UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL- UFRGS

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE- ICBS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: NEUROCIÊNCIAS

EFEITOS DE DIFERENTES MODELOS DE ESTRESSE CRÔNICO SOBRE PARÂMETROS NEUROQUÍMICOS E COMPORTAMENTOS DO TIPO ANSIOSO E DO TIPO DEPRESSIVO EM RATOS

Leonardo Machado Crema

Porto Alegre

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

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"*Se o conhecimento pode criar problemas, não é através da ignorância que podemos solucioná-los*"

Isaac Asimov (1920-1992)

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Abreviaturas

5-HT- serotonina

$5HT_{1A}$- subtipo de receptor de serotonina

A_{1A}, A_{2A}, A_{2B} e A_3- subtipos de receptores de adenosina

ACTH- Hormônio Adrenoconticotrópico

AMPc- Adenosina Monofosfato Cíclico

ATP- Trifosfato de Adenosina

BDNF- Fator Neurotrófico Derivado do Encéfalo

Bmax- Ligação total do ligante radioativo

CAT- Catalase

CREB- Elemento de Resposta a Ligação do AMPcíclico

CRH- Hormônio Liberador de Corticotropina

CRS- Modelo de Estresse Crônico Repetido

D_1 e D_2- Subtipos de Receptores de Dopamina

DA- Dopamina

DSM-IV- Manual Diagnóstico e Estatístico de Transtornos Mentais 4º Edição

ECVT- Eletroconvulsoterapia

EROs- Espécies Reativas de Oxigênio

GPx- Glutationa Peroxidase

HPA- Eixo Hipotálamo- Pituitária- Adrenal

IL-6- Citocina pró- inflamatória Interleucina do tipo 6

NA- Noradrenalina

NA^+,K^+,-ATPase- Sódio, Potássio - ATPase

PET- Tomografia por Emissão de Pósitrons

PTSD- Distúrbio do Estresse Pós- Traumático

REM- Rapid Eyes Movement- Movimento Rápido dos Olhos

SNC- Sistema Nervoso Central

SOD- Superóxido Dismutase

SRE- Sistema de Recompensa Encefálico

TCAs- Classe de Antidepressivos Tricíclicos

TNF- Teste do Nado Forçado

TNFα- Fator de Necrose Tumoral alpha

TSC- Teste do Consumo de Sacarose

UCMS- Modelo de Estresse Crônico Moderado Imprevisível

Resumo

Na presente tese, nós estudamos os efeitos do estresse crônico repetido (CRS) e do estresse crônico imprevisível moderado (UCMS) sobre comportamentos do tipo- ansioso e do tipo- depressivo na tentativa de estabelecer possíveis diferenças comportamentais sobre os modelos de estresse. Além disso, verificar os efeitos de ambos os modelos sobre parâmetros bioquímicos como a atividade da enzima Na^+, K^+-ATPase e o *binding* dos receptores de adenosina A_1 (A_1Rs) e A_{2A} ($A_{2A}Rs$) no hipocampo e estriado, respectivamente, de ratos machos adultos *Wistar*. Nos dois trabalhos apresentados neste estudo, os animais foram submetidos ao CRS e ao UCMS durante 40 dias e subsequentemente foram avaliados em uma série de tarefas comportamentais para estudo de comportamentos do tipo- ansioso e do tipo- depressivo. O primeiro artigo demonstrou que ratos submetidos ao CRS e UCMS apresentaram comportamento do tipo- ansioso, analisado pela diminuição na permanência nos quadrados centrais na tarefa do campo aberto. Além disso, foi demonstrada uma diminuição da atividade da enzima Na^+, K^+-ATPase na amígdala desses ratos, não sendo, todavia, observado alteração do imunoconteúdo da enzima. Adicionalmente, com o objetivo de elucidar as possíveis causas da diminuição da atividade da Na^+, K^+-ATPase,medimos diversos parâmetros de estresse oxidativo, porém não obtivemos qualquer diferença significativa nessas medidas, capaz de explicar, ao menos em parte, uma possível causa dessa diminuição da Na^+, K^+-ATPase na amígdala dos ratos estressados cronicamente. No segundo trabalho, somente o UCMS foi capaz de induzir comportamento do tipo- depressivo, verificado pelo aumento no tempo de imobilidade no teste do nado forçado. Este comportamento tem sido interpretado como desamparo aprendido. Desse modo, utilizamos somente o UCMS como variável para o consumo de solução de sacarose 1%. Este consumo foi monitorado semanalmente, durante oito semanas. De fato, UCMS foi capaz de induzir diminuição no consumo de solução de sacarose, comportamento entendido como anedonia, perda de motivação em situações prazerosas. Uma vez estabelecidas as diferenças comportamentais entre CRS e UCMS, verificamos alterações no sisterma adenosinérgico ao analisarmos os A_1Rs e os $A_{2A}Rs$. Demonstramos uma similaridade no *binding* de A_1Rs, aumentando Bmax com aumento do imunoconteúdo dos A_1Rs tanto no CRS quanto no UCMS. Interessantemente, quanto ao *binding* de $A_{2A}Rs$, o grupo UCMS mostrou-se diferente do CRS, com aumento de Bmax para $A_{2A}R$. Em suma, concluímos que os dois modelos de estresse crônico causaram alterações similares na atividade da Na^+, K^+-ATPase na amígdala de ratos, e ambos os grupos estressados aumentaram o comportamento do tipo- ansioso e sensibilização (*up-regulation*) de A_1Rs no hipocampo. Por outro lado, somente UCMS foi capaz de induzir desamparo aprendido, anedonia e aumento no *binding* de $A_{2A}Rs$ no estriado. Enfim, acreditamos que estas alterações neuroquímicas e comportamentais expostas na presente tese possam servir no refinamento do conhecimento básico para posteriores interesses no melhoramento de terapias farmacológicas sobre psicopatologias.

Abstract

The aim of this dissertation was to study the effects of Chronic Restraint Stress (CRS) and Unpredictable Chronic Mild Stress (UCMS) upon anxiety-like and depressive-like behaviors in order to establish possible behavioral differences between CRS and UCMS. In addition, we aimed to verify effects of both stress models upon biochemical parameters such as Na^+, K^+-ATPase activity and binding of the A_1 (A_1Rs) e A_{2A} (A_{2A}Rs) adenosine receptors in hippocampus and striatum, respectively, in adult male Wistar rats. In all studies, the animals were submitted to CRS and UCMS during 40 days; the control group was no submitted to any kind of stress, and subsequently all groups were submitted to behavioral tasks to evaluate anxiety-like and depressive-like behaviors. The first paper demonstrated that both stress models (CRS and UCMS) were able to increase anxiety-like behavior evaluated as the time in the central area of the open field task. Additionally, there was decreased Na^+, K^+-ATPase activity in amygdala of stressed rats. Besides that, there were no alterations in α 3 subunit immuncontent. We tried next to elucidate possible causes for the decreased Na^+, K^+-ATPase activity, and we measured several oxidative stress parameters, however no important differences were detected in this analysis, that could explain, at least in part, the possible causes of a decrease in Na^+, K^+-ATPase activity in amygdala of chronically stressed rats. On the other hand, only the UCMS group was able to induce depressive-like behavior, displayed by increased immobility time on the forced swimming test, which has been interpreted as learning helplessness. Therefore, we next studied if UCMS could lead to altered consumption of sucrose 1%, and this consumption was monitored weekly during eight weeks. Indeed, UCMS was able to induce decreased consumption of sucrose solution, a response that was considered as anhedonia, lost of motivation for pleasant situations. Once these behavioral differences between CRS and UCMS were detected, we studied possible alterations on the adenosinergic system, analyzing A_1Rs e A_{2A}Rs We showed similarities on the effects of both types of chronic stress on A_1Rs binding, since both increased Bmax as well as A_1Rs immunocontent. Interestingly, when we analyzed A_{2A}Rs binding, only UCMS increased A_{2A}Rs Bmax. Finally, we concluded that CRS and UCMS were capable of inducing similar alterations in Na^+, K^+-ATPase activity in amygdala of rats. Additionally, both stressed groups increased anxiety-like behavior and showed up-regulation in hippocampal A_1Rs. Besides, UCMS was able to induce learned helplessness, anhedonia and up-regulation in striatal A_{2A}Rs. It is expected that the behavioral and biochemical changes presented in this dissertation could refine the basic knowledge in this area, improving pharmacological therapies to treat psychopathologies.

1. INTRODUÇÃO

1.1. Definição, epidemiologia e impacto social da Depressão

O transtorno depressivo é uma séria doença incapacitante que ameça a qualidade de vida do indivíduo (Sullivan *et al.,* 2000;Duman, 2010) e apresenta um conjunto de etiologias: sócio-ambiental, genética e do desenvolvimento. Esses fatores isolados ou combinados induzem um estado de vulnerabilidade no indivíduo (Sullivan et al., 2000; Davidson, 2011). De acordo com o Manual Diagnóstico e Estatístico de Transtornos Mentais (*Diagnostic and Statistical Manual of Mental Disorders*, DSM-IV, 1994), a pessoa diagnosticada com depressão deverá apresentar juntamente com humor disfórico, perda de interesse ou prazer em praticamente todas as atividades, sintoma conhecido como anedonia. Além disso, o paciente deverá exibir pelo menos quatro sintomas adicionais por um período de no mínimo duas semanas (Williamns et al., 2000; Kessler et al., 2005; Davidson, 2011). Estes sintomas deverão causar significante sofrimento e prejuízo social, ocupacional e em outras áreas de funcionamento (Williams et al., 1995).

Dados levantados pela Organização Mundial da Saúde (*World Health Organization* - WHO) indicam a depressão entre os principais distúrbios psiquiátricos, afetando aproximadamente 121 milhões de indivíduos em todo o mundo, incluindo 17 milhões de brasileiros afetados por essa doença. Sua prevalência ao longo da vida varia de 5% a 25% dependendo da população investigada (Mitchell et al., 2009).

Para o ano de 2020, está previsto que o transtorno depressivo alcançará o segundo lugar no *ranking* como distúrbio incapacitante entre todas as idades e sexos (Sullivan et al., 2000). Dados levantados pela *WHO* demonstram que a depressão já está no quarto lugar entre as causas nesse *ranking*. Contudo, o tratamento da depressão e os custos diretos e indiretos representam um enorme prejuízo econômico

para a sociedade. Somente nos Estados Unidos, um estudo realizado em 1990, estima que os gastos anuais sejam entre $43- 53 billhões de dólares (Greenberg et al., 2001). Já em 2004, pesquisando 466 milhões de pessoas em 28 países da Europa foi estimado um gasto anual de 118 bilhões de Euros (Sobocki et al., 2006).

A depressão acomete pessoas de todas as idades, etnias e classes socioeconômicas (Brhlikova et al., 2011), trazendo prejuízos na qualidade das relações interpessoais e pode apresentar comorbidade com outras doenças psiquiátricas e neurológicas, como ansiedade, abuso de substâncias, doença de Parkinson, entre outros diversos distúrbios somáticos, os quais limitam atividades normais (Gotlib & Joormann, 2010).

1.2. Etiologia da Depressão

A depressão não é uma doença de causa única, e sim, resulta da combinação de fatores genéticos, bioquímicos, ambientais e psicológicos. Alguns subtipos de depressão como o distúrbio depressivo maior apresentam maior incidência em determinadas famílias, desse modo sugerindo uma influência genética (Bienvenu et al., 2011). Estudos genéticos sugerem que o risco para adquirir a doença resulta da influência de múltiplos genes, os quais podem sofrer interferências ambientais (Bienvenu et al., 2011). Além disso, episódios depressivos podem ser desencadeados por eventos traumáticos, (e.g. perda de um ente querido) ou qualquer situação percebida como estressante (Klauke et al., 2010). Diversos fatores psicossociais, como idade, gênero, classe social, educação e renda tem também sido identificados como importantes fatores que podem explicar a variabilidade na prevalência da doença. (Akhtar-Danesh & Landeen, 2007).

Comumente, as mulheres apresentam maior prevalência de depressão e ansiedade do que homens (taxa de risco ao longo da vida para transtorno depressivo maior de 10-25% em mulheres e 5-10% em homens). Isto pode ser o resultado de características hormonais e psicossociais singulares entre homens e mulheres, as quais podem explicar as altas taxas de depressão. Foi demonstrado que hormônios como os estrógenos, afetam estruturas límbicas que são importantes no controle das emoções e do humor. Os efeitos do estresse, violência, carência de suporte social, problemas familiares, baixa auto-estima e estilos cognitivos ruminativos têm se relacionado com a alta vulnerabilidade desta enfermidade em mulheres (Posmontier, 2008; Parker & Brotchie, 2010). O elevado risco de desenvolver esse transtorno, também está associado com a presença de condições médicas como doenças cardíacas e câncer. Além disso, a medicação usada para essas enfermidades pode produzir efeitos colaterais que podem contribuir para o desenvolvimento da depressão (Parker & Brotchie, 2010).

Apesar do forte impacto e prevalência da depressão na sociedade, existe ainda pouco conhecimento sobre sua patogenia. Isso pode ser devido a vários aspectos, como a dificuldade de pesquisar mudanças patológicas no encéfalo humano. As técnicas disponíveis para acessar as funções cerebrais consistem de estudos post-mortem e de neuroimagem (Tham et al., 2010), as quais fornecem importantes relatos sobre as regiões encefálicas envolvidas neste transtorno do humor, embora simples mudanças na atividade cerebral possam não explicar esta complexa doença em sua totalidade (Krishnan & Nestler, 2008).

A modulação das emoções, recompensa e funções executivas engloba diversas regiões e circuitos encefálicos, os quais são altamente interconectados. Entre essas

estruturas, o córtex pré-frontal, estriado ventral (incluindo o núcleo accumbens), amígdala e hipocampo têm um papel fundamental. Acredita-se que prejuízos morfofuncionais nessas áreas estejam relacionados à depressão, por essa razão, essas estruturas são consideradas alvos estratégicos para o tratamento antidepressivo (Maletic et al., 2007). Desse modo, as regiões encefálicas citadas acima parecem contribuir para diferentes mecanismos da doença (Maletic et al., 2007). Por exemplo, neocortex e hipocampo podem mediar sintomas cognitivos da depressão, como prejuízos de memória, sentimentos de desvalia, desesperança e culpa. Estudos prévios avaliando pacientes deprimidos demonstraram alterações no fluxo sanguíneo, redução do volume da substância cinzenta e densidade glial no córtex pré-frontal e no hipocampo (Krishnan &Nestler, 2008). Além disso, prejuízos no estriado e no núcleo *accumbens* podem ser responsáveis por déficits em memórias emocionais, além de induzir motivação reduzida e anedonia. O hipotálamo, também apresenta papel importante nesta doença, modulando funções neurovegetativas como o sono-vigília, distúrbios de apetite e regulação do eixo Hipotálamo-Hipófise- Adrenal. Adicionalmente, outra estrutura chave na regulação das respostas ao estresse e depressão é a amígdala, uma vez que alterações morfofuncionais e bioquímicas nesta estrutura encefálica estão implicadas na modulação do medo e da ansiedade (Rodrigues et al., 2009)

Estudos de imageamento por ressonância magnética funcional e tomografia por emissão de pósitrons têm demonstrado que sintomas depressivos associados com aumento da atividade da amígdala e córtex cingulado subgenual estão relacionados a emoções disfóricas (Krishnan & Nestler, 2008; Vago et al.,2011).

