UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS – NEUROCIÊNCIAS

Estudo de aspectos comportamentais, metabólicos e neuroquímicos envolvidos na regulação do consumo de alimento palatável em animais manipulados no período neonatal

Tese de Doutorado

Patrícia Pelufo Silveira

Porto Alegre, 2007.

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A espantosa realidade das coisas
É a minha descoberta de todos os dias.
Cada coisa é o que é,
E é difícil explicar a alguém quanto isso me alegra,
E quanto isso me basta.

Alberto Caeiro (Fernando Pessoa)

SUMÁRIO

ABREVIATURAS

ACTH	Hormônio adrenocorticotrófico
ADN	Ácido desoxirribonucléico
ADP	Adenosina difosfato
AGRP	Proteína relacionada ao gene cutia
AMP	Adenosina monofosfato
ANOVA	Análise de variância
ARC	Núcleo arqueado do hipotálamo
ARN	Ácido ribonucléico
ATP	Adenosina trifosfato
AVP	Vasopressina
BDNF	Fator neurotrófico derivado do cérebro
BDZ	Benzodiazepínicos
CART	Peptídeo relacionado à cocaina e à anfetamina
CB1	Receptor canabinóide tipo 1
CCK	Colecistocinina
CRH	Hormônio liberador de corticotrofina
CRH 1 e 2	Receptor para CRH tipos 1 e 2
DAT	Transportador de dopamina
EDTA	Ácido etilenodiamino tetra-acético
ELISA	Enzyme Linked Immuno Sorbent Assay
ECV	Estresse crônico variável
FSH	Hormônio estimulante folicular
GABA	Ácido gama-amino butírico

GHS-R 1a	Receptor para o secretagogo de hormônio do crescimento (grelina) tipo 1a
GLP-1	Peptídeo semelhante ao glucagon tipo 1
GR	Receptor para glicocorticóide
HPA	Hipotálamo-pituitária-adrenal
LH	Hormônio luteinizante
IRS	Substrato do receptor de insulina
MCH	Hormônio concentrador de melanina
N	Normal
NF-kB	Fator nuclear kappa B
NGFI-A	Fator de crescimento do nervo induzível pelo fator A
NPV	Núcleo paraventricular do hipotálamo
NPY	Neuropeptídeo Y
NTS	Núcleo do trato solitário
PET scan	Tomografia por emissão de pósitrons
POMC	Proopiomelanocortina
QUICKI	Índice quantitativo de sensibilidade à insulina
rpm	Rotações por minutos
SNC	Sistema nervoso central
SOCS-3	Supressor da sinalização de citocinas-3
TRH	Hormônio liberador de tireotrofina
UTI	Unidade de terapia intensiva
VMH	Núcleo ventro-medial do hipotálamo
VTA	Área tegmentar ventral
11β-HSD-2	11β-hidróxi-esteróide-desidrogenase tipo 2

5HT1A	Receptor para serotonina tipo 1A
5HT7	Receptor para serotonina tipo 7

RESUMO

A manipulação neonatal leva a uma série de alterações comportamentais e neuroendócrinas na vida adulta. Sabemos de nossos estudos anteriores que animais manipulados no período neonatal ingerem mais alimento palatável em relação a animais controle em uma tarefa de comportamento alimentar, sendo que o consumo de ração padrão não é diferente entre os grupos. Neste estudo, buscamos investigar as origens desse efeito sobre o comportamento alimentar, assim como descrever suas características, seu tempo de aparecimento e possíveis fatores causais e interferentes. Vimos que ratos manipulados apresentam uma curva de saciedade definida e resposta de saciedade ao recebem sacarose antes do teste, assim como menor nível plasmático de grelina. Demonstramos que animais manipulados no período neonatal apresentam maior incentivo para busca da recompensa do alimento doce numa tarefa de corredor, porém demonstram menor condicionamento de preferência de lugar e menor reação hedônica ao sabor doce, assim como menor metabolismo dopaminérgico no núcleo acumbens. Vimos também que o efeito da manipulação neonatal é evidente apenas se os animais são testados após a adolescência. A exposição precoce ao doce ou a um ambiente novo aumenta o consumo de animais controles semelhantemente à manipulação neonatal, eliminando as diferenças entre os grupos. Na adolescência, animais manipulados no período neonatal ingerem menos doce que animais controle, sem diferenças na hidrólise do ATP, ADP ou AMP no núcleo acumbens. Na vida adulta, animais manipulados no período neonatal consomem mais doce e apresentam menor hidrólise de AMP, um passo limitante para a síntese de adenosina. Verificamos ainda a interação entre a manipulação neonatal e a exposição ao estresse crônico variável (ECV) na vida adulta, observando que animais manipulados apresentam alterações basais como menor ganho de peso, maior consumo de doce, menor tempo de imobilidade no nado forçado, menor atividade da enzima

Na+,K+-ATPase no hipocampo e maior na amígdala. Os efeitos do estresse crônico (menor ganho de peso, exacerbação do consumo de doce e diminuição da atividade da enzima no hipocampo, na amígdala e no córtex parietal) são menos marcantes neste animais. Por fim, vimos que animais filhos de mães altamente cuidadoras têm alterações semelhantes às encontradas nos animais manipulados, sendo que possivelmente os achados da manipulação sobre o comportamento alimentar pode ser explicado pelo aumento do cuidado materno. Concluímos que as alterações do comportamento alimentar em ratos adultos manipulados no período neonatal possivelmente é resultado de uma interessante interação da atividade de vias homeostáticas (grelina) e hedônicas (dopamina) no núcleo acumbens; outras intervenções na infância como a exposição precoce ao doce ou a ambientes novos e até mesmo o aumento natural do comportamento materno alteram o consumo de doce na vida adulta. A manipulação neonatal, por suas características comportamentais e neuroquímicas, pode ser um interessante modelo para estudo de neuropsicopatologias como a esquizofrenia, a depressão e os transtornos alimentares. A compreensão dos mecanismos pelos quais experiências precoces na vida influenciam na saúde do adulto tem implicações para a identificação de populações de risco e introdução de medidas preventivas.

ABSTRACT

Neonatal handling leads to several behavioral and neuroendocrine alterations in adulthood. We know from our previous studies that neonatal handled animals have increased ingestion of palatable food in a behavioral feeding task compared to intact animals, although the consumption of standard lab chow is not different between the groups. In this study, we aimed to investigate the origins of this effect on feeding behavior, as well as to describe its characteristics, timing and possible causal and interfering factors. Handled rats presented a better defined satiation curve, an increased satiation response to sucrose ingestion prior to the test and decreased plasmatic ghrelin levels. We showed that neonatally handled rats demonstrate an enhanced incentive salience for sweet food in a runway task, but are less prone to show conditioned place preference and have less evident hedonic reaction to sweet food as well as lower dopaminergic metabolism in the nucleus accumbens. We also identified that the effect of neonatal handling is evident only if the animals were habituated and tested after puberty. The precocious exposure to sweet food or to a new environment increases the sweet food ingestion in intact animals with the same magnitude as it does in neonatally handled rats, diluting the differences between the groups. In the puberty, neonatally handled rats eat less sweet food than intact rats, having no differences in the hydrolysis of ATP, ADP or AMP in the nucleus accumbens. In adulthood, neonatally handled rats eat more sweet food and have a decreased hydrolysis of AMP, a step-limiting reaction to the formation of adenosine. We verified the interaction between neonatal handling and the exposure to a chronic variable stress in adulthood, observing that neonatally handled rats have some alterations at the baseline such as lower weight gain, increased sweet food ingestion, decreased immobility time in a forced swimming task, lower activity of Na+,K+-ATPase in the hippocampus and higher in the amygdala. The effects of the chronic variable stress (lower weight gain, increased sweet food

ingestion and a decrease in the enzyme activity in the hippocampus, amygdala and parietal cortex) are less evident in neonatally handled rats. At last, we saw that pups reared by mothers exhibiting high intensities of maternal care have the same pattern of alterations as neonatally handled rats do, suggesting that the findings on feeding behavior after neonatal handling can possibly be explained by an effect of the increased maternal care. We conclude that the alterations found on feeding behavior in neonatally handled rats in adulthood are possibly resultants from an interesting interaction between homeostatic and hedonic pathways' actions in the nucleus accumbens; other types of intervention in infancy such as the exposure to sweet food or to a new environment or even the natural variation in maternal care levels can influence feeding behavior later in life. Neonatal handling, for its behavioral and neurochemical characteristics, can be an interesting model to study neuropsichopathologies such as schizophrenia, depression and eating disorders. The comprehension of the mechanisms through which early life experiences influences adult health has implications to the identification of populations at risk and introduction of preventive measures.

1. INTRODUÇÃO

Mesmo sendo uma Ciência relativamente moderna (o primeiro Congresso Internacional aconteceu em Paris em 1912), a Pediatria sofre atualmente uma grande revolução. Desde os avanços nas imunizações iniciados em 1956 com a vacina antipólio, acompanhados de medidas simples de saneamento básico e uso de re-hidratação oral prevenindo a desidratação e as doenças diarréicas, a maioria das grandes causas de mortalidade infantil tem sido eficazmente erradicada, como em poucas outras áreas da Medicina.

Isso permitiu aos estudiosos realizarem aprimoramentos em aspectos mais específicos do cuidado pediátrico como o atendimento em salas de parto e o suporte técnico e farmacológico nas UTIs neonatais, aumentando a sobrevida de recém-nascidos muito doentes, prematuros ou de baixo peso (Anthony et al., 2004; Darlow et al., 2003; Harper et al., 2002). Atualmente, estima-se que a prevalência de recém-nascidos prematuros ou com baixo peso situe-se por volta de 10 a 15%, variando conforme a população estudada (Kilsztajn et al., 2003; Fang et al., 1999; Spencer et al., 1999). Como conseqüência de lidar com indivíduos em estágios do desenvolvimento fetal previamente pouco conhecidos, novas entidades patológicas foram identificadas, muitas como resultado da imaturidade, muitos derivando das terapias agressivas que foram desenvolvidas para o suporte dessa população especial.

Não é difícil supor que toda a instrumentação, assim como as intervenções farmacológicas e cirúrgicas necessárias para o suporte da vida frágil de um bebê previamente inviável possa trazer conseqüências ainda mais a longo prazo do que se imaginava a princípio. Porém uma idéia mais sutil é a de imaginar que, mesmo em situações supostamente fisiológicas, as adaptações metabólicas que o organismo materno sofre na tentativa de gerar um indivíduo em condições ambientais adversas (como a desnutrição) ou na vigência de doenças maternas (como a diabetes ou a até mesmo a depressão) pudessem também acarretar conseqüências permanentes para a saúde da prole. Ou ainda, que variações tênues do ambiente pós-natal pudessem determinar as características, o comportamento e o risco para doenças do indivíduo.

Já em meados da década de 30, enquanto estudavam as taxas de mortalidade na Inglaterra e na Suécia, pesquisadores surpreenderam-se com a constatação de que as condições ambientais precoces pareciam estar determinando a sobrevida de cada geração. Em seu artigo de 1934, Kermark et al. afirmam: "Nós chegamos então a um cenário (...) em geral inesperado(...). Cada geração após a idade de 5 anos parece carregar consigo a mesma mortalidade relativa por toda a vida, mesmo se considerarmos idades avançadas (...). A análise estatística se

comporta como se a expectativa de vida fosse determinada pelas condições que existiam durante os primeiros anos de vida da criança" (Kermark et al., 1934).

Na década de 70, Ravelli e colaboradores estudaram uma interessante população de 300.000 homens expostos à *Dutch Famine* durante a Segunda Guerra Mundial. Na vida adulta, esses indivíduos apresentavam padrões diferenciados de composição corporal dependendo da época em que tinham sido expostos à fome durante a vida intra-uterina. Se a mãe houvesse sofrido desnutrição durante o último trimestre da gestação, esse grupo tinha uma incidência extremamente baixa de obesidade. No entanto, se a desnutrição houvesse acontecido no primeiro semestre da gestação, a incidência de obesidade aumentava consideravelmente na prole adulta (Ravelli et al., 1976), sugerindo a existência de períodos críticos para o desenvolvimento do tecido adiposo.

Seguindo esse raciocínio, Barker e colaboradores (1989) desenvolveram a hipótese de que condições adversas intra-útero e durante a infância aumentavam o risco de doenças cardiovasculares na vida adulta. Para testá-la, estudaram registros de peso ao nascer e condições ambientais durante a infância de pessoas nascidas no início do século XX em Hertfordshire, Inglaterra, e correlacionaram com suas atuais condições de saúde cardiovascular (Barker et al., 1989). Como um grupo, pessoas nascidas com baixo peso permaneceram biologicamente diferentes de forma persistente até a vida adulta. Elas tem maior pressão arterial, e são mais propensas a desenvolver diabetes tipo II. Além disso, em seus estudos subseqüentes, esses e outros pesquisadores demonstraram que o baixo peso ao nascer está associado a um padrão alterado de lipídeos plasmáticos, redução da densidade óssea, respostas ao estresse diferenciadas, paredes ventriculares mais espessas, artérias menos elásticas, padrões de secreção hormonal específicos e maior incidência de depressão. Essas observações geraram a "Hipótese do Fenótipo Econômico" (*Thrifty Phenotype Hypothesis*), que propõe que o feto se adapta a um ambiente intrauterino adverso otimizando o uso de um suprimento energético reduzido para garantir a sobrevivência. Entretanto, favorecendo o desenvolvimento de alguns órgãos em sacrifício de outros, esse fenótipo levaria a alterações persistentes no crescimento e função dos tecidos (Hales & Barker, 1992).

Aproximadamente no mesmo período, um grupo independente de pesquisadores se concentrava em estudar os efeitos da dieta precoce em diferentes desfechos a longo prazo, especialmente em bebês prematuros. Descrevendo que o tipo de leite oferecido aos bebês influenciava o crescimento, o desenvolvimento neuropsicomotor e até mesmo o risco para doenças atópicas a longo prazo, Alan Lucas e colaboradores (também na Inglaterra) propuseram o uso da expressão "Programação" nesse contexto. Alinhavado por Dörner e colaboradores (Dörner, 1975) mas amplamente explorado por Lucas, esta expressão se refere ao conceito de que

um insulto ou estímulo aplicado num período crítico ou sensível pode ter efeitos duradouros ou persistentes sobre a estrutura ou função de um organismo (Lucas, 1991). Assim, o desenvolvimento e a gravidade de diversas condições patológicas na vida adulta dependem da vulnerabilidade genética do indivíduo, da exposição a fatores ambientais adversos, assim como do período de ocorrência do evento estressante (para uma revisão, veja Charmandari et al., 2003). Uma vez que a vida pré-natal, a infância e a adolescência são períodos críticos caracterizados por alta plasticidade neuronal (revisões em Khazipov & Luhmann, 2006; Crews et al., 2007), a exposição do indivíduo a um estímulo nesses períodos pode ser organizacional e levar a alterações persistentes no funcionamento do organismo.

Agregando informações desses dois grupos (Barker e Lucas), assim como dos estudos anteriores e subseqüentes de diversos outros centros de pesquisa em todo o mundo, formulou-se a Teoria da Origem do Padrão de Saúde e Doença do Adulto Relacionada ao Desenvolvimento (*Developmental Origins of Adult Health and Disease*, DOHaD - Gluckman & Hanson, 2004). Esse novo ramo de conhecimento se dedica a estudar as associações e mecanismos que levam o ambiente precoce a gerar maior risco para doenças na vida adulta, produzindo conhecimento de importância a diferentes grandes áreas da Medicina como a Obstetrícia, a Pediatria, a Clínica Médica, a Psiquiatria e a Saúde Comunitária.

1.1 O papel das Neurociências

Há muito mais tempo, vindo de um pólo distinto mas seguindo em uma mesma direção, as Neurociências têm dedicado parte de seus esforços estudando os efeitos de um ambiente adverso na fisiologia e na saúde do indivíduo. Já em 1911 Cannon e de La Paz propõem o papel da glândula adrenal no controle das funções do organismo em situações adversas. Em 1914 novamente Cannon, em um estudo clássico, baseia-se em teorias propostas por McDougall (1908) e explica que "a emoção do medo e a emoção da raiva são, na vida selvagem, provavelmente seguidas por atividades como fugir ou lutar" (Cannon, 1914).

Esse conceito foi um dos marcos para a definição de estresse, anos mais tarde. Em 1936, Hans Seyle afirma que quando repentinamente confrontado com uma situação crítica, o organismo apresenta uma "reação de alarme geral": aumento do timo, baço, linfonodos e fígado, desaparecimento do tecido adiposo, formação de

edema, perda do tônus muscular, diminuição da temperatura corporal, surgimento de erosões agudas no trato gastrointestinal, redução da adrenal, exoftalmia, lacrimejamento e salivação. Após um período de submissão contínua ao agressor, o organismo passaria a apresentar um esforço generalizado para adaptar-se às novas condições, sendo que a maioria dos órgãos e sistemas retornam praticamente ao normal. Logo, a síndrome como um todo foi chamada por Seyle "Síndrome da Adaptação Geral" (Seyle, 1936).

Não demorou muito para que o interesse pelo estudo das influências ambientais da vida precoce em diferentes desfechos na vida adulta fosse despertado. A partir da década de 50, estudiosos passaram a descrever elegantes experimentos demonstrando que o ambiente perinatal influencia diversos parâmetros na vida adulta, tais como o condicionamento comportamental, o crescimento, a resistência à infecção, a eficiência calórica e a resposta a estímulos agressivos (Levine et al., 1957; Dubos et al., 1966; Ader & Grota, 1969). Muitos destes experimentos, utilizando o paradigma da manipulação neonatal e outros, ajudaram a desenhar com mais clareza as respostas ao estresse, especialmente o funcionamento do eixo hipotálamo-pituitária-adrenal (HPA) e os sistemas de retroalimentação negativa dos glicocorticóides, que revisaremos a seguir.

1.2 Respostas ao estresse

O termo "estresse" tem sido largamente usado de várias formas. Como descrito acima, este conceito foi introduzido por Seyle no início do século XX como uma adaptação de um conceito existente na Física - estado de tensão sobre um material antes de se partir. A definição de estresse então deu-se como uma seqüência de reações a uma série de agressões direcionadas contra a integridade física e psicológica, ameaçando o estado de equilíbrio do organismo (homeostase). Ultimamente, a palavra "estresse" tem sido interpretada como o conjunto de respostas do organismo a um estressor. "Estressor" é definido como um desafio ao indivíduo, que perturba a homeostase e requer uma resposta fisiológica. Pode também ser apenas uma interpretação errônea da situação, percebida como ameaça, que resulta numa resposta comportamental e/ou hormonal (McEwen, 2002; Tsigos et al., 2002).

A resposta adaptativa a um estressor agudo inclui processos fisiológicos que redirecionam a utilização de energia entre os vários órgãos, mobilizando suas reservas e preparando o organismo para uma exposição estressante adicional, imprevisível. O aumento do suprimento energético aos órgãos-alvo (fundamentais para o enfrentamento ou a fuga, como o coração, cérebro e músculos) é feito principalmente pela liberação de catecolaminas e glicocorticóides que, em geral, ativam a gliconeogênese e a glicogenólise hepáticas, inibem a

captação de glicose por diversos tecidos e aumentam a proteólise (músculo) e a lipólise (tecido adiposo). Outras adaptações fisiológicas incluem aumento do tônus cardiovascular e freqüência respiratória, assim como inibição das funções vegetativas como comportamento alimentar, digestão, crescimento, reprodução e imunidade (Sapolsky et al., 2000; Habib et al., 2001)

A ativação da resposta ao estresse também inicia uma série de alterações comportamentais como aumento do estado de alerta e euforia, melhora aguda da cognição e memória para o evento estressor, assim como analgesia (Chrousos & Gold, 1992). Essas respostas comportamentais e fisiológicas são afetadas pela ativação de sistemas efetores primários como o sistema nervoso simpático (liberação de noradrenalina), sistema adrenomedular (liberação de adrenalina), sistema hipotalâmico-pituitário-adrenocortical (liberação do hormônio adrenocorticotrófico (ACTH) e glicocorticóides), sistema nervoso parassimpático (liberação de acetilcolina) e sistema renina-angiotensina (liberação de renina).

Vários outros sistemas contribuem para o restabelecimento da homeostase, como o eixo hipotálamo-hipófise-tireóide (resposta ao frio e calor), eixo hipotálamo-hipófise-gonadal (redução temporária da função reprodutiva), liberação do hormônio do crescimento e alterações na função imunológica. Todos estes sistemas agem diretamente, alterando a liberação ou os efeitos biológicos de muitos mediadores da resposta ao estresse agudo (ex.: neurotransmissores, hormônios, citocinas, etc.), ou indiretamente, alterando os níveis das variáveis monitoradas (ex.: pressão sangüínea, temperatura corporal, etc.), com subseqüente ajuste reflexivo determinado pela homeostase interna (McEwen, 2000). Apesar da importância da resposta individual e integrada de todos estes eixos, o eixo HPA e o sistema neurovegetativo são os mais estudados.

Todo o Sistema Nervoso Central (SNC) está direta ou indiretamente envolvido na manutenção da homeostase e participa na organização geral da resposta ao estresse. Diversas estruturas do prosencéfalo, incluindo o córtex pré-frontal, hipocampo, amígdala e septo, juntamente com as fibras nervosas condutoras dos estímulos sensoriais, lançam aferências mono e polissinápticas que convergem para o núcleo paraventricular hipotalâmico (NPV). Portanto, os componentes centrais da resposta ao estresse estão localizados no hipotálamo - incluindo principalmente o hormônio liberador de corticotrofina (CRH) e a arginina-vasopressina (AVP), o tronco encefálico, os neurônios do núcleo paraventricular hipotalâmico, assim como o locus ceruleus e outros grupos celulares catecolaminérgicos do bulbo e da ponte (sistema simpático central) (Chrousos, 1992; Tsigos & Chrousos, 1994).

A resposta ao estresse é ativada por estímulos externos como dor (Palkovits et al., 1999), recrutamento de sistemas de defesa inatos (Figueiredo et al., 2003) ou associações ligadas aos estímulos sensoriais, como o medo condicionado (Van de Kar et al., 1991). Além disso, distúrbios internos da homeostase sinalizados por meio dos sistemas cardiovascular, respiratório e das vísceras são capazes de acionar tais mecanismos. Esses distúrbios parecem ser sinalizados ao NPV através de neurônios do tronco encefálico, localizados na região do núcleo do tracto solitário (Swanson and Sawchenko, 1983). Uma porção importante desses neurônios são noradrenérgicos e adrenérgicos (Cunningham et al., 1990 and Cunningham and Sawchenko, 1988). Esses estímulos atingem a região medial parvocelular do núcleo periventricular hipotalâmico. Através do sistema simpático, o NPV estimula a medula das glândulas adrenais, levando à liberação de catecolaminas endógenas (adrenalina e noradrenalina), o que constitui uma resposta imediata inicial ao estresse (Ursin e Olff, 1993).

Este núcleo também possui neurônios hipofisiotrópicos que produzem e secretam CRH em resposta ao estresse, liberando este hormônio na vasculatura porta hipofisária que tem acesso à porção anterior da glândula pituitária. A ligação do CRH no seu receptor na pituitária induz a liberação do hormônio adrenocorticotrópico (ACTH) na circulação sistêmica. O principal alvo do ACTH é o córtex da glândula adrenal, onde ele estimula a síntese e secreção de glicocorticóides na zona fasciculada. Os glicocorticóides são os efetores periféricos do eixo HPA e promovem alterações fisiológicas através da ligação em seus receptores intracelulares (Munck et al., 1984; Bamberger et al., 1996).

Os glicocorticóides, cortisol em humanos e corticosterona em roedores, são a maior subclasse de hormônios esteróides que regulam processos metabólicos, cardiovasculares, imunológicos e comportamentais (Charmandari et al., 2005; Sapolsky et al., 2000). Os efeitos fisiológicos dos glicocorticóides são mediados por uma proteína citosólica de 94 kD, o receptor glicocorticóide (GR). O GR está amplamente distribuído no cérebro e tecidos periféricos. No estado inativo, o GR é parte de um complexo multiprotéico consistindo de várias moléculas de proteínas de choque térmico (*heat shock proteins;* Bamberger et al., 1996; Giguere et al., 1986; Cadepond et al., 1991). Ligando-se aos glicocorticóides, o GR transloca-se para o núcleo da célula, onde interage com elementos responsivos aos glicocorticóides específicos no ADN, de modo a alterar a transcrição de determinados genes (Pratt, 1990). O receptor ativado também inibe, via interações proteína-proteína, outros fatores de transcrição como o c-jun/c-fos e NF-kB, que são reguladores positivos da transcrição de vários genes envolvidos na ativação e crescimento de células do sistema imunológico e outros tipos celulares (Scheinman et al., 1995). Além disso, os glicocorticóides alteram a estabilidade do ARN mensageiro e portanto a tradução de várias proteínas, assim como alteram o potencial elétrico de neurônios. Na maioria dos vertebrados há um ritmo circadiano pronunciado da secreção de glicocorticóides, com picos relacionados ao início da fase ativa do ciclo diurno (Keller-Wood &. Dallman, 1984). O ritmo circadiano glicocorticóide é dependente do núcleo supraquiasmático, uma vez que lesões dessa estrutura levam a um nível aproximadamente constante e intermediário entre o pico e o nadir circadianos (Cascio et al., 1987; Moore &. Eichler, 1972).

A regulação das ações do eixo HPA é feita, em grande parte, por retroalimentação negativa dos glicocorticóides sobre componentes do SNC, aumentando ou diminuindo sua atividade de acordo com as necessidades fisiológicas (Marti et al., 1999). Várias estruturas cerebrais estão envolvidas nos processos de retroalimentação, dentre as quais destacam-se o hipotálamo, a amígdala, o córtex cerebral pré-frontal e o hipocampo (Campeau et al., 1998), sendo esta última estrutura uma das mais fortemente relacionadas à regulação do eixo, devido a sua alta concentração de receptores glicocorticóides.

1.3 O eixo HPA no início da vida e mecanismos de Programação

Os glicocorticóides têm importância fundamental na gestação em mamíferos, uma vez que estão envolvidos nas adaptações metabólicas maternas (Atkinson & Waddell, 1995). Além disso, atuam na coordenação entre a aptidão para o nascimento e o início dos mecanismos de parto. Durante a gestação, enquanto os esteróides lipofílicos facilmente atravessam a placenta, os níveis de glicocorticoides fetais são muito menores que os

maternos (Beitens et al., 1973; Klemcke, 1995). Isso se deve à atuação da enzima 11β-hidróxi-esteróide-desidrogenase tipo 2 (11β-HSD-2), altamente expressa na placenta. Esta enzima catalisa a conversão dos glicocorticóides fisiologicamente ativos cortisol e corticosterona em formas inertes como a cortisona (White et al., 1997). Na placenta, essa enzima forma uma barreira protetora do feto contra a exposição aos glicocorticóides maternos, embora ainda permita a passagem de cerca de 10 a 20% deles para o filhote (Venihaki et al., 2000). É interessante notar que há uma correlação positiva entre o peso de nascimento e a atividade dessa enzima em ratos (Benediktsson et al., 1993) e humanos (Stewart et al., 1995), sendo que a maior exposição aos glicocorticóides no período fetal tem sido proposta como o mecanismo de programação do maior risco para doenças na vida adulta em indivíduos nascidos com baixo peso (Seckl & Meaney, 2004), como hipertensão, diabetes e distúrbios psiquiátricos como a depressão e a ansiedade.

Em humanos, os níveis plasmáticos maternos de CRH (produzido pela placenta) aumentam exponencialmente conforme a gestação avança, tendo seu pico no momento do parto. Em partos prematuros, esse aumento é muito mais rápido (McLean et al., 1995). O CRH placentário atinge o feto, embora em concentrações menores que na mãe (Nodwell et al., 1999). No feto, há receptores de CRH na pituitária (Asa et al., 1991) e na adrenal (Smith et al., 1998). A estimulação da pituitária fetal pelo CRH aumenta a produção de ACTH e conseqüentemente de cortisol pela adrenal, amadurecendo o eixo HPA fetal e induzindo a formação de surfactante nos pulmões.

A responsividade do eixo HPA em mamíferos flutua no período perinatal, sendo moderadamente responsiva no momento do nascimento mas diminuindo em intensidade no período neonatal (Rokicki et al., 1990; Bergant et al., 1998). Em ratos, há um pico de corticosterona no último estágio fetal, seguido de pouca responsividade até o fim da segunda semana de vida, fato conhecido como Período Hiporresponsivo ao Estresse (Sapolsky &. Meaney, 1986; Guillet & Michaelson, 1978). Caracteristicamente, há uma exacerbação do mecanismo de retroalimentação negativa dos glicocorticóides na hipófise e diminuição da sensibilidade da adrenal ao ACTH neste período (Yoshimura et al., 2003).

Conforme propõe o conceito de Programação, a submissão do rato a um estímulo ou estressor nesses primeiros dias determina alterações neuroquímicas e comportamentais observáveis durante toda a vida. Embora "hiporresponsivos", esses animais respondem agudamente ao estresse de separação da mãe mesmo se não expostos a nenhum estressor adicional (Kuhn et al, 1990), sendo que a resposta aumenta progressivamente nas 24 horas subseqüentes. Além disso, nessa fase, os níveis de transcortina são muito baixos, sendo que a maior parte da

corticosterona circula em sua forma não-ligada e portanto biologicamente ativa (Henning, 1978). Logo, mesmo que a concentração total da corticosterona plasmática seja baixa no período hiporresponsivo, a concentração de corticosterona biologicamente ativa é relativamente alta, o que é suficiente para que o hormônio exerça suas ações biológicas e possivelmente atue programando o SNC de forma persistente.

Um dos modelos experimentais mais interessantes e intensamente estudados de intervenção nesse período crítico do desenvolvimento é a manipulação neonatal em ratos. Caracterizado pela separação dos filhotes da mãe por curtos períodos de tempo (não mais que 30 minutos) diariamente nos primeiros dias de vida, este protocolo gera nos filhotes alterações persistentes do funcionamento do eixo HPA, acompanhadas de alterações comportamentais, metabólicas e neuroquímicas. Em essência, esses animais apresentam menor medo quando expostos a ambiente diferente da caixa-moradia, com maior atividade e exploração (Levine et al., 1967). Há uma clássica persistência da exacerbação da retroalimentação negativa dos glicocorticóides na vida adulta (Ader & Grota, 1969), com redução da expressão de ARN mensageiro para CRH no hipotálamo e diminuição do conteúdo de CRH na eminência média (Plotsky & Meaney, 1992). Além disso, há maior concentração de receptores glicocorticóides no hipocampo (Meaney et al., 1989), com aumento da inibição mediada pelo hipocampo e diminuição da excitação mediada pela amígdala na resposta neuroendócrina do eixo HPA (de Kloet et al., 1998). Além disso, uma marcante diminuição da liberação de noradrenalina no núcleo paraventricular do hipotálamo em resposta a estresse por contenção (Liu et al., 2000) contribui para uma marcada supressão crônica da resposta de liberação de glicocorticóides frente ao estresse pelo eixo.

