UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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ALTERAÇÕES MORFOLÓGICAS DO SISTEMA NERVOSO CENTRAL INDUZIDAS PELA MANIPULAÇÃO NEONATAL

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"O destino é dos distraídos, a

sorte é dos guerreiros"

Ao meu esposo Adriano, cujo amor, apoio e paciência são indiscutíveis.

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SUMÁRIO

ABREVIATURAS

ACTH: hormônio adrenocorticotrófico

AVP: arginina vasopressina

BaLA: núcleo basolateral da amígdala

CeA: núcleo central da amígdala

CRF1: receptor do fator liberador de corticotrofina do tipo 1

CRH: hormônio liberador de corticotrofina

GR: receptor para glicocorticóide

HPA: hipotálamo-hipófise adrenal

HPG: hipotálamo-hipófise gonadal

LaA: núcleo lateral da amígdala

LC: *Locus coeruleus*

LH: hormônio luteinizante

NE: noradrenalina

NM: não manipulado

M: manipulado

OX: ocitocina

PVNm: região magnocelular do núcleo paraventricular do hipotálamo

PVNp: região parvocelular do núcleo paraventricular do hipotálamo

RNAm: ácido ribonucléico mensageiro

SHRP: período hiporresponsivo ao estresse

SON: núcleo supra-óptico do hipotálamo

RESUMO

A manipulação neonatal, uma estimulação ambiental no período neonatal, tem um papel marcante no desenvolvimento de respostas ao estresse pelo eixo hipotálamo-hipófise adrenal (HPA). Em ratos, esta estimulação consiste na manipulação dos filhotes por poucos minutos, geralmente durante as duas primeiras semanas de vida. Este período é considerado crítico para o desenvolvimento do sistema nervoso e também é considerado uma fase hiporresponsiva ao estresse. Esta intervenção no período neonatal causa um aumento da atividade exploratória em ambientes novos e aversivos em ratos adultos, interpretado como uma diminuição do medo. Além disso, os animais manipulados apresentam uma diminuição da secreção dos hormônios do estresse, como o hormônios adrenocorticotrófico, a corticosterona, prolactina e adrenalina e outros neurotransmissores tais como a serotonina. No hipocampo sabe-se que esta estimulação induz a um aumento do número de receptores para glicocorticóides. Esta tese teve por objetivo avaliar se a manipulação neonatal afeta o número de neurônios em diferentes núcleos do sistema nervoso central (PVNp, PVNm, SON, CeA, LaA, BaLA, áreas CA1, CA2 e CA3 do hipocampo) e células gliais (PVNp, PVNm, SON, CeA, LaA, BaLA). Os resultados mostraram que o estímulo aparentemente inofensivo a que os animais são submetidos nos dez primeiros dias de vida causou uma redução significativa do número de neurônios do Locus coeruleus em ratos machos e em fêmeas aos 11, 26, 35 e 90 dias de idade. O PVNp e o SON de animais manipulados no período neonatal também apresentaram uma redução significativa no número de neurônios. Entretanto, na área CA1 do lado esquerdo do hipocampo foi observado um aumento na

densidade de neurônios. A manipulação neonatal não provocou alterações quanto ao número de neurônios nas áreas CA2 e CA3 do hipocampo e nos diferentes núcleos da amígdala analisados (CeA, LaA e BaLA). A densidade de células gliais não foi significativamente diferente em animais manipulados quando comparados aos não manipulados. Desta forma, demonstramos que a manipulação neonatal induz a alterações morfológicas do sistema nervoso central provocando aumento ou redução do número de neurônios em núcleos que participam do circuito do estresse.

ABSTRACT

The neonatal handling, a early life environmental stimulation, play an essential role in the development of hypothalamo-pituitary-adrenal axis (HPA) response to stress. In rats, this stimulation consists of the manipulation of the pups for few minutes, generally during the first two weeks of life. This period is considered critical for the development of the nervous system and it has been referred as stress hyporesponsive period. This intervention in the neonatal period induces these animals, when adults, an increase exploratory activity, which is interpreted as decreased fearfulness to novels environments. Besides, the handled animals present a decrease of the secretion of the hormones of the stress, as the adrenocorticotrophin hormone and corticosterone for the adrenal, of the prolactin, of the adrenaline and of the serotonin. In the hippocampus, the neonatal handling induces an increase in glucocorticoid receptor density. This thesis had as objective to investigate if the neonatal handling affect the number of neurons in different nucleus of the central nervous system (PVNp, PVNm, SON, CeA, LaA, BaLA, CA1, CA2 and CA3 layers of the hippocampus) and glial cells (PVNp, PVNm, SON, CeA, LaA, BaLA). The results obtained in this thesis showed that the neonatal handling induces a significant reduction in the number of neurons in the *Locus coeruleus* in males and females rats with 11, 26, 35 and 90 days old. The PVNp and SON of animals handled in the neonatal period also present a significative reduction in the number of neurons. However, on the left side in the CA1 layer of the hippocampus was observed an increase in the density of neurons. The neonatal handling no induces a significant changes in relation to the number of neurons in the CA2 and CA3 layers of the hippocampus and in the different

nuclei of the amygdala analysed (CeA, LaA e BaLA). The density of glial cells was not significantly different in handled animals when compared to nonhandled animals. In this way, the neonatal handling induces to morphological changes in the central nervous system provoking increase or reduction of the number of neurons in nuclei that participate in the circuit of the stress.

1. INTRODUÇÃO

1. INTRODUÇÃO

1.1. Conceitos e respostas de estresse

Os seres vivos são formados por sistemas que integram funções complexas que buscam manter o organismo num estado de equilíbrio dinâmico e harmonioso denominado de homeostase. Este equilíbrio é necessário para a manutenção da vida e pode ser constantemente ameaçado por fatores extrínsecos e intrínsecos, ao que denominamos de estressores. Em situações de ameaça ou perigo, os organismos desencadeiam uma série de respostas adaptativas, tanto físicas quanto mentais, ao estresse. Essas respostas geram alterações fisiológicas e psicológicas que tem por objetivo o restabelecimento da homeostase (Chrousos & Gold, 1992).

O estresse é um conceito difícil de ser definido, uma vez que as interpretações variam muito pelos pesquisadores que trabalham nesta área. Hans Selye foi um dos pioneiros nos estudos abordando a fisiologia e patofisiologia do estresse, definindo-o como uma resposta não específica de um organismo a um estímulo. Em seus estudos foram enfatizados as respostas integradas de múltiplos sistemas de um corpo. Embora todos os órgãos sejam afetados quando um organismo rompe seu estado de homeostasia, existem aqueles que são os primeiros a sofrerem as alterações funcionais, tais como os sistemas neuroendócrino, cardiovascular, imune e gastrointestinal (Selye, 1976).

A exposição de um organismo a diferentes condições ambientais (usualmente referidas como estressores) resulta em uma série de respostas coordenadas que são organizadas para aumentar sua probabilidade de sobrevivência. Estas respostas coordenadas são referidas como respostas ao

estresse e são compostas de alterações comportamentais e neurovegetativas e da secreção de vários hormônios, incluindo o hormônio adrenocorticotrófico (ACTH), cortisol/corticosterona, catecolaminas adrenais, ocitocina, prolactina e renina (Van de Kar & Blair, 1999).

Os estressores podem ser agrupados em três categorias: a) estressores psicológicos, que se baseiam em respostas aprendidas como sendo indicação de perigo ou ameaça, tais como o medo, a ansiedade, a exposição a um ambiente novo ou o incontrolável; b) estressores físicos, causados por estímulos físicos, mas que mantém um forte vínculo psicológico, como a dor, o choque nas patas e a imobilização; c) estressores que alteram a homeostase cardiovascular, como hemorragias e exercícios (Van de Kar & Blair, 1999).

Em resposta ao estresse físico e psicológico é ativado o eixo hipotálamo-hipófise-adrenal (HPA). O estresse aumenta a secreção de CRH e de peptídeos liberados em conjunto com a arginina vasopressina (AVP) e a ocitocina, promovendo um aumento na síntese e liberação de ACTH e beta-endorfina pela adenohipófise (Antoni, 1986). O ACTH interage com receptores localizados no córtex da supra-renal promovendo um aumento na liberação de glicocorticóides no plasma (Aguilera *et al*, 2001). Os glicocorticóides podem promover mudanças importantes no metabolismo, que se caracterizam por aumentar o catabolismo e reduzir os processos anabólicos, tendo como conseqüência a lipólise, a glicogenólise e o catabolismo de proteínas. Desta forma, estas catecolaminas induzem alterações de inúmeras funções vegetativas que dão suporte necessário para o organismo restabelecer o equilíbrio e também mobilizam a produção de substratos energéticos durante o estresse (Kopin, 1995).

A divisão simpática do sistema neurovegetativo também é ativada, estimulando a liberação de adrenalina e de noradrenalina (NE) nos terminais sinápticos e na medula da supra-renal (Chrousos & Gold, 1992). Além da descarga periférica de noradrenalina, o estresse induz a secreção deste neurotransmissor em todo o sistema nervoso, sendo liberado, nesta situação, principalmente pelo *Locus coeruleus* (LC), uma vez que o conteúdo de NE está diminuído neste núcleo após um evento estressante (Konstandi *et al.*, 2000).

1.2. Desenvolvimento do sistema nervoso e período hiporresponsivo ao estresse

Em ratos, as duas primeiras semanas de vida representam um período crítico para o desenvolvimento do sistema nervoso, uma vez que, nesta fase da vida, ainda ocorrem processos vitais como migração, divisão, diferenciação, crescimento e morte de células neste sistema (Mistretta & Bradley, 1978, Schmidt *et al.*, 2002). Desta forma, a manutenção de uma baixa concentração de corticosterona durante o crescimento do rato é essencial para um desenvolvimento normal do sistema nervoso. A administração de glicocorticóides interfere no crescimento e diferenciação, promovendo alterações permanentes neste indivíduo (Levine, 2001). Altas concentrações de glicocorticóides causam diminuição da mitose, da mielinização e da neuromorfogênese (Bohn, 1980) e alterações comportamentais e neuroquímicas no adulto (Gonzáles *et al.*, 1990).

De fato, o que sabemos é que, durante as duas primeiras semanas de vida, o eixo HPA apresenta uma resposta reduzida a estímulos estressores (Haltmeyer *et al*, 1966), porém a duração exata deste período é alvo de controvérsias. Estudos *in vivo* e *in vitro* mostraram que a hipófise e a adrenal são capazes de liberar ACTH

e corticosterona, no 17° e 18° dia de vida fetal e no 2° dia pós-natal, respectivamente (Guillet *et al* 1978). Com estes dados concluiu-se que o período do nascimento até aos 14 dias de vida (período de desenvolvimento de estruturas neurais) não poderia ser considerado totalmente não responsivo ao estresse, por isso foi então denominado de período hiporresponsivo ao estresse. A imaturidade do eixo HPA durante as duas primeiras semanas de vida resulta em um embotamento da secreção de ACTH após a exposição a vários estressores. Contudo, foi observado que o CRH pode ser liberado em estágios iniciais da vida, ligando-se em seus receptores na adenohipófise e promovendo a liberação de ACTH. Sugere-se que o período hiporresponsivo ao estresse, no rato, seria causado por uma falta da habilidade da maioria dos estressores em induzir a liberação de CRH na circulação porta-hipofisária, de maneira suficiente para superar o aumento da sensibilidade da hipófise ao sistema de retroalimentação negativa de glicocorticóides (Abrahám & Kovacs, 2000; Walker *et al.*, 1986).

1.3. Manipulação neonatal

Como descrito acima, no período neonatal (duas primeiras semanas de vida) o sistema nervoso encontra-se em franco processo de diferenciação e desenvolvimento. Este fato mostra que o meio ambiente que cerca o indivíduo durante a infância pode afetar o desenvolvimento de estruturas neurais que estão envolvidas no estresse (Denenberg, 1964, Meaney *et al.*, 1996). A influência de fatores ambientais no desenvolvimento neural pode também ser observada em humanos, uma vez que crianças que sofreram abusos (como agressão) têm uma maior incidência de depressão, de ansiedade e de outras enfermidades psiquiátricas na vida adulta (Kaufman *et al.*, 2000). Estudos demonstraram que

estímulos estressantes, no início da vida, podem alterar vários sistemas de neurotransmissores implicados na etiologia de doenças psiquiátricas (Caldji *et al.*, 2000), sugerindo que o modelo experimental de estresse neonatal pode assim ser um instrumento importante no entendimento de condições psiquiátricas, como a depressão (Kaufman *et al.*, 2000).

A estimulação neonatal tem sido utilizada há algumas décadas como um modelo experimental para examinar os mecanismos através dos quais variações precoces do ambiente do animal afetam o desenvolvimento de sistemas neurais, dando origem a alterações comportamentais e neuroendócrinas estáveis (Levine, 1962, Denenberg, 1964).

Em ratos, a estimulação neonatal tipicamente consiste da manipulação dos animais por alguns minutos, em geral durante as duas primeiras semanas de vida (Figura 1). Este procedimento, aparentemente não nocivo ao indivíduo, tem como conseqüência em uma série de alterações comportamentais que se caracterizam basicamente por uma diminuição do medo a ambientes novos, na vida adulta. Além disso, sabe-se que estes animais, quando adultos, têm uma resposta menos acentuada da secreção de glicocorticóides pela supra-renal quando expostos a estímulos estressantes (Levine, 1993, Meaney *et al.*, 1993).

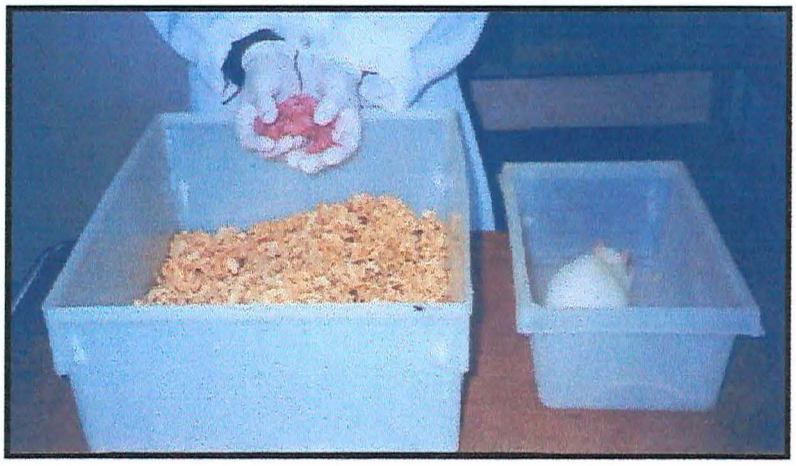

Figura 1. Modelo de manipulação neonatal.

Contudo, quando adultos, os níveis basais de corticosterona de animais manipulados e não manipulados na infância não difere entre si. As diferenças entre eles parecem ser devidas a uma sensibilidade diferencial do sistema nervoso central ao mecanismo de retroalimentação negativa da supra-renal (Levine, 1994). Desta forma, a manipulação durante as duas primeiras semanas de vida, em roedores, diminui a responsividade do eixo HPA ao estresse am animais adultos (Levine, 1993; Meaney *et al.*, 1996, Liu *et al.*, 2000). Estes efeitos são mediados pelas alterações da expressão de receptores para glicocorticóides em regiões do sistema nervoso central envolvidas na regulação do *feedback negativo*, como o hipocampo e o córtex frontal (Plotsky & Meaney, 1993; Meaney *et al*, 1993; Sapolky, 1994; Liu *et al.*, 2000). Estas diferenças com relação aos receptores para glicocorticóides modificam a sensibilidade do *feedback negativo*, tornando-o mais sensível nos animais manipulados (Meaney *et al.*, 1996).

A manipulação também induz à alterações comportamentais. Um efeito central da manipulação neonatal é a redução da inibição comportamental, expressa por um aumento da locomoção em ambientes novos (Denenberg, 1964; Meaney *et al.*, 1996; Padoin *et al.*, 2001). Foram realizados trabalhos em nosso laboratório relacionando a manipulação neonatal e os vários comportamentos, como atividade locomotora em ambientes novos e comportamentos aversivos. Os resultados mostraram que a manipulação diária dos filhotes, durante os 10 primeiros dias de vida, reduz o comportamento sexual de ratos machos e fêmeas adultos (Padoin *et al.*, 2001). Há dados que demonstram que a manipulação neonatal também induz uma significativa redução da ovulação, com fêmeas apresentando ciclos anovulatórios (Gomes *et al.*, 1999, Gomes *et al.*, 2004). Esses resultados sugerem

que, a manipulação além da alterar o eixo HPA, também interfere no eixo hipotálamo-hipófise-gonadal (HPG).

Há poucos dados da literatura sobre alterações morfológicas causadas pela manipulação neonatal. A redução do número de receptores para glicocorticóides no hipocampo (Meaney *et al.*, 1993) mostra que aqueles procedimentos aparentemente inofensivos, realizados logo após o nascimento, podem alterar morfologicamente as estruturas do sistema nervoso central relacionadas ao circuito de estresse.

1.4. Estruturas neurais envolvidas no circuito do estresse

Várias estruturas neurais estão envolvidas na organização de respostas aversivas ou de estímulos estressantes. Entre elas estão o hipotálamo, o sistema septo-hipocampal, a amígdala, o córtex cingulado e o pré-frontal e regiões do tronco encefálico que contêm agrupamentos de corpos neuronais catecolaminérgicos (grupos celulares A2/C2 do núcleo do trato solitário; os grupos celulares A1/C1 da medula ventro-lateral, os grupos celulares A6 do *Locus coeruleus*), o núcleo parabraquial, o núcleo cuneiforme e o núcleo dorsal da raphe (Hermann & Cullinan, 1997; López *et al.*, 1999; Van de Kar & Blair, 1999).

1.4.1. *Locus coeruleus*

O *Locus coeruleus* (LC) também é chamado de núcleo A6 e localiza-se bilateralmente ao IV° ventrículo, na porção dorsolateral, sendo considerado o principal núcleo noradrenérgico do sistema nervoso central (figura 2). O LC é formado por neurônios de 3 tamanhos distintos: pequenos, médios e grandes (figura 3) (Aston-Jones et al., 1995; Guillamón et al., 1988).

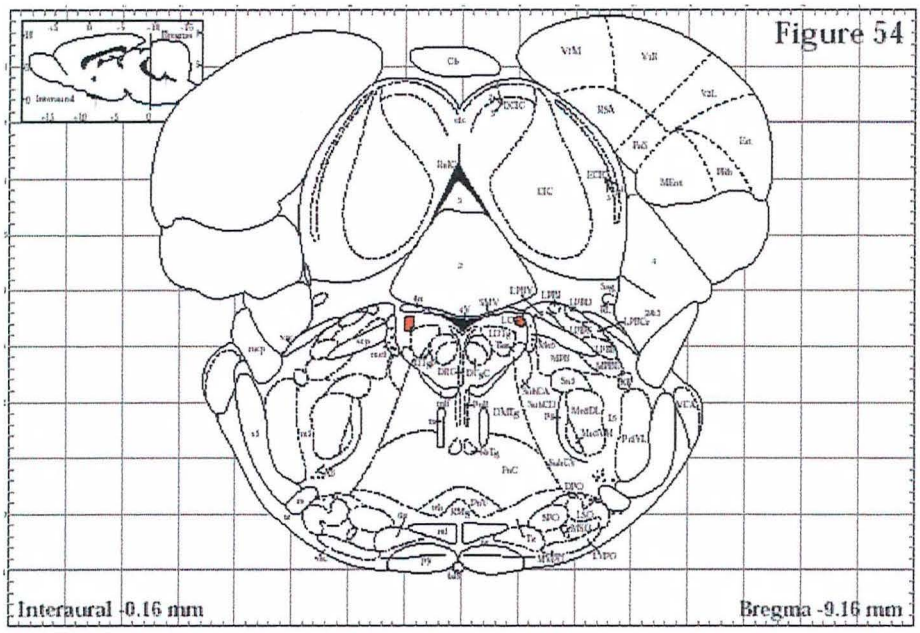

Figura 2. Localização do *Locus coeruleus* (vermelho), Paxinos & Watson, 1997.

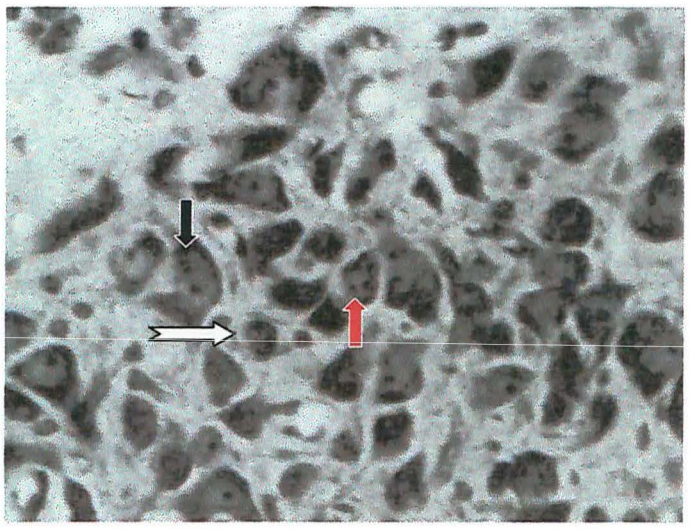

Figura 3. Neurônios do LC: pequeno (seta branca), médio (seta vermelha) e grande (seta preta) (400x).

Sabe-se que o LC é sensível ao estímulo da manipulação neonatal (Price et al., 1998). Este núcleo é essencial no controle da atividade do eixo hipotálamo-hipófise-gonadal. Lesões eletrolíticas deste núcleo causam uma diminuição do conteúdo de noradrenalina no hipotálamo, bloqueia a liberação pulsátil de LH, bloqueia o pico de gonadotrofinas pré-ovulatórias (Anselmo-Franci et al., 1997, 1999, Franci & Anselmo-Franci, 2002).

1.4.2. Núcleos hipotalâmicos relacionados ao estresse

Núcleo paraventricular do hipotálamo

O núcleo paraventricular do hipotálamo (PVN) tem um papel importante na coordenação das respostas ao estresse (Rivier *et al.*, 1982). Informações sensoriais relacionadas ao estresse convergem na secreção do hormônio liberador de corticotrofina (CRH) a partir de neurônios localizados no PVN para iniciar uma cascata de efeitos neuroendócrinos. O CRH estimula, por sua vez, a liberação do hormônio adrenocorticotrófico (ACTH) da adenohipófise e a conseqüente liberação de corticosterona pelo córtex da supra-renal. Esse processo acaba ativando o eixo HPA (Bhrun *et al.*, 1984; Herman *et al.*, 2002).

O PVN localiza-se lateralmente ao III ventrículo e é dividido, classicamente, em região magnocelular (PVNm) e parvocelular (PVNp) (Figura 4). Essas regiões são subdivididas em três magnocelulares (anterior, medial e posterior) e cinco parvocelulares (anterior, medial, lateral, dorsal e periventricular). Neurônios magnocelulares (figura 5) estão diretamente relacionados com a liberação dos peptídeos ocitocina e vasopressina da

neurohipófise dentro da circulação sistêmica, comandando, desta forma, a lactação, o parto, o controle da pressão sanguínea e o balanço eletrolítico. Esses peptídeos neurohipofisários magnocelulares liberados na circulação porta podem ser liberados numa situação de estresse e parecem modular a secreção de ACTH em determinadas circunstâncias (Plotsky *et al.*, 1985). Porém, a integração desses efeitos com a atividade do eixo HPA durante o estresse ainda precisam ser esclarecidas.

Neurônios do componente dorsolateral da parte medial do PVNp projetam-se para a eminência mediana e liberam o CRH. Os neurônios da região dorsal do PVNp, da parte ventral do componente medial do PVNp e do componente lateral do PVNp projetam-se para o tronco cerebral e para circuitos neurovegetativos da medula espinhal e parecem integrar o fluxo do sistema nervoso simpático e do parassimpático (Sawchenko & Swanson, 1981; Whitnall, 1993). Estas regiões do PVNp podem também regular a ativação do eixo HPA, indiretamente, através da modulação do tônus cardiovascular e, diretamente, via inervação para o próprio córtex da adrenal (Jasper & Engeland, 1997). Assim, enquanto os neurônios CRH-positivos agem como mediadores primários na secreção de ACTH, as populações de neurônios consideradas pré-autonômicas e também os neurônios magnocelulares contribuem na integração do PVN como moduladores das respostas do estresse (Herman *et al*, 2002).

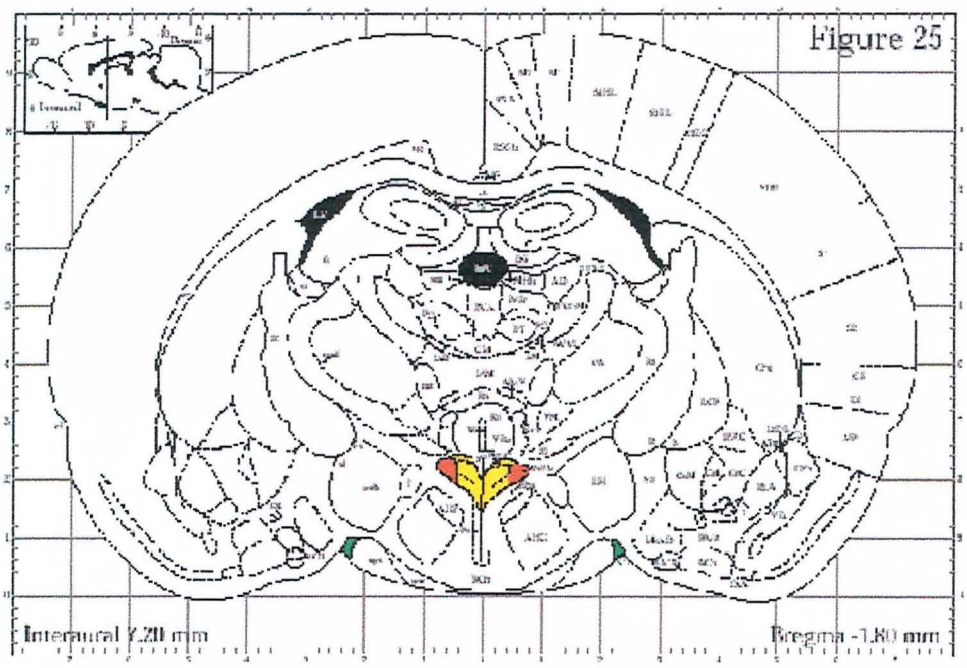

Figura 4. Localização da região magnocelular (vermelho) e parvocelular (amarelo) do núcleo paraventricular do hipotálamo e do núcleo supra-óptico (verde), Paxinos & Watson, 1997.

