UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

AVALIAÇÃO DA ATIVIDADE NEUROPROTETORA E ANTIDEPRESSIVA DO TRATAMENTO COM LÍTIO EM UM MODELO DE ESTRESSE CRÔNICO VARIADO

Tese de Doutorado

Ana Paula Santana de Vasconcellos

Porto Alegre, 2005

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Para Sônia e Athelson,
minha mãe e meu marido,
meu sul e meu norte,
meus portos seguros onde quer que eu esteja

"O pensamento lógico pode levar você de A a B, mas a imaginação te leva a qualquer parte do Universo."

Albert Einstein

"...É necessário, pois, a propósito disso, fazer uma das coisas seguintes: não perder a ocasião de instruir-se, ou procurar aprender por si mesmo, ou então, se não se for capaz nem de uma nem de outra dessas ações, ir buscar em nossas antigas tradições humanas o que houver de melhor e menos contestável, deixando-se assim levar como sobre uma jangada, na qual nos arriscaremos a fazer a travessia da vida, uma vez que não a podemos percorrer, com mais segurança e com menos riscos, sobre um transporte mais sólido: quero dizer, uma revelação divina."

Platão

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LISTA DE ABREVIATURAS

5HT	5-Hidroxitriptamina (Serotonina)
ACTH	Hormônio Adrenocorticotrófico
Akt/PKB	Proteína Cinase B
AMPA	α-Amino-3-Hidroxi-5-Metil-4-Isoxazolpropionato
ARNm	Ácido Ribonucléico mensageiro
ATP	Adenosina Trifosfato
Bcl-2	Célula de Linfoma B
BDNF	Fator Neurotrófico Derivado do Encéfalo
CA1	Corno de Amon 1
CA3	Corno de Amon 3
CAM-Cinase II	Cinase Dependente de Cálcio e Calmodulina II
CREB	Proteína Ligante de Elemento Responsivo ao AMP-cíclico
CRH	Hormônio Liberador de Corticotrofina
DCF	Diclorofluoresceína
DOPAC	Ácido 3,4-diidroxifenilacético
EAAC1	Transportador (Carreador) de Aminoácidos Excitatórios 1
ECV	Estresse Crônico Variado
ERNs	Espécies Reativas de Nitrogênio
EROs	Espécies Reativas de Oxigênio
GABA	Ácido Gama-Aminobutírico
GC	Glicocorticóide
GD	Giro Denteado
GFAP	Proteína Glial Fibrilar Ácida
GLAST	Transportador de glutamato e aspartato
GLT-1	Transportador de glutamato 1
GluR-1	Subunidade 1 do Receptor Glutamaérgico AMPA
GPx	Glutationa Peroxidase
GSK-3β	Glicogênio Sintase Cinase 3-beta
H_2O_2	Peróxido de Hidrogênio
HHA	Eixo Hipotálamo-Hipófise-Adrenal

Hsp	Proteína de Choque Térmico
LDH	Lactato Desidrogenase
LTD	Depressão de Longa Duração
LTP	Potenciação de Longa Duração
Na^+,K^+-ATPase	Bomba sódio-potássio-ATPase
NMDA	N-metil-D-aspartato
NPV	Núcleo Paraventricular do Hipotálamo
$O_2^{-\bullet}$	Ânion Superóxido
$OH^\bullet$	Radical Hidroxila
PKC	Proteína Cinase C
PMD	Psicose Maníaco-Depressiva
POG	Privação de Oxigênio e Glicose
SNC	Sistema Nervoso Central
SNV	Sistema Neurovegetativo
SOD	Superóxido Dismutase
THB	Transtorno de Humor Bipolar

RESUMO

Esta tese foi desenvolvida para investigar os mecanismos envolvidos nos efeitos protetores do tratamento com lítio sobre a memória espacial de ratos submetidos a um modelo de estresse crônico variado, bem como verificar os efeitos destes tratamentos em alguns parâmetros relacionados com depressão. Foram utilizados ratos Wistar (*Rattus norvegicus*), divididos em 4 grupos experimentais: controles, controles tratados com lítio, estressados e estressados tratados com lítio. Observamos que em 21 dias de tratamentos com estresse e lítio não há alteração na memória espacial, e que aos 30 dias o tratamento com lítio induz um efeito facilitador sobre a memória. A partir disso, os tratamentos tiveram duração de quarenta dias, após os quais as medidas comportamentais e bioquímicas foram desenvolvidas. Observamos que o estresse diminui a atividade da enzima Na^+, K^+-ATPase em membranas sinápticas hipocampais, e este efeito é prevenido pelo tratamento com lítio, bem como revertido tanto pela interrupção do estresse quanto pelo tratamento pós-estresse com lítio. A interrupção do estresse por um período de trinta dias também reverte os seus efeitos sobre a memória, assim como o faz o tratamento pós-estresse com lítio. Estes dados indicam que o déficit cognitivo induzido pelo estresse crônico é mediado por alterações plásticas, e a similaridade temporal com os efeitos sobre a atividade da enzima Na^+, K^+-ATPase sugere que ela esteja envolvida no prejuízo observado na memória espacial. Também avaliamos os efeitos destes tratamentos sobre a transmissão glutamatérgica hipocampal, e observamos que o estresse aumenta a liberação basal de glutamato e diminui a captação deste neurotransmissor por fatias hipocampais. Este efeito pode induzir excitotoxicidade glutamatérgica e colaborar no agravamento de outros insultos, como o aumento na morte celular observado após a privação de oxigênio e glicose. Por sua vez, o lítio aumentou a captação de glutamato por sinaptossomas, o que pode ser uma ferramenta adicional na neuroproteção após diversos tipos de insulto. Também houve um aumento na liberação de glutamato após estímulo, e este efeito pode, por um lado, estar facilitando os mecanismos de plasticidade sináptica, por outro, ter contribuído para o aumento na morte celular observado após privação de oxigênio e glicose. Foram realizadas medidas de estresse oxidativo, onde o tratamento com lítio induziu um relativo efeito antioxidante, demonstrado pela diminuição na formação de espécies oxidantes no hipocampo e pelo aumento da reatividade antioxidante total em hipocampo e hipotálamo, bem como pelo aumento na atividade da superóxido dismutase (SOD) e da glutationa peroxidase (GPX) em hipotálamo e hipocampo, respectivamente. Contudo, este efeito

antioxidante não foi eficiente na prevenção da peroxidação lipídica hipocampal provocada pelo estresse, e o aumento desproporcional na atividade da SOD em animais estressados e estressados + lítio é um possível causador da peroxidação dos lipídeos de membrana em hipocampo. Os tratamentos com estresse e lítio induziram aumento no consumo de alimentos doces, sem, contudo, alterar o consumo de ração padrão pelos animais. No entanto, somente animais tratados com lítio apresentaram aumento também no consumo de alimentos salgados, e o aumento no consumo de doces por este grupo foi muito mais acentuado do que nos animais estressados. Estes efeitos não parecem ser devidos a uma maior ansiedade, visto que não houve efeito ansiogênico dos tratamentos no labirinto em cruz elevado. Animais estressados não apresentaram a analgesia característica após exposição a um sabor doce agradável (leite condensado) na medida de latência de retirada da cauda; contudo, mostraram analgesia induzida por um sabor ácido e desagradável, o que caracteriza alguns dos efeitos clássicos da depressão. Já animais tratados com lítio apresentaram analgesia tanto após o sabor agradável quanto o aversivo, sugerindo uma maior sensibilidade a estímulos gustativos nestes animais, e o lítio preveniu a ausência de analgesia induzida por doce em animais estressados, o que pode ser tomado como um efeito antidepressivo deste tratamento. Por fim, observamos uma diminuição na atividade dopaminérgica ventro-estriatal de animais estressados, que pode estar envolvida na ausência de comportamento apetitivo condicionado por um estímulo palatável. Por outro lado, animais tratados com lítio apresentaram um aumento no tônus dopaminérgico, demonstrado pela aquisição de comportamento apetitivo e pela sensibilização cruzada com dietilpropiona, sem, contudo, apresentarem alteração no conteúdo total de dopamina no núcleo accumbens. Pode-se sugerir que o tratamento com lítio induza um aumento na liberação fásica de dopamina ou alteração em seus receptores, e estes efeitos podem estar envolvidos no aumentado interesse de animais tratados com lítio por alimentos novos.

ABSTRACT

These studies were undertaken to investigate the mechanisms involved in the protective effects of lithium treatment on spatial memory of rats submitted to chronic variate stress paradigm, and to verify the effects of these treatments in some depression related parameters. Adult male Wistar rats (*Rattus norvegicus*) were divided into 4 groups: control, control treated with lithium, stressed and stressed treated with lithium. We observed that 21 days of stress and lithium treatments did not alter spatial memory, and that 30 days of lithium treatment had a facilitative effect on memory. From these results on, all treatments lasted 40 days, and after that behavioral and biochemical measurements were performed. We observed that stress decreases Na^+, K^+-ATPase activity in hippocampal synaptic membranes and this effect is prevented by lithium treatment, as well as it is reversed by stress interruption and post-stress lithium treatment. Thirty days of stress interruption also reversed its effects on spatial memory, as well as does a post-stress lithium treatment. These data suggest that the cognitive deficit induced by chronic stress is mediated by plastic alterations, and the parallelism between the effects on spatial memory and on Na^+, K^+-ATPase activity suggests that this enzyme may be involved in the spatial memory damage observed in stressed animals. We also evaluated the effects of stress and lithium on hippocampal glutamatergic transmission, and we observed that stress increases basal synaptosomal release and decreases glutamate uptake in slices of hippocampus. These effects may favor glutamatergic excitotoxicity and contribute to neuronal loss after other kinds of insults, as it was observed after oxygen and glucose deprivation. Lithium increased synaptosomal glutamate uptake, what can represent an additional tool in neuroprotection against several kinds of insults. Lithium also increased stimulated glutamate release, what can be benefic for neuronal plasticity but can also contribute to neuroendangerment after oxygen and glucose deprivation. We performed some oxidative stress measurements, and lithium treatment induced an antioxidant effect, evidenced by a decreased formation of oxidative agents in hippocampus and an increased on total antioxidant reactivity in hippocampus and hypothalamus, as well as an increased activity of superoxide dismuthase (SOD) and gluthathione peroxidase (GPx) in hippocampus and hypothalamus, respectively. However, this antioxidant effect was not enough to prevent the hippocampal lipid peroxidation induced by stress, and the disproportional increase on SOD activity in stressed and stressed + lithium is a possible mediator for the lipid peroxidation observed in this structure. Stress and lithium treatments

induced an increased consumption of sweet foods, not altering standard chow consumption. However, just lithium-treated animals presented an increase in intake of savory foods, and their intake of sweet foods was notably higher than in stressed animals. These effects do not seem to be related to higher anxiety levels, since there was no anxiogenic effects of stress and lithium in the Plus Maze test. Stressed animals did not present sweet-induced analgesia, as evaluated by the tail-flick measurement, but they presented analgesia induced by an unpleasant flavor, characterizing some effects observed in depression. Conversely, lithium-treated animals presented analgesia after pleasant and unpleasant tastes, suggesting an increased sensibility to gustative stimuli in these animals, and lithium prevented the absence of sweet-induced analgesia observed in stressed animals, what can be taken as an antidepressive effect of this treatment. Finally, we observed a decreased ventral-striatal dopaminergic activity in stressed animals that can be involved in the absence of a palatable-conditioned appetitive behavior. On the other hand, lithium-treated animals presented an increased dopaminergic tonus, as showed by the acquired appetitive behavior and by the cross-sensitization with diethylpropione, without presenting any alteration in the total amount of dopamine in the nucleus accumbens. We can suggest that lithium treatment induces an increase on phasic dopamine release or an alteration in its receptors, and these effects can be involved in the increased interest of lithium-treated animals by new palatable foods.

INTRODUÇÃO

Os seres vivos estão constantemente em contato com situações que lhes exigem adaptação física e/ou psicológica. Exemplos disso são as alterações climáticas, que exigem um reequilíbrio térmico do organismo, alterações no estado nutricional, que levam o organismo a mobilizar-se para armazenar ou liberar energia, ou também, para seres que vivem em comunidade, alterações sociais, que levam o indivíduo à necessidade de reestruturar-se para se integrar melhor à sociedade. Tais alterações, por gerarem distúrbios no complexo equilíbrio orgânico conhecido como "homeostase", levam à ativação de forças adaptativas, que são cruciais para a sobrevivência do organismo e responsáveis por sua resistência às alterações do meio ambiente. Segundo Chrousos & Gold (1995), organismos unicelulares adaptam-se com mudanças apropriadas em sua bioquímica, organismos multicelulares adaptam-se através de complexas mudanças neurais, metabólicas e celulares, e organismos sociais, cuja sobrevivência depende da cooperação da comunidade, também desenvolvem ligações sociais com esta comunidade, e a manutenção destas ligações é crucial para a homeostase. Contudo, não são apenas eventos desagradáveis que desencadeiam uma resposta adaptativa: sentimentos de exultação, excitação, provocam no organismo a mesma série de reações, o que é denominado por alguns autores como um "bom estresse" (McEwen, 2000a).

Em 1936, o cientista e médico húngaro Hans Selye definiu o estresse como "Síndrome da Adaptação Geral", ou seja, a resposta adaptativa de um organismo à ação de agentes nocivos – os chamados agentes estressores. Ele postulou ainda que a resposta ao estresse seria dividida em três estágios: um primeiro de alarme, onde o agente estressor seria notado, um segundo de resistência, no qual o organismo estaria combatendo o agente estressor com sucesso, e, por fim, um estado de exaustão, onde o organismo esgotaria sua capacidade de resposta ao estresse, daí advindo os seus efeitos deletérios (Kopin, 1995). De fato, várias alterações - ou transtornos - estão associadas à hiperexposição a agentes estressores, o que denominamos estresse crônico (McEwen & Magariños, 1997). Exemplos disso são disfunções

hormonais, que vão desde alterações no crescimento a problemas reprodutivos, hipertensão, diabetes induzida por esteróides, etc. No entanto, são poucas as evidências que indiquem esta "falência" apontada por Selye. O estresse crônico não é patogênico em função das falhas nas defesas do organismo, mas em função das próprias defesas tornarem-se patogênicas (Sapolsky, 1992). Para entender isto, contudo, é necessário relembrar quais sistemas estão envolvidos na resposta ao estresse e os mecanismos pelos quais atuam sobre o organismo.

Neuroendocrinologia da resposta ao estresse

Frente ao estímulo estressante, as aferências sensoriais, oriundas do tronco cerebral e de diversas regiões da medula espinal, e/ou estímulos oriundos de estruturas prosencefálicas (e.g., a recordação de um momento estressante, ou a antecipação de um evento de mesma característica), atingem a região periventricular do hipotálamo. Este estimula o sistema simpático, levando à liberação de catecolaminas (adrenalina e noradrenalina) a partir dos gânglios simpáticos e da medula das glândulas adrenais, o que constitui uma resposta imediata inicial ao estresse (Ursin & Olff, 1993; Bear *et al*., 1996; Tsigos & Chrousos, 2002). Esta descarga catecolaminérgica tem como função principal a mobilização de energia e a supressão de todos os sistemas "desnecessários" na situação de alarme, como digestão, crescimento, reprodução e imunidade (Sapolsky, 2000; Leonard & Song, 1996), e é responsável pelos sintomas descritos por Walter Cannon no início do século passado, e que foram imortalizadas pelo paradigma da "luta ou fuga".

Historicamente, contudo, o sistema biológico que tem sido associado mais diretamente à resposta ao estresse em mamíferos é o eixo Hipotálamo-Hipófise-Adrenal (HHA), que possui componentes tanto cerebrais quanto endócrinos, constituindo, então, o circuito neuroendócrino de resposta ao estresse (Herman *et al*., 1996; Fuchs *et al*., 2001).

Diversas estruturas do prosencéfalo, incluindo o córtex pré-frontal, hipocampo, amígdala e septo, juntamente com as fibras nervosas transportadoras dos estímulos sensoriais, lançam aferências mono e polissinápticas que convergem para o núcleo paraventricular hipotalâmico (NPV), o qual age como um integrador final da resposta ao estresse (López *et al*., 1999; Tsigos & Chrousos, 2002). Estas células expressam importantes peptídeos, como arginina-vasopressina, ocitocina e, neste caso especialmente, o hormônio liberador da corticotrofina (CRH, do inglês corticotrophin releasing hormone) (Stratakis & Chrousos, 1995; Tsigos & Chrousos, 2002), que são liberados em resposta a estressores fisiológicos e psicológicos. Os axônios destes neurônios projetam-se para a zona externa da eminência média do hipotálamo, o que leva à liberação dos peptídeos no sistema porta - uma estrutura vascular especializada que liga o sistema nervoso central (SNC) à porção anterior da glândula hipófise (Hayden-Hixson & Nemeroff, 1993). Isto resulta na síntese e liberação hipofisária de diversos outros peptídeos derivados de um precursor comum: a pró-opiomelanocortina. Entre estes peptídeos estão incluídos o opióide endógeno β-endorfina e o hormônio adrenocorticotrófico (ACTH) (Akil & Morano, 1995), o qual, ao ser lançado na circulação sistêmica, é carregado até as glândulas adrenais, onde ativa a produção e liberação de glicocorticóides pelo córtex das mesmas (Lupien & Meaney, 1998). Estes hormônios exercem seus efeitos sobre vários tecidos, e sua ação está relacionada em grande parte à interação com receptores citosólicos, regulando eventos genômicos e alterando a transcrição de determinados genes (Sapolsky, 1992; Korte *et al*., 2005).

O nome "glicocorticóide" é devido à habilidade destes hormônios em promover a conversão de proteínas a fontes de carboidratos prontamente utilizáveis pelo organismo (McEwen, 2000a). São, portanto, hormônios hiperglicemiantes, e participam da regulação energética e da atividade metabólica corpórea estimulando a ingestão de alimentos e inibindo o armazenamento periférico de energia (Strack *et al*., 1995; Korte *et al*., 2005). Estes

hormônios são liberados no organismo em ciclos alternados, obedecendo ao ritmo circadiano, e encontrando-se aumentados nos momentos que precedem o despertar, o que estimula o comportamento exploratório e a busca por comida.

Parece claro que a ativação do eixo HHA, e conseqüente liberação de glicocorticóides, é adaptativa e essencial para a sobrevivência imediata do organismo quando em resposta a estímulos agudos. Contudo, a hiperativação deste sistema pode levar a alterações consideradas patológicas, ou resultantes de uma adaptação inadequada, gerando a situação deletéria denominada estresse crônico.

Estresse Crônico e Neurotoxicidade

A regulação das ações do eixo HHA é feita, em grande parte, por retroalimentação ("feed-back") dos glicocorticóides sobre componentes do SNC, de modo que sua atividade é aumentada ou diminuída de acordo com as necessidades fisiológicas (Marti *et al*., 1999). Várias estruturas cerebrais estão envolvidas nos processos de retroalimentação, dentre as quais se incluem os próprios componentes do eixo HHA, a amígdala, o córtex cerebral pré-frontal e o hipocampo (Campeau *et al*., 1998), sendo esta última estrutura uma das mais fortemente relacionadas à regulação do eixo, devido a sua alta concentração de receptores glicocorticóides. Lesões no hipocampo estão relacionadas ao desligamento defeituoso da resposta ao estresse e ao aumento do CRH e da vasopressina, com conseqüente hipersecreção de glicocorticóides. Por outro lado, a exposição prolongada a altos níveis de glicocorticóides lesa os neurônios hipocampais, reduzindo de modo permanente a sensibilidade à retroação e perpetuando o excesso de glicocorticóides e suas repercussões sobre o hipocampo (Halbe *et al*., 1996).

Diversos dados da literatura vêm apontando os efeitos neurotóxicos da exposição prolongada a glicocorticóides. De todas as estruturas cerebrais, parecem ser as camadas de células piramidais das regiões CA3 e CA4 do hipocampo as mais sensíveis a injúrias mediadas por glicocorticóides (McEwen, 1999; McKittrick, 2000). Estas injúrias podem se manifestar em diversos níveis, que incluem desde atrofia dendrítica e retração celular até morte neuronal (Watanabe *et al*., 1992; Sapolsky, 2000). Evidentemente, o nível da lesão provocada é dependente da intensidade e duração da exposição aos glicocorticóides, e aqui é importante ressaltar que as quantidades destes hormônios liberadas durante situações de estresse crônico são suficientes para causar atrofia dendrítica e perda celular (McEwen *et al*., 1999; McKittrick, 2000; Sapolsky, 2000). Este tipo de dano às células hipocampais também é observado em humanos: diversos estudos constataram que o hipocampo sofre atrofia após situações como estresse traumático, depressão recorrente e síndrome de Cushing, patologias estas que apresentam como característica comum um aumento da atividade do eixo HHA (Magariños *et al*., 1997). Indivíduos idosos também apresentam uma aumentada atividade do eixo HHA, o que pode provocar atrofia de neurônios hipocampais, e nesta linha de raciocínio foram desenvolvidos trabalhos com ratos velhos, demonstrando uma resposta exacerbada a situações de estresse agudo, justamente pela impossibilidade do hipocampo em ativar os processos de retroação negativa (Sapolsky, 1983; Smith, 1995).

Embora o dano celular nas regiões CA1 e Giro Denteado (GD) do hipocampo, mediado por altos níveis de glicocorticóides (GCs), não seja tão evidenciado quanto na região CA3, estes hormônios têm a propriedade de aumentar a vulnerabilidade destas estruturas frente a insultos induzidos por outros agentes lesivos (Stein-Behrens *et al*., 1992, 1994a e 1994b), ou também inibir o funcionamento normal dos neurônios destas regiões. Exemplos disso são a potencialização dos danos provocados na região CA1 por processos isquêmicos (Adachi *et al*., 2001; DeVries *et al*., 2001) e o aumento da vulnerabilidade destas células

frente a insultos induzidos por drogas convulsivantes, como o ácido caínico (Smith-Swintosky *et al*., 1996; Sapolsky 2000).

Os mecanismos pelos quais os GCs prejudicam as células nervosas são constante fonte de estudos, já tendo sido apontados diversos fatores que podem estar envolvidos nos processos de lesão. São eles:

a) Depleção Energética e Diminuição da Captação de Glicose

Este efeito faz parte das alterações clássicas do organismo em resposta ao estresse, i.e., a inibição da utilização de glicose por sistemas "desnecessários" na resposta de alarme. De fato, os glicocorticóides inibem em torno de 75% o transporte de glicose em células do sistema imunológico, de fibroblastos e adipócitos (Munck, 1971 apud Sapolsky,1998). Estes efeitos parecem ser dependentes de síntese protéica (Giaume *et al*., 1995): em poucos minutos os GCs estimulam a síntese de uma proteína seqüestradora de transportadores de glicose, carreando-os para sítios intracelulares ou redistribuindo-os para locais outros que não os seus sítios ativos; além disso, em questão de horas ou dias, os GCs inibem a transcrição dos genes para os transportadores de glicose, ou desestabilizam os níveis de ARNm para estes transportadores. Isto resulta, enfim, num decréscimo no transporte de glicose para o meio intracelular.

Nos últimos tempos, têm-se evidenciado estes mesmos efeitos inibitórios em estruturas do SNC, especialmente no hipocampo. Estudos indicam a possível atuação dos GCs via eventos genômicos lentos, uma vez que os insultos não são aparentes após exposição de culturas celulares a períodos de poucos minutos (Xavier, 1995; Chipkin, 1998; Sapolsky, 1998). Comparando, contudo, com os tecidos periféricos, este efeito é bem mais reduzido, localizando-se na faixa dos 25% (Sapolsky, 1992). Esta redução por si só não é suficiente para provocar perda celular, mas é capaz de exacerbar as lesões induzidas por outros insultos, bem como prejudicar mecanismos de plasticidade sináptica.

b) Toxicidade Glutamatérgica e Acúmulo de Cálcio

O glutamato, que é um dos aminoácidos protéicos, é também o principal neurotransmissor excitatório do SNC, perfazendo cerca de 60% do total de sinapses nele existentes. Altas concentrações de glutamato, contudo, podem ser deletérias às células nervosas, por provocar a denominada excitotoxicidade glutamatérgica, ou seja, uma situação de hiperestimulação destas células. Para evitar esta excitotoxicidade, existe um fino equilíbrio entre a liberação e a recaptação de glutamato: ele é liberado na fenda sináptica mediante estímulo, e rapidamente recaptado, a maior parte pelos astrócitos adjacentes, via co-transporte com sódio, por transportadores acoplados à atividade de uma bomba sódio-potassio-ATPase (Na+/K+-ATPase) (Smith *et al*., 1995; Anderson & Swanson, 2000). É lógico, portanto, que há a necessidade de uma alta concentração de sódio extracelular como coadjuvante para este co-transporte, o que não ocorre num período pós-excitatório extenso, em que o influxo neuronal de sódio é elevado (Sapolsky, 2000). Justamente neste ponto a ação dos GCs pode ser deletéria: ao diminuir a captação de glicose, diminui a fonte de substrato energético para o funcionamento das Na+/K+-ATPases, o que tende a diminuir a captação de glutamato (Reagan & McEwen, 1997). Somando-se a isto, em situações de depleção energética severa, a despolarização da membrana neuronal leva a uma maior concentração de sódio intracelular, e conseqüente efeito reverso da bomba: não só haverá falha na remoção do glutamato, como também aumento no efluxo das reservas citosólicas deste neurotransmissor (Rossi *et al*., 2000).

A elevação nos níveis de glutamato induz efeitos neurotóxicos basicamente pelo aumento dos níveis de cálcio intracelular, o que ocorre em parte pela ligação aos receptores ionotrópicos (i.e., receptor N-metil-D-aspartato – NMDA, e receptor α-amino-3-hidroxi-5-metil-4-isoxazolropionato – AMPA), permitindo a entrada de cálcio. O aumento exacerbado nos níveis intracelulares de cálcio desencadeia uma série de reações que poderão levar a

danos ou mesmo morte neuronal. Um exemplo disso é a gênese de radicais livres mediada por cálcio, o que é evidenciado pela indução de síntese de óxido nítrico, geração de xantina oxidase, ativação de fosfolipases que liberam ácido araquidônico das membranas e prejuízo da atividade mitocondrial (Sapolsky, 2000). Outro mecanismo pelo qual o cálcio pode ser lesivo é através da ativação de proteases como a calpaína, responsável pela destruição da proteína espectrina, uma das proteínas componentes do citoesqueleto (Vanderklish & Bahr, 2000). A atuação destas proteases pode ser um dos fatores responsáveis pela atrofia dendrítica observada frente a situações de estresse (Rajkowska, 2000; Souza *et al*., 2000; McEwen, 2000b).

Resumindo: o estresse estimula a liberação de glicocorticóides, que por sua vez inibem a captação de glicose em tecidos periféricos e no SNC. A diminuição de glicose, principal substrato energético para o funcionamento cerebral, leva a uma diminuição da atividade das ATPases existentes no sistema nervoso. Um grupo destas ATPases está associado a transportadores de glutamato – logo, sem combustível para seu funcionamento, haverá um acumulo de glutamato nas fendas sinápticas, o que implica também em aumento das concentrações de cálcio intracelular, tanto pela ativação de receptores glutamatérgicos ionotrópicos quanto pela mobilização das reservas intracelulares de cálcio.

Contudo, conforme citado anteriormente, apenas a diminuição na captação de glicose mediada pela hiperexposição a situações de estresse não seria suficientemente potente para provocar perda celular. Além disso, há situações em que a simples administração de GCs não é suficiente para mimetizar os efeitos do estresse crônico, o que indica que fatores encefálicos adicionais devem estar envolvidos nas alterações observadas após a exposição ao estresse (Smith, 1996; Sapolsky, 1998). Estudos correntes têm demonstrado que existem outros mecanismos envolvidos na ação deletéria do estresse crônico, como a diminuição nas defesas contra insultos neurológicos.

c) Diminuição das Defesas Neuronais

Diversos estudos têm relatado que o estresse crônico induz uma diminuição em diversos tipos de defesas neuronais. Como exemplo disso pode-se citar a marcante diminuição de enzimas antioxidantes, e.g. diminuição da atividade das enzimas Cu/Zn-superóxido dismutase e glutationa peroxidase (McIntosh & Sapolsky, 1996; McIntosh *et al*., 1998), provocada pela exposição a níveis suprafisiológicos de GCs. Outro exemplo é a diminuição na mobilização extracelular do ácido gama-aminobutírico (GABA, do inglês gamma-aminobutyric acid) e da adenosina durante insultos (Ravindran *et al*.. 1994 APUD Sapolsky, 1998), bem como a redução do potencial inibitório pós-sináptico gabaérgico, que funciona como uma resposta protetora aos insultos glutamatérgicos (McEwen, 2000b). Além disso, o estresse parece induzir alterações na síntese e eficácia de proteínas de choque térmico, como a hsp72 (hsp, do inglês Heat Shock Protein), cuja expressão é neuroprotetora em uma variedade de insultos (Sapolsky, 2000), e a hsp 27, que parece ser diferentemente modulada por situações de estresse agudo e estresse crônico (Barr & Dokas, 1999).

Os dados até aqui mencionados sugerem um efeito lesivo do estresse sobre diferentes estruturas cerebrais, especialmente estruturas do sistema límbico ou a ele relacionadas.

Esses efeitos deletérios do estresse também se manifestam em tarefas comportamentais: exposição de ratos adultos e primatas a elevadas concentrações de GCs, tais quais as observadas em situações de estresse crônico, resulta em danos cognitivos em diversas tarefas cujo desempenho está relacionado à função hipocampal, tais como exposição ao labirinto aquático (Bodnoff *et al*., 1995), ao labirinto radial de oito braços (Luine *et al*., 1993; Nishimura *et al*., 1999), ao labirinto em Y (Conrad *et al*., 1996), e ao labirinto de Barnes (McLay *et al*., 1998).

Além dos efeitos cognitivos citados acima, diversos estudos têm demonstrado um efeito ansiogênico do estresse na tarefa do labirinto em cruz elevado (File, 1996; Zurita *et al*.,

2000; Padovan *et al*., 2000), o que é evidenciado por um comportamento de "congelamento" e diminuição do número de entradas nos braços abertos. Paralelamente, a atividade exploratória na tarefa de exposição ao campo aberto também encontra-se alterada em animais cronicamente estressados (D'Aquila *et al*., 2000).

Estresse e Depressão

A depressão é uma desordem que chega a atingir um percentual de 5% da população, e pode ser classificada em dois tipos básicos: a depressão maior, ou unipolar, que é caracterizada pela anedonia e estado de constante melancolia do paciente, e o transtorno maníaco-depressivo, ou transtorno de humor bipolar, que intercala os períodos depressivos com surtos de mania, i.e., elevação do humor, irritabilidade, inquietação, etc. Cerca de 25% dos pacientes depressivos sofrem também de acessos de mania (Kandel, 1991).

A patofisiologia destes transtornos tem sido bastante estudada, e a exposição ao estresse é demonstrada como um importante fator envolvido na gênese e sustentação de estados depressivos. Não obstante, a relação entre os efeitos do estresse e da depressão é muito mais próxima. Sabe-se, por exemplo, que em grande parte dos pacientes deprimidos existe uma hiperatividade do eixo HHA e conseqüente hipersecreção de CRH e cortisol (Hatzinger, 2000; Parker *et al*., 2003), tal qual em situações de estresse. Reforçando esta idéia, 50% das pessoas que sofrem de síndrome de Cushing, a qual apresenta hipercortisolemia como característica marcante, sofrem de depressão, o que também ocorre com mais de 75% das pessoas submetidas a tratamento crônico com corticóides. Esses dados estabelecem uma forte relação entre a depressão e os níveis de glicocorticóides circulantes. E, concordando com isto, observa-se também nestas patologias uma acentuada atrofia dendrítica, especialmente nos neurônios piramidais apicais da região CA3 do hipocampo (Duman *et al*.,

1997, 1999, 2000; Sapolsky, 2000), semelhante à observada em situações de estresse. Além da atrofia dendrítica, estudos post-mortem em pacientes com transtornos de humor revelaram densidade alterada de células gliais e neuronais, bem como alterações no volume de estruturas como hipocampo e córtex pré-frontal (Rajkowska, 2000; Cotter *et al*., 2001).

Existem ainda outras semelhanças entre a patogenia da depressão e do estresse crônico. Exemplos importantes são as alterações nos sistemas neurotransmissores. De fato, outros sistemas neurotransmissores, além do glutamatérgico, encontram-se alterados após situações de estresse. O sistema serotoninérgico, por exemplo, encontra-se prejudicado em tais situações: experimentos in vitro e in vivo demonstraram que os glicocorticóides têm a propriedade de diminuir a expressão dos receptores 5HT1A pós-sinápticos, que são de especial interesse por estarem envolvidos em estados de ansiedade e depressão, em estruturas como hipocampo e córtex cerebral (Flügge, 1995; Nishi & Azmitia, 1996; Meijer *et al*., 1997; Carrasco & Van de Kar, 2003). Estes experimentos demonstraram ainda que os efeitos encontrados devem-se, aparentemente, à estimulação dos receptores mineralocorticóides, uma vez que se dão sob concentrações basais de hormônios glicocorticóides (Meijer *et al*., 1997).

Além destes, outros estudos indicam uma relação entre serotonina e os danos observados no hipocampo de animais cronicamente estressados, uma vez que o tratamento com tianeptina – uma droga estimuladora da captação de serotonina – inibe a atrofia dendrítica da região CA3 (McEwen *et al*., 1997). Postula-se ainda que a serotonina atue sinergicamente com o glutamato, interagindo pré- ou pós-sinapticamente com o glutamato liberado pelo estresse ou pelos glicocorticóides, potencializando a ligação aos receptores NMDA, bem como sua atividade (McEwen, 2000a). Curiosamente, a serotonina, juntamente com a noradrenalina, é o neurotransmissor mais fortemente relacionado à depressão, tanto que muitas drogas utilizadas como tratamento para esta patologia modulam a atividade serotoninérgica. Além disso, o aumento da função serotoninérgica dos receptores 5HT1A pós-

sinápticos parece ser requerido para a atividade da terapia antidepressiva (Duman *et al*., 1999, 2000).

Assim como se fez necessário para o estudo de outras patologias, foram desenvolvidos diversos modelos animais de depressão, que buscam mimetizar em animais similaridades nas respostas farmacológicas (i.e. validade preditiva) e comportamentais, ou sintomáticas (i.e. validade de face) observadas em pacientes deprimidos. Dentre os modelos de depressão existentes destaca-se o modelo de estresse crônico moderado como o que possui maior validade de face, preditiva e construtiva.

Paradigma de Estresse Crônico Moderado

O modelo de estresse crônico moderado (do inglês, chronic mild stress) foi desenhado a partir das observações de Katz (1982), nas quais, quando expostos durante um período prolongado de tempo a uma variedade de estressores relativamente graves, ratos reduziam seu consumo de soluções doces, o que foi sugerido como uma diminuição do impacto hedônico dessas soluções para os animais. Posteriormente, um pesquisador chamado Paul Willner propôs o modelo de estresse crônico moderado, diferindo do modelo de Katz pelo fato dos estressores utilizados serem mais amenos, objetivando, assim, proporcionar uma simulação mais realista dos estressores da vida diária (Willner, 2005). Esse modelo propõe que a exposição de ratos ou camundongos a uma variedade de estressores amenos, como períodos de restrição de água ou comida, alterações na temperatura ambiente, iluminação ambiente contínua, inclinação das caixas-moradia, mudanças nos companheiros de caixa, dentre outros estressores considerados “inócuos”, induz uma diminuição na responsividade a recompensas, tais como diminuição no consumo ou preferência por soluções de sacarose, assim como diminuição nas propriedades “recompensadoras” de uma variedade de reforços

naturais ou farmacológicos no paradigma de preferência de lugar condicionada (Muscat *et al*., 1992; Papp *et al*., 1991, 1993; Bekris *et al*., 2005). Somando-se a isto, outra característica importante desse modelo de estresse e depressão é o fato de que os comportamentos afetados (i.e. diminuição do consumo de soluções de sacarose ou incapacidade de aquisição de comportamentos motivados por alimentos palatáveis, e.g. Gambarana *et al*., 2003; Pijlman *et al*., 2003) são restaurados por fármacos utilizados na terapia antidepressiva, mas não por outras classes de medicamentos (Bekris *et al*., 2005, Willner, 2005).

Dentre os circuitos neurais envolvidos na resposta tanto ao estresse quanto a fármacos antidepressivos, o sistema dopaminérgico mesolímbico tem sido foco de muitos estudos (Cabib & Puglisi-Allegra, 1996; Di Chiara *et al*., 1999), e é de particular interesse devido a seu envolvimento em comportamentos relacionados à recompensa (D'Aquila *et al*., 2000). A dopamina desempenha um importante papel na modulção de várias manifestações comportamentais, como movimento, motivação e recompensa, e é bem estabelecido que quase todas as drogas de abuso, assim como as recompensas naturais (tais como alimentos ou soluções palatáveis) induzem ativação do sistema dopaminérgico (Berridge and Robinson, 1998; Nestler, 2002; Robinson and Berridge, 2000; Schultz, 2002; Wise, 2002).

A observação de que a dopamina é liberada no estriado ventral em resposta a recompensas naturais, bem como a drogas de abuso (Hajnal and Norgreen, 2001, 2002; Hernandez and Hoebel, 1988), corrobora com a hipótese de que a dopamina é uma mediadora da experiência prazerosa – ou hedônica – relacionada à recompensa (Wise, 1994). Consistente com isso, estudos demonstraram que ratos submetidos a um modelo de estresse crônico moderado apresentam diminuição na liberação basal de dopamina (Gambarana *et al*., 2003), assim como uma diminuição na resposta dopaminérgica após consumo de um alimento palatável no córtex pré-frontal e no núcleo accumbens. Isto sugere que esse modelo de estresse afeta tanto a liberação fásica quanto tônica de dopamina (Di Chiara & Tanda, 1997),

efeito este que possivelmente está envolvido na anedonia observada em animais (Willner, 2005) e em pacientes deprimidos (Di Chiara *et al*., 1999).

Lítio

O lítio (Li+) foi introduzido na terapêutica por Alexander Ure por volta de 1840, primeiramente para o tratamento da gota, que ele acreditava estar relacionada aos sintomas afetivos de mania e depressão. Foi somente em 1880, com os estudos de dois médicos americanos chamados John Aulde e Carl Lange, que o Li+ passou a ser utilizado para o tratamento da depressão, independentemente da gota. Contudo, após alguns anos de uso indiscriminado em tônicos ou mesmo como substituto do sal, o conseqüente alto índice de toxicidade fez com que este sal entrasse em desuso (Lenox *et al*., 1998).

Em 1949, John Cade, um psiquiatra australiano, publicou um estudo sobre o efeito do Li+ no tratamento da fase maníaca do transtorno de humor bipolar (THB), o que abriu novamente as portas da terapêutica para este sal. Em 1952, Mogens Schou reinvestigou a ação do Li+, comprovando a eficiência do mesmo para aplacar a agitação maníaca. O efeito profilático do Li+ sobre o THB foi descrito entre 1959-1960, simultânea e independentemente, por um psiquiatra inglês, G.P. Hartigan, e um psiquiatra dinamarquês, P.C. Baastrup, e vários ensaios clínicos confirmaram a eficácia do Li+ em prevenir tanto os episódios de mania como os de depressão no THB. A partir de 1969 (quando foi aprovado para uso clínico nos EUA), o Li+ começou a ser largamente utilizado no mundo inteiro para o tratamento e profilaxia da PMD (Schou, 1980). Estima-se que cerca de 0,1% da população mundial faça tratamento com Li+, que atualmente é também utilizado como adjuvante no tratamento da depressão maior, além de ser a droga de escolha para o tratamento do distúrbio bipolar (Manji *et al*., 1995; Schou *et al*.; 1998).

Os mecanismos de ação deste cátion monovalente são fontes de constantes estudos, o que é compreensível: nos últimos anos, têm sido descobertas muitas vias pelas quais se dá a sua ação. Sabe-se, por exemplo, que o Li+ atua sobre a maioria dos mecanismos de transdução de sinal (Manji *et al*., 1995, 2000a; Jope, 1999) dentre os quais pode-se dar uma ênfase especial ao ciclo do fosfatidilinositol: o Li+ inibe a reciclagem dos fosfatos de inositol através da inibição da enzima inositol monofosfatase, o que tem como conseqüência o acúmulo de diacilglicerol e inositol-trifosfato. Os níveis elevados de diacilglicerol, por sua vez, levam à ativação de proteínas cinases C (PKC), que são responsáveis por mediar as alterações de longo prazo nas funções celulares, alterando inclusive a expressão gênica (Berridge *et al*., 1989; Lenox *et al*., 1998; Shaldubina *et al*., 2001).

