in the periphery. Consistent with this view, there are studies indicating that in addition to infiltration or indirect signaling from the periphery, cytokines (including IL-1β, IL-6 and TNF-α), as well as their receptors, are constitutively produced in the CNS itself [49, 50]. Moreover, studies have shown an increase in cytokine mRNA expression in the brain of rats after stress [48, 51]. Among the possible causes to the increase in proinflammatory cytokines in hippocampus is the activation of microglia, the brain-specific tissue macrophage, which initiates the repair of brain injury by producing and releasing multiple cytoactive factors. Several factors can provoke the microglial activation, for instance, oxidative stress [52–54] activation of NMDA receptors [55, 56] and corticoid-mediated degeneration of neurons [57, 58]. All these events have been extensively reported in animal models of depression [59–65].

With regard to the lack of effect in MCP-1 levels, our results are in agreement with another study that shows that the mere exposure to the stress protocol did not significantly alter the MCP-1 concentration in the cortex of rats [66]. In contrast, there is a report demonstrating that the production of MCP-1, and its receptor CCR2, is down-regulated by glucocorticoids in the hippocampus of rats [67].

Chronic stress in rats, besides precipitating affective disorders such as depression and anxiety, has been shown to impair learning and memory and earlier studies suggest cholinergic dysfunction as the underlying cause in the behavioral deficits following stress [16–18]. On the other hand, the cholinergic signaling is notably involved in anti-inflammatory reactions [15]. Based on these data, in the present study, we investigated the AChE activity in the hippocampus of rats submitted to chronic stress. We observed that the activity of this enzyme was increased after the stress period. These results are in agreement with Rada et al. [68], who observed an increase in AchE expression in the brain of animals submitted to forced swimming and with studies that showed that antidepressants inhibited the activity of AchE [69, 70]. Considering the role of Ach in inhibiting the release of pro-inflammatory cytokines [15], we can speculate that this increase in AChe activity may be causing an impairment of ACh ability in regulating inflammatory processes, which would

explain, at least in part, the alterations in cytokines levels described above. Beyond that, the increase in AChe activity and consequent decrease in the levels of ACh can be related with the impairment of learning and memory that is common in depressed patients.

In summary, in the present study, we demonstrated that chronic variable stress induces immune activation by increasing cytokines levels in the hippocampus of rats, in addition to an increase in acetylcholinesterase activity. Our findings provide insights into the role of immune and cholinergic systems in the pathogenesis of depression and, if confirmed in humans, could represent possibilities to investigate new therapeutic approaches.

Acknowledgments This work was supported in part by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq—Brazil) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

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4.3 Artigo 3: Chronic variable stress impairs energy metabolism in prefrontal cortex and hippocampus of rats: prevention by chronic antioxidant treatment

Publicado em: Metababolic Brain Disease (2010) 25:169–176

Metab Brain Dis (2010) 25:169–176
DOI 10.1007/s11011-010-9194-x

ORIGINAL PAPER

Chronic variable stress impairs energy metabolism in prefrontal cortex and hippocampus of rats: prevention by chronic antioxidant treatment

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Received: 25 June 2009 / Accepted: 28 October 2009 / Published online: 27 May 2010

Abstract Since chronic stress has been used widely for studying clinical depression and that brain energy metabolism and oxidative stress might be involved in the pathophysiology of this illness, the objective of this study was investigate the activities of pyruvate kinase, complex II and IV (cytocrome c oxidase) in hippocampus and prefrontal cortex of rats submitted to chronic variable stress. We also evaluated if vitamins E and C administration could prevent such effects. During 40 days adult rats from the stressed group were subjected to one stressor per day, at a different time each day, in order to minimize predictability. The stressed group had gained less weight while its immobilization time in the forced swimming test was greater than that of the control group. Results showed that stressed group presented an inhibition in the activities of complex II and cytochrome c oxidase in prefrontal cortex, while in hippocampus just complex IV was inhibited. Pyruvate kinase activity was not altered in stressed group when compared to control. Vitamins E and C administration prevented the alterations on respiratory chain caused by stress. These data suggest that the impairment of energy metabolism and oxidative stress could be related with the pathogenic pathways in stress related disorders.

Keywords Chronic variable stress · Complex II · Cytochrome c oxidase · Pyruvate kinase

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Introduction

Stress may be described as any challenge, either internal or external, that has the potential to disturb the maintenance of homeostasis (Leonard 2005). The role of stress in psychiatric disorders is well demonstrated, in particular, epidemiological data have provided strong support to the idea that stressful life events play a role in the etiology of depression (Kendler et al. 1995) and anxiety (Shelton 2004). In this context, it has been described that when a prolonged and sustained stimulation caused by stress exceeds the body capacity to maintain homeostasis it can result in psychopathological sequels, including depression.

Chronic unpredictable mild stress, a well-validated animal model, has been widely used for studying clinical depression, as well as evaluating antidepressant effects of diverse drugs (Katz and Hersh 1981; Wilner 2005; Ni et al. 2008; Gamaro et al. 2008). In this model, rats are exposed to different weak stressors for several days. The exposure of experimental animals to stress is related to significant changes in animal behavior and especially the induction of anhedonic behavior (inability to experience pleasure) that

simulates the defining symptom of the melancholic subtype of major depression (Bekris et al. 2005).

Although the pathophysiology of depression is still poorly understood, several studies have demonstrated that deficiencies in energy metabolism may be involved in this disease (Harvey 2008). Pyruvate kinase is a thiol-containing enzyme critical for glucose metabolism, the main pathway that provides energy for brain function and plays a vital role in synaptic transmission. Recent evidence suggests that ATP synthesized by synaptic vesicle-associated pyruvate kinase is harnessed to transport glutamate, GABA, dopamine, and serotonin into synaptic vesicles. It is possible that ATP locally generated by glycolysis supports vesicular accumulation of neurotransmitters (Ishida et al. 2009), what has been suggested to be associated with depression (Tordera et al. 2007; Foley et al. 2006).

Together with the glycolytic pathway and the citric acid cycle, the production of cellular energy (in the form of ATP) via the process of oxidative phosphorylation is absolutely essential for normal cellular function. Deficiencies in respiratory complex activities are possibly associated with oxidant/antioxidant imbalance and are thought to underlie defects in energy metabolism and induce cellular degeneration (Calabrese et al. 2005). In addition, evidence shows that cortisol-induced changes of the mitochondrial membrane potential can result in the release of cytochrome c from the mitochondria to the cytoplasm, where the cytochrome c promotes the action of caspases which leads to apoptosis (Zhang et al. 2006). Mitochondrial changes and consequential cell death appear to be related with atrophy of the hippocampus and prefrontal cortex observed in depressed patients (Galecki et al. 2008; Lee et al. 2002).

Oxidative stress is characterized by an increased level of free radicals that disrupts the intracellular reduction-oxidation (redox) balance. These reactive species may damage a variety of cell macromolecules, including those that constitute the electron transport system, therefore disrupting mitochondrial function (Madrigal et al. 2006). The damage induced by reactive species in cells is normally held in check by natural enzymatic and nonenzymatic antioxidant systems, including vitamin E and C (Sies et al. 1995). It has been showed that oxidative stress can be associated with the pathophysiology of depression. In this context, clinical studies show that vitamins E and C are reduced in serum of depressed patients (Khanzode et al. 2003, Maes et al. 2000; Owen et al. 2005). Also, antidepressant drugs may show antioxidant properties (Herken et al. 2007; Khanzode et al. 2003 Bilici et al. 2001) and some antioxidants have antidepressant-like effects (Ferreira et al. 2008, Binfaré et al. 2009). In addition, it has been reported that rats subjected to chronic variable stress presented alterations in some parameters of oxidative stress, like TBARS levels, superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in different regions of rat brain (Vasconcellos et al. 2006).

Changes in energy metabolism have been associated with disturbances characteristic of depression (Rezin et al. 2009). A decrease in mitochondrial function was observed in forebrain after chronic stress in rats (Madrigal et al. 2001). In addition, clinical studies demonstrate a reduction in mitochondrial ATP production rates in muscle of depressed patients (Gardner et al. 2003). However, there are no reports on the brain energy metabolism in chronic stressed rats after vitamins E and C administration.

In the present study we investigate some parameters of energy metabolism in hippocampus and prefrontal cortex of rats submitted to chronic variable stress, an animal model of depression. The effect of administration of vitamins E and C in these parameters was also evaluated. We used prefrontal cortex and hippocampus because depressed patients present alterations in these cerebral structures (Lorenzetti et al. 2009 and Wayne et al. 2008).

Material and methods

Animals and reagents

Adult, male Wistar rats (60 days old; 200–270 g weight) were used. The experimentally-naive animals were housed in groups of 4-5 in home cages made of Plexiglas material (65×25×15 cm) with the floor covered with sawdust. They were maintained under a standard dark–light cycle (lights on between 7:00 and 19:00 h) at a room temperature of 22± 2°C. The rats had free access to food (standard rat chow) and water, except for the stressed group during the period when the stressor applied required no water. In the first set of experiments, after being randomized to assure all groups presented similar body weights, the animals were divided into two groups: control and stressed. In the next set, another group of animals were divided into four groups: control with saline administration (Control), control with vitamin administration (Vit), stressed with saline administration (Stressed) and stressed with vitamin administration (Stressed plus Vit). Animal care followed the "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985) and was approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul, Brazil (project number 2008102). All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

Stress model

Chronic variable stress (CVS) protocol was applied as described by Gamaro et al. (2003a) with some modifica-

tions in the types of stressors applied, as inclination of the home cages instead of food deprivation and damp bedding instead of forced swimming. Control animals were handled daily. A variable-stressor paradigm was used for the animals in the stressed group. This protocol differs from other chronic stress protocols that use only one stressor in that the different stressors used diminish adaptation to stress (Marin et al. 2007). Animals were subjected to one stressor per day, at different times each day, in order to minimize predictability. The following stressors were used: a) 24 h of water deprivation, b) 1 h to 3 h of restraint, as described below, c) 1.5 to 2 h of restraint at 4°C, d) flashing light during 120 to 210 min, e) isolation (2 to 3 days), f) inclination of the home cages at a 45° angle for 4–6 h, g) damp bedding (300 ml water spilled onto bedding during 1.5 to 2 h). Restraint was carried out by placing the animal in a 25× 7 cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. Exposure to flashing light was made by placing the animal in a 50 cm-high, 40× 60 cm open field made of brown polywood with a frontal glass wall. A 40 W lamp, flashing at a frequency of 60 flashes per minute, was used. Rats were submitted to chronic variate stress during 40 days as described in Table 1.

