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DANIELE BRAZ TORRES

Análise dos efeitos da restrição proteica *in utero* no BNST e na Amígdala de ratos: Estudo da estrutura dendrítica neural, de parâmetros funcionais e moleculares.

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Tese apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Ciências

Orientadora: Profa. Dra. Patricia Aline Boer

Coorientador: Prof. Dr. Jose Antonio Rocha Gontijo

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RESUMO

O núcleo da estria terminal (BNST) e a amígdala têm sido associados à modulação do comportamento ansioso e do medo. Devido à sua plasticidade, tanto a morfologia quanto a composição de neurotransmissores destas regiões podem ser influenciadas por eventos adversos durante o período perinatal. A restrição proteica *in utero*, com consequente exposição do feto a concentração elevada de glicocorticoides (GC), está associa à desregulação do eixo HPA e a resposta exarcebada ao estresse na vida adulta. Recentemente, um estudo conduzido em nosso laboratório demonstrou que a restrição proteica gestacional promove alterações neuroquímicas e morfológicas no BNST que se associam ao comportamento de ansiedade em ratos machos adultos. Entretanto, neste estudo não foi possível afirmar se as alterações encontradas são de fato decorrentes da programação gestacional ou secundárias a outros fatores desconhecidos que poderiam determinar sua ocorrencia na vida adulta. Assim, o objetivo do presente estudo foi investigar, em animais com idade precoce, os possíveis efeitos da restrição proteica gestacional durante o desenvolvimento do BNST e da amigdala. Nosso estudo mostrou que a prole submetida à restrição proteica gestacional apresenta diminuição significativa do peso corporal ao nascer, que persiste reduzido até o 14° dia de vida. Além disso, a quantificação das células do BNST e os estudos esteriológicis mostram redução significativa do número total de células e do volume da região antero-dorsal do BNST em animais do grupo LP, no 14º dia de vida em comparação ao grupo NP de mesma idade. Houve também redução no comprimento e na arborização dendrítica dos neurônios do BNST na prole LP. Demonstramos ainda redução na expressão de receptores de glicocorticóides (GR) e mineralocorticóides (MR) nos animais do grupo LP com 7 e 14 dias de vida, associada a redução na expressão do BNDF e aumento na expressão do receptor CRF1 no BNST de animais do grupo LP com 7 dias de vida, apesar de não ter havido alteração nos níveis plasmáticos de corticosterona. Nos animais do grupo LP com 14 dias observamos redução na expressão dso receptores 5HT1A e aumento na expressão dos receptores 5HT2A nos animais do grupo LP com 7 e 14 dias de vida. Nossos resultados também demonstraram aumento significativo

da concentração de norepinefrina e 5HIAA no BNST e redução do *turnorver* de dopamina e seu precurssor DOPAC no BNST, respectivamente, nos animais do grupo LP com 7 e 14 dias. Quanto à amigdala nós não observamos alterações significativas no comprimento e no número de ramificações dos dendritos de neurônios localizados na amigdala basolateral dos animais do grupo LP com 14 dias comparativamente aos animais do grupo NP de mesma idade. Da mesma forma, o número de células na amigdala foi similar nos animais dos grupos LP e NP no14º dia. Houve redução significativa na concentração de norepinefrina, epinefrina e dopamina bem como na expressão de CRF e BDNF na amigdala de animais do grupo LP no 7º dia, assim como houve redução na expressão de GR, MR e CRF na amigdala dos animais do grupo LP no14º dia. Em conclusão, esta é a primeira descrição na literatura da ocorrência de modulação da plasticidade, tanto com relação ao número de neurônios e outros tipos celulares quanto da neuroquímica do BNST precocemente, decorrente da restrição proteica gestacional. Além disso, as alterações neuroquímicas observadas na amigdala e no BNST podem contribuir para as alterações comportamentais induzidas pela restrição proteica gestacional bem como alterações em outras regiões cerebrais. Estas diferenças podem representar a adaptação que ocorre durante o desenvolvimento embrionário às altas concentrações de glicocorticóides maternos e pode estar relacionada à hiperatividade do eixo HPA e ao comportamento de ansiedade observado na idade adulta em indivíduos submetidos à restrição proteica gestacional.

Palavras chaves: BNST, amigdala, programação fetal, receptores esteróides, CRF, BDNF, serotonina, dopamina, catecolamina, análise dendrítica.