O papel do sistema serotoninérgico e do eixo HPA nos mecanismos da depressão estão bem documentados, entre outros sistemas regulatórios do encéfalo. Durante a depressão, o eixo HPA pode estar hiperativado, uma vez que cerca de 40% dos pacientes deprimidos apresentam níveis elevados do Hormônio Liberador de Corticotropina (CRH) e cortisol (Bremmer et al.,2000), uma anormalidade que pode ser revertida com antidepressivos e exercício físico (Dickerson & Kemeny, 2004). Baixa resposta da secreção do Hormônio Adrenocorticotrópico, (ACTH) ao teste da dexametasona está entre outra característica desse transtorno, a qual reflete a função alterada do eixo HPA (Hayley et al., 2005). Acredita-se que os elevados níveis de CRH, gradualmente desensibilizam os receptores de CRH que atenuam a resposta da hipófise. De acordo com outra hipótese, a resposta atenuada da liberação de ACTH pela hipófise parece ser devido à sensibilidade diminuída de receptores de serotonina (5-HT) e reduzida neurotransmissão serotoninérgica. A liberação de ACTH é regulada por receptores de serotonina 1A ($5\text{-}HT_{1A}$) presentes no hipotálamo e na hipófise (Keeney et al., 2006). Vários estudos indicam prejuízos na neurotransmissão serotoninérgica (Doherty & Gratton, 1996; Briones-Aranda et al., 2005), sendo que estudos farmacológicos e por PET demonstraram um diminuição na sinalização mediada por receptores $5\text{-}HT_{1A}$ em pacientes deprimidos (Anisman, 2009).

A hipótese monoaminérgica (Maletic et al., 2007; O'Mahony et al., 2011) postula que a depressão é causada pelo prejuízo no sistema monoaminérgico no encéfalo, caracterizado pela deficiência na neurotransmissão mediada por 5-HT, noradrenalina (NA) e dopamina (DA). Esta hipótese tem sido refinada com o passar dos anos e mais evidências experimentais e clínicas estão vindo a tona (Krishnan & Nestler, 2008; Anisman, 2009). A concentração de monoaminas pode ser alterada através de uma

perturbação na síntese, armazenamento ou liberação, ou estes podem manter-se inalterados, mas as atividades dos receptores e/ou mensageiros intracelulares podem estar alteradas. Monoaminas afetam diversas funções, as quais estão alteradas no curso da doença, prejudicando funções como: sono-vigília, apetite, motivação, atividade motora e recompensa. Outra hipótese está baseada em alterações no sistema de recompensa encefálico (SRE), circuito neural envolvido na resposta a experiências recompensadoras em animais, incluindo humanos, e postula-se que esse sistema possa sustentar mecanismos cerebrais desencadeadores da anedonia em transtornos depressivos (Naranjo et al., 2001; Nestler & Carlezon, 2006).

Finalmente, o papel de fatores neurotróficos na etiologia da depressão tem sido também discutido (Altar, 1999, Larsen et al., 2010). Fatores neurotróficos são conhecidos por serem potentes reguladores da plasticidade e sobrevivência de células neurais e gliais adultas. Assim, a hipótese neurotrófica sugere a diminuição de fatores neurotróficos, contribuindo para o déficit na função hipocampal durante o desenvolvimento da síndrome depressiva, sendo esta condição, revertida por tratamento antidepressivo e eletroconvulsoterapia (ECVT) (Kunugi et al., 2010.; Larsen et al., 2010). Esta hipótese tem dado maior enfoque ao fator neurotrófico derivado do encéfalo (BDNF), um dos principais fatores neurotróficos no encéfalo (Nestler et al., 2002), o qual apresenta níveis de concentração diminuída no soro e no hipocampo de pacientes que apresentaram estados depressivos como anedonia, além disso foi demonstrado diminuição dos níveis de BDNF no hipocampo de ratos submetidos ao estresse crônico imprevisível (Karege et al., 2002; Larsen et al., 2010), além de apresentar um importante papel na regulação do eixo HPA (Kunugi et al., 2010).

1.3. O papel do estresse na patogênese da depressão

O termo estresse foi originalmente definido na literatura biomédica por Hans Selye, na década de 1940 (Selye & Fortier, 1949), como uma reação não específica do organismo após uma reação a estímulos nocivos, chamados de estressores. Atualmente, estresse é caracterizado como um conjunto de respostas adaptativas (físicas, mentais ou emocionais) diante de eventos capazes de causar mudanças na homeostase do organismo, permitindo maximizar as chances de sobrevivência quando submetido a situações ameaçadoras. A resposta do organismo ao estresse começa no sistema nervoso central (SNC), o qual processa as informações sensoriais relacionadas a estressores externos. Se a situação é avaliada como potencialmente nociva, uma cascata de respostas neurais, hormonais e comportamentais será iniciada para lidar com a situação (Rodrigues et al., 2009).

Embora seja verdade que o estresse pode ter associação causal direta e indireta com a patogênese da depressão (Paykel, 2001), o impacto do estresse no organismo dependerá de várias características (tipo de estressor- agudo ou crônico, controlável ou incontrolável) e das condições do indivíduo afetado, como a habilidade de lidar com o problema e o histórico de eventos estressantes (Anisman & Merali, 2000; Paykel, 2001).

As alterações que ocorrem durante uma resposta ao estresse são frequentemente encontradas na depressão. O eixo HPA é ativado pelo estresse, o qual leva à liberação de glicocorticóides (Fig.1). Em pacientes depressivos, a concentração de glicocorticóides está frequentemente elevada, a qual caracteriza a disfunção no eixo HPA (Nestler et al., 2002; Krishnan & Nestler, 2008). Isto leva à hiperatividade do

sistema nervoso simpático e a hipertrofia das adrenais. Esses prejuízos causam um típico perfil caracterizado pela diminuição nos níveis de catecolaminas, como a noradrenalina, adrenalina e dopamina na corrente sanguínea, e aumento dos níveis plasmáticos de cortisol. O aumento dos níveis de cortisol é devido a liberação de CRH pelo hipotálamo, o qual estimula a síntese e liberação de ACTH proveniente da glândula hipófise (Ulrich-Lai & Herman, 2009). Este hormônio estimula a produção de glicocorticóides, cortisol em humanos e corticosterona em roedores, pelo córtex da glândula adrenal, causando a condição denominada hipercortisolemia e hipercorticosterolemia, respectivamente.

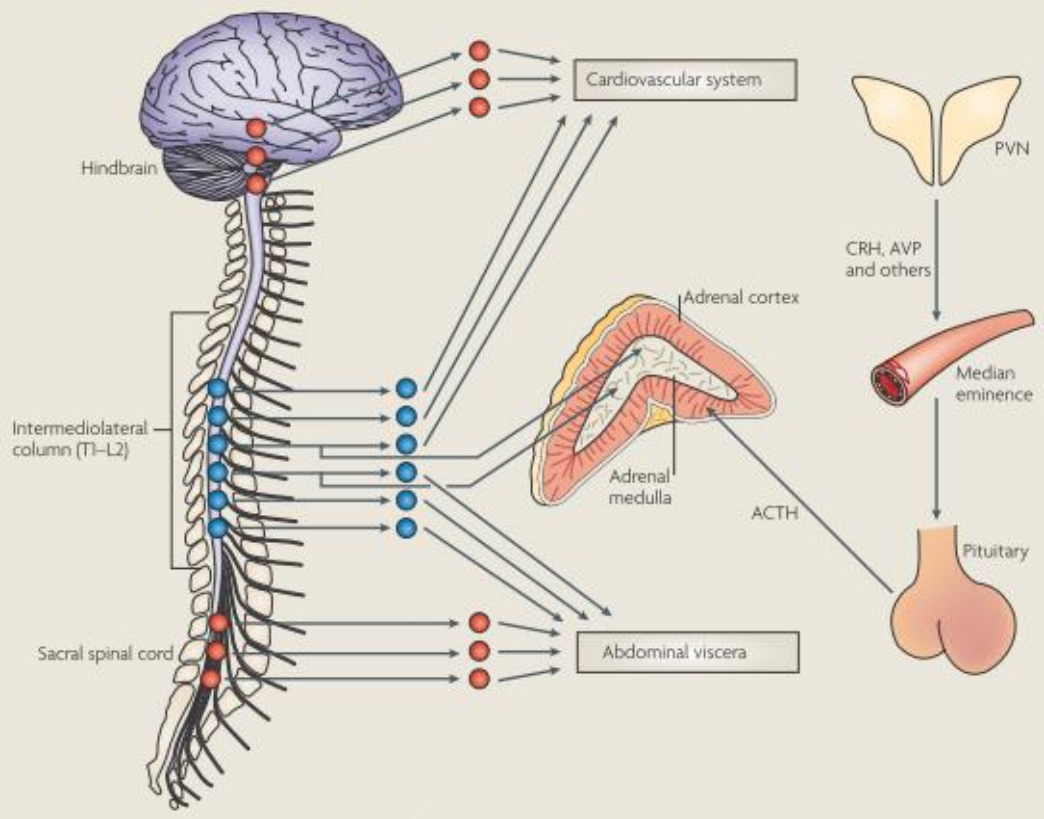

Figura 1. Eixo HPA e sistema nervoso neurovegetativo de resposta ao estresse (Ulrich-Lai & Herman, 2009)

Os glicocorticóides exercem um profundo efeito no metabolismo e também dramaticamente afetam o comportamento, atuando diretamente em diversas áreas encefálicas, por exemplo, causando prejuízos no hipocampo (Krishnan & Nestler, 2008). Estresses crônico e agudo podem também reduzir a expressão de BDNF, o que pode ser observado no hipocampo de humanos depressivos post-mortem (Nestler et al., 2002; Krishnan & Nestler, 2008).

1.4. Depressão e outras doenças psiquiátricas relacionadas ao estresse

Eventos estressores são conhecidos por favorecer o desenvolvimento de depressão em humanos (Kendler et al., 1999). No entanto, após situações traumáticas, a maioria das pessoas podem não se tornar deprimidas. Além disso, a maioria dos estressores para grande parte da população são considerados moderados. Estressores graves como guerra, estupro ou morte de um ente querido, em geral, não induzem depressão, mas ao invés disso levam ao desenvolvimento de síndromes como os distúrbios do estresse pós-traumático (PTSD), do pânico e da ansiedade generalizada. A depressão compartilha características fundamentais com essas doenças relacionadas ao estresse, mas há diferenças no que diz respeito aos sintomas da doença (Nestler et al., 2002); contudo a sobreposição de sintomas são suficientes para um falso diagnóstico. Entre os sintomas que podem sofrer sobreposição destacam-se: distúrbios de sono, irritabilidade, agitação, dificuldade de concentração, perda de controle, fadiga e angústia. Desse modo, levanta-se a necessidade de estudos buscando um melhor esclarecimento da natureza de sobreposição destes sintomas.

O mesmo acontece em modelos animais usados para mimetizar depressão. Para induzir sintomas do tipo-depressivo, alguns modelos consistem na exposição dos

animais a situações estressantes (Duman, 2010). A exposição ao estresse frequentemente resulta em diversas alterações comportamentais, neuroquímicas e neuroendócrinas, semelhante àquelas encontradas em humanos com depressão (Willner, 1997; Sapolsky, 2003; Rodrigues et al., 2009).

1.5. Pesquisa básica com modelos animais de depressão

A depressão é caracterizada por um amplo espectro de alterações, as quais não são reproduzíveis em sua totalidade em modelos animais (Cryan & Holmes, 2005). A maioria dos principais sintomas da depressão, como humor deprimido, sentimento de desvalia e pensamentos de suicídio, não pode ser facilmente medido em animais de laboratórios uma vez que lhes falta, por exemplo, a simples capacidade de relatar pensamentos entre outras considerações (Nestler et al., 2002). Contudo, existem diversos endo-fenótipos na depressão (Cryan & Slattery, 2007), os quais podem ser reproduzidos independentemente e avaliados em animais. Entre estes endo-fenótipos podemos citar: anedonia, ansiedade (sintoma com alta prevalência na depressão) distúrbios neuroendócrinos, principalmente relacionados ao eixo HPA como alterações de peso, alterações do ritmo circadiano, entre outras mudanças neuroanatômicas no encéfalo (Cryan & Slattery, 2007). Contudo, uma vez que estes sintomas podem ser encontrados em outras psicopatologias, deve-se ter cuidado na interpretação dos mesmos (Cryan & Slattery, 2007).

Com isso, modelos animais de depressão devem representar diversos aspectos da depressão nas espécies pesquisadas, comumente roedores (ratos e camundongos). Estes, representam uma valiosa ferramenta de estudo dos aspectos neurobiológicos da

depressão, como também no estudo de mecanismos de ação de fármacos antidepressivos (Willner, 1997; Deussing, 2006).

Embora humanos e roedores apresentem diferenças marcantes na anatomia encefálica, diversos circuitos que regem respostas comportamentais e fisiológicas, estão conservados entres estas espécies (Cryan & Holmes, 2005). Por meio de inferências baseadas em achados provenientes de modelos animais, nós podemos elucidar comportamentos, vias neurais e fatores genéticos relacionados ao transtorno depressivo e aperfeiçoar o entendimento do comportamento humano frente à doença.

Para isso o modelo deve apresentar uma razoável analogia com a patologia humana, sua sintomatologia (validade) e mudanças comportamentais que podem ser monitoradas objetivamente (Willner, 2005), podendo ser revertidas através do mesmo tratamento efetivo em humanos (validade preditiva). Adicionalmente, isto deve ser reprodutível em diferentes grupos de pesquisa (McKinney & Bunney, 1969), e ter etiologia similar entre o modelo e a patologia humana (Frazer & Morilak, 2005). Existe diversos testes que têm sido validados em modelos animais. Estes incluem tarefas que avaliam desamparo aprendido, ansiedade e anedonia.

1.6. Modelos de estresse

A depressão é fortemente influenciada por eventos estressantes e traumáticos ao longo da vida, sugerindo que pacientes deprimidos devam ter prejuízos em estratégias de lidar com situações aversivas (de Kloet et al., 2005). Devido a isso, a maioria dos modelos são baseados na exposição do animal a uma variedade de estressores.

Atualmente, modelos de estresse estão baseados em estresses fortes, moderados ou a combinação dos dois. Os modelos de estresse crônico parecem serem mais apropriados para modelos de depressão comparado a outros modelos, além disso estresse crônico e frustração crônica comumente induzem alterações neurobiológicas, as quais podem levar à depressão (Willner, 1997).

1.7. Paradigma do estresse crônico moderado

Willner e colaboradores desenvolveram em 1987 o paradigma do estresse crônico moderado, o qual incluía uma variedade de estressores moderados aplicados por um longo período (Willner et al., 1987). A apresentação de diferentes tipos de estressores é essencial para o modelo, ao invés de aplicar um único estressor de forma repetida que frequentemente induziria habituação comportamental (Muscat & Willner, 1992). Os estressores utilizados foram: maravalha suja, inclinação da caixa moradia, alterações no ciclo claro escuro, períodos de privação de água e comida e agrupamento (Willner et al., 1987). A duração da exposição de cada estressor variou entre poucas horas a um dia, durante 2 (duas) até 5 (cinco) semanas. Este modelo parece simular melhor a condição do ambiente humano, principalmente pela exposição do indivíduo a estressores diários moderados do que a eventos traumáticos. Algumas das anormalidades vistas no CMS (da sigla em inglês *Chronic Mild Stress*), designado neste trabalho como UCMS (da sigla em inglês Unpredictable Chronic Mild Stress) e adaptado no nosso laboratório por Manoli et al., (2000) apresentaram sintomas do tipo depressivos observados em humanos e que foram revertidos por tratamento crônico com antidepressivos (Manoli et al., 2000; Mitchell & Baker, 2010).