Outro possível mecanismo de ação do Li+ é a modulação de sistemas neurotransmissores (Lenox *et al*., 1998). Exemplos disto são os efeitos inibitórios do Li+ sobre os auto-receptores serotoninérgicos pré-sinápticos, os quais têm como função diminuir a disponibilidade de serotonina por mecanismos de retroação negativa (Shaldubina *et al*., 2001). Inibindo estes auto-receptores, haverá uma maior disponibilidade de serotonina, bem como aumento das quantidades liberadas por impulso (Lenox *et al*., 1998), o que, postula-se, seja responsável pela sua eficácia em acelerar os efeitos da terapia antidepressiva em pacientes com depressão maior.

Postula-se que o tratamento com lítio corrige o desbalanço nos sistemas neurotransmissores que contribuem para a patogenia de distúrbios depressivos, e evidências clínicas sugerem que este desbalanço se deve em parte a uma excessiva atividade dopaminérgica e reduzida atividade colinérgica (Basselin *et al*. 2005; Bymaster and Felder, 2002, Ichikawa *et al*., 2005). Paralelamente, foi observado que o Li+ protege neurônios estriatais em um modelo de Parkinson, em que há uma depleção de dopamina nos terminais

nervosos (Youdim and Arraf, 2004), sugerindo que este sal atue na modulação da atividade dopaminérgica.

A atividade glutamatérgica também parece ser influenciada pelo tratamento com lítio. Por exemplo, estudos demonstraram que o lítio administrado agudamente inibe a captação de glutamato em terminais pré-sinápticos de córtex cerebral de camundongos, tendo efeito contrário – i.e., estimulando a captação de glutamato – quando o tratamento é aplicado cronicamente (Dixon & Hokin, 1998). Por outro lado, Nonaka *et al*. (1998) demonstraram que o tratamento com lítio protege neurônios de culturas cerebelares contra a excitotoxicidade glutamatérgica, através da inibição do influxo de cálcio mediado por receptores NMDA, ao passo que estudos mais recentes indicam que o lítio modula a expressão de subunidades do receptor glutamatérgico do tipo AMPA (Du *et al*., 2004). Estes dados sugerem a modulação de receptores glutamatérgicos como outro possível mecanismo de ação deste sal.

Nos últimos anos tem-se dado uma atenção especial aos possíveis efeitos neuroprotetores do Li+ especialmente em função da descoberta de seus efeitos reguladores sobre proteínas pró- e anti-apoptóticas (Manji *et al*., 2000[a]). Estudos recentes têm demonstrado que o tratamento crônico com Li+ provoca aumento nos níveis da proteína citoprotetora bcl-2 em estruturas como hipocampo, córtex frontal e estriado (Chen *et al*., 1999; Manji *et al*., 1999, 2000b; Chen & Chuang, 1999). Essa proteína expressa no sistema nervoso de mamíferos localiza-se em diversas estruturas celulares, como a membrana mitocondrial externa, o retículo endoplasmático e a membrana nuclear, o que lhe permite atuar de maneira protetora nos mais diversos níveis, pela ação antioxidante, inibição dos efeitos da ação das caspases, inibição da liberação de cálcio e recaptação do mesmo, etc. Sabe-se que estas proteínas atuam inibindo tanto a morte celular apoptótica quanto a necrose celular, e estudos mais recentes apontam as bcl-2 como promotoras de regeneração celular.

Paralelamente, estudos têm demonstrado o efeito inibidor do tratamento com Li+ sobre a expressão da proteína pró-apoptótica p53 em células granulares do cerebelo bem como em células de neuroblastoma humano (Lu *et al*., 1999; Chen & Chuang, 1999). Além disso, o Li+ regula negativamente a expressão e atividade da proteína glicogênio sintase cinase-3β (GSK-3β). A inibição da atividade desta enzima pelo tratamento com lítio pode se dar tanto diretamente, através de fosforilação de seu sítio de magnésio, quanto indiretamente pela ativação da via AKt/PKB, e estes efeitos parecem ser um dos alvos cruciais na terapia com sais de lítio. A GSK-3β está diretamente relacionada à regulação dos níveis das proteínas tau e β-catenina fosforiladas, que estão envolvidas, por exemplo, na neurodegeneração observada na doença de Alzheimer, bem como na desestruturação do citoesqueleto de células nervosas mediante processos de lesão que desencadeiam cascatas apoptóticas (Lovestone *et al*., 1998; Manji *et al*., 1999; Muñoz-Montaño *et al*., 1999; Grimes & Jope, 2001; Mora *et al*., 2001).

Acredita-se que a soma destes mecanismos seja responsável pelos efeitos neuroprotetores do Li+ em diferentes modelos de patologias do sistema nervoso, que têm sido apontados por diversos estudos in vitro e in vivo. Nonaka e Chuang (1998) demonstraram o efeito neuroprotetor do Li+ em ratos submetidos à isquemia cerebral focal, e este efeito neuroprotetor sobre células submetidas à isquemia também foi demonstrado in vitro por Cimarosti *et al*. (2001), através da utilização de cultura organotípica hipocampal. Outros estudos, realizados por Volonte *et al*. (1994), demonstraram que o tratamento de culturas primárias de neurônios cerebelares e corticais com Li+ promove a sobrevivência de neurônios GABAérgicos (é interessante aqui relembrar o prejuízo observado na neurotransmissão GABAérgica mediante estados depressivos, conforme mencionado anteriormente). Além disso, o tratamento crônico de ratos com Li+ também tem a propriedade de aumentar a expressão do fator neurotrófico derivado do encéfalo (BDNF, o qual se encontra reduzido em

situações de estresse e na depressão) em estruturas como o hipocampo e os córtices temporal e frontal (Fukumoto *et al*., 2001).

Por fim, convém salientar que o efeito neuroprotetor do Li+ também é observado em cérebro de humanos in vivo: Moore *et al*., (2000b) demonstraram, por ressonância magnética tri-dimensional, que o tratamento de pacientes bipolares com Li+ aumenta o volume total da massa cinzenta encefálica. Estes mesmos autores apontaram, por espectroscopia magnética por ressonância de prótons, que o tratamento com Li+ aumenta a concentração de N-acetil-aspartato, um marcador de viabilidade e funcionamento neuronal, em diversas estruturas cerebrais humanas (Moore *et al*., 2000a). Estes dados são de extrema importância por demonstrarem a aplicabilidade e os efeitos terapêuticos do Li+ na clínica.

Estresse X Lítio

Em nosso laboratório, foi estabelecido um modelo de estresse crônico variado (ECV, Gamaro, 1998; Manoli *et al*., 2000) baseado em modelos pré-existentes de estresse e depressão (Willner *et al*.; 1987; Echandía *et al*., 1988; Konarska *et al*., 1990; Papp *et al*., 1991; Willner & Muscat, 1991; Murua & Molina, 1992; Jordan *et al*., 1994; Gamaro *et al*., 2003), que apresenta efeitos sobre diversos parâmetros bioquímicos e comportamentais. Por exemplo, demonstraram-se alterações de comportamento alimentar, com diminuição da ingestão de alimento doce por animais cronicamente estressados, e alterações na nocicepção, em que os animais submetidos ao estresse crônico responderam com hiperalgesia (Gamaro *et al*., 1998).

Este modelo de estresse serviu de base para o estabelecimento de um tratamento conjunto com estresse crônico variável (com algumas alterações em relação ao de Gamaro) e lítio: durante um período de 40 dias, animais foram submetidos aos dois tratamentos (para

melhor compreensão do modelo de estresse, ver anexo 1 – cronograma básico do modelo de estresse crônico variado), e ao final deste período, foram realizadas diferentes tarefas comportamentais para avaliação de memória, atividade locomotora e nocicepção. Dos resultados obtidos, os que mais nos chamaram a atenção foram os referentes ao labirinto aquático de Morris. Os efeitos lesivos do estresse sobre a memória espacial, que têm sido demonstrados por diversos autores (Bodnoff *et al*., 1995; Conrad *et al*., 1996; McLay *et al*., 1998; Nishimura *et al*., 1999), também foram observados, sendo denotados pelo aumento da latência para achar o local exato da plataforma, diminuição no número de cruzamentos sobre este local, e diminuição da razão entre o tempo gasto no quadrante alvo e o tempo gasto no quadrante oposto. Somando-se a estes resultados, foi observada a eficiência da administração crônica de lítio na prevenção dos efeitos do estresse, bem como aumento do número de cruzamentos pelos animais tratados com este sal e não submetidos ao estresse. Houve também um efeito marginalmente significativo do lítio sobre a latência para encontrar a plataforma, o que poderia indicar que o tratamento com este sal possa ter um efeito facilitador sobre a memória (Vasconcellos *et al*., 2003)

Embora trabalhos anteriores tenham demonstrado uma diminuição da atividade exploratória em animais cronicamente estressados (Garcia-Marquez & Armario, 1987), acreditamos que esta não tenha sido a causa dos resultados aqui citados, uma vez que o desempenho dos animais no campo aberto, uma das outras tarefas avaliadas, foi semelhante em todos os grupos. Além disso, os animais apresentaram desempenho adequado tanto nas sessões treino para a memória de referência quanto na memória de trabalho, o que não ocorreria se houvesse um déficit motor.

Posteriormente, foram realizadas medidas de imunoconteúdo das proteínas neuro-gliais beta-tubulina III e proteína glial fibrilar ácida (GFAP) – a primeira específica de

neurônios e a segunda específica de astrócitos – em animais submetidos aos tratamentos com estresse crônico variável e lítio.

A beta tubulina III é uma das tubulinas mais especializadas, especificamente usada para marcar nerônios. Estas proteínas formam a estrutura do corpo celular de neurônios, bem como dos seus dendritos, e o aumento de sua expressão está relacionado com o desenvolvimento e a regeneração celular, também podendo significar sobrevivência neuronal (Kimonides *et al*., 1999), uma vez que seu imunoconteúdo encontra-se diminuído após alguns processos de lesão. Devido à especificidade destas proteínas como marcadoras neuronais, alterações na quantidade das mesmas poderiam refletir plasticidade neural. Os dados que obtivemos com a imunodetecção de beta-tubulina III não apontaram diferenças entre os grupos nas regiões analisadas, a exceção do córtex, onde observamos uma diferença restrita ao hemisfério direito, com todos os grupos apresentando valores diminuídos quando comparados aos controles.

Estudos de contagem de células realizados anteriormente, com animais submetidos ao mesmo tratamento com Li+, mas por um período de 30 dias, demonstraram não haver diferenças no número de neurônios em córtex e hipocampo, quando avaliado pelas técnicas de Nissl e Hematoxilina-Eosina (Gehlen, 2002). Estes resultados concordam com os encontrados em nosso trabalho, uma vez que níveis inalterados de beta-tubulina poderiam ser interpretados como resultantes de também inalteradas densidades neuronais. Deve-se ainda salientar que a ausência de mudanças no conteúdo de beta-tubulina III nas estruturas analisadas não exclui a possibilidade de alterações neuronais mais especificamente relacionadas com as sinapses envolvidas nas respostas aos tratamentos com estresse e lítio, o que impõe a necessidade de pesquisas mais aprofundadas para compreender os mecanismos de injúria (e recuperação) destes tratamentos.

A GFAP, por sua vez, é uma proteína marcadora de astrócitos maduros e também da reatividade astrocitária, sugerindo-se que ela pode ser necessária para a formação de processos gliais estáveis em resposta a estímulos neuronais (Eddleston & Mucke, 1993). Além disso, sabe-se que os astrócitos desempenham um importante papel na "reorganização e plasticidade" morfológica no encéfalo de ratos adultos, através de uma gliose reativa que ocorre em resposta aos mais diversos insultos, como lesões mecânicas, químicas, e alguns modelos de isquemia, assim como processos fisiológicos, tais como a lactação e a exploração de ambientes ricos (Calvo *et al*., 1991; Norenberg, 1994; Louw *et al*., 1998). Trabalhos anteriores demonstraram que o tratamento crônico com lítio induz uma astrogliose reativa em hipocampo de ratos (Rocha e Rodnight, 1994; Rocha *et al*., 1998), e uma das hipóteses iniciais de nosso trabalho era a de que o lítio pudesse auxiliar nos processos cognitivos, bem como prevenir os danos mediados pelo estresse, por induzir uma astrogliose benéfica no hipocampo dos animais submetidos ao tratamento crônico, o que aumentaria a superfície de contato entre as células gliais e os neurônios, minimizando, assim, os postulados efeitos neurotóxicos do excesso de glutamato presente em situações de estresse através do aumento da captação glial deste neurotransmissor.

Os dados obtidos demonstraram diferentes tendências a aumento na GFAP imunodetectável em três estruturas cerebrais: no córtex, este aumento foi induzido unicamente pelo tratamento com lítio. No giro denteado, por sua vez, houve uma tendência a aumento em função do estresse. Já na amígdala, tanto o estresse quanto o lítio influenciaram a expressão desta proteína. Aparentemente, contudo, os efeitos do lítio sobre a memória espacial não foram mediados por astrogliose, uma vez que as estruturas cerebrais classicamente envolvidas com o aprendizado (i.e., as regiões do hipocampo) não apresentaram diferenças no imunoconteúdo de GFAP, podendo-se supor com isto que o efeito protetor do lítio se dê por outros mecanismos.

Embora representando uma medida indireta do número de neurônios e células gliais, os resultados obtidos com a avaliação das proteínas beta-tubulina III e GFAP permitiram postular que os efeitos do tratamento com lítio sobre a memória espacial de animais cronicamente estressados sejam mediados mais por alterações metabólicas e plásticas do que propriamente por prevenção de perda neuronal ou alteração na densidade de astrócitos. Para buscar uma melhor compreensão desta inferência, se tornou necessária uma investigação de possíveis alterações no funcionamento de proteínas presentes no sistema nervoso, relacionadas com os fenômenos abordados acima, bem como na atividade de diferentes sistemas neurotransmissores, e das implicações comportamentais mediadas por estas alterações.

OBJETIVOS

Conforme já mencionado, em trabalhos anteriores foi demonstrado que o tratamento crônico com lítio previne alguns dos efeitos comportamentais desencadeados pela exposição ao estresse crônico variado (ECV) por um período de 40 dias, dentre os quais se destaca a prevenção do déficit cognitivo avaliado no Labirinto Aquático de Morris.

Na tentativa de elucidar alguns dos possíveis mecanismos envolvidos nestes efeitos, o objetivo geral desta tese de doutorado foi avaliar os efeitos dos tratamentos com estresse e lítio sobre diferentes parâmetros neuroquímicos envolvidos em mecanismos cognitivos e/ou em mecanismos de lesão celular. Para tanto foram traçados os seguintes objetivos específicos:

1. Avaliar o efeito de períodos mais curtos de ECV (21 e 30 dias) sobre a memória espacial, bem como determinar a partir de que período de tratamento os efeitos protetores do lítio se manifestam;
2. Verificar o efeito do tratamento com lítio sobre a memória espacial de animais cronicamente estressados após a exposição ao estresse crônico;
3. Verificar a atividade da enzima Na+-K+ ATPase em hipocampo de animais submetidos aos tratamentos com estresse e lítio, bem como após a interrupção do ECV, e em animais cujo tratamento com lítio foi iniciado após quarenta dias de exposição ao estresse;
4. Estudar a atividade glutamatérgica em hipocampo de animais submetidos aos tratamentos citados acima, através da avaliação da captação e liberação de glutamato por sinaptossomas bem como a liberação de glutamato por fatias de hipocampo;
5. Avaliar a vulnerabilidade celular de fatias de hipocampo de animais tratados com estresse e lítio quando estas são submetidas à privação de oxigênio e glicose;
6. Verificar o estresse oxidativo no cérebro dos animais submetidos aos tratamentos com estresse e lítio.

Considerando ainda que o modelo de estresse em questão é também considerado um modelo de depressão, que o lítio é um reconhecido agente auxiliar na terapia antidepressiva, e que uma das características marcantes da depressão é anedonia, objetivamos também avaliar os efeitos do ECV e do lítio sobre a resposta hedônica dos animais a estímulos reconhecidamente apetitivos, bem como indiretamente avaliar a atividade dopaminérgica em animais submetidos a tais tratamentos. Assim sendo, também foram objetivos desta tese:

7. Avaliar o comportamento alimentar de animas submetidos aos tratamentos com estresse e lítio;
8. Avaliar a resposta nociceptiva dos animais submetidos aos tratamentos citados acima quando expostos a sabores agradáveis e desagradáveis;
9. Avaliar o comportamento dos animais em tarefas que envolvem atividade dopaminérgica, como condicionamento apetitivo e atividade motora induzida por análogos à anfetamina, e avaliar o conteúdo total de dopamina e de seus metabólitos no núcleo accumbens de ratos estressados e tratados com lítio.

TRABALHOS QUE COMPÕEM ESTA TESE

Esta tese está composta por seis artigos científicos. Os três primeiros estão relacionados à investigação dos mecanismos através dos quais o tratamento com lítio previne os efeitos do estresse crônico variável sobre a memória espacial, e os demais artigos referem-se ao estudo dos efeitos dos tratamentos com estresse e lítio sobre o comportamento alimentar, bem como uma possível implicação de alterações no sistema dopaminérgico nestes efeitos. Por fim, estão expostos alguns resultados que não foram incluídos nos artigos submetidos à publicação, referentes à avaliação da curva curso-temporal dos efeitos do estresse crônico variável e do lítio sobre a memória espacial avaliada no Labirinto Aquático de Morris.

No **artigo 1**, os objetivos foram 1) avaliar a atividade da enzima Na^+, K^+-ATPase em hipocampo de animais estressados e tratados com lítio, e 2) avaliar o efeito da interrupção do estresse, bem como da administração de lítio após quarenta dias de estresse (período necessário para que os déficits na memória espacial se manifestem) sobre a memória espacial e sobre a atividade da Na^+, K^+-ATPase no hipocampo. Foi observado que o estresse crônico provoca uma diminuição na atividade desta enzima, a qual é prevenida pelo tratamento com lítio, embora o lítio não induza *per se* alguma alteração na atividade enzimática. Além disso, tanto a interrupção do estresse quanto o tratamento com lítio após os quarenta dias de estresse revertem os déficits observados na memória espacial, bem como restauram a atividade da enzima Na^+, K^+-ATPase a níveis normais. Com estes resultados, foi possível inferir que os efeitos do estresse crônico variável sobre a memória são reversíveis, provavelmente oriundos de alterações na plasticidade sináptica e não de perda neuronal, e que a modulação da atividade da Na^+, K^+-ATPase pode estar envolvida nos efeitos do tratamento com lítio sobre a memória espacial de animais cronicamente estressados.

O **artigo 2** desta tese trata da investigação dos efeitos dos tratamentos crônicos com estresse e lítio sobre a atividade glutamatérgica hipocampal, avaliada pela captação e liberação de glutamato por sinaptossomas, bem como pela captação de glutamato em fatias hipocampais. Além disso, foi avaliada a vulnerabilidade celular de fatias hipocampais destes mesmos animais à privação de oxigênio e glicose. O lítio induziu um aumento na captação e na liberação induzida de glutamato por sinaptossomas de hipocampo, efeito este que pode estar envolvido com plasticidade sináptica, ao passo que o estresse provocou um aumento na liberação basal deste neurotransmissor, bem como diminuiu a captação de glutamato por fatias hipocampais. Adicionalmente, foi verificado que ambos os tratamentos aumentam a vulnerabilidade celular à privação de oxigênio e glicose, medida pela liberação da enzima lactato desidrogenase, embora somente em animais estressados ela esteja aumentada antes da exposição à privação. Estes resultados indicam um possível envolvimento da transmissão glutamatérgica nos efeito do lítio sobre a memória espacial, e também o envolvimento de excitoxicidade glutamatérgica na aumentada vulnerabilidade celular observada após os tratamentos com estresse e lítio.

O **artigo 3** inclui os resultados obtidos na avaliação do estresse oxidativo em córtex frontal, hipotálamo e hipocampo de animais estressados e tratados com lítio. Neste estudo, o córtex frontal não foi afetado, ao passo que no hipotálamo foi observada uma aumentada reatividade antioxidante total, juntamente com uma aumentada atividade da enzima Superóxido Dismutase (SOD), em animais tratados com lítio. No hipocampo, houve um aumento na peroxidação de lipídeos em ratos estressados, uma diminuição na formação de radicais livres em ratos tratados com lítio, e aumento na reatividade antioxidante total em animais estressados e tratados com lítio. A atividade da enzima Glutationa Peroxidase encontrou-se aumentada em hipocampo de animais tratados com lítio, bem como a SOD em

animais estressados. Adicionalmente, foi observada uma marcante interação entre os tratamentos com estresse e lítio na reatividade antioxidante total em hipocampo (que foi diminuída pela administração concomitante dos tratamentos), bem como na atividade da SOD tanto no hipocampo quanto no hipotálamo. Estes resultados indicam que o tratamento com lítio possui algumas propriedades antioxidantes, mas que não foram eficientes para prevenir o dano oxidativo em hipocampo de animais estressados.

No **artigo 4** estão descritos os efeitos dos tratamentos com estresse e lítio sobre o comportamento alimentar e sobre a ansiedade (avaliada no Labirinto em Cruz Elevado) de ratos. Foi observado que os tratamentos com estresse e lítio induzem um aumento no consumo de alimentos doces (Froot Loops da Kellogg's ®) quando comparados ao consumo de animais controle, mas que somente o tratamento com lítio aumenta o consumo de alimentos salgados (amendoim salgado). Nenhum dos tratamentos alterou o consumo de ração padrão, sugerindo uma preferência por alimentos mais palatáveis. Adicionalmente, foi observado um forte efeito ansiolítico do tratamento com lítio, evidenciado pelo aumento no tempo gasto nos braços abertos, bem como no número de entradas, no Labirinto em Cruz Elevado, e não houve efeito do estresse neste parâmetro. Estes dados sugerem que o aumento no consumo de doce observado em animais estressados não seja devido a uma maior ansiedade dos animais, e que o aumento na preferência por alimentos mais calóricos contribua para o ganho de peso observado em pacientes tratados com lítio.

No **artigo 5** foi verificado se os tratamentos com estresse e lítio são capazes de alterar a resposta nociceptiva dos animais após exposição a diferentes sabores. Esta avaliação foi feita através da medida da latência para retirada da cauda (Tail Flick), e se observou que a exposição ao estresse crônico inibe a antinocicepção induzida por um sabor doce agradável

(leite condensado), aumentando apenas a nocicepção em animais submetidos a um sabor desagradável (ácido acético). Por sua vez, o tratamento com lítio aumenta a nocicepção dos animais tanto após exposição ao sabor agradável quanto ao aversivo. Pode-se inferir que a ausência de analgesia induzida por um sabor doce e prazeroso em animais estressados representa um efeito anedônico do tratamento com estresse crônico variável, e que a percepção de diferentes sabores seja mais proeminente em animais tratados com lítio.

No **artigo 6**, o objetivo foi avaliar a neurotransmissão dopaminérgica em animais estressados e tratados com lítio através de tarefas comportamentais cujo desempenho envolve a participação deste neurotransmissor. Foram avaliadas a Preferência de Lugar Condicionada e a atividade locomotora induzida por dietilpropiona, bem como o conteúdo total de dopamina e de seus metabólitos no núcleo accumbens destes animais. Os resultados demonstraram que animais tratados com lítio apresentam aumento na atividade locomotora após administração de dietilpropiona quando comparados aos demais grupos, bem como desenvolvem preferência de lugar condicionada por um estímulo apetitivo, indicando um aumento no tônus dopaminérgico; por outro lado, animais estressados não desenvolvem condicionamento apetitivo. Ambos os tratamentos induziram uma diminuição nos níveis de DOPAC no núcleo accumbens, mas somente o estresse induziu diminuição da razão DOPAC/dopamina. Estes dados sugerem que o tratamento com lítio modula a atividade dopaminérgica, o que pode fazer parte do seu repertório de mecanismos de ação, e que alguns dos efeitos comportamentais do modelo de estresse utilizado, o qual é referido como um modelo de depressão, devam-se a uma atividade dopaminérgica diminuída no núcleo accumbens.

Artigo 1:

"Na^+,K^+-ATPase ACTIVITY IS REDUCED IN HIPPOCAMPUS OF RATS SUBMITTED TO AN EXPERIMENTAL MODEL OF DEPRESSION: EFFECT OF CHRONIC LITHIUM TREATMENT AND POSSIBLE INVOLVEMENT IN LEARNING DEFICITS"

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Na^+,K^+-ATPase activity is reduced in hippocampus of rats submitted to an experimental model of depression: Effect of chronic lithium treatment and possible involvement in learning deficits

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Abstract

This study was undertaken to verify the effects of chronic stress and lithium treatments on the hippocampal Na^+,K^+-ATPase activity of rats, as well as to investigate the effects of stress interruption and post-stress lithium treatment on this enzyme activity and on spatial memory. Two experiments were carried out; in the first experiment, adult male Wistar rats were divided into two groups: control and submitted to a chronic variate stress paradigm, and subdivided into treated or not with LiCl. After 40 days of treatment, rats were killed, and Na^+,K^+-ATPase activity was determined. In the second experiment, rats were stressed during 40 days, and their performance was evaluated in the Water Maze task. The stressed group was then subdivided into four groups, with continued or interrupted stress treatment and treated or not with lithium for 30 additional days. After a second evaluation of performance in the Water Maze, rats were killed and Na^+,K^+-ATPase activity was also measured. Results showed an impairment in Na^+,K^+-ATPase activity and in Water Maze performance of chronically stressed rats, which were prevented by lithium treatment and reversed by lithium treatment and by stress interruption. These results suggest that the modulation of Na^+,K^+-ATPase activity may be one of the mechanisms of action of lithium in the treatment of mood disorders.

Keywords: Chronic variate stress; Lithium; Na^+,K^+-ATPase; Hippocampus; Depression; Spatial memory; Water Maze

1. 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. It is present in high concentrations in brain cellular membranes, consuming about 40–50% of the ATP generated in this tissue (Erecinska & Silver, 1994).

The pathophysiology of some psychiatric disorders is believed to be associated with some perturbation of ion homeostasis, and earlier studies have shown that Na^+,K^+-ATPase activity is decreased in patients with depression and other psychiatric disorders (Hokin-Neaverson & Jefferson, 1989; Mynett-Johnson et al., 1998; Rybakowsky, Potok, Strzizewski, & Nowakowska, 1984; Wood et al., 1991). Nevertheless, little is known about this enzyme activity in experimental models of

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depression in animals, although we have previously observed a decreased Na^+,K^+-ATPase activity in the hippocampus of animals submitted to chronic mild stress (Gamaro, Manoli, Torres, Silveira, & Dalmaz, 2003). Exposure to chronic mild stress has been proposed as a model of depression in animal studies (Katz, 1981;Pucilowski, Overstreet, Rezvani, & Janowsky, 1993; Willner, 1990, 1991), and this effect was accompanied by anhedonic behavior, a characteristic of depressive states.

Additionally, we have shown that animals submitted to chronic variate stress present a cognitive deficit in spatial memory, as evaluated by the Morris Water Maze task (Vasconcellos, Tabajara, Ferrari, Rocha, & Dalmaz, 2003). Some authors believe that the impairing effects of chronic stress could result from a disruption in brain energy metabolism (Hoyer, Lannert, Latteier, & Meisel, 2004; Sadowski et al., 2004). This energy deficit could, consequently, affect Na^+,K^+-ATPase activity. Furthermore, a set of studies have suggested the involvement of Na^+,K^+-ATPase activity in memory consolidation (Sato et al., 2004; Wyse et al., 2004).

Lithium salts are widely used for the treatment of affective disorders, and increasing evidence supports the notion that lithium has neuroprotective effects in a variety of insults (Chen & Chuang, 1999; Jope, 1999; Manji, Moore, & Chen, 1999). We observed previously that the memory impairments in rats caused by exposure to chronic stress was prevented by concomitant lithium treatment (Vasconcellos et al., 2003). Although the mechanism of action of lithium remains unclear, it is already known that this cation is able to normalize erythrocyte Na^+,K^+-ATPase activity in patients with mood disorders (Hokin-Neaverson & Jefferson, 1989). Moreover, lithium prevents or delays neurochemical and behavioral effects elicited by ouabain, an inhibitor of Na^+,K^+-ATPase activity (El-Mallakh, Schurr, Payne, & Li, 2000; Hennion, El-Masri, Huff, & El-Mallakh, 2002; Li, el-Mallakh, Harrison, Changaris, & Levy, 1997), suggesting a possible modulation of Na^+,K^+-ATPase activity by this cation.

The aim of the present study was to verify the effect of an animal model of depression (chronic variate stress) on Na^+,K^+-ATPase activity in synaptic plasma membranes from rat hippocampus, and the action of lithium treatment on such effect. In addition, we also evaluated the effects of stress interruption on enzymatic activity and on spatial memory, as well as the effects of administration of lithium after repeated stress.

2. Experimental procedures

2.1. Animals

One hundred adult male Wistar rats (60 days old; 180–230 g in weight) were used. The experimentally naive animals were housed in groups of 4 or 5 in homecages made of Plexiglas material (65 × 25 × 15 cm) with the floor covered with sawdust. Animals were maintained under a standard dark-light cycle (lights on between 7:00 and 19:00 h) in a room temperature of 22 ± 2 °C. The rats had free access to food and water. All animal treatments were in accordance with the institutional guidelines and according to the recommendations of the International Council for Laboratory Animal Science (ICLAS), and all efforts were made to reduce the number of animals. Animals were further divided in subgroups, control and stressed, and receiving or not lithium treatment; see Table 1 for group divisions and experimental design.

2.2. Chronic variate stress model

Chronic variate stress was modified from other models of variate stress (Gamaro et al., 2003; Konarska, Stewart, & McCarty, 1990;Murua & Molina, 1992; Willner, 1990, 1991). The animals were divided into two groups: group 1 (control), that was kept undisturbed in their home cages during the first 40 days of treatment, and group 2 (chronically stressed). A chronic variate-stressor paradigm was used for the animals in the stressed group. The following stressors were used: (a) inclination of the home cages at a 45° angle for 4–6 h, (b) 10–15 min of noise, (c) 1–3 h of restraint, as described below, (d) 1.5–2 h of restraint at 4 °C, (e) forced swimming for 10 or 15 min, as described below, (f) flashing light during 2 to 4 h, and (g) isolation (2–3 days). Animals were exposed to stress starting at a different time everyday, in order to minimize its predictability. We have previously measured plasma corticosterone levels after this chronic stress model, and corticosterone basal levels (i.e., levels at the 40th day of exposure to stress, before being exposed to a stressor) were not different from control animals. After being exposed to a stressor, at the 40th day of treatment, corticosterone levels increased around 90%.

Restraint was carried out by placing the animal in a 25 × 7 cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. Forced swimming was carried out by placing the animal in a glass tank measuring 50 × 47 × 40 cm with 30 cm of water at 23 ± 2 °C. Exposure to flashing light was achieved by placing the animal in a 50 cm-high, 40 × 60 cm open field made of brown plywood with a frontal glass wall. A 100 W lamp, flashing in a frequency of 60 flashes per minute, was used.

2.3. Chronic lithium treatment

Lithium was administered through the chow. Lithium chloride (LiCl—2.5 mg/g of chow) and sodium

Table 1
Groups and experimental design for experiments 1 and 2

	Groups			Treatment duration			Experiment	
Experiment 1 20 male Wistar rats	Control (n = 5) Lithium treated (n = 5) Chronically stressed (n = 5) Stressed and treated with lithium (n = 5)			40 days of treatment			Evaluation of Na^+,K^+-ATPase activity	
	Group	Treatment duration	Experiment	Subdivision of the groups	Treatment duration	Experiment	Lithium and stress treatment continued	Experiment
Experiment 2 85 male Wistar rats	Control n = 25 Stress n = 60	40 days of treatment	First exposure to the Water Maze task	Control Control + lithium Stress continued Stress continued + lithium Stress interrupted + lithium Stress interruption	30 additional days of treatment	Second exposure to the Water Maze task	One week of interval	Evaluation of Na^+, K^+-ATPase activity

Experiment 1: rats were treated for 40 days with the procedures described under Section 2, constituting four experimental groups: control (1), lithium treated (2), chronically stressed (3), and chronically stressed plus lithium treatment (4).
Experiment 2: rats were treated for 40 days with the procedures described under Section 2, constituting two experimental groups: control and chronically stressed. At the end of this period, these animals were submitted to the first exposure to the Water Maze task. Afterwards, the control group was divided in two other groups: control (1) and lithium treatment (2), and the stressed group was subdivided in four other groups: stress continued (3), stress continued plus lithium (4), stress interrupted plus lithium (5), and stress interrupted (6). The animals were treated during 30 additional days, and then submitted to a second exposure to the Water Maze task. One week after Water Maze procedures, and 24 h after the last stress exposure, the animals were killed by decapitation and brains were removed for enzymatic measurements.

chloride (NaCl—17 mg/g) were added to the food, as described by Rocha and Rodnight (1994). This treatment has been previously used, and at the end of a period of 4 weeks or more animals present lithium levels in the range of 0.6–1.2 mM (Rocha & Rodnight, 1994; Vasconcellos et al., 2003), similar to the levels observed in treated patients.

2.4. Water Maze apparatus and procedures

This task was adapted from the paradigm originally described by Morris (1984). The Water Maze was a black circular pool (180 cm diameter, 60 cm high), filled with water (depth 30 cm; 24 ± 1 °C), placed in a room that was rich in consistently located spatial cues (including a large wood door, two prominent posters on one wall, and the experimenter). An escape platform (10 cm diameter) was placed in the middle of one of the quadrants, 1.5 cm below the water surface, equidistant from the sidewall and middle of the pool. The platform provided the only escape from the water and was located in the same quadrant in every trial. The position of the animal in the pool was recorded during the entire experiment. Four different starting positions were equally spaced around the perimeter of the pool. On each of the training days, all four start positions were used once in a random sequence (i.e. four training trials per day). A trial began by placing the animal in the water facing the wall of the pool at one of the starting points. If the animal failed to escape within 60 s, it was gently conducted to the platform by the experimenter. The rat was allowed to stay there for 10 s. The intertrial interval was 10 min. After each trial the rats were dried and were returned to their cages at the end of the session. Animals were trained for 5 days. Twenty-four hours after the last training session, the rats were submitted to a test session. Before this session, the submerged platform was removed. The retention test consisted of placing the animals in the water for 1 min. The latency in reaching the original position of the platform, the number of crossings in that place, and the time spent in the target quadrant compared to the opposite quadrant were measured. Training and test sessions were always performed between 13 and 17 h.

The protocol for the second exposure to the Water Maze task was identical to the explained above, except by the modification of the platform position during training.

2.5. Preparation of synaptic plasma membrane from hippocampus

After chronic treatments (Experiment 1; see Table 1) and 1 week after exposure to the Water Maze task (Experiment 2; see Table 1), animals were killed by decapitation without anesthesia, the brain was rapidly removed, and the hippocampus was dissected to prepare synaptic plasma membranes according to the method of Jones and Matus (1974), with some modifications (Wyse et al., 2000).

The hippocampus was homogenized in ten volumes of a 0.32 M sucrose solution containing 5 mM Hepes and 1 mM EDTA. The homogenate was centrifuged at 1000*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.

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

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 final 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. 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 (Wyse et al., 2000). Released inorganic phosphate (Pi) was measured by the method of Chan, Delfer, and Junger (1986). 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.

2.7. Protein measurement

Protein was measured by the method of Bradford (1976), with bovine serum albumin used as standard.

2.8. Statistical analysis

Data were expressed as means ± SEM. Na^+,K^+-ATPase activity was analyzed using one- or two-way analysis of variance, followed by the Duncan multiple range test when the *F* test was significant. Water Maze performance was analyzed using Student's *t* test (when comparing two groups) or one-way analysis of variance, followed by the Duncan's multiple range test when the *F* test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

3.1. Experiment 1: Effect of chronic stress and chronic lithium treatment upon hippocampal Na^+,K^+-ATPase activity

After the treatments, hippocampal Na^+,K^+-ATPase activity was measured. The effect of 40 days of chronic variate stress and concomitant chronic lithium treatment upon the Na^+,K^+-ATPase activity is shown in Fig. 1. A two-way analysis of variance showed a significant interaction between stress and lithium treatment [two-way ANOVA, $F(1,20) = 4.08$, $P < 0.05$, $N = 5$–7 animals/group]. A Duncan's multiple range test demonstrated that just the stressed group was different from the control group, indicating that lithium, although not presenting any effect by itself, prevented the stress-induced inhibition in the enzyme activity.

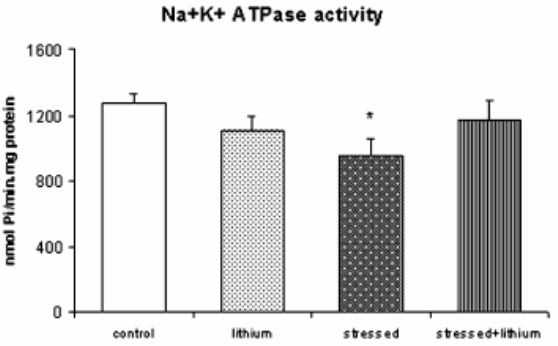

Fig. 1. Effect of chronic variable stress and chronic lithium treatment on Na^+,K^+-ATPase activity in synaptic plasma membranes from rat hippocampus. Control represents the normal group, lithium represents the group treated just with LiCl for 40 days, stressed represents the group submitted to a chronic variable stress paradigm for 40 days, and stressed+lithium represents the group stressed for 40 days and concomitantly submitted to lithium treatment. Data are expressed as means ± SEM, for 5–7 animals in each group. There was a significant interaction between stress and lithium treatments (two-way ANOVA, $P < 0.05$). *Significantly different from the control group (Duncan's multiple range test, $P < 0.05$).

3.2. Experiment 2: Effect of chronic stress interruption and post-stress chronic lithium treatment upon spatial memory in the Water Maze task and upon hippocampal Na^+,K^+-ATPase activity

To verify whether lithium treatment could reverse the cognitive impairment and the decrease in Na^+,K^+-ATPase activity after these effects of chronic stress were already established, rats were initially divided into two groups: control and chronically stressed. After 40 days of chronic stress exposure, animals were submitted to the Water Maze task (Fig. 2), and the stressed rats showed impaired performance in this task. Regarding the number of times that the animals crossed the platform location, results showed that chronically-stressed rats presented a decreased number of crossings [Student's *t* test; $t(31) = 2.804$; $P < 0.01$], as shown in Fig. 2A. These results are not related to reduced motor activity, since no difference is observed in the number of crossings and rearings between groups when these animals are exposed to an open field (data not shown). When analyzing the

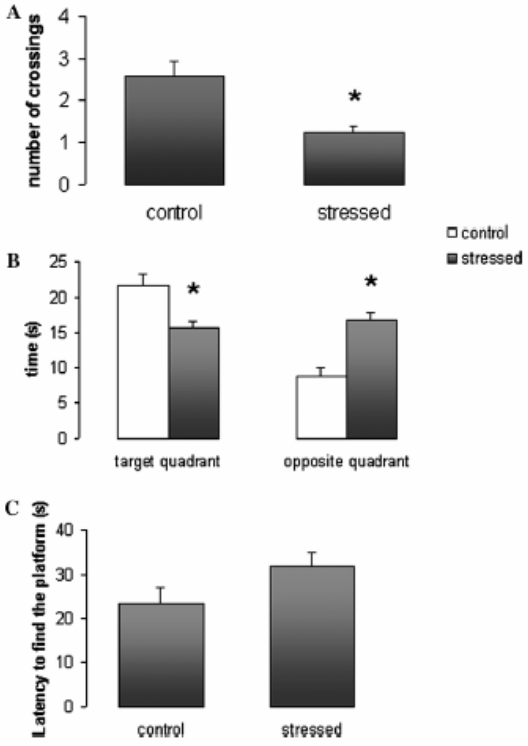

Fig. 2. Effect of treatment with chronic variable stress for 40 days on the performance of rats in the Morris Water Maze task. (A) Number of crossings performed by the animals at the exact location where the platform was; (B) time spent by the animals in the target quadrant and in the opposite quadrant; and (C) latency to find the original location where the platform was. Data are expressed as means ± SEM, $n = 28$ animals in the control group and 60 animals in the stressed group. *Significant effect of stress treatment (Student's t test for independent samples, $P < 0.01$).

