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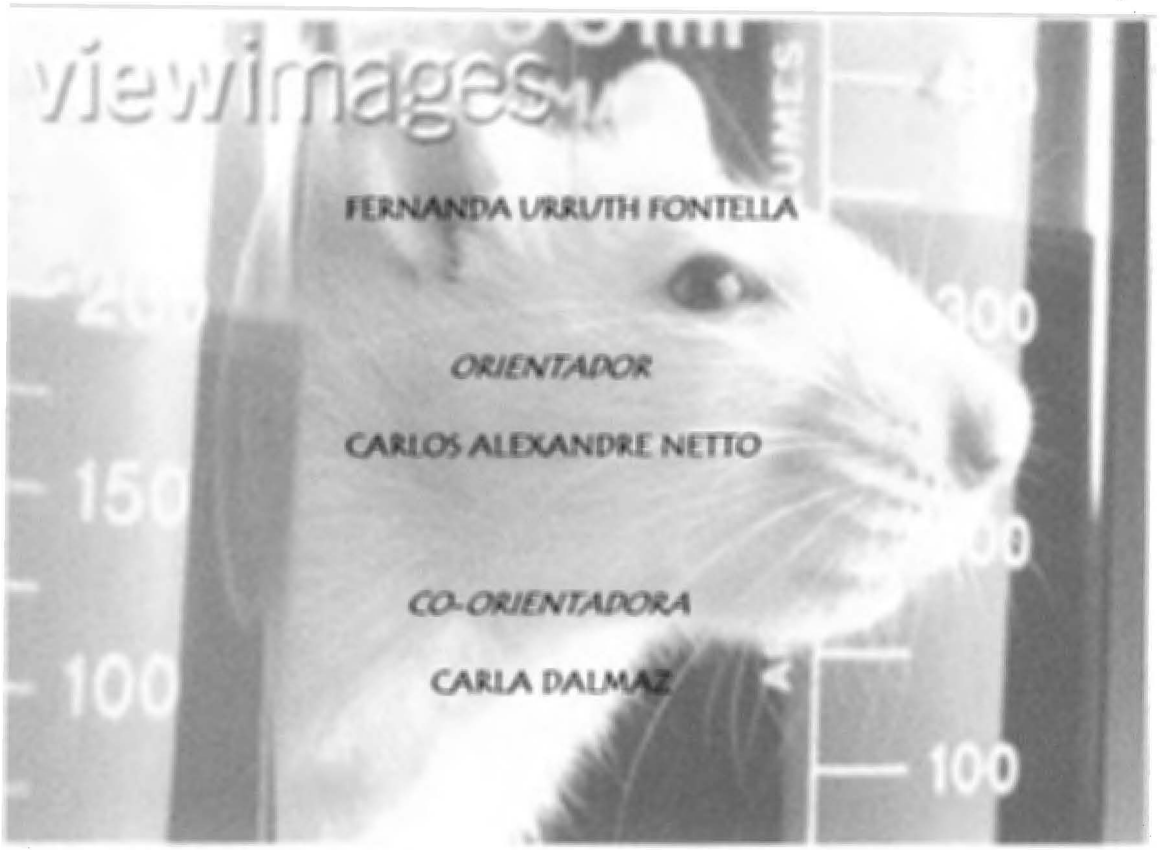
ESTUDOS COMPORTAMENTAIS E NEUROQUÍMICOS EM UM MODELO DE ESTRESSE REPETIDO EM RATOS



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2004

"Se as coisas são intangíveis... ora!

Não é motivo para não querê-las...

Que tristes os caminhos, se não fora

A presença distante das estrelas!"

Mário Quintana

Essas palavras foram a minha oração nestes últimos anos, mas agora elas fazem realmente sentido para mim.

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LISTA DE ABREVIATURAS

A1 - receptor pré-sináptico de adenosina
A2 - receptor de adenosina
ABAP – di-hidrocloreto de 2,2'azobis (2 amidinopropano)
ACTH - hormônio adrenocorticotrófico
ADP - difosfato de adenosina
AMP - monofosfato de adenosina
AMPA - α-amino-3-hidróxi-5-metil-4-asoxazolpropionato
ATP - trifosfato de adenosina
AVP - arginina-vasopressina
CA1, CA3 e CA4 - *cornu ammonis,* regiões do hipocampo
CAT - catalase
CRH - hormônio liberador da corticotrofina
DCF - 2'-7'-diclorofluoresceína
DNA - ácido desoxirribonucléico
DNPH - 2,4-dinitro-fenilhidrazina
EAA - do inglês, *excitatory aminoacid* (aminoácido excitatório)
EAO - espécies ativas de oxigênio
EROs - espécies reativas de oxigênio
GC - glicocorticóides
GD - giro denteado
Glu - glutamato
GPx - glutationa peroxidase
GSH - glutationa reduzida
GSSG - glutationa oxidada
i.p. - intra-peritoneal
K^+ - íon potássio
LDH - lactatodesidrogenase
LHHA - eixo límbico hipotalâmico-hipófise-adrenocortical
LTP - do inglês, *long-term potention* (potenciação de longa duração)
mRNA - do inglês, *ribonucleic acid* (RNA mensageiro)
Na^+ - íon sódio
NADPH - nicotinamida adenina dinucleotídeo-fosfato – forma reduzida.
NMDA - ácido N-metil-D-Aspártico
NPV - núcleo paraventricular hipotalâmico
NSE - do inglês, *neuron specific enolase* (enolase neurônio específica)
NTPDase - do inglês, *nucleoside triphosphate diphosphohydrolase*
OGD - do inglês, *oxygen and glucose deprivation* (privação de oxigênio e glicose)
OVX - ooforectomizada
S100 B - proteína produzida pelos astrócitos
SIA - do inglês *stress-induced analgesia* (analgesia induzida pelo estresse)
SNC - sistema nervoso central
SNV - sistema neuro-vegetativo
SOD - superóxido dismutase
TAR - do inglês, *total antioxidant reactivity* (reatividade antioxidante total)
TCA - do inglês, *trichloroacetic acid* (ácido tricloroacético)
TBARS - do inglês, *thiobarbituric acid reactive species* (espécies reativas ao ácido tiobarbitúrico)

RESUMO

A resposta ao estresse é essencial para as funções vitais do organismo, contudo, a prolongada exposição a um estímulo estressor pode ser deletéria, resultando em condições patológicas. Sabe-se, por outro lado, que a exposição repetida ao estresse também pode levar a adaptações. Experimentos realizados no nosso laboratório utilizando o modelo de estresse crônico por imobilização mostraram que esse tratamento altera a nocicepção de ratos de maneira gênero-dependente. Os objetivos dessa Tese são: a) observar alterações na resposta nociceptiva em animais repetidamente estressados em diferentes situações, bem como o efeito do sexo do animal, b) avaliar alterações na memória e c) na vulnerabilidade neuronal, além de alguns fatores neuroquímicos possivelmente envolvidos na modulação dessas respostas (tais como a dinâmica da transmissão glutamatérgica, o estresse oxidativo, a atividade de enzimas envolvidas na cascata extra-celular de degradação do ATP). Investigamos a nocicepção dos animais após exposição a diferentes estímulos gustativos e verificamos que os animais controle respondem com analgesia somente quando experimentam o sabor doce (estímulo agradável), enquanto os animais estressados parecem mais aptos a perceber um estímulo desagradável. Considerando as diferenças sexuais na resposta nociceptiva, nós avaliamos a produção de radicais livres, a lipoperoxidação e a reatividade antioxidante na medula de ratos machos e fêmeas, estressados cronicamente, assim como a influência do estradiol sobre a hidrólise de ATP, ADP e AMP em sinaptossomas preparados a partir da medula destes animais. Os resultados obtidos sugerem que o estresse repetido diminui a reatividade antioxidante tanto em machos quanto em fêmeas e que existe uma diferença sexual neste efeito, assim como na produção de radicais livres, sem que haja alterações na lipoperoxidação. As fêmeas estressadas, com ou sem reposição hormonal, apresentam menor limiar nociceptivo, com menores níveis de adenosina extracelular, efeito esse revertido pela reposição hormonal. Para avaliar os efeitos do estresse repetido sobre a memória, utilizamos três diferentes tarefas: labirinto aquático de Morris (memória espacial e memória de referência); esquiva inibitória e teste de reconhecimento de objetos. Observamos que este modelo de estresse é capaz de modificar a memória espacial, que depende da função hipocampal. Com relação aos parâmetros neuroquímicos possivelmente envolvidos nessas alterações comportamentais,

comportamentais, investigamos o efeito da exposição aguda e crônica ao estresse de restrição sobre a captação e liberação de glutamato em sinaptossomas de hipocampo de ratos, sobre o *binging* de [^{3}H]glutamato nas membranas sinápticas, sobre a hidrólise do ATP, ADP e AMP em sinaptossomas hipocampais e sobre o estresse oxidativo no hipocampo destes animais. Nós concluímos que animais expostos repetidamente ao estresse por contenção apresentam aumentada tanto a captação quanto a liberação basal de glutamato e diminuído o *binding* de [^{3}H]glutamato no hipocampo. Observamos, ainda, diferentes efeitos nos dois modelos de estresse estudados, com um significativo aumento da hidrólise de ATP, ADP e AMP em resposta ao estresse agudo e um aumento somente de uma ecto-ATPase induzida por estresse repetido. Este modelo de estresse repetido também induziu estresse oxidativo. Por fim, para avaliar a influência do estresse sobre a vulnerabilidade neuronal, nós submetemos fatias de hipocampo de ratos estressados, aguda ou repetidamente, a um modelo de privação de oxigênio e glicose (POG), e demonstramos que o estresse repetido aumenta a vulnerabilidade hipocampal a um insulto subseqüente, no caso a POG, com conseqüências principalmente sobre os neurônios. Também verificamos que o mecanismo responsável por esta maior susceptibilidade neuronal à POG não envolve a captação de glutamato.

Nós concluímos que o estresse repetido é capaz de alterar tanto as respostas nociceptivas como o desempenho nas tarefas de memória em ratos, que estas alterações na resposta à dor podem ser influenciadas por estímulos externos e/ou por hormônios e que ambos os comportamentos podem ter relação com alterações neuroquímicas ocorridas na medula espinal e no hipocampo, como a degradação extracelular do ATP, o estresse oxidativo e a captação de glutamato. Ainda, que essas alterações neuroquímicas podem aumentar a susceptibilidade das células a outros insultos. As conseqüências dessa vulnerabilidade podem incluir danos ainda maiores, que podem ser irreversíveis para o organismo.

Índice de Figuras e Tabela

Capítulo 8

1. INTRODUÇÃO

Há mais de 150 anos, Claude Bernard reconheceu que, para a manutenção da vida, é necessário manter um ambiente interno relativamente constante (Kopin, 1995). Muitos anos depois, Walter Cannon estudou os mecanismos necessários para manter os parâmetros fisiológicos dentro de limites toleráveis e criou o termo *homeostase* para descrever a manutenção deste estado estável por estes mecamismos (Cannon, 1932; 1935).

A palavra *estresse* vem do inglês *"stress"* (ou *strain).* Este termo foi usado inicialmente na física para traduzir o grau de deformidade sofrido por um material sólido quando submetido a um esforço ou tensão (compressão, alongamento ou torção). Um médico húngaro chamado Hans Selye, em 1936, transpôs este termo para a medicina e a biologia, significando esforço de adaptação do organismo para enfrentar situações consideradas ameaçadoras à sua vida e a seu equilíbrio interno (homeostase).

Assim, considera-se estressor qualquer estímulo capaz de ameaçar a homeostase do organismo e desencadear um conjunto de respostas orgânicas, mentais, psicológicas e/ou comportamentais que, em princípio, têm como objetivo adaptar o indivíduo à nova situação, gerada pelo estímulo estressor. O conjunto destas respostas é chamado de estresse e o "estado de estresse" está, então, relacionado com a resposta de adaptação ao estímulo (Pacák e Palkovits, 2001; Sapolsky et al., 1985), considerada essencial para as funções vitais do organismo (McEween, 1998; Pacák e Palkovits, 2001).

Em sua primeira publicação sobre estresse, Hans Selye descreve uma síndrome produzida por "agentes nocivos diversos, não-específicos", gerando respostas "não-específicas": "*Experimentos em ratos mostram que, se o organismo é severamente atacado por agentes nocivos agudos não-específicos, como a exposição ao frio, injúria cirúrgica, ..., uma síndrome típica aparece, com sintomas independentes da natureza do agente nocivo...*" (Selye, 1936). Neste trabalho, 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 (Selye, 1936, APUD Kopin, 1995). De fato, várias alterações - ou distúrbios - estão associadas à superexposição a

agentes estressores, o que denominamos **estresse crônico** (McEwen e Magariños, 1997). Exemplos disso são as 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. Na verdade, hoje sabe-se que 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).

A **resposta adaptativa** a um **estressor metabólico agudo** inclui processos fisiológicos importantes para redirecionar a utilização de energia entre os vários órgãos, assim como mobilizar suas reservas energéticas preparando o organismo para uma exposição estressante adicional, imprevisível. O aumento do suprimento energético a órgãos "cruciais" é feito preferencialmente pela liberação de catecolaminas e glicocorticóides que, em geral, ativam a gliconeogênese e a glicogenólise, inibem a captação de glicose e aumentam a proteólise e a lipólise. Esta descarga catecolaminérgica corresponde ao estágio de alarme, primeiro estágio da resposta ao estresse proposta por Selye (Sapolsky, 2000; Leonard e 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".**

A resposta adaptativa ao estresse também inclui **alterações comportamentais** como, por exemplo, mudança no limiar sensorial e cognitivo, aumento no estado de alerta, aumento da memória seletiva, inibição dos comportamentos alimentar e reprodutivo e analgesia induzida pelo estresse. Essas respostas comportamentais e fisiológicas são afetadas pela ativação de sistemas efetores primários como o sistema nervoso simpático (liberação de noradrenalina), sistema adrenomedular (liberação de adrenalina), sistema límbico hipotalâmico-hipófise-adrenocortical [LHHA; liberação do hormônio adrenocorticotrófico (ACTH) e glicocorticóides], sistema nervoso parassimpático (liberação de acetilcolina) e sistema renina-angiotensina (liberação de renina).

Além desses, vários outros sistemas contribuem para o restabelecimento da homeostase, como o eixo hipotálamo-hipófise-tireóide (resposta ao frio e calor), eixo hipotálamo-hipófise-gonadal (redução temporária da função reprodutiva), liberação do hormônio do crescimento e alterações na função imunológica. Todos estes sistemas agem diretamente, alterando a liberação ou os efeitos biológicos de muitos mediadores da

resposta ao estresse agudo (p.e., neurotransmissores, hormônios, citocinas, etc), ou indiretamente, alterando os níveis das variáveis monitoradas (p.e., pressão sangüínea, temperatura corporal, etc), com conseqüente ajuste reflexivo determinado pela homeostase interna (McEwen, 2000).

Apesar da importância individual de todos estes mecanismos, os sistemas fisiológicos responsivos ao estresse mais estudados são o eixo LHHA e o sistema neuro-vegetativo (SNV, em especial a resposta simpática da medula adrenal e dos nervos simpáticos), sendo ambos controlados pelos neurônios da região periventricular do hipotálamo, chamados "neurônios neurossecretórios parvocelulares" (López *et al.*, 1999). O eixo LHHA, que possui componentes tanto cerebrais quanto endócrinos, constitui 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)

Assim, frente a um estímulo estressante, as aferências sensoriais, oriundas do tronco cerebral e de diversas regiões da medula espinal, e/ou estímulos oriundos do sistema nervoso central (SNC, e.g. a recordação de um momento estressante, ou a antecipação de um evento de mesmas características) atingem a região periventricular do hipotálamo (**Figura 1**). Este, através do sistema simpático, estimula a medula das glândulas adrenais, levando à liberação de catecolaminas endógenas (adrenalina e noradrenalina), o que constitui uma resposta imediata inicial ao estresse (Ursin e Olff, 1993; Zigmond *et al.*, 1995; Bear *et al.*, 1996) e estimula o eixo LHHA através da expressão de importantes peptídeos, como arginina-vasopressina (AVP), ocitocina e, neste caso especialmente, o hormônio liberador da corticotrofina (CRH, do inglês *corticotrophin releasing hormone*) (Stratakis e Chrousos, 1995), que são liberados em resposta a estressores fisiológicos e psicológicos.

Ainda, os axônios dos neurônios neurossecretórios parvocelulares 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 SNC à porção anterior da

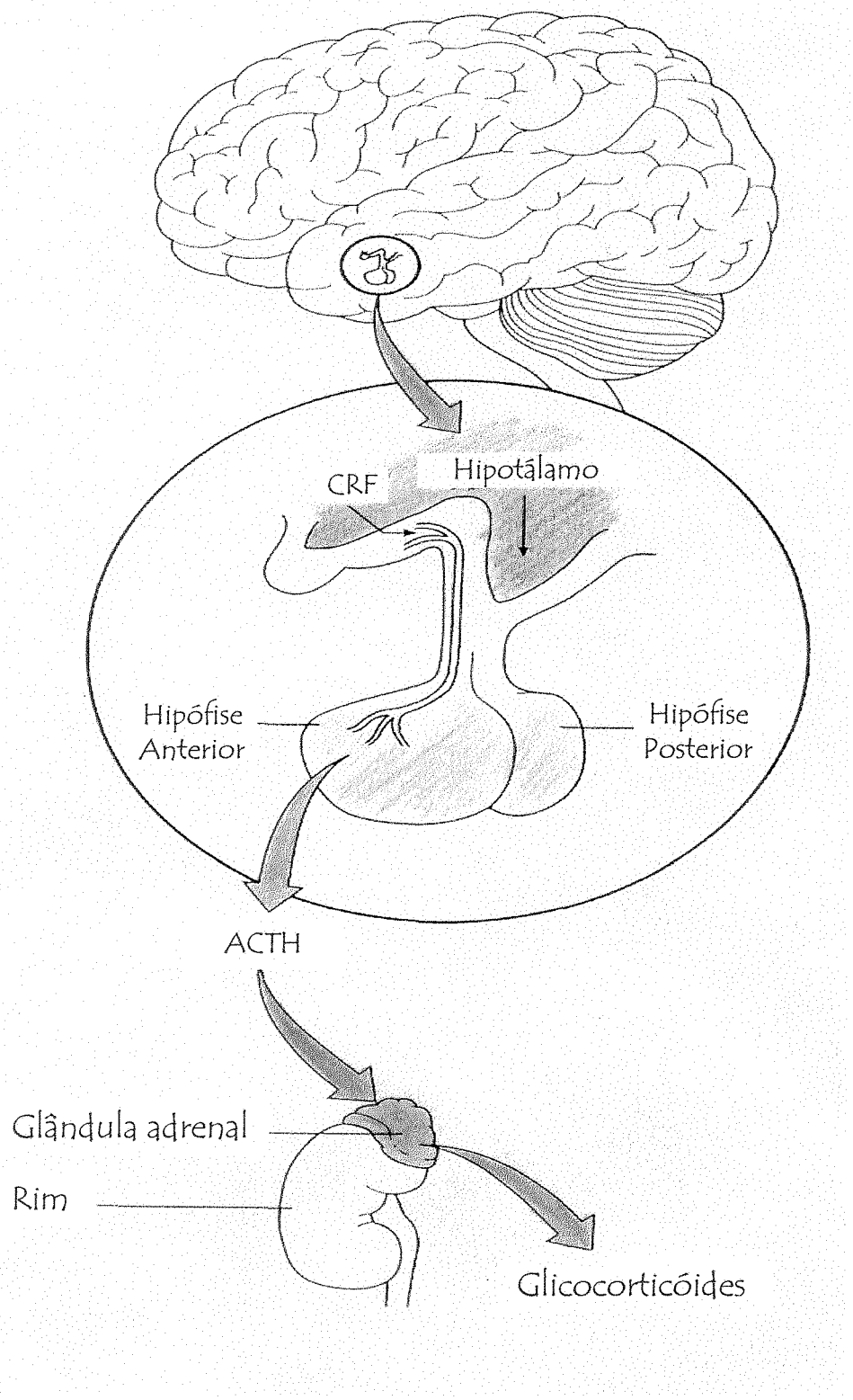


Figura 1: o eixo hipotálamo-hipófise-adrenal.

glândula hipófise (Hayden-Hixson e 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 os opióides endógenos e o ACTH (Akil e Morano, 1995), o qual, ao ser lançado na circulação sistêmica, ativa a produção e liberação de **glicocorticóides** (GC) pelo córtex das glândulas adrenais (Lupien et al., 1998). Estes hormônios exercem 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).

O nome "glicocorticóide" se deve à habilidade destes hormônios em promover a conversão de proteínas e lipídeos a fontes de carboidratos prontamente utilizáveis pelo organismo. 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). Em condições normais de homeostasia, estes hormônios são liberados no organismo em ciclos alternados, obedecendo a um relógio biológico, com altos níveis pela manhã em humanos e no início da noite em ratos, que são animais noturnos. Parece claro que a ativação do eixo LHHA, e a conseqüente liberação de glicocorticóides, é adaptativa e essencial para a sobrevivência imediata do organismo quando em resposta a estímulos agudos. Contudo, o estresse crônico pode levar a alterações específicas na atividade deste circuito, que podem ser consideradas patológicas, ou resultantes de uma adaptação inadequada.

A **regulação das ações do eixo LHHA** é feita, em grande parte, por retroalimentação (*"feed-back"*) dos glicocorticóides sobre componentes do SNC, aumentando ou diminuindo sua atividade de acordo com as necessidades fisiológicas (Marti *et al.*, 1999). Várias estruturas cerebrais estão envolvidas nos processos de retroalimentação, dentre as quais destacam-se o hipotálamo, componente do eixo LHHA, 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.

Dados da literatura vêm apontando os efeitos neurotóxicos da exposição prolongada aos glicocorticóides, As camadas de células piramidais das regiões CA3 e CA4 do hipocampo parecem ser, de todas as estruturas cerebrais, as mais sensíveis a injúrias

mediadas por glicocorticóides (McEwen, 1999; McKittrick, 2000). Estas injúrias podem se manifestar em diversos níveis, que incluem desde a atrofia dendrítica e a retração celular até a 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 o estresse traumático, depressões recorrentes e a síndrome de Cushing, patologias estas que apresentam como característica comum um aumento da atividade do eixo LHHA (Magariños *et al.*, 1997). Indivíduos idosos também apresentam uma atividade aumentada do eixo LHHA, o que pode provocar atrofia de neurônios hipocampais. Trabalhos desenvolvidos com ratos velhos demonstraram uma resposta exacerbada a situações de estresse agudo, justamente pela impossibilidade do hipocampo em ativar os processos de retroalimentação negativa (Smith, 1995; Sapolsky, 1999).

Outro efeito da exposição a níveis elevados de GC, como aqueles observados em situações de estresse, é a diminuição na proliferação de células granulares na região do Giro Denteado (GD), no hipocampo, assim como sua diferenciação em novas células nervosas, que acontece tanto nos cérebros em desenvolvimento (Tanapat *et al*, 1998) quanto na fase adulta (Gould e Tanapat, 1999; Gould *et al.*, 2000). Esse processo de diferenciação das novas células a neurônios e o equilíbrio entre a gênese e morte apoptótica destas células hipocampais é denominada neurogênese. As reais implicações destes novos neurônios não são ainda bem descritas (Scharff, 2000); contudo, há indícios de que estas células estejam envolvidas nos mecanismos de aprendizagem e memória, e que façam parte dos processos de plasticidade neural (Gould *et al.*, 1999; Hastings *et al.*, 2001; Snyder *et al.*, 2001). Esta ação supressiva na proliferação celular causada por níveis elevados de GC não parece ser direta, mas ocorrer através da via excitatória dependente de receptores NMDA (Gould e Tanapat, 1999).

Finalmente, existem enormes diferenças individuais na interpretação e na resposta aos estímulos estressores, assim como diferenças individuais na susceptibilidade a doenças,

nas quais o estresse possa ter alguma influência. Além da pré-disposição genética, que aumenta o risco de certas doenças, o processo de desenvolvimento, tais como estresse pré-natal ou experiências adquiridas da educação pós-natal, contribuem para as respostas fisiológicas e comportamentais a estressores. Ademais, experiências adquiridas ao longo da vida resultam em memórias de situações particularmente agradáveis ou desagradáveis que, combinadas com a genética e as influências do processo de desenvolvimento, resultam em diferenças importantes entre indivíduos no modo de reagir ao estresse e nas conseqüências que podem dele advir ao longo do tempo (McEwen, 2000).

1.1 Estresse e Dor

As respostas fisiológicas ao estresse incluem alterações na percepção e resposta à dor. A dor é uma experiência sensorial e emocional que envolve numerosas regiões do cérebro, principalmente aquelas relacionadas com os sistemas límbico e somatossensorial (Coghill et al., 1994). A conexão entre estes sistemas revela uma possível rota através da qual o estímulo somatossensorial pode ser integrado à nocicepção, o que permite diferentes interpretações do estímulo doloroso e a modulação da percepção da dor.

Embora a exposição aguda a uma variedade de estressores produza **analgesia** imediata em vários testes de sensibilidade à dor (Amir e Amit, 1978; Menendez *et al.*, 1993; Vaccarino e Kastin, 2000), o estresse prolongado pela exposição repetida a um estressor, tal como o frio ou a contenção, causa **hiperalgesia** (Satoh et al., 1992; Gamaro et al., 1998). Tais diferenças observadas na nocicepção após o estresse agudo ou crônico são consideradas parte do processo de adaptação. Vários sistemas neurotransmissores têm sido relacionados com este processo de "dessensibilização", tais como os opióides, serotonina, dopamina (Cancela et al., 1988; 1990; 1995; Gamaro et al., 2003), corticosteróides e ACTH, que têm função não só no estresse como também em modulação da dor (Lewis *et al.*, 1980; MacLennan *et al.*, 1982) e excitabilidade cerebral (McEwen *et al.*, 1986).

Os **mecanismos envolvidos na analgesia** induzida pelo estresse (SIA, do inglês *stress-induced analgesia*) envolvem, basicamente, dois circuitos neuroendócrinos: o eixo LHHA e o eixo neural simpático. Ainda que diferentes estímulos possam ativar estes eixos de forma variada, todos eles são ativados pela maioria dos estressores (Strausbaugh e Levine, 2000). A analgesia induzida pelo estresse pode gerar dois subtipos de resposta –

dependente ou independente do sistema opióide (Lewis, 1986, MacLennan et al.,1982). A natureza distinta das respostas ao estresse é sugerida pelo fato de não haver tolerância cruzada entre elas, ainda que ambas possam ser classicamente condicionadas (Strausbaugh e Levine, 2000).

Os subtipos de resposta induzida pelo estresse também variam de acordo com as **características do estressor**, tais como a severidade e o padrão temporal de administração (Vidal e Jacob, 1982). Em geral, estressores intermitentes e menos severos produzem analgesia opióide, enquanto estressores contínuos e de grande severidade produzem analgesia independente de opióide. Um exemplo disso é a demonstração de que ratos expostos a estresse agudo por imobilização aumentam a magnitude e a duração do efeito analgésico de fármacos opióides (Calcagnetti e Holtzman, 1992). É possível que o mecanismo responsável por estes efeitos envolva sítios espinais e supraespinais (Calcagnetti e Holtzman, 1992).

A **hiperalgesia,** ou **antinocicepção,** induzida pelo estresse crônico é definida como um aumento na sensibilidade a um estímulo nocivo ou uma diminuição da analgesia após a exposição ao estresse (Strausbaugh e Levine, 2000). Os estados hiperalgésicos são comumente observados após injúria de nervo central ou periférico, ou como manifestação de inflamação tecidual. Nessas situações, estímulos anteriormente não-nocivos passam a produzir dor, ou o estímulo nocivo é percebido com uma dor muito mais intensa (Levine *et al.*, 1986).

Embora os mecanismos envolvidos nos efeitos sobre a nocicepção induzida pelo estresse crônico sejam ainda menos conhecidos que aqueles relacionados ao estresse agudo, é possível que a hiperalgesia observada em animais estressados cronicamente envolva mudanças no processamento central da informação nociceptiva e não nas vias nociceptivas periféricas, como ocorre na analgesia induzida pelo estresse agudo. Da Silva-Torres e colegas (2003) observaram que ratos cronicamente estressados não apresentam o efeito analgésico característico após injeção de morfina (1,0 e 5,0 mg/kg, via i.p.), sugerindo que o estresse crônico possa estar provocando alterações na liberação de opióides endógenos. Do mesmo modo, a antinocicepção induzida pela novidade (Fanselow, 1985; Netto, *et al.*, 1987), mediada pela liberação de β-endorfina (Siegfried *et al.*, 1987; Netto *et al.*, 1987), não é observada em animais cronicamente estressados (Torres *et al*, 2001b).

Além disso, muitos trabalhos têm discutido as **diferenças sexuais** na percepção da dor, incluindo, por exemplo, a percepção da intensidade da dor aguda ou crônica e respostas a analgésicos (Bodnar et al., 1988; Kavaliers e Innes, 1987; 1992; Pollard e Dyer, 1985; Romero e Bodnar, 1986). A hipótese de haver relação entre os hormônios ovarianos e a sensibilidade à dor é um dos possíveis mecanismos envolvidos nessas diferenças. Os esteróides gonadais podem influenciar a via nociceptiva de diferentes formas, como através da regulação da liberação de neurotransmissores (Loscher et al., 1992) e de sua cascata de produção/degradação. O estrógeno, um dos hormônios sexuais envolvidos na regulação da analgesia e nocicepção (Frye et al., 1992; Murua e Molina, 1992; Morley et al., 1984), parece estimular a liberação de β-endorfina no cérebro (Wardlaw et al., 1982). Também é possível observar variação no limiar de dor em fêmeas nas diferentes fases do ciclo estral (Kennet et al., 1986; Sternberg, 1999; Martinez-Gomes et al., 1994).

Embora haja resultados conflitantes para os estudos experimentais e clínicos das diferenças sexuais na nocicepção, a maioria dos pesquisadores concorda que certos fatores, como a capacidade de percepção e mecanismos fisiológicos, possam explicar as diferenças sexuais relacionadas à percepção da dor e seu tratamento (Vallerand e Polomano, 2000). Ademais, considerando o efeito do estresse, os esteróides sexuais podem regular a síntese de CRH, um mecanismo pelo qual a nocicepção induzida pelo estresse pode ser modulada em fêmeas (Vamvakopoulos e Chrousos, 1993). Esse fato poderia estar envolvido nas diferenças sexuais na percepção da dor, observada em resposta a situações de estresse crônico (Gamaro et al., 1998).

1.2 Estresse e Memória

Talvez a primeira proposição da existência de alterações celulares subjacentes à formação de memórias tenha sido feita pelo psicólogo canadense Donald Hebb, no seu livro *Organizatlon of Behavior* publicado em 1949 (Hebb, 1949). Baseado nos trabalhos anatômicos de Lorente de Nó sobre a estrutura e as conexões entre os neurônios, Hebb propôs que durante a situação de aprendizagem um neurônio estimula outro, de tal forma que a sinapse entre eles se torna mais fortalecida, e isso poderia produzir alterações estruturais nessas células. De acordo com essa idéia, o armazenamento dessa informação

estrutural poderia explicar o fenômeno da memória. Entretanto, ele não tinha a menor idéia dos mecanismos neurais subjacentes à sua teoria.

Em 1973, os fisiologistas Timothy Bliss e Terje Lomo (Bliss e Lømo, 1973) demonstraram em neurônios localizados no hipocampo [uma estrutura do cérebro intimamente relacionada aos processos de memória (Kadar et al., 1990; Lebrun et al., 1990; Peinado-Manzano, 1990; Sutherland e McDonald, 1990)], que a estimulação elétrica de alta freqüência num axônio pré-sináptico durante alguns segundos produz um aumento na magnitude da resposta pós-sináptica. O aumento pode durar algumas horas em animais anestesiados ou vários dias ou mesmo meses em animais acordados. Esse fenômeno foi denominado pelos pesquisadores de potenciação de longa duração *(long-term potentiation* — LTP).

Do ponto de vista funcional, a LTP corresponde a um processo de facilitação do sistema nervoso, cujo estabelecimento depende da duração e da freqüência do estímulo repetitivo; ou numa analogia, depende do 'treinamento' e, portanto, de um processo de 'aprendizado'. Esses fenômenos seriam a base para a teoria neuropsicológica proposta por Hebb.

Entretanto, Bliss e Lomo haviam apenas demonstrado que a estimulação elétrica de alta freqüência de alguma forma tornava os circuitos neurais mais potentes. Mas quais seriam os mecanismos neurais responsáveis por este fenômeno? No hipocampo, vários estudos têm demonstrado a importância do aminoácido glutamato para o armazenamento da memória e produção de LTP. Isso ocorre através de uma cascata de eventos bioquímicos (Lynch, 2003; veja a Figura 2).

Figura 2: neurotransmissão glutamatérgica

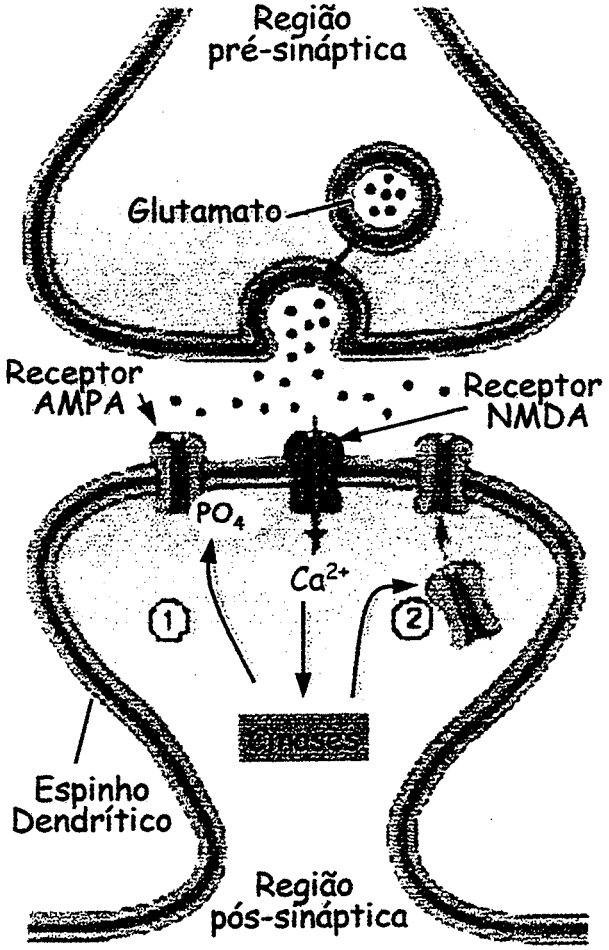


Figura2: Neurotransmissão glutamatérgica - Rota para a expressão de LTP em CA1. A membrana pós-sináptica é despolarizada através de receptores do tipo não-NMDA. Essa despolarização libera o bloqueio exercido pelo magnésio sobre os receptores NMDA, permitindo a entrada de cálcio (Ca^{2+}) para o interior da membrana pós sináptica. O cálcio estimula proteínas cinases cálcio-dependentes, levando à indução de LTP por (1) alterar a efetividade de receptores AMPA pós-sinápticos preexistentes ou (2) estimular a inserção de novos receptores AMPA.

A manutenção de LTP depende do aumento na quantidade de neurotransmissor liberada pelo terminal pré-sináptico. Assim, a indução de LTP depende de um mecanismo

pós-sináptico (entrada de cálcio através dos receptores de NMDA), enquanto a manutenção de LTP requer a participação de um mecanismo pré-sináptico (Lynch, 2003).

Hoje sabemos que a memória é produto de mudanças estruturais e bioquímicas que ocorrem a nível celular e tecidual durante a aquisição da informação. Funcionalmente, a memória é interpretada como uma capacidade mental, que possibilita a um indivíduo registrar, conservar e evocar experiências adquiridas anteriormente (idéias, imagens, acontecimentos, sentimentos, etc.). Para tanto, é preciso que ocorra primeiro o aprendizado, definido como a aquisição de informação através da experiência e, posteriormente, a memória propriamente dita, tida como o armazenamento e a evocação desta informação e de suas conseqüências. Assim, não existe memória sem aprendizado, e não há aprendizado sem experiências (Izquierdo, 1989).

Várias regiões do cérebro têm sido implicadas nos processos de memória (**Figura 3**): a amígdala, o hipocampo, o núcleo caudado, o hipotálamo e o tálamo, o córtex cerebral (Izquierdo & Medina, 1997). Este conjunto de estruturas integra um sistema modulador que influencia a decisão, por parte do sistema nervoso, diante de cada experiência, do que deve ser gravado e do que deve ser evocado (Izquierdo, 1989).

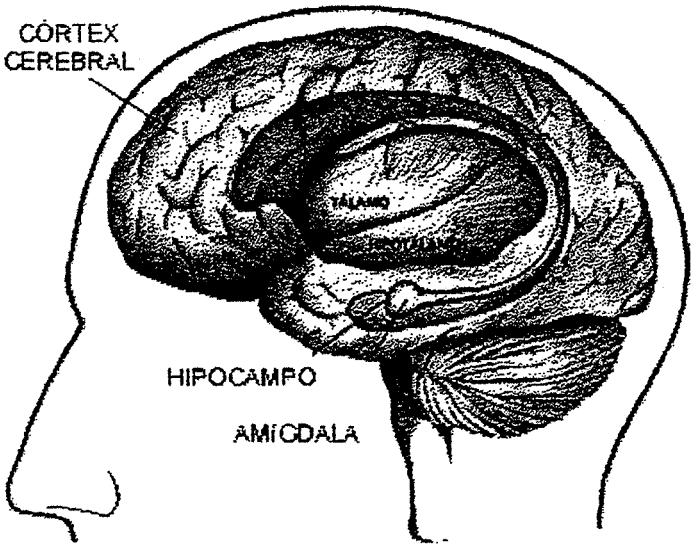


Figura 3: O hipocampo é a região do cérebro responsável pela memória temporária. Ele interage com o córtex cerebral e a memória adquire caráter permanente.

Há dois grandes grupos de memória que se podem subdividir ou não. Um é o da memória de procedimentos, de atos motores ou de concatenações de atos motores, como por exemplo, saber escrever à máquina. Essa memória tem uma localização cortical em parte, pelo menos inicialmente, mas depois envolve os gânglios basais e o cerebelo. A outra é a memória declarativa. É a memória de fatos, de eventos, de seqüências de fatos e eventos, de pessoas, de faces, de conceitos, de idéias, etc. As memórias declarativas se formam em primeiro lugar no hipocampo, que tem muitas fibras de conexão com o córtex entorrinal (Zanatta et al., 1996; Pereira et al., 2001).

Quando a memória é de tipo aversivo, ou envolve emoções ou algum grau de estresse, entram em jogo outras estruturas cerebrais adicionais, como a amígdala, que está no próprio lobo temporal, perto do hipocampo, e que tem conexões bidirecionais com o mesmo (Christian and Thompson, 2003). O hipocampo efetua uma série de processos bioquímicos que eventualmente servem para fortalecer suas conexões com outras estruturas. Dependendo do tipo de memória, a via envolverá, mais tarde, o córtex parietal associativo e os córtices associativos frontal, occipital e temporal (Zanatta et al., 1996; Pereira et al., 2001; Mello e Souza et al., 2000; Hoh et al., 2003).