Os roedores, como a maioria dos humanos, normalmente preferem consumir soluções doces (Willner et., 1987). Willner e colaboradores demonstraram que o CMS pode induzir significante redução no consumo de sacarose, comportamento designado como anedonia, um dos principais sintomas do transtorno depressivo. Desse modo, a reatividade à recompensa foi adotada como o objetivo neste paradigma (Willner, 2005). Esta diminuição tem duração superior a 8 (oito) semanas, podendo ser revertida através de terapia com antidepressivos tricíclicos (TCAs) (Willner et al., 1987).

Além disso, o modelo acima citado diminui comportamentos de agressividade, sexuais e de atividade locomotora em ratos durante a fase ativa (Yan et al., 2010). Alterações circadianas e do ritmo diurno (Gorka et al., 1996), distúrbios do sono, como mudanças no padrão de sono REM (do inglês, *Rapid Eyes Movement*) (Cheeta et al., 1997; Grønli, 2007) foram, também, observados, além de perda de peso (Willner & Jones, 1996), distúrbios na regulação simpática das funções cardíacas (Grippo et al., 2003), níveis séricos de citocinas como o fator de necrose tumoral α (TNF- α) (Grippo et al., 2005) e aumento da atividade do eixo HPA (Muscat & Willner, 1992).

1.8. Estresse Crônico e a enzima Na+,K+, ATPase

A enzima Na^+, K^+- ATPase ou bomba Na^+, K^+, pertence à classe de ATPases do tipo-P, a qual hidrolisa o ATP, gerando energia para o transporte iônico pela membrana plasmática. Esta enzima exporta íons Na^+ da célula e importa íons K^+, desse modo permitindo a geração e a manutenção da alta concentração de Na^+ extracelular e alta concentração de K^+ intracelular. A manutenção desses gradientes iônicos é fundamental para diversas funções celulares, incluindo a regulação do potencial de repouso e excitabilidade celular. O gradiente de Na^+ gerado pela Na^+, K^+- ATPase é

utilizado para múltiplos sistemas de transporte secundário, incluindo o transporte de glicose e aminoácidos, liberação de Ca^{2+} e H^{+} via canais iônicos que trocam Na^{+}/Ca^{2+} e Na^{+}/H^{+} (Michel et al., 2007; Newman et al., 2008; Illarionova et al., 2010). A atividade da Na^{+}, K^{+}- ATPase está sujeita a regulação fisiológica por moduladores endógenos e por alterações nos estados de fosforilação protéica. Prejuízos nessa enzima podem resultar também de um conjunto de situações como déficit de energia, aumento de Espécies Reativas de Oxigênio (EROs) e diferentes sinais de estresse celular (Zhang et al., 2008).

Estudos anteriores do nosso laboratório têm demonstrado que o estresse crônico, modelo de depressão, influencia a modulação da enzima Na^{+}, K^{+}- ATPase no cérebro de ratos. Gamaro e colaboradores, (2003) verificaram uma redução da atividade da enzima no hipocampo de ratos submetidos ao estresse crônico moderado por 40 (quarenta) dias. Apoiando o estudo de Gamaro, de Vasconcellos e colaboradores (2005) utilizando o mesmo modelo experimental de depressão replicaram uma diminuição similar na atividade da enzima Na+, K^{+}- ATPase no hipocampo, no entanto este efeito foi revertido através do tratamento crônico com lítio, sugerindo que este efeito possa ser um dos mecanismos de ação do tratamento com lítio em transtornos do humor (de Vasconcellos et al., 2005).

No entanto, é interessante ressaltar que não há estudos demonstrando alterações da enzima Na+, K^{+}- ATPase em outras estruturas límbicas de resposta ao estresse relacionadas com a regulação do humor. Desse modo, levanta-se a questão de como estariam estes parâmetros em outras estruturas do SNC utilizando modelos de estresse crônico como modelo de depressão.

1.9. Estresse Crônico e o Sistema Adenosinérgico

A adenosina é uma molécula neuromoduladora de sinalização extracelular que influencia a transmissão sináptica e modulando a atividade do sistema nervoso (Boison, 2007). A adenosina é formada dentro da célula como resultado da hidrólise do AMP (Adenosina Monofosfato), pela ação da enzima 5'- nucleotidase. No meio extracelular, a adenosina depende da taxa de hidrólise do ATP liberado de neurônios e de células gliais, quando esses estão sob estresse (Boison, 2007; Stone et al., 2009).

A adenosina modifica a função celular atuando em 4 (quatro) diferentes receptores acoplados a proteínas G (A_1, A_{2a}, A_{2B} e A_3) localizados na membrana celular de diversos tecidos, incluindo o tecido nervoso (Boison, 2007 ; Sebastião & Ribeiro, 2009). Sua principal via de sinalização intracelular envolve o controle da formação de AMPc, no qual a ativação de A_1 e A_3 inibem e a ativação de A_{2a} e A_{2B} estimulam a formação de AMPc (Boison, 2007; Stone et al., 2009). Além disso, a adenosina está envolvida na regulação de importantes funções do SNC como a memória, o alerta, a agressividade e a ansiedade (Sebastião & Ribeiro, 2009). No entanto, o papel da adenosina na modulação da depressão, a qual está associada a prejuízos na plasticidade estrutural e resiliência celular (Sebastião & Ribeiro, 2009), ainda não estão totalmente esclarecidos. Estudos recentes têm demonstrado que os efeitos antidepressivos do zinco no teste do nado forçado em camundongos envolvem ativação direta e indireta dos receptores de adenosina A_1 e A_{2A} (Lobato et al., 2008). Além disso, estudos de Kaster e colaboradores (2004) demonstraram efeitos do tipo antidepressivos induzidos pela administração aguda de adenosina, os quais são revertidos por antagonistas A_1 e A_{2A}. Portanto, considerando as propriedades neuroprotetoras da adenosina, particularmente associadas à ativação de receptores A_1

(Stone et al., 2009) e antidepressivas de antagonistas de receptores A_{2A}, isto nos leva a pensar em questões relacionadas a determinação da afinidade e da densidade desses receptores em estruturas regulatórias fundamentais na modulação do estresse, visando elucidar de maneira mais clara a relação dos receptores de adenosina com o transtorno depressivo, valendo-se do estudo em modelos animais de depressão.

2. OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo geral deste estudo foi verificar os efeitos de diferentes modelos de estresse crônico, incluindo um modelo de depressão sobre comportamentos do tipo ansioso e do tipo depressivo.

2.1.1. OBJETIVO ESPECÍFICO 1

Investigar os efeitos de diferentes modelos de estresse crônico, incluindo um modelo de depressão sobre comportamentos do tipo ansioso e a atividade da enzima Na^+, K^+- ATPase na amígdala de ratos

2.1.2. OBJETIVO ESPECÍFICO 2

Estudar os efeitos de diferentes modelos de estresse crônico, incluindo um modelo de depressão sobre comportamentos do tipo depressivo e o sistema adenosinérgico no encéfalo de ratos.

2.1.1. OBJETIVO ESPECÍFICO 1

Artigo: *Na^+, K^+- ATPase Activity is Reduced in Amygdala of Rats with Chronic Stress-Induced Anxiety-Like Behavior*- Publicado no *Neurochemical Research*

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ORIGINAL PAPER

Na^+, K^+ ATPase Activity Is Reduced in Amygdala of Rats with Chronic Stress-Induced Anxiety-Like Behavior

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Abstract In this study, we examined the effects of two chronic stress regimens upon anxiety-like behavior, Na^+, K^+-ATPase activity and immunocontent, and oxidative stress parameters (antioxidant enzymes and reactive oxygen species production) in the amygdala. Male rats were subjected to chronic unpredictable and to chronic restraint stress for 40 days. Subsequently, anxiety-like behavior was examined. Both stressed groups presented increased anxiety-like behavior. Reduced amygdalal Na^+, K^+-ATPase activity in the synaptic plasma membranes was also observed, without alterations in the amygdala immunocontent. In addition, when analyzing oxidative stress parameters, only superoxide dismutase activity was decreased in the amygdala of animals subjected to unpredictable stress. We conclude that both models of chronic stress lead to anxiety-like behavior and decreased amygdalal Na^+, K^+-ATPase activity, which appears not to be related to oxidative imbalance. The relationship between this decreased activity and anxiety-like behavior remains to be studied.

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Keywords Chronic stress · Depression · Anxiety · Amygdala · Na^+, K^+-ATPase · Oxidative stress

Introduction

Na^+, K^+-ATPase is the enzyme responsible for the active transport of sodium and potassium ions in the nervous system, maintaining and re-establishing, after each depolarization, the electrochemical gradient necessary for neuronal excitability and regulation of neuronal cell volume. This enzyme is present in high concentrations in brain cellular membranes, consuming about 40–50% of the ATP generated in this tissue [1]. This enzyme is composed of two essential subunits: the α subunit hydrolyzes ATP coupled with Na^+, K^+ transport, whereas the β subunit is required for protein folding and modulates substrate affinity. Different subunit isoforms exist and these show a high degree of amino acid identity [2–4].

Na^+, K^+-ATPase activity is known to be affected by the redox state of the cell, and reduced antioxidants or antioxidant enzymes activities are related to reduced Na^+, K^+-ATPase activity [5–8]. In this context, reactive oxygen species (ROS) are believed to be involved in tissue damage, resulting in a wide variety of insults (e.g., [9–11]). ROS can directly damage cellular proteins, DNA, and lipids, and thereby affect cellular functions [12]. The brain is especially vulnerable to free radical-induced damage because of its high oxygen consumption, abundant lipid content and relative paucity of antioxidant enzymes [13–15]; furthermore, oxidative injury has been associated with the etiopathology of several CNS disorders [13, 14]. In order to neutralize the effects of reactive species, the cell uses antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [15].

Inhibition of Na^+, K^+-ATPase activity is found in various neuropathological conditions, including cerebral ischemia [16], epilepsy [17], and neurodegenerative disorders [18–20]. Moreover, some psychiatric disorders are believed to be associated with perturbation of ion homeostasis, and earlier studies have shown that Na^+, K^+-ATPase activity is decreased in patients with psychiatric disorders, such as depression [21–25]. A preclinical study showed that intracerebroventricular administration of ouabain (a Na^+, K^+-ATPase inhibitor) in rats mimic some symptoms of bipolar disorder [26]. In addition, we have previously observed a decreased Na^+, K^+ ATPase activity in the hippocampus of animals submitted to unpredictable chronic mild stress [27, 28].

Some models of chronic mild stress have been proposed as models of depression in animals [27–29]. In these studies, rats are exposed to different weak stressors for several days, and the response to rewarding stimuli is diminished, which is interpreted as anhedonia [27, 30, 31]. However, different models of stress can lead to different effects. For instance, repeated restraint stress leads to alterations in feeding behavior with increased sweet food ingestion [32], which is interpreted as an expression of increased levels of anxiety, since this effect is reversed by diazepam [32]. In these studies, using repeated exposure to the same stressor, there is a certain degree of predictability when compared to models using different stressors, which may lead to habituation to the stressor [33, 34].

Reports have suggested that maladaptive changes in amygdala may be related to depression [35, 36] and anxiety behavior [37–41]. In addition, a recent study showed that animals with an impaired Na^+, K^+-ATPase activity [41, 42], mainly in the amygdala [41], exhibit increased fear/anxiety. It is known that this brain structure is related to stress responses, presents a high concentration of glucocorticoid receptors [43, 44], and is relevant to the control of non-hippocampal emotional processes, including anxiety behavior [37]. Therefore, the aim of the present study was to verify the effects of two models of chronic stress on anxiety-like behavior and the possible relationship with Na^+, K^+-ATPase activity and its immunocontent in the amygdala. We used these two models of chronic stress because of their distinct characteristics. Additionally, since this enzyme activity has been observed to be reduced in the hipppocampus of animals subjected to unpredictable stress, we also measured its activity in the hippocampus of animals subjected to repeated stress, to verify whether the effects observed are specific to the amygdala. Since Na^+, K^+-ATPase activity is altered by the redox state (e.g., [5–8, 26]), we also evaluated oxidative stress parameters, namely SOD, CAT and GPx activities, as well as ROS production in amygdala.

Experimental Procedures

Animals

Male Wistar rats (60 days at the beginning of the treatment, weighing 180–230 g) were used. Experimental 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 and maintained on a standard dark–light cycle (lights on between 7 and 19 h), at a room temperature of 22 ± 1°C. The rats had free access to food (standard lab rat chow) and water, except during the periods when restraint stress or water or food deprivation were applied. All animal treatments were in accordance with the institutional guidelines and according to the recommendations of the International Council for Laboratory Animal Science (ICLAS); all efforts were made to reduce the number of animals.

Stress Models

The animals were divided into three groups: Chronic Restraint Stress (CRS), Unpredictable Chronic Mild Stress (UCMS) and Control. Controls were kept undisturbed in their home cages during the entire period of treatment, receiving only ordinary daily care with daily supports of food and water. Chronic restraint stress was applied by placing the animal in a 25 × 7 cm plastic bottle, and fixing it with plaster tape on the outside so that the animal was unable to move. There was a 1.5 cm hole at one far end for breathing. The restraint procedure was performed between 12:00 and 14:00 h. The animals were stressed for 1 h/day, 5 days a week for 40 days [33]. Control animals were kept in their home cages. Unpredictable chronic mild stress protocol was adapted from Gamaro et al. [27]. The 40-day unpredictable chronic mild stress paradigm was used for the animals in the UCMS group (see Table 1). One of the following stressors were used each day: (1) 24 h of food deprivation; (2) 24 h of water deprivation; (3) 1–3 h of restraint, as described below; (4) 1.5–2 h of restraint at 4°C; (5) 3–6 h of home cage inclination at an angle of 45°; (6) 2–5 h of flashing light as described below; (7) isolation (2–3 days); (8) 5–10 min of forced swimming. Stress was applied in different schedules every day, in order to increase the unpredictability. Exposure to the flashing light was carried out by placing the animal in a 60 × 60 × 25 cm plywood box. A 40-W lamp, flashing at a frequency of 60 flashes/min, was used.

Exposure to the Open Field

A 50-cm high, 40 × 60-cm open field made of wood with a frontal glass wall was used [45]. The floor was subdivided

Table 1 Schedule of stressor agents used during the unpredictable chronic mild stress

Days	Stressors
1	1.5 h of restraint at 4°C
2	24 h of water deprivation
3	5 h of flashing light
4	2 h of restraint
5	24 h of food deprivation
6	24 h of isolation
7	24 h of isolation
8	4 h of home cage inclination
9	1.5 h of restraint
10	2 h of flashing light
11	24 h of water deprivation
12	24 h of food deprivation
13	24 h of isolation
14	24 h of isolation
15	Non stressor applied
16	10 min of forced swimming
17	3 h of restraint
18	6 h of home cage inclination
19	4 h of flashing light
20	24 h of water deprivation
21	2 h of restraint at 4°C
22	24 h of food deprivation
23	24 h of isolation
24	24 h of isolation
25	24 h of isolation
26	1 h of restraint
27	5 min of forced swimming
28	3.5 h of flashing light
29	3 h of home cage inclination
30	2 h of restraint at 4°C
31	Non stressor applied
32	24 h of food deprivation
33	3 h of restraint
34	4 h of flashing light
35	24 h of food deprivation
36	10 min of forced swimming
37	24 h of isolation
38	24 h of isolation
39	24 of water deprivation
40	10 min of forced swimming

with white lines into 12 equal 13.3- by 15.0-cm rectangles. The animals ($n = 15$/group) were submitted to this task before the stress session on the 40th day of chronic stress and were gently placed facing the left corner and allowed to explore the arena for 5 min. The number of line crossings, and the time spent in central squares was counted. The number of crossings was used as a measurement of exploratory behavior, while time spent in central squares was considered to evaluate anxiety-like behavior [46].