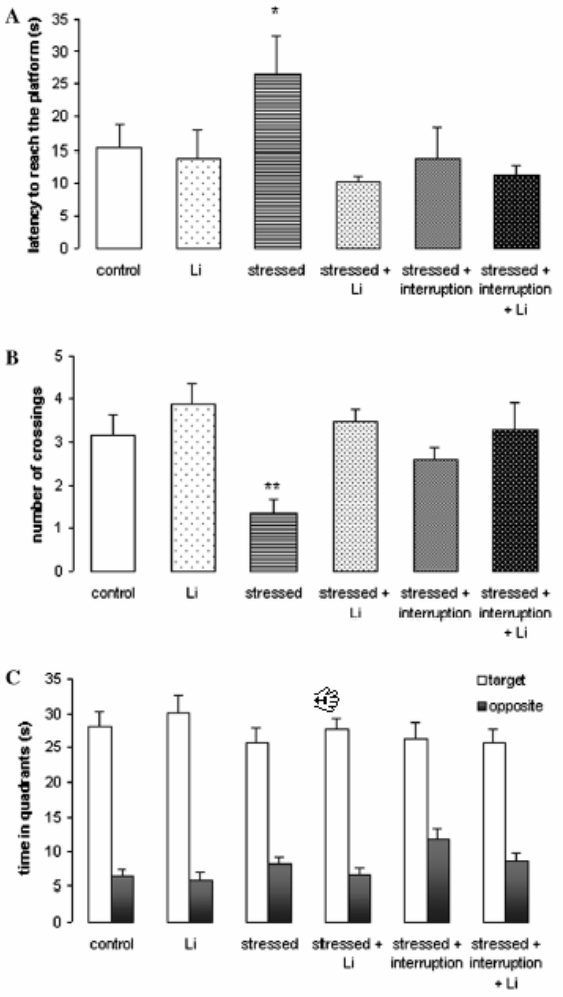

Fig. 3. Effect of stress interruption and of post-stress lithium treatment on the performance of rats in the Morris Water Maze task. (A) Latency to find the original location where the platform was; (B) number of crossings performed by the animals at the exact location where the platform was; and (C) time spent by the animals in the target quadrant and in the opposite quadrant. Control represents the normal group; Li represents the control group that received lithium for 30 days; stressed represents the group that continued to be stressed during all the experimental protocol; stressed + Li represents the stressed group that, after 40 days of stress, started to receive lithium for 30 days; stressed + interruption represents the group that had the stress treatment interrupted, and stressed + interruption + Li represents the group that had the stress treatment interrupted and started to receive lithium treatment. Data are expressed as means ± SEM, for 10–15 animals in each group. *Significantly different from the control and from all the other groups (one-way ANOVA, followed by Duncan's multiple range test, $P < 0.05$); **Significantly different from the control and from all the other groups (one-way ANOVA, followed by Duncan's multiple range test, $P < 0.001$).

time spent in the target and in the opposite quadrants, it was observed that chronic stress decreased time spent in the target quadrant [Student's t test; $t(44) = 2.932$; $P < 0.05$], while increasing time spent in the opposite quadrant [Student's t test; $t(81) = 4.610$; $P < 0.01$; Fig. 2B]. As may be observed in Fig. 2C, there was no significant difference in the latency to reach the original position of the platform [$t(50) = 1.219$; $P > 0.05$].

Afterwards, the stressed animals were divided into four additional groups, as displayed in Table 1, and the stress treatment was continued (groups 3 and 4) or discontinued (groups 5 and 6), with (groups 4 and 5) or without (groups 3 and 6) lithium treatment. The control group was divided into two groups, treated or not with lithium. Thirty days later, animals were again submitted to the Water Maze task (Fig. 3). The continuously stressed group presented an impaired performance in this task when compared to all the other groups, as demonstrated by an increased latency to find the original place where the platform was localized [$F(5, 71) = 2.60$; $P < 0.05$, followed by Duncan's multiple range test, $N = 10$–15 animals/group], and a decreased number of

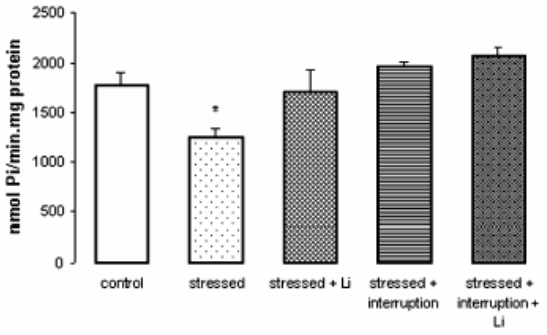

Fig. 4. Effect of stress interruption and of post-stress lithium treatment on Na^+,K^+-ATPase activity in hippocampal synaptic plasma membranes. Control represents the normal group; stressed represents the group that continued to be stressed during all the experimental protocol; stressed + Li represents the stressed group that, after 40 days of stress, started to receive lithium for 30 days; stressed + interruption represents the group that had the stress treatment interrupted, and stressed + interruption + Li represents the group that had the stress treatment interrupted and started to receive lithium treatment. Data are expressed as means ± SEM, $n = 5$ animals/group. *Significantly different from the control and from all the other groups (one-way ANOVA, followed by Duncan's multiple range test, $P < 0.005$).

crossings over the platform [$F(5,71) = 4.44$; $P = 0.001$, followed by Duncan's multiple range test]. Both these effects of stress were reversed by lithium treatment, as well as by stress interruption. There was no difference in the time spent in the target quadrant [$F(5,71) = 0.60$; $P > 0.05$].

One week after this behavioral task, animals were killed and hippocampal Na^+,K^+-ATPase activity was measured. As displayed in Fig. 4, the animals stressed during 70 days presented reduced enzymatic activity, when compared to other groups [$F(4,15) = 5.851$, $P < 0.005$, followed by Duncan's multiple range test, $N = 4$ animals/group].

4. Discussion

In the present study, we used a chronic stress model adapted from studies concerning models of depression in animals (Echandia, Gonzalves, Cabrera, & Fracchia, 1988; Konarska et al., 1990; Murua & Molina, 1992; Papp, Willner, & Muscat, 1991; Willner, 1990), which consists of exposing rats to different weak stressors for several days. We evaluated the effects of this chronic variate stress model on Na^+,K^+-ATPase activity in the hippocampus, since this region is particularly sensitive to stress effects (McEwen, 2000; Sapolsky, 2000). A decreased activity of Na^+,K^+-ATPase was observed in stressed animals, in agreement with previous observations both in animals (Gamaro et al., 2003) and in depressed patients (el-Mallakh & Wyatt, 1995). Since decreased activity of this enzyme seems to be an important characteristic of depressive disorders (el-Mallakh & Li, 1993; el-Mallakh & Wyatt, 1995), this finding further support this model as an animal model of depression.

A common feature of chronic stress exposure and major depression is the activation of the hypothalamus–pituitary–adrenal (HPA) axis, which culminates in the synthesis and release of glucocorticoids (Barden, 2004). Glucocorticoids present a negative feed-back upon corticosteroid receptors (GR), located at the level of pituitary, hypothalamus, and limbic brain areas (Barden, 2004). Sapolsky, Krey, and McEwen (1984) first demonstrated that elevated glucocorticoid levels lead to loss of hippocampal GR-containing cells that mediate the glucocorticoid-induced suppression of HPA axis, what could lead to its persistent hyperactivation. An extensive literature has shown that prolonged stress or prolonged exposure to glucocorticoids can have adverse effects on the rodent hippocampus, which may include neuronal atrophy and explicit memory deficits, and more recent findings suggest a similar phenomenon in the human hippocampus of patients with neuropsychiatric disorders, such as major depression (Sapolsky, 2000). What is not well established is whether the hippocampal atrophy arises from the neuropsychiatric disorder or precedes and predisposes toward it (Barden, 2004; Campbell & MacQueen, 2004; Sapolsky, 2000). The fact is that this atrophy, with a reduction in the number of branch points and synapses, may be involved in several chronic stress effects, including reduced Na^+,K^+-ATPase activity.

Several studies have demonstrated that major alterations of the HPA axis can be successfully reversed or prevented by treatments with antidepressants (Holsboer & Barden, 1996; Peiffer, Veillux, & Barden, 1991), which can act by increasing glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) expression and function, and this, in turn, is associated with enhanced negative feedback by endogenous glucocorticoids.

Considering that lithium treatment is able to increase GR in rat brain (Semba, Watanabe, Suhura, & Akanuma, 2000), we also verified the effect of lithium treatment on the reduction of Na^+,K^+-ATPase activity caused by stress. Although lithium did not present any effect by itself, it was able to prevent chronic stress effects on Na^+,K^+-ATPase activity, in such a way that animals that were chronically stressed and treated with lithium showed the same levels of enzyme activity as controls. This cation is able to normalize erythrocyte Na^+,K^+-ATPase activity in bipolar patients, a disturbance that is characterized by surges of mania intercalated with periods of depression (Hokin-Neaverson & Jefferson, 1989; Wood, Elphick, & Grahame-Smith, 1989), and these results of our work suggest that lithium treatment may be useful also in the treatment of major depression. Since Na^+,K^+-ATPase is essential to brain normal function, modulation of this enzyme might contribute to the therapeutic efficacy and neuroprotective effects of lithium.

To mimic clinical conditions, when drug therapy is applied after a mood disorder has been diagnosed, we evaluated the effects of chronic lithium treatment after the establishment of chronic stress effects. Lithium treatment was initiated after 40 days of chronic variate stress, when both behavioral (spatial memory) and neurochemical (Na^+,K^+-ATPase activity) deficits had already been installed. It was observed that, in this condition, lithium treatment was able to reverse the biochemical and behavioral alterations studied. This is an important property of this salt, since in most cases a treatment is required after the damage has been installed.

Different morphological and neurochemical effects have been reported in hippocampus and other brain structures after depressive states or prolonged exposure to stress situations. On the other hand, lithium treatment has been shown to reverse illness-related atrophy and to increase the brain gray matter volume in humans (Gray, Zhou, Du, Moore, & Manji, 2003; Moore, Bebchuk, Wilds, Chen, & Manji, 2000). Some mechanisms have been proposed to explain these effects, such as the robust increase the expression of the cytoprotective protein bcl-2 in the CNS (Chen et al., 1999), activation of signaling cascades utilized by endogenous growth factors, like the extracellular signal-regulated kinase (ERK), mitogen-activated protein (MAP) kinase pathway (Manji & Chen, 2002), and altered levels of brain-derived neurotrophic factor (BDNF). Beside the ability to normalize Na^+,K^+-ATPase activity, verified in this and other studies from the literature (El-Mallakh et al., 2000; Hennion et al., 2002), these data argues in favor of the possible trophic and neuroprotective effects of chronic lithium treatment.

We observed that interruption of chronic stress by itself was able to reverse stress effects upon memory and Na^+,K^+-ATPase activity, since the stress-interrupted group presented similar values of enzymatic activity and a similar performance in the Water Maze task when compared to the control group. Several studies have shown hippocampal neuronal loss and/or atrophy after chronic-stress situations, particularly after severe stress paradigms (Kuipers, Trentani, Den Boer, & Ter Horst, 2003; McEwen, 2002). Factors underlying this cellular remodeling include elevated glucocorticoids levels, as mentioned above, which are implicated in decreased neurogenesis and increased activity of excitatory amino acid neurotransmitter, what, by this way, could result in both potentially reversible remodeling and irreversible cell death (Campbell & MacQueen, 2004). Considering that the interruption of exposure to stress was able to reverse the effects observed, we believe that they were not due to neuronal loss, but to neurochemical changes induced by this chronic mild stress model in the rat hippocampus, such as the decreased Na^+,K^+-ATPase activity.

Chronic stress has been shown to induce spatial memory deficits (Bodnoff et al., 1995; Conrad, Galea, Kuroda, & McEwen, 1996; McLay, Freeman, & Zadina, 1998; Nishimura, Endo, & Kimura, 1999; Vasconcellos et al., 2003), and we showed in a previous work that chronic lithium administration is able to attenuate the effect of stress on memory (Vasconcellos et al., 2003). Performance in tasks that measure spatial memory, such as the Water Maze task, is strongly linked to hippocampal function (see Nichols, Zieba, & Bye, 2001, for a review). We found that hippocampal Na^+,K^+-ATPase is diminished in rats chronically stressed. Thereby, this decreased enzymatic activity could be interfering in the energy-dependent memory storage stages (Gibbs & Ng, 1977).

Although the fact that a parallelism of effects was verified between Na^+,K^+-ATPase activity and spatial memory, it does not necessarily mean that the reduced activity of this enzyme would be the only cause of the memory impairment observed. However, there is evidence of a role of Na^+,K^+-ATPase in long-term potentiation (Glushchenko & Izvarina, 1997), and it has been showed that the inhibition of this enzyme activity induces long-term depression (Reich, Mason, & Alger, 2004) as well as spreading depression—a transient breakdown of neuronal function concomitant with a massive failure in ion homeostasis (Kohling et al., 2003). Additionally, Na^+,K^+-ATPase inhibition can lead to memory impairment in the inhibitory avoidance and in the Water Maze tasks (dos Reis, de Oliveira, Lamers, Netto, & Wyse, 2002; Sato et al., 2004; Wyse et al., 2004; Zhan, Tada, Nakazato, Tanaka, & Hongo, 2004), and cognitive deficits have been reported in situations where Na^+,K^+-ATPase was reduced, such as Alzheimer disease and under oxidative stress (Hattori et al., 1998; Lehotsky et al., 1999). Since the Na^+,K^+-ATPase is crucial for maintaining ionic gradients in neurons and is reported to be critically involved in potassium buffering after periods of hyperstimulation (Xiong & Stringer, 2000), it is well acceptable that a reduction in this enzyme activity may impair neuronal activity and memory storage.

The regulation of Na^+,K^+-ATPase activity is a complex matter. This activity seems to be regulated by several factors, including hormones and neurotransmitters, such as catecholamines (Mallick & Adya, 1999; Nishi et al., 1999) and serotonin (Stepp & Novakoski, 1997). It should be observed that central catecholaminergic and serotonergic activity may be modulated by exposure to stress situations (Carrasco & Van de Kar, 2003). In addition, regulation of Na^+,K^+-ATPase activity can be divided into a "short-term" and a "long-term" control (Bertorello & Katz, 1993). Although the exact mechanisms underlying these results are not known, it is possible that the "long-term" control is involved, since both lithium and stress treatments were administered during several weeks. This long-term control could be due to an

altered number of pumps, brought about by an increase or decrease in protein synthesis or degradation. In this context, studies demonstrate that chronic lithium treatment may increase Na^+,K^+-ATPase number as measured by [^{3}H]ouabain binding in lymphocytes (Jenkins, Aronson, & Brearley, 1991; Wood et al., 1991). Additionally, lithium can exert direct effects on Na^+,K^+-ATPase activity, or indirect effects on intracellular sodium and calcium concentrations, besides interactions with second messenger transduction and formation, and it is possible that these proposed mechanisms are not mutually exclusive and may even be synergistic (el-Mallakh & Li, 1993; El-Mallakh et al., 2000). These possibilities should be tested in future studies.

In summary, the present study demonstrates that lithium treatment is able to prevent and reverse chronic variate stress effects both on Na^+,K^+-ATPase activity and on cognition. Considering the effects observed in these biochemical and behavioral parameters, it is possible that there is a correlation between Na^+,K^+-ATPase activity and memory deficits in chronically stressed animals.

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Artigo 2:

CHRONIC STRESS AND LITHIUM TREATMENTS ALTER HIPPOCAMPAL GLUTAMATE UPTAKE AND RELEASE IN THE RAT AND POTENTIATE NECROTIC CELLULAR DEATH AFTER OXYGEN AND GLUCOSE DEPRIVATION

De Vasconcellos et al.

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ABSTRACT

The glutamatergic system is thought to be involved in stress-induced changes of brain function, especially in the hippocampus. Several studies have suggested the involvement of glutamatergic neurotransmission in the effects of lithium treatment. In this investigation, we evaluated the effects of chronic variate stress and lithium treatments on glutamatergic release and uptake by synaptosomes and slices of hippocampus of Wistar rats, and the effects of these treatments on hippocampal vulnerability to oxygen and glucose deprivation (OGD). We found that [^{3}H]glutamate uptake was increased in synaptosomes of lithium-treated rats, and decreased in hippocampal slices of stressed animals. In basal conditions, we observed an increased [^{3}H]glutamate release in synaptosomes of chronically-stressed rats, and an increase in stimulated release in rats treated with lithium. Cellular vulnerability was quantified by measuring lactate dehydrogenase (LDH) in the incubation medium, and both stress and lithium treatments increased LDH release by slices submitted to OGD after 3 hours of reoxygenation, with no effects after 24 hours. We suggest that changes in the glutamatergic system are likely to take part in the mechanisms involved in nervous system plasticity following chronic lithium treatment, and that also may be in part responsible for the neuroendangerment observed after stress exposure.

Key words: chronic variate stress, lithium, synaptosomes, hippocampus.

Stress is known to influence a wide range of neuronal systems what, in the acute phase, result in beneficial endocrine and behavioral responses; however, repeated or severe stress can lead to adverse effects on neuronal function. The hippocampus is one brain structure that has been extensively studied with regard to the actions of stress and depression, and hippocampal neurons are reported to be damaged by chronic exposure to stress or activation of the hypothalamic-pituitary-adrenal (HPA) axis and increased levels of glucocorticoids (GCs). Dysfunction of the hippocampus could result in some of the vegetative and endocrine abnormalities, as well as cognitive and memory deficits, observed after prolonged stress exposure and in depressed patients (Duman et al., 1999).

Several mechanisms for the deleterious effects of GCs have been suggested. GCs are known to influence glucose uptake and cellular energy in peripheral tissues, and a decrease in glucose uptake has been reported in primary neuronal cultures treated with GCs (Horner et al., 1990). This could result in a decreased cellular metabolism that could, eventually, cause neurotoxicity or produce a heightened state of neuroendangerment to other types of insults (Hoyer et al., 2004; Sadowski et al., 2004). In addition, stress or glucocorticoid administration are reported to increase levels of glutamate in extracellular dialysate in the hippocampus (Sapolsky, 1996; Duman et al., 1999), and rats submitted to repeated restraint stress show an increased basal release of glutamate (Fontella et al., 2005).

Glutamate action is mediated via ionotropic and metabotropic receptors. Accumulation of glutamate in the synaptic cleft may lead to excitotoxic neuronal damage due to overstimulation of their receptors. Glutamate, acting via *N*-methyl-D-aspartate (NMDA) and non-NMDA ionotropic receptors, increases intracellular levels of Ca^{2+} and this sustained increase is known to underlie the excitotoxic effects of repeated seizures and ischemia (see Sattler and Tymianski, 2001, for a review). Based on these observations, it has been suggested

that enhanced glutamate release could also contribute to glucocorticoid-induced neuroendangerment.

Glutamate uptake is mediated by transporter proteins located in astrocytes and also in nerve terminals and this mechanism has been proven to play an important role in the termination of glutamatergic neurotransmission and prevention of excitotoxicity (Danbolt, 2001). Importantly, glutamate is removed from the synaptic cleft through high affinity sodium- and ATP-dependent glutamate transporters, which will be less active in a situation of energetic failure (Smith, 1995; Anderson and Swanson, 2000, Chen and Swansom, 2003).

Ischemia is defined as a severe reduction or complete blockage of blood flow (White et al., 2000) and is a pathophysiological event that frequently results in cerebral damage. Therefore, when higher glucocorticoid levels are combined with ischemia, there is an aggravation in cell loss seen *in vitro* (Payne and Schurr, 2001) or *in vivo* models of ischemia (Sapolsky, 1999; Krugers et al., 2000). Synaptic function, along with cellular integrity, can be preserved after hypoxia/ischemia by preventing the rise in corticosteroid levels by the use of the steroid synthesis inhibitor metyrapone (Adachi et al., 1998, 1999; Krugers et al., 2000), or by adrenalectomy (Morse and Davis, 1990; Antonawich et al., 1999). In addition, brain injury can be reduced by antagonists of NMDA receptors in several models of focal ischemia, suggesting that hypoxic neuronal loss is linked to excessive glutamate synaptic transmission (Albers et al., 1989).

Lithium salts have been in the first line of therapeutic drugs used to treat affective disorders, mainly bipolar disorder. Its clinical profile includes antimanic and antidepressant actions, as well as prophylaxis of both mania and depression, by reducing the frequency of bipolar episodes (Manji et al., 1999; Shaldubina et al., 2001). Increasing evidence supports the notion that lithium has neuroprotective effects in a variety of insults, such as glutamate-induced excitotoxicity, in cultured cells and animal models of diseases (Chen and Chuang,

1999; Jope, 1999; Manji et al., 1999, 2000). With respect to cerebral ischemia, studies have been performed in models *in vivo* and *in vitro*, and show that pretreatment with lithium is able to prevent neuronal loss after focal ischemia (Nonaka and Chuang, 1998), as well as prevent cultured neuron death in such situations (Cimarosti et al., 2001; Chuang et al., 2002). In addition, it is able to prevent apoptotic neuronal death when administered after an ischemic insult (Ren et al., 2003).

In this study, we aimed to investigate the effects of a chronic variate stress model, regarded as an animal model of depression, and of a concomitant chronic lithium treatment on hippocampal glutamatergic activity, which was measured by evaluation of glutamate release by synaptosomes and glutamate uptake by synaptosomes and slices of hippocampus. We also evaluated the effects of stress and lithium treatments on hippocampal vulnerability to oxygen and glucose deprivation, which is considered an *in vitro* model of cerebral ischemia.

EXPERIMENTAL PROCEDURES

Animals

One hundred and sixty five adult male Wistar rats (60 days at the beginning of the treatment) weighing 160-230 g were used. Experimentally naive animals were housed in groups of 4 or 5 rats in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust. They were maintained under a standard dark-light cycle (lights on between 7:00 a.m. and 7:00 p.m.), with a room temperature of 22 $\pm$ 2 °C. Rats had free access to food and water, except during the period when restraint stress or forced swimming were applied. All animal treatments were in accordance with the institutional guidelines and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS).

Experimental groups

The animals were divided in two groups. One group received standard rat chow and the other group had lithium chloride (LiCl - 2.5 mg/g of chow) and NaCl (17 mg/g) added to the food, as previously described (Vasconcellos et al., 2003). This treatment has been previously used, and at the end of a period of four weeks or more, animals remain healthy and present lithium levels in the range of 0.6 – 1.2 mM (Rocha and Rodnight, 1994; Vasconcellos et al., 2003, 2005), similar to the levels observed in treated patients. These groups were subdivided into two other groups: control and submitted to a chronic variate stress paradigm.

Chronic Variate Stress Model

Chronic variate stress was modified from other models of variate stress (Konarska et al., 1990; Willner, 1990, 1991; Murua and Molina, 1992; Gamaro et al., 2003). The following stressors were used: (a) inclination of the home cages at a 45^{o} angle for 4 to 6 h, (b) 10 to 15 min of noise, (c) 1 h to 3 h of restraint, as described below, (d) 1.5 to 2 h of restraint at 4^{o} C, (e) forced swimming for 10 or 15 min, as described below, (f) flashing light during 2 to 4 h, (g) isolation (2 to 3 days). Animals were exposed to stress starting at a different time every day, in order to minimize its predictability. The duration of stress and lithium treatments was of forty days.

Restraint was carried out by placing the animal in a 25 x 7 cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. Forced swimming was carried out by placing the animal in a glass tank measuring 50 x 47 x 40 cm with 30 cm of water at 23 $\pm$ 2^{o} C. Exposure to flashing light was achieved by placing the animal in a 50 cm-high, 40 x 60 cm open field made of brown plywood with a frontal glass wall. A 100 W lamp, flashing at a frequency of 60 flashes per minute, was used.

Synaptosomal preparation

After forty days of chronic variate stress and lithium treatments, the animals were sacrificed by decapitation and the brain was rapidly removed. The hippocampus was dissected out on ice and gently homogenized in 10 vol. of ice-cold medium consisting of 320 mM sucrose, 1 mM EDTA, and 0.25 mM dithiothreitol, pH 7.4, with a motor driven Teflon-glass homogenizer. Hippocampi of two animals were pooled and used for the preparation of each synaptosomal fraction, which was isolated on a discontinuous Percoll gradient, according to Dunkley et al. (1988). Briefly, the homogenate was centrifuged at 1000 g for 10 min and 2 ml of the supernatant collected (S1 fraction) were layered gently on a Percoll gradient comprised of 2 ml each of 23%, 15%, 10% and 3% Percoll (v/v) in a solution containing 320 mM sucrose, 1 mM EDTA and 0.25 mM dithiothreitol (final concentrations), with pH 7.4. The tubes were centrifuged for 5 min at 32,500 g and the interfacial fraction between 15% and 23% Percoll was carefully collected. This fraction was chosen because it is the most enriched in viable synaptosomes and has been recommended for use in biochemical and physiological studies. Protein concentration was measured according to the method of Lowry et al. (1951). The material was prepared fresh daily and maintained at 0 - 4 $^{\circ}$C throughout the experiment.

Synaptosomal glutamate uptake

Determination of glutamate uptake was performed as described by Migues et al. (1999). Synaptosomal preparations were washed twice, through resuspension in three volumes of 300 mM sucrose with 15 mM Tris/acetate buffer (pH 7.4) and centrifugation at 13,000 *g* for 15 min at 4 ^{o}C. The final pellet was resuspended in 300 mM sucrose with 15 mM Tris/acetate buffer (pH 7.4) and incubated in HBSS (Hepes/Cl buffered salt solution), pH 7.4 (containing 27 mM HEPES, 133 mM NaCl, 2.4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 12 mM Glucose, 1.0 mM $CaCl_2$) in the presence of 2 μM of L-[^{3}H]glutamic acid (Amersham International, UK, specific activity 1.97 x 10^6 GBq/mol), for 1 min at 37 °C. The reaction was stopped by filtration through GF/B filters. The filters were washed three times with 3 ml of

ice-cold 15 mM Tris/acetate buffer (pH 7.4) in 155 mM ammonium acetate, and the radioactivity retained on the filters was measured in a Wallac scintillation counter. Specific [^{3}H]glutamate uptake was calculated as the difference between uptake obtained in the incubation medium as described above and uptake obtained with a similar incubation medium containing choline chloride instead of NaCl (non-specific uptake). Na^+-independent uptake was less than 10% of the total. All measurements were made in triplicate.

Synaptosomal glutamate release

Glutamate release from synaptosomes was measured according to Migues et al. (1999), with minor modifications. Synaptosomal preparations were washed twice, through resuspension in three volumes of HBSS (pH 7.4) and centrifugation at 13,000 *g* for 15 min at 4^o C. The final pellet was resuspended in 500 μl HBSS (pH 7.4) and incubated in the same medium, for 15 min at 37 °C, in the presence of [^{3}H]glutamate (final concentration, 2 μM). Aliquots of labelled synaptosomes (1.4 mg protein) were centrifuged at 13,000 *g* for 1 min. Supernatants were discarded, and the pellets were washed 4 times in HBSS by centrifugation at 13,000 *g* for 1 min at 4 °C. In order to measure the basal release of [^{3}H]glutamate, the final pellet was resuspended in HBSS and incubated for 60 seconds at 37 °C. Incubation was terminated by immediate centrifugation (16,000 *g* for 1 min at 4 °C). Radioactivity present in supernatants and pellets was separately determined in a Wallac scintillation counter. The released [^{3}H]glutamate was calculated as a percentage of the total amount of radiolabel in the synaptosomal preparation in relation to the start of the incubation period. K^+-stimulated [^{3}H]glutamate release was assessed as described for basal release, except that the incubation medium contained 40 mM KCl (NaCl decreased accordingly) in order to induce synaptosomal depolarization. All measurements were made in triplicate.

Four to six synaptosomal fractions per group (hippocampi of two rats were pooled for each synaptosomal fraction) were prepared for experiments involving glutamate uptake and release, and protein content was assessed by the method described by Lowry et al. (1951).

Glutamate uptake by slices

Slices preparation: The animals were decapitated, their brains immediately removed and humidified with Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 1.11 glucose, in pH 7.2. Hippocampi were dissected onto Petri dishes with HBSS and slices (0.4 mm) were obtained using a McIIwain tissue chopper. Slices of hippocampus were separated with the help of a magnifying glass and transferred to 24-well culture plates: one plate was maintained at 35°C and the other was maintained on ice. The slices from the first plate were washed once with 1mL of 35°C HBSS and the second with 1mL of 4°C sodium-free HBSS for the analysis of non-specific uptake (see below).

Total uptake: Glutamate uptake was performed according to Frizzo et al. (2002), adjusted for hippocampus in accordance with incubation times (Thomazi et al., 2004). Slices were incubated at 35°C with 0.66 μCi mL^{-1} L-[^{3}H]glutamate and 100μM (final concentration) unlabeled glutamate in glucose-HBSS solution. Incubation was stopped after 5 minutes with two ice-cold washes of 1mL HBSS, immediately followed by the addition of 0.2 mL 0.5N NaOH; this material was then kept overnight.

Sodium-independent uptake (Non-specific uptake): To measure sodium-independent uptake, the same protocol described above was used, though with differences in the temperature and the medium used. Sodium-independent uptake was determined on ice (4°C), using N-methyl-D-glucamine instead of sodium chloride. The results were subtracted from the total uptake to obtain the specific uptake. Both the specific and non-specific uptakes were performed in triplicate. Protein content was measured following the method described by Peterson (1997).

Radioactivity quantification: Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409).

Preparation and incubation of slices for oxygen and glucose deprivation (OGD) experiment

Rats were decapitated, their hippocampi were quickly dissected out and transverse sections (400 μm) were rapidly obtained using a McIlwain tissue chopper. One slice was placed into each well of a 24-well culture plate (plates were paired: control and oxygen and glucose deprived – OGD) and preincubated for 15 minutes in a modified Krebs-Henseleit solution (preincubation solution) containing 120 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, 10 mM $MgSO_4$, 26 mM $NaHCO_3$, 1.18 mM KH_2PO_4 and 11 mM glucose (pH 7.4) at 37°C in an atmosphere of 5% CO_2 (95%O_2/5%CO_2) (Cárdenas et al., 2000).

Oxygen and glucose deprivation (OGD): The exposure to oxygen and glucose deprivation was based on the method described by Strasser (1995) with some modifications (Cimarosti et al., 2001) After the preincubation period, control slices were rinsed in a modified Krebs-Henseleit solution (incubation solution – control medium) containing (in mM): NaCl 120, KCl 2, $CaCl_2$ 2, $MgSO_4$ 1.19, $NaHCO_3$ 26, KH_2PO_4 1.18 and glucose 11 (pH 7.4). The slices corresponding to the control group were then incubated for another 60 minutes, in the same conditions. Slices corresponding to the "ischemic" experimental group were rinsed once with incubation solution without glucose (OGD medium), which was previously bubbled with N_2 for 30 seconds, and incubated in OGD medium for 60 minutes in an anaerobic chamber, saturated with N_2. During the procedure, the temperature was kept at 37^{o}C. After this period, the incubation solution was replaced with fresh control medium ("ischemic" slices were first rinsed once with control medium) and slices were incubated for 3

or 24 hours at 37 º C in an atmosphere of 5% CO_2 (95%O_2/5%CO_2) to simulate "reperfusion" period (Cárdenas et al., 2000).

Assessment of neural injury - LDH assay: Neural cell injury was quantified by the measurement of lactate dehydrogenase (LDH) released from damaged cells into the extracellular fluid. LDH efflux occurs from either necrotic or apoptotic cells, and is proportional to the number of damaged cells (Koh and Choi, 1987).

LDH activity was determined using a commercial kit (Doles Reagentes); the activity of this enzyme was assessed in the bathing fluid. Following the conversion of exogenously added lactate to pyruvate, 1,10-phenantroline is converted to a colored complex, after a chain of reactions resulting from the NADH formed by the enzymatic reaction, which is measured using a spectrophotometric method (490 nm).

We used an entire slice of each animal for assessing the total amount of LDH, and LDH release in control and OGD experiments was expressed as the percentage of the total amount of LDH in one slice.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM) and were analysed using two-way or three-way ANOVA, as indicated.

RESULTS

Experiment 1 - Effect of chronic variable stress and chronic lithium treatment on hippocampal glutamate uptake and release.

Chronic variable stress induced an increased [^{3}H]glutamate release under basal conditions [Two-way ANOVA, $F(1, 12) = 10.669$; $P < 0.01$], and no effect of lithium treatment [$F(1, 11) = 0.000$; $P > 0.05$] nor interaction [$F(1, 12) = 0.995$; $P > 0.05$] were

observed (Figure 1). On the other hand, lithium treatment increased K^+-stimulated [^{3}H]glutamate release [$F(1, 12) = 5.399$; $P < 0.05$]. There was no effect of chronic stress [$F(1, 12) = 2.845$; $P > 0.05$], but a significant interaction between lithium and stress treatments [$F(1, 12) = 5.488$; $P < 0.05$] was displayed in this parameter (Figure 1).

Insert Figure 1

A two-way ANOVA showed a significant effect of chronic lithium treatment on [^{3}H]glutamate uptake in hippocampal synaptosomes [$F(1, 20) = 8.987$; $P < 0.01$] (Figure 2). When applying a Duncan's multiple range test, the chronic lithium-treated animals were observed to present increased glutamate uptake when compared to the control group. There was no effect of chronic variate stress on neuronal glutamate uptake, [$F(1, 20) = 0.004$; $P > 0.05$], nor interaction between these variables [$F(1, 20) = 3.804$; $P > 0.05$].

Insert Figure 2

In order to verify the effects of chronic stress and lithium on neuronal and glial glutamate uptake, we also evaluated neurotransmitter uptake in slices of hippocampus. Figure 3 shows that the exposure to chronic stress significantly decreased glutamate uptake in slices [Two-way ANOVA, $F(3, 33) = 9.008$; $P < 0.005$], with no effect of lithium treatment [$F(3, 33) = 1.563$; $P > 0.05$] nor interaction [$F(3, 33) = 0.031$; $P > 0.05$] of these treatments.

Insert Figure 3

Experiment 2 - Effect of OGD on hippocampal slices obtained from animals

subjected to chronic variable stress and chronic lithium treatment

To evaluate whether previous exposure to chronic variate stress and lithium treatments interferes with the neuronal damage induced by OGD, we assayed LDH activity after 60 minutes of OGD followed by 3 or 24 hours of reoxygenation. Figure 4 shows, as expected, that there was a significant effect of OGD on LDH release into the medium after 3 hours of reoxygenation [three-way ANOVA, $F(1, 40) = 51.185$, $P < 0.001$]. Furthermore, both chronic variate stress and lithium treatments increased LDH release after OGD exposure when compared to the control group [three-way ANOVA, $F(1, 40) = 6.023$, $P < 0.02$ for lithium treatment and $F(1, 40) = 14.459$, $P < 0.001$ for chronic stress]. There were no interactions between treatments.

Insert Figure 4

After twenty-four hours of reoxygenation, the effect of OGD on LDH release was still observed in all groups [three-way ANOVA, $F(1, 40) = 23.726$, $P < 0.001$] (Figure 5). Nevertheless, there was an absence of effects of chronic variate stress and lithium treatments at this time [three-way ANOVA, $F(1, 40) = 1.213$, $P > 0.05$ for chronic stress treatment and $F(1, 40) = 1.337$, $P > 0.05$ for lithium treatment].

Insert Figure 5

DISCUSSION

The results reported herein demonstrate the effects of chronic variate stress in animals upon glutamate neurotransmission and the interaction between stress and chronic lithium treatment in this parameter. It was observed that stress and lithium alter differently the

glutamate release in nerve endings: while stress increases basal glutamate release, lithium leads to an increased release after high potassium stimulation, which mimics a neuronal depolarization.

Basal glutamate release is an important factor related to plasticity of synapses in the nervous system. A study from McKinney et al. (1999) describes the importance of spontaneous miniature synaptic potentials in the maintenance of dendritic spines. In this study they suggest that the low level of AMPA receptor stimulation, produced by glutamate spontaneously released from presynaptic buttons, is necessary to maintain spine morphology at mature synapses. This stabilization of spine morphology by spontaneously released glutamate is thought to occur through actions on actin filaments in the spine (Matus et al., 2000). Therefore, the increased spontaneous release of glutamate may be related to the plasticity induced by stress in the nervous system, possibly as a form of adaptation to the noxious stimulus that stress may represent. Indeed, it could represent a maladaptation, since the elevated basal levels of released glutamate could induce an excitotoxic effect.

Additionally, release of cytoplasmic glutamate may occur by reversal of the Na^+-coupled reuptake carrier. For instance, under conditions of energy failure, such as ischemia or hypoglycemia, the electrochemical gradient is greatly reduced and glutamate transporters may function by carrying glutamate from the cytoplasm to the exterior, thus contributing to glutamate excitotoxicity (Nicholls and Atwell, 1990). In this way, reversed transport may explain the results found in this study concerning basal glutamate release.

In this study, we have demonstrated that chronic lithium treatment is able to induce an enhancement in K^+-stimulated release of glutamate by hippocampal synaptosomes. An earlier report has already demonstrated a similar effect of lithium treatment in mouse and monkey cerebral cortex slices (Dixon et al., 1994). In this study, the authors observed an increased accumulation of glutamate in the incubation medium of slices acutely treated with

lithium chloride that could be due to a decreased glutamate uptake and/or metabolism, in addition to an increased release (Dixon et al., 1994; Dixon and Hokin, 1997). Here, the increase in release of glutamate is exclusively due to presynaptic nerve endings release, since a synaptosomal fraction was used for incubation with high K^+ levels. This effect could account for the chronic therapeutic effects of lithium treatment in mania episodes by inducing a regulation of glutamate receptors expression. In fact, recent studies by Du et al. (2004) demonstrated that chronic (but not acute) treatment of rats with therapeutically relevant concentrations of lithium and valproate, another antimanic agent, regulates synaptic expression of AMPA receptor subunit glutamate receptor 1 (GluR1), suggesting an effect of these drugs in the regulation of glutamate-mediated synaptic plasticity, which may be involved in these therapeutic effects.

Increasing glutamate release and modulating receptors expression can also lead to effects on memory. We observed, in previous studies, that chronic lithium treatment is able to prevent and reverse the deleterious effects of chronic stress on spatial memory evaluated in the Morris Water Maze task, in which the performance is strongly dependent on the hippocampus (Vasconcellos et al., 2003; 2005). The most acceptable molecular model for acquisition, storage and consolidation of memories has been long-term potentiation (LTP) (Lynch, 2004; Blitzer et al., 2005), and the critical event leading to induction of LTP appears to be the influx of calcium ions in the postsynaptic spine, which is usually mediated by NMDA receptors (Lynch et al., 2004; Baudry and Lynch, 2001). A report by Yu, Son and colleagues (2003) showed that lithium treatment facilitates hippocampal LTP induction. Thus, the enhanced glutamate pulse after each depolarization may be, at least in part, responsible for the previously observed effects of lithium upon spatial memory, perhaps by facilitation of LTP induction.

When evaluating glutamate uptake, we observed that lithium treatment increases synaptosomal uptake, while chronic variate stress had no effect on this parameter. Additionally, hippocampal slices had a decreased glutamate uptake after forty days of stress, with no effect of lithium.

Evidence from the literature suggests that different types of stress, including restraint and exposure to ether, or increased glucocorticoid concentrations, may increase extracellular glutamate levels in the hippocampus, and that glucocorticoids impair the uptake of glutamate by hippocampal astrocytes (Lowy et al., 1993, 1995; Venero and Borrell, 1999; Stein-Bahrens et al., 1994, 1992). The removal of glutamate from the synaptic cleft is a crucial step in terminating glutamate neurotransmission and sparing excitotoxicity, and sodium-dependent uptake is the major mechanism for the regulation of synaptic glutamate levels (Yeh et al., 2005) This uptake is mediated by a family of transporters, including GLAST, GLT-1 and EAAC1 (Danbolt, 2001; González et al., 2002). These transporters show differential patterns of expression: GLAST and GLT-1 are primarily expressed by glial cells, while EAAC1 are generally considered neuronal transporters (for review, see Sims and Robinson, 1999; Danbolt, 2001). GLT-1 and GLAST, the glial transporters, play a main role in removing released glutamate, and their lack of function may lead to several deleterious effects (Mitani and Tanaka, 2003; Rothstein et al., 1996; Maragakis and Rothstein, 2001, 2004; Tanaka et al.; 1997). In this study, we observed a decreased glutamate uptake in slices of hippocampus of stressed rats, with no alterations in synaptosomal uptake. In this sense, it is conceivable that this effect is mediated by a decrease, or dysfunction, of astroglial glutamate transporters.