Assim, a cadeia de fenômenos da memória é constituída por esta seqüência de eventos: 1) experiência nova, 2) aquisição do comportamento ou informação, 3) armazenamento, 5) evocação (Izquierdo e Netto, 1985b). A memória pode ser modificada por tratamentos farmacológicos e/ou comportamentais que atuam sobre os processos de aquisição, consolidação e/ou evocação (Gold & McGaugh, 1975; Izquierdo et al., 1984).

O hipocampo possui a mais alta concentração de receptores de corticosterona do cérebro (McEwen, 2001). As evidências de que a exposição crônica ou a níveis aumentados de cortisol está associada com mudanças significantes na anatomia e funcionamento do hipocampo (Lupien et al.,1998; McEwen, 2001) têm levados muitos pesquisadores a proporem uma ligação entre a exposição a diversos níveis de estresse crônico e perda de memória (Davies e Underwood, 2000).

Vários hormônios liberados em resposta ao estresse – os hormônios do eixo LHHA, hormônios da medula adrenal, incluindo peptídeos opióides, assim como as catecolaminas centrais - têm sido implicados nos mecanismos de aprendizado e memória em ratos

(Introini-Collison e McGaugh, 1986; Gold e van Buskirk, 1978; Izquierdo e Netto, 1985a; McGaugh et al., 1984; Roozendaal, 2002).

A potenciação de longa duração (LTP) que, conforme visto acima, é considerada por muitos como um mecanismo de armazenamento da memória, está marcadamente reduzida no hipocampo de animais expostos ao estresse (Foy et al., 1987). Além disso, sabe-se que a atividade de neurônios piramidais nessa estrutura é afetada pela corticosterona (McEwen et al., 1999; Souza et al., 2000). A diminuição da neurogênese no hipocampo de animais submetidos a diferentes modelos de estresse, bem como à administração de GC, (Tanapat *et al*, 1998; Gould e Tanapat, 1999; Gould *et al.*, 2000), como já mencionado, também parece estar relacionada com mecanismos de aprendizado, memória e plasticidade neural (Gould *et al.*, 1999; Hastings *et al.*, 2001; Snyder *et al.*, 2001).

Os efeitos deletérios do estresse também são observados nas tarefas comportamentais: exposição de primatas e ratos adultos a elevadas concentrações de GCs, como aquelas observadas em situações de estresse, resulta em danos cognitivos em diversas tarefas que avaliam a memória espacial, 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). Neste tipo de tarefa, os animais parecem ser capazes de formar mapas cognitivos do ambiente que os rodeia, através do comportamento exploratório, e utilizar-se destes mapas para orientarem-se de forma flexível neste ambiente (Vasconcellos et al., 2003).

Assim sendo, a capacidade de reter e recuperar informações é um processo que produz alterações no nosso comportamento, permitindo que o indivíduo se situe no presente, considerando o passado e o futuro. Ela fornece as bases para todos os nossos conhecimentos, habilidades, sonhos, planos e anseios. Memória é, portanto, um aspecto central da existência humana. O conhecimento de sua natureza e bases biológicas, bem como de fatores que possam influenciá-la, como o estresse, é essencial para se entender a mente humana e desenvolver terapias relacionadas com as doenças cognitivas.

1.3 Vulnerabilidade neuronal

Os neurônios do cérebro de mamíferos adultos são células pós-mitóticas em sua maioria incapazes de regenerar-se após injúrias metabólicas. Desta forma, lesões cerebrais de qualquer natureza têm conseqüências permanentes e podem ocasionar sérias disfunções neurológicas (Jacobs e Fehling, 2003).

Estudos em animais sugerem que a **exposição prolongada a glicocorticóides ou ao estresse** tem conseqüências deletérias para o hipocampo (Sapolsky et al., 1989; Sapolsky, 2000; McEwen, 2001). Tem sido relatada atrofia de dendritos após exposição a um desses fatores, e esta pode evoluir para morte neuronal (Sapolsky *et al.*, 1988; McEwen e Magarinos, 1997.; Stein e Sapolsky, 1988; Sapolsky *et al.*, 1985). Um possível mecanismo seria a depleção de fontes energéticas, uma vez que os glicocorticóides diminuem a captação de glicose em cultura de tecido hipocampal, incluindo neurônios e células gliais (Horner et al., 1990). Em nosso laboratório, observamos uma diminuição na captação de glicose em fatias de hipocampo e de córtex cerebral de ratos, 24h após a exposição ao estresse agudo, enquanto nenhum efeito foi abservado após o estresse crônico (Torres et al., 2001a).

Por outro lado, dados da literatura sugerem que os glicocorticóides não matam os neurônios hipocampais, mas exacerbam a toxicidade de outros insultos, possivelmente acelerando a diminuição nos níveis de ATP nestas células durante períodos de aumento na demanda energética (Armanini et al., 1990; Tombaugh e Sapolsky, 1992 a, 1992 b; Elliott et al., 1993; Elliott e Sapolsky, 1993; Lawrence e Sapolsky, 1994; Torres et al., 2001a). Embora o dano celular nas regiões CA1 e Giro Denteado (GD) do hipocampo, mediado por altos níveis de GCs, não seja tão evidenciado quanto na região CA3, como já foi comentado, estes hormônios tem 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).

A **isquemia cerebral** é definida como um estado patológico em que o fluxo sangüíneo está comprometido, diminuindo o fornecimento de oxigênio e glicose ao cérebro e prejudicando a remoção de produtos do metabolismo (Sweeney et al, 1995); este evento freqüentemente resulta em dano cerebral (White et al, 2000). Devido à alta taxa no metabolismo aeróbico, à falta de estoques teciduais de oxigênio e à pequena reserva de fosfatos ou carboidratos altamente energéticos, o cérebro é muito sensível à isquemia e à hipóxia (Schmidt-Kastner e Freund, 1991). Assim, a presença de maiores níveis circulantes de glicocorticóides no momento da isquemia aumenta o dano celular (Sapolsky e Pusinelli, 1985; Smith-Swintosky *et al.*, 1996; Rami *et al.*, 1998; Adachi *et al.*, 1998), sendo que os mecanismos propostos envolvem um aumento na liberação de glutamato na região de CA1 do hipocampo (Chen *et al.*, 1998) e/ou uma diminuição na latência de despolarização da membrana induzida pela isquemia, com aumento na concentração de cálcio intracelular (Adachi *et al.*, 1998). Além disso, a função sináptica, juntamente com a integridade celular, pode ser preservadas após a hipóxia/isquemia pela inibição do aumento nos níveis de corticosteróide, com o uso de metirapone, um inibidor da síntese deste esteróide (Adachi et al., 1998, 1999; Krugers et al., 2000) ou pela adrenalectomia (Morse e Davis, 1990; Antonawich et al, 1999).

Numa situação de isquemia, como citado acima, o neurônio perde o suprimento de O_2 e de glicose. Porém, quando o fluxo é restaurado, é essencial para a sua recuperação que o metabolismo energético seja também plenamente restabelecido. Para isto é preciso que a célula seja capaz de captar toda a glicose necessária. Como um dos principais efeitos dos glicocorticóides sobre as células, amplamente demonstrado *in vitro,* é o de reduzir a captação de glicose, o estresse poderia alterar o metabolismo neuronal da célula submetida à isquemia (Horner et al., 1990) o que implicaria no desencadeamento e/ou agravamento deste processo. É interessante salientar que alguns outros fatores influenciados por estes hormônios são considerados importantes eventos causadores da morte neuronal isquêmica. Por exemplo, a **excitotoxicidade** causada pela forte e sustentada estimulação dos neurônios pós-sinápticos, em decorrência de um excesso de neurotransmissores excitatórios na fenda sináptica, está entre as principais teorias de morte neuronal isquêmica (Schmidt-Kastner e Freund, 1991). Num organismo estressado, esta excitotoxicidade pode ser potenciada, já que os glicocorticóides, como demonstrado *in vitro,* podem reduzir a recaptação destes

neurotransmissores excitatórios (Chou et al., 1994; Stein-Behrens et al., 1992). Além disso, as exposições ao estresse ou a níveis aumentados de glicocorticóides estão associadas a um aumento no metabolismo, que pode levar a um aumento na produção de espécies ativas de oxigênio e a danos oxidativos, particularmente em áreas do cérebro como o hipocampo, o qual apresenta altas concentrações de receptores para glicocorticóides (Armanini *et al.*, 1990). Assim, a ligação de glicocorticóides nesses receptores pode aumentar o influxo de cálcio citosólico, que leva a dano oxidativo dependente de cálcio e degeneração neuronal (Armanini *et al.*, 1990).

1.4 Estresse crônico – alguns mecanismos moleculares relacionados

Os mecanismos pelos quais o estresse influencia o funcionamento do SNC são constante objeto de estudo, já tendo sido apontados diversos fatores que podem estar envolvidos nos processos de lesão/proteção deste sistema. Neste trabalho discutiremos o estresse oxidativo, a cascata de degradação do ATP e a transmissão glutamatérgica.

1.4.1 Estresse oxidativo

A exposição ao estresse ou a níveis aumentados de glicocorticóides está associada a um aumento no metabolismo, que pode levar a um aumento na produção de espécies ativas de oxigênio (EAO) e a danos oxidativos, conforme citado anteriormente. As EAO podem danificar diretamente proteínas celulares, DNA e lipídeos, afetando todas as funções celulares (Cochrane, 1991). O tecido nervoso é altamente suscetível a danos causados por processos de lipoperoxidação. Possivelmente isso se deva ao seu elevado consumo de oxigênio e seu alto conteúdo de substratos facilmente oxidáveis, principalmente ácidos graxos poliinsaturados e catecolaminas (Anderson et al., 1985). Dentre as estruturas cerebrais, o hipocampo é particularmente vulnerável à ação das EAO devido à sua alta concentração de receptores para glicocorticóides (Armanini *et al.*, 1990). Assim, a ligação de glicocorticóides nesses receptores pode aumentar o influxo de cálcio citosólico, que leva a um dano oxidativo dependente de cálcio e degeneração neuronal (Armanini *et al.*, 1990).

A exposição a níveis supra-fisiológicos de GCs também é capaz de interferir com a atividade de sistemas antioxidantes enzimáticos e não-enzimáticos (Liu et al., 1994; Sudakov e Sosnovsky, 1996), como a atividade de enzimas antioxidantes Cu/Zn-

superóxido dismutase e glutationa peroxidase (McIntosh e Sapolsky, 1996; McIntosh *et al.*, 1998). Por outro lado, é interessante observar que existem relatos de que glicocorticóides podem agir como *scavengers* de radicais livres (Mizoi *et al.*, 1986).

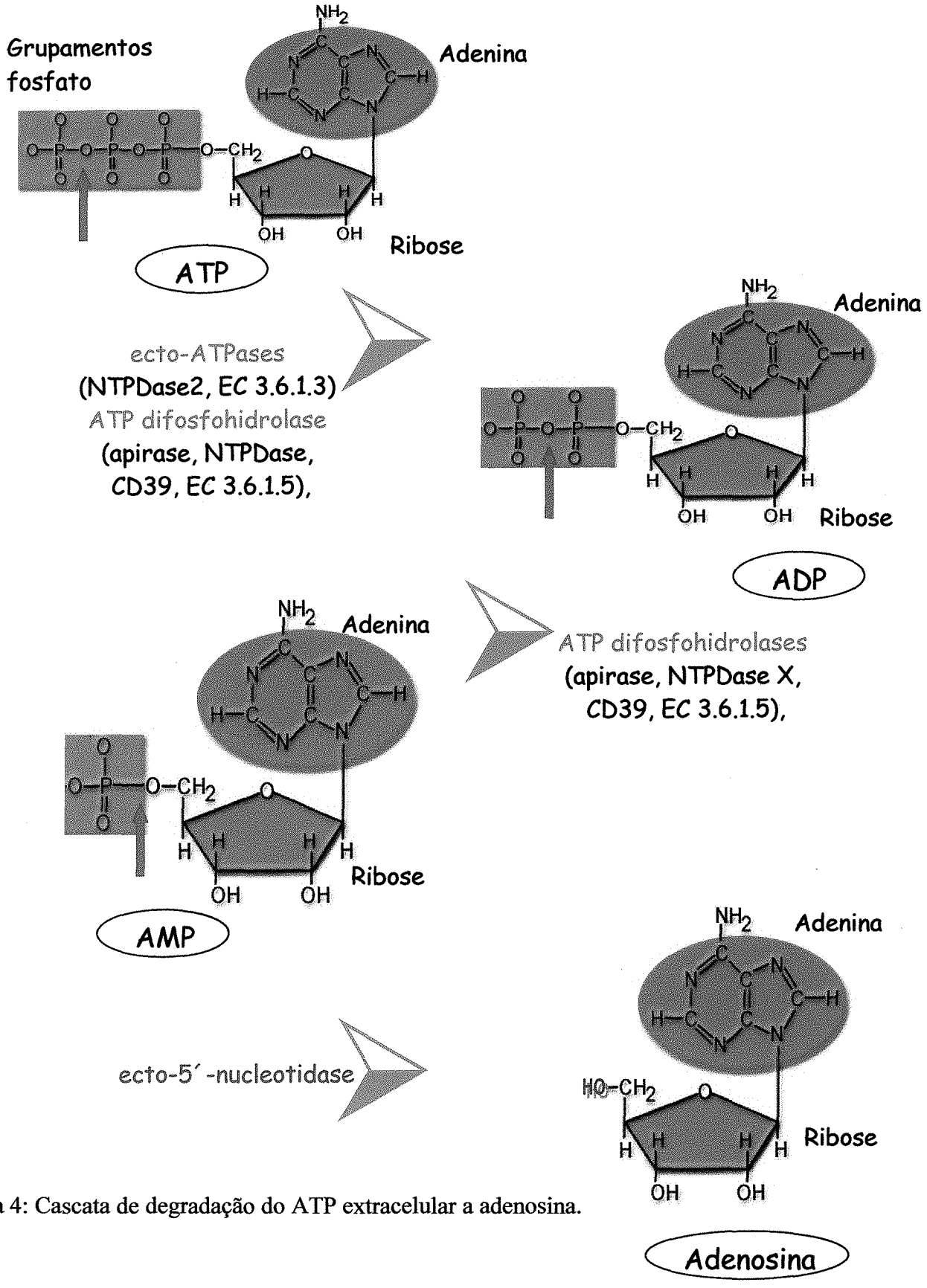
Os estudos relatados acima sugerem que a exposição a níveis elevados de glicocorticóides é capaz de afetar diversos processos no hipocampo de ratos, incluindo efeitos sobre a memória, além de alterar a nocicepção. Esses estudos, porém, foram realizados, em sua maior parte, utilizando administração de glicocorticóides, tanto *in vivo* quanto *in vitro*, ou após exposição de animais ao estresse agudo. O estresse crônico, por outro lado, provavelmente induz alterações plásticas nas estruturas relacionadas com a resposta ao estresse, que podem protegê-las ou, talvez, torná-las mais vulneráveis.

Em vistas disso, nós procuramos investigar a possível participação do estresse oxidativo sobre a nocicepção e memória de animais submetidos ao tratamento de estresse crônico repetido, através da medida de produtos formados por ação dos radicais livres (DCF TBARS) e a atividade antioxidante (enzimáticas e/ou não-enzimática) nas estruturas relacionadas com estes sistemas (medula espinal e hipocampo, respectivamente).

1.4.2 Cascata de degradação do trifosfato de adenosina (ATP)

O ATP é um neurotransmissor que atua no sistema nervoso central e periférico. (Edwards et al., 1992). Em situações fisiológicas, o ATP existe em baixas concentrações, no entanto, em condições patológicas, grandes quantidades de ATP extracelular pode causar morte celular (Inoue, 2002).

O ATP liberado na fenda sináptica pode ser hidrolisado a adenosina, um agente neuromodulador e neuroprotetor (**Figura 4**). As enzimas que promovem esta conversão parecem ter um papel na modulação e no controle da transmissão sináptica excitatória (Cunha, 2001a; Cunha e Ribeiro, 2000). A adenosina tem várias funções no SNC, as quais envolvem o tônus inibitório da neurotransmissão e ações neuroprotetoras em condições patológicas (Latini e Pedata, 2001). Sua função como um mensageiro transcelular para sinalizar alterações metabólicas também é bem estabelecida. As ações neuroprotetoras da adenosina são atribuídas à ativação de receptores A1 pré-sinápticos, o que reduz a liberação de neurotransmissores e deprime a atividade neural no SNC (Phillips e Wu, 1981; Dragunow, 1988; Brundege e Dunwiddie, 1997). A adenosina tanto pode ser liberada



gura 4: Cascata de degradação do ATP extracelular a adenosina.

diretamente através de transportadores bidirecionais como pode originar-se extracelularmente pelo catabolismo de nucleotídeos da adenina, ATP, ADP e AMP (Cunha e Ribeiro, 2000).

Conforme observado na **Figura 4**, o ATP é hidrolisado a adenosina por uma cadeia de ecto-nucleotidases (Cunha et al., 1992; Sarkis e Salto, 1991). Esta cadeia de enzimas inclui ecto-ATPases (NTPDase2, EC 3.6.1.3), ATP difosfoidrolases (apirase, NTPDase, CD39, EC 3.6.1.5) e a ecto-5′-nucleotidase (CD73, EC 3.1.3.5). Em recentes estudos foi demonstrado que o ATP pode ser hidrolisado a adenosina pela ação conjugada de uma ATP difosfoidrolase (NTPDase3, CD39, ecto-apirase, EC 3.6.1.5) e uma 5′-nucleotidase (*lymphocyte surface protein*, CD73, EC 3.1.3.5) (Sarkis e Salto, 1991; Battastini et al., 1995), no sistema nervoso central e periférico. A ação conjunta das ecto-nucleotidases controla a disponibilidade dos ligantes (ATP, ADP, AMP e adenosina) para os receptores de nucleotídeos e nucleosídeos e, conseqüentemente, a duração e extensão da ativação destes receptores (Chen e Guidotti, 2001). Assim, estas ecto-nucleotidase têm dupla função: remover um sinal (ATP) e gerar outro (adenosina). Esta cascata pode ter um efetivo papel na regulação de vários processos, uma vez que atuam na neuroplasticidade em diferentes situações patofisiológicas (Agteresch et al., 1999; Cunha et al, 2001; Bonan et al., 2000). Estas enzimas podem também ter uma ação protetora através da manutenção da relação ATP/ADP e adenosina em níveis fisiológicos (Agteresch et al., 1999).

Alguns autores sugerem que a reação catalisadas pela 5'-nucleotidase seja o passo limitante na via de degradação de ATP extracelular a adenosina (para revisão, veja Cunha e Ribeiro, 2000). É importante lembrar que esta enzima é inibida pelo ATP e/ou ADP (Cunha e Sebastião, 1991). Então, somente quando os níveis de ATP e ADP atingem valores abaixo do limiar de inibição da 5'-nucleotidase, a adenosina irá ser formada em quantidades importantes.

Além disso, a adenosina possui um papel homeostático que ocorre virtualmente em todos os tipos de células (para revisão, veja Cunha 2001b). Este fato pode ser observado em trabalhos que demonstram o aumento da concentração extracelular de adenosina após situações metabólicas estressantes, incluindo hipóxia e isquemia (Latini e Pedata, 2001; Pearson et al., 2001) ou glicoprivação pela administração de 2-deóxi-D-glicose (Minor et al., 2001).

A exposição a estressores como cirurgia e estimulação elétrica causa mudança na concentração de ATP (Kennedy et al., 1997) e adenosina extracelular (Latini e Pedata, 2001). Também já foram observadas alterações na atividade das enzimas envolvidas na hidrólise deste nucleotídeo na medula de ratos machos expostos ao estresse repetido (Torres et al., 2002a). Este efeito, no entanto, não foi observado em fêmeas (Torres et al., 2002a).

Ademais, há um acúmulo de evidências sugerindo que a adenosina e o ATP atuam como neuromoduladores da dor na medula espinal (Post, 1984; Rhee et al., 2000; Tsuda et al., 1999a). O ATP atua no corno dorsal da medula espinal após ser liberado de uma subpopulação de nervos aferentes primários envolvidos na trajetória da dor (Fyffe e Perl, 1984; Sawynok e Sweeny, 1989) e a administração intratecal de antagonistas do purinoceptor-P2 têm sido associada como uma ação antinociceptiva (Driessen et al., 1994; Tsuda et al., 1999a; 1999b).

Baseados nessas evidências experimentais e da literatura, nós utilizamos nosso modelo de estresse crônico repetido para avaliar a influência da hidrólise dos nucleotídeos da adenina sobre a nocicepção, medindo a atividade das ectonucleotidases em sinaptossomas de medula espinal. Considerando que as atividades dessas enzimas se mostraram alteradas apenas em machos, nós conduzimos nossas investigações utilizando ratas fêmeas ooforectomizadas, e ooforectomizadas com reposição hormonal, o que nos permitiu analisar o efeito dos hormônios femininos de uma maneira geral e do estradiol, especificamente. Avaliamos também a hidrólise de nucleotídeos da adenina em sinaptossomas (medindo-se a atividade de ectonucleotidases) preparados a partir do hipocampo de ratos estressados repetidamente. Com estes resultados pretendemos verificar o efeito da cascata de degradação do ATP nessa estrutura importante para a memória.

1.4.3 Trasmissão glutamatérgica

O glutamato captado pelas células pode ser usado para fins metabólicos (síntese de proteínas, metabolismo energético, fixação de amônia) ou como transmissor. Este aminoácido é considerado o principal mediador de sinais excitatórios no SNC de mamíferos e está envolvido em muitos aspectos do funcionamento normal do cérebro, incluindo cognição, memória e aprendizado (Danboldt, 2001). O Glutamato também tem um papel

importante no desenvolvimento do SNC, incluindo indução e eliminação de sinapses, migração, diferenciação e morte celular (Danbolt, 2001).

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, principalmente pelos astrócitos adjacentes, através de cotransporte com sódio por transportadores acoplados a uma bomba sódio-potassio-ATPase (Na^+/K^+-ATPase) (Smith, 1995; Anderson e Swanson, 2000; Danbolt, 2001).

A elevação nos níveis de glutamato produz efeitos neurotóxicos basicamente pelo aumento dos níveis de cálcio intracelular, o que ocorre em parte pela ligação aos receptores ionotrópicos (i.e., NMDA e AMPA). O aumento exacerbado nos níveis intracelulares de cálcio desencadeia uma série de reações que poderão levar a danos ou mesmo morte neuronal. Exemplo disso é a gênese de radicais livres mediada por cálcio (Sapolsky, 2000).

Assim, 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.

Nesse trabalho avaliamos o efeito do estresse repetido sobre a captação e liberação de glutamato em sinaptossomas e por fatias de hipocampo, a densidade de receptores de glutamato.

2. OBJETIVOS

Gerais

Estudar os efeitos da exposição de ratos ao estresse repetido sobre a nocicepção, a memória e a vulnerabilidade neuronal a outros insultos, visando compreender seus mecanismos.

Específicos

1. Estudo dos efeitos do estresse sobre a nocicepção

1.1 Estudo Comportamental

a. Verificar o efeito da exposição a diferentes sabores sobre a nocicepção em ratos submetidos ao estresse repetido.

1.2. Estudo neuroquímico

Verificar o efeito da exposição ao estresse repetido sobre a medula espinal, avaliando:

a. Parâmetros relacionados ao estresse oxidativo, comparando-se ratos machos e fêmeas, uma vez que os efeitos do estresse repetido sobre a nocicepção são gênero-dependentes;

b. A hidrólise de nucleotídeos da adenina em sinaptossomas de medula espinal de ratas fêmeas ooforectomizadas e ooforectomizadas com reposição hormonal, medindo-se a atividade de ectonucleotidases.

2. Estudo dos efeitos do estresse sobre a memória.

2.1 Estudo Comportamental

a. Verificar o efeito do estresse repetido sobre o desempenho dos animais em diferentes tarefas comportamentais para avaliação da memória: labirinto aquático, esquiva inibitória e reconhecimento de objetos.

2.2. Estudo neuroquímico

Verificar o efeito da exposição ao estresse repetido sobre o hipocampo, avaliando:

a. A liberação de glutamato por sinaptossomas e a captação desse neurotransmissor por sinaptossomas e por fatias;

b. A união de glutamato a receptores em membranas;

c. A hidrólise de nucleotídeos da adenina em sinaptossomas, medindo-se a atividade de ectonucleotidases;

d. Alterações em parâmetros relacionados ao estresse oxidativo.

3. Estudo da vulnerabilidade neuronal

Verificar a vulnerabilidade do hipocampo de ratos submetidos ao estresse repetido, estudando:

a. O grau de perda celular em fatias de hipocampo submetidas à privação de oxigênio e glicose (POG);

b. A caracterização das células afetadas pela POG, através da utilização de diferentes substratos e medidas de proteínas específicas: a enolase neurônio-específica, NSE; e a proteína S100B, secretada por astrócitos;

c. A captação de glutamato em fatias de hipocampo submetidas à POG.

3. RESULTADOS

Esta tese deu origem a oito trabalhos que serão apresentados em forma de capítulos, organizados da seguinte maneira: os três primeiros capítulos referem-se aos efeitos do estresse sobre a medula espinal e os cinco últimos, demonstram as alterações observadas no hipocampo

Estudo dos efeitos do estresse sobre a medula espinal.

O Capítulo I: Artigo "Taste modulation of nociception differently affects chronically intermittent stressed rats". De acordo com a literatura, alguns trabalhos do nosso laboratório nos mostram que os animais submetidos ao estresse repetido apresentam alteração na nocicepção, como parte do processo de adaptação a este tratamento. Além disso, vários autores têm sugerido o possível envolvimento de experiências prazerosas ou desagradáveis sobre a percepção da dor. Em vista disso, nós resolvemos realizar este trabalho onde avaliamos a resposta nociceptiva após a ingesta de doce (estímulo agradável) ou ácido acético (estímulo desagradável) por animais estressados cronicamente. Nós verificamos diferentes respostas nociceptivas destes animais quando expostos a estímulos agradáveis ou desagradáveis, onde os animais controle respondem com analgesia somente quando experimentam o sabor doce, enquanto os animais estressados parecem mais aptos a perceberem o estímulo desagradável.

Publicado: Fontella, FU; Nunes, ML; Crema, LM; Balk, RS; Dalmaz, C; Netto, CA. Taste modulation of nociception defferently affects chronically stressed rats. *Physiology and Behavior* **80:** 557-561, 2004.

Capítulo II: Artigo: "Gender differences in oxidative stress on oxidative stress". Considerando as alterações na nocicepção observadas após a exposição ao estresse repetido, a diferença sexual nestas respostas, e o possível envolvimento dos hormônios sexuais na lipoperoxidação, largamente descrito na literatura, nós realizamos este trabalho

que consistiu na avaliação da produção de radicais livres, na lipoperoxidação e na reatividade antioxidante em medula de ratos machos e fêmeas, estressados cronicamente. Os resultados obtidos sugerem que o estresse repetido diminui a reatividade antioxidante tanto em machos quanto em fêmeas e que existe uma diferença sexual nesta diminuição, assim como na produção de radicais livres, sem que haja alterações na lipoperoxidação. Estes efeitos podem ser responsáveis, ao menos em parte, pelas alterações induzidas pelo estresse e é possível que as diferenças hormonais possam contribuir para estas alterações.

Publicado: Tabajara, A.S.; Fontella, F.U.; Torres, I.L.T.; Dalmaz, C. Gender Differences in Oxidative Stress in Spinal Cord of Rats Submitted to Repeated Restraint Stress. *Neurochemical Research*, **28**: 1315-1320, 2003.

Capítulo III: Manuscrito "Repeated Stress Effects on Ecto-Nucleotidase Activities of Female Spinal Cord Synaptosomes are Dependent on Estradiol Levels". Uma vez evidenciada as diferenças sexuais na nocicepção após exposição ao estresse repetido, nós investigamos a influência do esteróide gonadal, 17-β-estradiol, em fêmeas submetidas a este tratamento. Nós também investigamos a influência deste hormônio sobre a hidrólise de ATP, ADP e AMP em sinaptossomas preparados a partir da medula destes animais, já que tanto o ATP quanto a adenosina podem atuar como neuromodularores da dor e sua degradação/produção recebem influência tanto do estresse quanto do estradiol. Os resultados obtidos aqui nos mostram que as fêmeas estressadas, com ou sem reposição hormonal, apresentam menor limiar nociceptivo quando comparadas com as controles. Também se verificou uma diminuição nos níveis do adenosina extracelular (pela diminuição da atividade as 5'-nucleotidase) após o estresse repetido, um efeito revertido pela reposição hormonal. Estas observações sugerem que a sensibilidade à dor depende de

um sistema regulatório complexo e que a modulação da neurotransmissão pelos nucleotídeos da adenina na medula espinhal envolve a interação do hormônio 17-β-estradiol e da exposição ao estresse. Entretanto, a redução da produção de adenosina extracelular não é a única explicação da hiperalgesia observada após a exposição ao estresse repetido, uma vez que a reposição estradiol normaliza a sua produçãomas não é suficiente para reverter a resposta hiperalgésica ao estresse repetido.

Submetido: *Hormones and Behavior*

Estudo dos efeitos do estresse sobre a memória.

Capítulo IV: Manuscrito "Effect of repeated restraint stress on different memory tasks in hippocampal synaptic membranes". Este trabalho investigou o efeito do estresse repetido sobre a memória de ratos adultos utilizando três diferentes tarefas: Morris water maze (memória especial e memória de referência); esquiva inibitória e reconhecimento de objeto. Os resultados deste trabalho nos sugere que o estresse repetido é capaz de modificar a memória especial, a qual depende a função hipocampal.

Submetido: *Brazilian Journal of Medical and Biological Research*

Capítulo V: Artigo "Repeated restraint stress alters hippocampal glutamate uptake and release in the rat". Considerando as evidências da literatura de que, tanto o estresse agudo quanto o tratamento crônico com corticosterona interferem no metabolismo do glutamato, nós investigamos neste trabalho o efeito da exposição aguda e crônica ao estresse de contenção sobre a captação e liberação de glutamato em sinaptossomas de hipocampo de ratos. Além disso, também investigamos o efeito destes tratamentos sobre o *binging* de [^{3}H] glutamato nas membranas sinápticas destes animais. Com este trabalho

pudemos concluir que o estresse repetido de contenção aumenta tanto a captação quanto a liberação basal de glutamato, além de uma diminuição no *binding* de [^{3}H] glutamato nas membranas sinápticas. Uma vez que estes efeitos não foram observados após o estresse agudo, imaginamos que este poderia ser uma resposta adaptativa neuroprotetora ao tratamento crônico.

Aceito em março/2004: *Neurochemical Research*

Capítulo VI: Artigo "Acute and Chronic Stress Alter Ecto-Nucleotidase Activities in Synaptosomes from the Rat Hippocampus ". Considerando que os nucleotídeos da adenina, assim como a adenosina, estão envolvidos na modulação de vários processos fisiológicos e patológicos e que seus níveis podem estar alterados em situações de estresse, este trabalho avaliou o efeito do estresse agudo e repetido sobre a atividade das ATP difosfohydrolase and 5'-nucleotidase, via hidrólise do ATP, ADP e AMP em sinaptossomas hipocampais de ratos adultos. Nós pudemos observar diferentes efeitos nos dois modelos de estresse estudados, com um significante aumento hidrólise de ATP, ADP and AMP em resposta ao estresse agudo, sugerindo a ativação de uma ATP-difosofoidrolase e da 5'-nucleotidase; e um aumento somente de uma ecto-ATPase induzida por estresse repetido, possivelmente resultante de uma adaptação deste tratamento.

Aceito em abril/2004: *Pharmacology Biochemistry and Behavior*

Capítulo VII: Manuscrito "Repeated restraint stress induces oxidative damage in hippocampus of rat". Uma vez que várias evidências da literatura relatam o envolvimento do estresse emocional na formação de espécies ativas de oxigênio e na diminuição das defesas antioxidantes, fatores que podem levar ao dano oxidativo no cérebro, nós realizamos este trabalho a fim de avaliar o efeito do nosso modelo de estresse repetido sobre a produção de radicais livres, sobre a lipoperoxidação, sobre a capacidade

antioxidante total (potencial e reatividade antioxidante), assim como, sobre a atividade de enzimas antioxidantes no hipocampo de ratos. Os nossos resultados nos permitiram concluir que este modelo de estresse repetido induz estresse oxidativo. Essas alterações poderiam estar relacionadas com os efeitos deletérios ao hipocampo, referido por vários autores após o estresse prolongado.

Aceito em outubro/2004: *Neurochemical Research.*

Capítulo VIII: Manuscrito "Acute and repeated restraint stress influences cellular damage in rat hippocampal slices exposed to oxygen and glucose deprivation". Nós realizamos este estudo afim de avaliar a influência do estresse sobre a vulnerabilidade neuronal. Para tanto, submetemos slices de hipocampo de ratos estressados aguda ou repetidamente a uma modelo de isquemia *in vitro.* A partir disso, nós medimos o dano celular utilizando métodos que avaliam a integridade da membrana; a secreção celular de uma proteína produzida por astrócitos e liberada em situações de injúria; assim como captação de glutamato pelas faitas de hipocampo de ratos estressados submetidas à isquemia *in vitro.* Nós observamos que o estresse repetido aumenta a vulnerabilidade hipocampal a um insulto subseqüente, no caso a isquemia, e que as células prejudicadas pela soma destes fatores são principalmente neurônios. Também concluímos que o mecanismo responsável por esta maior susceptibilidade neuronal à isquemia não envolve a captação de glutamato.

Submetido: *Brain Research Bulletin*

***Capítulo I*:** Artigo 1

Taste modulation of nociception differently affects chronically intermittent stressed rats

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Taste modulation of nociception differently affects chronically stressed rats

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Abstract

Stress responses cover a wide range of physiological changes, including alterations in the perception of and response to pain. Animals submitted to repeated stress present altered nociception and this effect is part of this process of adaptation; in addition pleasant and unpleasant experiences with tastes and odors have been shown to affect distinct behavioral aspects, such as pain perception. The aim of the present study is to verify the responses of repeatedly stressed rats (1 h of daily immobilization during 40 days) to pleasant and unpleasant tastes on nociception, when compared to control animals. An increase in the tail-flick latency (TFL) was observed 5 min after exposure to a sweet taste in the control group, whereas no effect was observed in chronically stressed animals. When submitted to an unpleasant taste (5% acetic acid), the chronically stressed group presented an increase in TFL, whereas no effect was observed in the control group. In conclusion, chronically stressed animals present different nociceptive responses to sweet and acid tastes; although control animals suitably respond to a sweet stimulus, stressed animals seem to be more apt to react to the unpleasant stimulus.

Keywords: Nociception; Taste; Stress; Chronic stress; Restraint; Tail flick; Pain; Rats

1. Introduction

Chronic stress is known to produce a series of physiological and behavioral changes. Physiological responses to stress include alterations in the perception and response to pain. Although acute exposure to a variety of stressors produces immediate analgesia in several pain tests (SIA) [1], prolonged stress by repeated exposure to stressors, such as cold or restraint, has been reported to cause hyperalgesia [2,3]. Such different effects observed in nociception after acute and chronic stress are considered part of a process of adaptation, which includes several biochemical and physiological processes. Various neurotransmitter systems have been suggested to play a role in stress desensitization process, such as opioids, serotonin and dopamine [4–8].

Besides presenting decreased pain thresholds [3], chronically stressed animals show tolerance to morphine antinociceptive effect [9] and no novelty-induced antinociception [10], a phenomenon attributed to opioid activation [11–13]. These studies suggest an alteration in the pattern of opioid activity in the chronic restraint model. In addition, alterations in dopamine transmission in chronically stressed animals have also been shown (e.g., Ref. [7]), and this neurotransmitter is involved in mediating nociceptive response, either directly or indirectly through its interaction with endogenous opioid peptide systems [14].

Taste experiences are naturally pleasant or unpleasant. Both pleasant and unpleasant experiences with tastes and odors have been shown to affect different behavioral aspects, such as mood and emotions [15,16], peripheral autonomic responses [17,18], and also the perception of pain [19–22]. Recent work has revealed that intake (both chronic and acute) of palatable foods and fluids can significantly alter the behavioral consequences of opioid agents on nociception [23–26]. Based on these data, it has been suggested that intake of palatable foods and fluids alters the

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behavioral actions of drugs or transmitter systems on nociception [23–26].

While showing increased nociception [3], stressed rats present altered consumption of sweet food [27], so we hypothesized that repeatedly restrained animals may also respond differently to pleasant and unpleasant tastes. The objective of the present study is to determine the effect of sweet and acid flavors on nociception in repeatedly stressed rats.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (60 days at the beginning of the treatment, 200–230 g) from our breeding stock were used. Experimentally naive animals were housed in groups of four to five in home cages made of Plexiglas material (65 × 25 × 15 cm) with the floor covered with sawdust. They were maintained on a standard 12-h dark/light cycle (lights on between 0700 and 1900 h) at room temperature (22 ± 1 °C). The rats had free access to food (standard lab rat chow) and water, except during the period of exposure to the stressor. All animal procedures were approved by the institutional Ethical Research Committee.

2.2. Chronic restraint stress procedure

Animals were divided into two groups: control (20 rats) and stressed (18 rats), which were housed in different cages. Stressed animals were submitted to restraint 1 h daily, 5 days per week for 40 days (total time) [27]. Restraint was done 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 in the far end for breathing. The control group was not submitted to stress, being kept in their home cages. The immobilization procedure was performed between 1200 and 14:00 h.

2.3. Tail-flick measure

All tail-flick measurements were done in the afternoon. Nociception was assessed with the tail-flick apparatus [28]. Rats were wrapped in a towel and placed on the apparatus. The light source positioned below the tail was focused on a point 2–3 cm rostral to the tip of the tail. Deflection of the tail activated a photocell and automatically terminated the trial. The tail-flick latency (TFL) represented the period of time (seconds) from the beginning of the trial to the tail deflection. A cutoff time of 10 s was used to prevent tissue damage. Shortly after the last session of the treatment (40 days) and 24 h before the first measurement, the animals were exposed to the tail-flick apparatus to familiarize them with the procedure, since the novelty of the apparatus can itself induce antinociception [12].

2.4. Exposure to different tastes

To determine the effect of pleasant and unpleasant tastes on nociception, animals were submitted to both tastes, in a random order of exposure. The procedure was as follows:

(a) Effect of sweet taste on nociception: The animals were placed in a lighted rectangular box (40 × 15 × 20 cm) with a glass ceiling; floor and sidewalls were made of wood. Ten Froot Loops (Kellogg's pellets of wheat, cornstarch and sucrose) were placed in one corner of the box. Animals were submitted to a 3-min trial, once a day during 3 days, in order to become familiarized with this food [27]. On the next day, the animals were submitted to the tail-flick test (basal latency), and returned to their home cages, where they received three pellets of Froot Loops for a 5-min period. Then, TFL was measured again. Animals presenting a basal TFL higher than 10 s (four controls and one stressed) or the ones that did not try the sweet food (one stressed) were discarded.