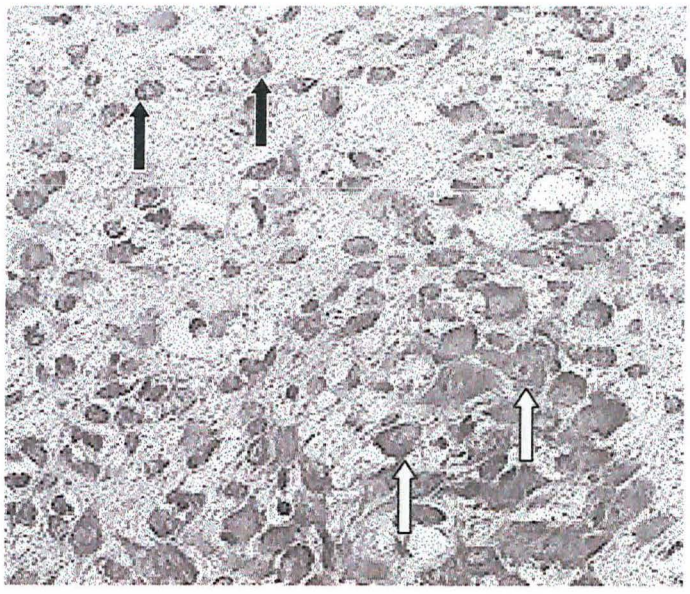

Figura 5. Neurônios parvocelulares (seta preta) e magnocelulares (seta branca) do PVN (480x).

Núcleo supra-óptico do hipotálamo

O núcleo supra-óptico (SON) constitui uma parte do sistema de neurosecreção magnocelular do hipotálamo. Seus neurônios sintetizam ocitocina (OX) e arginina-vasopressina (AVP), que são transportados através do trato supra-óptico neurohipofisário para o lobo posterior da hipófise onde são então liberados na circulação sistêmica (Castel *et al.*, 1984).

De acordo com Leránth *et al.* (1975), o SON é dividido anatomicamente em três regiões: a *pars principalis*, que consiste de uma massa de neurônios grandes distribuídos desde a borda lateral do quiasma óptico até o trato óptico, correspondendo a 80% dos neurônios do núcleo supra-óptico; a *pars intraoptica*, que é representada por neurônios localizados entre aqueles da *pars principalis* e a terceira região chamada de *pars tuberalis*. Esta última é composta de neurônios localizados medialmente aos tractos ópticos. A região abordada em nosso estudo foi somente a *pars principalis* (figura 4), uma vez que é nela que se encontram a grande maioria dos neurônios magnocelulares responsáveis pela produção de ocitocina e vasopressina (figura 6).

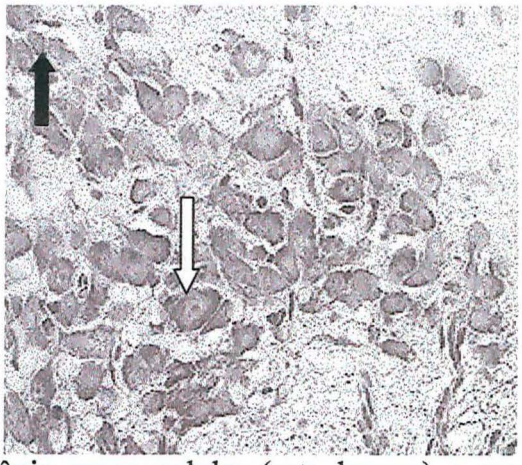

Figura 6. Neurônio magnocelular (seta branca) e parvocelular (seta preta) do núcleo supra-óptico (480x).

As funções da ocitocina em fêmeas estão diretamente relacionadas à reprodução. Durante o parto e a lactação, a ocitocina é liberada pelos neurônios magnocelulares do SON e do PVN para a circulação sistêmica e para diferentes regiões cerebrais (Neumann *et al.*, 1993). No tecido cerebral, a ocitocina está envolvida com funções relacionadas à reprodução, incluindo comportamentos sexuais (Kow *et al.*, 1994) e maternais (Pedersen & Prandge, 1979). Sabe-se também que uma variedade de estressores que estimulam o eixo HPA induzem a secreção de ocitocina (Nishioka *et al.*, 1998). Em condições de repouso a ocitocina age como um inibidor endógeno na atividade do eixo HPA, enquanto que em condições de estresse a ocitocina parece estimular um aumento da liberação dos hormônios deste eixo (Neumann, 2002). Os locais onde a ocitocina é liberada no cérebro em resposta a estímulos estressores incluem os dendritos e o pericário de neurônios ocitocinérgicos do núcleo paraventricular (Nishioka *et al.*, 1998), o qual contém neurônios que sintetizam CRH e ADH e se projetam para a zona externa da eminência mediana para controlar a secreção de ACTH (Herman *et al*, 2002).

1.4.3. Núcleos da amígdala relacionados ao estresse

A amígdala, assim como os núcleos hipotalâmicos acima descritos, também participa na elaboração das respostas relacionadas ao estresse. A amígdala é um componente essencial do prosencéfalo basal de mamíferos e está implicada numa variedade de funções regulatórias e comportamentais. Dentre estas funções estão a emoção e a memória, os comportamentos sociais, tais como a agressão e a reprodução, e a modulação de sistemas autonômicos e neuroendócrinos (Alheid *et al.*, 1995; Shepard *et al.*, 2003).

A organização estrutural da amígdala têm sido muito estudada. Sah *et al.* (2003), dividiu a amígdala dentro de três grupos: 1) o grupo basolateral ou profundo (complexo basolateral), que inclui o núcleo lateral, o núcleo basal (algumas vezes também referido como núcleo basolateral) e o núcleo basal acessório (que também é conhecido como núcleo basomedial); 2) o grupo superficial ou cortical, que inclui o núcleo cortical e o núcleo do trato olfatório lateral; 3) o grupo centromedial composto pelo núcleo medial e pelo núcleo central da amígdala. De acordo com alguns autores (Alheid *et al.*, 1995; Alheid & Heimer, 1988) a amígdala centromedial inerva o núcleo da base da estria terminal (BNST) e as regiões caudodorsal do núcleo pálido ventral. Desta forma, estas duas regiões têm conexões eferentes similares para projeções descendentes da amígdala, sendo as mesmas incluídas no complexo amigdalóide. Através da inclusão destas regiões no complexo centromedial, este passou a ser denominado de amígdala estendida (Figura 7).

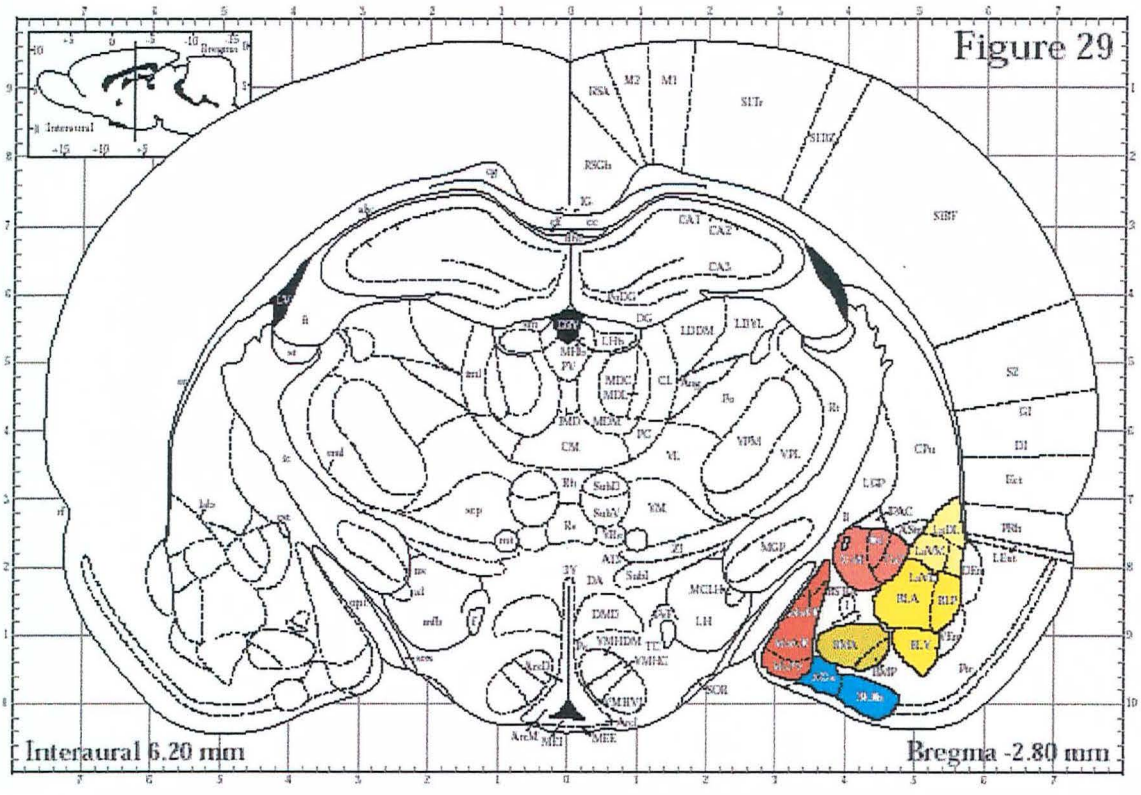

Figura 7. Núcleos da amígdala: grupo basolateral (amarelo), grupo cortical (azul) e grupo centromedial (vermelho).

Utilizando a coloração de Nissl, os limites dos núcleos da amígdala são facilmente distinguidos com base na sua citoarquitetura. Neurônios do núcleo basolateral são maiores apresentando um diâmetro do soma neuronal entre 20 a 35 μm (figura 8) e menos densamente marcados do que os neurônios do núcleo lateral (15 a 30 μm - figura 9). Os neurônios do núcleo central da amígdala apresentam um soma neuronal entre 15 a 25 μm de diâmetro (figura 10).

Núcleo basolateral e lateral da amígdala

Os núcleos basolateral e lateral da amígdala desempenham um papel importante nas respostas ao medo inato e ao medo-aprendido. Lesões destes núcleos reduzem dramaticamente e, em muitos casos, abolem completamente respostas comportamentais e neurovegetativos para o estímulo do medo condicionado (Davis 2000, LeDoux, 2000).

Núcleo central da amígdala

O núcleo central da amígdala medeia sinais e sintomas específicos do medo que agem em conjunto para intervir na produção de respostas para o mesmo Quando um animal é exposto a um estímulo que provoca medo, a CeA, através de estímulos aferentes da BaLA e LaA, projeta seus axônios a diversos núcleos hipotalâmicos e do tronco cerebral, causando efeitos comportamentais nestes animais. O estímulo da CeA que é recebido pelo hipotálamo lateral causa taquicardia, dilatação da pupila, elevação da pressão sanguínea, enquanto que aqueles estímulos da CeA que se dirigem para o PVN tem como conseqüência a liberação de corticosteróides (Walker *et al.*, 2003). Deste modo, podemos verificar que estes núcleos da amígdala interferem nas respostas a estímulos estressantes que provocam medo, sendo que um dos mais evidentes efeitos da manipulação neonatal é justamente a redução do medo nestes animais quando adultos e quando expostos a um ambiente novo ou frente a um estímulo estressor, como a presença do gato (Padoin *et al*, 2001).

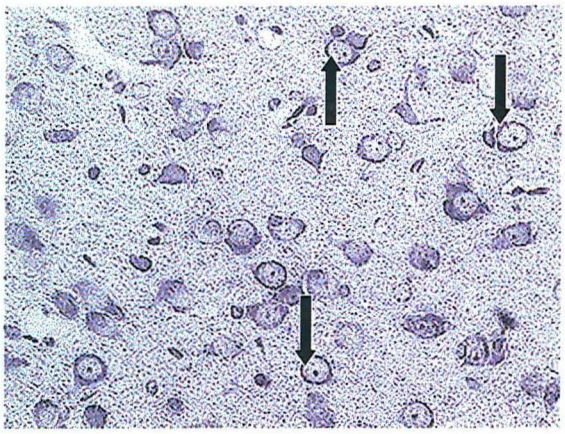

Figura 8. Neurônios (setas) do núcleo basolateral da amígdala (480x).

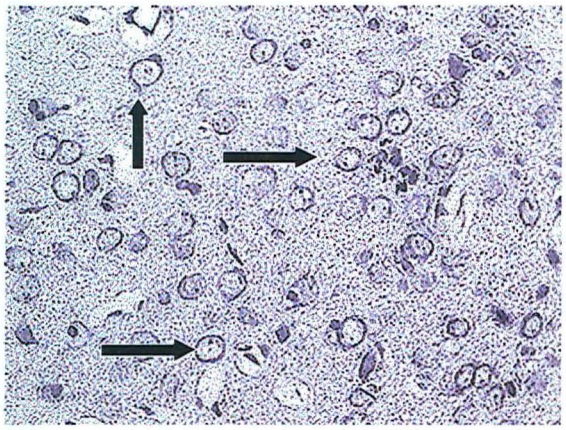

Figura 9. Neurônios (setas) do núcleo lateral da amígdala (480x).

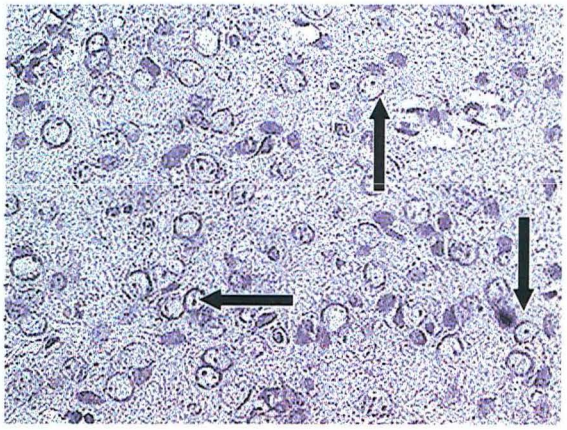

Figura 10. Neurônios (setas) do núcleo central da amígdala (480x).

1.4.3. Hipocampo

O hipocampo, uma estrutura do sistema nervoso central localizado no lobo temporal de cada hemisfério cerebral, está diretamente relacionado a funções de aprendizagem e memória, participando também da neurocircuitaria do estresse (Roozendal *et al.*, 2003). Em termos citoarquitetônicos, o hipocampo é formado por dois grandes agrupamentos de células que divididos em duas camadas celulares principais: as células granulares, que formam o giro denteado, e a camada de células piramidais CA1, CA2 e CA3 do próprio hipocampo (Swanson *et al.*, 1987) (figura 11,12).

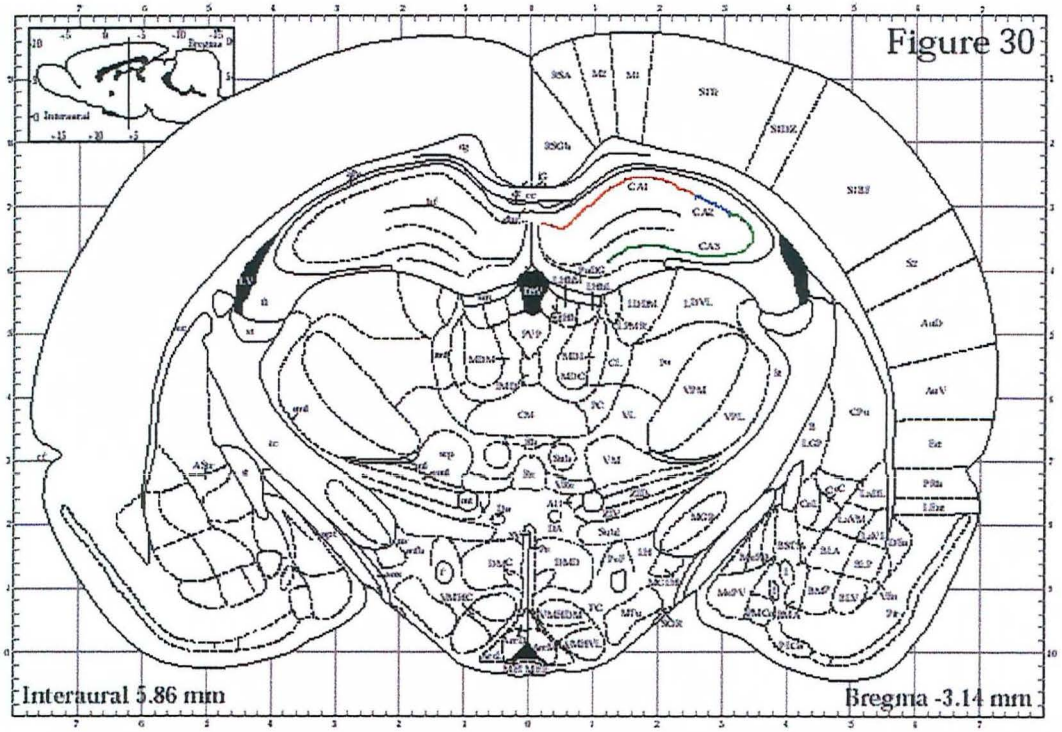

Figura 11. Camada CA1, CA2 e CA3 do hipocampo, conforme o atlas de Paxinos & Watson, 1997.

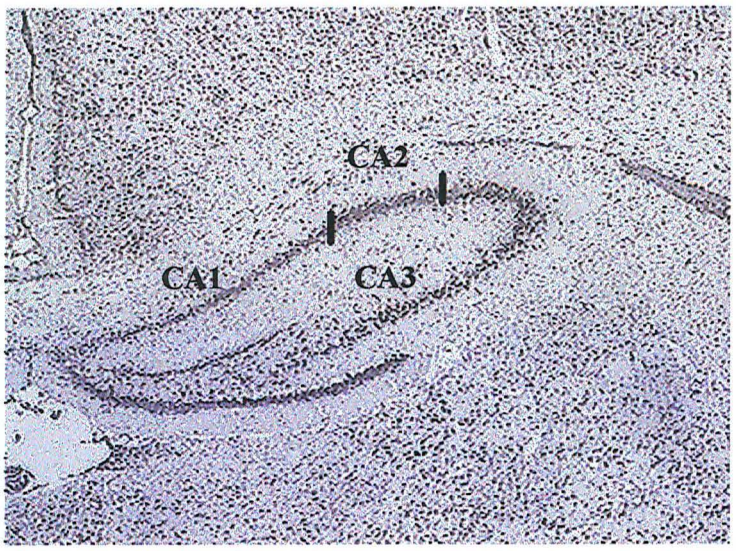

Figura 12. Fotomicrografia dos neurônios da região do CA1, CA2 e CA3 do hipocampo (48x).

A diminuição do número de receptores para glicocorticóides no hipocampo (Meaney *et al.*, 1993) induzido pela manipulação neonatal mostra que este núcleo do sistema nervoso central é afetado por estímulos externos que alteram o estado de homeostasia do organismo. Existem várias moléculas que podem ser liberadas pelo sistema nervoso em decorrência de um estímulo de estresse. Dentre elas está os glicocorticóides secretados pela glândula supra-renal e que, por sua vez, depois de serem liberados na circulação sistêmica e penetrarem na barreira hemato-encefálica agem nos *receptores* do hipocampo (Pavlides & McEwen, 1999). O hipocampo é um dos principais mediadores do mecanismo de retroalimentação negativa sobre o eixo HPA, também medeia processos cognitivos e comportamentais modulados por corticosteróides. O CRH também foi identificado em populações de células da camada piramidal do hipocampo de ratas jovens (Chen *et al.* 2001a) e receptores CRF1 foram encontrados principalmente nas áreas CA1 e CA3 do hipocampo (Van Pett *et al.*, 2000).

1.5. Relação neurônio e glia

O papel das células da glia nas diferentes funções desempenhadas pelo sistema nervoso central vêm sendo muito discutido e estudado. Sabe-se que essas células gliais desempenham funções importantes relacionadas à migração e à maturação dos neurônios, à formação da bainha de mielina, à regulação das concentrações iônicas, ao metabolismo de transmissores químicos, à integração sináptica, ao suprimento de energia para os neurônios e às respostas para injúria cerebral (Bezzi *et al.*, 2001; Kettenmann & Ramsom, 1995).

Assim a hipótese desta tese é relacionar os efeitos comportamentais e neuroendócrinos no animal adulto que receberam uma estimulação neonatal com uma alteração estável, provavelmente plástica, no sistema neuroendócrino ou em estruturas neurais que modula os eixos hipotalâmicos-hipofisiários. Testamos a hipótese de que o número de células nervosas e células gliais que formam os sistemas moduladores do eixo hipotálamo-hipófise adrenal seria afetado pela manipulação neonatal. Acreditava-se que a manipulação neonatal induz a uma redução do número de neurônios do *Locus coeruleus*, de núcleos hipotalâmicos (PVN e SON) e da amígdala (CeA, LaA e BaLA), uma vez que estes núcleos estão relacionados à diminuição da atividade do eixo HPG e HPA. No hipocampo era esperado encontrar um aumento na densidade de neurônios uma vez que isto poderia explicar o aumento de receptores para glicocorticóides. Acreditava-se que as células gliais poderiam estar alteradas em ratas adultas manipuladas no período neonatal, especialmente em regiões onde eventualmente não ocorresse redução do número de neurônios, uma vez que que estas células atuam diretamente na integração sináptica, na nutrição e proteção dos neurônios.

2. OBJETIVOS

2. Objetivos

2.1. Objetivo geral

Verificar se a manipulação neonatal induz à alterações morfológicas de núcleos do sistema nervoso central que participam do circuito do estresse: *Locus coeruleus*, núcleo paraventricular do hipotálamo, núcleo supra-óptico, núcleos central, lateral e basolateral da amígdala e as três camadas de células piramidais do hipocampo (CA1, CA2, CA3), em ratas Wistar em diferentes idades.

2.2. Objetivos específicos

a. Localizar o LC e realizar a contagem do número de neurônios, bem como verificar o volume deste núcleo em ratos machos e fêmeas manipulados e não manipulados no período neonatal, aos 11, 26, 35 e 90 dias de idade.

b. Verificar o diâmetro de neurônios do LC diferenciando os neurônios pequenos, médios e grandes em ratos machos e fêmeas manipulados e não manipulados no período neonatal aos 11, 26, 90 dias de idade.

c. Localizar os núcleos hipotalâmicos PVNp, PVNm e SON e realizar a contagem do número de neurônios, assim como verificar o volume de cada núcleo em ratas manipuladas e não manipuladas aos 11 e 90 dias de idade.

d. Localizar os núcleos central, lateral e basolateral da amígdala e realizar a contagem do número de neurônios destas regiões e verificar o volume de cada um destes núcleos em ratas manipuladas e não manipuladas aos 11 e 90 dias de idade.

e. Localizar as áreas CA1, CA2 e CA3 do hipocampo e realizar a contagem do número de neurônios e verificar o volume de cada área em ratas manipuladas e não manipuladas aos 11 e 90 dias de idade.

f. Investigar se a manipulação neonatal altera a densidade de células gliais nos núcleos paraventricular do hipotálamo, supra-óptico, lateral, central e basolateral da amígdala em ratas adultas.

3. RESULTADOS -TRABALHOS REALIZADOS

3. TRABALHOS REALIZADOS

3.1. A manipulação neonatal reduz o número de células no *Locus coeruleus* de ratos (Lucion, A. B., Pereira, F. M., Winkelmann. E. C., Sanvitto, G. L, Anselmo-Franci, J. A. Neonatal handling reduces the number of cells in the *Locus coeruleus* of rats, Behavioral Neuroscience, 117(5) 384-903, 2003)

O núcleo *Locus coeruleus* (LC) também chamado de núcleo A6 contém o maior número de neurônios noradrenérgicos do sistema nervoso central. Durante um estímulo de estresse o LC participa do controle da secreção do hormônio luteinizante (LH), sendo desta forma um importante modulador do eixo hipotálamo-hipófise-gonadal. Sabendo-se que um dos efeitos induzidos pela manipulação neonatal refere-se a diminuição da ovulação de ratas que foram manipuladas no período neonatal (Gomes *et al.*, 1999) e que a NE liberada pelo LC modula a secreção de LHRH que induz a secreção de LH (importante indução da ovulação), o objetivo deste trabalho foi verificar se a manipulação neonatal afeta o número de neurônios do LC em ratos machos e fêmeas em diferentes idades (11, 26, 35 e 90 dias de idade).

Este trabalho foi o pioneiro em relação a alterações morfológicas relacionadas ao número de células causadas pela manipulação neonatal desenvolvido em nosso laboratório. O mesmo foi realizado de forma solidária e com igual participação com a aluna de Mestrado Francine Martins Pereira.

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Neonatal Handling Reduces the Number of Cells in the Locus Coeruleus of Rats

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Janete A. Anselmo-Franci
Universidade de São Paulo

Neonatal handling induces long-lasting effects on behaviors and stress responses. The objective of the present study was to analyze the effects of neonatal handling (from the 1st to the 10th day after delivery) on the number of cells and volume of locus coeruleus (LC) nucleus in male and female rats at 4 different ages: 11, 26, 35, and 90 days. Results showed significant reductions in the number of cells and the volume of the LC nucleus in neonatally handled males and females compared with nonhandled rats. Environmental stimulation early in life induced a stable structural change in a central noradrenergic nucleus, which could be one of the causal factors for the behavioral and hormonal alterations observed in adulthood.

The environment that surrounds an individual during infancy determines the development of responses to stress (Anisman, Zaharia, Meaney, & Merali, 1998; Denenberg, 1964; Francis, Caldji, Champagne, Plotsky, & Meaney, 1999; Liu et al., 1997; Meaney et al., 1996). The effects of environmental interventions during the postnatal period may constitute the foundation of an individual's vulnerability to diseases resulting from stress throughout life (Heim, Owens, Plotsky, & Nemeroff, 1997; Henry & Wang, 1998; Huether, 1998; Kaufman, Plotsky, Nemeroff, & Charney, 2000; Pryce, Bettschen, Bahr, & Feldon, 2001). In rats, stimulation of the pups by human handling has been used as an experimental procedure to examine the mechanisms by which variations in the environment during the neonatal period could affect the development of neural systems (Ábrahám & Kovács, 2000; Anand, Coskun, Thrivikraman, Nemeroff, & Plotsky, 1999; Anand & Scalzo, 2000; Moore, Dou, & Juraska, 1992; Papaioannou, Dafni, Alikaridis, Bolaris, & Stylianopoulou, 2002; Veenman et al., 1999; Zhang et al., 2002), giving rise to stable behavioral and neuroendocrine changes (Caldji, Francis, Sharma, Plotsky, & Meaney, 2000; Caldji et al., 1998; Denenberg, 1964; Gschanes, Eggenreich, Windisch, & Crailsheim, 1998; Kaufman et al., 2000; Levine, 1962; Meerlo, Horvath, Nagy, Bohus, & Koolhaas, 1999).