With respect to the effects of lithium upon synaptosomal glutamate uptake, although in a very less extent, they may represent a useful cue to prevent excitotoxicity, as well as promote neuronal plasticity, since changes in glutamate transporter activity have been implicated in synaptic plasticity including long-term potentiation and depression (Brasnjo and

Otis, 2001; Levenson et al., 2001). Several intracellular signaling pathways can regulate glutamate transporter activity within minutes, through mechanisms that are not only dependent on *de novo* transporter synthesis, and involve translocation of transporters from intracellular sites to the cell surface. These signaling pathways include PKC, arachidonic acid and phosphatidylinositol 3-kinase (PI3K) (Sims and Robinson, 1999; Danbolt, 2001). PKC, for example, increases EAAC1 activity by increasing cell surface expression and catalytic efficiency of the transporter (González et al., 2002; Fournier et al., 2004). Lithium is known to alter several signal transduction pathways, including the PI3K pathway and PKC family of kinases (Jope, 1999; Manji et al., 1999; Manji and Chen, 2002) and an increased expression or translocation of EAAC1 transporters may be a mechanism by which lithium treatment alters glutamate uptake in hippocampal synaptosomes.

With regard to hippocampal vulnerability to oxygen and glucose deprivation, our initial premise was that lithium, by acting on glutamate neurotransmission and on several signal transduction pathways, could prevent some deleterious effects of OGD in control and chronically-stressed rats. As was observed, this was not the case. After 3 hours of reoxygenation, hippocampal slices of rats treated with lithium presented higher levels of LDH release in response to OGD when compared to the control group, although there were no differences in basal release, and this increase in LDH release is possibly the consequence of cell damage or death. It is important to note that this effect is not due to the physical presence of the salt, because experiments with *in vitro* addition of lithium were performed and there was no difference in LDH release after OGD exposure (data not shown).

Lithium increased stimulated glutamate release and this effect may be useful in facilitating synaptic plasticity. In contrast, this increased glutamate release may favour the induction of a necrotic cellular death, since the calcium influx through NMDA glutamate receptors seems to be essential for the deleterious effects of cerebral ischemia (Sattler and

Tymianski, 2001). Indeed, doses of lithium higher than 0.6 – 1.2 mM, which is the range utilized in therapeutics, are known to be very toxic and it is possible that the increased glutamate release observed here contributes to this toxicity.

Nevertheless, it should be considered that in the majority of investigations studying the effects of lithium on ischemia models, the evaluation of cellular death was made at least 24 hours after the ischemic insult. At this point, several apoptotic cascades have been initialized, and it is well established that apoptosis-mediated neuronal death is the main contributor to delayed loss of neurons from the penumbral region of infarcts (Slevin et al., 2005; Love, 2003). Chronic lithium treatment has been demonstrated to markedly increase the levels of the neuroprotective and antiapoptotic protein bcl-2 in rat frontal cortex, hippocampus and striatum, and ratios of Bcl-2/Bax, a proapoptotic protein, are increased by approximately 5-fold after lithium treatment (Chen and Chuang 1999; Manji et al., 2000). Lithium also inhibits glycogen synthase kinase 3beta (GSK-3beta) activity, which is an apoptotic promoter and is involved in neurodegenerative diseases (Grimes and Jope, 2001). In this sense, it is possible that a beneficial effect of lithium had not been observed in function of the time of the analyses, when the amount of LDH released originates from necrotic cellular death, since it seems that this salt is able to protect neurons mainly against apoptotic death.

The measurement of LDH released into the medium after 3 hours of reoxygenation indicates that repeated stress increases the effect of OGD on this parameter. This result may be interpreted as an increased vulnerability of these cells to ischemia. These results are in agreement with previous reports, which suggest that repeated stress may increase vulnerability of hippocampal neurons to several insults, including hypoxia-ischemia (McEwen 1999; Fontella et al., 2005). Chronic exposure to elevated levels of glucocorticoids has been demonstrated to result in cellular loss as well as in reduced neuronal plasticity and regeneration (Daw et al., 1991; McEwen and Sapolsky, 1995; Scheff et al., 1980), and some

mechanisms suggested to play a role in this increased vulnerability of hippocampal cells include decreased glucose uptake (Horner et al., 1990), increased glutamate tonus (McEwen 1999, 2000, 2001) and increased production of oxygen reactive species (ROS; Sapolsky, 2000). In this study, we observed that chronic stress increases basal release of glutamate and decreases its uptake in hippocampal slices. The consequent accumulation of glutamate in the synaptic cleft could represent, in this model, an explanation for the increased cellular vulnerability observed in the hippocampus of chronically stressed rats.

Finally, when analyzing the LDH release after 24 hours of reperfusion, the absence of effect of both treatments, stress and lithium, compared to the increased LDH activity observed after 3 hours of reoxygenation, may suggest that these treatments accelerated cellular death, but did not interfere in the total amount of cellular death observed after 24 hours. However, we should also consider that 24 hours of reoxygenation is a too long time for hippocampal slices, possibly inducing a generalized cellular death, in which case it might not be possible to observe tenuous alterations caused by the treatments with stress and lithium.

In conclusion, this study demonstrates that chronic stress damages glutamate uptake in slices of hippocampus and increases basal release of glutamate by hippocampal nerve endings, and that these effects may account for the increased hippocampal vulnerability to an ischemic insult. In contrast, the exposure to chronic lithium treatment increased neuronal presynaptic glutamate uptake, but had no effect on the uptake by hippocampal slices and increased the induced glutamate release. Despite the possible beneficial effect of the modulation of glutamatergic system on synaptic plasticity, lithium treatment increased cellular vulnerability to OGD, demonstrating an absence of neuroprotective effect in this model of injury. Taken together, these results demonstrate changes in the glutamatergic system that are likely to take part in the mechanisms involved in nervous system plasticity following repeated stress exposure and lithium treatment. A better understanding of these

processes may lead to the development of novel therapeutic approaches for stress disorders, as well as to new insights into mechanisms of synaptic plasticity.

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FIGURES:

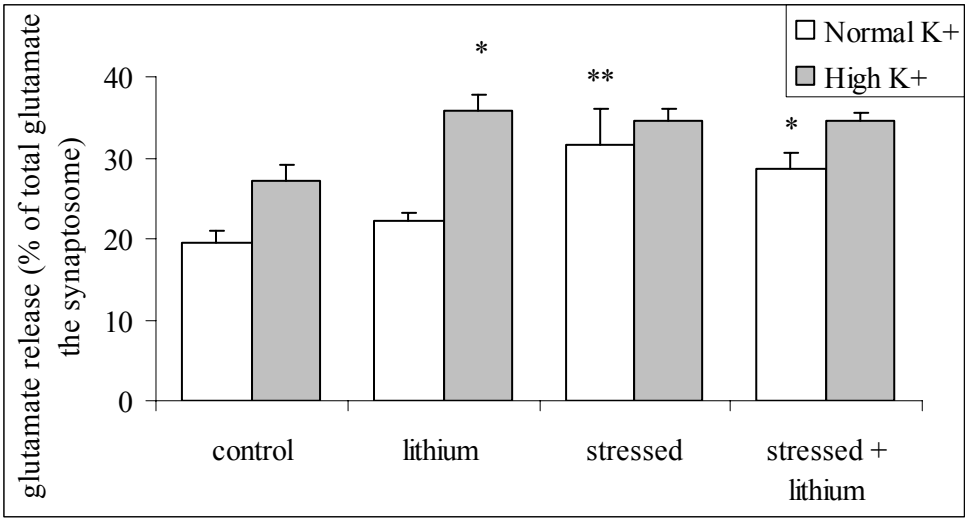

Figure 1: Effect of chronic variate stress and chronic lithium treatments on glutamate released by synaptosomes from rat hippocampus. Control represents the normal group, lithium represents the group treated just with LiCl for 40 days, stressed represents the group submitted to a chronic variate stress paradigm for 40 days, and stressed+lithium represents the group stressed for 40 days and concomitantly submitted to lithium treatment. Data are expressed as mean $\pm$ S.E.M., for 4 animals in each group. There was a significant effect of chronic stress treatment on basal release of glutamate (Two-way ANOVA, $P < 0.01$) and a significant effect of lithium treatment, as well as a significant interaction between treatments on stimulated release of glutamate (Two-way ANOVA, $P < 0.05$).

* Significantly different from the control group (Duncan´s multiple range test, $P < 0.05$).

** Significantly different from the control and lithium groups (Duncan´s multiple range test, $P < 0.05$).

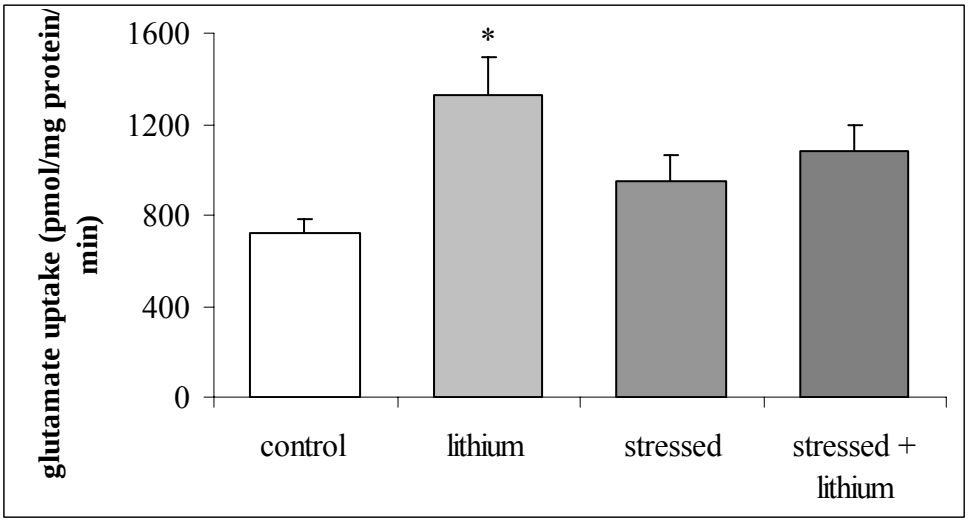

Figure 2: Effect of chronic variate stress and chronic lithium treatment on glutamate uptake by synaptosomes from rat hippocampus. Control represents the normal group, lithium represents the group treated just with LiCl for 40 days, stressed represents the group submitted to a chronic variable stress paradigm for 40 days, and stressed + lithium represents the group stressed for 40 days and concomitantly submitted to lithium treatment. Data are expressed as mean $\pm$ S.E.M., for 9-10 animals in each group. There was a significant effect of chronic lithium treatment on glutamate uptake (Two-way ANOVA, $P < 0.01$).

* Significantly different from the control group (Duncan´s multiple range test, $P < 0.05$).

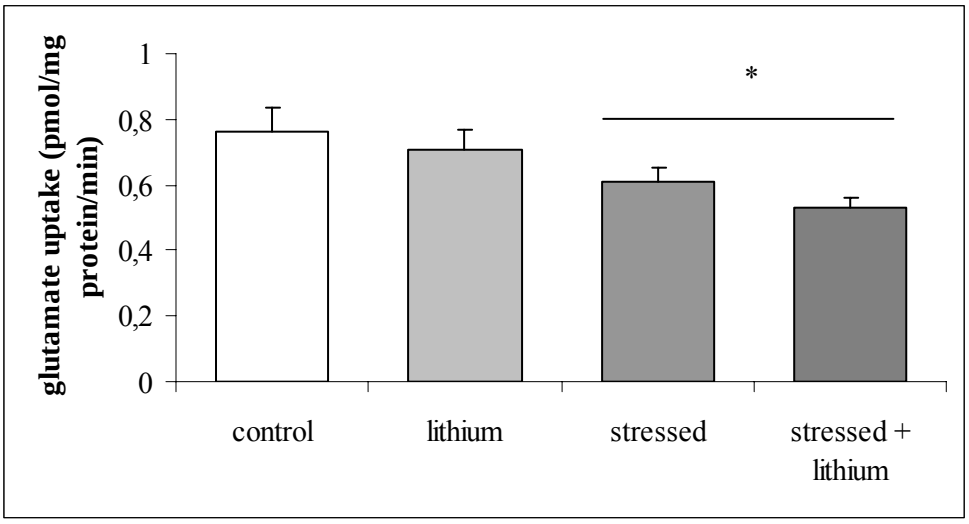

Figure 3: Effect of chronic variate stress and chronic lithium treatments on glutamate uptake by slices from rat hippocampus. Control represents the normal group, lithium represents the group treated just with LiCl for 40 days, stressed represents the group submitted to a chronic variable stress paradigm for 40 days, and stressed+lithium represents the group stressed for 40 days and concomitantly submitted to lithium treatment. Data are expressed as mean $\pm$ S.E.M., for 6 animals in each group. There was a significant effect of chronic stress on glutamate uptake (Two-way ANOVA, $P < 0.01$).

* Significantly different from the control group (Duncan´s multiple range test, $P < 0.05$).

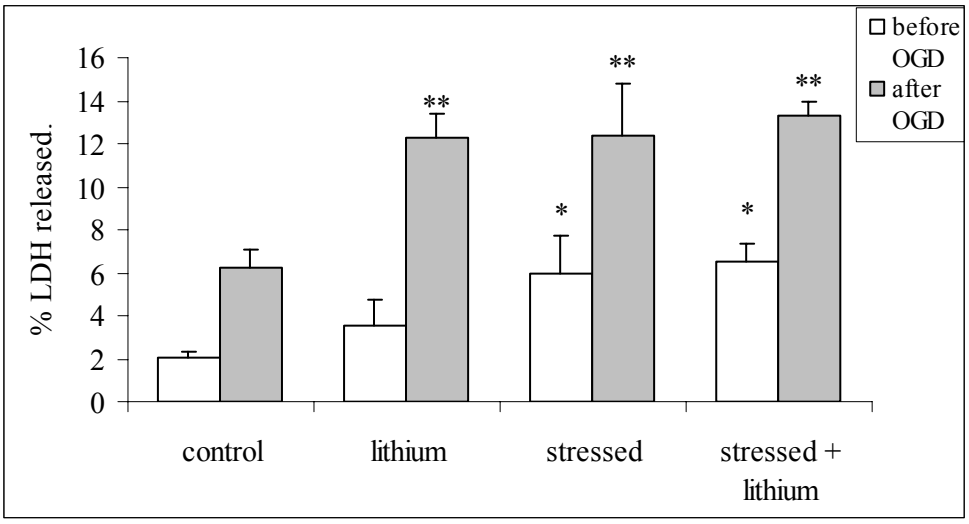

Figure 4: Effect of chronic variate stress and chronic lithium treatment on LDH released to the medium by control slices or slices exposed to OGD for 60 minutes and submitted to 3 hours of reoxygenation. Control represents the normal group, lithium represents the group treated just with LiCl for 40 days, stressed represents the group submitted to a chronic variate stress paradigm for 40 days, and stressed + lithium represents the group stressed for 40 days and concomitantly submitted to lithium treatment. Data are expressed as mean $\pm$ S.E.M., for 6 animals in each group. There was a significant effect of OGD (Three-way ANOVA, $P < 0.001$) and of lithium and stress treatments on LDH release after OGD (Three-way ANOVA, $P < 0.02$ and <0,001, respectively).

* Significantly different from the control group (Duncan´s multiple range test, $P < 0.001$).

** Significantly different from the control group which was submitted to OGD (Duncan´s multiple range test, $P < 0.001$).

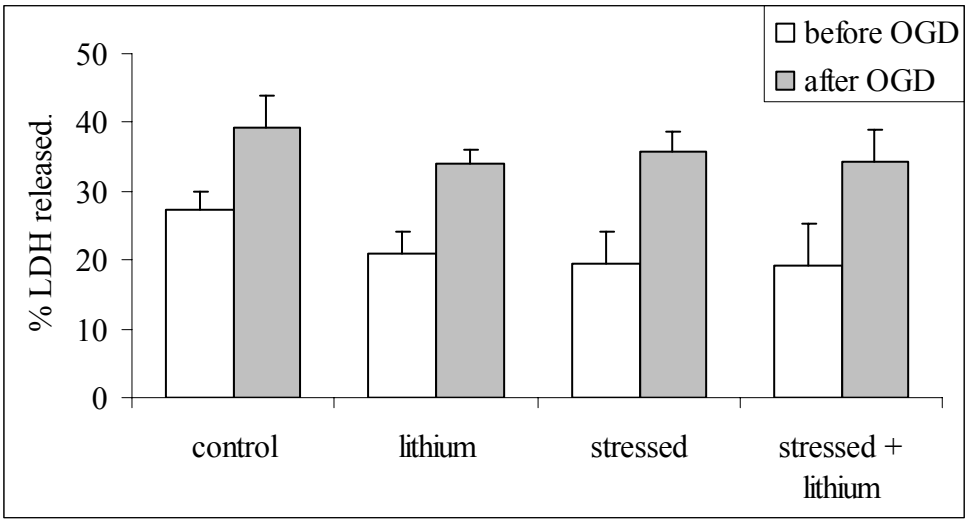

Figure 5: Effect of chronic variate stress and chronic lithium treatment on LDH released to the medium by control slices or slices exposed to OGD for 60 minutes and submitted to 24 hours of reoxygenation. Control represents the normal group, lithium represents the group treated just with LiCl for 40 days, stressed represents the group submitted to a chronic variate stress paradigm for 40 days, and stressed + lithium represents the group stressed for 40 days and concomitantly submitted to lithium treatment. Data are expressed as mean $\pm$ S.E.M., for 6 animals in each group. There was a significant effect of OGD (Three-way ANOVA, $P < 0.001$) and no effect of lithium and stress treatments.

Artigo 3:

CHRONIC LITHIUM TREATMENT HAS ANTIOXIDANT PROPERTIES BUT DOES NOT PREVENT OXIDATIVE DAMAGE INDUCED BY CHRONIC VARIATE STRESS

De Vasconcellos et al.

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CHRONIC LITHIUM TREATMENT HAS ANTIOXIDANT PROPERTIES BUT DOES NOT PREVENT OXIDATIVE DAMAGE INDUCED BY CHRONIC VARIATE STRESS

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ABSTRACT

This study was undertaken to verify the effects of chronic stress and lithium treatments on some oxidative stress parameters in distinct brain structures. Adult male Wistar rats were divided into two groups: control and submitted to a chronic variate stress paradigm, and subdivided into treated or not with LiCl. After 40 days of treatment, rats were killed, and lipid peroxidation (assessed by TBARS levels), production of free radicals (evaluated by the DCF test), total antioxidant reactivity (TAR) levels, and SOD and GPx activities were evaluated in hippocampus, hypothalamus, and frontal cortex. The results showed that stress increased lipid peroxidation and that lithium decreased free radicals production in hippocampus; both treatments increased TAR and presented interaction in this structure. In hypothalamus, lithium increased TAR and no effect was observed in frontal cortex. Stress increased SOD activity in hippocampus, while lithium treatment increased GPx in hippocampus and SOD in hypothalamus; significant interaction was observed between stress and lithium on SOD activity. We concluded that lithium present antioxidant properties but is not able to prevent oxidative damage induced by chronic variate stress.

Running title: Stress, lithium and oxidative damage

Key words: lithium, chronic variate stress, oxidative stress, SOD, GPx, antioxidant enzymes, lipoperoxidation, free radicals.

INTRODUCTION

Stress is known to influence a wide range of neuronal systems what, in the acute phase, result in beneficial endocrine and behavioral responses; however, repeated or severe stress can lead to adverse effects on neuronal function (McEwen, 2000). Several brain structures are involved in the stress response, e.g. hypothalamus, frontal cortex, and hippocampus, and may be affected by chronic exposure to stress (McEwen, 2005; Pacak and Palkovits, 2001). Among these structures, the hippocampus has been the most extensively studied with regard to the actions of stress and depression, and hippocampal neurons are reported to be damaged by chronic exposure to stress or activation of the hypothalamic-pituitary-adrenal (HPA) axis and increased levels of glucocorticoids (GCs). Dysfunction of the hippocampus could result in some of the vegetative and endocrine abnormalities, as well as cognitive and memory deficits, observed after prolonged stress exposure and in depressed patients (Duman et al., 1999).

The neuroendangerment observed after stress exposure or elevated GCs levels have been linked to an increased generation of reactive oxygen species (ROS) (McIntosh and Sapolsky, 1996). ROS are believed to be involved in tissue damage resulting from a wide variety of insults. These substances can directly damage cellular proteins, DNA, and lipids, and thereby affect all cellular functions (Cochrane, 1991). The nervous system is extremely sensitive to peroxidative damage, since it is rich in oxidizable substrates, has a high oxygen tension and low antioxidant capacity (Anderson et al., 1985; Metodiewa and Koska, 2000). Membrane lipids are highly susceptible to this kind of injury, and this event may damage cell membranes and interfere with the activity of membrane-associated enzymes. Furthermore, it induces changes in membrane fluidity and potential and in its permeability to ions (Sandhir et al. 1994). Moreover, the localization of major antioxidant defense systems in glial cells, rather

than in neurons, may cause the nerve cells to be more susceptible to oxidants present in the brain (Bondy, 1992).

The possible damage induced by ROS in cells is normally held in check by natural enzymatic and nonenzymatic antioxidant systems (Halliwell and Cross, 1994). These cellular defenses reduce the steady-state concentrations of free radical species and repair oxidative cellular damage. The antioxidant defense system includes enzymes, such as superoxide dismutase (SOD), which converts superoxide radicals into H_2O_2, and glutathione peroxidase (GPx), which breaks down peroxides, notably those derived from the oxidation of membrane phospholipids. This removal of superoxide and H_2O_2 reduces the generation of hydroxyl radicals, which are formed by the iron-catalyzed Fenton reaction or by the Haber-Weiss reaction (Kehrer, 2000). There are also non-enzymatic antioxidants (carotenoids, vitamin E, glutathione) with important roles in defense mechanisms. Exposure to stress situations has been proposed to impair antioxidant defenses, leading to oxidative damage by changing the balance between oxidant and antioxidant factors (McIntosh et al, 1998a, 1998b, Fontella et al., 2005).

Lithium salts have been in the first line of therapeutic drugs used to treat affective disorders, mainly bipolar disorder (Manji et al., 1999; Shaldubina et al., 2001), and increasing evidence supports the notion that lithium has neuroprotective effects in a variety of insults, such as glutamate-induced excitotoxicity, in cultured cells and animal models of diseases (Chen and Chuang, 1999; Jope, 1999; Manji et al., 1999, 2000). Chronic lithium treatment has been demonstrated to markedly increase the levels of the neuroprotective and antiapoptotic protein bcl-2 in rat frontal cortex, hippocampus and striatum (Chen and Chuang 1999; Manji et al., 2000). Lithium also inhibits glycogen synthase kinase 3beta (GSK-3beta) activity, which is an apoptotic promoter and is involved in neurodegenerative diseases (Grimes and Jope, 2001), and both bcl-2 and GSK-3beta seem to be involved in the prevention and/or

induction of the deleterious effects induced by oxidative stress processes (Schafer et al., 2004; Pugazhenthi et al., 2003; Hochman et al., 1998). Therefore, it is conceivable that lithium treatment may exert some of its neuroprotective effects by attenuating oxidative stress. The aim of this work was evaluate the effects of chronic variate stress and lithium treatments on different parameters of oxidative stress in brain structures like hippocampus, hypothalamus and cerebral frontal cortex.

EXPERIMENTAL PROCEDURE

Chemicals

Thiobarbituric acid and Trolox were obtained from Merck, ABAP was obtained from Wako Chemicals USA, Inc., 2'-7'-dichlorofluorescein diacetate (DCFH-DA), 2'-7'-dichlorofluorescein (DCF), trichloroacetic acid (TCA), 2,4-dinitrophenylhydrazine (DNPH), guanidine hydrochloride, 5-amino-2,3-dihydro-1,4-phtalazinedione (luminol) and H_2O_2 stock solution were purchased from Sigma Chemical Co.

Animals

Sixty adult male Wistar rats (60 days at the beginning of the treatment) weighing 160-230 g were used. Experimentally naive animals were housed in groups of 4 or 5 rats in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust. They were maintained under a standard dark-light cycle (lights on between 7:00 a.m. and 7:00 p.m.), with a room temperature of 22 + 2 °C. Rats had free access to food and water, except during the period when restraint stress or forced swimming were applied. All animal treatments were in accordance with the institutional guidelines and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS).

Experimental groups

The animals were divided in two groups. One group received standard rat chow and the other group had lithium chloride (2.5 mg LiCl /g of chow) and NaCl (17 mg/g) added to the food, as previously described (Vasconcellos et al., 2003). This treatment has been previously used, and at the end of a period of four weeks or more, animals remain healthy and present lithium levels in the range of 0.6 – 1.2 mM (Rocha and Rodnight, 1994; Vasconcellos et al., 2003, 2005), similar to the levels observed in treated patients. These groups were subdivided into two other groups: control and submitted to a chronic variate stress paradigm.

Chronic Variate Stress Model

Chronic variate stress was modified from other models of variate stress (Konarska et al., 1990; Willner, 1990; Murua and Molina, 1992; Gamaro et al., 2003). The following stressors were used: (a) inclination of the home cages at a 45^{o} angle for 4 to 6 h, (b) 10 to 15 min of noise, (c) 1 h to 3 h of restraint, as described below, (d) 1.5 to 2 h of restraint at 4^{o} C, (e) forced swimming for 10 or 15 min, as described below, (f) flashing light during 2 to 4 h, (g) isolation (2 to 3 days). Animals were exposed to stress starting at a different time every day, in order to minimize its predictability.

Restraint was carried out by placing the animal in a 25 x 7 cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. Forced swimming was carried out by placing the animal in a glass tank measuring 50 x 47 x 40 cm with 30 cm of water at 23 + 2^{o} C. Exposure to flashing light was achieved by placing the animal in a 50 cm-high, 40 x 60 cm open field made of brown plywood with a frontal glass wall. A 100 W lamp, flashing at a frequency of 60 flashes per minute, was used.

Rats were submitted to chronic variate stress and/or treated with lithium chloride during forty days.

Tissue Preparation

Rats were killed by decapitation, on the day following the last day of stress exposure. Hippocampus, hypothalamus and cerebral cortex were dissected out and instantaneously placed in liquid nitrogen and stored at -70 ^{o}C until biochemical measurements, when the tissues were homogenized in 10 volumes of ice-cold phosphate buffer (0.1 M, pH 7.4) containing 140 mM KCl and 1 mM EDTA. The homogenate was centrifuged at 960 x g for 10 min and the supernatant was used.

Reactive oxygen species (ROS) formation

To assess the free radical levels, 2'-7'-dichlorofluorescein diacetate (DCFH-DA) was used as a probe. This method does not determine the presence of specific free radicals, because DCFH may be oxidized by several reactive intermediates (Wang and Joseph, 1999). An aliquot of the sample was incubated with DCFH-DA (100 μM) at 37^{o}C for 30 min; chilling the reaction mixture in ice terminated the reaction. The formation of the oxidized fluorescent derivative (DCF) was monitored at excitation and emission wavelengths of 488 nm 525 nm, respectively, using a fluorescence spectrophotometer (Hitachi F-2000). The free radicals content was quantified using a DCF standard curve and results were expressed as pmol of DCF formed/mg protein. All procedures were performed in the dark, and blanks containing DCFH-DA (no homogenate) and homogenate (no DCFH-DA) were processed for measurement of autofluorescence. Data were expressed as percentage of the control group.

Assay of lipid peroxidation

The formation of thiobarbituric acid reactive substances (TBARS) was used as an indicator of lipoperoxidation. Malondialdehyde (MDA), a product of lipoperoxidation, reacts with two molecules of thiobarbituric acid (TBA) at low pH and high temperature to form a pink-colored complex. Therefore, the formation of thiobarbituric acid reactive substances (TBARS) was expressed as MDA equivalents / mg of protein. This test was based on the

method described by Buege and Aust (1987). Aliquots of samples were incubated with 10% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA). The mixture was heated (30 min) on a boiling water bath. Afterwards, n-butanol was added and the mixture was centrifuged. The organic phase was collected to measure fluorescence at excitation and emission wavelengths of 515 and 553 nm (18), respectively. 1,1,3,3-Tetramethoxypropane, which is converted to malondialdehyde (MDA), was used as standard. Data were expressed as percentage of the control group.

Total Antioxidant Reactivity (TAR) Assay

This assay is based on luminol-enhanced chemiluminescence measurement, induced by an azo initiator (Evelson et al. 2001; Lissi et al. 1992, 1995). The reaction mixture contained 2 mM 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP), a source of peroxyl radicals, and 6 mM luminol in glycine buffer (0.1 M, pH 8.6). The chemiluminescence generated was measured in a scintillation counter (Beckman) working out of coincidence mode. The addition of Trolox (antioxidant standard, 200 nM) or samples decreases chemiluminescence levels, and TAR values were determined by assessing the initial decrease of luminescence calculated as the ratio "Io/I", where "Io" is the chemiluminescence (CL) in the absence of additives, and "I" is the CL after addition of the 200 nM Trolox, or the samples TAR values were expressed as equivalents of Trolox concentration per mg of protein. Data were expressed as percentage of the control group.

Enzymatic activities

Superoxide dismutase activity. SOD activity was determined using a RANSOD kit (Randox Laboratories Ltd., UK) which is based on a procedure previously described by Delmas-Beauvieux et al. (1995). This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye that is assayed spectrophotometrically

at 505 nm at 37°C. One unit of SOD activity is defined as the amount of enzyme that inhibits the rate of the formazan dye formation by 50% and the results were expressed as Units/mg protein.

Glutathione peroxidase activity. Glutathione peroxidase (GPx) activity was determined according to Wendel (1981). The reaction was carried out at 25°C in 600 μl of solution containing 100 mM pH 7.7 potassium phosphate buffer, 1 mM EDTA, 0.4 mM sodium azide, 2 mM GSH, 0.1 mM NADPH, 0.62 U of GSH reductase. The activity of selenium-dependent 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 rate. GPx activity was expressed as Units (nmol NADPH oxidized/minute)/mg protein.

Protein Assay

The total protein concentrations were determined using the method described by Lowry et al. (1951) with bovine serum albumin as the standard.

Statistical analysis

Data were analyzed using two-way analysis of variance (ANOVA) when evaluating possible interactions between chronic variate stress and lithium, with *post hoc* analysis performed by the Duncan's multiple range test. A difference was considered significant when $P < 0.05$. Results are expressed as mean ± standard error of the mean (SEM).

RESULTS

There were no significant effects of chronic variate stress and lithium treatments on oxidative stress in the frontal cortex. Table 1 shows the effects of stress and lithium on the ROS formation, as evaluated by the DCF test, on lipid peroxidation, evaluated by TBARS

test, and on the total antioxidant reactivity, indicating that neither of the treatments alter the oxidative status of this structure (Two-way ANOVA, $P > 0.05$ for all parameters).

Hypothalamic oxidative stress was also assessed by the measurements of lipid peroxidation and total antioxidant reactivity. In this case, we observed an absence of effect of both treatments on lipid peroxidation values (Figure 1A), but lithium treatment significantly increased TAR in this structure [Two-way ANOVA, $F(1, 18) = 4.253$, $P < 0.05$; Figure 1B], while there was no effect of stress [Two-way ANOVA, $F(1, 18) = 0{,}001$, $P > 0.1$] nor interaction between treatments [Two-way ANOVA, $F(1, 18) = 0.848$, $P > 0.1$] in this parameter. There were no effects of lithium or stress treatments on DCF test in this structure (Figure 1C).

Considering the altered TAR observed in lithium treated animals, we decided to verify the activity of antioxidant enzymes, i.e. superoxide dismuthase (SOD) and gluthathione peroxidase (GPx) in the hypothalamus of stressed and lithium-treated rats. We observed that SOD activity was significantly increased by lithium treatment [Two-way ANOVA, $F(1, 16) = 5.654$, $P < 0.05$; Figure 2A], and that there was a significant interaction between stress and lithium treatments, since the stressed + lithium group presented values higher than the other treatments alone [Two-way ANOVA, $F(1, 16) = 4.727$, $P < 0.05$]. There was no effect of stress in this parameter [$F(1, 18) = 3.462$, $P < 0.05$]. GPx activity was not significantly altered by stress or lithium treatments [Two-way ANOVA, $F(1, 19) = 0.015$, $P > 0.1$ for stress and $F(1, 19) = 0.959$, $P > 0.1$ for lithium treatment; Figure 2B]; however there was a significant interaction between stress and lithium treatments [Two-way ANOVA, $F(1, 19) = 6.282$, $P < 0.05$].

In hippocampus, it was observed that lithium treatment significantly decreased the ROS formation [Two-way ANOVA, $F(1, 19) = 8.038$, $P < 0.01$, Figure 3A], and there was an absence of effect of chronic stress or interaction between treatments [Two-way ANOVA, F

(1, 19) = 0.221, P > 0.1 for stress and F (1, 19) = 0.318, P > 0.1 for the interaction]. On the other hand, chronic stress induced an increased lipid peroxidation [Two-way ANOVA, F (1, 17) = 6.524, P < 0.05, Figure 3B], with no effect of lithium, nor interaction, in this parameter [Two-way ANOVA, F (1, 17) = 3.299, P > 0.05 for lithium and F (1, 17) = 0.321, P > 0.1 for interaction].

When evaluating the TAR in the hippocampus of chronically stressed and lithium-treated rats, we observed a strongly significant interaction, since both treatments separately increased the antioxidant reactivity, but when put together the animals presented values similar to the control group [Two-way ANOVA, F (1, 19) = 0.060, P > 0.1 for stress effects, F (1, 19) = 1.325, P > 0.1 for lithium effects and F (1, 19) = 32.489, P < 0.001 for the interaction, Figure 3C]. A post-hoc analysis showed that both stress- and lithium-treated animals have increased TAR values when comparing to the control and stress + lithium groups (Duncan's multiple range test, P < 0.001).

To better characterize the effects of stress and lithium in the hippocampus, we also measured SOD and GPx activities. We observed that both stress and lithium treatments induced an altered activity of SOD, and that there was a significant interaction between treatments [Two-way ANOVA, F (1, 17) = 22.076, P < 0.001 for stress effects, F (1, 17) = 6.549, P < 0.02 for lithium effects and F (1, 17) = 8.413, P < 0.01 for the interaction, Figure 4A]. GPx activity was also increased by lithium treatment [Two-way ANOVA, F (1, 18) = 6.013, P < 0.03], but there was no effect of stress, nor interaction, on this enzyme activity [F (1, 18) = 0.842, P > 0.1 for stress effects and F (1, 18) = 0.233, P > 0.1 for the interaction, Figure 4B].

DISCUSSION

This study was performed to evaluate the effects of chronic variate stress and lithium treatments on oxidative stress parameters in different brain structures. We observed that chronic exposure to stress induced an increase in the oxidative status in hippocampus, as showed by the increased lipid peroxidation, and that lithium treatment presented an antioxidant effect, demonstrated by decreased ROS generation in hippocampus and increased antioxidant reactivity. The effects of both treatments were region-specific and the hippocampus was the most affected structure; these results agree with several other works showing that the hippocampus, as well as hippocampus-related behaviors, are strongly affected by stress exposure and lithium treatment (Vasconcellos et al., 2003, 2005).

The results of this study agree with other reports showing that exposure to GCs or to stress may lead to oxidative injury in various tissues. Glucocorticoids in cell cultures increase ROS production, as measured by the DCF test (McIntosh and Sapolsky, 1996), and an increased oxidative damage has been proposed as one of the mechanisms through which glucocorticoids increase the vulnerability of different brain regions, particularly the hippocampus, to metabolic insults (McIntosh and Sapolsky, 1996; McIntosh et al., 1998a, 1998b). Besides the reported effects of glucocorticoids, exposure to stress has also been shown to increase lipid peroxidation in plasma of acutely stressed rats (Liu et al., 1994; Oishi et al., 1999), and in hippocampus, after repeated restraint stress (Fontella et al., 2005). Effects of a different model of chronic variate stress on TBARS in brain have also been observed, where distinct brain regions show different responses to stress (Manoli et al., 2000). Therefore, different models of chronic stress apparently lead to different results concerning induction of oxidative stress. The present findings support the idea that stress produces oxidants, and it is possible that the oxidative damage in stress could contribute, at least in part, to stress-related diseases, such as depression.

Exactly how increased lipoperoxidation occurs in the hippocampus of stressed animals, in the absence of any alteration in free radicals content (evaluated by the DCF test) is not clear. Measurements were made one day after the last stress procedures, in order to verify alterations induced by chronic stress, and not responses to any particular stress session. Therefore, it is possible that increased free radicals production occurs during exposure to some determined stressor (s) (since different stressors were used in this model), and that these levels may return to normal at the time of the sacrifice. In addition, glucocorticoids (which have been reported to increase ROS production, as measured by the DCF test, see 1) have already returned to basal levels at the time of the sacrifice. Similar results, i.e., increased TBARS levels without increased ROS formation in the DCF test, were also observed after another model of chronic stress (Fontella et al., 2005).

On the other hand, lithium treatment decreased free radicals generation (as measured by DCF test) in hippocampus, with no effects in the other structures examined. Additionally, an increased TAR was observed in hippocampus and hypothalamus after lithium treatment; in hippocampus, however, a significant interaction with stress treatment occurred, and the group stress + lithium presented TAR levels similar to the control group. These results are in agreement with other studies, which suggest an antioxidant effect of lithium (Shao et al., 2005; King and Jope, 2005). Despite these effects, lithium treatment was not able to prevent stress-induced increase in macromolecules damage, such as lipoperoxidation, in hippocampus.

The analysis of antioxidant enzymes indicates that lithium treatment was able to increase glutathione peroxidase activity in hippocampus homogenates, as well as it increased SOD activity in hypothalamus, further suggesting an antioxidant effect of lithium chronic treatment. We cannot explain at the present the altered activity of these enzymes in one structure, and the lack of alteration in the other, after in vivo lithium administration; however

other studies have also observed variable alterations in SOD or GPx activities after chronic lithium treatment in rats when different tissues were studied (Kielczykowska et al., 2004).

In addition, chronic stress induced an increased SOD activity in hippocampus, maybe as an adaptation to this chronic situation. The most intriguing result, however, was the interaction observed between lithium and stress treatments on SOD activity, which was observed both in hippocampus and hypothalamus. In both structures, SOD activity levels were potentiated when both treatment were applied together. It is interesting to note that lithium added in vitro (in the range of concentrations from 0.1 to 10 mM) did not affect this enzyme activity (data not shown), suggesting that the in vivo action of lithium on SOD activity is probably not a simple enzyme activation induced by the presence of this salt in the medium, but possibly involves an indirect effect, which is induced in vivo.

Although the precise mechanisms involved in the unexpected potentiation of SOD activity in rats receiving both lithium and stress treatments, as observed in this study, are not known, it has been reported that lithium increases plasmatic levels of corticosterone (Levine and Saltzman, 2005; Husum and Mathe, 2002) suggesting that a further activation of the HPA axis in chronically stressed rats may be induced by lithium, possibly causing this interaction. However, this potentiation may also suggest that these two treatments act through different and additive mechanisms to induce this increased SOD activity. These effects could be either on the number of molecules and/or on the degree of activation of this enzyme. Further studies are necessary to elucidate these mechanisms.

SOD and GPx are responsible for degradation of $O_2^{\cdot-}$ and H_2O_2, respectively. The balance between SOD and GPX activities has been suggested to be more important than the absolute amount of any one of them, for the protection against free radicals (Ceballos-Picot et al.,1992; Erakovic et al., 2000). In vitro, GPx was demonstrated to offer greater protection than SOD against oxidative stress, maybe because it may use different peroxides besides

H_2O_2 (Masella et al., 2005; Wesbrot-Lefkowitz et al., 1998). An important point, however, is that SOD transforms $O_2^{\cdot-}$, producing H_2O_2, which needs to be detoxified by another enzyme, such as GPx. An imbalance between these two activities, such as that observed in the stressed and stressed + lithium group, may result in the accumulation of H_2O_2, which undergoes Fenton's reaction, generating hydroxyl radicals which may lead to lipid peroxidation (Michiels et al., 1994). This increased production of free radicals, specially in the stressed + lithium group (in which the interaction induces a higher increase in SOD activity) could be responsible for the consumption of antioxidants in the tissue, leading to a decreased total antioxidant reactivity, as measured by TAR test in the hippocampus.