(b) Effect of an unpleasant taste on nociception: In the first day, animals were habituated to being alone in a home cage, without food and with a drinking bottle of water, during 5 min. On the next day, the animals were submitted to the tail-flick test (basal latency) and placed alone in the home cage of the previous day, without food and with a drinking bottle containing 5% acetic acid, for a period of 5 min. Only the animals that tried the bottle content were used in this experiment. Afterwards, TFL was measured again. The animals that presented a basal

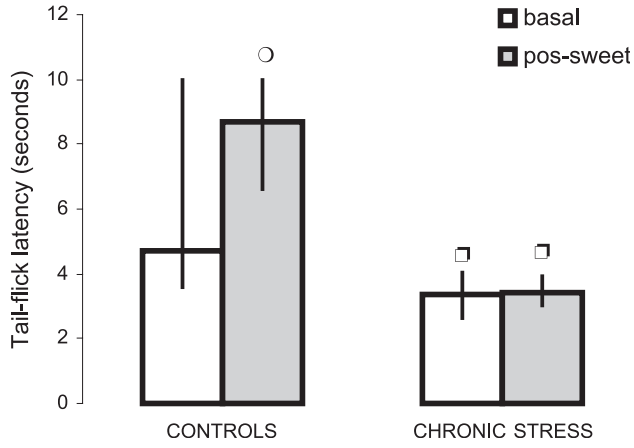

Fig. 1. Nociceptive response before (basal) and after exposure to sweet food in rats previously submitted to chronic stress. Number of animals = 16 per group. Data are expressed as medians (interquartile range) of TFL. ❍ Significant difference, as compared to the basal measurement of the control group (Wilcoxon matched-pairs test, $P < .01$). ❒ Significantly different from control group latencies, Mann–Whitney U test, $P < .05$.

TFL higher than 10 s (one control) were not used in this experiment.

3. Statistical analysis

Data are expressed as median (interquartile range) of TFL. The comparison between two groups was made by Wilcoxon matched-pairs signed-ranks test (for repeated measures) or by Mann–Whitney *U* test (for independent samples). A *P* value of .05 was considered statistically significant.

4. Results

Results showing the effect of a sweet taste on nociception in control and stressed animals are presented in Fig. 1. According to the basal measures, the chronically stressed animals were hyperalgesic (basal TFL from chronic stress group compared to the basal TFL from control group; Mann–Whitney *U* test, $P<.05$). Five minutes after exposure to a sweet taste, an increase in TFL was observed in the control group (Wilcoxon matched-pairs test, $P<.01$), whereas no effect was seen in chronically stressed animals (Wilcoxon matched-pairs test).

The effect of the unpleasant taste on nociception in animals submitted to chronic intermittent stress is shown in Fig. 2. Stressed animals presented decreased TFL after 40 days of treatment (Mann–Whitney *U* test, $P<.001$). Five minutes after drinking from a bottle containing 5% acetic acid, no effect was observed in the control group (Wilcoxon matched-pairs test), whereas chronically stressed animals presented an increase in nociceptive threshold, i.e., the TFL was increased (Wilcoxon matched-pairs test, $P<.01$).

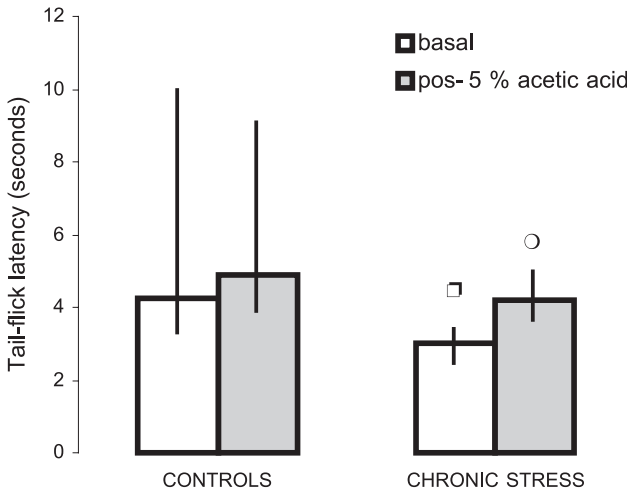

Fig. 2. Nociceptive response before (basal) and after exposure to 5% acetic acid in rats previously submitted to chronic stress. Number of animals = 19–20 per group. Data are expressed as medians (interquartile range) of TFL. ❍ Significant difference compared to the basal measurement of the chronic stressed group (Wilcoxon matched-pairs test, $P<.01$). ❒ Significant difference from control group latencies (Mann–Whitney *U* test, $P<.001$).

5. Discussion

Chronic stress may lead to different effects on nociception and on perception of sweet and acid flavors. Confirming the working hypothesis, the presented results demonstrate that pleasant and unpleasant tastes differently affect pain threshold of repeatedly stressed rats as compared to control animals. Hyperalgesia was induced by repeated restraint stress procedures, as observed in the basal measures, a finding that agrees with previous reports [3,9]. The control group showed increased TFL after tasting sweet food, confirming results from other authors [25,29], whereas stressed animals did not present any alteration in pain threshold after exposure to sweet food. This effect of chronic stress had not yet been reported.

The possibility that Froot Loops is more satiating than the acetic acid solution and that satiation could partly account for the difference in reaction to the two flavors must also be considered, since it is unclear how exactly this sweet taste operates to alter nociception. However, as the effect was measured just 5 min after exposing the animals to the flavors, it is hardly conceivable that the nutritive properties of Froot Loops could account for its effects on nociception.

On the other hand, when animals were exposed to an unpleasant taste, only the stressed group presented an alteration in tail-flick latencies, i.e., an analgesic effect. These results suggest that chronic stress shifted the dose–response function: a threshold sweet taste does not affect nociception and a mild acidic taste is functionally more effective in stressed than in control rats.

Negative emotional processing, including aversive stimuli in both the olfactory and gustatory modalities, is known to be mediated by several central nervous pathways, which are not exactly the same as the ones that mediate responses to pleasant stimuli [15,30]. Pain is a sensory and emotional experience that is likely to involve activation of numerous regions of the brain, particularly those linked with both somatosensory and limbic systems [21]. Such connections may provide one route through which somatosensory input may be integrated with nociception in order to allow a different appreciation of the meaning and dangers of painful stimuli, modulating pain perception at different levels. In this context, bidirectional changes in affective state (i.e. reward or aversion production), associated with pharmacological manipulation, have been reported to modulate nociception at the spinal cord level.

Because the animals have not been previously exposed to acetic acid, the analgesic effect observed could be tentatively explained by the novelty. In fact, nociception has been

reported to be influenced by novelty [11–13], an effect shown to be blocked by chronic stress [10]. Whereas control rats present an increased TFL after exposure to a novel environment [10–13], chronically stressed animals did not show this effect [10]. Considering data presented in Fig. 2, the fact that controls had no change on nociception after exposure to acid taste suggest that the novelty effect in this response of the stressed rats is not a significant one.

The fact that chronically stressed animals are able to respond to acid taste with an antinociceptive response, differently from control animals, might be related to physiological consequences of stress exposure. For example, the response to a new stressor after chronic stress may be intact [31] or potentiated, a phenomenon termed facilitation [32–34]. Furthermore, the finding that only stressed animals presented altered pain threshold after exposure to an aversive taste suggests that these animals are more sensitive than controls to this type of sensations, possibly because of an exacerbated perception to this taste.

Different neurotransmitter systems seem to be involved in nociceptive response to pleasant and unpleasant stimuli of chronically stressed animals. As previously mentioned, because endogenous opioids may be involved in the analgesic effect of sucrose, it is possible that the absence of response to sweet substances might be related to the reduced function of opioid system already reported in this model of chronic stress [9].

In addition, chronic stress exposure has been reported to alter dopamine reactivity. This effect occurs in a similar way as the alteration on the nociceptive response to motivational stimuli, i.e., in a manner dependent on the aversive or rewarding nature or the stimulus. Evidence for an impairment of dopamine transmission in rats submitted to chronic stress have been reported [35,36] and chronic stressed rats present changes in the responsiveness of dopamine transmission to aversive and rewarding stimuli [36]; interestingly, stimulatory dopamine response to aversive stimuli is potentiated and the response to rewarding stimuli is reduced [36]. Concerning dopamine role in nociception, it has been suggested that dopamine plays a direct role in this process [37,38]; additionally, it may have an indirect role, interacting with endogenous opioid systems [39]. Therefore, it is possible that the different nociceptive effects to rewarding and aversive stimuli may be the result of altered motivational impact of the stimuli on mesolimbic dopaminergic neurons.

In conclusion, chronically stressed and control animals present different nociceptive responses to sweet and acid tastes. Since the perception of different stimuli and the response to nociception involves many aspects, including mood and emotions [40,41], it is possible that the different responses observed between control and stressed rats might be related to such aspects; control animals respond to a sweet stimulus in a more suitable manner, whereas stressed rats are more apt to perceive, and react to, the unpleasant stimulus. Alterations in transmitter systems involved in nociception and in the perception of rewarding or aversive stimuli, such as opioid or dopamine systems, may underlie these effects.

Acknowledgements

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Capítulo II: Artigo 2

Gender differences in oxidative stress in spinal cord of rats submitted to repeated restraint stress

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Gender Differences in Oxidative Stress in Spinal Cord of Rats Submitted to Repeated Restraint Stress

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Behavioral and neurochemical gender-specific effects have been observed following repeated stress. The aim of this study is to verify the effects of repeated restraint stress on free radical production (evaluated by DCF test), lipoperoxidation (evaluated by TBARS levels), and total antioxidant reactivity (TAR) in the spinal cord of male and female rats. Results demonstrate no effect on lipoperoxidation; chronic stress decreased TAR both in male and female spinal cord. In addition, gender differences were observed both in TAR and in the production of free radicals, both being increased in females. These results may be relevant to the gender-specific differences observed after exposure to repeated stress.

KEY WORDS: Chronic stress; gender; lipid peroxidation; free radicals; TAR.

INTRODUCTION

Reactive oxygen species (ROS), which include hydrogen peroxide and other chemical forms known as free radicals, such as superoxide anion and hydroxyl and peroxyl radicals, are produced as part of many normal and essential biological processes (1). A diversity of antioxidant systems, both enzymatic and nonenzymatic, operate to control excessive levels of ROS (1). A cell is generally able to maintain an appropriate balance between oxidants and antioxidants in normal conditions. When this balance is perturbed and shifts toward oxidative stress by either an increase in oxidants or a decrease in antioxidants, the cell becomes more susceptible to injuries, such as trauma, for example (2,3).

Nervous tissue is highly susceptible to injury by the lipid peroxidative process, probably because of its high oxygen consumption and its high content of easily oxidable substrates, mainly polyunsaturated fatty acids and catecholamines (4). Extensive experimental evidence suggests that peroxidative degradation of neuronal membrane phospholipids occurs following central nervous system (CNS) trauma (4,5). In spinal cord, increased susceptibility to lipid peroxidation (LP) was observed after some types of injury, such as trauma and ischemia-reperfusion (6,7).

There is strong evidence to suggest that in the brain, high levels of glucocorticoids (GCs), hormones released from the adrenal cortex in stress situations, may induce deleterious effects, including damage to neurons (8). These events have been linked to an increased generation of ROS, which can directly damage cellular proteins, DNA, and lipids, and thereby affect all cellular functions (9). Immobilization stress is followed by an increase in free radical levels, especially in LP, in plasma and many structures of brain, such as cerebral cortex,

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cerebellum, hippocampus, and midbrain, compared to the unstressed controls (10,11). In addition, a decreased activity of the antioxidant enzymes copper/zinc superoxide dismutase (Cu/Zn SOD) and glutathione peroxidase (GSPx) in the brain of rats treated with GCs has also been observed (12). Stress may thus impair antioxidant defenses, leading to oxidative damage, considerably changing the balance between oxidative and antioxidant factors in brain (10,11).

Experimental evidence suggests that exposure to repeated stress induces physiological effects, with gender differences, on the spinal cord. For example, some enzyme activities are altered in chronically stressed male rats, an effect that was not observed in females (13). Repeatedly stressed male rats also exhibit decreased tail-flick latency, characterizing a hyperalgesic response, an effect that was not observed in females (14). These observations suggest a gender-specific susceptibility of the spinal cord to repeated stress. In addition, sex hormones have been suggested to play a role in lipid peroxidation, because estradiol inhibits the formation of lipid peroxides in some tissues (15).

In an attempt to characterize the complex biochemical conditions involved in the action of free radicals in the spinal cord of repeatedly stressed male and female rats, we evaluated the production of free radicals, lipid peroxidation, and the total antioxidant reactivity (TAR) levels in this tissue.

EXPERIMENTAL PROCEDURE

Subjects. Experimentally naive adult male and female Wistar rats (60 days old) were used. They were housed in groups of five in home cages made of Plexiglas (65 × 25 × 15 cm) with the floor covered with sawdust. Animals were maintained in a controlled environment (12:12 light/dark cycle, temperature 22 ± 1°C) before and throughout the experimental period. Rats had free access to food (standard laboratory rat chow) and water, except during the period when restraint stress was applied. The restraint procedure was performed between 10:00 and 12:00 h.

Repeated-Restraint Stress Procedure. Both male and female animals were divided in two groups: stressed and control. Restraint was applied by placing the animals in a 25 × 7 cm plastic bottle, and fixing it with plaster tape on the outside, so that the animals was unable to move. There was a 1-cm hole at one far end for breathing. After the stress procedure the animals were returned to the home cages. Control animals were kept in their home cages during the period of treatment. The animals were stressed 1 h/day, 5 days a week for 40 days (16). 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 minimize animal suffering and reduce the number of animals.

Preparation of the Samples. After 40 days of treatment, the animals were sacrificed by decapitation 24 h after the last exposure to stress. Their spinal cords were quickly dissected out and frozen in liquid N_2. Samples were stored at −70°C until analysis, when they were homogenized in 10 vol (w:v) ice-cold 0.1 M phosphate buffer (pH 7.4). An aliquot of the total homogenate was separated to measure TBARS. The remainder of the homogenates was centrifuged at 960 × *g* to remove the particulate fraction. Supernatants were used to assay TAR and DCF. Protein content was determined both in the total homogenates and in the supernatants by the Lowry method using bovine serum albumin as standard (17).

Reactive Oxygen Species (ROS) Formation. To assess the production of free radicals we used 2′-7′-dichlorofluorescein diacetate (DCFH-DA) as a probe. An aliquot of the sample (25 μl) was incubated with 0.1 mM DCFH-DA at 37°C for 30 min. DCFH-DA is hydrolyzed to DCFH, because the diacetate group is cleaved by esterases. This compound is oxidized by ROS to form the fluorescent compound, DCF. The formation of the oxidized fluorescent derivative (DCF) was monitored at an excitation wavelength of 488 nm and an emission wavelength of 525 nm using a fluorescence spectrophotometer (Hitachi F-2000). Background fluorescence was corrected by the inclusion of parallel blancks. The formation of ROS was quantified using a 2′-7′-dichlorofluorescein (DCF) standard curve, and results were expressed as nmol DCF/mg protein (18,19).

Assay of Lipid Peroxides. Lipid peroxidation was assessed by the thiobarbituric acid reactive substances (TBARS) assay (7,20,21), and expressed as MDA equivalents per milligram of protein. The aldehyde groups react with two molecules of thiobarbituric acid at low pH and high temperature to form a pink-colored complex. Briefly, aliquots of the samples were incubated with 10% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA). The mixture was heated in a boiling water bath for 15 min and, after it was cooling, *n*-butanol was added. The mixture was shaken and centrifuged and the organic phase was collected to measure the fluorescence intensities. The fluorescence was read at excitation and emission wavelengths of 515 and 553 nm, respectively. We used 1,1,3,3-tetramethoxypropane as a standard.

Total Antioxidant Reactivity (TAR) Assay. The method used was based on (22,23). The reaction mixture contained the free radical source (2 mM ABAP) and 6 mM luminol in glycine buffer (0.1M, pH 8.6). Incubation of this mixture at 20°C generates an almost constant light intensity that was measured in a scintillation counter (Beckman) working in the out of coincidence mode. The TAR values were determined by measuring the initial decrease of luminol luminescence calculated as the ratio Io/I, where Io is the luminescence intensity in the absence of additives, and I is the luminescence intensity after addition of a small aliquot of the sample. For determination of antioxidant reactivity, 1 μl of the sample was added to the system. TAR values as equivalents of Trolox concentration were obtained by comparison of the ratio Io/I of the samples with that of 20 nM Trolox.

Statistical Analysis. Data were expressed as mean ± standard error of the mean, and were analyzed by a two-way ANOVA followed by the Student-Newman-Keuls test, or by Student's t test.

RESULTS

Figure 1 shows the effects of repeated restraint stress on total antioxidant reactivity (TAR) assay, in spinal cord homogenates of male and female rats. A two-way ANOVA showed an effect of stress [$F(1,31) = 17.14$; $P < 0.001$], because chronic stress decreased TAR in both, male and female groups, as

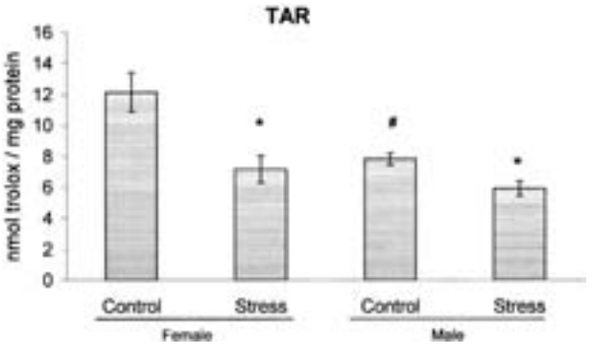

Fig. 1. Effects of repeated restraint stress on total antioxidant reactivity (TAR) in spinal cord of male and female rats. Results are expressed as nmol trolox/mg protein. Columns represent mean (n = 8–10) ± SEM. * Significantly different from the control group (two-way ANOVA, followed by Student's t test, $P < 0.01$). # Significantly different from females (two-way ANOVA, followed by the Student's t test, $P < 0.01$).

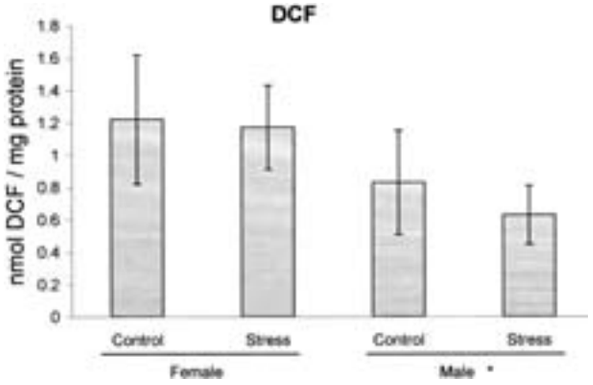

Fig. 3. Effect of repeated restraint stress on free radical production, using DCFH-DA as a probe, in rat spinal cord of females and males. Results are expressed as nmol of DCF/mg protein and are mean ± SEM. of 8–10 samples in each group. The mean values in females were 1.2 ± 0.4 (control); 1.2 ± 0.3 (stress), and in males were 0.8 ± 0.3 (control); 0.6 ± 0.2 (stress) nmol of DCF/mg protein. * Significantly different from females, as determined by two-way ANOVA, followed by the Student-Newman-Keuls test ($P < 0.01$).

shown by Student's t test ($P < 0.01$ in both cases). There was also an effect of sex [$F(1,31) = 11.30$; $P < 0.005$], especially in the control groups (comparing control male and female rats, Student's t test shows significantly higher levels of TAR in the female group, $P < 0.01$). There was a marginal interaction between stress and sex (two-way ANOVA, $P = 0.072$). These results suggest that repeated restraint exposure may decrease antioxidant defenses in spinal cord of rats and that this parameter is higher in females.

When we measured lipid peroxidation in spinal cord homogenates of stressed and control rats, on the other hand, a two-way ANOVA showed no effect of treatment and no difference between male and female rats ($P > 0.05$ in both cases), although there was a marginal interaction between sex and treatment ($P = 0.067$) (Fig. 2).

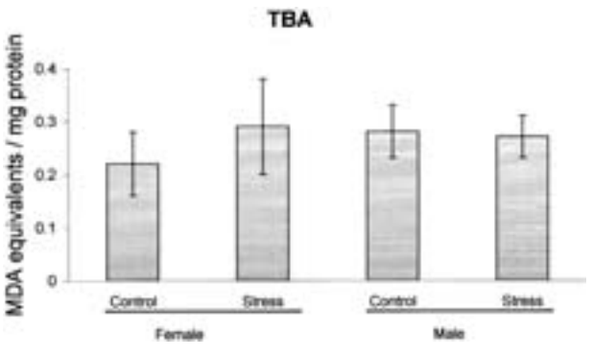

Fig. 2. Effect of repeated restraint stress on TBARS levels in rat spinal cord homogenates. Results are expressed as MDA equivalents/mg protein and are mean (n = 8–10) ± SEM. The mean values for female were 0.2 ± 0.06 (control); 0.3 ± 0.09 (stress), and for male were 0.3 ± 0.05 (control); 0.3 ± 0.04 (stress) MDA equivalents/mg of protein. No differences were found using two-way ANOVA.

A significant difference in the generation of free radicals was observed between the male and female groups, that is, higher DCF levels were observed in spinal cord homogenates of female rats (Fig. 3). A two-way ANOVA showed no effect of stress ($P > 0.05$) and a significant effect of sex ($P < 0.001$). However, this effect was more pronounced when comparing stressed males and females (Student's t test, $P < 0.001$; in the case of control male and females, $P = 0.052$). These results suggest a higher production of oxidants in female spinal cord.

DISCUSSION

The present study demonstrated that repeated stress had no effect upon lipid peroxidation in the spinal cord homogenates of rats. Conversely, a significant decrease in the generation of free radicals was observed in the male group, compared to the female group, because higher DCF levels were observed in spinal cord homogenates of female rats.

Repeated stress decreased TAR in both male and female groups, with higher TAR differences in spinal cord homogenates of females, compared to males, because the reduction in TAR was more pronounced in female spinal cord.

The TAR assay has been developed to measure total antioxidant reactivity of biological samples

(22,23). This test measures both nonenzymatic antioxidants, such as GSH, ascorbic acid, α-tocopherol, β-carotone, and antioxidant reactivity resulting from enzymatic action (22). It is essential to measure all these antioxidants in assessing antioxidant status. However, the number of different antioxidants in biological samples makes it difficult to measure each of them separately. In addition, the possible interaction among different antioxidants could also make the measure of individual antioxidants less representative of the overall antioxidant status (24). This is the reason why methods to evaluate the total antioxidant capacity were developed. The TAR assay we employ in this study is widely used (23,25–28). The principle of this method is that peroxyl radicals produced from 2,2′-azobis (2-aminopropane) dihydrochloride oxidize luminol, leading to the formation of luminol radicals that emit light (22). Antioxidants in the sample determine a reduction in this chemiluminescence that is proportional to the total antioxidant potential of the sample. The interest in this type of determination resides in that they provide information about the evaluation of the capacity of a biological fluid to prevent the damage associated to free radical processes.

Exposure to GCs or stress has been reported to induce formation of ROS and lead to oxidative injury in various tissues (29,30). In our study, no alteration in LP (measured by TBARS) or in free radical production (evaluated by DCF production) was observed after repeated stress. Whereas immobilization stress or exposure to GCs may lead to an increase in ROS production (8,12), exposure to chronic stress, as reported here, may induce regulatory changes in GCs release or their receptors (31). It should be noted that in the chronic restraint stress model, after 40 days of treatment, the increase in corticosterone levels after exposure to stress is much smaller than after the first stress session (32). Variable chronic stress, but not repeated restraint stress, induces increased lipid peroxidation of rat lungs after 40 days of treatment (Torres et al., personal communication), an effect possibly related to the lack of previsibility, which characterizes a variable stress model, contrary to this model, in which repeated stress may lead to an adaptation, with progressively decreased responses to the stressor. Effects of chronic stress on TBARS in different brain structures have also been observed, and distinct brain regions show different responses to stress(33).

Several biochemical processes have been identified following spinal cord injury (SCI) by trauma or excitotoxicity, including the extracellular accumulation of glutamate, increased concentrations of intracellular Ca^{2+}, overproduction of ROS, and LP (34–36). Although previous results have pointed to biochemical and physiological alterations in spinal cord (13,14), this model did not induce an imbalance in the oxidative stress system. An alternative explanation would be that a high total antioxidant reactivity in this structure is enough to protect against ROS production. In this sense, the decreased antioxidant reactivity in stressed rats may represent an amount of antioxidants consumed in order to maintain the oxidative balance.

In this study, we also observed sex differences, both in TAR and in free radical production. These differences may be due to either organizational factors (sexually dimorphic structures may present different plastic alterations in response to chronic stress in males and females), or activation factors (sexual hormones may influence response to oxidative stress), or both. Effects of sex hormones on oxidative stress have been reported, particularly the effects of estradiol (37–41).

Estrogens and their precursors can be released by the adrenal glands, as well as by ovaries (42), and their receptors have been identified in the rat spinal cord, where they have been shown to vary in density across the estrous cycle (43). Estrogens have antioxidant properties and can inhibit lipid peroxidation in vitro, which might contribute directly to their neuroprotective effect (37–39), and the antioxidant mechanism of estrogen protection has been suggested to be independent of receptor binding (39,40). In addition, significant effects of gender on hydrogen peroxide production in plasma were found, with men having greater values than women (41).

On the other hand, besides their neuroprotective effects, estrogens have been demonstrated to be neurotoxic in several studies. For example, the formation of hydroxy radicals has been demonstrated to be induced by environmental estrogen-like chemicals (44). Estradiol has also been shown to have neurotoxic effects on β-endorphin neurons, an effect prevented by treatment with the antioxidant vitamin E, suggesting that the neurotoxic effect is mediated by free radicals (45).

No effect was observed on LP, as assessed by the TBARS assay. This is the simplest method for studying the effects of different treatments on LP, and it can be applied to crude biological systems. Although its specificity has been questioned (46,47), this particular assay is widely used for ex vivo and in vitro measurements (48,49), and it has been accepted as an empirical window in the complex process of LP (47).

Exactly how increased free radical production occurs in females whilst no alteration in damage was

observed in lipids is not clear. It is possible that the increased antioxidant reactivity observed in females may help to explain this. The TAR indicates the quality of antioxidants present in the homogenate. This measurement constitutes a useful index of the capacity of a compound or fluid to modulate the damage associated with an enhanced production of free radicals (22). Therefore increased TAR in the female spinal cord may compensate the increased production of free radicals in such a way that no alteration in LP will result.

In conclusion, the present results suggest that repeated restraint stress may induce decreased antioxidant reactivity both in male and female spinal cord, and this may be a factor involved in chronic stress-induced effects. In addition, gender differences have been observed both in antioxidant reactivity and in the production of free radicals, although no difference was observed in LP. It is possible that hormonal differences may account for these alterations. The exact biochemical mechanism involved in the differences in TAR and DCF observed between male and female spinal cords, which are the antioxidants (both enzymatic and nonenzymatic) involved in this effect—and if there is a relationship between these differences and sexual hormones—still requires more detailed study. On the basis of the present results, gender-based investigations are warranted.

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Capítulo III: Manuscrito 3

Repeated Stress Effects on Ecto-Nucleotidase Activities of Female Spinal Cord Synaptosomes are Dependent on Estradiol Levels

Submetido: *Hormones and Behavior*

Repeated Stress Effects on Ecto-Nucleotidase Activities of Female Spinal Cord Synaptosomes are Dependent on Estradiol Levels

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Running title: repeated stress, estradiol and nucleotidase activities in female spinal cord.

Abstract

It has been reported that animals submitted to repeated restraint stress present different adaptation responses, which are dependent on the gender of the animal, including repeated stress effects on nociception and on adenine nucleotide hydrolysis. We report the effect of chronic administration of a gonadal esteroid (17-β-estradiol) on ATP, ADP and AMP hydrolysis in spinal cord synaptosomes of adult ovariectomies (OVX) Wistar rats submitted to repeated restraint stress, during 40 days. We also measured pain threshold in these animals using the tail-flick test. The results show that tail-flick latencies were decreased in both stressed groups, OVX and OVX rats receiving estradiol replacement therapy, indicating reduced pain threshold after exposure to repeated stress. Repeated restraint stress caused no effect on ATPase nor ADPase activities. On the other hand, AMP hydrolysis in spinal cord synaptosomes from repeatedly stressed rats was decreased in OVX rats, as compared to non stressed OVX ones, indicating reduced extracellular adenosine production; this effect was reversed by hormonal replacement. These observations suggest that pain sensitivity to noxious stimuli is affected by repeated stress and that modulation of neurotransmission by adenine nucleotides in spinal cord may be altered by the interaction of sexual hormones and psychological factors, such as exposure to stress.

Keywords:

Stress, Repeated stress, ATPase-ADPase activities, 5′- nucleotidase activity, Spinal cord, Estradiol, Nociception

Animals submitted to repeated restraint stress present different adaptation responses that are dependent on the gender of the animal (Gamaro et al. 1998, Torres et al., 2002 a), including the expression of nociception (Bodnar et al., 1988; Kavaliers and Innes, 1987; 1992; Pollard and Dyer, 1985; Romero and Bodnar, 1986). In a previous study, chronically stressed male rats showed a decrease in tail-flick latency both in the basal state and just after exposure to restraint, characterizing an hyperalgesic effect, while chronically stressed females did not present the same response (Gamaro et al., 1998). This raises the possibility of gender differences in a variety of components associated with the regulation of nociception. For example, evidence indicates that estrogen is also involved in the regulation of analgesia and nociception (Frye et al., 1992; Murua and Molina, 1992; Morley et al., 1984) and appears to stimulate the release of brain β-endorphin (Wardlaw et al., 1982). In addition, pain thresholds may vary in females in different phases of the estrous cycle (Sternberg, 1999; Martinez-Gomes et al., 1994).

Altered hydrolysis of adenine nucleotides has been observed in synaptosomes from spinal cord of repeatedly stressed male rats, however this effect was not observed in repeatedly stressed females (Torres et al., 2002a). Adenine nucleotides are found in a variety of biological systems, being involved in different physiological and pathological functions (Vizi and Sperlaugh, 1999). In addition to its well-established role in cellular metabolism, extra cellular ATP and its breakdown product adenosine have pronounced effects in a variety of biological processes, including neurotransmission (Agteresch et al., 1999; Cunha, 2001; Millan, 1999). There is increasing evidence that adenosine and ATP may act as pain neuromodulators in the spinal cord (Post, 1984; Rhee et al., 2000; Tsuda et al., 1999a). Some researches have demonstrated that ATP acts on dorsal horn neurons in

the spinal cord after being released from a subpopulation of small primary afferent nerves involved in pain pathways (Fyffe and Perl, 1984; Sawynok and Sweeny, 1989), and that intrathecal administration of P2-purinoceptor antagonists has been associated with an antinociceptive action (Driessen et al., 1994; Tsuda et al., 1999a; 1999b).

ATP, as well as other nucleotide agonists for P_2-puriceptors, could be degraded by ectonucleotidases (Zimmermann, 1996). A cascade of these enzymes may play a role in the effective regulation of processes related to nociceptive thresholds and may also have a protective function by keeping extracellular ATP and adenosine within physiological levels (Cunha et al., 1992; Sarkis and Salto, 1991). This chain of enzymes includes ecto-ATPases (NTPDase2, CD39L1, EC 3.6.1.3), ATP diphosphohydrolase (NTPDase1, CD39, ecto-apyrase, EC 3.6.1.5) and ecto-5′-nucleotidase (lymphocyte surface protein, CD73, EC 3.1.3.5). In central and peripheral nervous system, ATP is hydrolyzed to adenosine by the conjugated action of an ATP diphosphohydrolase and a 5′-nucleotidase (Sarkis and Salto, 1991; Battastini et al., 1995). These ectonucleotidases, acting together, control the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors, and consequently, the duration and extent of receptor activation. Therefore, this cascade of ectonucleotidases is an enzymatic pathway with a double function of removing one signal (ATP) and generating a second one (adenosine). This cascade may play a role in the effective regulation of several processes, since they have considerable plasticity in different pathophysiological conditions (Agteresch et al., 1999; Cunha et al, 2001; Bonan et al., 2000).

It is also important to observe that, although evidence suggest gender differences in the components involved on regulation of analgesia, most papers concerning ATP or adenosine effects on nociception/antinociception were done using male stressed rats.

Studies comparing the effects of chronic stress on males and females have showed changes in nociception (Gamaro et al., 1998) and altered hydrolysis of adenine nucleotides in male spinal cord (Torres et al., 2002a), while no effect was observed in females, possibly because of variations in the estrous cycle phases. We investigate the hypothesis that repeated stressed females may present the same effect observed on nociception in males when the fluctuations of gonadal hormones are controlled; if that is so, it would be important to study if the mechanisms putatively to be involved in this effect of chronic stress in spinal cord in males would also be observed in females. On this basis, the present study aimed to investigate the effect of chronic administration of a gonadal esteroid (17-β-estradiol) on ATP, ADP and AMP hydrolysis on spinal cord synaptosomes and on pain threshold of adult ovariectomies female Wistar rats submitted to repeated restraint stress.

MATERIAL AND METHODS

Subjects. Adult female Wistar rats (60 days at the beginning of the treatment, weighing 150-165 g) were used. Experimental animals were housed in groups of 5 in home cages made of Plexiglas material (65 x 25 x 15 cm) with the floor covered with sawdust and maintained on a standard dark-light cycle (lights on between 7 a.m.-7 p.m.), at room temperature of 22 ± 1°C. The rats had free access to food (standard lab rat chow) and water, except during the period when restraint stress was applied. All animal treatments were in accordance with the institutional guidelines and according to the recommendations of the International Council for Laboratory Animal Science (ICLAS); all efforts were made to reduce the number of animals.

Surgery. Ovariectomy was performed as follows: rats were anesthetized with ketamine (75 mg/kg) and xilazine (10 mg/kg). Abdominal skin was cut, the peritoneum was opened, both

ovarian arteries were ligated, and both ovaries were removed. One week later, they were again submitted to anesthesia and received a subcutaneous implant of a sillastic pellet, containing 10 µl of 5% 17-β-estradiol or oil (vehicle). The animals were assigned to one of the following groups: group 1, OVX rats (oil pellet); group 2, OVX rats (oil pellet) submitted to repeated restraint stress; group 3, OVX rats receiving estrogen replacement therapy (17-β-estradiol pellet); group 4, OVX rats receiving estrogen replacement therapy (17-β-estradiol pellet) and submitted to repeated restraint stress. Stress procedures begun three days later and lasted for 40 days. The pellet was replaced by a new one on day 25th of stress treatment.

Repeated restraint stress procedure. The animals were divided into two groups: stressed and control. Restraint was applied by placing the animals in a 25 x 7 cm plastic bottle, and fixing it with plaster tape on the outside so that the animal was unable to move. There was a 1.5 cm hole at one far end for breathing. The restraint procedure was always performed between 09 a.m. and 11 a.m. The animals were stressed 1 h/day, 5 days a week for 40 days (Gamaro et al., 1998). Control animals were kept in their home cages.

Tail-flick measure. Nociception was assessed with the tail-flick apparatus (D'Amour and Smith, 1941). Rats were wrapped in a towel and placed on the apparatus. The light source positioned below the tail was focused on a point 2 - 3 cm rostral to the tip of the tail. Deflection of the tail activated a photocell and automatically terminated the trial. The tail-flick latency (TFL) represented the period of time (seconds) from the beginning of the trial to the tail deflection. A cut-off time of 10 s was used to prevent tissue damage. Shortly after the last session of the treatment (40 days) and twenty-four hours before the first measurement, the animals were exposed to the tail-flick apparatus to familiarize them with

the procedure, since the novelty of the apparatus can itself induce antinociception (Netto et al., 1987).

Sub cellular fractionation. Approximately 24 h after the last stress session, the animals were killed by decapitation and the spinal cord was rapidly removed and gently homogenized in 10 vol. of ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA, and 5 mM HEPES, pH 7.5, with a motor driven Teflon-glass homogenizer. The synaptosomes were isolated as described previously (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 ml of the crude mitochondrial fraction was mixed with 4 ml 8.5% Percoll solution and layered onto an isosmotic Percoll/sucrose discontinuous gradient (10/20%). After 20 minutes of centrifugaion at 12.000 X g at 4° C, the synaptosomes that banded at the 10/20% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0-4°C throughout preparation.

Enzyme assays. The reaction medium used to assay ATP and ADP hydrolysis in synaptosomal preparation was essentially as previously described (Battastini et al., 1995) and contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris-HCl buffer, pH 8.0, in a final volume of 200 µl. The reaction medium used to assay 5′-nucleotidase activity contained 10 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5 and 0.15 M sucrose in a final volume of 200µL (Heymann et al., 1984). The synaptosomal fraction (10-20 µg protein) was added to the reaction mixtures (ATP, ADP and AMP hydrolysis) and preincubated for 10 minutes and incubated for 20 minutes at 37°C. The reaction was initiated by the addition of ATP, ADP or AMP to a final

concentration of 1.0 mM and was stopped by the addition of 0.2 of mL 10% trichloroacetic acid (TCA). The samples were chilled on ice for 10 minutes and 100 µl samples were taken for the assay of released inorganic phosphate (Pi). Inorganic phosphate (Pi) released was determined as previously described by Chan and coworkers (1986).

The incubation times and protein concentration were chosen to ensure the linearity of the reaction (results not shown). Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct the nonenzymatic hydrolysis of the substrates. All samples were assayed in triplicate. Enzyme activities were generally expressed as nanomoles of Pi released per minute per milligram of protein and absorbance was measured at 630 nm.

Protein determination. Protein was determined by the Coomassie Blue method, according to Bradford (1976) using bovine serum albumin as standard.

Statistical analysis. Data are expressed by mean $\pm$ standard error of the mean and analyzed using a two-way ANOVA, followed by Duncan's test when indicated. A P-value ≤ 0.05 was considered statistically significant.

RESULTS

Effect of repeated restraint stress and 17-β-estradiol on tail-flick latency in female rats

Tail-flick latencies, measured 22 hours after the last exposure to restraint stress, were decreased in both stressed groups, OVX rats (oil pellet) and OVX rats receiving estrogen replacement therapy (17-β-estradiol pellet), (two-way ANOVA, $F(1,33)=4.932$, $P=0.033$ for stress treatment and $F(1,33)=0.007$, $P=0.935$ for estradiol treatment), compared to the respective controls (Figure 1). No interaction between treatments was observed ($F(1.33)=0.622$, $P=0.436$).

Effect of repeated restraint stress and 17-β-estradiol treatment on ATP, ADP and AMP hydrolysis in rat spinal cord synaptosomes

After repeated restraint stress during 40 days, no effect was observed on ATPase activity [two-way ANOVA, $F(1,15) = 0.083$, $P=0.777$ for repeated stress and $F(1,15)=0.455$, $P=0.510$ for estradiol treatment] nor on ADPase activity [two-way ANOVA, $F(1,15) = 0.002$, $P=0.968$ for repeated stress and $F(1,15)=1.094$, $P=0.312$ for estradiol treatment] in synaptosomes from spinal cord 22 hours after last exposure to stress, as shown in Figure 2. On the other hand, AMP hydrolysis in spinal cord synaptosomes from repeatedly stressed rats was decreased in group 2 (OVX rats submitted to repeated restraint stress) compared to group 1 (OVX rats), and this effect was reversed by hormonal replacement, since OVX rats that were submitted to repeated restraint stress and received estrogen replacement therapy presented levels of activity similar to control groups. This reversion is evidenced by a significant interaction between these treatments [two-way ANOVA, $F(1,15)= 4.179$, $P = 0.05$ for estradiol treatment; $F(1,15)= 5.394$, $P = 0.035$ for the interaction] (Figure 3).