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The neonatal handling procedure also shows the extraordinary plasticity of the stress response-mediating neural systems. In rats, neonatal stimulation typically consists of the manipulation of pups for a few minutes, generally during the first 2 weeks of life. Handling is an apparently mild environmental intervention involving brief removal of the pups from the nest for about 3–15 min. However, under natural conditions, the development of a rat typically occurs in dark holes with very few interventions from the environment and, obviously, nothing even close to human handling (Daly, 1973). Maternal deprivation is another type of environmental manipulation in the postnatal period that can induce long-lasting effects on behavior and hypothalamic–pituitary–adrenal axis responsiveness (Anisman et al., 1998; Lehmann et al., 2002; Liu, Caldji, Sharma, Plotsky, & Meaney, 2000; Plotsky & Meaney, 1993; Suchecki, Mozaffarian, Gross, Rosenfeld, & Levine, 1993; Van Oers, de Kloet, Whelan, & Levine, 1998). In contrast to handling, maternal deprivation consists of prolonged separation of the pups from the mother, varying from 3 to 24 hr each session.

The changes in adult rats resulting from neonatal handling can be characterized by the reduced response to stressful stimuli, expressed by lower corticosterone (Levine, 1962; Levine, Haltmeyer, Karas, & Denenberg, 1967; Meaney et al., 1993; Plotsky & Meaney, 1993), prolactin (Meerlo et al., 1999; Núnez, Ferré, Escorihuela, Tobeña, & Fernández-Teruel, 1996), and adrenaline secretion (Meerlo et al., 1999), and by an increased sensitivity of hypothalamic–pituitary–adrenal axis feedback, which has been related to the increased population of glucocorticoid receptors in the hippocampus and frontal cortex (Liu et al., 1997; Meaney & Aitken, 1985; Meaney et al., 1993; Meaney et al., 1994).

Besides the stress-related hormonal changes, neonatal handling can also induce behavioral alterations. A core effect of neonatal handling is reduced behavioral inhibition, expressed by increased locomotion in novel environments (Denenberg, 1964; Meaney et al., 1996; Padoin, Cadore, Gomes, Barros, & Lucion, 2001). Recently, we studied the effects of neonatal handling on several behaviors besides locomotor activity in novel and aversive envi-

ronments. We showed that daily handling of the offspring during the first 10 days of life reduced sexual behavior of male and female rats (Padoin et al., 2001) and induced a significant decrease in ovulation, with most of handled females having anovulatory estrous cycles (Gomes, Frantz, Sanvitto, Anselmo-Franci, & Lucion, 1999). These results raise some doubts regarding the supposedly positive effects of neonatal stimulation (Costela, Tejedor-Real, Mico, & Gilbert-Rahola, 1995; Gonzalez, Rodriguez Echandia, Cabrera, & Foscolo, 1994). Furthermore, these results show that neonatal handling may affect the activity of the hypothalamic–pituitary–gonadal axis in addition to activity of the hypothalamic–pituitary–adrenal axis.

The locus coeruleus (LC) nucleus, also called the A6 nucleus, is located in the dorsolateral tegmentum of the pons, bilateral to the fourth ventricle, and it is the main cluster of noradrenergic neurons in the central nervous system. The nucleus has a broad and diffuse action on several structures of the nervous system. In rats, the LC contains about 1,500 neurons (Aston-Jones, Shipley, & Grzanna, 1995; Foote, Bloom, & Aston-Jones, 1983; Guillamón, de Blas, & Segovia, 1988). Previous studies have identified fusiform medium-sized neurons that project mainly into the cerebral cortex and hippocampus. Large multipolar neurons send projections into the spinal cord, and the multipolar neurons of the anterior region of the LC project into the hypothalamus (Aston-Jones et al., 1995; Cunningham & Sawchenko, 1998; Loughlin, Foote, & Grzanna, 1986; Swanson, 1976). The LC activity is remarkably synchronized, producing a coordinated release of noradrenaline (NA) throughout the central nervous system.

Besides being activated by stressful stimuli (Kaufman et al., 2000; Pacák & Palkovits, 2001; Van Bockstaele, Bajic, Proudfit, & Valentino, 2001), the LC participates in the control of luteinizing hormone (LH) secretion, thus being an important modulator of the hypothalamic–pituitary–gonadal axis. Electrical stimulation of the LC potentiates the release of LH induced by stimulation of the medial preoptic area (MPOA; Gitler & Barraclough, 1987). Moreover, we have demonstrated that electrolytic lesion of the LC decreases the content of NA in the medial basal hypothalamus and blocks the pulsatile release of LH in ovariectomized rats, the preovulatory gonadotropin surges, as well as the surges induced by steroids in ovariectomized rats (Anselmo-Franci, Franci, Krulich, Antunes-Rodrigues, & McCann, 1997; Anselmo-Franci, Rocha-Barros, Franci, & McCann, 1999; Franci & Antunes-Rodrigues, 1985; Helena, Franci, & Anselmo-Franci, 2002). These blockades of hormonal surges are accompanied by an increase in the LH-releasing hormone (LHRH) content in the preoptic area, suggesting that the LHRH is not released by the time of gonadotropin surges in the lesioned animals (Helena et al., 2002). In addition, we observed an increased number of neurons immunoreactive for Fos protein in the LC during the period of the preovulatory gonadotropin surges, as well as during those surges induced by steroids in ovariectomized rats (unpublished data), which suggest that the activation of this nucleus may be important for the occurrence of gonadotropin surges.

The central hypothesis of the current study is that neonatal stimulation might induce structural changes in the central nervous system, which would in turn account for the alterations observed in adult life. In order to explain the behavioral and neuroendocrine effects of early handling in an adult animal, it is plausible to raise the hypothesis that early-life stimulation leads to stable, probably structural, alterations, which could explain the behavioral and neuroendocrine effects of neonatal handling (Aguiar, Cadore, Padoin, Barbosa-Coutinho, & Lucion, 1997; Caldji et al., 2000; Hermel et al., 2001; Liu, Diorio, Day, Francis, & Meaney, 2000; Meaney et al., 1996; Sturrock, Smart, & Tricklebank, 1983; Vaid et al., 1997; Veenman et al., 1999). A trace left by the neonatal stimulation in the nervous system could be the cause of the behavioral changes observed in adulthood. Thus, it is plausible to assume that the anovulatory estrous cycles of the rats that were handled during the neonatal period (Gomes et al., 1999) could be due to reduced activity of the LC. In fact, a previous study (Liu, Caldji, et al., 2000) showed that neonatal handling diminishes the release of NA into the paraventricular hypothalamus, indicating decreased LC activity. Thus, the objective of the present study was to analyze the effects of neonatal handling on the number of cells and volume of the LC nucleus of male and female rats at different ages.

Method

Subjects and Housing

Pregnant female Wistar rats were brought from the colony of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil) to an animal room in our laboratory. About 7 days before delivery, the females were housed individually, and the presence of pups was checked for twice a day (beginning and end of the light period). On the day of birth (Day 0), the number of pups was culled to 8 per dam by randomly removing some of them with minimal contact with the remaining pups and the dam. After weaning (Postnatal Day 21), the pups were housed in same-sex groups of 2–4 per cage (41 cm long × 34 cm wide × 17 cm high) according to body weight. All rats were maintained on a 12-hr light–dark cycle (lights on from 6:00 a.m. to 6:00 p.m.), the room temperature was 22 ± 1 °C, and water and food (Rodent chow, Nutrilab, Colombo, Brazil) were available at all times. Male and female rats were studied at four different ages: 11 (neonatal period), 26 (prepubertal), 35 (peripubertal), and 90 (adult) days of age. The rats in each experimental group (nonhandled and handled) and of each age ($n = 6$) were nonsiblings (each subject from a different litter), but males and females came from the same litter. Adult females were studied in diestrus. The estrous cycle was verified by taking vaginal smears over a period of 15 days, and only females with at least three regular cycles were studied. In our laboratory, vaginal opening was observed at 36 days of age in nonhandled (control) females and at 41 days in handled ones.

Neonatal Handling and Experimental Groups

At each of the ages studied, the rats were divided into two groups: nonhandled ($n = 6$ for each sex and age), in which the pups were not handled either by the researchers or by the caretakers from the 1st to the 10th day postnatal, with bedding being changed twice a week from the 10th day on, and handled ($n = 6$ for each sex and age), in which the litter and the mother in their home cage were taken to a quiet room next to the animal facility, with the same light period and temperature. First, the mother was placed in another cage next to the home cage, and the experimenter then gently handled all pups at the same time using both hands, covered with fine latex gloves, for 1 min. After handling, all pups were returned to the nest at the same time and the mother was placed back in the home cage. The pups were handled at a distance of about 1 to 2 m from the mother, and the total time of mother–infant separation was approximately 2 min. This procedure was repeated from the 1st to the 10th postnatal day, during the light period of the daily photoperiod cycle, avoiding a fixed temporal pattern of stimulation across the 10 days.

A total of 48 rats of each sex were divided into two groups and four ages, with 6 rats of each age. They were used for cell counting and LC nucleus volume measurement. The neuron soma diameters were measured in 36 of the 48 rats of each sex, as only three ages (11, 26, and 90 days) were analyzed. Experiments were performed in accordance with the National Institutes of Health guidelines for animal research and were approved by the Research Committee of the Federal University of Rio Grande do Sul.

Histological Procedures

Rats were anesthetized with xylazine (0.1 ml/g body weight im) and ketamine (0.1 ml/g body weight im) and then carefully perfused through the heart with 100 ml phosphate-buffered saline with heparin for 20 min, followed by 200 ml of 4% (wt/vol) paraformaldehyde diluted in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 30 min. The brains were removed and postfixed for 72 hr in the same fixing solution at 4 °C. After fixation, the brains were washed for 1 hr in running water and placed in 70% alcohol - overnight, and then dehydrated with alcohol and xylol. The brains were embedded in paraplastic resin (Histosec; Merck, Darmstadt, Germany) in the final position. Serial 15-μm thick coronal sections of the brains were cut with a microtome and mounted in series on Entellan (Merck) on slides which were then coverslipped. The tissue sections were stained with Cresyl-violet. The LC was identified according to a rat brain atlas of Paxinos and Watson (1997).

Morphological Measures

In order to characterize the cells to be counted, we measured the size (in microns) of the cell body of the small, medium, and large neurons. We used a light microscope (Zeiss Axioscop2; Zeiss, Goettingen, Germany) with a 40× lens, a video camera (CCD video camera module; Sony, Tokyo, Japan) attached to a computer (Apple Macintosh 8600–300), and the NIH Image 1.62F image analyzing system (Rasband, 1996) to measure the largest diameter of the neuron soma in microns. A total of 15 neurons of each size (small, medium, or large) per rat were chosen to be measured on the basis of a clear and distinct cell membrane, nucleus, and nucleolus. Five neurons—multipolar, fusiform, or ovoid—were chosen from each of the three parts into which the LC nucleus was divided, that is, anterior, middle and posterior. The mean size of these 15 neurons was calculated for each group of neurons (small, medium, or large) and for each rat. Cell body size was measured on the same sections that were later used for neuron count, and the volume of the LC nucleus was calculated.

We counted all nucleated medium- and large-sized neurons in the sections of the right-side LC nucleus. A previous study (Swanson, 1976) showed no significant difference in the number of LC neurons between the two sides of the brain. Beginning on the second posterior section, we counted each fifth section rostrally. The sectioned fields were 60 μm apart to avoid duplicate counts of the same cell. This quantification was performed separately by two experimenters, with the microscope fitted with a 40× lens and a 10× eyepiece with a 1-mm^2 reticule to permit an easier count. The total estimated number of medium and large neurons in the LC was calculated according to the following formula: $Nt = ns \times P$, where Nt is the total number of estimated units in the structure, ns the number of units counted in all sampled sections of the nucleus, and P the period during which the sections were sampled (Konigsmark, 1970). The cell nucleus was the unit counted. The estimated number of cells of each rat was averaged between the two experimenters, who were blind to the neonatal treatment and age. The variation between the results of the two LC counts performed at separate times by the experimenters was about 4.5%, indicating good reliability.

The area of the sampled sections was measured with the same brain imaging system as described above, a 10× lens, and an object micrometer for the size equivalence of the real object and the image. The serial sections were identified, digitized, and displayed on the computer monitor. The borders of the LC nucleus were traced by using the computer mouse, and the area of each cross-section was obtained. The volume of the right-side LC nucleus, expressed in cubic millimeters, was estimated by multiplying the sum of the areas by the interval between sections (60 μm).

Statistical Analysis

The values of the diameter (in microns) of the cell body of the LC neurons, the total estimated number (Nt) of medium and large neurons in the LC, and the volume (in cubic millimeters) of the LC nucleus were expressed as means (± *SEM*). Results were compared between the groups of equal gender (nonhandled and handled) and among the ages studied by two-way analysis of variance (ANOVA; group and age effect). If a significant main or interaction effect was detected, a post hoc Newman–Keuls test comparison was used to measure individual treatment group differences. In all cases, the accepted level of significance was $p < .05$. It was not an objective of the study to analyze sex differences.

Results

Cell Size in Males

Table 1 presents the soma diameter size of neurons in the LC of male rats. Cells were classified into three groups according to size. Significant main effects for age, $F(2, 30) = 11.11$, $p < .01$, but not group, $F(1, 30) = 1.63$, $p > .05$, on the size of small cells were observed. No significant interaction was detected between groups (nonhandled and handled) or ages (11, 26, and 90 days), $F(2, 30) = 1.06$, $p > .05$. Post hoc analysis revealed that the main effect for age was accounted for by a significant larger soma diameter in 11-day-old males than in 26- and 90-day-old males (Newman–Keuls test, $p < .05$). For the soma diameter of medium-sized cells in males, no significant main effect was detected for age, $F(2, 30) = 2.21$, $p > .05$, but there was an almost significant main effect of group, $F(1, 30) = 4.15$, $p = .051$. There was no significant Group × Age interaction effect, $F(2, 30) = 0.06$, $p > .05$, on the soma diameter of medium size cells in males. No significant main effects of age, $F(2, 30) = 0.81$, $p > .05$; group, $F(1,$

Table 1
Soma Size (in Microns) of Small, Medium, and Large Neurons in the Right Locus Coeruleus of Male Rats

	Age (days)		
Cell size and group	11	26	90
Small			
Nonhandled	3.75 ± 0.08	3.35 ± 0.10	3.36 ± 0.15
Handled	3.82 ± 0.06	3.60 ± 0.08	3.33 ± 0.08
Medium			
Nonhandled	17.17 ± 0.30	16.48 ± 0.20	16.87 ± 0.39
Handled	16.66 ± 0.30	16.10 ± 0.12	16.28 ± 0.39
Large			
Nonhandled	27.13 ± 0.48	28.53 ± 0.76	27.28 ± 0.76
Handled	27.08 ± 0.47	27.13 ± 0.50	27.24 ± 0.56

Note. Values are means (± *SEM*) of the largest diameter of the cell body at three different ages. Nonhandled control rats were kept undisturbed during the neonatal period, and handled rats were gently manipulated for 1 min daily from the 1st to the 10th day after delivery. Two-way ANOVA (between groups and among ages) showed significant main effect of age on the size of small cells, but no significant main effect of group or the Group × Age interaction. $n = 6$ per group.

30) = 1.02, $p > .05$; or interaction between groups and ages, $F(2, 30) = 0.84$, $p > .05$, were detected on the soma diameter of large-sized cells in males.

Cell Size in Females

Table 2 presents the soma diameter size of neurons in the LC of female rats. A significant main effect of age, $F(2, 30) = 77.21$, $p < .01$, and an almost-significant main effect of group, $F(1, 30) = 3.14$, $p = .08$, were detected on the size of small cells. No significant interaction was detected between groups and ages studied, $F(2, 30) = 0.82$, $p > .05$. Post hoc analysis revealed that the main effect of age was accounted for by a significantly larger soma diameter in 11-day-old females than in the 26- and 90-day-old females (Newman–Keuls test, $p < .05$ for both comparisons). No significant main effects of age, $F(2, 30) = 0.74$, $p > .05$; group, $F(1, 30) = 0.75$, $p > .05$; or the interaction between groups and ages, $F(2, 30) = 0.47$, $p > .05$, were observed on the soma diameter of medium-sized cells of female rats. A significant main effect of age, $F(2, 30) = 4.28$, $p < .05$, but not group, $F(1, 30) = 0.50$, $p > .05$, was detected on the soma diameter of large-sized cells in female rats. The interaction between group and age, $F(2, 30) = 2.74$, $p = .08$, tended toward significance. Post hoc analysis revealed that the main effect for age was accounted for by a significantly larger soma diameter in 90-day-old females than in 11- and 26-day-old females (Newman–Keuls test, $p < .05$ for both comparisons).

Cells were not labeled to determine cell type. However, the literature suggests that small neurons are interneurons, whereas medium and large cells are neurons in the LC that project to other brain areas (Aston-Jones et al., 1995). Neurons were easily differentiated from glial cells, which showed a larger nucleus and a deeply stained nucleolus. Glial cells are small, being approximately the same size as the smallest neurons, which were not counted in the present study.

Table 2
Soma Size (in Microns) of Small, Medium, and Large Neurons in the Right Locus Coeruleus of Female Rats

	Age (days)		
Cell size and group	11	26	90
Small			
Nonhandled	3.71 ± 0.08	2.90 ± 0.06	2.98 ± 0.07
Handled	3.71 ± 0.08	3.05 ± 0.04	3.12 ± 0.04
Medium			
Nonhandled	17.83 ± 0.39	16.47 ± 0.18	16.47 ± 0.13
Handled	16.79 ± 0.19	16.60 ± 0.18	16.87 ± 0.20
Large			
Nonhandled	27.66 ± 0.61	27.55 ± 0.41	27.55 ± 0.41
Handled	26.22 ± 0.41	27.23 ± 0.42	28.13 ± 0.50

Note. Values are means (± *SEM*) of the largest diameter of the cell body at three different ages. Nonhandled control rats were kept undisturbed during the neonatal period, and handled rats were gently manipulated for 1 min daily from the 1st to the 10th day after delivery. Two-way ANOVA (between groups and among ages) showed a significant main effect of age on the size of small and large cells, but no significant main effect of group or the Group × Age interaction. $n = 6$ per group.

Number of Cells in Males

Figure 1 illustrates the estimated number of medium and large neurons in the right-side LC of male rats. Significant main effects of age, $F(3, 40) = 2.89$, $p < .05$, and group, $F(1, 40) = 60.33$, $p < .01$, were detected on the number of cells. No significant interaction was detected between groups (nonhandled and handled) and the ages studied (11, 26, 35, and 90 days), $F(3, 40) = 1.11$, $p > .05$. Post hoc analysis revealed that the main effect of age was accounted for by increased number of cells in 26-day-old compared with 90-day-old males (Newman–Keuls test, $p < .05$).

LC Volume in Males

Figure 2 illustrates the estimated volume (in cubic millimeters) of the right-side LC nucleus in male rats. Significant main effects of age, $F(3, 40) = 45.43$, $p < .01$; group, $F(1, 40) = 27.21$, $p < .01$; and a Group × Age interaction, $F(3, 40) = 12.46$, $p < .01$, were detected. Post hoc analysis revealed that LC nucleus volume in nonhandled males increased from Day 11 to Day 26, and then decreased on Day 35 compared with Day 26 (but was still larger than on Day 11), finally increasing to its maximum size on Day 90 (Newman–Keuls test, $p < .05$ for all comparisons). In handled males, the volume of the LC nucleus increased from Day 11 to Day 26 and kept approximately the same size at the other ages studied (Newman–Keuls test, $p < .01$ comparing Day 11 with Days 26, 35, and 90). Post hoc analysis also revealed that the volume of LC nucleus decreased in handled males compared with nonhandled males aged 11 and 90 days (Newman–Keuls test, $p < .05$ for both comparisons).

Number of Cells in Females

Figure 3 shows the estimated number of medium and large neurons in the right-side LC of female rats. Significant main effects of age, $F(3, 40) = 3.48$, $p < .05$, and group, $F(1, 40) = 85.27$, $p < .01$, were detected on the number of cells. No significant interaction was detected between group and age, $F(3, 40) = 0.82$, $p > .05$. Post hoc analysis revealed that the main effect of age was accounted for by a decreased number of cells on Day 35 compared with Days 26 and 90 (Newman–Keuls test, $p < .05$ for both comparisons).

LC Volume in Females

Figure 4 illustrates the estimated volume (in cubic millimeters) of the right-side LC nucleus in female rats. Significant main effects of age, $F(3, 40) = 7.92$, $p < .01$, and group, $F(1, 40) = 17.89$, $p < .01$, were detected on the volume of the LC nucleus. No significant interaction was detected between group and age, $F(3, 40) = 0.67$, $p > .05$. Post hoc analysis revealed that the main effect for age was accounted for by a decreased LC nucleus volume on Day 35 compared with all other ages (Newman–Keuls test, $p < .05$ for all comparisons).

Discussion

The results obtained in the present study show that daily neonatal handling during the first 10 days of life induces a reduction in the number of cells in the LC of male and female rats. The

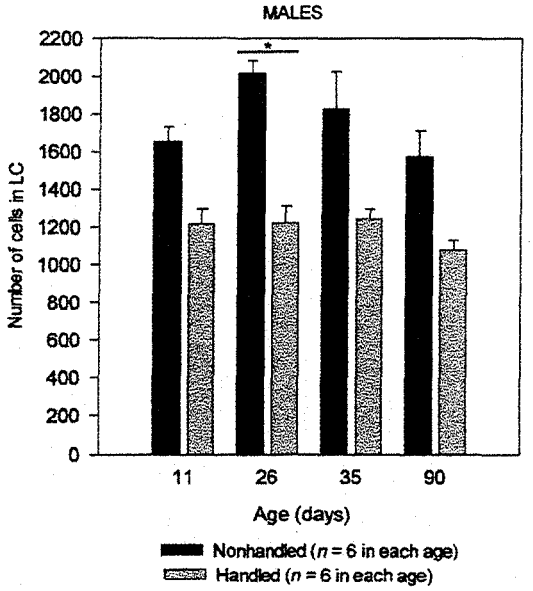

Figure 1. Effects of neonatal handling on the number of cells in the right locus coeruleus (LC) nucleus of male rats at four different ages. The estimated number of cells is reported as mean (± *SEM*). Nonhandled control rats were kept undisturbed during the neonatal period, and handled rats were gently manipulated for 1 min daily from the 1st to the 10th day after delivery. Two-way ANOVA (between groups and among ages) showed significant main effects for age and group, but no significant Group × Age interaction. Asterisk indicates a significant difference from the 90-day-old group (Newman–Keuls test, $p < .05$).

number of cells in the 11-day-old handled rats was lower than that of the nonhandled group at the same age, and this lower level was maintained almost constant during the other periods analyzed, without the fluctuations observed in the control group and described in a previous study (Pinos et al., 2001). At all ages studied, the reduction in the number of cells was quite marked. Although different experimental approaches (Konigsmark, 1970; Mayhew, 1992; West, 1999) to cell counting were used in the present study and in a previous one (Pinos et al., 2001), the results were similar. Moreover, both studies show the abrupt decrease of cells on Postnatal Day 35 in the nonhandled males and females. Because the same cell counting method was used in both groups and the purpose of the study was to compare the number of cells between handled and nonhandled rats, the procedures used may be appropriate to estimate the effects of the neonatal stimulation on the development of the LC nucleus.

In an as-yet-unexplained manner, early-life environmental stimulation prevented the normal development of the LC nucleus that is characterized by the increase in the number of neurons and volume from birth to adulthood in both male and female rats (Pinos et al., 2001). Neonatal handling reduced the number of cells in the 11-day-old rats and prevented its expected increase. Thus, we may infer that handling may have reduced the number of neurons, possibly by acting on cell death and/or proliferation.

This plastic alteration in a structure functionally related to the stress system could cause the attenuated responses to stressors and the behavioral changes originating from the neonatal stimulation procedure of handling. In fact, a previous study showed that neonatal handling increases the expression of alpha2-receptor binding levels in the noradrenergic neurons in the LC. These inhibitory autoreceptors could explain the reduced stress response of NA in the paraventricular hypothalamic nucleus (PVN) of adult male rats (Liu, Caldji, et al., 2000). Moreover, handling also increased $GABA_A$ and central benzodiazepine receptor levels in the LC of rats, which would contribute to the behavioral differences, especially the expression of fear in adulthood (Caldji et al., 2000). Other studies (Lehmann et al., 2002; Liu et al., 2000) showed that in both young adult and aged rats, the hippocampus of those rats reared by dams that performed high levels of maternal licking/grooming and arched-back nursing had a higher expression of NMDA receptor and brain-derived neurotrophic factor mRNA, as well as increased synaptogenesis, but not neuron density, which could account for the enhancement of spatial learning.

Most studies on neonatal handling deal with the neural basis of stress, probably because of the prominent effects of overt stimulation during the neonatal period on stress-related responses. However, Gomes et al. (1999) demonstrated that neonatal handling can also cause a significant reduction of ovulation in female rats. The LC is an integrative nucleus related to both stress responses and reproductive functions (Anselmo-Franci et al., 1997, 1999; Kaufman et al., 2000; Van Bockstaele et al., 2001).