Preparation of Synaptic Plasma Membrane from Amygdala and Hippocampus

Twenty-four hours after the last session of chronic stress, the animals were killed by decapitation ($n = 6$/group) without anesthesia, the brain was rapidly removed, and amygdala and hippocampus were dissected to prepare synaptic plasma membranes according to the method of Jones and Matus [47], with some modifications [16]. The structures were homogenized in ten volumes of a 0.32 M sucrose solution containing 5 mM Hepes and 1 mM EDTA. The homogenate was centrifuged at 1,000*g* for 20 min and the supernatant removed and centrifuged at 12,000*g* for a further 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris–HCl buffer, pH 8.1), incubated at 0°C for 30 min, and applied on a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8, and 1.0 M. After centrifugation at 69,000*g* for 2 h, the fraction at the 0.8–1.0 M sucrose interface was taken as the membrane enzyme preparation.

Na^+, K^+ ATPase Activity Evaluation

The reaction mixture for the Na^+, K^+ ATPase assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris–HCl buffer, pH 7.4, in a total volume of 200 µl. The reaction was started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3.0 mM. The control was assayed under the same conditions with the addition of 1.0 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays [16]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. [48]. Enzyme specific activity was expressed as nmol Pi released per min per mg of protein. All assays were performed in duplicate and the mean was used for statistical analysis. In this experiment, protein was measured by the method of Bradford [49], with bovine serum albumin used as standard.

Western Blot Analysis

For immunoblotting studies, a further group of animals was stressed (as described above) and rats were decapitated, their brains removed and amygdala dissected out and frozen at −70°C until use ($n = 5$–6/group). Brains from controls were processed similarly. Protein samples were prepared by homogenization in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.1% sodium dodecyl-sulfate (SDS). Aliquots were taken for protein determination and β-mercaptoethanol was added to a final

concentration of 5%. Samples containing 50 mg of protein were resolved by 10% SDS–PAGE. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes using a semi-dry apparatus (Bio-Rad Trans-Blot SD). The membranes were blocked for 1 h with 5% powdered milk in Tris-buffered saline plus 0.1% Tween-20, followed by incubation overnight at 4°C with anti-Na^+,K^+-ATPase (1:3000 dilution, α3 Subunit, mouse monoclonal, Sigma) diluted in the same blocking solution. Subsequently, the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse antibody also diluted in blocking solution (1:1000). All blots were re-probed with β-actin antibody (1:4000 dilution, mouse monoclonal, Sigma) as an internal control. Immunoreactive bands were revealed by an enhanced chemiluminescence kit (ECL Amersham from GE Healthcare), and detected using X-ray films. The immunoblot films were scanned and the digitalized images analyzed with Optiquant software (Packard Instrument).

Preparation of the Samples for Oxidative Stress Measurements

Six animals per group were used in these measurements. Animals were killed by decapitation 24 h after the last exposure to stress. Amygdala were quickly dissected out and stored at −70°C until analysis, when they were homogenized in 10 vol (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA for most determinations. To measure catalase activity, samples were homogenized in 10 vol (w:v) ice-cold 10 mM potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 3,000 rpm for 10 min at 4°C and the supernatant was used.

Superoxide Dismutase Activity

Superoxide Dismutase (SOD) activity was determined using a RANSOD kit (Randox Labs., USA), which is based on the procedure described by Delmas-Beauvieux et al. [50]. This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a formazan dye that is assayed spectrophotometrically at 492 nm at 37°C. The inhibition in production of the chromogen is proportional to the activity of SOD present in the sample; one unit of SOD causes 50% inhibition of the rate of reduction of INT under the conditions of the assay.

Catalase Activity

Catalase (CAT) is an enzyme able to degrade peroxides, including hydrogen peroxide (H_2O_2), and its activity assessment is based upon establishing the rate of H_2O_2 degradation spectrophotometrically at 240 nm at 25°C [51]. CAT activity was calculated in terms of micromoles of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

Glutathione Peroxidase Activity

Glutathione Peroxidase (GPx) activity was determined according to [52], with modifications. The reaction was carried out at 37°C in a solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione and 0.4 U glutathione reductase. The activity of GPx was measured taking tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was calculated as pmol NADPH oxidized per minute per mg protein.

Reactive Oxygen Species Content

To assess the free radicals content, we used 2′-7′-dichlorofluorescein diacetate (DCFH-DA) as a probe. An aliquot of the sample was incubated with DCFH-DA (100 mM) at 37°C for 30 min. The reaction was terminated by chilling the reaction mixture on ice. The production of the oxidized fluorescent derivative Reactive Oxygen Species Content (DCF) was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, using a fluorescence spectrophotometer (Hitachi F-2000). The ROS content was quantified using a DCF standard curve and results were expressed as pmol DCF produced/mg protein. All procedures were performed in triplicate, in the dark, and blanks containing DCFH-DA were processed for measurement of autofluorescence [53].

Protein Assay

The total protein concentrations were determined using the method described by Lowry et al. [54] with bovine serum albumin as the standard.

Statistical Analysis

Comparisons between stressed and control groups were performed by one way ANOVA, followed by Duncan's multiple range test when appropriate. A value of $P < 0.05$ was considered to be significant.

Results

Both unpredictable chronic mild stress and chronic restraint stress were able to increase anxiety-like behavior,

evaluated as the time in the central squares of the open field, as shown in Fig. 1a [one-way ANOVA, $F(2, 42) = 11.91$, $P < 0.01$]. As shown in Fig. 1b, there were no alterations in locomotion when we analyzed the total number of crossings in the open field task [one-way ANOVA, $F(2,42) = 0.48$, $P > 0.05$].

Unpredictable chronic mild stress and chronic restraint stress were both able to decrease the activity of Na^+, K^+-ATPase in the amygdala (one-way ANOVA, $F(2,14) = 5.989$, $P < 0.02$), as shown in Fig. 2a. Additionally, in order to verify whether the effect observed is specific to this structure, we also measured this enzyme's activity in hippocampus of animals subjected to repeated stress, since it has been reported that Na^+, K^+-ATPase is reduced in the hippocampus of animals subjected to UCMS [27]. There was no difference in Na^+, K^+-ATPase activity in rats subjected to this experimental stress model [Student's t test, $t(8) = 0.77$; $P > 0.05$].

There were no alterations in the immunocontent of the α3 subunit of this enzyme in the amygdala, as evaluated by Western Blotting analysis [displayed in Fig. 2b; $F(2,15) = 0.629$, $P > 0.05$]. Since Na^+, K^+-ATPase activity may be reduced in situations involving oxidative stress [5–8], we also evaluated antioxidant enzyme activities and reactive oxygen species production in the amygdale of these animals. Exposure to UCMS induced a decreased activity of SOD in amygdala, as shown in Fig. 3a [$F(2,14) = 6.227$, $P < 0.02$, followed by Duncan multiple range test, $P < 0.05$], while no effect was observed after exposure to CRS. No significant differences were observed in the other enzyme activities [$F(2,14) = 3.118$ for GPx and $F(2,14) = 0.046$ for CAT; $P > 0.05$; see Fig. 3b and c]. ROS production in amygdala was not affected by any of these stress models, as evaluated by the DCF test [$F(2,13) = 0.541$, $P > 0.05$; see Fig. 4].

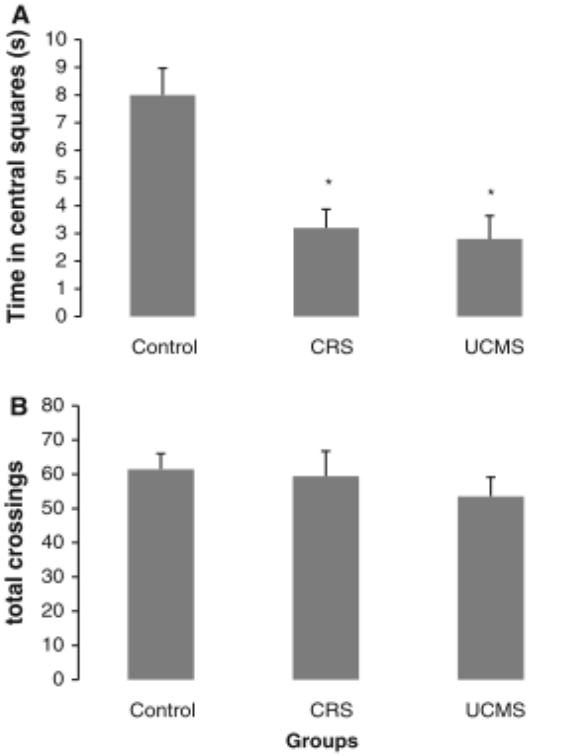

Fig. 1 Effects of exposure to two chronic stress models, chronic restraint stress (CRS) and unpredictable chronic mild stress (UCMS), for 40 days on behavior in the open field. **a** Anxiety-like behavior. A one-way ANOVA showed a significant decrease in time spent in central squares in both stressed groups. **b** Locomotor behavior. There was no effect on the number of crossings (one-way ANOVA, $P > 0.05$). Data are expressed as means ± standard error of the mean. $N = 15$/group. * Significant difference from control group ($P < 0.05$; Duncan multiple range test)

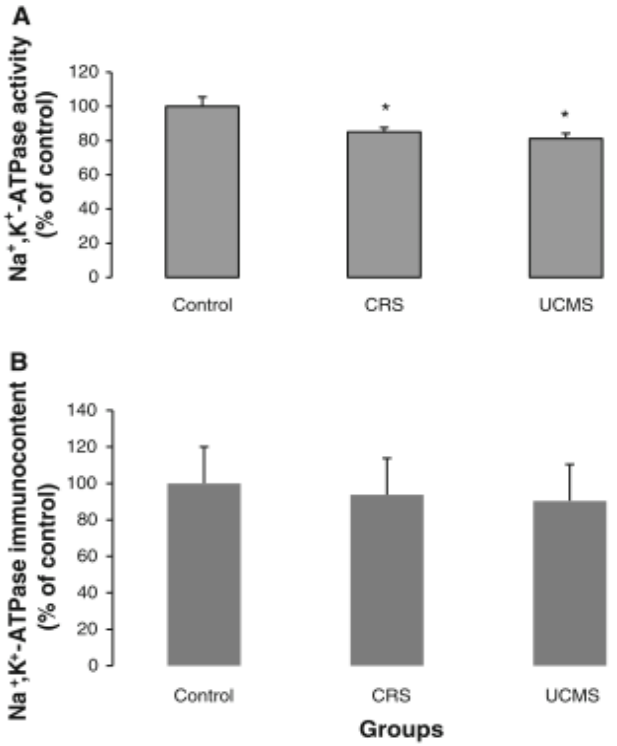

Fig. 2 Effect of exposure to two models of chronic stress, chronic restraint stress (CRS) and unpredictable chronic mild stress (UCMS) on Na^+, K^+-ATPase in the amygdala. **a** Na^+, K^+-ATPase activity. A one-way ANOVA showed a significant decrease in Na^+, K^+-ATPase activity in both stressed groups. **b** Na^+, K^+-ATPase immunocontent. Data represent relative optical density. There was no effect on the immunocontent. Data are expressed as means ± standard error of the mean. $N = 5$–6/group. * Significant difference from control group ($P < 0.05$, Duncan multiple range test)

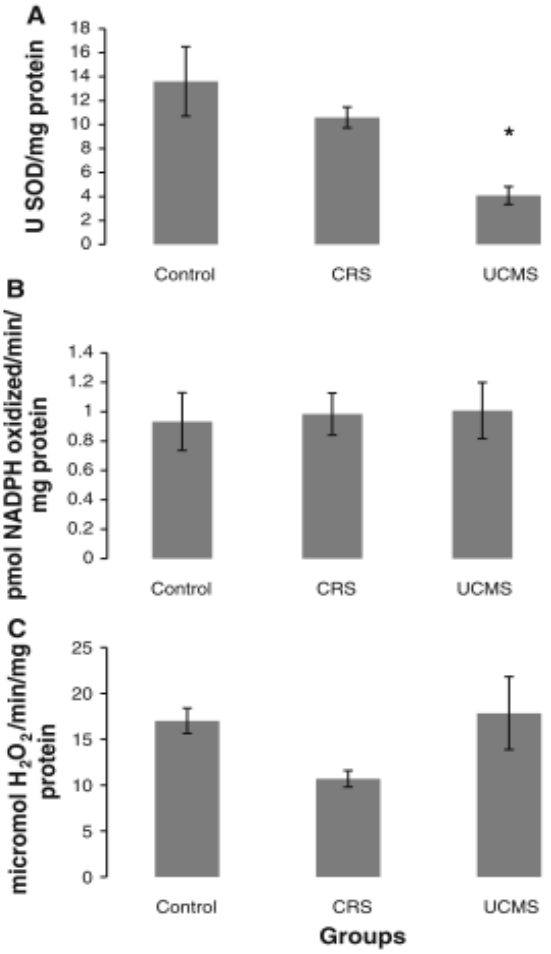

Fig. 3 Effect of exposure to two models of chronic stress, chronic restraint stress (CRS) and unpredictable chronic mild stress (UCMS) on antioxidant enzymes activities in the amygdala. **a** Superoxide dismutase activity. One-way ANOVA showed a significant decrease in this enzyme activity in the UCMS group, as compared to the control. **b** Gluthatione peroxidase activity. There was no significant effect. **c** Catalase activity. There was no effect on catalase activity. Data are expressed as means ± standard error of the mean. $N = 4$–5/group. * Significant difference from control group ($P < 0.05$, Duncan multiple range test)

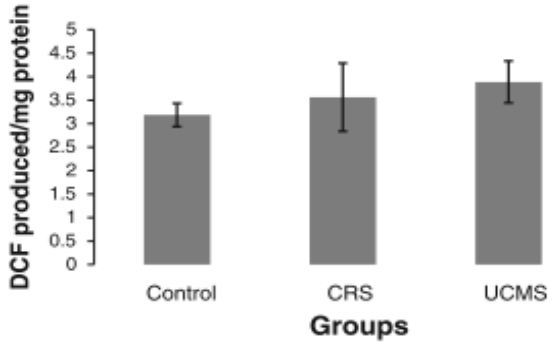

Fig. 4 Effect of exposure to two models of chronic stress, chronic restraint stress (CRS) and unpredictable chronic mild stress (UCMS) on ROS production. There was no effect on the DCF analysis. Data are expressed as means ± standard error of the mean. $N = 4$–5/group

Discussion

In the present study, we observed an increased anxiety-like behavior in the open field task in both models of chronic stress. Additionally, there was a decrease in Na^+, K^+-ATPase activity in the amygdala of rats submitted to 40 days of both models of stress. On the other hand, there were no alterations in the immunocontent of the α3 subunit. With regard to antioxidant enzymes, just UCMS was able to decrease SOD activity, but not the other parameters of oxidative stress studied. To our knowledge, this is the first study to compare the effects of two different models of chronic stress, carried out at the same time, on such parameters.

Some models of unpredictable stress, such as the one used here (UCMS), have been proposed as models of depression in animals [29, 30, 55]. We have observed that UCMS animals show a higher immobility time (unpublished results) than both controls and CRS animals. Another characteristic of animals subjected to UCMS is that they present a decreased appetite for sweet food [56], an effect interpreted as an expression of anhedonia [31]. In UCMS models, rats are exposed to different weak stressors for several days. The absence of predictability concerning the stressor applied is an important characteristic of this model, and may be related to the different effect observed in these animals when compared to other models, in which repeated stress is used and a higher consumption of sweet food is observed [32, 57]. Thus, we also submitted the animals to predictable CRS to analyze possible differences.

