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# Avaliação do Comportamento Alimentar em Ratos Submetidos ao Estresse Crônico Variado - Interações Hormonais e Neuroquímicas

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que, mesmo distante,deve estar muito orgulhosa

de mais esta conquista em minha vida.

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# SUMÁRIO

# LISTA DE ABREVIATURAS

α: Receptores noradrenérgicos do tipo alfa
ACTH: Hormônio adrenocorticotrópico
AGRP: Peptídio relacionado ao gene cotia
$\beta_2$: Receptores noradrenérgicos do tipo beta-2
CCK: Colecistocinina
CRH: Hormônio liberador de corticotrofina
$D_2$: Receptor dopaminérgico do tipo D2
DOI: agonista seletivo de receptores serotoninérgicos $5HT_{2A/2C}$
DOPAC: Ácido 3,4-dihidroxifenil-acético
GABA: Ácido gama-amino butírico
Gene ob: Gene que, quando em homozigoze, codifica a falta de produção de leptina pelo adipócito.
HPA: Eixo hipotálamo-hipófise-adrenal
HPG: Eixo hipotálamo-hipófise-gonadal
MCH: Hormônio concentrador de melanina
NE: Norepinefrina
NPY: Neuropeptídeo Y
OVX: Ovarectomia
POMC: Pró-ópio melanocortina
PVN: Núcleo paraventricular do hipotálamo
5HT: Serotonina
$5HT_{1A}$: Receptores serotoninérgicos do tipo 1A
$5HT_{1B}$: Receptores serotoninérgicos do tipo 1B
$5HT_2$: Receptores serotoninérgicos do tipo 2
$5HT_{2A}$: Receptores serotoninérgicos do tipo 2A
$5HT_{2B}$: Receptores serotoninérgicos do tipo 2B
$5HT_{2C}$: Receptores serotoninérgicos do tipo 2C
$5HT_D$: Receptores serotoninérgicos do tipo D

# RESUMO

Alterações sobre o comportamento alimentar têm sido relatadas após exposição a situações de estresse. O modelo de estresse crônico variado causa uma diminuição do consumo de alimento doce. Nesta tese, estudamos o comportamento alimentar em animais submetidos ao estresse crônico variado e avaliamos alguns parâmetros relacionados ao comportamento alimentar em animais cronicamente estressados, tais como monoaminas no encéfalo e níveis séricos de leptina, os quais estão sabidamente envolvidos no controle da ingestão de alimento. Além de utilizarmos ratos machos, ratos fêmeas foram estudadas, assim como a possível interação entre hormônios ovarianos e os efeitos do estresse crônico sobre o comportamento alimentar, pois as respostas fisiológicas e comportamentais ao estresse são muitas vezes sexualmente dimórficas. Uma vez que o estresse crônico variado tem sido sugerido como um modelo de depressão em animais, também avaliamos a atividade da $Na^+,K^+$-ATPase em membranas plasmáticas sinápticas e os efeitos do antidepressivo fluoxetina. Observou-se diminuição na ingestão de alimento doce após 30 ou 40 dias de estresse, mas não após 20 dias. Durante o tratamento, não houve alteração no consumo de água e de ração padrão. Animais machos submetidos ao estresse crônico variado por 40 dias apresentaram aumento no metabolismo de serotonina e dopamina hipocampais. Observaram-se também um aumento na atividade dopaminérgica no córtex-frontal e uma diminuição no hipotálamo. Na amígdala, não foram observadas alterações nos níveis dos neurotransmissores estudados. As estruturas centrais estudadas podem estar envolvidas de forma direta ou indireta na regulação do apetite e do humor. Ratas fêmeas estressadas cronicamente apresentaram diminuição na atividade da enzima $Na^+,K^+$ -ATPase no hipocampo, que foi revertida após tratamento de 60 dias de tratamento com fluoxetina, e este resultado está de acordo com sugestões de que a atividade da $Na^+,K^+$-ATPase esteja reduzida em transtornos depressivos e, portanto, reforçam este modelo como um modelo de depressão em animais. Adicionalmente, avaliamos a interação entre níveis de estradiol e estresse crônico variado em

relação à ingestão de alimento doce e nos níveis de leptina, um hormônio secretado por células adiposas, com papel na regulação do apetite. Embora os animais (ratas ovariectomizadas) recebendo reposição com estradiol tenham apresentado menor ganho de peso, apresentaram aumento no consumo de alimento doce. Os animais do grupo estressado apresentaram níveis aumentados de leptina aos 30 dias de estresse, acompanhado da diminuição no consumo de doce. Nesses animais, a reposição com estradiol preveniu tanto a redução no consumo de alimento doce quanto o aumento nos níveis de leptina, sugerindo uma interação entre estresse crônico e reposição com estradiol no que tange ao comportamento alimentar, especificamente o consumo de alimento doce, e que essa interação pode estar relacionada com alterações nos níveis de leptina. A interação entre tratamento crônico com fluoxetina e níveis de leptina também foi estudada nesses animais cronicamente estressados. Após 40 dias de estresse, iniciou-se o tratamento com fluoxetina, que causou uma redução nos níveis de leptina dos animais controles, sem apresentar alterações nos níveis de leptina dos estressados. Podemos concluir que o estresse crônico variado pode afetar os níveis de leptina e que esse efeito é dependente do período de exposição ao estresse e da presença de estradiol, sendo também modulado por fluoxetina. O efeito do estresse crônico variado sobre o comportamento alimentar manifesta-se em machos e em fêmeas, e necessita mais de 20 dias de exposição ao estresse para se manifestar. É possível que os efeitos relatados nesta tese estejam relacionados, pelo menos em parte, com níveis alterados de leptina no soro e com neurotransmissão serotoninérgica e dopaminérgica alterada no encéfalo. No entanto, o mecanismo envolvido neste efeito e a relação entre esses parâmetros ainda necessita de mais estudos.

# ABSTRACT

Exposure to stress may cause either an increase or a decrease in food intake. It was observed that chronic variate stress decrease the ingestion of sweet food when compared to control rats. In this thesis, we aimed to study feeding behavior in chronically stressed rats and evaluate some parameters related to their feeding behavior, such as brain monoamines and leptin levels in serum, which are known to be involved in the control of food intake. Besides using male rats, females were studied. It was evaluated the possible interaction between stress effects on feeding behavior and ovarian hormones, since behavioral and physiological responses to stress are sometimes sexually dimorphic. Since chronic variate stress has been suggested as a model of depression, we also evaluated $Na^+,K^+$-ATPase activity in hippocampal synaptic plasma membranes, and the effects of an antidepressive, fluoxetine. Chronic variate stress decreased sweet food intake at 30 and 40 but not at 20 days of treatment. During the treatment, there were no differences in the consumption of water and regular food between stressed and control animals. Increased levels of DOPAC were observed in the frontal cortex and in the hippocampus, and an increased 5-HIAA/5-HT ratio was also observed in this latter structure. In the hypothalamus, levels of HVA and DOPAC were decreased, as well as the DOPAC/DA ratio, while no difference was found in amygdala. Therefore, chronic variate stress caused decreased dopaminergic neurotransmission in hypothalamus, and increased dopaminergic neurotransmission in the cortex and hippocampus, with increased serotonergic activity also in hippocampus. Some of these modifications may be related to alterations in feeding behavior. Reduction of hippocampal $Na^+,K^+$-ATPase activity was also observed. Treatment with fluoxetine increased this enzyme activity, and reversed the effect of stress. Chronic fluoxetine decreased the ingestion of sweet food. This result is in agreement with suggestions that reduction of $Na^+,K^+$-ATPase activity is a caracteristic of depressive disorders. In addition, we evaluated the interaction between estradiol levels and chronic variate stress on the intake of sweet food and on serum levels of leptin, a hormone secreted by the adipose cells with a role in the regulation of body weight. Although animals receiving estradiol replacement presented smaller weight gain, they

showed an increased consumption of sweet food. Estradiol replacement in the stressed group prevented both the reduction observed in sweet food intake and the increase in leptin levels, suggesting an interaction between chronic stress and estradiol replacement in feeding behavior, concerning sweet food consumption, and that this interaction may be related to altered leptin levels. The interaction between chronic fluoxetine treatment and leptin levels in animals submitted to chronic variate stress was also studied. After 30 days of chronic stress, the animals presented increased leptin levels compared to controls, as well as decreased consumption of sweet food. After this first stress period, the animals received daily injections of saline or fluoxetine (8mg/kg, i.p.). On the 60th day of fluoxetine treatment leptin levels were decreased in fluoxetine-treated animals indicating no effect of stress. In addition, chronic fluoxetine treatment induced a strong reduction in leptin levels. We conclude that chronic variate stress may affect leptin levels, and that this effect is dependent on the time of stress exposure, and on the presence of estradiol, being also modulated by fluoxetine. It is possible that the chronic variate stress effects reported herein on feeding behavior may be related, at least in part, with altered leptin levels in serum and altered serotonergic and dopaminergic neurotransmission in the brain. Nevertheless, the neurobiological mechanism involved in this effect and the relationship between all these parameters still require further studies.

# Capítulo I - INTRODUÇÃO

## 1.1 Estresse

Na década de 20, Walter Cannon criou o conceito de "luta ou fuga", que baseava-se na resposta do organismo a uma situação adversa, levando a uma ativação do sistema nervoso simpático e liberação de adrenalina. Em 1936, Hans Selye introduziu o conceito de "estresse" em biologia, e propôs que a resposta ao estresse seria como uma síndrome produzida por agentes nocivos diversos, e seria uma resposta não específica para um agente não específico. Selye considerou que o primeiro estágio do estresse seria uma reação geral de alarme, denominada "síndrome da adaptação geral". Esta definição foi de grande importância para estudos posteriores em relação ao estresse, pois serviu de substrato para outras indagações em relação ao tema.

Em mamíferos, a resposta ao estresse envolve vários processos, incluindo, além da ativação simpática, que resultará em liberação de adrenalina, o componente mediado pelo eixo hipotálamo-hipófise-adrenal (HPA) (Cullinan et al., 1995). O estresse é um complexo processo com retroalimentação e mecanismos de controle, semelhante a quaisquer outros dos sistemas auto-controladores presentes em nosso organismo. Estes mecanismos afetam muitos processos orgânicos, podendo funcionar em situações de alarme sempre que exista um real ou aparente desafio à homeostase do organismo (Ursin e Olff, 1993).

Segundo Ursin e Olff (1993), o conceito de estresse é composto, multidimensional, constituído de três principais elementos que podemos identificar como: a) estímulo estressante, ou estressor; b) sistema de processamento do estímulo, incluindo experiências de estresse subjetivas, e c) resposta ao estresse.

Não podemos encarar estes três elementos como estáticos, pois eles interagem entre si, ficando difícil separá-los. A experiência e as características das vias de entrada (*inputs)* sensoriais relacionadas com a percepção do estresse poderiam ser incluídas como mais um elemento em sua conceituação. Levine e Ursin (1991) relatam a importância da carga emocional relacionada ao estímulo estressante. O aspecto emocional é de muita importância e varia com aspectos particulares de cada indivíduo. Desta forma podemos visualizar a interrelação do sistema límbico com os sistemas de resposta ao estresse.

Várias vias de entrada (*inputs*) relacionadas com o estresse convergem para os neurônios do núcleo paraventricular hipotalâmico (PVN). Estes neurônios, que sintetizam, entre outros, o hormônio liberador de corticotrofina (CRH) e arginina-vasopressina, projetam-se para a eminência média, onde seus produtos são liberados na circulação porta, agindo na hipófise anterior e resultando na síntese e liberação de hormônio adrenocorticotrófico (ACTH) e outros peptídeos derivados de um precursor comum, a pro-ópio-melanocortina (POMC). O ACTH, por sua vez, ativa a biossíntese e liberação de glicocorticóides do córtex da adrenal (corticosterona nos roedores e cortisol nos primatas). Estes esteróides possuem uma atuação extremamente ampla, mediada por receptores especializados que afetam a expressão e regulação de genes, resultando em mudanças em vários processos metabólicos. Entre os eventos observados em resposta aos glicocorticóides, incluem-se, por exemplo, alterações nas respostas imunológicas e processos inflamatórios, além da resposta ao estresse. Muitos destes processos são requeridos para uma adaptação e preparação do organismo

para lidar com a situação estressante, incluindo mudanças na forma de obtenção de energia e no metabolismo (Cullinan et al., 1995; Akil & Morano, 1995).

A ativação do eixo hipotálamo-hipófise-adrenal representa a manifestação primária do estresse, mas deve-se levar em conta que aspectos adicionais da resposta do sistema nervoso ao estresse, e suas interações potenciais, devem ser considerados para um completo entendimento dos efeitos observados. O aumento no plasma dos níveis de catecolaminas e cortisol (um dos glicocorticóides) em conjunto aumentará a produção de glicose (Berne & Levy, 1992). A adrenalina liberada ativará as vias da glicogenólise, enquanto que o cortisol exercerá sua ação para suprir os substratos de aminoácidos para a gliconeogênese. Ambos os processos têm como finalidade a defesa imediata do organismo e irão desviar os "gastos" de energia periféricos para um melhor desempenho (Marks & Marks, 1996; Berne & Levy, 1992). A ativação do sistema nervoso simpático em resposta a um evento estressante causa um aumento da pressão arterial, broncodilatação e vasoconstrição periférica. Atividades como as dos tratos gastrintestinal e urinário são diminuídas (Berne & Levy, 1992). Podemos perceber a importância destes hormônios diante de uma situação estressante.

Em resposta a um estresse agudo, os glicocorticóides são liberados a fim de adaptar o organismo à situação estressante, porém uma liberação contínua destes hormônios poderia levar a situações patológicas (Sapolsky, 1986).

O estresse agudo apresenta uma resposta fisiológica relativamente bem caracterizada. Por outro lado, para o estudo da integração dos vários componentes do estresse e de seus possíveis efeitos deletérios torna-se importante o estudo de modelos de tratamento crônico. Sabe-se que o estresse

crônico induz alterações comportamentais, neuroquímicas e endócrinas diferentes daquelas causadas pelo estresse agudo (Martí et al., 1993). O modelo de estresse crônico oferece a oportunidade de examinar a plasticidade do organismo, revelando diferentes possibilidades de adaptações (Akil & Morano, 1995).

Os efeitos do estresse crônico diferem em relação a natureza do agente estressor, duração do regime de estresse, idade e espécie de animal. Outras características fisiológicas dos estressores como controlabilidade e previsibilidade são importantes (Marti et al., 1993). Existem relatos em relação à regularidade da exposição ao estresse crônico, mostrando sua importância na determinação de variáveis fisiológicas como níveis plasmáticos de corticosterona, glicose e ácidos graxos livres (Martí et al., 1993). Nesse contexto de efeitos deletérios da exposição crônica ao estresse, Sapolsky e colaboradores propõem que as alterações causadas pelo estresse crônico são comparáveis às alterações causadas pelo envelhecimento (Sapolsky et al., 1985).

Em nosso laboratório, desenvolvemos um modelo de estresse crônico por imobilização em ratos. Neste modelo, os animais são estressados cinco vezes por semana, sempre por imobilização, durante 1h a cada dia. Alguns efeitos sobre a memória foram observados (Xavier, 1995), sendo tarefa-específicos (amnésia na tarefa de exposição ao campo aberto, enquanto não se observou efeito nas tarefas de esquiva ativa de duas vias e esquiva inibitória).

Um determinante crítico para respostas fisiológicas e comportamentais dos animais cronicamente estressados é a previsibilidade do agente estressor, o que, de certa forma, ocorre no modelo descrito acima. Vários autores sugerem que as respostas fisiológicas ao estresse podem ser "amortecidas" se o animal tem

informação prévia relativa a começo, duração ou término de cada sessão de estresse (Konarska et al., 1990). Por esta razão, também desenvolvemos em nosso laboratório um modelo que tem como característica principal a imprevisibilidade, o modelo de estresse crônico variável. Este modelo foi idealizado com base em trabalhos já descritos na literatura, porém com algumas modificações (Willner et al., 1987; Konarska et al., 1990; Echandía et al., 1988; Muscat at al., 1992; Jordan et al., 1994; Willner and Muscat, 1991; Papp et al., 1991; Murua e Molina, 1992).

Assim sendo, conforme visto acima, a resposta ao estresse é parte integral de um sistema biológico adaptativo. Respostas comportamentais ou fisiológicas são necessárias para que seres humanos e outros animais enfrentem e sobrevivam, dentro de limites dinâmicos, os freqüentes desafios do ambiente (Charvat et al., 1964). Essas respostas levam, assim, a várias alterações comportamentais.

## 1.2 Comportamento Alimentar e Estresse

O comportamento alimentar é fundamental para a manutenção do metabolismo energético, sendo necessário para a sobrevivência do organismo. O controle da ingestão de alimento é um processo complexo, multifacetado, que está relacionado às demandas energéticas do organismo. Uma série de mecanismos neuroquímicos e metabólicos apresenta importantes papéis em relação ao comportamento alimentar. O sítio de regulação do comportamento alimentar é basicamente o hipotálamo (a região do cérebro responsável pelo controle da

homeostasia do organismo). O núcleo paraventricular (PVN) tem-se mostrado particularmente um importante foco de regulação dentro do eixo do hipotálamo medial, governando tanto o comportamento alimentar quanto o metabolismo energético, além da atuação de outros núcleos hipotalâmicos (Bishop et al., 2000). Esta regulação é mediada por complexas interações entre os órgãos periféricos e o sistema nervoso central e ainda sofre modulações de estímulos ambientais.

Dentre as influências periféricas, podemos ressaltar a ação dos hormônios liberados no trato gastrintestinal (colecistocinina, grelina, somastotatina e bombesina) (Beck, 2000, Brunetti et al., 2002, Attele et al., 2002; Baba et al., 2000, Beck, 2001, Yamada et al., 2002) e peptídos liberados pelo pâncreas, como glucagônio e insulina (Orosco, 2000; Strack et al.,1995; Geary, et al., 1996). Os hormônios esteróides, testosterona, estradiol e os glicocorticóides, apresentam importante influência sobre o comportamento alimentar (Tempel et al.,1992, Bonavera, et al., 1994; Hrupka et al., 1996, Geary & Asarian, 1999, Mystkowski & Schwartz, 2000). A leptina, hormônio peptídico liberado das células adiposas, também é um importante sinalizador periférico envolvido com o comportamento alimentar (Prolo et al., 1998, Loftus1999; Buchanan et al., 1998). Como exemplos de regulação central podemos citar a influência de mediadores químicos como o CRH e neurotransmissores como a serotonina, a dopamina, a noradrenalina e o GABA (Brown & Coscina,1994, Koob & Heinrichs 1999;. Currie et al., 2002; Wirtshafter, 2000, Bishop et al., 2000, Inui, 1999, Zhang et al., 2003, Benoit et al., 2003, Ahn & Phillips 2002, Backberg et al., 2003), além da presença de neuropeptídios como o neuropeptídio Y (NPY), o peptídio relacionado ao gene cotia (*agouti-gene related peptide*, AGRP), o hormônio concentrador de melanina

(MCH), as orexinas A e B, a galanina, a urocortina e a neurotensina (Kokot & Ficek 1999, Wirth & Giraudo 2000, Ahima & Osei, 2000, Sahu et al., 2001 Suply et al., 2001, Rodgers et al., 2002; Kasting et al., 2002, Latchman, 2002). O comportamento alimentar também sofre influências de fatores ambientais que poderão atuar no nível emocional, adicionando colorido emocional a uma resposta fisiológica, como, por exemplo, o olfato, a percepção do gosto, a visualização da comida, entre outros fatores. Importante ressaltar que os fatores ambientais não se restringem a produzir respostas meramente fisiológicas. Eles podem atuar como reguladores, podendo causar modificações em relação ao comportamento alimentar, como no caso de um estímulo estressante.