The LC projects to the preoptic and the suprachiasmatic areas, which are involved in the regulation of the cyclic secretion of hormones in the anterior pituitary (Anselmo-Franci et al., 1997;

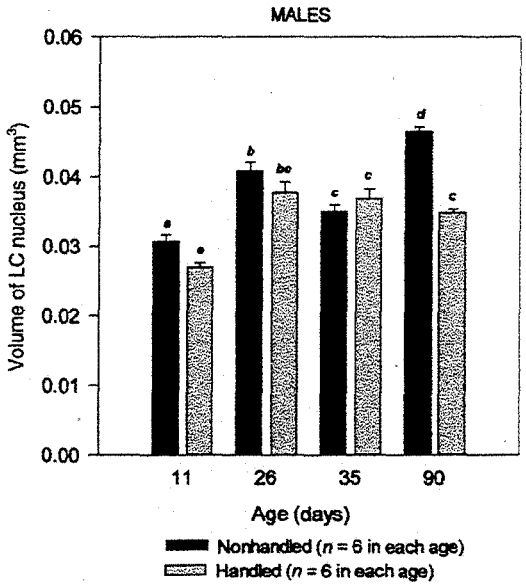

Figure 2. Effects of neonatal handling on the volume (in cubic millimeters) of the right locus coeruleus (LC) nucleus of male rats at four different ages. The estimated volume is reported as mean (± *SEM*). Nonhandled control rats were kept undisturbed during the neonatal period, and handled rats were gently manipulated for 1 min daily from the 1st to the 10th day after delivery. Data were analyzed with a two-way ANOVA (between groups and among ages) followed by the Newman–Keuls test (significance accepted at $p < .05$). ANOVA showed significant interaction between the variables. Values with a letter in common were statistically indistinguishable ($p \geq .05$) on the basis of post hoc contrasts.

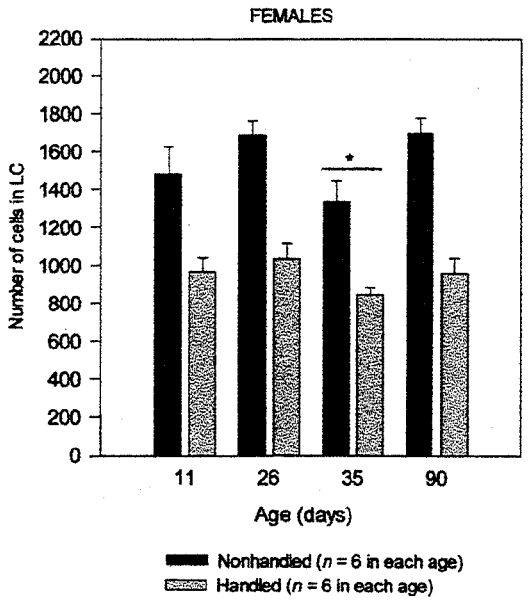

Figure 3. Effects of neonatal handling on the number of cells in the right locus coeruleus (LC) nucleus of female rats at four different ages. The estimated number of cells is reported as mean (± *SEM*). Nonhandled control rats were kept undisturbed during the neonatal period, and handled rats were gently manipulated for 1 min daily from the 1st to the 10th day after delivery. Two-way ANOVA (between groups and among ages) showed significant main effects of age and group, but no significant Group × Age interaction. Asterisk indicates a significant difference from the 26- and 90-day-old groups (Newman–Keuls test, $p < .05$).

Jones & Moore, 1977). The stimulatory effect of LC on the hypothalamus and preoptic area, specifically on LH secretion, seems to be necessary for ovulation. It is interesting to note that there is an inverse relationship between the size of partial electrolytic lesions of the LC and the amplitude and number of LH pulses (Anselmo-Franci et al., 1999). In the present study, the reduction in the number of neurons in the LC of female rats handled during the neonatal period may have decreased the stimulatory activity of the LC on preoptic and hypothalamic areas, with a consequent reduction of LH secretion. In this regard, a reduced preovulatory LH surge could explain the anovulatory cycles observed in neonatally handled females (Gomes et al., 1999). The increased expression of the adrenergic alpha2 inhibitory autoreceptors (Liu, Caldji, et al., 2000) would also tend to diminish the stimulatory effect of LC NA on the pulsatile secretion of LHRH in the preoptic and medial basal hypothalamus.

The present study shows that handling during the postnatal period may have caused a stable change in the development of the nucleus. The LC responds to peripheral sensory input, even at earlier stages of development (Dent, Smith, & Levine, 2001; Nakamura, Kimura, & Sakaguchi, 1987). During the neonatal period, the LC is more sensitive to somatosensory stimuli, such as airpuffs and tactile stimulation, than to noxious ones (Nakamura et al., 1987). Thus, taking these results into consideration, we may infer that the handling procedure can increase the activity of LC neurons, either directly by the impact of the environmental stimulation produced by human handling, or by the increased maternal licking and grooming of the pups observed after the handling procedure. Further studies should be designed in order to determine whether the behavioral and neuroendocrine effects of neonatal handling are due to the increased maternal caregiving, as proposed by previous studies (Hennessy, Vogt, & Levine, 1982; Levine, 2001; Liu et al., 1997; Pryce, Bettschen, & Feldon, 2001; Villescas, Bell, Wright, & Kufner, 1977), or to possible deleterious actions of overexposure to environmental stimulation provoked by human manipulation.

An interesting research line related to the present study has shown dual effects of early-life environmental stimulation on the early olfactory learning process (Price, Darby-King, Harley, & McLean, 1998). According to the results of the cited study, stimulation increases activity of noradrenergic neurons through the beta-adrenergic receptors, with a consequent activation of cAMP pathways. This activation apparently modulates neural plasticity changes and thus plays a crucial role in learning and memory processes (Frank & Greenberg, 1994; Price et al., 1998). An inverted U-shape curve was demonstrated between activation of the central noradrenergic system and olfactory learning. In the first 2 weeks after delivery, the overt stimulation of the pups by handling and the increased maternal behavior after the external intervention reduce olfactory learning in rats. One stimulus (stroking, for instance) regulates cAMP levels within an optimal range and increases olfactory learning, whereas an additional noradrenergic stimulation (increased maternal licking, for instance) may

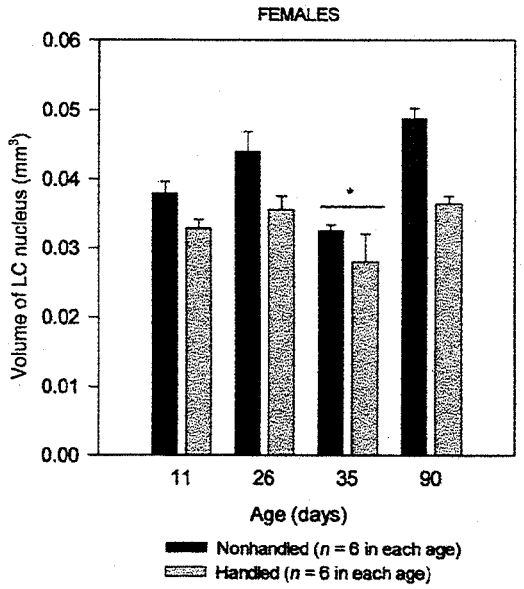

Figure 4. Effects of neonatal handling on the volume (in cubic millimeters) of the right locus coeruleus (LC) nucleus of female rats at four different ages. The estimated volume is reported as mean (± *SEM*). Nonhandled control rats were kept undisturbed during the neonatal period, and handled rats were gently manipulated for 1 min daily from the 1st to the 10th day after delivery. Two-way ANOVA (between groups and among ages) showed significant main effects of age and group, but no significant Group × Age interaction. Asterisk indicates a significant difference from the 11-, 26-, and 90-day-old groups (Newman–Keuls test, $p < .05$).

block the effect of stroking and thus inhibit learning (Price et al., 1998). Overt pup stimulation may play an inhibitory role in cellular proliferation in the LC nucleus in a manner similar to that described for the long-lasting effects on olfactory learning, as shown in a previous study in which an inverted U-shaped curve between early life stimulation and learning was observed (Price et al., 1998).

In the present study, the reduction in the number of cells in the LC may have been related to the neurotrophic effects of cAMP. A previous study (Sklair-Tavron & Segal, 1993) showed that cAMP was able to induce significant increases in the number of tyrosine hydroxylase cells in LC cell cultures through beta-adrenergic receptor agonists (Sklair-Tavron & Segal, 1993) and other brain areas (Wagner, Seidler, Lappi, McCook, & Slotkin, 1995) during critical periods of development. The presynaptic input programs the coupling of beta-adrenergic signals to nuclear events through adenylate cyclase and second messengers like cAMP that can activate the expression of basic regulators of cell differentiation, like ornithine descarboxylase (ODC). ODC catalyzes the initial step in the synthesis of polyamines, which in turn are related to nucleic acid and protein synthesis and consequently to cell growth and development (Slotkin, Fergunson, Cada, McCook, & Seidler, 2000). On the other hand, several studies (Kuhn & Schanberg, 1998; Schanberg & Kuhn, 1985; Wang, Bartolome, & Schanberg, 1996) have shown that long periods of maternal deprivation can decrease the expression of ODC in various tissues, including the brain. Conversely, stroking the pups with brushes, a manipulation that mimics the tactile stimulation produced by the natural maternal licking/grooming, can increase ODC activity above control levels (Schanberg & Kuhn, 1985). Stroking can also reverse effects of maternal deprivation, such as the increased ACTH response and decreased corticotropin-releasing hormone mRNA in the PVN of 12-day-old rat pups (Van Oers et al., 1998). According to these data, we would expect an increased number of cells as a result of the neonatal handling procedure, because the manipulation per se and the increased maternal behavior induced by human manipulation increase ODC activity.

However, a previous study (Baamonde et al., 1999) showed that neonatal handling decreases binding properties of beta-adrenoceptors in several brain areas of young rats and decreases the basal content of cAMP in adult rats. Considering these reduced binding properties of beta-adrenoceptors in neonatally handled animals, we suggest that the decreased number of cells in the LC observed in the present study could be due to changes in the polyamine chain. A lower activity of beta-adrenoceptors would cause a lower activation of cAMP, which in turn would reduce the activity of ODC, whose mRNA is expressed in the LC of rats (Kilpelainen, Rybnikova, Hietala, & Pelto-Huikko, 2000), and consequently decrease polyamine synthesis. The effects of handling on the binding properties of beta-adrenoceptors in the LC could explain the long-lasting effects of neonatal handling on the number of cells in the LC nucleus.

As an alternative explanation, we may also assume that, since handling can increase corticosterone on Postnatal Day 2, as previously shown (Denenberg, Brumaghim, Haltmeyer, & Zarrow, 1967; McCormick, Kehoe, & Kovacs, 1998), the reduction in the number of cells in the LC may be caused by this peak in plasma corticosterone. In fact, stressful stimulation during the neonatal period can reduce the number of granule neurons in the developing dentate gyrus of rats (Tanapat, Galea, & Gould, 1998). During the stress hyporesponsive period, from approximately Day 4 to Day 14 in rodents, adrenal response to stressors is minimal or nonexistent (Levine, 2001). However, if a corticosterone disturbance does occur during that period, it can affect the cell survival program by shutting off polyamine biosynthesis (Gilad, Gilad, Eliyayev, & Rabey, 1998). Nonetheless, handling does not seem to be able to increase plasma corticosterone during the stress hyporesponsive period (Sapolsky & Meaney, 1986; Walker, Perrin, Vale, & Rivier, 1986), except on the 2nd day after delivery when it may induce a significant raise in plasma corticosterone (Denenberg et al., 1967; McCormick et al., 1998). The effects of neonatal handling would be restricted to this limited period of time, a fact that would make this explanation rather unlikely.

In conclusion, the present results show that neonatal handling can induce a long-lasting stable structural change in the LC nucleus. They agree with previous study results (Price et al., 1998), in the sense that overt stimulation during the neonatal period and long-lasting effects might not necessarily be expressed by a positive linear relationship. Because of the stimulatory effect of the LC on the hypothalamus, and thus on the hypothalamic–pituitary–adrenal and the hypothalamic–pituitary–gonadal axis, the reduced number of cells in this nucleus could be one of the causal factors for the attenuated stress response and the anovulatory estrous cycles shown to occur in neonatally handled rats.

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Call for Nominations

The Publications and Communications (P&C) Board has opened nominations for the editorships of ***Comparative Psychology***, ***Experimental and Clinical Psychopharmacology***, ***Journal of Abnormal Psychology***, ***Journal of Counseling Psychology***, and ***JEP: Human Perception and Performance*** for the years 2006–2011. Meredith J. West, PhD, Warren K. Bickel, PhD, Timothy B. Baker, PhD, Jo-Ida C. Hansen, PhD, and David A. Rosenbaum, PhD, respectively, are the incumbent editors.

Candidates should be members of APA and should be available to start receiving manuscripts in early 2005 to prepare for issues published in 2006. Please note that the P&C Board encourages participation by members of underrepresented groups in the publication process and would particularly welcome such nominees. Self-nominations also are encouraged.

Search chairs have been appointed as follows:

- ***Comparative Psychology***, Joseph J. Campos, PhD
- ***Experimental and Clinical Psychopharmacology***, Linda P. Spear, PhD
- ***Journal of Abnormal Psychology***, Mark Appelbaum, PhD, and David C. Funder, PhD
- ***Journal of Counseling Psychology***, Susan H. McDaniel, PhD, and William C. Howell, PhD
- ***JEP: Human Perception and Performance***, Randi C. Martin, PhD

To nominate candidates, prepare a statement of one page or less in support of each candidate. Address all nominations to the appropriate search committee at the following address:

Karen Sellman, P&C Board Search Liaison
Room 2004
American Psychological Association
750 First Street, NE
Washington, DC 20002-4242

The first review of nominations will begin December 8, 2003. The deadline for accepting nominations is **December 15, 2003.**

3.2. Efeitos da manipulação neonatal sobre o número de neurônios do hipotálamo em ratas (Winkelmann-Duarte, E.C., Fernandes, M. C.; Bittencourt, L. C.; Pereira, G. A. M; Samios, V. N.; Schuh, A. S.; Achaval, M. E.; Xavier, L. L.; Sanvitto, G. L.; Mandarim-de-Lacerda, C. A.; Lucion, A. B. Effects of neonatal handling on the number of neurons in the hypothalamus in female rats. Submetido à revista Developmental Brain Research em novembro de 2004).

O PVN está diretamente relacionado a variações na liberação de CRH quando um animal é exposto a um estímulo de estresse. Este núcleo também recebe aferências de outros núcleos do sistema nervoso central que através da liberação de seus neurotransmissores ou hormônios inibem ou estimulam o eixo HPA, participando da modulação deste eixo. Dentre estes núcleos está o supra-óptico do hipotálamo, que apresenta neurônios ocitocinérgicos relacionados a funções reprodutivas e neurônios vasopressinérgicos que participam da homeostase do fluido corporal. Desta forma, o objetivo deste trabalho foi verificar se a manipulação neonatal altera o número de neurônios dos núcleos paraventricular do hipotálamo e supra-óptico que efetivamente participam dos circuitos do estresse.

Effects of neonatal handling on the number of neurons in the hypothalamus in female rats

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Early-life environmental stimulation can exert profound impact on behaviors and neuroendocrine functions in adulthood. In order to explain the effects induced by a procedure performed months earlier, it is plausible to raise the hypothesis that the neonatal stimulation (handling) may lead to stable, probably structural alterations in the central nervous system. The purpose of the present study was to examine the effects of neonatal handling on the number of cells and the volume of the hypothalamic paraventricular nucleus (pPVN, parvocellular and mPVN, magnocellular regions) and supraoptic nucleus (SON) in female rats at 11 and 90 days of age. Daily handling during the first 10 days of life induced a stable

reduction in the number of cells in the pPVN, but not in the mPVN, and in the SON in both sides. Handled females at the age of 11 and 90 days showed fewer cells than nonhandled ones (21% and 27% loss for right and left side and 41% and 36% for right and left side, respectively to the age) in the pPVN; and (30% and 33% at the age of 11 days and 21% and 30% at the age of 90 days, respectively to the side of the brain) in the SON. Immunohistochemistry for GFAP showed no difference between the handled and nonhandled adult females, indicating that the reduction in the number of cells was apparently due to neuronal loss, rather than glial cells. Results showed long-lasting structural changes in specific areas of the central nervous system induced by an apparently mild environmental stimulation during the neonatal period. The reduction in the number of cells in pPVN and the SON may explain the reduced activity of the HPA axis in the neonatal handled animals.

Key words: neonatal handling, hypothalamic paraventricular nucleus, supraoptic nucleus, number of neurons, GFAP.

Introduction

Early-life environmental stimulation induces long-lasting effects on behaviors and neuroendocrine functions [10, 11, 16, 28]. In rats, the manipulation of the pups for few minutes, usually during the first two weeks of life is a procedure seemingly not noxious, but can exert a profound impact on fear/anxiety behaviors and stress responses in adulthood, characterized by increased exploratory activity in novel and aversive environments, which has been

interpreted as decreased fearfulness (28, 61), and a reduced stress response (38). Besides the well-established effects of neonatal handling on stress related functions, previous studies presented evidence that the same procedure can reduce sexual behavior and ovulation in rats (21, 46).

In order to explain the behavioral and neuroendocrine effects induced in an adult animal by a procedure that was performed months earlier, it is plausible to raise the hypothesis that the neonatal handling may lead to stable, probably structural alterations in the central nervous system. The trace left in the nervous system by the neonatal experimental stimulation procedure and, as a consequence, the increased maternal licking/grooming behavior could cause the behavioral and hormonal changes observed in adulthood. Indeed, the demonstration that neonatal handling is related to an increased density of glucocorticoid receptors in the hippocampus and frontal cortex (31, 37, 39, 52) is essential to explain the reduced stress response in adulthood. Neonatal handling also induces changes in hippocampal $GABA_A$ receptors that were associated with stress response in adulthood (23). Handling the pups was able to reverse the effects of neonatal hypoxia/ischemia on the volume reduction of the hippocampus of rats (51). On the other hand, [32] showed that daily neonatal handling during the first 10 days of age causes a reduction in the number of cells in the *Locus coeruleus* (LC) of male and female rats at several ages (11, 26, 35 and 90 days old), presenting evidence for a stable and long-lasting structural change. In the same line, Liu et al. [30] showed that neonatal manipulation diminishes the release of noradrenaline (NE) into the paraventricular hypothalamus, indicating a decrease in the LC activity due an increased expression in alfa2 adrenoceptors. LC is involved with both stress and reproductive functions [1, 15, 36].

On the other hand, early life environmental stimulation may also affect glial cells, besides the neurons. Glial cells are relevant in several CNS functions, such as migration and maturation of neurons, myelinization, regulation of ionic concentrations, metabolism of chemical transmitters, synaptic integration, energy supply to neurons, and response to brain injury [5]. Moreover, neuronal-glial interaction can affect the synaptic patterning in the developing brain as well in the adult CNS [19, 42].

The PVN is a pivotal structure in the organization of the responses to stress stimuli. It is the major corticotrophin releasing hormone (CRH) nucleus in central nervous system [2, 6, 48]. The PVN can be divided in two distinct regions: the magnocellular and the parvocellular [3, 59]. The magnocellular neurons are directly related to the release of the neurohypophysial peptides oxytocin and vasopressin, which are hormones that also respond to several stress stimuli [43]. On the other hand, the parvocellular neurons in the PVN express CRH [29, 57] and project to the median eminence and also to several brain structures like the central and medial amygdaloid nuclei and the noradrenergic nuclei in the brainstem [7, 33, 54]. The SON, as the PVN, is involved in the organization of stress responses [43]. Most neurons in the SON produce either oxytocin (OT) and vasopressin (VP) and project to the pituitary [3]. In adulthood, the SON expresses CRH neurons, although in a lesser amount than the PVN.

The purpose of the present study was to examine the effects of neonatal handling on the number of cells and the volume of the hypothalamic paraventricular nucleus (PVN, magnocellular and parvocellular regions) and supraoptic nucleus (SON) in female rats at 11 and 90 days of age. Moreover, the expression of the glial fibrillary acidic protein (GFAP) in the PVN and SON of

90-day-old handled and nonhandled females was analyzed. We aimed to test the hypothesis that early life stimulation can induce stable structural changes in the central nervous system, which would account for the behavioral and neuroendocrine alterations observed in adult animals. We studied females in order to reveal possible causes for the stress-related and also reproductive alterations, such as the reduced ovulation and sexual receptiveness.

Material and Method

Animals:

Pregnant females from colonies of the Federal University of Rio Grande do Sul were brought to the animal room in our laboratory. Approximately 7 days before delivery, they were housed individually and the presence of pups was checked for twice a day (beginning and end of the light period). The day of birth was considered day 0. In the next day, the number of pups was culled to 8 per dam by randomly removing some of them with minimal contact with the remaining pups and the dam. After weaning (postnatal day 21), the females were housed in same-sex groups of 2-4 per cage (41 cm long x 34 cm wide x 17 cm high). The animals were maintained on a 12-hr light-dark cycle (lights on from 6:00 a.m. to 6:00 p.m.), the room temperature was 22 ± 1 ° C, and water and food (Rodent chow, Nutrilab, Colombo, Brazil) were available at all times. Experiments were performed in accordance with the National Institute of Health (NIH) guidelines (1986) and were approved by the University Research Committee.

Neonatal Handling and Experimental groups:

The pups were divided into two groups: nonhandled, they were not manipulated either by the researchers or by the caretakers from the 1st to the 10th postnatal day, with bedding being changed twice a week from the 10th day on; and handled, the pups were touched for 1 min during the first day after birth. First, the litter and the mother in their home cage were taken to a quiet room next to the animal facility, with the same light period and temperature. The mother was placed in another cage next to the home cage, and then the experimenter gently handled all pups at the same time using both hands, covered with fine latex gloves, for 1 min. After handling, all pups were returned to the nest at the same time and the mother was placed back in the home cage. The pups were handled at a distance of about 1 to 2 m from the mother, and the total time of the mother-infant separation was approximately 2 min. This procedure was repeated from the 1st to the 10th postnatal day (neonatal period), during the light period of the daily photoperiod cycle [21, 32, 46, 55].

In the experiment in which the neurons were estimated by the dissector method, the females of both groups were studied at 2 different ages: 11 (neonatal period) and 90 (adult) days old. The number of animals in each experimental group (nonhandled and handled) and age (11 and 90 days old) was 6. In the experiment in which the expression of GFAP was estimated by optical density (OD), adult nonhandled and handled females were studied, and the number of animals in each group was 5. In the experimental groups, the animals were nonsiblings (each subject from a different litter). Adult females were studied in diestrus. The estrous cycle was verified by taking vaginal smears over a period of 15 days, and only females with at least 3 regular cycles were studied.

Histological procedures for cell counting

Rats were anaesthetized with xilasine (0.1ml/g body weight, i. m.) and ketamine (0.1ml/g body weight, i. m.) and were perfused with saline phosphate buffer (PBS) with heparin (50 ml in the 11-day and 100 ml in the 90-day-old rats) followed by paraformaldehyde 4% diluted in phosphate buffer 0.1M (pH 7.4) at 4° C at the same flow rate and total amount. The perfusion rate was approximately 1 drop in 7 seconds for the 11- day and 1 drop in 5 seconds for the 90-day-old females. After the perfusion, the brain was extracted from the skull, weighed, and placed in the same fixing solution for 72 h. After fixation, the brain was washed for 1 h in running water and then dehydrated in different concentrations of ethanol (70%, 80%, 90%, 95% and absolute ethanol) and cleared with xylol. They were included in the final position with a paraplastic resin (Histosec - Merck). Coronal serial sections (6 μm thick) were obtained with a microtome and serially collected on glued slides. The tissue was stained with cresyl violet. After staining, the sections were dehydrated through and ethanol series, and coverslipped with Entellan (Merck).

Immunohistochemistry for GFAP

Adult females (90 days old) were anaesthetized with thiopental sodium (50mg/Kg body weight, i. p.) and were perfused with 100ml of saline phosphate buffer with heparin followed by paraformaldehyde 4% diluted in phosphate buffer 0.1M (pH 7.4) at 4° C at the same flow rate and total amount. After the perfusion, the brain was extracted from the skull, and placed in the same fixing solution for 4h. After fixation, the brain was cryoprotected by 15% sucrose/PBS solution until sinking followed by 30% sucrose/PBS, and then quickly frozen in cooled

isopentane. Coronal serial sections (50 μm thick) of the PVN and the SON were obtained in the cryostat, using the atlas Paxinos and Watson for anatomical references. The samples were collected in PBS solution with an interval of 100 μm. The tissue was processed for GFAP immunohistochemistry using the antibody peroxidase-antiperoxidase (PAP) procedure. Free-floating sections were treated in 10% methanol and 3% H_2O_2 for 30 min and washed carefully. Then, the sections were pre-incubated in 3% normal goat serum (NGS) in PBS containing 0.3% Triton X-100 (PBS-Tx, Sigma Chemical Co.) for 30 min and incubated with polyclonal GFAP antiserum raised in rabbit (Sigma Chemical Co.) diluted 1:150 in 3% NGS in PBS-Tx for 48 h at 4°C. After washing several times with PBS-Tx, sections were incubated in a rabbit anti-rabbit IgG diluted 1:50 in PBS-Tx at room temperature for 2 h. Sections were washed again in PBS and incubated in a rabbit PAP (Sigma Chemical Co.) diluted 1:500 in PBS for 2 h at room temperature. The immunohistochemical reaction was revealed by incubating the sections in a histochemical medium that contained 0.06% 3'3-diaminobenzidine (DAB, Sigma Chemical Co.) dissolved in PBS for 10 min and then, in the same solution containing 1μM of 3% H_2O_2 per ml of DAB medium for 10 min. Afterwards, the sections were rinsed in PBS, dehydrated in ethanol, cleared with xilene and covered with Entellan and coverslips. Control sections were prepared omitting the primary antibody by replacing it with PBS. All animals used in this experiment were processed (perfusion and immudetection) in the same day and in the same solutions to avoid changes in the background and differences in the chromogen reaction [50].

The structures of the central nervous system, hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus (SON) were identified according to a rat

brain atlas [47]. The main references used to locate the PVN were the presence of the fornix and the 3rd ventricle. The parvocellular region was situated within 0.80 and 2.12 mm and the magnocellular region within 1.80 and 2.12 mm in relation to the bregma suture. The optic tract was used as a reference to localize the SON (0.80 and 1.80 mm in relation to the bregma suture). The cells in the decussation and in the retrochiasmatic nucleus of the SON were not analyzed.

Estimation of the volume of the nuclei

The PVN was divided in the parvocellular (pPVN) and the magnocelular (mPVN) regions. According to the rat brain atlas [47], the anterior parvocelular (PaAP), medial parvocellular (PaMP), ventral (PaV), posterior parvocelular (PaPo), dorsal capsule (PaDC) were considered as the parvocelular region (pPVN). The anterior magnocellular (PaAM) and lateral magnocellular (PaLM) were considered as the magnocellular region (mPVN). The volume of the SO was evaluated but not the decussation of the nucleus neither the supraoptic retrochiasmatic nucleus. The volume of each nucleus was estimated according to the Cavalieri principle (14, 34) using a light microscope (Zeiss Axioscop2) with planachromatic objective, a video camera (CCD video camera module) and a computer (Apple Macintosh 8600-300) with the image analysis system (NIH Image 1.62f, Rasband, 1996).