The open field has been suggested as a good model of the normal anxiety that animals experience when confronted with a stressful or threatening situation [46]. Since our aim was to evaluate the basal anxiety shown by chronically-stressed or control animals, we evaluated the animals' behavior in this task. In addition, differences in brain excitability due to changes in Na^+, K^+-ATPase activity have been suggested to lead to changes in behavior in the open field [58]. Interestingly, both regimens of chronic stress (the one used to induce depressive-like behavior—UCMS—and another that does not induce this type of behavior) had similar effects concerning anxiety-like behavior and amygdalal Na^+, K^+-ATPase activity. The present results agree with previous data from the literature that show increased anxiety-like behavior in animals submitted to chronic stress [59–61].

The brain mechanisms related to the induction of anxiety-like behavior are complex. However, a key main structure related to these functions is the amygdala [37, 42, 62], which

was the focus of our study. Structural and/or functional changes in the amygdala are associated with several psychiatric conditions in humans, including anxiety disorders, and imaging studies have repeatedly emphasized the central role of the amygdala in anxiety (e.g., [38–40]). Also, prolonged stress situations or exposure to high levels of glucocorticoids have been demonstrated to induce amygdalal plasticity [62, 63], such as an increase in the dendritic arborization, spine production, and synaptic connectivity [63, 64].

We have previously observed decreased Na^+, K^+-ATPase activity in hippocampus of rats subjected to a similar UCMS model. In order to verify whether the effects observed herein are specific to the amygdala or are common to all CNS structures, we also measured Na^+, K^+-ATPase activity in the hippocampus of CRS rats, and no differences were observed between the CRS and control groups. Therefore, the reduction observed in the amygdala is not a general observation in this model, although in the case of UCMS both structures were affected [27, 65].

Molecularly, chronic stress alters several neurotransmitters [66, 67] and enzymes in the amygdala that may be associated with anxiety disorders [68, 69]. In the present study, we demonstrated a significant decrease in Na^+, K^+-ATPase activity in the amygdala. This activity is believed to be regulated by several factors, including hormones [70, 71] and neurotransmitters [72–75]. In agreement with the present results, Moseley et al. [42] showed that deficiency in the Na^+, K^+-ATPase alpha isoform can be associated with increased anxiety behavior.

Interestingly, although there was no alteration in the Na^+, K^+ ATPase immunocontent, both chronic stress models decreased this enzyme activity, suggesting that chronic stress was able to alter the functionality of this enzyme, instead of protein quantity. The immunocontent was evaluated using the anti-α3 subunit of Na^+, K^+-ATPase, since the alpha subunit has the ATPase activity and the ouabain binding site, and the alpha 3 isoform is neuron-specific [76, 77]. Additionally, glucocorticoids have been shown to be positive modulators of the Na^+, K^+-ATPase alpha 3 subunit mRNA in a few brain regions, mainly in the amygdala [78], and this isoform has been reported to be altered in animal models related to changes in behavior [79, 80]. On the other hand, other subunits of this enzyme are present in the brain [81–83] and may be involved in the reduction observed in enzyme activity.

Since Na^+, K^+-ATPase activity has been shown to be related to the redox status of the cell [5–8], and chronic stress has been reported to induce oxidative imbalance in some brain structures [84, 85], in the present study we also measured antioxidant enzymes activities, as well as ROS production (through the DCF test). We observed a decrease in SOD activity in the amygdala of animals subjected to UCMS, suggesting some type of oxidative status change, but only in this stress model. No significant effect of any of the stress models used on ROS production was observed when measured by the DCF test. Therefore, it is unlikely that the oxidative imbalance could explain the reduced activity of Na^+, K^+-ATPase observed in both models of chronic stress. However, the fact that SOD activity was only reduced by UCMS suggests that unpredictable chronic stress is more effective in altering oxidative stress than repeated stress, possibly because this later model induces some degree of habituation to the stressor [33, 34].

The decreased Na^+, K^+-ATPase activity observed in the present study could also be due to the direct influence of high concentrations of stress hormones [86]. For example, similarly to corticoids, ouabain, previously known as a cardiotonic steroid capable of inhibiting Na^+, K^+-ATPase, has recently been identified as an endogenous compound produced by the adrenals and hypothalamus and found circulating in the plasma [87, 88]. Additionally, it has been suggested that this endogenous digitalis-like factor is also released under stress conditions [88, 89].

In conclusion, both models of chronic stress lead to anxiety-like behavior and decreased amygdalal Na^+, K^+-ATPase activity, which appears not to be related to oxidative imbalance, since only the UCMS group showed a reduction in SOD activity. Although the effects, observed herein, on enzyme activity and behavior suggest some correlation, it is not possible to draw causality from these results. Therefore, the relationship between decreased Na^+, K^+-ATPase activity and anxiety-like behavior remains to be studied.

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2.1.2. OBJETIVO ESPECÍFICO 2

Artigo a ser submetido: The role of hippocampal A_1 and striatal A_{2A} adenosine receptors on the depressive- like behaviors induced by chronic stress in rats.

The role of hippocampal A_1 and striatal A_{2A} adenosine receptors on the depressive- like behavior induced by chronic stress in rats

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Abstract

In this study, we examined the effects of two chronic stress regimens upon depressive-like behavior, and A_1 and A_{2A} adenosine receptors binding and immunocontent. Male rats were subjected to unpredictable chronic mild stress (UCMS) or to chronic restraint stress (CRS) for 40 days. Subsequently, depressive-like behavior (forced swim and consumption of sucrose) were evaluated, and A_1 adenosine or A_{2A} adenosine receptors were examined in the hippocampus or striatum, respectively. UCMS animals showed depressive-related behaviors (anhedonia and leaning helplessness). This group also showed increased hippocampal A_1 adenosine receptor binding and immunocontent in hippocampus, as well as increased striatal A_{2A} adenosine receptors binding in striatum, without any alteration in the immunocontent. On the other hand, chronic restraint stress group only displayed an increase in A_1 adenosine receptor binding and no alteration on the other parameters evaluated. We suggest that an imbalance on adenosine receptors, mainly the upregulation in striatal A_{2A} adenosine receptors displayed by the UCMS group, could be associated with depressive-related behavior.

Keywords Chronic stress – Depression – Hippocampus – Striatum – Adenosine - A_1 receptor - A_{2A} receptor – Binding – Western Blotting

Introduction

Depression is a serious disorder often manifested with symptoms at the psychological, behavioural and physiological levels. Numerous attempts have been made in order to set up animal models of depression or at least of some disease aspects ([Cryan et al., 2002], [Holmes, 2003] and [Yadi et al., 2000]). Most of these animal models share the common feature of stress in the form of various stress procedures or even aversive events, and chronic stress models appear more suitable for the experimental investigation of depression than acute stress models ([Katz et al., 1981] and [Willner et al., 1987]). Prolonged exposure of experimental animals to a variety of mild stressors is associated with to significant changes in the animal behavior and to the induction of helplessness learning in the model of forced swimming test (FST) (Dichter et al., 2009). This task simulates one main symptom of the melancholic subtype of major depression which envolves several brain structures related to the modulation of depressive behaviors such as hippocampus, amygdala, hypothalamus and prefrontal cortex ([Kieran, N., 2010], [Mozhui et al., 2010] [Noschang, et al., 2009] and [Zambello et al., 2010]). Further, recent studies have suggested functional and biochemical alterations in the striatum induced by chronic stress, proposing that this structure is involved in the modulation of depressive behaviors (Marais, L., 2009; Dichter, G. S., 2009)

In the present study, we focused the hipocampal A_1 and striatal A_{2A} receptors of the adenosinergic system. Adenosine is a brain neuromodulator and its extracellular concentration is increased in noxious brain conditions (Fredholm et al., 2005), namely during stress (Scaccianoce et al., 1989). Its analogues have been shown to induce "behavioral despair" in animal models of depression (El Yacolbi et al., 2003). It can either inhibit or facilitate neuronal activity by activation of metabotropic A_1 receptors or

A_{2A} receptors, respectively (Fredholm et al., 2005), both of which are predominantly located in synapses in the limbic regions and neocortex ([Rebola et al., 2003] and [Rebola et al., 2005]). However, the regulation of adenosine receptors is dynamic and is known to be modified by chronic brain insults (Cunha, 2005). Furthermore, it is well established that blocking of A_{2A} receptors affords a robust neuroprotection in chronic noxious conditions in the adult brain (Cunha, 2005). Interestingly, A_{2A} adenosine receptor antagonists have been proposed as potential anti-depressants (El Yacoubi et al., 2003). This is in accordance with the ability of adenosine to control corticotrophin and cortisol/corticosterone release ([Chau et al., 1999], [Geiger and Glavin, 1985] and [Jegou et al., 2003]). Given that glucocorticoids modify the expression of adenosine receptors ([Gerwins and Fredholm, 1991] and [Svenningsson and Fredholm, 1997], we investigated the consequences of chronic restraint stress and unpredictable chronic mild stress on the modulation of A_1 and A_{2A} adenosine receptors in the hippocampus and striatum, respectively.

Experimental Procedures

Animals

Seventy-six adult male Wistar rats (60 days at the beginning of the treatment, weighing 150–190 g) were used. Experimental 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 and maintained on a standard dark–light cycle (lights on between 7 and 19 h), at room temperature of 22 ± 1 °C. The rats had free access to food (standard lab rat chow) and water, except during the periods when restraint stress and water deprivation were applied. All animal treatments were in accordance with the institutional guidelines and

according to the recommendations of the International Council for Laboratory Animal Science (ICLAS); all efforts were made to reduce the number of animals.

Stress Models

The animals were divided in three groups: Chronic Restraint Stress (CRS), Unpredictable Chronic Mild Stress (UCMS) and Control. Controls were kept undisturbed in their home cages during the entire period of treatment receiving only ordinary daily care with daily supports of food and water. Chronic restraint stress was applied by placing the animal in a 25 × 7 cm plastic bottle, and fixing it with plaster tape on the outside so that the animal was unable to move. There was a 1.5 cm hole at one far end for breathing. The restraint procedure was performed between 12 and 14 h. The animals were stressed 1 h/day, 5 days a week for 40 days (Gamaro et al., 1998). Control animals were kept in their home cages. Unpredictable chronic mild stress protocol was adapted from (Gamaro et al., 1998). The 40-day unpredictable chronic mild stress paradigm was used for the animals in the UCMS group (see Table 1). One of the following stressors were used each day: (1) 24 h of damp sawdust; (2) light during the night; (3) 1–3 h of restraint (see procedures above) as described above; (4) 1.5–2 h of restraint at 4°C; (5) 3-6 h of home cage inclination in a angle of 45°; (6) 2-5 h of flashing light as described below; (7) isolation (2–3 days); (8) 1-4 h of cage placed at 4°C. Stress was applied at different schedules every day, in order to increase the unpredictability. Exposure to flashing light was made by placing the animal in a 60 cm x 60 cm x 25 cm plywood made box. A 40-W lamp, flashing at frequency of 60 flashes/min, was used.

Depressive-like behavior: forced swimming test

24 hours after the last session of chronic stress, animals were placed individually into plexiglass cylinders (height 40 cm, diameter 18 cm) filled with 25 ± 1 °C water. Two different sessions were performed with a 15 min to habituation followed by a 5 min test 24 h later. The time of immobility was determined when no additional activity was observed other than the movements necessary to keep the rats' head above the water. Swimming was considered when the rats showed active swimming movements, e.g., moving around in the cylinder.

Sucrose solution intake

Rats were trained to consume 1% (w/v) sucrose solution before starting the UCMS protocol. Sucrose consumption test was performed only in the UCMS group. Habituation consisted of three 1 h sessions (Monday, Wednesday and Friday), in which animal could select between two bottles: 1% sucrose solution or tap water. The sessions were realized 20 h after food and water deprivation. The tests were conducted for 1 hour, once a week (Wednesday) during 8 weeks, to evaluate the preference (Moreau et al., 1994). During this period the animals were stressed daily, and the food and water removed the night before the test.

Preparation of total membranes from hippocampus and striatum

The rats were killed by decapitation, brain removed and hippocampus and striatum were dissected. Briefly, the two hippocampi and striate from one were homogenized at 4 °C in buffer of homogenization containing sucrose solution 0.32 M, 50 mM Tris–HCl, 2 mM EGTA and 1 mM dithiothreitol, pH 7.6, centrifuged at 3.000x*g* for 10 min at 4 °C. The supernatants were collected, centrifuged at 14.000x*g* for 20 min at 4 °C and the pellet was resuspended at 4 °C in 500 µl in incubation buffer containing 50 mM Tris-HCl

(pH 7.4) and 2mM $MgCl_2$ for A_1 adenosine receptor or 10 mM $MgCl_2$ A_{2A} adenosine receptor, (modified from Cunha et al.,1999).

Binding studies

Hippocampal and striatal membranes were incubated with adenosine deaminase (Calbiochem) for A_1 (2 U/ml) and A_{2A} (4 U/ml) adenosine receptors, respectively, in buffer containing 50 mM Tris-HCl, $MgCl_2$ (2 mM for A_1 and 10 mM for A_2 receptors), pH 7.4, for 30 min at 25 °C, in order to eliminate endogenous adenosine from membrane preparations. Binding of the selective A_1 receptor antagonist, [^{3}H]-1,3-dipropyl-8 cyclopentylxanthine (DPCPX) (specific activity of 120 Ci/mmol; from Perkin Elmer) was evaluated for 2 h at 25 °C, with 40–100 µg of protein in a final volume of 300 µl in a solution containing 50 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.4, as previously described by León et al., (2002), with some modifications. A saturation curve was constructed using five different concentrations of [^{3}H]-DPCPX (0.1, 0.5, 1, 5, 10 nM). To evaluate the binding of A_{2A} receptors, we used a selective agonist, [^{3}H]-2-[4-(2-p-carboxyethyl)phenylamino]-5'-N-ethylcarboxiamidoadenosine (CGS 21680) 40.5 Ci/ mmol; from Perkin Elmer). Binding was measured for 4 h at 25 °C with 30-100 µg of protein in a final volume of 300 µl in the solution containing 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4, as previously described by Cunha et al., (1999), with some modifications. A saturation curve was constructed using five different concentrations of [^{3}H]-CGS 21680 (1, 2.5, 5, 10, 20 nM). Specific binding was determined by subtraction of the non-specific binding, which was measured in the presence of a specific concentration 10,000 times higher of 2-Cl-Adenosine (CADO) (from Research Biochemical Inc., Sigma-Aldrich). Each binding assay data point was performed in triplicate. The binding reactions were stopped by rapid vacuum filtration through glass

fiber filters (GF/C filters) which were immediately washed three times with 4 mL ice-cold buffer. The filters were then placed in scintillation vials and 1 ml of scintillation liquid (from Perkin Elmer) was added. Radioactivity was determined after at least 12 h with a counting efficiency of 55–60%. The samples were counted for 2 min. The specific binding from saturation experiments was fitted by non-linear regression to a one binding site equation using the software (GraphPad Inplot) to determine the binding parameters (dissociation constant, Kd, and maximal number of binding sites, *B*max).

Western blot analysis

For immunoblotting studies, hippocampal and striatal total membranes were prepared by homogenization in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.1% sodium dodecyl-sulfate (SDS). Aliquots were taken for protein determination and β-mercaptoethanol was added to a final concentration of 5%. Samples containing 50 mg of protein were resolved by 10% SDS-PAGE. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes using a semi-dry apparatus (Bio-Rad Trans-Blot SD). The membranes were blocked for 1h with 5% powdered milk in Tris-buffered saline plus 0.1% Tween-20, followed by incubation overnight at 4 °C with anti-A_1 and A_{2A} receptors (1:1000 dilution, mouse monoclonal, Sigma) diluted in the same blocking solution. Subsequently, the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse antibody also diluted in blocking solution (1:1000). All blots were re-probed with β-actin antibody (1:4000 dilution, mouse monoclonal, Sigma) as an internal control. Immunoreactive bands were revealed by an enhanced chemiluminescence kit (ECL Amersham from GE Healthcare), and detected using X-ray films. The immunoblot films were scanned and the digitalized images analyzed with Optiquant software (Packard Instrument).