After 40 days of stress, forced swimming test was performed according to Porsolt et al. 1977, in order to confirm the ability of this stress to increase the immobility time, an indicative of depressive behavior. The test involves two individual exposures to a cylindrical tank with water in which rats cannot touch the bottom of the tank or escape. The tank is made of clear Plexiglas, 50 cm tall, 30 cm in diameter, and filled with water (22-23°C) to a depth of 30 cm. Water in the tank was changed after each rat swimming test section. For the first exposure, rats were placed in the water for 15 min (pre-test session). Twenty-four hours later, rats were placed in the water again for a 5 min session (test session), and the immobility time was recorded in seconds.

Body weight was measured at different times during treatment, since several works reported that chronic stress induced significant reduction in body weight gain (Konarska et al. 1990; Harro et al. 2001).

Table 1 Stressors applied during chronic treatment

	Stressor applied
1	Cold restraint (1.5 h)
2	Inclination of home cages (4h)
3	Flashing light (2h)
4	Restraint (2h)
5	Isolation
6	Isolation
7	Isolation
8	Damp bedding (2h)
9	Inclination of home cages (6h)
10	No stressor applied
11	Flashing light (2h)
12	Water deprivation (24h)
13	Restraint (3h)
14	Damp bedding (3h)
15	Inclination of home cages (4h)
16	Cold restraint (2h)
17	Flashing light (3h)
18	Restraint (2.5h)
19	Damp bedding (3h)
20	Isolation
21	Isolation
22	Isolation
23	Cold restraint (1.5h)
24	Water deprivation (24h)
25	Inclination of home cages (4h)
26	Restraint (3h)
27	Flashing light (3h)
28	Restraint (1h)
29	Damp bedding (2h)
30	No stressor applied
31	Water deprivation (24h)
32	Inclination of home cages (6h)
33	Flashing light (2h)
34	Cold restraint (2h)
35	Isolation
36	Isolation
37	Isolation
38	Flashing light (3h)
39	Damp bedding (2h)
40	Restraint (3h)

Vitamin E and C treatment

During the stress period, animals were treated with a daily intraperitoneous administration of vitamins E (40 mg/Kg) and C (100 mg/Kg) (Wyse et al. 2002) or with saline (0.9% NaCl). Approximately 24 h after the last stress session, the animals were sacrificed by decapitation and hippocampus and prefrontal cortex were rapidly removed.

Tissue preparation

Brains were dissected on ice. To remove prefrontal cortex, regions from infralimbic (IL) prelimbic (PrL), and cingulate (Cg) cortices (see Gabbott et al. 2005) were dissected according to Paxinos and Watson (1986). In order to

remove hippocampus, both dorsal and ventral regions of this structure were dissected (according to Paxinos and Watson 1986).

For determination of pyruvate kinase activity, hippocampus and prefrontal cortex were homogenized with a Teflon-glass homogenizer in five volumes of ice-cold SETH buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris-HCl), pH 7.4. The homogenate was centrifuged at 800×g and the pellet was discarded. The supernatant was centrifuged at 10,000×g for 10 min. The supernatant solution containing cytosol and other cellular components such as endoplasmic reticulum and lysosomes was collected and used to enzymatic assay.

For complex II and cytochrome c oxidase activity determination, prefrontal cortex and hippocampus were homogenized (1:10, w/v) in SETH (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 UI/mL heparin) buffer, pH 7.4. The homogenates were centrifuged at 800×*g* for 10 min and the supernatants were used for enzyme activity determination.

All steps were performed at 4°C. The tissue preparation was stored for no more than 1 week at -70°C when the assay was not carried out immediately.

Piruvate kinase activity

Pyruvate kinase activity was assayed essentially as described by Leong et al. (1981). The incubation medium consisted of 0.1 M Tris–HCl buffer, pH 7.5, 10 mM $MgCl_2$, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7.0 unit of L-lactate dehydrogenase, 0.1% (v/v) Triton X-100, and 10 μL of the mitochondria-free supernatant in a final volume of 0.5 mL. Unless otherwise stated, the reaction was started after 30 min of pre-incubation by the addition of 1.0 mM phosphoenolpyruvate (PEP). All assays were performed in duplicate at 25°C. Results were expressed as μmol of pyruvate formed per min per mg of protein.

Complex II activities

Immediately before the assay, the samples were frozen and thawed three times to break mitochondrial membranes. The activity of succinate: DCIP oxiredutase (CII) were measured in homogenates following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm with 700 nm as reference wavelength ($\mathring{a}$=19.1 $mM^{-1}cm^{-1}$) in the presence of phenazine methasulphate (PMS), according to Fischer et al. (1985). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 μM DCIP was pre-incubated with 40–80 μg homogenate protein at 30°C for 20 min. Subsequently, for complex II activity, was added 4 mM sodium azide in order to inhibit the activity of complex I and 7 μM rotenone to inhibit the complex IV activity. The reaction was initiated by addition of 40 μM DCIP and was monitored for 5 min.

Cytochrome c oxidase activity

The activity of cytochrome c oxidase was measured according to Rustin et al. (1994). Enzymatic activity was measured by following the decrease in absorbance due to oxidation of previously reduced cytochrome c at 550 nm with 580 nm as reference wavelength ($\mathring{a}$=19 .1 $mM^{-1}cm^{-1}$). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-α-D-maltoside, 2–4 μg homogenate protein and the reaction was initiated with addition of 0.7 μg reduced cytochrome c. The activity of cytochrome c oxidase was measured at 25°C for 10 min.

Protein determination

Protein content was determined by the method described by Lowry et al. (1951) using bovine serum albumin as standard.

Statistical analysis

Data are mean±S.D. for five or six animals in each group and were analyzed by Student's *t* test for comparison of two means or by ANOVA followed by the Duncan multiple range test when the *F*-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer. A $p<0.05$ was considered significant.

Results

After 40 days of stress, body weight was evaluated and forced swimming test was performed. Stressed animals presented a lower body weight [Control: 60.5±16.9 g; Stressed: 29±12.6 g ($t(18)=4.7$; $p<0.01$)] and an increase in immobility time [Control: 75.9±7.6 s; Stressed: 104.8±32.7 s ($t(18)=2.2$; $p<0.05$)], indicating that these animals presented depressive behavior.

As shown in Fig. 1, chronic stress did not modify pyruvate kinase activity in hippocampus and prefrontal cortex of rats [$t(9)=0.78$; $p>0.05$ and $t(9)=1.4$; $p>0.05$]. We also examined the effect of stress on activity of complex II and cytocrome c oxidase. As can be observed in Fig. 2a, the activity of complex II was inhibited in prefrontal cortex [$t(10)=4.9$; $p<0.05$], but was not altered in hippocampus [$t(8)=1.01$; $p>0.05$]. Figure 2b shows that cytocrome c oxidase was inhibited both in hippocampus [$t(10)=4.1$; $p<0.05$] and prefrontal cortex [$t(13)=3.5$; $p<0.05$] when compared with the control group.

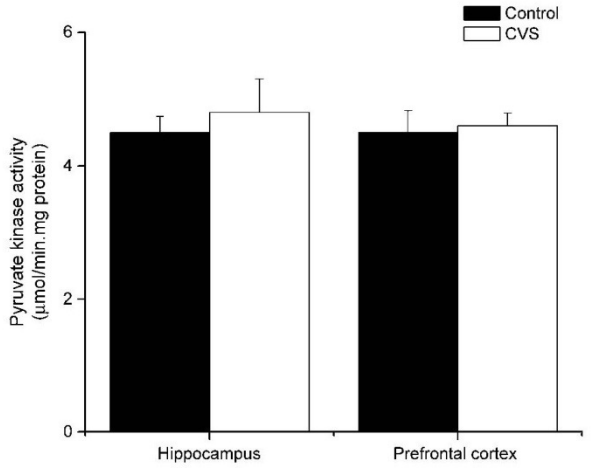

Fig 1 Effect of chronic variable stress on pyruvate kinase activity in rat hippocampus and prefrontal cortex. Data are mean±S.D. for five or six animals in each group. Different from control, *$p<0.05$ (*independent Student's t-test*)

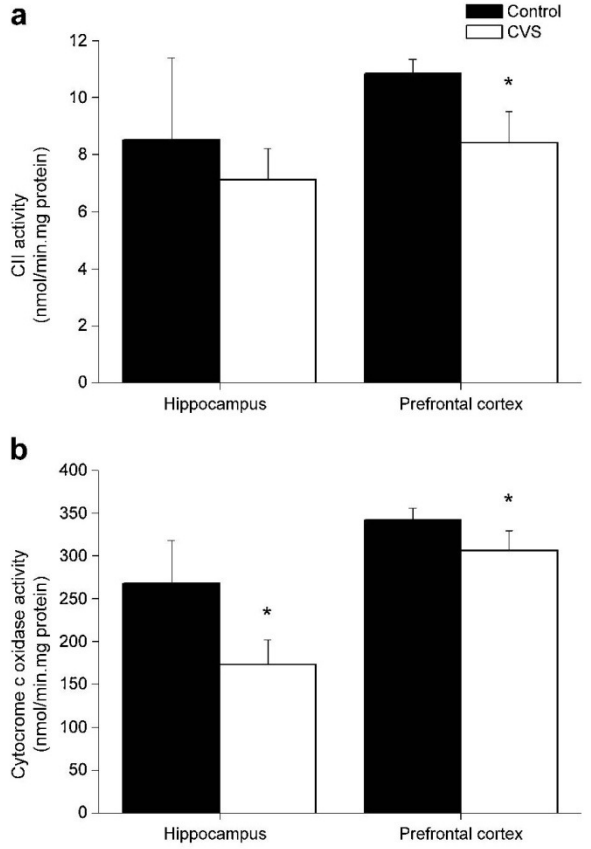

Fig 2 Effect of chronic variable stress on complex II **a** and cytochrome c oxidase **b** activities in rat hippocampus and prefrontal cortex. Data are mean±S.D. for five or six animals in each group. Different from control, *$p<0.05$ (*unpaired Student's t-test*)

In order to evaluate whether oxidative stress could be involved in the inhibition of complex II and cytocrome c oxidase caused by stress, we investigated the effect of administration of antioxidants, vitamins E and C in rats submitted to stress. Vitamins treatment during the 40 days of stress model induction was able to prevent the inhibition of CII in prefrontal cortex [$F(3,18)=7.8$; $p<0.01$] (Fig. 3). Vitamins E and C also prevented the inhibition of cytocrome c oxidase activity in hippocampus [$F(3,18)=8.5$; $p<0.01$] and prefrontal cortex [$F(3,16)=5.7$; $p<0.01$] (Fig. 4a and b, respectively).