Estimation of the number of cells

In order to characterize the cells to be counted, we measure the size (in microns) of the cells body of 30 neurons of the PVNp, PVNm and SON. We used

the same light microscope, a video camera and a computer with the image analysis system descripted by the volume analysis of the nuclei. The largest diameter of the soma of the neurons in the pPVN varied from 6 to 12 μm; in the mPVN from13 to 25 μm; and in the SON from 15 to 25 μm. Glial cells were smaller with a larger nucleus and a deeply stained nucleolus compared to the neurons.

The disector method was used to estimate the total number of cells in each nucleus (56). The optical disector is based on two parallel sections (lookup and lookdown planes). These planes were determined over a frame of known surface. The numerical density (Nv) of neurons (number of neurons per mm^3) was determined from 10 random disector pairs for each rat. The total number of cells (N) was calculated by multiplying the Nv by the volume of the nucleus, previously estimated according the Cavalieri principle. For reasons of efficiency, we analyzed the nuclei of the neurons (one nucleus represented one neuron). The number of cells nuclei were counted inside a frame of 2304 μm^2 in the lookup plane provided that they did not intersect the right and inferior exclusion edges of the frame (forbidden lines) [22]. The thickness of the dissector (t) was 3 μm for the PVN and SON, automatically controlled with the motorized stage of the Leica DMRBE microscope.

Optical density (OD)

The intensity of reaction product of GFAP immunohistochemistry was measured by optical densitometry, using a Nikon Eclipse E-600 (125x) microscope coupled to a Pro-Series High performance CCD camera and Image

Pro Plus Software 4.1 (Media Cybernetics, USA). The images obtained from the sections were digitized and converted in an 8-bit gray scale (0-255). To estimate the optical density generated by GFAP immunoreaction in the pPVN, mPVN and SON, the areas of interest (AOI) were 43264 μm^2, 16378 μm^2 and 17401 μm^2, respectively. The size of AOIs was determined to avoid picture elements (pixels) from outside of nucleus analyzed, but also to gather a significant number of pixels from the different sub-regions of the SON, mPVN, and pPVN.

Both, left and right sides of brains were analyzed, and at least 5 readings were performed in each nucleus per side. All lighting conditions and magnifications were held constant. Moreover, the investigator was unaware of the experimental groups from which the slices were obtained during the analysis. The optical density was obtained using the Lamber-Beer formula: $OD = \log_{10} 1/ T$, where OD is the Optical Density and T is the Transmitance.

Statistical Analysis

The values of the N (estimated number of cells in the nucleus), the volume of the nucleus (mm^3), the Nv (numerical density, number of cells/mm^3), and the OD (optical density) were expressed as medians (interquartile range). Results were compared among groups (nonhandled and handled at 11 and 90 days of age) by the Kruskal-Wallis nonparametric analysis of variance. If appropriate, a post hoc Mann-Whitney comparison was used to measure individual differences. The body and brain weight were compared between the handled and nonhandled groups by the Student's *t* test. In all cases, the accepted level of significance was $p < 0.05$.

Results

Figure 1 shows a representative low magnification view of the areas studied: pPVN and SON (1a), PVN and SON (1b) and mPVN and SON (1c). Tissue is from a nonhandled rat.

Figure 2 shows the estimated number of cells and the volume of the parvocellular region of the PVN. In the right side of the pPVN, the number of cells of the handled group at both ages was lower than the nonhandled ones, but no significant difference was detected comparing the 90-day and the 11-day-old females ($H(3)=9.78$, $p=0.02$; followed by the Newman-Keuls test). The volume of the right pPVN showed no difference among the groups ($H(3)=2.51$). In the left side, as in the right side, the number of cells of the handled group at both ages was lower than the nonhandled one; moreover, in 90-day-old handled females, the number of cells was lower than in the 11-day-old ones, but this age difference was not detected in the nonhandled group ($H(3)=12.09$ $p=0.007$; followed by the Newman-Keuls test). The volume of the left pPVN showed no difference among the groups ($H(3)=3.70$).

Figure 3 shows the estimated number of cells and the volume of the magnocellular region of the PVN. In the right and the left side, the number of cells almost reached significant difference among groups ($H(3)=7.46$, $p=0.06$ and $H(3)=7.33$, $p=0.06$, respectively to the side). Comparing handled and nonhandled groups, no significant difference was detected in the volume of the right-side mPVN; but the volume decreased at 90 days as compared to 11 days of age in both handled and nonhandled groups ($H(3)=14.15$, $p=0.0003$; followed by the Newman-Keuls test). The volume of the left mPVN of the 90-day-old handled

females was lower than the nonhandled ones, but no difference was detected in the 11-day-old handled females compared to the nonhandled ones. Figure 2b also shows that the volume of the left mPVN at age of 90 days was lower than at 11 days in both groups ($H(3)=12.85$, $p=0.005$; followed by the Newman-Keuls test).

Figure 4 shows the estimated number of cells and the volume of the supraoptic nucleus (SON). A tendency ($p<0.10$) to decrease the number of cells in the handled group in both sides was observed at the age of 11 days, however, probably due to the high variability of the sample at the left side, a significant difference was not reached. However, in the right side, the number of cells of the handled group at the age of 90 days was lower than the nonhandled one, but not at the age of 11 days. Moreover, 90-day-old handled and nonhandled females showed increased number of cells compared to 11-day-old ones ($H(3)=17.45$, $p<0.001$, followed by Newman-Keuls test). Figure 3 also shows that the volume of the right SON in the handled females at the age of 90 days was lower than the nonhandled ones at the same age. Moreover, the volume in both groups at age of 90 days was lower than at 11 days ($H(3)=10.94$, $p=0.01$; followed by the Newman-Keuls test). In the left side, as in the right, the number of cells of the handled group at the age of 90 days was lower than the nonhandled ones, but not 11 days. 90-day-old nonhandled, but not handled females, showed increased number of cells compared to 11 days ($H(3)=11.27$, $p=0.01$). Figure 4d also shows that the volume of the left SON almost reached significance among the groups ($H(3)=7.04$, $p=0.07$).

Table 1 shows the numerical density (NV) of cells in the nuclei. In the right pPVN, the NV of the handled group at both ages was lower than the nonhandled one, but no significant difference was detected comparing the 90-day

and the 11-day-old females ($H(3)=13.14$, $p=0.004$; followed by the Newman-Keuls test). In the left pPVN, the NV of the handled group at both ages was lower than the nonhandled one, and handled females at the age of 90 days showed decreased NV compared to the age of 11 days ($H(3)=13.80$, $p=0.003$). The NV in the right SON of 90-day-old females of both groups was significantly higher than the 11-day-old ones, but no difference between handled and nonhandled groups was detected ($H(3)=10.18$, $p=0.02$). In the left SON, the NV showed no difference among the groups ($H(3)=5.92$).

Table 2 shows the optical density (OD) of GFAP imunohistochemistry in the 90-day-old females. No difference was detected comparing the handled with nonhandled groups in each side of the nuclei analyzed.

The mean (±SEM) body weight (g) of the 11-day and 90-day-old handled (23.6±1.2 and 232.1±8.7, respectively) was not different from the nonhandled (20.7±1.2 and 223.5±11.2; $t(10)=1.67$ and $t(10)=0.61$, respectively). The mean (±SEM) brain weight (g) of the 11-day-old handled (1.13±0.03) was significantly higher than the nonhandled at the same age (1.03±0.02; $t(10)=2.66$). The mean (±SEM) brain weight (g) of the 90-day-old handled (1.76±0.07) was not different from the nonhandled at the same age (1.68±0.03; $t(10)=0.91$).

Discussion

Handling during the first 10 days of life induced a stable reduction in the number of cells in the parvocellular region of the PVN in both sides. Handled females at the age of 11 and 90 days showed fewer cells than nonhandled ones (21% and 27% loss for right and left side and 41% and 36% for right and left side,

respectively to the age). The cell loss was detected shortly after the manipulation period and it was stable throughout life. Since the optical density of GFAP in the pPVN showed no significant difference between handled and nonhandled adult females, the reduction in the number of cells was probably due to a reduction in the number of neurons. On the other hand, handling did not change the number of magnocellular cells and the volume of this region of the PVN, except 90-day-old handled females in which the left mPVN volume was smaller than the nonhandled ones. The optical density of GFAP was not different between adult handled and nonhandled females. The handling procedure and probably the increased maternal licking/grooming behavior after handling affected the number of cells in the parvocellular region of the PVN, which expresses several neuromodulators, mainly CRH-neurons, but not the magnocellular region, which projects neurons to the posterior lobe of the pituitary.

The anatomical and histological subdivisions of the PVN vary in different studies [3, 25, 58, 60]. Nonetheless, all studies agree in distinguishing magnocellular and parvocellular neurons, based on the average size of the cells [45]. In rats, [26] estimated 6100 magnocellular and 11500 parvocellular neurons, while Swanson and Sawchenko [59] estimated 7000 parvocellular neurons. These differences could be due to several factors: the method to estimate the number of cells, the staining procedure, and the rat strain, for instance [20]. In the present study, the same counting procedures for all groups were applied and thus the comparisons may be appropriate to estimate the effects of the neonatal handling on the development of the nuclei studied.

In order to explain the observed reduction in the number of cells, as early as 11 days postpartum, it is necessary to know whether the PVN responds to the

handling procedure. Previous studies [4, 18] have shown that neonatal handling from day 1 postpartum decreases CRH-mRNA expression in the PVN, beginning on day 9 through day 45 postpartum. Moreover, rat pups respond to cold stress by increasing plasma corticosterone and phosphorylation of CRE binding protein [12]. Thus, CRH neurons in the PVN do respond to environmental stimulation, including handling, during the neonatal period. However, the causes for cell loss remain to be determined.

In the SON, handling reduced the number of cells in the right and left side compared to nonhandled (30% and 33% at the age of 11 days and 21% and 30% at the age of 90 days, respectively to the side of the brain). Since the optical density of GFAP in the SON showed no significant difference between handled and nonhandled adult females, the reduction in the number of cells was probably due to a reduction in the number of neurons, like the effects of neonatal handling in the pPVN. The volume of the nucleus of the handled females at the age of 90 days was lower than the nonhandled ones, specially the right SON.

It is not known whether the SON responds to environmental stimulation during the postnatal period. Nevertheless, by a yet unrevealed manner, the handling procedure reduced number of cells in the SON. The number of neurons in the control nonhandled females increased from the 11th to the 90th day in both groups, concurrently to an increase in the volume of the nucleus, which is in agreement with previous report [13]. There was even a greater increase throughout life in the handled females compared to nonhandled ones, especially in the right side (23% versus in the right versus 8% in the left side). Thus, apparently the primary effects of handling on the number of cells occurred within the neonatal period. The proliferation of cells that occur in the SON from the neonatal

period to adulthood was little affected by the handling procedure. We may infer that the observed decrease in the number of neurons in the SON appears to be due to cell loss rather than suppression of neurogenesis. However, in the pPVN, the reduction in the number of cells was more pronounced in adulthood than one after the last handling stimulation, suggesting that both cell loss and reduction in neurogenesis might have occurred.

We may conclude that neonatal handling reduces the number of cells in structures related to the organization of stress responses. The cell loss appears to be specific to certain structures and not widespread in the brain. In the PVN, only the parvocellular neurons decreased. It is possible that by the time of the handling stimulation some nuclei were in a process of cell division and differentiation rendering them more likely to be affected by the environmental stimulation than others quiescent at that time.

The reduction in the number of cells could be related to the possible increase in CRH induced by handling. It has been shown that the administration CRH during the neonatal period can cause a reduction in the number of neurons in the CA3 hippocampus of adult rats, which is related to the glutamatergic mechanism and enhanced intracellular calcium [8]. However, it is difficult to conceive that changes in CRH could cause for the reduction in the number of cells in the pPVN due to the fact that handling in fact reduces CRH mRNA expression in the PVN [18]. Moreover, handling does not seem to alter plasma corticosterone in the stress hyporesponsive period [53], except on the 2nd day after delivery when it may induce a significant raise in plasma corticosterone [28, 40]. Nevertheless, the effects of neonatal handling would be restricted to this limited period of time, which would make the explanation rather unlikely.

Another possible explanation for the reduction in the number of cells in the PVN and SON is the effect of the noradrenergic system. During brain development neurotransmitters, like noradrenaline, can exert trophic functions in cell replication, differentiation and cell death [27]. The effects of environmental stimulation can act on beta-adrenoreceptors [64] and increase cell replication, through the activation of intracellular mechanisms involving c-AMP. However, an overt stimulation of beta-adrenorecptors can in fact induce cell death. Thus, the decrease in the number of cells observed in the present study could be due to an excess of environmental stimulation, due to the experimental manipulation and the increased licking/grooming by dam after the handling procedure.

The reduction in the number of cells could also be due to the reduction of DNA methylation in the neonatal stimulated rats. Indeed, previous studies [65] have shown that early life stimulation decreases the DNA methylation, which is related to gene silencing through chromatin remodeling [35]. Considering the inverse relationship between environmental stimulation upon the pups and the activity-dependent gene regulation of neural plasticity, we may suggest that neonatal handling reduces the number of cells by decreasing DNA methylation. However, the mechanisms through which DNA methylation would be altered and the reason why some cells were affected by the neonatal handling procedure and others not remain to be established.

In conclusion, results show stable structural changes in the central nervous system induced by apparently a mild environmental stimulation during the neonatal period. The impact of handling on cell loss affect several brain structures, but does not appear to be unspecific and uniformly widespread. In adulthood, the reduction in the number of cells was apparently due to neuronal

loss, rather than glial cells. The effects of the reduction in the number of cells in pPVN and the SON could explain the reduced activity of the HPA axis in the neonatal handled animals.

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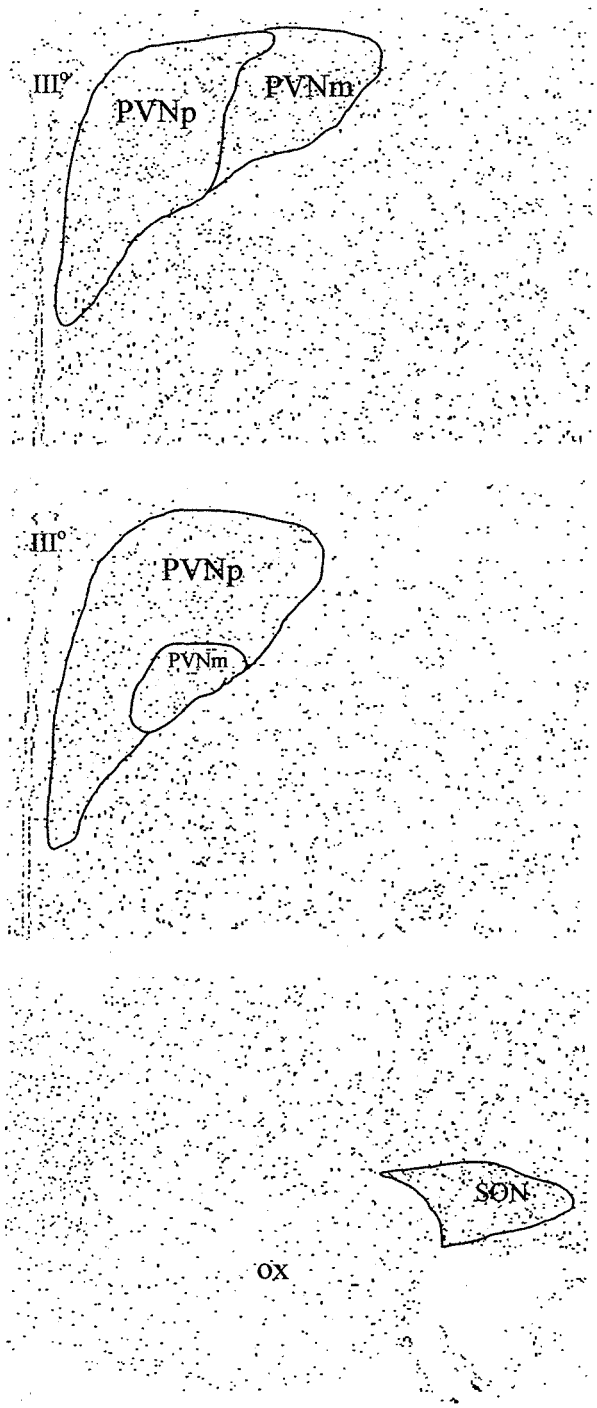

Figure 1 - Low magnification view and location of pPVN, mPVN and SON where cells were counted and the volume of the nuclei were analyzed in female rats. Ox: chiasm optic, III°: third cerebral ventricle.

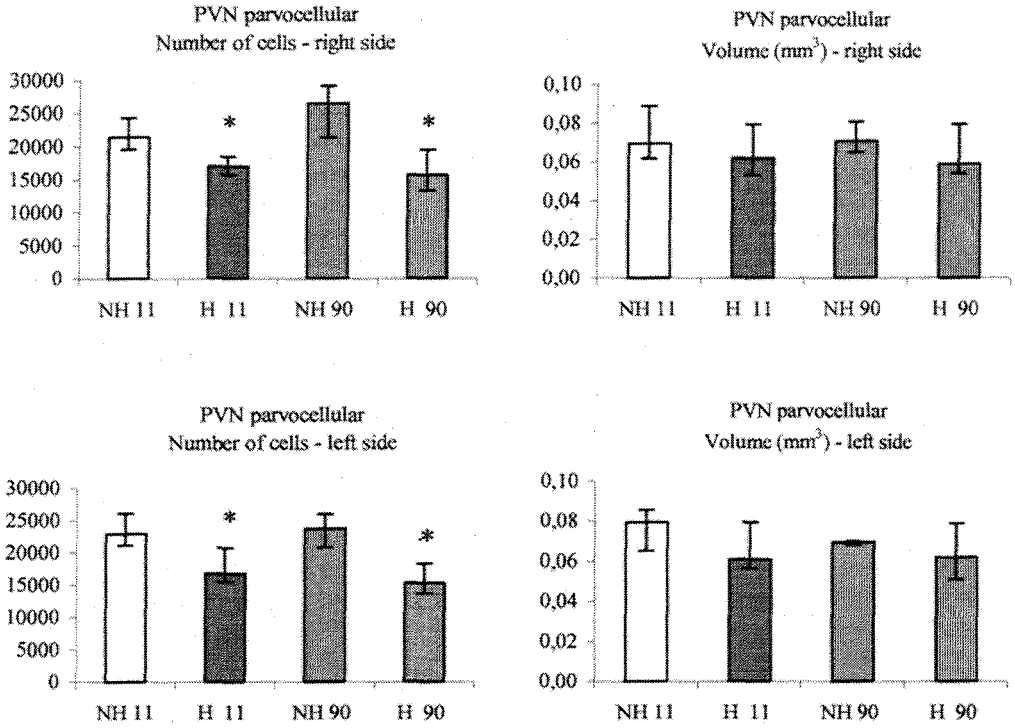

Figure 2 - Effects of neonatal handling on the number of cells and volume of the right and left side parvocellular region of the PVN (pPVN) in female rats at 11 and 90 days of age. The estimated number of cells and volume (mm^3) are reported as median (interquartile range). Nonhandled control rats were kept undisturbed during the neonatal period and handled animals were gently manipulated for 1 min daily from the 1st to the 10th day after delivery (n represents the number of animals in each age). In the right and left side, the number of cells of the handled group at both ages was lower than the nonhandled group. In the left side of 90-day-old handled females, the number of cells was lower than in the 11-day-old ones. The volume of the left and the right pPVN showed no difference between the groups and ages.

* Indicates significant difference between the handled and nonhandled groups at the same age (Newman-Keuls test, $p < 0.05$).

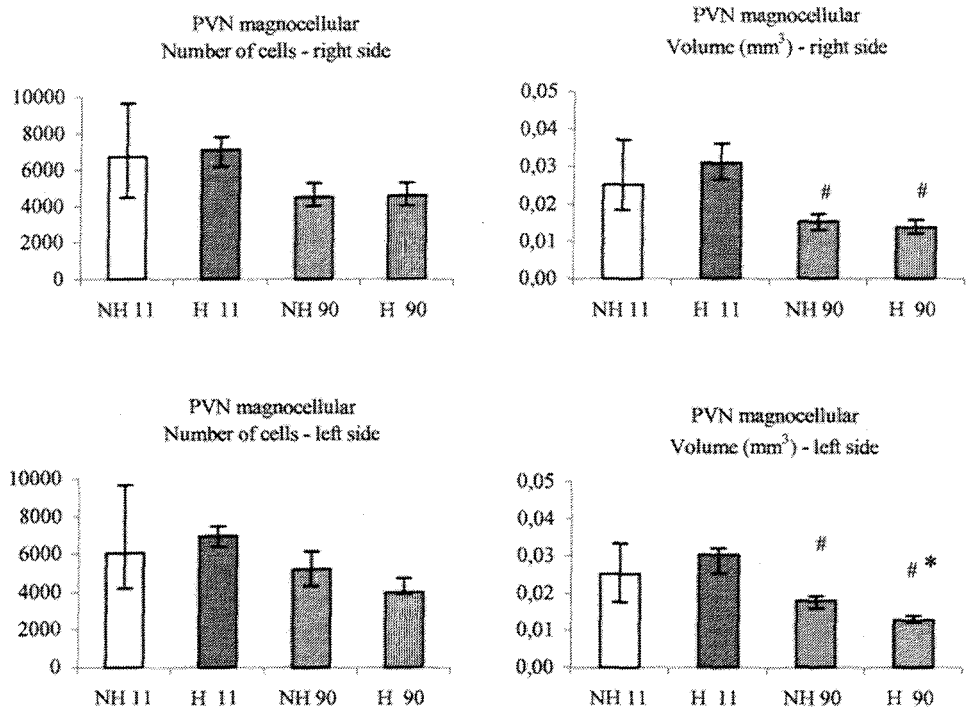

Figure 3 - Effects of neonatal handling on the number of cells and volume of the right and left side magnocellular region of the PVN (mPVN) in female rats at 11 and 90 days of age. The same procedures as described in Figure 2 were adopted. In the right and the left side, the number of cells did not reach significant difference between the groups at both ages. The volume of the right mPVN was not different between the groups at both ages. The volume of the left mPVN of the 90-day-old handled females was smaller than the nonhandled ones, but no difference was detected in the 11-day-old handled females compared to the nonhandled ones. The volume of the right and left mPVN at the age of 90 days was lower than at 11 days in both groups.

* Indicates significant difference between the handled and nonhandled groups at the same age (Newman-Keuls test, $p < 0.05$).

# Indicates significant difference between the 11 and 90-day-old females of the same group (Newman-Keuls test, $p < 0.05$).

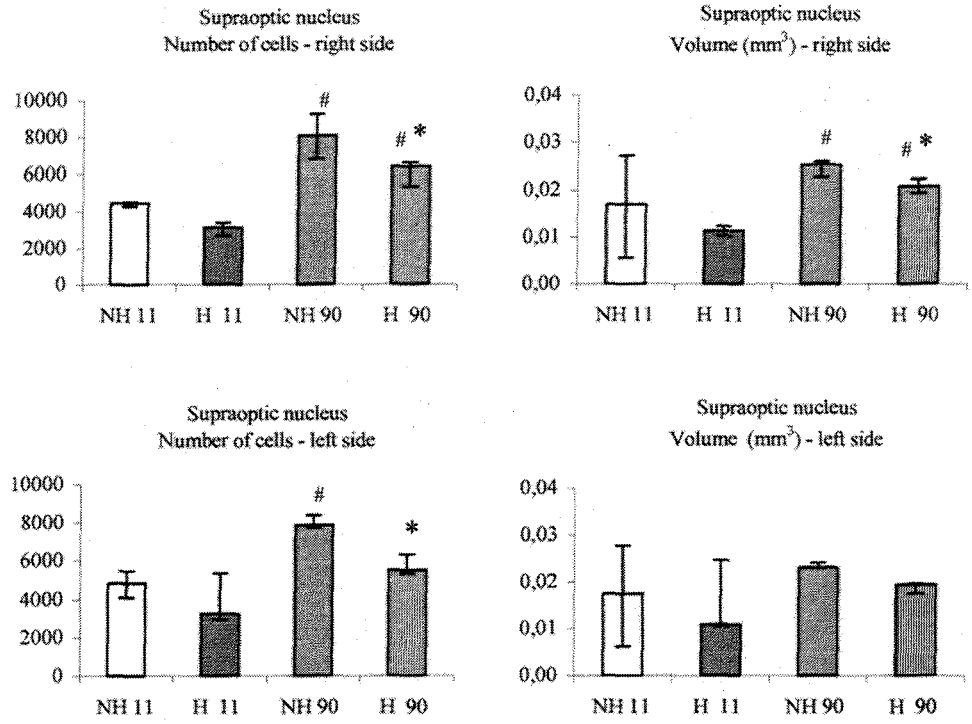

Figure 4 - Effects of neonatal handling on the number of cells and volume of the right and left side of the SON in female rats at 11 and 90 days of age. The same procedures as described in Figure 2 were adopted. In the right and left side, the number of cells of the handled group at the age of 90 days was lower than the nonhandled one, but not at the age of 11 days. When the ages were compared, the 90-day-old handled and nonhandled females showed more cells than 11-day-old ones in both sides. The volume of the right SON in the handled females at the age of 90 days was smaller than the nonhandled ones; and the volume in both groups at age of 90 days was smaller than at 11 days.

* Indicates significant difference between the handled and nonhandled groups at the same age (Newman-Keuls test, $p < 0.05$).

# Indicates significant difference between the 11 and 90-day-old females of the same group (Newman-Keuls test, $p < 0.05$).

Nucleus of CNS	Age and Group	Nv - right side	Nv – left side
pPVN	11 NH	311053 (274884-318287)	296586 (289352-318287)
	11 H	267651 (231482-274884) *	274884 (260417-274884) *
	90 NH	361690 (303819-390625)	325521 (303819-390625)
	90 H	245949 (245949-260417) *	238715 (217014-260417) *#
mPVN	11 NH	253183 (245949-260417)	238715 (231482-260417)
	11 H	231482 (217014-231482) *	231482 (202546-260417) *
	90 NH	311053 (274884-261690) #	296586 (289352-303819) #
	90 H	325521 (303819-332755) #	303819 (303819-303819) #
SON	11 NH	260417 (245949-274884)	267651 (260417-274884)
	11 H	260417 (231482-274884)	267651 (260417-303819)
	90 NH	325521 (318287-332755) #	332755 (318287-347222)
	90 H	296586 (274884-332755) #	282118 (274884-332755)

Table 1 - Effects of neonatal handling on the numerical density (NV) of cells in the pPVN, mPVN and SON in female rats at 11 and 90 days of age. The numerical density (number of cells per mm^3) is reported as median (interquartile range). The same procedures as described in Figure 2 were adopted. In the right and left pPVN, the Nv of the handled group at both ages was lower than the nonhandled one, and handled females at the age of 90 days showed decreased Nv compared to the age of 11 days. The NV in the right SON of 90-day-old females of both groups was significantly higher than the 11-day-old ones, but no difference between handled and nonhandled groups was detected. In the left SON, the NV showed no difference among the groups and ages.