O contato maternal parece ser fundamental para o desenvolvimento de tais alterações em ratos submetidos à manipulação neonatal (Cirulli et al., 2003). O fato de retirar os filhotes da ninhada gera na mãe um aumento nos cuidados quando do retorno deles para a caixa-moradia logo após a manipulação neonatal (Branchi et al., 2001; Pryce et al., 2001). Além disso, variações naturais do cuidado materno se correlacionam com a reatividade dos filhotes ao estresse na vida adulta, sendo que filhotes de mães altamente cuidadoras serão menos responsivos (semelhantemente a animais manipulados no período neonatal) em relação a filhotes de mães pouco cuidadoras (Liu et al., 1997).

O mecanismo pelo qual o cuidado materno leva a essas alterações persistentes tem sido descrito em detalhes por Meaney e colaboradores em uma série de estudos desde a década de 90. Ultimamente, eles propõem que esses eventos pós-natais (manipulação e aumento do cuidado materno) atuem através de vias serotoninérgicas ascendentes do núcleo da rafe (Smythe et al., 1994) que induzem a expressão de receptores glicocorticóides no

hipocampo (Mitchell et al., 1990; Yau et al., 1997a). A serotonina atua provavelmente através do receptor 5HT7, que é regulado por glicocorticóides (Yau et al., 1997b), e positivamente ligado ao AMP cíclico (Meaney et al., 2000). Ocorre então a estimulação de fatores de transcrição associados ao AMPc como o NGFI-A (Fator de Crescimento do Nervo Induzível pelo Fator A) (Meaney et al., 2000). Embora a afinidade do NGFI-A ao seu sítio de reconhecimento na seqüência de ADN responsável pela produção de GR seja baixa, a estimulação tátil promove uma grande elevação nos níveis deste fator de transcrição, aumentando portanto a chance de ligação (Encio & Detera-Wadleigh, 1991). A ligação do NGFI-A resulta em recrutamento de histonas-acetiltransferases, que aumentam a acetilação das histonas, facilitando o acesso de desmetilases e a desmetilação do sítio promotor do GR (Carvin et al., 2003). O sítio promotor desmetilado exibirá alta afinidade ao NGFI-A mesmo durante a vida adulta, resultando em uma maior atividade do promotor de GR induzida por NGFI-A no hipocampo, uma maior produção de receptores glicocorticóides nessa estrutura e, portanto, um mecanismo de retroalimentação negativa mais eficiente.

Além de alterações na resposta ao estresse agudo, animais manipulados no período neonatal também apresentam respostas atenuadas ao estresse crônico repetido (Papaioannou et al., 2002b), com menor indução de desamparo aprendido após choque inescapável (Costela et al., 1995), possivelmente por alterações específicas na neurotransmissão noradrenérgica (Tejedor-Real et al., 1998). Há descrição de menor inibição comportamental com maior exposição ao predador em um campo aberto, aumento no comportamento materno agressivo (Padoim et al., 2001), menor indução de medo condicionado relacionado ao contexto (Madruga et al., 2006) e melhor memória espacial (Meaney et al., 1988; Tang 2001; Bilbo et al., 2007).

Estudos sugerem que esse modelo de intervenção neonatal está associado a uma menor vulnerabilidade à depressão na vida adulta (Costela et al., 1995; Papaioannou et al., 2002b; Plotsky et al., 2005; Arborelius & Eklund, 2007) e à reversão de efeitos adversos do estresse pré-natal (Lemaire et al., 2006) e da hipóxia-isquemia (Rodrigues et al., 2004) no dano neuronal hipocampal, sem alterar o desfecho comportamental nem a atrofia dendrítica causada por lesões maiores como a remoção do córtex medial frontal (Gibb & Kolb, 2005). Há relatos de respostas comportamentais atenuadas após injeção de substâncias aditivas como a cocaína (Brake et al., 2004), embora o consumo deste psicoestimulante seja maior nos primeiros dias de exposição (Marquardt et al., 2004). Em relação ao etanol, há menor preferência e consumo nesses animais quando comparados aos controles (Jaworski et al., 2005).

Fêmeas manipuladas no período neonatal demonstram menor receptividade sexual, menores níveis de prolactina no proestro (Gomes et al., 2005) e maior número de ciclos anovulatórios na vida adulta (Gomes et al., 1999). Além disso, apresentam menor secreção de estrogênio, LH e FSH no proestro (Gomes et al., 2005). O comportamento sexual é diminuído também em machos (Padoim et al., 2001).

Vários estudos descrevem a associação entre a manipulação neonatal e alterações persistentes de diversos sistemas neurotransmissores em diferentes áreas cerebrais. Por exemplo, há descrição de aumento do número de receptores 5HT1-A no hipocampo de ratos adultos machos manipulados no período neonatal (Stamatakis et al., 2006), assim como maiores níveis de serotonina no hipotálamo desses animais após o estresse agudo (Papaioannou et al., 2002A). Há maior metabolismo dopaminérgico no hipotálamo de machos adultos manipulados na vida precoce (Papaioannou et al., 2002A), sem alteração no número de neurônios expressando tirosina-hidroxilase nessa estrutura (Hermel et al., 2001). Outro estudo mostra menor quantidade de receptores D3 no núcleo acumbens desses animais (Brake et al., 2004). Alterações em sistemas como o GABA/BDZ (Caldji et al., 2000; Jaworski et al., 2005), opióide (Ploj et al., 2001, 2003) e noradrenérgico (Liu et al., 2000) também já foram descritas em estruturas cerebrais específicas.

Em nossos estudos prévios, observamos que a manipulação neonatal aumenta o consumo de alimentos palatáveis (doce e salgado) na vida adulta. Esse efeito não é acompanhado de alterações do consumo de ração padrão, água ou soluções palatáveis doces e salgadas (Silveira et al., 2004). Além disso, a preferência por doce é evidente mesmo em épocas mais tardias da vida, e este maior consumo de doce não é revertido por injeção de diazepam logo antes do teste. Da mesma forma, esses animais não apresentam comportamento compatível com ansiedade em testes como o labirinto em cruz elevado e o teste de transição claro/escuro (Silveira et al., 2005).

1.4 Regulação do comportamento alimentar

Tão essencial à sobrevivência e à manutenção da homeostase, a alimentação é finamente controlada por meio de uma complexa e intricada rede de mecanismos. Como exemplo ilustrativo da importância do comportamento alimentar, sabe-se que ratos mesmo com o tronco encefálico isolado continuam a regular a ingestão alimentar e a demonstrar respostas afetivas aos alimentos palatáveis (Grill, & Kaplan, 2001, 2002). Basicamente, o comportamento alimentar pode ser divido em diferentes fases para melhor compreensão de seus mecanismos: Na fase de **iniciação**, o "valor" de um objetivo alimentar disponível ou o estado interno de alguma maneira atraem a atenção do indivíduo para a alimentação. A presença de um estímulo direto do alimento como a

visão ou o olfato podem disparar a fase de iniciação sem necessariamente haver a presença de um estado interno adjuvante. Uma vez que a atenção seletiva é alcançada e a motivação para a ingestão alimentar é grande, inicia-se a fase de **procura**. Este comportamento requer planejamento, aprendizado e memória e depende essencialmente de processos corticais cognitivos. A fase de **consumo** começa quando o alimento é finalmente presente e ingerido. Embora essa fase envolva uma série de comportamentos estereotipados, também é caracterizada pela degustação dos alimentos ingeridos e de seus nutrientes a nível cefálico e gastrointestinal, assim como pela formação de associações entre os vários atributos sensoriais do alimento. Na seqüência, tomam parte da ação os mecanismos de **saciedade**, por fim levando ao **término** da refeição, que inclui o fim do consumo *per se* mas também a sensação das conseqüências da absorção e pós-absorção, assim como o armazenamento dessas sensações em forma de memória associativa para posterior comparação (revisado em Berthoud, 2002).

Os sistemas de aferência de informação para o cérebro em relação ao alimento incluem estímulos externos, como as aferências visuais, olfativas, auditivas e táteis, e estímulos internos, que subdividem-se em pré-gástricos (principalmente sabor), gástricos (distenção) e pós-gástricos (ou pré-absortivos). Há ainda estímulos pós-absortivos, que dividem-se em (a) mecanismos de transporte de nutrientes e a liberação de hormônios locais como a colecistocinina (CCK), agindo através da circulação sangüínea ou de nervos sensoriais viscerais; (b) nutrientes, metabólitos e hormônios agindo em sensores do sistema porta hepático, como a glicose; (c) passos de processamento metabólico e seus mensageiros locais e hormônios agindo nos sensores do fígado, como o ATP e o glucagon e (d) metabólitos, hormônios e outros fatores originários de vários tecidos circulando no sangue ou no sistema linfático e ativando sensores diretamente no cérebro, como a glicose, aminoácidos, insulina e leptina (Berthoud, 2002).

1.4.1 Sistemas neurais envolvidos na regulação do comportamento alimentar

O hipotálamo é uma estrutura chave na regulação do comportamento alimentar. Em mamíferos, esta estrutura consiste de mais de 40 áreas e núcleos histologicamente distintos, e muitos deles ainda podem ser subdivididos em subnúcleos. Além de controlar a alimentação, o hipotálamo está envolvido em outros processos como a ingestão hídrica, comportamentos defensivos e agressivos, comportamento sexual, regulação da temperatura corporal e defesa imunitária. No que tange ao comportamento alimentar, vários de seus núcleos recebem aferências e enviam eferências a diversas partes do encéfalo e medula espinhal, porém os núcleos mais

intensamente envolvidos nesse controle são o núcleo arqueado, o hipotálamo lateral e ventromedial e o núcleo paraventricular.

O núcleo arqueado do hipotálamo (ARC) é uma região que recebe aferências de outros núcleos como o núcleo periventricular e área pré-óptica medial, assim como do hipotálamo lateral (Guan et al., 2001; Horvath, et al.,1999). Aferências extra-hipotalâmicas incluem o córtex (DeFalco et al., 2001) a amígdala, o núcleo próprio da estria terminal e núcleos do tronco encefálico como o núcleo parabraquial e o núcleo do trato solitário (Li et al., 1999; Ricardo & Koh, 1978). Seus neurônios são anatomicamente posicionados próximos a capilares fenestrados na base do hipotálamo, o que os coloca em contato com importantes hormônios como a leptina (Glaum et al., 1996), o hormônio do crescimento (Kamegai et al., 1996), esteróides sexuais (Tong et al., 1990), insulina e glicose (Muroya et al., 1999) e grelina (Wang et al., 2002), para os quais possuem receptores (Benoit et al., 2000; Cone et al., 2001). Esse núcleo envia sinais a outros núcleos do hipotálamo e para sítios extra-hipotalâmicos como núcleos talâmicos mediais, núcleo próprio da estria terminal, núcleos da rafe, substância cinzenta periaquedutal e núcleo parabraquial lateral. Dois subtipos de neurônios foram identificadas no ARC, ambos contendo o neurotransmissor inibitório GABA (Horvath et al., 1997, Hentges et al., 2004). Uma das populações neuronais expressa a proopiomelanocortina (POMC) e o peptídeo relacionado à cocaína e à anfetamina (CART), e quando ativada leva a uma diminuição do apetite e aumento do gasto energético (Boston et al., 1997; Ellacott & Cone, 2004; Cone, 2005). Em contraste, a outra população de células, contendo neuropeptídeo Y (NPY) e Proteína Relacionada ao Gene Cutia (AGRP), leva a uma resposta orexigênica e menor gasto energético (Clark et al., 1984). Tanto os neurônios contendo POMC quanto os neurônios contendo NPY expressam receptores para leptina e grelina (Baskin et al., 1999; Riedger et al., 2003). A leptina aumenta a atividade dos neurônios POMC e inibe neurônios NPY (Baskin et al., 1999), enquanto a grelina age fazendo o oposto (Traebert et al., 2002).

O hipotálamo lateral, por sua vez, recebe aferências de várias áreas corticais e límbicas como a amígdala, o hipocampo e o núcleo accumbens, assim como dos núcleos paraventricular e arqueado (especialmente de neurônios contendo NPY na área perifornicial). Ele se projeta para todo o córtex, hipocampo, amígdala, gânglios da base, tálamo, ponte e medula espinhal, assim como para os outros núcleos do próprio hipotálamo. Nesse núcleo encontram-se duas populações de neurônios que contêm tanto orexina (peptídeo envolvido no estado de vigília, atenção e no comportamento alimentar, Chen et al., 1999; Date et al., 1999) como o hormônio concentrador de melanina (MCH, outro potente estimulante da ingestão alimentar, Bittencourt et al., 1992).

O núcleo ventromedial do hipotálamo (VMH) possui alta expressão de receptores para a leptina e tem sido descrito como o núcleo responsável pela mediação das ações da leptina na homeostase (Mercer et al., 1996; Fei et al., 1997). Eferências do VMH são na sua maioria excitatórias, aumentando a atividade das células POMC, e durante o jejum esses estímulos diminuem (Sternson et al., 2005). Infusões de NPY nesse núcleo aumentam o consumo alimentar, e o jejum aumenta os níveis de NPY nessa região (Bouali et al., 1995; Kalra et al., 1999). Uma característica notável desse núcleo é a expressão acentuada e específica de fator neurotrófico derivado do encéfalo (BDNF). O BDNF tem sido apontado como inibidor do apetite, sendo um fator regulatório no ajuste do balanço energético controlado pela sinalização de leptina (Rios et al., 2001; Nakagawa et al., 2002, 2003).

O núcleo paraventricular do hipotálamo recebe aferências de outros núcleos hipotalâmicos como a área pré-óptica medial, órgão subfornicial e núcleos arqueado, dorsomedial e lateral. Além disso, aferências do tronco encefálico como o núcleo do trato solitário (principalmente noradrenérgicas, Sawchenko & Swanson, 1982), locus ceruleus e núcleo da rafe (principalmente serotoninérgicas, Sawchenko et al., 1983), assim como do núcleo próprio da estria terminal e amígdala também atingem o NPV (Sawchenko & Swanson, 1983). As eferências endócrinas mais reconhecidas desse núcleo são provenientes dos neurônios magnocelulares para a pituitária posterior, secretando ocitocina e vasopressina, assim como dos neurônios parvocelulares secretando CRH e TRH. Eferências não-endócrinas incluem a maioria dos outros núcleos hipotalâmicos e núcleos autonômicos pré-ganglionares no mesencéfalo, prosencéfalo e medula espinhal, assim como neurônios pré-ganglionares simpáticos e parassimpáticos inervando o pâncreas (Jansen et al., 1997) e estruturas diencefálicas e telencefálicas como o tálamo e a amígdala.

Outras estruturas são também importantes na regulação do comportamento alimentar. Por exemplo, o córtex sensório-visceral dissemina importantes informações nutricionais da cavidade oral e trato gastrointestinal para áreas corticais envolvidas na geração de representações e associações polimodais, tanto diretamente como através da amígdala para processamento emocional ou através do estriado ventral para aspectos motivacionais. O córtex olfatório primário tem propriedades semelhantes em relação ao olfato, com a possibilidade de estocagem de memórias através da formação hipocampal. O hipocampo, por sua vez, além de envolvido no aprendizado e memória dos aspectos relacionados ao comportamento alimentar como a qualquer outro comportamento, também parece ter um papel específico na alimentação (Clifton et al., 1998). A amígdala é a única região cerebral além do córtex gustatório e do hipotálamo lateral a receber aferências gustatórias diretas do núcleo do trato solitário e do núcleo parabraquial, mas também recebe informações provenientes de áreas corticais, hipotalâmicas,

hipocampais e do estriado ventral, sendo responsiva a uma variedade de peptídeos e neurotransmissores envolvidos no comportamento alimentar como os opióides (Giraudo et al., 1998) e a enterostatina (Lin & York, 1997).

Estruturas do tronco encefálico integram as grandes vias de aferências víscero-sensoriais e eferências motoras, sendo que suas áreas mais estudadas em relação ao comportamento alimentar são o núcleo do trato solitário (NTS) e área postrema e o núcleo parabraquial. O NTS e a área postrema são extremamente hábeis para detectar hormônios e outros fatores circulantes, além de receberem aferências dos receptores víscero-sensoriais e gustatórios via neurônios aferentes primários vagais, glossofaríngeos, faciais e trigeminais, possuindo uma população significativa de neurônios expressando POMC e sendo também responsivos à urocortina (Grill et al., 2000). O núcleo parabraquial localiza-se na ponte e integra várias modalidades sensoriais, como gustação (Spector, 1995), quimio e mecanossensação visceral (Baird et al., 2001) e dor (Gauriau & Bernard, 2002), via projeções recíprocas a várias áreas do tronco, do prosencéfalo e diencéfalo, servindo como uma interface entre o controle reflexo medular e a regulação integrativa dos sistemas neurovegetativos.

1.4.2 O sistema mesolímbico e a regulação do apetite

Mesmo na ausência de fome, o prazer e a sensação de recompensa associados ao alimento podem estimular o consumo. O núcleo acumbens tem sido amplamente relacionado a comportamentos direcionados e aprendizado instrumental apetitivo (Baldwin et al., 2002; Corbit et al, 2001). O desejo ou a saliência da comida nesse tipo de tarefa é determinado pelo estado nutricional e pelo valor hedônico do alimento, assim como pela interação dos dois fatores (Berridge, 1991). Além disso, evidências recentes sugerem que a atividade dos neurônios dopaminérgicos da área tegmental ventral (VTA) que se projetam para o núcleo acumbens pode ser modulada por sinalizadores do estado energético como a leptina, a insulina e a grelina (Abizaid et al., 2006; Jerlhag et al., 2006; Figlewicz, 2003), revelando a potencial importância desse sistema na regulação da ingestão alimentar.

De acordo com Berridge e Robinson (1998), a saliência ("querer") e a sensação hedônica ("gostar") relacionadas ao alimento representam processos distintos no circuito da motivação e da recompensa. Segundo essa teoria, os neurônios dopaminérgicos da área tegmental ventral (VTA) projetando-se para o núcleo acumbens determinam seletivamente o nível de saliência do alimento, enquanto sensação hedônica ligada ao alimento palatável está associada com sistemas opióides e GABA/benzodiazepínicos difusos distribuídos nos núcleos gustatórios do tronco encefálico, no estriado ventral e possivelmente em outras áreas como a amígdala, o córtex

límbico e o hipotálamo. Realmente, antagonistas opióides injetados no núcleo acumbens reduzem o consumo de alimentos doces mas não de substâncias menos palatáveis (Zhang et al., 2003). No entanto, interessantes estudos em humanos utilizando tomografia por emissão de pósitrons (*PET scan*) mostram que o consumo alimentar associa-se à liberação de dopamina no estriado dorsal, e que a quantidade liberada do neurotransmissor se correlaciona com o grau de prazer associado à alimentação (Small et al., 2003), sugerindo que a regulação dos mecanismos hedônicos pode ser mais complexa.

Vários estudos demonstram que a administração de canabinóides endógenos estimula a ingestão alimentar em modelos animais (Koch, 2001). Esse efeito é possivelmente mediado via receptor canabinóide tipo 1 (CB1) no hipotálamo, onde se co-localiza com a CART, MCH e orexina (Cota et al., 2003). É interessante notar que há receptores CB1 também em adipócitos onde eles parecem estimular a lipogênese (Cota et al., 2003).

Outros sistemas podem ser também capazes de modular tanto circuitos homeostáticos quanto hedônicos no controle do comportamento alimentar. A serotonina, por exemplo, pode influenciar diretamente a rota da melanocortina no ARC via receptores 5HT. O sistema noradrenérgico por sua vez influencia o apetite através de seus receptores α_1- e β_2- adrenérgicos inibindo o apetite, enquanto a ativação do receptor α_2-adrenérgico estimula o apetite.

1.4.3 Regulação periférica do comportamento alimentar

O controle periférico do comportamento alimentar é dado principalmente por hormônios produzidos no tecido adiposo (como a leptina, a adiponectina e a resistina) hormônios pancreáticos (como a insulina e o polipeptídeo pancreático) e hormônios produzidos no trato gastrointestinal (como o peptídeo YY, a grelina, o GLP-1, a oxintomodulina, a bombesina e a colecistocinina) . Neste trabalho, nosso foco recai sobre três hormônios, cada um produzido em um dos tecidos: a leptina, a insulina e a grelina.

A leptina é um homônio peptídico que influencia a homeostase energética e as funções endócrinas e imunes. É o produto do gene *ob* expresso predominantemente em adipócitos (Zhang et al., 1994), mas também em menores níveis no epitélio gástrico (Bado et al., 1998) e na placenta (Masuzaki et al., 1997). Os níveis circulantes de leptina refletem tanto estoques energéticos como balanço energético agudo. O jejum suprime os níveis circulantes (Frederich et al., 1995, Maffei et al., 1995), o que é revertido pela alimentação ou administração de insulina. Sua sinalização ocorre através de receptores com domínio transmembrana único da família dos receptores para citocinas (Tartaglia et al., 1995), podendo ser classificados como receptores do tipo longo, curto e

solúvel (Tartaglia, 1997; Ge et al., 2002). A forma longa está envolvida nos efeitos da leptina no comportamento alimentar, agindo através da ativação da rota JAK-STAT e induzindo a expressão do supressor da sinalização de citocinas-3 (SOCS-3). A expressão do SOCS-3 é aumentada pela leptina no hipotálamo em regiões que possuem a forma longa do receptor *ob* (conhecido como Ob-Rb). O aumento da expressão de SOCS-3 inibe a leptina (regulando os níveis hormonais e a expressão de receptores), sendo um dos mecanismos propostos como responsáveis pela resistência às ações do hormônio que acontece, por exemplo, na obesidade. Por sua vez, a forma curta do receptor *ob* parece estar envolvida no transporte da leptina através da barreira hemato-encefálica (El Haschimi et al., 2000), enquanto a forma solúvel liga-se ao hormônio circulante modulando sua disponibilidade biológica e atividade (Ge et al., 2002).

A insulina é um hormônio pancreático que, como a leptina, correlaciona-se com o balanço energético a longo prazo (Bagdade et al., 1967; Woods et al., 1974). No entanto, ao contrário do hormônio produzido no tecido adiposo, seus níveis plasmáticos flutuam drasticamente conforme as refeições, aumentando de forma rápida após a alimentação (Polonsky et al., 1988). A insulina e seus agentes miméticos agem como um sinal anorexigênico no SNC, diminuindo o consumo alimentar e o peso corporal quando administrados centralmente (Air et al., 2002). Sua passagem pela barreira hemato-encefálica se dá por um processo saturável mediado por receptores (Baura et al., 1993), e como a produção central de insulina é ínfima (Woods et al., 2003; Banks, 2004), níveis circulantes periféricos devem ter ações similares à administração central. Sua sinalização ocorre via receptor na membrana plasmática, composto de uma subunidade extracelular α que se liga ao hormônio e uma subunidade intracelular β com atividade tirosina-cinase intrínseca que, quando ativada, inicia uma cascata de reações de fosforilação intracelulares que regulam interações protéicas e atividade de enzimas. Os substratos mais importantes do receptor de insulina (IRS) são IRS-1 e IRS-2, que quando fosforilados ligam-se e ativam cinases celulares, iniciando rotas de sinalização divergentes envolvidas na mediação da ação celular da insulina (Baskin et al., 1994).

A grelina é um potente fator estimulante do apetite, sendo produzida e liberada primariamente pelas células oxínticas do estômago, mas também pelo duodeno, íleo, ceco e cólon (Date et al., 2000; Sakata et al., 2002), sendo expressa da mesma forma no SNC (Cowley et al., 2003). Estruturalmente, a grelina possui 28 aminoácidos com adição de uma cadeia lateral acila (ácido n-octanóico) ao seu terceiro resíduo serina, que é essencial à sua ligação com o receptor GHS-R 1a e aos seus efeitos no comportamento alimentar (Kojima et al., 1999). Seus níveis plasmáticos flutuam com o ritmo circadiano e são muito influenciados pela alimentação,

tipicamente apresentando seu pico logo antes das refeições, e queda abrupta ao início da ingestão alimentar (Murakami et al., 2002; Ariyasu et al., 2001) causada pelo consumo calórico e por sinais como a glicose mas não pela ingestão hídrica, sugerindo que a distensão gástrica não influencia a secreção de grelina (Tschop et al., 2000).

1.5 O eixo HPA e o comportamento alimentar

O estado emocional afeta o comportamento alimentar, e diferentes alimentos influenciam nas respostas ao estresse, numa complexa via dupla de regulação e tênue equilíbrio (revisada em Gibson et al., 2006). Estudos em humanos demonstram que as experiências emocionais podem levar a um aumento de ingestão de alimento, em especial doce e rico em calorias (Oliver et al., 2000). Períodos de maior sobrecarga de trabalho associam-se a maior consumo de calorias e gorduras, principalmente em pessoas que usualmente fazem dietas (McCann et al., 1990; Wardle et al., 2000; Michaud et al., 1990). A variação individual da intensidade da resposta ao estresse se correlaciona com o grau de influência do estresse no comportamento alimentar (Epel et al., 2001). Em modelos animais, vários pesquisadores demonstram que a exposição crônica a agentes estressores pode alterar o consumo de alimento e o peso corporal. Por exemplo, animais submetidos ao estresse de choque inescapável diminuem a ingestão de alimento e o peso corporal (Dess et al., 1989), enquanto a exposição ao ruído e o estresse social aumentam o consumo alimentar (Krebs et al., 1996; Bhatnagar et al., 2006). O estresse crônico repetido por contenção aumenta a ingestão de alimento doce (Ely et al., 1997) sem alterar o consumo de ração padrão, e a administração de um fármaco ansiolítico reverte esse efeito. A influência do estresse no comportamento alimentar, intensificando ou atenuando o apetite ou ainda aumentando o consumo de macronutrientes ou sabores específicos varia conforme a intensidade e a duração do agente estressor (Marti et al, 1994).

Como vimos nas seções anteriores, a resposta ao estresse inclui a liberação de CRH do hipotálamo. O CRH tem sua ação mediada por receptores CRH-1 e CRH-2, e sua ligação principalmente neste último tem um efeito inibitório sobre o comportamento alimentar (Koob e Heinrichs, 1999). Os glicocorticóides têm um efeito permissivo sobre o consumo alimentar, como bem exemplificado na hiperfagia e obesidade associadas à síndrome de Cushing e anorexia ligada à doença de Adison. Agindo no SNC, possivelmente modulam a ingestão de alimento através da ativação do NPY (Dallman et al., 1993). A remoção dos glicocorticóides por adrenalectomia suprime o consumo alimentar em 10-20% e diminui o ganho de peso (Bhatnagar et al., 2000), assim como inibe a obesidade induzida pelo NPY (Dallman et al., 2004). Esses efeitos da adrenalectomia são revertidos pela administração de glicocorticóides (Freedman et al., 1985). Como existe uma sobreposição importante em

neurônios alvo de glicocorticóides, insulina e leptina, sugere-se que estes hormônios atuem de modo coordenado na regulação do apetite e gasto energético.

É intrigante, porém, que a dieta também possa influenciar a resposta ao estresse. Por exemplo, após uma noite de jejum, uma sobrecarga de carboidratos (mas não de proteínas ou gordura) aumenta a secreção de cortisol induzida por estresse (Gonzalez-Bono et al., 2002). Por outro lado, após 10 dias de consumo de uma dieta rica em carboidratos, há uma diminuição nos níveis basais de cortisol em relação a indivíduos recebendo uma dieta rica em proteínas (Anderson et al., 1987). Em ratos, a ingestão de uma solução de glicose por vários dias inibe a produção de CRH central (Dallman et al., 2003). Dietas forçosamente ricas em gordura aumentam a secreção de glicocorticóides basal e induzida por estresse (Tannenbaum et al., 1997), assim como reduzem resposta vegetativa ao estresse quando comparadas com dietas ricas em carboidratos (Buwalda et al., 2001). Dietas hipercalóricas atenuam a resposta do eixo HPA ao estresse (Strack et al., 1997). Por sua vez, o jejum aumenta a secreção de ACTH e corticosterona, reduzindo a retroalimentação negativa do eixo HPA (Dallman et al., 1999). Tem sido proposto que os glicocorticóides e a insulina possam estimular o consumo de alimentos altamente calóricos ("*comfort foods*"), que por sua vez protegeriam o eixo HPA da disfunção associada ao estresse e conseqüente depressão e ansiedade (Dallman et al., 2003).

1.6 Hipóteses Gerais de Estudo

Levando em consideração o fato que animais manipulados no período neonatal apresentam uma clássica alteração na atividade do eixo HPA, com aumento da retroalimentação negativa dos glicocorticóides e menores respostas ao estresse, e ainda um maior consumo de alimentos palatáveis em relação a animais controle, nossas hipóteses para explicação deste fenômeno eram: (a) alteração nos mecanismos homeostáticos que regulam o comportamento alimentar com provável mecanismo de saciedade diferenciado nestes animais; (b) alteração nos mecanismos hedônicos envolvidos na preferência alimentar e

consumo de alimentos palatáveis; (c) estabelecimento das alterações em idades precoces determinando a preferência pelo consumo de doce desde a infância; (d) envolvimento das vias relacionadas à resposta ao estresse na expressão do comportamento alimentar diferenciado, com possível alteração de outros desfechos relacionados ao estresse crônico em animais manipulados e (e) os efeitos da manipulação sobre o comportamento alimentar sendo possivelmente explicados pelo aumento do cuidado materno nestes animais.

2. OBJETIVOS

Gerais

Estudar os efeitos da manipulação neonatal e do cuidado materno sobre o comportamento alimentar e sua regulação na vida adulta dos filhotes avaliando tanto vias homeostáticas como hedônicas, assim como sobre o consumo alimentar e outros parâmetros em resposta à exposição a um modelo de estresse crônico variável.

Específicos

CAPÍTULO I - Estudo dos efeitos da manipulação neonatal sobre a saciedade e hormônios ligados ao comportamento alimentar

1.1 Estudos comportamentais

Verificar o efeito da manipulação neonatal sobre a ingestão de alimento doce na caixa moradia, assim como numa tarefa de exposição repetida a alimentos ricos em carboidratos simples e complexos para observação da saciedade. Avaliar da mesma forma a saciedade após o consumo de uma solução de sacarose.