In conclusion, the present findings suggest that lithium has antioxidant properties, while chronic stress presents pro-oxidant effects, mainly in hippocampus. Nevertheless, chronic lithium was not able to prevent lipoperoxidation induced by stress exposure, and presented an interaction with chronic stress, leading to a imbalance of SOD and GPx enzymatic activities, which should be taken into account when considering lithium therapy.

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TABLES:

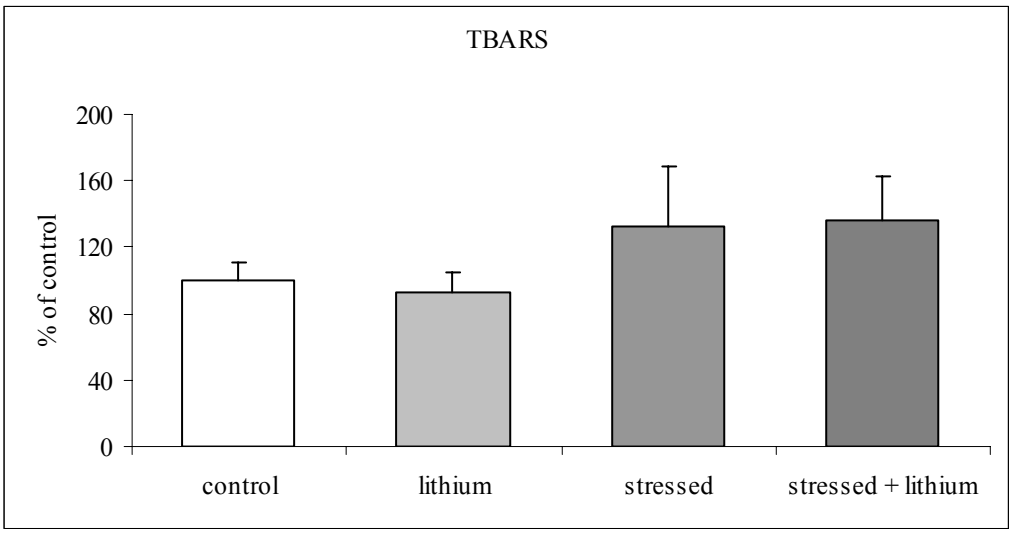
	Control	Lithium	Stressed	Stressed + Lithium
TBARS	100.13 ± 3.5	103.07 ± 6.4	82.55 ± 6.22	104.58 ± 7.48
DCF	100.12 ± 2.7	121.36 ± 11.2	104.83 ± 5.2	99.99 ± 7.68
TAR	100.01 ± 8.5	111.61 ± 7.6	146.13 ± 20.7	110.90 ± 13.7

Table 1: Effects of chronic variate stress and chronic lithium treatments on oxidative stress parameters in frontal cortex of rats. Data are expressed as mean + standard error of the mean of percentage of the control gorup, N of 6 animals/group.

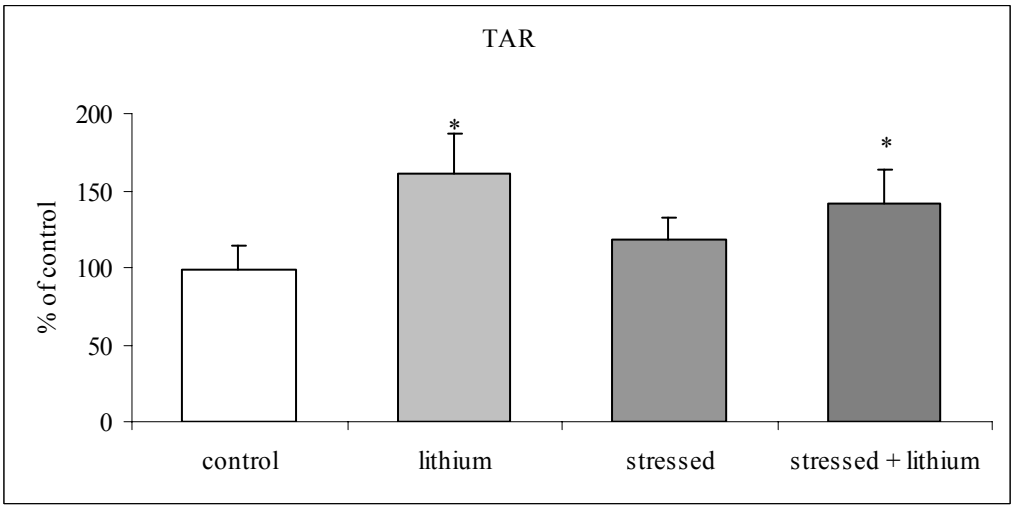
There was no effect of chronic variate stress and lithium treatments on lipid peroxidation (TBARS), on free radicals formation (DCF) nor on antioxidant reactivity (TAR) in this structure (Two-way ANOVA, $P > 0.05$ for all parameters)

FIGURES:

1A



1B



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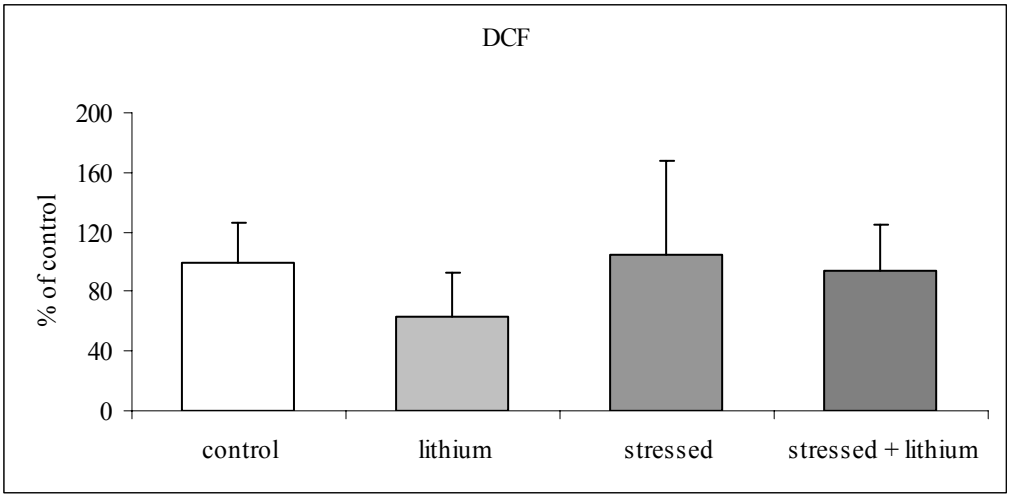


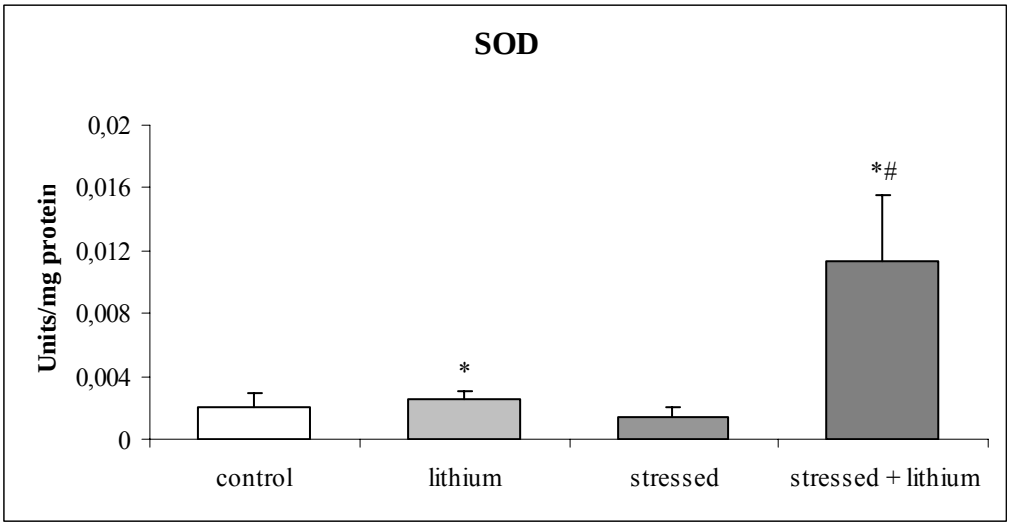
Figure 1: Effects of chronic variate stress and chronic lithium treatments on oxidative stress parameters in hypothalamus of rats. Data are expressed as mean + standard error of the mean, N of 5-6 animals/group.

1A: Thiobarbituric acid reactive substances in hypothalamus homogenates of chronically stressed and lithium-treated rats. There were no significant differences among groups.

1B: Total antioxidant reactivity in hypothalamus homogenates of chronically stressed and lithium treated rats. *: Significantly different from control and stressed groups (Two-way ANOVA, $P < 0.05$)

1C: Reactive oxygen species (ROS) formation, assessed by the DCF test, in hypothalamus homogenates of chronically stressed and lithium-treated rats. There were no significant differences among groups.

2A



2B

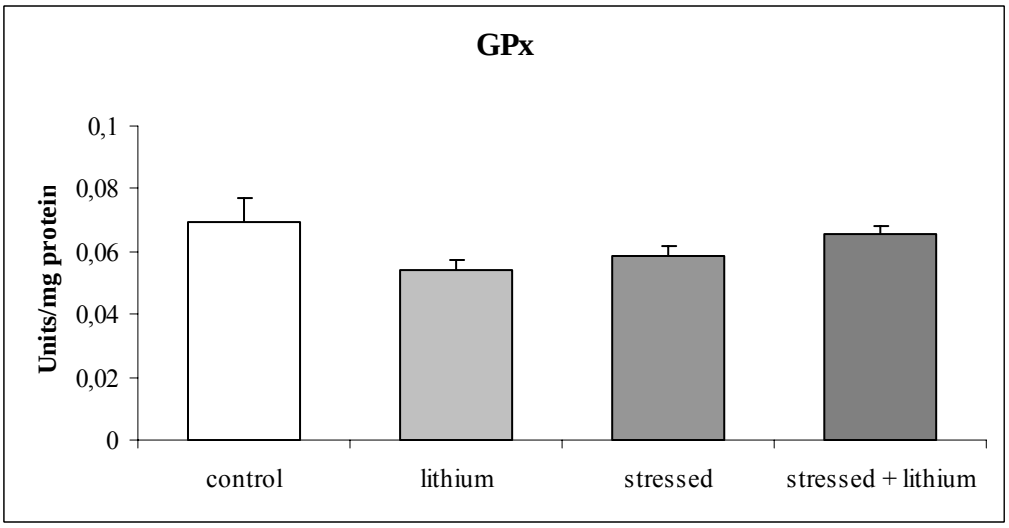
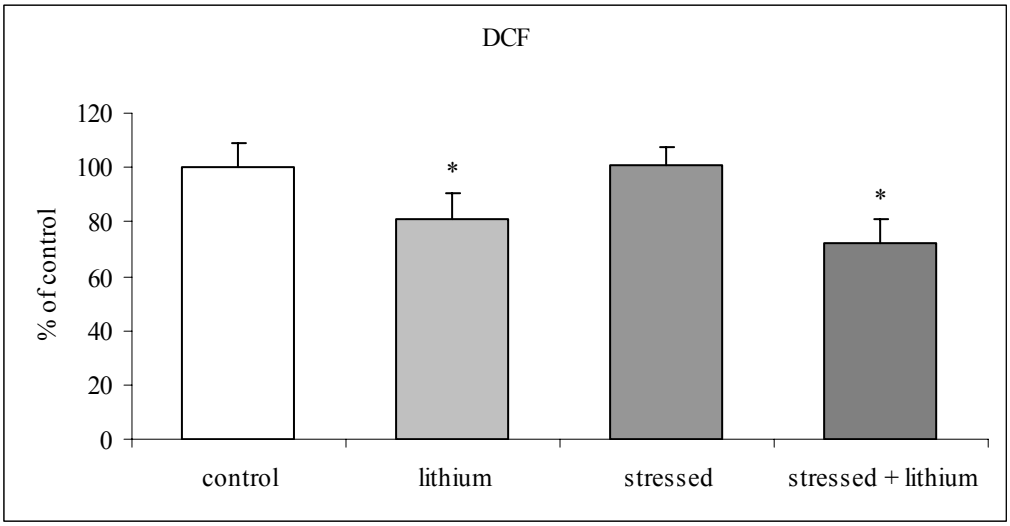


Figure 2: Effects of chronic variate stress and chronic lithium treatments on the activity of antioxidant enzymes in hypothalamus of rats. Data are expressed as mean + standard error of the mean, N of 5-6 animals/group.

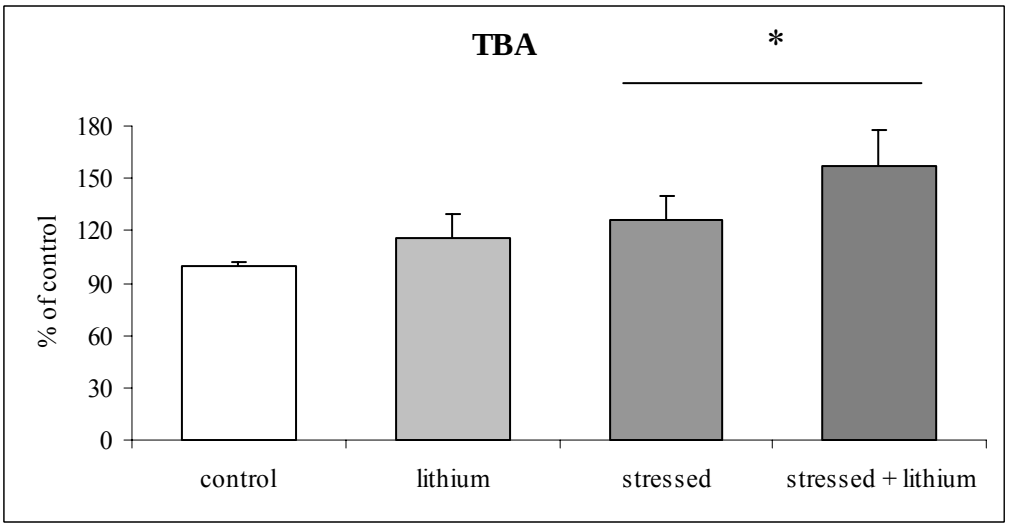
2A: Superoxide dismuthase activity in hypothalamus of chronically stressed and lithium-treated rats. *There was a significant effect of lithium treatment (two-way ANOVA, P=0.03 and *#there was a significant interaction between treatments (two-way ANOVA, $P < 0.05$).

2B: Gluthathione peroxidase activity in hypothalamus of chronically stressed and lithium treated rats. There were no significant differences among groups, but a significant interaction was observed between stress and lithium treatments (two-way ANOVA, $P<0.05$).

3A


DCF
% of control
120
100
80
60
40
20
0
*
*
control
lithium
stressed
stressed + lithium

3B


TBA
*
% of control
180
150
120
90
60
30
0
control
lithium
stressed
stressed + lithium

3C

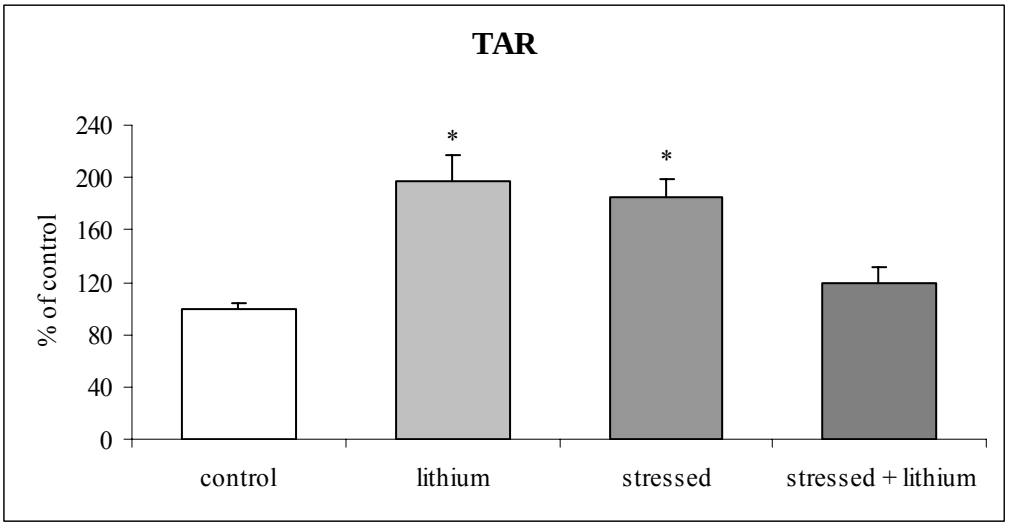


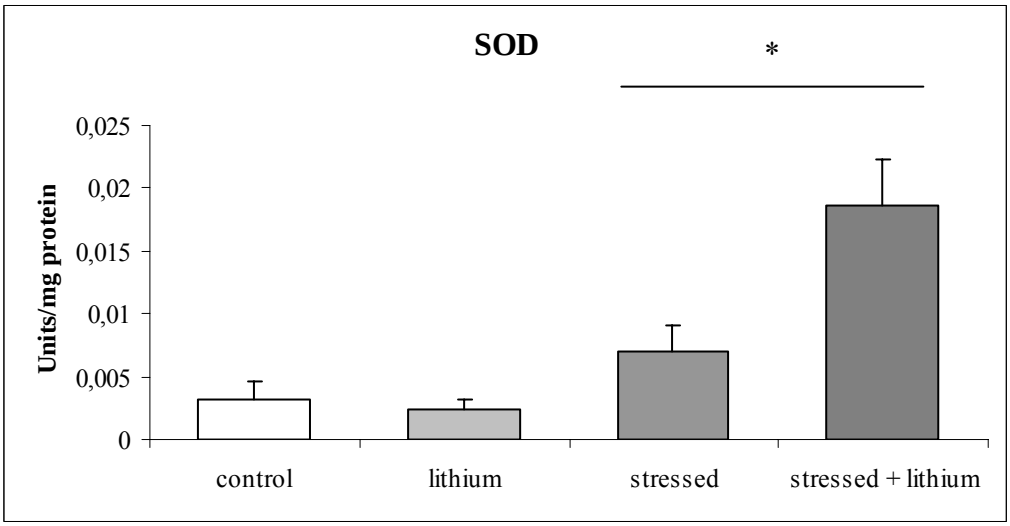
Figure 3: Effects of chronic variate stress and chronic lithium treatments on oxidative stress parameters in hippocampus of rats. Data are expressed as mean + standard error of the mean of percentage of the control group, N of 6 animals/group.

3A: Reactive oxygen species (ROS) formation, assessed by the DCF test, in hippocampus homogenates of chronically stressed and lithium-treated rats. There was a significant effect of lithium treatment in this parameter. *: Significantly different from control and stressed groups (Two-way ANOVA, $P < 0.01$)

3B: Thiobarbituric acid reactive substances in hippocampus homogenates of chronically stressed and lithium-treated rats. There was a significant effect of stress in this parameter. *: Significantly different from control and lithium-treated groups (Two-way ANOVA, $P < 0.03$).

3C: Total antioxidant reactivity in hypothalamus homogenates of chronically stressed and lithium treated rats. There was a significant interaction between stress and lithium treatments. *: Significantly different from control and stressed + lithium groups (Two-way ANOVA, $P < 0.001$)

4A



4B

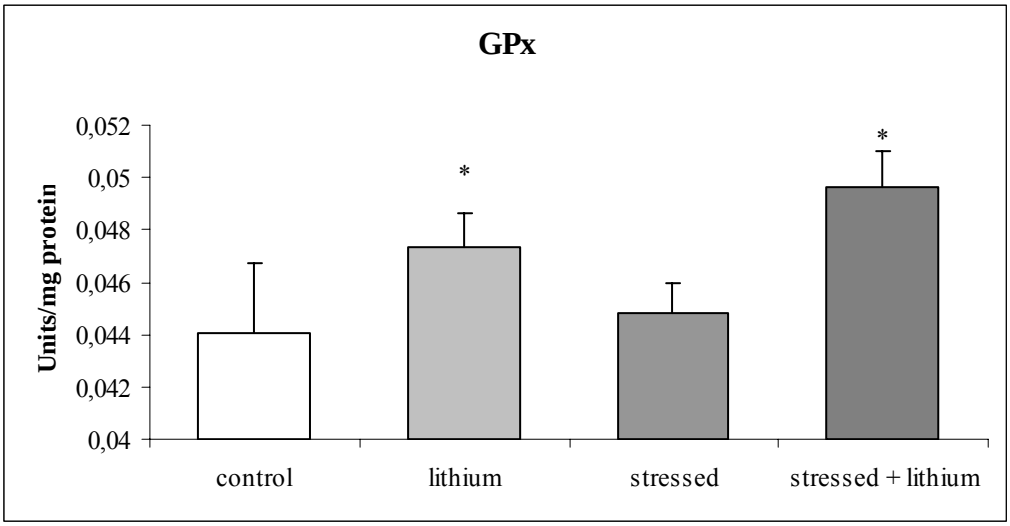


Figure 4: Effects of chronic variate stress and chronic lithium treatments on the activity of antioxidant enzymes in hippocampus of rats. Data are expressed as mean + standard error of the mean, N of 5-6 animals/group.

4A: Superoxide dismuthase activity in hippocampus of chronically stressed and lithium-treated rats. *There was a significant effect of chronic stress (two-way ANOVA, $P<0.001$)

and a significant interaction between stress and lithium treatments (two-way ANOVA, $P<0.01$).

4B: Gluthathione peroxidase activity in hippocampus of chronically stressed and lithium treated rats. There was a significant effect of lithium treatment in this parameter. *: Significantly different from control and stressed groups (Two-way ANOVA, $P < 0.03$).

Artigo 4:

CHRONIC VARIATE STRESS AND LITHIUM ALTER FEEDING BEHAVIOR OF RATS

De Vasconcellos et al.

A ser submetido.

CHRONIC VARIATE STRESS AND LITHIUM ALTER FEEDING BEHAVIOR OF RATS

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ABSTRACT:

The control of feeding behavior is a complex mechanism that includes appetite, motivation and caloric demands of the organism, and may be altered by different factors, such as availability of nutrients and emotional status. Exposure to stress can alter food intake and preference and this effect can be, or not, related to the anxiety levels of the animals. The purpose of this study was to characterize the feeding behavior of rats submitted to a chronic variate stress paradigm and chronic lithium treatment, and to verify if there is a relationship between feeding behavior and anxiety levels. Adult male Wistar rats were divided into two groups; control and submitted to a chronic variate stress paradigm, and subdivided into treated or not with LiCl. After forty days of treatment, the ingestion of standard lab chow, sweet (Froot Loops Kellogg's ®) and savory (salty peanuts) food was evaluated, as well as the behavior in the elevated Plus Maze. Both stress and lithium treatments were observed to increase sweet food ingestion, not altering standard lab chow consumption, while only lithium increased the ingestion of savory food. Stress did not affect the time spent in the open arms of the Plus Maze, while lithium had an anxiolytic effect in this task. These results suggest that the increase in sweet food consumption observed in stressed and lithium-treated animals is not an anxiety related effect, and that the increased interest for more caloric foods may be the cause for the gain of weight observed in lithium-treated patients.

Key words: Chronic Variate Stress, Lithium, Feeding Behavior, Sweet Food Ingestion, Plus Maze, Anxiety.

INTRODUCTION

Feeding behavior and energy balance are controlled by a complex network of interacting feedback mechanisms that involve the hypothalamus, the brain stem, higher brain centers, and, in the periphery, stomach, gut, liver, pancreas, thyroid and adipose tissue [45]. Of these structures, the hypothalamus appears to be the master regulator of energy metabolism, responding to several hormonal signals as well as directly responding to nutritional levels [29],[39]. In addition to metabolic and nutritional aspects, food intake has an intrinsic hedonic factor, since eating is a source of pleasure and has been shown to activate reward circuits that are also involved in the response to drugs of abuse [12][68]]. All these aspects make feeding behavior a complex matter, and the increase in feeding-related disorders, such as obesity, has led to a strong emphasis in research in this field.

Emotional changes, such as exposure to stress situations, can influence feeding behavior, and several studies have demonstrated that chronic exposure to stressors may alter food intake and body weight of rats [14][48][15][18][48]. Different types of stressor may present different effects on the consumption of foods. For example, physical and emotional kinds of stress differently affect saccharine drinking, indicating a different response to rewarding stimuli [48], and inescapable shock affects food intake and reduce weight gain, with shocked rats gaining significantly less weight than control or submitted to stress by restraint [14]. Previous studies from our laboratory demonstrate that animals that are repeatedly stressed by restraint present increased ingestion of sweet food [15]. This effect was reversed by diazepam administration, indicating that anxiety is an important factor influencing eating behavior in this stress model. In another set of studies, it has been shown that neonatal stress, which leads to permanent alterations in hypothalamus-pituitary-adrenal (HPA) axis responsiveness, induces an increase in palatable food consumption, an effect that

was not related to anxiety [57][58]. In contrast, rats submitted to chronic mild stress, which has been proposed as model of depression in animals studies [36][49][72][73], present a diminished response to rewarding stimuli, as demonstrated by tests showing reduced sucrose consumption [38][3], as well as a decrease in sweet food ingestion [18]. Taken together, these data indicate that different models of chronic stress may lead to different effects on feeding behavior.

Studies in humans have provided further evidence of overeating induced by emotional experiences [76]. Increased consumption of carbohydrates has been observed following exposure to stress [41], with patients exposed to stress presenting the tendency to overeat carbohydrates to make themselves feel better, and suggesting that the pleasurable consequence (reinforcement) that results from ingesting carbohydrate-rich food may serve to insure this type of feeding behavior. Nevertheless, feeding during times when glucocorticoids are high also stimulates insulin secretion [10] and, together, the increase in both hormones may ultimately result in a remodeling of energy stores from muscle to fat, especially in the abdominal region [11][52]. Thus, although eating leads to a "feeling better" emotion, this mechanism leads to an increased risk of developing obesity and obesity-related diseases (cardiovascular, diabetes) since these diseases are particularly associated with abdominal obesity [52][54][70].

Lithium salts are in the first line of therapeutic drugs used to treat affective disorders, mainly bipolar disorder, although its mechanism of action remains unclear. Its clinical profile includes antimanic and antidepressant actions, as well as prophylaxis of both mania and depression, by reducing the frequency of bipolar episodes [44][56]. Despite being widely used and generally chosen for the treatment of affective disorders, patients submitted to lithium treatment present a high rate of forsaking/treatment interruption, and one of the main motives that lead patients to give up treatment is weight gain.

We have previously shown that lithium is able to prevent some behavioral effects induced by a chronic variate stress model [65]. In this study, we aimed to investigate the effects of a chronic variate stress paradigm on the feeding behavior of rats and the interaction between stress and lithium treatments in this parameter. Since some studies have related feeding behavior to the level of anxiety that animals experience, we also evaluated the effects of stress and lithium treatments on behavior in the Plus Maze test.

MATERIAL AND METHODS:

Subjects:

One hundred and sixty five adult male Wistar rats (60 days at the beginning of the treatment) weighing 160-230 g were used. Experimentally-naive animals were housed in groups of 4 or 5 rats in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust. They were maintained under a standard dark-light cycle (lights on between 7:00 a.m. and 7:00 p.m.), with a room temperature of 22 + 2 °C. Rats had free access to food and water, except during the period when restraint stress or forced swimming were applied, or during the period of habituation to a different food (see below). All animal treatments were in accordance with the institutional guidelines and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS).

Chronic Variate Stress Model

Chronic variate stress was modified from other models of variate stress [18][37][46][72][73]. The animals were submitted to one of the following stressors each day: (a) inclination of the home cages at a 45^{o} angle for 4 to 6 h, (b) 10 to 15 min of noise, (c) 1 h to 3 h of restraint, as described below, (d) 1.5 to 2 h of restraint at 4^{o} C, (e) forced swimming

for 10 or 15 min, as described below, (f) flashing light during 2 to 4 h, (g) isolation (2 to 3 days). Animals were exposed to stress starting at a different times everyday, in order to minimize its predictability.

Restraint was carried out by placing the animal in a 25 x 7 cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. Forced swimming was carried out by placing the animal in a glass tank measuring 50 x 47 x 40 cm with 30 cm of water at 23 + 2^{o} C. Exposure to flashing light was achieved by placing the animals in a 50 cm-high, 40 x 60 cm open field made of brown plywood with a frontal glass wall. A 100 W lamp, flashing at a frequency of 60 flashes per minute, was used.

Experimental groups

The animals were divided in two groups: control and submitted to a chronic variate stress paradigm. These groups were subdivided in two other groups, treated or not with lithium. The groups treated with lithium had lithium chloride (LiCl - 2.5 mg/g of chow) and NaCl (17 mg/g) added to the food, as described before [65]. This treatment has been previously used, and at the end of a period of four weeks or more animals remain healthy and present lithium levels in the range of 0.6 – 1.2 mM [51][65], similar to the levels observed in treated patients. The groups not treated with lithium received standard rat chow with NaCl (17 mg/g), in order to standardize the taste of the food, since taste could interfere in the preference for new flavors.

In another experiment, we evaluated the effects of stress interruption on feeding behavior. For this study, rats were divided in two groups, control and chronically stressed for forty days. After that, stressed animals were subdivided into two more groups: one had stress

interrupted, and the other continued to be stressed. After thirty additional days of treatment, rats were submitted to the behavioral evaluations.

Feeding behavior evaluation:

At the end of the chronic treatments, rats were exposed to the behavioral tests. For evaluation of standard lab chow ingestion, rats were placed in single home cages and the ingestion of food was measured for three consecutive days. Ingestion was measured by leaving a determined amount of food in the cage and checking the remainder the next day. The first day of measurement was not considered in the analysis to exclude the effect of acute social isolation stress.

For sweet food ingestion, the animals were placed in a lightened rectangular box (40 x 15 x 20 cm) with floor and side walls made of wood and a glass ceiling. Ten Froot loops (Kellogg's ® - pellets of wheat and corn starch and sucrose) were placed in one extremity of the box. Each animal was submitted to five habituation trials of three minutes each, on different days, to familiarize them with the new food [15], and this habituation was performed under food restriction (80% of habitual ingestion of standard lab chow), in order to increase the interest for the new food. On the sixth day, animals fed *ad libitum* were exposed to a test session, when the number of ingested pellets was measured after a three-minute period. Only animals that tasted the new food during the habituation period were evaluated. A protocol was established in such a way that when the animals ate part of a Froot loop (e.g. 1/3 or 1/4), this fraction was considered.

For savory food ingestion, 10 g of salty peanuts were placed in the same apparatus. Habituation was established in another 5 days, and testing was performed in the same manner as described above. Peanut consumption was evaluated by weighing the total amount before and after the test session.

Plus maze test

The elevated plus maze apparatus was made of wood and consisted of two opposed open arms (50 X 10 cm), two opposed enclosed arms with no roof (50 X 10 X 40 cm), and an open square (10 X 10 cm) in the center. The maze was elevated 50 cm above the floor. The behavioral test was conducted in the observational room using red light illumination. The animal was placed into the center of the plus-maze, facing one of the open arms, and remained in the apparatus for five minutes. We analyzed the number of entries and the time spent in open or enclosed arms.

Statistical Analysis:

For chow intake, gain of weight and Plus Maze results, data were expressed as mean + standard error of the mean. Chow intake and Plus Maze data were analyzed using two-way ANOVA, and gain of weight was evaluated using repeated-measures ANOVA. For sweet and savory food consumption, data were expressed as medians (interquartile range). The comparison among all groups was made by Kruskal-Wallis test, and comparisons between two independent groups were made by Mann-Whitney U test. A *P*-value of 0.05 was considered statistically significant.

RESULTS:

Body weight:

Body weight was measured at the beginning of chronic stress and lithium treatments, and then measured once a week. The body weight gain curve is shown in Figure 1. A repeated-measures ANOVA showed that all groups gained weight across time ($F(3.54, 127.6) = 456.628$; $P < 0.001$, N=10 animals per group). A significant interaction between time

and stress was also observed (F (3.54, 127.6) = 3.539; *P* = 0.01) and between time and lithium treatment (F (3.54, 127.6) = 26.085; *P* < 0.001), since both treatments induced a delay in the gain of weight, with lithium-treated animals gaining significantly less weight in the first two weeks of treatment (F (1, 36) = 6.689; *P* = 0.014).

Standard lab chow ingestion:

Figure 2 shows the effects of chronic stress and lithium treatments on standard lab chow consumption. A two-way ANOVA shows that there was no significant effect of stress (F (1, 45) = 2.175) or lithium (F (1, 45) = 1.492), nor any interaction between these treatments in this parameter (F (1, 45) = 0.920).

Evaluation of sweet and savory food ingestion:

When evaluating sweet food ingestion, a significant difference was observed among groups in the number of sweet pellets consumed (Kruskal-Wallis test, χ^2 (3) = 25.864, P < 0.001), while there was no difference in the latency to cross the maze and reach the food (Kruskal-Wallis test, χ^2 (3) = 2.255, P = 0.521). Both chronic stress and lithium induced an increase in sweet food consumption when compared to the control group (Mann Whitney U test, P < 0.001 for the lithium treated group and P = 0.015 for the stressed group, Figure 3A). Indeed, the increase induced by lithium treatment was much stronger then the effect induced by chronic variate stress (Mann Whitney U test, P < 0.001). Additionally, the group receiving both treatments presented increased consumption of sweet food, similar to that observed in the lithium treatment (Mann Whitney U test, P < 0.001 compared to control and stressed groups; P = 0.427 compared to lithium-treated group).

We also evaluated the effects of chronic stress and lithium treatments on the consumption of salty peanuts. We observed a significant difference between groups (Kruskal-Wallis test, χ^2 (3) = 29.066, P < 0.001). While chronic stress had no effect on salty peanut intake, lithium treatment had a significant effect on consumption, as is displayed in Figure 3B

(Mann Whitney U test, $P < 0.001$ for the lithium-treated group and $P > 0.1$ for the stressed group).

Since we observed an increased interest in sweet food in the stressed group, we also evaluated the effects of stress interruption on sweet food consumption. It was observed that, even thirty days after the interruption of stress exposure, the animals still presented enhanced intake of sweet food (Kruskal-Wallis test, $P = 0.036$, Figure 3C).

Behavioral evaluation in the Plus Maze test:

Eating behavior disorders have been postulated to be related to anxiety and/or stress[13][15]. In this experiment, we investigate the effects of chronic variate stress and lithium treatments on the Plus Maze task. A two-way ANOVA showed a significant increase in the percentage of time that lithium-treated rats spent in the open arms [$F(1, 34) = 12.060$; $P < 0.001$], with no effect of chronic stress, as well as no interaction between stress and lithium treatments in this parameter [$F(1, 34) = 0.148$ for stress; $F(1, 34) = 1.749$; for interaction]. There was no effect of these treatments in the percentage of time spent in the enclosed arms (Figure 4A).

There was also a significant difference in the number of entries into the open arms, with an increase induced by lithium in the number of entries [$F(1, 34) = 14.963$; $P < 0.001$] and a decrease induced by stress [$F(1, 34) = 5.827$; $P = 0.02$], as may be observed in Figure 4B. There was no interaction between stress and lithium [$F(1, 34) = 1.949$], and no effect of these treatments on the number of entries into the enclosed arms.

DISCUSSION

In this study, we observed that chronic variate stress, which was adapted from some other models of chronic mild stress [18][37][46][72][73], induces an increase in the consumption of sweet food, and that this increase was specific for sweet food, since there was no effect on the consumption of savory food and standard lab chow. This alteration in feeding behavior was not accompanied by an anxiety-like behavior, since stressed rats, although presenting a small decrease in the number of entries, did not diminish the percentage of time spent in the open arms of the plus maze task, which is considered an index of anxiety of the animals. Previous studies from our laboratory demonstrated the effects of different models of stress on sweet consumption. For example, chronic-restraint stress induced an increase in sweet food consumption [15][64]. Intriguingly, while we found an increased preference for sweet food, a similar chronic variate stress model decreased it [18]. It is important to note, however, that the stress model used by Gamaro et al. (2003) differs from the model used in this study, since it included periods of 24 h of food deprivation, once a week. Periods of food restriction have been reported to induce an increased release of serotonin in the hypothalamus, which is the regulatory center for energy balance [22]. In pharmacological studies, drugs that increase postsynaptic serotonergic stimulation routinely decrease food consumption in mammalian species ranging from rodents to human and nonhuman primates [1][8],[17][25][67]. The general consensus developed, therefore, is that 5HT serves an inhibitory role in feeding [59]. Periods of food restriction could induce a compensatory mechanism by increasing 5-HT secretion, thereby reducing feeding behavior. It may be suggested that, in chronic variate stress models, using food deprivation as a stressor could lead to a different outcome, at least in studies related to feeding behavior.

The central mechanisms involved in stress-induced overeating are very complex. Many agents stimulate food intake, such as α-adrenoceptors agonists, beta-endorphin, dynorphin, neuropeptide Y (NPY) and galanine [6][5]. The main effectors of the HPA axis, corticotrophin-releasing hormone (CRH) and glucocorticoids, also affect feeding behavior and energy expenditure. CRH participates in the regulation of appetite and energy expenditure [33][34], inhibiting food intake, for example, by modulation of NPY activity [26]. Glucocorticoids, which promote a negative feed-back on CRH neurons, may also induce alterations in appetite. Therapeutic doses of glucocorticoids in humans increase energy intake, an effect that may be related to their ability to act directly or indirectly on the central regulation of appetite [40][62]. Glucocorticoid levels within the physiological range can interfere with the action of leptin, a satiety signal released by adipocytes, reversing its effects partly independently of NPY [61].

The effects of pharmacological and behavioral treatments on the hedonic response to feeding are another important aspect of eating behavior. Consistent with this, it should be taken into account that stress may increase the release of endogenous opioids, which are proposed to play a role in the control of food intake by providing the hedonic/pleasurable aspects of food intake [4][7][40]. Besides opioids, the dopaminergic system mediates the incentive salience aspect of feeding. Since corticosterone facilitates induction of drug addiction, possibly by a sensitization-associated phenomenon involving dopamine [21], it is plausible that stress may induce a greater sensitivity to the reinforcing effects of palatable foods, thus increasing sweet food consumption.

It is interesting to note that stressed animals prefer to ingest sucrose compared to saccharin [14], favoring the idea that hedonic and metabolic factors influence feeding behavior, since sucrose is a palatable and highly energetic substrate. It has been proposed that carbohydrates may promote feed back on the HPA axis, based on the findings that sucrose

ingestion normalizes feeding, energy balance and central corticotrophin-releasing hormone expression in adrenalectomized rats [40]. Considering these modulatory actions of carbohydrate ingestion on both basal and stress-induced activity in the HPA axis, it is conceivable that the increase in sweet food consumption may be an adaptive response to stress, in an attempt to normalize HPA axis and restore basal levels of circulating glucocorticoids.

In addition, thirty days of stress interruption were not able to reverse the effects of stress on sweet food consumption, indicating that the alterations induced by chronic stress on feeding behavior are long lasting. Several reports from the literature show that the consequences of stress are long lasting. Handling during the neonatal period leads to alterations in feeding behavior that persist through adulthood [57]. In adult animals, behavioral, physiological and morphological consequences of stress are often long lasting (e.g., [2],[9][69]). The specific pathways, activated by this chronic stress model, that underlie the development of this long-term effect on feeding behavior remain to be tested.

In this study, lithium treatment was observed to have anxiolytic properties, since rats treated with lithium spent more time in the open arms of the plus maze task, and presented an increased number of entries into the open arms. This was an expected result, since lithium is used as an adjuvant to the treatment of anxiety and depressive disorders [63]. These results are not related to any alteration in motor activity, since no differences were observed in the number of crossings or rearings between groups when these animals were exposed to an open field (data not shown). In previous studies, we observed that lithium treatment is able to prevent and reverse some behavioral and biochemical alterations induced by chronic-variate stress, such as learning deficits and decreased Na^+,K^+-ATPase activity [65],[66]. Considering these results, our initial hypothesis was that lithium could also interfere with the effects of stress on feeding behavior. Nevertheless, this was not the case. We observed that lithium-

treated rats gained less weight in the first weeks of treatment, but that later they started to gain weight in a similar fashion to the control group. This effect is probably due to the initial adaptation to the new food (i.e. standard chow added with lithium and sodium chloride) and, as was observed, does not reflect an altered food intake. Despite this, when exposed to sweet or savory palatable foods, rats treated with lithium presented a significant increase in palatable food consumption, and this increase was significantly higher than that induced by chronic stress.