DISCUSSION

In the present work, sex steroid replacement strategies were undertaken in ovariectomized rats to determine how a specific hormone (17-β-estradiol) affect the nociceptive response to repeated stress, as well as the effects of stress on adenine nucleotide hydrolysis. Exposure to repeated restraint stress caused a reduction in tail-flick latency, suggesting that ovariectomized female rats present hiperalgesia after chronic intermittent stress, similar to the effect observed in males (Gamaro et al., 1998). Therefore, the absence of effect of chronic stress on nociception in females, as previously reported,

was probably due to estrous cycle phase differences. Anyway, this absence of hyperalgesia in repeatedly stressed females when the estrous cycle was not controled (Gamaro et al., 1998) can not be caused by estradiol alone, since its replacement did not interfere with the effects of chronic stress on nociception.

Allthough several data support the hypothesis of a relationship between ovarian steroids and pain sensitivity, results from those studies are somewhat contradictory, maybe due to the influence of gonadal steroids on nociceptive pathways through different mechanisms, such as the regulation of neurotransmitter release (Loscher et al., 1992), and the possible alteration in cascades of neurotransmitters degradation/production. In the present study, no effect of estradiol was observed on nociception.

Since female rats presented hiperalgesia after repeated stress and there is evidence that adenosine and ATP may act as pain neuromodulators in the spinal cord (Post, 1984; Rhee et al., 2000; Tsuda et al., 1999a), the second experiment was done to evaluate the cascade of ATP hydrolysis to adenosine in synaptosomes from this structure, using female OVX rats. Although no significant difference was observed in ATP or ADP hydrolysis, repeated stress caused a decrease in AMP hydrolysis in OVX rats. On the other hand, 17β-estradiol-OVX rats, either submitted or not to stress exposure, exhibit similar 5'-nucleotidase activity as that seen in OVX controls, suggesting that the presence of estradiol prevents this effect of chronic stress exposure.

Previous studies have shown that estrogen is associated with changes in ATP-diphosphohydrolase activity and/or 5'-nucleotidase activities in some tissues. For example in rat uterus and mammary gland, ATP-diphosphohydrolase activity is slightly increased by estradiol administration (Valenzuela et al., 1992; Faas et al., 1997). High levels of estradiol

have also been reported to be associated with increased activity of 5'-nucleotidase in different tissues (Yoneyama et al., 2002; Garcia et al., 1985; Ramalingam et al., 1993). In the present experiment, estradiol replacement by itself did not induced changes in the activity of these enzymes in synaptosomes from spinal cord; however, this hormone did prevent the decrease in 5'-nucleotidase activity induced by exposure to repeated restraint stress.

These data suggest that 5'-nucleotidase activity may be modulated by stress in spinal cord synaptosomes. The reaction catalyzed by 5'-nucleotidase is the rate-limiting step in this extracellular pathway from ATP to adenosine (for a review, see Cunha and Ribeiro, 2000). It is important to observe that this enzyme is inhibited by ATP and/or ADP (Cunha and Sebastião, 1991), in such a way that only when ATP and ADP levels decrease below the threshold of inhibition of 5'-nucleotidase will adenosine be formed in an important amount. Since in the case of repeatedly stressed rats both ATPase and ADPase activities are similar to controls, and 5'-nucleotidase activity is reduced, it is possible that ADP and ATP acumulate, while reducing adenosine.

Animal studies have demonstrated adenosine- and adenosine analog-mediated inhibitory influences on presumed nociceptive reflex responses (Sawynok, 1998; Sollewi, 1997). The dorsal spinal cord contains both A1 and A2 adenosine receptors (Choca et al., 1987; Sawynok and Sweeny, 1989). Adenosine modulation by A1/A2 receptors occurs in different regions of the CNS and different effects have been observed when distinct brain regions are considered (Lamber and Tayler, 1991; Thompson et al.,1993). The adenosine receptors of the A_1-subtype are associated with a modulatory effect on pain transmission at the spinal cord level (Keil and DeLander, 1996) and A_1 agonists appear to act presynaptically inhibiting the release of neurotransmitters or postsynaptically reducing

neuronal excitability (Hass and Green, 1988; Lamber and Tayler, 1991). Therefore, since a decreased activity of 5'-nucleotidase will probably imply on reduced extracelular adenosine levels, altering the regulation of nociceptive thresholds at the spinal cord level (Jurna, 1984; Sawynok et al., 1986), this effect could be responsible, in part, for the hyperalgesia observed in repeatedly stressed animals.

This reduced extracellular adenosine production, however, is not the only possible explanation for the hyperalgesia observed herein, since estradiol replacement reverses the decrease observed in the activity of 5'nucleotidase in stressed rats, but failed to modify the hyperalgesic response to repeated stress.

In previous studies, a decrease in ADPase activity and increased 5'nucleotidase activity was observed in synaptosomes from spinal cord (Torres et al., 2002a). These effects were very distinct from the ones of the present study using females. Therefore, the altered activity of the enzymes involved in the cascade of degradation of adenine nucleotides after repeated stress in synaptosomes from spinal cord is gender-specific.

The mechanisms through which repeated stress can modulate 5'-nucleotidase activity still remain unclear. However, since this is a chronic situation, it is possible that this mechanism could be the result of an adaptation to repeated stress and may involve an decrease in mRNA expression of the enzyme.

In summary, present results show a reduction in pain threshold after exposure to repeated stress in OVX female rats, with or without estradiol replacement, although no interaction was observed between estradiol administration and chronic stress as regards to nociception. However, decreased 5'-nucleotidase activity was detected in response to chronic stress, suggesting reduced extracellular adenosine production in this condition; additionally, an interaction between estradiol and chronic stress was observed regarding this

enzyme activity: the stress effect was reversed by estradiol replacement. These observations are consistent with the concept that pain sensitivity depends on a complex regulatory system and that modulation of neurotransmission by adenine nucleotides in spinal cord may be altered by the interaction of sexual hormones and psychological factors, such as exposure to stress.

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Figures

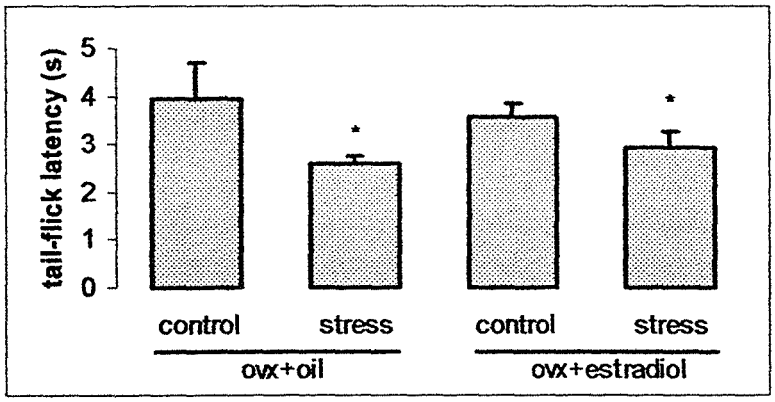

Figure 1: Effect of repeated restraint stress during 40 days on nociceptive response in female OVX rats, with or without estradiol replacement. The data are expressed as mean ± S.E.M., in seconds. N = 9-10 animals/group.

* There was a significant effect of stress treatment (two-way ANOVA, $P < 0.05$, followed by Student's t test).

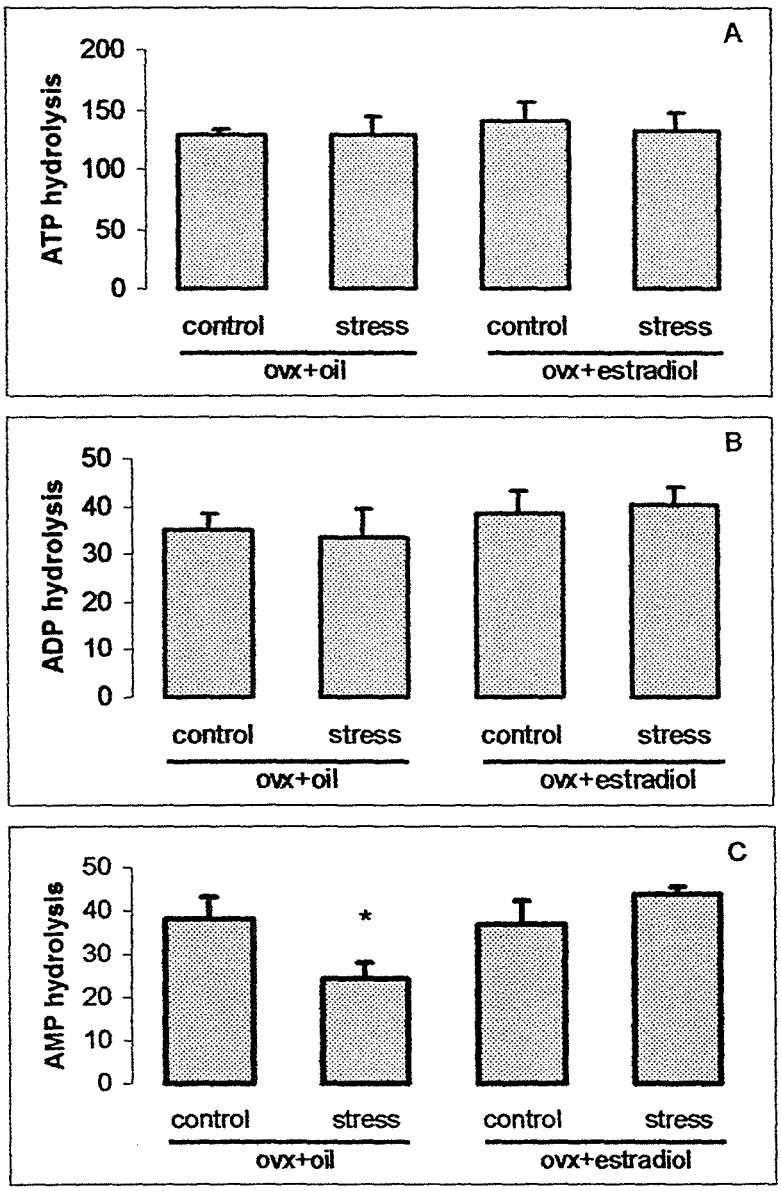

Figure 2. Effect of repeated restraint stress on (A) ATP, (B) ADP and (C) AMP hydrolysis in synaptosomes from spinal cord (n= 4-5 animals/group) of OVX female rats, receiving or not estradiol replacement. Values are mean ± SEM of specific activity (nmoles of phosphate production/min/mg protein). There was no difference between groups for ATP or ADP hydrolysis (two-way ANOVA, $P > 0.05$). For AMP hydrolysis, a two-way ANOVA showed an interaction between stress and estradiol replacement ($P < 0.05$).

* Significant difference from control group (OVX + oil) and from stressed group

receiving estradiol (two-way ANOVA, followed by Duncan multiple range test, $P < 0.05$).

Capítulo IV: Manuscrito 4

Effect of repeated restraint stress on different memory tasks

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Effect of repeated restraint stress on different memory tasks

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Running title: Repeated restraint stress, memory

Abstract

Exposure to stress may induce neural damage and, consequently, alteration on memory. However, chronically stressed animals may adapt to the stress situation, specially if the same stressor is repeated, and it can result modified behavior. The present study investigated the effect of repeated restraint stress in rats on memory evaluated by different behavioral tasks: water maze, inhibitory avoidance and object recognition. The findings suggest that repeated stress has different effects on rat behavior when different tasks are considered. In the water maze test, the stressed animals presented impairment of memory, indicated by reduced number of crossings on the platform location in the test session, but their memory scores were similar to the control group in the other tasks. The findings suggest that repeated exposure to stressors is capable of modifying memory, depending on the task. The spatial memory, which is dependent on hippocampal function, was impaired in this stress model; neverthless, more intensive stress exposure or different chronic stress models may lead to several degrees of memory impaiment.

Key words: Repeated stress, Memory, Inhibitory Avoidance, Morris Water Maze, Object Recognition

Introduction

Stress response is an important part of an adaptive biological system. In response to stressful stimuli many hormones and neurotransmitters are released in order to help the organism to function in a dynamic and challenging environment (Ursin and Olff, 1993): In this situation, adrenomedullary epinephrine is released and the hypothalamic-pituitary-adrenocortical (HPA) axis is activated, releasing hormones such as CRH, ACTH and glucocorticoids (Sapolsky, 1992). Several of these hormones can influence memory storage, either facilitanting or depressing it (Gold and Van Buskirk, 1978; Izquierdo and Dias, 1983; Bohus and Kloet, 1981; Roozendaal et al., 1997; Dachir et al., 1995). In addition, exposure of adult rats and primates to continuously-elevated concentrations of glucocorticoids, or to different stress situations, are known to result in cognitive impairments. Impaired performance has been observed in several tasks such as the Morris water maze [Bodnoff et al., 1995], radial maze [Nishimura et al., 1999], Y-maze [Conrad et al., 1996], and the Barnes maze [McLay et al., 1998]. Performance in these mazes is strongly linked to hippocampal function, and it has been suggested that chronic stress could lead to aging-like processes in the hippocampus [see Nichols et al., 2001, for a review]. Early studies concerning chronic stress effects on brain showed alterations in hippocampal morphology, with loss of pyramidal neurons following exposure to sustained elevations of corticosteroid levels [McEwen et al., 1999; Souza et al., 2000]. Later stereological studies failed to detect significant neuronal loss in the hippocampus after chronic exposure to glucocorticoids [Fuchs and Flügge, 1998]. A conclusion that has been drawn is that behavioral tests are more sensitive to hippocampal impairment than cell counts in the hippocampus.

Most of the studies cited above showing altered memory performance investigated chronic administration of corticosterone (Bodnoff et al., 1995; McLay et al., 1998) or stress models using variate stress (Vasconcellos et al., 2003) or exposure to models of high intensity stress, such as 6 daily hours of immobilization (Beck and Luini, 1999). However, chronically stressed animals may adapt to the stress situation, specially if the same stressor is repeated, and it was suggested that previous exposure to chronic stress modified behavior in a novel stress situation (Garcia-Marquez and Armario, 1987; Cancela et al., 1995). Therefore, it seems reasonable that a previous history of repeated stress would modify behavior in novel situations such as memory tasks.

Since repeated stress may lead to an adaptation, presenting different effects compared to models of corticosterone administration or models of chronic stress exposure with unpredictable stressors, the present study investigated the effect of repeated restraint stress on memory. Considering that different tasks evaluate different types of memory processing, we assessed behavior of animals using three different tasks: water maze (reference spatial memory), recognition of objects and inhibitory avoidance.

Material and Methods

Animals

Adult male Wistar rats (60 days at the beginning of the treatment, 200-230 g) from our breeding stock were used. Experimentally naive animals were housed in groups of 4-5 in home cages made of Plexiglas material (65 x 25 x 15 cm) with the floor covered with sawdust. They were maintained on a standard 12-h dark/light cycle (lights on between 7.00h – 19.00h) at room

temperature (22 ± 1°C). The rats had free access to food (standard lab rat chow) and water, except during the period of exposure to the stressor. All animal procedures were approved by the institutional Ethical Research Committee.

Chronic restraint stress procedure

Animals were divided into two groups: control and stressed, which were housed in different cages. Stressed animals were submitted to restraint 1h daily, 5 days per week for 40 days (total time) (Ely et al., 1997). Restraint was carried out by placing the animal in 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 in the far end for breathing. The control group was not submitted to stress, being kept in their home cages. The immobilization procedure was performed between 12:00h and 14:00h.

Behavioral procedures

The animals were submitted to the behavioral tasks at the end of the 40th day of repeated stress. Different animals were used for inhibitory avoidance, object recognition, and water maze tasks.

Water maze apparatus and procedures

Spatial discrimination task: 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 on 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 inter-trial interval was 10 min. After each trial the rats were dried, and returned to their cages at the end of the session. Animals were trained for 5-6 days, until the control group presented a criterion of mean latency of 10 seconds to find the platform. Twenty-four h 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.

Working memory task: Eight days after the reference memory testing phase, the animals were submitted to a working memory task. The tank was the same as described above. Animals were submitted to this task for four days, four trials per day. The escape platform was located in the same position during the four trials of a session, but it was placed in a different position the next day. In the first trial (acquisition), the animals had to find the platform in a new position. The animal was allowed to stay on the platform 10 s before it was returned to the home cage. In the subsequent trials (retrieval), which were administrated at intervals of 40 min, the platform was in its previous position but the animals started from a different place from the preceding trial.

Step-down inhibitory avoidance

The animals were trained and tested for the one-way step-down inhibitory task using a 50 x 25 x 25-cm plywood box with a glass-wall front and a floor consisting of l-mm bronze bars

spaced 10 mm apart (Gamaro et al., 1999). The left end of the grid was covered with a 5-cm high, 25-cm wide, and 7.5-cm long wood platform. During the training trial, the animals were gently held by the body and lowered onto the platform with their noses pointing to the left corner, at which point a timer was activated. The timer measured the latency to step down (i.e., placing all four paws on the grid) at which point an intermittent footshock (0.4 mA, 60 Hz) was delivered until the animal climbed back onto the platform. In the test session, the animals were again placed on the platform. No footshock was given in the testing session. A 180-s ceiling was imposed on testing session latency measurements; latencies $\geq$ 300-s were counted as 300-s. Differences in testing-training session latencies were used as retention scores.

Spontaneous object recognition

This task was similar to the task described by Ennaceur and Aggleton (1997). The apparatus consisted of a 50-cm high, 40 x 60-cm box made of wood with a frontal glass wall, the inside of which was painted beige. The objects to be discriminated were made of plastic, so that they could be cleaned between sessions. Behavioural testing: all rats were given two habituation sessions in which they were allowed 5 min to explore the apparatus (with no objects present). Each test consisted of two phases, a sample phase and a choice phase. At the start of each sample phase two identical objects (A1 and A2) were placed in the back corner of the box, 10 cm from the side wall. A rat was then placed in the middle of the box and the total time spent exploring the two objects was recorded by the experimenter with two stop watches. Exploration of an object was defined as directing the nose to the object at a distance of less than 2 cm. Turning around or sitting on the object was not considered as exploratory behaviour. The rat was removed from the open field either after 3 min had elapsed. The rat was then placed in its own cage. After a 15 min retention delay the rat was reintroduced to the open field for 3 min. In this choice phase the test

box now contained an identical copy of the familiar object (A) and a new object (B). These were placed in the same locations as the sample stimuli. As far as could be ascertained the objects had no natural significance for the rats and they had never been associated with a reinforcer.

Performance in this test was evaluated by comparing the total time spent exploring objects during the sample and choice phases. The following measures were analyzed (Ennaceur and Aggleton, 1997): total time spent in exploring the two identical objects during the sample phase and total time spent exploring the two objects in the choice phase; a discrimination index, which is the difference in time spent exploring the two objects in the choice phase (i.e. time with B minus time with A); and the discrimination ratio, which is the difference in exploration time, expressed as a ratio of the total time spent exploring the two objects in the choice phase. This ratio makes it possible to adjust for individual or group differences in the total amount of exploration time.

Biochemical measurements

Statistical analysis

Non-parametric data (i.e., latency to step-down from the platform in the inhibitory avoidance task, latency to find the platform in the water maze task) are reported as median (interquartile range) and were analyzed by the Friedman test (for related samples) or the Mann-Whitney U test (for comparisons between 2 independent samples). Parametric data are reported as mean $\pm$ standard error of the mean, and were analyzed by Student's t test. Comparisons between time in target and opposite quadrant were made by the Student paired t-test. A difference was considered significant when $P < 0.05$.

Results

Effect of repeated stress on Morris water maze

Results from the spatial learning task are shown in Figure 1 and 2. No significant difference was observed in the performance during the training sessions between the different groups (Figure 1). A Mann-Whitney U test showed no difference between the groups in every day of training ($P > 0.05$ in all cases), and a Friedman test showed that both groups presented learning during the sessions ($P < 0.001$ in both cases). In the retention test, an effect of repeated stress on memory was verified, as shown in Figure 2. A Student's t test showed that repeatedly-stressed rats presented a decreased number of crossings on the platform location [Figure 2A; $t(38) = 2.34$; $P < 0.05$]; No difference was observed on the latency in reaching the original position of the platform [Figure 2B; $t(38) = 0.68$; $P > 0.05$] or on the time spent in the target and in the opposite quadrants [Figure 2C; $t(38) = 0.66$ for the target quadrant and $t(38) = 1.52$ for the opposite quadrant; $P > 0.05$ in both cases], and both groups presented a preference for the target quadrant in this session, as shown in Figure 2C [Student's t test for dependent samples, $t(19) = 13.2$ for the control group and $t(19) = 7.36$ for the stressed group; $P < 0.001$ in both cases].

Results from the working memory task were evaluated by calculating the median latency of trials 1 to 4, during the four days of training, and analyzing the differences between the trials. A Friedman test showed a significant effect between trials, meaning that the animals learned the task [$\chi^2(3)=13.37$; $P < 0.005$ for the control group; $\chi^2(3)=26.44$; $P < 0.001$ for the stressed group] (Figure 3). No effect of repeated stress treatment was observed in this task, since there was no difference between the latency to find the platform in any trial (Mann-Whitney U test, $P > 0.05$ in all trials).

Effect of repeated restraint stress on the inhibitory avoidance task

In the step-down inhibitory avoidance task, both groups presented memory, since latency in the test session was significantly different from training session (Wilcoxon Signed Ranks test, $Z = 3.18$ for the control group, $P < 0.005$; $Z = 2.08$ for the stressed group, $P < 0.05$). There was no significant difference between control and repeatedly stressed groups in the training or in the test session (Mann-Whitney U test, $Z = 1.61$ for the training session and $Z = 1.49$ for the test session; $P > 0.05$ in both cases), and although the control group presented an more significant difference between training and test session, there was no significant difference in performance between groups (Figure 4).

Effects of repeated restraint stress on object recognition test

There was no difference between the performance of repeatedly stressed and control rats in the object recognition test, as displayed in Table 1. A Student's t test compared the total time required for exploration of the two objects in the sample phase and the total time of exploration of the two objects in the choice phase. There was no difference between the groups in the sample phase [$t(22) = 0.179$; $P > 0.05$], and there was a marginal significantly difference in the choice phase [$t(2) = 2.02$, $P = 0.055$], since the stressed groups spent more time exploring objects in the choice phase. Nevertheless, no difference was observed in the discrimination index and in the discrimination ratio [Student's t test, $t(22) = 0.322$ for the index and $t(22) = 0.093$ for the ratio; $P > 0.05$ in both cases]. Both groups spent more time in exploring the novel object than the familiar one [Student's t test for dependent samples, $t(11) = 2.574$ for the control group and $t(11) = 2.673$ for the stressed group; $P > 0.05$ in both cases].

Discussion

It has been reported that chronic stress may induce spatial memory deficits (Bodnoff et al., 1995; Nishimura et al., 1999; Conrad et al., 1996; McLay et al., 1998). Consistent with these reports, we also observed decreased spatial memory in the water maze task after repeated restraint stress, as evaluated by decreased number of crossings. This memory impairment, however, was small, and no effect was observed on other parameters of memory in the water maze task, such as the latency in finding the platform, and the ratio between time spent in the target quadrant and the opposite quadrant. Additionally, no effects were seen on other memory tasks (inhibitory avoidance and object recognition tasks).

Several studies have suggested that exposure to glucocorticoids, hormones released by the adrenal cortex during stress, may increase the vulnerability of hippocampal cells to certain insults that can damage hippocampal neurons (Sapolsky, 1999; 2000). The hippocampus plays important roles in cognition and neuroendocrine function (Jarrard, 1993), and is known to be important for the consolidation of memories in different tasks, including the water maze task (White et al., 2003; Ferbinteanu et al., 2003), studied in this report. In contrast to the water maze task, the object recognition task, a non-spatial, non-aversive memory task, is not believed to be hippocampal dependent, because fornix and hippocampal lesions do not significantly affect performance in this task (Ennaceur and Aggleton, 1994; Rothblat and Kromer, 1991). The data reported here suggest that repeated stress has different effects on rat behavior when different tasks are considered.

It should be noted that while deleterious effects of excess glucocorticoid on the brain have been shown, the extent to which brain function itself is compromised in a situation of repeated

stress depends on some variables, such as the intensity and predictability of the stressor used. That is possible the reason why the present repeated stress model seems do not compromise cognitive function as much as other models studied, such as the variate stress model (Vasconcellos et al., 2003), whose characteristics is the unpredictability of the stress situation. The animals repeatedly exposed to the same aversive agent present a physiological adaptation to that stressor, and are known to release less corticosterone after the stress session compared to acute stress (Torres et al., 2001), at least in this model of 1 hour restraint. On the other hand, studies using repeated restraint during 6 hours/day have reported to find impairment in memory tasks, including object recognition (Beck and Luini, 1999). It is possible that this more intensive stress schedule induces greatest decrements in memory, compared to the present model.

The possibility of an adaptation of these animals to chronic stress should be considered; in such a way that the different tasks would cause a different degree of stress in chronically stressed and control animals. Studies have demonstrated that prolonged exposure to different kinds of stress causes hyperresponsiveness of the hypothalamus-pituitary-adrenal axis to a new stimulus, although there is a desensitization of the relative responses of the pituitary ACTH or serum corticosterone to the primary stressor (Tizabi and Aguilera, 1992; Bhatnagar and Dallman, 1998; Torres et al., 2001).

These results led us to conclude that repeated exposure to stressors is capable of modifying memory, depending on the task; spatial memory, which is dependent on hippocampal function, was impaired in this model; more intensive stress exposure, and different chronic stress models may, therefore, lead to several degrees of memory impaiment.

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Table 1: Measures of exploration and discrimination in the object recognition task. Data expressed as mean ± standard error of the mean of total time exploring objects in session 1 (t1) and in session 2 (t2), discrimination index and discrimination ratio. N = 12 animals per group.

	t1(s)	t2(s)	discrimination index (s)	discrimination ratio
control	26.2 ± 3.4	25.1 ± 4.0	9.8 ± 3.8	0.30 ± 0.16
stressed	27.1 ± 3.4	37.8 ± 4.8	11.6 ± 4.4	0.29 ± 0.09

There was no difference between groups (Student's t test, $P > 0.05$).

Legends to figures:

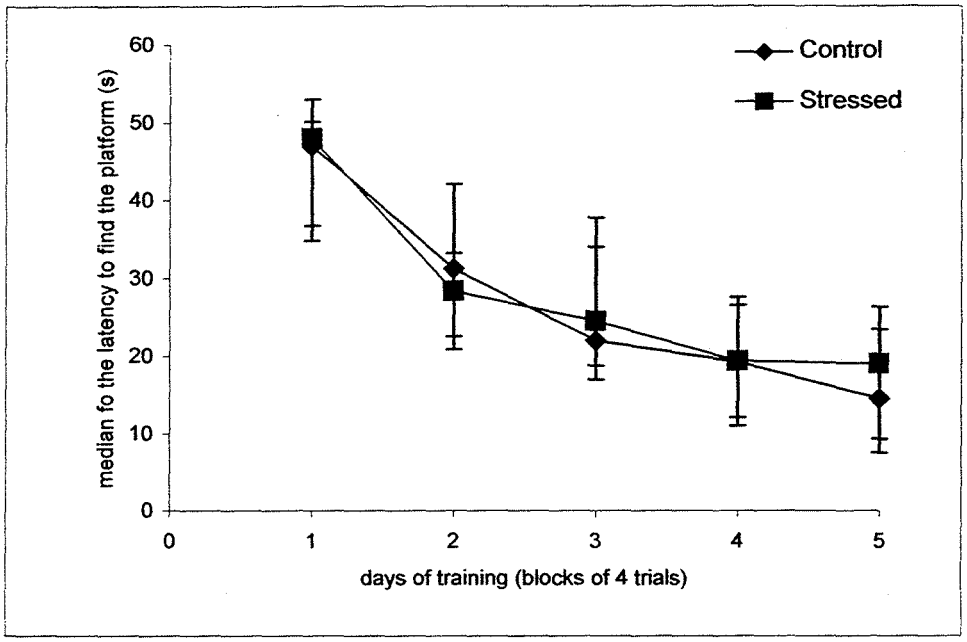

Figure 1. Effect of repeated restraint stress on performance during five days of training in a water maze task. Each session consisted of four trials; in a session, the inter-trial interval was 10 min. A ceiling of 60 seconds was used. Data are expressed as median latency (in seconds) in reaching the platform in each trial. N = 21 animals in the control group and 22 animals in the stressed group. There are no significant differences between groups (Mann-Whitney U test, $P > 0.05$), and both groups learned the task (Friedman test, $P < 0.001$).

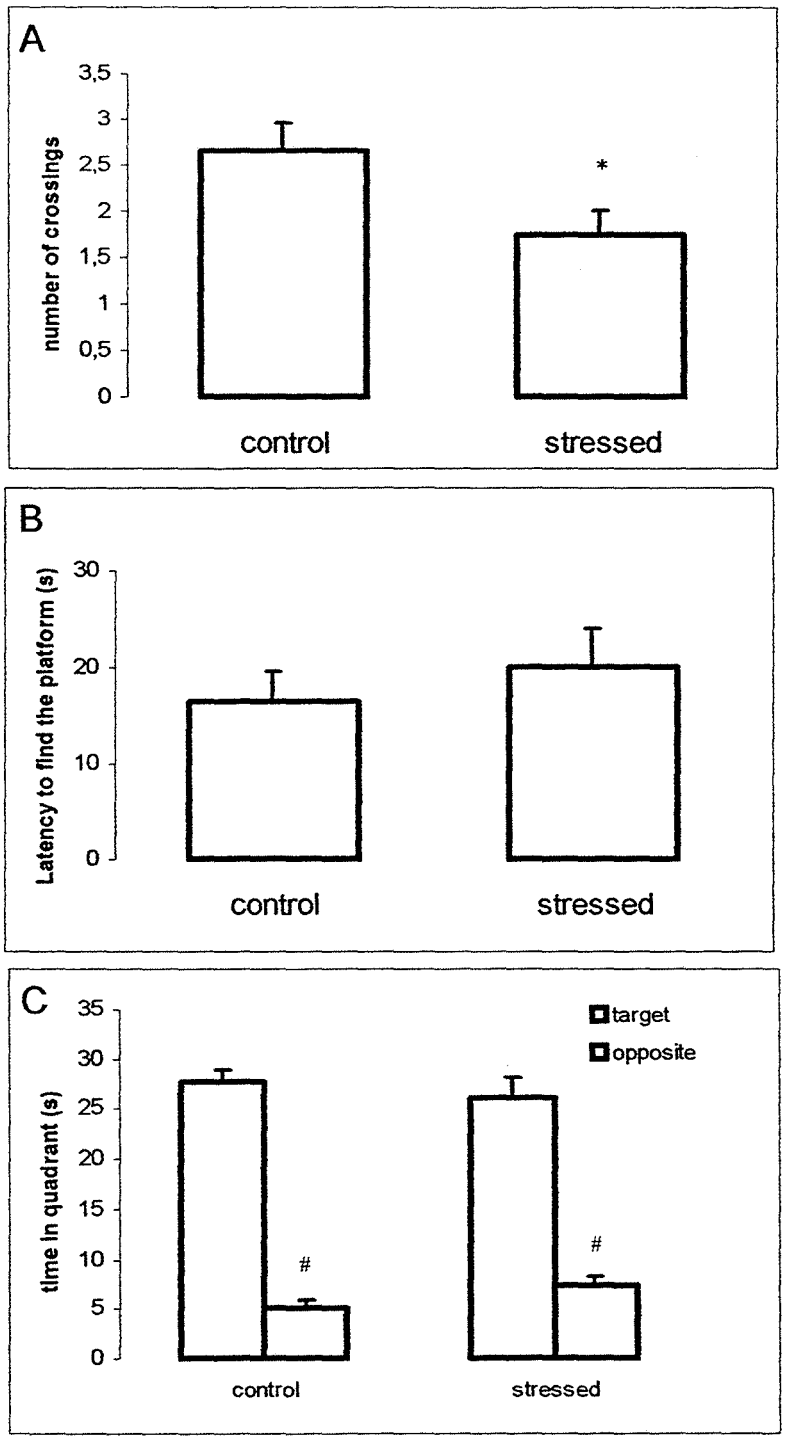

Figure 2. Effect of repeated restraint stress on performance of spatial reference memory in a water maze task during the retention test (test session). Data are expressed as mean ± S.E.M. (A) Number of crossings in the area of the original location of the platform; (B) Latency in finding the original platform location, in seconds; (C) Time spent in the target quadrant vs. time spent in the opposite quadrant. N = 21 animals in the control group and 22 animals in the stressed group.

* Significantly different from the number of crossings in the control group (Student's t test; $P < 0.05$).

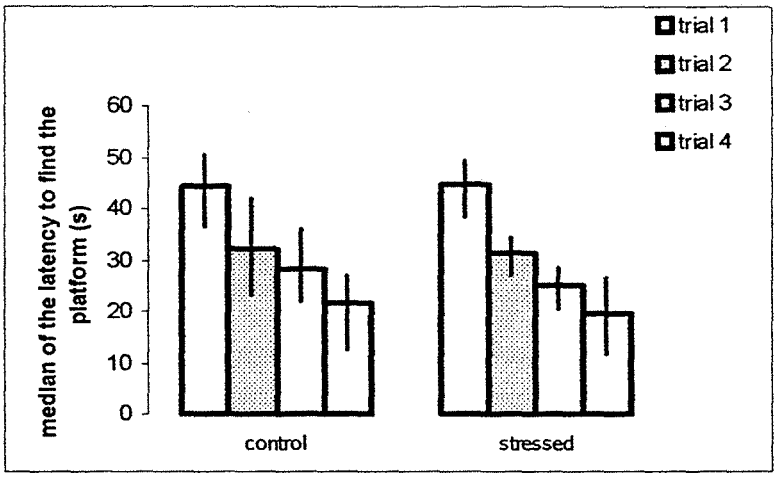

Figure 3. Effect of repeated restraint stress on working memory evaluated in a water maze task. Data are expressed as mean ± S.E.M. of the mean latency in seconds in finding the platform in each trial during the four days of training. N = 21 animals in the control group and 22 animals in the stressed group. There was a significant difference between trials (Friedman test, $P < 0.005$). There was no difference between groups (Mann-Whitney U test, $P > 0.05$ in all trials).

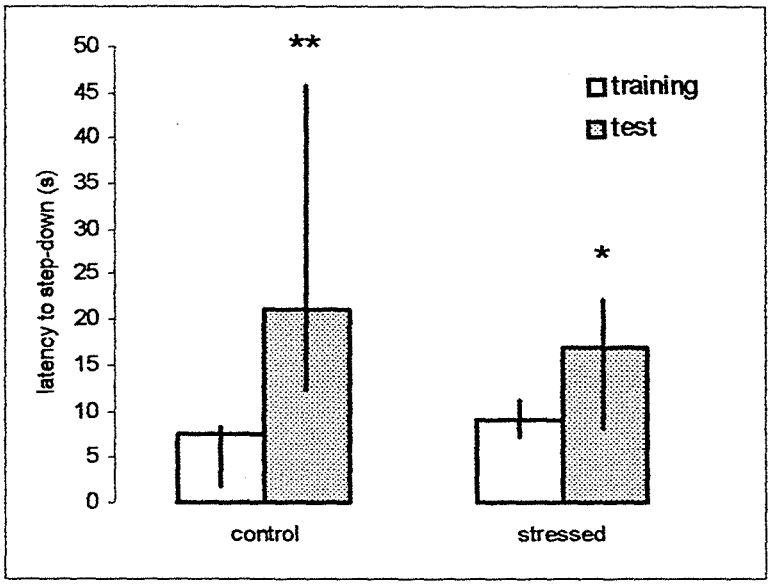

Figure 4. Effect of repeated restraint stress on performance in a step-down inhibitory avoidance task. Data are reported as median (interquartile range) of the latency to step-down (N = 10-13 animals/group). There was no difference between groups (Mann-Whitney U test; $P > 0.05$).

Capítulo V: Artigo 5

Repeated restraint stress alters hippocampal glutamate uptake and release in the rat

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REPEATED RESTRAINT STRESS ALTERS HIPPOCAMPAL GLUTAMATE UPTAKE AND RELEASE IN THE RAT

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Running title: Chronic stress, glutamate uptake and release

ABSTRACT

Glutamatergic mechanisms are thought to be involved in stress-induced changes of brain function, especially in the hippocampus. We hypothesized that alterations caused by the hormonal changes associated with chronic and acute stress may affect glutamate uptake and release from hippocampal synaptosomes in Wistar rats. It was found that [^{3}H]glutamate uptake and release by hippocampal nerve endings, when measured 24 h after 1 hour of acute restraint, presented no significant difference. The exposure to repeated restraint stress for 40 days increased neuronal presynaptic [^{3}H]glutamate uptake as well as basal, and K^+-stimulated glutamate release, when measured 24 h after the last stress session. Chronic treatment also caused a significant decrease in [^{3}H]glutamate binding to hippocampal membranes. We suggest that changes in the glutamatergic system are likely to take part in the mechanisms involved in nervous system plasticity following repeated stress exposure.

Key words: restraint stress, chronic stress, glutamate uptake, glutamate release, glutamate binding, hippocampus.

INTRODUCTION

Stress responses are experienced by all animals and comprise physiologic changes considered to be adaptative to the organism (1). Prominent among these responses is the release of glucocorticoids by the adrenal glands, which serves both to alert the organism to environmental or physiologic changes and to maintain homeostasis (2). However, both inadequate control of the stress response or repeated exposure to stress situations represent a severe threat to the health and well being of the organism. Exposure to chronic stress can promote the development of physiological and psychological dysfunctions (systemic diseases, affective disorders and neurodegenerative disease) and also appears to be a major factor influencing the course and outcome of diseases (2 - 6).

Repeated stress may lead to deleterious effects in the brain (7 - 9). Exposure to high levels of glucocorticoids or to repeated stress decreases the number of apical dendrite branch points in the CA3 pyramidal layer of the hippocampus (7, 8, 10, 11) and this atrophy may be reversed by N-methyl-D-aspartate receptor blockers, suggesting an involvement of EAA in this effect (9, 12). In addition, the hippocampus plays important roles in cognition and neuroendocrine function (13) and contains a high concentration of receptors to corticosteroids (14).

Glutamate (Glu) is considered the major excitatory neurotransmitter in the mammalian nervous system and its action is mediated via ionotropic and metabotropic receptors. These receptors mediate most of the excitatory neurotransmission in the mammalian central nervous system. Their appropriate activation is therefore essential for normal brain function, whereas their overstimulation potentially leads to excitotoxicity (15). Accumulation of Glu in the synaptic cleft may lead to excitotoxic neuronal damage

due to over stimulation of glutamate receptors. The Na^+-dependent, high affinity uptake of glutamate is mediated by transporter proteins located in astrocytes and also in nerve terminals and this mechanism has been proved to play an important role in the termination of glutamatergic neurotransmission and prevention of excitotoxicity (15).

The changes in hippocampal function caused by stress have been attributed to an increased glutamatergic tonus. Exposure to acute stress increases extracellular levels of glutamate in the hippocampus, the prefrontal cortex and the nucleus accumbens (16, 17), while adrenalectomy attenuates stress-induced outflow of glutamate in hippocampus and prefrontal cortex and glucocorticoid replacement abolishes this effect (18). Additionally, acute restraint stress exposure has been shown to lead to increased glutamate uptake and release in synaptosomes from limbic forebrain regions (19).