1.2 Estudos metabólicos

Verificar o efeito da manipulação neonatal sobre os níveis plasmáticos basais de leptina, insulina, grelina, glicose e corticosterona, assim como sobre a deposição de gordura abdominal.

CAPÍTULO II - Estudo dos efeitos da manipulação neonatal sobre o metabolismo dopaminérgico no núcleo acumbens, e sobre comportamentos relacionados à atividade dopaminérgica nesta estrutura

2.1 Estudos comportamentais

Verificar o efeito da manipulação neonatal sobre o condicionamento de preferência ao lugar utilizando doce como recompensa, sobre o esforço realizado para obtenção da recompensa numa tarefa de corredor e sobre as respostas hedônicas ao sabor do doce.

2.2 Estudo farmacológico

Verificar o efeito da manipulação neonatal sobre o consumo de doce após a injeção de um agente mimético da dopamina, o metilfenidato.

2.3 Estudo neuroquímico

Verificar o metabolismo dopaminérgico no núcleo acumbens através da medida do neurotransmissor e seus metabólitos utilizando cromatografia.

CAPÍTULO III - Estudo dos efeitos da manipulação neonatal sobre o consumo de doce em diferentes idades

Verificar o efeito da manipulação neonatal sobre o consumo de doce em diferentes idades. Avaliar o efeito da exposição precoce ao doce ou a outros estímulos como a exposição a brinquedos no consumo de doce na vida adulta.

CAPÍTULO IV - Estudo dos efeitos da manipulação neonatal sobre a atividade de ectonucleotidases no núcleo acumbens em diferentes idades

Verificar o efeito da manipulação neonatal sobre o sistema adenosinérgico, um potencial regulador da dopamina no núcleo acumbens em ratos de diferentes idades e sua correlação com o consumo de alimento doce.

CAPÍTULO V - Estudo dos efeitos da exposição à manipulação neonatal na infância e a um modelo de depressão (estresse crônico variável) na vida adulta

Verificar a interação entre a manipulação neonatal e o estresse crônico variável na vida adulta em parâmetros como o peso corporal, consumo de alimento doce, nado forçado, medidas plasmáticas basais de corticosterona, insulina, glicose e cálculo de um índice de resistência à insulina (QUICKI), assim como avaliação da atividade da Na^+,K^+ATPase em diferentes estruturas cerebrais como hipocampo, amígdala e córtex parietal.

CAPÍTULO VI - Estudo dos efeitos das variações naturais de cuidado materno sobre o comportamento alimentar na vida adulta

Verificar se variações naturais do cuidado materno influenciam o consumo de alimentos palatáveis na vida adulta, assim como os níveis plasmáticos basais de grelina, leptina, insulina e corticosterona, o peso corporal e a deposição de gordura adominal.

3. MÉTODOS E RESULTADOS

Esta tese deu origem a cinco trabalhos que são apresentados em forma de capítulos além de um capítulo extra com resultados adicionais não organizados em forma de artigo científico. Seguem-se breves resumos dos capítulos antecedendo cada trabalho científico:

3.1 CAPÍTULO I

Estudo dos efeitos da manipulação neonatal sobre a saciedade e hormônios ligados ao comportamento alimentar.

Nossos estudos anteriores demonstram que animais manipulados no período neonatal ingerem mais alimento palatável em relação a animais controle em uma tarefa de comportamento alimentar. Neste estudo, resolvemos avaliar o consumo de doce durante uma exposição repetida e prolongada para estudo da saciedade. Vimos que animais manipulados no período neonatal consomem mais alimento doce, tanto se a exposição ocorre na caixa moradia quanto no corredor utilizado para realização da tarefa comportamental. Estes ratos também ingerem mais doce quando repetidamente expostos a este alimento, apresentando uma curva de saciedade que parece não ser tão evidente em animais controle. Além disso, ratos manipulados apresentam resposta de saciedade se recebem sacarose antes do teste, enquanto animais controle mantém o mesmo consumo independente de receber ou não sacarose. No entanto, não ha diferenças entre os grupos no consumo de um alimento rico em carboidratos complexos, assim como no peso corporal, gordural abdominal e níveis plasmáticos de insulina, glicose, leptina, e corticosterona após 6 horas de jejum. Nessas condições, ratos manipulados tem menor nível plasmático de grelina.

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SATIETY ASSESSMENT IN NEONATALLY HANDLED RATS

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Satiety assessment in neonatally handled rats – We have previously demonstrated that neonatal handling increases sweet food ingestion. In the present study, we examined whether food intake, using different kinds of food, is altered in neonatally handled animals, with or without inducing satiety using a sucrose solution. Abdominal fat, glycemia and hormones linked to appetite including leptin, ghrelin and insulin were also measured. We tested palatable food consumption in the homecage to verify whether environmental cues could influence ingestion. Nests of Wistar rats were either (1) non-handled or (2) handled (10 min/day). Handling was performed on days 1-10 after birth. When adults, rats were habituated to sweet food (Froot Loops – Kellogs ®) and to palatable fiber pellets (Fiber One ® – Nestlé). Sweet food consumption was increased in the neonatally handled group, when tested in the homecage, and also in the satiety experiment. These rats displayed a satiety curve when compared to the control group, which ate less but constantly. Handled rats exposed to a sucrose solution decreased sweet food ingestion, which did not occur in the control group. When exposed to a food with complex carbohydrates, these differences disappeared. There were no differences in body weight, abdominal fat or in glycemia, as well as no differences in plasma levels of insulin or leptin. However, ghrelin was decreased in neonatally handled rats. Neonatally handled rats demonstrated an increased consumption of sweet food, satiety responses to sucrose, as well as decreased levels of plasma ghrelin. It is possible that signaling mechanisms related to satiety, both peripherally and/or centrally may contribute to these behavioral findings.

Key words: feeding, stress, neonatal handling, satiety, insulin, leptin, ghrelin, abdominal fat.

INTRODUCTION

The control of feeding behavior is complex and involves interactions between several signaling systems. Homeostatic inputs such as energy status and demands of the organism are received and processed in the arcuate and paraventricular nucleus of the hypothalamus, as well as in the nucleus of the solitary tract. Neuronal circuits may inhibit food intake, via the expression of the neuropeptides pro-opiomelanocortin [36] and cocaine- and amphetamine- regulated transcript [5], or stimulate food intake, via the expression of neuropeptide Y and agouti-related peptide [43, 45].

Pleasurable sensations are also linked to the ingestion of food, especially if it is palatable. It is known that oral sucrose stimulation increases dopamine in the nucleus accumbens [12], a region of the limbic system associated with auto-stimulation and reward mechanisms. Increased dopamine in this nucleus is related to a greater ingestion of sweet food [11], in a positive feedback fashion that perpetuates until local or peripheral signals interrupt the cycle [42].

Hedonic and homeostatic components are in close contact with regard to feeding behavior. The sensation of reward is influenced by energy status, since the subjective palatability of food is increased in the fasted state, in comparison to the fed state [47].

Therefore, signals of energy status such as leptin and insulin are able to influence reward pathways [8]. Insulin is a major metabolic hormone produced by the pancreas and acts as an adiposity signal [38]. In the central nervous system, it acts as an anorexigenic signal, decreasing food intake and body weight [15]. Furthermore, insulin may modulate the hedonic aspects of feeding behavior [7]. Leptin is secreted from adipose tissue, and influences energy homeostasis, immune and neuroendocrine function. Food restriction leads to a suppression of leptin levels, which can be reversed by refeeding [9, 13]. Production of leptin correlates positively with adipose tissue mass [13], therefore circulating leptin levels are involved both with signaling of energy stores and food intake. With respect to sweet food ingestion, leptin is believed to suppress behavioral responses to sweet substances through its action on specific receptors in taste cells [39].

In addition, another peptide hormone suggested to be involved in food regulation is ghrelin. This peptide is an orexigenic factor released primarily from the oxyntic cells of the stomach [37]. Circulating ghrelin levels are high during a period of fasting, and fall after eating [44]. It has been recently demonstrated that ghrelin increases food ingestion when injected in the ventral tegmental area or in the nucleus accumbens, and ghrelin has been proposed to have a role in the hedonic responses to food [28].

All these mechanisms may be altered on an individual basis by different factors such as available nutrients, previous meals, experience and stress [6]. An example of such a factor is neonatal handling. This experimental paradigm, although not very stressful for the pups, increases maternal care [35] and leads to enduring behavioral alterations that persist into adulthood. These effects include greater exploration when exposed to a different environment from that of the homecage [17], diminished neuroendocrine responses to stress

[29, 30], and decreased innate and learned fear responses [18]. These animals also show an increased ingestion of palatable food which is not accompanied by an increase in lab chow ingestion [41], suggesting that they prefer to ingest according to the taste of the food, or to what this taste represents. Although neonatal handling procedures vary among different laboratories, brief episodes of separation from the mother (from 1 to 30 minutes) during infancy (which may vary from a few days to the first three weeks of life) are largely known to be associated with the above mentioned effects regarding anxiety and responses to stress.

In this study, our goals were to verify whether the time to reach satiety was affected by neonatal handling, if the type of carbohydrate used (simple or complex) would interfere with the time to reach satiety and if we could increase satiety using a sucrose solution before the test. We also wanted to confirm that neonatal handling increases sweet food ingestion; as such we measured the consumption in the homecage to eliminate environmental influences. Finally, we measured hormones that influence feeding behavior, including plasma levels of insulin, leptin and ghrelin following 6 hours of fasting. We also measured glycemia and abdominal fat weight. Our hypotheses were that neonatally handled rats might present an altered satiety for sweet food and that it could be accompanied by an alteration in the hormones linked to appetite, since these animals eat more palatable food when exposed to them [41].

MATERIAL AND METHODS

Subjects:

Pregnant Wistar rats bred at our own animal facility were randomly selected. They were housed alone in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust and were

maintained in a controlled environment: lights on between 07:00h and 19:00h, temperature of 22 ± 2°C, cage cleaning once a week, food and water provided ad libitum. The day of birth was considered as day 0. All litters were culled within 24 h of birth to eight pups and were maintained undisturbed except for handling procedures, which were carried out between 10:00h and 15:00h. Several litters were submitted to the handling procedures in the same day, so that it is included in this period the time to set up the incubator, to bring the cages from the facility and briefly habituate the dams to the new room, to perform careful removal of the pups from the nest, the time of handling per se, the returning of the pups to the dam and, again after a brief period, to return the cage to the facility room. The researcher also changed gloves between the manipulation of each litter to avoid any kind of odor to be spread from nest to nest.

Litters were weaned on postnatal day 21. Two male pups from each litter were assigned to each experiment. After weaning, rats were housed four to five per cage. Sixty-nine male rats were used in the different experiments, derived from 18 different litters. Rats had free access to food (standard lab rat chow) and water, except during the period when the behavioral tasks were applied. Tasks were performed between 11:00h and 15:00h.

Neonatal Handling model [41]*:*

Nonhandled group: Pups were left undisturbed with the dam until weaning. It was stated on the cage that these animals should not be touched, not even for cage cleaning. Dirty sawdust was carefully removed from one side of the cage, without disturbing the mother and the nest, and replaced by clean sawdust at that side by the principal researcher.

Handled group: The dam was gently pulled to one side of the cage and the pups were removed from their home cage and were placed into a clean cage lined with clean paper towel. This cage was placed into an incubator at 34° C next to the dam's cage. After 10 minutes, pups were returned to their dams. This procedure was performed from day 1 to 10 following birth, and then pups were left undisturbed until the 21st day of life.

Habituation to the new foods

Starting on day 60 of life, rats were habituated to a novel environment containing new foods [41]. During this period, they were placed in a lightened rectangular box (40 x 15 x 20 cm) with floor and side walls made of wood and a glass ceiling. Ten Froot loops (Kellogg's ® - pellets of wheat, cornstarch and sucrose) or, on another occasion, a previously weighed amount of fiber (Fiber One® – Nestlé – wheat, cornstarch, and aspartame) were placed in one extremity of the box. The animals were habituated to this environment during 5 days, 3 min each day, under food restriction (receiving about 80% of habitual ingestion). This procedure was performed because all the experiments described in this study (except for the sweet food consumption in the homecage) were performed inside this specific box [41]. After this habituation period, the animals received rat chow ad libitum. All the following tests were performed with the animals in the fed state.

Sweet food ingestion in the homecages

Animals were transferred to another cage similar to their homecage. All rat chow was removed from their cages. Afterwards, animals were returned to their original homecage one by one, and twenty Froot Loops pellets were offered, for ten minutes. The amount ingested was measured.

Repeated food intake to address satiety:

On the test day, each animal was submitted to 8 exposures to the box for evaluation of feeding behavior described above (see *Habituation to the new foods*), for 3 minutes each, every 5 minutes. After 3 minutes in the box, where it could eat the palatable food, the rat was returned to his home cage for 2 minutes, then again to the box for the feeding evaluation, and the cycle was repeated 8 times. This procedure was performed with the animals in the fed state. A protocol was established so that when the animals ate part of the Froot loops (eg.: 1/3 or 1/4), this fraction was included.

On another occasion, rats were submitted to the same procedure using a different kind of palatable food (Fiber One®). The amount ingested was measured by weighing the pellets before and after each exposure. Table 1 displays the nutritional compounds/100g of each food provided in these tests.

Table 1 – please insert about here.

In another experiment, animals were water restricted for 24 hours. Thirty minutes before the test, handled and nonhandled rats were assigned to receive either a solution of 5% sucrose for twenty minutes and then water for ten minutes, or just water for the whole period of thirty minutes. The four groups of rats (non-handled +water, non-handled +sucrose, handled +water and handled +sucrose) were then subjected to the same protocol of repeated food intake to verify satiety for Froot loops as previously described.

Blood collection and abdominal fat dissection:

Animals were weighed and following six hours of fasting they were sacrificed by decapitation. Trunk blood was collected into heparinized tubes for insulin, leptin, glucose and corticosterone determination. Blood to be used for ghrelin assessment was collected into tubes containing aprotinin and EDTA. The tubes were centrifuged at 4^{o}C and plasma was separated and frozen until the day of analysis. Hormonal measurements were performed with commercial rat ELISA kits: Cayman Chemical Co., Ann Arbor, MI, USA, for insulin, Linco Research Inc., St. Charles, MO, USA for leptin, Linco Research Inc., St. Charles, MO, USA, for ghrelin, Cayman Chemical, Ann Arbor, MI, USA, for corticosterone. Plasma glucose was measured by the glucose oxidase method using a commercial kit, BioSystems, Barcelona, Spain. The two major portions of abdominal fat (epididymal and retroperitoneal adipose tissue depots) were dissected and weighed separately.

Statistical analysis:

Data were expressed as mean $\pm$ standard error of the mean, and were analyzed by Student t Test or by Repeated Measures ANOVA followed by the Student-Newman-Keuls' test [4]. Significance level was accepted as different when the *P* value was equal or less than .05. Sample size varies in each experiment and is shown individually in the Results section.

RESULTS

Sweet food ingestion in the homecage

Figure 1 displays this result, demonstrating that neonatally handled rats ate more sweet food in the homecage in comparison to the non-handled group [Student´s t test, t(14) = -2.196, *P*<0.045, n = 7-9/group].

Figure 1 – please insert about here.

Repeated food intake to address satiety:

Neonatally handled rats ate more sweet food during the eight exposure periods to Froot loops (a food with simple carbohydrates) (Repeated measures ANOVA [F (1, 28) = 2.230, *P*=0.05], n = 13-17/group). The total amount of sweet food ingested was also higher in the handled group. Non-handled rats eat less but in a more constant fashion, while handled rats eat more but reach satiety by the 7th exposure period, with ingestion decreasing dramatically from this moment on. Please see Figure 2 and Table 2.

Figure 2 – please insert about here.

When using a palatable food with complex carbohydrates and fiber (Fiber One), there were no differences between groups in the eight measurements (Repeated measures ANOVA [F (1, 17) = 0.683, *P*=0.42], n = 9-10/group), nor in the total amount of food eaten. Figure 3 and Table 2 display these results.

Figure 3 – please insert about here.

Table 2 – please insert about here.

After receiving a sucrose solution, groups behaved differently with regard to sweet food consumption. A repeated measures ANOVA shows a significant effect of time [F(1, 36) = 10.259, P < 0.01, n= 7-12/group] and a significant interaction between handling and time [F(1, 36) = 5.310, P < 0.05]. While both subgroups of non-handled animals ate a similar and constant amount of sweet food, handled rats

receiving sucrose solution before the test showeded satiety to sweet food earlier than handled rats receiving water. It is important to note that the amount of sucrose solution ingested before the test was not different between the groups (14.04±1.77 ml for the nonhandled group and 14.46± 0.46 ml for the handled group, Student T test *P*=0.923).

Figure 4 – please insert about here.

Plasma measurements and abdominal fat:

There were no differences between groups in body weight [Student´s t test, t(67) = 0.884, *P*=0.38, n=34-35/group], in the total abdominal fat weight [Student´s t test, t(23) = -0.208, *P*=0.837, n=12-13/group], in plasma glucose [Student´s t test, t(37) = -1.657, *P*=0.106, n=19-20/group], plasma insulin [Student´s t test, t(11) = 0.423, *P*=0.545, n=6-7/group], plasma leptin [Student´s t test, t(13) = -0.686, *P*=0.505, n=7-8/group], and plasma corticosterone [Student´s t test, t(12) = 1.00, *P*=0.339, n=5-9/group]. Ratios such as glucose to insulin [Student´s t test, t(10) = 0.514, *P*=0.618] and leptin to total abdominal fat [Student´s t test, t(11) = -0.633, *P*=0.540] were also not different between groups. However, plasma ghrelin levels were diminished in the neonatally handled group [Student´s t test, t(13) = 4.435, *P*=0.001, n=7-8/group]. Please refer to table 3.

Table 3 – please insert about here.

DISCUSSION

In this study, we demonstrate that neonatal handling induces increased sweet food ingestion and a satiety response to sucrose in comparison to nonhandled rats. We also show that neonatal handling decreases plasma levels of ghrelin after six hours of fasting in adulthood, without altering body weight, abdominal fat, glucose, insulin and leptin levels.

Increased sweet food consumption in neonatally handled rats has been previously observed [41]; however, in these studies, an apparatus was used that was different from the homecage where the animals usually eat. Since it is known that neonatal handling leads to a differential response to stress [21], and

possibly to a differential response to environmental cues, the finding that sweet food consumption is increased in the animals' homecage excludes the possibility that neonatally handled rats eat more because of the exposure to a different environment (the behavioral apparatus) during the test. Additionally, in the satiety experiment, neonatally handled rats reached satiety by the 7th exposure to sweet pellets. This probably occurs because these animals eat more, triggering a cluster of events involved in satiety processes [14, 26, 32]. It should be observed, however, that the total amount of food consumed by handled animals was much higher than the amount consumed by control animals. Additionally, it should also be observed that these experiments were performed with the animals fed "ad libitum"; therefore, hunger was not playing a role in the increased consumption.

The effects of carbohydrates on satiety are probably not mediated only by mechanisms sensitive to their effect on blood glucose concentrations [2], but also to the release of satiety peptides [46]. It is not usually easy to observe satiety after sucrose solution ingestion in rats [23, 24], as observed in the behavior of the nonhandled group. It is intriguing that a prior exposure to a sucrose solution was able to inhibit the sweet food consumption only in handled animals. Therefore, neonatally handled rats seem to possess quite effective, and perhaps more sensitive satiety mechanisms related to an overload of sugar, despite showing preference to this type of food when they are exposed to it during a short period. This hypothesis, however, is in disagreement with a previous finding of a blunted feeding suppressant response to cholecystokinin in handled animals [19].

As stated in the introduction section, sucrose increases dopamine in the nucleus accumbens [12], being related to a greater ingestion of sweet food [11], in a positive feedback fashion. In this study, when using a food made with complex carbohydrate (that would take longer to increase glycemia), there were no differences in the amount consumed between groups. It is possible that the accumbens dopamine response to sweet food is different between these groups. It is interesting that some studies concerning handling-induced changes related to ethanol consumption and cocaine self-administration have shown that these animals are more resistant to these addictive behaviors [33, 22].

Additionally, some reports demonstrated that aspartame does not taste sweet or even good to rats, and lines selected for high and low saccharin consumption do not necessarily match with those that ingest more or less aspartame solution, respectively [3]. On the other hand, in this study, we used a snack, and texture is an important aspect to consider [27]. In addition, animals received food *ad libitum* on the day before the experiment, and since the majority of them ate at the test session, we believe this food might taste good. In summary, the results from this experiment reinforce the idea that neonatally handled animals have food preferences that are different from controls in such a way that only certain types of food, when offered, are more ingested by these animals.

Since neonatally handled rats have been shown to exhibit a specific pattern of HPA axis responses [21], altered glucocorticoid levels could differentially influence the fat depots in the two groups [31]. In the present study, similarly to previous findings, basal levels of these hormones were not different between the groups [20], We also did not find any difference in the abdominal fat depots. Since there were no differences in the body weight, neither in abdominal fat or in glycemia after six hours of fasting, we may presume that neonatal handling is not associated with a major metabolic alteration

Body weight in neonatally handled animals is an issue still to be addressed. In the present study, we did not find any difference in body weight between the groups. However, other authors have already reported an increase [30] or no difference in body weight [34] in neonatally handled rats. Body weight is dependent on the conditions of each facility, and could be affected by simple variables such as the rat chow used or the size of the cage. Neonatally handled rats could display differential responses to these influences on body weight, as they do respond differently regarding body weight when exposed to chronic stress [30].

Circulating insulin and leptin levels are both involved with signaling of energy stores and food intake [7, 13, 38, 39]. In this study, basal measurements of these hormones after six hours of fasting did not show differences between groups. We cannot exclude, however, alterations in other responses involving these hormones, such as their receptors

levels and intracellular signaling cascades. Nevertheless, the lack of effect of neonatal handling on body weight and abdominal fat is in agreement with unaltered leptin plasma levels.

Neonatally handled rats exhibited, in adulthood, decreased plasma levels of ghrelin. This hormone has been shown to be an orexigenic factor [37], and it has been proposed to have a role in chronic energy balance [25] and in the hedonic responses to food when injected into the ventral tegmental area [28]. Therefore, altered levels of ghrelin at the moment of food ingestion could affect reward-based food intake in neonatally handled rats.

Despite its first attributed role concerning appetite modulation, ghrelin has been recently shown to exhibit other functions, specially in mediating neuroendocrine and behavioral responses to stressors [1]. Asakawa and coworkers demonstrated that ghrelin administration increases anxiety in the plus maze, and that this effect was possibly mediated by corticotrophin-releasing hormone (CRH). Furthermore, peripherally administered ghrelin increases CRH mRNA expression in the hypothalamus and increases serum corticosterone levels [1]. Therefore, the reduced ghrelin levels observed in neonatally handled animals may be involved in the reduced neuroendocrine response to stress reported in these animals [29, 30].

In summary, the disruption of the mother-infant relationship at an early age may lead to persistent alterations throughout adulthood, including modified feeding behavior and some on metabolic aspects, such as decreased ghrelin levels. It is important to stress that the alterations reported here were observed under basal conditions, i.e., were not produced in response to stimuli, such as stressors, as were many of the already known effects of neonatal handling. Therefore, handling during the neonatal period may induce

alterations not related to stimulation of the hypothalamus-pituitary-adrenal axis. We suggest that early life experiences may determine individual differences in food choices that could also be involved in the pathophysiology of eating disorders and their correlates.

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LEGENDS TO FIGURES

Figure 1: Sweet food consumption in the homecage. Data are expressed as mean ± S.E.M. for grams of pellets consumed. Neonatally handled rats showed a greater ingestion of sweet food compared to nonhandled rats (Student's t Test, $P = 0.045$*).

Figure 2: Repeated food intake using a palatable food with simple carbohydrate. Data are expressed as mean ± S.E.M. for grams of pellets consumed. Neonatally handled rats ate more sweet food than nonhandled rats (Repeated Measures ANOVA, $P = 0.05$* for group).

Figure 3: Repeated food intake using a complex carbohydrate palatable food. Data are expressed as mean ± S.E.M. for grams consumed. There is no difference between groups (Repeated Measures ANOVA, $P = 0.42$ for group).

Figure 4: Repeated sweet food intake after ingestion of 5% sucrose. Data are expressed as mean ± S.E.M. for grams of pellets consumed. There is an effect of time and a significant interaction between handling and time (Repeated Measures ANOVA, $P < 0.01$ and $P < 0.05$, respectively)

TABLES

Table 1: Nutritional composition/100g of the palatable food used in the studies performed.

Food	Energy (kcal)	Total protein (g)	Total carbohydrate (g)	Total fat (g)	Crude fiber (g)
Froot loops Kellogg's ®	390	6	85 (54% simple, 46% complex)	3	2
Fiber One Nestlé®	225	7.5	42.5 (whole complex, sweetened with aspartame)	2.5	40

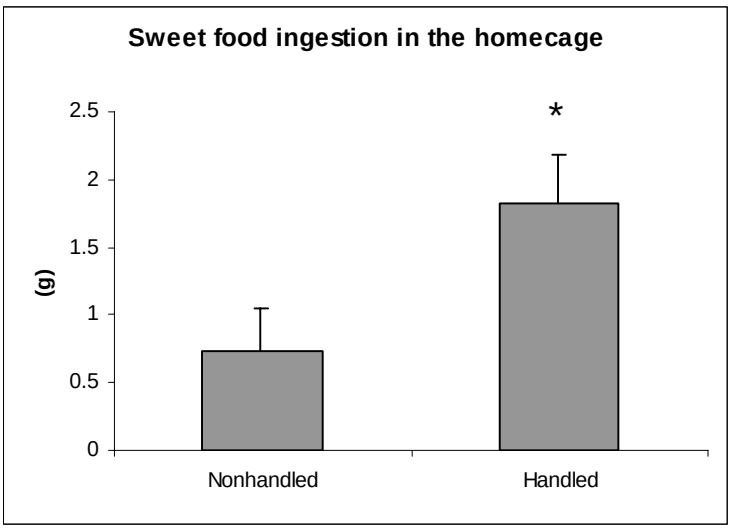
Table 2: Mean consumption of macronutrients during repeated food consumption. Data are expressed as mean ± S.E.M. for each measurement. Neonatally handled rats showed increased ingestion of simple carbohydrate sweet food in comparison to the nonhandled group (Repeated measures ANOVA, $P < 0.05$*).

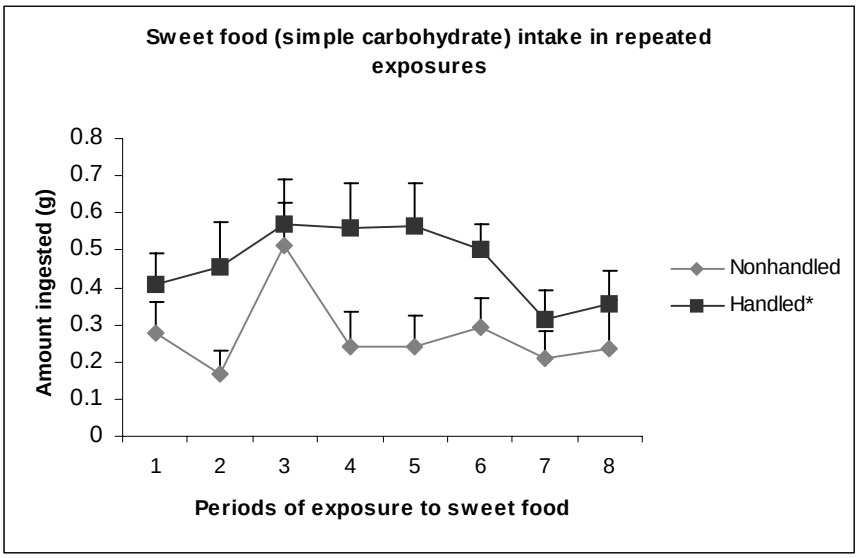
Food	Group	Energy (kcal)	Protein (g)	Carbohydrate (g)	Fat (g)
Froot loops Kellogg's ®	Nonhandled	11.15±1.35	0.17±0.02	2.43±0.29	0.08±0.01
	Handled	14.71±1.80*	0.23±0.03*	3.20±0.39*	0.11±0.14*
Fiber One Nestlé®	Nonhandled	5.86±0.8	0.19±0.27	1.11±0.15	0.06±0.01
	Handled	4.74±1.07	0.16±0.36	0.89±0.20	0.06±0.01

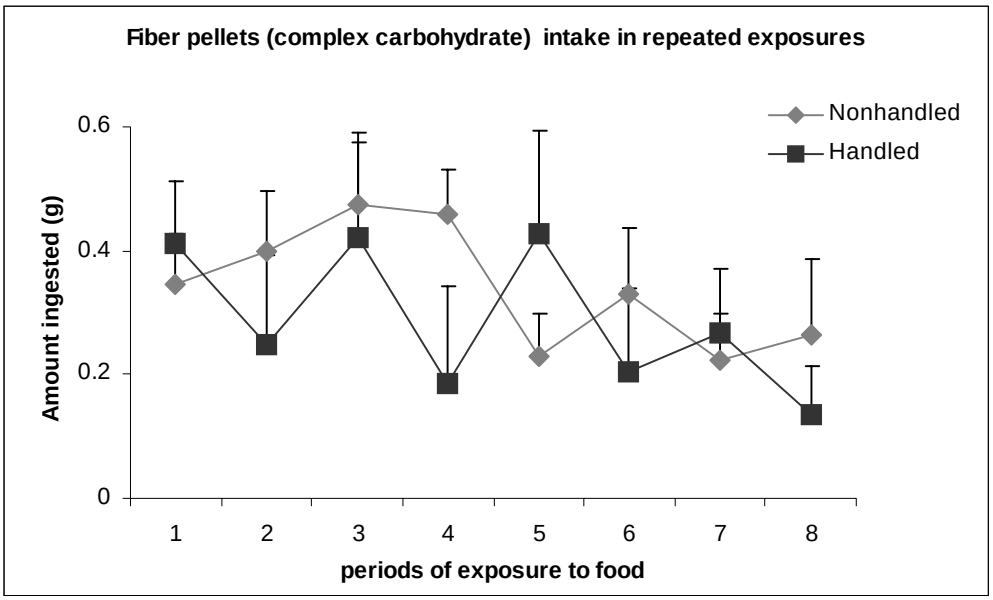
Table 3: Plasma glucose, insulin, leptin, ghrelin, and corticosterone measurements and total abdominal fat in nonhandled and neonatally handled rats. Data are expressed as mean ± S.E.M. for each measurement.