One of the side effects of lithium in the treatment of bipolar disorder and other mood disorders is weight gain [78], increasing the risk of patients developing diabetes, cardiovascular diseases and other obesity-related diseases [28]. As observed in the present study, rats treated with lithium presented an increased preference for more caloric (i.e. sweet and fatty foods) and palatable foods, with no increase in normal chow intake. Although lithium-treated rats did not present a higher weight gain, it is important to consider that they did not have free access to these palatable foods, which were used just during the testing sessions. Therefore, a possible explanation for the weight gain in patients treated with lithium is an increased preference for palatable foods in an environment where seductive and highly caloric foods are readily available.

The mechanism underlying the effect of lithium treatment on palatable food intake is not determined. Chronic treatment with lithium has been shown to increase mRNA and peptide levels of neuropeptide Y (NPY), [30],[31][77], which presents a stimulatory effect on feeding behavior, besides being considered to be involved in the pathogenesis of affective disorders. Lithium treatment has also been shown to increase corticosterone levels [30], and some of its therapeutic effects may be promoted by a negative feed-back of corticosterone on the HPA axis with decreasing CRH mRNA expression [20]. As described before,

corticosterone induces an increased preference for sweet foods. Therefore, increased levels of corticosterone may contribute to the effects of lithium on the preference for sweet foods.

Additionally, it is known that lithium inhibits glycogen synthase kinase 3β (GSK3) activity, both directly and indirectly through the Akt/PKB signaling pathway [42]. Lochhead *et al*. [43] showed that GSK3 inhibition decreases the expression of genes encoding enzymes involved in gluconeogenesis and glucose release from liver cells, resulting in increased glucogen synthesis and decreased blood glucose levels. This possibly decreased glycemia could also help to explain the increase in sweet food intake described in the present study.

Since lithium effects on feeding behavior were restricted to palatable food, i.e. food rich in fat and sugar, there is another important approach to be considered, which is the reward value associated with food intake. Almost all drugs of abuse elicit the activation of dopaminergic systems, as do "natural" rewards such as palatable foods [4][47][50][53][55][60][75]. The observations that dopamine (DA) is released in the striatum in response to natural and drug rewards [23][24][27], and that animals self-administer many different classes of drugs that all enhance DA release [71], are consistent with the hypothesis that DA mediates the hedonic or pleasurable experience of reward [74]. More recent studies, however, have attributed to dopamine a role in the development of addiction by influencing the learning about the environmental events that predict reward [47][50][55]. Several studies have shown that lithium decreases dopamine release in the nucleus accumbens, a structure known to be involved in the reward system [16][32], but despite of this effect, lithium-treated rats do acquire palatable-food-sustained appetitive behaviors [19]. Lithium treatment has also been shown to modulate dopamine receptors mRNA [35]. Therefore, another possibility that may underlie the effect of lithium on palatable food intake may be an alteration in the interpretation of stimuli, leading rats to perceive sweet or savory food with a different salience of stimuli when they are presented to them.

In summary, this study provides new data concerning the effects of stress and lithium treatments on feeding behavior, and these results may contribute to understanding the weight gain seen in patients submitted to lithium therapy. Additional studies are necessary to elucidate the mechanisms by which lithium promotes the increased preference observed for palatable foods.

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FIGURES:

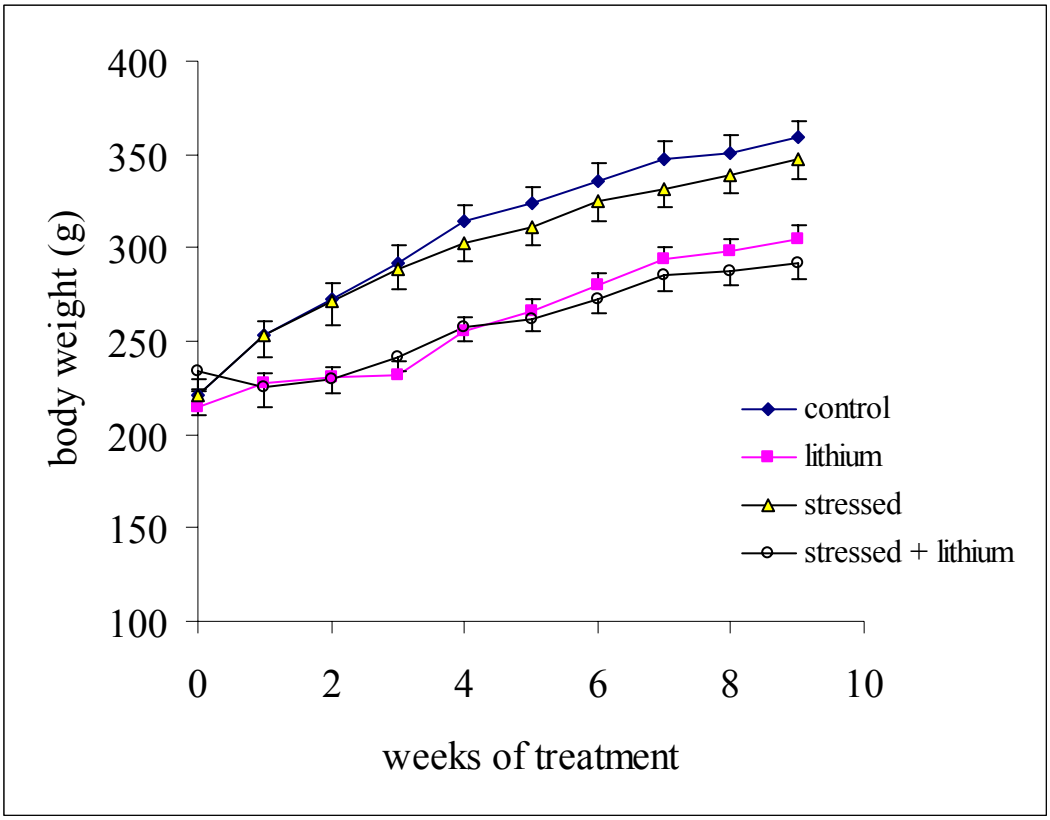


Figure 1: Effects of chronic-variate stress and lithium treatments on weight gain in rats. Data are expressed as mean + standard error of the mean of body weight in grams. N=10 animals/group. Control represents the control group, lithium represents the group treated just with LiCl for 40 days, stressed represents the group submitted to a chronic variate stress paradigm for 40 days, and stressed + lithium represents the group stressed for 40 days and concomitantly submitted to lithium treatment. Lithium-treated rats gained significantly less weight in the first two weeks (repeated measures ANOVA, $P < 0.001$), and after that their gain of weight was similar to that of controls. Stressed rats also gained weight differently to the control group (repeated measures ANOVA, $P < 0.01$).

*: Lithium treated groups are significantly different ($P < 0.01$) from control and stressed groups.

**: Lithium treated groups are significantly different ($P < 0.001$) from control and stressed groups.

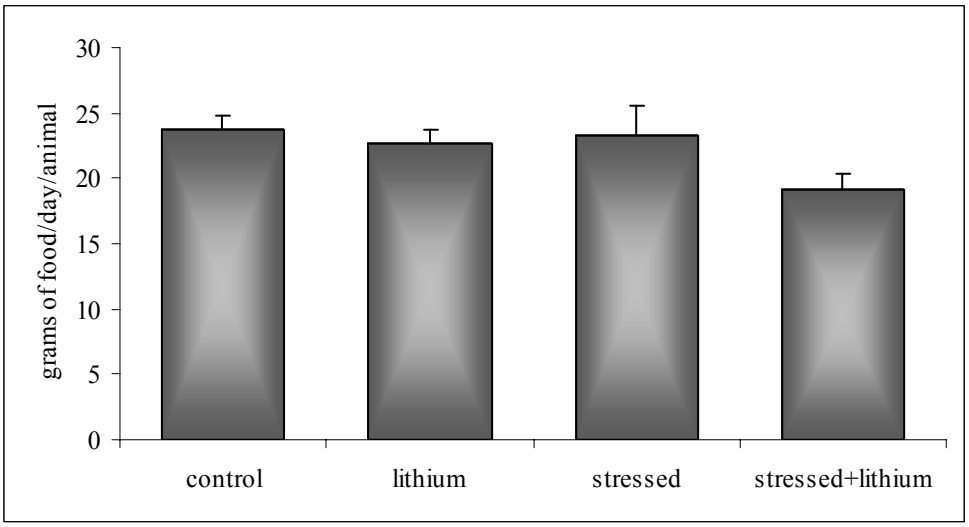
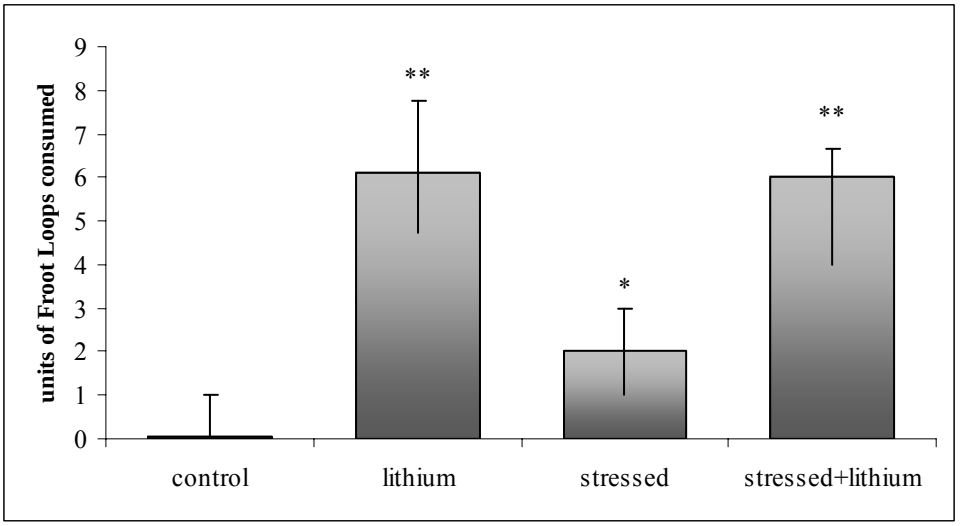
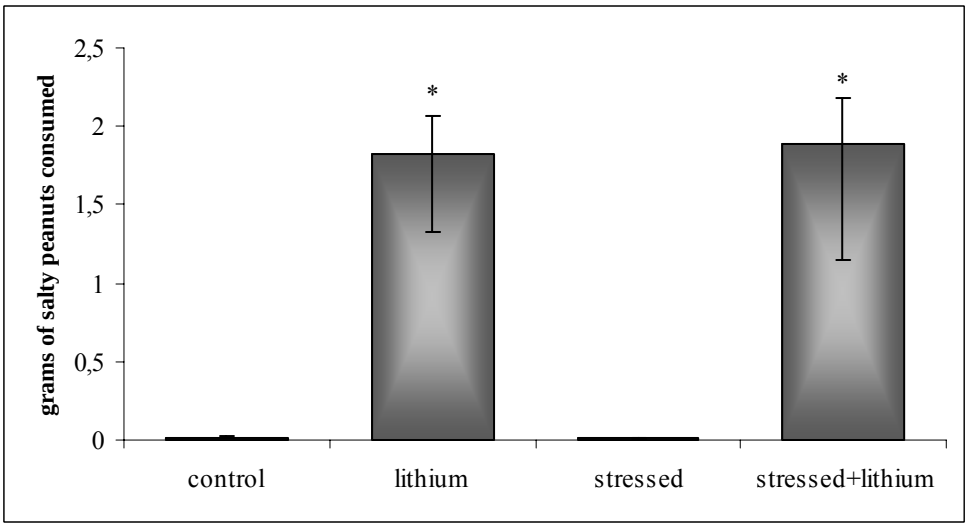


Figure 2: Effects of chronic-variate stress and lithium treatments on standard lab chow ingestion. Control represents the normal group, lithium represents the group treated just with LiCl for 40 days, stressed represents the group submitted to a chronic-variate stress paradigm for 40 days, and stressed + lithium represents the group stressed for 40 days and concomitantly submitted to lithium treatment. Data are expressed as mean + S.E.M. for grams of food/24 h/animal. N=6-18 animals/group. There was no significant difference in chow ingestion between the groups (Two-way ANOVA, $P > 0.1$).

3A


units of Froot Loops consumed
9
8
7
6
5
4
3
2
1
0
**
*
**
control
lithium
stressed
stressed+lithium

3B


grams of salty peanuts consumed
2,5
2
1,5
1
0,5
0
*
*
control
lithium
stressed
stressed+lithium

3C

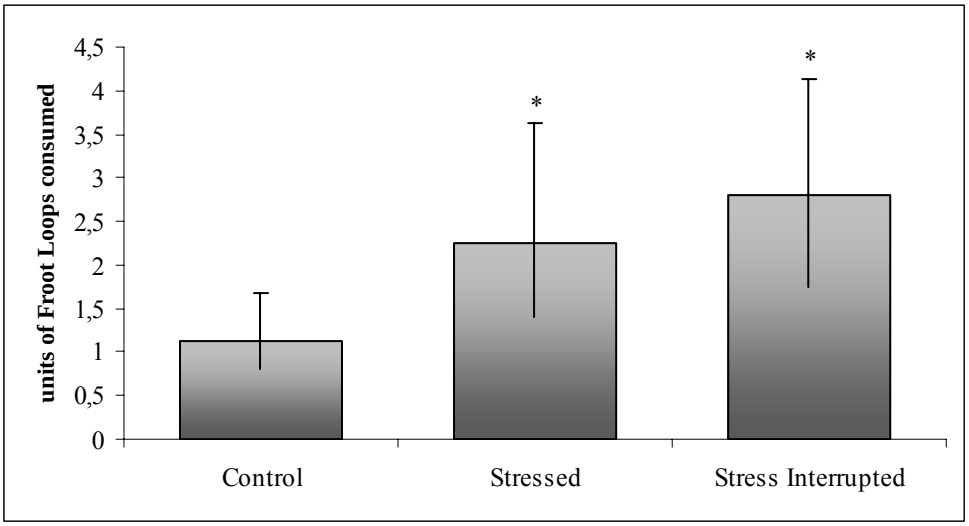


Figure 3: Effects of chronic variate stress and lithium treatments on sweet (A) and salty food (B) ingestion, and effect of stress interruption on sweet food intake (C). Control represents the normal group, lithium represents the group treated just with LiCl for 40 days, stressed represents the group submitted to a chronic-variate stress paradigm for 40 days, and stressed + lithium represents the group stressed for 40 days and concomitantly submitted to lithium treatment. Data are expressed as median (interquartile range) of units of Froot Loops® consumed (figures 3A and 3C) or grams of salty peanuts ingested (figure 3B).

A) There was a significant effect of chronic-variate stress (Mann-Whitney test, $P < 0.02$) and of chronic-lithium treatment (Mann-Whitney test, $P < 0.001$, N=10 animals/group) on Froot Loops® consumption when compared to the control group.

*: Significantly different from the control group.

**: Significantly different from the control and stressed groups.

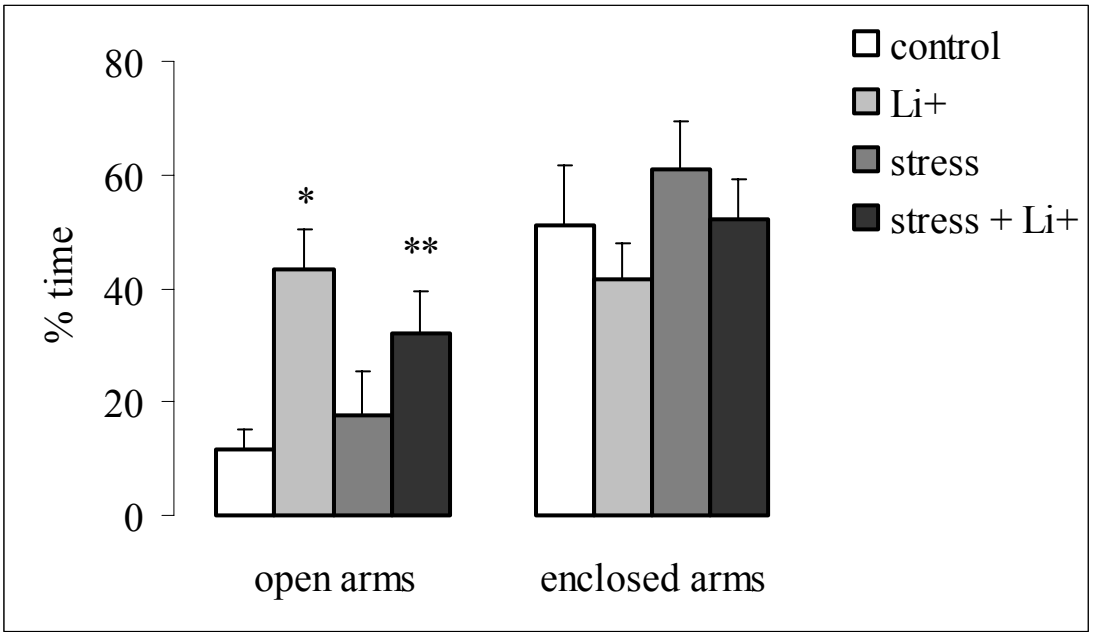
B) There was a significant effect of chronic-lithium treatment (Mann-Whitney test, $P < 0.001$, N=10 animals/group) on salty peanut consumption when compared to the control group.

*: Significantly different from the control and stressed groups.

C) Thirty days of stress interruption did not reverse the effects observed on sweet food consumption (Mann-Whitney test, $P < 0.05$ for Stressed and Stress Interrupted groups, when compared to the control group; N=10 animals/group).

*: Significantly different from the control group.

4A



4B

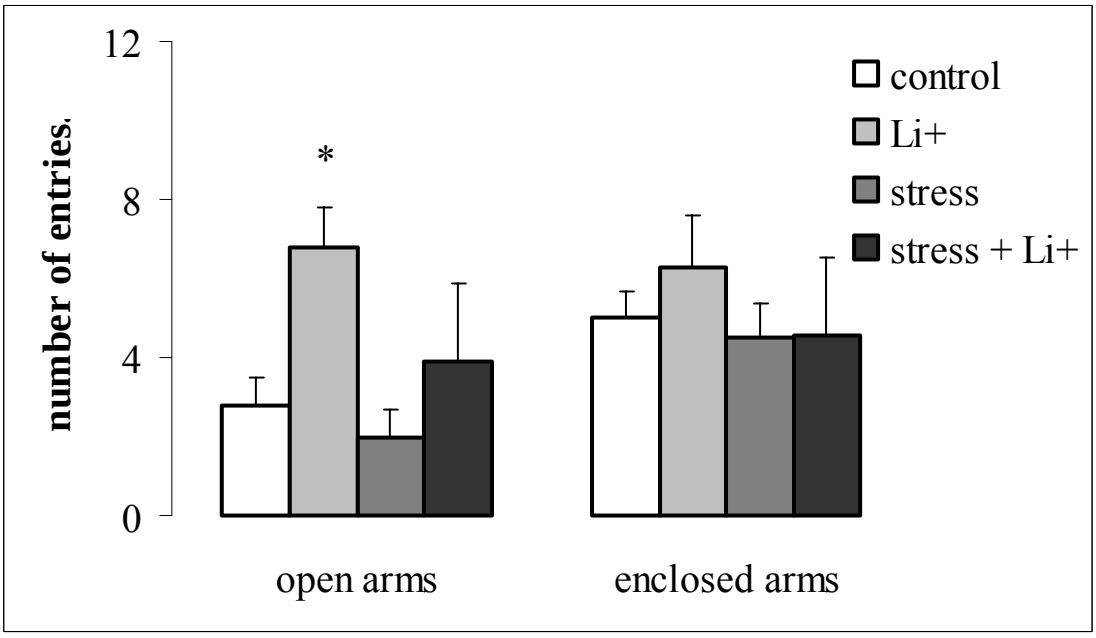


Figure 4: Effects of chronic-variate stress and lithium treatments on the performance in the elevated plus maze. Control represents the normal group, lithium represents the group treated

just with LiCl for 40 days, stressed represents the group submitted to a chronic-variate stress paradigm for 40 days, and stressed + lithium represents the group stressed for 40 days and concomitantly submitted to lithium treatment.

(A) Mean + S.E.M. of the time spent in the open arms during the 5 minutes of exposure to the elevated-plus maze. There was a significant effect of lithium treatment on time spent in the open arms (Two-way ANOVA, $P < 0.001$, N=10 animals/group).

*: Significantly different from the control and stressed groups (Duncan's multiple range test, $P < 0.01$)

**: Significantly different from the control group (Duncan's multiple range test, $P < 0.01$)

(B) Mean + S.E.M. of the number of entries into the open and enclosed arms. Stress significantly decreased the number of entries in the open arms (Two-way ANOVA, $P < 0.05$, N=10 animals/group), while lithium increased this parameter (Two-way ANOVA, $P < 0.001$, N=10 animals/group).

*: Significantly different from all the other groups (Duncan's multiple range test, $P < 0.001$)

Artigo 5:

THE NOCICEPTIVE RESPONSE OF STRESSED AND LITHIUM-TREATED RATS IS DIFFERENTLY MODULATED BY DIFFERENT FLAVORS

De Vasconcellos et al.

Aceito para publicação na revista “Physiology and Behavior”, 2006.

Artigo 6

BEHAVIORAL AND NEUROCHEMICAL ASSESSMENT OF DOPAMINERGIC PATHWAYS IN CHRONICALLY STRESSED AND LITHIUM-TREATED RATS

De Vasconcellos et al.

Submetido à revista “Pharmacology, Biochemestry and Behavior”

BEHAVIORAL AND NEUROCHEMICAL ASSESSMENT OF DOPAMINERGIC PATHWAYS IN CHRONICALLY STRESSED AND LITHIUM-TREATED RATS

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ABSTRACT

We observed, in previous studies, that stress and lithium treatments induce an increased consumption of sweet and palatable foods. In this study, we characterized the involvement of dopaminergic activity on these effects. Adult male Wistar rats were divided into four groups; control, control treated with lithium, stressed and stressed treated with lithium. A chronic variate stress paradigm was used, and lithium was added to the chow. After forty days of treatment, we observed that lithium-treated groups presented increased conditioned place preference in response to a sweet palatable flavor and increased motor activity after diethylpropion administration, suggesting an increased dopaminergic tonus, while the stressed group did not. When evaluating the total amount of dopamine (DA) and its metabolites by HPLC in the nucleus accumbens, we observed decreased dihydroxyphenylacetic acid (DOPAC) levels and a decreased DOPAC/DA ratio, while lithium decreased DOPAC but not the DOPAC/DA ratio. It suggests a decreased dopaminergic activity induced by stress. These results suggest that modulation of dopaminergic neurotransmission may be a part of the repertory of actions of lithium treatment, and that some effects of chronic variate stress, which is related to depressive states, may be related to a decreased dopaminergic activity in the nucleus accumbens.

Key-words: Chronic variate stress; lithium; dopamine, nucleus accumbens, conditioned place preference, diethylpropion.

INTRODUCTION

Emotional changes, such as exposure to stress situations, may influence a wide range of neuronal systems, and such effects can be manifested at a behavioral level. For example, studies have shown that animals that are exposed to repeated episodes of unavoidable stress are unable to acquire a motivated behavior sustained by a palatable food (Gambarana et al 2003), also presenting a hypo-reactivity to aversive and pleasurable stimuli (Gambarana et al 1995, Papp et al 1992, Moreau et al 1992). These effects represent the basis for models of depression (Gambarana et al 2001, Willner 1995) and are strongly related to the dopaminergic activity in the mesolimbic system (Lucas et al 2004).

The neurotransmitter dopamine (DA) plays an important role in the modulation of various forms of animal and human behavior, such as movement, motivation and reward (Nowak et al 2005, Vezina et al 2002), and it has been observed that dopamine is released in the ventral striatum in response to natural (e.g. palatable foods) and drug rewards (Hajnal and Norgreen, 2001, 2002; Hernandez and Hoebel, 1988). A structure that functions as an interface between "motivation and action" is the nucleus accumbens, which appears to play a crucial role in behaviors related to natural reinforces, such as food ingestion, sexual activity and instrumental learning (Cardinal et al., 2002; Kelley and Berridge, 2002; Koob et al.; 1978; Robbins and Everitt, 1996). Its major dopaminergic innervation, arising from the ventral tegmental area, plays a key role in many of these functions (Schultz, 2000).

Lithium salts have been the first line of therapeutic drugs used to treat affective disorders, mainly bipolar disorder (Manji et al., 1999; Shaldubina et al., 2001), and its action seems to be partly mediated by modulation of dopaminergic activity, (Basselin et al. 2005; Bymaster and Felder, 2002, Ichikawa et al., 2005). Besides its already established neuroprotective effects in a variety of insults in cultured cells and animal models of diseases

(Chen and Chuang, 1999; Jope, 1999; Manji et al., 1999, 2000), recent studies demonstrate that lithium protects striatal neurons in a model of Parkinson disease (Youdim and Arraf, 2004), in which there is a depletion of dopamine in striatal nerve terminals.

We have previously established in our laboratory a chronic variate stress paradigm, modified from other models of variate stress (Konarska et al., 1990; Willner, 1990, 1991; Murua and Molina, 1992; Gamaro et al., 2003), and this model has been shown to induce behavioral and neurochemical alterations, some of which are reversed or prevented by lithium treatment (Vasconcellos et al., 2003, 2005). We have also observed that both stress and lithium treatments increase the consumption of sweet food; furthermore, stress- and lithium-treated animals also respond differently to pleasant and unpleasant tastes (Vasconcellos et al., 2006). Considering the involvement of dopaminergic transmission in the neuronal processing of these stimuli (Nowak et al 2005; Vezina et al 2002; Berridge and Robinson, 1998; Robinson and Berridge, 2000; Schultz, 2002), we decided to evaluate the effects of stress and lithium treatments on the behavior of rats in tasks that are known to involve dopaminergic activity, such as locomotion after administration of diethylpropion, which is an amphetamine-like drug (McFarland et al., 2004) and conditioned place preference (Papp, 1989). We also evaluated the concentration of dopamine and its metabolites in the nucleus accumbens of chronically stressed and lithium-treated rats.

MATERIAL AND METHODS

Animals

Eighty-six adult male Wistar rats (60 days at the beginning of the treatment) weighing 160-230 g were used. Experimentally naive animals were housed in groups of 4 or 5 rats in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust. They were maintained under a standard dark-light cycle (lights on between 7:00 a.m. and 7:00

p.m.), with a room temperature of 22 ± 1 °C. Rats had free access to food and water, except during the period when restraint stress or forced swimming were applied. All animal treatments were in accordance with the institutional guidelines and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS).

Experimental groups

Animals were divided in two groups. One group received standard rat chow and the other group had lithium chloride (LiCl [VETEC, Brazil] - 2.5 mg/g of chow) and NaCl (Reagen, Brazil) (17 mg/g) added to the food, as previously described (Vasconcellos et al., 2003). This treatment has been previously used, and at the end of a period of four weeks or more, animals remain healthy and present lithium levels in the range of 0.6 – 1.2 mM (Rocha and Rodnight, 1994; Vasconcellos et al., 2003, 2005), similar to the levels observed in treated patients. Five days after the beginning of lithium treatment, these groups were subdivided into two other groups: control and submitted to a chronic variate stress paradigm.

Chronic Variate Stress Model

Chronic variate stress was modified from other models of variate stress (Konarska et al., 1990; Willner, 1990, 1991; Murua and Molina, 1992; Gamaro et al., 2003). The following stressors were used: (a) inclination of the home cages at a 45^{o} angle for 4 to 6 h, (b) 10 to 15 min of noise, (c) 1 h to 3 h of restraint, as described below, (d) 1.5 to 2 h of restraint at 4^{o} C, (e) forced swimming for 10 or 15 min, as described below, (f) flashing light during 2 to 4 h, (g) isolation (2 to 3 days). Animals were exposed to only one stressor every day, with stress starting at a different time in order to minimize its predictability. The exposure to stress situations continued during at least 40 days, and was not interrupted during the behavioral

evaluations. Please see Table 1 for the sequence of stressors applied during all the experimental procedures.

Restraint was carried out by placing the animal in a 25 x 7 cm plastic tube and adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole at the far end for breathing. Forced swimming was carried out by placing the animal in a glass tank measuring 50 x 47 x 40 cm with 30 cm of water at 23 $\pm$ 2^{o} C. Exposure to flashing light was achieved by placing the animal in a 50 cm-high, 40 x 60 cm open field made of brown plywood with a frontal glass wall. A 100 W lamp, flashing at a frequency of 60 flashes per minute, was used. All animal treatments were in accordance with the institutional guidelines and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS).

Conditioned place preference

After forty days of chronic stress and lithium treatments, thirty-two rats were trained in the conditioned place preference (CPP) paradigm. CPP apparatus consisted of two compartments of 35 x 10 cm, with a removable 10-cm divider between them. The two compartments were distinguished by white vs. black walls, by flooring texture and by a lamp on the white side. On the first day, rats were placed in the apparatus for 15 min with free access to both compartments to evaluate the natural preference for each side. The time spent in the compartments was scored. No food was available in the apparatus on the first day of exposure.

From the second day on, twenty grams of condensed sweet milk were used as a 'reward' during CPP training days. During the 6-day training period, rats were placed on alternating sides of the two-compartment corridor for a 30-min training session. These compartments were closed, so the animals did not have access to the other compartment. In the non-preferred side (lighter side), rats received the condensed sweet milk (Nestlé) and in

the other side rats received no treatment. The first and second days of habituation were performed with animals maintained under food restriction (80% of habitual ingestion of standard lab chow), in order to increase the interest for the new food offered in the CPP apparatus. On the test day (Day 8), rats were placed in the apparatus for 15 min with free access to both compartments, again without condensed sweet milk available. The time spent in the condensed sweet milk-paired compartment was registered, and a difference between time spent in condensed sweet milk-paired side in the last session and the time spent in the condensed sweet milk-paired side in the first session was evaluated as indicative of the conditioning of a place preference.

One group of control animals was exposed to the same procedure, however no condensed milk was offered during the habituation days. The difference between time spent by these animals in the lightened side in the the eighth day and in first day was used as a basal value, and data of control, stressed- and lithium-treated rats were expressed as percentage of the basal value.

During these days of exposure to CPP, the animals continued receiving lithium and being exposed to stress situations, however stress was always applied at least one hour after exposure to this behavioral apparatus.

Locomotor response to diethylpropion

Twenty-four rats were submitted to a challenge with the low potency psychostimulant phenylethylamine diethylpropion (1 phenyl-2-diethylamine-1-propanone hydrochloride), which typically causes an increase in locomotor activity (Reimer et al., 1995; Gevaerd et al., 1999), like amphetamine and other phenylethylamines. On the first day, rats received 1 ml/kg saline i.p. Immediately afterwards, they were allowed to remain in a neutral box for ten minutes, and then were gently put in a wooden box (53.5 x 37.5 x 50 cm), with the frontal side made of glass and the floor divided into 12 squares of 12.5 x 12.5 cm. The number of

crossings and rearings were measured for a 30 min period. On day two, rats were injected with 10 mg/kg diethylpropion (DEG – Brazil) i.p. and the same procedures were performed. The observations were made by direct check of the experimenter, and all observations for each animal were made by the same experimenter.

Dopamine and metabolites measurement

The animals were sacrificed by decapitation 24 h after the last stress session, to minimize its immediate effects on neurotransmitter metabolism. These animals were not previously submitted to behavioral tasks. Brains were quickly removed and placed on an inverted Petri dish on ice, where the nucleus accumbens was dissected according to the atlas by Paxinos and Watson (1998). After this, the structure was weighed and rapidly frozen and stored at -70ºC until assayed.

On the day of the assay, tissue samples were homogenized with an ultra turrax in 10 volumes of perchloric acid 0.10 M (Merck) and centrifuged at 4^{o} C for 40 minutes at 1400 x g. The pellet was discarded, and the supernatants were filtered in filters with diameter pores of 0.2 □m. Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxytyramine (3-MT) were analyzed in the resultant filtrate using high performance liquid chromatography coupled with electrochemical detection (HPLC-EC), with a sensibility (I range) of 100 µA and an oxidation potential 0.85V.

The instrumentation used included a Waters model 5100 pump, a manual Rheodyne injector (loop of 20µl), and a Waters electrochemical detector, model 464, and a Spherisord ODS C18 column (250 x 3.9 mm). The mobile phase was modified from DiBussolo et al. (1983), and consisted of 100 mM formic acid (Merck), 1mM citric acid (Merck), 0.36 mM sodium 1-heptansulfonate (Akros), 0.1 mM EDTA (SIGMA) and 2.5% diethylamine (Merck), with pH adjusted to 4.2 – 4.5 and added with acetonitrile (Merck, HPLC grade) 98:8 (v:v). The flow rate was 0.8 ml/min. Calibration curves were made previously, and they were linear

in the range measured. Data were acquired by a PC using Empower Software, and compared to 20 μl samples of an external calibrating standard solution containing 250 ng/ml of each, DA, DOPAC, HVA and 3-MT (all from SIGMA). Concentrations of these substances in the samples were calculated and expressed as ng/g wet tissue, and the activity (turnover) of the dopaminergic system were expressed as DOPAC/DA ratio. Homovanillic acid (HVA) was not detectable in our samples.

Statistical analysis:

Data were expressed as mean ± standard error of the mean. Data obtained in CPP evaluation and in the measurements of the content of dopamine and its metabolites in the nucleus accumbens was evaluated by Two-way ANOVA (using lithium and stress treatments as independent factors), followed by Duncan's multiple range test when applicable. Results obtained in the evaluation of locomotor activity before and after diethylpropion administration were analyzed by Repeated Measures ANOVA (using activity after saline and after diethylpropion injections to evaluate within-subjects effects and lithium and stress treatment as between-subjects factors), followed by the Student-Newman-Keuls' test.

RESULTS

Conditioned place preference

A two-way ANOVA showed no effects of chronic lithium or stress treatments during the first day of exposure to this apparatus, but there was an interaction between these treatments, since lithium treated animals (but not the lithium + stress group) displayed an increased time in the lighter side when compared to the control group (data not shown). Therefore, conditioned place preference was evaluated by the difference between time spent by animals in the condensed-sweet milk paired side in the day one and in the day eight, using

animals habituated to this apparatus without the presence of an appetitive stimulus as a basal measurement. Data was expressed as percentage of the basal measurement value. A two-way ANOVA showed a significant effect of chronic lithium treatment in the conditioned place preference [F (1, 28) = 6.95, $P < 0.05$], but there was no effect o chronic stress or interaction between treatments in this parameter [F (1, 28) = 0.01, $P > 0.1$ for stress and (1, 28) = 2.66, $P > 0.1$ for interaction].

Locomotor response to diethylpropion

All groups presented an increased number of crossings after diethylpropion administration [Repeated Measures ANOVA, F (1, 20) = 325.646, $P<0.001$ for the within-subjects factor], n = 6 animals/group), and there was a significantly higher effect of lithium treatment on this parameter compared to the control or stressed group, since lithium-treated rats performed significantly more crossings than did the other groups [F (1, 20) = 15.399, $P < 0.001$ for lithium treatment]. There was no effect of chronic stress [F (1, 20) = 0.744, $P > 0.1$] nor any interaction between treatments [F (1, 20) = 2.506, $P > 0.1$] in locomotor activity (Figure 2A).

Rearings were also increased by the drug [Repeated Measures ANOVA, F (1, 20) = 70.277, P < 0.001, n = 6 animals/group] in all groups, with no specific effect of stress [F (1, 20) = 0.482, $P > 0.1$], lithium treatment [F (1, 20) = 0.053, $P > 0.1$], nor any interaction between treatments [F (1, 20) = 2.951, $P > 0.1$] (Figure 2B).

Dopamine and metabolites measurements

The effects of chronic variate stress and lithium treatment on dopamine and its metabolite levels in nucleus accumbens are shown in Table 2. Chronic variate stress was shown to induce a decrease in DOPAC content [Two way ANOVA, F (1, 20) = 7.070, $P < 0.02$], with a marginal effect of lithium treatment [Two way ANOVA, F (1, 20) = 3.038, $P =$

0.087] and no interaction between these treatments [Two way ANOVA, F (1, 20) = 2.028, $P > 0.1$]. When performing a post-hoc test, a Duncan's multiple range test demonstrated that DOPAC levels of all groups are decreased when compared to the control group [$P < 0.03$]. A Two way ANOVA showed no effect of chronic stress [F (1, 20) = 0.764, $P > 0.1$], lithium treatment [F (1, 20) = 0.990, $P > 0.1$] nor interaction between treatments [F (1, 20) = 1.216, $P > 0.1$] in the total amount of dopamine in the nucleus accumbens. There was also an absence of effect in the levels of 3-MT in all groups analyzed [F (1, 20) = 1.251, $P > 0.1$ for the effect of stress, F (1, 20) = 2.352, $P > 0.1$ for the effect of lithium, and F (1, 20) = 0.600, $P > 0.1$ for interaction between treatments].

The ratio between DOPAC/DA was also evaluated, and we observed a significant effect of chronic variate stress on decreasing this parameter [Two way ANOVA, F (1, 20) = 7.439, $P < 0.02$], with no effect of lithium treatment [F (1, 20) = 0.990, $P > 0.1$], nor interaction between stress and lithium [F (1, 20) = 2.391, $P > 0.1$] (Figure 3).

DISCUSSION

The present study was performed to investigate the effects of chronic variate stress and chronic lithium treatment on dopamine-related behaviors, i.e. conditioned place preference and locomotion after diethylpropion administration, and on the content of dopamine and its metabolites in the nucleus accumbens, which has long been known to play a crucial role in behaviors related to natural reinforces, such as food ingestion (Cardinal et al., 2002; Kelley and Berridge, 2002; Koob et al.; 1978; Robbins and Everitt, 1996).

In the evaluation of the total amount of DA and its metabolites in the nucleus accumbens of stressed rats, we observed decreased DOPAC levels and a decreased DOPAC/DA ratio, suggesting a decreased dopaminergic activity induced by chronic stress.

Furthermore, stress was unable to induce cross-sensitization in the diethylpropione challenge, and did not induce an altered behavior in the place-preference conditioning task; in contrast, lithium treatment decreased DOPAC but not the DOPAC/DA ratio in the nucleus accumbens, and induced a behavioral sensitization in both tasks involving dopaminergic activity.

The nucleus accumbens is composed of two major sub-areas, namely the core (tissue surrounding the anterior commissure) and the shell, a region extending around the core, and these two distinct regions show differences in their afferent inputs and efferent projections (Deutch and Cameron, 1992). The general notion is that the accumbens core is more allied to voluntary motor functions, while the shell lies more in the regulation of visceral and motivational mechanisms (Kelley, 2004). Although the results obtained in dopamine and metabolites measurements in the nucleus accumbens cannot be directly related to the behavioral alterations observed, since there was no differentiation between core and shell regions, it gives some idea of ventral striatum dopaminergic metabolism, indicating an alteration in the chronically stressed group. Further studies would be necessary to better evaluate alterations in dopaminergic transmission after chronic exposure to stress and lithium treatments.

The conditioned place preference (CPP) is a behavioral paradigm that assesses the reward value of stimuli, and it has been shown that the integrity of dopaminergic pathways is necessary for the development of place preference (Papp, 1989). We observed that lithium-treated rats presented a strong place preference conditioned by an appetitive gustatory stimulus, suggesting a lower threshold for a reward, which was demonstrated by the increased in the difference of time spent by the animals in the lighter side of the CPP apparatus, while control and stressed groups did not. Lithium treated rats spent more time in the lighter side of the CPP already during the first exposure. We previously observed that lithium presents an anxiolytic effect when evaluating the behavior in the plus maze (unpublished data), and it is

plausible to suppose that this increased time in the lighter side of the apparatus before conditioning is due to a lithium-induced reduction in anxiety. Besides, we believe that this is not the main influence on the effects observed in place preference conditioning, since the stress + lithium groups did not present a preference for the lighter side before habituation, but displayed an increased time in this side after conditioning.

Dopamine is known to have an important influence on the acquisition of appetitive behaviors, and DA release in the nucleus accumbens in response to an unfamiliar taste has been correlated to the formation and consolidation of a gustatory short-memory trace, the duration of which is crucial in associating a taste with possible incoming postingestive changes (Fenu et al., 2001). Thus, the increased release in the mesolimbic DA in response to a novel palatable food seems to be necessary for associating it with a contingent postingestive modification, rather than properly be involved in the reward value of this new taste. In the present study we evaluated the amount of basal DA in the tissue, what does not exclude the possibility of a difference in the DA released when rats were exposed to the appetitive stimulus, what can be verified in future studies.