Estudos em animais e humanos demonstraram que a ingestão de alimento pode aumentar ou diminuir em resposta ao estresse, e que tal fato dependerá da característica do estressor (duração, tipo e intensidade) (Macht et al., 2001; Greeno & Wing, 1996). Vários estudos demonstraram que a exposição crônica a agentes estressores pode alterar o consumo de alimento e o peso corporal. Por exemplo, em animais submetidos ao estresse de choque inescapável, ocorreu uma diminuição na ingestão de alimento, acompanhada de uma redução no peso corporal, quando comparados com animais estressados por restrição e animais controle (Dess et al., 1988). Em nosso laboratório, observamos que animais submetidos ao estresse crônico repetido (por contenção) apresentaram um aumento na ingestão de alimento doce (Ely et al., 1997). Este aumento na ingestão de doce, sem haver, no entanto, aumento no consumo de ração padrão, está relacionado à característica do agente estressor (tipo e intensidade). Este

efeito foi revertido pela administração de um ansiolítico, o diazepam. Sugere-se, então, que este modelo de estresse pode causar ansiedade no animal.

Uma vez que, no modelo de estresse crônico citado acima, o agente estressor é mantido durante 40 dias e o animal é exposto sempre em uma hora determinada, por um tempo constante, tal característica pode gerar um maior grau de adaptação do animal ao estresse e, desta forma, o efeito sobre o comportamento alimentar é diferente do observado em outros modelos de estresse que utilizam outros agentes estressores com características diferenciadas (Katz, 1981; Willner, 1991; Konarska et al., 1990, Willner et al., 1987; Ferreti et al., 1995).

Em modelos de estresse crônico variado, podemos observar um efeito diferenciado em relação ao comportamento alimentar (Gamaro et al., 2003). Este fato deve-se a características do agente estressor.

Sabe-se que em situações de estresse ocorre ativação do eixo hipotálamo-hipófise-adrenal, culminando com a liberação de uma série de hormônios e neurotransmissores que visam a manutenção da homeostase. A resposta primária ao estresse se dá por meio da liberação de CRH do hipotálamo. Este, por sua vez, tem um efeito inibitório sobre o comportamento alimentar (Koob & Heinrichs, 1999). A ativação do sistema nervoso simpático e a atuação do ACTH na glândula adrenal acarretam a liberação de adrenalina e glicocorticóides. Por serem hormônios que estimulam a gliconeogênese e a glicogenólise, sinalizam a falta de glicose no sangue, juntamente com a liberação de glucagônio (Marks & Marks, 1996). Além disso, os glicocorticóides, agindo centralmente, modulam a ingestão de alimento por meio de um estímulo que potencializa a ação do NPY, NE e

galanina, via receptores tipo 2 no PVN e em outros sítios hipotalâmicos (Ahima & Osei, 2001). Estudos relatam o envolvimento dos esteróides produzidos pela adrenal, como a corticosterona e a aldosterona, estimulando a ingestão de um alimento específico. Em animais adrenalectomizados, que apresentam um efeito de supressão do comportamento alimentar, implantes de corticosterona no PVN estimulam o consumo de carboidratos, enquanto implantes de aldosterona estimulam o consumo de gordura (Tempel & Leibowitz, 1989). Na periferia, porém, tais hormônios atuam como inibidores de processos que armazenam energia no organismo (Strack et al., 1995).

Estudos em humanos têm demonstrado que as experiências emocionais podem levar a um aumento de ingestão de alimento (Yates, 1992). Em resposta a uma exposição ao estresse, podemos observar um aumento no consumo de carboidratos (Levine & Marcus, 1997). Pacientes, quando submetidos ao estresse, apresentam uma tendência de comer mais carboidratos, pois estes podem estar relacionados a uma melhora do bem-estar do indivíduo. E, como conseqüência disso, os pacientes acabam tornando-se obesos. Acredita-se que existe alguma propriedade neste tipo de alimento que atua via secreção de insulina e na razão de triptofano no plasma, que aumenta a liberação de serotonina, a qual está envolvida com o controle do humor (Wurtman & Wurtman, 1995), além de controlar o comportamento alimentar em mamíferos. Em estudos farmacológicos, fármacos que alteram de uma forma direta ou indireta os níveis pós-sinápticos de serotonina, causando um estímulo de tais vias, determinam um decréscimo no consumo de alimento em mamíferos de diferentes espécies (Halford e Blundell, 2000; Arkle & Ebenezer, 2000; Finn et al., 2001; Vickers et al., 2001; Brown et al.,

2001). Em contraste, substâncias que bloqueiam receptores serotoninérgicos pós-sinápticos, ou que causam a diminuição da neurotransmissão serotoninérgica pela ativação de autorreceptores, geralmente aumentam a ingestão de alimento. O consenso geral desenvolvido é que a serotonina apresenta um papel inibitório no comportamento alimentar (Simansky, 1996). Já a noradrenalina, atuando sobre receptores $\alpha$ no PVN, estimula a ingestão de carboidratos (Halmi, 1995, Leibowitz et al., 1985). Porém, quando a estimulação ocorre em receptores $\beta_2$ no hipotálamo perifornicial, causa uma diminuição no comportamento alimentar (Leibowitz, 1987, Bishop et al., 2000). É importante ressaltar que muitos dos neurotransmissores envolvidos na regulação do comportamento alimentar apresentam uma determinada ação influenciada pela região e pelo tipo de receptores aos quais se ligarão, como no caso da noradrenalina, da serotonina e da dopamina. Assim, geralmente a ativação de receptores $5HT_{1A}$ causa hiperfagia, enquanto a ativação de receptores $5HT_{1B}$ determinam efeitos hipofágicos (Collin et al., 2000). Existem também relatos sobre o envolvimento de receptores $5HT_{2C}$ na regulação do comportamento alimentar, em que pré-tratamento com DOI, agonista de receptores $5HT_{2C}$ e $5HT_{2A}$, inibe o comportamento alimentar estimulado pelo NPY no PVN (Collin et al 2000, Currie et al, 2002, Bouwknecht et al., 2001; Datla e Curzon, 1997). A modulação da serotonina pode ocorrer via uma inibição do NPY, envolvendo receptores do tipo $5HT_{2A}$ ou $5HT_{2C}$, ou por outros mecanismos que não envolvam a participação de tal neuropeptídio, que estão relacionados com receptores $5HT_{1A,B}$ e $5HT_D$ (Currie, et al., 2002). Já o neurotransmissor GABA inibe os disparos de neurônios dopaminérgicos, inibindo o comportamento

alimentar. No caso dos opióides endógenos, estes estimulam o comportamento alimentar quando atuam no PVN (Hoebel, 1979), em geral influenciando a ingestão de alimentos com alto teor em gordura (Rosmos et al., 1987).

## 1.3 Estresse e Leptina

A leptina é um hormônio peptídico descoberto por Zhang e colaboradores. (1994), constituído por 167 aminoácidos (Prolo et al., 1998), produzido e liberado pelas células do tecido adiposo branco via transcrição do gene *ob* (Caldefie-Chézet et al., 2001; Prolo et al., 1998; Zhang et al., 1994). A principal função de tal hormônio consiste na modulação de sinais de saciedade, por meio da inibição da síntese de NPY, que estimula o apetite e reduz o gasto energético (Wang et al., 1998; Yokosuka et al., 1998). Existem várias isoformas para os receptores de leptina, sendo que o receptor que se acredita mediar os efeitos sobre o comportamento alimentar encontra-se distribuído em núcleos hipotalâmicos diferenciados como o ventromedial e o lateral (Mercer et al., 1996 citado Loftus, 1999). O núcleo arqueado é um de seus sítios de ação, sendo também sítio de produção do NPY (Thorsell et al., 2002; Buchanan et al., 1998; Prolo et al., 1998; Jang et al., 2000). Devido ao amplo espectro de ação da leptina, uma série de funções regulatórias tem sido proposta, além do efeito anorexigênico, como a regulação da homeostase energética e da função reprodutiva (Buchanan et al., 1998). Estudos recentes sugerem uma interação da leptina com outros sistemas endócrinos, fornecendo informações sobre o tamanho das reservas de gordura, atuando como um fator que permite ao organismo desencadear situações de alta

demanda energética, como a puberdade e a reprodução, somente quando o tamanho das reservas energéticas for suficiente para garantir o sucesso da espécie (Casabiell et al., 2001). Além disso, a leptina pode ser produzida em pequenas quantidades no tecido muscular, na placenta e no estômago (Loftus, 1999, Wang et al., 1998; Lepercq et al., 1998, Bado et al., 1998). Importante ressaltar que os níveis de leptina encontram-se geralmente mais altos em fêmeas do que em machos, de maneira proporcional à quantidade de tecido adiposo. Tal dado sugere uma interação entre o tecido adiposo e o sistema reprodutivo, sendo possivelmente a liberação de leptina modulada de diferentes formas em machos e fêmeas, por hormônios androgênicos e estrogênicos (Casabiell et al., 2001, Prolo et al., 1998, Mystkowski & Schwartz, 2000).

Tem sido sugerido um papel da leptina na resposta neuroendócrina ao estresse (Sandoval & Davis, 2003; Prolo et al.,1998; Inui, 1999). Existem evidências que sugerem uma via de regulação da leptina sobre o eixo hipotálamo-hipófise-adrenal (HPA), em que o ACTH inibe a secreção de leptina pelo tecido adiposo e, por sua vez, a leptina aumenta a expressão e a secreção do ACTH (Nowak et al., 2002). O tratamento prolongado com baixas doses de leptina causa diminuição na produção de ACTH, provavelmente mediado pelo hipotálamo, e inibe a resposta da corticosterona ao estresse (Nowak et al., 2002). Adicionalmente, estudos com animais submetidos ao estresse por contenção em um período de 3 e 10 dias demonstram que os níveis de leptina encontram-se mais baixos. Neste estudo, a infusão de leptina antes e durante o estresse não impediu o efeito de perda de peso dos animais, mas diminuiu os níveis de corticosterona em resposta ao estresse (Harris et al., 2002). A falta de

glicocorticóides circulantes e /ou aumento da concentração de ACTH no plasma determinam uma diminuição da leptina (Spinedi & Gaillard, 1998, Nowak et al., 2002, Solano & Jacobson, 1999), apoiando uma íntima relação bi-direcional entre o circuito HPA e o metabolismo do tecido adiposo. Este fato também está relacionado com o tipo de estresse associado a muitos fenótipos de obesidade (Spinedi et al., 1998).

## 1.4 Hormônios sexuais e resposta ao estresse

A ativação neuroendócrina é uma das principais características da resposta ao estresse. Essa resposta é uniforme, mas depende do estímulo estressante envolvido e de muitos outros fatores, incluindo o gênero do indivíduo. Os hormônios gonadais podem exercer influência nas respostas comportamentais dos indivíduos, uma vez que eles estimulam o eixo HPA, resultando em um dimorfismo sexual em resposta ao estresse (Kelly et al., 1999). Machos diferem de fêmeas em relação à resposta a um estímulo doloroso (Gamaro, et al., 1997, Aloisi, 1997). Existem também diferenças entre os gêneros em relação ao comportamento alimentar, até porque os hormônios sexuais atuam de maneira oposta na regulação do comportamento alimentar, pois geralmente a testosterona atua estimulando o apetite, enquanto o estrogênio atua inibindo o mesmo (Mystkowski & Schwartz, 2000). A influência dos hormônios gonadais está relacionada também com a incidência de algumas patologias, como no caso de desordens afetivas e distúrbios alimentares (Faraday, 2002; Kornstein et al., 2002).

Em ratos, os níveis de corticosterona e ACTH, bem como a atividade funcional do eixo HPA, são maiores em fêmeas do que em machos, em condições basais e de estresse (Jezova et al., 1996). A exposição a choques nas patas e a injeção aguda de álcool, atuando como agentes estressores, causam um aumento na secreção de ACTH, corticosterona e hormônios sexuais. Fêmeas submetidas ao choques secretam significativamente mais ACTH quando comparadas aos machos, sendo esta diferença abolida após o procedimento de retirada dos ovários, o que demonstra o envolvimento dos hormônios sexuais na ativação do eixo HPA, uma vez que tais hormônios podem influenciar a atividade das glândulas pituitária e adrenal em algumas circunstâncias (Rivier, 1999).

Em animais submetidos ao estresse por contenção, agudo ou crônico, observaram-se diferenças significativas em determinadas áreas cerebrais para receptores glicococorticóides, em que fêmeas apresentam mecanismos distintos de regulação de receptores glicocorticóides e mineralocorticóides no hipocampo após estresse crônico: existe maior propensão a ocorrerem alterações na expressão dos receptores glicocorticóides no hipotálamo das fêmeas do que no de machos em resposta ao estresse por contenção (Karandrea et al., 2000).

Estudos recentes em humanos e animais demonstram o envolvimento do eixo HPA e do eixo hipotálamo-hipófise-gônadas (HPG) em processos de aprendizagem e memória (Luine, 2002), uma vez que o estresse crônico pode causar uma resposta diferenciada dependente do sexo dos animais. Ratos machos estressados cronicamente apresentaram déficit de memória, quando comparados a fêmeas, em testes relacionados com memória espacial (Luine, 2002). Houve também alterações nos níveis de neurotransmissores e seus

metabólitos de maneira diferenciada em fêmeas e machos, sugerindo que as respostas das aminas podem contribuir para as diferenças sexuais do estresse sobre a memória. No modelo de estresse variado empregado neste trabalho, porém, não observamos alterações na memória de animais machos (Gamaro, 1998).

Em fêmeas ocorrem variações hormonais nas diferentes fases do ciclo estral (Aloisi, 1997; Freeman, 1994; Geary et al., 1994), o que pode alterar o nível de certos neurotransmissores que podem modificar o comportamento do animal. No pró-estro, por exemplo, observa-se um aumento nos níveis de acetilcolina em comparação com as demais fases do ciclo (Aloisi, 1997), bem como uma diminuição nos níveis de noradrenalina no hipocampo e um aumento da atividade serotoninérgica no sistema septo-hipocampal, o que está associado a diferenças sexuais em resposta a ansiedade, comportamentos defensivos e no comportamento de esquiva inibitória. Sabe-se que em fêmeas a ingestão de alimento é afetada pelos hormônios ovarianos, sendo que níveis altos de estradiol inibem a ingestão de alimento e diminuem o peso corporal (Bonavera et al., 1994; Mystkowski & Schwartz, 2000). Desta forma, existem variações na ingestão de alimento, bem como tamanho e número de refeições durante o ciclo estral e este fato deve-se à flutuação dos níveis dos hormônios ovarianos. A ingestão de alimento é diminuída no pró-estro, quando os níveis de estrogênio estão elevados. O contrário é observado nas fases de metaestro e diestro, em que os níveis de estrogênio caem e a ingestão aumenta (Varma et al., 1999). A progesterona exerce um efeito estimulante sobre o apetite, podendo muitas vezes reverter o efeito inibitório causado pelo estradiol (Mystkowski & Schwartz, 2000). Mudanças

em relação ao consumo de alimento e a preferência por alimentos adocicados em mulheres no período pré-menstrual têm sido relatadas (Kuga et al., 1999), além de alterações na percepção de sacarose, cujo limiar está diminuído durante a pré-ovulação e aumentado durante a menstruação e pós-ovulação (Than et al., 1994).

## 1.5 Estresse e Transtornos Afetivos

Tem sido sugerido que anormalidades na resposta do eixo hipotálamo-hipófise-adrenal podem estar associadas com uma variedade de estados patológicos. Transtornos afetivos são alguns dos problemas que podem aparecer, por exemplo, na síndrome de Cushing e na doença de Addison, patologias estas que refletem alterações nos níveis de glicocorticóides pelo mau funcionamento da glândula adrenal, acarretando distúrbios metabólicos na utilização de energia e na resposta ao estresse (Michelson et al., 1995). No caso da depressão maior, por exemplo, existe uma hiper-ativação do eixo HPA onde ocorre um aumento na produção de cortisol e possível excesso de CRH (Michelson et al., 1995).

Além disso, sabe-se que o estresse pode precipitar o aparecimento de doenças afetivas ou ser um fator de predisposição para estas (Malysko et al., 1994; Ferreti et al., 1995). Os glicocorticóides afetam o comportamento e o humor. Alterações no eixo HPA são encontradas em pacientes deprimidos, o que sugere um papel importante destes hormônios na etiologia da depressão e de outras doenças afetivas (Chalmers et al., 1993). O sistema serotoninérgico também tem um papel importante em inúmeras funções cerebrais, incluindo a reação ao estresse, a depressão e a ansiedade (Nishi & Azmitia, 1996).

Existe uma interrelação entre transtornos afetivos e o estresse. O modelo de estresse crônico variável, por exemplo, tem sido utilizado na investigação experimental da depressão (Ferreti et al., 1995), uma vez que este modelo mostrou-se capaz de induzir anedonia, um dos efeitos mais importantes apresentados em transtornos afetivos dessa natureza. Em geral, este efeito é testado por medidas de consumo de sacarose (Willner et al., 1987), condicionamento de preferência de lugar (Papp et al., 1991; Willner et al., 1992) e auto-estimulação intracraniana (Moreau et al., 1992).

A exposição a estresses agudos e crônicos pode estar associada a desordens neurológicas e psicológicas, que têm sido atribuídas, em parte, a efeitos neurotóxicos do estresse em neurônios hipocampais (Moghaddam et al., 1994). O estresse agudo altera a neurotransmissão das catecolaminas (dopamina, noradrenalina e serotonina) (Malysko et al., 1994; Paris et al., 1987; Nishi & Azmitia, 1996; Chaouloff, 1993; Cuadra et al., 2001). Pouco se sabe sobre alterações causadas a longo prazo, como situações de estresse crônico, sobre a neurotransmissão serotoninérgica. Considerando a eficácia de inibidores da recaptação de tal neurotransmissor no tratamento da depressão, é possível que a serotonina possa estar envolvida com o desenvolvimento ou na expressão do estresse relacionado ao estado depressivo.

Os glicocorticóides liberados por meio da resposta ao estresse induzem a atividade da enzima triptofano-hidroxilase no núcleo da rafe, estimulando a síntese e a utilização de serotonina nos terminais sinápticos (Chalmers et al., 1993; Singh et al., 1990; Chaouloff, 1993). Os glicocorticóides podem também modular diretamente a neurotransmissão serotoninérgica por meio da regulação dos níveis

de receptores (Biegon et al., 1985). Vários estudos demonstraram que os receptores para serotonina, no caso os $5HT_{1A}$, são sensíveis aos níveis de glicocorticóides circulantes (Nishi & Azmitia, 1996).