Discussion

In the present study, we investigated the effect of chronic stress on some parameters of energy metabolism, such as activities of pyruvate kinase and of key enzymes of the respiratory chain in prefrontal cortex and hippocampus of rats. After 40 days of chronic variable stress, we verified that the animals presented higher immobility time in the forced swim test, suggesting a depressive-like state; the activity of pyruvate kinase was not altered in this animal model. However, we did not discard the involvement of other enzymes of glycolytic pathway in brain of rats subjected to stress model.

We observed a reduction in the activities of complex II and cytocrome c oxidase in prefrontal cortex of stressed rats, while in hippocampus just cytocrome c oxidase activity was inhibited. Our results are in agreement with pre-clinical and clinical studies that demonstrated a mitochondrial dysfunction in forebrain after chronic stress in rats (Madrigal et al. 2001) and a reduction in mitochondrial

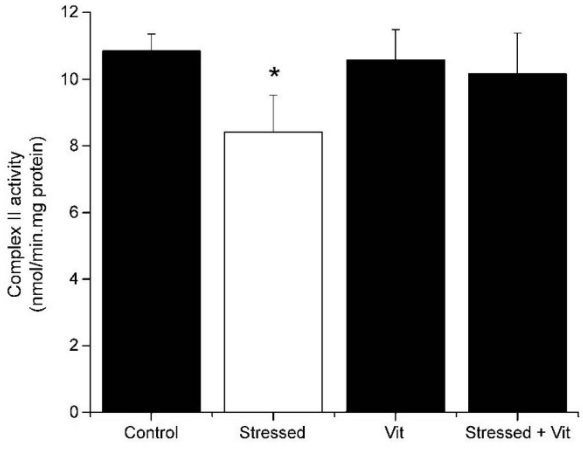

Fig. 3 Effect of chronic variable stress, vitamins E and C (*Vit*) and stress plus vitamins E and C (*Stressed+Vit*) on complex II activity in prefrontal cortex of rats. Data are mean±S.D. for five or six animals in each group. Different from control, *$p<0.01$ (*Duncan's multiple range test*)

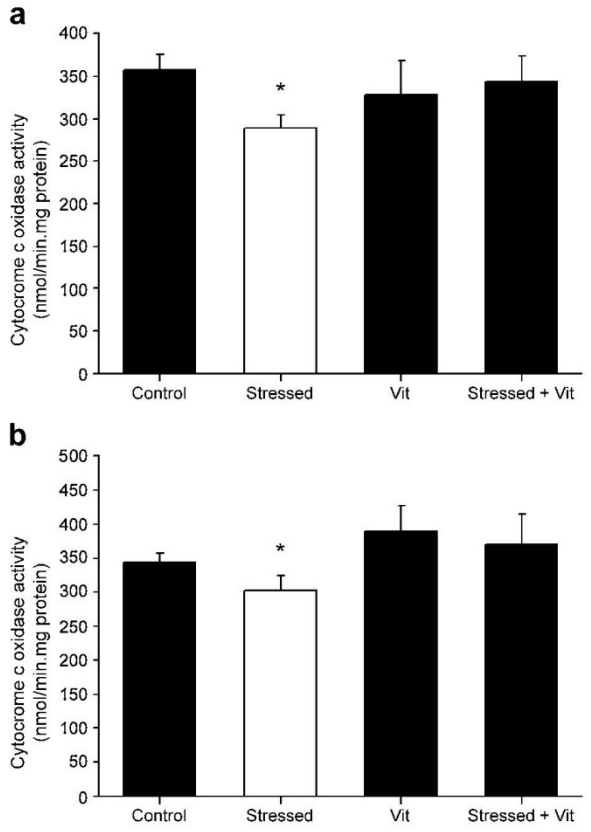

Fig. 4 Effect of chronic variable stress, vitamins E and C (*Vit*) and stress plus vitamins E and C (*Stressed+Vit*) on complex IV activity in hippocampus **a** and prefrontal cortex **b** of rats. Data are mean±S.D. for five or six animals in each group. Different from control, $*p<0.01$ (*Duncan's multiple range test*)

ATP production rates in muscle of depressed patients (Gardner et al. 2003).

Morphological alterations, mainly volumetric reductions, have been verified both in hippocampus and prefrontal cortex of patients with depression (Lorenzetti et al. 2009 and Wayne et al. 2008). However, the causes and consequences of this reduction seem to be different in each brain region, as demonstrated in several studies that presented region-specific alterations in rats (Vasconcellos et al. 2006; Rezin et al. 2008; Ni et al. 2008). The different effects of stress on hippocampus and prefrontal cortex verified in our results could be due to the corticosteroid receptors, since hippocampus has more corticosteroid receptors than prefrontal cortex, what makes it more sensitive to alterations in glucocorticoid signaling, as demonstrated by clinical (Maletic et al. 2007) and preclinical studies (Lanfumey et al. 2008). On the other hand, it has been showed that the blood flow is reduced in some regions of prefrontal cortex of depressed patients (Manji et al. 2001) what could cause a reduction of oxidative metabolism, as found in the present study. Other studies from the literature point to prefrontal cortex as a susceptible structure: for example, Lucca et al. (2009), in a similar CVS model, observed an increase in superoxide production both in hippocampus and prefrontal cortex, while increased lipoperoxidation was detected only in prefrontal cortex.

Previous studies, using the same animal model of the present study, demonstrated an impairment in Na^+,K^+-ATPase activity in hippocampus of rats (Gamaro et al. 2003b). Considering that this enzyme consume about 40–50% of the ATP generated in the brain, the inhibition of Na^+,K^+-ATPase activity might be related to reduction of respiratory chain observed in our study. Additionally, it has been shown that the cognitive deficit observed in rats subjected to chronic variable stress can result from a disruption in brain energy metabolism (Hoyer et al. 2004 and Sadowski et al. 2004).

Considering that chronic stress induces oxidative damage in rats (Vasconcellos et al. 2006), we evaluated the effect of the chronic administration of vitamins E and C on the energy parameters altered by stress in the present study. Our results demonstrated that vitamins E and C administered concomitant with the application of stress model was able to prevent the inhibition of complex II and cytocrome c oxidase activities, indicating a possible involvement of oxidative stress in such alterations.

It is well known that mitochondrial oxidative phosphorylation system generates free radicals and that the electron transport chain itself is vulnerable to damage by free radicals (Dudkina et al. 2008). Therefore, oxidative damage induced by stress may be either the cause or the consequence of the mitochondrial dysfunction. On the other hand, an initial formation of large amounts of reactive oxygen and/or nitrogen species during stress may initiate lipid peroxidation (Braughler and Hall 1989). In this context, vitamins E and C could break the propagation of lipoperoxidation in biological membranes, and thus prevent the inhibition of complexes II and IV. In this way, our results are in agreement with Vatassery (2004) who suggest that α-tocopherol might have a specific role in maintaining oxidative phosphorylation through preventing the propagation of lipid peroxidation. In addition, these vitamins could compensate the reduction of Glutathione (Bolaños et al. 1996) and the activities of GPx and SOD observed in rat brain after chronic stress exposure (Vasconcellos et al. 2006).

Our findings suggest that the impairment of energy metabolism observed in this experimental depression model was probably caused by oxidative stress, what could be related to the pathogenic pathways in stress related disorders. These results, as well as other studies describing metabolic disturbances in major depression, add evidence for metabolic changes in depression, and emphasize the importance of a broader knowledge of the neurobiology of

this disease. These results are in agreement with studies that show antidepressive-like effect of vitamins with antioxidant properties. (Binfaré et al. 2009; Bragin et al. 2005). Although extrapolation of findings from animal experiments to humans is difficult, it is conceivable that these vitamins might serve as an adjuvant therapy in order to avoid progression of brain damage in depressed patients.

Acknowledgments This work was supported in part by grants from CNPq—Brazil. We would like to thank Mr. Lucas Mocelin Pitrez for his technical assistance.