Repeated, intermittent exposure to psychostimulants results in long-lasting, progressive sensitization of the behavioral effects of a subsequent amphetamine challenge. This phenomenon has been linked to a persistent hyperactivity of the mesolimbic dopamine system (McFarland et al., 2004). Several studies demonstrate that sensitization of the dopamine receptor by direct or indirect-acting dopamine agonists is expressed as increased locomotor activity and augmented stereotyped behaviors (Itzhak and Martin, 1999, Wunderlich et al., 2004). Therefore, we submitted stressed and lithium treated rats to a challenge with diethylpropion, an amphetamine analog. We observed that, although not presenting any difference in the basal locomotion (i.e. after saline i.p.), lithium treated rats presented a significantly increased locomotor activity compared to the other groups. This

result confirms the initial hypothesis that lithium treatment can sensitize dopaminergic pathways. The fact that we did not observe any effect of lithium on the basal amount of DA and its metabolites in the ventral striatum, and that other studies have shown that lithium does not increase basal dopaminergic release (Gambarana et al., 2003; Masi et al., 2000), may suggest that the effects of lithium on dopaminergic pathways may also involve alterations in DA receptor expression. Kameda et al. (2001) described an effect of lithium in increasing dopamine D2 mRNA expression, an effect that could be involved in the increased sensitivity to diethylpropione, and further studies are necessary to elucidate the possible involvement of DA receptors, as well as DA transporters, in the behavioral sensitization to dopaminergic drugs induced by lithium treatment.

The behavioral sensitization phenomenon can also be observed after repeated exposure to stress (Goeders, 2002; Kalivas and Duffy, 1989). In the present study, however, we failed to observe such alterations, since the increased locomotor activity observed in chronically stressed rats after diethylpropione challenge was not different from that observed in control animals.

Studies concerning the effects of chronic stress on dopamine release, as well as sensitization to drugs of abuse, are somewhat conflicting. Some studies have shown that chronic stress exposure produces a sensitization of dopaminergic pathways, similar to that obtained after chronic psychostimulant treatments (Goeders, 2002; Kalivas and Duffy, 1989; Leyton and Stwart, 1990); however, it has been proposed that only unpredictable chronic stressors produce behavioral sensitization to psychostimulants, while predictable stressors, such as restraint stress, do not (Cabib and Puglisi-Allegra, 1996; Haile et al., 2001). Although unpredictable, the chronic variate stress model used in the present study is adapted from models of depression, and a characteristic of depressive states is the anhedonia induced by a blunted dopaminergic response in structures related to reward and motivation (Di Chiara et

al.; 1999). Therefore, it is conceivable that the model of stress used here, which decreased dopamine turnover, as observed in the DOPAC/DA ratio, also inhibits the development of cross-sensitization in stressed animals.

In conclusion, we propose that the dopaminergic system may be linked to the increased “palatable food” ingestion observed in lithium treated rats, since it appears to promote a sensitization of the dopaminergic system that is not directly related to the total amount of dopamine in the ventral striatum. Otherwise, the chronic variate stress model used in this study does not induce cross-sensitization with psychostimulant drugs, but leads to a reduction in dopaminergic activity in the nucleus accumbens, what agrees with the observed effects in some other models of depression.

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TABLES:

Day of treatment	Stressor applied
1	Restraint (2 hs)
2	Flashing light (4 hs)
3	Forced swimming (15 min)
4	Cold restraint (1h 30 min)
5	Isolation
6	Isolation
7	Isolation
8	Inclination of home cages (6 hs)
9	Noise (10 min)
10	Forced swimming (3 min, cold water)
11	Cold restraint (1 h)
12	Restraint (2 hs)
13	Flashing light (6 hs)
14	No stressor applied
15	Forced swimming (15 min)
16	Inclination of home cages (5 hs)
17	Cold restraint (1h 30 min)
18	Noise (10 min)
19	Isolation
20	Isolation
21	Isolation
22	Restraint (2 hs)
23	Forced swimming (3 min, cold water)
24	Flashing light (6 hs)
25	Noise (10 min)
26	Cold restraint (1h)
27	Inclination of home cages (5 hs)
28	No stressor applied
29	Forced swimming (15 min)
30	Restraint (1 h)
31	Flashing light (6 hs)
32	Cold restraint (1h 30 min)
33	Isolation
34	Isolation
35	Isolation
36	Inclination of home cages (6 hs)
37	Noise (10 min)
38	Forced swimming (3 min, cold water)
39	Flashing light (5 hs)
40	Restraint (2 hs)
41	No stressor applied
42	Inclination of home cages
43	Noise (10 min)
44	Forced swimming (3 min, cold water)
45	Cold restraint (1 h)
46	Restraint (2 hs)
47	Flashing light (6 hs)
48	No stressor applied
49	Forced swimming (15 min)
50	Cold restraint

Table 1: Chronic Variate Stress model – sequence of stressors applied during all the experimental procedures.

	Control	Lithium	Stressed	Stressed + Lithium
Dopamine	1202.3 ± 123.8	1218.8 ± 237.4	1506.06 ± 37.2	1183.69 ± 146.2
DOPAC	3809.6 ± 988.1	1959.4 ± 602.5*	1424.3 ± 79.0*	1237.9 ± 140.6*
3-MT	1962.7 ± 401.0	1336.7 ± 234.6	2056.0 ± 32.8	1850.1 ± 278.1

Table 2: Effects of chronic variate stress and lithium treatments on the total amount of dopamine and its metabolites in the nucleus accumbens. Data are expressed as mean ± standard error of the mean (ng/mg wet tissue), N = 6 animals/group.

There was a significant effect of stress and lithium treatments on the total amount of DOPAC in the Nucleus Accumbens (Duncan's multiple range test, $P < 0.02$), and no effect of the treatments on the levels of all other compounds analyzed. *: Different from the control group.

FIGURES

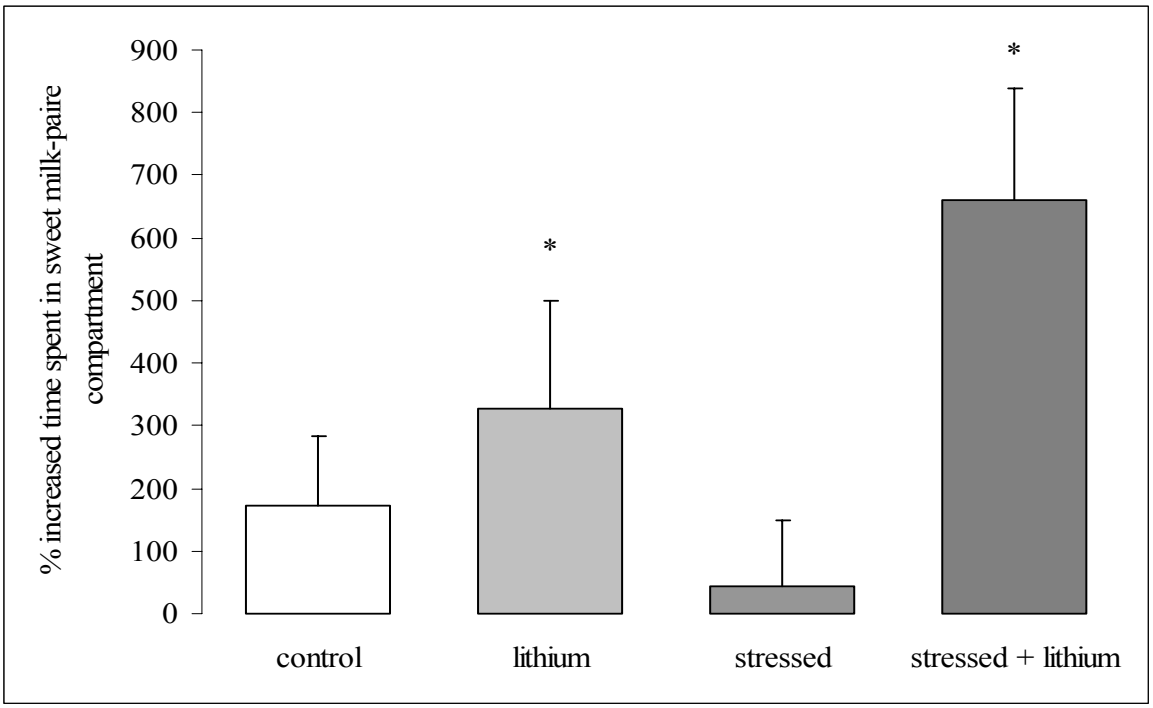
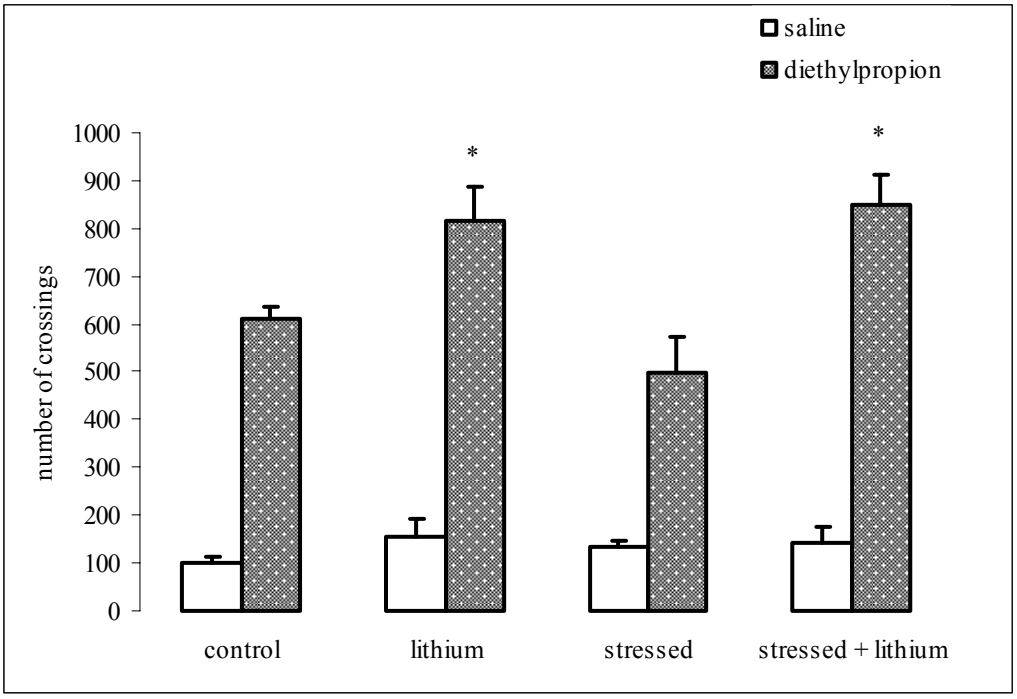


Figure 1: Effects of chronic-variate stress and lithium treatments on the conditioned place preference task. Data are expressed as the difference between time spent by animals in the condensed-sweet milk paired side in the day eight and in day one, using animals habituated to this apparatus without the presence of an appetitive stimulus as a basal measurement. Data was expressed as mean $\pm$ standard error of the mean, as percentage of the basal measurement value. Control represents the control group, lithium represents the group treated just with LiCl for 40 days, stressed represents the group submitted to a chronic variate stress model for 40 days, and stressed + lithium represents the group stressed for 40 days and concomitantly submitted to lithium treatment. There was a significant effect of lithium treatment on the time spent in the condensed sweet milk paired side (Two-way ANOVA, $P < 0.02$), and no effect of stress in this parameter. *: Different from the control and stressed groups.

1A


saline
diethylpropion
number of crossings
1000
900
800
700
600
500
400
300
200
100
0
*
*
control
lithium
stressed
stressed + lithium

1B

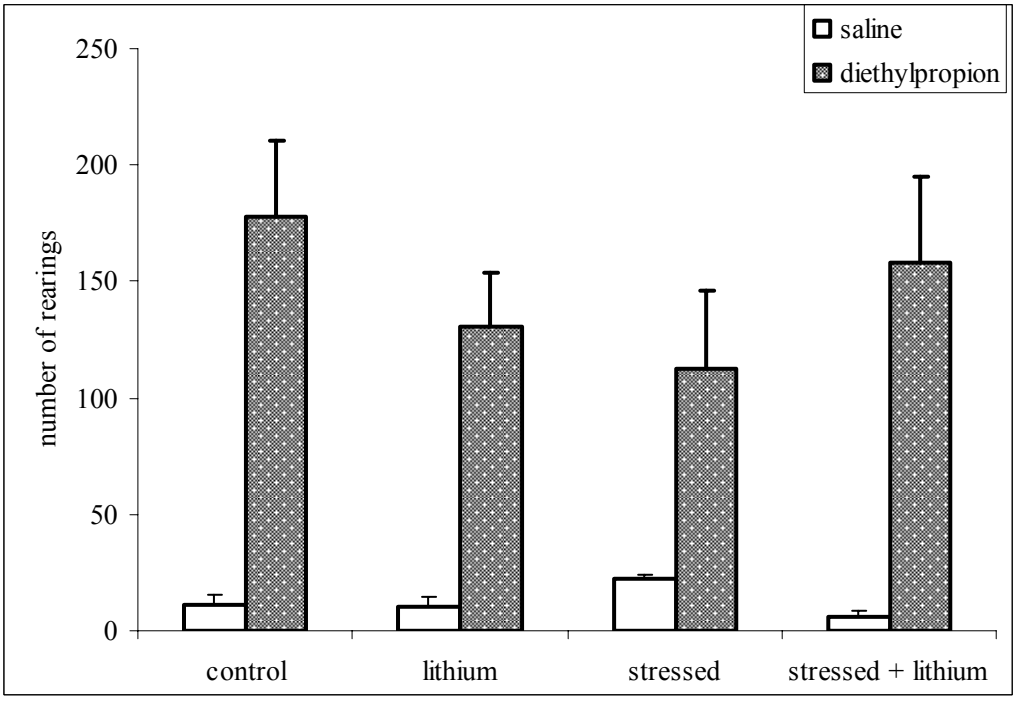

saline
diethylpropion
number of rearings
250
200
150
100
50
0
control
lithium
stressed
stressed + lithium

Figure 2: Effects of chronic-variate stress and lithium treatments on the locomotor response to diethylpropion. Data are expressed as mean ± standard error of the mean of number of crossings (A) and number of rearings (B), N = 6 animals/group. Control represents the control group, lithium represents the group treated just with LiCl for 40 days, stressed represents the group submitted to a chronic variate stress paradigm for 40 days, and stressed + lithium represents the group stressed for 40 days and concomitantly submitted to lithium treatment.

A) There was a significant effect of diethylpropion treatment (Repeated Measures ANOVA, $P < 0.0001$) and a significant effect of lithium treatment (Repeated Measures ANOVA, $P < 0.001$), with lithium-treated animals performing more crossings than the other groups in the test session. *: Different from groups that did not receive lithium treatment.

B) There was a significant effect of session (Repeated Measures ANOVA, $P < 0.0001$) and no effects of stress and lithium treatments on the number of rearings.

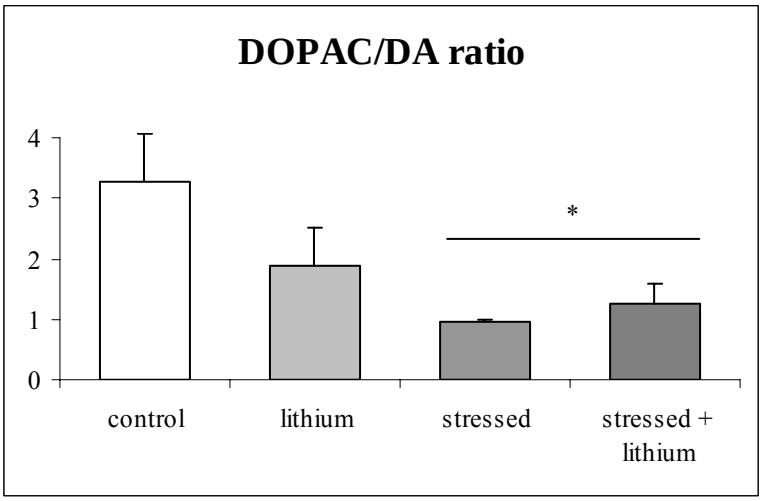
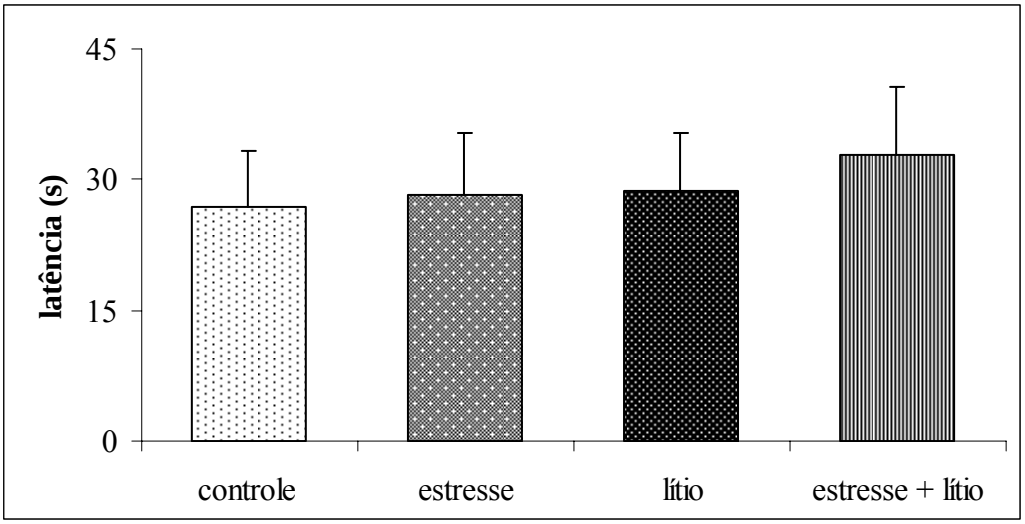


Figure 3: Effects of chronic variate stress and lithium treatments on the DOPAC/DA ratio in the nucleus accumbens. Data are expressed as mean $\pm$ standard error of the mean of the DOPAC/DA ratio . N = 6 animals/group. There was a significant effect of stress treatment on the DOPAC/DA ratio (Two-way ANOVA, $P < 0.02$), and no effect of lithium in this parameter.

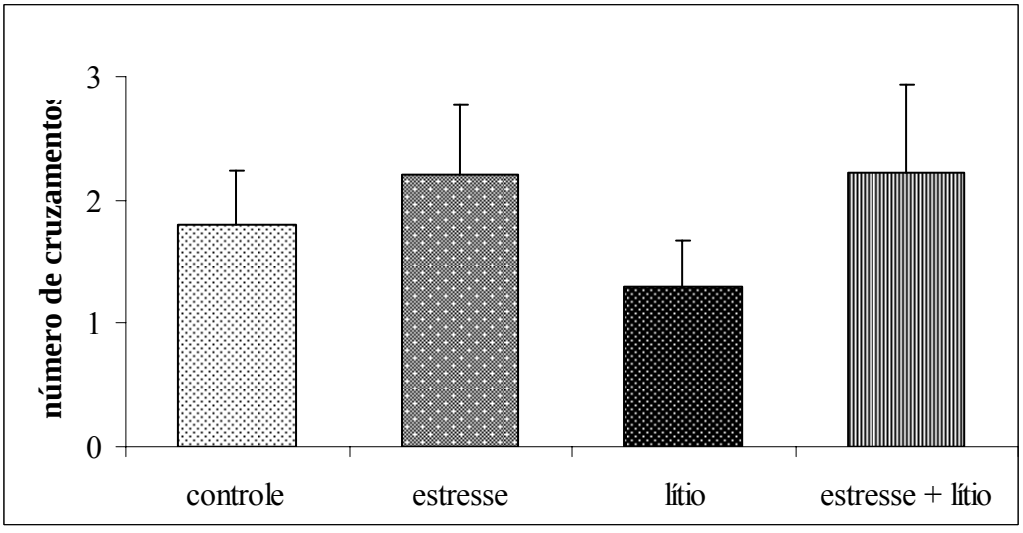
Resultados não publicados ou submetidos:

AVALIAÇÃO CURSO-TEMPORAL DOS EFEITOS DO ESTRESSE E DO LÍTIO SOBRE A MEMÓRIA ESPACIAL NO LABIRINTO AQUÁTICO

1A


45
30
15
0
latência (s)
controle
estresse
lítio
estresse + lítio

1B


3
2
1
0
número de cruzamentos
controle
estresse
lítio
estresse + lítio

1C

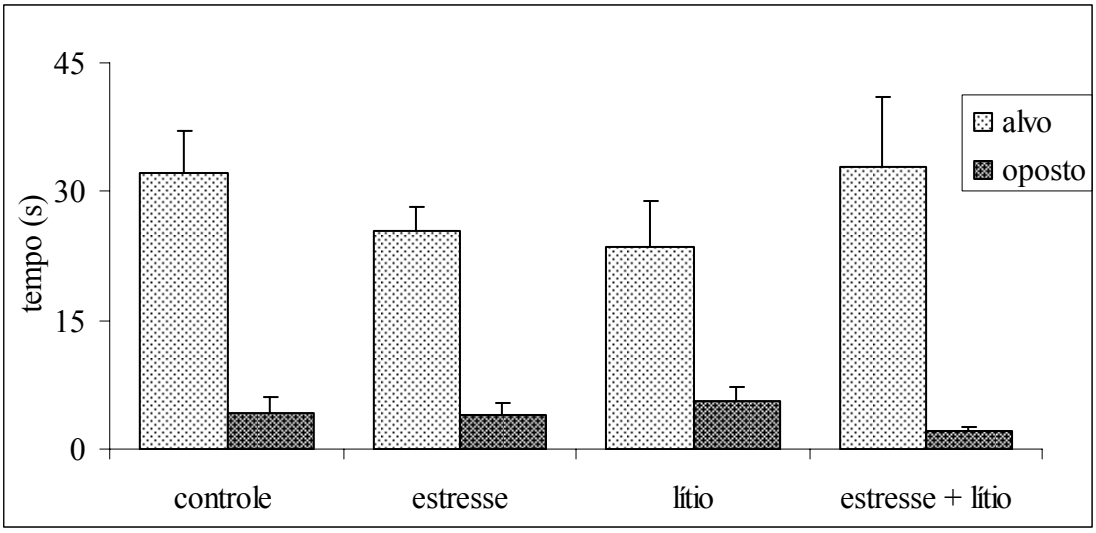
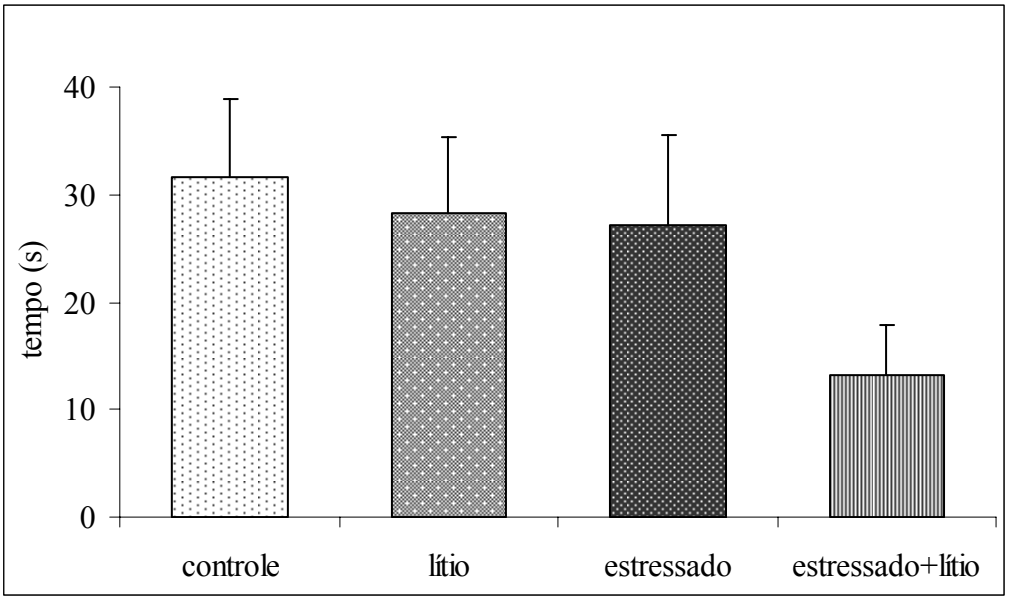
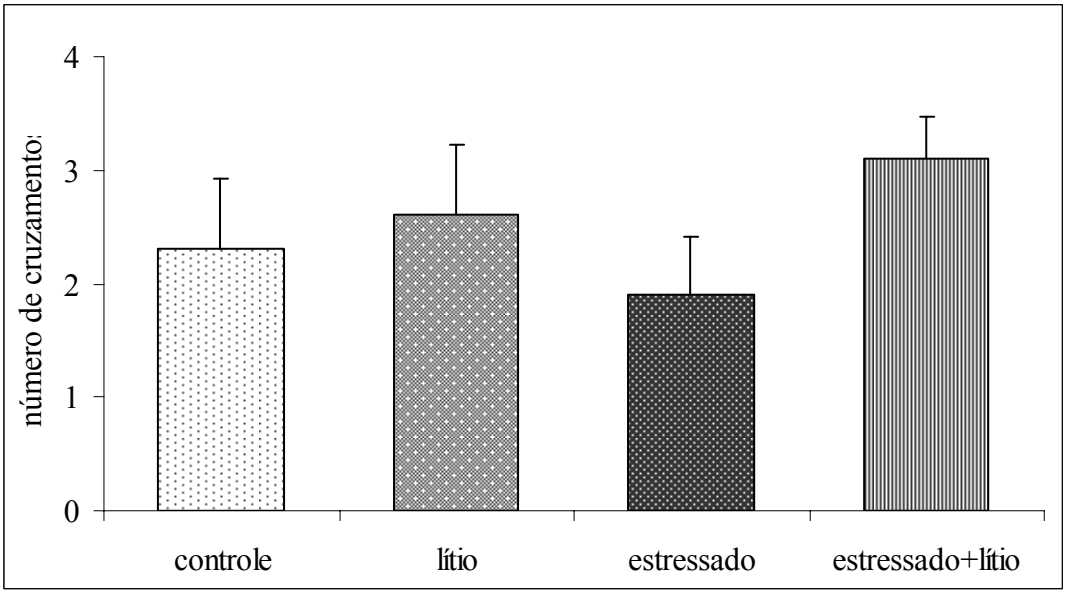


Figura 1: Efeito de 21 dias de tratamentos com estresse crônico variável e lítio sobre a memória espacial de ratos no Labirinto Aquático de Morris. Os dados estão expressos como média $\pm$ erro padrão, N=10 animais/ grupo. Não houve diferença significativa na latência para o primeiro cruzamento (1A), no número de cruzamentos efetuados pelos animais (1B), nem nos tempos gastos nos quadrantes alvo e oposto (1C, ANOVA de duas vias, $P > 0,01$ para todos os parâmetros).

2A


40
30
20
10
0
tempo (s)
controle
lítio
estressado
estressado+lítio

2B


4
3
2
1
0
controle
lítio
estressado
estressado+lítio

2C

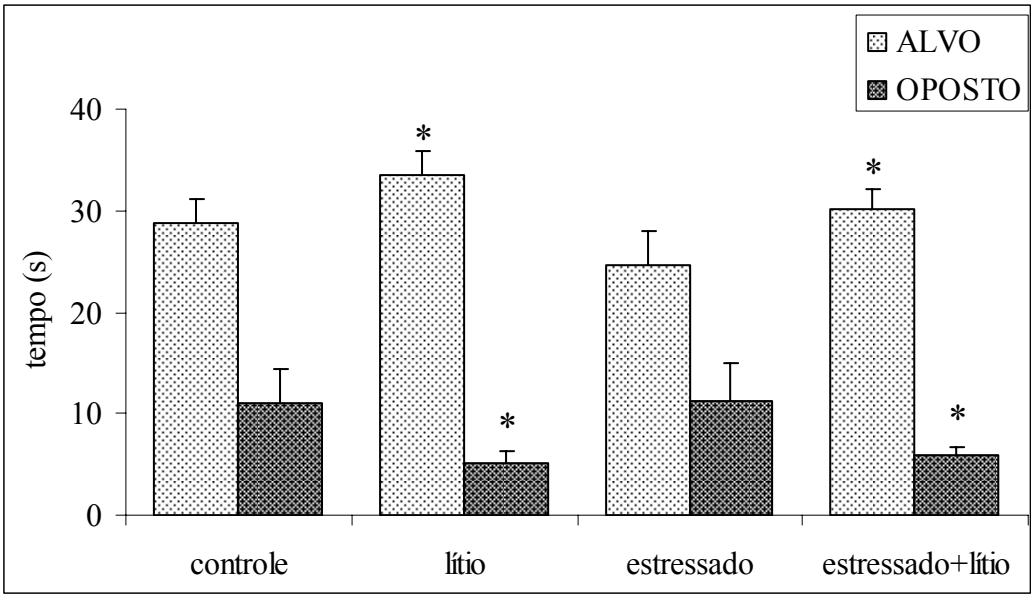


Figura 2 Efeito de 30 dias de tratamentos com estresse crônico variável e lítio sobre a memória espacial de ratos no Labirinto Aquático de Morris. Os dados estão expressos como média $\pm$ erro padrão, N=10 animais/ grupo. Não houve efeito dos tratamentos sobre a latência para realizar o primeiro cruzamento (2A) nem sobre o número de cruzamentos (2B, ANOVA de duas vias, $P > 0,05$ para ambos). Houve efeito do tratamento com lítio sobre os tempos nos quadrantes, aumentando o tempo dos animais no quadrante alvo e diminuindo o tempo no quadrante oposto (ANOVA de duas vias, $P < 0,05$ em ambos os casos).

*: Diferença estatisticamente significativa dos animais tratados com lítio e seus respectivos controles.

DISCUSSÃO GERAL

O objetivo geral dos estudos desta tese de doutorado foi investigar alguns dos mecanismos que possivelmente estão envolvidos nos efeitos do modelo de estresse crônico variável utilizado – o qual é uma adaptação do paradigma de estresse crônico moderado – sobre a memória espacial avaliada no Labirinto Aquático de Morris. A premissa inicial, e que foi de fato observada, era de que o estresse induziria um déficit cognitivo. Conforme mencionado, este déficit foi prevenido e revertido pelo tratamento com lítio.

O lítio foi escolhido para este trabalho em função de ser utilizado como adjuvante na terapia antidepressiva e, paralelamente, estar sendo descrito como uma droga neuroprotetora contra diversos insultos, incluindo isquemia cerebral e insultos induzidos por drogas convulsivantes (Manji *et al*., 1999). Esta peculiaridade, não apresentada por muitas outras classes de antidepressivos, faz do lítio um potencial agente terapêutico útil na profilaxia e tratamento de diversos outros distúrbios neurológicos além da depressão, incluindo doenças neurodegenerativas como as doenças de Alzheimer e de Parkinson (Bauer *et al*., 2003; Chen *et al*., 2004).

Uma vez estabelecido que o tratamento com lítio previne o déficit na memória espacial induzido pelo estresse, foi feita uma avaliação curso temporal destes efeitos. Observamos que a ação dos tratamentos é tempo-dependente, uma vez que 21 ou 30 dias de estresse não surtiram efeito sobre a memória. Por sua vez, 30 dias de tratamento com lítio foram suficientes para induzir um melhor desempenho dos animais no labirinto aquático, o que foi evidenciado por um aumento do tempo gasto no quadrante alvo e diminuição do tempo gasto no quadrante oposto. Em função destes resultados, padronizamos a duração do estresse em 40 dias, uma vez que tempos inferiores não induziram déficit cognitivo, para investigação dos possíveis mecanismos envolvidos nestes efeitos, e o tempo de tratamento com lítio (quando este foi administrado após a indução dos déficits na memória) foi padronizado em 30 dias.

No artigo 1 desta tese, foi observado que o tratamento com lítio tanto previne quanto reverte os déficits na memória espacial, uma vez que animais tratados com este sal durante 30 dias, após os 40 dias de estresse, apresentaram comportamento semelhante ao dos animais controle no labirinto aquático. Estes dados apresentam uma interessante relevância clínica, uma vez que o combate a doenças neurológicas ou neurodegenerativas dificilmente é feito de forma profilática, normalmente sendo iniciado após os danos serem instaurados.

A hipótese inicial deste trabalho era de que os efeitos observados sobre a memória não fossem provocados por perda neuronal, baseado na ausência de alterações nos marcadores neurogliais beta-tubulina III e GFAP. O primeiro trabalho dessa tese corroborou com esta hipótese, uma vez que a interrupção do estresse *per se* já é capaz de reverter os déficits cognitivos produzidos após 40 dias de tratamento. Isto indica que os efeitos do estresse sobre a memória sejam mais de ordem plástica do que propriamente por morte neuronal.

Observamos ainda que o estresse diminui a atividade da enzima Na^+, K^+- ATPase em membranas sinápticas de hipocampo. Este resultado já havia sido observado anteriormente em pacientes deprimidos (el-Mallakh & Wyatt, 1995), bem como em animais submetidos ao mesmo modelo de estresse aqui utilizado, porém fêmeas (Gamaro et al., 2003). Isto representa uma relação adicional entre este modelo e a depressão. Em ambas as situações – estresse e depressão – se observa uma hiperativação do eixo HHA, o que, conforme já mencionado, pode induzir atrofia neuronal, especialmente em estruturas altamente suscetíveis como o hipocampo (Sapolsky, 2000). Não é bem estabelecido, contudo, se a atrofia dos neurônios hipocampais é oriunda de desordens psiquiátricas (como a depressão) ou se precede e predispõe a elas (Barden, 2004; Campbell & MacQueen, 2004); contudo, o fato é que esta atrofia amplamente documentada, com diminuição dos pontos de ramificação dendrítica e sinapses, pode estar envolvida em muitos dos efeitos do estresse crônico, incluindo déficits cognitivos e alteração na atividade da Na^+, K^+- ATPase.

Embora não tendo alterado por si só a atividade desta enzima, o tratamento com lítio preveniu e reverteu a diminuição induzida pelo estresse, o que aponta a modulação da atividade da Na^+, K^+- ATPase como um possível mecanismo adicional de ação do lítio. Interessante observar que, após 30 dias de interrupção do estresse, a atividade enzimática também foi normalizada, indicando novamente que as alterações induzidas pelo estresse não são permanentes.

Apesar de o paralelismo nos efeitos dos tratamentos com estresse e lítio sobre a memória espacial e sobre a atividade da Na^+, K^+-ATPase não ser suficiente para estabelecer uma relação causa-efeito, muitos autores têm apontado déficits cognitivos induzidos pela inibição desta enzima (Dos Reis *et al*., 2005; Sato *et al*., 2004; Wyse *et al*., 2004; Zhan *et al*., 2004). A atividade da Na^+, K^+-ATPase também tem sido relacionada com potenciação de longa duração (LTP, do inglês *long-term potentiation*; Glushchenko & Izvarina, 1997), bem como sua inibição com depressão de longa duração (LTD, do inglês *long-term depression*; Reich *et al*., 2004), e déficits cognitivos têm sido apontados em situações onde a atividade desta enzima encontra-se reduzida, como na Doença de Alzheimer e em danos oxidativos (Hattori *et al*., 1998; Lehotsky *et al*., 1999). Uma vez que a atividade da Na^+, K^+-ATPase é crucial para a manutenção do gradiente iônico neuronal, é concebível que sua redução possa prejudicar o funcionamento dos neurônios hipocampais e, conseqüentemente, a memória espacial de animais cronicamente estressados.

Diversas proteínas têm sua atividade acoplada às bombas Na^+, K^+-ATPase, e um exemplo importante são os transportadores de glutamato: quando liberado na fenda sináptica, o glutamato é rapidamente recaptado, a maior parte pelos astrócitos adjacentes, via co-transporte com sódio, por transportadores acoplados à atividade de uma bomba Na^+, K^+-ATPase (Anderson & Swanson, 2000). Portanto, a diminuição na atividade da enzima Na^+,

K^+-ATPase também poderia estar sendo prejudicial no modelo de estresse estudado por facilitar a indução de excitotoxicidade glutamatérgica.

No artigo 2 desta tese estudamos os efeitos dos tratamentos com estresse e lítio sobre alguns aspectos da atividade glutamatérgica hipocampal. Desta feita, verificamos que o estresse provoca um aumento na liberação basal de glutamato por sinaptossomas, sem, contudo, alterar a captação pelos mesmos, e observamos ainda uma diminuição na captação de glutamato em fatias de hipocampo de ratos estressados.

Alguns pesquisadores sugerem que diferentes tipos de estressores, bem como um aumentado nível de glicocorticóides, induzem aumento nos níveis extracelulares de glutamato no hipocampo (Lowy *et al*., 1993, 1995; Venero and Borrell, 1999; Stein-Bahrens *et al*., 1994, 1992). A remoção do glutamato da fenda sináptica é um passo crucial no término da neurotransmissão glutamatérgica e prevenção de excitotoxicidade, e a captação sódio-dependente é o mecanismo principal de regulação dos níveis sinápticos de glutamato (Yeh *et al*., 2005). Esta captação é mediada por uma família de transportadores que apresentam padrões diferenciados de expressão e distribuição no sistema nervoso: GLAST e GLT-1 são principalmente expressos em células gliais, especialmente nos astrócitos, enquanto os transportadores EAAC1 são geralmente encontrados nas membranas neuronais (Sims & Robinson, 1999; Danbolt, 2001; González *et al*., 2002). Por serem os mais abundantes, GLT-1 e GLAST desempenham o papel mais importante na remoção do glutamato liberado, e falhas na sua função podem levar a diversos efeitos deletérios (Rothstein et al., 1996; Tanaka et al.; 1997; Mitani and Tanaka, 2003; Maragakis and Rothstein, 2001, 2004). Considerando a observada ausência de alteração na captação pela fração sinaptossomal – o que equivaleria à captação neuronal de glutamato – pode-se inferir que o efeito do modelo de estresse aqui utilizado sobre a captação de glutamato em fatias hipocampais seja mediado principalmente por alteração na atividade de transportadores gliais (efeito este possivelmente mediado, ao

menos em parte, pela diminuição na atividade das Na^+, K^+, ATPases), e que a diminuição na captação, aliada à aumentada liberação basal, pode representar uma importante contribuição aos mecanismos de lesão provocada pelo estresse.

Observamos também um aumento na captação, bem como aumentada liberação de glutamato induzida por alta concentração de potássio, em sinaptossomas de animais tratados com lítio. Embora a captação de glutamato por sinaptossomas não seja a ferramenta principal na remoção deste neurotransmissor da fenda sináptica após períodos de despolarização, ela pode representar um mecanismo adicional nos efeitos neuroprotetores do tratamento com lítio em situações que envolvem hiperestimulção glutamatérgica (e.g. Nonaka *et al*., 1998; Chen & Chuang, 1999).

Por sua vez, a liberação de glutamato, com a conseqüente ativação de receptores ionotrópicos e metabotrópicos, está notoriamente envolvida em mecanismos de plasticidade sináptica, e é amplamente aceito que a formação de memórias é dependente de fortalecimento entre as sinapses e aumento de sua eficiência. Dos mecanismos envolvidos no fortalecimento entre as sinapses, o mais estudado é a LTP, e acredita-se que para a ocorrência de LTP seja necessário o acionamento de mecanismos pós-sinápticos – tais como estimulação de receptores NMDA, aumento no influxo de cálcio, ativação de cinases específicas como a cinase dependente de cálcio e calmodulina (CaM cinaseII) e inserção de novos receptores AMPA na membrana pós-sináptica – como também, e paralelamente, de eventos pré-sinápticos, como a liberação de quantidades maiores de glutamato pelo neurônio pré-sináptico (Squire & Kandel, 2000; Lynch, 2004; Blitzer *et al*., 2005). Desta forma, pode-se supor que o aumento na liberação estimulada de glutamato em animais tratados com lítio esteja envolvido com mecanismos de plasticidade sináptica.