In contrast, the effect of a chronic stress regimen on glutamatergic neurotransmission is not well determined. Repeated exposure to an aversive stressful situation can lead to a process of adaptation to that stimulus, hence chronically stressed animals do not show the same behavior, and do not experience the same physiological consequences, that animals exposed to acute stress do (20 - 26). Therefore, considering the evidence indicating that both acute stress and chronic corticosterone treatment interferes with glutamate metabolism (16, 17, 27), we herein investigated the effects of acute or repeated exposure of rats to restraint stress on glutamate uptake and release by hippocampal synaptosomes and on [^{3}H]glutamate binding on hippocampal synaptic membranes.

2. Experimental Procedure

2.1. Subjects:

Sixty adult male Wistar rats (60 days at the beginning of the treatment) weighing 180-230 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 (standard lab rat chow) and water, except during the period when restraint stress was applied. The stress procedure was performed between 1:00 and 3:00 p.m. All animal treatments were in accordance with the institutional guidelines and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS).

2.2. Stress model:

2.2.1. Chronic treatment - Rats were divided in two groups: stressed and controls. Restraint stress was carried out by placing the animal in a 25 x 7 cm plastic bottle, adjusted with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole in one far end for breathing. The animals were stressed 1h/day, 5 days a week for 40 days. Control animals were kept in their home cages.

2.2.2. Acute treatment - Animals were submitted to a single same exposure, whilst the control group was not touched.

2.3. Synaptosomal preparation

Approximately 24 h after the last stress session (chronic) or 24 h after the single session (acute), the animals were sacrificed by decapitation and the brain was rapidly removed. This time point was chosen in order to evaluate chronic stress effects on the parameters studied, so the same time point was observed after acute stress. 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 three 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. (28). 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 (final concentrations) 320 mM sucrose, 1 mM EDTA and 0.25 mM dithiothreitol, 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 (28). Protein concentration was measured according to the method of Lowry et al. (29). The material was prepared fresh daily and maintained at 0 - 4 °C throughout the experiment.

2.4. Na^+-dependent high affinity glutamate uptake:

Determination of Na^+-dependent high affinity glutamate uptake was performed as described by Leal et al. (30). 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 °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.

2.5. Glutamate release:

Glutamate release from synaptosomes was measured according to Migues et al. (31), 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 °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.

2.6. Brain membrane preparation:

All binding assays were performed using brain synaptic membranes prepared as described (32) and stored at -70 °C until the day of the binding assay. The membranes were rapidly thawed in a water bath (37 °C), homogenized with three volumes of assay buffer, pH 7.4, and centrifuged three times at 27,000 g for 15 min. The final pellet was resuspended in the same buffer in order to yield a protein concentration of 1-2 mg/ml and was used for the binding assay.

2.7. [^{3}H]Glutamate binding assay:

Glutamate binding was determined as described in Nogueira et al. (33). Membranes were incubated in 0.5 mL reaction mixture containing 50 mM Tris/HCl, pH 7.4, and 40 nM [^{3}H]glutamate. Incubation was carried out at 30 °C for 30 minutes and the reaction was stopped by centrifugation at 27,000 g for 15 minutes. The pellet and the wall of the tube were quickly and carefully washed with ice-cold Milli-Q water. SDS/NaOH (0.1%) was added to the dry pellet and incorporated radioactivity was determined in a Wallac scintillation counter.

2.8. Statistical analysis:

Data are expressed as mean $\pm$ standard error (SEM) and were analysed using Student t tests for dependent or independent samples, as indicated. Four or five synaptosomal fractions per group (hippocampi of three rats were pooled for each synaptosomal fraction) were prepared for experiments involving glutamate uptake and release. For the glutamate binding assay experiments we used 11 membrane preparations

per group.

RESULTS

Experiment 1 - Effect of acute restraint stress on hippocampal synaptosomal preparations: in vitro glutamate uptake and release.

Exposure to acute stress or to increased corticosterone levels increases extracellular glutamate levels in brain (17, 16). In these conditions, the Na^+-dependent, high affinity uptake of glutamate, mediated by transporter proteins located in astrocytes and also in nerve terminals, plays an important role in the termination of glutamatergic neurotransmission and prevention of excitotoxicity (15). In order to evaluate whether exposure to acute stress interferes with the neuronal presynaptic glutamate uptake, we assayed [^{3}H]glutamate uptake into hippocampal synaptosomal preparations. The effect of acute stress on [^{3}H]glutamate release in basal (physiological extracellular K^+ concentration) or depolarized (40 mM KCl) conditions was evaluated. Additionally, we evaluated Na^+-dependent high affinity glutamate uptake by hippocampal synaptosomes from rats subjected to acute exposure to restraint stress, and sacrificed 24 hours afterwards. There was no effect of acute stress on [^{3}H]glutamate uptake [$t(5.9) = 0.42$; $P > 0.05$] (Figure 1A) nor on [^{3}H]glutamate release under basal [$t(15) = 0.33$; $P > 0.05$] or depolarized [$t(15) = 1.52$; $P > 0.05$] conditions (Figure 1B).

Experiment 2 - Effect of chronic restraint stress on hippocampal synaptosomes: in vitro glutamate uptake and release.

Chronically stressed animals do not experience all the hormonal consequences that

animals exposed to one single stress episode do (34, 35), and this phenomenon of adaptation to chronic stress may be reflected in several biochemical and physiological processes. Considering the evidence indicating that both acute stress and chronic corticosterone treatment interferes with glutamate metabolism (16, 17, 27), we next investigated the effects of repeated exposure of rats to restraint stress on glutamate uptake and release by hippocampal synaptosomes. Exposure to chronic stress increased neuronal presynaptic Na^+-dependent high affinity glutamate uptake, when measured 24 h after the last restraint session [$t(10) = 2.59$; $P < 0.05$] (Figure 2A). The effect of chronic stress on [^{3}H]glutamate release in basal or depolarized (40 mM KCl) conditions was also evaluated (Figure 2B). Chronic stress caused an increase in basal glutamate release [$t(4.91) = 6.27$; $P < 0.01$], as well as in K^+-stimulated release ($t(8) = 3.22$; $P < 0.02$). However, no significant difference between control and stressed animals was observed in the increase in [^{3}H]glutamate released induced by the high K^+ medium, when glutamate basal levels were subtracted (the increase was 17.5 + 2.4 % for controls and 12.6 + 2.1 % for stressed animals; $t(8) = 1.55$, $P > 0.05$).

Experiment 3 - Effect of chronic restraint stress on [^{3}H]glutamate binding to hippocampal synaptic membranes

Glutamatergic neurotransmission depends on the amount of glutamate present in the synaptic cleft (which depends on glutamate uptake and release), and also on the glutamate binding to its receptors, which mediate most of the excitatory neurotransmission (15). Therefore, we measured [^{3}H]glutamate binding to hippocampal synaptic membranes. Figure 3 shows in vitro [^{3}H]glutamate binding on hippocampal membranes. Exposure to chronic stress caused a 36% inhibition of [^{3}H]glutamate binding, when measured 24 h after

the last restraint session [t(10) = 3.47; P = 0.006].

DISCUSSION

The results reported here demonstrate the different effects of acute or chronic stress in animals upon glutamate neurotransmission. No effect was observed after acute restraint on the glutamatergic parameters evaluated, either on glutamate uptake or on its release in hippocampal synaptosomes. In contrast, after repeated-restraint stress, the Na^+-dependent high affinity glutamate uptake was enhanced in nerve endings, as well as basal glutamate release, while [^{3}H]glutamate binding was significantly decreased in rat hippocampal synaptic membrane preparations, all these measurements were performed 24 h after the last restraint session.

It should be noted that all measurements were made 24 h after exposure to stress. This time was chosen in order to compare the effects of acute stress with those of chronic stress. Thus, if alterations occurred in glutamate release or uptake by hippocampal nerve endings after acute restraint stress, as is suggested by some data from the literature, they were no longer present 24 h later. Measurements of extracellular levels of glutamate in the hippocampus, taken after 1-hour restraint stress, demonstrate that glutamate is released during stress, however, extracellular glutamate returns to basal levels within 2 hours (36). Evidence from the literature suggests that different types of stress, including restraint and ether exposure, or increased glucocorticoid concentrations, may increase extracellular glutamate levels in the hippocampus, and this effect occurs in parallel with a rise in corticosterone in this brain structure (16, 27, 37, 38). Other studies using glucocorticoids have indicated that these hormones rapidly enhance the Na^+-dependent high affinity glutamate uptake in nerve endings and in neuroblastoma clone SK-N-SH cells, even at

physiological concentrations (39). Since these are immediate effects, they are thought to be exerted through a non-genomic mechanism of action, probably via G-proteins (27).

Although several Na(+)-dependent glutamate transporters have been identified, the glutamate transporter in glutamatergic nerve terminals has not been molecularly identified and the exact stoichiometry of the transport cycle is not known (15). With respect to repeated-restraint stress, our results indicate that the Na^+-dependent high affinity Glu uptake is enhanced in hippocampal synaptosomes, as well as basal glutamate release 24 h after the last restraint session. It is important to note that, 24 h after restraint exposure, glucocorticoid levels had returned to normal and the increase in their levels after the 40^{th} day of exposure to stress is much lower than that after the first exposure (38), suggesting a physiological adaptation of the organism to the stressor. The long-lasting effects observed herein may involve genomic effects of glucocorticoids (12, 39), although we can not exclude that other factors related to repeated-stress exposure may be inducing these alterations.

The increase in glutamate uptake in synaptosomes from repeatedly-stressed rats may be a protective mechanism, since an increased uptake of glutamate, as observed here, may have an important role in the modulation of glutamatergic neurotransmission and prevention of excitotoxicity in cases of repeated stress (15). It is possible that this increase in glutamate uptake into synaptosomes is associated with a greater number of glutamate transporter sites in the presynaptic membranes, or with functional changes of these transporters. In this respect, increasing evidence indicates that glutamate transporters may also undergo functional changes, depending on increased oxidation or reduction of reactive sulphydryls present in their structure (41,42), in such a way that they are vulnerable to the action of biological oxidants, resulting in reduced uptake function. Nevertheless, exposure

to chronic stress has been reported to increase (43, 44), or have no effect (45), upon oxidative stress in hippocampus. Therefore, an altered redox state of the transporters seems not to be the best explanation for the increased uptake observed in the present experiment. Thus, analysis of oxidative stress in this particular cellular fraction may be of interest.

Although we have measured only the glutamate uptake by synaptosomes, which is part of total glutamate uptake, it is important to consider that glial glutamate transporters play an important role in maintaining extracellular glutamate concentrations below neurotoxic levels in the brain (15, 46, 47). Contamination of the fraction studied by glial particles is possible, but not probable, since glutamate uptake in hippocampal slices, which has been measured in our laboratory, is not altered after chronic stress, in contrast to the effects observed in synaptic endings. In addition, glucocorticoids are known to impair the uptake of glutamate by hippocampal astrocytes (48), further suggesting that the stress effect observed herein probably is not due glial transporters, which could be contaminating synaptosomal fractions.

A recent study concerning the effects of 21 days of repeated stress on glutamate uptake in synaptosomes from cerebral cortex found a lower uptake (49) immediately after the stress section, in contrast with the results of the present study. It is also important to consider that different stress intensities and periods may alter glutamate dynamics differently in the nervous system. This provides evidence that chronic stress modifies the synaptic uptake of glutamate in a time-dependent and regionally selective manner.

An increased basal glutamate release was also observed. This 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 (50). In this way, reversed transport may explain the results found in this study concerning basal glutamate release. Conversely, to measure glutamate release, the synaptosomal fraction is first incubated in the presence of [^{3}H]glutamate to produce labelled synaptosomes. Therefore, the increased basal release may be the result of increased uptake, since stressed animals present higher glutamate uptake.

Glutamate release evoked by depolarizing K^+ concentrations on the control and stressed animals was not significantly different when glutamate basal levels were discounted. This suggests that, although basal glutamate release is higher in stressed animals, vesicular glutamate release is not different.

Additionally, the present results also indicate that exposure to repeat stress decreased the [^{3}H]glutamate binding to rat hippocampal synaptic membrane preparations. Results from other studies have also shown that repeated administration of corticosterone can selectively decrease binding sites for excitatory amino acids in hippocampal tissue (51). Since activation of glutamate receptors can trigger the death of neural cells, particularly when they are coincidentally subjected to adverse conditions, the reduction in [^{3}H]glutamate binding may function as a protective mechanism, considering the increased basal release of glutamate observed herein. On the other hand, since the glutamatergic system is involved in several physiological processes such as learning and plasticity [51], this inhibitory effect of repeated stress on [^{3}H]glutamate binding and the higher [^{3}H]glutamate uptake may disturb proper brain functioning and may be related to behavioural effects observed after chronic stress, especially in memory tasks (12, 52 - 55).

In conclusion, this study demonstrates that glutamate uptake or release by hippocampal nerve endings, when measured 24 h after 1-hour acute restraint, presented no

significant difference. In contrast, the exposure to repeated restraint stress increased neuronal presynaptic glutamate uptake, as well as basal glutamate release, and decreased [^{3}H]glutamate binding by synaptic membranes, indicating that these changes are part of the brain's response to chronic stress. It is possible that increased basal glutamate release by nerve endings induces a higher uptake and decreased glutamate receptor density as neuroprotective mechanisms. 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. 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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Legends and Figures:

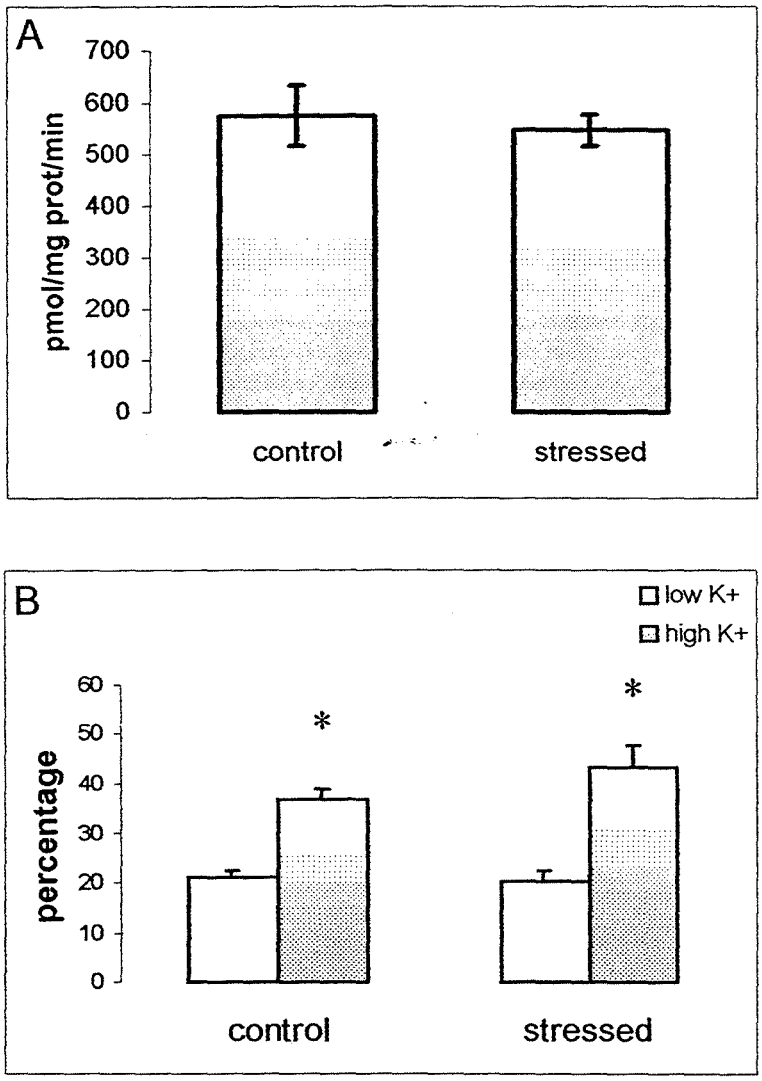

Figure 1. Effect of acute restraint stress on glutamate uptake by (A) and release from (B) synaptosomes from hippocampus, 24 hours after stress. Values are means of 4-5 separate experiments each run in triplicate and error bars indicate S.E.M.

* Significantly different from glutamate release in low K^+ medium (Student t test for dependent samples; $P < 0.02$).

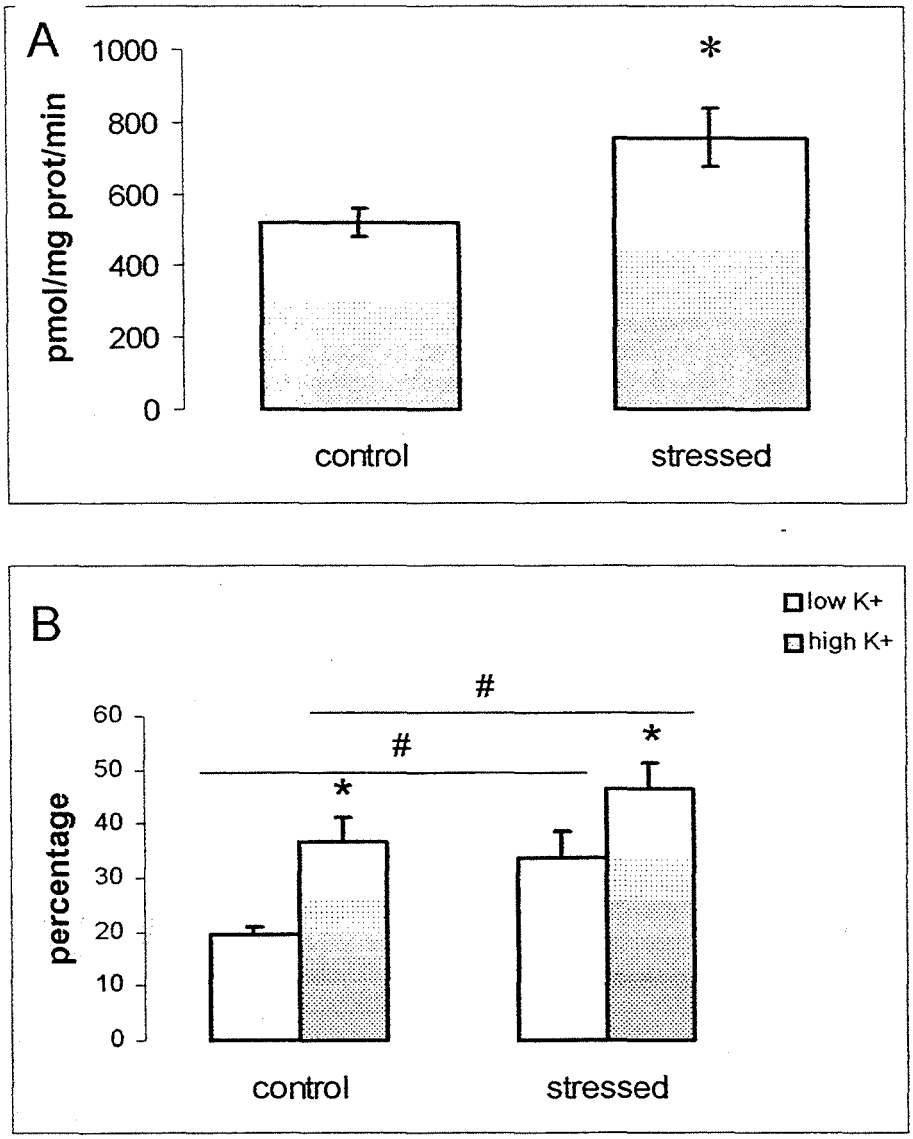

Figure 2. Effect of chronic restraint stress on glutamate uptake by (A) and release from (B) synaptosomes from hippocampus, 24 hours after the last stress session. Values are means of 4-5 separate experiments each run in triplicate and error bars indicate S.E.M.

Significantly different from control group (Student's t test, $P < 0.02$).

* Significantly different from glutamate release in low K^+ medium (Student's t test for dependent samples, $P < 0.02$).

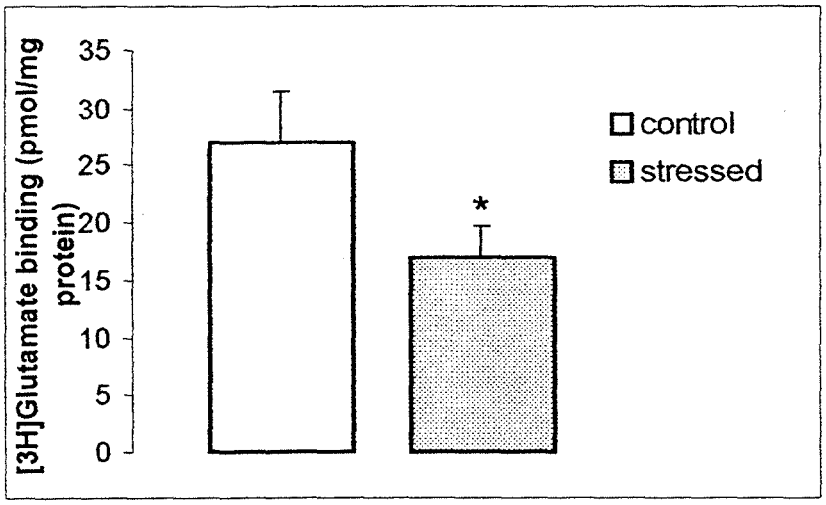

Figure 3. Effect of chronic restraint stress on [^{3}H]glutamate binding. An aliquot of synaptic membrane preparation from control and repeatedly-stressed animals was incubated with 40 nM [^{3}H]glutamate at 30 °C for 30 min. [^{3}H]Glutamate binding was 27.0 ± 4.5 and 16.8 ± 2.9 pmol/mg/protein for control and restraint stress groups, respectively. Data are mean ± S.E.M. for 11 separate determinations performed in triplicate.

* Significantly different from control group (Student's t test, $P < 0.005$).

Capítulo VI: Artigo 6

Acute and Chronic Stress Alter Ecto-Nucleotidase Activities in Synaptosomes from the Rat Hippocampus

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Acute and Chronic Stress Alter Ecto-Nucleotidase Activities in Synaptosomes from the Rat Hippocampus

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Running head: stress and nucleotidase activities

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Abstract

A constant hyperactivity of the stress response has long been recognized as maladaptive and pathological. The hippocampus, a brain structure important in mediating this response, is known to be affected by chronic stress, a situation which has been reported to induce changes in adenine nucleotide hydrolysis in the rat. The enzymes catalyzing the hydrolysis of ATP to adenosine in the synaptic cleft are thought to have a role in modulating and controlling excitatory synaptic transmission. The present study aimed to investigate the effect of acute and repeated restraint stress on the ATP, ADP and AMP hydrolysis in rat hippocampal synaptosomes. Adult male Wistar rats were submitted to acute or chronic stress and ATPase-ADPase, and 5'nucleotidase activities were assayed in the hippocampal synaptosomal fraction. Acute stress induced increased hydrolysis of ATP (21%), ADP (21%) and AMP (40%). In contrast, ATP hydrolysis was increased by 20% in repeatedly-stressed rats, without changes in the ADP or AMP hydrolysis. Therefore, acute stress increases ATP diphosphohydrolase activity which, in association with 5'-nucleotidase, contributes to the elimination of ATP and provides extracellular adenosine. Interestingly, increased ecto-ATPase activity in response to chronic stress reveals an adaptation to this treatment.

Keywords:

Stress, Chronic stress, ATPase-ADPase activities, 5'- nucleotidase activity, Hippocampus

Introduction

Activation of the stress response is essential for vital functions (McEwen, 1998; Pacák and Palkovits, 2001), however the deleterious effects of a constant hyperactivity of this response have long been recognized as maladaptive and pathological. Chronic stress may lead to impaired feedback mechanisms (Mizoguchi et al., 2001), cognitive impairments and disturbances of the activity of the limbic-hypothalamus-pituitary-adrenal (LHPA) axis (Sapolsky et al., 1986). Several sudies have shown that chronic corticosterone administration may lead to a significant atrophy of CA3 neurons in the hippocampus, structural alterations and volume reduction of the hippocampal formation (Sapolsky et al., 1985; Uno et al., 1989; Woolley et al., 1990; Watanabe et al., 1992; Magarinos et al, 1996, 1997; McEween, 1997) and an increased vulnerability to metabolic insults (Uno et al., 1989; Watanabe et al., 1992; Magarinos and McEwen, 1995). The hippocampus has been extensively studied following exposure to chronic stress, since it is one of the most important brain structures mediating the stress response. The high concentration of receptors for glucocorticoids in the hippocampus (McEwen et al., 1968, 1986) modulates glucocorticoid release through a negative feedback loop, exerting inhibitory effects on the LHPA axis (Sapolsky et al., 1986).

Some studies have reported that after the exposure to stressors, such as shear and electrical stimulation, changes occur in the concentrations of extracellular ATP (Kennedy et al., 1997) and adenosine (Latini and Pedata, 2001). Alterations in activities of enzymes involved in nucleotide hydrolysis have also been reported in spinal cord and serum after repeated-restraint stress (Torres et al., 2002a; 2002b).

ATP is recognized as a neurotransmitter in the peripheral, as well as in the central nervous system (CNS) (Edwards et al., 1992). In physiological situations, extracellular

ATP exists at low concentrations, however, in pathological conditions large quantities of extracellular ATP may cause cell death (Inoue, 2002).

The ATP released in the synaptic cleft can be hydrolyzed to adenosine, a neuroprotective and neuromodulatory agent. The enzymes that promote this conversion are proposed to have a role in modulating and controlling excitatory synaptic transmission (Cunha, 2001a; Cunha and Ribeiro, 2000). Adenosine has several functions within the CNS, including the inhibitory tone of neurotransmission and neuroprotective actions in pathological conditions (Latini and Pedata, 2001), and is particularly well suited to function as a transcellular messenger to signal metabolic imbalance. The neuroprotective actions of adenosine are attributed to the activation of presynaptic A1 receptors, which reduce neurotransmitter release and depress the neuronal activity in the central nervous system (Phillips and Wu, 1981).

ATP is hydrolyzed to adenosine by an extracellular chain of ectonucleotidases (Cunha et al., 1992; Sarkis and Salto, 1991). This chain of enzymes includes ecto-ATPases (NTPDase2, EC 3.6.1.3), ATP diphosphohydrolase (ecto-apyrase, NTPDase1, CD39, EC 3.6.1.5) and ecto-5′-nucleotidase (CD73, EC 3.1.3.5). While NTPDase1 hydrolyzes equally well ATP and ADP, NTPDase2 prefers ATP as a substrate (the ratio ATPase/ADPase for this enzyme is approximately 10). The enzyme 5′- nucleotidase is not able to promote the hydrolysis of ATP or ADP. This enzyme promotes the hydrolysis of nucleosides monophosphated, like AMP.

It has been recently demonstrated that ATP is hydrolyzed to adenosine by the conjugated action of an ATP diphosphohydrolase and a 5′-nucleotidase (Sarkis and Salto, 1991; Battastini et al., 1995), both in the central and peripheral nervous systems. The concerted action of ectonucleotidases controls the availability of ligands (ATP, ADP, AMP

and adenosine) for both nucleotide and nucleoside receptors and, consequently, the duration and extent of receptor activation (Chen and Guidotti, 2001). Therefore, this ectonucleotidase pathway has a double function of removing one signal (ATP) and of generating a second one (adenosine). This cascade may play a role in the effective regulation of several processes, since it shows considerable plasticity in different pathophysiological situations (Agteresch et al., 1999; Cunha et al., 2001; Bonan et al., 2000). These enzymes may also have a protective function by keeping extracellular ATP/ADP and adenosine at physiological levels (Agteresch et al., 1999).

Since adenine nucleotides, as well as adenosine, are involved in the modulation of several physiological and pathological processes and their levels may be altered by stress in some situations, we hypothesized that the enzymes involved in the degradation of ATP to adenosine may have their activities altered after chronic stress as an adaptation to protect the tissue against an excessive excitatory transmission induced by this treatment. Therefore, we investigated the effect of acute and repeated restraint stress on the ATP diphosphohydrolase and 5'-nucleotidases activities via ATP, ADP and AMP hydrolysis in hippocampal synaptosomes of adult male Wistar rats.

MATERIAL AND METHODS

Subjects

Adult male Wistar rats (60 days at the beginning of the treatment, weighing 200-230 g) were used. Experimentally naive animals were housed in groups of 5 in home cages made of Plexiglas material (65 x 25 x 15 cm) with the floor covered with sawdust and maintained on a standard dark-light cycle (lights on between 7 a.m. to 7 p.m.), at a room temperature of

22 ± 2°C. The rats had free access to food (standard lab rat chow) and water, except during the period when restraint stress was applied. The restraint procedure was always performed between 10 a.m. and 12 a.m. 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 used in the experiment.

Acute stress procedure

The animals were divided into two groups: stressed and control. Restraint stress was applied by placing the animals in a 25 x 7 cm plastic bottle, and fixing it with plaster tape on the outside so that the animal was unable to move. There was a 1-cm hole at one far end for breathing. The animals were stressed for 1 h, 22 h before the assay. Control animals were kept in their home cage.

Chronic restraint stress procedure

The animals were divided into two groups: stressed and control. Restraint was applied using the same procedure as described above. The animals were stressed 1 h/day, 5 days a week for 15 or 40 days (Ely et al., 1997). Control animals were kept in their home cages.

Subcellular fractionation

Approximately 22 h after the last stress session (chronic stress) or the single session (acute stress), the animals were killed by decapitation and the hippocampi were rapidly removed and gently homogenized in 5 vol. of ice-cold medium, consisting of 320 mM sucrose, 0.1 mM EDTA (ethylenediaminetetraacetic acid), and 5 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid], pH 7.5, with a motor driven Teflon-glass homogenizer. Synaptosomes from hippocampus were prepared as described previously (Nagy and

Delgado-Escueta, 1984). Briefly, 0.5 ml of the crude mitochondrial fraction were mixed with 4.0 ml of an 8.5% Percoll solution and layered onto a Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. Synaptosomal fractions were then washed twice at 12 000 x *g* for 20 min with the same ice-cold medium to remove the contaminating Percoll. The synaptosome pellet was then ressuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0-4° C throughout preparation.

Enzyme assays

The reaction medium used to assay ATP and ADP hydrolysis in synaptosomal preparation was as previously described (Battastini et al., 1991) and contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris-HCl buffer, pH 8.0, in a final volume of 200 µl. The reaction medium used to assay 5′-nucleotidase activity contained 10 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5 and 0.15 M sucrose in a final volume of 200µL (Heymann et al., 1984).

The synaptosomal fraction (10-20 µg protein) was added to the reaction mixture, preincubated for 10 minutes and then incubated for 20 minutes at 37°C. The reaction was initiated by the addition of ATP, ADP or AMP to a final concentration of 1.0 mM, and was stopped by the addition of 0.2 mL 10% trichloroacetic acid (TCA). The samples were chilled on ice for 10 minutes and 100 µl samples were taken for the assay of released inorganic phosphate (Pi). Inorganic phosphate released was determined as previously described by Chan et al. (1986).

The incubation times and protein concentration were chosen to ensure the linearity of the reactions (results not shown). The Chan method is largely used to evaluate the ATP diphosphohydrolase and 5'-nucleotidase activities and presents an adequate sensitivity to the purpose of our work (Bonan et al., 2000; Sarkis and Salto, 1991; Torres et al., 2001, 2002b); these enzyme activities are well established and characterized in synaptosomal preparations (Battastini et al., 1991; 1995). Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct for non-enzymatic hydrolysis of substrates. All samples were assayed in triplicate. Enzyme activities are expressed as nanomoles of Pi released per minute per milligram of protein.

Protein determination:

Protein was determined by the Coomassie Blue method, according to Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

Data are expressed as mean $\pm$ standard error mean and analyzed by Student's t test.

RESULTS

Effect of acute restraint stress on ATPase-ADPase and 5'-nucleotidase activities in rat hippocampal synaptosomes

ATPase and ADPase activities, as well as 5'-nucleotidase activity, in synaptosomes from hippocampus were increased 22 hours after exposure to restraint stress (Student's t test, ATP ($t(8) = 3.23$); ADP ($t(8) = 3.39$); AMP ($t(8) = 4.87$); $P < 0.05$ in all cases; test for equality of variances, $P > 0.05$ in all cases), as compared to controls (Figure 1). The

ATPase/ADPase ratio was the same in both groups (mean ± S.D.: control, 2.64 ± 0.31; after acute stress, 2.64 ± 0.39).

Effect of chronic restraint stress on ATPase-ADPase and 5'-nucleotidase activities in rat hippocampal synaptosomes

Repeated restraint stress caused an increase in ATPase activity in synaptosomes from hippocampus of around 20% (in relation to controls), 22 hours after the last exposure to stress (Student's t test, $t(4.07) = 4.82$, $P < 0.01$, for 15 days of restraint and $t(11) = 2.45$; $P < 0.05$, for 40 days of restraint) (test for equality of variances, $P > 0.05$ for 40 days of treatment; $P < 0.05$ for 15 days of treatment; therefore, in this case, t test was performed for equal variances not assumed). The same effect was observed after both 15 and 40 days of stress. On the other hand, no effect was observed on ADPase activity (Student's t test, $t(7) = 0.29$ for 15 days of stress and $t(10) = 0.73$ for 40 days; $P > 0.05$ in both cases). In addition, 5'-nucleotidase activity was also not altered in synaptosomes from repeatedly-stressed rats (Student's t test, $t(7) = 1.25$ for 15 days of stress and $t(11) = 0.30$ for 40 days; $P > 0.05$ in both cases) (test for equality of variances, $P > 0.05$ in all these cases) (Figure 2). The ATPase/ADPase ratio was significantly different in the repeated stressed animals compared to controls: 2.50 ± 0.13 (mean ± S.D.) for the control group, and 3.01 ± 0.17 after 15 days of repeated stress (Student's t test, $t(7) = 5.04$; $P < 0.005$). After 40 days of treatment, although the stressed group presented a higher ratio, there was no significant difference compared to controls ($P > 0.05$).

Discussion

In the present study, we observed changes in adenine nucleotide hydrolysis after the induction of acute and repeated stress. Additionally, the modifications observed in enzyme activities were distinct in relation to acute and chronic stress, suggesting the involvement of different enzymes in both situations studied. Animals submitted to acute stress showed a significant increase in ATP, ADP and AMP hydrolysis when compared to their respective controls. Since ADP is considered to be a substrate marker for ATP diphosphohydrolase activity (Battastini et al., 1991), this result suggests increased activity of this enzyme after exposure to acute stress. Moreover, the activation observed in ATP (21%) and ADP hydrolysis (21%), was very similar, and the parallelism in kinetic behavior (similar profile for both substrates) is also characteristic of ATP diphosphohydrolases described in the literature (Sarkis and Salto, 1991; Frassetto et al., 1993).

AMP hydrolysis was also significantly increased after acute stress, indicating an increase in the 5'-nucleotidase activity and probably in adenosine levels resulting from the extracellular ATP hydrolysis cascade. It has been proposed that the reaction catalyzed by 5'-nucleotidase is the rate limiting step in this extracellular pathway from ATP to adenosine (for a review, see Cunha and Ribeiro, 2000). It should be noted that this enzyme is inhibited by ATP and/or ADP (Cunha and Sebastião, 1991). Thus, only when ATP and ADP levels decrease below the threshold of inhibition of 5'-nucleotidase will adenosine be formed in a significant amount. Since, in the case of acutely stressed rats, both ATPase and ADPase activities are increased, these nucleotides will be rapidly converted to AMP. Therefore, ATP and ADP levels will decrease and AMP, the substrate to 5'-nucleotidase, will be increased, as well as this enzyme activity. These results could suggest that, after a single exposure to restraint stress, a long-term (at least 22 hours) effect on enzymes involved in

extracellular ATP breakdown will result in increased extracellular adenosine in the hippocampus.

Several reports have documented an increase in the extracellular concentration of adenosine upon stressful metabolic challenges, including hypoxia and ischemia (Latini and Pedata, 2001; Pearson et al., 2001), as well as glucoprivation by 2-deoxy-D-glucose administration (Minor et al., 2001). The term "retaliatory metabolite" has been used for this homeostatic role of adenosine, which occurs in virtually all cell types (for a review, see Cunha 2001b). Although adenosine may act on A_2 receptors causing increased neurotransmitter release in hippocampus (Cunha et al., 1992), most reports of adenosine action in this structure have shown inhibition of hippocampal neurons. A_1 agonists appear to act presynaptically, inhibiting the release of neurotransmitters or postsynaptically reducing neuronal excitability (Hass and Greene, 1988; Lamber and Teyler, 1991). Since the hippocampus is well documented as being particularly sensitive to cellular injury (Inoue, 1998), this result suggests the presence of a physiological response in order to protect this structure from damage caused by exposure to stressors.

In contrast to the effects observed after acute stress, repeatedly stressed rats presented a significant difference only in ATP hydrolysis, with no significant changes in ADPase or 5'-nucleotidase activities in synaptosomes from hippocampus. This pattern of activity for these enzymes was already observed after 15 days of repeated restraint, and was still present after 40 days of stress.

The apparent dissociation observed between the two substrates (ATP and ADP), in this case, may be due to the simultaneous presence of at least two different ectoenzymes involved in ATP hydrolysis, an ecto-ATP diphosphohydrolase (NTPDase3) and an ecto-ATPase (NTPDase2) (Kegel et al., 1997; Zimmermaen, 1996). Conversely, only one

enzyme, an ecto-ATP diphosphohydrolase (NTPDase3), is involved in ADP hydrolysis. When we have similar changes in ATP and ADP hydrolysis, it is tentative to think in the enzyme ATP diphosphohydrolase, but when we have changes only in ATP hydrolysis, the more probable enzyme involved is an ecto-ATPase. Therefore, the increase observed in ATP hydrolysis, and not in ADP hydrolysis, suggests the participation of an ecto-ATPase in modulation of the response to chronic stress. The presence of different populations of nucleotidases is also supported by the increased ATPase/ADPase ratio observed in synaptosomes from chronically stressed rats. The presence of an ecto-ATPase was demonstrated in the central nervous system, where it is coexpressed with the ATP diphosphohydrolase (Kegel et al., 1997). These enzymes have been identified in molecular terms and differ in their preference for their substrates (Zimmermann, 1996).

The increase of ATP hydrolysis in repeated stress may constitute a protective mechanism, due to the potential neurotoxic effect of this nucleotide (Schulze-Lohoff et al., 1998). The activation of ATP hydrolysis may be a protective mechanism, since ATP acts as an excitatory neurotransmitter, and a number of neurodegenerative events are associated with increased excitatory neurotransmission (Danbolt, 2001). Hence, the activation of the ecto-ATPase could be an adaptive response of the chronically stressed rats. The mechanisms through which repeated stress may modulate ecto-ATPase activity still remain unclear. However, since this is a chronic situation, it is possible that this mechanism involves an increase in expression of mRNA for this enzyme.

The differences here observed in enzymes activities after acute and chronic stress suggest an adaptation. Chronically stressed animals do not experience all the hormonal consequences that animals exposed to one single stress episode do (Hashiguchi, et al., 1997; Torres et al., 2001), and this phenomenon of adaptation to chronic stress may be

reflected in several biochemical and physiological processes. Glucocorticoids levels are also different after acute and chronic stress: the increase in corticosterone is smaller when the animals are submitted to repeated stress, as compared to the increase observed after acute stress (Torres et al., 2001).