* Indicates significant difference between the handled and nonhandled groups at the same age (Newman-Keuls test, $p < 0.05$).

# Indicates significant difference between the 11 and 90-day-old females of the same group (Newman-Keuls test, $p < 0.05$).

Nucleus of CNS	Group	OD – GFAP right side	OD – GFAP left side
pPVN	NH	33.9 (30.7/35.0)	34.0 (30.9/35.7)
	H	32.0 (31.8/33.6)	32.0 (32.0/33.5)
mPVN	NH	28.8 (28.6/32.8)	29.0 (28.8/29.4)
	H	29.8 (29.6/32.0)	28.8 (28.4/30.2)
SON	NH	45.1 (44.7/46.9)	45.7 (43.5/48.0)
	H	46.5 (44.5/47.9)	43.9 (43.9/45.0)

Table 2 - Effects of neonatal handling on the optical density (OD) of GFAP in the pPVN, mPVN and SON of female rats at 90 days of age. The OD is reported as median (interquartile range). The same procedures as described in Figure 2 were adopted. No significant differences were detected between groups and ages.

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3.3. Efeitos da manipulação neonatal sobre o número de neurônios e células gliais da amígdala (Winkelmann-Duarte, E. C.; Fernandes, M. C.; Schuh, A. S.; Pereira, G. A. M.; Achaval, M. E., Xavier, L. L., Sanvitto, G. L.; Lucion, A. B. Effects of neonatal handling on the number of neurons and glial cells in the amygdala, a ser submetido)

A amígdala é formada por diferentes núcleos que participam das respostas ao estresse. Dentre estes núcleos estão a amígdala central (CeA), lateral (LaA) e basolateral (BaLA). A LaA e BaLA modulam através da CeA as respostas do medo, sendo que a CeA atua também na ativação do eixo HPA por apresentar neurônios CRH-positivos. Assim sendo, a redução do número de neurônios destes núcleos poderia explicar, em parte, a redução da atividade do eixo HPA observado em ratas manipuladas no período neonatal.

As células gliais desempenham funções importantes relacionadas com a migração e maturação dos neurônios, à formação da bainha de mielina, à regulação das concentrações iônicas, ao metabolismo de transmissores químicos, à integração sináptica, ao suprimento de energia para os neurônios, nas respostas para injúria cerebral e no desenvolvimento do sistema nervoso central (Bezzi *et al.*, 2001; Kettenmann & Ramsom, 1995).

Desta forma, o objetivo deste trabalho foi verificar se a manipulação neonatal afeta o número de neurônios e de células da glia na CeA, LaA e BaLA em ratas aos 11 e 90 dias de idade.

Effects of neonatal handling on the neurons and glial cells in amygdala

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Abstract

The central (CeA), lateral (LaA) and basolateral (BaLA) nuclei of amygdala mediating stress circuits. The amygdala present projections to the hypothalamus that participate in the control of the function of the hypothalamic-pituitary-adrenal axis. Neonatal handling no induces a reduction on the number of neurons in the central, lateral and basolateral nucleus of the amygdala. Glial cells are relevant for the development of the central nervous system (CNS) participating to the migration and maturation of the neurons. Adult female, handled in the neonatal period were used to the imunohistochemistry for the astrocytic marker glial fibrillary acidic protein (GFAP) in CeA, LaA, BaLA. The results showed that the neonatal handling no induces a stable changes on the glial cells in all nuclei analysed. This suggest that the nucleus of the amygdala no is affected morphologically by this intervention and that the reduction of the HPA (hypothalamic paraventricular axis) observed in animals handled in the neonatal period do not have relations with changes on the number of cells (neurons and glial cells) in different nuclei of the amygdala.

Key words: neonatal handling, amygdala, number of neurons, GFAP.

Introduction

In rats, neonatal handling has been used as an experimental procedure to examine the mechanisms by which variations in the environmental during the neonatal period could affect the development of neural systems (Abrahám & Kovács, 2000; Anand et al., 1999; Anand & Scalzo, 2000, Moore et al., 1992; Papaioannou et al., 2002; Veenman et al., 1999; Zhang et al., 2002). This procedure, seemingly inoffensive for the animal, it causes deep and stable changes in many functions in the central nervous system (CNS). It typically consists of the manipulation of pups for a few minutes, generally during the first 2 weeks of life. This period is considered critical for the development of the nervous system and it is also known that of the 4 to the 14 days the responses of the adrenal for the stress are basically absent (Levine et al 2001; Mistretta & Bradley, 1978; Schmidt et al., 2002).

In relation to the behaviors responses to the neonatal intervention, it is known that the neonatal handling take a increased exploratory activity in novel and aversive environmets, which has been interpreted as decreased fearfulness (Levine, 1967; Tejedor-Real et al., 1998) and a reduced stress response (Levine, 1993; Meaney et al., 1993). The changes in adult rats resulting from neonatal handling can be characterized by the reduced hormones responses to stressfull stimuli. Among the hormones altered in animals that neonatally was stimulated they are the prolactin (Meerlo et al., 1999; Nunez et al., 1996; Severino et al., 2004), the serotonin (Smythe et al., 1994) the adrenaline (Meerlo et al., 1999) and the corticosterone (Levine 1962; Levine et al., 1967; Meaney et al., 1993; Plostky & Meaney, 1993).

Therefore, it is well determined that the neonatal handling, as a crucial early life experience, plays an essential role in the development of hypothalamo-pituitary-adrenal (HPA) axis response to stress (Abrahám & Kovács, 2000; Beane et al., 2002; Levine et al., 2001). Normally, stress-related sensory information conveyed to corticotrophin-releasing hormone (CRH)-secreting neurons in the hypothalamic paraventricular nucleus (PVN) to iniciate neuroendocrine stress cascade. CRH stimulates the adrenocorticotropin hormone (ACTH) release from the pituitary and subsequently the secretion of corticosterone from the adrenal cortex. The CRH-cells are stimulated by cortical, limbic, thalamic, hypothalamic areas and in the regions of brain stem in response to internal and external changes (Abrahám & Kovács, 2000; Larsen & Mikkelsen, 1995; Sawchenko et al., 1996).

The neuroendocrine and behavioral changes induced by the neonatal handling can be coming of morphologic alterations of structures of the CNS activated during a stress stimuli. The reduction of the activity of the HPA axis induced by that intervention in the neonatal period appears to result, in part at least, from the increase in glucocorticoid receptor density in the hippocampus and frontal cortex, which permits enhanced negative-feedback controle of this axis (Liu et al., 1997; Meaney & Aitken, 1985; Meaney et al., 1993; Meaney et al., 1994). Besides, handled rats neonatally have a reduced number of neurons in many structures of the CNS that participate in the stress circuits, such as the parvocellular region of the PVN, the hypothalamic supraoptic nucleus (SON) (Winkelmann-Duarte et al., 2004a) and the *Locus coeruleus* (Lucion et al., 2003).

Recent reports suggest that the glial cells, especially astrocytes, are intimately involved in the active control of neuronal activity and synaptic neurotransmission and that takes to many researchers to analyze the role of the

glia in the regulation of neural integration in the CNS (Bezzi & Volterra, 2001; Auld & Robitaille, 2003). Glial cells (particularly astrocytes with their processes that contact or even enfold a synapse) modulate the eficacy of synaptic transmission (Mitterauer et al., 1996; Mitterauer, 2003; Fields & Stevens-Graham, 2002). Besides, the glial cells are relevant for CNS functions such as migration and maturation of neurons, myelin ensheating, regulation of ionic concentrations, metabolism of chemical transmitters, synaptic integration, energy supply to neurons, and response to brain injury (Bezzi & Volterra, 2001; Kettenmann & Ransom, 1995; Magistretti, 1999). Neuronal-glial interactions can affect the synaptic patterning in the developing brain as well in the adult CNS (Garcia-Segura et al., 1996; Mong et al., 1999).

The changes of the neurons number (reduction of the number of neurons in parvocellular region of PVN, SON and LC) (Lucion et al., 2003; Winkelmann et al., 2004) caused by the neonatal handling, an intervention in the period more critic of the development of CNS showed that the neonatal handling induces a morphological changes in different nuclei of the CNS involved by stress circuits. In this way, the proposal of this study is to verify the manipulation it causes changes in the number of neurons and glial cells in the central, lateral and basolateral nuclei of the amygdala, once it is already known that in several nuclei of the central nervous system (CNS) related to the stress these alterations were observed.

Material and Methods

Animals:

Pregnant females from colonies of the Federal University of Rio Grande do Sul were brought to the animal room in our laboratory. Approximately 7 days before delivery, they were housed individually and the presence of pups was checked for twice a day (beginning and end of the light period). The day of birth was considered day 0. In the next day, the number of pups was culled to 8 per dam by randomly removing some of them with minimal contact with the remaining pups and the dam. After weaning (postnatal day 21), the females were housed in same-sex groups of 2-4 per cage (41 cm long x 34 cm wide x 17 cm high). The animals were maintained on a 12-hr light-dark cycle (lights on from 6:00 a.m. to 6:00 p.m.), the room temperature was 22 ± 1 ° C, and water and food (Rodent show, Nutrilab, Colombo, Brazil) were available at all times. Experiments were performed in accordance with the National Institute of Health (NIH) guidelines (1986) and were approved by the University Research Committee.

Neonatal Handling and Experimental groups:

The pups were divided into two groups: nonhandled, they were not manipulated either by the researchers or by the caretakers from the 1st to the 10th postnatal day, with bedding being changed twice a week from the 10th day on; and handled, the pups were touched for 1 min during the first day after birth. First, the litter and the mother in their home cage were taken to a quiet room next to the animal facility, with the same light period and temperature. The mother was placed in another cage next to the home cage, and then the experimenter gently handled all pups at the same time using both hands, covered with fine latex

gloves, for 1 min. After handling, all pups were returned to the nest at the same time and the mother was placed back in the home cage. The pups were handled at a distance of about 1 to 2 m from the mother, and the total time of the mother-infant separation was approximately 2 min. This procedure was repeated from the 1st to the 10th postnatal day (neonatal period), during the light period of the daily photoperiod cycle (Lucion et al., 2003, Severino et al 2004).

Histological procedures for cell counting

In the experiment for the cell counting were used females ob both groups (handled and not handled) with 11 and 90 (adult) days old. The number of animals used for each groups was 6 and the aninals were nonsiblings (each subject from a different litter). Adult females were studied in diestrus only when they have 3 regular cycles.

Rats were anaesthetized with xilasine (0.1ml/100g body weight, i. m.) and ketamine (0.1ml/100g body weight, i. m.) and were perfused with saline phosphate buffer (PBS) with heparin (50 ml in the 11-day and 100 ml in the 90-day-old rats) followed by paraformaldehyde 4% diluted in phosphate buffer 0.1M (pH 7.4) at 4° C at the same flow rate and total amount. The perfusion rate was approximately 1 drop in 7 seconds for the 11- day and 1 drop in 5 seconds for the 90-day-old females. After the perfusion, the brain was extracted from the skull, weighed, and placed in the same fixing solution for 72 h. After fixation, the brain was washed for 1 h in running water and then dehydrated in different concentrations of ethanol (70%, 80%, 90%, 95% and absolute ethanol) and cleared with xylol. They were included in the final position with a paraplastic resin (Histosec - Merck). Coronal serial sections (6 μm thick) were obtained with

a microtome and serially collected on glued slides. The tissue was stained with cresyl violet. After staining, the sections were dehydrated through and ethanol series, and coverslipped with Entellan (Merck).

Estimation of the volume of the nuclei

The amygdala is divided in different nuclei and each nuclei have your subdivisions. According the atlas Paxinos & Watson (1997), the medial (CeM), lateral (CeL) and central (CeC) subdivisions were considered as the central nucleus of the amygdala (CeA). The dorsolateral (LaDL), ventrolateral (LaVL) and ventromedial (LaLM) were the lateral nucleus of the amygdala (LaA). The anterior (BLA), posterior (BLP) and ventral (BLV) formed the basolateral nucleus of the amygdala (BaLA). The volume of each nucleus was estimated according to the Cavalieri principle (Mandarim-de-Lacerda, 2003, Cruz-Orive, 2004) using a light microscope (Zeiss Axioscop2) with planachormatic objective, a video camera (CCD video camera module) and a computer (Apple Macintosh 8600-300) with the image analysis system (NIH Image 1.62f, Rasband, 1996).

Estimation of the number of cells

In order to characterize the cells to be counted, we measure the size (in microns) of the cells body of 30 neurons in the CeA, LaA and BaLA nuclei of adult nonhandled females rats. We used the same light microscope, a video camera and a computer with the image analysis system descripted by the volume analysis of the nuclei. In the CeA the largest diameter of the soma of the neurons varied from 15 to 25 μm; in the LaA from 15 to 30 μm; and in the BLA from 20

to 35 μm. Glial cells were smaller with a larger nucleus and a deeply stained nucleolus compared to the neurons.

The disector method was used to estimate the total number of cells in each nucleus (Sterio, 1984). The optical disector is based on two parallel sections (lookup and lookdown planes). These planes were determined over a frame of known surface. The numerical density (Nv) of neurons (number of neurons per mm^3) was determined from 10 random disector pairs for each rat. The total number of cells (N) was calculated by multiplying the Nv by the volume of the nucleus, previously estimated according the Cavalieri principle. For reasons of efficiency we analysed the nuclei of the neurons (one nucleus represented one neuron. The number of cells nuclei were counted inside frame 2.304 μm^2 in the lookup plane provided that they did not intersect the right and inferior exclusion edges of the frame (forbidden lines) (Gundersen, 1977). The thickness of the disector (t) was 3 μm for the CeA and 5 μm for the LaA and BaLA, automatically controlled with the motorized stage of the Leica DMRBE microscope.

Immunohistochemistry for GFAP

The females of both groups were studied at 90 (adult) days old. The number of animals in each experimental group (nonhandled and handled) was 5, and they were nonsiblings (each subject from a different litter). Adult females were studied in diestrus to avoid unpredictable variations in the results due to different levels of sex steroids in circulation. The estrous cycle was verified by taking vaginal smears over a period of 15 days, and only females with at least 3 regular cycles were studied.

Tissue preparation

Adult females (90 days old) were anaesthetized with thyopental (50mg/Kg body weight, i. p.) and were perfused with 100ml of saline phosphate buffer with heparin followed by paraformaldehyde 4% diluted in phosphate buffer 0.1M (pH 7.4) at 4° C at the same flow rate and total amount. After the perfusion, the brain was extracted from the skull, and placed in the same fixing solution for 4h. After fixation, the brain was cryoprotected by 15% sucrose/PBS (phosphate buffer saline) solution until sinking and posteriorlly by 30% sucrose/PBS, then quickly frozen in cooled isopentane. This brain was stayed for 3 hours in the criostato for -20°C and soon after it took place cuts on a cryostat (Microm HM 505E) of 50 μm of different structures of the CNS (central, lateral and basolateral nucleus of the amygdala). This nucleus are presents from -1.80 to -3.80 bregma reference of the Atlas Paxinos & Watson (1997). The samples were collected in PBS solution in an interval of 100 μm. It was processed for GFAP immunohistochemistry following the antibody peroxidase-antiperoxidase (PAP) (Sterneberger, 1979). Free-floating sections were treated in 10% metanol and 3% H_2O_2 for 30 min and washed carefully. Then, the sections were preincubated in 3% normal goat serum (NGS) in PBS containing 0.3% Triton X-100 (PBS-Tx, Sigma Chemical Co.) for 30 min and incubated with polyclonal GFAP antiserum raised in rabbit (Sigma Chemical Co.) diluted 1:150 in 3% NGS in PBS-Tx for 48 h at 4°C. After washing several times with PBS-Tx, sections were incubated in a rabit anti-rabbit IgG diluted 1:50 in PBS-Tx at room temperature for 2 h. Sections were washed again in PBS and incubated in a rabbit PAP (Sigma Chemical Co.) diluted 1:500 in PBS for 2 h at room temperature. The immunohistochemical reaction was revealed by incubating the sections in a histochemical medium that contained

0.06% 3'3-diaminobenzidine (DAB, Sigma Chemical Co.) dissolved in PBS for 10 min and then, in the same solution containing 1μM of 3% H_2O_2 per ml of DAB medium for 10 min. Afterwards, the sections were rinsed in PBS, dehydrated in ethanol, cleared with xilene and covered with Entellan and coverslips. Control sections were prepared omitting the primary antibody by replacing it with PBS. All animals used in this experiment were processed (perfusion and immudetection) in the same day and in the same solutions to avoid changes in the background and differences in the chromogen reaction (Rasia-Filho et al., 2002).

Optical density (OD)

The intensity of reaction product of GFAP imunohistochemistry was measured by optical densitometry, using a Nikon Eclipse E-600 (125x) microscope coupled to a Pro-Series High performance CCD camera and Image Pro Plus Software 4.1 (Media Cybernetics, USA). The images obtained from the sections were digitized and converted in an 8-bit gray scale (0-255). To estimate the optical density generated by GFAP immunoreaction in the different nucleus of CNS (CeA, LaA and BaLA) were employed the same area of interest, (AOI) for each nucleus analyzed, ranging 46136 μm^2. The size of AOIs was determined to avoid picture elements (pixels) from outside of nucleus analysed, but also to gather a significant number of pixels from the different subregions of the CeA, LaA and BaLA.

Both, left and right sides of brains were analyzed, and at least 5 readings were performed in each nucleus *per* side. All lighting conditions and magnifications were held constant. Moreover, the investigator was unaware of the experimental groups from which the slices were obtained during the analysis. The

optical density was obtained using the Lamber-Beer formula: OD= $\log_{10}$ 1/ T, were OD = Optical Density and T = Transmitance.

Statistical Analysis

The values were expressed as medians (interquartile range). Differences among groups (nonhandled and handled at 11 and 90 days of age) were tested with Kruskal-Wallis nonparametric analysis of variance. If appropriate, a Mann-Whitney test was used to measure individual differences. In all cases, the accepted level of significance was $p < 0.05$.

Results

In the handling group, animals showed no difference when compared with the controls in the number of cells and the volume of the different nuclei of amygdala analysed. Figure 1 showed the number of cells and volume in the central nucleus of the amygdala (CeA). The results demonstrated that the handled animals have a tendence a reduction of the number of cells at 11 and 90 days old in the right side ($H(3)= 4.28$, $p= 0.232$) and in the left side ($H(3)= 6.733$, $p= 0.081$). The volume was similar in the handled and nonhandled groups in the right side ($H(3)= 1.42$, $p= 0.70$) and in the left side ($H(3)= 6.08$ $p= 0.10$).

The number of cells and the volume of the lateral nucleus of the amygdala (LaA) was demonstrated in the figure 2. The statistic analysis showed that the neonatal handling no affect this nucleus in both age studied. In the rigth side the results of the number of cells was ($H(3)= 2.28$, $p= 0.51$ and left side $H(3)= 4.24$,

p= 0.232. The statistic analysis of the volume of the LaA was H(3)= 1.76, p= 0.62 in the right side and H(3)= 1.14, p= 0.76 in the left side.

Figure 3 showed the number of cells and the volume in the basolateral nucleus of the amygdala (BaLA). As well as in other nuclei of the amygdala, the intervention submited in the animals in the neonatal period no affected the BaLA. In the right side the results was H(3)= 1.75, p= 0.62 and left side was H(3)= 4.60, p= 0.20 for the number of cells and H(3)= 5.42, p= 0.49 for volume total of the nucleus.

Table 1 shows the numerical density (Nv) of cells in the nuclei. In the CeA no significant difference were detected comparing the groups and ages (rigth side: H(3)= 3.49, p= 0.32 and left side: H= 0.34, p= 0.95). Also no have significant difference when Nv of the groups and ages were comparing in the lateral (rigth side: H(3)= 7.28, p= 0.06 and left side: H= 4.07, p= 0.25) and basolateral (rigth side: H(3)= 7.10, p= 0.07 and left side: H= 3.09, p= 0.37) nucleus of the amygdala.

The median (interquartile range) of the dates of the glial cells obtained by optic density of GFAP imunohistochemistry in adult females rats (around 90 days old) was showed in the table 2. No differences was detected comparing the handled with nonhandled groups in each side of the nuclei analysed.

Discussion

The results obtained in the present study show that daily neonatal handling during the first 10 days of life no affect the number of neurons in female rats with 11 and 90 days old that were handled in the neonatal period and the density of glial cells in adult female rats also was not affected by this intervention in the

different analyzed nuclei of the amygdala (CeA, LaA, BaLA). This suggest that neonatal handling does not reduces the number of cells in all structures related to the organization of stress responses, but the cell loss appears to be specific and not widespread in the brain.

A structure involved with stress events and several behaviors is the amygdala (Davis, 1998; Davis, 2000) being an important component in the basal forebrain of mammals implicated in a bewildering variety of behavioral and regulatory functions. These include emotion and memory, social behavior such as reproduction and agression, and modulation of the autonomic and neuroendocrine systems, being involved in the expression of condicioned fear and anxiety (Alheid et al., 1995; Davis, 1998; Fendt & Fanselow, 1999; Shepard et al., 2003). The architectonic organization and connectivity of the amygdala have been extensively reviewed (Alheid et al., 1995; McDonald, 1998). Sah et al. (2003) using the nomenclature, classificated the amygdala nuclei into three groups: 1) the superficial or cortical-like group, which includes the cortical nuclei and nucleus of the lateral olfactory tract; 2) the deep or basolateral group (basolateral complex), which includes the lateral nucleus, the basal nucleus (sometimes called the basolateral nucleus), and acessory basal nucleus (which is also know as the basomedial nucleus); 3) the centromedial group composed of the medial and central nuclei. The basolateral amygdaloid complex (lateral, basolateral and basomedial amygdala nuclei) have a key paper in the fear and fear-learning and lesions in these nuclei reduces behavioral and autonomic responses to condicioned fear stimuli (Walker et al., 2003; Davis 2000; LeDoux, 2000; Iwata et al., 1987; Davis, 1992; Makino et al., 1999). This way, it suggests that the neonatal handling

could affect these nuclei, once, it was already demonstrated that this stimuli in this period takes the decrease of the fear in adult rats (Padoin et al.,2001).

In the CeA, handling induced a margin not significant reduction in the number of cells at the age of 11 (12% in the right and 18 % in the left side) and 90 days (19 % in the right and 18% in the left side). On the other hand, the number of cells and the volume of the basolateral and lateral nuclei of amygdala were not affected by the neonatal handling.

One of the characteristics more evident of the effects of the neonatal handling is that the animals that were handled in the neonatal period present a reduction of the adrenal corticosterone secretion when these are exposed to a stress stimuli (Levine, 1962; Levine et al., 1967; Meaney et al., 1993; Plotsky & Meaney, 1993). This reduction of the corticosterone is related a reduction in the HPA axis and in this way, directlly related with the secretion of CRH by the hypothalamic neurons. The amygdala also have CRH-positive neurons. Previous study showed that neonatal handling decreases CRH-mRNA in the PVN from day 9 through day 45 postpartum (Avishai-Eliner et al 2001, Fenoglio et al 2004) but increase CRH-mRNA in the central amygdala nucleus (Fenoglio et al 2004). Thus, handling can activate CRH-neurons within the neonatal period (Chen et al 2001, Fenoglio et al 2004, Jutapakdeegul et al 2003).

However, changes in central CRH as a possible cause for the observed cell loss seems unlikely. In fact, in the PVN handling decreases CRH, which is the opposite effect to reduce the cell number. In the central amygdala, the effects of handling on the activation of CRH activation were described to begin on day 9 postpartum, after daily handling since day 1 (Fenoglio et al 2004). However, we ended our handling procedure on day 10 after delivery, therefore it is unlikely that

the expected increase of CRH in the CeA could be the cause for the observed tendency to decrease the number of cells in that nucleus. Few works tell the number of neurons of these three nuclei of the amygdala. In female Sprague-Dawley rats, the CeA presents around 32000 neurons the LaA has around 22000 neurons, The volume of the CeA and LaA was similar ($5.0x10^{08}$ μm^3). In the BaLA the number of neurons is around 20000 and the volume approximately $7.0x10^{08}$ μm^3 (Salm et al., 2004). In Wistar females rats not found data in the literature on the number of neurons and volume of these three nuclei of the amygdala studied in this experiments.

However, we can speculate the hypothesis of the glial cells be altered in the period in that the animals were handled, once it is well described that the same interferes in the development of the central nervous system through its direct relationships with the migration and maturation neuronal (Bezzi & Volterra, 2001; Kettenmann & Ramson, 1995).

The effect of corticosterone might be linked to its interaction with the glucocorticoid receptor and elevated glucocorticoid levels are frequently found in aged rats and may contribute to age-related memory and learning deficits (McEwen, 2000; Porter & Landfield, 2002). In the literature, studies relate the neuronal-glial interaction with the action of the corticosterone. It is know that the hippocampus contains the highest density of corticosteroids receptors in CNS and that glucocorticoids may modulate the expression of GFAP following injury, inhibiting yours expression (O'Calaghan et al., 1991). In this way, the reduction of the corticosterone observed in adult rats that were handled in the neonatal period could induce to a increase of the expression of glial cells in nuclei that

present CRH-positive neurons (hypothalamus and amygdala), once this hormone induces the secretion of corticosterone by the adrenal gland.

There are evidence suggesting that brief pup experience in the neonatal period can lead to long-term functional changes in the brain and this are associated with increase of *c-fos* expression indicating an increase in neural activity (Modney & Hatton, 1994). However, given the fact that *c-fos* expression can also occur in nonneural cells such as astrocytes and olygodendrocytes (Arenander & De Vellis, 1992). Therefore, the neonatal handling, a intervention considered a experience to the pups, could be associated with dysfuction or loss of glial cells, especially astrocytes, once these cells can lead to neuronal death (Chen & Swanson, 2003). Since astrocytes play a central role in maintaining neuronal viability both under normal conditions and during stress such ischemia, study of the astrocytic response to stress is essential to understand many types of brain patology (Ouyang & Giffard, 2004).