A $Na^+,K^+$-ATPase é a enzima responsável pelo transporte ativo dos íons sódio e potássio no sistema nervoso central, mantendo o gradiente iônico necessário para a excitabilidade neuronal e a regulação do volume celular. Esta enzima encontra-se em altas concentrações em membranas celulares, consumindo cerca de 40-50% da energia gerada pelo ATP naquele tecido (Erecinska & Silver, 1994). Sua atividade está diminuída em pacientes portadores de doenças psiquiátricas, como, por exemplo, pacientes com depressão bipolar (Naylor et al., 1980; Hokin-Neaverson e Jefferson, 1989; Mynett-Johnson et al., 1998; Wood et al., 1991).

Alguns modelos de estresse crônico variado têm sido propostos como sendo modelos de indução de depressão em animais (Pucilowski et al., 1993; Katz et al., 1981; Willner, 1991). Nestes modelos, os animais são expostos a diferentes estressores por vários dias. A resposta a estímulos de recompensa apresenta-se diminuída nos animais submetidos ao estresse, e tal fato é demonstrado por testes que mostram redução no consumo de soluções palatáveis, como soluções contendo sacarose, o que pode ser interpretado como anedonia, um dos sintomas do estado depressivo.

Fluoxetina e os antidepressivos que atuam por meio do mecanismo de inibição da recaptação da serotonina são freqüentemente usados para o tratamento da depressão (Bergstrom et al., 1988). A serotonina (5-HT) está intimamente envolvida na patofisiologia de doenças afetivas (Van Praag et al.,

1990), e sua remoção da fenda sináptica é dependente da captação de sódio por transportadores localizados na membrana plasmática das células pré- sinápticas (Worrall & Willians, 1994; Lesch et al., 1993). Existe uma associação entre estes e a atividade da enzima $Na^+,K^+$-ATPase (Lesch et al., 1993). Dados recentes de Zanatta e colaboradores (2001) demonstraram que a administração crônica de fluoxetina aumenta significantemente a atividade da enzima $Na^+,K^+$-ATPase no cérebro de animais normais.

## 1.6 Objetivos

Tendo em vista o exposto acima, são objetivos desta tese:

- avaliar os níveis de aminas biogênicas em machos submetidos ao estresse crônico variado e relacionar com alterações no comportamento alimentar;
- avaliar os efeitos do estresse crônico variável sobre o comportamento alimentar em fêmeas;
- determinar a atividade da enzima $Na^+,K^+$-ATPase no hipocampo de fêmeas estressadas cronicamente e em fêmeas estressadas e submetidas a um tratamento crônico com fluoxetina;
- relacionar o efeito comportamental com os níveis séricos de leptina nas fêmeas tratadas com fluoxetina;
- avaliar os efeitos dos hormônios ovarianos (especialmente o estradiol) em relação ao comportamento alimentar, na vigência da ovariectomia;
- relacionar as alterações causadas pelo estresse crônico variado com o comportamento alimentar e os níveis de leptina de fêmeas ovarectomizadas.

## 1.7 Organização dos trabalhos que compõem esta tese

O Capítulo II aborda os efeitos do tratamento de estresse crônico variado sobre os níveis de dopamina e serotonina em diferentes estruturas cerebrais em ratos machos. Os resultados publicados em *Neurochemistry International* (42:107-114, 2003) descrevem também o efeito do estresse crônico variado sobre o comportamento alimentar, causando a diminuição do consumo de alimento doce, acompanhada pela diminuição da neurotransmissão dopaminérgica no hipotálamo e do aumento da neurotransmissão serotonérgica no hipocampo, sendo também analisados os níveis destes neurotransmissores em outras estruturas cerebrais.

No Capítulo III foram analisados os efeitos do estresse crônico variado sobre o comportamento alimentar em fêmeas. Outro parâmetro avaliado foi a atividade da enzima $Na^+,K^+$-ATPase hipocampal que apresentou-se inibida nos animais submetidos ao estresse. Os resultados publicados em *Neurochemical Research*, (28: 1339-1344, 2003) demostraram que o tratamento com fluoxetina reverte a ação causada pelo estresse sobre a atividade da enzima.

No Capítulo IV foram avaliadas a influência do estresse sobre os níveis de leptina e a interação entre o tratamento crônico com fluoxetina com os níveis de leptina dos animais submetidos ao estresse. O artigo que foi submetido para revista *Physiology & Behavior* demostra que o efeito inibitório do estresse sobre o apetite, que ocorre com os animais submetidos ao estresse, parece estar relacionado aos níveis aumentados de leptina. O tratamento com fluoxetina diminuiu os níveis de

leptina nos animais estressados, não apresentando estes animais o efeito do estresse sobre o comportamento alimentar.

O Capítulo V busca relacionar os efeitos do estresse sobre os níveis de leptina em animais ovarectomizados que receberam reposição hormonal de estradiol. Os animais submetidos ao estresse apresentaram diminuição no consumo de doce, porém os animais do grupo estressado que recebeu estradiol apresentaram um aumento no consumo de doce. Os resultados do artigo, publicados na revista *Pharmacology Biochemistry and Behavior* (76: 327-333,2003) descrevem a possível interação entre a reposição de estradiol sobre os efeitos do estresse e níveis de leptina, que também parece ser modulada pelo estradiol.

## Capítulo II – ARTIGO 1

Efeitos do Estresse Crônico Variado sobre o comportamento alimentar e níveis de monoaminas em diferentes estruturas cerebrais de ratos

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# Effects of chronic variate stress on feeding behavior and on monoamine levels in different rat brain structures

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**Abstract**

Chronic variate stress was seen to decrease the ingestion of sweet food when compared to control rats. Brain monoamines are known to be involved in the control of food intake, serotonin appears to be involved in the mechanisms of satiety, and dopamine in mediating appetite or approach behaviors triggered by incentive stimuli associated with rewards. The effect of chronic variate stress on cerebral levels of monoamines was also studied in rats. Increased levels of DOPAC were observed in the frontal cortex and in the hippocampus and an increased 5-HIAA/5-HT ratio was also observed in this latter structure. In the hypothalamus, levels of HVA and DOPAC were decreased, as well as the DOPAC/DA ratio, while no difference was found in amygdala. During the treatment, there were no differences in the consumption of water and regular food between stressed and control animals. An increase in the adrenal weight was observed at the end of the treatment. The results suggest that emotional changes, such as exposure to stress situations can influence feeding behavior, chronic variate stress causes decreased ingestion of sweet food and decreased dopaminergic neurotransmission in hypothalamus. Increased dopamine metabolite levels in the cortex and hippocampus were also observed and some of these modifications may be related to alterations in feeding behavior.


*Keywords:* Chronic variate stress; Feeding behavior; Reward; Monoamines; Glucocorticoids; Dopamine; Serotonin; Frontal cortex

## 1. Introduction

Emotional changes, such as exposure to stress situations can influence feeding behavior, and several studies have demonstrated that chronic exposure to stressors may alter food intake and body weight of rats. For example, inescapable shock can affect food intake and reduce weight gain with shocked rats gaining significantly less weight than restrained and not treated rats (Dess et al., 1988). Previously, we observed that animals repeatedly stressed by restraint show increased ingestion of sweet food (Ely et al., 1997). It should be pointed out, however, that there is a certain degree of predictability in studies using repeated restraint, when compared to models using different stressors (Katz et al., 1981; Willner, 1991; Konarska et al., 1990; Willner et al., 1987; Ferreti et al., 1995). In addition, some models of chronic mild stress have been proposed as models of depression in animals studies (Pucilowski et al., 1993; Katz et al., 1981; Willner, 1990, 1991). In these models, rats are exposed to different weak stressors for several days. The response to rewarding stimuli is diminished, as demonstrated by tests showing reduced sucrose consumption, which is interpreted as anhedonia. Thus, different models of stress can lead to different effects concerning feeding behavior.

Studies in humans have provided further evidence of overeating induced by emotional experiences (Yates, 1992). Increased consumption of carbohydrates has been observed following exposition to stress (Levine and Marcus, 1997). Sometimes, patients exposed to stress present the tendency to overeat carbohydrates to make themselves feel better. This effect is believed to be related to the property of carbohydrate consumption, acting via insulin secretion and the "plasma tryptophan ratio" to increase serotonin release, which is also involved in functions, such as mood control (Wurtman and Wurtman, 1995) and in controlling feeding behavior in mammals. In pharmacological studies, drugs that increase post-synaptic serotonergic stimulation routinely decrease food consumption in mammalian species ranging from rodents to human and non-human primates (Halford and Blundell, 2000; Arkle and Ebenezer, 2000; Finn et al., 2001; Vickers et al., 2001; Brown et al., 2001).

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In contrast, agents that block post-synaptic serotonin receptors or that diminish serotonergic neurotransmission by activating autoreceptors often increase food intake. The general consensus developed, therefore, is that 5-HT serves an inhibitory role in feeding (Simansky, 1996).

Besides serotonin, dopamine is also believed to be involved in the control of food intake (Orosco and Nicolaidis, 1992), and stress has been shown to alter normal serotonergic and dopaminergic neurotransmission (Malyszko et al., 1994; Paris et al., 1987; Nishi and Azmitia, 1996; Chauloff, 1993; Cuadra et al., 2001). Although little is known about the long-term alterations in these systems caused by chronic stress, the efficacy of serotonin selective reuptake inhibitors in treating depression suggests that serotonin may be involved in the development or expression of stress-related depression. In addition, a role for dopamine as part of the biochemical basis of depression has also been suggested (Willner, 1983; Zacharko and Anisman, 1991; Kapur and Mann, 1992; Tanda et al., 1996).

At the neurochemical level it is important to correlate changes in behavior with neurobiological modifications, such as altered monoamine activity. The aim of the present study was to verify the effect of a chronic variate stress model on feeding behavior (sweet food and standard chow intake) and on monoamine (serotonin, dopamine and their metabolites) levels in different brain regions related to food intake and involved in the stress response.

## 2. Experimental procedures

### 2.1. Chemicals

Chemicals for high-performance liquid chromatographic analysis were purchased from Baker (Pittsburg, PA, USA) and were of analytical grade.

### 2.2. Animals

Adult male Wistar rats (60 days old; 180–230 g of weight) were used. Twenty-nine animals were used in the behavioral measurements and 19 rats were used to measure monoamine levels. The experimentally naive animals were housed in groups of four or five in home-cages made of Plexiglas material (65 cm × 25 cm × 15 cm) with the floor covered with sawdust. They were maintained under a standard dark-light cycle (lights on between 7:00 and 19:00 h) at room temperature of 22 ± 2 °C. The rats had free access to food (standard rat chow) and water, except for the stressed group during the period when the stressor applied required no food or water. During the treatment, the ingestion of rat chow and water was monitored. Body weight was measured at the beginning and at the end of the treatment. After sacrificing the animals, the adrenal gland weight was evaluated as an indirect parameter of hypothalamic-pituitary-adrenal axis activation. 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 as well as to reduce the number of animals.

### 2.3. Stress model

Chronic variate stress was modified from other models of variate stress (Willner et al., 1987; Konarska et al., 1990; Papp et al., 1991; Muscat et al., 1992; Murua and Molina, 1992). The animals were divided in two groups: control and stressed. Controls were kept undisturbed in their home cages during the 40 days of treatment. A 40-day variate-stressor paradigm was used for the animals in the stressed group. Individual stressors and length of time applied each day are listed in Table 1. The following stressors were used: (a) 24 h of food deprivation; (b) 24 h of water deprivation;

Table 1
Schedule of stressor agents used during the chronic treatment

| Day of treatment | Stressor used | Duration |
|---|---|---|
| 1 | Water deprivation | 24 h |
| 2 | Food deprivation | 24 h |
| 3 | Isolation | 24 h |
| 4 | Isolation | 24 h |
| 5 | Isolation | 24 h |
| 6 | Flashing light | 3 h |
| 7 | Food deprivation | 24 h |
| 8 | Forced swimming | 10 min |
| 9 | Restraint | 1 h |
| 10 | Water deprivation | 24 h |
| 11 | No stressor applied | – |
| 12 | No stressor applied | – |
| 13 | Restraint + cold | 2 h |
| 14 | Flashing light | 2.5 h |
| 15 | Food deprivation | 24 h |
| 16 | Forced swimming | 15 min |
| 17 | Isolation | 24 h |
| 18 | Isolation | 24 h |
| 19 | Isolation | 24 h |
| 20 | Water deprivation | 24 h |
| 21 | Food deprivation | 24 h |
| 22 | Flashing light | 3 h |
| 23 | Restraint | 2 h |
| 24 | Isolation | 24 h |
| 25 | Isolation | 24 h |
| 26 | Restraint + cold | 1.5 h |
| 27 | Forced swimming | 10 min |
| 28 | Flashing light | 3.5 h |
| 29 | No stressor applied | – |
| 30 | Food deprivation | 24 h |
| 31 | Restraint | 3 h |
| 32 | Flashing light | 2 h |
| 33 | Water deprivation | 24 h |
| 34 | Restraint + cold | 2 h |
| 35 | Forced swimming | 15 min |
| 36 | Isolation | 24 h |
| 37 | Isolation | 24 h |
| 38 | No stressor applied | – |
| 39 | Flashing light | 3 h |
| 40 | Forced swimming | 10 min |

(c) 1–3 h of restraint, as described later; (d) 1.5–2 h of restraint at 4 °C; (e) forced swimming during 10 or 15 min, as described later; (f) flashing light during 120–210 min; (g) isolation (2–3 days). Stress application started at different times everyday, in order to minimize its predictability.

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

### 2.4. Consumption of sweet food, water and lab chow

After 40 days of treatment, consumption of sweet food was measured in 29 animals (14 controls and 15 stressed). The animals were placed in a lightened rectangular box (40 cm × 15 cm × 20 cm) with a glass ceiling, floor and side walls made of wood. Ten Froot loops (Kellogg's®—pellets of wheat and corn starch and sucrose) were placed in one extremity of the box. Animals were submitted to five 3 min trials, one per day, in order to become familiarized with this food (Ely et al., 1997). After being habituated, the animals were exposed to two test sessions, 3 min each, when the number of ingested pellets was measured. A protocol was established so that when the animal ate part of the Froot loops (e.g. 1/3 or 1/4) this fraction was considered. These two evaluations were made with the animals submitted to fasting (during a period of 22 h prior to the behavioral task) or with animals fed ad libitum. These evaluations were made since food deprivation, which is used in many behavior tasks as a motivating stimulus, may also be an acute stressor (Katz et al., 1981). Food deprivation increases serum corticosterone levels and decreases dopaminergic activity after eating food (Pothos et al., 1995).

Food and water intake were evaluated in stressed and control rats. The measurements were made before each daily stress session. Data from days when the stressor applied was food or water deprivation were not considered, as well as data obtained immediately following these days. Food intake was measured by weighing the amount of standard chow put into the feeders and weighing the amount that remained in them, and is expressed as grams of food consumed per animal in 24 h. Since the animals were housed in groups of four or five per cage, food intake in each of the 10 cages used in this study was measured and the mean intake was averaged across 10-day blocks and expressed as grams of food consumed per animal. Liquid intake was measured as the difference between the amount of water put in the drinking bottle and the remaining amount, and is expressed as the mean intake of liquid per rat in 24 h.

### 2.5. Monoamine measurement

The animals were sacrificed by decapitation 24 h after the last stress session, to minimize its immediate effects on neurotransmitter metabolism. Brains were quickly removed and placed on an inverted Petri dish on ice, where hippocampus, frontal cortex (approximately between 1.2 and 3.2 mm anterior to Bregma), hypothalamus (including the preoptic area), and amygdala were dissected, according to the atlas by Paxinos and Watson (1998) and frozen in aluminum foil (−70 °C). On the day of the assay, tissue samples were weighed and sonicated in 700 μl PCA (percloric acid) (amygdala and hypothalamus) or 800 μl PCA (hippocampus and frontal cortex) for 3 s and centrifuged at 4 °C for 15 min at 10,000 × *g*. The pellet was discarded and dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) tissue concentrations were analyzed in the supernatants using high pressure liquid chromatography coupled with electrochemical detection (HPLC-EC) (sensitivity 10 and 20 nA and oxidation potential 0.85 V) as previously described (Jonsson et al., 1980; Claustre et al., 1986). Chromatoghraphic separations were performed using a C18 column (250 mm × 4.6 mm) packed on microparticulate (6 μm). The mobile phase consisted of 0.15 M citric acid, 0.015% sodium octyl sulfate, 3.2% acetonitrile (v/v); 1.2% tetrahydrofurane (v/v); 1.0 mM EDTA in double distilled water, pH 3.0. The mobile phase was filtered through a 0.2 μm filter, degassed under vacuum and delivered at a flow rate of 1.0 ml/min. The position and height of the peaks in tissue homogenates were measured and compared to 50 μl samples of an external calibrating standard solution containing 5 ng each of DA, DOPAC, HVA, 5-HT and 5-HIAA. Concentrations of these substances in the samples were calculated and expressed as ng/g wet tissue. The activity (turnover) of the dopaminergic and serotonergic systems were expressed as DOPAC/DA, HVA/DA, and 5-HIAA/5-HT rations.

### 2.6. Statistical analysis

Data were expressed as mean ± S.E.M. Chow and water intake, and sweet food intake were analyzed using repeated measures ANOVA. Body weight, adrenal weight, as well as monoamine levels were analyzed using Student's *t*-test.

## 3. Results

Food and water intake were evaluated in stressed and control rats (data not shown). Analyzing food and water intake, repeated measures ANOVA showed no effect of time, no difference between the groups, and no interaction between group and time ($P > 0.05$ in all cases).

Body weight before and after the treatment in 29 animals is shown in Fig. 1. For body weight, repeated measures

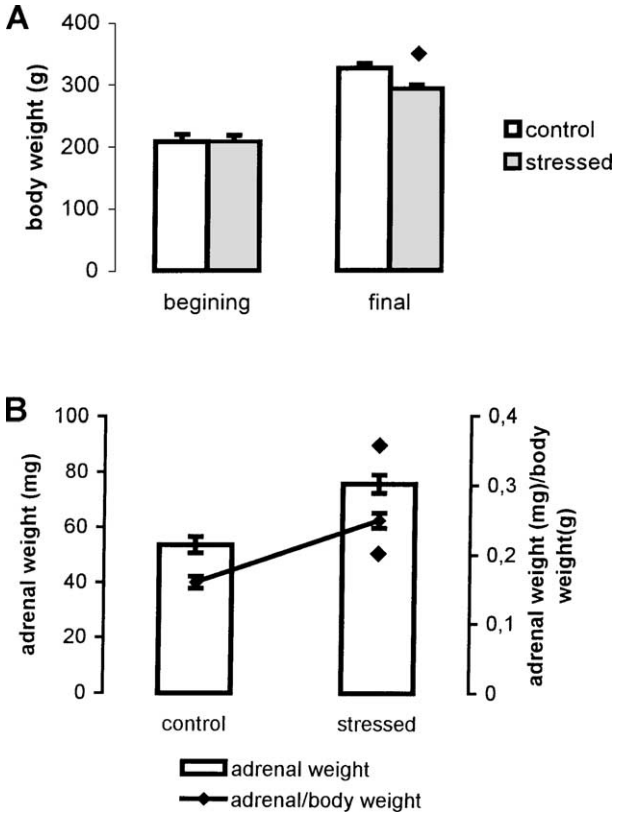


Fig. 1. Body weight and adrenal weight after chronic variate stress during 40 days. (A) Body weight before and after chronic stress. (B) Adrenal weight and adrenal/body weight ratio after the treatment. Data expressed as mean ± S.E.M. (◆) Significantly different from control group (Student's *t*-test, $P < 0.05$).