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4.4 Artigo 4: Antioxidants prevent memory deficits provoked by chronic variable stress in rats

Publicado em: Neurochemical Research (2011) 36:2373–2380

Neurochem Res (2011) 36:2373–2380
DOI 10.1007/s11064-011-0563-6

ORIGINAL PAPER

Antioxidants Prevent Memory Deficits Provoked by Chronic Variable Stress in Rats

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Received: 7 June 2011 / Revised: 21 July 2011 / Accepted: 27 July 2011 / Published online: 7 August 2011

Abstract Learning and memory deficits occur in depression and other stress related disorders. Although the pathogenesis of cognitive impairment after stress has not been fully elucidated, factors such as oxidative stress and neurotrophins are thought to play possible roles. Here we investigated the effect of treatment with vitamin E (40 mg/kg) and vitamin C (100 mg/kg) on the effects elicited by chronic variable stress on rat performance in Morris water maze. Brain-derived neurotrophic factor (BDNF) immunocontent was also evaluated in hippocampus of rats. Sixty-day old Wistar rats were submitted to different stressors for 40 days (stressed group). Half of stressed group received administration of vitamins once a day, during the period of stress. Chronically stressed rats presented a marked decrease in reference memory in the water maze task as well as a reduced efficiency to find the platform in the working memory task. Rats treated with vitamins E and C had part of the above effects prevented, suggesting the participation of oxidative stress in such effects. The BDNF levels were not altered in hippocampus of stressed group when compared to controls. Our findings lend support to a novel therapeutic strategy, associated with these vitamins, to the cognitive dysfunction observed in depression and other stress related diseases.

Keywords Chronic variable stress · Memory · Antioxidants · BDNF

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Introduction

Central stress deregulation may result in an increased vulnerability to neuropsychiatric disorders. While the stress response is essential for maintenance of homeostasis, maladaptive responses to stress can lead to disease, including an elevation in risk factors for depression and anxiety [1–3]. This way, the chronic variable stress (CVS) paradigm has been proposed as an animal model of depression [4–6].

Long-term exposure to stress or glucocorticoids produces numerous changes in hippocampal structure that include altered neurochemistry, excitability, neuronal morphology and even cell death [7–12]. Since the hippocampus plays a very important role in processing new information leading to learning and memory, chronic stress can be associated to cognitive deficits in animals submitted to several tasks, such as the Morris water maze [13], radial maze [14], Y-maze [15], and the Barnes maze [16]. However, the pathogenesis of the cognitive impairment observed in stress related disorders has not been fully elucidated.

Oxidative stress and reactive oxygen species seem to be involved in memory and cognitive impairments present in

aging and neurodegenerative diseases [17–19]. Regarding depression and stress, studies have been demonstrated that serotonine depletion [20], as well as corticosterone administration [21] promote oxidative stress and consequently cause memory deficits in rats. Although the stress-induced impairment in learning is extensively studied, very few studies have looked into the possible ways of preventing this stress-induced deficit. On the other hand, antioxidants including vitamins E and C can enhance learning and memory and prevent memory deficits in other experimental conditions like Alzheimer's disease [22], aging and menopause [19, 23] and aminoacidopathies [24, 25].

Neuronal plasticity mediated by changes in synaptic morphology and function is well known to be involved in learning and memory, as well as in the adaptation to stress [26]. There is a growing body of evidence demonstrating that acute stress decreases the expression of brain-derived neurotrophic factor (BDNF) in limbic structures that control mood [26–29], and that antidepressant treatment reverses or blocks the effects of stress [30], but there are few studies demonstrating the relationship between chronic stress and BDNF expression and function.

In light of these reports, we examined whether supplementation with vitamins C or E could protect against learning and memory deficits assessed by the water maze task in rats submitted to CVS model of depression. The effect of stress on BDNF levels in hippocampus was also evaluated.

Experimental Procedure

Animals and Reagents

Male Wistar rats (60 days old) were obtained from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in an air conditioned constant temperature (22 ± 1°C) colony room, with free access to water and 20% (w/w) protein commercial chow, except for the stressed group during the period when the stressor applied required no water. Animal care followed the ''Principles of Laboratory Animal Care'' (NIH publication 85–23, revised 1985) and was approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul, Brazil (project number 2008102). All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA. Animals were randomly assigned to one of the following groups: control with saline administration, stressed with saline administration, control with vitamin E plus C administration and stressed with vitamin E plus C administration.

Stress Model

CVS protocol was applied, as described by Gamaro et al. [31], with some modifications in the types of stressors applied, such as inclination of the home cages instead of food deprivation and damp bedding instead of forced swimming. Control animals were handled daily. A variable stressor paradigm was used for the animals in the stressed group. This protocol differs from other chronic stress protocols that use only one stressor in that the different stressors used diminished adaptation to stress [32]. Animals were subjected to one stressor per day, at different times each day, in order to minimize predictability. The following stressors were used: (a) 24 h of water deprivation, (b) 1–3 h of restraint, as described below, (c) 1.5–2 h of restraint at 4°C, (d) flashing light during 120–210 min, (e) isolation (2–3 days), (f) inclination of the home cages at a 45° angle for 4–6 h, (g) damp bedding (300 mL water spilled onto bedding for 1.5–2 h). Restraint was carried out by placing the animal in a 25 × 7 cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. Exposure to flashing light was carried out by placing the animal in a 50 cm-high, 40 × 60 cm open field made of brown plywood with a frontal glass wall. A 40 W lamp, flashing at a frequency of 60 flashes per minute, was used. Rats were submitted to CVS for 40 days, as described in Table 1.

Vitamin E and C Treatment

During the 40 days of stress, the vitamins group were treated with a daily intraperitoneous (i.p.) administration of a solution containing vitamins E (40 mg/Kg) and C (100 mg/Kg) dissolved in saline (0.9% NaCl) and polysorbate-80 (2.0% Tween 80®) [33]. The saline group received a daily i.p. injection of 0.9% NaCl solution (10 mL/Kg).

Behavioral Testing

Behavioral testing was started when animals reached 100 days of life; the Morris water maze (MWM), an apparatus widely employed for the study of spatial learning and memory tasks [34–36] was used. Behavioral experiments were conducted between 7 and 12 h a.m. The water maze consisted of a black round tank, 200 cm in diameter and 100 cm high, filled to a depth of 50 cm with water maintained at constant temperature of 23°C. The tank was theoretically divided into four equal quadrants for the purpose of analysis. Several distal visual cues were placed on the walls of the room. Trials were recorded by a video

Table 1 Schedule of stressor agents

Day of treatment	Stressor applied
1	Cold restraint (1.5 h)
2	Inclination of home cages (4 h)
3	Flashing light (2 h)
4	Restraint (2 h)
5	Isolation
6	Isolation
7	Isolation
8	Damp bedding (2 h)
9	Inclination of home cages (6 h)
10	No stressor applied
11	Flashing light (2 h)
12	Water deprivation (24 h)
13	Restraint (3 h)
14	Damp bedding (3 h)
15	Inclination of home cages (4 h)
16	Cold restraint (2 h)
17	Flashing light (3 h)
18	Restraint (2.5 h)
19	Damp bedding (3 h)
20	Isolation
21	Isolation
22	Isolation
23	Cold restraint (1.5 h)
24	Water deprivation (24 h)
25	Inclination of home cages (4 h)
26	Restraint (3 h)
27	Flashing light (3 h)
28	Restraint (1 h)
29	Damp bedding (2 h)
30	No stressor applied
31	Water deprivation (24 h)
32	Inclination of home cages (6 h)
33	Flashing light (2 h)
34	Cold restraint (2 h)
35	Isolation
36	Isolation
37	Isolation
38	Flashing light (3 h)
39	Damp bedding (2 h)
40	Restraint (3 h)

camera mounted above the center of the tank. Videotapes were analyzed using dedicated software (ANY-maze ®).

Reference Memory Task

The task consisted of six training and one test sessions. In the acquisition phase, rats had daily sessions of four trials per day for 6 days to find the platform, submerged 2 cm under the water surface, placed on the center of one of the quadrants of the tank during all training days. For each trial, the rat was placed in water facing tank wall, in one of the four starting locations (N, S, W and E). The order of starting position varied in every trial and any given sequence was not repeated on acquisition phase days. Rats were allowed to search for the platform during 60 s and, in the case of failure, they were gently guided to it; all animals were allowed to remain on the platform for 10 s. Latency to find the platform was measured in each trial. The interval between trials was of 5–10 min. One day after the last training trial, animals were subjected to a probe trial in which the platform was removed; four parameters were then recorded, namely latency to cross over the location of the platform, the number of target crossings and the time spent in target (the quadrant in which the platform was located in training sessions) and opposite quadrants. Such parameters were taken as a measure for spatial memory [36].

Working Memory Task

After 1 week, the working memory version of Morris water maze was run. The task consisted of four consecutive trials per day, with a 5 min inter-trial interval, when the animals were placed in the tank facing the wall and allowed to search for the submerged platform, positioned on the center of one of the quadrants. Platform position changed every subsequent day during the four testing days. Latencies to find the platform in every first, second, third and fourth trials were calculated considering all testing days so to assess working memory performance [36].

Analysis of BDNF Immunocontent

Mature BDNF protein was assessed 24 h after the behavioral test using the E-Max ELISA kit (Promega) according to the manufacturer's recommendations. Briefly, hippocampus were individually homogenized (1:10 w/v) in lysis buffer containing: 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), Igepal (1%), glycerol (10%), 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM sodium vanadate, 0.1 mM EDTA, and 0.1 mM EGTA, and centrifuged for 3 min at 14,000 rpm at 4°C. Supernatant was diluted (1:5 v/v) in sample buffer and incubated on a 96-well flat-bottom plates previously coated with anti-BDNF monoclonal antibody and blocked with Block and Sample buffer. After sample incubation, plates were incubated with polyclonal anti-human antibody for 2 h and horseradish peroxidase for 1 h. Then color reaction with tetramethylbenzidine was quantified in a plate reader at 450 nm. The standard BDNF curve, ranging from 0 to 500 pg/mL, was performed in each plate.

Protein Determination

Protein was measured by the method of Lowry [37], using bovine serum albumin as standard.

Statistical Analysis

Data from acquisition phase (training days) in the Morris water maze and from working memory were analyzed by factorial ANOVA for repeated measures considering the days and trials as the repeated measure, respectively. Post hoc Duncan multiple range tests were run when indicated. Data from probe trial session were analyzed by two-way ANOVA. BDNF immunocontent was analyzed by Student's t test; $P < 0.05$ was considered significant. Descriptive statistics data were expressed as mean ± SEM. All analyses were performed using the Statistical Package for the Social Science (SPSS) software in a PC-compatible computer.