The principalis glial cell present in the CNS is the astrocyte and it is expressed in great quantity in the amygdaloid nuclear complex. The description of the interaction neuronal-glial in the amygdala is related mainly to the gonadal steroids (Drekic et al., 1995; Rasia-Filho et al., 2002). Different reactivity to estrogen in various regions of neonatal and juvenile rat in amygdala may be related to the different events of the neurogenesis process. The glia actively participate in the process of cell death during the development because surrounding glial cells remove degenerate neurons (Cowan et al., 1984).

The neonatal handling no affect the number of neurons in different nuclei of the amygdala analysed (CeA, LaA and BaLA). In this study we also observed that this intervention in the neonatal period no affect the expression of GFAP in

these similar nucleus, showing that in these case the glial cells were maintained without changes, in spite of knowing that mainly the CeA presents CRH-positive neurons and that these could be indirectly acting in the axis HPA.

In conclusion, the neonatal handling no induces a stable changes in the number of cells (glial and neurons) in the amygdala. Although the glial cells are involved in the functions such as migration and maturation of neurons, and response to brain injury (Bezzi & Volterra, 2001; Kettenmann & Ransom, 1995; Magistretti, 1999), the stimulation in the animals in the neonatal period, a critical phase for the development of the nervous system, no affect morphologically this nuclei and probably the reduction of the activity of the HPA axis is related to the reduction of the number of neurons in the parvocellular region of the PVN (Winkelmann-Duarte et al., 2004), once this is the principal nucleus of CNS that have CRH-positive neurons.

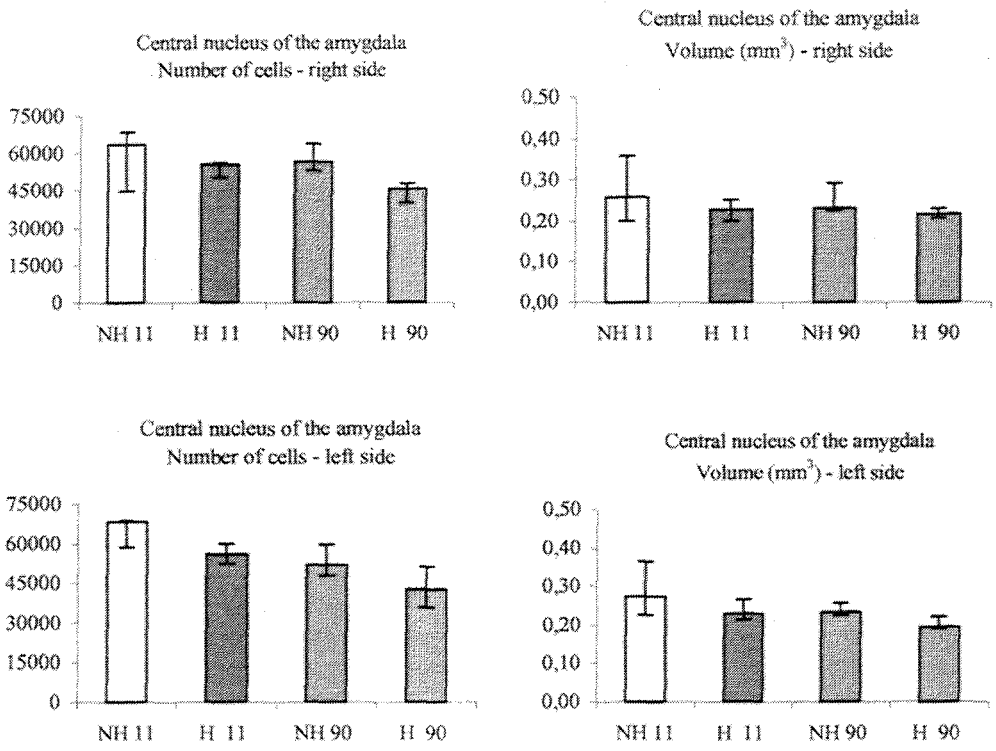

Figure 1. Effects of neonatal handling on the number of cells and volume of the right and left side central nucleus of the amygdala (CeA) in female rats at 11 and 90 days of age. The estimated number of cells and volume (mm^3) are reported as median (interquartile range). The neonatal handling caused no significant changes in the number of cells and the volume of the CeA, but there is a tendency to reduced the number of cells mainly in the adult female rats in the right side (The significance accepted was < 0.05).

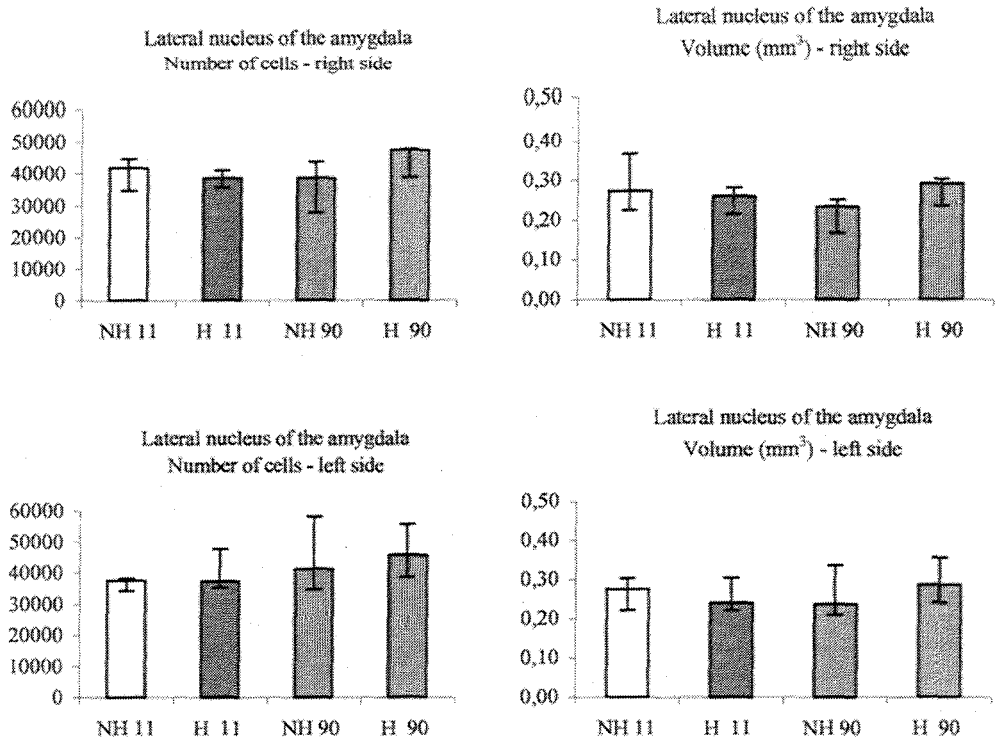

Figure 2. Effects of neonatal handling on the number of cells and volume of the right and left side lateral nucleus of the amygdala (LaA) in female rats at 11 and 90 days of age. The dates obtained in this nucleus showed that the neonatal handling no affect the LaA. The Kruskall Wallis test did not show differences between the different groups and ages analysed ($p > 0.05$).

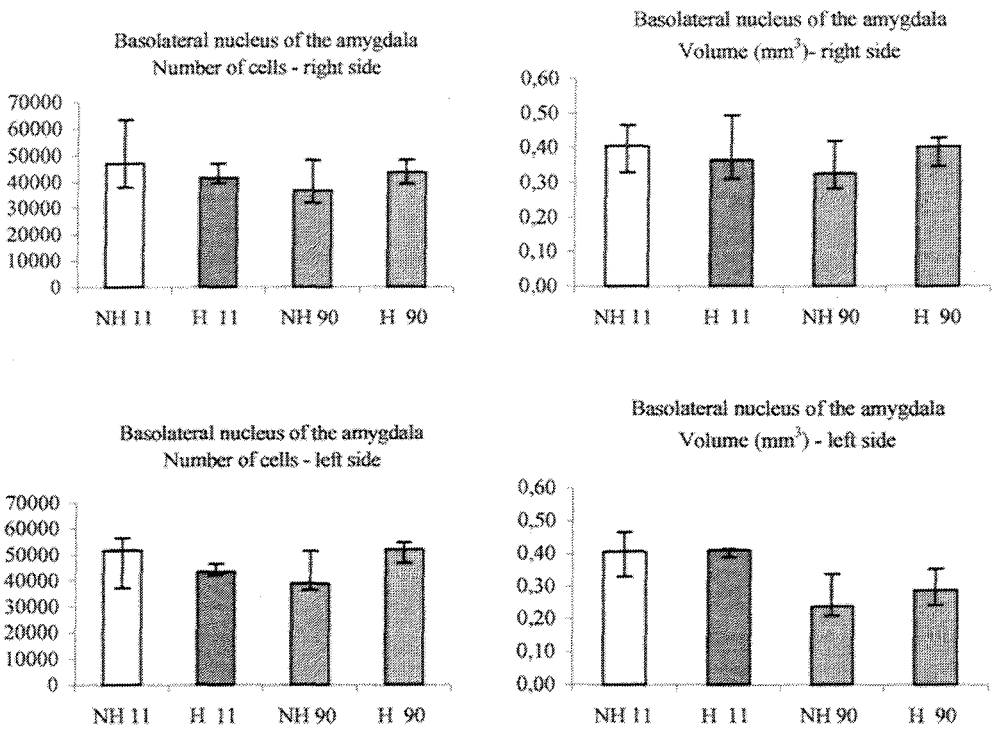

Figure 3. Effects of neonatal handling on the number of cells and volume of the right and left side basolateral nucleus of the amygdala (BaLA) in female rats at 11 and 90 days of age. The neonatal handling is a stimuli insufficient to promote structural changes in the BaLA ($p > 0.05$).

Nucleus of CNS	Age and Group	Nv - right side	Nv – left side
CeA	11 NH	220631 (209780-282118)	235132 (188079-253183)
	11 H	235098 (231481-253183)	224248 (224248-231482)
	90 NH	224248 (217014-231482)	224248 (202546-231482)
	90 H	209780 (188079-217013)	217014 (188079-236742)
LaA	11 NH	138889 (121528-156250)	138889 (112847-164930)
	11 H	143229 (138889-156250)	147569 (138889-156250)
	90 NH	164931 (156250-164931)	169271 (156250-173611)
	90 H	164931 (156250-164931)	156250 (147569-164931)
BaLA	11 NH	117188 (104167-130208)	117188 (112847-121528)
	11 H	104167 (104167-112847)	108507 (104167-112847)
	90 NH	112847 (112847-121528)	117188 (112847-121528)
	90 H	112847 (112847-112847)	117188 (104167-121528)

Table 1. Effects of neonatal handling on the numerical density (NV) of cells in the CeA, LaA and BaLA and female rats at 11 and 90 days old. The numerical density (number of cells per mm^3) is reported as median (interquartile range). There were no significant differences in relationship to the groups and ages in all nucleus of the amygdala analysed.

Nucleus of CNS	Group	OD - GFAP right side	OD - GFAP left side
CeA	NH	21.13(20.63-21.25)	20.13(19.88-21.00)
	H	21.50(21.25-21.88)	21.25(20.88-21.38)
LaA	NH	19.20(19.00-19.70)	21.10(20.40-21.80)
	H	20.20(20.10-20.20)	22.10(21.70-23.00)
BaLA	NH	20.20(20.00-20.30)	19.20(18.80-19.30)
	H	20.60(20.40-20.80)	20.20(19.30-20.70)

Table 2. Effects of neonatal handling on the optical density (OD) of GFAP in the CeA, LaA and BaLA of female rats at 90 days old. The dates is reported as median (interquartile range).

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3.4. Efeitos da manipulação neonatal sobre a densidade de neurônios no hipocampo em ratas (Winkelmann-Duarte, E. C.; Schuh, A. S.; Sanvitto, G. L.; Lucion, A. B. Effects of neonatal handling on the density of neurons in the hippocampus of female rats).

O hipocampo é uma estrutura do sistema nervoso central que participa dos efeitos diretos causados por estímulos de estresse. Avishai-Eliner *et al.* (2001) mostrou que a redução da expressão do CRH hipotalâmico observado em resposta ao estresse é seguido por um aumento dos níveis de RNAm para glicocorticóides no hipocampo. A uma redução da atividade do eixo hipotálamo-hipófise-adrenal observada em animais que foram manipulados no período neonatal, pode ser explicada, em parte, por um aumento da densidade de receptores para glicocorticóides, observado principalmente na área CA1 do hipocampo e isto leva a hipótese de que este aumento do número destes receptores poderia ocorrer por um aumento no número de neurônios das diferentes áreas do hipocampo. Portanto, o objetivo deste trabalho foi verificar se a manipulação neonatal altera o número de neurônios das áreas CA1, CA2 e CA3 do hipocampo em ratas aos 11 e 90 dias de idade.

The neonatal handling induces a changes on the number of neurons in the CA1 field of the hippocampus of female rats

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Abstract

Neonatal handling plays an essential role in the development of hypothalamo-pituitary-adrenal axis response to stress. The reduction of the activity of the HPA axis induced by that intervention appears to result, at least in part, from the increase in glucocorticoid receptor density in the hippocampus and frontal cortex, which allows for enhanced negative-feedback controle of this axis. In the present study we analysed the effects of the neonatal handling on the density of neurons in the CA1, CA2 and CA3 fields of the hippocampus in females rats. We observed an increase on the density of neurons in female rats handled in the neonatal period in the left side of the CA1 field of the hippocampus in adult females (90 days old).

Key words: neonatal handling, density of neurons, hippocampus.

Introduction

The neonatal handling, an early life environmental stimulation, play an essential role in the development of hypothalamo-pituitary-adrenal axis (HPA) response to stress (Levine 1962, Denenberg, 1964, Beane et al., 2002). The

neonatal handling of rats has been used to investigate the impact of early development experiences on neuroendocrine and behavioral functions plasticity (Abrahám & Kovacs, 2000; Beane et al., 2002; Papaioannou et al., 2002). This procedure, typically consists of the manipulation of pups for a few minutes, generally during the first 2 weeks of life. This period is considered critical for the development of the nervous system and it is also known that of the 4 to the 14 days the responses of the adrenal for the stress are basically absent ad has been referred to as "stress hyporesponsive period" (SHRP) (Levine et al 2001; Mistretta & Bradley, 1978; Schmidt et al., 2002).

Handling is apparently mild environmental intervention involving brief removal of the pups from the nest for about 3-15 minutes. Even so, in rats, early postnatal handling reduces emocional responses in adulthood; the reduction is expressed an increase exploratory activity, which is interpreted as decreased fearfulness to novels environments (Fernandez-Teruel et al., 1991; Levine et al., 1967; Tejedor-Real et al., 1998). Another effects of the neonatal handling is reduced behavioral inhibition, expressed by increased locomotion in novel environments (Denenberg 1964; Meaney et al., 1996; Padoin et al., 2001). In relation to the reproduction, the daily handling of the offspring during the first 10 days of life reduces sexual behavior of male and female (Padoin et al., 2001) and induces a significant decrease in ovulation, with most of handled females having anovulatory estrous cycles (Gomes et al., 1999). The neuroendocrine alterations well-known for the neonatal handling are the decrease of adrenocorticotrophin hormone (ACTH) and the corticosterone for the adrenal (Levine 1962; Levine et al., 1967; Meaney et al., 1993; Plostky & Meaney, 1993), of the prolactin (Meerlo

et al., 1999; Nunez et al., 1996; Severino et al., 2004), of the serotonin (Smythe et al., 1994) and of the adrenaline (Meerlo et al., 1999).

Few dates was described related to morphologic changes induced by the neonatal handling. Lucion et al. (2003) demonstrated a reduction of the number os neurons in the *Locus coeruleus* (LC) in all the studied ages (11, 26, 35 and 90 days old). Besides being activated by stressful (Kaufman et al., 2000; Pacak & Palkovits, 2001; Van Bockstaele, et al., 2001), the LC participates in the controle of luteinizing hormone (LH) secretion, thus being an important modulator of the HPA axis. The number of neurons also was reduced in the parvocelular region of the paraventricular nucleus and in the supraoptic nucleus of the hypothalamus (Winkelmann et al., 2004a). The reduction of the activity of the HPA axis induced by that intervention appears to result, in part at least, from the increase in glucocorticoid receptor density in the hippocampus and frontal cortex, which permits enhanced negative-feedback controle of this axis (Liu et al., 1997; Meaney & Aitken, 1985; Meaney et al., 1993; Meaney et al., 1994). This way, the hippocampus presents alterations induced by neonatal handling. This structure is located in the medial temporal area and in terms of cytoarchitectonics, the hippocampus consists of two C-shaped interlocking principal cellular layers: the granular cell layer of the dentate gyrus and the pyramidal cell layer of the hippocampus proper (Gottlieb & Cowan, 1973; Swanson et al., 1987).

The hypothesis of this study was to verify if the neonatal stimulation induce a structural changes in a structure of the central nervous system directly related to the neural circuits mediating stress. In the present experiments we investigated the effect of neonatal handling on the number of neurons of the CA1, CA2 and CA3 fields of the hippocampus in females rats, to investigate that the

increase of the glicocorticoids receptors in this nucleus, it could be explained through an increase of the number of its neurons.

Material and methods

Subjects and Housing:

Pregnant females from colonies of the Federal University of Rio Grande do Sul were brought to the animal room in our laboratory. Approximately 7 days before delivery, they were housed individually and the presence of pups was checked for twice a day (beginning and end of the light period). The day of birth was considered day 0. In the next day, the number of pups was culled to 8 per dam by randomly removing some of them with minimal contact with the remaining pups and the dam. After weaning (postnatal day 21), the females were housed in same-sex groups of 2-4 per cage (41 cm long x 34 cm wide x 17 cm high). The animals were maintained on a 12-hr light-dark cycle (lights on from 6:00 a.m. to 6:00 p.m.), the room temperature was 22 ± 1 ° C, and water and food (Rodent show, Nutrilab, Colombo, Brazil) were available at all times. Experiments were performed in accordance with the National Institute of Health (NIH) guidelines (1986) and were approved by the University Research Committee.

Neonatal Handling and Experimental groups

The pups were divided into two groups: nonhandled, they were not manipulated either by the researchers or by the caretakers from the 1st to the 10th postnatal day, with bedding being changed twice a week from the 10th day on; and handled, the pups were touched for 1 min during the first day after birth. First,

the litter and the mother in their home cage were taken to a quiet room next to the animal facility, with the same light period and temperature. The mother was placed in another cage next to the home cage, and then the experimenter gently handled all pups at the same time using both hands, covered with fine latex gloves, for 1 min. After handling, all pups were returned to the nest at the same time and the mother was placed back in the home cage. The pups were handled at a distance of about 1 to 2 m from the mother, and the total time of the mother-infant separation was approximately 2 min. This procedure was repeated from the 1st to the 10th postnatal day (neonatal period), during the light period of the daily photoperiod cycle (Padoin et al 1997, Gomes et a1999, Lucion et al 2003, Severino et al 2004).

The females of both groups were studied at 2 different ages: 11 (neonatal period) and 90 (adult) days old. The number of animals in each experimental group (nonhandled and handled) and age (11 and 90 days old) was 6, and they were nonsiblings (each subject from a different litter). Adult females were studied in diestrus. The estrous cycle was verified by taking vaginal smears and only females with at least 3 regular cycles were studied.

Histological procedures

Rats were anaesthetized with xilasine (0,1ml/100g body weight, i. m.) and ketamine (0,1ml/100g body weight, i. m.) and were perfused with saline phosphate buffer with heparin (50 ml in the 11-day and 100 ml in the 90-day-old rats) followed by paraformaldehyde 4% diluted in phosphate buffer 0,1M (pH 7.4) at 4° C at the same flow rate and total amount. The perfusion rate was approximately 1 drop in 7 seconds for the 11- day and 1 drop in 5 seconds for the

90-day-old females. After the perfusion, the brain was extracted from the skull, weighed, and placed in the same fixing solution for 72 h. After fixation, the brain was washed for 1 h in running water and then dehydrated in different concentrations of alcohol (70%, 80%, 90%, 95% and absolute alcohol) and xylol. They were included in the final position with a paraplastic resin (Histosec - Merk). Coronal serial sections (6 μm thick) were obtained with a microtome and serially collected on glued slides. The tissue was stained with cresyl violet. After staining, the sections were dehydrated through and alcohol series, cleared with xylene, and coverslipped with Entellan (Merk). The cell layer of the CA1, CA2 and CA3 fields of the hippocampus were identified according to a rat brain atlas of Paxinos & Watson (1997). Pyramidal cells of the CA3 subfield were recognize as having a larger size than those of the CA1 subfield; they have a distinct nucleolus within an ovoid nucleus and had elongated perikarya. The transition to the CA1 subfield, the so-called CA2 subfield, was recognized as narrow zone containing large, loosely organized pyramidal cells, contrasting with the characteristically tightly-packed pyramidal cells of CA1. Glial cells, characterized by their much smaller size compared to the neighbouring neurons as well as peculiar dense bodies and large nuclei surrounded by a sparse cytoplasm, were not count in this experiment (Sousa et al., 1998).

Estimation of the volume

The volume of each area of the hippocampus (CA1, CA2, CA3) was estimated according to the Cavalieri principle. We used an optic microscope (Zeiss Axioscop2) with a 10X lens and a video camera (CCD video camera module) attached to a computer (Apple Macintosh 8600-300) and to an image

analyzing system (NIH Image 1.62f, Rasband, 1996). The area of the nucleus, expressed in mm^2, was measured starting from the 3rd section and an interval of 40. The volume (mm^3) of the right and left-side CA1, CA2 and CA3 fields were estimated by multiplying the sum of the areas by the interval between sections (240 μm).

Estimation of the density of cells

The neurons of the CA1, CA2 and CA3 areas analysed were counted starting from the 3rd section of each area and an interval of 40. We counted all nucleated neurons in the sections of the right and left side. The intervals of the sections were 240 μm. This quantification was performed separately by two experimenters, with the microscope fitted with a 40X lents and a 10X eyepiece with 13288 μm^2 of the test area. The results was expressed in density of cells/mm^2.

Statistical Analysis

Results were compared among groups (nonhandled and handled at 11 and 90 days of age) by the analysis of variance (ANOVA) test followed by Neuman-Keuls post hoc analysis when appropriate. The values of the density of cells/mm^2 and the volume of each area of the hippocampus (CA1, CA2 and CA3) were expressed as means±SEM. In all cases, the accepted level of significance was $p < 0.05$.

Results

Analysis of variance (ANOVA) followed by Newmann Keuls test revealed a significant effect of the neonatal handling upon the total number of cells in the CA1 area of the hippocampus, only in the left side, $F(1\text{-}20) = 6.60$, $p = 0.018$. In the right side of the CA1 area the neonatal handling no induces a differences significant relation to the number of cells, $F(1\text{-}20) = 1.67$, $p = 0.20$. When the different ages were compared no significant differences in the number of cells was founded in the dates analysed (left side $F(1\text{-}20) = 2.47$, $p = 0.13$, right side $F(1\text{-}20) = 0.65$, $p = 0.43$). In the number of cells in the CA1 area of the hippocampus there was no significant Group and Age interction effect in the left side $F(1\text{-}20) = 0.08$, $p = 0.76$ and in the right side $F(1\text{-}20) = 0.01$, $p = 0.89$,. The volume of this area present significant main effects of age in left side, $F(1\text{-}20) = 8.14$, $p = 0.009$ and in the right side, $F(1\text{-}20) = 13.88$, $p = 0.001$. The neonatal handling no induces a changes in the volume in the CA1 area (left side $F(1\text{-}20) = 0.26$, $p = 0.61$, right side $F(1\text{-}20) = 10.3$, $p = 0.32$. The volume of the CA1 area of the hippocampus there was no significant Group and age interaction effect in the left side $F(1\text{-}20) = 0.26$, $p = 0.61$ and in the right side $F(1\text{-}20) = 0.10$, $p = 0.75$ (Figure 1).

Figure 2 show the number of cells and the volume of the CA2 area of the hippocampus. Handled animals present similar number of cells when compared with nonhandled animals showing that the neonatal handling no induces a changes in this area (right side $F(1\text{-}20) = 1.25$, $p = 0.27$), but in the left side ($F(1\text{-}20) = 2.64$, $p = 0.11$) there was a tendence a increase de number of cells when the different groups were compared. The age no presents significant changes in the

left side F(1-20) = 1.96 p = 0.17 and in the right side F(1-20) = 2.54, p = 0.12). In relation to the volume, animals with 11 days old is more larger when compared with the volume of the CA1 area of animals 90 days old in the left side, F(1-20) = 38.27, p = 0.000005, and in the right side F(1-20) = 52.45, p = 0.000001. When the handled animals were compared with nonhandled animals, no significant differences were obtained (left side F(1-20) = 0.05, p = 0.81, right side F(1-20) = 0.45, p = 0.50). In the CA2 area of the hippocampus there was not Group and Age interaction effects on the number of cells (left side F(1-20) = 0.09, p = 0.75, right side F(1-20) = 0.24, p = 0.62) and on the total volume is this area (left side F(1-20) = 1.23, p = 0.27, right side F(1-20) = 0.82, p = 0.37).

In the CA3 area of the hippocampus the neonatal handling also no induces a changes in the number of cells in the different groups (left side F(1-20) = 1.22, p = 0.28, right side F(1-20) = 2.64, p = 0.11) and in the different ages (left side F(1-20) = 0.58, p = 0.45, right side F(1-20) = 0.48, p = 0.49). The volume also no have changes in the different groups (left side F(1-20) = 0.64, p = 0.43, right side F(1-20) = 1.11, p = 0.30) and in the different ages (left side F(1-20) = 0.08, p = 0.77, right side F(1-20) = 0.24, p = 0.62) . To the opposing the CA1 and the CA2 areas of the hippocampus the CA3 area present a similar volume in animals 11 days old when compared with animals 90 days old in the left side (Figure 3).