Protein Assay

The total protein concentrations were determined using the method described by Lowry et al., 1951 for binding studies and by Peterson, 1979 with bovine serum albumin as the standard.

Statistics Analysis

Comparisons between experimental and control groups were performed by One way ANOVA followed by Duncan's multiple range test when appropriate and repeated measures ANOVA for sucrose consumption test. A value of $P< 0.05$ was considered to be significant.

Results

In this study, only unpredictable chronic mild stress was able to increase depression-like behavior, evaluated as the immobility time in the forced swimming test as shown in Fig. 1 [one –way ANOVA, $F(2,68)= .8.80$, $p< 0.05$] On the other hand, there was no significant difference on that task in CRS group ($p> 0.05$). Therefore, for the next behavioral evaluation (sucrose consumption task), we used only UCMS.

As displayed in Figure 2, UCMS rats initiate the task drinking more sucrose solution (first days of stress exposure) and diminished the consumption along the time, in such a way that during the last weeks of treatment, they consumed significantly less sucrose solution, compared to controls [repeated measures ANOVA [$F (7,210)= 6,03$, $p< 0.05$].

Rats submitted to UCMS and CRS presented an increase in the Bmax in hippocampal A_1 adenosine receptors. These data are shown in Fig 3

[one-way ANOVA, Bmax: $F (2.7)= 6,42$, $p<0.05$ and Kd: $F (2.6)= 0.51$, $p>0.05$]. In addition, both stress models (CRS and UCMS) were able to increase A_1 adenosine receptor immunocontent in this structure. Additionally, A_1 receptor immunocontent was

increased in both stressed groups when compared to controls, however UCMS showed higher immunocontent than the CRS group, as evaluated by Western Blotting analysis, as showed in Fig. 4. [one- way ANOVA, [$F(2,8)= 18.5$, $p<0.05$].

When we evaluated the striatal A_{2A} adenosine receptors, only the UCMS group had increased A_{2A} receptor binding, as showed in Fig. 5 [one- way ANOVA, Bmax: $F(2.14)= 7.27$, $p<0.05$ and Kd: $F(2.15)= 1.46$, $p>0.05$]. However, a Western Blot analysis did not evidenced altered immunocontent of striatal A_{2A} adenosine receptors in any of the stressed groups (one- way ANOVA $F(2,6)= 2.7$, $p>0.05$), as showed in Fig 6.

Discussion

In the present study, we observed an increase in depressive-like behavior in the forced swimming task and weekly sucrose consumption only in animals subjected to the unpredictable mild chronic stress model. Additionally, UCMS group showed increased A_1 adenosine receptors (A_1Rs) binding (Bmax) and immunocontent in the hippocampus. This group also displayed increased A_{2A} adenosine receptors (A_{2A}Rs) in striatum, as demonstrated by increases of Bmax, without any alteration in the immunocontent. On the other hand, chronic restraint stress only displayed an increase in A_1 adenosine receptor binding and no alteration on the other measurements.

This is the first study comparing the effects of two different models of chronic stress carried out at the same time on such parameters. Data from literature had demonstrated that some models of unpredictable stress, such as the models used here, could be proposed as models of depression in animal studies([Pucilowski et al., 1993] and [Willner, 1991]). In the UCMS model, rats are exposed to different weak stressors for several days. We observed that UCMS animals showed a higher immobility time

than both control and CRS groups. These data agree with studies from the literature, showing that animals submitted to UCMS present diminished sucrose consumption ([Quan et al., 2011] and [Taliaz et al., 2011]). This is interpreted as lack of pleasure or anhedonia (Cox et al., 2011). The absence of predictability concerning the stressor applied is an important characteristic of this model, and may be related to the different effect observed in these animals when compared to other models; therefore, we also submitted the animals to predictable CRS to analyze possible differences.

The brain mechanisms related to the induction of depressive-like behavior are extremely complex. However, one of the most well studied structures related to this behavior is the hippocampus ([Taliaz et al., 2011], [Zhang et al., 2011] and [Wang et al., 2011]). Structural and/or functional changes in the hippocampus are associated with several mood disorders, including major depression, and imaging studies have repeatedly emphasized the central role of the hippocampus in depression ([Lucassen et al., 2010] and [Wang et al., 2011]). Also, prolonged stress situations or exposure to high levels of glucocorticoids have been demonstrated to induce hippocampal plasticity ([Lucassen et al., 2010] and [van Hasselt et al., 2011]), such as a decrease in the dendritic arborization, spine production, and synaptic connectivity [35, 36]. Another structure focused in our study was the striatum. Data from the literature have suggested a main role of this structure in depressive-like behavior ([Jahng, 2011] and [Hollis et al., 2011]), mainly that concerning anhedonia.

One important finding of this study is the altered density in hippocampal A_1 adenosine receptor and striatal A_{2A} adenosine receptors in UCMS. According to previous works, the modification of the density of adenosine receptors is related with the adaptation of the adenosine modulatory system in chronic noxious brain conditions

([Coelho et al., 2006] and [Rebola et al., 2005]). In fact, when rats are submitted to a subchronic restraint stress for 7 days, a down-regulation (i.e. decreased function) of A_1 adenosine receptor is observed in hippocampus (Cunha et al., 2006). In contrast, we demonstrated that both chronic stress models for 40 days produced an up-regulation (i.e. increased function) in hippocampal A_1 adenosine receptor, showed by binding and western blot studies, without any alteration on the receptor affinity (Kd), as shown in Figure 3. Indeed, endogenous corticosteroids positively regulate the expression of the A_1 adenosine receptor in rat brain (Svenningsson and Fredholm, 1997), and more specifically restraint chronic stress for 14 days increased the binding of A_1 adenosine receptor in rat hypothalamus, demonstrating a possible relationship between HPA axis and A_1 adenosine receptor modulation (Anderson et al., 1988). Thus, we could suggest that adaptation of A_1 adenosine receptor in the hippocampus is time-dependent during aversive situations. In addition, Tagliari (2010) demonstrated that UCMS was able to increase the levels of important proinflammatory cytokines such as IL-1β, IL-6 and TNF-α, in hippocampus of rats. Therefore, it has been proposed that interleukin-6 (IL-6) can upregulate neuronal adenosine A_1 receptors, demonstrating that such mechanism is involved in the enhancement of A_1 receptor-mediated signaling in the brain under excitotoxic situations, with a beneficial impact on neuronal survival (Biber et al., 2008). We could suggest that the adenosine A_1 receptors up-regulation seen herein could be involved in protecting hippocampal cells from insults induced by chronic stress.

Additionally, we choose the striatum, once A_{2a} adenosine receptors are predominantly located in several basal ganglia structures ([Svenningsson, 1999] and [Rebola et al., 2005]) and activation of A_{2A} receptors interferes with effects mediated by most of the principal neurotransmitters in striatum, in particular, the inhibitory interaction

between adenosine acting on A_{2A} receptors and dopamine acting on D_2 receptors. Also, striatum (caudate-putamen, nucleus *accumbens* and olfactory tubercle) is the major component of the basal ganglia in which excitatory glutamatergic inputs from the cortex, thalamus, and limbic areas are integrated with dopaminergic inputs from the mesencephalon (Nestler et al., 2002). The striatum plays a critical role in integrating sensory, emotional, motivational and motor components of ongoing action (Nestler et al., 2002). Thus, another important finding here is the striatal A_{2A} adenosine receptors up-regulation in the UCMS group, which also demonstrated depression-related behaviors such as decrease of sucrose consumption (anhedonia) and increase of immobility in the forced swimming task (learned helplessness). Furthermore, recently the CRS have been proposed to counteract depressive-like behaviors and deleterious neurochemical effects induced by unpredictable chronic stress (Parihar et al., 2011). Herein, CRS was unable to induced helplessness learning on the forced swimming test.

UCMS induced anhedonia and learned helplessness and induced an increased Bmax of striatal A_{2A} adenosine receptors binding. However, it was not followed by an increased A_{2A} receptor immunocontent. This result seems consistent with published data that show that glucocorticoids are unable to regulate A_{2A} adenosine receptors (Svenningsson and Fredholm, 1997). A_{2A} adenosine receptors modulation is complex, since A_{2A}Rs may form homomers and/or heteromers (including oligomers) with other receptors such as A_1Rs, D_2 dopamine receptors, metabotropic glutamate receptor 5 (mGlu5) and CB_1 canabinoid receptors ([Kachroo et al., 2005], [Salamone et al., 2009] and [Martire et al., 2011]). These receptors interactions could led to modifications in the quaternary structure of A_{2A}Rs, acting as allosteric efectors (Gracia et al., 2011).

In addition, A_{2A}Rs could interact with the so-called 'accessory' proteins such as α-actinin, ARNO/ cytohesin-2, USP4, NECAB2 and calmodulin), those proteins can bind in the extended tail of A_{2A}Rs C-terminus and to initiate receptor desensitization and endocytosis of A_{2A}Rs ([Gsandtner et al., 2005], [Burgueño et al., 2003] and [Zezula & Freissmuth, 2008]). Thus, although the precise regulatory role of these interactions remains to be established, the increases of Bmax could be due to alosteric activation by interactions with other proteins.

Indeed, Huang et al demonstrated that activation of A_{2A} receptors in the ventral medial striatum is a critical mediator of reserpine-induced depression, and a selective A_{2A} antagonist CSC was also able to ameliorate the rat performance in the swimming test (Huang et al., 2004). El Yacoubi's data support a possible potential role of A_{2A} receptor antagonist as novel anti-depressants, since the D_2 receptor-like antagonist (haloperidol) was able to prevent the antidepressant effect resulting from A_{2A} receptor blockade or inactivation. These results led to the hypothesis that these effects of A_{2A} adenosine receptors might involve adenosine-dopamine interactions, concerning drugs acting on dopaminergic signaling to manage mood disorders ([El Yacoubi et al, 2001] and [El Yacoubi et al, 2003]). However, additional mechanisms such as the A_{2A} receptor interaction with other neurotransmitter systems in forebrain regions (but outside the striatum) or the ability of this receptor to control glial metabolism and neuroinflammation should also be explored by future studies.

In conclusion, both chronic stress models generated an up-regulation in hippocampal A_1 adenosine receptors, while only unpredictable chronic mild stress (UCMS) was able to induce depressive-like behavior and it was capable of up-regulating A_{2A} adenosine receptors on striatum. Although the effects observed herein on

adenosine receptors and behavior suggests some correlation, it is not possible to draw causality from these results. Therefore, the relationship between striatal A_{2A}, hippocampal A_1 adenosine receptors and depressive-like behavior must be further studied.

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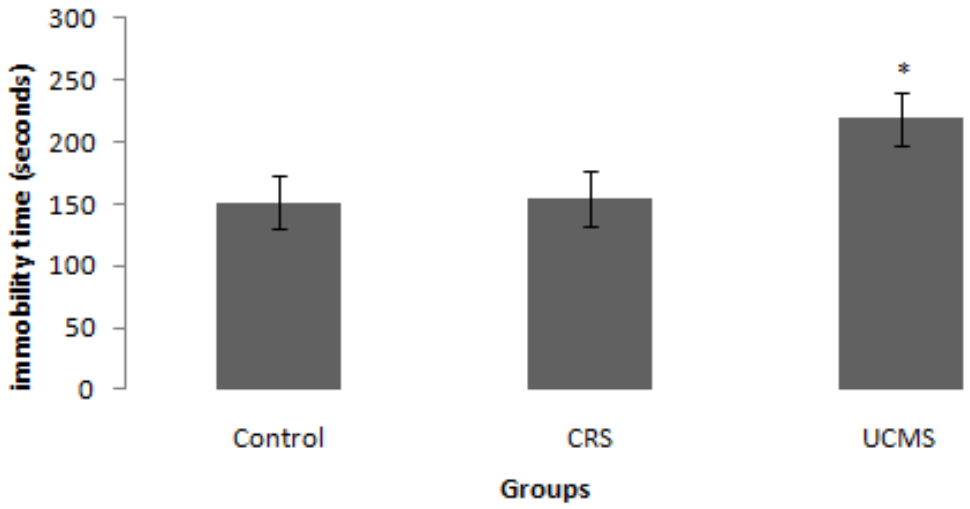

Figure 1. Effects of exposure to two chronic stress models, chronic restraint stress (CRS) and unpredictable chronic stress (UCMS), for 40 days on the depressive-like behavior using the forced swimming task (one-way ANOVA showed a significant increase in the immobility time (seconds) only on the UCMS $P< 0.05$). Data are expressed as means $\pm$ standard error of the mean. N = 23-24/ group.

* Significant difference from control and CRS groups ($P < 0.05$; Duncan multiple range test)

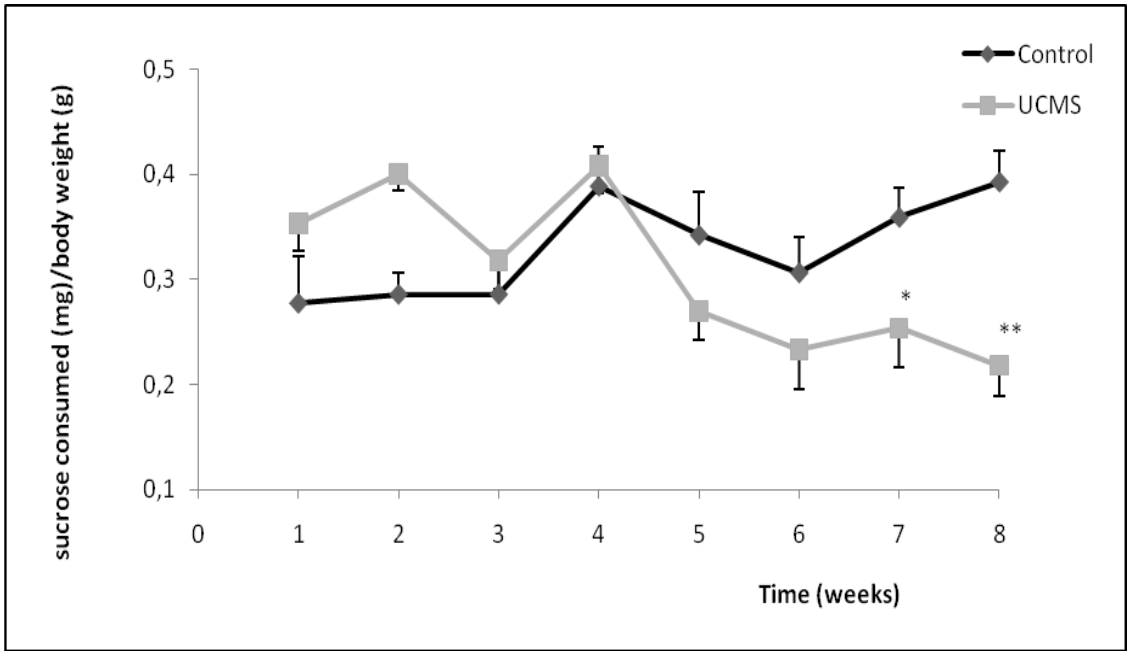

Figure 2 Effects of exposure to two chronic stress models, chronic restraint stress (CRS) and unpredictable chronic stress (UCMS), for 40 days on the depressive-like behavior, evaluating chronic sucrose consumption (one-way ANOVA showed a significant decrease in the sucrose intake on the UCMS $P< 0.05$). Data are expressed as means $\pm$ standard error of the mean. N = 8/ group.