Results

Results from the spatial learning task are shown in Figs. 1 and 2. A significant difference between the different groups was observed in the performance during the training sessions (Fig. 1). Repeated measures ANOVA showed that there was a significant *days x stress* interaction [F = 4.79, $P < 0.001$]. Duncan's post hoc test showed that groups differed on days 4 ($P < 0.05$), 5 and 6 ($P < 0.01$) of training, with stressed rats presenting higher latencies to find the platform; vitamins *per se* did not alter this parameter, however prevented the effects of stress on day 4 onwards. Chronic stress also caused impairment in the retention test. A two-way ANOVA showed that chronically stressed rats presented increased latency in reaching the original position of the platform [F(1, 44) = 9.80; $P < 0.01$] (Fig. 2a); no difference between the groups ($P > 0.05$) was observed regarding the number of times that the animals crossed the platform location (Fig. 2b). When analyzing the time spent in the target quadrant (Fig. 2c) and the time spent in the opposite quadrant (Fig. 2d), results showed that chronic stress decrease the time spent in target quadrant [F(1, 44) = 12.74; $P < 0.01$] and increased time spent in the opposite quadrant [F(1, 44) = 4.27; $P < 0.05$]. Vitamins E and C were unable to prevent the damage caused by stress ($P > 0.05$ in all cases).

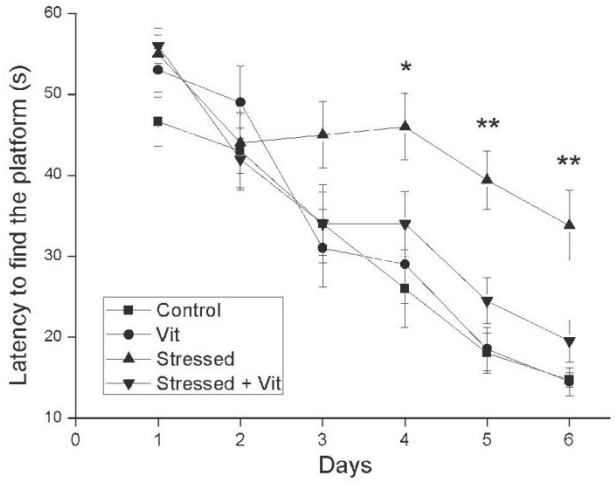

Fig. 1 Effect of chronic variable stress and vitamins E and C administration on spatial reference memory. Data show latencies for finding the platform across blocks of four trials on each day in acquisition phase. Data are expressed as mean ± SEM for 10–14 animals in each group. Different from other groups, *$P < 0.05$; **$P < 0.01$ (Repeated measures ANOVA, followed by Duncan's test). *Vit* Vitamin E plus C

Results from the working memory task were evaluated by calculating the mean latency of trials 1–4, during the 4 days of training, and analyzing the differences between the trials. A repeated-measures ANOVA showed that there was an interaction between stress and trials [F = 6.91; $P < 0.001$]. A post hoc test showed a significant difference in stressed and control rat performance in trials 2, 3 and 4 ($P < 0.05$). Besides, vitamins E and C were able to prevent this effect (Fig. 3).

Mature BDNF levels in hippocampus of stressed and control rats were measured 24 h after behavioral testing. Table 2 shows that BDNF immunocontent was not affected in hippocampus (t(5) = 1.173; $P > 0.05$) when compared to control group.

Discussion

The CVS model of depression is considered to have good predictive validity (behavioural changes are reversed by chronic treatment with a wide variety of antidepressants), face validity (almost all demonstrable symptoms of depression have been demonstrated), and construct validity (CVS causes a generalized decrease in responsiveness to rewards, comparable to anhedonia, the core symptom of the melancholic subtype of major depressive disorder). In the CVS paradigm, rats are subjected to a variety of mild stressors for prolonged periods of time (e.g., several weeks) [38].

The purpose of this work was to evaluate the effects of treatment with antioxidants on hippocampal-dependent function in rats submitted to CVS, since previous works have demonstrated that chronic stress may induce spatial memory deficits [13, 15, 39]. For this, we used the MWM, a robust and reliable test that is strongly correlated with hippocampal synaptic plasticity [40].

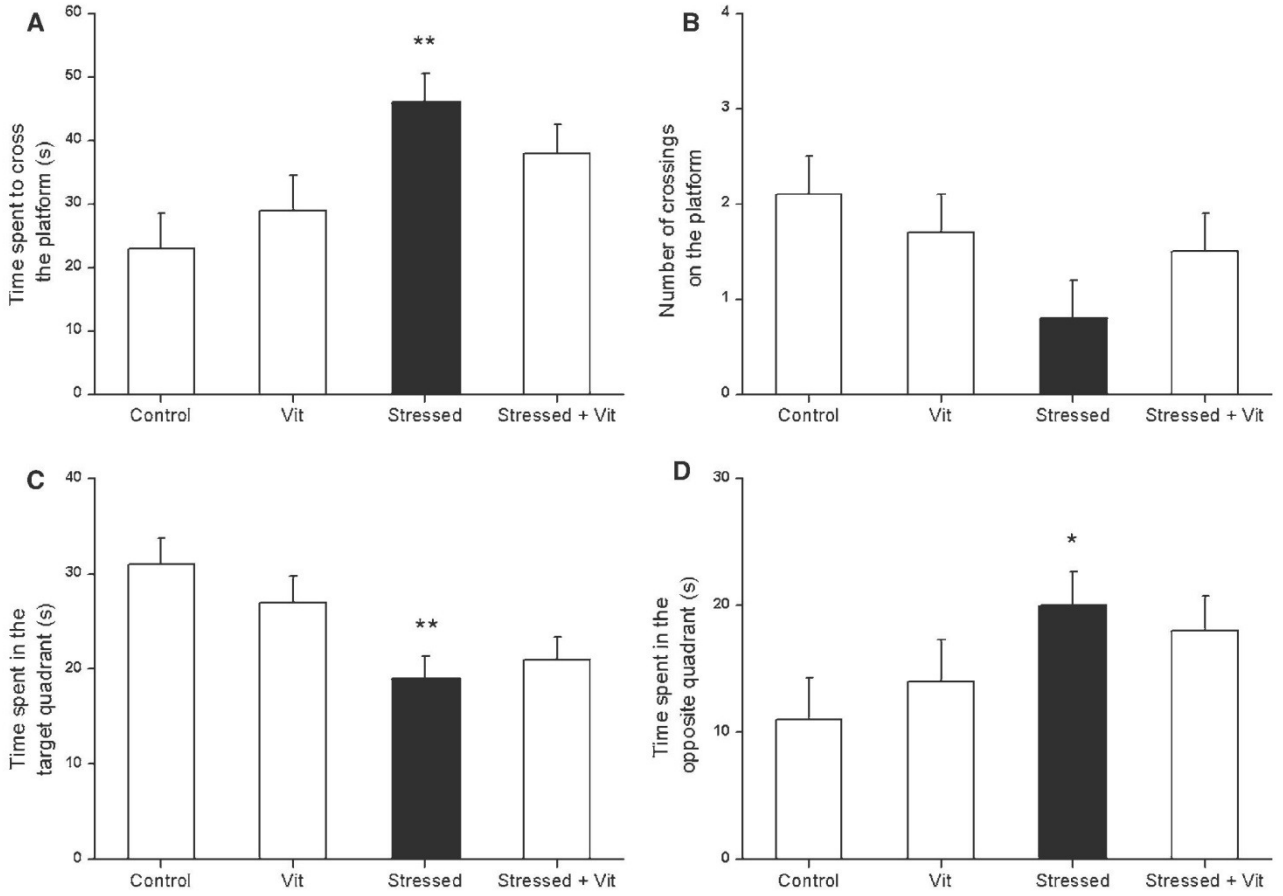

Fig. 2 Effects of chronic variable stress and vitamins E and C administration on performance of spatial memory test session parameters of Morris water maze: latency to cross the platform (**a**), number of crossings over the platform position (**b**), time spent in the target quadrant (**c**) and the time spent in the opposite quadrant (**d**). Data are expressed as mean ± SEM for 10–14 animals in each group. Different from other groups, $^{*}P < 0.05$; $^{**}P < 0.01$ (Two-way ANOVA). *Vit* Vitamin E plus C

Consistent with the reports cited above, we also observed decreased reference memory in the water maze task after CVS, as evaluated by a significant difference in the place learning during the acquisition phase and by impaired memory retention observed in the test session (increased latency in finding the platform and decreased ratio between time spent in the target quadrant and the opposite quadrant). Vitamins E and C administration prevented the impairment in memory caused by stress in the acquisition phase, but did not prevent the damage in the retention test. It is important to observe that altered exploratory or motor activity is probably not the cause for the effects observed, since performance in the open field or the swim speed was not affected by either of the treatments (data not shown).