Discussion

The results obtained in the present study show that daily neonatal handling during the first 10 days of life induces a increase in the number of cells in the left side in the CA1 layer of the hippocampus. Therefore, the hippocampus, as well as

parvocellular region of the paraventricular nucleus hypothalamic, supra-optic nucleus (Winkelmann et al., 2004a) and *Locus coeruleus* (Lucion et al., 2003), it is one of the structures of the central nervous system affected by the neonatal handling. It is known that the neonatal stimulation increases postnatal neurogenesis, prevents hippocampal neuronal loss associated with stress and aging and improves cognitive function, that is to say, that early stimulation results in animals that appear to be better adapted to the every day stress of handling which lead to more adaptive adult ability of coping with novel stimulations (Meaney et al., 1988; Sapolsky, 1992; Pham et al., 1997). This way, the increase of the density of cells in CA1 field of the hippocampus are in agreement with the data described in the literature. The number of neurons in the hippocampus varies among 350000 to 400000 in the CA1 field and around 250000 neurons in the CA3 field (Sousa et al., 1999, Herguido et al., 1999). The volume of the CA1 field is 1.63 mm^3 and in the CA3 field is 2.44 mm^3 (Sousa et al., 1999). In the CA2 area of the hippocampus we didn't find dates related with the number of cells and the volume.

The neonatal handling influences hormonal and behavioral responses throughout life (Levine, 1964; Heim et al., 1997). The reduction of the hypothalamic corticotropin-releasing hormone (CRH) alters the regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Plotsky & Meaney, 1993). The levels of CRH mRNA in the PVN, where CRH release elicits ACTH and glucocorticoid secretion, are reduced in early-life handled rats (Avishai-Eliner, et al., 2001; Plotsky & Meaney, 1993). In addition, glucocorticoid receptor expression in hippocampal CA1 is increased in early-life handled rats, consistent with increased sensitivity to circulating corticosterone and more efficient glucocorticoid-

mediated negative feedback (Herman et al., 1989; Meaney et al., 1993). The increase of the number of neurons in the pyramidal layers in the hippocampus, could explain, in parts, the increase of the number of the glucocorticoids receptors (GR), taking to a reduction of the activity of the HPA axis.

Avishai-Eliner et al. (2001) showed an early reduction of hypothalamic CRH expression with subsequent changes in the stress response that are followed by increased hippocampal glucocorticoid-mRNA levels. The mechanisms mediating this chain of events may be divided in two general alternatives: First, reduced hypothalamic CRH may influence hippocampal GRs directly, via a neuroendocrine feedback loop. Thus diminished CRH release during stress, with consequent reduction of glucocorticoid secretion, disinhibits (up-regulates) hippocampal GR expression (Herman et al., 1989; Pfeiffer et al., 1991). This molecular cascade leads to a new steady-state, consisting of the reduced HPA tone observed in adult rats handled during the neonatal period. The early-life handling/sensory input may influence as yet unknown targets in the stress circuit, via complex multineurotransmitter mechanisms. This primary modulation would then alter hippocampal GR and hypothalamic CRH with different velocities or at different time-points. The actual interval, days 23-45, when GR-mRNA up-regulation occurs also coincides with puberty. Thus, potential interactions of these processes with sex hormones may be considered (Avishai-Eliner et al., 2001).

Another molecule that influence the hippocampal GR density is the serotonin. Actually, early works have proposed that the hypothermia that results from maternal separation stimulates, through thyreoid hormones, hippocampal and cortical serotoninergic systems, stimulation of serotonin $(5HT)_{2A}$ receptors would then promote an increase in GR binding capacity in these two brain regions

(Meaney et al., 1994; Durand et al., 1998). Dates showed that the prior blockade of 5-HT_{2A} receptors prevents handling-induced increased in hippocampal GR density (Micthell et al., 1990) and the number of hippocampal and cortical 5-HT_{2A} receptors is decreased by neonatal handling (Smythe et al., 1994). Therefore, serotonin and glucocorticoid receptors interact not only anatomically, but also functionally.

Besides, previous studies already demonstrated that glucocorticoids influences the process of consolidation of the memory in the context of the conditioned fear (Pugh et al., 1997b). This suggests that through the influence of the control of the development of the axis HPA about the corticosterone secretion, the neonatal handling can increase the dependent processes of memory of the hippocampus present in the context of the learned fear. Beane et al. (2002) demonstrated that when rats suffer stress for retention and they are handling later on it happens a faster return of the corticosterone to its basal concentrations than in nonhandled rats. These results show that the neonatal handling increases the processes of development of the recent memory involved in the conditioning of the fear and it confirms effects previously described of this intervention in the neonatal period on the corticosterone starting from the control of the axis HPA. Rodrigues et al. (2004) showed that the tactile stimulation produce functional/behavioral protection, preventing hippocampal damage in rats submitted to neonatal hypoxia-ischemia.

Therefore, the neonatal handling can be considered a positive intervention in the life of the animal and it is for this reason that some researchers consider this stimulation as being a benefit and not a middle of causing damages in the acting of the functions of the several systems of the body of the animal. On the other

hand, we have to take in consideration that in spite of the neonatal handling increases the number of glucocorticoid receptors in the hippocampus, that takes to a decrease of the corticosterone secretion for adrenal and consequently a reduction of the activity of the axis HPA and this can increase the dependent processes of memory of the hippocampus present in the context of the learned fear, when the animals are less afraid, the life risk it increases and this could be considered a negative point caused by the neonatal handling.

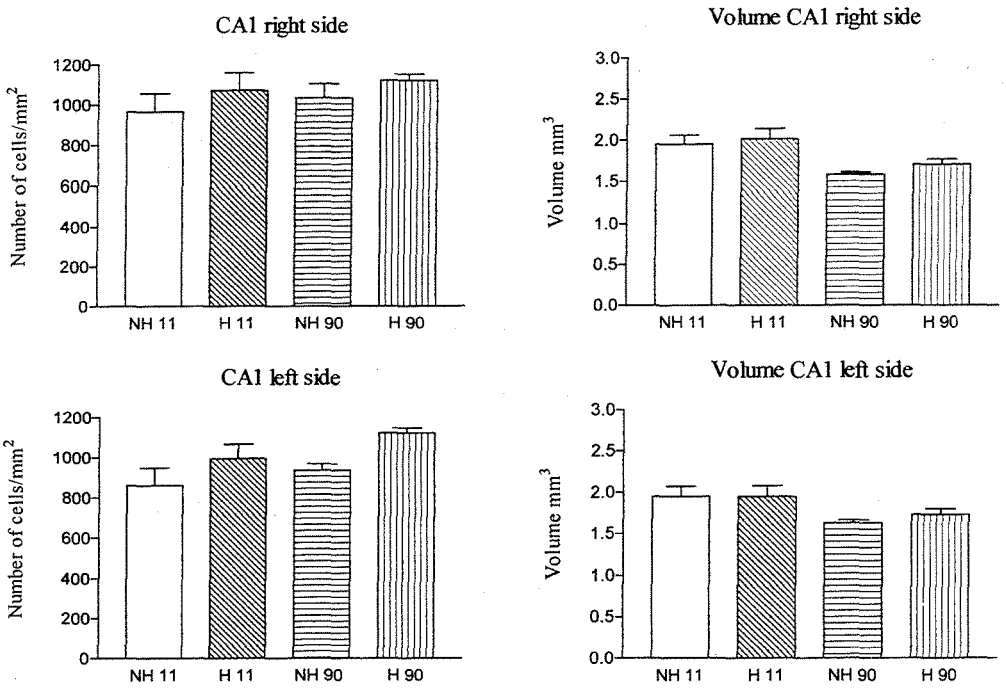

Figure 1. Effects of neonatal handling on the number of cells in the right and left side of the CA1 area of the hippocampus in the 11 and 90-days old animals handled and nonhandled in the neonatal period. There was a increase on the density of cells in the left side of this field and the total volume in the left and right side of the CA1 layer is smaller in adult females rats compared with 11 days old. ANOVA showed no significant interaction between the variables.

NH = nonhandled animals

H = handled animals

11 = 11 days old

90 = 90 days old

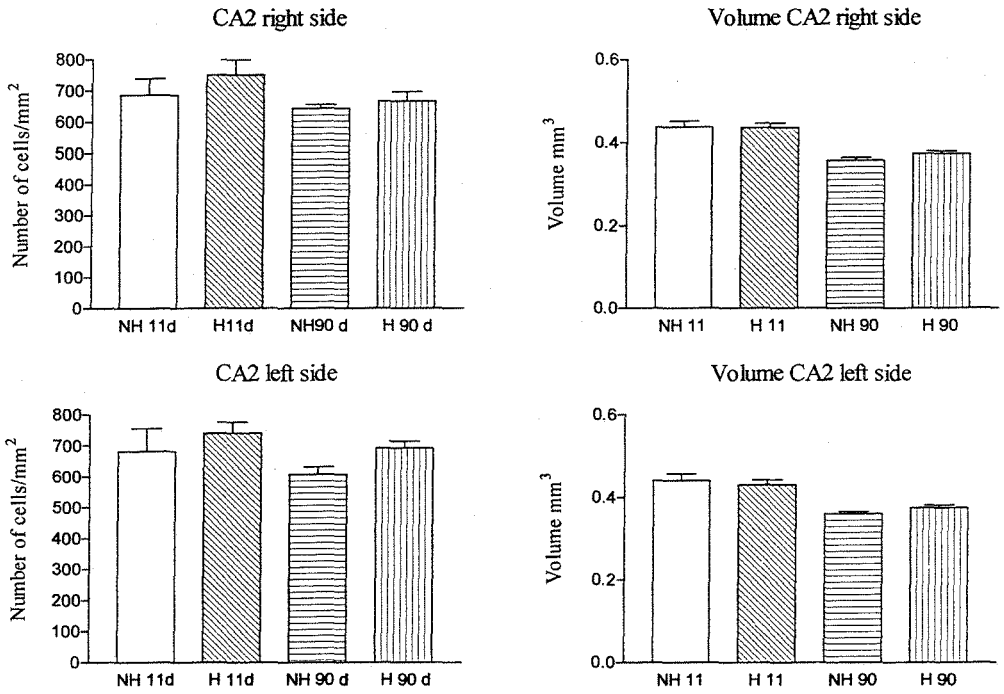

Figure 2. Effects of neonatal handling on the number of cells in the right and left side of the CA2 area of the hippocampus in the 11 and 90-days old animals handled and nonhandled in the neonatal period. There was no significant changes on the number of cells in this area. The total volume in the left and right side of the CA2 area is smaller in adult females rats compared with 11 days old.

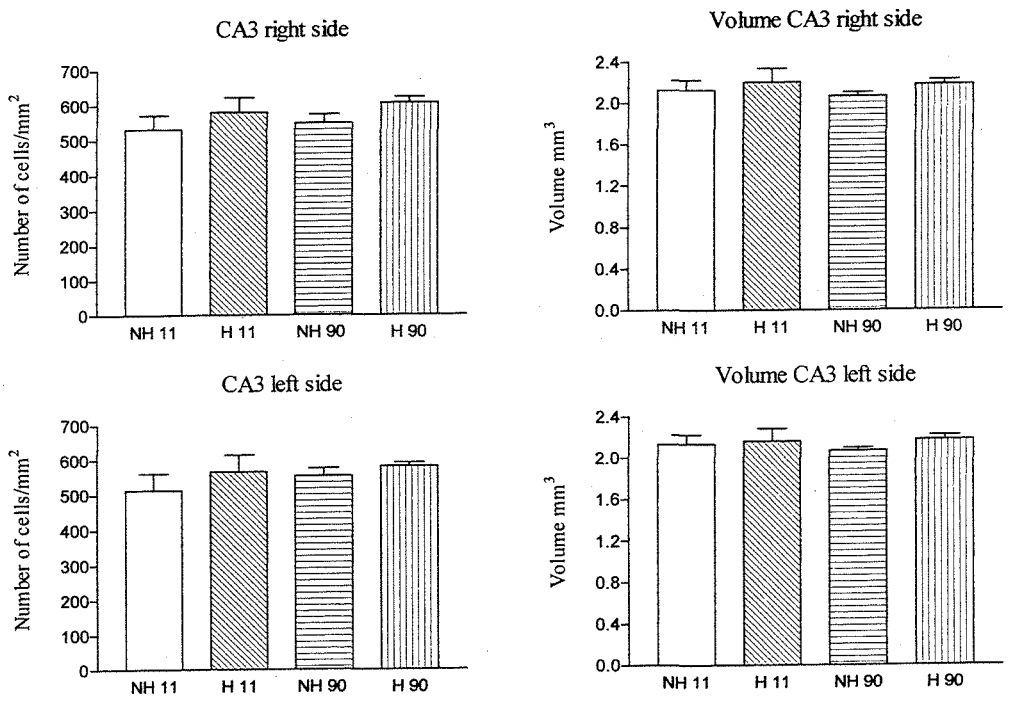

Figure 3. Effects of neonatal handling on the number of cells in the right and left side of the CA3 area of the hippocampus in the different groups (handled and nonhandled) and in different ages (11 and 90 days old). There was no significant changes on the number of cells and the total volume in this area.

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4. CONCLUSÕES

4. Conclusões

4.1. A partir dos resultados obtidos no trabalho intitulado **"A manipulação neonatal reduz o número de células do Locus coeruleus em ratos"**, podemos concluir que:

A manipulação neonatal, realizada durante os dez primeiros dias de vida, causa alterações morfológicas estáveis no SNC, sendo que no Locus coeruleus (LC) ocorre uma diminuição do número de neurônios em ratos machos e fêmeas que foram manipulados no período neonatal em todas as idades estudadas (11, 26, 35 e 90 dias de idade).

A noradrenalina liberada pelo LC estimula os neurônios da área pré-óptica medial a liberarem LHRH (hormônio liberador do hormônio luteinizante). Este hormônio ativa por sua vez o eixo HPG (hipotálamo-hipófise-gonadal). Em ratas manipuladas não ocorre pico de LH na tarde do proestro, possivelmente devido ao conteúdo diminuído de LHRH na APOM na tarde do proestro. Assim, se existe menos neurônios no LC pode ocorrer uma liberação menor de noradrenalina reduzindo a estimulação da área pré-óptica medial o que poderia estar inibindo o eixo HPG causando a diminuição da ovulação.

4.2. A partir dos resultados obtidos no trabalho intitulado **"Efeitos da manipulação neonatal sobre o número de neurônios do hipotálamo em ratas"**, sugerimos que:

A manipulação neonatal induziu a uma redução significativa do número de neurônios da região parvocelular do PVN. Sendo muitos dos neurônios parvocelulares do PVN considerados secretores de CRH, isto poderia explicar a

redução da liberação de corticosterona quando estes animais são expostos a um estímulo de estresse. Um outro efeito da manipulação neonatal é a uma redução do número de neurônios do SON que apresenta uma grande quantidade de neurônios secretores de ocitocina que atuam no eixo HPA inibindo a sua resposta ao estresse.

O estímulo realizado nos filhotes no período de desenvolvimento do sistema nervoso não causou alterações na densidade de astrócitos no PVNp, PVNm e SON em ratas adultas, mostrando que as células gliais não apresentam alterações estáveis provocadas pela manipulação neonatal, apesar destas células estarem diretamente relacionadas ao desenvolvimento do sistema nervoso atuando na migração celular e maturação dos neurônios.

4.3. A partir dos resultados obtidos no trabalho intitulado **"Os efeitos da manipulação neonatal sobre os neurônios e células gliais na amígdala"** podemos concluir que:

A manipulação neonatal não leva a alterações estruturais da CeA, LaA e BaLA, uma vez que o número de neurônios destes núcleos não foi significativamente diferente entre os animais manipulados comparados aos animais não manipulados, sugerindo que, a diminuição do medo observado em ratas adultas manipuladas no período neonatal não pode ser explicada por uma alteração no número de neurônios destes núcleos. O comportamento do medo está relacionado 'a atividade dos neurônios dos núcleos da amígdala, principalmente a CeA.

As células gliais parecem também não serem afetadas pela intervenção da manipulação submetida aos animais no período neonatal, uma vez que a

densidade de glias, especificamente astrócitos não foi alterada nos diferentes núcleos da amígdala estudados neste experimento.

4.4. Os resultados obtidos no trabalho intitulado **"Efeitos da manipulação neonatal sobre a densidade de neurônios no hipocampo em ratas"** podem levar as seguintes conclusões:

A região CA1 do hipocampo sofreu alterações morfológicas causadas pela manipulação neonatal. Ao contrário do que foi encontrado no PVNp e SON, os estímulos que os animais receberam no período neonatal causaram um aumento da densidade de neurônios nesta área do hipocampo no lado esquerdo em ratas adultas. Sugere-se que este aumento de células pode explicar a maior eficiência do feedback negativo do eixo HPA a estímulos estressantes.

A manipulação neonatal não causa alterações semelhantes para todas as estruturas envolvidas no circuito do estresse, uma vez que, no PVNp e SON observou-se uma redução do número de neurônios em ratas manipuladas e em contrapartida, no lado esquerdo da área CA1 do hipocampo de ratas adultas a manipulação induziu a um aumento no número de neurônios, sendo que outros núcleos (PVNm, CeA, LaA e BaLA) que também se relacionam com os efeitos comportamentais induzidos pela manipulação neonatal não apresentaram alterações.

5. PERSPECTIVAS

5. Perspectivas

A partir dos resultados obtidos nesta tese, continuarei realizando trabalhos para verificar outras alterações morfológicas, além do número de células neuronais e gliais, que podem ser encontradas no sistema nervoso central, induzidas pela manipulação neonatal.

A partir dos dados apresentados nos quatro artigos que compõe esta tese, pode-se avaliar se a redução do número de neurônios nos núcleos PVNp SON e também no LC pode ser induzida por apoptose ou necrose. Para isso deverão ser analisadas características celulares, através da microscopia eletrônica que podem descrever o processo de morte neuronal.

A outra hipótese que pode explicar a redução ou aumento do número de neurônios de uma estrutura é uma alteração da proliferação celular. Estas alterações podem ser analisadas através da imunohistoquímica do BrDU. Desta forma, também tenho a proposta de investigar se este evento pode ser modulado pelo procedimento da manipulação neonatal.

Vários outros núcleos do SNC participam das respostas ao estresse. Sendo assim, também pretendo continuar investigando o número de células em outros núcleos afetados pelo estresse.

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Anexo 1

Estudos preliminares de contagem de neurônios utilizando um método mais antigo

O número de neurônios do núcleo paraventricular (região magnocelular - PVNm, e parvocelular - PVNp) do hipotálamo e do núcleo central da amígdala (CeA), do lado esquerdo foi estimado através de um outro método de contagem de células.

Os cortes histológicos utilizados para esta contagem foram os mesmos descritos nos artigos intitulados "Effects of neonatal handling on the number of neurons in the hypothalamus in female rats" e "Effects of neonatal handling on the number of neurons and glial cells in the amygdala". Desta forma, os procedimentos de manipulação, perfusão, inclusão, cortes do material e coloração estão descritos nestes artigos acima citados.

Para a realização da contagem do número de neurônios do lado esquerdo dos núcleos paraventricular do hipotálamo (PVNm e PVNp) e do núcleo central da amígdala foi utilizado um microscópio óptico (Zeiss Axioscop2) com uma lente de 10X e uma câmera de vídeo (CCD) acoplada a um computador (Apple Macintosh 8600-300) e um sistema de análise de imagem (NIH Image 1.62f, Rasband, 1996).

As imagens do núcleo a ser analisado (PVNp, PVNm, e CeA) foram capturadas e eram visualizadas na tela do computador. A contagem foi realizada ao longo do núcleo com um intervalo de 10 cortes, sendo que o primeiro corte a ser contado de cada núcleo foi considerado o terceiro. Foram contados todos os

neurônios do corte que apresentavam o nucléolo visível. Desta forma não era necessário verificar o volume do núcleo, pois para estimar o número total de neurônios era necessário somar o número de células contadas pelo intervalo de cortes, ou seja, $Nt = ns \times p$, onde o Nt é o número de células estimado na estrutura, o ns é o número de unidades contadas e o p corresponde o intervalo entre os cortes usados na amostra (Konigsmark, 1970).

Resultados

O número de neurônios (mediana - intervalo interquartil) do PVNp em ratas aos 11 dias de idade não manipuladas foi de 22940 (21390-24315) e nas manipuladas foi de 23432 (21660-25105). Aos 90 dias de idade o número foi de 19075 (18295-21020) nas não manipuladas e 15035 (13815-16350) nas ratas manipuladas no período neonatal.

O teste de Kruskal-wallis mostrou que ocorre uma redução do número de neurônios parvocelulares em ratas manipuladas aos 90 dias de idade. O número de neurônios do PVNp em ratas aos 90 dias de idade que foram manipuladas no período neonatal foi significativamente menor do que aos 11 dias de idade (Fig. a).

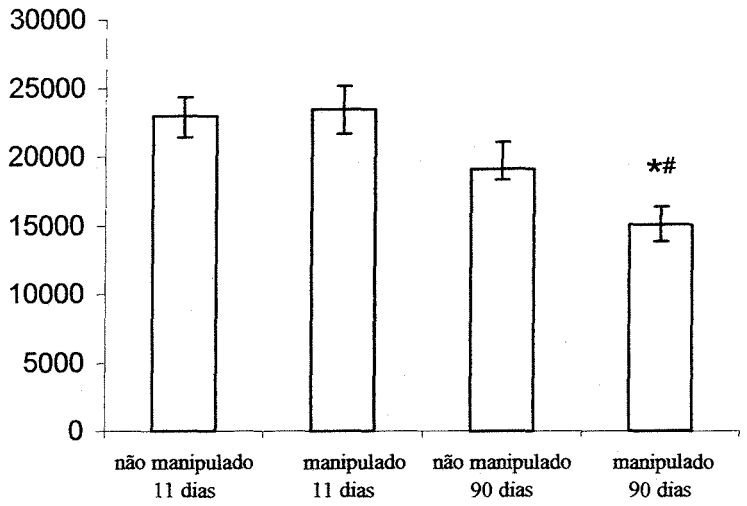

Fig. a. Número total de neurônios da região parvocelular do PVN de ratas manipuladas e não manipuladas aos 11 e 90 dias de idade. Os grupos e suas respectivas idades estão indicadas abaixo de cada coluna do gráfico.

A figura b mostra o número de neurônios magnocelulares encontrado no PVN. Aos 11 dias de idade foram encontrados 6031 (3009-9664) neurônios em ratas não manipuladas e 4282 (3975-4820) nas manipuladas. Aos 90 dias este número foi de 3347 (3055-3880) nas ratas não manipuladas e 3005 (2580-3380) nas manipuladas. Não foram encontradas diferenças significativas no número de neurônios do PVN magnocelulares quando comparados os diferentes grupos e idades estudadas.

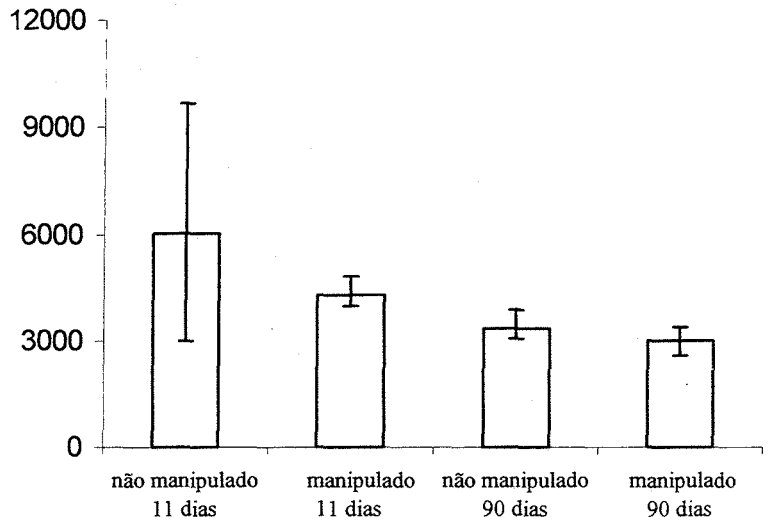

Fig. b. Número de neurônios da região magnocelular do núcleo paraventricular do hipotálamo.

A partir dos estudos da CeA foi verificado que o número de neurônios deste núcleo não foi afetado pela manipulação neonatal. Aos 11 dias de idade as ratas não manipuladas apresentavam 36850 (3550-44180) neurônios e nas manipuladas 38335 (35860-44120) neurônios. Aos 90 dias este número reduziu significativamente para 23135 (21310-28600) nas não manipuladas e para 21640 (18940-22470) nas ratas manipuladas (fig. c).

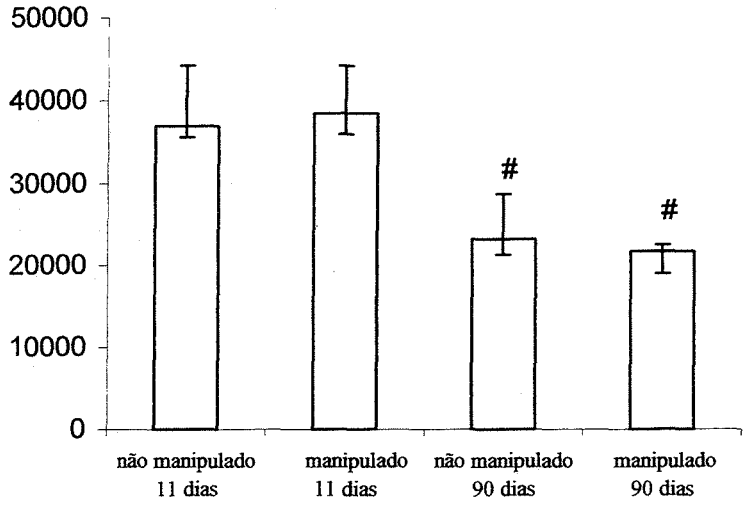

Fig. c. Número total de neurônios do núcleo central da amígdala em ratas aos 11 e 90 dias de idade manipuladas e não manipuladas no período neonatal.

A partir dos resultados obtidos com a utilização deste método de contagem de células podemos concluir que a manipulação neonatal não afeta o número de células nos núcleos central da amígdala e região magnocelular do núcleo paraventricular do hipotálamo, uma vez que a redução do número de neurônios foi observado somente na região parvocelular do PVN.

Apesar de também ter sido encontrado uma diminuição do número de neurônios em ratas manipuladas no PVNp, os valores obtidos com este método de contagem são diferentes daqueles descritos através do método do disector óptico. Isto mostra que os valores obtidos em experimentos de contagem de células usando-se métodos diferentes poderão resultar em valores diferentes. Cada método de contagem descrito na literatura apresenta vantagens e desvantagens, sendo o do disector óptico considerado o mais adequado, pois analisa a presença do elemento (núcleo ou nucléolo) em três dimensões, impedindo uma dupla

contagem da mesma unidade (Cruz-Orive, 1999; Pakkenberg & Gundersen, 1995; Mandarim-De-Lacerda, 2003).