ANOVA showed an effect of time [$F(1, 27) = 130.9$; $P < 0.001$], since both groups gained weight and a marginal interaction between time and treatment [$F(1, 27) = 3.56$; $P = 0.070$]. Adrenal gland weight was measured as an index of chronic stress. Student's *t*-test indicates that adrenal/body weight ratio was increased in stressed rats [$t(17) = 6.38$; $P < 0.005$ for the adrenal/body weight ratio].

The effect of chronic variate stress upon the intake of Froot loops in fasted and non-fasted rats is shown in Fig. 2. A repeated measures ANOVA revealed a significant effect of the chronic treatment [$F(1, 27) = 5.13$, $P < 0.05$], where the stress determined a decreased intake. The fasted animals showed an increased mean intake when compared to normally fed animals [$F(1, 27) = 31.02$, $P < 0.001$]. There was no interaction between stress treatment and fed state [$F(1, 58) = 0.89$; $P > 0.05$], i.e. stress decreased sweet food consumption either in the fast or in the fed state. When consumption is related to body weight, this decreased ingestion is still observed, with a 19% reduction in the stressed rats in the fed state, but a smaller (around 5%) reduction in the fast state.

The effect of chronic variate stress on serotonin and 5-HIAA levels in different brain structures, as well as the

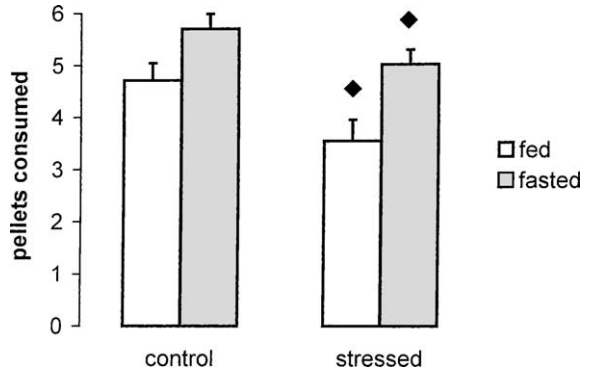


Fig. 2. Mean intake of sweet food (Froot loops) after chronic variate stress (mean ± S.E.M.). $N = 14$ (control group) and 15 (stressed group). A repeated measures ANOVA showed an effect of treatment ($P < 0.05$) and fed state ($P < 0.001$). (◆) Significantly different from control group ($P < 0.05$).

5-HIAA/5-HT ratio, is shown in Table 2. A Student's *t*-test revealed a significant effect of the chronic treatment on 5-HIAA/5-HT ratio in hippocampus, with an increased ratio in chronic stressed animals ($t(17) = 2.1$; $P < 0.05$). This stress model had no effect upon any of the other structures analyzed (Student's *t*-test, $P > 0.05$).

The effect of chronic variate stress on dopamine and its metabolite levels in different brain structures, as well as the DOPAC/DA and HVA/DA ratios, is shown in Table 3. A Student's *t*-test revealed a significant effect of the chronic treatment on DOPAC and HVA levels in hypothalamus ($t(17) = 3.2$, $P < 0.005$ for DOPAC; $t(17) = 2.55$, $P < 0.05$ for HVA), which were decreased, in addition to a decreased ratio of DOPAC/DA ($t(17) = 2.33$, $P < 0.05$). DOPAC levels were increased in hippocampus ($t(17) = 2.38$, $P < 0.05$) and frontal cortex ($t(17) = 3.72$, $P < 0.005$). No effect was observed in amygdala ($P > 0.05$ in all cases).

Table 2
The 5-HT, 5-HIAA levels, and 5-HIAA/5-HT ratio in amygdala (AMY), hypothalamus (HPT), hippocampus (HPC), and frontal cortex (CTX) in control and chronic stressed animals

| | 5-HT | 5-HIAA | 5-HIAA/5-HT |
|---|---|---|---|
| Control group ($N = 10$) | | | |
| AMY | 626.0 ± 36.1 | 543.8 ± 32.3 | 0.89 ± 0.07 |
| HPT | 624.7 ± 48.6 | 637.8 ± 59.7 | 1.07 ± 0.12 |
| HPC | 354.1 ± 39.0 | 376.2 ± 32.0 | 1.11 ± 0.10 |
| CTX | 434.0 ± 55.1 | 300.7 ± 16.1 | 0.87 ± 0.18 |
| Chronic stressed group ($N = 9$) | | | |
| AMY | 626.1 ± 24.5 | 585.1 ± 31.9 | 0.94 ± 0.04 |
| HPT | 645.3 ± 33.6 | 576.1 ± 54.3 | 0.92 ± 0.09 |
| HPC | 277.0 ± 35.6 | 379.2 ± 34.7 | 1.44 ± 0.11* |
| CTX | 485.8 ± 79.8 | 322.6 ± 24.7 | 0.84 ± 0.14 |

Values are mean ± S.E.M.
* Significant difference from control group by Student's *t*-test, $P < 0.05$.

Table 3
Dopamine (DA), DOPAC and HVA levels, and DOPAC/DA and HVA/DA ratios in amygdala (AMY), hypothalamus (HPT), hippocampus (HPC), and frontal cortex (CTX) in control and chronic stressed animals

| | DA | DOPAC | HVA | DOPAC/DA | HVA/DA |
|---|---|---|---|---|---|
| Control group ($N = 10$) | | | | | |
| AMY | 1135.9 ± 125.6 | 446.1 ± 64.5 | 204.8 ± 25.4 | 0.38 ± 0.03 | 0.20 ± 0.04 |
| HPT | 235.1 ± 23.7 | 100.4 ± 10.2 | 130.4 ± 25.8 | 0.51 ± 0.10 | 0.67 ± 0.18 |
| HPC | 56.3 ± 12.9 | 25.8 ± 2.9 | 34.4 ± 14.3 | 0.72 ± 0.19 | 0.84 ± 0.45 |
| CTX | 43.4 ± 6.3 | 34.2 ± 3.9 | 121.4 ± 53.2 | 0.94 ± 0.16 | 4.37 ± 2.50 |
| Chronic stressed group ($N = 9$) | | | | | |
| AMY | 1081.4 ± 171.2 | 332.1 ± 47.1 | 176.3 ± 22.0 | 0.33 ± 0.04 | 0.21 ± 0.05 |
| HPT | 245.6 ± 59.4 | 53.7 ± 10.45* | 55.6 ± 14.0† | 0.25 ± 0.04† | 0.29 ± 0.08 |
| HPC | 40.4 ± 8.0 | 52.6 ± 11.4† | 58.0 ± 12.5 | 2.45 ± 1.27 | 2.22 ± 0.93 |
| CTX | 68.60 ± 13.46 | 87.45 ± 13.78† | 103.7 ± 27.4 | 1.71 ± 0.42 | 2.32 ± 0.85 |

Values are mean ± S.E.M.
* Significant difference from control group by Student's *t*-test, $P < 0.005$.
† Significant difference from control group by Student's *t*-test, $P < 0.05$.

## 4. Discussion

In this study, we observed a decreased appetite for sweet food in response to chronic variate stress, whereas there was no alteration in the intake of habitual rat chow in chronically-stressed rats. Both groups (control and stressed) gained weight. The adrenal hypertrophy observed may be considered an index of the effectiveness of the stress model.

When considering changes in body weight or adrenal weight, chronic variate stress has been observed to possibly induce a significant reduced gain in body weight, as well as an increase in the adrenal weight after 14 (Harro et al., 2001) or 28 days (Konarska et al., 1990). These last authors found increased HVA levels in the frontal cortex of animals submitted to chronic variable stress, but not reduced sucrose intake or preference in non-deprived animals. Animals exposed to chronic variable stress which were tested 1 week later for sucrose preference (1%) showed a reduction in sucrose preference only when they were later exposed to restraint (Zurita et al., 2000). Data from our laboratory show reduced consumption of sweet food after at least 30 days of stress. Therefore, it appears that animals submitted to chronic variable stress may present other effects, such as increased adrenals after just 2 weeks of stress, while behavioral changes, such as the one observed here may need more time to develop.

Some studies (Dess et al., 1988; Hargreaves, 1990; Konarska et al., 1990; Harro et al., 2001) have reported that chronic exposure to stressors of a certain severity decreases food intake and body weight of rats. Despite this, the type, duration or severity of stress and the predictability of the stressor applied may modify the responses to stress (Hargreaves, 1990; Paré and Redei, 1993; Pucilowski et al., 1993; Marti et al., 1994). Morley et al. (1986) proposed that stress can lead to either decreased or increased feeding, depending on the nature of the stressor. For instance, repeated restraint stress leads to alterations in feeding behavior with increased sweet food ingestion (Ely et al., 1997), which is interpreted as an expression of increased levels of anxiety in chronically stressed animals, since this effect is reversed by diazepam (Ely et al., 1997). On the other hand, some models of chronic mild stress have been reported to lead to a wide range of behavioral disturbances (Katz et al., 1981; Willner, 1991; Basso et al., 1993), including decreased responses to rewarding stimuli, as demonstrated by decreased sucrose consumption (Willner et al., 1987; Ferreti et al., 1995) and place preference conditioning (Papp et al., 1991, 1992).

The chronic mild stress paradigm consists of exposing rats to different weak stressors for several days and it is believed to be an animal model of decreased response to rewards (anhedonia) (Willner, 1990; Dáquila et al., 1994), although some authors have not observed a reliable decrease in sucrose consumption after chronic mild stress (Matthews et al., 1995; Nielsen et al., 2000), possibly because of different sensitivity to stress by different rat strains. The chronic stress model used in the present study was adapted from various models of chronic mild stress (Willner et al., 1987; Echandia et al., 1988; Konarska et al., 1990; Papp et al., 1991; Muscat et al., 1992; Murua and Molina, 1992) although some different stressors were used. The absence of predictability of the stressor applied is an important characteristic of this model and may be related to the different effects observed in these animals when compared to other models in which repeated stress is used and higher consumption of sweet food is observed (Ely et al., 1997; Silveira et al., 2000).

Brain monoamine levels change after acute stress, and chronic exposure to stress may present different effects on these neurotransmitters. For example, in the prefrontal cortex, increased extracellular levels of dopamine are observed after acute stress (Detch et al., 1990; Imperato et al., 1992; Sorg and Kalivas, 1993). On the other hand, chronic stress over several days (Imperato et al., 1992; Mangiavacchi et al., 2001) reduces DA output. Studies suggest that mechanisms

involving central dopamine and serotonin systems are necessary for a normal eating response to sweet tasting stimuli independently of hunger perception (Blundell, 1991; Orosco and Nicolaidis, 1992; Ahn and Phillips, 1999; Orosco et al., 2000; Collin et al., 2000; Voigt et al., 2000; Pitts and Horvitz, 2000).

In this study, an increased serotonergic activity was observed in hippocampus in chronic stressed animals. Since the hippocampus is a brain structure with a high density of glucocorticoid receptors, this increased serotonergic activity may be a result of glucocorticoid action in this structure (Chalmers et al., 1993; Nishi and Azmitia, 1996). An increase in the levels of glucocorticoid hormones, one of the main biological responses to stress, is known to alter serotonin metabolism in the central nervous system and periphery (Paris et al., 1987; Malyszko et al., 1994; Nishi and Azmitia, 1996). Increased 5-HT turnover and tryptophan hydroxylase activity, the rate limiting biosynthetic enzyme for serotonin, appear to be sensitive to circulating corticosteroid levels (Singh et al., 1990; Chalmers et al., 1993; Chauloff, 1993). Electrophysiological evidence indicates a direct interaction between 5-HT and corticosteroid-responsive hippocampal neurons (Azmitia et al., 1984; Joels and de Kloet, 1992; Joels et al., 1995; Joels et al., 1997). Removal of circulating corticosteroids by adrenalectomy results in anatomically specific decreased indices of 5-HT metabolism, while stressful procedures, which raise corticosteroid levels, cause increases in 5-HT turnover (Chalmers et al., 1993; Malyszko et al., 1994; Nishi and Azmitia, 1996).

Enhancing activity at post-synaptic serotonergic receptors reduces the amount of food eaten during a meal and decreases the rate of eating (Simansky, 1996). In this study, however, the only structure presenting increased serotonergic activity was the hippocampus. In the hippocampus, 5-HT is thought to influence mood and also to interact with the brain-pituitary-adrenal axis (Siegel, 1993). In addition, the hippocampus has been implicated in the organization of meal pattern (Clifton, 2000), with little evidence of an influence on body weight regulation. It is possible that the altered serotonergic activity in hippocampus is not related to the decreased sweet food consumption observed after chronic stress, since 5-HT has been shown to be involved in the modulation of feeding behavior mainly in other structures, such as the hypothalamus (Leibowitz et al., 1990; Orosco and Nicolaidis, 1992; Simansky, 1996; Collin et al., 2000; Vry and Schereiber, 2000) and the amygdala (Parker and Coscina, 2001; Parker et al., 2001).

In the present study, we observed increased levels of DOPAC in hippocampus and frontal cortex, suggesting an increased catabolism of DA to DOPAC by intraneuronal monoamine oxidase, which may reflect increased metabolism of dopaminergic neurons in these structures. This finding agrees with other reports in the literature which report that exposure to mild stress increases the dopaminergic activity in several brain regions (Thierry et al., 1976; Jedema and Moghaddam, 1994; Roth et al., 1998). It has been suggested that the changes in dopamine activity may be related to coping attempts made by the animal (Cabib and Puglisi-Allegra, 1994). In addition, it has been suggested that DA activity in medial prefrontal cortex is influenced by the sensory incentive properties of food, and increases, signaling the relative salience of foods (Ahn and Phillips, 1999). This observation contrasts with our observation that increased metabolism of dopamine is observed together with decreased appetite for sweet food. On the other hand, DA transmission in prefrontal cortex is also enhanced by aversive stimuli (Di Chiara et al., 1999); as a result, the alteration observed in frontal cortex in the present study may be an effect of the exposure to the stress model.

Decreased dopaminergic activity (as shown by decreased DOPAC/DA and HVA/DA ratios) was observed in hypothalamus. Dopaminergic and serotonergic activities in hypothalamus are related to feeding behavior (Orosco and Nicolaidis, 1992; Collin et al., 2000; Fetissov et al., 2000; Vry and Schereiber, 2000). Dopamine is involved in motivation and it is believed to mediate appetite or approach behaviors triggered by incentive stimuli associated with rewards (Berridge and Robinson, 1998; Ahn and Phillips, 1999). DA is also involved in mechanisms of inhibition of food intake, mainly on lateral hypothalamus, while in the medial hypothalamic nuclei it is associated with stimulation of food intake (Fetissov et al., 2000). Although the physiological significance of dopamine release in the hypothalamus in food intake regulation remains incompletely understood, evidence points to a relationship with the process of hunger and satiety (Orosco and Nicolaidis, 1992; Fetissov et al., 2000). Since brain monoamines respond differently to acute or chronic stress, we do not know at which time of stress treatment changes in hypothalamic monoamines develop, or if they coincide with changes in sweet food consumption. More detailed studies in specific hypothalamic regions in this stress model are needed to help to understand the effects of chronic stress on monoamines and its relation with eating behavior.

Dopamine seems to regulate food intake by modulating food reward (Martel and Fantino, 1996; Balcioglu and Wurtman, 1998). We do not yet know what the effect of dopamine receptor antagonists would be on sweet food intake in chronically stressed rats, but certainly this is an experiment that would bring some light to the mechanisms by which chronic variable stress acts on feeding behavior. It is possible that one of the effects of chronic mild stress is to modify, at the neurobiological level, the motivational and/or reinforcing properties of sweet food, in this case decreasing the activity of those systems involved in motivation and reward.

These results suggest that chronically stressed rats that show decreased motivation toward normally pleasurable stimuli had a significant decrease in dopaminergic activity in hypothalamus. It is possible that alterations in monoamine activity may help to explain some of the effects of chronic stress on behavior. The exact neurobiological mechanism

involved in this effect after chronic stress, and if there is a relationship between brain monoamines and the anorexic effect of chronic stress, still requires further study.

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## Capítulo III – ARTIGO 2

Redução da atividade da enzima $Na^+$, $K^+$ - ATPase hipocampal em ratos submetidos a um modelo experimental de depressão

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# Reduction of Hippocampal $Na^+$, $K^+$-ATPase Activity in Rats Subjected to an Experimental Model of Depression

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The effect of a model of depression using female rats on $Na^+$, $K^+$-ATPase activity in hippocampal synaptic plasma membranes was studied. In addition, the effect of further chronic treatment with fluoxetine on this enzyme activity was verified. Sweet food consumption was measured to evaluate the efficacy of this model in inducing a state of reduced response to rewarding stimili. After 40 days of mild stress, a reduction in sweet food ingestion was observed. Reduction of hippocampal $Na^+$, $K^+$-ATPase activity was also observed. Treatment with fluoxetine increased this enzyme activity and reversed the effect of stress. Chronic fluoxetine decreased the ingestion of sweet food in both groups. This result is in agreement with suggestions that reduction of $Na^+$, $K^+$-ATPase activity is a caracteristic of depressive disorders. Fluoxetine reversed this effect. Therefore it is possible that altered $Na^+$, $K^+$-ATPase activity may be involved in the pathophysiology of depression in patients.

**KEY WORDS:** $Na^+$, $K^+$-ATPase; fluoxetine; chronic mild stress; hippocampus; depression.

## INTRODUCTION

$Na^+$, $K^+$-ATPase is the enzyme responsible for the active transport of sodium and potassium ions in the nervous system, maintaining the ionic gradient necessary for neuronal excitability and regulation of neuronal cell volume. It is present in high concentration in brain cellular membranes, consuming about 40%–50% of the ATP generated in this tissue (1), and its activity is decreased in patients with bipolar affective disorder and other psychiatric disorders (2–5). Nevertheless, no determinations of the enzyme activity in experimental models of depression in animals have been carried out.

Some models of chronic mild stress have been proposed as models of depression in animals studies (6–8). In these models, rats are exposed to different weak stressors for several days. The response to rewarding stimuli is diminished, as demonstrated by tests showing reduced sucrose consumption, which is interpreted as anhedonia. It is interesting to consider that, although in humans women are more sensitive to this condition than men (9), most of the studies using animal models of depression has being done using males.

Fluoxetine, an antidepressant that selectively inhibits serotonin reuptake (10), is widely used in the treatment of depression. Serotonin (5-HT) has been implicated in the pathophysiology of affective disorders (11), and it is removed from the synaptic cleft by sodium-dependent uptake through transporters localized in the plasma membrane of presynaptic cells (12,13), in a close association with $Na^+$, $K^+$-ATPase activity (12). In this context, recently we have shown that chronic administration of fluoxetine significantly increased $Na^+$, $K^+$-ATPase activity in brain of normal rats (14).