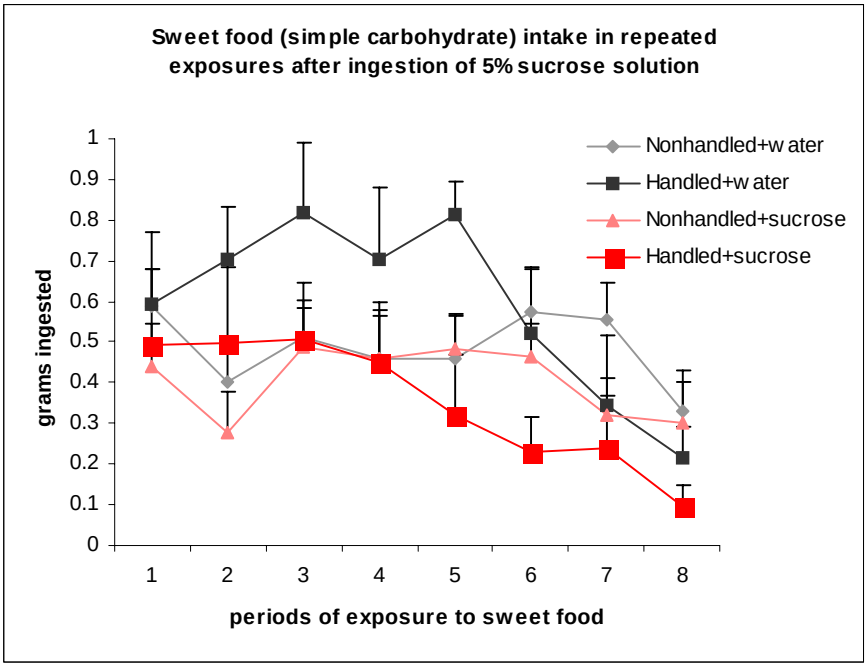
Neonatally handled rats showed decreased levels of plasma ghrelin in comparison to the nonhandled group (Student's t test, $P = 0.001$*).

Measurement	Nonhandled group	Neonatally handled group
Body weight (g)	319.1 ± 5.8	309.5 ± 9.2
Total abdominal fat (g)	6.6 ± 0.7	6.5 ± 0.4
Plasma glucose (mg/dl)	114.6 ± 2.6	112.6 ± 4.0
Plasma insulin (ng/ml)	3.2 ± 0.7	2.6 ± 0.6
Plasma leptin (ng/ml)	1.1 ± 0.7	1.3 ± 0.3
Plasma ghrelin (fmol/ml)	35.4 ± 2.9	17.0 ± 2.9*
Plasma corticosterone (ng/ml)	138.7 ± 23.3	101.4 ± 27.1


Sweet food ingestion in the homecage
2.5
2
1.5
1
0.5
0
(g)
*
Nonhandled
Handled


Sweet food (simple carbohydrate) intake in repeated exposures
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Amount ingested (g)
1
2
3
4
5
6
7
8
Periods of exposure to sweet food
Nonhandled
Handled*


Fiber pellets (complex carbohydrate) intake in repeated exposures
Nonhandled
Handled
Amount ingested (g)
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
periods of exposure to food


Sweet food (simple carbohydrate) intake in repeated exposures after ingestion of 5% sucrose solution
Nonhandled+water
Handled+water
Nonhandled+sucrose
Handled+sucrose
grams ingested
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
periods of exposure to sweet food

3.2 CAPÍTULO II:

Estudo dos efeitos da manipulação neonatal sobre o metabolismo dopaminérgico no núcleo acumbens e sobre comportamentos relacionados à atividade dopaminérgica nesta estrutura.

Neste estudo, demonstramos que animais manipulados no período neonatal apresentam maior incentivo para busca da recompensa do alimento doce numa tarefa de corredor, porém demonstram menor condicionamento de preferência de lugar utilizando o doce como recompensa. Além disso, a maior ingestão de alimento doce em animais manipulados no período neonatal repetidamente reproduzida em nossos estudos é acompanhada de uma menor reação hedônica ao consumir uma solução doce. Quando injetados com metilfenidato e expostos ao doce no estado de jejum, estes animais não respondem aumentando o consumo de doce como os ratos controle, demonstrando também menor metabolismo dopaminérgico no núcleo acumbens.

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EARLY LIFE EXPERIENCE ALTERS BEHAVIORAL RESPONSES TO SWEET FOOD AND ACCUMBAL DOPAMINE METABOLISM

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Running title: Dopamine, behavior and neonatal handling.

SILVEIRA PP, PORTELLA AK, ASSIS SACN, NIETO FB, DIEHL LA, CREMA LM, PERES W, COSTA G, SCORZA C, QUILLFELDT JA, LUCION AB, DALMAZ C. **Early life experience alters behavioral responses to sweet food and accumbal dopamine metabolism. -** Neonatal handling persistently alters behavioral parameters and responses to stress. Such animals eat more sweet food in adult life, without alterations in lab chow ingestion. Here, we show that neonatally-handled rats display greater incentive salience to a sweet reward in a runway test; however they are less prone to conditioned place preference and show less positive hedonic reactions to sweet food. When injected with methylphenidate (a dopamine mimetic agent), non-handled rats increase their sweet food ingestion in the fasted state, while neonatally-handled rats do not respond. A lower dopamine metabolism in the nucleus accumbens was observed in handled animals. We suggest that early handling leads to a particular response to positive reinforcers such as palatable food, in a very peculiar fashion of higher ingestion but lower hedonic impact, as well as higher incentive salience, but diminished dopaminergic metabolism in the nucleus accumbens.

Key words: feeding behavior, sweet food, neonatal handling, dopamine, conditioned place preference, methylphenidate, hedonic impact, taste reactivity, nucleus accumbens.

Interventions in early life are associated with persistent alterations in behavior, neurochemistry and susceptibility for diseases in adulthood. Animal models of perinatal manipulations have become useful tools for the understanding of psychopathologies and stress responses. Neonatal handling is one of these experimental paradigms associated with several persistent alterations. When exposed to repeated, brief separations from the mother during the early postnatal period, rats present less fear in an environment different from the

homecage as adults (1), as well as decreased sexual activity (2) and reduced CRF mRNA, ACTH and corticosterone in response to stress (22, 23).

These animals also ingest more sweet or savory pellets than controls when exposed to them in both corridors (3) and in homecages (21), without differences in the consumption of regular lab chow or fiber pellets. However, satiety to sweet food seems to be adequate in neonatally-handled rats (21). Taken together, these data suggest that either the motivation for approaching or the hedonic impact of the reward represented by the palatable food might be affected by the neonatal environment.

Sweet food is a natural reward and has potent motivating proprieties, being used in several behavioral tasks as a reinforcer. Sucrose licking is known to increase accumbens dopamine (DA), and a quantitative relationship has been demonstrated between the concentration-dependent effect of orosensory stimulation by sucrose during eating and the overflow of dopamine in the nucleus accumbens (7). Additionally, repeated access to sucrose increases dopamine turnover in the accumbens (8). On the other hand, increased dopamine in this nucleus is related to a greater ingestion of sweet food (6), in a positive feedback fashion that perpetuates until local or peripheral signals interrupt the cycle (5).

Although the source of continuous debate, accumbal dopamine is believed to have a gating function, regulating the information flow from the limbic structures such as amygdala and hippocampus to the motor nuclei (24). Therefore, when concerning food ingestion, accumbal dopamine seems to interfere with the approach or to modulate the perceived incentive salience of the reward (25). Interestingly, besides their peculiar feeding behavior regarding sweet food, neonatally-handled rats seem to have also a less responsive DA neurotransmission in the nucleus accumbens. For instance, they do not display sensitivity to cocaine-induced locomotor activity and have a blunted rise in nucleus accumbens DA levels after a mild stressor (4). In addition, a reduced density of dopamine D3 receptors has been described in this brain region in neonatally-handled rats (4).

Aiming to better understand why neonatally-handled rats are more prone to ingest palatable food in different situations, we performed a series of behavioral tests to evaluate the conditioning proprieties, the hedonic impact and the incentive value of sweet food in these animals. We also examined the sweet food ingestion after an acute injection of a DA mimetic agent (methylphenidate) and the DA metabolism in the nucleus accumbens using chromatography.

METHODS

Rat treatments: All animal treatments were approved by the Institutional Ethical Committee (Ethical Committee, UFRGS, # 200270) and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS).

Early life experience: Pregnant Wistar rats bred at our own animal facility were randomly selected. They were housed alone in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust and were maintained in a controlled environment until offspring: lights on between 07:00h and 19:00h, temperature of 22 $\pm$ 2°C, cage cleaning once a week, food and water provided. All litters were culled within 24 h to eight pups and were maintained intact unless for handling procedures, which were carried out between 10:00h and 15:00h. Included in this period were the time to set up the incubator, to bring the cages from the facility and briefly habituate the dams to the new room, to perform careful removal of the pups from the nest, the time of handling per se, the return of the pups to the dam and, again after a brief period, to return the cage to the facility room. The researcher also changed gloves for the manipulation of each litter to avoid the spread of any kind of odor from nest to nest.

In the non-handled group, pups were left undisturbed with the dam until weaning. It was stated on the cage that these animals should not be touched, not even for cage cleaning. Dirty sawdust was carefully removed from one side of the cage, without disturbing the mother and the nest, and replaced by clean sawdust at that side by the principal researcher. In the handled group, pups were removed from their home cage and

placed into a clean cage lined with clean paper towel, inside an incubator at 34° C for 10 minutes, being returned to their dams (which stayed in the home cage, next to the incubator) afterwards. This procedure was carried out for the first ten days of life, after which pups were left undisturbed until weaning.

Weaning was on postnatal day 21. One or two male pups were used per litter per experiment. Rats were housed about four to five per cage in home cages similar to those described above. Ninety-one experimental male rats were used in the different experiments, derived from 32 different litters. Rats had free access to food (standard lab rat chow) and water, except during the period when the behavioral tasks were applied. Tasks were performed between 13:00h and 16:00h.

Habituation to sweet food: Animals were placed in a lightened rectangular box (40 x 15 x 20 cm) with floor and side walls made of wood and a glass ceiling. Ten Froot loops (Kellogg's ® - pellets of wheat and cornstarch and sucrose) were placed in one extremity of the box. Each animal was submitted to 5 habituation trials of 3 minutes each, on different days. This procedure was performed under food restriction (80% of habitual ingestion of standard lab chow), and after three minutes in the behavioral apparatus, the number of ingested pellets was measured. A protocol was established so that when the animals ate part of the Froot loops (ex.: 1/3 or 1/4), this fraction was considered. For every behavioral experiment described below, a different set of animals was used but all rats were habituated to the sweet food for 5 days in the previous 10-15 days before the experiment.

Conditioned Place Preference: *Ten days after being habituated to sweet food, rats were trained in the CPP paradigm. For the entirety of this procedure, rats remained under food restriction. The CPP apparatus consisted of two compartments of 35 x 10 cm, with a removable 10-cm divider between them. The two compartments were distinguished by white vs. black walls, by flooring texture and by a lightened lamp on the white side. On the first day, rats were placed in the apparatus for 15 min with free access to both compartments to evaluate the natural preference for each side. The time spent in the compartments was scored. No food was available on the first day of exposure. All rats clearly preferred the dark side on day 1, staying there for the majority of the 15 minutes.*

From the second day onward, twenty pellets of Froot Loops were used as sucrose 'reward' pellets during CPP training days. On alternate days of the 6-day training period, rats were placed on alternating sides of the two-compartment corridor for a 30-min training session. On the non-preferred side, rats received the sucrose pellets and on the other side rats received no treatment.

On the test day (Day 8), rats were placed in the apparatus for 15 min with free access to both compartments, again without food available. The time spent in the sucrose-paired compartment was registered. A difference between time spent in the sucrose-paired side in the last session and the time spent in the sucrose-paired side in the first session is indicative of the conditioning of a place preference.

Runway task: Rats were maintained under food restriction while the experiment was performed. The apparatus was a straight-alley maze (184-cm long, 18-cm wide, 20-cm high), constructed from metal plates. The maze runway floor was covered with soft paper and the open ceiling permitted the observation of the animal by the researcher. The apparatus consisted of a start set (32 cm), continuous to a runway segment (120 cm), and a goal set (32 cm) with a food cup available. Points in the walls signaled at 32 cm into the runway and 32 cm on arrival from the goal set, so that runway latency based on the 120 cm between the points was recorded. The task consisted of six trials per day with a 30s intertrial interval. For each trial, rats received 10 half Froot Loops pellets for six days. The mean time to reach the goal in each day was considered for the analysis. Initially, the rats were allowed 60 s to complete a trial. If a trial was not completed, the rat was gently encouraged down the alley to the goal set and the animals were then allowed to consume the reward for one minute.

Taste reactivity test: On the first day, rats were placed in a transparent test chamber and gently held by a researcher for one minute. A 58 x 42 cm mirror positioned on the floor of the chamber reflected a view of the rat's face and mouth into the close-up lens of a video camera to permit videotaping of affective facial reactions. This procedure was repeated daily during 6 days, in order to habituate the animal. From the 2nd to the 6th days, a 0.20 ml volume of water was delivered into the animal mouth through an automatic dispenser. On the following day (test), a solution of 0.1 M sucrose and 1 M sucrose was offered at different times (2 hours of interval between tests). Affective reactions elicited by the taste solution were videotaped for subsequent analysis. They were scored in video analysis (frame-by-frame) and expressed as total time in seconds (1 frame = 1/30s). A positive hedonic 'liking' total was compiled by adding scores for rhythmic tongue protrusions, lateral tongue protrusions, and paw licks. A negative aversive 'disliking' total was compiled by adding scores for gapes, headshakes, forelimb flails, paw treading, and chin rubs (26).

Sweet food ingestion after methylphenidate injection: Rats received 2.5 mg/kg methylphenidate i.p., and sweet food ingestion was measured 30, 60 and 120 min after injection (called "fed test" in the Results section). After three days, the same was performed with the animals fasted for the preceding 24 hours (called "fasted test" in the Results section). The dose and the intervals to evaluate sweet food ingestion after the drug injection were chosen based on the literature description of the time to start action and mean plasma half life in rats (9-12).

Monoamines measurement in the nucleus accumbens: The animals were sacrificed by decapitation. Brains were quickly removed and nucleus accumbens was dissected, according to Paxinos et al. (27), and kept at -70º C until use. On the day of the assay, tissue samples were weighed and suspended in 0.1 M $HClO_4$ (1:50 w:v), sonicated for 5 seconds and finally centrifuged at 15 000 rpm for 15 minutes at 4°C. The pellet was discarded and dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) tissue concentrations were analyzed in the supernatants (50 μl) using high pressure liquid chromatography coupled with electrochemical detection (HPLC-ED, BAS, USA). The sensitivity of the amperometric detector was 5 and 20 nA and oxidation potential was fixed at 0.75V (28, 29).

Chromatoghraphic separations were performed using a C18 reverse phase column (150 x 4.6 mm, Phenomenex, USA) packed on microparticulate (5 μm). The mobile phase consisted of 0.15 M citric acid,

0.015% sodium octyl sulfate, 1.6 % acetonitrile (v:v); 3 % tetrahydrofurane (v:v), 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 1.2 ml/minute. 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 of each DA, DOPAC and HVA. Concentrations of these substances in the samples were calculated and expressed as ng/g wet tissue. The activity (turnover) of the dopaminergic system was expressed as DOPAC/DA and HVA/DA.

Statistical analysis: Data were expressed as mean ± standard error of the mean, and were analyzed by Student's t Test (taste reactivity, DA metabolism; non-handling X handling) or by Repeated Measures ANOVA (CPP, Runway test, methylphenidate injection; early life experience X measures) (13). Significance level was accepted as different when the *P* value was equal or less than 0.05. An adequate sample size for each behavioral task was estimated based on previous pilot studies and is shown individually in the Results section.

RESULTS

Effects of early experience on adult behavior

For the conditioned place preference, the mean ± standard error for the time spent on the dark side on day 1 was 13.91±0.24 minutes for intact rats and 14.11±0.25 minutes for neonatally-handled rats, with no statistical difference between groups observed (P=0.580). Both groups preferred the lightened (sugar-paired) side of the corridor on the test session (effect of the session, [$F_{1,18}$ = 18.79, P<0.0001, N=8-12], ***Fig. 1****). However, rats that were neonatally-handled in the first 10 days of life remained less time in the sugar-paired side of the corridor than non-handled rats, especially in the test session (effect of the group [$F_{1,18}$ = 6.16, P = 0.023] and interaction session X group [$F_{1,18}$ = 4.37, P = 0.05]).*

Figure 1 – please insert about here.

Neonatally-handled rats also ran faster to get to the sweet food on the runway test (effect of the group [$F_{1, 15}$ = 8.133, P = 0.012, N=8-9], **Fig. 2**), although both groups decreased their time to reach the sweet

food as the days passed by (effect of the days, [$F_{1, 15} = 10.678$, P = 0.005]). There was no interaction between group and days.

Figure 2 – please insert about here.

Positive affective or 'liking' reactions elicited by 0.1 M sucrose solution ingestion were diminished in neonatally-handled rats, almost reaching statistical significance ([$t_{23} = 1.872$, P=0.074, N=10-15], **Fig 3A**). The effect was more prominent when using a 1 M sucrose solution, for which handled rats elicited significantly less positive reactions than non-handled rats ([$t_{23} = 2.065$, P=0.05]). Aversive 'disliking' reactions to both sucrose solutions were very low and not different between groups (sucrose 0.1 M [$t_{23} = -0.602$, P=0.553] and sucrose 1 M [$t_{23} = 1.114$, P=0.277], **Fig 3B**).

Effects of methylphenidate on sweet food consumption

In the fed state (rats receiving lab chow *ad libitum* before the test), neonatally-handled rats ate more sweet food in comparison to non-handled animals (effect of the group, [$F_{1, 20} = 0.844$, P = 0.011, N=10-12]), but no effect of the drug was observed ([$F_{1, 20} = 0.124$, P = 0.728], **Fig 4A**), nor interactions.

After 24 hours of fasting, non-handled rats respond to methylphenidate with an increase in sweet food consumption, whereas handled rats fail to respond to the drug (interaction time X group, [$F_{1, 20} = 4.634$, P = 0.044], **Fig 4B**). There is no effect of the group as an overall [$F_{1, 20} = 2.579$, P = 0.124], signifying that, in the fasted state, the drug was able to abolish the differences between groups by increasing the sweet food ingestion in the non-handled group.

Figure 4 – please insert about here.

Early life experience and dopamine metabolism in the nucleus accumbens

Neonatally-handled rats showed an increased total amount of DA in the nucleus accumbens ([$t_{21} = -2.363$, P=0.028, N=9-14], **Fig 5**), but also a decreased DOPAC [$t_{20} = 6.546$, P<0.0001, N=9-13] and HVA [$t_{21} = 5.072$, P<0.0001, N=10-13]. The DOPAC/DA ratio was statistically diminished in neonatally-handled rats

in comparison to non-handled rats [Non-handled 1.12±0.13 and Handled 0.42±0.03, $t_{20} = 6.124$, $P<0.0001$], as well as the HVA/DA ratio [Non-handled 0.18±0.02 and Handled 0.07±0.01, $t_{20} = 6.519$, $P<0.0001$], suggesting a decreased dopaminergic metabolism in the nucleus accumbens in these animals.

Figure 5 – please insert about here.

DISCUSSION

In general, the present findings suggest that the early experience influences the development of individual behavioral differences concerning the hedonic impact and incentive value of the sweet food during adulthood. Our data also show that repeated once-daily periods of brief maternal separation during the first 10 days of life can lead to a decreased accumbal dopamine metabolism and to a failure to respond to an acute dose of a dopamine mimetic agent.

The conditioned place preference (CPP) is a behavioral procedure in which an association between a stimulus of positive valence and the place in which the stimulus is experienced during training is formed (33). Neonatally-handled rats, although known to eat more sweet food when exposed to it in different situations (3, 21), showed less conditioned place preference to sweet food in comparison to non-handled rats. It is interesting to note that systemic Cis-Flupenthixol (DA antagonist which binds to dopamine D1-, D2- and D3 receptors) affects associative processes in a CPP paradigm using sucrose solution as a reward, but has no effect on sucrose consumption per se (30). Another study showed that systemic haloperidol during the conditioning phase blocks the establishment of food place preference in hungry rats even though the animals keep consuming the food in the drugged state (31).

Additionally, it has been shown that systemic haloperidol is unable to alter a food conditioned runway behavior, in such a way that the animals still run at the same speed to reach the food goal after use of the drug. Thus, it seems that the same dopamine antagonist treatments that disrupt food reinforcement do not prevent food-seeking behavior (32). In our experiments, neonatally-handled rats showed a similar profile to rats in studies using systemic haloperidol treatment, with less conditioning on the CPP but preserved or rather faster food-seeking behavior on the runway task. This was accompanied by a decreased dopamine metabolism in the nucleus accumbens, which may be in accordance with the state post use of a DA antagonist such as haloperidol.

Methylphenidate can induce place preference, providing rewarding proprieties by itself (20). Therefore, the fact that handled rats do not respond to this drug may contribute to the general idea that these animals are less sensitive to the rewarding effects of some reinforcers. Methylphenidate is a psychostimulant drug, widely used for the treatment of attention deficit hyperactivity disorder. In the brain, it increases the extracellular levels of dopamine and norepinephrine in a manner similar to cocaine and amphetamine. The mechanism of action is thought to include dopamine-reuptake inhibition (14), and it has been used as a tool in animal experiments for characterizing dopamine-behavior relationship without a serotonin effect (15). In our experiments, we showed that methylphenidate had no impact on sweet food ingestion in the fed state. In this situation, rats have more insulin available in the plasma, and as insulin stimulates dopamine reuptake (stimulating dopamine transporter - DAT, (16), this hormone could be counterbalancing the effects of the drug.

In the fasted state, however, non-handled rats responded to methylphenidate, increasing sweet food ingestion, while handled rats failed to do so. After starvation, DAT

mRNA, as well as its function, decreases in the ventral tegmental area/substantia nigra pars compacta (17), and this inhibition is probably potentiated by the action of the drug (14). Since handled rats did not respond, it is possible that the dopamine transporter presents some alteration in its function in handled rats; however, a similar concentration of DAT has already been reported in the nucleus accumbens and striatum of handled and non-handled rats (4).

Food restriction is associated with an increased reward value of abused drugs (18). Studies suggest that food restriction may enhance the functional activity of dopamine receptors (19). Therefore, an alteration in dopamine receptors (either number or affinity), or in signal transduction mechanisms, may also account for this difference between handled and non-handled animals. This possibility deserves further investigation.

The original anhedonia hypothesis states that low dopamine activity may produce anhedonia, leading individuals to overconsume food or drug rewards as an attempt to compensate (34). The results from the present study agree with this view, and other studies have demonstrated that sweet food ingestion is associated with an increase in DA release in the nucleus accumbens (7). Nevertheless, recent important studies have extensively addressed the role of DA in reinforcement and reward, showing that depletions in accumbal DA reduce the motor effort that the rat would expend in obtaining food reward, but do not diminish approach or intake when food is easily available (35, 36). In addition, massive chemical lesions of the dopamine system, rendering the animals incapable of initiating behaviors aimed at obtaining food, do not affect the hedonic-like reactions to sucrose placed in their mouth (37). Therefore, it seems that dopamine transmission in the nucleus accumbens is involved in the process of evaluation of the effort to obtain a goal, and not in the hedonic reactivity to the reward. In our study, neonatally-handled rats are more avid in

the search for a food goal in the runway test, but show decreased dopamine metabolism in the nucleus accumbens. However, we measured the DA metabolism in the baseline state, which does not exclude the possibility that these animals, when facing the task, have a greater DA release in accordance with an enhanced incentive salience for sweet food. It should also be taken into account that, when challenged with a dopamine mimetic agent, these animals respond less to sweet food intake, which could mean that the neurotransmission is blunted even in response to sweet food presentation. Interestingly, these animals showed decreased positive affective reactions to a sucrose solution, suggesting that the hedonic effects of sweet food are less prominent for them.

The compilation of these data suggest a paradigm of greater sweet food ingestion but decreased hedonic impact and decreased conditioning proprieties of the sweet taste, with decreased DA metabolism in the nucleus accumbens and a lower response to methylphenidate in neonatally-handled rats. Interestingly, a similar paradigm is found in experiments with accumbal amphetamine infusion, which does not enable the acquisition of food-reinforced operant responding in ad libitum-fed rats (38), suggesting that the DA dependent processes of approaching/evaluation of the effort and the other aspects involved in hedonic responses are finely regulated, demanding a specific degree of acumbal dopaminergic activation, and the pharmacological augmentation of accumbal dopamine transmission could artificially fragment the coordinated activation of the multiple neurochemical systems in which the dopaminergic tonus is involved (39). Tracing a parallel, one could propose that neonatally-handled rats have a peculiar regulation of the mesolimbic dopamine neurotransmission, leading to an aberrant attribution of salience and reward prediction.

Mesolimbic dopamine transmission contributes to the neural mechanisms that drive the circadian timing of opioid-dependent feeding (41). Other studies have proposed a signaling pathway between the VTA and the nucleus accumbens in which opioids and DA facilitate feeding in an interdependent manner (40). The altered DA neurotransmission in the nucleus accumbens in neonatally-handled rats could modulate the opioid regulation of feeding and lead to the behavioral findings described.

Another interesting finding is that neonatally-handled rats have diminished plasma ghrelin in comparison to non-handled rats (21). When acting on its receptors in the VTA, ghrelin enhances dopamine neuronal activity, synapse formation, and dopamine turnover in the nucleus accumbens, as well as triggering feeding (42, 44). Furthermore, ghrelin has also been suggested to have a role in mediating neuroendocrine and behavioral responses to stressors (43); therefore, the reduced ghrelin levels observed in neonatally-handled animals may be involved with the reduced neuroendocrine response to stress reported in these animals (22, 23), as well as with the reduced dopaminergic activity, as observed in the present study.

In conclusion, we propose that neonatally-handled rats have a peculiar pattern of response to a reward stimuli such as sweet food, in which a greater ingestion is associated with a lower hedonic reactivity and diminished conditioning to sweet food. The paradoxical lower DA metabolism in the nucleus accumbens and greater incentive salience facing sweet food place this animal model as an interesting subject of study to broaden our knowledge about rewarding proprieties of palatable food. In addition, the understanding that early life events can persistently program an individual's responses to food and lead to a particular fashion of hedonic reaction/consumption is of extreme importance in times of obesity epidemics and abundant availability of calorically dense foods such as fats and sweets.

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LEGENDS TO FIGURES

Figure 1: Conditioned place preference – time spent in the sugar-paired side. Data are expressed as mean ± S.E.M. for time (seconds). Both groups increased their time spent in the sugar-paired side (# - effect of session, Repeated Measures ANOVA, $P < 0.0001$), but handled rats stayed less time on this side than non-handled rats in the test session (* - effect of the group, Repeated Measures ANOVA, $P = 0.007$ and interaction session X group $P = 0.05$).

Figure 2: Runway test. Data are expressed as mean ± S.E.M. for time (milliseconds). Handled rats run faster than non-handled ones. (Repeated-Measures ANOVA, $P = 0.012$ for group*). There is an effect of the sessions (both groups decreased the time to reach the goal as the days passed by), but no session X group interaction.

Figure 3: Taste reactivity test. Data are expressed as mean ± S.E.M. for time (seconds). A – Total time of hedonic positive reactions. Handled show less positive hedonic reactions than non-handled ones when exposed to sucrose, reaching statistical significance when using the 1% solution (Student's T Test, $P = 0.05$ for group*). B- Total time of aversive reactions. There is no difference between groups.

Figure 4: Sweet food ingestion after 2.5 mg/kg i.p. methylphenidate. Data are expressed as mean ± S.E.M. for food pellets ingested. A: Fed state - There was no effect of the drug (Repeated-Measures ANOVA, $P = 0.728$), with handled rats eating more sweet food (* - effect of the group on Repeated-Measures ANOVA, $P = 0.011$). B: Fasted state – There was an interaction between group and drug (Repeated-measures ANOVA, $P = 0.044$), meaning that methylphenidate increased sweet food ingestion only in non-handled rats and abolishing the group effect drug (Repeated-measures ANOVA, $P = 0.124$)

Figure 5: Dopamine metabolism in the nucleus accumbens measured by chromatography. Data are expressed as mean ± S.E.M. for tissue levels in ng/g. *Neonatally-handled rats have an increased dopamine content (Student's T Test, $P = 0.028$) but a decreased DOPAC (Student's T Test, $P < 0.0001$) and HVA (Student's T Test, $P < 0.0001$), suggesting a decreased dopaminergic metabolism in the nucleus accumbens of these animals.