A facilitação da indução de LTP pelo tratamento com lítio já foi relatada por outros pesquisadores, que atribuíram este efeito à regulação positiva de proteínas relacionadas à

plasticidade sináptica, como CaM Cinase, CREB, tirosina cinase, etc (Son et al., 2003; Yu *et al*., 2003). Conforme exposto no anexo 1 desta tese, verificamos também os efeitos dos tratamentos com estresse e lítio sobre a indução de LTP no giro denteado, e os dados preliminares indicaram uma facilitação mediada pelo tratamento com lítio. Por sua vez, animais estressados apresentaram aumentados limiares para indução de LTP, ao passo que animais estressados e tratados com lítio apresentaram resposta à estimulação tetânica em intensidade similar à necessária para induzir LTP no giro denteado de animais controle. É possível que este efeito do lítio seja mediado, ao menos em parte, pelo aumento na liberação de glutamato após estimulação, e que isto possa contribuir para a facilitação da memória espacial observada no labirinto aquático.

É importante ressaltar, contudo, que há alguma controvérsia com relação aos mecanismos envolvidos nos efeitos do lítio sobre a plasticidade sináptica. Nonaka e colaboradores. (1998) demonstraram que o tratamento com lítio protege neurônios contra excitotoxicidade via inibição do influxo de cálcio mediado por receptores NMDA, o que representa um efeito oposto a um dos passos fundamentais na ocorrência da LTP: a ativação de receptores NMDA e conseqüente influxo de cálcio. Além disso, Du e colaboradores (2004) observaram que o tratamento com lítio inibe a expressão da sub-unidade GluR-1 do receptor AMPA em hipocampo, podendo diminuir sua inserção na membrana pós-sináptica, o que também poderia prejudicar a indução de LTP. De qualquer forma, estes dados apontam que o lítio atua modulando a neurotransmissão glutamatérgica, e seus efeitos sobre a plasticidade hipocampal necessitam estudos adicionais para serem melhor caracterizados.

O acúmulo de glutamato na fenda sináptica, que pode ter como conseqüência a indução de excitotoxicidade glutamatérgica, é acompanhado pela abertura de canais de cálcio na membrana pós-sináptica, e estudos evidenciam que o aumento dos níveis intracelulares de cálcio pode levar à gênese de espécies reativas de oxigênio (EROs) e nitrogênio (ERNs), ou

mais comumente denominados “radicais livres”. Isto é evidenciado pela indução de síntese de óxido nítrico, geração de xantina oxidase, ativação de fosfolipases que liberam ácido araquidônico das membranas e prejuízo da atividade mitocondrial (Sapolsky, 2000; Lewen *et al*., 2000), e é bem estabelecido que as EROs e ERNs estão envolvidas na morte de neurônios e astrócitos, tanto por necrose quanto por apoptose (Sonee, 2003; Rouach, 2004; Ha & Park, 2005).

Ácidos graxos poliinsaturados são as macromoléculas mais sensíveis a danos por EROs, devido à presença de elétrons altamente instáveis próximos a suas ligações duplas (Barja, 2004). No artigo 3 desta tese observamos que a exposição prolongada ao estresse induz um aumento na peroxidação de lipídeos de membrana no hipocampo. Estes dados concordam com outros estudos, que demonstraram aumento na lipoperoxidação hipocampal de animais submetidos cronicamente ao estresse (Fontella et al., 2005), confirmando a vulnerabilidade desta estrutura em tais situações. Considerando ainda os diversos trabalhos na literatura que descrevem a indução de estresse oxidativo após excitotoxicidade glutamatérgica (Vergum *et al*., 2001; Chong *et al*., 2005), é possível que a peroxidação lipídica observada neste trabalho seja um efeito final da aumentada liberação basal de glutamato induzida por este modelo de estresse.

Por outro lado, o tratamento com lítio induziu uma diminuição na geração de radicais livres, avaliada pela oxidação do DCF, sem, contudo, prevenir os efeitos do estresse sobre a peroxidação lipídica. Isto levou à possibilidade de uma alteração nas defesas antioxidantes em hipocampo de animais estressados, e de fato observamos que tanto os tratamentos com estresse quanto com lítio aumentam a reatividade antioxidante total em hipocampo. Ainda observamos que os dois tratamentos interagem significativamente neste parâmetro, uma vez que a reatividade antioxidante hipocampal de animais estressados e tratados com lítio é similar a dos animais controle.

Para melhor compreensão destes efeitos, fizemos a avaliação da atividade das enzimas antioxidantes Superóxido Dismutase (SOD) e Glutationa Peroxidase (GPx) nas estruturas cerebrais em que foram observadas alterações na reatividade antioxidante total, i.e. hipotálamo e hipocampo. Desta feita, constatamos que a GPx está aumentada em hipocampo de animais tratados com lítio, ao passo que a SOD apresentou-se mais ativa no hipotálamo destes animais. Por outro lado, o estresse induziu um aumento significativo na atividade da SOD no hipocampo, o que pode ser efeito de uma adaptação induzida pelo estresse. No entanto, o resultado mais intrigante deste trabalho foi a forte interação observada entre os tratamentos com estresse e lítio sobre a atividade da SOD: tanto no hipocampo quanto no hipotálamo, esta enzima apresentou-se muito mais ativa quando os dois tratamentos foram aplicados juntos. Embora não compreendendo as "causas" desta interação (que pode estar associada ao aumento no número de moléculas da enzima ou na atividade das mesmas), é interessante especular suas "conseqüências".

A principal fonte de EROs no organismo é o metabolismo oxidativo da mitocôndria, que consome aproximadamente 90% do oxigênio inalado (Gilgun-Sherki *et al*., 2002). Cerca de 2% deste oxigênio é convertido a ânions superóxido ($O_2^{\cdot -}$), que são as EROs mais abundantemente produzidas na cadeia respiratória e cuja degradação produz peróxido de hidrogênio (H_2O_2; Maier & Chan, 2002). Por sua vez, os radicais hidroxila ($HO^{\bullet}$), que são as EROs mais reativas em sistemas biológicos e principais responsáveis pela peroxidação de lipídeos de membrana (Keller & Mattson, 1998), são produzidos a partir do peróxido de hidrogênio na presença de íons ferro, através das reações de Fenton (Maier & Chan, 2002; Chong *et al*., 2005). Os radicais hidroxila também podem ser gerados alternativamente a partir do peroxinitrito, o qual é formado através da interação entre ânions superóxido e óxido nítrico (Fubbini & Hubbard, 2003).

As enzimas SOD e GPx são responsáveis pela degradação de $O_2^{\cdot-}$ e H_2O_2, respectivamente, e o balanço entre as atividades dessas enzimas parece ser mais importante na prevenção do estresse oxidativo do que propriamente a quantidade absoluta de alguma delas (Ceballos-Picot et al., 1992; Erakovic et al., 2000). Pelo observado neste trabalho, a atividade da SOD está desproporcionalmente elevada quando comparada com a atividade da GPx tanto no hipocampo quanto no hipotálamo dos animais estressados e tratados com lítio, o que poderia estar induzindo um acúmulo de H_2O_2 e, conseqüentemente, geração de radicais $HO^{\bullet}$ via reação de Fenton. Isto poderia explicar o aumento na peroxidação lipídica observado em hipocampo de animais estressados (devido ao aumento na atividade da SOD e, conseqüentemente, na produção de H_2O_2, o que poderia gerar radicais $HO^{\bullet}$), bem como a interação entre os tratamentos observada na reatividade antioxidante total desta estrutura (uma vez que os sistemas antioxidantes estariam sendo utilizados para neutralizar os radicais formados).

Nos experimento referentes à avaliação da vulnerabilidade celular, os quais fazem parte do artigo 2, foi observado que os tratamentos com estresse e lítio aumentam a liberação da enzima lactato desidrogenase (LDH) por fatias de hipocampo após exposição à privação de oxigênio e glicose (POG). Isto pode ser relacionado a um aumento no dano ou morte celular, uma vez que a LDH é uma enzima citosólica, provavelmente liberada após ruptura da membrana plasmática. Somando-se todos os resultados obtidos, algumas explicações para estes efeitos são possíveis.

Primeiramente, foi observado que o estresse aumenta a liberação basal de glutamato e diminui a captação deste neurotransmissor em fatias de hipocampo, em parte possivelmente via inibição da atividade de transportadores associados a Na^+, K^+, ATPases. Isto pode provocar uma hiperatividade glutamatérgica, com conseqüente ativação de receptores NMDA e aumento no influxo de cálcio. Somando-se a isto, o tratamento com estresse induz aumento

na atividade da enzima SOD, sem alterar a atividade da GPx, possibilitando maior formação de radicais $HO^{\bullet}$. Estas espécies são extremamente reativas, sendo potenciais indutoras de lipoperoxidação – a qual, de fato, encontra-se aumentada em hipocampo de animais estressados.

Em situações em que há uma despolarização generalizada, como é o caso durante a privação de oxigênio e glicose, a liberação de glutamato é ainda mais pronunciada. Em um sistema com captação ineficiente, as chances de morte neuronal por excitotoxicidade são maiores. Somando-se a isto, há um aumento na peroxidação de lipídeos de membrana, tornando-as mais vulneráveis em um processo de necrose celular. Tomados juntos, estes efeitos podem ser responsáveis pela aumentada vulnerabilidade celular observada 3 horas após a POG.

Também foi observada uma aumentada liberação de LDH em hipocampo de animais tratados com lítio. Este efeito contrariou a premissa inicial, de que o tratamento com lítio teria efeitos neuroprotetores em situações de lesão. Uma possível explicação para este efeito seria o aumento na liberação de glutamato mediante estímulo – que pode ser benéfica para os mecanismos de plasticidade celular, mas em situações de hiperestimulação pode contribuir para excitotoxicidade glutamatérgica. Paralelamente, o tratamento com lítio não foi capaz de prevenir os efeitos do estresse sobre a peroxidação lipídica hipocampal, embora apresentando uma relativa atividade antioxidante, o que corrobora com a idéia de vulnerabilidade dos neurônios hipocampais mesmo mediante o tratamento com lítio. Contudo, é importante salientar que a avaliação da LDH liberada foi feita 3 horas após a POG, um período no qual há uma morte celular predominantemente necrótica, enquanto na maioria dos trabalhos que observaram proteção pelo tratamento com lítio em modelos de isquemia cerebral a análise foi feita no mínimo 24hs após o insulto (para uma revisão, ver Chuang *et al*., 2002). Neste período, há uma importante contribuição de morte celular por apoptose (Slevin et al., 2005;

Love, 2003). Considerando que o lítio tem uma marcante ação anti-apoptótica (Chen & Chuang 1999; Manji *et al*., 2000), é possível que seus efeitos protetores em situações de isquemia cerebral só sejam verificáveis após períodos maiores de reperfusão.

Conforme mencionado na introdução, o modelo de estresse utilizado neste trabalho é baseado no paradigma de estresse crônico moderado, o qual é amplamente aplicado como um modelo de depressão. Assim sendo, o segundo grande objetivo desta tese foi avaliar os efeitos antidepressivos do tratamento com lítio sobre alguns parâmetros notoriamente alterados em animais submetidos a modelos de depressão, tais como a diminuição do interesse por alimentos palatáveis e inibição da aquisição de comportamentos apetitivos estimulados por estes alimentos.

O primeiro estudo realizado neste sentido está descrito no artigo 4, em que avaliamos os efeitos dos tratamentos com estresse e lítio sobre o comportamento alimentar e a possível relação com o nível de ansiedade dos animais, a qual foi verificada no Labirinto em Cruz Elevado. Este trabalho foi assim desenhado porque, em estudos anteriores no laboratório, havia sido observado que a exposição a outro modelo de estresse repetido (40 dias de imobilização, 1 hora por dia) induz um aumento no consumo de alimento doce (Foot Loops, Kellog's®), o qual é revertido pela administração de diazepam (Ely *et al*., 1997). Por outro lado, em um modelo de estresse crônico variado similar ao que utilizamos neste trabalho, houve diminuição no consumo de doce (Gamaro *et al*., 2003)

Nos estudos desta tese, observamos que tanto o estresse quanto o lítio aumentam o consumo de alimentos doces, sem, contudo, aumentar o consumo de ração padrão. Entretanto, o aumento na ingestão de doce pelos animais tratados com lítio foi mais acentuado, e somente estes animais apresentaram aumento no consumo de alimentos salgados.

Na avaliação no labirinto em cruz elevado, observamos um efeito claramente ansiolítico do tratamento com lítio, sem alterações expressivas no comportamento dos animais

estressados. Isto sugere que os efeitos observados no comportamento alimentar não são devidos a uma ansiedade aumentada dos animais. Além disso, o aumento específico para outros tipos de alimento (alimentos palatáveis), diferentes da ração padrão, indica que esse efeito está mais relacionado à sensação que o alimento proporciona do que a um aumento na ingestão provocado por maior fome.

Um ponto importante observado neste estudo foi o de que o lítio aumenta a ingestão de alimentos mais calóricos – no caso, carboidratos simples e gorduras. Este efeito do lítio difere do efeito de outras drogas antidepressivas, que não apresentam efeito estimulador *per se* no consumo de alimentos ou soluções doces, e sim apenas revertem os efeitos induzidos pelos modelos animais de depressão (Bekris *et al*., 2005). Estes dados são relevantes especialmente ao considerarmos que, na clínica, um dos principais efeitos colaterais do tratamento com lítio é o ganho de peso corporal (responsável, muitas vezes, pela desistência no tratamento), que está relacionado ao aumento no risco de incidência de síndrome metabólica (Zimmermann *et al*., 2003; Himmerich *et al*, 2005).

Por outro lado, embora utilizando um modelo de estresse que é baseado em modelos de depressão, os animais estressados aumentaram o consumo de doce. Estes dados contrariam a premissa da anedonia induzida pelos modelos de depressão, manifestada pela diminuição no consumo de alimentos ou soluções palatáveis. Comparando os diferentes modelos de estresse crônico moderado existentes na literatura (para uma revisão, ver Willner, 2005), bem como o modelo desenvolvido por Gamaro et al. (2003) em nosso laboratório, uma diferença observada com relação ao modelo utilizado neste trabalho é a ausência de períodos de restrição alimentar, e é sugerido que a privação de alimentos induz um aumento na liberação de serotonina no hipotálamo (Gur et al., 2003), o que pode induzir supressão do apetite (Arkle & Ebenezer, 2000; Halford & Blundell, 2000). Assim sendo, a ausência de privação alimentar

no modelo desta tese pode ser, ao menos em parte, responsável pelo aumento de consumo de alimentos doces.

Por outro lado, Laugero (2001) propôs que os carboidratos podem promover uma retroalimentação sobre o eixo HHA, baseado em resultados anteriores demonstrando que a ingestão de sacarose normaliza a ingestão alimentar, o balanço energético e a expressão do hormônio liberador de cortocotrofina em ratos adrenalectomizados (Laugero *et al*., 2001). Aliado a isto, foi demonstrado que animais estressados apresentam preferência por soluções de sacarose a soluções de sacarina (Dess, 1992). Isto favorece a idéia de que fatores metabólicos influenciem a preferência alimentar de animais estressados, talvez como uma resposta adaptativa na tentativa de normalizar a atividade do eixo HHA e restabelecer o nível basal de glicorticóides circulantes.

No artigo 5 desta tese verificamos a resposta nociceptiva de animais estressados e tratados com lítio quando expostos a um sabor agradável (leite condensado) ou a um sabor desagradável (ácido acético). Os resultados mostraram que enquanto o estresse aumenta o limiar nociceptivo após um estímulo desagradável sem alterar, contudo, a resposta ao estímulo agradável, o tratamento com lítio torna os animais mais responsivos a estímulos tanto prazerosos quanto aversivos.

É amplamente descrito que a ingestão de soluções de sacarose induz analgesia, bem como amplifica o efeito analgésico de agonistas opióides (Kanarek et al,. 2000; Kanarek & Homoleski, 2000; Segato et al., 2005). Estes efeitos também são observados em humanos neonatos (Morash & Fowler, 2004), sendo sugerido que o efeito analgésico está diretamente relacionado ao valor hedônico da sacarose (Pepino & Mennella, 2005). Estudos recentes demontraram que o consumo de sacarose ativa neurônios da matéria cinzenta periaquedutal e do núcleo magno da rafe, duas estruturas do tronco cerebral envolvidas na modulação descendente da dor (Anseloni et al., 2005), bem como indicam o envolvimento de opióides

endógenos e das atividades monoaminérgica e serotoninérgica na analgesia induzida por sacarose (Rebouças *et al*., 2005).

Embora não tenhamos observado uma diminuição no consumo de alimento doce em ratos estressados, houve ausência de analgesia após o consumo de leite condensado, indicando um prejuízo na resposta central a estes alimentos que pode influenciar também a resposta hedônica a estímulos gustativos prazerosos. Por outro lado, animais estressados apresentaram analgesia após exposição ao sabor ácido e desagradável, corroborando com mais uma característica da depressão que á a aumentada reatividade, ou hipervalorização, de estímulos aversivos (American Psychiatry Association, 1994, APUD Di Chiara, 1999).

Por sua vez, os animais tratados com lítio apresentaram a analgesia característica após o consumo de leite condensado, e o lítio preveniu a ausência de resposta analgésica de animais estressados. Curiosamente, o tratamento com lítio também induziu um aumento no limiar nociceptivo dos animais após exposição ao sabor desagradável. Aparentemente, estes efeitos não são mediados pela ação do sistema opióide, uma vez que diversos estudos demonstram que o lítio atenua a analgesia opióide, bem como previne a síndrome de abstinência induzida por naloxone (Dehpour *et al*., 1994, 1995; Johnston & Westbrook, 2004). Considerando que animais tratados com lítio apresentam um marcante interesse por novos alimentos, é possível que exista uma alteração na saliência com que estes animais percebem diferentes sabores, e que isso possa influenciar aspectos motivacionais na busca por novos alimentos.

A dopamina desempenha um importante papel na modulação de diversas manifestações comportamentais, como movimento, recompensa e motivação (Nowak et al 2005, Vezina et al 2002), e é bem estabelecido que a ingestão de alimentos palatáveis, bem como o uso de drogas de abuso, induz a ativação do sistema dopaminérgico (Berridge and Robinson, 1998; Nestler, 2002; Schultz, 2002; Wise, 2002). O fato de a dopamina ser liberada

no estriado ventral em resposta a drogas de abuso e a alimentos corrobora com a hipótese de que este neurotransmissor seja mediador do valor hedônico relacionado a uma recompensa (Hajnal & Norgreen, 2001, 2002).

Postulamos que alterações no sistema dopaminérgico possam estar relacionadas aos efeitos dos tratamentos com estresse e lítio sobre o consumo de alimentos palatáveis, através da alteração do valor de recompensa associado a estes alimentos. Para testar tal hipótese, resolvemos avaliar o comportamento destes animais em tarefas que sabidamente envolvem atividade dopaminérgica, como a tarefa de preferência de lugar condicionada por um estímulo gustativo apetitivo e a atividade locomotora após administração de dietilpropiona. Também avaliamos a atividade dopaminérgica, através da medida do conteúdo total de dopamina e de seus metabólitos, no núcleo acumbens, que é conceituado como uma importante "interface entre motivação e ação", tendo uma função essencial em comportamentos relacionados a reforços naturais como ingestão alimentar e comportamento sexual (Robbins and Everitt, 1996; Cardinal *et al*., 2002; Kelley and Berridge, 2002).

Conforme descrito no artigo 6, observamos que os animais tratados com lítio adquirem preferência de lugar condicionada por um estímulo apetitivo (no caso, leite condensado), bem como apresentam uma sensibilização cruzada com análogos da anfetamina quando comparando com os animais controle, a qual foi manifestada pelo aumento no número de cruzamentos no campo aberto após injeção de dietilpropiona. Não houve alteração nos níveis de dopamina, mas observamos uma diminuição nos níveis de DOPAC no núcleo acumbens de animais tratados com lítio, o que pode ser resultado da menor liberação basal de dopamina, como descrita por outros pesquisadores (Gambarana *et al*., 1999, 2003).

Apesar de não haver alteração nos níveis de dopamina de animais tratados com lítio, os resultados obtidos indicam um claro fortalecimento do tônus dopaminérgico. É importante salientar que o fato de não haver aumento na liberação basal deste neurotransmissor não

impede que a liberação fásica esteja aumentada, conforme descrito por Gambarana e colaboradores (2003), e que o aumento na liberação de dopamina após um estímulo (como, por exemplo, a exposição a um sabor palatável ou administração de uma droga análoga à anfetamina) seja responsável pela aquisição do comportamento apetitivo e da sensibilização cruzada à dietilpropiona.

Somando-se a isto, a possibilidade de o tratamento com lítio induzir uma alteração na densidade de receptores e/ou transportadores de dopamina não pode ser menosprezada, especialmente ao considerarmos que este sal atua em diversas cascatas de transdução de sinal e altera a expressão de determinados genes (para uma revisão, ver Manji *et al*., 1995). Embora a alteração nos transportadores dopaminérgicos seja pouco descrita, Kameda e colaboradores (2001) observaram que o lítio aumenta a expressão do mRNA do receptor dopaminérgico D2. Estas possibilidades devem ser investigadas mais profundamente para melhor compreensão dos efeitos do lítio sobre a atividade dopaminérgica.

Por sua vez, animais estressados cronicamente não adquiriram o comportamento apetitivo induzido por leite condensado, bem como não desenvolveram sensibilização cruzada com dietilpropiona. Ainda, a avaliação do conteúdo de dopamina demonstrou uma diminuição nos níveis de DOPAC, bem como diminuição da relação DOPAC/dopamina no núcleo acumbens, indicando uma diminuição no *turnover* deste neurotransmissor.

Modelos de estresse crônico moderado induzem um estado do tipo anedônico, o qual se assemelha a alguns dos sintomas da depressão maior. Alguns estudos demonstraram diminuição da atividade dopaminérgica estriatal em animais submetidos a este modelo (Dziedzicka-Wasylewska *et al* 1997; Bekris *et al*., 2005), bem como uma resposta dopaminérgica prejudicada após a exposição a sabores doces (DiChiara *et al*., 1999). Somando-se a isto, o tratamento crônico com drogas antidepressivas produz uma variedade de alterações na transmissão dopaminérgica, como a sensibilização das respostas

comportamentais a agonistas dos receptores D2/D3 no núcleo accumbens, e acredita-se que estas alterações sejam cruciais para os efeitos antidepressivos dessas drogas em modelos de depressão como nado forçado ou o estresse crônico moderado (Willner, 1997).

Neste sentido, é possível que a ausência de comportamento apetitivo nos animais estressados seja uma resposta anedônica induzida pelo modelo, a qual pode ser causada por uma disfunção no sistema dopaminérgico estriatal. Considerando ainda que o tratamento com lítio modificou esta resposta em animais estressados, uma vez que o grupo estressado + lítio adquiriu preferência de lugar condicionada, pode-se inferir que esta habilidade seja parte do repertório de ações antidepressivas deste sal.

Concluindo, embora o modelo de estresse crônico variado utilizado nesta tese tenha induzido aumento no consumo de alimento doce, a ausência de condicionamento apetitivo, bem como de analgesia induzida por um estímulo gustativo prazeroso, podem ser indicativos da anedonia induzida pelo modelo, possivelmente relacionada à diminuição na atividade dopaminérgica no estriado ventral. Pode-se supor que o aumento no consumo de doce destes animais seja uma resposta de adaptação metabólica, numa tentativa de normalizar a atividade do eixo HHA. Por outro lado, o desempenho de animais tratados com lítio em tarefas que envolvem atividade dopaminérgica leva a crer que há um aumento na atividade deste neurotransmissor, que pode ser em função de alterações na liberação fásica, na densidade de receptores ou nos transportadores de dopamina, e é possível que esta alteração nas vias dopaminérgicas seja em parte responsável pelo aumentado interesse por alimentos novos apresentado por estes animais.

CONCLUSÕES

1 – Os efeitos do estresse crônico variável e do lítio sobre a memória espacial são tempo-dependentes: 30 dias de tratamento com lítio são suficientes para induzir um efeito facilitador na memória, ao passo que somente após 40 dias de estresse os déficits cognitivos são observados;

2 – O estresse diminui a atividade da enzima Na^+, K^+-ATPase em membranas sinápticas hipocampais, e este efeito é prevenido pelo tratamento com lítio, bem como revertido tanto pela interrupção do estresse quanto pelo tratamento pós-estresse com lítio;

3 – A interrupção do estresse por um período de trinta dias também reverte os seus efeitos sobre a memória, assim como o faz o tratamento pós-estresse com lítio. Estes dados indicam que o déficit cognitivo induzido pelo estresse crônico é mediado por alterações plásticas, e a similaridade temporal com os efeitos sobre a atividade da enzima Na^+, K^+-ATPase sugere que ela possa estar envolvida no prejuízo observado na memória espacial;

4 – O estresse aumenta a liberação basal de glutamato e diminui a captação deste neurotransmissor por fatias hipocampais, efeito este que pode induzir excitotoxicidade glutamatérgica e colaborar no agravamento de outros insultos. O aumento da morte celular em fatias de hipocampo de animais estressados após a privação de oxigênio e glicose confirma esta hipótese;

5 – O lítio aumenta a captação de glutamato por sinaptossomas, o que pode ser uma ferramenta adicional na neuroproteção após diversos tipos de insulto. Também há um aumento na liberação de glutamato após estímulo, e este efeito pode, por um lado, facilitar os

mecanismos de plasticidade sináptica, por outro, aumentar a chance da dano após privação de oxigênio e glicose;

6 – O tratamento com lítio induziu um relativo efeito antioxidante, demonstrado pela diminuição na formação de agentes oxidantes no hipocampo e pelo aumento da reatividade antioxidante total em hipocampo e hipotálamo, bem como pelo aumento na atividade da SOD e da GPX em hipotálamo e hipocampo, respectivamente. Contudo, este efeito antioxidante não foi eficiente na prevenção da peroxidação lipídica provocada pelo estresse no hipocampo, e o aumento desproporcional na atividade da SOD em animais estressados e estressados + lítio é um possível causador do aumento na peroxidação dos lipídeos de membrana;

7 – Os tratamentos com estresse e lítio induziram aumento no consumo de alimentos doces, sem, contudo, alterar o consumo de ração padrão pelos animais. No entanto, somente animais tratados com lítio apresentaram aumento também no consumo de alimentos salgados, e o aumento no consumo de doces por este grupo foi muito mais acentuado do que nos animais estressados. Estes efeitos não parecem ser devidos a uma maior ansiedade, visto que não houve efeito ansiogênico dos tratamentos no labirinto em cruz elevado.

8 – Animais estressados não apresentaram a analgesia característica após exposição a um sabor doce agradável (leite condensado) na medida de latência de retirada da cauda; contudo, mostraram analgesia induzida por um sabor ácido e desagradável, o que caracteriza alguns dos efeitos clássicos da depressão. Já animais tratados com lítio apresentaram analgesia tanto após o sabor agradável quanto o aversivo, sugerindo uma maior sensibilidade a estímulos gustativos nestes animais, e o lítio preveniu a ausência de analgesia induzida por doce em animais estressados, o que pode ser tomado como um efeito antidepressivo deste tratamento;

9 – Houve uma diminuição na atividade dopaminérgica ventro-estriatal de animais estressados, que pode estar envolvida na ausência de comportamento apetitivo condicionado por um estímulo palatável. Por outro lado, animais tratados com lítio apresentaram um aumento no tônus dopaminérgico, demonstrado pela aquisição de comportamento apetitivo e pela sensibilização cruzada com dietilpropiona, sem, contudo, apresentarem alteração no conteúdo total de dopamina no núcleo accumbens. Pode-se sugerir um aumento na liberação fásica de dopamina ou alteração em seus receptores, e estes efeitos podem estar envolvidos no aumentado interesse de animais tratados com lítio por alimentos novos.

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ANEXO

ESTRESSE PREJUDICA A PLASTICIDADE SINÁPTICA HIPOCAMPAL: EFEITO ATENUADO PELO TRATAMENTO COM LÍTIO

De Vasconcellos et al.

Trabalho realizado em colaboração com o **Prof. Dr. Oscar Ramirez**, Depto. Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba – Argentina**.**

Apresentado como cartaz na XIX Reunião Anual da Federação das Sociedades de Biologia Experimental – FESBE, em Águas de Lindóia, agosto de 2004.

ESTRESSE PREJUDICA PLASTICIDADE SINÁPTICA HIPOCAMPAL:

EFEITO ATENUADO PELO TRATAMENTO COM LÍTIO.

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INTRODUÇÃO

A platicidade sináptica desempenha um papel fundamental na modulação e fortalecimento de conexões sinápticas durante o desnvolvimento e também no cérebro adulto, e é amplamente aceita como um passo importante no processo de formação da memória. Um dos principais modelos de fortalecimento entre as conexões sinápticas é a Potenciação de Longa Duração (LTP), considerada como um correlato de memória e aprendizagem por suas características temporais tão semelhantes às de aquisição e consolidação da memória.

Ao contrário do estresse agudo, que é facilitador de processos de memória e da plasticidade sináptica, o estresse crônico prejudica a memória espacial e também a plasticidade sináptica hipocampal. Estudos anteriores em nosso laboratório demonstraram que animais submetidos a um modelo de estresse crônico variável apresentam déficits na memória espacial, avaliada no labirinto aquático de Morris, e que os efeitos cognitivos observados nestes ratos são prevenidos pelo tratamento concomitante com lítio.

Os sais de lítio são largamente utilizados no tratamento de distúrbios psiquiátricos, sendo a droga de escolha para o tratamento do distúrbio bipolar e também como adjuvante no tratamento da depressão maior. Diversos trabalhos têm demonstrado uma proeminente ação neuroprotetora da terapia com lítio.

O objetivo deste trabalho foi verificar o efeito dos tratamentos com estresse e lítio sobre a plasticidade sináptica hipocampal.

MATERIAIS E MÉTODOS

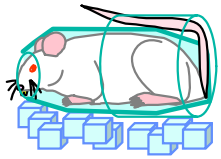
RATOS WISTAR MACHOS E ADULTOS

CONTROLES | **ESTRESSADOS**

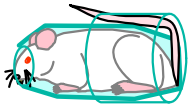
Com Lítio | **Sem Lítio** | **Com Lítio** | **Sem Lítio**

40 DIAS DE ESTRESSE

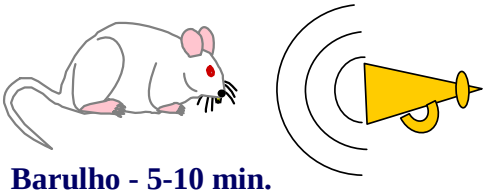
Luz piscante - 2-4 hs



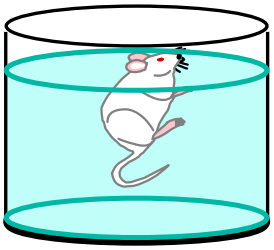
Imobilização + frio 60-90 min.



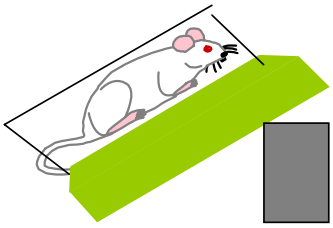
Imobilização - 60-90 min.



Barulho - 5-10 min.



Nado forçado - 3-15 min.



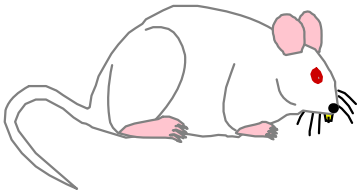
Inclinação - 2-6 hs

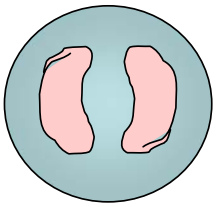
Isolamento - 48-72 hs.

7 AGENTES ESTRESSORES

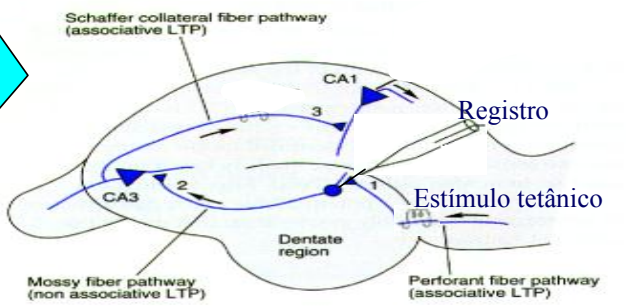
MATERIAIS E MÉTODOS

Decapitação

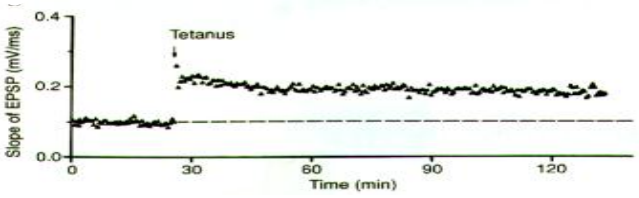




Dissecção dos hipocampos, fatiados na expessura de aprox. 400 µm

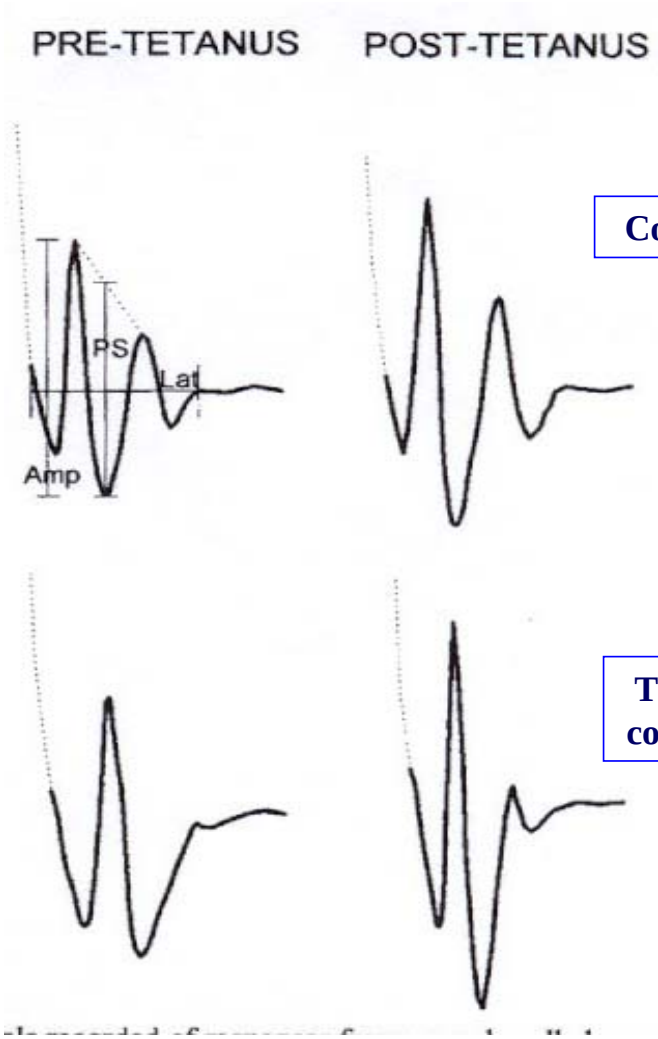


Estimulação tetânica da Via Perforante e indução de LTP no Giro Denteado

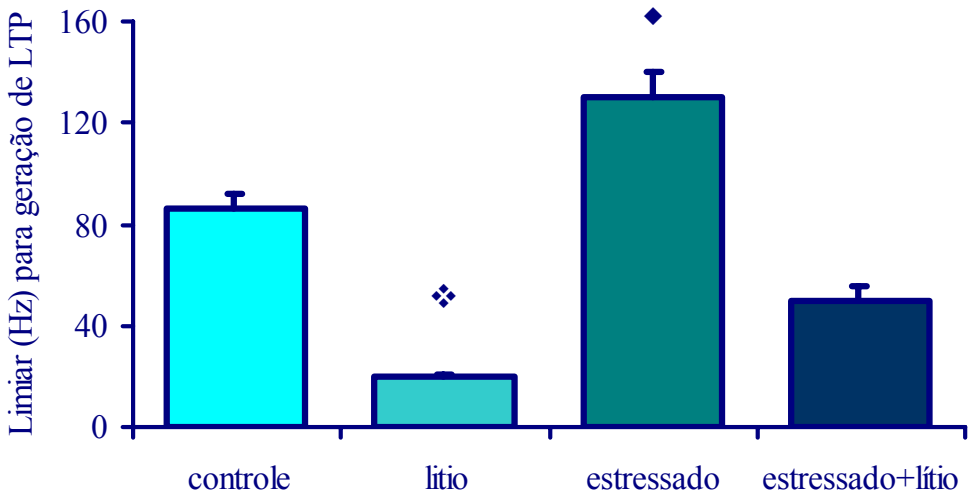


MATERIAIS E MÉTODOS

Considerou-se geração de LTP quando a estimulação induziu um aumento de 30% na amplitude ou inclinação dos PEPS ou dos potenciais em ponta de população (*population spikes*).



RESULTADO



Efeitos dos tratamentos com estresse e lítio sobre o limiar em Hz para geração de LTP em fatias de hipocampo. Dados expressos como média ± erro padrão, N = 3-5 por grupo.

❖ : Efeito significativo do tratamento com lítio (ANOVA de duas vias, *P* < 0,05).

◆ : Efeito significativo do estresse (ANOVA de duas vias, *P* < 0,05).

DISCUSSÃO

Os resultados deste trabalho demonstraram que o estresse crônico prejudica a plasticidade sináptica hipocampal, o que foi avaliado pelo aumento na intensidade de estímulo necessária para geração de LTP. Este efeito foi prevenido pelo tratamento concomitante com lítio, assim como o lítio *per se* induziu uma diminuição nos limiares para geração de LTP. Estes resultados apontam alterações na plasticidade sináptica como possíveis causas dos efeitos desses tratamentos sobre a memória.

Os mecanismos de ação do tratamento com lítio são fontes de constantes estudos. Sabe-se que este sal induz diversos efeitos benéficos, como aumento na expressão e síntese de proteínas neuroprotetoras, fatores neurotróficos e aumento da neurogênese hipocampal. Curiosamente, todos estes eventos têm sido relacionados com facilitação da memória.

Paralelamente, o lítio atua sobre diversos mecanismos de transdução de sinal, incluindo geração de AMP cíclico e, por consequência, ativação de PKA. Também, por estas e outras rotas, o lítio induz aumento na fosforilação de CREB, o qual tem sido relacionado com plasticidade sináptica e alguns tipos de memória. Por fim, trabalhos de nosso laboratório demonstraram que o tratamento com lítio induz um aumento na liberação de glutamato pós-impulso, o que poderia colaborar para o fortalecimento das conexões sinápticas.

ABSTRACT

CHRONIC STRESS IMPAIRS HIPPOCAMPAL SYNAPTIC PLASTICITY: EFFECT ATTENUATED BY LITHIUM TREATMENT. **Vasconcellos, A.P.S.**1; Maglio, L.E.**2; Almirón, R.S.**2; Carlini, V.**2; Rocha, E.R.1; Ramirez, O.A.2; Dalmaz, C1. 1PPG-Neurociências, ICBS – UFRGS, Brazil; 2Depto. Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba – Argentina.**

Objectives: Memory formation is dependent of alterations in the synaptic efficacy, and one model of long-lasting enhancement of synaptic transmission efficacy is LTP, which is considered the base for some forms of learning and memory. It is well known that chronic stress impairs spatial memory formation and hippocampal synaptic plasticity, and earlier studies from our laboratory showed that the cognitive deficits observed in chronically stressed rats is prevented by lithium treatment. The aim of this work was verify the effects of stress and lithium treatment on hippocampal synaptic plasticity.

Methods and Results: Adult male Wistar rats were divided into two groups, control and chronically stressed, treated either with normal chow or with chow containing LiCl for 40 days. Stress treatment was a chronic variable stress model, which consists in the exposure of the animals to seven different stressors, in a variable way once a day, every day. At the end of the treatment, rats were killed, brains were removed, hippocampi were dissected and transverse slices, approximately 400μm thick, were obtained for electrophysiological analysis. Induction of LTP was evaluated by insertion of a stimulating electrode in the perforant path, and a recording microelectrode was inserted in the dentate granule cell body layer. Only cells showing a stable response were analyzed, and LTP was considered to have been produced when the amplitude and slope of excitatory post-synaptic potential and evoked population spikes were increased in at least 30%, remaining stable for 1 hour. Results showed an increase in the thresholds for LTP induction in hippocampus of chronically stressed rats, and this effect was prevented by lithium treatment. Rats just treated with lithium presented a diminished threshold for LTP induction (Two-way ANOVA, $P < 0,05$).

Conclusions: These results suggest that modulation of synaptic plasticity and facilitation of LTP induction may be a mechanism of action of lithium in preventing cognitive impairments induced by chronic stress.