A growing body of evidence suggests that reactive oxygen species can function as small physiological molecules involved in functional and structural changes necessary for synaptic plasticity and that memory processes depend on the redox status of neurons [17]. However,

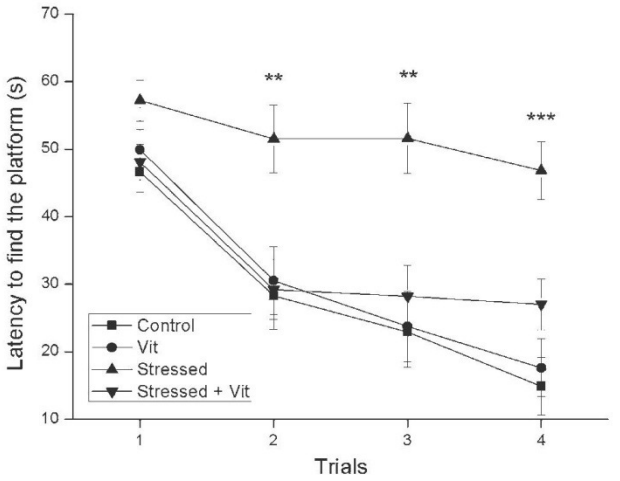

Fig. 3 Effects of chronic variable stress and vitamins E and C administration on spatial working memory. Data show mean latencies to find the platform on each trial during the 4 days. Data are expressed as mean ± SEM for 10–14 animals in each group. Different from other groups, $^{**}P < 0.01$; $^{***}P < 0.001$ (Repeated measures ANOVA, followed by Duncan's test). *Vit* Vitamin E plus C

Table 2 Effect of chronic variable stress on BDNF concentration in hippocampus of rats

Groups	BDNF concentration (pg/mg protein)
Control	13.4 ± 2.9
Stressed	17.5 ± 1.4

Results are expressed as mean ± SEM for experiments performed in duplicate

although reactive oxygen species undoubtedly are very important physiological mediators of plasticity and signaling, they can become detrimental to neuronal function when they accumulate excessively in the brain [17–19]. In this sense, it has been demonstrated that chronic stress provokes increase in oxidative stress and also decreases antioxidant defense status in the brain [41–44], which may form the basis for impaired memory. In the present study, our hypothesis was that the memory deficits observed after chronic stress might have arisen as a result of increased reactive oxygen species formation and subsequent oxidative injury to neurons, and therefore could be prevented by antioxidant treatment. Our results showed that administration of vitamins E and C during the period of stress was able to prevent the impairment observed in the acquisition phase of the task, but did not have any effect in the damage observed in the retention test.

Differential effects on acquisition versus retention in the water maze have already been observed [13, 45, 46], and have been attributed to different characteristics between training and test sessions. For instance, when the platform is absent in the test, there is higher "attentional demand" relative to acquisition trials [46]. Additionally, glucocorticoids secreted during stressful events influence differently memory consolidation and retrieval [47]. These reports indicate that there are different mechanisms involved in the different memory phases, in other words, we can suppose that the impairment observed in our study could have different causes in training and test sections. Therefore, oxidative stress seems to be involved in the damage observed in the acquisition phase, since antioxidants were able to at least partially prevent this damage. On the other hand, in the retrieval phase, the observed impairment cannot be attributed to oxidative stress only.

To assess working or trial-dependent learning and memory, a different method which is also called matching-to-sample was performed. In this task, the platform is relocated every day and the animal is given four trials per day. We observed that stressed animals presented higher latencies in all trials, except the first. This is explained by the different strategies used by the animals to reach the platform in the first and in the next trials. During the sample trial (first trial), the animal learns the new location of the platform by trial-and-error, and in the subsequent trials the animal recalls the sample trial. Our results indicate that stress impairs working memory and that treatment with vitamins E and C was able to prevent this effect.

The neurotrophin BDNF has been implicated in the regulation of neuroplasticity, gene expression, and synaptic function in the adult brain, as well as in the pathophysiology of neuropsychiatric disorders and the mechanism of action of antidepressants [48–51]. In order to investigate other possible mechanism underlying the memory deficits observed in stressed rats, we decide to analyze the levels of BDNF in hippocampus of stressed rats after the water maze task. We observed that BDNF levels were not altered in stressed animals when compared to controls. Our results are in agreement with previous studies that show that the exposure of rats to chronic mild stress does not change BDNF mRNA levels in the hippocampus [52–54]. On the other hand, there are studies demonstrating that stress can cause damage and atrophy of neurons in the hippocampus [55–57] and recently, other works suggested that different stress paradigms decrease the expression of BDNF in hippocampus [26–29] and that antidepressant treatment reverses or blocks the effects of stress [30].

Although data presented in this study disagree with other findings, it is important to point out that the impact of stressors is dependent upon the characteristics of the stressor, such as severity, chronicity, and predictability [58]. In this sense, it is important to note that most studies supporting a role for BDNF in depression have been performed in behavioral models of depression that have low face validity with regard to the human disease, such as the learned helplessness and the forced swim test, and that even studies considered chronic lasted only 21 days. The original hypothesis regarding the role of BDNF in depression was based on different observations showing that chronic stress alters hippocampal structure, but it must be remembered that hippocampal dendritic retraction takes time to develop [59, 60] and self corrects following recovery from stress [61–63], suggesting that stress-induced functional outcomes most often reflect moderate changes from structural reorganization, rather than severe outcomes caused by cell loss [61]. Beside this, it has been shown that hippocampus-dependent learning in the Morris water maze, contextual fear and passive avoidance tests is associated with a rapid and transient increase in BDNF mRNA expression in this brain structure [50, 64, 65] and that the BDNF assay was performed 18 days after the last stress section. All these conflicting evidences indicate that the exact role of endogenous BDNF contents after stress, as well as its roles in learning and memory remains a matter of further investigation.

Alterations on the neurocognitive level are observed in many neuropsychiatric diseases, and the search for new

treatment possibilities to cure such diseases, or at least, alleviate the associated neurocognitive dysfunction, continues to be a major point of focus in contemporary neuroscience. In this context, although the administration of vitamins E and C was not able in completely preventing the memory impairment caused by stress, our results are quite promising, demonstrating that antioxidants, possibly in association with other antidepressants drugs, may help to counteract some of the deleterious effects of stress on cognition.

Acknowledgments This work was supported in part by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq—Brazil) and Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS).

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5 DISCUSSÃO

Estudos epidemiológicos demonstram que a depressão é o transtorno psiquiátrico mais prevalente ao longo da vida em diversas regiões do mundo. Estima-se que aproximadamente 16% dos indivíduos adultos sofrerão pelo menos um episódio depressivo (KESSLER et al., 2005). Na maioria dos casos, a doença é crônica e recorrente (MALETIC et al., 2007). Além disso, acredita-se que a depressão também é uma doença progressiva, na qual um episódio prévio diminui o limiar para o aparecimento de um novo (MONROE and HARKNESS, 2005; MALETIC et al., 2007). Atualmente, sabe-se que a depressão é uma síndrome neuropsiquiátrica, hereditária em aproximadamente 40% dos casos (KENDLER et al., 2006; UHER, 2011), e caracterizada por alterações celulares e moleculares relativamente sutis, observadas em uma complexa rede de substratos neurais (KRISHNAN and NESTLER, 2010).

Por ser uma patologia clínica e etiologicamente muito heterogênea, diferentes mecanismos patofisiológicos podem estar implicados em diferentes pacientes com diagnóstico de depressão segundo o DSM-IV. Assim, diferentes teorias têm sido propostas para explicar a patogênse da depressão, incluindo hipóteses baseadas na deficiência das aminas biogênicas (CHARNEY, 1998; HIRSCHFELD, 2000), na disfunção do eixo HPA (DINAN, 1994; PARIANTE and LIGHTMAN, 2008), em distúrbios da neurogênse (JACOBS et al., 2000; SAHAY and HEN, 2007; ZHAO et al., 2008) e mais recentemente, na teoria inflamatória (MAES et al., 2009; ZUNSZAIN et al., 2011; SONG and WANG, 2011). Os GC, hormônios adrenais liberados em resposta a uma variedade de estressores, têm um papel importante em várias dessas hipóteses. Assim, o ECV tem sido amplamente utilizado como modelo animal de depressão, já que além de mimetizar muitos sintomas observados em pacientes, também promove um aumento nos níveis séricos de corticosterona.

No presente trabalho, utilizamos o modelo de ECV para avaliar alguns parâmetros bioquímicos e comportamentais em ratos. Primeiramente, avaliamos parâmetros de estresse oxidativo (TBARS, CAT, SOD e GPx) em sangue de animais submetidos ao estresse crônico, já que a depressão não é uma doença de manifestações puramente psicológicas, mas também apresenta efeitos deletérios em múltiplos órgãos e sistemas periféricos (CHARNEY and MANJI, 2004).

Nossos resultados demonstraram que ratos adultos submetidos ao modelo de ECV durante 40 dias, tiveram um aumento nos níveis plasmáticos de TBARS, considerado um marcador de peroxidação lipídica. A lipoperoxidação induz alterações nas propriedades das membranas biológicas tais como distúrbios na estrutura, perda de função e alterações de permeabilidade, que podem resultar em morte celular e neurodegeneração (MISOEK, 2007; NIKI, 2008). Os produtos da lipoperoxidação ainda podem reagir com outras macromoléculas importantes para a célula como proteínas e as bases nitrogenadas do DNA (MARNETT, 2002; NAIR et al., 2007). Considerando que a lipoperoxidação é uma consequência da formação de RL e que estes podem ser neutralizados pelas enzimas antioxidantes, também avaliamos a atividade das enzimas SOD, CAT e GPx em eritrócitos de ratos. Os animais estressados apresentaram um aumento significativo da razão SOD/CAT em relação aos controles, representando um desequilíbrio entre as duas enzimas, que provavelmente resulta em aumento nos níveis de H_2O_2 (o produto da reação catalisada pela SOD); o H_2O_2 pode reagir com metais de transição e gerar o radical hidroxil, o mais potente radical formado intracelularmente (KELNER et al., 1995; MATÉS et al., 1999). Por outro lado, não houve alteração na atividade da GPx em eritrócitos de ratos estressados.

Nossos dados corroboram com o crescente número de evidências que sugerem que o estresse oxidativo é um dos principais mecanismos responsáveis pela má adaptação ao estresse crônico em ratos (OLIVENZA et al., 2000; MADRIGAL et al., 2001; LUCCA et al., 2009). Estudos clínicos também têm evidenciado um aumento em parâmetros de estresse oxidativo em soro de pacientes deprimidos (KHANZODE et al., 2003; CUMURCU et al., 2009), demonstrando inclusive uma correlação positiva entre a gravidade da doença e a atividade da SOD em eritrócitos (BILICI et al., 2001).