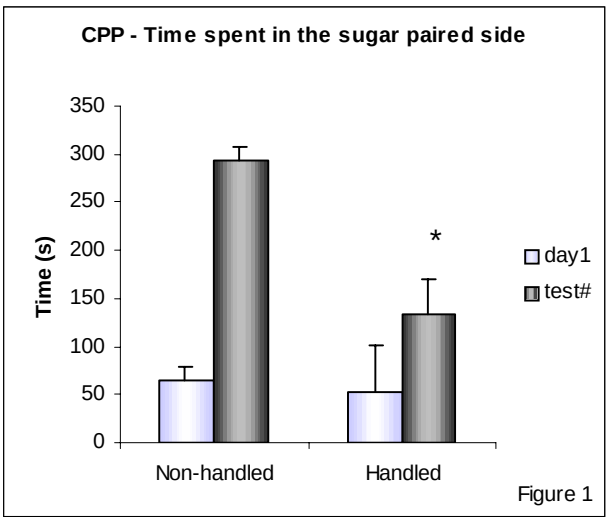

Figure 1

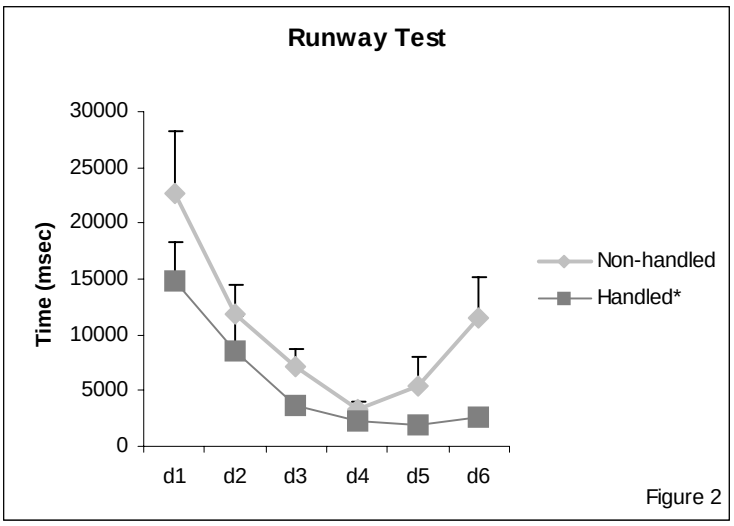

Figure 2

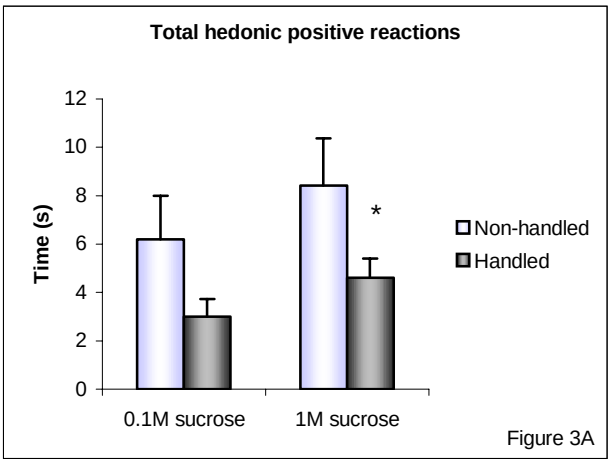

Figure 3A

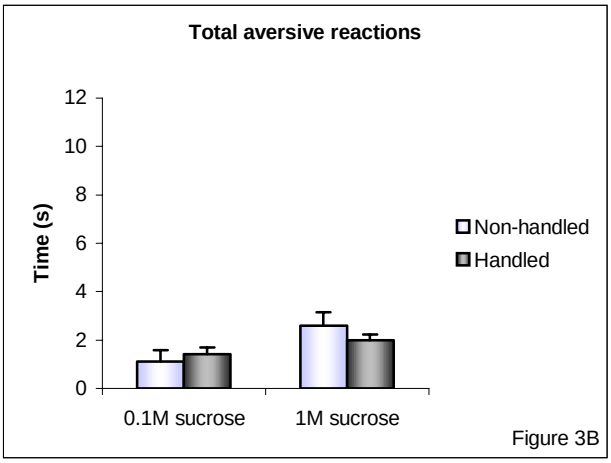

Figure 3B

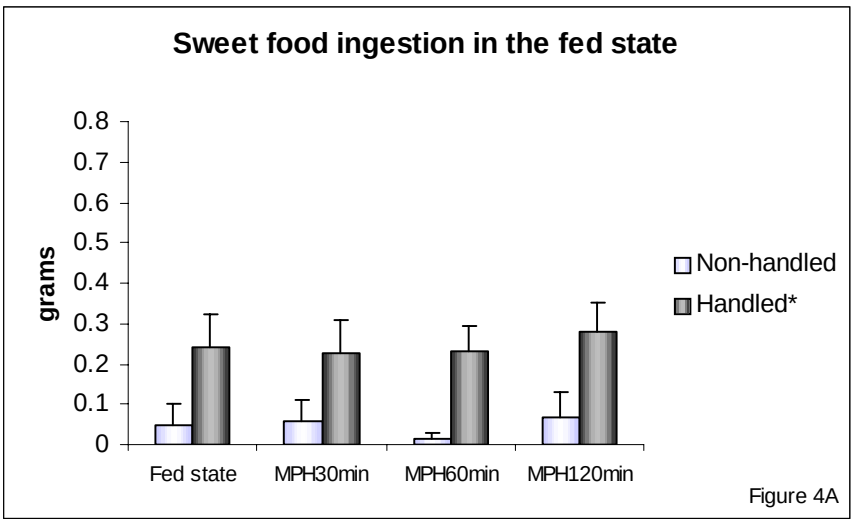

Figure 4A

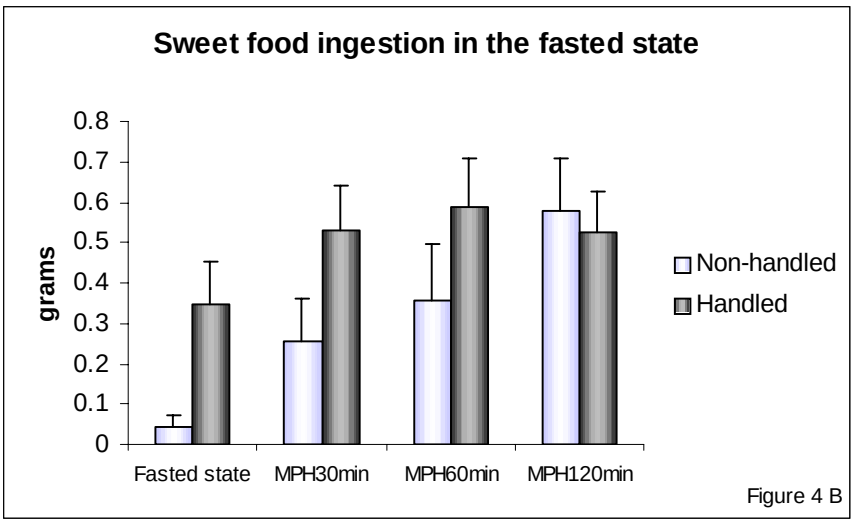

Figure 4 B

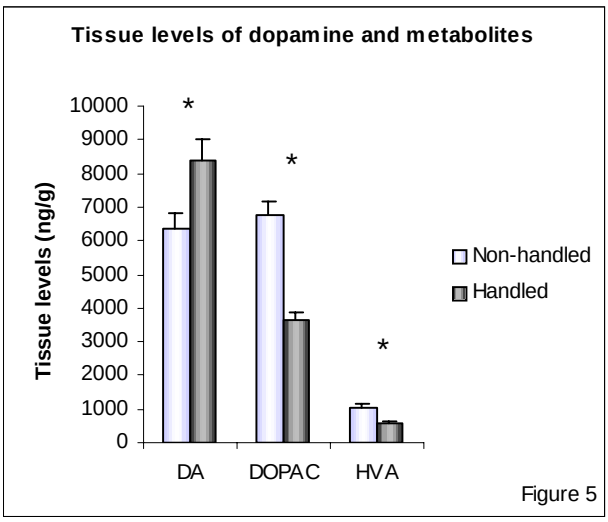
Tissue levels of dopamine and metabolites
Tissue levels (ng/g)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
*
DA
DOPAC
HVA
Non-handled
Handled

Figure 5

3.3 CAPÍTULO III:

Estudo dos efeitos da manipulação neonatal sobre o consumo de doce em diferentes idades.

Neste estudo avaliamos o consumo de doce em animais adolescentes manipulados no período neonatal. Além disso, estudamos o efeito dessa exposição precoce ao doce e de outros estímulos (como exposição a brinquedos) no consumo deste alimento na vida adulta. Vimos que o efeito da manipulação neonatal é evidente apenas se os animais são testados após a adolescência. A exposição precoce ao doce aumenta o consumo de animais controles semelhantemente à manipulação neonatal, eliminando as diferenças entre os grupos. Além disso, a simples exposição dos animais a um ambiente novo na infância aumenta o consumo de doce na vida adulta.

Submetido: Silveira PP; Portella AK; Crema L; Correa M; Nieto FB; Diehl L; Lucion AB; Dalmaz C. Both infantile stimulation and exposure to sweet food lead to increased sweet food ingestion in adult life. *Physiology and Behavior*

BOTH INFANTILE STIMULATION AND EXPOSURE TO SWEET FOOD LEAD TO AN INCREASED SWEET FOOD INGESTION IN ADULT LIFE

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Running head : Early stimulation and sweet consumption

ABSTRACT

SILVEIRA PP; PORTELLA AK; CREMA L; CORREA M; NIETO FB; DIEHL L; LUCION AB; DALMAZ C. **Both infantile stimulation and exposure to sweet food lead to increased sweet food ingestion in adult life** – We have reported that neonatal handling leads to increased sweet food preference in adult life. Our aim was to verify if these differences in feeding behavior appear before puberty, and whether other types of

intervention in periadolescence (such as exposure to toys) could interfere with sweet food consumption later in life. Nests of Wistar rats were (1) non-handled or (2) handled (10 min/day) on days 1-10 after birth. Males from these groups were subdivided in two subgroups: one was habituated to sweet food (Froot Loops – Kellogs ®) in a new environment for four days and tested for sweet food preference at age 27 days, before submitting to a new habituation and test for sweet food ingestion again in adult life. The other subgroup was habituated and tested only in adulthood. In another set of experiments, neonatally non-handled rats were exposed or not to a new environment with toys in periadolescence, and tested for sweet food ingestion as adults. Neonatal handling increases sweet food consumption only if the habituation and tests are performed after puberty. Interestingly, infant exposure to sweet food had a similar effect to neonatal handling, since controls that were exposed to sweet food at age 22 to 27 days increased their ingestion as adults. Exposure to toys in periadolescence had the same effect. We suggest that an intervention during the first postnatal days or exposure to an enriched environment later in the pre-pubertal period leads to behavioral alterations that persist through adulthood, such as increased sweet food ingestion.

Key words: feeding behavior, periadolescent stimulation, palatable food ingestion, sweet food ingestion, neonatal handling, environmental enrichment.

INTRODUCTION

The first two weeks of life in a rat correlate to the perinatal period in humans. In this period, the development of various systems continues to occur, including the central nervous system (CNS). These first days are called the stress hyporesponsive period (1), when there is an exacerbation of glucocorticoid negative feedback in the pituitary and a decreased sensitivity of the adrenal gland to the adrenocorticotropic hormone (ACTH) (2).

Since it is a critical period of differentiation, the submission of an animal to brief handling during these days determines neurochemical and behavioral alterations that persist throughout life. Essentially, these animals present less fear in a new environment, increased activity and exploration (3). These findings agree with neuroendocrine data, such as a persistently increased negative feedback of glucocorticoids (4), reduction of mRNA corticotropin-releasing hormone (CRH) expression in hypothalamus and decreased CRH in median eminence (5).

In general, these alterations can be observed only after puberty (6), suggesting the need of an exposure to a particular pattern of hormonal secretion for the behavioral effects of the neonatal intervention to be expressed. However, early-life environmental intervention leads to structural alterations beginning in the neonatal period and being persistent through the whole life (7, 8).

We have previously observed that neonatal manipulations can alter the feeding behavior in adult life. More specifically, early handling leads to an increased ingestion of palatable food, such as sweet and savoury snacks in adult life, without alteration in standard lab chow ingestion and no effect on body weight (9). This increased ingestion of sweet food is not related to anxiety (10). Comparable findings were described in Sprague-Dawley rats that were handled for 15 minutes in the first 3 weeks of life (11). Longer periods of maternal separation can also affect feeding behavior, increasing rebound hyperphagia after a period of food restriction, especially in females (12).

To better characterize the effect of neonatal handling on sweet food ingestion, we tried to determine if these alterations occur before puberty, and if the precocious exposure to sweet food can alter the ingestion of this type of food later in life. Finally, we tried to verify if another intervention in infancy, such as exposure to a diverse environment, could also lead to a different pattern of sweet food consumption in adulthood.

MATERIAL AND METHODS

Subjects:

Pregnant Wistar rats bred at our own animal facility were randomly selected. Animals were housed alone in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust and were maintained in a controlled environment: lights on between 07:00h and 19:00h, temperature of 22 $\pm$ 2°C, cage cleaning once a week, food and water provided *ad libitum*. The day of birth was considered as day 0. All litters were culled within 24 h to eight pups and were maintained undisturbed except for handling procedures, which were carried out between 10:00h and 12:00h. Included in this period were the time to set up the incubator, to bring the cages from the facility and briefly habituate the dams to the new room, to perform

careful removal of the pups from the nest, the time of handling per se, the return of the pups to the dam and, again after a brief period, to return the cage to the facility room. The researcher also changed gloves for the manipulation of each litter to avoid the spread of any kind of odor from nest to nest.

Litters were weaned on postnatal day 21. Two male pups from each litter were assigned to each experiment. After weaning, rats were housed four to five per cage. Eighty male rats were used in the different experiments, derived from 23 different litters. Rats had free access to food (standard lab rat chow) and water, except during the period when the behavioral tasks were applied. Tasks were performed between 11:00h and 15:00h.

Neonatal Handling model (9, 10)*:*

Non-handled group: Pups were left undisturbed with the dam until weaning. It was stated on the cage that these animals should not be touched, not even for cage cleaning. Dirty sawdust was carefully removed from one side of the cage, without disturbing the mother and the nest, and replaced by clean sawdust at that side by the principal researcher.

Handled group: The dam was gently pulled to one side of the cage and the pups were removed from their home cage and were placed into a clean cage lined with clean paper towel. This cage was placed into an incubator at 34° C next to the dam's cage. After 10 minutes, pups were returned to their dams. This procedure was performed from day 1 to 10 following birth, and then pups were left undisturbed until the 21st day of life.

Sweet food ingestion test:

Animals were placed in a lightened rectangular box (40 x 15 x 20 cm) with floor and side walls made of wood and a glass ceiling. Ten Froot loops (Kellogg's ® - pellets of wheat and cornstarch and sucrose) were placed at one extremity of the box. Each animal was submitted to 4-5 habituation trials under food restriction (80% of habitual ingestion of standard lab chow), during 3 minutes each, in different days, in order to discard novelty (from the environment or the food) as a factor on the test day. When the animals were 27 days of age, they were exposed for 3 min to the apparatus, where the number of ingested pellets was measured. A protocol

was established so that when the animals ate part of the Froot loops (ex.: 1/3 or 1/4), this fraction was considered. This evaluation was made with the animals fed *ad libitum* on the previous day.

In this experiment, one subgroup of animals handled during the neonatal period and one subgroup of non-handled rats were submitted to the behavioral procedure described above, and the other subgroup was subjected to this same procedure only at 90 days of age. Additionally, the group exposed to sweet food during the peripuberal period was subjected again to all these procedures at 90 days of life.

Experiment 2

In experiment 2, rats without neonatal intervention were exposed to a lightened rectangular box (40 x 15 x 20 cm) with floor and side walls made of wood and a glass ceiling. Three different plastic toys were placed at one extremity of the box. They remained in this place for 3 minutes before being gently placed again in the home cage. This procedure was performed on days 22-27, 37 and 45 of life. Toys changed in color and shape each day and were washed after every session. Rats could interact with the toy as much as they wanted to, sniffing or even moving it around the box. The control group was left undisturbed since birth. Both groups were habituated and tested as adults (90 days of life) for sweet food ingestion.

Statistical analysis:

Data were expressed as mean ± standard error of the mean, and were analyzed, according to the experiment, using Student's t test or two-way ANOVA (13). The significance level was accepted as different when the *P* value was equal or less than 0.05. Sample size was estimated based on pilot studies, varying in each experiment, and is shown individually in the Results section.

RESULTS

Experiment 1 – Effect of handling during the neonatal period on sweet food consumption at different ages

The test of sweet food consumption at 27 days of age was marginally significant; neonatally handled rats ate less than non-handled rats [Student´s t test, $t(22) = 1.96$, $P=0.063$, n = 9-15]. Please refer to Figure 1.

Please insert Figure 1 about here.

In the test session performed in adulthood, rats exposed to sweet food only in adult life ate different amounts of sweet pellets when comparing handled and non-handled animals: handled rats ate more than non-handled animals in the absence of hunger [Student´s t test, t(28) = -2.63, $P<0.014$, n = 14-16] (see Figure 2). However, the test for sweet food consumption in adulthood when rats were previously exposed to sweet food in periadolescence showed no effect of neonatal intervention, with both neonatal groups eating equally and a significant interaction between exposure to sweet food in periadolescence and neonatal intervention [Two way ANOVA, F(1, 52)=5.589, $P=0.025$], in which neonatally nonhandled rats increase their ingestion in adulthood if exposed to sweet food in periadolescence, although neonatally handled rats seem to keep the same pattern of ingestion independently of the exposure to palatable food in periadolescence. In fact, all rats habituated to the new food early in life exhibit, as adults, an increased ingestion of sweet food compared to non-handled rats that were not exposed to sweet food [Two way ANOVA, F(1, 52)=4.256, $P=0.048$]. The magnitude of the increased consumption in the exposed group was similar to the increase caused by neonatal handling itself in the nonexposed group (Figure 2).

Please insert Figure2 about here.

Experiment 2 – Sweet food ingestion in adulthood after exposure to toys in periadolescence

The test for sweet food consumption in adulthood, using rats that were exposed to toys, had the same effect as the exposure to sweet food in periadolescence, increasing sweet food ingestion later in life [Student´s t test, t(29) = -2.86, $P=0.008$, n = 13-18]. Please see Figure 3.

Please insert Figure 3 about here.

DISCUSSION

In this study, we verified that the increased sweet food ingestion caused by neonatal handling is apparent only after puberty, and persists throughout adulthood (9). Interestingly, some psychiatric and eating disorders, like schizophrenia and anorexia/bulimia, also express themselves clinically after puberty. Other behavioral changes caused by neonatal stress that appear just after puberty have already been reported, as well as the altered responsiveness to stress (6). It is possible that some neural pathways are persistently programmed in these animals. Additionally, analogous to schizophrenia and anorexia/bulimia, these pathways could be related to an altered dopaminergic and/or adenosinergic transmission (14-17).

We do not yet know why these animals present this alteration in feeding behavior. One possible explanation would be an altered dopaminergic neurotransmission. Neonatal handling is related to alterations in the dopaminergic metabolism: an increased dopaminergic metabolism in the hypothalamus has been reported (18), as well as altered dopamine (DA) neurotransmission in the nucleus accumbens (19). Palatable food consumption promotes dopamine release in the nucleus accumbens and prefrontal cortex (20). In addition, repeated exposure to sweet food can increase the turnover of dopamine in the nucleus accumbens (21). Increases in nucleus accumbens DA initiate feeding behavior, even in satiated rats (22). Therefore, altered dopamine neurotransmission may play a role in the increased palatable food ingestion described in the present study.

Another interesting result, observed here, is that the early exposure of the animal to sweet food also leads to an increased ingestion of this type of food in adult life. A question that arose at this point concerned the possibility that this increased ingestion could be caused by the ingestion of the food itself or by the environmental stimulation that the rat was submitted to when being exposed to a new set, with a new food available. Experiment

2 helped to clarify this question: we observed that the simple exposure of the animal to a new environment during this phase of life was able to increase sweet food ingestion in the adulthood, to levels similar to those observed in animals exposed to neonatal handling, as previously described (9). These alterations in sweet food preference may have similar mechanisms of action, since the effect of neonatal handling is diluted after infantile stimulation, demonstrated by the finding that non-handled rats exposed to sweet food in periadolescence eat as much as handled rats exposed to sweet food in periadolescence. It is noteworthy that, although periadolescent rats ate small amounts of sweet food, the amount ingested was similar to that of adult animals when considering body weight (as a ratio of consumption to body weight, data not shown).

Periadolescent rats have been studied as a model for the analysis of risk factors of several kinds of disorders in humans, such as attention-deficit-hyperactivity-disorder (ADHD) (23) and drug abuse (24). Periadolescent stimulation increases acetylcholine synthesis in the cortex and hippocampus after a training experience (25). Peculiar responses in corticosterone have been reported when the animals are exposed to several kinds of stressors at this age, compared to adult rats or mice (26, 27). Additionally, animals at this specific age also have particular responses to some drugs, such as dopaminergic agonists (28) and morphine (29). Interestingly, manipulation of the animals in this period of life can lead to neuroendocrine and behavioral alterations in adulthood of rats (30, 31). This intervention also seems to act in the modulation of the effects of early adverse events such as prenatal exposition to cocaine (32), maternal separation (33), neonatal anoxia (34) and low maternal care (35).

The ingestive pattern of an animal in this critical period of periadolescence also seems to modify preferences later in life. For example, the preference for alcohol in

adulthood may be enhanced, depending on the age that the rat first experienced alcohol effects (36). It has been demonstrated that exposure to sucrose at weaning may modify the preference for sweet solutions later in life, and that this effect is related to altered dopaminergic content in hypothalamus (37). Pre-weaned rats are capable of learning the association of an arbitrary flavor with the postingestive effects of nutrients and then demonstrate a preference for that flavor after weaning (38).

It is possible that rats exposed to sweet food early in life present a decreased reward effect of sweet food ingestion in adulthood, needing to ingest more to feel better. In accordance with this hypothesis, mice exposed to nicotine during adolescent development showed a decrease in cocaine's rewarding effects and an increase in cocaine's motor activating effects, even with low and repeated doses (39). A decreased reward effect is also evident in obese individuals: they tend to increase palatable food ingestion in response to depressive symptoms, and this fact is related to mesolimbic dopaminergic neurotransmission (40).

In conclusion, infantile environmental stimulation, in the presence of sweet food or even toys, can alter feeding behavior later in life. These procedures have similar effects to those caused by neonatal handling. It is possible that these precocious interventions, independently of the age applied, could lead to the same alterations in feeding behavior, which will be expressed only after puberty.

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LEGENDS TO FIGURES

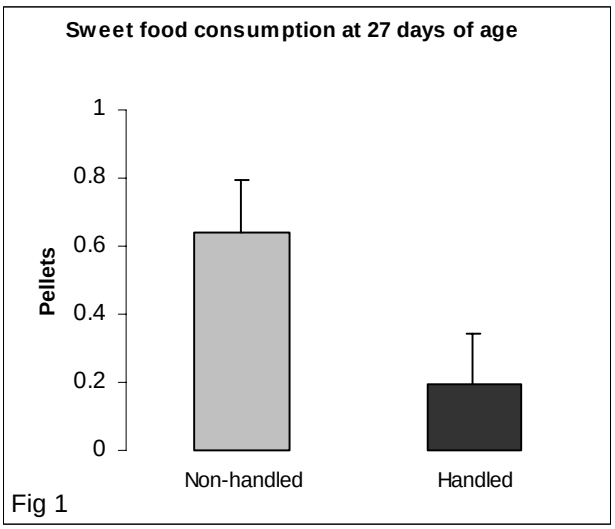
Figure 1: Sweet food consumption at 27 days of age. Data are expressed as mean ± S.E.M. for pellets consumed.

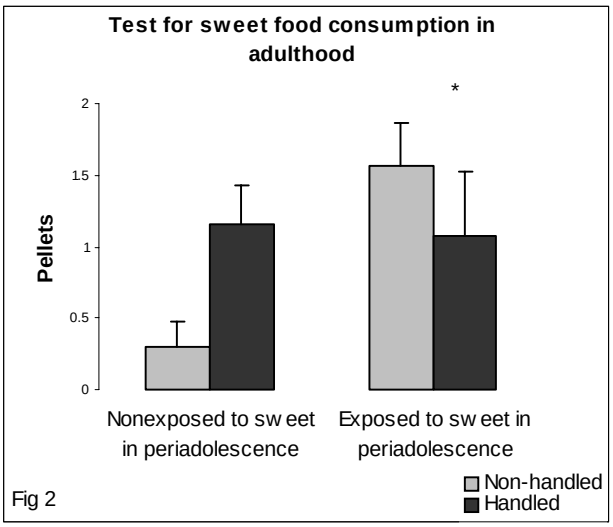
Figure 2: Test for sweet food consumption in adulthood in rats exposed or not to sweet food in periadolescence. Data are expressed as mean ± S.E.M. for pellets consumed. * Increased consumption in

relation to rats that were non-handled non-exposed to sweet food in periadolescence (Two Way ANOVA, $P<0.05$).

Figure 3: Sweet food ingestion in adulthood in nonhandled rats exposed or not to toys in periadolescence. Data are expressed as mean $\pm$ S.E.M. for pellets consumed.

***** Increased in relation to intact rats (Student's t test, $P=0.008$)


Sweet food consumption at 27 days of age
Pellets
1
0.8
0.6
0.4
0.2
0
Non-handled
Handled
Fig 1


Test for sweet food consumption in adulthood
Pellets
2
1.5
1
0.5
0
*
Nonexposed to sweet in periadolescence
Exposed to sweet in periadolescence
Non-handled
Handled
Fig 2

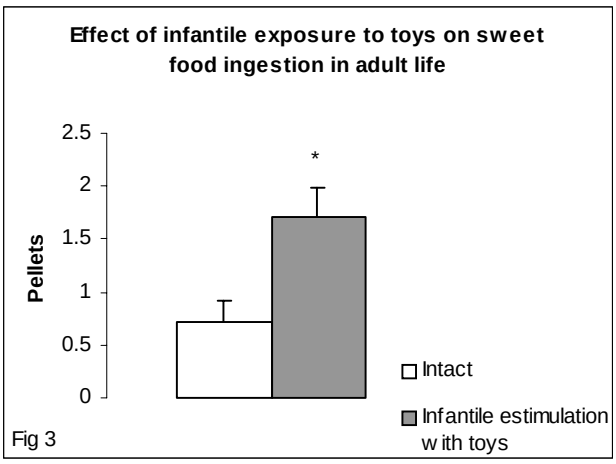

Effect of infantile exposure to toys on sweet food ingestion in adult life
Pellets
2.5
2
1.5
1
0.5
0
*
Intact
Infantile estimulation with toys

Fig 3

3.4 CAPÍTULO IV:

Estudo dos efeitos da manipulação neonatal sobre a atividade de ectonucleotidases no núcleo acumbens em diferentes idades.

Neste estudo avaliamos o sistema purinérgico no núcleo acumbens de animais manipulados no período neonatal, uma vez que esta estrutura é um local de interação entre os sistemas adenosinérgico e dopaminérgico. Vimos que, na adolescência, animais manipulados no período neonatal ingerem menos doce que animais controle, sem diferenças na hidrólise do ATP, ADP ou AMP no núcleo acumbens. Na vida adulta, animais manipulados no período neonatal consomem mais doce e apresentam menor hidrólise de AMP, um passo limitante para a síntese de adenosina.

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NEONATAL HANDLING, SWEET FOOD INGESTION AND ECTONUCLEOTIDASE ACTIVITIES IN NUCLEUS ACCUMBENS AT DIFFERENT AGES

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Running head: Neonatal handling and ectonucleotidase activities in nucleus accumbens

SILVEIRA PP; COGNATO G; CREMA L; PEDERIVA FQ; BONAN CD; SARKIS JJ; LUCION AB; DALMAZ C. **Neonatal handling, sweet food ingestion and ectonucleotidase activities in nucleus accumbens at different ages** – Neonatal handled rats ingest more sweet food than non-handled ones, but it was documented only after puberty. Here, we studied the purinergic system in the nucleus accumbens, a possible target for the alteration in the preference for palatable food. We measured the ATP, ADP and AMP hydrolysis mediated by ectonucleotidases in synaptosomes of the nucleus accumbens in periadolescent and adult rats from different neonatal environments: non-handled and handled (10 min/day, 10 first days of life). Before adolescence, we found a decreased ingestion of sweet food in the neonatally handled group, with no effect on ATP, ADP or AMP hydrolysis. In adults, we found a greater ingestion of sweet food in the neonatally handled group, with no effect on ATPase or ADPase activities, but a decreased AMP hydrolysis. The nucleus accumbens is a site of intensive interaction between the adenosinergic and dopaminergic systems. Therefore, adenosine may modulate accumbens' dopamine neurotransmission differently in neonatally handled rats.

Key words: neonatal handling, adenosine, nucleus accumbens, dopamine, ATPase–ADPase activities; 5′-nucleotidase activity.

INTRODUCTION

Mother-infant interactions promote an adequate environment for neurodevelopment (1-2) and are very important for the establishment of a healthy adult life (3). Precocious interventions in this relationship may lead to persistent alteration of several aspects, including behavioral (4), neurochemical (5) and neuroendocrine responses to stress (6).

Previous studies from our laboratory have shown that neonatal handling, a brief and apparently innocuous separation from the mother in the neonatal period, can lead to increased sweet food consumption in adulthood, without differences in standard lab chow ingestion (7). Since this alteration occurs only with palatable food, it appears that hedonic mechanisms must be involved. These rats also present a faster and consistent search for sweetened snacks, although they are less prone to place conditioning related to this food (Silveira et al., unpublished results).

Sucrose licking is known to increase accumbens dopamine (8), and a quantitative relationship has been demonstrated between the concentration-dependent rewarding effect of orosensory stimulation by sucrose during eating and the overflow of dopamine in the nucleus accumbens (9). In addition, repeated access to sucrose increases dopamine turnover in the accumbens (10).

The co-expression of adenosine A_{2A} and dopamine D2 receptors in the same GABAergic medium spiny neurons is a characteristic feature of the nucleus accumbens (11). These receptors appear to present synergy for protein kinase A (PKA) signaling in response to ethanol (12), and there have been suggestions that adenosine in the nucleus accumbens plays a significant role in activity reward, reinforcement and drug-seeking behavior (13, 14).

Adenine nucleotides are thought to be an important potential source of extracellular adenosine (15, 16). These nucleotides are hydrolyzed by an extracellular cascade of enzymes, which includes ecto-ATPase (NTPDase2, CD39L1, EC 3.6.1.3), ATP

diphosphohydrolase (NTPDase1, CD39, ecto-apyrase, EC 3.6.1.5) and ecto-5'-nucleotidase (lymphocyte surface protein, CD73, EC 3.1.3.5) (17, 18). In the central and peripheral nervous systems, ATP is hydrolyzed to adenosine by the conjugated action of NTPDases and 5′-nucleotidase (19, 20). These ectonucleotidases, acting together, control the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors and, consequently, the duration and extent of receptor activation.

Previous findings have suggested that the neonatal handling-induced increase in sweet food consumption may be observed just in adulthood, and not in younger rats. At the neurochemical level, it is important to correlate changes in behavior with neurobiological modifications, such as altered neurotransmission. In this paper, we aimed to verify the sweet food ingestion in neonatally handled rats before and after puberty and to determine ATP, ADP and AMP hydrolysis in synaptosomes from the nucleus accumbens in these two ages.

EXPERIMENTAL PROCEDURE

Subjects:

Pregnant Wistar rats bred at our own animal facility were randomly selected. They were housed alone in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust and were maintained in a controlled environment until offspring: lights on between 07:00h and 19:00h, temperature of 22 $\pm$ 2°C, cage cleaning once a week, food and water provided. All litters were culled within 24 h to eight pups and were maintained intact unless for handling procedures, which were carried out between 10:00h and 15:00h.

Weaning was on postnatal day 21. Two male pups were used per litter per experiment. Rats were housed four to five per cage. Fifty-two experimental male rats were

used in the different experiments, derived from 21 different litters. Rats had free access to food (standard lab rat chow) and water, except during the period when the behavioral tasks were applied. Tasks were performed between 13:00h and 16:00h.

All animal treatments were approved by the Institutional Ethical Committee (Ethical Committee, UFRGS, # 200270) and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS).

Neonatal Handling model (7)*:*

Non-handled group – Pups were left undisturbed with the dam until weaning. It was stated on the cage that these animals should not be touched, not even for cage cleaning. Dirty sawdust was carefully removed from one side of the cage, without disturbing the mother and the nest, and replaced by clean sawdust at that side by the principal researcher.