* Significant difference from control for the weeks number 7 and 8. ($P < 0.05$; Duncan multiple range test)

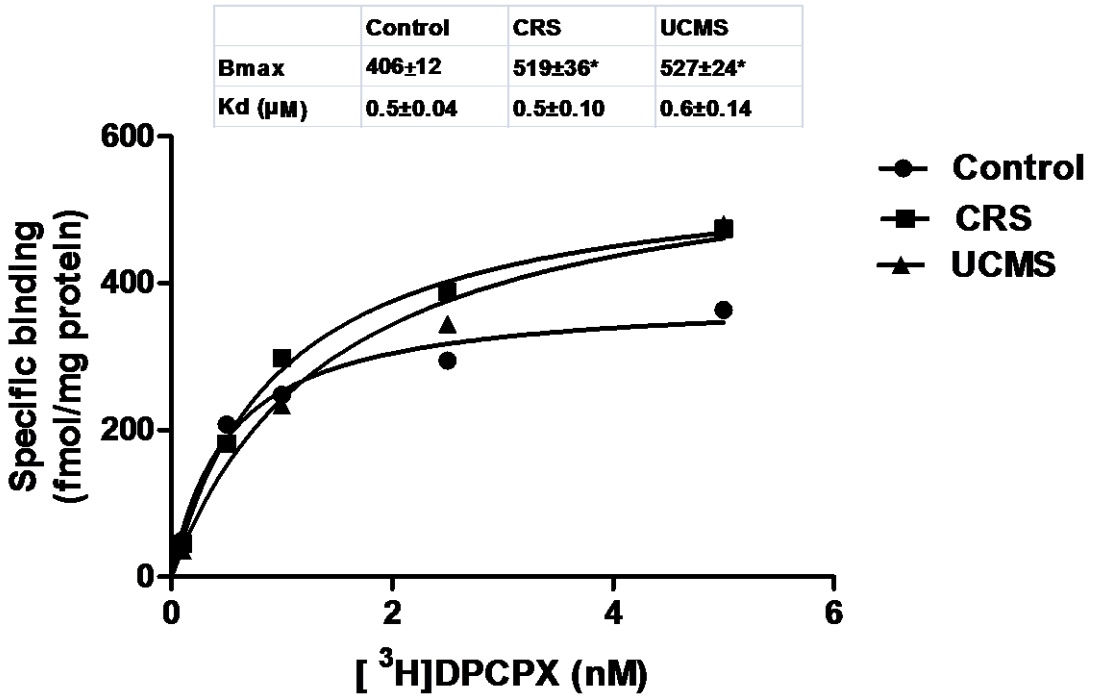

Figure 3 Effects of exposure to two chronic stress models, chronic restraint stress (CRS) and unpredictable chronic stress (UCMS), for 40 days on A_1 adenosine receptor binding in the hippocampus. One-way ANOVA showed a significant increase in Bmax in both stressed groups. There was no effect on the Kd between groups. Data are expressed as means ± standard error of the mean. N = 4-5/group. * Significant difference from control ($P < 0,05$, Duncan multiple range test)

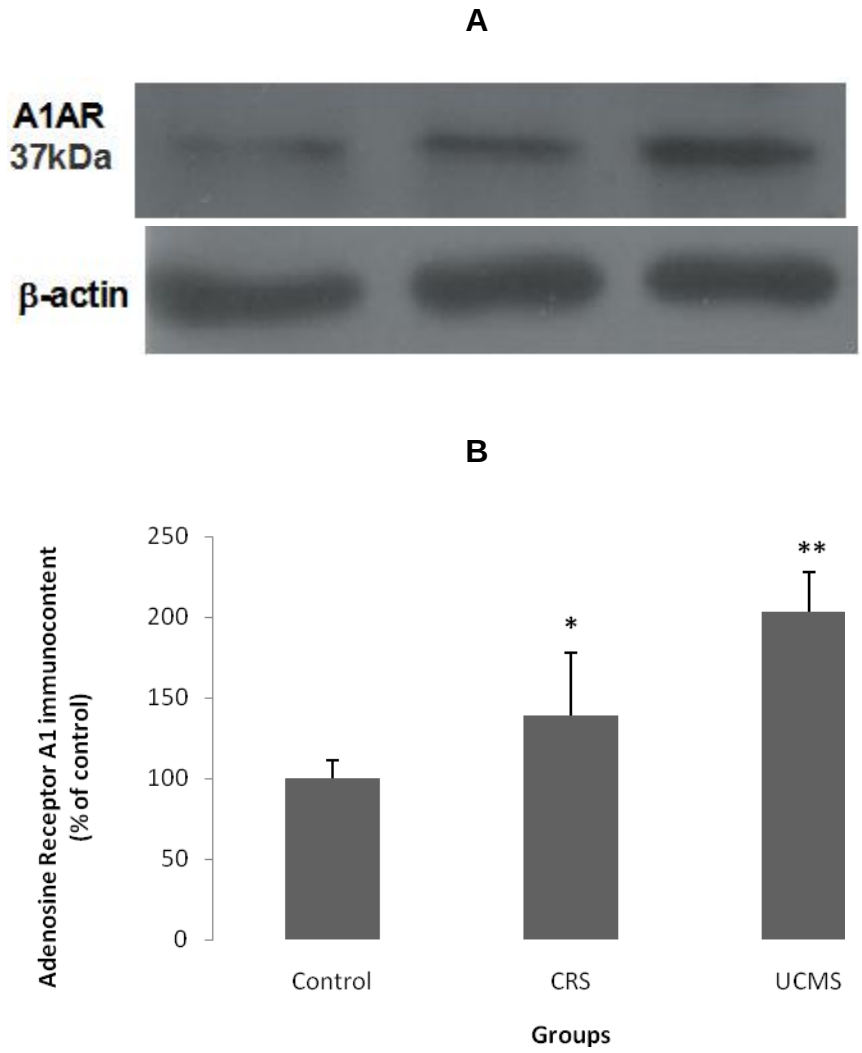

Figure 4 A_1 receptor immunocontent. A. Data represent relative optical density. B. A one-way ANOVA showed a significant difference in the A_1 receptor immunocontent ($P < 0.05$). Data are expressed as means ± standard error of the mean for stressed groups/ control group percentage. N = 4-5/group. * Significant difference from control and UCMS group. ** Significant difference from control and CRS groups ($P < 0.05$, Duncan multiple range test).

	Control	CRS	UCMS
Bmax	281±17	234±9	356±18*
Kd (μM)	4.5±0.5	6.5±0.4	5.6±0.6

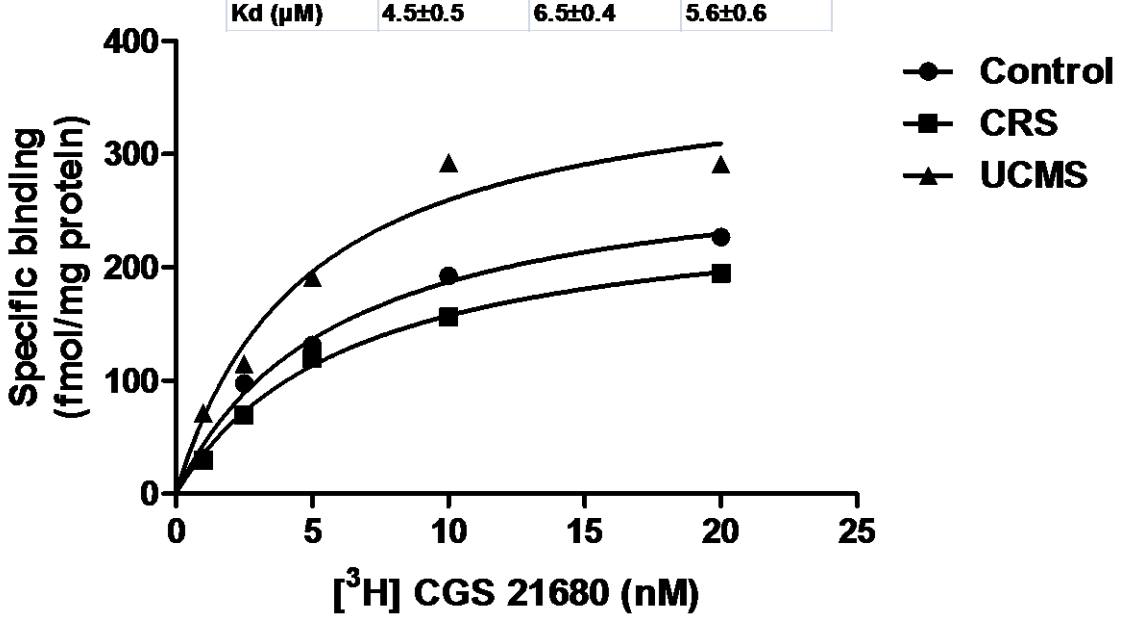

Figure 5 Effects of exposure to two chronic stress models, chronic restraint stress (CRS) and unpredictable chronic stress (UCMS), for 40 days on A_{2A} receptor binding in striatum. A one-way ANOVA showed a significant increase in A_{2A} receptor Bmax only in UCMS group. There was no effect on the Kd between groups. Data are expressed as means $\pm$ standard error of the mean. N = 4-5/group. * Significant difference from control and CRS groups. ($P < 0.05$, Duncan multiple range test)

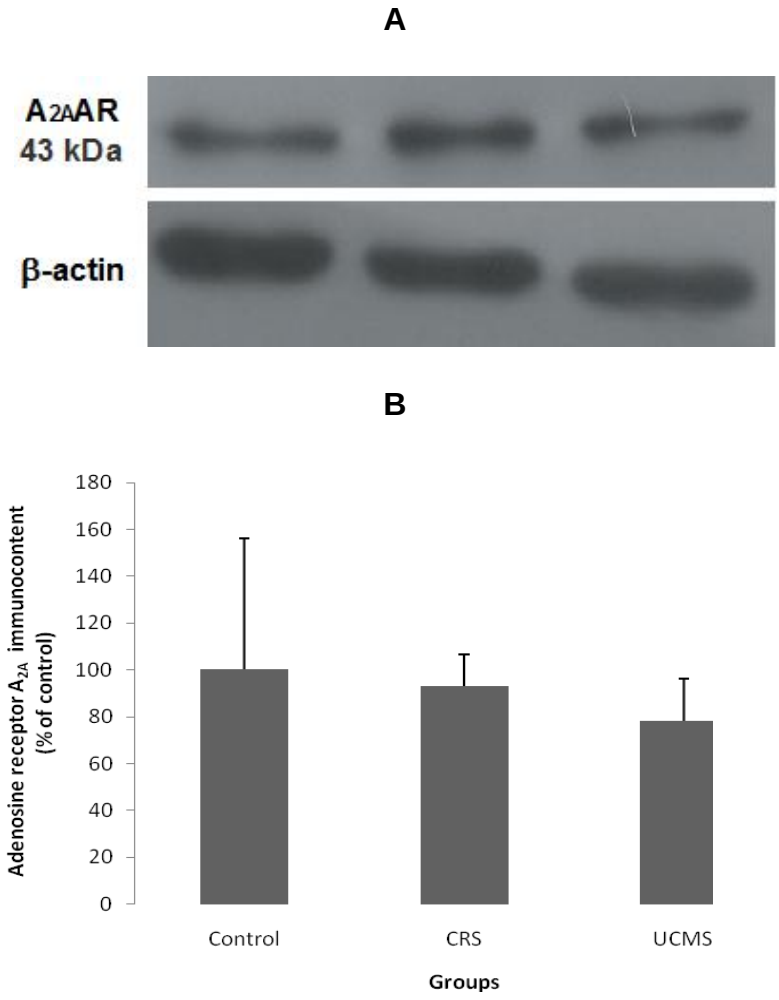

Figure 6 A_{2A} receptor immunocontent. A. Data represent relative optical density. B. There was no effect in A_{2A} receptor immunocontent. Data are expressed as means ± standard error of the mean for stressed groups/ control group percentage. N = 4-5/group. ($P > 0.05$ Duncan multiple range test)

Tabela 1 Schedule of stressor agents used during the unpredictable chronic mild stress.

Days	Stressors
1	1.5 h of restraint at 4°C
2	Light during the night
3	5 h of flashing light
4	2 h of restraint
5	4 h of cage placed at 4°C
6	24 h of isolation
7	24h of isolation
8	4 h of home cage inclination
9	1.5 h of restraint
10	2 h of flashing light
11	Light during de night
12	3h cage placed at 4°C
13	24 h of isolation
14	24 h of isolation
15	Non stressor applied
16	12h of damp sawdust
17	3 h of restraint
18	6 h of home cage inclination
19	2 h of restraint at 4°C
20	light during the night
21	2h of flashing light

22	2h cage placed at 4°C
23	24 h of isolation
24	24 h of isolation
25	24 h of isolation
26	1 h of restraint
27	24 h of damp sawdust
28	3.5 h of flashing light
29	3 h of home cage inclination
30	2 h of restraint at 4°C
31	Non stressor applied
32	24 h of food deprivation
33	3 h of restraint
34	4 h of flashing light
35	24 h of food deprivation
36	16 h of 12h of damp sawdust
37	24 h of isolation
38	24 h of isolation
39	Light during the night
40	20 h of damp sawdust

3. DISCUSSÃO

A presente tese apresentou resultados distribuídos em dois artigos relacionados à influência de diferentes modelos de estresse crônico em ratos Wistar [Estresse Crônico Repetido (CRS) e Estresse Crônico Moderado Imprevisível (UCMS)] sobre respostas comportamentais e bioquímicas em estruturas fundamentais para a modulação do humor e das emoções, como hipocampo, amígdala e estriado (Surget et al., 2011; Jones et al., 2011; Uchida et al., 2011), dando enfoque a comportamentos implicados às psicopatologias como depressão e ansiedade.

O primeiro artigo, publicado em 2010 na revista *Neurochemical Research,* demonstrou aumento do comportamento do tipo ansioso na tarefa do campo aberto, verificado pela diminuição no tempo de permanência dos animais submetidos aos dois modelos de estresse no setor central do aparato. Além disso, demonstramos que as amígdalas desses animais apresentaram diminuição na atividade da enzima Na^+, K^+-ATPase e esta alteração parece não ter relação com um desequilíbrio oxidativo. Importante de salientar que este é o primeiro relato de diminuição da atividade dessa enzima na amígdala de ratos estressados. A amígdala tem sido importante alvo nos estudos de distúrbios como ansiedade, transtorno do estresse pós-traumático e abuso de drogas. Seria interessante a utilização de testes comportamentais que tivessem como foco a amígdala, bem como manipulação de proteínas específicas (e.g, CREB e BDNF, entre muitas outras) para explorar seus possíveis papéis na ansiedade e na ação ansiolítica (Yu & Chen, 2011). No caso do presente estudo optamos pela enzima Na^+, K^+- ATPase, enzima essencial na manutenção do gradiente eletroquímico das células neurais (discutido detalhadamente no primeiro artigo).

Estudos prévios do nosso laboratório relataram diminuição da atividade da Na^+, K^+-ATPase em modelos experimentais de depressão, porém estas alterações

ocorreram no hipocampo de ratos (Gamaro et al., 2003; de Vasconcellos et al., 2005). Dados da literatura demonstram que a expressão da Na^+, K^+- ATPase pode ser regulada por glicocorticóides, por exemplo, utilizando um tratamento agudo com dexametasona, corticóide exógeno, em ratos adrenalectomizados, verificou-se aumento da transcrição de RNAm para as subunidades α-3 e β-1 da Na^+, K^+- ATPase em diversas regiões encefálicas, incluindo amígdala medial e lateral (Grillo et al., 1994).