Glucocorticoids might be involved in the changes of activities of adenine nucleotides hydrolysis observed in the present study. These hormones have already been reported to alter the activity of other enzymes involved in the hydrolysis of ATP. High levels of glucocorticoids or exposure to acute stress have been reported to increase Na^+, K^+-ATPase activity in brain and other tissues (Shaheen et al., 1996; Rodrigo et al., 2002), while decreasing Ca^{2+}-ATPase activity (Bhargava et al, 2000, 2002), although other non-glucocorticoid-dependent factors appear also to be involved in the response of these enzymes to stress (Bhargava et al., 2000). The enzymes cited above are involved in the intracelular degradation of ATP and, therefore, in the energetic demands of the cell. The enzymes studied herein, on the other hand, are ectoenzymes and are involved in the modulation of neurotransmission by controlling adenine nucleotides/nucleoside.

Other mediators of the stress response could possibly be involved in the effects observed herein. Several data provide evidence that the transcription factor, cAMP response element-binding protein (CREB), and the neurotrophin, brain derived-neurotrophic factor (BDNF) levels, are changed in response to stress (D'Sa and Duman, 2002; Rossant et al., 1999; Hatalski and Baram, 1997; Duman et al., 2000), and suggest that chronic stress may be associated with a disruption of mechanisms that govern cell survival and neural plasticity in the brain (Duman et al., 2000). For example, CREB binding is critical for basal expression of 5'-nucleotidase; however, it is not presently known how tightly this enzyme expression is regulated (Synnestvedt et al., 2002). Conversely,

adenosine protective effects are suggested to be mediated by protein kinase C, and protein kinase A activation by means of cAMP and activation of CREB (Lee and Emala, 2002). In addition, extracellular ATP co-operates with growth factors in different tissues (Wagstaff et al., 2000; Huwiler et al., 2000). Therefore, altered extracellular levels of ATP and adenosine may produce changes in multiple signalling pathways.

In previous studies, using synaptosomes from other central nervous system structures, no effect was observed after repeated stress, either upon ATP or ADP hydrolysis in hypothalamus and cerebral cortex (Torres et al., 2002b), however, a decrease in ADPase activity was observed in spinal cord (Torres et al., 2002a). Therefore, the increased ATPase activity after repeated stress, observed in this study in synaptosomes from hippocampus, is structure-specific. Such alterations that can be observed in particular tissues or structures may reflect the different functions of the nucleotides/nucleoside and enzymes in different regions of the central nervous system.

In conclusion, the results reported herein demonstrate a significant increase in ATP, ADP and AMP hydrolysis in response to acute stress, suggesting the participation of an ATP diphosphohydrolase which, in association with 5'-nucleotidase, contributes to the elimination of ATP and also provides a source of extracellular adenosine as a neuroprotective agent. On the other hand, repeated stress elicits an increase in ecto-ATPase activity alone, possibly representing a protective adaptation of this treatment. Furthermore, the differences observed in altered ecto-nucleotidase activities in response to both kinds of stresses studied may be used as biochemical tools to characterize chronic and acute stress in hippocampal synaptosomes.

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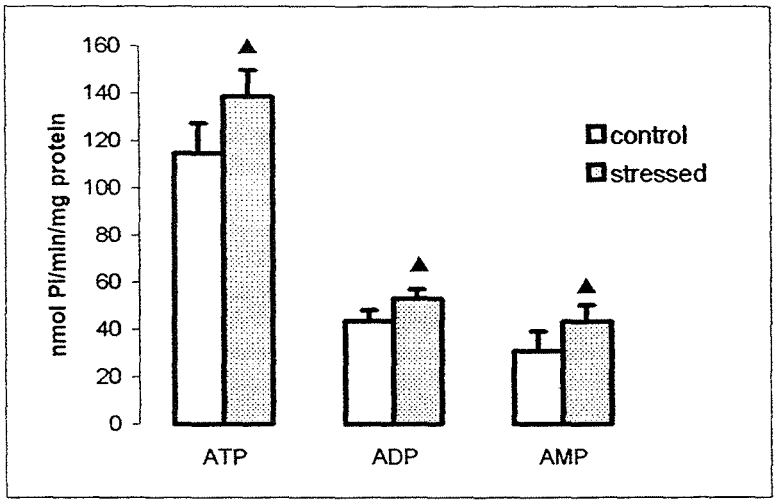

Figure 1. Effect of acute stress on ATP and ADP hydrolysis, and on 5'-nucleotidase activity in synaptosomes from rat hippocampus measured 22 hours after stress exposure (n= 4-5 animals/group). Values are mean ± standard deviation of specific activity (nmoles of phosphate production/min/mg protein).

▲ Different from control group (Student's t test, $P < 0.02$).

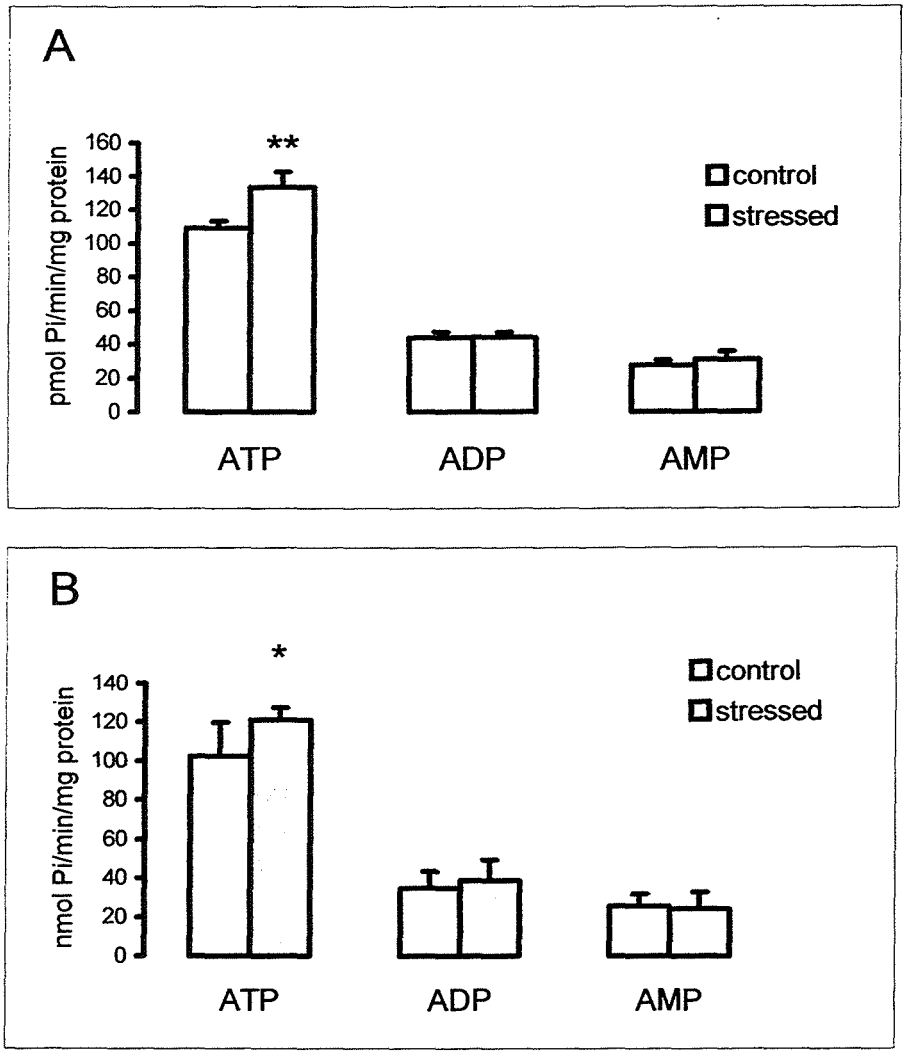

Figure 2. Effect of 15 days (A) or 40 days (B) of repeated stress on ATP and ADP hydrolysis, and on 5'-nucleotidase activity in synaptosomes from rat hippocampus (n= 4-6 animals/group). Values are mean ± standard deviation of specific activity (nmoles of phosphate production/min/mg protein).

* Different from control group (Student's t test, $P < 0.05$).

** Different from control group (Student's t test, $P < 0.01$).

Capítulo VII: Artigo 7

Repeated restraint stress induces oxidative damage in hippocampus of rat

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REPEATED RESTRAINT STRESS INDUCES OXIDATIVE DAMAGE IN HIPPOCAMPUS OF RAT

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Running read: Repeated Restraint Stress and Oxidative Stress

ABSTRACT

It has been shown that emotional stress may induce the oxidative damage, and considerably change the balance between pro-oxidant and antioxidant factors in the brain. The aim of this study was to verify the effect of repeated restraint stress (RRS; 1 h/day during 40 days) on several parameters of oxidative stress in hippocampus of adult Wistar rats. We evaluated the lipid peroxide levels (assessed by TBARS levels), the production of free radicals (evaluated by the DCF test), the total radical-trapping potential (TRAP) and the total antioxidant reactivity (TAR) levels, and the antioxidant enzyme activities (SOD, GPx and CAT) in hippocampus of rats. The results showed that RRS induced a increase in TBARS levels and in GPx activity, while TAR was found reduced. We concluded that RRS induces oxidative stress in the rat hippocampus, and that these alterations may contribute to the deleterious effects observed by several authors after prolonged stress.

Key words: chronic stress, lipoperoxidation, free radicals, total antioxidant reactivity, ROS, antioxidants, GPx.

INTRODUCTION

Physical and psychological stressors cause release of glucocorticoids (GCs), steroid hormones released by the adrenals; these substances play an adaptative role, mobilizing energy to critical tissues during an emergency and suppressing unessential anabolism. Nevertheless, there is strong evidence suggesting that, in the brain, high levels of GCs may produce deleterious effects, including damage to neurons (1). These events have been linked to an increased generation of reactive oxygen species (ROS) (1). 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 (2).

In addition to GCs administration, exposure to emotional stress has been shown to be accompanied by activation of free radical-mediated processes, particularly lipid peroxidation (3, 4). Restraint stress, often used as a model of emotional stress in rats, is followed by an increase in lipid peroxidation (3, 5).

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 (6, 7). Membrane lipids are highly suceptible to this kind of injury, and this event can damage the 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 (8). 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 (9).

Damage induced by ROS in cells is normally held in check by natural

enzymatic and nonenzymatic antioxidant systems (10). 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, catalase (CAT), which has been shown to be responsible for detoxification of H_2O_2, and glutathione peroxidase (GPx), that breaks down peroxides, notably those derived from the oxidation of membrane phospholipids. The removal of superoxide and H_2O_2 blocks the generation of hydroxyl radicals, which are formed by the iron-catalyzed Fenton reaction or by the Haber-Weiss reaction (11). 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 (12, 13).

Unlike acute stress effects on lipoperoxidation, the actions of repeated restraint stress are less known. In the present study we evaluated the effects of repeated restraint stress on the production of free radicals, the lipoperoxidation, as well as the total antioxidant capacity, including the total radical-trapping potential (TRAP) and the total antioxidant reactivity (TAR) levels, in addition to antioxidant enzyme activities (SOD, GPx and CAT) in hippocampus of rats.

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.

Subjects: Experimentally naive adult male Wistar rats (60 days old; 180-230 g body mass) were used. They were housed in groups of five in cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust. They were maintained in a controlled environment (lights on between 7:00 and 19:00h, temperature of 22 $\pm$ 2° C) before and throughout the experimental period. Rats had free access to food (standard lab rat chow) and water, except during the period when stress was applied. The restraint procedure was performed between 10:00 and 13:00h. The institutional Research Committee approved all animal procedures.

Repeated restraint: The animals were divided in two groups: stressed and control. The restraint stressed group was taken to a different room, where restraint was carried out by placing the animal in a 25 x 7 cm plastic bottle, adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1-cm hole in one far end for breathing. The animals were stressed 1h/day, 5 days a week for 40 days (14). The restraint procedure was performed between 10:00 h and 13:00 h.

Preparation of the samples: The animals were killed by decapitation 24 h after the last exposure to stress. Their hippocampi were quickly dissected out and frozen in liquid N_2. Samples were stored at minus 70° C until analysis, when they were homogenized in 10 vol (w:v) ice-cold 0.1 M phosphate buffer (pH 7.4), containing 140 mM KCl and 1 mM EDTA. The homogenate was centrifuged at 960xg for 10 min and the supernatant, a suspension of mixed and preserved organelles, was used.

Free radical content: To assess the free radicals content we used 2'-7'-dichlorofluorescein diacetate (DCFH-DA) as a probe. This method does not determine the presence of specific free radicals, because DCFH may be oxidized by several reactive intermediates (15). An aliquot of the sample was incubated with DCFH-DA (100 μM) at 37°C for 30 min. The reaction was terminated by chilling the reaction mixture in ice. 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 was performed in the dark, and blanks containing DCFH-DA (no homogenate) were processed for measurement of autofluorescence (16).

Determination of TBA reactive substances (TBARS): 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 methods described by Buege and Aust (17). 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.

Total radical-trapping potential (TRAP) Assay: This assay is based on luminol-enhanced chemiluminescence measurement, induced by an azo initiator (19-21). The reaction mixture contained 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP) 10 mM, source of peroxyl radicals, and luminol (4 mM) in glycine buffer (0.1M, 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 (5.0 µl or 1.0 µl of sample) decreases chemiluminescence to basal levels for a period (induction time) proportional to the concentration of antioxidants. The TRAP values were calculated as equivalents of Trolox concentration per mg of protein.

Total Antioxidant Reactivity (TAR) Assay: The reaction mixture contained 2 mM ABAP and 6 mM luminol in glycine buffer. TAR values were determined by assessing the initial decrease of luminescence calculated as the ratio "Io/I", where "Io" is the CL in the absence of additives, and "I" is the CL after addition of the 20 nM Trolox, or the samples (1 µl) (21). TAR values were expressed as equivalents of Trolox concentration per mg of protein.

Superoxide dismutase activity: SOD activity was determined using a RANSOD kit (Randox labs., USA) which is based on the procedure described by Delmas-Beauvieux et al. (22). 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. The inhibition on production of the chromogen is proportional to the activity of SOD present in the sample.

Catalase activity: The homogenate was incubated with ethanol (10%) and Triton (10%). The activity was assayed at 25°C by determining the rate of degradation of H_2O_2 at 240 nm in 10 mM potassium phosphate buffer (pH 7.0). The extinction coefficient of 43.6 mM^{-}

$^{1}cm^{-1}$ was used for calculation. One unit is defined as one pmol of H_2O_2 consumed per minute and the specific activity is reported as units per mg protein (23).

Glutathione peroxidase activity: GPx activity was determined according to Wendel (24). 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*-butyl-hydroperoxide 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 nmol NADPH oxidized per minute per mg protein.

Protein Assay: The total protein concentrations were determined using the method described by Lowry et al. (25) with bovine serum albumin as the standard.

Statistical analysis: Data were expressed as mean ± standard error of the mean, and were analyzed by Student's t test for independent samples. A difference was considered significant when $P < 0.05$.

RESULTS

The results show that chronic stress during 40 days induced a significant increase in lipoperoxidation in the hippocampus, as evidenced by the TBARS test [Student's t test, $t(20) = 2.20$; $P < 0.05$; Figure 1A]. No differences were observed on free radicals production by the DCF test 24 h after the last stress session [Student's t test, $t(15) = 1.01$; $P > 0.05$; Figure 1B].

When evaluating antioxidant capacity, decreased total antioxidant reactivity (TAR) levels were observed in the stressed group [Student's t test, $t(17) = 3.26$; $P < 0.005$;

Figure 2A], while a non-significant decrease was seen in total radical-trapping potential [TRAP; Student's t test, $t(21) = 0.12$; $P > 0.05$; Figure 2B].

The effects of repeated stress on the activities of SOD, CAT, and GPx are depicted in Figure 3. There were no changes in SOD and CAT activities [Figure 3A and 3C; Student's t test, $t(17) = 0.98$ for SOD and $t(17) = 1.38$ for CAT; $P > 0.05$ in both cases). However, the GPx activity was significantly higher in hippocampus of repeatedly stressed rats as compared to controls (Figure 3B; Student's t test, $t(14) = 2.66$; $P < 0.02$).

DISCUSSION

We have here reported alterations on oxidative homeostasis induced by repeated restraint stress. These alterations included augmented lipid peroxidation, as well as the consumption of antioxidant defenses, evidenced by reduced total antioxidant reactivity. This decrease was probably due to reduced nonenzymatic antioxidants, since antioxidant enzymes activities were increased or not affected by this chronic treatment.

The results of this study agree with other reports showing that exposure to GCs or to stress may induce formation of ROS and lead to oxidative injury in various tissues, including blood (26) and brain (1, 27, 28). A significant increase in lipoperoxidation was observed in plasma after one session of immobilization stress (26). Effects of chronic stress on TBARS in brain have also been observed and distinct brain regions show different responses to stress (e.g., 4). In addition, significant increases in levels of protein oxidation were found in different regions of the brain compared to the unstressed controls (28). 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 degenerative diseases.

Glucocorticoids have been shown to increase the vulnerability of different brain regions, particularly the hippocampus, to metabolic insults, potentially by altering the neuronal defense capacity against oxidative damage (1, 12, 13). The impairment of antioxidant enzyme defenses in hippocampus, cortex, and cerebellum has been reported as a possible component of GC-mediated neuroendangerment (29, 30).

It is important to point out, however, that after 40 days of exposure to restraint stress, glucocorticoid release by the stress situation is decreased, when compared to the first exposure to this type of stress (31). Repeated exposure to the same aversive event leads to a process of adaptation to that stimulus. Therefore, animals exposed repeatedly to restraint will present different physiological and behavioral responses, and the changes observed in the present study possibly represent adaptation to this chronic situation, as the increased activity of glutathione peroxidase.

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. It is possible that increased free radicals production occurs during stress and that these levels may return to normal 24 hours later, when the animals are sacrificed. Lipoperoxidation may still be present, since it may not be so easy to recover from damage to lipids.

In our study, repeated stress induced a decrease in TAR in hippocampus 24 after last exposure to stress, with no difference in TRAP. According to Lissi et al. (21), in a complex biological sample, the total TRAP capacity corresponds to the concentration of antioxidants and the number of free radical trapped by each molecule, which represents the quantity of the antioxidant. However, in a system exposed to continuous inputs of free radicals, the relevant parameter is the capacity of the medium to regulate the steady-state

concentration of damaging free radicals, and its quality must be considered, what is given by TAR. Therefore, the decreased TAR observed herein suggests higher vulnerability to oxidants in this tissue.

We also observed an increment on GPx activity, an enzyme that catalizes the reaction of hydroperoxides with reduced glutathione (GSH), producing oxidized glutathione (GSSG). Corticosterone has been shown to decrease GPx activity and reduced glutathione (GSH) levels in hippocampal cells culture (30). Therefore, the increased GPx activity observed in the present study is possibly an adaptation to repeated stress. The reaction catalized by GPx is considered an important mechanism for breaking lipid peroxidizing chain reactions. The activity of GPx in the hippocampus has been reported to increase after excitotoxic injury (32). An increase in GPx activity indicates an increase in cellular peroxides. Together with the results of TBARS, the data support the idea that repeated stress is capable of inducing reactive oxygen species.

GSH, which is oxidazed by GPx, has long been known to be involved in detoxifying reactions in animals' tissues, plants, and microorganisms (33). Because of its high intracellular concentration (0.1-10 mM), GSH serves as the major reducing peptide within all cells, contributing its sulfhydryl group to buffering and removal of free radicals generated during metabolic processes such as respiration and during oxidative stress (34). Hippocampal GSH is also significantly depleted by excitotoxic injury (32, 35). In agreement with our data, these studies support the theory that a loss of GSH may occur due to an oxidative event as one mechanism of utilization. Under normal cellular conditions, as GSSG is formed, it is immediately reduced to GSH by glutathione reductase, using NADPH as a coenzyme. In situations where oxidative stress occurs, the capacity of this enzyme may be overwhelmed. Additionally, decreased NADPH levels have been reported

after corticosterone treatment (30), another mechanism that could induce a reduction in GSH levels and, therefore, in TAR.

The decreased TAR observed herein is not related to decreased antioxidant enzymes activties, since they are not changed or are even increased (see GPx); so, the reduction in TAR possibly means decreased non-enzymatics antioxidants, such as GSH (30), which is consumed for more oxidant species produced by repeated restraint stress (see lipoperoxidation). The action of GSH as a scavenger of free radicals is made possible thanks to GPx activity, which was encountered augmented in this situation. This effect is likely to have functional implications, since the enhancement of GSPx activity by adding exogenous GSH protects against excitotoxicity (35).

Although their involvement in tissue damage, oxygen free radicals influence brain function by altering signal transduction across membranes, by changing the properties of several receptors, which have functionally important disulfide bonds whose redox state induces conformational changes. In this respect, increasing evidence indicates that glutamate transporters may also undergo functional changes, depending on increased oxidation or reduction of reactive sulphydryls present in their structure (36, 37), in such a way that they are vulnerable to the action of biological oxidants, resulting in reduced uptake function. In this sense, different levels of free radicals in brain structures after exposure to chronic stress may influence responses to these neurotransmitters and be a part of the constellation of factors involved in the adaptation to chronic stress.

We conclude that exposure to repeated stress induces oxidative stress in hippocampus. These changes may depend on the chronicity and also on the model of chronic stress and may contribute to the effects observed by several authors after prolonged stress.

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LEGENDS TO FIGURES

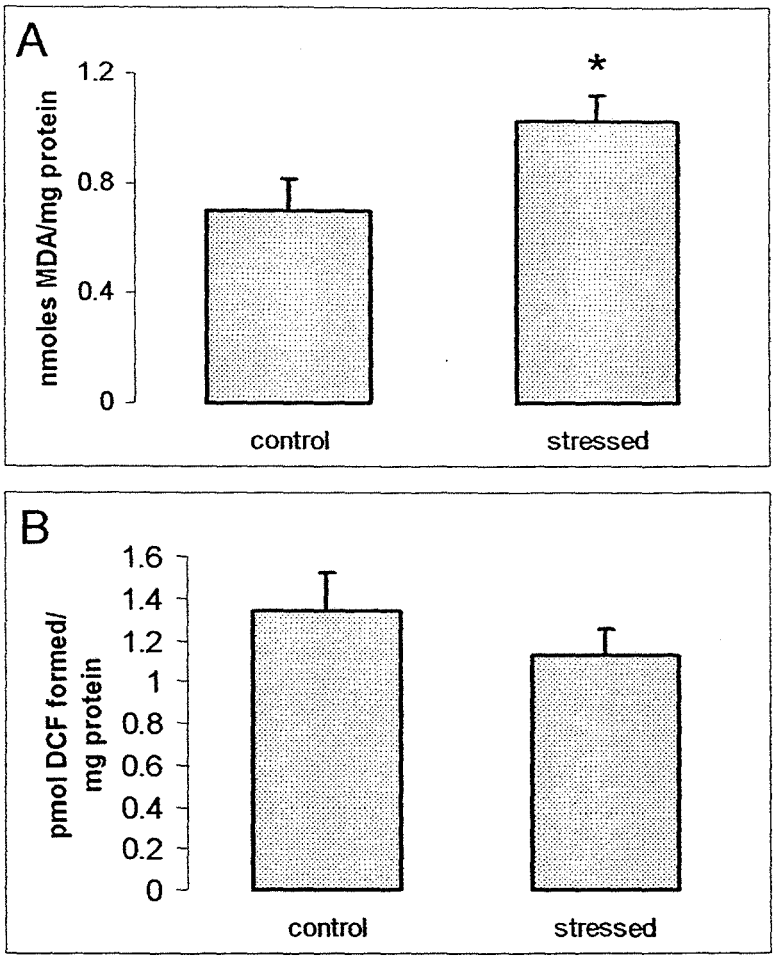

Figure 1. Effect of exposure to repeated restraint stress on lipoperoxidation (A), using the TBARS assay, and on free radical production (B), using DCFH-DA as a probe, in rat hippocampus. Results are expressed as mean ± standard error of the mean (n = 8-11 samples per group).

* Significantly different from control group (Student's t test, $P < 0.05$).

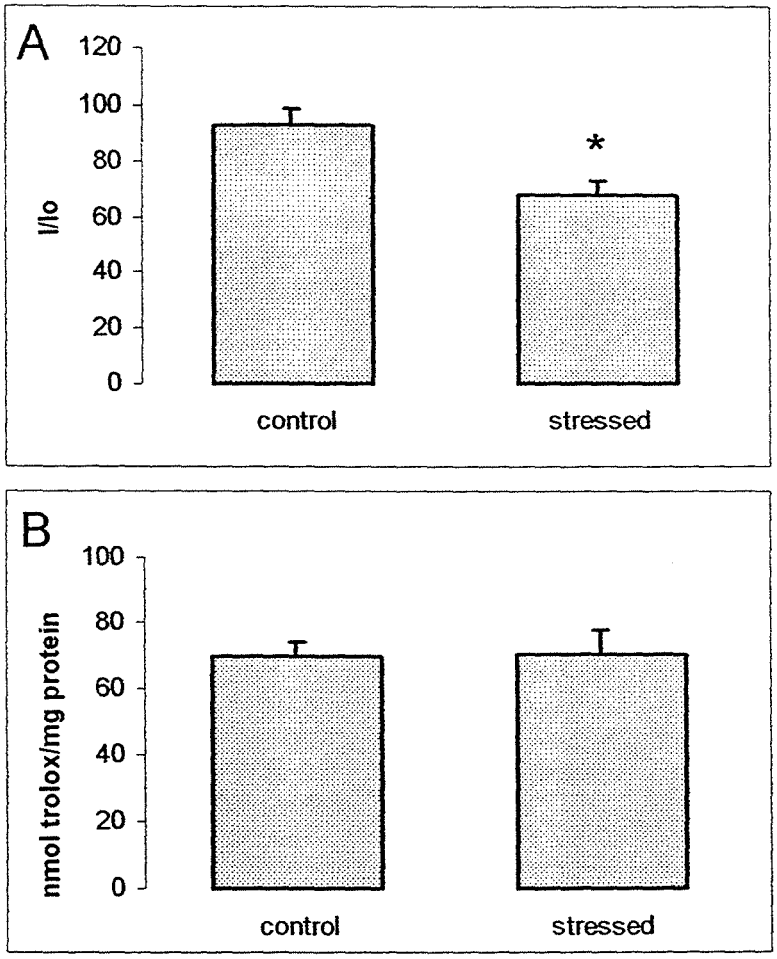

Figure 2. Effect of exposure to repeated restraint stress on TAR (A), and on TRAP (B) in rat hippocampus. (A) Results are expressed as mean ± standard error of the mean of the ratio "Io/I", where "Io" is the chemiluminescence in the absence of additives, and "I" is the chemiluminescence after addition of the Trolox, or the samples, and calculated as equivalents of Trolox concentration per mg of protein (B) TRAP values were expressed as equivalents of Trolox concentration per mg of protein for the induction time. N = 9-12 samples per group.

* Significantly different from control group (Student's t test, $P < 0.005$).

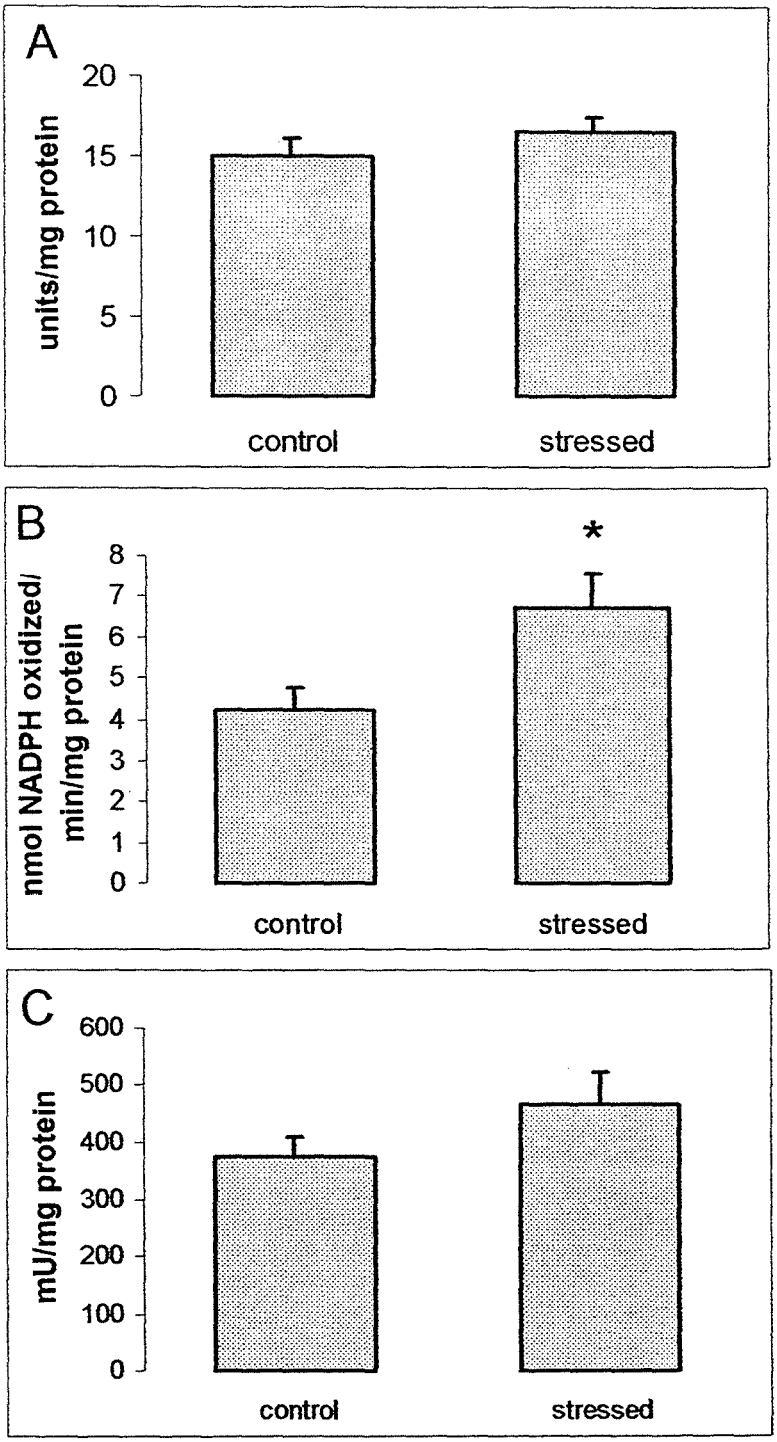

Figure 3: Effect of exposure to repeated restraint stress on antioxidant enzymes activities, superoxide dismutase (A), glutathione peroxidase (B) and catalase (C) in rat hippocampus. Results are expressed as mean ± standard error of the mean. N = 8-10 samples per group.

* Significantly different from control group (Student's t test, $P < 0.02$).

Capítulo VIII: Manuscrito 8

Acute and repeated restraint stress influences cellular damage in rat hippocampal slices exposed to oxygen and glucose deprivation

Submetido: *Brain Research Bulletin*

Acute and repeated restraint stress influences cellular damage in rat hippocampal slices exposed to oxygen and glucose deprivation

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Abstract

Several studies have shown that high corticosteroid hormone levels increase neuronal vulnerability. Here we evaluate the consequences of in vivo acute or repeated restraint stress on cellular viability in rat hippocampal slices suffuring an *in vitro* model of ischemia. Cellular injury was quantified by measuring lactate dehydrogenase (LDH) and neuron-specific enolase released into the medium. Acute stress did not affect cellular death when oxygen and glucose deprivation (OGD) was applied both immediately or 24 hours after restraint. The exposure to OGD, followed by reoxygenation, resulted in increased LDH in the medium. Repeated stress potentiated the effect of OGD both, on LDH and neuron-specific enolase released to the medium. There was no effect of repeated stress on the release of S100B, an astrocytic protein, released to the medium. Additionally, no effect of repeated stress was observed on glutamate uptake by the tissue. These results suggest that repeated stress increases the vulnerability of hippocampal cells to an in vitro model of ischemia, potentiating cellular damage induced by OGD, and that the cells damaged by the exposure to repeated stress + OGD are mostly neurons. The uptake of glutamate does not seem to participate on the mechanisms responsible for rendering the neurons more susceptible to ischemic damage after repeated stress.

Theme: A. Developement and regeneration (Neuronal death)

Key words: stress, oxygen/glucose deprivation, hippocampus, LDH, NSE, glutamate uptake

INTRODUCTION

Evidence from literature suggests that exposure to persistently high levels of corticosterone or repeated exposure to stressors present adverse effects on various brain regions, specially the hippocampus [15, 16, 40, 52, 55, 56]. These hormones do not kill hippocampal neurons, but exacerbate the toxicity of other insults, possibly accelerating the decline in ATP levels in neurons during periods of increased energy demand [4, 15, 16, 29, 59, 64, 65]. For example, acute, short exposure to glicocorticoids can significantly exacerbate postischemic outcome [47, 48].

Ischemia is defined as a severe reduction or complete blocking of the blood flow [68] 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 caused by in vitro [47] or in vivo models of ischemia [27, 53]. In addition, synaptic function along with cellular integrity can be preserved after hypoxia/ischemia by preventing the rise in corticosteroid levels by the use of steroid synthesis inhibitor metyrapone [1, 2, 27] or by adrenalectomy [3, 43].

Most of the avaiable studies concerning with the effects of glucocorticoids on ischemic events have used acute administration of, or in vitro exposure to, these hormones [6, 64], or reduced glucocorticoid release by the use of synthesis inhibitors or adrenalectomy [25, 27, 55, 58]. Chronic treatment with glucocorticoids has been shown to cause different results on ischemia-induced neuronal damage in hippocampus: some behavioral and histological studies observed aggravated damage [24, 51, 61], while others suggest a reduction in brain damage following hypoxia/ischaemia [26, 33]. However, chronic exposure to stressors present several different aspects in relation to chronic administration of glucocorticoids: First, it is well known that repeated exposure to the same

aversive event during stress can lead to a process of adaptation to that stimulus [4, 5, 7, 8, 21, 28, 34]. According to that, it is important to note that the levels of corticosterone observed following acute restraint stress are higher than those that follow the same restraint when it is repeated several times [65]. Second, besides releasing glucocorticoids, stress response involves a large number of neurotransmitters, neuropeptides and neuromodulators in various brain regions [for a review, 46].

Our hypothesis is that the exposure to stress will possibly increase suceptibility to ischemic injury. Both acute and intermittent stress will be used, since adaptation to stress may lead to a different outcome in this condition. The present study aimed to evaluate the consequences of acute or repeated exposure to restraint stress on cellular function after oxygen and glucose deprivation (OGD), an in vitro model of ischemic events [9, 42, 44], in rat hippocampal slices. Neuronal injury was quantified by evaluating lactate dehydrogenase (LDH) activity and neuron-specific enolase (NSE) levels released to the bathing medium; levels of S100B, a protein released by astrocytes in conditions of injury were also evaluated in the medium. Additionally, damage induced by both procedures, stress and ischemia, have been suggested to be due, at least in part, to increased glutamatergic tonus [30, 32, 41, 63, 67]. In such situation, transporter proteins which uptake glutamate play an important role in the termination of glutamatergic neurotransmission and prevention of excitotoxicity [12]. Therefore, we also evaluated glutamate uptake by hippocampal slices from rats subjected to repeated exposure to restraint stress, and sacrified 24 hours afterwards, when these slices were exposed to OGD conditions.

2. MATERIALS AND METHODS

2.1. Subjects:

Sixty-four adult male Wistar rats (60 days at the beginning of the treatment) weighing 180-230 g were used. The experimentally naive animals were housed in groups of 4 to 5 rats, in homecages made of Plexiglas material (65 x 25 x 15 cm) with the floor covered with sawdust. They were maintained under standard dark-light cycle (lights on between 7:00 and 19:00h), with room temperature of 22 ± 2° C. Animals had free access to food (standard lab rat chow) and water, except during the period when restraint stress was applied. The restraint procedure was performed between 13:00 and 15:00 h. All animal treatments were in accordance with the institutional guidelines and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS); all efforts were made to minimize animal suffering as well as to reduce the number of animals.

2.2. Stress procedure:

2.2.1. Acute treatment - The animals were submitted to a single exposure (1 hour restraint) and the control group was not stressed. Restraint was carried out by placing the animal in a 25 x 7 cm plastic bottle, adjusting it with plaster tape on the outside, so that the animal was unable to move. There was a 1 cm hole in one far end for breathing.

2.2.2. Repeated stress treatment - Animals were separated in two groups: stressed and control. Rats were stressed 1 hour/day, 5 days a week for 8 weeks [18]. Control animals were kept in their homecages.

2.3 Preparation and incubation of slices:

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 5% CO_2 (95%O_2/5%CO_2) [9].

2.4 Oxygen and glucose deprivation (OGD)

The exposure to oxygen and glucose deprivation was based on the method described by Strasser [60], with some modifications [11]. After the preincubation period, control slices were incubated in a modified Krebs-Henseleit solution (incubation solution – control medium) containing (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 around 37°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 hours at 37 ° C in an atmosphere 5% CO_2 (95%O_2/5%CO_2) to simulate a "reperfusion" period [9]. In a second experiment, this same methodology was used, but 10 mM lactate was added to the incubation medium instead of glucose.

2.5 Assessment of neural injury - LDH assay

Neural cell injury was quantified by the measurement of lactate dehydrogenase (LDH) released from damaged cells into the extracelular fluid [23]. LDH efflux occurs from either necrotic or apoptotic cells, and is proportional to the number of damaged cells [22, 23, 31].

LDH activity was determined using a commercial kit (Doles Reagentes, Goiânia, Brazil); the activity of this enzyme was assessed in the bathing fluid. Following the conversion of exogenouslly added lactate to pyruvate, occurs the conversion of 1,10-phenantroline 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).

Protein content was measured in aliquots of the dissociated slices following the method described by Peterson [49].

2.6. *Neuron-specific enolase (NSE) determination*: Assay for NSE was made by ELISA (CanAg Diagnostics AB, Gothenburg, Sweden). Incubation medium was collected after 3h reperfusion and stored at -20° C. Samples (25 ul) were applied on microtiter plates previously coated with monoclonal anti-NSE, and incubated overnight at room temperature. After washing, peroxidase-conjugated anti-NSE was added and incubation continued for 1 h. The plate was washed and peroxidase substrate was added and the plate incubated for further 5 min in the dark. Absorbance was measured at 620 nm on a microtiter plate reader.

2.7. *S100B determination*: Assay for S100B was carried out by ELISA as described by Tramontina et al. [66]. Briefly, incubation medium was collected after 3h reperfusion and stored at -20 °C. Samples (50 ul) were applied on microtiter plates previously coated with monoclonal anti-S100B (Sigma) in carbonate buffer and blocked with 1% bovine serum albumin. After washing, peroxidase-conjugated anti-S100 (polyclonal DAKO) diluted 1:1000 was added and incubation continued for 1 h. The plate was washed and 0.2 ml of

peroxidase substrate (Sigma Fast OPD) was added and the plate incubated for further 30 min in the dark. Absorbance was measured at 492 nm on a microtiter plate reader.