Handled – Pups were removed from their home cage and were placed into a clean cage lined with clean paper towel, inside an incubator at 34° C. After 10 minutes, pups were returned to their dams. This procedure was carried out for the first ten days of life, after which pups were left undisturbed until the 21st day of life.

Sweet food ingestion:

For sweet food ingestion, animals were placed in a lightened rectangular box (40 x 15 x 20 cm) with floor and side walls made of wood and a glass ceiling. Ten Froot loops (Kellogg's ® - pellets of wheat and cornstarch and sucrose) were placed in one extremity of the box. Each animal was submitted to four exposures, of 3 minutes each, on different days, and the total number of ingested pellets across the days was measured. This procedure was performed under food restriction (80% of habitual ingestion of standard lab chow). A protocol was established so that when the animals ate part of the Froot loops (ex.: 1/3 or 1/4), this fraction was considered. This protocol was started when the animals reached 23 days of age or at 60 days of age.

Animal preparation and subcellular fraction:

Animals were sacrificed by decapitation and their brains were removed and placed in ice-cold isolation medium (320 mM sucrose, 5 mM HEPES, pH 7.5, and 0.1 mM EDTA) and were cut coronally. Nucleus accumbens of both hemispheres were immediately dissected on ice and gently homogenized 1:10 (w:v) in ice-cold isolation medium with a motor-driven Teflon-glass homogenizer. In adults, accumbens from two animals were pooled. In the case of young animals, we used three animals for each pooled sample. The synaptosomal fraction was isolated as previously described (21). Briefly, 0.5 ml of crude mitochondrial fraction was mixed with 4.0 ml of an 8.5% Percoll solution and layered onto an isosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. Synaptosomal fractions were washed twice at 15000 x g for 20 min with the same ice-cold medium to remove the contaminating Percoll and the synaptosome pellet was resuspended. The material was prepared fresh daily and maintained at 0-4°C throughout preparation.

Enzyme Assays:

The reaction medium used to assay the ATP and ADP hydrolysis was essentially as described previously (22). The reaction medium contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM TRIS-HCl buffer, pH 8.0, in a final volume of 200 μl. The synaptosome preparation (10 μg protein) was added to the reaction mixture and preincubated for 10 minutes at 37°C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM and was stopped after 30 minutes by the addition of 200 μl 10% trichloroacetic acid. The released inorganic phosphate (Pi) was measured as previously described (23).

The reaction medium used to assay the 5'-nucleotidase activity (AMP hydrolysis) contained 10 mM $MgCl_2$, 0.1 M Tris-HCl, pH 7.0 and 0.15 M sucrose in a final volume of 200 μl (24). The synaptosome preparation (10-20 μg protein) was preincubated for 10 minutes at 37°C. The reaction was initiated by the addition of AMP to a final concentration of 1.0 mM and was stopped after 60 minutes by the addition of 200 μl 10% trichloroacetic acid; the released inorganic phosphoate (Pi) was measured as previously described

(23). In all enzyme assays, incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions (22, 24). Other conditions, such as medium reaction, pH and cation concentrations were used to assure the optimal enzyme activities (22, 24). Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct non-enzymatic hydrolysis of the substrates. All samples were run in triplicate. The mean specific activity obtained for control animals in each experimental day was considered 100% for comparisons. Protein was measured by the Coomassie Blue method, using bovine serum albumin as standard (25).

Statistical analysis:

Data were expressed as mean ± standard error of the mean, and were analyzed by Student's t test or by Two-way ANOVA (26). The significance level was accepted as different when the *P* value was equal or less than 0.05. Sample size varies in each experiment and is shown individually in the Results section.

RESULTS

Sweet food consumption

When exposed to sweet food between 23-26 days of age, neonatally handled rats ate less sweet food than non-handled rats [Student´s t test, t(22) = 2.215, *P*=0.037, n = 9-15/group]. At adulthood, neonatally handled rats ate more sweet food than the control group [Student´s t test, t(21)=-2.069, *P*=0.05, n = 11-12/group]. A Two-way ANOVA demonstrated an effect of the age, in which rats eat more as they get older [F(1, 46) = 40.303, P = 0.005]. There is also interaction between group and time, demonstrating that neonatally handled rats change their pattern of sweet food consumption as the time pass by, while the nonhandled group keep the same pattern [F(1, 46) = 35.970, P = 0.007]. Figure 1 displays these results.

Please insert Figure 1 about here

Ectonucleotidases activities

ATPase activity is not different between groups at 21 days of age [Student´s t test, t(8) = 0.727, *P*=0.488, n = 5/group], nor at 60 days of age [Student´s t test, t(16) = -0.36, *P*=0.721, n = 8-9/group]. The same lack of effect occurs with ADPase activity at 21 days of age [Student´s t test, t(8) = 1.310, *P*=0.226] and

60 days of age [Student´s t test, t(15) = -0.86, *P*=0.403]. AMP hydrolysis is not different between groups at 21 days of age [Student´s t test, t(8) = 0.184, *P*=0.859], but it is decreased by 14.5% in neonatally handled rats at 60 days of age [Student´s t test, t(15) = 2.11, *P*=0.05]. Figure 2 demonstrates these results.

Please insert figure 2 about here

Body weight

Mean body weight at 21 days was 45.09+11.90 g for non-handled rats and 41.08+2.40g for handled rats. In adulthood, the mean body weight was 319.93+21.94 g for non-handled rats and 297.92+74.87g for the handled group. There was no statistical difference between the groups concerning body weight in the different ages [Student's t test, *P*>0.05].

DISCUSSION

In this paper, we verified that the alteration in sweet food consumption in adulthood of neonatally handled rats is accompanied by a decrease in 5′-nucleotidase activity in the nucleus accumbens. The reaction catalyzed by 5'-nucleotidase is the rate-limiting step in the extracellular pathway from ATP to adenosine (for a review, see 27). Since AMP hydrolysis is the major source of extracelullar adenosine (15, 16), we suggest that animals that suffered neonatal handling present a decrease in extracellular adenosine levels in this structure when adults.

On the other hand, there is a decrease in sweet food consumption in the neonatally handled rats before adolescence. This effect could possibly be related to an increased serotonin content found in several structures (hypothalamus, hippocampus and striatum) in neonatally handled animals at this age, but not in adulthood (28), since serotonin is a known neurotransmitter linked to decreased appetite (29). Another possible explanation would be a higher dopamine metabolism rate in the hypothalamus, which was found in neonatally handled rats during puberty (28). This finding was associated with decreased sweetened solution ingestion in other studies (30, 31). At the same time, neonatally handled rats are not different from controls regarding ATP, ADP and AMP hydrolysis.

Adenosine modulates dopamine and other neurotransmitters, such as glutamate (32, 33), in the nucleus accumbens. This modulation could be involved in the present behavioral findings (34). Some studies demonstrate that a functional dopamine/adenosine interaction in the nucleus accumbens is necessary to induce the reinforcing effects of rewards (35), and that adenosine is involved in the sweet taste perception (36, 37). Therefore, since neonatally handled rats show a decreased adenosine function at this site in adulthood, this could mean that these animals present a lower perception of the rewarding effects of sweet food, due to a blunted dopaminergic tonus in the accumbens, in such a way that they may increase consumption of palatable food trying to reach a higher activation of this circuit.

If this is so, the question arises as to why these animals switch from a state of decreased ingestion in puberty to an increased consumption in adulthood? A similar fashion of shifting is found with respect to responses to stress: early handling induces long-lasting behavioral and stress-related hormonal changes, although these are not stable throughout life, being detectable mostly after puberty (38).

Interestingly, hetero-oligomerization of adenosine and dopamine receptors (A2A/D2) may be involved in the psychostimulant-induced behavioral sensitization (39), and neonatally handled rats are less prone to develop such a state of sensitization (40). In addition, this early-life intervention is also associated with a reduced D3 dopamine receptor binding and mRNA levels in the nucleus accumbens-shell (40), and there is evidence of functional A2A/D3 heteromeric complexes (41). Therefore, the decreased 5′-nucleotidase activity observed in this study and, consequently, a decrease in adenosine levels, may help to explain other behavioral and neurochemical findings presented by neonatally handled rats.

Some neurochemical alterations occur during periadolescence in the nucleus accumbens (42-44). There have been descriptions of changes in nucleotide-metabolizing enzymes in the central nervous system as a function of the developmental stage (45-47). Since neonatally handled rats present a diminished level of 5'-nucleotidase in adulthood, these effects could be related to the behavioral effects observed. It is known that adenosine modulation occurs only after puberty (48); therefore, the effects of dopamine modulation by adenosine (and the effects on feeding behavior) are possibly observed only in adulthood.

Although there were no differences concerning body weight, it remains to be determined if the preference for palatable food in the neonatally handled group is related to an increased vulnerability to obesity. As already demonstrated (7), these animals eat the same amount of lab chow than do controls. The

increased consumption is specific for palatable food, and only a continuous exposure to this type of food could answer the question above, which is out of the scope of this study. In addition, it is possible that differences in body weight appear between the groups in older rats.

In summary, neonatal handling leads to persistent behavioral and neurochemical alterations in adulthood, which appear only after puberty. An increased ingestion of sweet food, if associated with a differential accumbens function, may mean an increased vulnerability to compulsive eating (49) and its consequences, such as obesity and its correlates.

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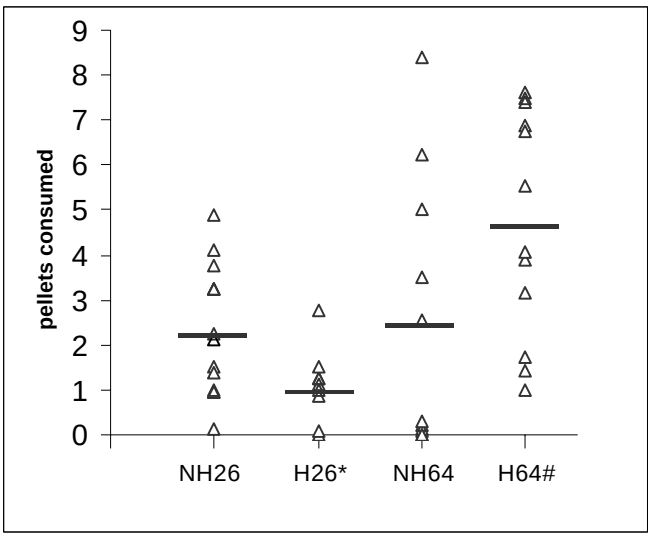
LEGENDS TO FIGURES

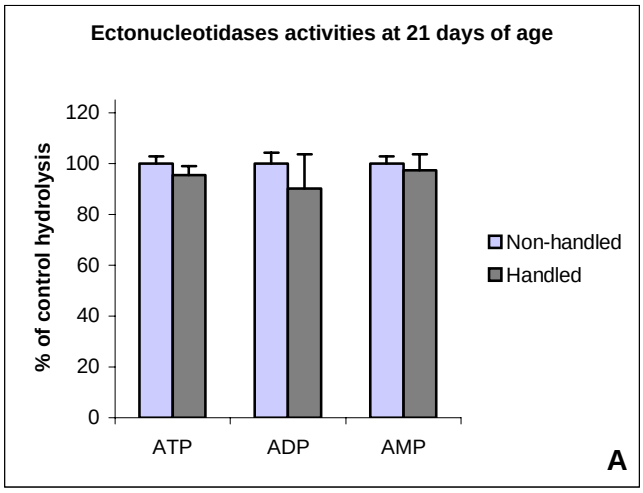
Figure 1: Sweet food consumption in neonatally handled rats before and after puberty. Data are expressed for each rat for the total number of pellets consumed during the four sessions. The black bar indicates the mean consumption in each group.

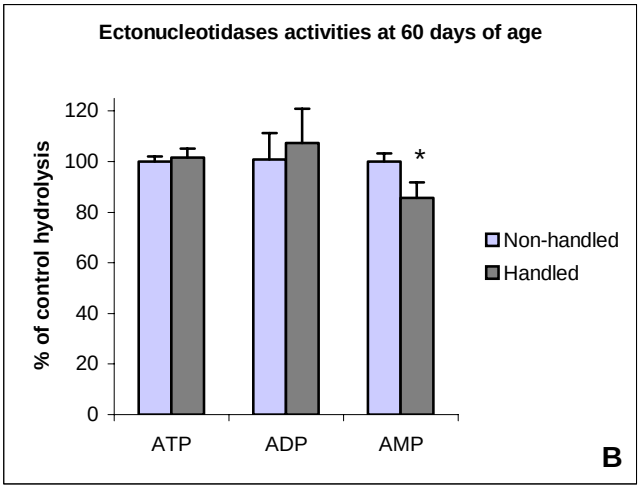
* Decreased consumption in relation to non-handled rats (Student's t test, P=0.037).

Increased consumption in relation to non-handled rats (Student's t test, P=0.05).

Figure 2: ATPase, ADPase and 5′-nucleotidase activities in the nucleus accumbens of young (A) and adult (B) neonatally handled rats. Data are expressed as mean $\pm$ S.E.M. for % of activity in control animals. *There is a decrease in 5′-nucleotidase activity in adulthood in neonatally handled rats (Student's t test, P=0.015).


pellets consumed
9
8
7
6
5
4
3
2
1
0
NH26
H26*
NH64
H64#


Ectonucleotidases activities at 21 days of age
% of control hydrolysis
120
100
80
60
40
20
0
ATP
ADP
AMP
Non-handled
Handled
A


Ectonucleotidases activities at 60 days of age
% of control hydrolysis
120
100
80
60
40
20
0
*
ATP
ADP
AMP
Non-handled
Handled
B

3.5 CAPÍTULO V:

Estudo dos efeitos da exposição à manipulação neonatal na infância e a um modelo de depressão (estresse crônico variável) na vida adulta.

Neste estudo verificamos a interação entre a manipulação neonatal e a exposição ao estresse crônico variável (ECV) na vida adulta, avaliando desfechos emocionais, metabólicos e neuroquímicos relacionados à depressão. Vimos que a manipulação neonatal leva a uma série de alterações persistente na vida adulta, como menor tempo de imobilidade na tarefa do nado forçado, maior consumo de doce e menor ganho de peso, menor atividade da Na^+,K^+-ATPase no hipocampo e maior na amígdala. Por sua vez, o também ECV diminui o ganho de peso e aumenta o consumo de doce, porém afeta os animais manipulados de forma mais tênue. O ECV também foi associado com uma diminuição da atividade da enzima Na^+,K^+-ATPase (semelhante ao que ocorre em transtornos de humor) no hipocampo, amígdala e córtex parietal, sendo que a manipulação neonatal foi capaz de atenuar esta diminuição no córtex e na amígdala. Não efeito da manipulação ou do ECV em outras variáveis metabólicas como a corticosterona basal e a resistência à insulina.

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EARLY LIFE HANDLING PROTECTS AGAINST SOME OF THE EMOTIONAL, METABOLIC AND NEUROCHEMICAL ALTERATIONS IN A RAT MODEL OF DEPRESSION

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ABSTRACT

Silveira PP; Portella AK; Diehl LA, Rosa LO, Nunes E, Benetti CS, Zugno AI, Scherer EBS, Mattos CB, Wyse ATS, Lucion AB, Dalmaz C. **Early life handling protects against some of the emotional, metabolic and neurochemical alterations in a rat model of depression**

Background: There is increasing evidence that early life events can influence neurodevelopment and later susceptibility to disease. Chronic variable stress (CVS) has been used as a model of depression. The objective of this study was to evaluate the interaction between early handling and chronic variable stress in adulthood, analyzing emotional, metabolic and neurochemical aspects related to depression.

Methods: Pups were (1) handled (10 min/day) or (2) left undisturbed from day 1 to 10 after birth. When adults, the groups were subdivided and the rats were submitted or not to CVS, which consisted of daily exposure to different stressors for 40 days, followed by a period of behavioral tasks, biochemical (plasma corticosterone and insulin sensitivity) and neurochemical (Na+, K+-ATPase activity in hippocampus, amygdala and parietal cortex) measurements.

Results: CVS diminishes the body weight gain and increases the sweet food ingestion in non-handled rats, affecting the handled group to a minor extent. Neonatally-handled rats also demonstrated shorter immobility times in the forced swimming test, independently of the stress condition. There was no difference concerning basal corticosterone or insulin sensitivity between the groups. Na^+,K^+-ATPase activity was decreased in hippocampus and increased in the amygdala of neonatally-handled rats. CVS decreased the enzyme activity in the three structures, mainly in the non-handled group. ***Conclusions***: These findings suggest that early life handling increases the ability to cope with chronic variable stress in adulthood, with animals showing less susceptibility to neurochemical features associated with depression, confirming the relevance of the precocious environment to vulnerability to psychiatric conditions in adulthood.

Key words: neonatal handling, depression, chronic stress, insulin sensitivity, Na^+,K^+-ATPase activity

During recent years, much research has been focused on early life events and their effects in adulthood. The association between low birth weight and a later risk for cardiovascular disease (Barker et al 1989; Rich-Edwards et al 1997; Eriksson et al 2001) and depression (Thompson et al 2001; Gale and Martyn 2004) is well known, as is the association between precocious nutritional experiences, growth rate and cognition (O´Connor et al 2003). These findings are often suggested to be mediated by the programming of the hypothalamus-pituitary-adrenal (HPA) axis activity (Thompson et al 2001; Gale and Martyn 2004; Jones et al 2006; Seckl 2004).

Growing evidence supports the idea that maternal care and the mother-infant interaction play a major role in these associations and programming the risk for diseases later in life. It is well known from animal research that the amount of care received in the first few days of life determines behavioral, hormonal and neurochemical aspects of the stress response, influencing especially the HPA axis activity (Liu et al 1997; Menard et al 2004; Zhang et al 2005). Studies in humans confirm that early adversity is associated with

an increased prevalence of depressive symptoms and anxiety (McCauley et al 1997), as well as altered stress responses (Heim et al 2000). Moreover, the self-reported early life parental bonding correlates with the mesolimbic dopamine release in response to stress in adulthood (Pruessner et al, 2004). However, most clinical studies are based on correlations and suffer from the enormous amount of variability in the environment that humans face during their life course.

Neonatal handling is an interesting experimental approach in which brief and repeated periods of separation from the mother are associated with an intensified maternal care when the pups are returned to the nest (Branchi et al 2001; Pryce et al 2001). This model has been used as a tool to study the physiology and the outcomes of a particular neonatal experience (Lucion et al 2003; Panagiotaropoulos et al 2004; Meaney et al 1989). In adulthood, these animals show decreased stress responses in the face of an acute stress situation (Meaney et al 1989), as well as chronic stress paradigms (Panagiotaropoulos et al 2004; Ladd et al 2005).

In addition to differential HPA axis activity, neonatally-handled rats have a decreased susceptibility to learned helplessness (Costela et al 1995), altered reproductive systems both in males (Mazaro R and Lamano-Carvalho 2006) and females (Gomes et al 2005), and altered sexual behavior in both genders (Padoim et al 2001). They also demonstrate an altered feeding behavior, ingesting more palatable food when exposed to it in comparison to controls (Silveira et al 2004) and having specific alterations in hormones linked to appetite and satiety (Silveira et al 2006, McIntosh et al., 1999). These animals also generally do better in memory tasks (Tang 2001, Meaney et al 1989).

Interestingly, major depression is a state in which the same broad range of aspects cited above is affected. For instance, depressed patients show the characteristic helplessness

associated with altered HPA axis activity (Wolff et al 1964; Samson et al 1992; Croes et al 1993; Ilgen and Hutchison 2005). Descriptions of co-morbidity between depression and feeding disturbances are also common (Gerke et al 2006, Dunkley & Grilo 2006). Recently, reports of insulin resistance detected in patients diagnosed with depression have been published (Timonen et al 2006). Negative mood states are typically associated with altered sexual function (Bancroft et al 2003, Kuffel & Heiman 2006) and memory impairments (Raes et al, 2006).

The chronic variable stress (CVS) protocol has been proposed as an animal model of depression for its validity in different profiles related to the depressed state (Willner, 2005). The protocol involves repeated exposures to different mild stressors over a certain period of time (Katz, 1982, Willner et al, 1987, Gamaro et al, 2003). This procedure has been widely studied over the last three decades, typically being associated with reduced consumption of a preferred dilute sucrose solution (Willner et al, 1987) or sweet pellets (Gamaro et al, 2003a), and reversal of this state by chronic treatment with antidepressant drugs (Willner et al, 1987).

At the neurochemical level, a decreased Na^+,K^+-ATPase activity in the hippocampus of animals submitted to chronic mild stress has been previously demonstrated and this was reversed by chronic antidepressant treatment (Gamaro et al, 2003b). This finding agrees with earlier studies, which have shown that Na^+,K^+-ATPase activity is decreased in patients with depression and other psychiatric disorders (Hokin-Neaverson & JeVerson, 1989, Wood et al, 1989, Goldstein et al., 2006). Na^+,K^+-ATPase is an integral membrane protein complex responsible for establishing the electrochemical gradients of Na^+ and K^+ ions across the plasma membranes of mammalian cells. This complex is present in high

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

In light of the suggestion that neonatal handling and depression affect the same general domains, we decided to observe the interaction between early handling and adult chronic variable stress on outcomes linked to depression, such as: sweet food ingestion and forced swimming test, body weight, insulin sensitivity, basal corticosterone and Na^+,K^+-ATPase levels in different brain structures. Our hypothesis was that neonatally-handled animals would respond differently to chronic stress exposure, possibly being more resilient through increased maternal care effects.

Methods and Materials

Subjects. **Pregnant Wistar rats bred at our own animal facility were randomly selected. They were housed alone in home cages made of Plexiglas (65 x 25 x 15 cm) with the floor covered with sawdust and were maintained in a controlled environment until offspring: lights on between 07:00h and 19:00h, temperature of 22 ± 2°C, cage cleaning once a week, food and water provided. All litters were culled within 24 h to eight pups and were maintained intact unless for handling procedures, which were carried out between 10:00h and 12:00h. Included in this period were the time to set up the incubator, to bring the cages from the facility and briefly habituate the dams to the new room, to perform careful removal of the pups from the nest, the time of handling per se, the return of the pups to the dam and, again after a brief period, to return the cage to the facility room. The researcher also changed gloves for the manipulation of each litter to avoid the spread of any kind of odor from nest to nest.**

Weaning was on postnatal day 21. One or two male pups were used per litter per experiment. Rats were housed four per cage in home cages similar to those described above. Fifty-two experimental male rats were used in the different experiments, derived from 16 different litters. Rats had free access to food (standard lab rat chow) and water,

except during the period when the behavioral tasks were applied. Tasks were performed between 13:00h and 16:00h.

Neonatal Handling model**. In the non-handled group, pups were left undisturbed with the dam until weaning. It was stated on the cage that these animals should not be touched, not even for cage cleaning. Dirty sawdust was carefully removed from one side of the cage, without disturbing the mother and the nest, and replaced by clean sawdust at the same side by the principal researcher. In the handled group, pups were removed from their home cage and placed into a clean cage lined with clean paper towel, inside an incubator at 34° C for 10 minutes, being returned to their dams (which stayed in the home cage, next to the incubator) afterwards. This procedure was carried out for the first ten days of life, after which pups were left undisturbed until weaning.**

Chronic variable stress protocol**. Chronic variable stress model was modified from other models of mild stress (Gamaro et al., 2003 a, b, Vasconcellos et al., 2005). At the age of 100 days, the animals were weighed and subdivided in four groups: non-handled control and chronically stressed, neonatally handled control and chronically stressed. A variate-stressor paradigm was used for the animals in the stressed groups. The following stressors were used: (i) 24 h of food deprivation; (ii) 24 h of water deprivation; (iii) 1 h of restraint, as described below; (iv) 1 to 3h exposure to cold (4°C); (v) 10–15min of noise; (vi) flashing light during 120–210 min as described below; (vii) inclination of the home cages at a 45° angle for 4–6 h, and (viii) isolation (2–3 days). Stress exposure started at different times every day, to minimize its predictability. Please refer to table 1 to see the distribution of the stressors over the 40 days period of CVS exposure.**

Table 1 - Please insert about here

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. Exposure to flashing light was made by placing the cage 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 in a frequency of 60

flashes/min, was used. The animals were weighed again at the end of the 40 days of chronic stress treatment. The habituation and test for sweet food started on the next day, and the stress protocol was kept for the stressed group during this period. Following this period, the forced swimming test was performed and since the test itself is stressful (Andrade et al, 2007), the animals were not stressed on these two days (see details below).

Habituation and test for sweet food ingestion. *To habituate the rats to the sweet food, we used the protocol described previously by our group (Silveira et al 2004, 2006). Briefly, animals were exposed to ten Froot loops (Kellogg's ® - pellets of wheat and cornstarch and sucrose) in a lightened rectangular box (40 x 15 x 20 cm) with floor and side walls made of wood and a glass ceiling, during 5 days, 3 minutes per day. This was done under food restriction (receiving about 80% of habitual ingestion). From our previous experience, after this habituation period each animal eats a mean of 500mg of sweet food. Animals that never ate any Froot loops during habituation were not considered for the analysis. In the testing day, the animals were transferred to another cage similar to their homecage. All rat chow was removed from their cages. Afterwards, animals were returned to their original homecage one by one, and twenty Froot Loops pellets were offered, for ten minutes. The amount ingested was measured.*

Forced swimming test. Two trials were given to the rats in which they were forced to swim in an inescapable polyvinyl carbonate cylinder aquarium, 60 cm in height and 30 cm in diameter filled with 30 cm tap water at 24°C. Rats were placed into the tank for 15 min on day 1 to induce a state of "helplessness." The rats were then dried off with a towel, and placed back into their home cage. Twenty-four hours after this initial 15 min test, a 5 min test was conducted (Porsolt et al, 1978) After placing rats individually in the pool, they display vigorous activity and then adopt an immobile posture characterized by floating with the head just above the water surface, making very little (but enough to keep from drowning) movement with their body. This immobility behavior was scored using a chronometer on the test day.

Plasma collection and biochemical measurements. Animals were sacrificed by decapitation 24 h after the last stress session, being fasted in the previous 6 hours. The trunk blood was collected into heparinized tubes for insulin, glucose and corticosterone determination. The tubes were centrifuged at 4^{o}C and plasma was separated and frozen until the day of analysis. Hormonal measurements were performed with commercial rat ELISA kits: Cayman Chemical Co., Ann Arbor, MI, USA for corticosterone evaluation, and Alpco Diagnostics, Mercodia AB, Uppsala, Sweden to measure insulin. Plasma glucose was measured by the

glucose oxidase method using a commercial kit, BioSystems, Barcelona, Spain. Insulin resistance was evaluated using the Quantitative Insulin Sensitivity Check Index (QUICKI), defined by 1/[log(fasting insulin) + 1 log(fasting glucose)] (Katz et al, 2000, Potenza et al, 2005).

Neurochemical studies. After decapitation, the brain was quickly removed and the hippocampus, amygdala and parietal cortex were dissected. For preparation of synaptic plasma membranes and determination of Na^+,K^+-ATPase activity, the structures were homogenized in 10 vol. 0.32 M sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4. After homogenization, synaptic plasma membranes were prepared and the activity of Na^+,K^+ATPase was determined. Synaptic plasma membranes were prepared according to the method of Jones and Matus (1974) with some modifications (Wyse et al., 1995, Wyse et al., 2000). They were isolated using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000 × g for 110 min, the fraction between the 0.8 and 1.0 sucrose interface was taken as the membrane enzyme preparation. The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 μl. The reaction was initiated by the addition of ATP. Controls were carried out 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, according to the method of Tsakiris and Deliconstantinos (1984). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein. Protein was measured by the method of Lowry et al. (1951) or Bradford (1976) using bovine serum albumin as standard.

Statistical analysis. **Data were expressed as mean ± standard error of the mean, and were analyzed by Two Way ANOVA (Downe & Heath, 1970). The significance level was accepted as different when the *P* value was equal or less than 0.05. Sample size varies in each experiment and is shown individually in the Results section.**

Results

Body weight gain

The weight gain during the 40 days of treatment is displayed in Figure 1. There was an effect of the group, in which neonatally-handled rats in general gain less weight than non-handled rats [Two-Way ANOVA, $F(1, 36) = 13.691$, $P = 0.001$, n= 7-12/group], as well as an effect of chronic stress that decreased the weight gain [$F(1, 36) = 16.166$, $P < 0.0001$]. An interaction between the neonatal intervention and the chronic stress exposure in adulthood was also observed, where the decrease in the weight gain observed in animals subjected to both treatments did not represent the sum of both effects [$F(1, 36) = 4.983$, $P = 0.032$].

Figure 1 – please insert about here.

Sweet food ingestion and forced swimming test

Chronic stress was observed to increase the sweet food ingestion [Two-Way ANOVA, $F(1, 36) = 10.144$, $P = 0.003$, n=7-12/group], while no effect of the neonatal intervention was detected [$F(1, 36) = 3.492$, $P = 0.071$]. There was an interaction between the neonatal environment and chronic stress exposure [$F(1, 36) = 5.836$, $P = 0.021$], since the consumption in the group subjected to both treatments did not reflect an addition of the individual effects presented by the two factors (see Figure 2).

Figure 2 – please insert about here.

The handled rats were less prone to demonstrate immobility behavior in the forced swimming test (effect of the neonatal group seen by Two-Way ANOVA, [$F(1, 42) = 8.370$, $P = 0.006$, n=10-12/group]. There was no effect of the chronic stress [$F(1, 42) = 0.8$, $P = 0.377$] and no interactions. Please refer to Figure 3.

Figure 3 – please insert about here.

Plasma glucose, insulin and corticosterone

Plasma glucose was not different between groups [Two-Way ANOVA, $F(1, 31) = 0.491$, $P = 0.489$, n=7-9/group] nor stress condition [$F(1, 31) = 0.140$, $P = 0.711$], and no interactions were seen. Insulin was not affected by the neonatal environment [$F(1, 31) = 1.005$, $P = 0.325$]. Although insulin was decreased in rats submitted to chronic stress [$F(1, 31) = 4.349$, $P = 0.046$], the insulin resistance index (QUICKI) showed no differences between neonatal groups [$F(1, 31) = 0.64$, $P = 0.803$] or stress condition [$F(1, 31) = 1.453$, $P = 0.238$] and no interactions. Basal plasma corticosterone was also not affected by the early life experience [Two-Way ANOVA, $F(1, 26) = 2.699$, $P = 0.114$, n=6-8/group] nor chronic stress in adulthood [$F(1, 26) = 0.007$, $P = 0.933$], and no interactions were noted. See Table 2.

Table 2 – please insert about here.