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Considering that most animal models of depression have used male animals, although females are usually more susceptible to depression, in the present study we studied a model of depression (chronic mild stress) using female rats. We also studied the effect of this model of depression on $Na^+$, $K^+$-ATPase activity in synaptic plasma membranes from rat hippocampus, with or without chronic fluoxetine administration. In addition, sweet food consumption was measured to evaluate the efficacy of this model of chronic stress in inducing a state of reduced response to rewarding stimili, both after the chronic stress treatment and during fluoxetine treatment.

## EXPERIMENTAL PROCEDURE

*Animals.* Thirty-six female Wistar rats (60 days old; 180–230 g of weight) were used for behavioral measures. Eighteen animals were used in biochemical measures. The experimentally naive animals were housed in groups of four or five in home cages made of Plexiglas (65 × 25 × 15 cm) with the floor covered with sawdust. They were maintained under standard dark-light cycle (lights on between 7:00 and 19:00 h) at a room temperature of 22° ± 2°C. The rats had free access to food (standard rat chow) and water, except for the stressed group during the period when the stressor applied required no food or water. All animals 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 used.

*Stress Model.* Chronic mild stress model was modified from other models of mild stress (15–17). The animals were divided in two groups: control and stressed. Controls were kept undisturbed in their home cages during the first 40 days of treatment. A variate-stressor paradigm was used for the animals in the stressed group. The following stressors were used: (i) 24 h of food deprivation; (ii) 24 h of water deprivation; (iii) 1 h to 3 h of restraint, as described below; (iv) 1.5 to 2 h of restraint at 4°C; (v) forced swimming during 10 or 15 min, as described below; (vi) flashing light during 120–210 min; and (vii) isolation (2–3 days). Stress application started at different times every day, to minimize its predictability.

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

*Consumption of Sweet Food.* After 40 days of treatment, consumption of sweet food was measured in 36 animals (17 controls and 19 stressed). The animals were placed in a lightened rectangular box (40 × 15 × 20 cm) with a glass ceiling and floor and side walls made of wood. Ten Froot Loops (Kellogg's®—pellets of wheat, cornstarch, and sucrose) were placed in one end of the box. Animals were subjected to five 3-min trials, one per day, to become familiarized with this food (18). During these habituation trials, the animals were under food restriction; that is, starting 24 h before the first trial, laboratory chow offered daily was 90% of normal. After being habituated, the animals received laboratory chow ad libitum, and 2 days later they were exposed to a test session, 3 min each, when the number of ingested pellets was measured. A protocol was established so that when the animal ate part of the Froot Loops (e.g., $\frac{1}{3}$ or $\frac{1}{4}$) this fraction was considered.

*Pharmacological Treatment.* Fluoxetine (8.0 mg/kg) or vehicle (10% Tween 80 in saline) were administered daily IP, between 9 and 10.00 A.M., during a total of 60 days, in animals subjected or not to chronic stress. This dose of fluoxetine was chosen according to the literature (19).

*Preparation of Synaptic Plasma Membrane from Hippocampus.* The brain was rapidly removed, and the hippocampus was dissected to prepare synaptic plasma membranes according to the method of Jones and Matus (20), with some modifications (21). The hippocampus was homogenized in 10 volumes of a 0.32-M sucrose solution containing 5 mM HEPES and 1 mM EDTA. The homogenate was centrifuged at 1000 × *g* for 20 min and the supernatant removed and centrifuged at 12000 × *g* for a further 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris-HCI buffer, pH 8.1), incubated at 0°C for 30 min, and applied on a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8, and 1.0 M. After centrifugation at 69,000 × *g* for 2 h, the fraction at the 0.8–1.0 M sucrose interface was taken as the membrane enzyme preparation.

*$Na^+$, $K^+$-ATPase Activity Assay.* The reaction mixture for the $Na^+$, $K^+$-ATPase assays contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCI, and 40.0 mM Tris-HCI buffer, pH 7.4, in a final volume of 200 μl. The reaction was started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3.0 mM. Control was assayed under the same conditions with the addition of 1.0 mM ouabain. $Na^+$, $K^+$-ATPase activity was calculated by the difference between the two assays (21,22). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (23). Enzyme-specific activities were expressed as nmol Pi released per minute per milligram of protein. All assays were performed in duplicate, and the mean was used for statistical analysis.

*Protein Determination.* Protein was measured by the method of Bradford (24) with bovine serum albumin used as standard.

*Statistical Analysis.* Data were expressed as mean ± SEM. Sweet food intake was analyzed using Student's t test for independent samples. $Na^+$, $K^+$-ATPase activity was analyzed using two-way ANOVA followed by Duncan's multiple range test when the *F* test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software.

## RESULTS

The effect of 40 days of chronic mild stress upon the intake of Froot Loops in animals fed ad libitum is shown in Fig. 1, in the bars representing the consumption of sweet food pellets before the fluoxetine treatment. There was a significant effect of the chronic treatment [$t(29.32) = 6.29$, $P < 0.001$], where the stress determined a decreased intake. After being subjected to the feeding behaviour test, animals in both groups were divided into two other groups (receiving daily administration of saline or fluoxetine 8 mg/kg IP), in such a way that four groups were obtained: control plus saline, control plus fluoxetine, stressed plus saline, and

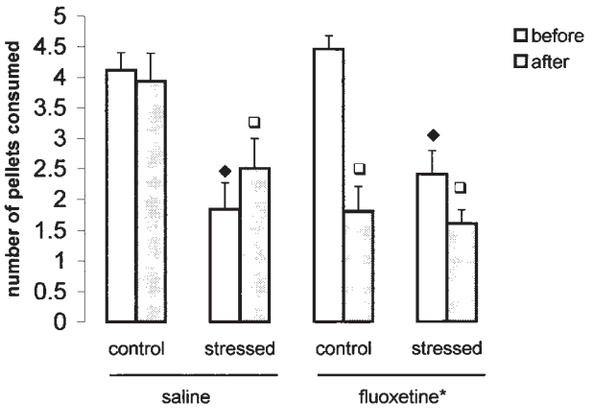


**Fig. 1.** Mean intake of sweet food (Froot Loops) after chronic mild stress (mean ± SEM), before and after administration of saline or fluoxetine (8 mg/kg) IP (n = 12–13 animals/group). A two-way ANOVA showed an effect of stress exposure ($P < 0.05$) and of fluoxetine treatment ($P < 0.001$). ◆ Stressed group differs significantly from control group before fluoxetine administration (Duncan's $P < 0.05$). ❑ Significantly different from control group receiving saline (Duncan's $P < 0.05$). * Significant effect of fluoxetine treatment (two-way ANOVA, $P < 0.01$).

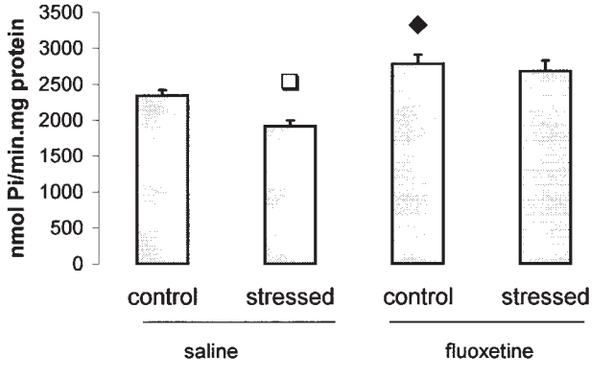


**Fig. 2.** Mean ± SEM of $Na^+$, $K^+$-ATPase activity (nmol inorganic phosphate released per min per mg protein) in hippocampal synaptic membranes of control and stressed rats, treated or not with fluoxetine (8 mg/kg) (n = 4–5 animals/group). A two-way ANOVA showed an effect of stress exposure ($P < 0.01$) and of fluoxetine treatment ($P < 0.05$). ❑ Significantly different from all other groups (Duncan, $P < 0.05$). ◆ Significantly different from control group receiving saline (Duncan, $P < 0.05$).

stressed plus fluoxetine. During the period when drugs were administered, stress continued to be applied regularly to the stressed animals. Figure 1 also shows that, after 28 days of treatment, the consumption of sweet food pellets was decreased by the fluoxetine treatment [two-way ANOVA, $F(1,32) = 14.99$, $P < 0.002$]. There was also an effect of the chronic stress treatment [two-way ANOVA, $F(1,32) = 4.34$, $P < 0.05$], but the stressed group receiving fluoxetine presented a consumption of sweet pellets similar to that of the control group receiving fluoxetine, as can be observed in Fig. 1. After 60 days of treatment, results present the same pattern as those presented in Fig. 1 for 28 days of treatment (data not shown).

Figure 2 shows that $Na^+$, $K^+$-ATPase activity presented a significant decrease of around 20% in rats subjected to this experimental model of depression [two-way ANOVA, $F(1,13) = 24.85$, $P < 0.001$ for the stress effect]. Fluoxetine per se significantly increased this enzyme activity around 20% [two-way ANOVA, $F(1,13) = 4.80$, $P < 0.05$ for the fluoxetine effect]. On the other hand, fluoxetine treatment reversed the decreased $Na^+$, $K^+$-ATPase activity caused by this model of chronic stress.

## DISCUSSION

In the present study, we observed a decreased appetite for sweet food in response to chronic mild stress. Similar models of chronic mild stress have been reported to lead to a wide range of behavioral and physiological disturbances (7,25,26), including decreased responses to rewarding stimuli, as demonstrated by decreased sucrose consumption (15,27) and place preference conditioning (26,28). Most of these studies, however, were done using males. In addition, it has been reported that Wistar male rats are more resistent to the chronic variable stress-induced behavioral effects when concerning hedonic measures (27). In the present study, we show that Wistar female rats subjected to variable stress actually present this reduced consumption of sweet food, which agrees with studies in humans showing that females are usually more susceptible to depression (9).

The chronic mild stress paradigm consists of exposing rats to different weak stressors for several days, and it is believed to be an animal model of decreased response to rewards (anhedonia) (8,17), although in certain cases some authors have not observed a reliable decrease in sucrose consumption after chronic mild stress (27,29), maybe because of different sensitivity to stress by different rat strains. The chronic stress model used in the present study was adapted from some models of chronic mild stress (15–17,26,30,31), which are considered models of depression in animals. The absence of predictability of the stressor applied is an important characteristic of this model and may be related to the different effects on sweet food consumption observed in these animals when compared to other models, in which the same stressor is repeatedly used (18,32).

Fluoxetine treatment started after 40 days of stress. During the treatment, an effect of fluoxetine was observed in the number of sweet pellets ingested, which were decreased both in the control and in the chronically stressed group. 5-HT has been implicated in the control of eating behavior and body weight. Enhanced activity at postsynaptic serotonergic receptors reduces the amount of food eaten during a meal, decreases the rate of eating and weight gain, and increases energy expenditure, both in animals and in humans (33,34). Fluoxetine is also known to produce anorectic effects (35,36), and its potency as an anorectic agent paralleles its potency as inhibitor of 5-hydroxytryptamine (5-HT) uptake in vivo (35). This drug preferentially inhibits the ingestion of carbohydrate, more than fat or protein (37). A negative feedback loop exists between the consumption of carbohydrates and the turnover of 5-HT in the hypothalamus. That is, carbohydrate ingestion enhances the synthesis and release of hypothalamic 5-HT, which in turn serves to control the size of carbohydrate-rich meals (38).

Sweet food consumption was similar in both fluoxetine-treated groups, stressed or control, in such a way that no addition of effects (stress and fluoxetine) was observed in the group subjected to both treatments. The fact that the decrease in sucrose consumption by fluoxetine treatment was unchanged by the addition of stress might represent a ceiling effect. This is not the case, however. When tested after 15 days of treatment with fluoxetine, a different pattern of consumption is observed. In the saline-treated groups, consumption was decreased by stress (control group: $5.2 \pm 0.5$, stressed group $2.8 \pm 0.8$), whereas in the fluoxetine-treated groups, consumption of sweet food was decreased by fluoxetine treatment and there was a further decrease induced by stress (control group + fluoxetine: $1.0 \pm 0.3$, stressed group + fluoxetine: $0.5 \pm 0.2$). Therefore, at 15 days of treatment, the antidepressive effect of fluoxetine was not yet evident. These observations may suggest that fluoxetine treatment for a longer period is able to prevent the effect of stress on this behavior.

$Na^+$, $K^+$-ATPase activity was decreased in hippocampus of animals subjected to chronic mild stress, which agrees with reports from findings in depressed patients (39). Because reduction of $Na^+$, $K^+$-ATPase activity seems to be an important caracteristic of depressive disorders (39,40), this finding further support this model of chronic mild stress as an animal model of depression. In addition, an increase of this enzyme activity was observed with fluoxetine treatment. This effect agrees with previous results (14), in which increased activity of this enzyme was observed after fluoxetine administration in vivo. Fluoxetine treatment reverted the effect of chronic stress on $Na^+$, $K^+$-ATPase activity, in such a way that animals which were chronically stressed and treated with fluoxetine showed the same levels of enzyme activity than controls. This is the first time that a reduction of $Na^+$, $K^+$-ATPase activity was demonstrated in an animal model of depression, or after exposure to chronic stress; in addition, $Na^+$, $K^+$-ATPase activity was reversed to the control levels in stressed animals. Theses results help to correlate changes in behavior with neurobiological modifications at the neurochemical level, which is an important further step in animal models of diseases.

Regulation of $Na^+$, $K^+$-ATPase activity is a complex matter. This activity seem to be regulated by several factors, including hormones and neurotransmitters (41–43), such as catecholamines (44) and serotonin (45). It should be observed that central catecholaminergic (25,46) and serotonergic (47,48) activity may be modulated by exposure to stress situations. In addition, regulation of $Na^+$, $K^+$-ATPase activity can be divided into a "short-term" and a "long-term" control (49). Although the exact underlying mechanisms responsible for our results are not known, it is possible that the "long-term" control is involved, because both fluoxetine and stress treatments were administered during several weeks. This long-term control could be due to an increased number of pumps, brought about by a increased protein synthesis or a decreased protein degradation. This possibility should be tested in future studies.

It is important to note that the present study evaluated the effects of repeated stress and fluoxetine treatment in hippocampus, a region particularly sensitive to stress effects (50). The systems mediating the 5-HT regulatory mechanisms on the $Na^+$, $K^+$-ATPase activity have been shown to depend on the brain area and on the particular physiological conditions (45); thus it is possible that different effects may be found in other brain regions in these conditions.

Considering repeated stress, different morphological and neurochemical effects have been reported in hippocampus. Depression, particularly stress-associated cases, may result from atrophy of pyramidal neurons in the hippocampus (51). This atrophy, with reduction in the number of synapses, may be involved in several repeated stress effects, including this reduced $Na^+$, $K^+$-ATPase activity. Antidepressant treatments could reverse this atrophy (52). This possibility deserves further investigation.

In conclusion, $Na^+$, $K^+$-ATPase activity was inhibited in a depression model in female rats. This result is in agreement with other evidence from the literature, suggesting decreased activity of this enzyme to be an

important caracteristic of depressive disorders (39,40), in such a way that altered activity of $Na^+$, $K^+$-ATPase may be involved in the pathophysiology of depression in patients. In addition, it was observed that fluoxetine reversed this inhibition. In this context, evidence from the literature shows increased activity of $Na^+$, $K^+$-ATPase in serotoninergic-rich regions of the brain, such as cortex, striatum, and hippocampus (53). It is important to note, however, that chronic administration of other drugs used for the treatment of bipolar illness such as haloperidol, carbamazepine, and lithium also increase membrane $Na^+$, $K^+$-ATPase activity in rat brain (39,54). Therefore it is possible that this enzyme activity might contribute to the therapeutic efficacy of fluoxetine, because brain $Na^+$, $K^+$-ATPase activity is decreased in bipolar patients.

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## Capítulo IV – ARTIGO 3

Efeitos do tratamento de fluoxetina sobre os níveis de leptina em ratos submetidos a um modelo de estresse crônico

*Phisiology & Behavior* (submetido em agosto 2003)

# EFFECTS OF FLUOXETINE TREATMENT ON LEPTIN LEVELS IN RATS SUBMITTED TO A MODEL OF CHRONIC STRESS

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**Running title**: fluoxetine, leptin and chronic stress

## Abstract

GAMARO, G.D.; PREDIGER, M.E.; LOPES, J. ; BASSANI, M.G. ; DALMAZ, C EFFECTS OF FLUOXETINE TREATMENT ON LEPTIN LEVELS IN RATS SUBMITTED TO A MODEL OF CHRONIC STRESS Physiol Behav 56(6) 000-000,2003. Stress situations can influence hormones levels and change behavioral and physiological patterns. Alterations in feeding behavior are sexually dimorphic and have been related to changes in monoamine levels. Leptin may interact with stress hormones and with the brain serotonergic system, possibly affecting feeding behavior of stressed rats. The aim of this study is to evaluate the interaction between chronic fluoxetine treatment and leptin levels in animals submitted to chronic variate stress. Adult female Wistar rats were divided into control and stressed groups. After 30 days of chronic stress, the animals presented increased leptin levels compared to controls, as well as decreased consumption of sweet food. After this first stress period, the animals were subdivided into two groups that received daily injections of saline or fluoxetine (8mg/kg, i.p.). Body weight was evaluated before, and after 2, 4 and 8 weeks of fluoxetine treatment. Significant stress x time and fluoxetine x time interactions were observed, signifying that the difference in weight gain was higher at the beginning than during the last days of treatment. On the 60th day of fluoxetine treatment leptin levels were decreased in fluoxetine-treated animals indicating no effect of stress. We conclude that chronic variate stress may affect leptin levels, and that this effect is dependent on the time of stress exposure. In addition, chronic fluoxetine treatment induced a strong reduction in leptin levels. The neurobiological mechanism involved in this effect after chronic fluoxetine administration and the relationship between feeding behavior and the effects reported herein require further study.

**Key words**: fluoxetin; chronic variate stress; leptin; female rats

## Introduction

Exposure to stress may cause either an increase or a decrease in food intake, depending on the nature of stress (1-4). Models of chronic variate stress have been observed to induce a decreased appetite for sweet food or palatable solutions (3;5). However, although, in humans, women are more sensitive to disturbances in feeding behavior than men (6,7), most of the studies in animal models have been performed in males (8,9).

Some models of chronic mild stress have been proposed as models of depression in animals studies (5,10,11,12). In these models, rats are exposed to different weak stressors for several days. The response to rewarding stimuli is diminished, as demonstrated by tests showing reduced sucrose consumption, which is interpreted as anhedonia (5;10). Stress has been shown to alter normal serotonergic and dopaminergic neurotransmission (13,14). Although less is known about the long-term alterations in serotonergic neurotransmission caused by chronic stress, the efficacy of serotonin selective re-uptake inhibitors in treating depression suggests that serotonin may be involved in the development or expression of stress-related depression.

In pharmacological studies, drugs that either directly or indirectly increase postsynaptic serotonergic stimulation routinely decrease the consumption of food by mammalian species ranging from rodents to human and nonhuman primates (15-17). In contrast, agents that block postsynaptic serotonin receptors or that diminish serotonergic neurotransmission by activating autoreceptors often increase food intake (for a review, see 18). Fluoxetine, a selective inhibitor of serotonin

reuptake, is indicated for patients with depression. Furthermore, it may be used in patients with obesity because it causes weight loss both in laboratory animals and humans (19, 20).