2.8. Glutamate Uptake

2.8.1. Total uptake: Glutamate uptake was performed according to the protocol of Frizzo et al. [17], adaptated for hippocampus. Slices were transferred to 24 wells-culture plates, one being maintained in a 35°C and other in a 0°C temperature. The slices of the first plate were washed once with 1mL of 35°C glucose-Hank's balanced salt solution (HBSS), containing 137 mM NaCl; 0.63 mM $Na_2HPO_4.7H_2O$; 4.17 mM $NaHCO_3$; 5.36 mM KCl; 0.44 mM KH_2PO_4; 1.26 mM $CaCl_2.2H_2O$; 0.41 mM $MgSO_4.7H_2O$; 0.49 mM $MgCl_2.6H_2O$ and 1.11 mM glucose, and pH adjusted to 7.2 with HCl; the second plate was washed with 1mL of 0°C HBSS sodium-free for the unspecific analyses. Slices were incubated with 0.66 $\mu CimL^{-1}$ L-[^{3}H]glutamate (Amersham) and 100μM unlabeled glutamate in glucose-HBSS solution. Incubation was stopped after 5 minutes with two ice-cold washes of 1mL HBSS, immediately followed by addition of 0.5N NaOH. An aliquot was used to protein determination and the to the remaining medium was added cintilation liquid and radioactivity was measured. Radioactivity incorporated was measured through scintillation counting.

2.8.2. Sodium-independent uptake (unspecific): The methodology used to measure unspecific uptake was the same described above excepting for the temperature and medium used. Sodium-independent uptake was determined at 0°C, using N-methyl-D-glucamine instead of sodium chloride, and its result was subtracted from the total uptake to obtain the specific one. Both specific and unspecific uptake was performed in triplicate and the mean was used for calculation.

2.9. Statistical analysis:

Data are expressed as mean ± standard error (SEM) and were analyzed using a two-way ANOVA, followed by Duncan's multiple range test when indicated.

RESULTS

Experiment 1 - Effect of OGD to hippocampal slices obtained immediately and 24 hours after 1 hour of restraint stress

In order to evaluate whether exposure to acute stress affects cellular damage induced by OGD, we assessed LDH activity in hippocampal slices from rats submitted to one single exposure of restraint stress, and sacrified immediately or 24 hours afterwards. Figure 1A shows that, in slices obtained from control rats and from animals sacrified immediately after acute restraint, OGD caused increased LDH release into the medium [two-way ANOVA, $F(1, 15) = 70.54$, $P < 0.001$], but no difference was observed after stress exposure when compared to control [$F(1, 15) = 2.42$, $P > 0.05$]. Acute stress did not interfere with the effects of OGD [$F(1, 15) = 0.57$, $P > 0.05$ when tested for interaction].

Similar results were found after 24 hours: there was no significant difference in LDH release between stressed and control groups (Figure 1B); the only significant effect was that caused by OGD [two-way ANOVA, $F(1, 14) = 20.19$, $P < 0.001$ for OGD and $F(1, 14) = 0.75$, $P > 0.05$ for the stress treatment]. No interaction between stress and OGD was observed [$F(1, 14) = 0.02$, $P > 0.05$].

Experiment 2 - Effect of OGD in hippocampal slices from rats previously submitted to repeated restraint stress

This experiment aimed to evaluate whether previous exposure to repeated restraint stress interferes with the neuronal damage induced by OGD. After 3 hours of reoxygenation following 60 minutes of OGD, LDH activity was assayed in the medium. Figure 2 shows that both treatments (OGD and stress) affected LDH released into the medium [two-way ANOVA, $F(1, 28) = 5.49$, $P < 0.05$ for the stress treatment and $F(1, 28) = 22.60$, $P < 0.001$ for the OGD treatment]. Slices from repeated stressed animals had LDH levels similar to controls, however the group submitted to both treatments presented higher levels of LDH in the medium than those of all other groups.

Experiment 3 - Effect of OGD in hippocampal slices from rats previously submitted to repeated restraint stress, when lactate was used as substrate

Glucose can be potentially taken up and utilized by neurons as well as astroglia, however neurons might preferentially utilize lactate both in vivo and in vitro, as substrate [62]. In order to evaluate if different substrates would result in distinct cellular damage when slices from animals subjected to repeated stress are submitted to OGD, we assessed LDH activity in hippocampal slices from rats subjected to repeated exposure to restraint stress, and sacrified 24 hours afterwards, when lactate is added to the medium instead of glucose. Figure 3 shows that OGD caused increased LDH released into the medium [two-way ANOVA, $F(1, 15) = 33.43$, $P < 0.001$], and the same effect was observed after stress exposure [$F(1, 15) = 10.94$, $P < 0.001$; no interaction between the variables was observed: $F(1, 15) = 1.53$; $P > 0.05$]. The group submitted to both treatments presented higher levels of LDH released to the medium than all other groups.

Experiment 4 - Effect of OGD in hippocampal slices from rats previously submitted to repeated restraint stress on neuron-specific enolase (NSE) and on S100B levels in the medium

In order to evaluate if cellular damage induced by OGD reflects neuronal or astroglial death, we measured both NSE content and the levels of S100B, a protein produced and released by astrocytes [45, 50] in the medium. Figure 4A shows that OGD caused increased NSE released into the medium [two-way ANOVA, $F(1, 17) = 10.86$, $P < 0.001$], and the same effect was observed after stress exposure [$F(1, 17) = 5.51$, $P < 0.05$; a significant interaction between the OGD and stress was observed: $F(1, 17) = 5.36$; $P < 0.05$]. Here again, the group submitted to both treatments presented higher NSE activity in the medium than all other groups. No effect of OGD or repeated stress was observed (Figure 4B) on S100B levels in the medium [two-way ANOVA, $F(1, 14) = 0.29$, $P > 0.05$ for OGD; $F(1, 14) = 1.33$, $P > 0.05$ for the stress treatment; there was no interaction: $F(1, 14) = 1.19$, $P > 0.05$].

Experiment 5 - Effect of OGD on glutamate uptake by hippocampal slices from rats previously submitted to repeated restraint stress.

Figure 5A shows that, immediately after OGD, there was a decrease in glutamate uptake by hippocampal slices [two-way ANOVA, $F(1, 15) = 20.13$, $P < 0.001$]. No effect of stress exposure was observed [$F(1, 15) = 0.21$, $P > 0.05$]. The same pattern of effect was observed with 3 hours of reperfusion [two-way ANOVA, $F(1, 15) = 22.97$, $P < 0.001$ for OGD; $F(1, 15) = 0.04$, $P > 0.05$ for stress treatment; $F(1, 15) = 0.43$; $P > 0.05$ for the interaction] (Figure 5B).

DISCUSSION

Aerobic metabolism is the major source of energy in the brain, but it may be compromised by the interruption of substrate delivery and disturbances in cerebral metabolism, as the condition resulting from ischemia. The exposure of hippocampal slices to an in vitro model of ischemia (OGD), followed by reoxygenation, resulted in increased LDH in the medium, which is a consequence of cell damage or death.

The consequences of acute or repeated exposure to stress on cellular integrity after oxygen and glucose deprivation (OGD) in rat hippocampal slices were investigated. We observed that acute and intermittent repeated stress induced different states of suceptibility to ischemic injury. Acute stress did not interfere with the effects of OGD exposure, i.e., when the slices were exposed to OGD immediately or 24 hours after acute stress, just the effect of OGD was observed. On the other hand, slices from hippocampus of repeatedly stressed animals presented a higher susceptibility to OGD.

The increase in damage observed after OGD when animals were previously submitted to repeated stress is possibly related to neuronal death, since the same results were observed after incubation with glucose (utilized by neurons and astroglia) or lactate (preferentially utilized by neurons); besides, neuron specific enolase (NSE) released to the medium after OGD was higher in slices from repeatedly stressed rats.

The same mediators suggested to be involved in neuronal damage caused by stress exposure, e.g., glucocorticoids, EAA, and NMDA receptor activation [54], participate in neuronal damage and death that is caused in pyramidal neurons by ischemia [30, 51]. This is believed to be one explanation for the fact that stress and acute corticosterone treatment increase ischemic damage [3, 37, 38]. In this model of OGD, however, no effect of acute

restraint stress on LDH release in the medium was observed, suggesting that acute stress did not increase in vitro cell death. A possible explanation for the differences observed between effects of corticosterone on *in vivo* and those of acute stress on *in vitro* ischemia is the different time used to observed the damage induced by these processes. Additionally, the stress response comprises more than the release of glucocorticoids; rather, other hormones and neurotransmitters are released, which may result in a different effect.

The measurement of LDH released into the medium 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. This effect is further evidenced by the increased NSE present in the medium, which is much higher when the slices are submitted to both situations, stress and OGD. This results are in agreement with previous reports, which suggest that repeated stress may increase vulnerability of hippocampal neurons to several insults, including hypoxia-ischemia [37]. Chronic exposure to elevated titers of glucocorticoids has been demonstrated to result in cell loss as well as in reduced neuronal plasticity and regeneration [13, 36, 57]. Some mechanisms suggested to play a role in this increased vulnerability of hippocampal cells include decreased glucose uptake [19], increased glutamate release [37-39], and increased production of oxygen reactive species [ROS; 10, 35].

On the other hand, based on S100B released in the medium, no astrocyte damage seems to occur after 3 hours of reperfusion. This protein is an important marker for monitoring damage and activation of astrocytes [50], and it is also a glial modulator of neuronal synaptic plasticity [45], since secreted glial S100B exerts trophic or toxic effects depending on its concentration: at nanomolar concentrations, S100B stimulates neurite outgrowth and enhances survival of neurons during development. In contrast, micromolar

levels of extracellular S100B in vitro stimulate the expression of proinflammatory cytokines and induce apoptosis [for a review, see 14]. Clinical studies have shown that serum levels of S100B correlate with the degree of brain ischemia when measured several hours later, while no correlation is observed until 15 h [20]. In contrast to NSE, no specific increments of peripheral levels of S100B were observed immediately after brain damage in stroke [69]. This delayed release of S100B, found in in vivo situations, has been attributed to the putative role of this protein in the brain repair mechanism. Thus, it would be possible that, using in vivo models of ischemia, altered levels may be observed with longer times of reperfusion.

The changes in hippocampal function caused by stress or by ischemia have been attributed to an increased glutamatergic tonus. Exposure to acute stress increases extracellular levels of glutamate in some brain structures, including the hippocampus [32, 41]. Accumulation of Glu in the synaptic cleft may lead to excitotoxic neuronal damage due to over stimulation of these receptors. The Na^+-dependent, high affinity uptake of glutamate is mediated by transporter proteins located mainly in astrocytes and this mechanism has been proved to play an important role in the termination of glutamatergic neurotransmission and prevention of excitotoxicity [12]. A compromised glutamate uptake in animals submitted to chronic stress could contribute to the higher damage induced by OGD exposure in these animals. This is not the case, however, since glutamate uptake was decreased only by OGD, with no effect of chronic stress in this parameter. Therefore, although exposure to acute stress may alter glutamatergic neurotransmission [32, 41], the ability of the tissue, mainly astrocytes, in reuptaking glutamate from the medium is not compromised after chronic stress, independent of exposing cells to OGD.

In conclusion, the present results suggest that (1) the effects of OGD on cell death in hippocampal slices, are not altered both immediately or 24 h after acute stress; (2) repeated restraint stress seems to worsen cellular damage induced by OGD; (3) The cells damaged by exposing to repeated stress + OGD are mainly neurons. (4) The mechanisms responsible for rendering the neurons more susceptible to damage after repeated stress do not involve glutamate uptake. The mechanisms involved in these effects of repeated stress in a situation of OGD remain to be explored.

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Legends and figures

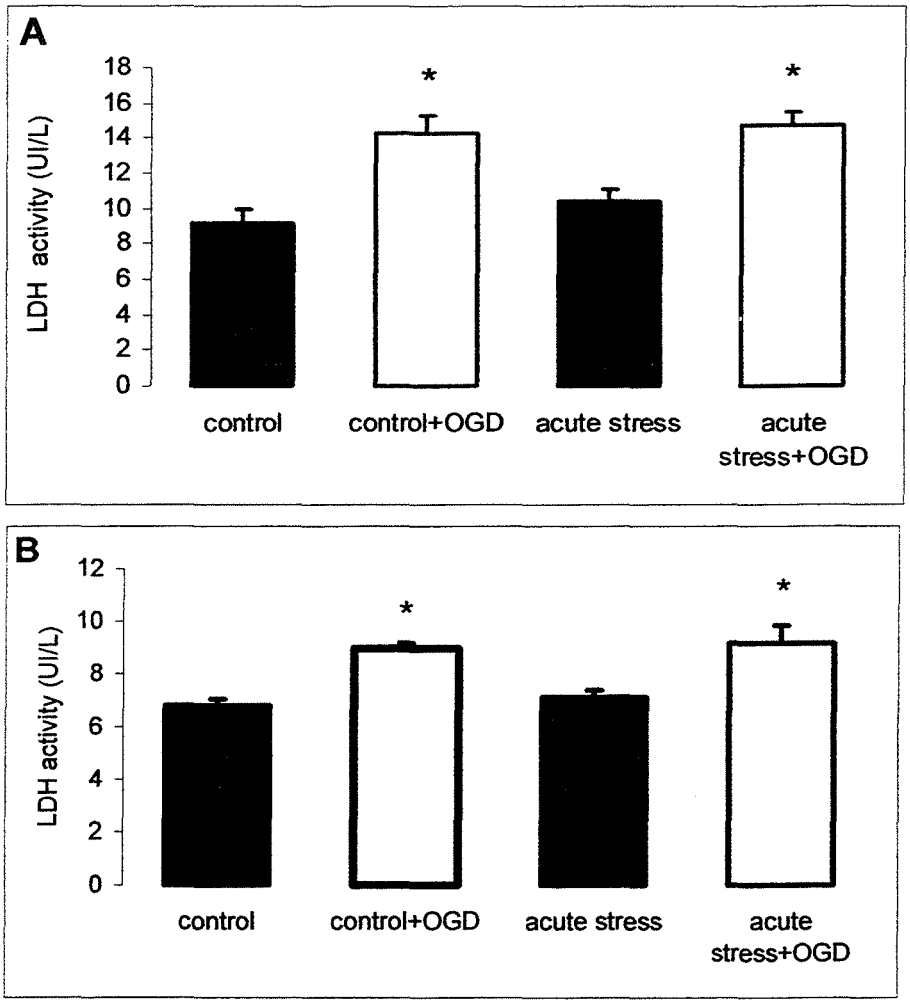

Figure 1. Effect of acute stress on hippocampal slices exposed to OGD for 60 minutes (A) immediately after acute restraint and (B) twenty-four hours after acute restraint. Results are presented as mean ± SEM of UI/l (n = 4-5/group). A two-way ANOVA showed no effect of stress ($P > 0.05$) and a significant effect of OGD ($P < 0.001$).

* Significantly different from control and acute stress groups, not submitted to OGD (Duncan test, $P < 0.05$).

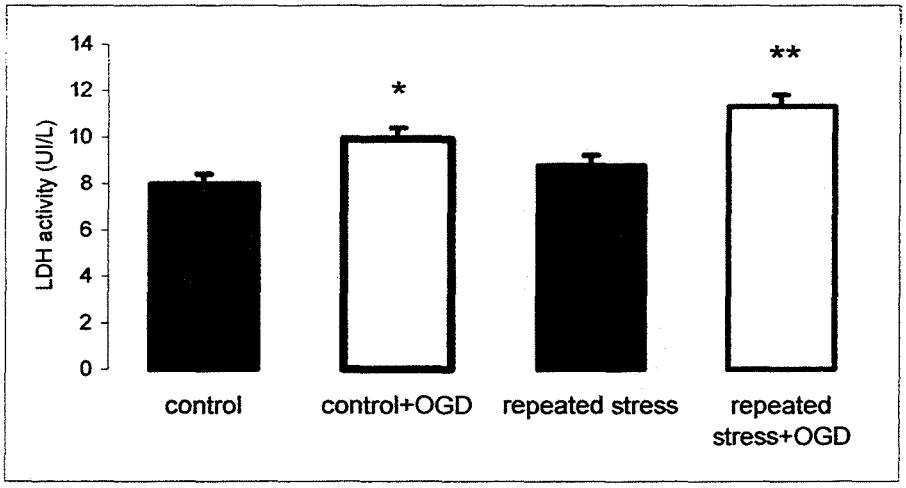

Figure 2. Effect of repeated stress on hippocampal slices exposed to OGD for 60 minutes. Results are presented as mean ± SEM of UI/l (n = 8/group). A two-way ANOVA showed that both treatments (OGD and stress) affected LDH released into the medium ($P < 0.05$ for the stress treatment and $P < 0.001$ for the OGD treatment).

* Significantly different from control and chronic stress groups, not submitted to OGD (Duncan test, $P < 0.05$).

** Significantly different from all other groups (Duncan test, $P < 0.05$).

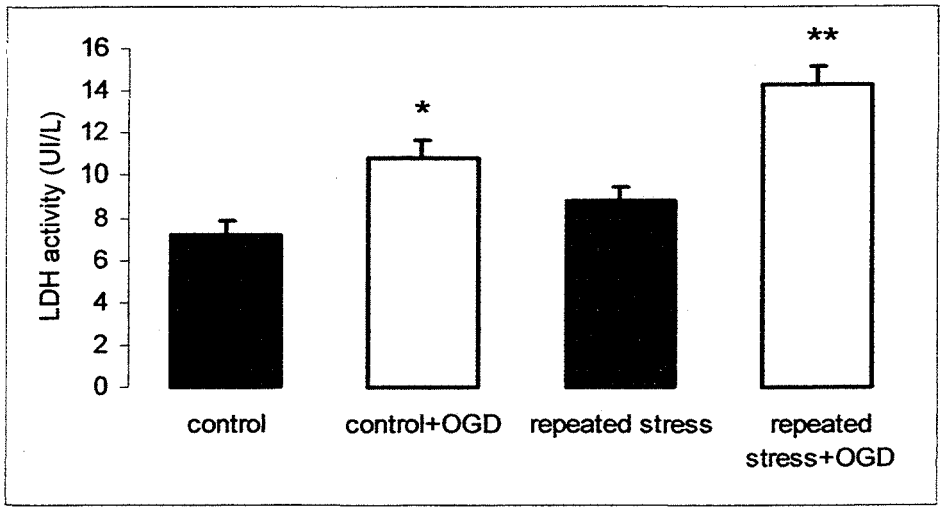

Figure 3. Effect of repeated stress on hippocampal slices exposed to OGD for 60 minutes, using lactate as a substrate. Results are presented as mean ± SEM of UI/l (n = 4-5/group). A two-way ANOVA showed that both treatments (OGD and stress) affected LDH released into the medium ($P < 0.001$ in both cases).

* Significantly different from control and chronic stress groups, not submitted to OGD (Duncan test, $P < 0.05$).

** Significantly different from all other groups (Duncan test, $P < 0.05$).

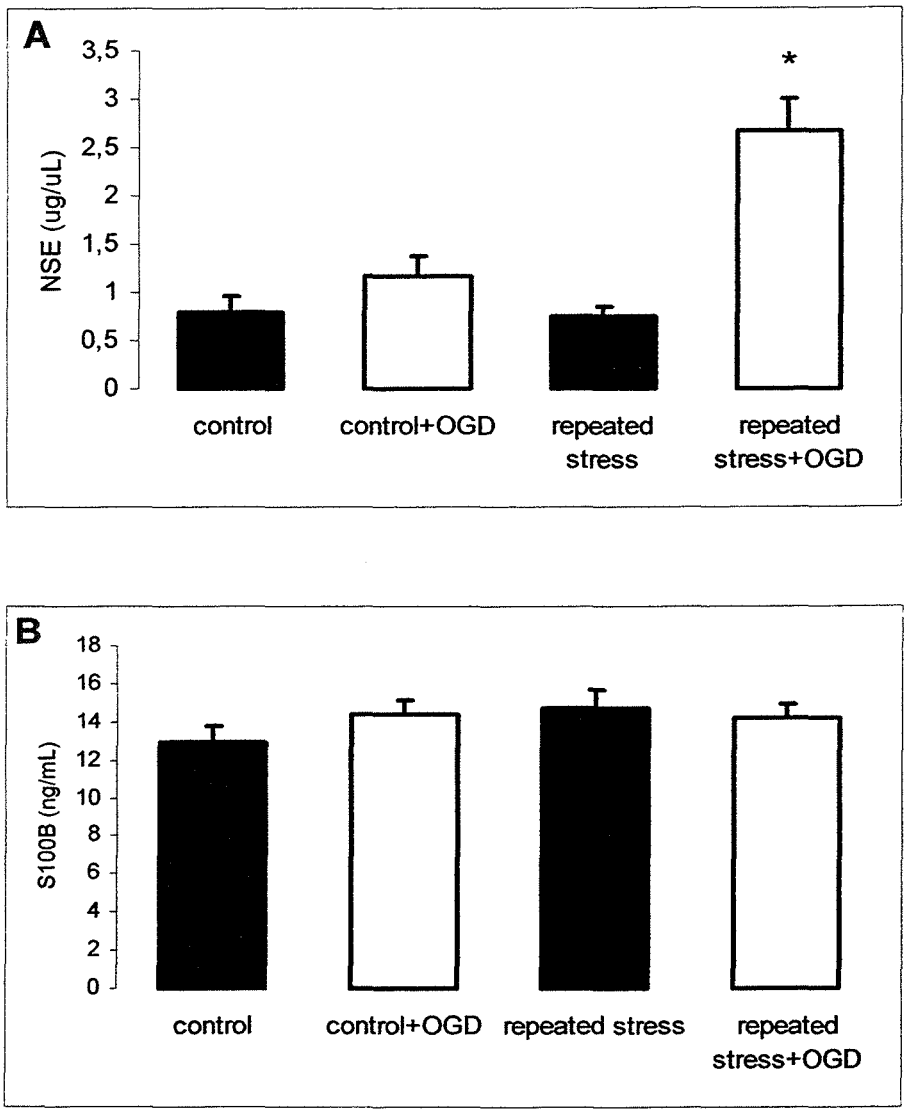

Figure 4. Effect of repeated stress, and repeated stress + OGD on (A) NSE and (B) S100B levels in the medium. Results are presented as mean ± SEM of UI/l (n = 5-6/group). A two-way ANOVA showed that both treatments (OGD and stress) affected NSE released into the medium ($P < 0.001$ for OGD exposure and $P < 0.05$ for repeated stress), with a significant interaction between the OGD and stress ($P < 0.05$). No effect of OGD or repeated stress was observed on S100B levels (two-way ANOVA, $P > 0.05$).

* Significantly different from all other groups (Duncan test, $P < 0.05$).

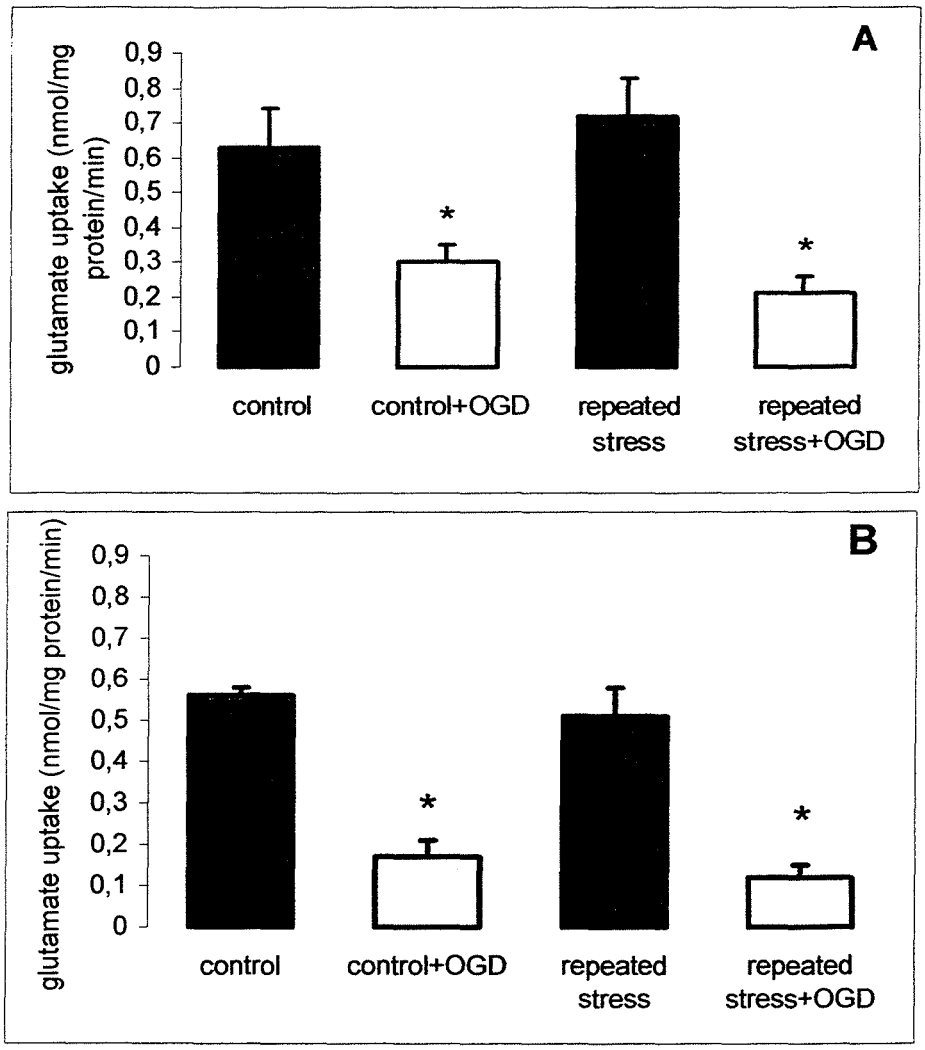

Figure 5. Effect of repeated stress in hippocampal slices exposed to OGD on glutamate uptake (A) immediatelly or (B) after 3 h of reperfusion. Results are presented as mean ± SEM of UI/l (n = 4-5/group). A two-way ANOVA showed that OGD caused a decreased glutamate uptake ($P < 0.001$), while no effect of repeated stress was observed ($P > 0.05$), at both times evaluated.

4. DISCUSSÃO

4.1 Efeitos do estresse sobre a medula espinhal

Embora a exposição aguda a uma variedade de estressores produza analgesia imediata em vários testes de sensibilidade à dor (Menendez *et al.*, 1993; Vaccarino e Kastin, 2000), o estresse prolongado pela exposição repetida a um estressor, tal como o frio ou a contenção, causa hiperalgesia (Satoh et al., 1992; Gamaro et al., 1998). De acordo com esses trabalhos, outros estudos de nosso laboratório mostram que animais machos submetidos a esse modelo de estresse repetido apresentam aumento na nocicepção, como parte do processo de adaptação a esse tratamento.

A dor é uma experiência sensorial e emocional que envolve numerosas regiões do SN, principalmente aquelas relacionadas com os sistemas límbico e somatossensorial (Coghill et al., 1994). A conexão entre estes sistemas revela uma possível rota através da qual o estímulo somatossensorial pode ser integrado à nocicepção, o que permite diferentes interpretações do estímulo doloroso e a modulação da percepção da dor.

Nesta linha de raciocínio, trabalhos de vários autores têm sugerido a possível influência de experiências prazerosas ou desagradáveis sobre a percepção da dor. Em vista disso, nós resolvemos realizar nosso primeiro trabalho sobre este tema, onde avaliamos a resposta nociceptiva após a ingestão de doce (estímulo agradável) ou solução de ácido acético (estímulo desagradável) por animais estressados cronicamente.

Nós verificamos diferentes respostas nociceptivas destes animais quando expostos a estímulos agradáveis ou desagradáveis, onde os animais controle respondem com analgesia somente quando experimentam o sabor doce, enquanto os animais estressados parecem mais aptos a perceberem o estímulo desagradável. Nós também observamos hiperalgesia induzida

pelo estresse repetido por contenção, o que concorda com outros trabalhos (Gamaro et al., 1998; da Silva Torres et al., 2003). O aumento na latência para retirada da cauda no aparato de tail-flick por animais controle após a ingestão de alimento doce também reforça os resultados de outros autores (Segato et al., 1997; Abdollahiet et al., 2000), porém nenhum efeito ocorreu nos animais estressados.

Por outro lado, somente os animais estressados apresentaram analgesia após a ingestão de solução de ácido acético. Estes resultados sugerem uma espécie de alteração na curva dose-resposta pelo estresse crônico: a nocicepção não é alterada por um sabor doce, enquanto um sabor mais ácido é funcionalmente mais efetivo em alterar este parâmetro. Por outro lado, o processamento emocional negativo, incluindo estímulos aversivos tanto ao sistema olfatório quanto ao gustatório, parece ser mediado por várias vias no SNC, as quais não são as mesmas que medeiam as respostas a estímulos prazerosos (Zald e Pardo, 1997; Zald et al., 1998).

Uma vez que os animais não haviam sido expostos anteriormente ao ácido acético, poderíamos tentar explicar esse efeito como semelhante ao efeito analgésico observado em resposta à novidade. De fato, a nocicepção é influenciada pela novidade (Dalmaz et al., 1991, Netto et al., 1987, Siegfried et al., 1987), um efeito que é bloqueado pelo estresse crônico (Torres et al., 2001b): animais controle mostram um aumento na latência de retirada da cauda após exposição a um ambiente novo (Torres et al., 2001b; Dalmaz et al., 1991, Netto et al., 1987, Siegfried et al., 1987) enquanto os animais estressados cronicamente não apresentam este efeito (Torres et al., 2001b). Considerando os resultados obtidos nos experimentos relacionados ao efeito do sabor da solução de ácido acético sobre a nocicepção, o fato dos controles não apresentarem alteração da nocicepção após a exposição ao sabor ácido nos sugere que o efeito da novidade nessa resposta não é significante. Outra possibilidade para explicar este resultado pode ser relacionada com as conseqüências fisiológicas da exposição ao estresse. Por exemplo, a

resposta a um novo estressor pode estar intacta (Marti and Armario, 1998) ou aumentada, um fenômeno chamado de facilitação (Garcia et al., 2000; Marti et al., 1994; Andres et al., 1999). Portanto, os achados de que somente os animais estressados apresentam alteração no limiar de dor após a exposição ao sabor aversivo sugerem que estes animais são mais sensíveis que os controles a este tipo de sensação, e respondem de forma aumentada. É ainda possível que esses animais apresentem uma percepção exacerbada a este sabor.

Diferentes neurotransmissores têm se mostrado envolvidos na resposta a estímulos prazerosos ou desagradáveis em animais cronicamente estressados. Os opióides, por exemplo, parecem estar envolvidos no efeito analgésico da sacarose, podemos sugerir que a ausência de resposta a substâncias doces esteja relacionada com a diminuição na função do sistema opióide já demonstrada neste modelo de estresse crônico (da Silva Torres et al., 2003).

O estresse crônico também altera a reatividade dopaminérgica, diminuindo a transmissão mediada por dopamina (Di Chiara e Tanda, 1997; Di Chiara et al., 1999) e induzindo mudanças na resposta desta transmissão frente estímulos aversivos ou de recompensa (Di Chiara et al., 1999). Este efeito ocorre de maneira similar à alteração da resposta nociceptiva ao estímulo motivacional, ou seja, de uma maneira dependente da natureza do estímulo. Alguns trabalhos sugerem uma ação direta da dopamina sobre a nocicepção (Frussa-Filho et al., 1996; Suandeau e Costentin, 1995). A dopamina, porém, parece ter ainda uma ação indireta, interagindo com o sistema opióide endógeno (Kamei e Saitoh, 1996). Deste modo, é possível que diferentes efeitos nociceptivos a estímulos aversivos ou de recompensa possam resultar de um diferente impacto motivacional destes estímulos em neurônios mesolímbicos dopaminérgicos.

Como observamos acima, os animais estressados repetidamente por contenção apresentam hiperalgesia em relação aos controles. Por outro lado, muitos trabalhos têm discutido as diferenças sexuais na percepção da dor, incluindo, por exemplo, a percepção da intensidade da

dor aguda ou crônica e respostas a analgésicos (Bodnar et al., 1988; Kavaliers e Innes, 1987; Pollard e Dyer, 1985; Romero e Bodnar, 1986). Essas diferenças têm sido observadas especialmente em resposta a situações de estresse crônico (Gamaro et al., 1998). Portanto, considerando as alterações na nocicepção observadas após a exposição ao estresse repetido, a diferença sexual nestas respostas, e o possível envolvimento dos hormônios sexuais na lipoperoxidação, demonstrada pela diminuição da lipoperoxidação em alguns tecidos induzida pelo estradiol (Huh et al., 1994), nós realizamos nosso próximo trabalho, que consistiu na avaliação da produção de radicais livres, da lipoperoxidação e da reatividade antioxidante total em medula espinal de ratos machos e fêmeas, estressados cronicamente.

Os resultados obtidos nos mostram que o estresse repetido diminui a reatividade antioxidante na medula espinal, tanto em machos quanto em fêmeas, e que existe uma diferença sexual nesta diminuição, com uma TAR significantemente maior na medula de fêmeas em comparação com machos. É interessante observar que o efeito do gênero na TAR é observada especialmente nos animais controle, havendo uma interação marginal entre estresse e gênero, observada por uma redução na TAR mais pronunciada em fêmeas após o estresse repetido.

Não observamos efeito do estresse repetido sobre a lipoperoxidação em ratos machos. Por outro lado, obtivemos um significante aumento nos níveis de DCF na medula das ratas fêmeas, quando comparadas com machos.

A exposição ao estresse ou a GCs, como já foi comentado, provoca danos oxidativos em vários tecidos. No nosso estudo, no entanto, o estresse repetido não causou alteração na lipoperoxidação (medida pelos níveis de TBARS) ou na produção de radicais livres (medida pela produção de DCF). Por outro lado, um aumento nos níveis de TBARS tem sido mostrado em diferentes estruturas cerebrais após exposição ao estresse (Manoli et al, 2000), inclusive no presente trabalho de tese. Uma explicação plausível seria uma TAR aumentada nesta estrutura, a

medula espinal, o que a protegeria contra a produção de EROs. Neste caso, a diminuição da reatividade antioxidante nos animais estressados poderia representar o consumo de antioxidantes com o intuito de manter o balanço oxidativo.

As diferenças sexuais que nós observamos na TAR e na produção de radicais livres podem ser originadas tanto por fatores concernentes à organização (por exemplo, diferentes alterações plásticas na resposta ao estresse crônico em machos e em fêmeas), quanto à ativação por hormônios sexuais (estes podem influenciar a resposta ao estresse oxidativo) ou por ambos. Nesse contexto, um dos hormônios sexuais que têm efeito sobre o estresse oxidativo é o estradiol (Lacord et al., 1995; Niki e Nakano, 1990; Howard et al., 2001; Lacy et al., 2000). Os estrógenos e seus precursores podem ser liberados pelas glândulas adrenais, assim como pelos ovários (Fajer et al., 1971). Seus receptores foram identificados na medula espinal de ratos, onde mostram variação na densidade de acordo com a fase do ciclo estral (Bradshaw et al., 2000). Esses hormônios têm propriedades antioxidantes e podem inibir a lipoperoxidação *in vitro*. Este fato pode contribuir diretamente para o seu efeito neuroprotetor (Lacor et al., 1995; Niki e Nakamo, 1990). Relatos de efeitos do gênero sobre a produção de hidroperóxido plasmáticos, onde os homens apresentam maior produção destas substâncias que as mulheres (Lacy et al., 2000) reforçam esta teoria. Porém, apesar de seus efeitos neuroprotetores, alguns efeitos neurotóxicos dos estrógenos também têm sido demonstrados. Por exemplo, substâncias químicas semelhantes ao estrógeno induzem a formação do radical livre hidroxil (Obada e Kubota, 2000); estrógenos também apresentam efeito neurotóxicos sobre neurônios beta-endorfínicos, efeito prevenido pelo tratamento com a vitamina E, o que sugere que este efeito seja mediado por radicais livres (Desjardins et al., 1992).

Não está claro como um aumento da produção de radicais livres, observado em fêmeas, não altera o dano em lipídeos. No entanto, é possível que o aumento na reatividade antioxidante,

também observada nestas amostras, possa ajudar na explicação deste fenômeno. Como já foi mencionado, a medida da TAR nos fornece um valor muito útil da capacidade de um componente ou fluido de modular o dano associado com o aumento da produção de radicais livres (Lissi et al., 1995). Dessa forma, o aumento na TAR observada na medula de fêmeas em relação aos machos poderia compensar o aumento na produção de radicais livres, o que preveniria alterações na lipoperoxidação. Os resultados obtidos neste estudo sugerem que o estresse repetido diminui a reatividade antioxidante tanto em machos quanto em fêmeas e que existe uma diferença sexual nesta diminuição, assim como na produção de radicais livre, sem que haja alterações na lipoperoxidação. Estes efeitos podem ser responsáveis, ao menos em parte, pelas alterações induzidas pelo estresse e é possível que as diferenças hormonais possam contribuir para estas alterações.

Finalmente, no último trabalho nós investigamos a influência do esteróide gonadal, 17-β-estradiol, em fêmeas ooforectomizadas (OVX), submetidas ao estresse repetido por contenção, sobre a nocicepção. Nós também investigamos a influência deste hormônio sobre a hidrólise de ATP, ADP e AMP em sinaptossomas preparados a partir da medula destes animais, uma vez que o ATP e a adenosina podem atuar como neuromodularores da dor e sua degradação/produção recebem influência tanto do estresse quanto do estradiol.

Os resultados aqui obtidos nos mostram que as fêmeas estressadas, com ou sem reposição hormonal, apresentam menor limiar nociceptivo quando comparadas com as controles, de modo semelhante aos efeitos observados em machos (Gamaro et al., 1998). Entretanto, a ausência de efeito do estresse crônico sobre a nocicepção, como descrito anteriormente em fêmeas (Gamaro et al., 1998), possivelmente se deva às variações induzidas por diferenças nas fases do ciclo estral destas ratas. De qualquer forma, a ausência de hiperalgesia observada no estudo de

Gamaro et al. (1998), quando a fase do ciclo não é controlada, não deve ser causada somente pelo estradiol, visto que a reposição deste hormônio não interfere com os efeitos do estresse crônico sobre a nocicepção.

Também verificamos uma diminuição da atividade da 5'-nucleotidase em sinaptossomas de medula espinal, sugerindo uma diminuição nos níveis de adenosina extracelular após o estresse repetido, um efeito revertido pela reposição hormonal.

Como já foi mencionado, alguns relatos envolvendo uma relação entre esteróides ovarianos e vias nociceptivas incluem o sistema opióide (Danzebrink et al., 1995), a regulação da liberação de neurotransmissores (Loscher et al., 1992) e a possível interação com cascatas de degradação/produção de neurotransmissores, como a observada neste estudo. Além disso, os hormônios gonadais parecem alterar a nocicepção afetando circuitos supraespinais mais que os espinais (Ceccarelli et al., 2003) e, como o teste de *tail flick* avalia um reflexo espinal, é possível que a depleção destes hormônios e/ou a reposição de estradiol atue por meio de diferentes mecanismos, que podem ser observados de acordo com o teste utilizado para medida de nocicepção.

Considerando os efeitos do estresse, os hormônios esteróides podem regular a síntese do hormônio liberador de corticotrofina (CRH), um mecanismo pelo qual a nocicepção induzida pelo estresse poderia ser modulada em fêmeas (Vamvakopoulos e Chrousos, 1993).