Na^+,K^+-ATPase activity in different brain structures

Chronic stress induced a decrease in Na^+,K^+-ATPase activity in the three brain structures analyzed [Two Way ANOVA – hippocampus: $F(1, 19) = 18.150$, $P = 0.001$, n=5-6/group; amygdala: $F(1, 22) = 4.552$, $P = 0.046$, n=5-6/group; parietal cortex: $F(1, 18) = 5.848$, $P = 0.029$, n=4-5/group]. Decreased Na^+,K^+-ATPase activity was observed in the hippocampus of neonatally-handled rats [$F(1, 19) = 6.277$, $P = 0.023$,] without interaction between the two interventions (Figure 4A). On the other hand, in the amygdala, the enzyme activity was increased in neonatally-handled rats [$F(1, 22) = 5.182$, $P = 0.035$,], without interactions (Figure 4B). In the parietal cortex, there was no effect of the early life experience [$F(1, 18) = 0.977$, $P = 0.339$]. However, an interaction between the early experience and chronic stress exposure in adulthood was observed [$F(1, 18) = 6.992$, $P = 0.018$], due to the fact that Na^+,K^+-ATPase activity was decreased by chronic stress in non-handled rats but not in the handled ones (Figure 4C).

Figure 4 – please insert about here.

DISCUSSION

In this study, we showed that neonatal handling induces a series of persistent alterations in adulthood, from behavioral to neurochemical parameters. Handled animals demonstrated shorter immobility time in the forced swimming test, greater sweet food ingestion, slower weight gain and a specific pattern of Na^+,K^+-ATPase activity in different brain regions (lower in the hippocampus and higher in the amygdala). We also observed that these rats were less prone to have their basal condition influenced by the exposure to a chronic variable stress in adulthood.

The current results agree with other studies, which have suggested that neonatal handling is associated with a greater ability to cope with a repeated chronic forced swimming stress in males but not in females (Papaioannou et al, 2002), which show more vulnerability to depression after chronic stress. These effects possibly occur due to sex specific alterations in serotonin 1A sub-type receptor mRNA, protein and binding sites, with females having higher levels than males (Stamatakis et al, 2006). Neonatal handling is also associated with a reduced helplessness behavior using inescapable shock (Costela et al, 1995), possibly through noradrenergic mediation (Tejedor-Real et al, 1998).

In our experiments, neonatally-handled rats have a peculiar behavior when faced with palatable food, ingesting more sweet food and savory pellets in relation to non-handled rats, but also demonstrating a more sensitive mechanism of satiation and altered plasma ghrelin levels (Silveira et al., 2004, 2006). The current study demonstrated that the effect of neonatal handling increasing the sweet food consumption was diluted after chronic variable stress exposure, mainly because CVS increased the ingestion in non-handled rats, while handled ones essentially kept the same pattern independently of the stress situation. Although a classic effect of the CVS paradigm is to decrease the sweet food preference, some reports have also found an increase in sucrose solution ingestion after chronic variable stress (Murison & Hansen, 2001). After a chronic stress exposure, the glucocorticoids, insulin and signals from the abdominal fat depots are linked to the enhanced consumption of highly palatable “comfort food” (Pecoraro et al, 2004), leading to metabolic disturbances if this type of food is constantly available (Dallman et al., 2004). Therefore, the fact that neonatally handled rats do not change their baseline status regarding palatable food ingestion and body weight may imply that they are less prone to suffer from these metabolic outcomes after a chronic stress exposure in adulthood.

Insulin resistance is another feature currently associated with depression (Timonen et al 2006). Hypercortisolemia has been suggested as the main factor leading to a disturbed glucose utilization in depressed patients (Weber-Hamann et al., 2005). In our study, although CVS decreased plasma insulin levels, we did not find a difference regarding the insulin resistance index QUICKI between the groups.

Evidences show that neonatally-handled rats have a decreased response to acute stress, demonstrating plasma corticosterone levels that return to baseline faster after an acute insult, with a more efficient glucocorticoid negative feedback due to an increased level of glucocorticoid receptors in the hippocampus (Meaney et al 1989) and decreased in the amygdala (Fenoglio et al., 2004). Interestingly, Na^+,K^+-ATPase has subunits that are responsive to glucocorticoids (Thompson et al., 2001). In this study, we showed that neonatally handled rats had a decreased enzyme activity in the hippocampus and increased in the amygdala. The differential pattern of central glucocorticoid distribution in neonatally handled rats could be accounting for the differential pattern of Na^+,K^+-ATPase activity seen in the baseline in these animals.

The brain Na^+,K^+-ATPase activity decreases with age, and it seems to contribute to the age-related brain deterioration (Kaur et al., 1998). Accumulating evidence proposes that this enzyme may be involved in the etiology of mood disorders in animal models in the brain (Gamaro et al, 2003) and peripheral tissues in humans (Hokin-Neaverson & Jefferson, 1989, Nurnberger et al., 1982). Recently, the first report of a decreased brain activity in the Na^+,K^+-ATPase in the parietal cortex in depressed patients was published (Goldstein et al., 2006). Interestingly, in our study, CVS induced a decrease in the enzyme activity in this brain region, and neonatal handling was able to protect against this effect. In the hippocampus and amygdala, CVS also decreased the Na^+,K^+-ATPase activity as previously demonstrated (Gamaro et al., 2003), but this effect was less evident in neonatally handled rats for their baseline characteristics described above.

As stated in the Introduction, it is worthy of note that most of the effects of neonatal handling on the stress responses may be explained by the induction of an increase in maternal care levels. Naturally occurring variations in the amount of care received during the first few days of life determine behavioral, hormonal and neurochemical aspects of the HPA axis activity (Liu et al 1997; Menard et al 2004; Zhang et al 2005). Maternal care regulates the hippocampal glucocorticoid receptor gene expression through epigenetic processes such as acetylation and methylation (Weaver et al 2004). As a result, the adult offspring raised in an environment with high amounts of maternal care show decreased startle responses, increased open-field

exploration (Caldji et al 1998; Francis et al 1999) and reduced plasma ACTH and corticosterone responses to acute stress (Liu et al 1997) in comparison to the adult offspring from environments with low maternal care.

Interestingly, it is common for patients with depression to demonstrate a hyperactive HPA axis (Plotsky et al., 1998), and treatment with antidepressants is usually associated with normalization of HPA axis activity (Salee et al., 1995). Although we did not find a difference in basal corticosterone between the groups in our study, we could propose that neonatal handling leads to a persistently differential functioning of several systems, resulting in a characteristic pattern of activity in behavioral, metabolic and neurochemical outcomes. Our results suggest that, when exposed to a rat model of depression, handled animals have a lower tendency to have their baseline status affected by the chronic adversity, possibly due to an enhancement in maternal care levels during the neonatal period (Branchi et al., 2001).

In summary, we propose early life experience influences the ability to cope with chronic stress in adulthood. In the case of neonatal handling, it seems that this brief, repeated separation from the mother in the postnatal period was able to modulate the way that these animals deal with some effects induced by the exposure to a rat model of depression in adult life, probably acting through increased maternal care effects. This experimental paradigm is, therefore, an important model to study the pathophysiology of the mood disorders and to contribute to the enlightenment of future therapeutic approaches.

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LEGENDS TO FIGURES AND TABLE

Figure 1: Weight gain after the chronic stress period. Data are expressed as mean $\pm$ S.E.M. for grams of body weight gained between the start and the end of chronic variable stress (40 days). *Neonatally-handled rats showed a decreased body weight gain in relation to non-handled ones (Two-Way ANOVA, $P = 0.001$). #CVS decreases weight gain in both groups ($P < 0.0001$), but an interaction between early experience and chronic stress exposure was also observed, $P = 0.032$.

Figure 2: Sweet food consumption in the homecage. Data are expressed as mean ± S.E.M. for grams of pellets consumed. #CVS leads to a greater ingestion of sweet food (Two-Way ANOVA, P = 0.003). An interaction was observed between the early environment and adult stress exposure (P=0.021).

Figure 3: Immobility time in the forced swimming test. Data are expressed as mean ± S.E.M. of times in seconds. * A decreasing effect of neonatal handling on immobility time may be observed (Two-Way ANOVA, P = 0.006), but no effect of the CVS nor interactions.

Figure 4: Na+, K+-ATPase activity (nmol inorganic phosphate released per min per mg protein) in different brain structures. Data are shown as mean ± S.E.M. A-Hippocampus – both *neonatal handling (Two Way ANOVA, P=0.023) and #CVS (P=0.001), decreased the enzyme activity. No interactions were seen. B-Amygdala – * neonatal handling increased the enzyme activity (P=0.035), while #CVS decreased it (P=0.046), without interactions. C- Parietal cortex – #CVS decreased the enzyme activity (P=0.029), and there was an interaction between CVS and the neonatal experience (P=0.018).

Table 1: Schedule of stressor agents used during the chronic treatment.

Table 2: Plasma glucose, insulin, QUICKI and corticosterone measurements in nonhandled and neonatally handled rats with or without chronic stress exposure in adulthood. Data are expressed as mean ± S.E.M. for each measurement. QUICKI=Quantitative Insulin Sensitivity Check Index (see text for details). #CVS decreased the plasma insulin levels (Two-Way ANOVA, $P = 0.046$).

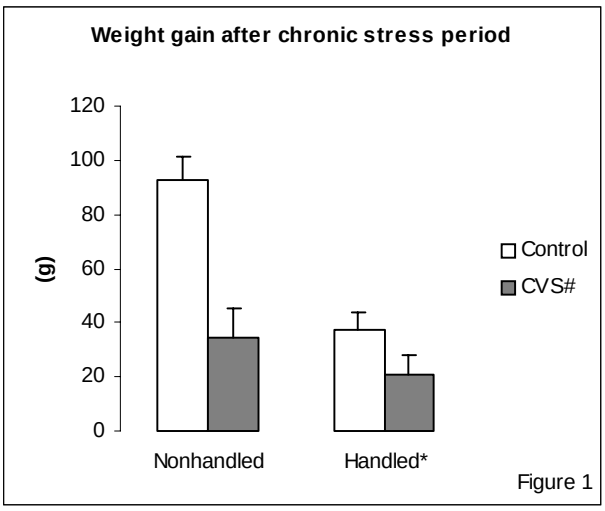


Figure 1

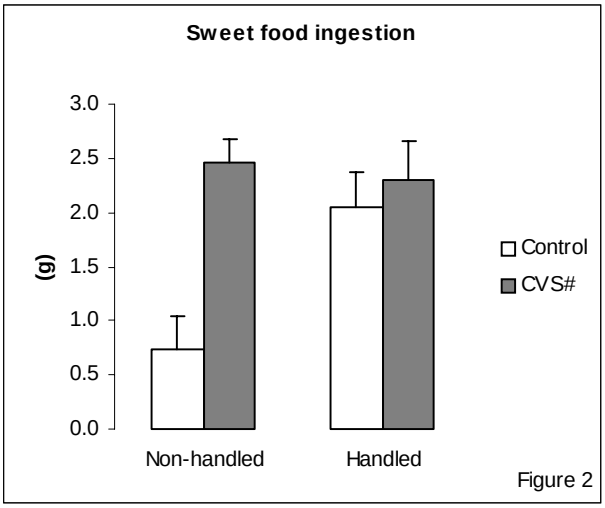
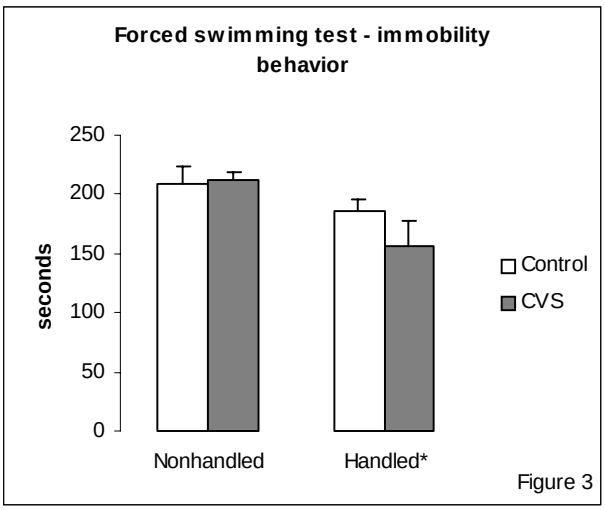


Figure 2


Forced swimming test - immobility behavior
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seconds
Nonhandled
Handled*
Control
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Figure 3

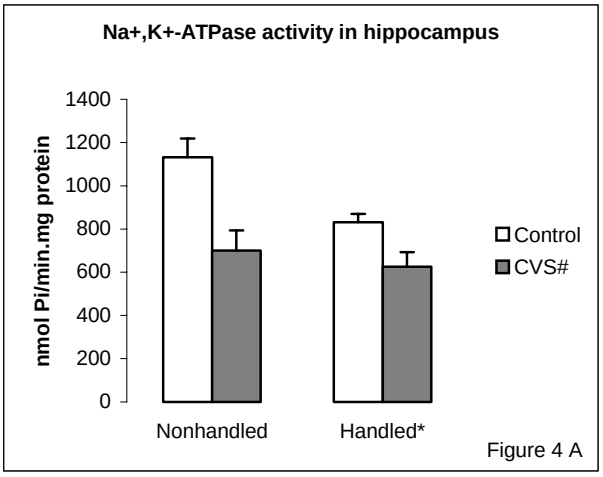


Figure 4 A

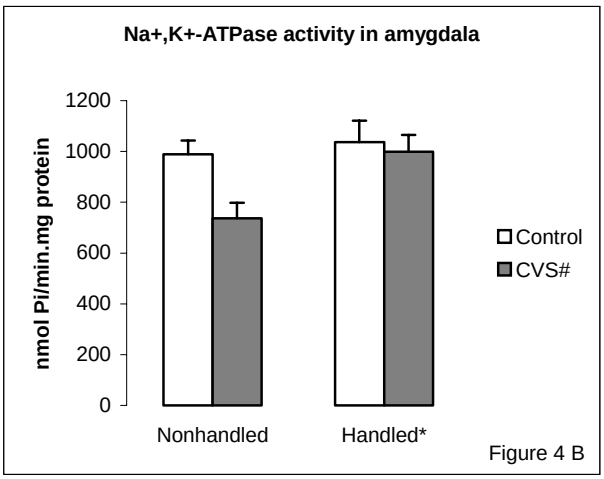


Figure 4 B

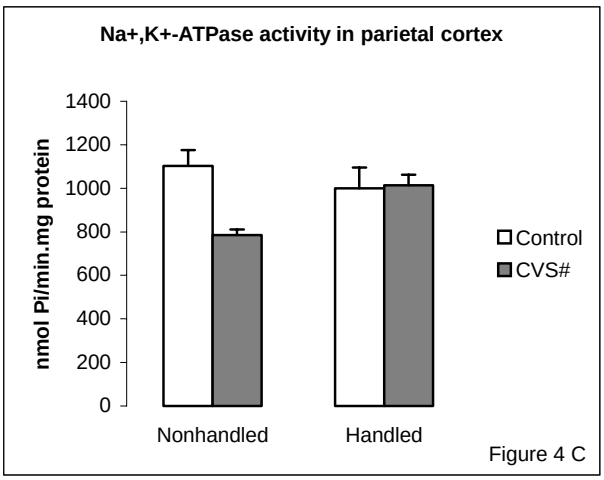


Figure 4 C

Table 1

Day of treatment	Stressor used
1	Noise (15min)
2	Flashing light (4h)
3	Water deprivation (24h)
4	Inclination of homecages (4h)
5	Isolation
6	Isolation
7	Isolation
8	Food deprivation (24h)
9	No stressor applied
10	Exposure to cold (1h)
11	Noise (15 min)
12	Restraint (1h)
13	Flashing light (4h)
14	No stressor applied
15	Inclination of homecages (5h)
16	Food deprivation (24h)
17	Noise (15 min)
18	Restraint (1h)
19	Isolation
20	Isolation
21	Isolation
22	Exposure to cold (1h)
23	Flashing light (4h)
24	Water deprivation (24h)
25	No stressor applied
26	Isolation
27	Isolation
28	Isolation
29	Inclination of homecages (5h)
30	Exposure to cold (1h)
31	Food deprivation (24h)
32	Noise (15 min)
33	Isolation
34	Isolation
35	Isolation
36	No stressor applied
37	Water deprivation (24h)
38	Inclination of homecages (5h)
39	Flashing light (3h)
40	Restraint (1h)

Table 2

Measurement	Nonhandled group		Neonatally handled group	
	Control	CVS	Control	CVS
Plasma glucose (mg/dl)	114.6 ± 4.3	120.5 ± 3.0	115.3 ± 5.9	113.0 ± 5.2
Plasma insulin (ng/ml)	2.1 ± 0.6	0.9 ± 0.2#	2.6 ± 0.6	1.5 ± 0.6#
QUICKI	0.46 ± 0.03	0.5 ± 0.02	0.47 ± 0.06	0.53 ± 0.04
Plasma corticosterone (ng/ml)	225.0 ± 52.8	248.6 ± 37.1	180.8 ± 30.6	163.8 ± 29.1

3.6 CAPÍTULO VI

Os seguintes dados são provinientes de um estágio de doutorado no exterior no laboratório do Dr. Michael Meaney (Developmental Neuroendocrinology Laboratory, Douglas Hospital Research Centre, Departments of Psychiatry, and Neurology and Neurosurgery, McGill University, Montreal, Canada).

Neste estudo avaliamos se variações naturais do cuidado materno nos primeiros dias de vida influenciam o consumo de alimentos palatáveis na vida adulta dos filhotes, assim como o peso corporal, a deposição de gordura abdominal e níveis plasmáticos de grelina, insulina, leptina e corticosterona. Além disso, avaliamos o consumo e a preferência alimentar desses animais oferecendo alimento doce e ração padrão continuamente por sete dias. Animais provenientes de mães altamente cuidadoras ingerem mais alimento palatável (doce e salgado) em relação a filhotes de mães pouco cuidadoras. No entanto, durante uma exposição prolongada, estas diferenças são observáveis apenas nos dois primeiros dias. Não há diferenças nos níveis basais de insulina, leptina, corticosterona, e deposição de gordura abdominal, mas animais filhos de mães altamente cuidadoras têm maior peso corporal nesta idade e apresentam menores níveis de grelina em relação aos filhotes de mães pouco cuidadoras.

Apresentado sob forma de resultados adicionais.

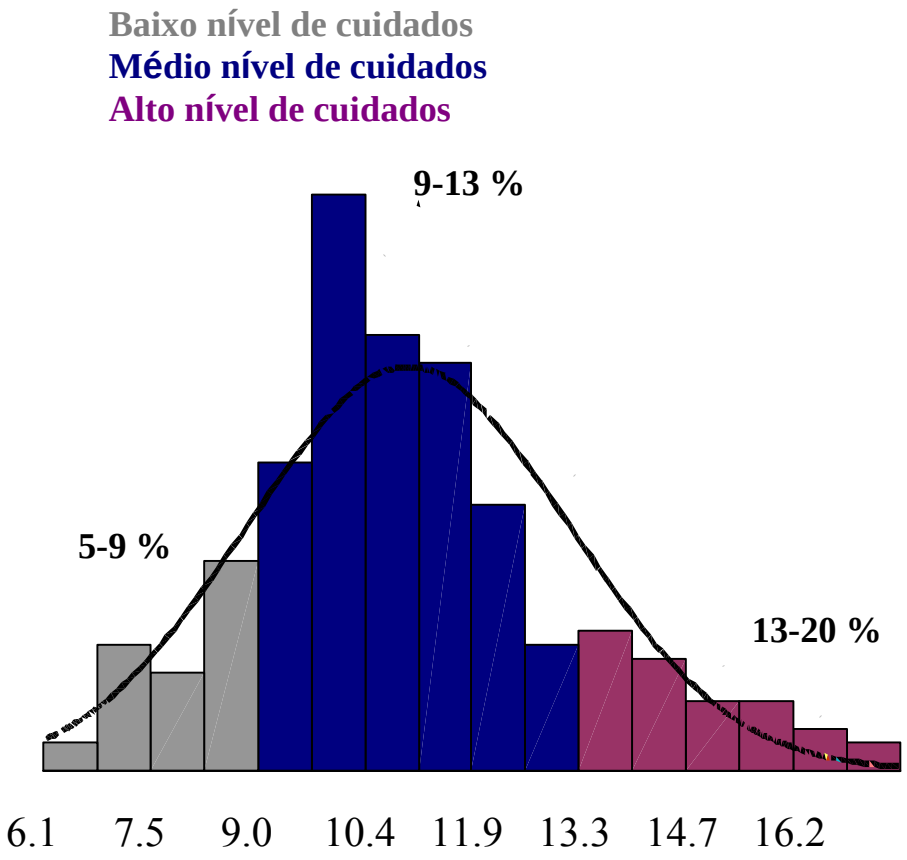
MATERIAIS E MÉTODOS

Animais experimentais

Ratas Long-Evans provenientes do Centro de Pesquisas do Hospital Douglas eram mantidas isoladas em caixas-moradia de policarbonato contendo maravalha, num ciclo normal claro/escuro de 12 horas, com ração padronizada e água "ad libitum". Seus filhotes foram agrupados conforme a intensidade de cuidado maternal durante a infância (ver abaixo). O desmame ocorrereu aos 21 dias e então os animais foram agrupados em 2 filhotes da mesma ninhada, sendo não mais do que dois filhotes de cada ninhada usados por grupo. Os procedimentos foram realizados de acordo com os padrões do *Canadian Council for Animal Care* e aprovados pelo *McGill University Animal Care Comitee.*

Observação dos Cuidados Maternais

O comportamento maternal de cada genitora era observado 5 vezes ao dia (07:00h, 10:00h, 13:00h, 17:00h e 20:00h), nos primeiros 6 dias após o parto, sendo o dia de nascimento considerado como dia 0. Para cada período de observação, os seguintes parâmetros de cuidados maternais eram observados, por um período de 112 minutos: a) freqüência de lambidas, b) freqüência de comportamento de amamentação (dorso arqueado) nos diferentes níveis, c) com ou sem contato com o filhote, d) mãe recolocando os filhotes no ninho e e) ninhada espalhada. Ao desmame, os filhotes são categorizados conforme a percentagem total do tempo de cuidado da mãe. Mães com alto nível de cuidado ("*High*") demonstram percentagens de tempo no mínimo de 1 desvio padrão acima da média da coorte inteira, enquanto mães com baixo nível de cuidado ("*Low*") demonstram percentagens de tempo no mínimo de 1 desvio padrão abaixo da média da coorte inteira. O cuidado materno se distribui na população de modo normal, sendo que o gráfico de distribuição típico é o seguinte:



Consumo de alimentos palatáveis

Quando adultos, os animais foram colocados numa caixa de madeira retangular de 40 X 15 X 20 cm. Vinte rosquinhas doces ou salgadas (Froot Loops, da Kellogg's® ou O´Grilled ®) foram deixadas numa das extremidades da caixa, dependendo do tipo de alimento que se queria testar. Cada animal foi submetido a 5 dias de habituação com 5 minutos de exposição ao alimento novo. Durante esse período os ratos permaneceram em restrição alimentar. No sexto dia foi feito o teste, semelhante à habituação porém com os animais alimentados à vontade nas 24 horas prévias.

Exposição prolongada ao doce – um grupo de animais foi exposto ao Froot Loops + ração padrão à vontade, ambos na caixa moradia, por 7 dias. Era colocada uma quantidade conhecida de alimento doce no primeiro dia e no dia seguinte o alimento era novamente pesado, sendo que a diferença correspondia ao consumo.

Peso corporal

Os animais eram pesados sendo colocados gentilmente em uma caixa de acrílico sobre uma balança de precisão de 1g previamente zerada e o peso era verificado.

Gordura abdominal e coleta de plasma

Os animais foram sacrificados por decapitação e o sangue coletado em tubos plásticos contendo 1 ml de aprotinina e EDTA 1:9, sendo após centrifugados a 2500 rpm por 10 minutos para obtenção do plasma. Este foi armazenado a –80°C até as análises bioquímicas. Para medida da grelina o plasma foi misturado a uma solução de ácido clorídrico 1 N para facilitar a conservação do peptídeo.

Os dois maiores depósitos de gordura intra-abdominal (epididimal e perirrenal) foram dissecados e pesados em balança de precisão 0.0001.

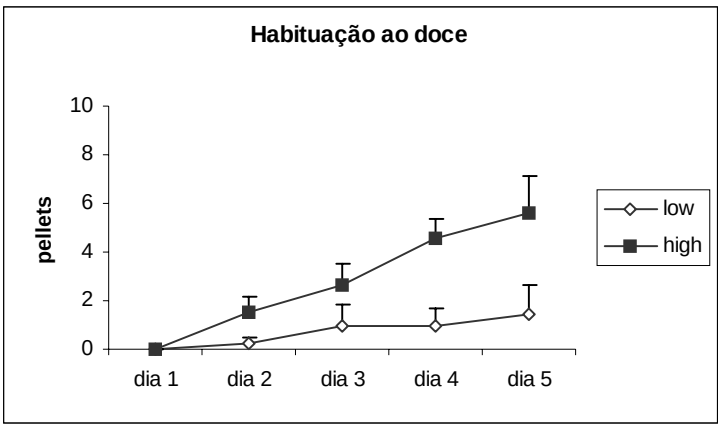
Medidas bioquímicas

Foram usados kits de ELISA da empresa LINCO RESEARCH para as medidas de leptina, grelina e insulina. A corticosterona foi medida por radioimunoensaio utilizando padrões próprios do laboratório.

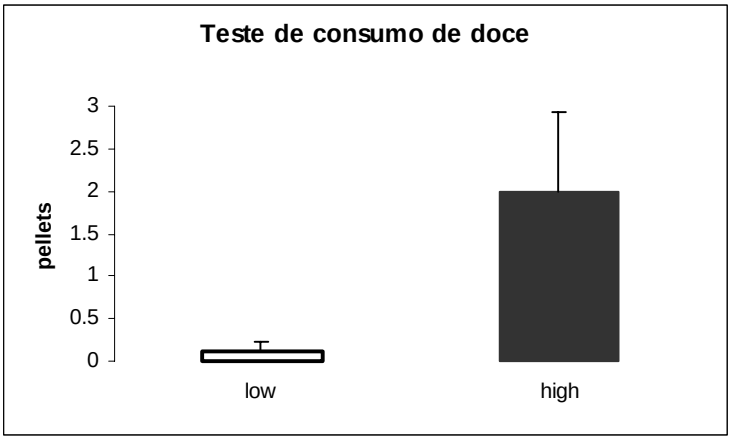
RESULTADOS

Consumo de alimentos palatáveis

Consumo de doce - Durante a habituação há efeito dos dias, uma vez que os ratos aumentam o consumo conforme os dias passam (ANOVA de medidas repetidas, $P<0.0001$). Há também efeito do grupo, em que animais filhos de mães altamente cuidadoras (*High*) ingerem mais doce que animais filhos de mães pouco cuidadoras (*Low*) ($P<0,03$). Há também interação dias X grupo, em que o aumento com o passar dos dias parece ser mais evidente em animais *High* ($P<0,01$). O teste demonstra uma tendência de diferença entre os grupos (Teste t de Student, $P=0,073$). N=9-10 por grupo.



Habituação ao alimento doce. Os dados são apresentados como média ± erro padrão. Efeito dos dias (P<0,0001), do grupo (P=0,03) e interação grupo X dias (P=0,01).



Teste de consumo de doce. Os dados são apresentados como média ± erro padrão. Diferença entre os grupos quase atinge significância estatística (P=0,073).

Consumo de alimento palatável salgado - Durante a habituação há efeito dos dias, uma vez que os ratos aumentam o consumo conforme os dias passam (ANOVA de medidas repetidas, P<0,0001). Há também efeito do grupo, em que animais filhos de mães altamente cuidadoras (*High*) ingerem mais salgado que animais filhos de mães pouco cuidadoras (*Low*) (P=0,015). Não há interação dias X grupo. O teste demonstra diferença significativa entre os grupos (Teste t de Student, P=0,009). N=9-10 por grupo.

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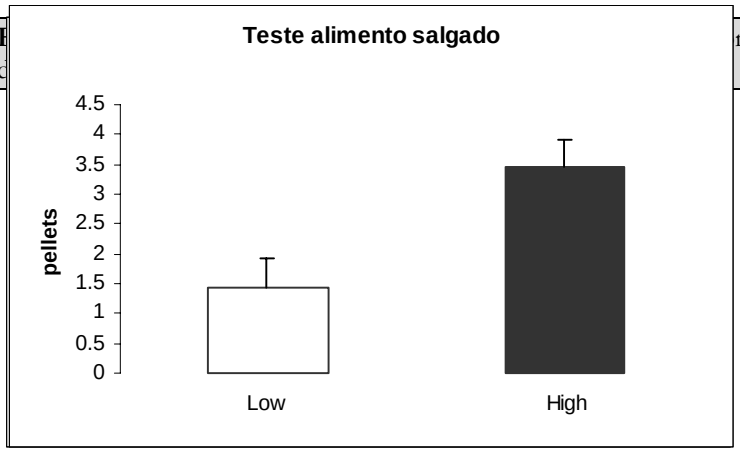
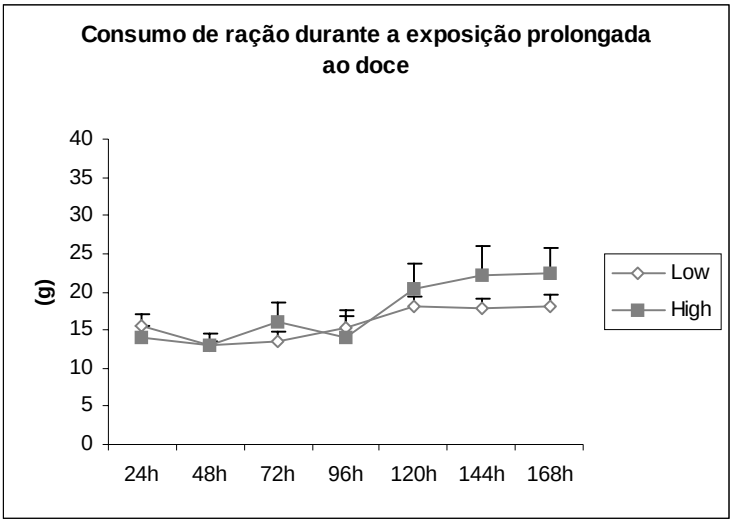


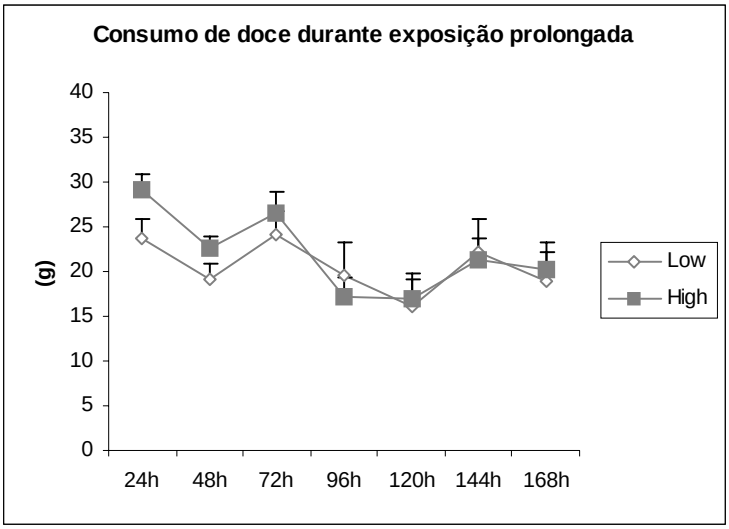
Teste do consumo de alimento salgado. Os dados são apresentados como média ± erro padrão. Efeito do grupo (P=0,009).