Diferentes mecanismos podem ser responsáveis pelo aumento no EO observado após estresse. Sabe-se que uma das ações dos GC durante a resposta ao estresse é promover a liberação de grandes quantidades de aminoácidos excitatórios, como aspartato e glutamato (MOGHADDAM, 1993). O glutamato liberado liga-se aos receptores NMDA e promove o influxo de Ca^{2+} para a célula. Os GC também provocam aumento nas concentrações

intracelulares de Ca^{2+} por inibir seu efluxo via Ca^{2+}/ATPase e trocador Na^{+}/Ca^{2+} (McEWEN and SAPOLSKY, 1995; YOU et al., 2009). O excesso de cálcio intracelular ativa certas enzimas dependentes de Ca^{2+} e resulta em produção de radicais livres (LIPTON, 1999).

Outro possível mecanismo envolvido na indução de EO após estresse é o aumento nos níveis de HCY, já que diversos estudos têm demonstrado que esse aminoácido é capaz de induzir estresse oxidativo (WYSE et al., 2002; FARACI and LENTZ, 2004; MATTÉ et al., 2009). Pacientes deprimidos também apresentam alterações no metabolismo da metionina (MET)/HCY (TOLMUNEN et al., 2004; LEVINE et al., 2008; JENDRICKO et al., 2009). Baseados nesses dados, investigamos os níveis plasmáticos de HCY em ratos cronicamente estressados. De acordo com nossos resultados, animais estressados apresentam níveis de HCY mais elevados do que os animais controles. Embora na literatura existam resultados controversos a respeito da correlação entre HCY e depressão, nossos dados estão de acordo com trabalhos que demonstraram um aumento dos níveis plasmáticos de HCY em pacientes deprimidos (BOTTIGLIERI et al., 2000; REIF et al., 2003; TOLMUNEN et al., 2004; RESLER et al., 2008; ALEXOPOULOS et al., 2010; WILHELM et al., 2010). Alguns estudos têm sugerido que alterações nos níveis de HCY em pacientes deprimidos podem ser decorrentes de deficiências nos níveis de folato ou de vitamina B12 (BOTTIGLIERI et al., 2000; REFSUM et al., 2006; KIM et al., 2008), que são importantes cofatores do metabolismo da MET/HCY. Os níveis plasmáticos de folato em ratos submetidos ao ECV também foram avaliados, entretanto nenhuma alteração significativa foi observada.

Considerando que prévios estudos do nosso laboratório demonstraram que a enzima BuChE pode ser inibida por HCY (MATTÉ et al., 2006; SCHERER et al., 2007) e que esta inibição é mediada, pelo menos em parte, pela geração de radicais livres (STEFANELLO et al., 2005), nós decidimos investigar a atividade da BuChE em soro de animais após 40 dias de estresse. Os resultados mostraram que a enzima está inibida nos animais estressados quando comparados com os controles. Estes resultados corroboram com os de Schaullreuter e Elwary (2007), que demonstraram que o aumento na produção

de H_2O_2 causado pelo desequilíbrio entre as atividades das enzimas SOD e CAT pode inibir as colinesterases séricas.

Já descrevemos anteriormente que a resposta do organismo ao estresse é caracterizada por três eventos relacionados: 1) ativação do sistema nervoso simpático (mecanismos de fuga ou luta), que leva à liberação de catecolaminas (SAPOLSKY, 1996); 2) aumento nos níveis plasmáticos de GC (SAPOLSKY, 1996) e 3) liberação de grandes quantidades de glutamato (MOGHADDAM, 1993). Estes mediadores da resposta do organismo a um estressor podem induzir uma resposta de fase aguda similar à desencadeada quando o organismo reage contra um agente infeccioso (MOGHADDAM, 1993). Por esta razão, muitos pesquisadores têm sugerido que episódios repetidos de estresse podem resultar em doenças inflamatórias (MOGHADDAM, 1993; LUCAS et al., 2006; GARCIA-BUENO et al., 2008). Por este motivo, neste trabalho nós avaliamos os efeitos do estresse sobre alguns marcadores de resposta inflamatória (IL-6, TNF-α, NO e PCR) em soro de ratos submetidos ao ECV. Os resultados mostraram que o modelo de ECV não alterou nenhum dos parâmetros estudados. Embora muitos estudos indiquem uma relação entre depressão e inflamação (SCHIEPERS et al., 2005; DANTZER, 2006; MAES et al., 2009), estudos usando modelos animais de estresse ainda apresentam resultados inconsistentes devido aos diferentes paradigmas de estresse empregados, ao tempo de exposição e aos mediadores avaliados. Por outro lado, os GC liberados em resposta ao estresse podem atuar como potentes anti-inflamatórios em determinadas situações (VINSON, 2009). Neste sentido, o papel da inflamação na patogênese da depressão e de outras condições relacionadas ao estresse é contraditório e sujeito a diferentes interpretações. Ainda é preciso determinar se o aumento de citocinas observado em alguns pacientes tem uma relação causal com o aparecimento dos sintomas ou é conseqüência de lesões cerebrais provocadas por outras causas.

Considerando que os dados da literatura a respeito do envolvimento do SI na depressão ainda são inconclusivos, decidimos investigar o efeito do ECV sobre alguns parâmetros do SI (IL-1 β, IL-6, TNF- α e MCP-1) em hipocampo de ratos. Resultados demonstraram que o ECV aumentou significativamente os

níveis de IL-1 β, IL-6 e TNF- α. O grupo estressado não apresentou qualquer alteração nos níveis de MCP-1 quando comparado ao grupo controle.

Esses resultados parecem não corresponder à chamada hipótese das citocinas (SCHIEPERS et al., 2005), a qual sugere que citocinas produzidas por uma ativação periférica seriam responsáveis pela variedade de alterações comportamentais, neuroendócrinas e neuroquímicas que são associadas à depressão. Levando em conta nosso estudo prévio que não indicou aumento nos níveis plasmáticos de citocinas pró-inflamatórias, podemos inferir que o aumento destes parâmetros em hipocampo de ratos estressados é secundário a um dano neuronal anterior e não causado pela passagem de citocinas produzidas perifericamente. Neste sentido, existem estudos comprovando que algumas citocinas (incluindo IL-1 β, IL-6 e TNF- α) e também seus receptores podem ser constitutivamente produzidos no SNC (HANISH, 2002). Além disso, trabalhos também demonstraram um aumento na expressão do mRNA das citocinas IL-1 β, IL-6 em cérebro de ratos estressados (NGUYEN et al., 1998; MIYAHARA et al., 2000). As citocinas produzidas dentro do SNC contribuem para o desenvolvimento e plasticidade neuronal, sinaptogênese e reparo tecidual (ALOISI et al., 1995; MUNOZ-FERNANDEZ and FRESNO, 1998; BEATTIE et al., 2002), entretanto, também podem ser produzidas por ativação da microglia causada por diversos fatores como estresse oxidativo (HENSLEY et al., 2006; INAMORATTO et al., 2009), ativação de receptores NMDA (DE et al., 2005) e neurodegeneração induzida por GC (FRANK et al., 2006). Como discutido anteriormente, todos estes eventos já foram reportados em modelos animais de depressão (DE KLOET, et al., 2005; VASCONCELLOS et al., 2006; LUCASSEN et al., 2006).

Como já havíamos identificado uma alteração no sistema colinérgico em soro de animais estressados (inibição da BuChE), optamos por avaliar a atividade da enzima AChE no hipocampo desses animais. Nossos resultados revelaram que a atividade da AChE está aumentada em hipocampo após 40 dias de estresse. Esses dados corroboram com os achados indicando que, além de transtornos afetivos, o estresse causa prejuízos cognitivos e que disfunções colinérgicas estão envolvidas nas alterações de aprendizado e memória em ratos (SRIKUMAR et al., 2006). Considerando o papel da

acetilcolina em inibir a liberação de citocinas anti-inflamatórias (BOROVIKOVA et al., 2000), supomos que o aumento na atividade de degradação pela AChE provoque um prejuízo na regulação dos processos inflamatórios pela acetilcolina, que corresponde ao aumento das citocinas observados anteriormente. Além disso, a diminuição da atividade da BuChE em soro pode estar ocorrendo como um mecanismo compensatório a esta maior degradação no cérebro.

Dando continuidade aos nossos estudos, investigamos o possível envolvimento do metabolismo energético cerebral nos danos neurológicos causados pelo estresse. Considerando que doenças mitocondriais hereditárias podem causar sintomas depressivos e que alterações mitocondriais parecem estar relacionadas com a morte celular e consequente atrofia do hipocampo e córtex pré-frontal observadas em pacientes deprimidos, nós avaliamos as atividades de importantes enzimas constituintes da cadeia transportadora de elétrons (complexos II e IV) em hipocampo e córtex pré-frontal de ratos submetidos ao modelo de ECV. Os resultados indicaram que a atividade do complexo II foi inibida apenas em hipocampo, enquanto que o complexo IV foi inibido nas duas estruturas estudadas. De forma geral, disfunções mitocondriais contribuem para neurodegeneração tanto por induzir apoptose quanto por gerar ER de oxigênio (DUDKINA et al., 2008). Por outro lado, o EO também pode ser causa da inibição da cadeia respiratória, já que os complexos enzimáticos estão inseridos na bicamada lipídica, que é danificada pela lipoperoxidação e ainda porque os RL são capazes de danificar diretamente a estrutura protéica das enzimas (MADRIGAL et al., 2006). A inibição da cadeia respiratória, além de induzir neurodegeneração, também pode prejudicar as atividades de outras enzimas que são altamente dependentes de ATP. Estudos anteriores demonstraram que a atividade da Na^+, K^+-ATPase está inibida em ratos submetidos ao ECV (GAMARO et al., 2003) e desta forma, nossos resultados podem explicar, pelo menos em parte, tal alteração.