A atividade da Na^+, K^+- ATPase parece estar envolvida em distúrbios do humor. De fato, Jornada (2010) administrou ouabaína I.P (inibidor especifico da Na^+, K^+- ATPase) para induzir modelo de mania, caracterizado por hiperlocomoção em ratos. Este comportamento pode estar relacionado com comportamentos do tipo ansioso (Long et al., 2010). Estes dados reforçariam nossos estudos ao estabelecer uma possível correlação entre a diminuição da atividade da Na^+, K^+- ATPase no hipocampo e na amigdala com comportamentos do tipo ansioso. A inibição da atividade da Na^+, K^+- ATPase por ouabaína no hipocampo e na amígdala também foi acompanhada com diminuição dos níveis de BDNF nessas estruturas (Jornada et al., 2010). Níveis diminuídos de BDNF têm sido encontrados no encéfalo de pacientes com depressão maior e em ratos submetidos a estresse crônico (Schimidt & Duman, 2011). Além disso, administração periférica de BDNF produziu efeitos antidepressivos tanto em nível celular quanto comportamental. Neste ponto de vista, sugerimos que diminuição da atividade da Na^+, K^+- ATPase na amígdala dos animais submetidos ao CRS e ao UCMS poderia se correlacionar com baixos níveis de BDNF nesta estrutura.

Adicionalmente, nós tentamos estabelecer alguma relação da diminuição da atividade da Na^+, K^+- ATPase com o desequilíbrio oxidativo, uma vez que estudos demonstram que a inibição da Na^+, K^+- ATPase no encéfalo de ratos por injeções intra-

cérebro-ventricular de ouabaína causou aumento do ácido tiobarbitúrico, aumento dos níveis de espécies reativas e produção de carbonilas em proteínas , além de alterar a atividade de enzimas antioxidantes como superóxido dismutase (SOD) e catalase (CAT) no córtex pré-frontal e hipocampo. No entanto, no presente estudo, somente o grupo UCMS apresentou uma inibição da atividade da SOD na amígdala, sem alterações nos outros parâmetros analisados, tornado imprecisa a correlação entre um desequilíbrio oxidativo e a diminuição da atividade da Na^+, K^+- ATPase.

Interessantemente, no segundo trabalho, demonstramos diferenças comportamentais entre os dois modelos experimentais. O grupo UCMS apresentou aumento do tempo de imobilidade na tarefa do nado forçado, e estudos prévios vêm demonstrando que este parâmetro pode ser correlato de desamparo, um dos principais sintomas da depressão (Tagliari et al., 2010; Karanges et al., 2011). Usamos, então, o UCMS como modelo do distúrbio depressivo para investigarmos o consumo de sacarose. De fato, o grupo UCMS apresentou diminuição ao longo de oito semanas no consumo de solução de sacarose, podendo ser, também, um correlato de comportamento do tipo depressivo, denominado anedonia (Dagytė et al., 2011; Jahng, 2011).

Na tentativa de conhecer o papel do sistema adenosinérgico sobre os efeitos dos dois modelos de estresse crônico (CRS e UCMS), observamos aumento do Bmax e do imunoconteúdo de receptores A_1 de adenosina (A_1Rs) no hipocampo dos ratos submetidos a ambos os modelos de estresse.

A adenosina modula a transmissão sináptica por meio da interação com sistemas de neurotransmissores glutamatérgico, colinérgico, GABAérgico e dopaminérgico (Kurokawa et al., 1996; Latini et al., 1996; Mori & Shindou, 2003; Popoli

et al., 2003), através dos seus quatro receptores acoplados a proteínas G, são eles: A_1, $A_{2A,}$ A_{2B} e A_3 (Jacobson & Gao, 2006)

A_1Rs estão expressos amplamente no encéfalo e a adenosina liga-se a eles em concentrações fisiológicas. Esses receptores estão envolvidos na modulação das ações fisiológicas desse nucleosídeo, incluindo regulação da liberação de diversos neurotransmissores, principalmente glutamato, modulando efeitos protetores contra danos induzidos por hipóxia e isquemia. (Reppert et al*.,* 1991; Dunwiddie & Masino, 2001). Por esse motivo, a maioria dos efeitos induzidos pela adenosina no encéfalo adulto ocorre pela ativação dos A_1Rs neuronais (Fredholm et al., 2005). De acordo, agonistas de A_1Rs induziram neuroproteção e antagonistas de A_1Rs exacerbaram danos cerebrais em animais adultos (de Mendonça et al*.,* 2000). Desse modo, o aumento de A_1Rs hipocampais nos ratos submetidos aos CRS e UCMS parece ser uma tentativa de proteção ou prevenção de insultos causados por estresse crônico e depressão no hipocampo, como diminuições do volume hipocampal, da sinaptogênese, da arborização dendrítica, de espinhos dendríticos, além de morte neuronal nesta estrutura (Joëls et al., 2004, Zhao et al., 2007; McEwen, 2007). A regulação dos A_1Rs hipocampais pode ser modulada pela exposição a altos níveis de glicocorticóides. Svenningsson & Fredholm (1997) demonstraram que ratos submetidos a injeções de dexametasona, corticóide exógeno, apresentaram aumento da transcrição de A_1Rs. Adicionalmente, recentes estudos indicam que UCMS, similar ao utilizado neste trabalho, aumentou os níveis de citocinas pró-inflamatórias, entre elas, a Interleucina-6 (IL-6), no hipocampo de ratos (Tagliari et al., 2011).

Biber et al. (2008) identificaram que a IL-6 é um importante fator na regulação da expressão e funcionalidade de A_1Rs no hipocampo, estimulando neuroproteção em

situações nocivas tanto *in vivo* quanto *in vitro*. No mesmo estudo, o aumento dos níveis de IL-6 potencializou a capacidade dos A_1Rs de inibirem a transmissão sináptica excitatória induzida por glutamato durante exposição à hipóxia em fatias de hipocampo. Embora os mecanismos pelos quais IL-6 potencializa a função neuroprotetora de A_1Rs na modulação da transmissão sináptica glutamatérgica e na sobrevivência neuronal possam não estar necessariamente conectados. Dessa maneira, parece razoável considerarmos a importância da regulação de A_1Rs hipocampais induzidas por CRS e UCMS como uma forma de modulação dos insultos neuroquímicos relacionados ao estresse crônico como a excitotoxicidade glutamatérgica. Por exemplo, ratos que foram submetidos ao UCMS apresentaram aumento da liberação e diminuição da captação de glutamato no hipocampo, acompanhados do aumento de morte celular após privação de oxigênio e glicose (de Vasconcellos-Bittencourt et al., 2011).

Por outro lado, foi evidenciado somente no grupo UCMS aumento do Bmax dos A_{2A}Rs no estriado de ratos, sem alteração no imunoconteúdo desses receptores. Diferentemente dos A_1Rs, os receptores, os A_{2A}Rs não estão amplamente distribuídos em todo encéfalo, ao invés disso apresentam maior densidade no estriado e sua regulação não parece sofrer influência de glicocorticóides (Svenningsson & Fredholm, 1997; Fredholm et al., 2005). O bloqueio da função dos A_{2A}Rs por antagonistas ou por deleção do gene que codifica este receptor tem sido reportado como possível alvo para o desenvolvimento de novos antidepressivos, promovendo neuroproteção (El Yacoubi et al., 2003; Cunha, 2005), motivos pelos quais têm sido grande alvo de estudos. Este peculiar aumento dos A_{2A}Rs no estriado de ratos submetidos ao UCMS, bem como aumento de comportamentos depressivos, demonstram novas diferenças entre esses dois modelos de estresse crônico, além de apoiar o UCMS como um modelo

experimental confiável para estudar distúrbios relacionados à depressão, e talvez o aumento do Bmax de A_{2A}Rs possa representar uma alteração relacionada a distúrbios depressivos. Dados da literatura indicam que a regulação dos A_{2A}Rs é complexa, como discutido no segundo artigo. Além disso, estes receptores possuem características funcionais e estruturais que aumentam essa complexidade. A_{2A}R tem uma longa cauda C- terminal, maior que 120 resíduos de aminoácidos, tornando o acoplamento de proteína G confinado a específicos micro-domínios de membrana, o que torna dispensável o acoplamento de proteínas G(s) (Zezula & Freissmuth, 2008). Isto deixaria a região C- terminal com diversos domínios disponíveis para o acoplamento de cinases e β- arrestina, que iniciariam processos de desensitização e de endocitose desses receptores. Recentemente, a longa região C- terminal dos A_{2A}Rs tem sido proposta como sitio de ligação para diversas “proteínas acessórias”, além desses receptores formarem complexos heteroméricos com outros receptores (Zezula & Freissmuth, 2008), como está discutido no segundo artigo. Embora os A_{2A}Rs apresentem sítios de regulação alostérica para diversas proteínas, existem determinadas regras para este acoplamento de determinados fatores regulatórios, o que permite que os receptores A_{2A}Rs induzam respostas biológicas diferentes dependendo do contexto celular, e da natureza do(s) estímulo(s). Dessa forma, os receptores A_{2A}Rs parecem ser detectores de coincidência de sinais integradores (Hernandez- Deviez et al., 2002, 2004).

Desse modo, dada a complexidade da regulação dos A_{2A}Rs exposta acima, torna-se difícil estabelecermos uma relação de casualidade direta entre a alta regulação dos A_{2A}Rs estriatais nos animais submetidos ao UCMS e os efeitos comportamentais observados. No entanto, tal sugestão parece plausível, uma vez que

estudos demonstraram que a adenosina é capaz de modular comportamentos do tipo depressivos através dos receptores A_{2A}Rs (Kaster et al., 2004). Além disso, A_{2A}Rs no núcleo *accumbens* parecem regular a ativação de comportamentos que exijam esforço, modulando a atividade da via ventral estriado-palidal associada a comportamentos motivados (Mingote et al., 2008). Além disso, adenosina agindo nos A_1Rs e A_{2A}Rs induziu efeitos anti-imobilidade no teste do nado forçado com a mediação do sistema opióide (Kaster et al., 2007). Estes estudos parecem apoiar nossos dados e estabelecem possíveis correlações entre o aumento da regulação dos A_{2A}Rs no estriado com comportamentos como desamparo e anedonia, demonstrados nesse estudo, além de apoiar um possível mecanismo de ação dos antagonistas A_{2A}Rs utilizados em modelos experimentais de depressão como possíveis antidepressivos (Kaster et al., 2007; Lobato et al., 2008)

Todos esses resultados tomados em conjunto reforçam que o UCMS é um confiável, válido e preditivo modelo de depressão, uma vez que os animais submetidos cronicamente a estressores fracos e imprevisíveis apresentaram um conjunto dos principais sintomas diagnósticos correlacionados a depressão como comportamento de desamparo, de anedonia (perda de prazer) e de ansiedade. Observar que tanto UCMS (modelo de depressão, quanto CRS (Estresse Crônico Repetido) apresentaram de forma similar o comportamento do tipo ansioso.

Quantos aos parâmetros bioquímicos aqui mensurados, verificamos que houve diminuição na atividade da enzima Na^+, K^+- ATPase na amígdala, aumento de receptores A_1 no hipocampo de ambos os modelos de estresse, e, por outro lado, aumento de receptores A_{2A} no estriado, e é interessante observar que este aumento ocorreu somente no grupo experimental utilizado como modelo de depressão neste

trabalho (UCMS). Desse modo, é possível que distúrbios na enzima Na^+, K^+- ATPase e nos receptores de adenosina A_1 e A_{2A} no SNC tenham um papel na modulação de comportamentos relacionados a distúrbios do humor, porém não podemos inferir correlações diretas entre os parâmetros neuroquímicos demonstrados neste trabalho com a psicopatologia humana da depressão. Os parâmetros neuroquímicos alterados nesse estudo e a relação com o distúrbio depressivo precisam ser elucidados com maior profundidade, no intuito de aumentar o conhecimento básico sobre psicopatologias relacionadas ao estresse. Esta base científica, futuramente, poderá servir de substrato para o avanço de pesquisas biomédicas na formulação e melhoramento de psicofármacos.

4. CONCLUSÕES

No presente estudo, concluímos que os dois modelos de estresse crônico (CRS e UCMS) induziram aumento do comportamento do tipo ansioso de maneira similar. Por outro lado, contrariamente ao CRS, o UCMS foi capaz de aumentar comportamentos do tipo depressivo, como anedonia e desamparo em ratos machos adultos *Wistar*.

Na tentativa de estabelecer possíveis correlações neuroquímicas com estes comportamentos, sugerimos uma possível relação da baixa atividade da Na^+, K^+-ATPase na amígdala na indução de comportamentos do tipo ansioso demonstrados nos modelos de estresse crônico, previsível (CRS) e imprevisível (UCMS). Além disso, não obtivemos resultados concretos que possamos concluir que a diminuição da atividade da Na^+, K^+-ATPase na amígdala esteja relacionado com aumento de estresse oxidativo nesta estrutura.

Além disso, de acordo com o segundo trabalho, concluímos que os comportamentos do tipo depressivo foram induzidos pelo estresse crônico imprevisível (UCMS). Dessa forma, sugerimos que esses comportamentos possam estar correlacionados com alterações nos receptores de adenosina, particularmente no aumento da regulação dos A_{2A}Rs no estriado. Por outro lado, não podemos afirmar um envolvimento direto da alta regulação dos A_1Rs no hipocampo com aumento de comportamentos do tipo depressivo, pois houve esse aumento em ambos os modelos de estresse.

Em suma, a presente tese sugere a possível existência entre ansiedade e diminuição da atividade da enzima Na^+, K^+-ATPase na amígdala, bem como depressão e aumento da regulação de A_{2A}Rs no estriado. Dessa maneira, ressaltamos que essas alterações demonstradas nesta tese, provavelmente possam ser úteis como alvos estratégicos no refinamento do conhecimento básico sobre distúrbios do humor como a ansiedade e a depressão, bem como no desenvolvimento de terapias farmacológicas alternativas para estes psicopatologias.

5. PERSPECTIVAS

- Verificar os efeitos da administração de inibidores e ativadores da Na^+, K^+-ATPase sobre comportamentos do tipo ansioso e do tipo depressivo.

- Estudar os efeitos dos dois modelos de estresse (CRS e UCMS) e a administração de ouabaína (inibidor seletivo da Na^+, K^+-ATPase) sobre a densidade da enzima Na^+, K^+-ATPase em diferentes subnúcleos e regiões da amígdala, do hipocampo, do estriado, do córtex pré-frontal e do hipotálamo.

- Pesquisar os efeitos dos dois modelos de estresse (CRS e UCMS) e de diferentes classes de antidepressivos como os tricíclicos, os inibidores seletivos da captação de serotonina, os inibidores seletivos da recaptação de noradrenalina e dopamina e o envolvimento de seus respectivos receptores na modulação da atividade e densidade da enzima Na^+, K^+-ATPase em diferentes subnúcleos e regiões da amígdala, do hipocampo, do estriado, do córtex pré-frontal e do hipotálamo.

- Investigar os efeitos do UCMS e de injeções de antagonistas de receptores A_1Rs, A_{2A}Rs, D1 e D2 no hipocampo de ratos sobre comportamentos do tipo depressivo e ansioso e analisar a densidade e a funcionalidade desses receptores em diferentes subnúcleos e regiões da amígdala, do hipocampo, do estriado, do córtex pré-frontal e do hipotálamo.

- Estudar o envolvimento dos receptores A_1Rs, A_{2A}Rs, D1 e D2, na habituação neuroendócrina ao estresse crônico, através de medidas plasmáticas dos níveis de corticosterona e de ACTH, utilizando antagonistas e agonistas desses receptores.

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