Exposição prolongada ao doce – Não há efeito do grupo no consumo de ração durante os dias de exposição ao doce (ANOVA de medidas repetidas, P=0,590). Há efeito dos dias, em que os animais gradualmente aumentam o consumo de ração com o passar do tempo. A interação entre grupo e dias não atingiu significância (P=0,08).

Não há diferenças entre os grupos no consumo de doce em geral (ANOVA de medidas repetidas, P=0,689). Há efeito dos dias, em que os animais gradualmente diminuem o consumo de doce com o passar do tempo. Uma interação entre grupo e dias quase atingiu significância (P=0,06). Vê-se na figura que animais *High* comem mais doce nos dois primeiros dias de exposição prolongada (análise isolada de cada dia através de teste t de Student, P<0,05 para dias 1 e 2). N=8 por grupo.



Consumo de ração durante exposição prolongada ao doce. Os dados são apresentados como média ± erro padrão. Efeito dos dias (P<0,05), sem diferenças entre os grupos. A interação grupo X dias não atinge significância (P=0,08).

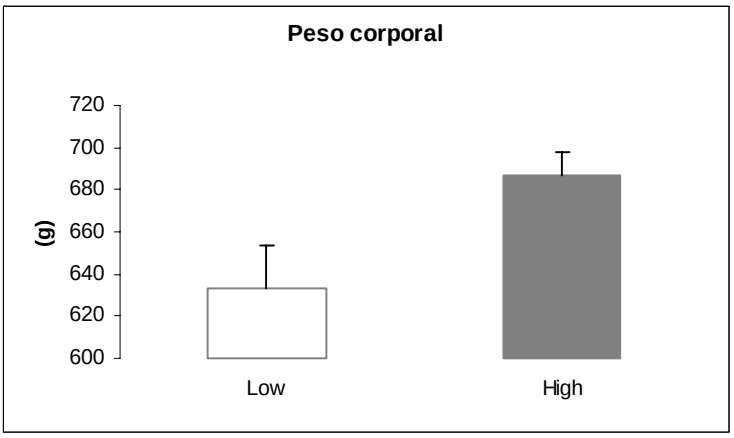


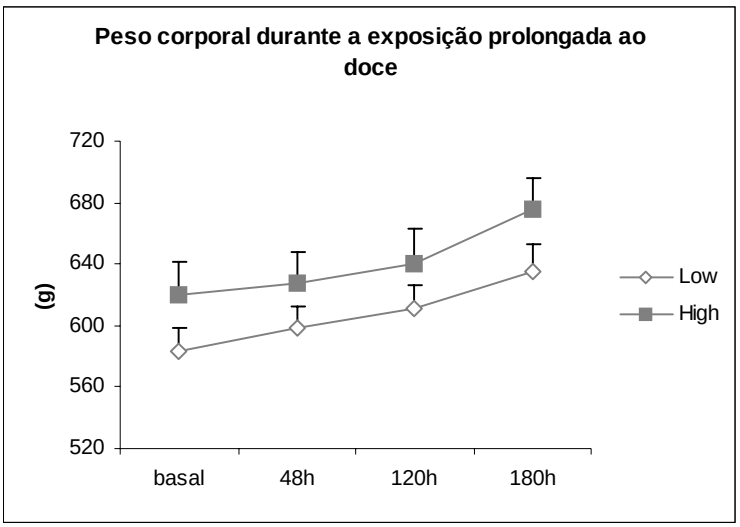
Consumo de doce durante a exposição prolongada. Os dados são apresentados como média ± erro padrão. Efeito dos dias (P<0,05), sem diferenças entre os grupos. A interação grupo X dias quase atinge significância (P=0,06). Análise isolada de cada dia revela que animais *High* ingerem significativamente mais doce nos dois primeiros dias de exposição.

Peso corporal

Ratos *High* são mais pesados que o grupo *Low* (Teste t de Student, P=0,032). Durante a exposição crônica ao doce, o mesmo padrão se mantém (ver figura) porém não se encontram diferenças estatísticas (ANOVA de medidas repetidas, P=0,2 para grupo). Há efeito do tempo no ganho de peso corporal durante a exposição prolongada ao doce (P<0,0001), sem interação grupo X dias.

Peso corporal . Os dados são apresentados como média ± erro padrão. Animais *High* pesam mais que animais *Low*, P=0,03.

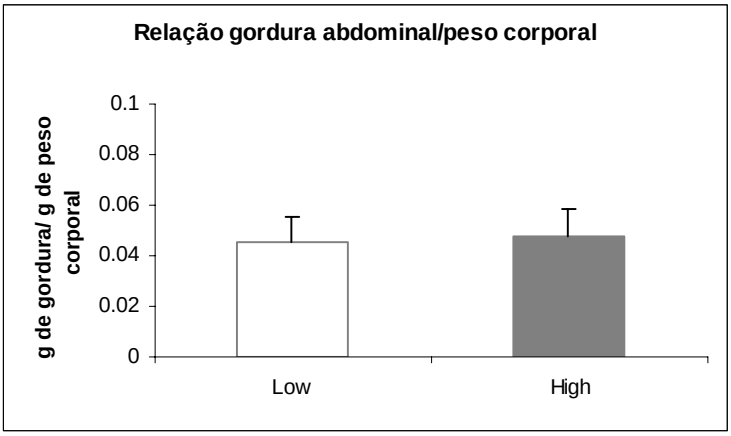




Peso corporal durante a exposição crónica ao doce. Os dados são apresentados como média ± erro padrão. Há efeito dos dias, sem efeito do grupo e sem interação.

Gordura abdominal

Não há diferenças no peso de gordura abdominal entre os grupos (Teste t de Student, P=0,11), nem na relação gordura abdominal/peso corporal (P=0,678).



Relação gordura abdominal/peso corporal. Os dados são apresentados como média ± erro padrão. Não há diferenças entre os grupos.

Medidas bioquímicas

Animais *high* têm menor grelina plasmática em relação ao grupo *low* (Teste t de Student, P=0,025). A leptina plasmática quase atingiu significância, sendo maior em animais *High* (P=0,055). Não há diferenças nos níveis de insulina (P=0,286) e corticosterona (P=0,960) entre os grupos.

Medida	*Grupo* "Low"	*Grupo* "High"
Grelina plasmática (fmol/ml)	8.76 ± 2.57	2.11 ± 1.23 *
Leptina plasmática (ng/ml)	16.11 ± 1.97	22.80 ± 2.52
Insulina plasmática (ng/ml)	4.34 ± 0.38	5.90 ± 1.28
Corticosterona plasmática (μg/dl)	21.11± 3.38	21.34 ± 3.09

Medidas bioquímicas comparando animais filhotes de mães altamente cuidadoras e mães pouco cuidadoras. Dados expressos como média ± erro padrão da média, N=7-10/grupo. *Animais *High* apresentam menor grelina plasmática em relação a animais *Low*.

4. DISCUSSÃO

O objetivo deste trabalho foi analisar em maior detalhe a associação entre a manipulação neonatal e o aumento no consumo de alimentos palatáveis na vida adulta. Para isso, estudamos aspectos hedônicos e homeostáticos envolvidos no controle do comportamento alimentar, assim como avaliamos diferentes fases do desenvolvimento e exposição a um ambiente adverso na vida adulta de animais manipulados no período neonatal. Vimos da mesma forma os efeitos de diferentes intensidades de cuidado materno no período neonatal sobre o consumo de alimentos palatáveis e variáveis metabólicas na vida adulta.

Tomando como base nossos estudos anteriores, que demonstravam que animais manipulados apresentam um aumento no consumo de alimentos palatáveis na vida adulta não acompanhado por maior consumo de ração padrão (Silveira et al., 2004), acreditávamos que mecanismos hedônicos pudessem ser os maiores envolvidos nesse efeito. Porém, conforme vimos no capítulo I, o consumo de uma solução de glicose antes do teste da saciedade foi capaz de inibir o consumo de doce em animais manipulados, mas não em animais intactos. Este experimento nos sugere dois aspectos importantes: primeiro, uma vez que todos os animais beberam aproximadamente o mesmo volume antes do teste (água ou solução glicosada, dividindo os dois grupos originais em quatro), possivelmente a distenção gástrica não seja um sinal importante na inibição do consumo de doce observada. A parede estomacal é cercada com sensores neurais de tensão (Berthoud &Powley, 1992), distensão (Phillips & Powley, 2000), e volume (Ritter, 2004). Estes mecanorreceptores comunicam-se com o cérebro através de nervos sensoriais vagais e espinhais (Ritter, 2004, Schwartz et al., 1999), usando uma rede complexa de neurotransmissores e neuromoduladores que incluem o glutamato, a acetilcolina, o óxido nítrico, o peptídeo relacionado ao gene da calcitonina, a substância P, a galanina e a CART (Ritter, 2004). Peptídeos relacionados à bombesina (por exemplo, peptídeo relacionado à gastrina e neuromedina B), que são produzidos por neurônios gástricos

mioentéricos, podem diminuir o consumo de alimentos quando administrados a animais e humanos. Sabemos da literatura que animais manipulados no período neonatal possuem inibição da ingestão de um alimento palatável após injeção de bombesina, de modo similar a animais intactos (McIntosh et al., 1999).

Em segundo lugar, a resposta de inibição do consumo nesses animais pela glicose abre a possibilidade de que tanto mecanismos homeostáticos quanto hedônicos (ou até mesmo ambos) possam estar envolvidos nos efeitos descritos. A glicose inibe os neurônios que expressam orexina e estimula os neurônios que expressam o hormônio concentrador de melanina (MCH) no hipotálamo lateral (Burdakov et al., 2005). No arqueado (ARC), há descrição de ações excitatórias da glicose nos neurônios anorexigênicos POMC (Ibrahim et al., 2003) e de inibição dos neurônios NPY (Muroya et al., 1999). É interessante notar que normalmente ratos não respondem a uma sobrecarga de glicose com inibição do apetite (Mook et al., 1993). Logo, o fato de animais manipulados responderem à ingestão de glicose inibindo o consumo poderia estar ligado à maior sensibilidade em algum desses sistemas homeostáticos. Por outro lado, o consumo de uma solução doce induz a liberação de dopamina no núcleo acumbens (Hajnal & Norgren, 2002), importante rede do sistema mesolímbico envolvido em processos de adição e, portanto, hedônicos.

Vimos também no capítulo I que, apesar de animais manipulados no período neonatal ingerirem mais Froot loops (carboidratos simples), não há diferenças no consumo de carboidratos complexos entre os grupos. Os carboidratos simples serão metabolizados a glicose de forma rápida, enquanto os carboidratos complexos serão metabolizados num tempo mais prolongado. Se a glicose aumenta o metabolismo dopaminérgico no núcleo acumbens como vimos acima, poderíamos imaginar que animais manipulados ingerem mais carboidratos simples na tentativa de estimular o sistema mesolímbico através da glicose. Esta hipótese seria

ainda mais interessante se levarmos em conta os resultados do capítulo II, em que vimos um menor metabolismo dopaminérgico no núcleo acumbens de animais manipulados.

Considerando apenas estes achados, a proposta de que animais manipulados ingerem mais doce buscando atingir maior atividade dopaminérgica no núcleo acumbens (e portanto maior prazer) concordaria com a hipótese da "Síndrome de deficiência de recompensa" (Comings & Blum, 2000). Segundo esta hipótese, indivíduos com uma atividade inadequada da dopamina no "centro de recompensa do cérebro" (sic) seriam predispostos a maior risco de engajamento em atividades de excesso comportamental (consumo de grandes quantidades de álcool ou carboidratos), o que por sua vez estimularia a produção e o uso de dopamina pelo cérebro. Entretanto, ao aceitar esta hipótese, forçosamente deve-se aceitar a "Hipótese da Anedonia", proposta por Roy Wise há mais de duas décadas (Wise et al. 1978; Wise 1982). Sua principal idéia é de que a dopamina atua como mediadora do prazer produzido pela alimentação e por outras recompensas como atividade sexual e uso de drogas, com uma redução na percepção do prazer quando a função dopaminérgica é inibida.

Entretanto, nos últimos anos, vários grupos de pesquisa têm contestado a hipótese da anedonia, propondo funções alternativas para a dopamina no que tange sua relação com a recompensa. Como exemplo, temos a teoria da predição da recompensa (Schultz et al. 1997; Schultz 2002), na qual a dopamina serviria como um sinal codificador de desvios ou erros entre a recompensa esperada e a recebida. Na teoria da saliência do incentivo, a dopamina sinalizaria o valor de incentivo do estímulo recompensador (Berridge and Robinson 1998), fazendo a distinção entre "querer" e "gostar": a dopamina seria necessária para "querer" o estímulo e buscá-lo, mas não para apreciá-lo ou sentir prazer no consumo. Nesta linha, Salamone e colaboradores propõem que um prejuízo na neurotransmissão dopaminérgica levaria a um

distúrbio na tendência motivacional de empreender esforço na obtenção de alimento (Salamone et al. 1991; Salamone and Correa 2002).

Portanto, os demais experimentos do capítulo II são úteis na tentativa de estabelecer o funcionamento das vias mesolímbicas dopaminérgicas em animais manipulados no período neonatal. Vimos que, apesar de consumirem mais alimento doce, estes animais expressam menos reações hedônicas ao sabor doce num teste de reatividade ao sabor. Além disso, apesar de demonstrarem mais esforço correndo rapidamente para obtenção do doce na tarefa do corredor, são menos dispostos a serem condicionados ao lugar associado ao doce, assim como apresentam menor metabolismo dopaminérgico no núcleo acumbens. Isso nos demonstra que possivelmente estes animais têm uma regulação diferenciada da resposta ao prazer do doce, levando a um padrão paradoxal se comparado às teorias atuais do papel da dopamina na recompensa.

Tivemos outros resultados apontando para essa possível regulação diferenciada do sistema dopaminérgico mesolímbico. Por exemplo, vimos no capítulo I que a manipulação neonatal está associada a menores níveis plasmáticos de grelina. Apesar de parecer um resultado contraditório – a grelina, sendo orexigênica, encontra-se em menores quantidades em animais manipulados, que comem mais doce – a análise mais aprofundada deste achado nos revela pontos interessantes. Por exemplo, recentemente tem sido demonstrado o papel da grelina na regulação da dopamina no sistema mesolímbico. Quando ligada a neurônios no VTA, a grelina dispara a atividade neuronal dopaminérgica, a formação de sinapses e o metabolismo dopaminérgico no núcleo acumbens (Abizaid et al., 2006). Quando injetada nesta área, a grelina estimula a alimentação (Naleid et al., 2005), sugerindo mais uma vez que fatores homeostáticos podem regular a resposta hedônica ao alimento, como no caso da glicose discutido acima. Esses dados nos propõem que a resposta de animais manipulados à

apresentação de uma recompensa possa estar sendo regulada por mecanismos que integram fatores periféricos como a grelina modulando fatores centrais como a dopamina mesolímbica.

Se considerarmos os resultados da resposta à injeção de metilfenidato no consumo de doce do capítulo II, temos mais uma vez a sugestão de uma regulação diferenciada do sistema dopaminérgico mesolímbico em animais manipulados no período neonatal. Neste experimento, vimos que no estado alimentado não há efeito da droga, sendo que apenas o efeito da manipulação neonatal aumentando o consumo de doce é observado. No entanto, em jejum, quando os níveis plasmáticos de insulina são baixos (a insulina pode contrapôr os efeitos do metilfenidato no estado alimentado – ver detalhes no capítulo IV), observamos que animais controle aumentam o consumo de doce após a injeção de metilfenidato, enquanto animais manipulados não respondem à droga. O metilfenidato age no transportador de dopamina (DAT), inibindo a recaptação do neurotransmissor e aumentando a disponibilidade de dopamina. Se a dopamina no núcleo acumbens for mesmo responsável pelo consumo alterado de doce em animais manipulados, o fato de não haver resposta à droga juntamente a um metabolismo atenuado deste neurotransmissor no acumbens nos sugere que o metabolismo deve ser tão diminuído que, mesmo amplificado pelo uso do metilfenidato, não atinge níveis suficientes para influenciar o consumo. Ou ainda, que estes animais necessitariam uma atividade dopaminérgica extraordinariamente maior que os animais controle para que ocorra interferência no consumo.

Poderíamos ainda citar o capítulo IV como mais um exemplo de sugestão de uma regulação diferenciada da atividade dopaminérgica mesolímbica em animais manipulados no período neonatal. Neste estudo, vimos que na vida adulta o aumento do consumo de doce nestes animais é acompanhado de uma diminuição da atividade da 5'-nucleotidase em sinaptossomas do núcleo acumbens, uma enzima envolvida na formação de adenosina (ver

detalhes no capítulo IV). Há evidências de que existe uma interação funcional entre a dopamina e os receptores A2A de adenosina em áreas estriatais, incluindo o núcleo acumbens (Chen et al. 2001). Antagonistas adenosinérgicos são capazes de reverter os efeitos de uma antagonista dopaminérgico na locomoção (Ishiwari et al., 2007) e em tarefas de condicionamento (Farrar et al., 2007). Mais uma vez, embora nosso resultado possa parecer contraditório quando pensamos que a menor atividade adenosinérgica deveria acompanhar uma maior atividade dopaminérgica, isso não parece ser verdadeiro no que se refere à resposta a substâncias aditivas como o etanol, agonistas opióides e canabinóides. Nessas situações, os dois sistemas parecem agir em sinergia nas vias intracelulares. Neurônios expressando receptores A2 e D2 ao mesmo tempo, como os do núcleo acumbens, são caracterizados por hipersensibilidade ao etanol e outras substâncias aditivas, com uma ativação simultânea da sinalização dopaminérgica; a sinergia requer adenosina e parece ser mediada pela liberação de dímeros beta-gama de uma proteína G inibitória via ativação do D2 (Yao et al., 2002; 2003). Por estes motivos, a adenosina no núcleo acumbens tem sido proposta como tendo um papel significativo nos comportamentos relacionados à recompensa e reforço, com a sinergia A2/D2 possivelmente relacionada ao comportamento de busca e ao consumo voluntário de álcool (Mailliard & Diamond, 2004). É interessante notar que baixas doses de antagonista A2 aumentam o consumo de álcool, o que seria consistente com a possibilidade de que ratos aumentam a ingestão na tentativa de superar um bloqueio parcial de A2, segundo os autores (Arolfo et al., 2004). Se extrapolarmos os achados destes estudos usando etanol para o consumo de doce, poderíamos propôr que animais manipulados no período neonatal, tendo uma discreta menor formação de adenosina no núcleo acumbens, aumentam o consumo de alimento doce na tentativa de compensar a menor atividade do sistema. Mais uma vez, voltaríamos à teoria da "Síndrome da deficiência de recompensa" discutida acima.

Conforme visto nos capítulos III e IV, animais manipulados no período neonatal ingerem mais doce apenas na vida adulta, e não em fases anteriores como a adolescência. Alguns estados patológicos como a esquizofrenia e os transtornos alimentares (anorexia e bulimia) também na maioria das vezes têm seu aparecimento apenas após a adolescência. A esquizofrenia tem sido ligada a um déficit na atividade do sistema adenosinérgico (Lara & Souza, 2000), atividade dopaminérgica mesolímbica errática, atribuição de valor aberrante a estímulos irrelevantes e idiossincráticos, e preferência marcante por alimentos palatáveis (para uma revisão, veja Elman et al., 2006), sendo intrigante a semelhança desta descrição com os achados deste trabalho em ratos manipulados.

Descrevemos nos capítulos III e VI que outras intervenções na infância, como a exposição ao doce, a brinquedos ou o aumento do cuidado materno também levaram a um aumento no consumo de doce na vida adulta. Portanto, é possível que os efeitos observados após a manipulação neonatal não sejam especificamente causados por esta intervenção ou pelo período em que a intervenção é aplicada, embora não saibamos se os mecanismos que levam animais intactos a aumentarem o consumo de doce na vida adulta após a simples exposição ao doce ou a brinquedos na infância são os mesmos que levam animais manipulados a ingerirem mais doce na vida adulta.

Quanto à concordância de resultados entre os animais manipulados no período neonatal e os animais que receberam maior cuidado materno nos primeiros dias de vida descrita no capítulo VI, nossos dados contribuem com os achados de outros autores que afirmam (a) muitos dos efeitos da manipulação neonatal ocorrem através de um aumento do cuidado materno quando os filhotes são devolvidos à ninhada após a manipulação (Branchi et al., 2001; Pryce et al., 2001); (b) filhotes de mães altamente cuidadoras têm o mesmo perfil de menor resposta ao estresse agudo e persistência da exacerbação dos mecanismos de

retroalimentação negativa dos glicocorticóides encontrado em animais manipulados no período neonatal (Liu et al., 1997) e (c) filhos de mães altamente cuidadoras têm comportamento sexual inibido tanto em machos quando em fêmeas da mesma forma que animais manipulados (comunicação pessoal). Portanto, é possível que os achados da manipulação neonatal sobre o consumo de doce e níveis plasmáticos de grelina sejam mediados pelo aumento do cuidado materno. Entretanto, é importante notar que utilizamos ratos Wistar para a manipulação neonatal e ratos Long-Evans para verificar os efeitos do cuidado materno. Além disso, embora os animais manipulados possam ser semelhantes aos filhotes de mães altamente cuidadoras ("high"), o grupo intacto não necessariamente sofreu falta de cuidado materno como o grupo "low". O mais provável é que animais do grupo intacto tenham recebido uma intensidade média de cuidados maternos. Portanto, se os efeitos observados no consumo de doce e nível plasmático de grelina são mesmo devido ao cuidado materno diferenciado, as comparações realizadas entre animais "high" e "low" serão como uma visão ampliada das comparações entre manipulados e intactos, uma vez que a diferença entre intensidade de cuidados maternos entre o primeiro par é maior que no segundo. Talvez por isso algumas variáveis não diferentes entre manipulados e intactos, como a leptina plasmática e o peso corporal foram marginal ou completamente estatisticamente significativas entre animais "high" e low".

Ainda do capítulo VI poderíamos ressaltar que o experimento da exposição prolongada ao doce nos demonstra que animais "high" comem mais doce apenas nos primeiros dois dias, sendo capazes de regular e diminuir o seu consumo a partir de então. Embora não tenhamos realizado um experimento semelhante em animais manipulados, este resultado parece estar de acordo com o experimento da saciedade do capítulo I, em que animais manipulados ingerem mais doce porém são capazes de regular a ingestão e demonstram saciedade após determinado período. É possível que, embora à primeira vista parecendo um comportamento problemático,

o maior consumo de doce por animais manipulados seja transitório e ocorra apenas no início da exposição ao alimento, e que portanto estes animais tenham um interessante mecanismo de regulação que os permite experimentar e ingerir maior quantidade, regulando o consumo a partir de então.

Em face destes resultados, poder-se-ia argumentar que animais manipulados apresentam menor neofobia ao corredor de realização da tarefa de comportamento alimentar e/ou ao novo alimento uma vez que em geral respondem menos ao estresse e têm menos medo de situações novas, e que por isso o consumo ficaria semelhante entre os grupos após alguns dias de exposição contínua ao doce. O primeiro experimento do capítulo I nos ajuda a negar esta hipótese, sendo que a ingestão de doce foi medida na própria caixa moradia, um ambiente que não seria novo para o grupo intacto, e ainda assim a diferença é evidente. Além disso, o resultado não pode ser explicado por neofobia ao próprio Froot loops por parte do grupo intacto, uma vez que todos os animais foram habituados ao doce antes destes experimentos por, no mínimo, 5 dias. Logo, é pouco provável que o aumento de consumo de doce demonstrado por animais manipulados possa ser explicado por menor neofobia.

Outra questão que inevitavelmente nos interpela é se o maior consumo de doce com possível auto-regulação através de mecanismos eficazes de saciedade durante a exposição prolongada ao alimento é adaptativa ou não. Pode parecer extremamente vantajoso poder experimentar e consumir uma maior quantidade de alimento palatável sem aparentemente sofrer maior ganho de peso e suas conseqüências metabólicas. No entanto, em condições não experimentais, os indivíduos não são expostos repetidamente ao mesmo tipo de alimento palatável, e sim possuem uma variedade imensa de sabores e texturas disponíveis. Resta-nos saber se animais manipulados manteriam seu consumo aumentado de alimento palatável por mais tempo se este variasse conforme o passar dos dias e conforme a diminuição de interesse

dos animais por aquele alimento, ou se ainda assim seriam capazes de regular o consumo e apresentar mecanismos eficazes de saciedade.

Por outro lado, não temos dúvida que a manipulação neonatal leva a uma série de alterações persistentes, levando a um padrão de funcionamento comportamental e neuroquímico diferenciado, e que estes animais apresentam menor tendência de sofrer interferência do estresse crônico variável sobre estas características. Há menor influência do ECV no peso corporal e no consumo de doce em animais manipulados, assim como a diminuição da atividade da Na^+,K^+-ATPase no córtex parietal causada pelo ECV foi revertida pela manipulação neonatal.

Portanto, a despeito dos efeitos observados pela manipulação *per se*, é evidente que esses animais não sofrem as influências da exposição ao estresse crônico como acontece com os animais intactos. Possíveis explicações para estes achados são: **(a)** a manipulação neonatal, como sabemos, "programa" uma menor resposta neuroendócrina ao estresse agudo na vida adulta (Plotsky & Meaney, 1992), possivelmente levando também a uma resposta atenuada ao estresse crônico variável, com efeitos deletérios menos evidentes nestes animais. De nosso conhecimento, há apenas um estudo avaliando a resposta ao ECV em animais manipulados, demonstrando que esta intervenção não altera as respostas neuroendócrinas de animais manipulados ao estresse, embora leve a um aumento na produção de ARN mensageiro para CRH no NPVdo hipotálamo (Ladd et al., 2005). Entretanto, este estudo não utilizava o grupo "intacto" como controle, sendo portanto difícil comparar seus achados com nossos estudos; **(b)** as alterações induzidas pelo ECV em animais intactos (menor ganho de peso, maior consumo de doce, diminuição da atividade da Na^+,K^+-ATPase no hipocampo) já são vistas em animais manipulados no basal, portanto para estes a exposição ao ECV não teria grande efeito adicional. Porém, estaríamos propondo que os mecanismos que levam às alterações causadas

pelo ECV na vida adulta de intactos são os mesmos das alterações causadas pela manipulação neonatal. Esta assumpção é enfraquecida pelo fato de que a manipulação num período vulnerável programa a atividade de determinados sistemas de forma persistente, enquanto vários efeitos do estresse crônico desaparecem após um período de suspensão do estresse (Vasconcellos et al., 2005); **(c)** a manipulação neonatal poderia estar associada a uma alteração na interpretação dos estímulos do ambiente. Assim, os animais responderiam de forma particular ao estresse crônico por interpretá-lo como sendo um estímulo não nocivo, talvez por um funcionamento cognitivo ou sensorial diferencial nestes animais. Esta hipótese é interessante se levarmos em conta dados como menor "ansiedade" quando expostos ao labirinto em cruz elevado (Severino et al., 2004), a maior exposição ao predador (Padoin et al., 2001) e a própria menor reatividade ao estresse agudo – é possível que estas respostas sejam simplesmente uma interpretação "alternativa" dos estímulos ambientais, encarados pelos animais manipulados como pouco ameaçadores. **(d)** a manipulação neonatal estaria associada a uma "rigidez comportamental", ou seja, suas características tem uma base biológica tão bem sedimentada que intervenções posteriores menos específicas como o uso sistêmico de fármacos (ex.: metilfenidato no capítulo II e diazepam em Silveira et al., 2005) ou a exposição ao estresse não conseguem influenciar nessas características. Além disso, em nosso teste de reação hedônica ao doce descrito no capítulo II, o estímulo do sabor é associado a uma menor reatividade afetiva nestes ratos. Estas duas últimas hipóteses (c e d) nos fazem mais uma vez lembrar das interpretações incorretas e bizarras de estímulos (Javitt et al., 2000) e emoções (Flack et al., 1998), do embotamento afetivo, "pensamento nebuloso" e alterações no estado de alerta classicamente descritos em quadros disfóricos e estados patológicos como a esquizofrenia.

5. CONCLUSÕES

A manipulação neonatal se associa a uma série de alterações relativas ao comportamento alimentar como maior consumo de alimentos palatáveis e resposta de saciedade diferencial e eficaz. Os menores níveis plasmáticos de grelina e menor metabolismo dopaminérgico no núcleo acumbens nos sugerem que uma integração entre mecanismos homeostáticos e hedônicos possa estar envolvida nestes achados.

A fase da vida parece ser um fator importante determinando os efeitos da manipulação neonatal no comportamento alimentar. Além disso, nosso trabalho propõe que estes efeitos possam estar sendo mediados pelo aumento do cuidado materno induzido pelo procedimento de manipulação neonatal. Sugerimos também que outras intervenções na infância têm potencial para afetar o comportamento alimentar na vida adulta, embora não seja claro se diferentes intervenções precoces compartilham os mesmos mecanismos para levar ao mesmo desfecho na vida adulta, ou se cada intervenção tem seu mecanismo específico.

Animais manipulados no período neonatal guardam semelhanças com algumas entidades clínicas e parecem ter uma resposta particular à exposição ao estresse crônico variável, sendo portanto um possível modelo animal interessante para estudo de diferentes psicopatologias.

Assim, vimos que uma intervenção num período crítico do desenvolvimento tem efeitos persistentes sobre sistemas de controle do comportamento alimentar e sobre o risco para determinadas doenças na vida adulta. A compreensão dos mecanismos pelos quais a vida precoce influencia no padrão de saúde do adulto tem implicações importantes para a identificação de populações de risco e busca de medidas preventivas.

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