Nós não observamos diferenças na hidrólise de ATP ou ADP em sinaptossomas de medula espinal em resposta ao estresse repetido, enquanto houve uma diminuição na hidrólise de AMP nas ratas OVX. Por outro lado, as ratas OVX que receberam reposição hormonal com estradiol não exibiram a mesma redução da atividade da 5'-nucleotidase em sinaptossomas de medula espinal, sugerindo que a presença do estradiol previne este efeito da exposição ao estresse repetido. Aqui é importante lembrar que os receptores de estrógeno não estão presentes

somente nas estruturas supraespinais, mas também nas camadas superficiais do corno dorsal da medula, a área onde ocorre o processamento inicial da sensação da dor (Papka et al., 2001).

Estes resultados sugerem que a atividade da 5'-nucleotidase em sinaptossomas de medula espinal pode ser modulada pelo estresse. Este efeito pode ser responsável, em parte, pelos efeitos hiperalgésicos em animais cronicamente estressadados, uma vez que a liberação de adenosina parece ter importância funcional na regulação do limiar de dor ao nível da medula espinal (Jurna, 1984; Sawynok et al., 1986), incluindo a mediação de um componente da analgesia medular pela morfina e pela serotonina (De Lander e Hopkins, 1986; 1987).

Muitos trabalhos demonstram que o estrógeno está associado com mudanças na atividade da ATP-difosfoidrolase e/ou 5'-nucleotidade em diferentes tecidos (Valenzuela et al., 1992; Faas et al., 1997; Yoneyama et al., 2002; Garcia et al., 1985; Ramalingam et al., 1993). No presente estudo, a reposição hormonal não induziu alterações na atividade destas enzimas em sinaptossomas de medula espinal. Este hormônio, entretanto, previne a redução na atividade da 5'-nucleotidase induzida pela exposição ao estresse repetido.

Estudos feitos em animais mostram a influência inibitória mediada pela adenosina ou por análogos desse nucleosídeo na resposta reflexa nociceptiva (Sawynok, 1998; Sollewi, 1997). O corno dorsal da medula contém os dois receptores de adenosina: A_1 e A_2 (Choca et al., 1987; Sawynok e Sweeny, 1989). A modulação da neurotransmissão pela adenosina através desses dois receptores ocorre em diferentes regiões do SNC, de modo que diferentes efeitos são observados quando distintas regiões são consideradas (Lamber e Tayler, 1991; Thompson et al., 1993). Os receptores A_1 estão associados com efeitos modulatórios da transmissão da dor na medula (Keil e DeLander,1996). Os agonistas A1 parecem agir pré-sinapticamente inibindo a liberação de neurotransmissores ou pós-sinapticamente reduzindo a excitabilidade neuronal (Hass e Selbach, 2000; Stone, 2002).

Considerando que as ratas expostas ao estresse repetido apresentaram atividades de ATPase e ADPase similares aos controles, com inibição na atividade da 5'-nucleotidase, é possível que a quantidade de ATP/ADP acumulados seja suficiente para manter a atividade da 5'nucleotidase inibida (conforme citado acima, essa enzima é inibida por ATP e ADP), o que causa uma diminuição nos níveis de adenosina extracelular. É claro que, como já foi mencionado nesta Discussão, os baixos níveis de adenosina extracelular não são a única explicação para a hiperalgesia observada neste trabalho, pois a reposição de estradiol reverte o efeito inibitório do estresse repetido sobre a atividade da 5'-nucleotidase, mas não é suficiente para reverter o efeito hiperalgésico induzido pelo estresse.

Outro fator que deve ser considerado é o fato de que outros trabalhos utilizando sinaptossomas de hipotálamo e córtex cerebral de ratos machos expostos ao estresse repetido não mostraram qualquer alteração na hidrólise de ATP ou ADP (Torres et al., 2002b); a atividade da ADPase, porém, se mostrou diminuída e a atividade da 5'-nucleotidase se mostrou aumentada em sinaptossomas de medula espinal de animais submetidos ao mesmo tratamento (Torres et al., 2002a). Portanto, a redução na hidrólise do AMP observada neste estudo em sinaptossomas de medula espinal é gênero-específica.

Os mecanismos através dos quais o estresse repetido pode modular a atividade da 5'-nucleotidase ainda não estão claros. Entretanto, como estamos analisando uma situação crônica, é possível que esses mecanismos envolvam um aumento na expressão do mRNA para esta enzima.

Resumindo, os resultados aqui obtidos nos mostram que as fêmeas estressadas, com ou sem reposição com estradiol, apresentam menor limiar nociceptivo quando comparadas com as controles. Também se sugere uma diminuição nos níveis de adenosina extracelular (pela diminuição da atividade da 5'-nucleotidase) após o estresse repetido, um efeito revertido pela

reposição hormonal. Adicionalmente, este efeito poderia estar relacionado com a diminuição na TAR na medula, observada após o estresse repetido. Essas observações sugerem que a sensibilidade à dor depende de um sistema regulatório complexo e que a modulação da neurotransmissão pelos nucleotídeos da adenina na medula espinal depende de uma interação do hormônio 17-β-estradiol com a exposição ao estresse. Entretanto, a redução da produção de adenosina extracelular não é a única explicação da hiperalgesia observada após a exposição ao estresse repetido, uma vez que a reposição de estradiol normaliza a sua produção, mas não é suficiente para reverter a resposta hiperalgésica ao estresse repetido.

4.2 Efeitos do estresse sobre o hipocampo

A literatura nos fornece muitas evidências de que o estresse crônico causa déficit de memória (Bodnoff et al., 1995; Nishimura et al., 1999; Conrad et al., 1996; McLay et al., 1998). De acordo com esses dados, nós também observamos diminuição da memória espacial em ratos estressados repetidamente. Este efeito, entretanto, foi pequeno, notado pela diminuição no número de cruzamentos no local da plataforma, um dos parâmetros avaliados pelo labirinto aquático de Morris. Os ratos estressados cronicamente não diferiram dos controles em outras tarefas de memória (esquiva inibitória e reconhecimento de objeto).

O hipocampo tem um papel importante na cognição e na função neuroendócrina (Jarrard, 1993) e também é sabidamente crucial para a consolidação da memória em diferentes testes, incluindo o labirinto aquático (White et al., 2003; Ferbinteanu et al., 2003). Por outro lado, a tarefa de reconhecimento de objetos, um teste de memória que não é aversivo nem envolve memória espacial, parece não ser dependente do hipocampo, uma vez que lesões nesta estrutura e no fórnix não afetam significativamente o desempenho nesta tarefa (Ennaceur e Aggleton,

1994; Rothblat e Kromer, 1991). Assim sendo, o estresse repetido, conforme utilizado no modelo empregado neste trabalho de tese, parece afetar em certo grau apenas a memória espacial.

Embora o efeito deletério do excesso de glicocorticóides no cérebro tenha sido amplamente demonstrado, a extensão do comprometimento deste órgão em situações de estresse repetido depende de algumas variáveis como a intensidade e a previsibilidade do agente estressor usado. Talvez esse seja um dos motivos do pequeno comprometimento cognitivo observado após o estresse crônico repetido, diferentemente do que se observa após o estresse crônico variado (Vasconcellos et al., 2003), cuja principal característica é a imprevisibilidade da exposição ao estresse. Além disso, os animais expostos ao modelo de estresse repetido por contenção apresentam uma adaptação fisiológica ao estressor, como pode ser observado pelos níveis diminuídos de corticosterona plasmática nesses animais após uma sessão de estresse, quando comparada com os níveis em animais expostos a um estresse agudo (Torres et al., 2001a). Por outro lado, estudos usando um modelo de estresse mais severo, como 6 horas de contenção/dia, observaram uma perda de memória, inclusive na tarefa de reconhecimento de objetos ((Beck E Luini, 1999).). Possivelmente este estresse mais intenso tenha conseqüências igualmente mais marcantes sobre a memória, comparado com o modelo utilizado neste trabalho.

A partir deste trabalho, nós iniciamos a investigação de fatores que estariam relacionados com a memória e que fossem afetados pelo estresse ou pela corticosterona, liberada em resposta a um agente estressor.

Considerando as evidências da literatura de que tanto o estresse agudo quanto o tratamento crônico com corticosterona interferem no metabolismo do glutamato no hipocampo e que estes efeitos ocorrem em paralelo com o aumento de corticosterona nesta estrutura (Lowy et al., 1993; Moghaddam, 1993; Venero e Borrel, 1999; Stein-Behrens et al., 1994), nós

imaginamos que as alterações hormonais associadas à exposição ao estresse agudo ou crônico poderiam ter o mesmo efeito.

O glutamato (Glu) é considerado o maior mediador de sinais excitatórios no SNC de mamíferos, sendo sua ação mediada por receptores ionotrópicos e metabotrópicos. Este aminoácido está envolvido em muitos aspectos do funcionamento normal do cérebro, incluindo cognição, memória e aprendizado (Fonnum, 1984; Headley e Grillner, 1990; Danboldt, 2001). O acúmulo de glutamato na fenda sináptica, porém, pode lesar os neurônios por excitotoxicidade. Assim, a captação de glutamato, realizado por proteínas localizadas nos astrócitos e nos terminais nervosos, é o mecanismo da terminação da neurotransmissão glutamatérgica e prevenção da excitotoxicidade (Danbolt, 2001). Assim, nós resolvemos investigar o efeito da exposição aguda e crônica ao estresse repetido por contenção sobre a captação e liberação de glutamato em sinaptossomas de hipocampo de ratos. Além disso, também investigamos o efeito destes tratamentos sobre o binging de [^{3}H] glutamato nas membranas sinápticas destes animais.

Com este trabalho pudemos concluir que o estresse repetido por contenção aumenta tanto a captação quanto a liberação basal de glutamato, além de causar uma diminuição no *binding* de [^{3}H] glutamato nas membranas sinápticas. Uma vez que estes efeitos não foram observados após o estresse agudo, imaginamos que este poderia ser uma resposta adaptativa neuroprotetora ao tratamento crônico. Todas estas medidas foram feitas 24h após a exposição ao estresse, com o intuito de avaliarmos somente a resposta adaptativa ao estresse repetido, sem a interferência das alterações hormonais observadas logo após a exposição à contenção. Os experimentos utilizando o modelo de estresse agudo, da mesma forma, foram feitos a fim de podermos comparar os efeitos dos dois tratamentos. A ausência de resultados observados neste trabalho 24h após a exposição ao estresse agudo está de acordo com dados literatura, que mostram um aumento na medida de glutamato extracelular feita no hipocampo após 1 hora de contenção, sendo o retorno

aos seus níveis basais observado 2 horas após (McEween, 2002). Uma vez que esses efeitos são imediatos, eles parecem ocorrer por mecanismos não genômicos, provavelmente via proteína G (Venero e Borrel, 1999).

Em relação ao aumento da captação e da liberação basal de glutamato, observadas 24 horas após a última sessão de estresse crônico repetido, é importante notar que este período pós-estresse é suficiente para que os níveis de corticosterona retornem aos seus valores basais e que o aumento nestes hormônios, após a 40ª exposição à contenção, é muito menor do que o aumento observado após a primeira exposição (Torres et al., 2001a). Esses fatos nos sugerem uma adaptação fisiológica do organismo ao agente estressor. Também podemos pensar que os efeitos observados aqui, ocorridos 24 horas após a última sessão de estresse, possam envolver efeitos genômicos causados pelos glicocorticóides (McEween, 1999; McEween e Magarinos, 1997), embora nós não possamos excluir outros fatores relacionados à exposição repetida ao estresse que também poderiam induzir estas alterações.

O aumento da recaptação de glutamato, em sinaptossomas de hipocampo de ratos estressados repetidamente, pode ser um mecanismo protetor, envolvendo a modulação da neurotransmissão glutamatérgica e prevenção da neurotoxicidade (Danbolt, 2001). Este efeito pode estar associado com um aumento no número de sítios de transporte de glutamato nas membranes pré-sinápticas, ou com mudanças funcionais destes transportadores.

Embora neste trabalho nós tenhamos medido a captação de glutamato somente em sinaptossomas neuronais, é importante considerar que esta é apenas uma parte da captação total e que a glia tem um papel relevante na manutenção da concentração de glutamato abaixo de níveis neurotóxicos para o cérebro (Danbolt, 2001; Rothstein et al., 1996; Tanaka et al., 1997). Stein-Behrens e colegas (1992) demonstraram que os glicocorticódes diminuem a captação de glutamato em astrócitos hipocampais, o que sugere que os efeitos do estresse observados aqui

não sejam causados por transportadores gliais, os quais poderia estar contaminando nossa fração sinaptossomal. Essa consideração é reforçada pelo fato de não haver aumento na captação de glutamato por fatias de hipocampo após este modelo de estresse crônico (ver adiante, nesta tese).

Além disso, comparando um trabalho realizado em ratos expostos a 21 dias de estresse repetido, cuja captação de glutamato se mostrou diminuída em sinaptossomas de córtex cerebral, medida imediatamente após a sessão de estresse (Olivenza et al., 2000), um resultado contrário ao observado neste trabalho de tese, podemos sugerir que diferentes intensidades e períodos de estresse são capazes de alterar diferentemente a dinâmica glutamatérgica no SNC e que estas alterações são região e tempo dependentes.

Quanto ao aumento na liberação basal de glutamato por nós observada, devemos considerar o seguinte: esta liberação de glutamato citoplasmático pode ocorrer pela recaptação reversa feita pelos próprios transportadores aclopados ao Na^+. Por exemplo, em condições de falta de energia, como em uma isquemia ou uma hipoglicemia, o gradiente eletroquímico é grandemente reduzido e os transportadores de glutamato podem funcionar carregando glutamato do citoplasma para o exterior (Nicholls e Atwell, 1990), o que contribuiria com a excitotoxicidade glutamatérgica. Por outro lado, para que possamos medir a liberação de glutamato, é necessário que a fração sinaptossomal seja incubada na presença de [^{3}H] glutamato para produzir sinaptossomas marcados. Assim, um aumento da liberação basal poderia ser resultado do aumento da captação de glutamato por esta fração, também observada após o estresse repetido.

O ATP é outro neurotransmissor que atua no sistema nervoso central e periférico. (Edwards et al., 1992). Em situações fisiológicas, o ATP existe em baixas concentrações. No entanto, em condições patológicas, grandes quantidades de ATP extracelulares podem causar morte celular (Inoue, 2002). Como já foi descrito na Introdução, o ATP liberado na fenda

sináptica pode ser hidrolisado a adenosina, um agente neuromodulador e neuroprotetor (Figura 2), através de enzimas que parecem ter um papel na modulação e no controle da transmissão sináptica excitatória (Cunha, 2001a; Cunha e Ribeiro, 2000). Desse modo, considerando que os nucleotídeos da adenina, assim como a adenosina, estão envolvidos na modulação de vários processos fisiológicos e patológicos e que seus níveis podem estar alterados em situações de estresse, nós avaliamos o efeito do estresse agudo e do estresse repetido sobre a atividade das enzimas ATP difosfoidrolase e 5'-nucleotidase, via hidrólise do ATP, ADP e AMP, em sinaptossomas hipocampais de ratos adultos. Nós pudemos observar diferentes efeitos nas duas condições estudadas, sugerindo o envolvimento de diferentes enzimas nestas situações. Nossos experimentos mostraram um significante aumento na hidrólise de ATP, ADP e AMP em resposta ao estresse agudo, sugerindo a ativação da ATP-difosofoidrolase e da 5'-nucleotidase; e um aumento somente de uma ecto-ATPase induzido por estresse repetido, possivelmente resultante de uma adaptação deste tratamento.

Com relação ao aumento na hidrólise de ATP, ADP e AMP obtido em resposta ao estresse agudo, esse resultado sugere uma ativação da enzima ATP-difosfoidrolase, uma vez que o ADP é considerado o substrato marcador para a atividade dessa enzima (Battastini et al., 1991). Além disso, a ativação similar observada na hidrólise do ATP (21%) e do ADP (21%) sugere um paralelismo no comportamento cinético (perfil similar para ambos os substratos), o que também é característica da ATP difosfoidrolase (Sarkis e Salto, 1991; Frassetto et al., 1993). O estresse agudo também induziu um aumento na atividade da 5'-nucleotidase e, provavelmente, nos níveis de adenosina da cascata de hidrólise do ATP extracelular. A reação catalisada pela 5'-nucleotidase é considerada um passo limitante na via de formação da adenosina a partir do ATP extracelular, uma vez que ela pode ser inibida pelo ATP e/ou ADP (para uma revisão a esse respeito, veja Cunha e Ribeiro, 2000). Desse modo, a adenosina será formada em quantidades

significativas somente em situações de níveis baixos de ATP e ADP, inferiores ao limiar de inibição da 5'-nucleotidade. No caso do estresse agudo, as atividades de hidrólise de ATP e de ADP estão aumentadas, o que permite a rápida conversão do ATP a ADP, e do ADP a AMP. Com isso, os níveis de ATP e ADP estarão diminuídos e o AMP, substrato para a 5'-nucleotidase, estará aumentado, assim como a atividade dessa enzima.

Esses resultados nos permitem sugerir que, após uma única exposição ao estresse por contenção, um efeito a longo prazo (aproximadamente 22 horas) envolvendo a degradação enzimática do ATP extracelular irá resultar no aumento da adenosina extracelular no hipocampo. Muitos trabalhos têm documentado um aumento na concentração de adenosina extracelular após situações metabólicas estressantes, tais como hipóxia e isquemia (Latini e Pedata, 2001; Pearson et al., 2001), ou como glicoprivação pela administração de 2-deoxi-D-glicose (Minor et al., 2001). O termo "metabólito retaliatório" tem sido usado para este papel homeostático da adenosina, a qual acorre em todos os tipos de célula (para uma revisão, veja Cunha 2001b). Embora esse nucleosídeo possa se ligar aos receptores A_2 causando um aumento na liberação de neurotransmissor no hipocampo (Cunha et al., 1992), a maioria dos trabalhos mostram uma ação inibitória da adenosina nesta estrutura. Os agonistas A_1 parecem agir nos neurônios pré-sinápticos, inibindo a liberação de neurotransmissor, ou em neurônios pós-sinápticos, reduzindo a excitabilidade neuronal (Hass e Selbach, 2000; Stone, 2002). Uma vez que o hipocampo é particularmente sensível a diversas injúrias (p.e., Inoue, 1998), este resultado sugere a presença de uma resposta fisiológica, a fim de proteger esta estrutura do dano causado pela exposição a agentes estressores.

Também nesse trabalho, em contraste aos efeitos observados após o estresse agudo, ratos repetidamente estressados apresentaram aumento somente de uma ecto-ATPase, sem nenhuma mudança significante na atividade da ADPase e da 5'-nucleotidase em sinaptossomas de

hipocampo de ratos. A aparente dissociação entre os dois substratos (ATP e ADP), neste caso, poderia ser devida à presença simultânea de duas diferentes ecto-enzimas envolvidas na hidrólise do ATP; uma ecto-ATP difosfoidrolase (NTPDase3) e uma ecto-ATPase (NTPDase2) (Kegel et al., 1997; Zimmermaen, 1996). Aqui é importante ressaltar que somente uma enzima, uma ecto-ATP difosfoidrolase (NTPDase3), está envolvida na hidrólise do ADP. Portanto, é razoável pensar que o aumento observado somente na hidrólise do ATP, e não na hidrólise do ADP, após o estresse repetido, envolva a participação de uma ecto-ATPase, excluindo-se uma alteração na atividade da ecto-ATP difosfoidrolase, na modulação da resposta a este tratamento. O aumento da hidrólise do ATP após o estresse repetido também pode constituir um mecanismo protetor, devido ao potencial efeito neurotóxico deste nucleotídeo (Schulze-Lohoff et al., 1998), o qual atua como um neurotransmissor excitatório, envolvido em eventos neurodegenerativos (Danbolt, 2001). Mais uma vez, as diferenças observadas neste trabalho na atividade das enzimas após exposição ao estresse agudo ou crônico sugerem uma adaptação do organismo a estas situações.

Ainda, em estudos medindo a atividade destas enzimas em outras estruturas do SNC de ratos expostos ao estresse repetido, realizados anteriormente em nosso laboratório, não foram observados efeitos na hidrólise do ATP ou do ADP em hipotálamo e córtex cerebral (Torres et al., 2002b), enquanto uma diminuição na atividade da ADPase foi observada em sinaptosomas de medula (Torres et al., 2002a). Esses resultados nos fazem acreditar que o aumento da atividade da enzima ATPase em sinaptossomas de hipocampo após o estresse repetido, observado nesse estudo, é específico para esta estrutura, e que tais alterações observadas em tecidos ou estruturas particulares, podem refletir as diferentes funções dos nucleotídeos/nucleosídeo e das enzimas estudadas nessas regiões do SNC.

O estresse oxidativo é outro fator amplamente relacionado a doenças neurodegenerativas (McIntosh e Sapolsky, 1996). A literatura relata o envolvimento do estresse emocional, bem

como da administração de GCs, na formação de espécies ativas de oxigênio e na diminuição das defesas antioxidantes, fatores que podem levar ao dano oxidativo no cérebro (Liu e Mori, 1994; Manoli et al., 2000; Sosnovsky e Kozlov, 1992). Nós realizamos, então, um trabalho visando avaliar as possíveis alterações na homeostase oxidativa induzidas pelo estresse repetido, utilizando os seguintes parâmetros: produção de radicais livres, lipoperoxidação, capacidade antioxidante total (potencial e reatividade antioxidante) e atividade de enzimas antioxidantes (CAT, SOD e GPx) no hipocampo de ratos. Os nossos resultados nos permitiram concluir que este modelo de estresse repetido induz estresse oxidativo. Estas alterações incluem o aumento na lipoperoxidação, assim como o consumo de defesas antioxidantes, evidenciado pela reduzida reatividade antioxidante total. Esta diminuição provavelmente se deva à redução de antioxidantes não enzimáticos, uma vez que as enzimas antioxidantes estudadas tiveram suas atividades aumentada ou inalteradas pelo tratamento crônico.

Os resultados por nós observados concordam com os dados da literatura, que mostram que a exposição a CGs ou ao estresse pode induzir a formação de EROs e levar ao dano oxidativo em vários tecidos, incluindo sangue (Oishi et al., 1999) e cérebro (Sudakov e Sosnovsky, 1996; Liu et al., 1996; McIntosh e Sapolsky, 1996). O efeito do estresse na produção de TBARS também tem sido observado, sendo que distintas regiões do cérebro mostram diferentes respostas a este tratamento (e.g., Manoli et al., 2000). Além disso, também um significante aumento nos níveis de oxidação de proteínas foi observada em diferentes regiões do cérebro de animais estressados, quando comparados com os controles (Liu et al., 1996). Assim, os nossos resultados suportam a idéia de que o estresse produz oxidantes, e é possível que o dano oxidativo possa contribuir, ao menos em parte, com as alterações induzidas pelo estresse crônico.

A diminuição nas defesas antioxidantes no hipocampo, córtex e cerebelo tem sido

sugerida como um possível componente no dano neuronal mediada por GCs (McIntosh e Sapolsky, 1996; McIntosh et al., 1998; Abraham et al., 2001; Patel et al., 2002). Essa diminuição da capacidade de defesa neuronal contra o dano oxidativo seria a causa do aumento da vulnerabilidade de diferentes regiões do cérebro, particularmente o hipocampo, a insultos metabólicos.

Exatamente como ocorre o aumento da lipoperoxidação no hipocampo de animais estressados, na ausência de qualquer alteração no conteúdo de radicais livres (avaliado pela medida de DCF), não nos é clara. É possível que ocorra um aumento na produção de radicais livres durante o estresse, e que estes níveis retornem ao normal 24 horas após a exposição, quando os animais foram sacrificados. Os produtos da lipoperoxidação poderiam ainda estar presentes por não ser tão fácil a recuperação de danos aos lipídeos.

Em nosso estudo, o estresse repetido também induziu uma diminuição na TAR no hipocampo 24h após a exposição ao estresse, sem que a TRAP tenha sido alterada. De acordo com Lissi et al. (1995), em uma amostra biológica complexa, a capacidade antioxidante total corresponde à concentração de antioxidante e ao número de radicais livres "neutralizados" por cada molécula, a qual representa a quantidade de antioxidante. Entretanto, em um sistema exposto a um contínuo ataque de radicais livres, mais importante é a capacidade do meio de regular, com sua concentração basal de antioxidantes, os danos causados pelos radicais livres; tornando-se mais importante considerar a qualidade das substâncias antioxidantes presentes no tecido, o que é medido pela TAR. Assim, a diminuição na TAR no hipocampo de ratos estressados repetidamente sugere uma maior vulnerabilidade deste tecido a substâncias oxidantes.

Nós também observamos um aumento na atividade da GPx, uma enzima que cataliza a redução de hidroperóxidos, usando glutationa reduzida (GSH) e produzindo glutationa oxidada

(GSSG). Esta reação catalizada pela GPx é considerada um importante mecanismo de interrupção da reação em cadeia de peroxidação de lipídeos. A atividade de GPx no hipocampo parece aumentar após injúria excitotóxica (Gilberti e Trombetta, 2000) e um aumento na atividade desta enzima indica um aumento nos peróxidos celulares. Junto com o resultado de TBARS, estes dados nos sugerem que o estresse repetido é capaz de induzir a produção de EROs.

A GSH, a qual é oxidada pela GPx, é sabidamente envolvida nas reações de detoxificação em tecidos de animais, plantas e microorganismos (Meister, 1988). Devido à sua alta concentração intracelular (0.1-10 mM), a GSH serve como o principal peptídeo redutor nas células, contribuindo com seu grupamento sulfidril no tamponamento e remoção de radicais livres gerados durante processos metabólicos, como a respiração, assim como durante o estresse oxidativo (Voehringer, 1999). A GSH hipocampal também é significantemente diminuída após uma injúria excitotóxica (Gilberti e Trombetta, 2000; Saija et al., 1994). Em concordância com os nossos resultados, esses estudos propõem a teoria de que a perda de GSH poderia ocorrer durante um evento oxidativo como um mecanismo de proteção, devido a sua utilização. Em condições normais, a GSSG é formada na célula e imediatamente reduzida a GSH pela glutationa redutase, usando NADPH como coenzima. Em situações de estresse oxidativo, a capacidade desta enzima em realizar esta reação pode estar comprometida. Além disso, Patel e colegas (2002) demonstraram uma diminuição nos níveis de NADPH após o tratamento com corticosterona, outro mecanismo que poderia induzir uma redução nos níveis de GSH e, conseqüentemente, na TAR.

Em resumo: a diminuição na TAR por nós observada não parece estar relacionada com a atividade de enzimas; então, esta redução possivelmente se deva à diminuição de antioxidantes não enzimáticos, como, por exemplo, da GSH (Patel et al., 2002), a qual estaria sendo consumida

pelo aumento de espécies oxidantes produzidas pelo estresse repetido (veja o resultado de lipoperoxidação). A ação da GSH como um "scavenger" de radicais livres é realizada possivelmente graças à atividade da GPx, a qual se encontra aumentada nesta situação. Esses efeitos poderiam ter implicações funcionais, uma vez que o aumento da atividade da GPx pela adição de GSH exógena, demonstrada por Saija e colegas (1994), protege contra a excitotoxicidade.

Além de seu envolvimento no dano de tecidos, alterando macromoléculas, os radicais livres de oxigênio também influenciam a função cerebral alterando a transdução de sinal através da membrana, e mudando as propriedades de vários receptores que possuem ligações dissulfeto funcionais, cujo estado redox induz mudanças conformacionais. A respeito disso, crescentes evidências indicam que os transportadores de glutamato também podem sofrer mudanças funcionais, dependendo do aumento na oxidação ou redução de grupamentos sulfidrila presentes em sua estrutura (Trotti et al., 1997; 1998), tornando-os vulneráveis a oxidantes biológicos e podendo resultar na diminuição da captação por estes transportadores. Assim, diferentes níveis de radicais livres nas estruturas cerebrais após o estresse crônico poderiam influenciar a resposta a esses neurotransmissores e, desta forma, fazer parte de uma constelação de fatores envolvidos na adaptação ao estresse crônico. No caso do modelo estudado neste trabalho de tese, porém, os efeitos observados sobre o transporte de glutamato por sinaptossomas provavelmente não são devidos a alterações no estresse oxidativo, pois aumento do estresse oxidativo, como observado, poderia prejudicar o transporte de glutamato, e observamos o efeito contrário, isto é, um aumento tanto da captação quanto da liberação basal desse neurotransmissor.

Para finalizar os nossos estudos neuroquímicos sobre os efeitos do estresse repetido no cérebro e, conseqüentemente, sobre os possíveis mecanismos do prejuízo na memória, nós

realizamos um trabalho visando avaliar a influência do estresse repetido por contenção sobre a vulnerabilidade neuronal. Para tanto, submetemos slices de hipocampo de ratos estressados aguda ou repetidamente a um modelo de isquemia *in vitro* (OGD). A partir disso, nós medimos o dano celular utilizando métodos que avaliam a integridade da membrana (LDH e NSE), a secreção celular de uma proteína produzida por astrócitos e liberada em situações de injúria (S100B), assim como a captação de glutamato em fatias de hipocampo de ratos estressados, submetidas à isquemia *in vitro*.

Como sabemos, o metabolismo aeróbico é a principal fonte de energia no cérebro e pode ser comprometido pela interrupção do fornecimento de substratos e por alterações no metabolismo no cérebro, como as condições resultantes de uma isquemia. A exposição de fatias hipocampais a um modelo de isquemia in vitro (OGD), seguido de um período de reoxigenação, resultou no aumento na liberação de LDH no meio, a qual é conseqüência de dano ou morte celular. Quando investigamos as conseqüências do estresse agudo ou repetido sobre a integridade celular após a OGD, nós observamos que o tecido de animais submetido a estes tratamentos induz diferente susceptibilidade aos danos causados pela isquemia. O estresse agudo não interfere com os efeitos da exposição à OGD, ou seja, quando as fatias de hipocampo foram submetidos à OGD imediatamente ou 24 horas após a exposição ao estresse agudo, somente o efeito da OGD foi observado. Por outro lado, as fatias de hipocampo de ratos repetidamente estressados apresentaram uma maior susceptibilidade à OGD. Ainda, o aumento no dano observado após este tratamento parece ser relacionado à morte de neurônios, uma vez que os mesmos resultados foram observados quando essas fatias foram incubadas com glicose (utilizada por neurônios e glia como fonte de energia) ou lactato (utilizado preferencialmente por neurônios); somado a isso, também observamos uma liberação aumentada de enolase neurônio-

específica (NSE) no meio após a OGD quando os animais foram previamente expostos ao estresse repetido.

Os mesmos mediadores possivelmente envolvidos no dano neuronal causado pela exposição ao estresse, p. e., glicocorticóides, EAA e ativação de receptores NMDA (Sapolsky, 2000), participam no dano e morte de neurônios piramidais pela isquemia (Rami et al., 1998; Lee et al., 2000). Acredita-se que esta seja uma das explicações para o fato do tratamento agudo e crônico com coricosterona aumentar o dano isquêmico (Antonawich et al., 1999; McEwen, 1999, 2001). No modelo de OGD utilizado aqui, entretanto, nenhum efeito foi observado após o estresse agudo por contenção, sugerindo que o tratamento agudo não aumenta a morte celular *in vitro*. Uma possível explicação para as diferenças observadas entre os efeitos da corticosterona *in vivo* e os efeitos do estresse agudo sobre a isquemia *in vitro* são os diferentes tempos usados para observar o dano induzido por este processo. Além disso, a resposta ao estresse compreende mais que a liberação de glicocorticóides, como, por exemplo, a liberação de neurotransmissores, os quais poderiam resultar em diferentes efeitos.

Conforme já foi comentado, a medida de LDH no meio nos mostrou que o estresse repetido aumenta os efeitos causados pela OGD neste parâmetro, o que poderia se interpretado como um aumento da vulnerabilidade destas células à isquemia. Esses resultados foram confirmados pelo aumento da NSE no meio, o qual é muito mais alto quando o tecido é submetido a ambas as situações, estresse e OGD. Estes resultados estão de acordo com dados da literatura que demonstram uma maior vulnerabilidade de neurônios hipocampais a insultos, incluindo hipóxia-isquemia (Sapolsky, 1999). Tem sido sugerido que a exposição crônica a altos níveis de GCs resulta na redução da plasticidade neuronal (Scheff et al., 1980; Daw et al., 1991; McEwen e Sapolsky, 1995). Alguns mecanismos sugeridos para esta vulnerabilidade aumentada de células hipocampais expostas a GCs incluem a diminuição da captação de glicose (Horner et

al., 1990), o aumento na liberação de glutamato (McEwen, 1999, 2000, 2001) e o aumento da produção de espécies ativas de oxigênio (ROS; Chan, 2001). Como vimos nesta tese, alguns desses mecanismos também poderiam ser responsáveis pela maior vulnerabilidade de fatias de hipocampo de ratos repetidamente estressados à OGD.

Por outro lado, com base na liberação de S100B no meio, nenhum dano a astrócitos parece ocorrer 3 horas após a reperfusão. Esta proteína é um importante marcador para monitoramento de dano e ativação de astrócitos (Petzold et al., 2003) e é também um modulador glial de plasticidade sináptica neuronal (Nishiyama et al., 2002). Uma vez secretada, a S100B glial exerce efeitos tróficos ou tóxicos dependendo de sua concentração: concentrações nanomolares de S100B estimulam o crescimento de neuritos e aumentam a sobrevivência de neurônios durante o desenvolvimento do SNC; já níveis micromolares de S100B extracelular estimulam, *in vitro*, a expressão de citocinas pró-inflamatórias e induzem apoptose (para uma revisão, veja Donato, 2001).

Estudos clínicos têm demonstrado correlação entre os níveis sorológicos de S100B e o grau de isquemia cerebral, quando esta proteína é medida várias horas após, enquanto nenhuma correlação é observada até 15 horas após o evento isquêmico (Jonsson et al., 2001). Em contraste à NSE, nenhum aumento específico nos níveis periféricos de S100B foram observados imediatamente após dano neuronal causado por trauma (Wunderlich et al., 1999) ou *status epilepticus* (Leutmezer et al., 2002). Essa menor liberação de S100B, encontrada em situações *in vivo*, tem sido atribuída ao papel desta proteína nos mecanismos de reparo no cérebro. Assim, é possível que, utilizando modelos de isquemia *in vivo*, possamos observar níveis elevados de S100B com tempos mais longos de reperfusão.

Quanto ao tônus glutamatérgico, cujo aumento causado tanto pelo estresse quanto pela isquemia leva a alterações na função hipocampal, podemos comentar que o aumento de

glutamato na fenda sináptica pode causar um dano neuronal excitotóxico, devido à superestimulação de seus receptores. O transporte de alta afinidade do glutamato, dependente de Na^+, é mediado por proteínas transportadoras localizadas principalmente em astrócitos, e este mecanismo tem mostrado ter um papel importante na terminação da neurotransmissão glutamatérgica e prevenção da excitotoxicidade (Danbolt, 2001). Um comprometimento da captação de glutamato em animais submetidos ao estresse crônico poderia contribuir para o aumento do dano induzido pela exposição à OGD nesses animais. Esta explicação, porém, não serviria para os danos observados nos nossos experimentos, já que a captação de glutamato foi comprometida somente pela OGD, sem que houvesse interferência do estresse crônico neste parâmetro; adicionalmente, a captação de glutamato por sinaptossomas, como vimos acima, está aumentada após esse modelo de estresse repetido. Então, embora sejam relatadas alterações na neurotransmissão glutamatérgica causada pelo estresse agudo (Lowy et al., 1993; Moghaddam, 1993), a habilidade do tecido, principalmente dos astrócitos, na recaptação de glutamato do meio não é comprometido após o estresse crônico, independente da exposição das células à OGD.

Em conclusão, nosso estudo a respeito da vulnerabilidade do hipocampo de animais estressados cronicamente à isquemia *in vitro* sugere que: (1) os efeitos da OGD na morte cellular de fatias de hipocampo não são alteradas quando este tratamento ocorre imediatamente ou 24h após o estresse agudo; (2) o estresse repetido por contenção piora o dano celular induzido pela OGD; (3) As células mais afetadas pela exposição ao extresse repetido + OGD são principalmente neurônios; (4) Os mecanismos responsáveis por tornar os neurônios do hipocampo mais suscetíveis ao dano após a isquemia não envolvem a captação glutamatérgica total, realizada principalmente pela glia. Não sabemos se a alteração do tônus glutamatérgico observado em sinaptossomas de neurônios hipocampais (aumento da liberação basal de glutamato) poderia contribuir para este efeito, mas fica aqui uma sugestão para futuras

investigações. Outro fator que poderia contribuir, ao menos em parte, para este efeito potencializador de dano observado após o estresse repetido poderia ser o maior estresse oxidativo observado no hipocampo de animais submetidos a este tratamento.

Assim, mesmo que o animal possa, através de diferentes mecanismos, adaptar-se à exposição a um estressor repetido sem que haja comprometimento das funções do organismo, essas alterações podem torná-lo mais susceptível a outros eventos estressantes.

5. CONCLUSÕES

A partir dos resultados obtidos neste trabalho de tese, podemos concluir que a exposição ao estresse repetido por contenção pode levar a alterações na plasticidade das duas estruturas estudadas: medula espinal e hipocampo.

Com relação aos efeitos sobre a nocicepção,

- animais estressados apresentam diferente impacto de estímulos aversivos ou de recompensa com relação à nocicepção, e respondem de forma diferente do grupo controle a sabores agradáveis e desagradáveis, sendo que esse efeito ocorre de uma maneira dependente da natureza do estímulo;

Com relação aos efeitos neuroquímicos sobre a medula espinal,

- o estresse repetido diminui a reatividade antioxidante de forma gênero-dependente;
- o estresse repetido diminui o limiar nociceptivo em fêmeas, com ou sem reposição com estradiol;

Essa diminuição do limiar nociceptivo após o estresse repetido, a possível diminuição nos níveis de adenosina extracelular (sugerida pela diminuição da atividade as 5'-nucleotidase), assim como a diminuição da reatividade antioxidante na medula espinal podem ser fênomenos interrelacionados; porém, essas provavelmente não são as únicas explicações para a resposta hiperalgésica ao estresse repetido.

Com relação aos efeitos sobre a memória,

- o estresse repetido causa prejuízo da memória espacial, sem prejuízo aparente em outras tarefas de memória;

Com relação aos efeitos neuroquímicos sobre o hipocampo,

- o estresse repetido promove alterações na atividade glutamatérgica e um aumento no estresse oxidativo nessa estrutura;
- outros efeitos plásticos são observados, os quais podem conferir uma maior neuroproteção contra a exposição repetida a um estressor: aumento na recaptação neuronal de glutamato (possivelmente sem alteração na captação glial), diminuição no número de receptores glutamatérgicos em membranas hipocampais e aumento na hidrólise do ATP extracelular. Paralelamente, essas mesmas alterações que podem proteger contra os efeitos de estressores repetidos, podem levar a prejuízos quanto a funções hipocampais, como é o caso da consolidação da memória.

Com relação aos efeitos sobre a vulnerabilidade neuronal,

- as adaptações acima citadas, possivelmente neuroprotetoras, não impedem, porém, que o hipocampo de animais repetidamente estressados se torne mais suscetível a outros insultos, como uma isquemia, pois a exposição de fatias hipocampais à privação de oxigênio e glicose (*in vitro*) causou maior morte neuronal em fatias de hipocampo de animais estressados em relação aos controle.

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