Leptin is a hormone secreted mainly by the adipose cells and has a role as a metabolic adaptator in the regulation of body weight. It is believed to establish a feedback loop between the energy reserves and the hypothalamic centers that control food intake (21-23). Recent data suggest that, in addition, leptin interacts with other endocrine systems to provide critical information about the size of the fat stores (22;24). This hormone participates in the expression of CRH in the hypothalamus, interacts in the adrenals with ACTH and its levels are regulated by glucocorticoids (25,26). Therefore, leptin is probably influenced by activation of the hypothalamo-pituitary-adrenal (HPA) axis, when animals are exposed to stress situations.

Acute treatment with fluoxetine causes a decrease in leptin levels, which has been suggested to be a mechanism for offsetting the appetite suppressing effects of fluoxetine (27). An interaction between leptin and the serotonergic system has recently been suggested by studies showing that i.p. leptin administration increases 5HT metabolism in the rat hypothalamus (28). Leptin appears to have a direct role in the cells of the dorsal raphe nucleus (17,29), therefore, apart from directly affecting hypothalamic neurons and, thereby, regulating body weight, leptin may also affect behavior mediated via the brain serotonergic system. Thus, leptin may affect body weight indirectly via projections

from the serotonergic raphe neurons to several hypothalamic regions containing multiple – 5HT receptors.

The aim of this study was to investigate the leptin response in rats submitted to chronic fluoxetine treatment (60 days), and the effects of chronic variate stress on leptin levels after chronic fluoxetine treatment.

**Experimental procedures**

Animals

Adult, female Wistar rats (60 days old; 200-270 g of weight) were used. The 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. They were maintained under a standard dark-light cycle (lights on between 7:00 and 19:00 h) in a room temperature of 22 $\pm$ 2°C. The rats had free access to food (standard rat chow) and water, except for the stressed group during the period when the stressor applied required no food or water. The animals were divided in two groups: control and stressed. After 30 days of stress, sweet food consumption was evaluated, in order to confirm the ability of this stress treatment to induce anhedonia, a symptom of depression. Serum leptin levels were also measured. Afterwards, both groups were subdivided in saline and fluoxetine treated, and the animals were submitted to fluoxetine treatment for 60 days.

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.

Stress Model:

Chronic variate stress was modified from other models of variate stress (30-33), and followed the protocol already described (3,34). The animals were divided in 2 groups: Control and Stressed. Controls were handled daily. A 100-day variate-stressor paradigm was used for the animals in the stressed group. The following stressors were used: a) 24 h of food deprivation, b) 24 h of water deprivation, c) 1 h to 3 h of restraint, as described below, d) 1.5 to 2 h of restraint at 4° C, e) forced swimming during 10 or 15 min, as described below, f) flashing light during 120 to 210 min, g) isolation (2 to 3 days).

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

Pharmacological treatment:

After 40 days of chronic stress treatment, each group (control and stressed) was subdivided into two other groups: (1) Fluoxetine (8.0 mg/kg) or (2) vehicle

(10% tween 80 in saline) were administered daily i.p., between 9 and 10:00 a.m., for a total of 60 days, in animals subjected, or not, to chronic stress. This dose of fluoxetine was chosen according to the literature (35).

Leptin Measurement

The animals were sacrificed by decapitation 24 h after the last stress session. Trunk blood was collected and serum separated and frozen until the day of the analysis. Measurement of serum leptin was performed with a commercial mouse leptin ELISA kit (Crystal Chem. Inc., Chicago, IL, USA).

**Statistical analysis**

Data were expressed as mean $\pm$ standard error of the mean (S.E.M.). Leptin levels were was analyzed using Student's t test for independent samples (first experiment) or two-way ANOVA (second experiment). Body weight was analyzed using repeated measures ANOVA. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software.

**Results**

After 30 days of treatment, some of the animals were sacrificed and serum leptin levels were measured in female rats subjected to chronic variate stress and control treatment. Results are shown in Figure 1. Leptin levels were increased in stressed rats (Student's t test, $t(8) = 2.71$; $P < 0.05$). At this time of the treatment, sweet food consumption was decreased in chronically stressed animals (data not shown), confirming the effect of this type of stress in this parameter.

At 40 days of treatment, both groups were subdivided and treated with saline or fluoxetine (8 mg/kg, i.p.). Body weight was evaluated in the four groups before fluoxetine treatment started, and after 2, 4 and 8 weeks of treatment. Weight gain during this treatment is shown in Figure 2. Repeated measures ANOVA presented a marginal effect of stress treatment [$F(1.16) = 4.12$; $P = 0.059$]. There was no effect of fluoxetine [$F(1,16) = 0.90$; $P > 0.05$] and no significant fluoxetine x time interaction [$F(1,16) = 0.10$; $P > 0.05$]. The animals gained weight with a significant effect of time [$F(3,48) = 115.48$; $P < 0.01$] and also significant stress x time interaction [$F(3,48) = 17.95$; $P < 0.01$], and fluoxetine x time interaction [$F(3,48) = 3.59$; $P = 0.02$], signifying that the difference in weight gain compared to controls was higher at the beginning of the treatments, both for stressed and fluoxetine-treated animals, when compared to the last days of treatment.

The effect of chronic fluoxetine treatment on serum leptin levels, both in controls and in animals subjected to variate stress is shown in Figure 3. A two-way ANOVA revealed a significant effect of fluoxetine treatment [$F(1,16) = 7.49$; $P < 0.02$], with no effect of the stress treatment [$F(1,16) = 0.009$; $P > 0.05$], and no interaction between stress exposure and fluoxetine treatment [$F(1, 16) = 0.155$, $P > 0.05$].

**Discussion**

In the present study, rats chronically stressed during 30 days presented an increase in leptin levels. At the same time, chronic stress induced depressive effects on ingestive behavior, particularly concerning sweet food (3 and the present

study). Hormonal factors, including glucocorticoids, the hormones secreted during stress, are known to modulate leptin secretion (36). Dexamethasone, for example, is a powerful stimulator of leptin secretion and leptin mRNA expression in rat adipose tissue in vitro. Therefore, it is presumed that dexamethasone regulates leptin at the transcriptional level (37), in such a way that it is probably a long lasting effect. The present results also show no significant effect of chronic variate stress on leptin levels after 100 days of treatment. Therefore, it appears that the effect of chronic variate stress on leptin levels is dependent on the time of stress treatment.

It was also shown that chronic fluoxetine administration decreases serum leptin levels, both in control rats and in rats subjected to chronic variate stress. This observation suggests that the effect previously observed by an acute or sub-acute (7 days) administration of fluoxetine on leptin levels (27) is not amenable to habituation, since the same effect was observed after 60 days of treatment. Thus, this study suggests that a chronic fluoxetine treatment is able to induce a pronounced suppressive effect upon the leptin released to serum.

Apart from directly affecting hypothalamic neurons and, thereby, regulating body weight, leptin interacts with dorsal raphe neurons, and it has been suggested that it also may affect behavior via the brain serotonergic system (29), known to play a role in the regulation of food intake in the hypothalamus, where it interacts with NPY (38). On the other hand, the mechanism by which chronic treatment with fluoxetine (which increases extracellular 5HT by preventing its reuptake) is able to decrease leptin levels is not known, although it has been suggested that the effect of 5HT on food intake, besides being mediated through NPY neurones, could also involve an inhibitory action on leptine secretion in the white adipose tissue (27).

Conversely, these reduced levels of leptin could be a reflection of a reduced food ingestion in fluoxetine-treated animals, since leptin is believed to provide information about the size of the fat stores (22;24), establishing a communication between the energy reserves and the hypothalamic centers that control food intake (21-23). Fluoxetine decreases appetite by 5HT receptor-mediated mechanisms (39). Administration of D- fenfluramine, which also increases extracellular 5HT, increases lipidic oxidation in obese rats (40). As a result of these events (decreased appetite and increased lipid oxidation), the leptin levels must be decreased. In this study, however, all animals gained weight across time during fluoxetine treatment, although the weight gain differed among the groups. The interaction between time and stress indicates that stressed females gain weight differently to non-stressed females, and this is consistent with other studies (31;41;42). In addition, fluoxetine-treated animals gained weight differently in relation to animals receiving saline, although at the end of the treatment fluoxetine-treated animals presented body weights similar those of saline-treated animals. Therefore, a decreased food ingestion is probably not the direct explanation for reduced leptin levels under these circumstances.

In conclusion, chronic variate stress may affect leptin levels, although this effect is dependent on the time of stress exposure. In addition, chronic fluoxetine induced a strong reduction in leptin levels, even at 60 days of treatment. The neurobiological mechanism involved in this effect of chronic fluoxetine administration and the relationship between feeding behavior and the effects reported herein require further study.

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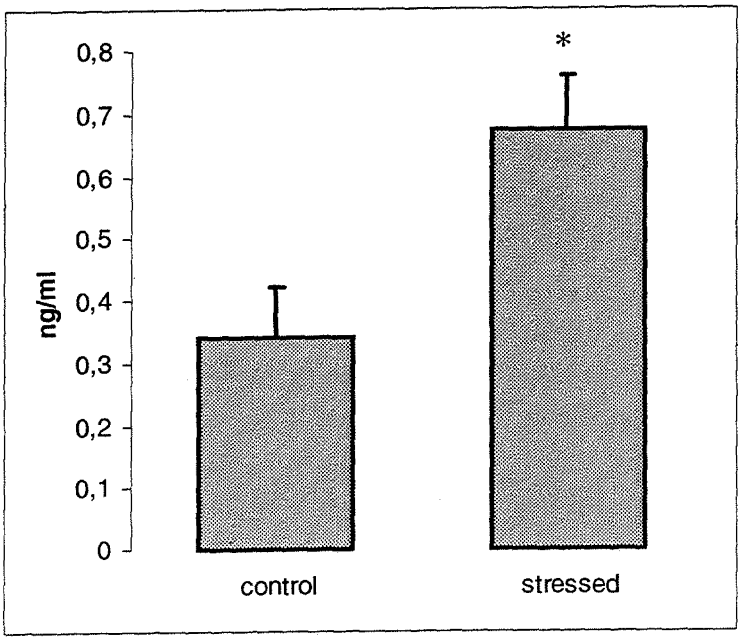


**Figure 1**: Mean serum leptin levels in female rats after 30 days of chronic variate stress. Data expressed as mean ± S.E.M. N = 4-6/group.

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

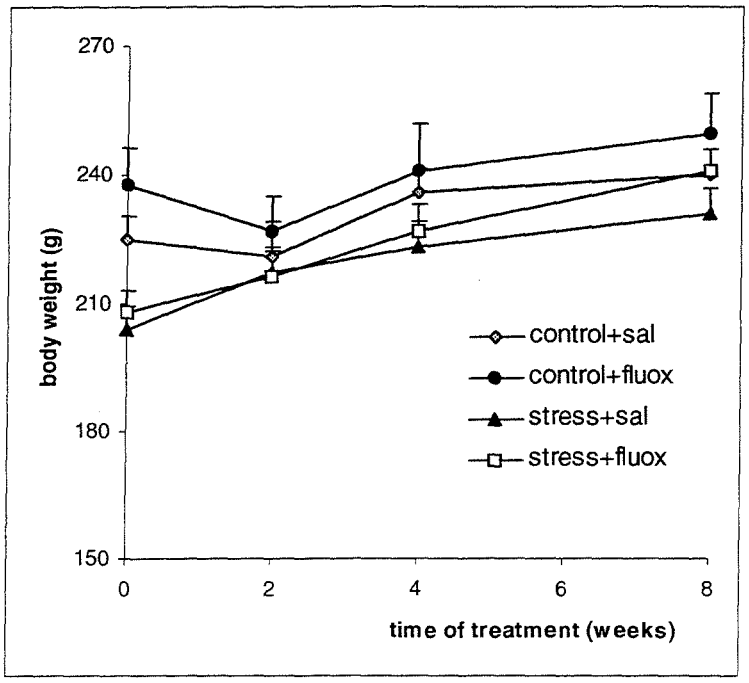


**Figure 2**: Mean body weight during chronic fluoxetine treatment (8 mg/kg), measured during 60 days of treatment. Data expressed as mean ± S.E.M. N = 5 animals/group. Repeated measures ANOVA presented a marginal effect of stress treatment [$F(1,16) = 4.12$; $P = 0.059$]. There was no effect of fluoxetine. There was a significant effect of time [$F(3,48) = 115.48$; $P < 0.01$] and also a significant stress x time interaction [$F(3,48) = 17.95$; $P < 0.01$], and a fluoxetine x time interaction [$F(3,48) = 3.59$; $P = 0.02$].

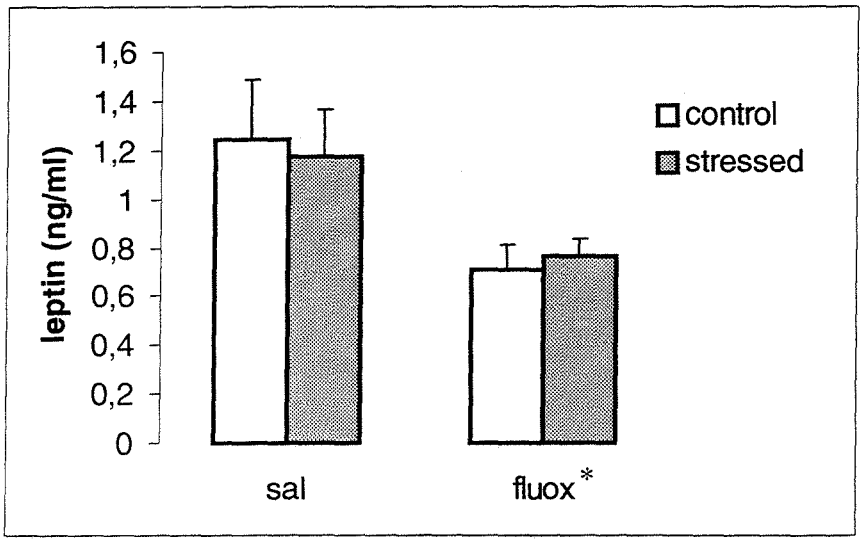


**Figure 3**: Serum leptin levels in female rats subjected to chronic variate stress during 100 days, with the concomitant administration of i.p. fluoxetine (8 mg/kg) during the last 60 days. Data expressed as mean ± S.E.M. N = 5 animals/group. *A two-way ANOVA revealed a significant effect of fluoxetine treatment [$F(1,16) = 7.49$; $P < 0.02$]. There was no effect of stress treatment [$F(1,16) = 1.16$; $P > 0.05$], and no interaction between stress exposure and fluoxetine treatment [$F(1, 16) = 0.155$, $P > 0.05$].

## Capítulo V– ARTIGO 4

Interação entre reposição de estradiol e estresse crônico sobre o comportamento alimentar e níveis de leptina

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# Interaction between estradiol replacement and chronic stress on feeding behavior and on serum leptin

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**Abstract**

Exposure to stress may cause either an increase or a decrease in food intake. Behavioral and physiological responses to stress, including alterations in feeding behavior, are sexually dimorphic. This study aimed to evaluate the interaction between estradiol levels and chronic variate stress on the intake of sweet food and on serum levels of leptin, a hormone secreted by the adipose cells with a role in the regulation of body weight. Adult female Wistar rats were used. After ovariectomy, the animals received estradiol replacement (or oil) subcutaneously. Rats were then divided in controls and stressed (submitted to 30 days of variate stress). Consumption of sweet food and of serum leptin was measured. Although animals receiving estradiol replacement presented smaller weight gain, they showed an increased consumption of sweet food. Chronic variate stress decreased sweet food intake at 30, but not at 20, days of treatment. Estradiol replacement in the stressed group prevented both the reduction observed in sweet food intake and the increase in leptin levels. These results suggest that there is an interaction between chronic stress and estradiol replacement in feeding behavior concerning sweet food consumption, and this interaction may be related to altered leptin levels.


*Keywords:* Estradiol; Chronic variate stress; Feeding behavior; Sweet food; Leptin; Female rats

## 1. Introduction

Food intake depends on several internal and external variables. Emotional changes such as those induced by the exposure to stress situations can influence feeding behavior, causing either an increase or a decrease in food intake, depending upon the nature of the stress (Varma et al., 1999; Ely et al., 1997; Gamaro et al., 2003a; Torres et al., 2002). Several studies have demonstrated that chronic exposure to stressors may alter food intake and body weight of rats (Harris et al., 2002; Kant and Bauman, 1993; D'áquila et al., 1997; Gamaro et al., 2003a,b). Inescapable shock, for example, can affect food intake and reduce weight gain with shocked rats gaining significantly less weight than restrained and untreated rats (Dess et al., 1988). Moreover, models of chronic mild stress have been reported to have different effects on feeding behavior depending on the model. Animals repeatedly stressed by restraint show increased ingestion of sweet food (Ely et al., 1997), while models of chronic variate stress have been observed to induce a decreased appetite for sweet food or palatable solutions (Gamaro et al., 2003a,b; Willner, 1991). It should be pointed out, however, that although in humans women are more sensitive to disturbances in feeding behavior than men (Kornstein, 2002; Oliver and Wardle, 1998), most of these studies in animal models have been carried out in males (Kelly et al., 1999; Harris et al., 2002).

Several sources of data indicate that behavioral and physiological responses to stress are sexually dimorphic, including alterations in feeding behavior (Ely et al., 1997; Faraday, 2002; Kelly et al.,1999; Than et al., 1994). Ovariectomy produces an increase in body weight and in meal size in rats (Butera et al., 1993; Bonavera et al., 1994), which is attenuated by estradiol treatment for 7 days (Shimizu et al., 1996), and the normal cyclic pattern of ovarian estradiol secretion leads to tonic and phasic inhibitions of feeding (Geary and Asarian, 1999).

Leptin is a hormone secreted mainly by the adipose cells with a role as a metabolic adaptor in the regulation of body weight. It is believed to establish a feedback loop between the energy reserves and the hypothalamic centers that

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control food intake (Prolo et al., 1998; Loftus, 1999; Inui, 1999). Recent data suggest also that leptin interacts with other endocrine systems to provide critical information about the size of the fat stores (Lotfus, 1999; Sandoval and Davis, 2003). As well as participating in the expression of CRH in the hypothalamus, leptin also interacts with ACTH in the adrenals and its levels are regulated by glucocorticoids (Spinedi and Gaillard, 1998; Oates et al., 2000). Thus, this hormone is probably influenced by activation of the hypothalamo–pituitary–adrenal (HPA) axis when animals are exposed to stress situations. The fact that leptin levels are always higher in females, even after correcting for body fat content (Chudek et al., 2002; Machinal-Quelin et al., 2002), suggests that the interaction between the adipose tissue and the reproductive system is modulated in a different way in males and females by androgenic and estrogenic hormones. While some androgens are inhibitors of leptin secretion, estradiol induces a strong stimulation in adipose tissue (Kristensen et al., 1999; Machinal-Quelin et al., 2002).

Although previous studies indicate that exposure to stress presents different effects on feeding behavior, depending on the gender, little is known about the ability of ovarian hormones to modulate the responses induced by chronic stress on feeding behavior or how they influence leptin levels in these animals. The study presented herein was conducted to investigate the interaction between estradiol levels and chronic variate stress on feeding behavior, on the intake of sweet food, and on serum leptin levels.