Devido ao íntimo envolvimento entre EO e a cadeia respiratória, também investigamos se a administração dos antioxidantes vitaminas E e C durante o período de indução do modelo de estresse seria capaz de prevenir a inibição do metabolismo energético. A administração das vitaminas E e C foi capaz de

prevenir a inibição dos complexos II e IV observadas nos animais estressados. As vitaminas E e C são capazes de bloquear a propagação da lipoperoxidação em membranas biológicas e assim prevenir a alteração das enzimas (WYSE et al., 2002; VATASSERY, 2004; DELWING et al., 2006). Além disso, estas vitaminas podem compensar a redução da glutationa e das atividades da GPx e SOD observadas em ratos submetidos ao estresse (VASCONCELLOS et al., 2006).

Além da cadeia transportadora de elétrons, o ATP pode ser gerado localmente por enzimas da via glicolítica, por exemplo, pela piruvato quinase. Estudos têm indicado que a atividade da piruvato quinase também é importante para o transporte de neurotransmissores como serotonina e glutamato nas vesículas sinápticas (ISHIDA et al., 2009). Nossos resultados não indicaram qualquer alteração na atividade da enzima, entretanto, não podemos descartar que outros componentes da via glicolítica estejam inibidos, o que pode corroborar para disfunções do metabolismo energético.

Tendo em vista que pacientes deprimidos e animais submetidos ao modelo de depressão geralmente apresentam danos neurológicos e distúrbios cognitivos e que, como já demonstramos, o estresse crônico alterou parâmetros bioquímicos importantes para os processos cognitivos, nós também avaliamos o desempenho de ratos estressados na tarefa do labirinto aquático de Mórris e o efeito das vitaminas E e C sobre tais alterações (VASCONCELLOS et al., 2003).

Trabalhos têm demonstrado que o EO e as ER estão envolvidos nos prejuízos cognitivos e de memória observados durante o envelhecimento e em doenças neurodegenerativas (BICKFORD et al., 2000; DUMONT et al., 2009; MASSAAD and KLANN, 2011). Tendo em vista os danos cognitivos causados por estresse e depressão, estudos mostraram que tanto a depleção de serotonina (CIOBICA et al., 2010) quanto a administração de corticosterona (SATO et al., 2010) promovem EO e consequentemente causam prejuízos de memória em ratos. Por outro lado, antioxidantes, incluindo as vitaminas E e C são capazes de prevenir os déficts de memória observados em outros modelos experimentais como da doença de Alzheimer (YAMADA et al., 1999), menopausa (BICKFORD et al., 2000; MONTEIRO et al., 2005) e de

aminoacidopatias (REIS et al., 2002; WYSE et al., 2002, DELWING et al., 2006). Assim, os antioxidantes surgem como candidatos para atuar em conjunto com os antidepressivos clássicos na prevenção dos prejuízos cognitivos causados pelo estresse.

Os testes comportamentais foram realizados 24 horas após a última sessão de estresse e consistiram em duas fases: na primeira, os animais foram treinados para encontrar uma plataforma submersa no tanque utilizando pistas dispostas nas paredes da sala. A seguir, foi realizada uma sessão de teste (sem a plataforma) que avaliou os seguintes parâmetros: tempo gasto no quadrante alvo e no quadrante oposto ao alvo, número de cruzamentos pelo local onde estava a plataforma e a latência para passar pela primeira vez no local da plataforma. A segunda fase consiste na avaliação da memória de trabalho. Neste teste a plataforma é realocada em um quadrante diferente a cada dia de treino. Da mesma forma que em trabalhos anteriores (VASCONCELLOS et al., 2006), nós também observamos um prejuízo na memória de referência em ratos submetidos ao modelo de ECV. Nós observamos diferenças significativas tanto na fase de aquisição quanto na retenção de memória, observada por um aumento na latência para encontrar a plataforma e por uma menor razão entre o tempo gasto no quadrante alvo e o tempo gasto no quadrante oposto. Os antioxidantes foram capazes de prevenir apenas os déficits observados durante a fase de aquisição. Esses resultados sugerem que o EO pode causar danos cognitivos, entretanto, não pode ser considerado o único mecanismo envolvido. Além disso, as diferenças observadas nas duas fases de memória estudadas podem ser consequência das diferenças entre as estruturas cerebrais envolvidas em cada uma das fases (SQUIRE and KANDEL, 2003), das diferentes demandas de atenção entre as fases (CZECH et al., 2000) e dos diferentes efeitos dos GC na fase de aquisição e na retenção (ROOZENDAAL, 2002).

A avaliação da memória de trabalho indicou que os animais estressados apresentaram uma maior latência para encontrar a plataforma do que os controles em todas as tentativas, exceto a primeira. É preciso observar que na primeira tentativa os animais aprendem o local da plataforma por tentativa e erro, enquanto que nas tentativas subsequentes o rato precisa lembrar-se do

que aprendeu anteriormente. O tratamento com as vitaminas foi capaz de prevenir o dano observado no grupo estressado.

Além do dano provocado pelo EO, outros mecanismos também podem estar envolvidos nos prejuízos de aprendizado e memória, por exemplo, o aumento na atividade da AChE observado anteriormente, já que a acetilcolina é um neurotransmissor fundamental para os processos de memória e cognição (MICHEAU and MARIGHETTO, 2011). A síndrome do comportamento doentio, provocada pelo aumento nas citocinas pró-inflamatórias no cérebro, também pode ter um papel importante no prejuízo cognitivo observado após estresse (MAES et al., 2009).

Como o hipocampo é uma estrutura tradicionalmente relacionada a processos cognitivos como aprendizado e memória (RIEDEL and MICHEAU, 2001), decidimos investigar o efeito do estresse sobre o imunoconteúdo de BDNF nesta estrutura cerebral. O BDNF tem sido implicado na regulação da plasticidade neural, na expressão gênica e na função sináptica, assim como no mecanismo de ação dos antidepressivos (YAMADA et al., 2002; LEE and SON, 2009; MUSAZZI et al., 2009). A determinação do imunoconteúdo de BDNF em hipocampo foi feita após a realização dos testes comportamentais. Nossos resultados demonstraram que não houve diferença significativa nos níveis de BDNF entre os grupos controle e estresse no período avaliado. Os trabalhos que avaliam conteúdo e expressão de BDNF após estresse apresentam resultados conflitantes, demonstrando diminuição (FUCHIKAMI et al., 2010) ou nenhuma alteração (ALLAMAN et al., 2008). As alterações na neurogênese e na expressão do BDNF variam de acordo com fatores, como as características dos estressores e a duração do estresse (ANISMAN and MATHESON, 2005), além disso, essas alterações ocorrem dentro de um período específico de tempo e podem ser revertidas após a interrupção do estresse (McLAUGHLIN et al., 2007). Estas observações sugerem que as alterações funcionais características da depressão refletem mais uma moderada reorganização estrutural do que consequências de morte neuronal (CONRAD et al., 1999).

Através da combinação de técnicas bioquímicas e comportamentais, nossos resultados demonstraram que múltiplos órgãos e sistemas participam na adaptação do organismo ao estresse e que o entendimento dos

mecanismos envolvidos é fundamental para o desenvolvimento de novas terapias para a depressão.

6 CONCLUSÕES

ESTUDOS BIOQUÍMICOS

- A indução do modelo experimental de ECV provocou um aumentou nos níveis de TBARS e na razão SOD/CAT e não alterou a atividade da GPx em sangue de ratos.
- O ECV não alterou os níveis séricos dos marcadores de resposta inflamatória, IL-6, TNF-α, PCR e NO.
- Animais estressados apresentaram uma menor atividade da enzima BuChE em soro em relação aos animais controles.
- Os animais submetidos ao estresse apresentaram um aumento nos níveis plasmáticos de HCY em relação ao grupo controle, entretanto, os níveis de folato não foram alterados.
- Os níveis das citocinas IL-1 β, IL-6 e TNF-α e a atividade da AChE foram aumentados em hipocampo de ratos após a indução do estresse crônico, mas não houve alteração nos níveis de MCP-1.
- O grupo estressado apresentou uma inibição nas atividades dos complexos II e IV da cadeia respiratória em córtex pré-frontal, enquanto que em hipocampo apenas a atividade do complexo IV está diminuída.
- A atividade da piruvato quinase não foi alterada pelo estresse em nenhuma das estruturas estudadas.
- A administração crônica de vitaminas E e C durante a indução do modelo de estresse preveniu a inibição das enzimas da cadeia respiratória.
- Não houve alteração no conteúdo de BDNF em hipocampo de ratos submetidos ao ECV.

ESTUDOS COMPORTAMENTAIS

- Animais submetidos ao ECV apresentaram prejuízo na fase de aprendizado da memória de referência, pior performance no teste de retenção da memória de referência e prejuízo na memória de trabalho.
- A administração de antioxidantes preveniu o prejuízo na fase de aprendizado da memória de referência e na memória de trabalho, mas não alterou o teste de retenção da memória de referência.

CONCLUSÃO GERAL

Embora ainda existam muitas dúvidas a respeito da patofisiologia da depressão e dos distúrbios causados por estresse, demonstramos no presente trabalho que o estresse crônico em ratos induz estresse oxidativo e altera a atividade da BuChE em sangue de ratos, induz inflamação e aumenta a atividade da AChE em hipocampo. Além disso, o paradigma de estresse crônico provocou alterações de metabolismo energético e de memória que foram prevenidas pela administração crônica de antioxidantes. Estes achados em conjunto podem auxiliar na elaboração de uma teoria que considere as inter-relações entre diferentes órgãos e sistemas para explicar a patofisiologia da depressão e abrir caminhos para novas alternativas de tratamento.

7 PERSPECTIVAS

1- Avaliar o efeito da administração concomitante de antioxidantes com antidepressivos clássicos sobre parâmetros bioquímicos e comportamentais alterados pelo modelo de estresse.

2- Avaliar se a administração de antioxidantes após a indução do modelo de estresse é capaz de reverter os danos neurológicos já instalados.

3- Investigar possíveis alterações epigenéticas provocadas pelo modelo de estresse através da determinação dos níveis de metilação do DNA.

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