## 2. Experimental procedures

### 2.1. Animals

Adult female Wistar rats (60 days old; 180–230 g of weight) were used. Thirty-eight rats were used in the behavioral measurements and 28 of these animals were used to measure leptin levels. The animals used to evaluate leptin levels in each group were randomly chosen. The experimentally naive animals were housed in groups of four or five in home cages made of Plexiglas material ($65 \times 25 \times 15$ cm) with the floor covered with sawdust. They were maintained under a standard dark–light cycle (lights on between 7:00 and 19:00 h) at a room temperature of $22 \pm 1$ °C. The rats had free access to food (standard rat chow) and water, except for the stressed group during the period when the stressor applied required no food or water. During the first days of treatment, the ingestion of rat chow was monitored. Body weight was measured at the beginning, at the middle, and at the end of the treatment. 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 as well as to reduce the number of animals.

### 2.2. Surgery

Overiectomy (OVX) was performed in the morning. Rats were anesthetized with 120 mg/kg ketamine HCl (Dopalen: Agribrands, Campinas, SP, Brazil) and 16 mg/kg xylazine (Anasedan: Agribrands), and bilateral ovariectomy was performed through a single abdominal incision. After a recovery period of at least 1 week, the animals were submitted to estradiol replacement or to a sham surgery.

### 2.3. Estradiol replacement

Briefly, 15 mm medical grade tubing (1.02 mm i.d. × 2.16 mm o.d.; Medicone, Multiplast, Porto Alegre, RS, Brazil) was filled with 10 μl of 5% (w/v) β-estradiol 3-benzoate (Sigma, St. Louis, MO) in corn oil and sealed with silicone. Capsules were soaked in sterile saline overnight and implanted subcutaneously between the scapulae under anesthesia. Sham animals were implanted with capsules containing just oil.

### 2.4. Stress model

Chronic variate stress was modified from other models of variate stress (Willner et al., 1987; Konarska et al., 1990; Papp et al., 1991), as described in Gamaro et al. (2003a). Animals with estradiol replacement and sham animals were subdivided in two groups: control and stressed. Controls were kept undisturbed in their home cages during the treatment, except for the cleaning of the cages and the exposure to the behavioral proceedings to measure consumption of sweet food. A 30-day variate-stressor paradigm was used for the animals in the stressed group. The following stressors were used: (a) 24 h of food deprivation, (b) 24 h of water deprivation, (c) 1–3 h of restraint, as described below, (d) 1.5–2 h of restraint at 4 °C, (e) inclination of home cage during 4 or 5 h, as described below, (f) flashing light during 120–210 min, and (g) isolation (2–3 days). Stress application started at different times everyday in order to minimize its predictability.

Restraint was carried out by placing the animal in a $25 \times 7$-cm plastic tube and adjusting it with plaster tape on the outside so that the animal was unable to move. There was a 1-cm hole at the far end for breathing. Exposure to flashing light was performed by placing the animal in a 50-cm high, $40 \times 60$-cm open field made of brown plywood with a frontal glass wall. A 40-W lamp, flashing at a frequency of 60 flashes per minute, was used.

### 2.5. Consumption of sweet food

After 20 and 30 days of treatment, consumption of sweet food was measured. The animals were placed in a lightened rectangular box ($40 \times 15 \times 20$ cm) with a glass ceiling, floor, and sidewalls made of wood. Ten Froot loops (Kellogg's: pellets of wheat and cornstarch and sucrose) were

placed in one extremity of the box. Animals were submitted to 3-min trials, one per day during 5 days, in order to become familiarized with this food (Ely et al., 1997). After being habituated, the animals were exposed to two test sessions, 3 min each, when the number of ingested pellets was measured. A protocol was established so that when the animal ate part of the Froot loops (e.g., 1/3 or 1/4), this fraction was considered.

### 2.6. Leptin measurement

The animals were sacrificed by decapitation 24 h after the last stress session. Trunk blood was collected and serum separated and frozen until the day of the analysis. Measurement of serum leptin was performed with a commercial mouse leptin ELISA kit (Crystal Chem., Chicago, IL).

### 2.7. Statistical analysis

Data were expressed as mean ± S.E.M. Data were analyzed using two-way ANOVA.

## 3. Results

Body weight was evaluated in the four groups before stress and estradiol treatments started, after 15 and after 30 days of treatment. Weight gain during the treatment is shown in Fig. 1. Repeated measure ANOVA showed an effect of estradiol treatment [$F(1,16) = 15.31$; $P = .01$], showing that rats treated with estradiol gained less weight than animals receiving just the vehicle. There was a significant Estradiol × Time interaction [$F(1,16) = 5.94$; $P < .05$] and also a significant Stress × Time interaction [$F(1,16) = 9.98$; $P < .01$], signifying that the difference in weight gain, compared to controls, was higher at the beginning of the treatments both for stressed and estradiol-treated animals.

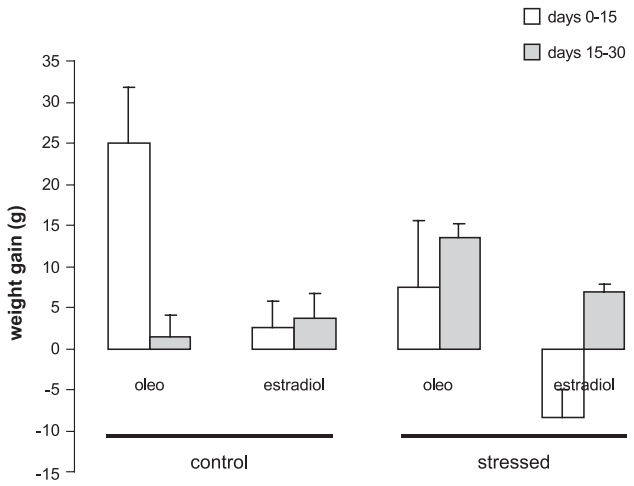


Fig. 1. Mean weight gain during the first 15 days of treatment and during the last 15 days of treatment. Data expressed as mean ± S.E.M. of weight gain in grams. A repeated measures ANOVA revealed a significant effect of estradiol replacement [$F(1,16) = 15.31$; $P = .01$], a significant Estradiol × Time interaction [$F(1,16) = 5.94$; $P < .05$], and a significant Stress × Time interaction [$F(1,16) = 9.98$; $P < .01$].

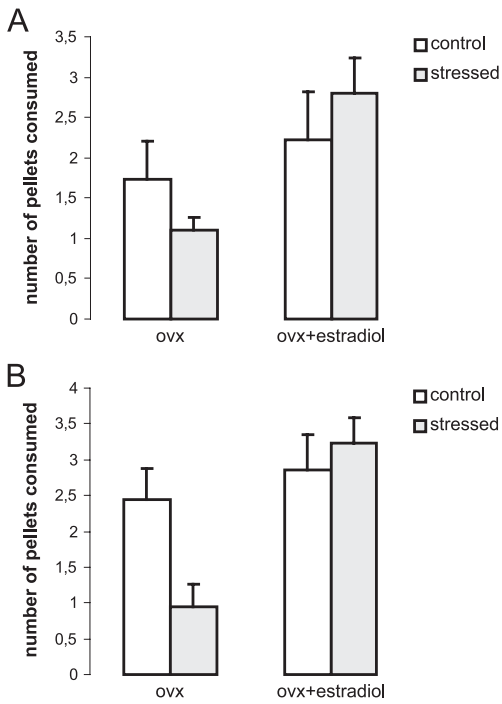


Fig. 2. Mean intake of sweet food (Froot loops) in ovariectomized female rats with or without estradiol replacement after chronic variate stress. (A) After 20 days of exposure to stress; (B) after 30 days of exposure to stress. Data expressed as mean ± S.E.M. $N = 9–10$ animals/group. A two-way ANOVA revealed a significant effect of estradiol replacement at both times of treatment [20 days, $F(1,34) = 6.270$, $P < .02$; 30 days, $F(1,34) = 11.872$, $P < .002$]. At 30 days of treatment (B), a significant interaction between estradiol replacement and exposure to chronic stress was also observed [$F(1,34) = 5.682$, $P < .02$].

The effect of chronic variate stress upon the intake of Froot Loops in ovariectomized female rats with or without estradiol replacement is shown in Fig. 2. Consumption of this type of sweet food was measured both at 20 and 30 days of stress treatment. A two-way ANOVA revealed a significant effect of estradiol replacement at both times of treatment [$F(1,34) = 6.270$, $P < .02$ for 20 days of treatment, and $F(1,34) = 11.872$, $P < .002$ for 30 days of treatment], with the hormone determining an increased intake. At 30 days of treatment, a significant interaction between estradiol replacement and exposure to chronic stress was also observed [$F(1,34) = 5.682$, $P < .02$], i.e., while chronic variate stress induced decreased consumption in OVX animals, this effect was not observed in rats with estradiol replacement.

The effect of chronic varied stress in decreasing sweet intake could also be secondary to a decrease in body weight. Therefore, we compared the ratio consumption of sweet food/body weight. The results presented a similar effect to the consumption only (OVX group: 0.0116 ± 0.0034; OVX + estradiol: 0.0124 ± 0.0037; OVX + stress: 0.0038 ± 0.0016; OVX + stress + estradiol: 0.0149 ± 0.002). A two-way

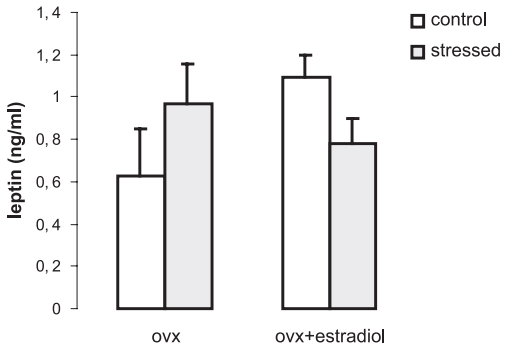


Fig. 3. Serum leptin levels in ovariectomized female rats with or without estradiol replacement after 30 days of chronic variate stress. Data expressed as mean ± S.E.M. $N = 5–9$ animals/group. A two-way ANOVA revealed a significant interaction between stress exposure and estradiol replacement [$F(1,24) = 4.396$, $P < .05$], with no effect of the treatments (stress and estradiol replacement) when separately analyzed.

ANOVA revealed a significant effect of estradiol replacement [$F(1,34) = 6.270$, $P < .02$ for 20 days of treatment, and $F(1,19) = 4.956$, $P < .05$], with the hormone determining an increased ratio. A marginal interaction between estradiol replacement and exposure to chronic stress was also observed [$F(1,19) = 3.753$, $P = .068$].

The effect of chronic variate stress on serum leptin levels in ovariectomized female rats with or without estradiol replacement is shown in Fig. 3. In OVX rats not submitted to chronic stress, estradiol replacement increased leptin levels (marginal significance, Student's $t$ test, $P = .06$). A two-way ANOVA revealed a significant interaction between stress exposure and estradiol replacement [$F(1,24) = 4.396$, $P < .05$], with no effect of the treatments (stress and estradiol replacement) when separately analyzed.

## 4. Discussion

All animals used in this study gained weight with time; however, the weight gain differed among the groups. The interaction between time and stress indicates that stressed females gain weight differently than nonstressed females, and this is consistent with other studies (Konarska et al., 1990; Harro et al., 2001; Bowman et al., 2002). In addition, estradiol-treated rats weighed less than their controls, a characteristic of the hypophagic effect of estrogen (Bowman et al., 2002).

The results of the present study show that the effect of chronic variate stress on sweet food consumption in ovariectomized rats can be modulated by hormonal replacement. This observation suggests a role of estradiol in the anorectic properties of chronic variate stress with respect to ingestion of sweet food.

Consistent with previous findings, when males were used (Gamaro et al., 2003a), chronic variate stress decreased ingestion of sweet food after 30 days of treatment. Non-ovariectomized females submitted to the same procedure also present a reduction in sweet food intake (Gamaro et al., 2003b). Feeding behavior after exposure to stress may depend on the nature and the predictability of the stressor (Hargreaves, 1990; Paré and Redei, 1993; Pucilowski et al., 1993; Martí et al., 1994; Morley et al., 1986). Models of chronic mild stress have been reported to lead to behavioral disturbances (Katz et al., 1981; Willner, 1991; Basso et al., 1993), including decreased responses to rewarding stimuli, as demonstrated by decreased sucrose consumption (Willner et al., 1987; Ferretti et al., 1995) and place preference conditioning (Papp et al., 1991, 1992). The absence of predictability of the stressor applied is an important characteristic of this model and may be related to the different effects observed in these animals when compared to other models in which repeated stress is used and higher consumption of sweet food is observed (Ely et al., 1997; Silveira et al., 2000). The present results also show no significant effect of stress after 20 days of treatment, although a nonsignificant decrease in sweet food ingestion may be observed. Therefore, it appears that behavioral changes such as the one observed here in animals submitted to chronic variable stress may require some time to develop.

Other studies have observed higher intakes of very sweet mash and lower rates of eating in animals submitted to chronic exposure to mild unpredictable stress relative to controls (Sampson et al., 1992). These results are somewhat different from the results obtained herein, possibly because the session length was very different. Furthermore, in the present data, it is important to point out that the measurement of sweet food consumption was made in a 3-min section. Therefore, the consumption observed represent the initial drive of the animals in relation to sweet food, and agreeing with other studies, this effect may represent decreased reactivity to sweetness following chronic exposure to stress.

In addition to its role in the stress response, the HPA axis and particularly the glucocorticoid hormone corticosterone have been shown to play an important role in appetitively motivated behavior (Barr et al., 2000; Piazza and Le Moal, 1997). Corticosterone is known to modulate feeding behavior, having a stimulatory effect on food intake, particularly on carbohydrates intake (Tempel and Leibowitz, 1989). This effect of corticosterone enhancing carbohydrate intake is opposite to the effect observed in the present study. It is possible that in rats exposed to chronic stress during a long treatment, opponent processes prevail, causing a hedonic shift in the other direction (Solomon and Corbit, 1974). On the other hand, activation of HPA axis also releases CRH, which is known to reduce feeding behavior (Koob and Heinrichs, 1999). Presently, it remains unclear if some of these different effects of HPA axis stimulation on feeding behavior is, at least in part, responsible for the present results.

It should be observed, however, that the pattern of consumption responses exhibited following stress treatment was dependent on whether or not subjects received hor-

monal replacement. After 30 days of variate stress, the suppressive effects of this treatment on sweet food intake was evident in the OVX group; however, it was not exhibited by the group receiving estradiol replacement. An overall effect of estradiol was also observed. Surprisingly, estradiol replacement induced a small, but significant, increase in the consumption of sweet food, although no effect was observed in the standard chow consumption (data not shown). In addition, weight gain was smaller in animals treated with estradiol in accordance with the known anorectic effect of this hormone. Thus, under the conditions of this task, estradiol replacement led to an increased appetite for sweet palatable food, although it reduced weight gain.

This differential pattern of ingestive behavior by the OVX + estradiol stressed group suggests that this model of chronic variate stress is able to induce a pronounced suppressive effect on ingestion in ovariectomized rats not receiving hormonal replacement; while in those rats receiving hormonal replacement, this effect of chronic variate stress was prevented. It has been hypothesized that chronic variate stress reduces intake of sweet food by promoting decreased responses to rewarding stimuli, a characteristic of a state of depression (anhedonia) (Willner, 1991; Murua and Molina, 1992). In the present study, estradiol replacement modified the effect of stress treatment on sweet food ingestion by increasing ingestive responses, an effect observed particularly in the stressed group. This effect may be related to an altered response to the sweet stimulus. Although estradiol is known to reduce intake (Ganesan, 1994; Wade and Schneider, 1992), gustatory and food habit changes have been observed during the menstrual cycle in women (Alberti-Fidanza et al., 1998) and the estral cycle in rats (Clarke and Ossenkopp, 1998b). Sensitivity to sweet taste increases with an increase of estradiol. At the same time, in these studies, with the highest estradiol values, there was a tendency towards lower energy intake, predominantly provided by carbohydrates (such as bread) (Alberti-Fidanza et al., 1998).

Estradiol replacement has been shown to decrease meal size in rats (Geary et al., 1994; Hrupka et al., 1997). Studies of meal microstructure after estradiol replacement have shown that the rate of licking was not changed during the beginning of the meal but was significantly slower during the remainder of the meal. Burst size, cluster size, and interburst interval were lower after estradiol replacement during 5–7 min of the test meal (Hrupka et al., 1997). In addition, estradiol is known to reduce meal size and food intake in female rats, at least in part, by increasing the satiating potency of CCK (Clarke and Ossenkopp, 1998a; Eckel et al., 2002), possibly by increasing the central processing of the vagal CCK satiation signal (Eckel et al., 2002), and this mechanism of action would explain why estradiol effects do not occur during the first minutes of a meal. Since the task used in this study to evaluate feeding behavior lasted for just 3 min, it is possible that longer periods would present different effects, with the manifestation of the anorectic effects of estradiol. In these first minutes, however, the main effect was an increase in consumption, maybe as a result of altered sensitivity to sweet taste, particularly in the chronically stressed group. In this case, estradiol prevented the reducing effect of chronic variate stress on sweet food consumption.

The effect of chronic variate stress to decrease sweet intake has also been hypothesized to be secondary to a decrease in body weight. The present data are relevant to this debate (e.g., Willner et al., 1996). Therefore, we evaluate the ratios sweet food consumption/body weight. The results show the same pattern of effect of the results observed in Fig. 2, suggesting that the reduction in body weight is not enough to explain the decreased consumption of sweet food.

In OVX rats not submitted to chronic stress, estradiol replacement increased leptin levels, consistent with the known anorectic effects of estradiol (Ganesan, 1994; Wade and Schneider, 1992). Females are characterized by significantly higher plasma leptin concentration than males (Chudek et al., 2002). Recent studies have shown that 17β-estradiol increases ob mRNA expression and leptin release in more than 80% (Tanaka et al., 2001; Machinal-Quelin et al., 2002), although others have reported that plasma concentration of estradiol contributes to leptinemia only to a minor degree (Chudek et al., 2002).

In the present study, chronically stressed rats presented a nonsignificant increase in leptin levels; while at the same time, chronic stress induces depressive effects on ingestive behavior. Hormonal factors, including glucocorticoids, the hormones secreted during stress, are known to modulate leptin secretion (Considine et al., 1997). Dexamethasone, for example, is a powerful stimulator of leptin secretion and leptin mRNA expression in rat adipose tissue in vitro. The time lag and the similarity of these two parameters indicate that dexamethasone presumably regulates leptin at the transcriptional level (Kristensen et al., 1999) in such a way that it probably has a long-lasting effect. In addition, in our chronically stressed group, no effect of estradiol replacement was observed as opposed to the effect observed in the control group (as expressed by the significant interaction between the treatments). These results suggest an interaction between the mechanisms of action of these two treatments with regard to their effects on serum leptin levels. Interestingly, estradiol replacement, in the stressed group, prevented both the reduction observed in sweet food intake and the increase in leptin.

Naturally, although it is possible that leptin could play a role in the behavioral effects described in the present study, it becomes clear, by the analysis of the two sets of data, behavioral and biochemical, that leptin levels are not completely consistent with the behavioral effects, particularly the estradiol groups. This interpretation is further complicated by the fact that the neural mechanisms by which estradiol interferes with food intake remain to be determined. The effects observed herein may be related to the known effects of estradiol on leptin biosynthesis and secre-

tion (Mystkowski and Schwartz, 2000) and indicate that, at least in estradiol-treated groups, other factors are certainly involved in the observed effects.

Estradiol treatment has been observed to decrease anxious behavior (Bowman et al., 2002), an effect which may be mediated, at least in part, by modifications in the 5-HT system in response to estradiol treatment (Williams and Uphouse, 1989; Bowman et al., 2002). As observed above, estradiol increases the secretion of leptin. In addition, a greater effect of estrogen on leptin secretion has been reported in dexamethasone-stimulated cells (Kristensen et al., 1999). Conversely, the effects of leptin on food intake have been suggested to be mediated in part by the midbrain serotonergic systems. Leptin can be selectively accumulated by serotonergic neurones in the raphe nuclei (Fernandez-Galaz et al., 2002), and its perfusion induces a significant increase in 5-HIAA overflow from the hypothalamus (Hastings et al., 2002). In addition, leptin treatment regionally down-regulates serotonin transporter binding sites in the brain (Charnay et al., 2000). Thus, a possible interaction between estradiol-induced leptin secretion and the serotonergic system would be a potential mechanism by which estradiol may interfere with the effects of chronic stress observed herein since some models of chronic-mild stress (such as the one used in the present study) have been proposed as models of depression in animals studies (Pucilowski et al., 1993; Katz et al., 1981; Willner, 1990, 1991) and serotonin has been implicated in the pathophysiology of depression (Van Praag et al., 1990). This possibility requires further study.

In conclusion, an alteration in leptin levels may contribute, at least in part, to the interaction observed between chronic stress and estradiol replacement in feeding behavior. The exact neurobiological mechanism involved in this effect, after chronic stress, remains to be clarified. In addition, the existence of a relationship between estradiol and the anorexic effect of chronic stress also requires further study.

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# Capítulo VI– DISCUSSÃO GERAL

Situações estressantes alteram o comportamento alimentar de maneira diferenciada de acordo com a característica do agente estressor, podendo causar aumento ou diminuição do apetite (Hargreaves , 1990; Macht et al., 2001 Martí et al., 1994; Pucilowski et al., 1993; Ely et al., 1997; Gamaro et al., 2003). Em situações de estresse, ocorre a ativação do eixo hipotálamo-hipófise-adrenal (HHA), causando a liberação de glicocorticóides entre outros hormônios (Akil & Morano, 1995). Os glicocorticóides, por sua vez, atuam no sistema nervoso central sobre o metabolismo da serotonina e da dopamina (Malyszko et al., 1994; Paris et al., 1987; Nishi & Azmitia, 1996; Chaouloff,1993; Cuadra et al., 2001). Sabe-se também que tais hormônios influenciam o comportamento alimentar (Tempel & Leibowitz, 1989; Strack et al., 1995), além de estimularem a liberação de leptina (Caldefie-Chézet et al., 2001).

O modelo de estresse abordado nessa dissertação apresenta a característica de imprevisibilidade e, por esta razão, tem sido sugerido como um modelo animal de indução de depressão (Pucilowski et al., 1993; Katz, 1981; Willner, 1991). A depressão pode ser determinada a partir da presença de um de seus sintomas, como a diminuição do consumo de soluções palatáveis, podendo também ser interpretada como uma diminuição da resposta à recompensa (anedonia) (Willner, 1987; D'áquila et al., 1994).

Os animais submetidos a tal modelo de estresse apresentaram uma diminuição no consumo de alimento doce, não sendo observadas alterações no consumo de ração padrão, como demonstrado no Capítulo II. Esta alteração é específica para o alimento doce, podendo ser resultante de uma modificação na neurotransmissão dopaminérgica e serotonérgica, pois sabe-se que ambos

neurotransmissores estão envolvidos na resposta normal para um estímulo de sabor adocicado, independente da percepção de fome do animal (Blundell, 1991, Ahn & Phillips, 1999). A dopamina está envolvida na regulação do comportamento alimentar relacionada com os mecanismos de recompensa e motivação (Orosco & Nicolaidis, 1992; Ahn & Phillips, 1999; Pitts & Horvitz, 2000; Berridge & Robinson, 1998). Já a serotonina está envolvida na regulação do humor e do apetite, apresentando, nesse último, um efeito inibitório, uma vez que atua nos mecanismos de saciedade do indivíduo (Simansky, 1996; Orosco et al., 2000). Estudos demonstram aumento em alguns tipos de receptores serotonérgicos, como $5HT_{1B}$ e $5HT_2$, após tratamento com corticosterona ou ACTH (Kuroda et al., 1992; Takao et al., 1997). A ativação desses receptores específicos causa efeitos hipofágicos (Collin et al., 2000). A ação inibitória da serotonina sobre o comportamento alimentar pode ocorrer via inibição do neuropeptídio Y (NPY), quando ocorre a atuação dos receptores do tipo $5HT_{2A}$ ou $5HT_{2C}$, ou por outros mecanismos que não envolvam a participação de tal neuropeptídio, que estão relacionados com outros receptores da família, como os receptores $5HT_{1A,B}$ e $5HT_D$ (Currie, et al., 2002).

Como demonstrado no Capítulo II, os animais submetidos ao estresse crônico variável apresentaram um aumento no metabolismo da serotonina no hipocampo. Tal estrutura cerebral é alvo para ação dos glicocorticóides por apresentar alta densidade de receptores para esses hormônios. Este aumento na atividade serotoninérgica pode ser devido à ação desses hormônios no hipocampo (Chalmers et al., 1993; Nishi & Azmitia, 1996),. pois sabe-se que a liberação de glicocorticóides é geralmente a resposta hormonal

do organismo ao estresse e que a ação de tais hormônios altera o metabolismo da serotonina no sistema nervoso central e na periferia (Malyszko et al., 1994, Paris et al., 1987; Nishi & Azmitia, 1996). Os níveis de glicocorticóides circulantes podem alterar a utilização da serotonina por meio da atividade da enzima triptofano hidroxilase, importante para a biotransformação do triptofano em serotonina (Chalmers et al., 1993; Singh et al., 1990; Chaouloff, 1993). Evidências eletrofisiológicas indicam uma interação direta entre serotonina e neurônios hipocampais responsivos aos glicocorticóides (Azmitia et al, 1984, Joels & de Kloet, 1992; Joels et al., 1995; Joels et al., 1997). Importante ressaltar que o hipocampo recebe várias inervações do núcleo da rafe, local de síntese de serotonina (Oleskevich and Descarries, 1990). Embora a serotonina hipotalâmica esteja mais relacionada a alterações no humor do que tenha relação direta com a modulação sobre o apetite, este neurotransmissor também interage com o eixo cérebro-pituitária-adrenal (Siegel et al., 1993). Além disso, o hipocampo tem sido relacionado com a organização do padrão de refeição (Clifton, 2000), podendo influenciar de maneira discreta a regulação do peso corporal. É possível que a atividade aumentada de serotonina no hipocampo possa não estar diretamente relacionada com a diminuição no consumo de doce observado nos animais submetidos ao estresse crônico. A 5-HT está envolvida na modulação do comportamento alimentar, principalmente em outras estruturas, como por exemplo, o hipotálamo (Leibowitz et al., 1990; Collin et al., 2000; Orosco & Nicolaidis, 1992; Simansky, 1996; Vry & Schereiber, 2000) e a amígdala (Parker & Coscina, 2001; Parker et al., 2001).

Os animais submetidos ao estresse crônico variável apresentaram, concomitantemente com a diminuição na atividade serotonérgica, uma diminuição na neurotransmissão dopaminérgica, porém em estrutura cerebral diferenciada. Os animais apresentaram diminuição na atividade dopaminérgica hipotalâmica. Sabe-se que o hipotálamo é o centro regulador do apetite (Bishop et al., 2000) Resultado oposto foi observado em córtex frontal e hipocampo, com um aumento no metabólito da dopamina, DOPAC, nessas duas estruturas, também envolvidas na regulação do comportamento alimentar. Este aumento está de acordo com dados da literatura que demonstram aumento da atividade dopaminérgica em várias estruturas cerebrais, principalmente no sistema dopaminérgico mesolímbico após a exposição ao estresse moderado (Thierry et al., 1976; Roth et al., 1998; Jedema & Moghaddam, 1994). Tem sido demonstrado um aumento na transmissão dopaminérgica no córtex pré-frontal em situações de estímulos aversivos (Di Chiara et al., 1999). A diminuição no consumo de doce pode estar relacionada com tal efeito aversivo causado pelo modelo de estresse.

A dopamina pode estar envolvida com mecanismos de inibição do apetite no hipotálamo lateral, mas pode também exercer efeito contrário no hipotálamo medial (Fetissov et al., 2000). A administração de fármacos que aumentem a concentração de dopamina ou que sejam agonistas dos receptores $D_2$ é anorexigênica (Towell et al., 1988; Aou et al., 1994; Rusk & Cooper, 1988)

A dopamina parece regular a ingestão de alimento modulando vias mesolímbicas relacionadas com mecanismos desenvolvidos por meio da recompensa (Balcioglu & Wurtman, 1998; Martel & Fantino, 1996). Baseado nesse fato, podemos inferir como um dos efeitos do estresse crônico variável a

modificação das propriedades motivacionais e/ou de reforço para alimentos doces. Nesse caso, causa uma diminuição na atividade dos sistemas envolvidos com motivação e recompensa. O modelo de estresse crônico variado causou alteração no metabolismo de serotonina e dopamina, acompanhada de diminuição no consumo de alimento doce.

Sabe-se que, em humanos, as mulheres são mais suscetíveis a desordens alimentares e afetivas. Por esta razão, passamos a analisar os efeitos deste modelo de estresse sobre o comportamento alimentar em fêmeas (Kornstein., 2002). As variações hormonais ovarianas durante o ciclo estral têm sido relatadas como importantes fatores de regulação da resposta ao estresse e na modulação do comportamento alimentar (Bonavera et al., 1994; Mystkowski e Schwartz, 2000).

Dados da literatura demonstram modificações de ingestão de alimentos ou aumento de alimentos de sabor adocicado em mulheres; durante a ovulação (Than et al.,1994, Kuga et al.,1998).

Ao submetermos animais fêmeas ao estresse crônico variável, como demonstrado no Capítulo III, pudemos observar alterações no comportamento alimentar, como a diminuição no consumo de alimento doce, da mesma forma observada em animais machos, além da diminuição na atividade da enzima $Na^+,K^+$-ATPase. Ambos parâmetros estão relacionados com sintomas da depressão (Willner, 1991; Van Praag et al., 1990). O estresse é um agente que altera a homeostase do organismo e modifica metabolismo de catecolaminas e serotonina. O estresse pode ser considerado um fator de predisposição à depressão (Chalmers et al., 1993; Nishi & Azmitia, 1996).

Sendo constatado tal efeito, iniciou-se o tratamento com um antidepressivo inibidor seletivo da recaptação de serotonina, fluoxetina, durante 60 dias. O tratamento com fluoxetina aumentou a atividade da enzima, revertendo o efeito do estresse. Esta enzima é regulada por hormônios e neurotransmissores, como catecolaminas e serotonina (Van Praag et al., 1990; Farman, et al., 1994 ; Awaiss et al., 2000; Hernandez, 1992; Valentino & Van Bockstaele, 2001), sendo ambas também moduladas por estresse (Chaouloff,1993 ; Malyszko et al., 1994). Como relatado anteriormente, o hipocampo é alvo para ação do glicocorticóides que atuam sobre o metabolismo da serotonina, uma vez que os níveis desse neurotransmissor encontram-se alterados nessa região. A serotonina está envolvida com a regulação do humor, sendo o hipocampo uma estrutura importante nesse processo. A atividade da enzima $Na^+,K^+$-ATPase no hipocampo está diminuída nos animais estressados. O tratamento com com fluoxetina reverte tal efeito causado pelo estresse sobre a atividade da enzima.

Sabe-se que a alteração causada pelo estresse no comportamento alimentar está relacionada com a modificação das vias serotoninérgicas e dopaminérgicas pelos hormônios do estresse, principalmente os glicocorticóides (Gamaro, et al., 2003). O comportamento alimentar pode ser modulado por vários hormônios e neuropepetídeos dentro dos quais a leptina e o NPY apresentam papéis antagônicos na regulação do comportamento alimentar. A diminuição do apetite causada pelo estresse pode estar relacionada também com um aumento nos níveis de leptina, um hormônio liberado pelos adipócitos com função da regulação do apetite (Sandoval & Davis, 2003; Yokosuka et al., 1998). A secreção de leptina é modulada pela ação dos glicocorticóides. Estudos prévios com

administração de dexametasona demonstraram um aumento nos níveis de leptina e na expressão do mRNA em culturas de células provenientes de tecido adiposo de rato (Kristensen et al., 1999). Este dado demonstra um efeito de longa duração, uma vez que atua em nível de transcrição. Sendo o estresse crônico um tratamento de longa duração, observamos um aumento nos níveis de leptina, acompanhado pela diminuição no consumo de alimento doce, como descrito no Capítulo IV.

Dados da literatura demonstram o envolvimento da serotonina com a regulação dos níveis de leptina, bem como diminuição nos níveis de leptina nos animais submetidos a um tratamento crônico com fluoxetina. Sabe-se que a fluoxetina inibe a recaptação da serotonina , aumentando sua concentração na fenda sináptica. Dryden e colaboradores, 1999 demonstraram o efeito da fluoxetina em estresse agudo ou sub-agudo (7 dias). Os resultados obtidos nessa tese, relatados no Capítulo IV, demonstram que, ao prolongarmos o tempo de tratamento com fluoxetina para 60 dias, ainda encontramos o efeito inibitório causado pelos tratamentos de menor período de duração. Os níveis de leptina permaneceram mais baixos nos animais dos grupos controle e estressado tratados com fluoxetina. Sugere-se que o efeito causado pela administração de fluoxetina sobre os níveis de leptina sérica seja passível de habituação com o passar do tempo. O efeito do estresse sobre os níveis de leptina, que aos 30 dias apresentam-se elevados, diminui com o aumento do tempo de estresse para 100 dias. Sugere-se uma habituação da liberação de leptina com o aumento do tempo de estresse.

Ao trabalharmos com fêmeas, não podemos deixar de lado os efeitos dos esteróides sexuais em relação ao comportamento alimentar. Os estrogênios, por exemplo, apresentam um efeito catabólico sobre o metabolismo. Estes hormônios inibem a ingestão de alimento, enquanto a testosterona apresenta um efeito ativador do apetite dose dependente, em que doses altas causam inibição da ingestão de alimento (Mystkowski e Schwartz, 2000). No Capitulo V dessa tese, demonstramos os efeitos do estresse em animais ovarectomizados (OVX), com e sem reposição hormonal. Nesta parte do trabalho, pudemos constatar um efeito do estresse sobre o comportamento alimentar tempo-dependente, uma vez que os animais que foram submetidos apenas a 20 dias de estresse não apresentavam alteração no comportamento alimentar. Porém, os animais OVX que receberam estradiol apresentaram um aumento no consumo de doce, tanto aos 20 quanto aos 30 dias de estresse, sendo que, aos 30 dias de estresse, pudemos observar nos animais OVX sem reposição o efeito inibitório do estresse sobre o comportamento alimentar. Estudos demonstram que os níveis de corticosterona podem estimular o consumo de carboidratos, sendo relacionado com mecanismos de recompensa (Tempel & Leibowitz, 1989 ). Porém não podemos esquecer que a resposta ao estresse ocorre através da ativação do eixo HPA. Esta resposta é iniciada pela liberação do CRH hipotalâmico que exerce efeito inibitório sobre o comportamento alimentar. Porém o efeito clássico do estradiol na redução do peso corporal e do comportamento alimentar não foi observado no Capítulo V (Hrupka et al., 1997; Geary & Asarian, 1999). Os animais submetidos ao estresse por 30 dias que receberam reposição hormonal com estradiol apresentaram um aumento no consumo de alimento doce. Deve-se observar que os mecanismos pelos quais

o estradiol atua no sistema nervoso central não são bem esclarecidos na literatura, além do fato de que, em nosso trabalho, o tempo de exposição ao alimento doce aplicado pela tarefa talvez não seja suficiente para determinar alterações em nível central do estradiol sobre o comportamento alimentar. Sabe-se que um dos mecanismos pelos quais o estradiol parece atuar é via estimulação vagal da CCK, tal efeito não ocorre nos primeiros momentos de uma refeição (Butera et al., 1993).

Como descrito no Capítulo V, também foram medidos os níveis de leptina nos animais submetidos ao estresse e a reposição hormonal. Os animais controles que receberam estradiol apresentaram níveis aumentados de leptina, porém os animais estressados não apresentaram alterações no nível de tal hormônio. Podemos sugerir que o efeito do estresse sobre o comportamento alimentar parace não ser dependente dos níveis de leptina, pelo menos nesse modelo de estresse. É importante ressaltar que o estradiol parece nesse modelo reverter o efeito do estresse sobre o consumo de doce.

# Capítulo VII - CONCLUSÕES

A partir dos resultados dessa tese podemos concluir o que segue:

O modelo de estresse crônico variável em animais machos induz uma diminuição do consumo de alimento doce, podendo tal efeito estar relacionado com as alterações observadas no metabolismo de dopamina e serotonina em estruturas do sistema nervoso central envolvidas com a regulação do comportamento alimentar.

A alteração no metabolismo de serotonina no hipocampo e a diminuição do consumo de alimento doce corroboram com os dados da literatura que sugerem a utilização de tal modelo como indutor de depressão.

O efeito do modelo de estresse crônico variado sobre o comportamento alimentar utilizando fêmeas foi o mesmo efeito observado em machos.

O efeito do estresse sobre o comportamento alimentar é tempo dependente, ou seja, somente observamos a diminuição no consumo de doce após um período maior que 20 dias de estresse.

O modelo de estresse crônico variado pode ser considerado um modelo experimental de depressão, uma vez que diminui os níveis da enzima $Na^+$, $K^+$ - ATPase em membranas do hipocampo dos animais submetidos ao estresse, pelo menos em fêmeas. O tratamento com o antidepressivo, fluoxetina, reverte o efeito do estresse sobre a atividade da enzima.

O modelo de estresse crônico variado causa aumento nos níveis de leptina aos 30 dias de estresse , o que pode estar relacionado com o efeito inibitório sobre o comportamento alimentar.

O tratamento crônico com fluoxetina é eficaz para reverter o efeito do estresse sobre a atividade da $Na^+,K^+$ ATPase, mas não sobre os níveis de leptina nos animais submetidos ao tratamento de estresse crônico variado por 100 dias.

O grupo de animais ovariectomizados que receberam reposição de estradiol apresentaram aumento no consumo de doce aos 20 e aos 30 dias de estresse. Os animais do grupo estressado que receberam estradiol apresentaram, aos 30 dias de estresse, uma diminuição dos níveis de leptina. Uma possível interação pode estar ocorrendo entre estradiol e a liberação de leptina, relacionada com a alteração observada no comportamento alimentar.

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