ABSTRACT

The bed nucleus of the stria terminalis (BNST) and amygdala have been associated with the modulation of anxiety-like behavior and fear. Due to their plasticity, its morphology and neurotransmitter compounds may be affected by perinatal adverse events such as protein restriction *in utero*, when the fetus is exposed to high maternal glucocorticoids (GC) levels, leading to a deregulation of HPA axis and exacerbated stress answer in adult life. We have recently demonstrated that gestational protein-restricted intake causes neurochemical and morphological changes in the BNST associated with anxiety-like behavior in male offspring adulthood. However, our study did not allow asserting the full mode whether these disorders are from gestational programming or secondary to other factors that could determine these changes in adulthood. Thus, our objective was investigating the possible effects of gestational protein restriction on development of BNST and amygdala in early age. The current study shows a significant decrease in body birth weight that remains up to 14 day of age, in gestational protein-restricted offspring. Otherwise, BNST cell quantification and stereology studies show a significant reduction of the total cells number associated with reduced volume of the anterodorsal BNST division in 14 d-old LP offspring compared to age-matched NP group. Also, we found reduction of the dendritic length and arborizations in the BNST neurons of the LP offspring. The present work demonstrates a decreased expression of gluco- and mineralocorticoid receptors (GR and MR) in 7 and 14-d old accompanied, by fall in BDNF and enhanced CRF1 receptor expression in the BNST of the 7-d old LP offspring, despite of unchanged corticosterone plasma level. Additionally, the 14 d-old LP offspring presents a reduced $5HT_{1A}$ receptor subtype levels, reciprocally accompanied, by increased $5HT_{2A}$ receptors in the 7 and 14-d old LP. Our findings also show a significant increase in the BNST norepinephrine and 5HIAA levels and, reduced dopamine turnover and 3,4-DOPAC BNST levels, respectively in 7-d old LP and 14 d-old LP. In complementary study we did not observe any significant changes in the basolateral amygdala neuronal dendrites length and ramifications in 14 d-old NP compared with age-matched gestational protein-restricted offspring.

Also, the amygdala cells number was similar in 14 day-old LP and NP offspring. We demonstrate a significant decrease in the amygdala content of norepinephrine, epinephrine, dopamine, CRF and BDNF in 7 day-old rats, as well as reduction in GR, MR and CRF expression in 14 day-old LP offspring. In conclusion, as far as we know is the first description of the modulation of neuron and non-neuron plasticity and neurochemistry of the BNST in the early life, by gestational protein-restricted intake. Also, the BNST and amygdala neurochemical changes observed in the current study may contribute to behavioral alterations induced by gestational protein restriction and these may be a primer for alterations in other brain regions. These findings may represent the adaptation during embryonic development to elevate maternal corticosteroids exposure and could be related to HPA axis hyperactivity and anxiety-like behavior observed in maternal protein-restricted offspring adulthood.

Keywords: BNST, amygdala, fetal programming, steroid receptors, CRF, BDNF, serotonin, dopamine, catecholamine, dendritic analysis

LISTA DE SIGLAS E ABREVIATURAS

OMS Organização Mundial da Saúde

BPN baixo peso ao nascer

RCIU retardo do crescimento fetal intra-utero

SNC sistema nervoso central

11β- HSD$_2$ enzima 11β-hidroxiesteróide desidrogenase tipo 2 placentária

HHPA eixo hipocampo-hipotálamo-pituitária-adrenal

GC glicocorticoides

MR Receptores de mineralocorticóides

ACTH Hormônio adrenocorticotrofico

PVN núcleo paraventricular

BNST núcleo da estria terminal

CRF hormônio liberador de corticotrofina

VTA núcleo ventral tegmentar

CeA núcleos central da amigdala

CeM núcleo medial da amigdala medial

BNSTL divisão lateral do BNST

MeA amígdala medial

BNSTM divisão medial do BNST

BLA núcleo basolateral da amígdala

BLAC parte caudal do BNST

TDAH déficit de atenção e hiperatividade

CRF1 fator liberador de corticotropina receptor 1

5-HT serotonina

5-HT1-7 família de receptores de serotonina dos tipos 1 a 7

5-HT1A receptor de serotonina tipo 1A

BDNF fator neurotrófico derivado do cérebro

ICVS Instituto de Ciências da Vida

DGAV Direção Geral de Alimentação e Veterinário

NP *Normal Protein*

LP *Low Protein*

DAG distância ano-genital

µm micrometros

NP7D *Normal Protein* sete dias

NP14D *Normal Protein* quatorze dias

LP7D Low Protein sete dias

LP14D *Low protein* quatorze dias

PFA paraformolaldeído

WB *western blotting*

RIPA Radio Immuno Preciptation Assay Buffer

rpm rotações por minuto

HPLC / **CE** cromatografia líquida de alto desempenho, combinada com detecção eletroquímica

COBEA Colégio brasileiro de experimentação animal

CEEA comissão de ética na experimentação animal

CEMIB Unicamp Centro Multidisciplinar para Investigações Biológicas da Unicamp

RIA radioimunoensaio

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1. INTRODUÇÃO

Embora o número de pessoas que passam fome no mundo tenha caído ele ainda é inaceitável, abrangendo 795 milhões de pessoas A prevalência da desnutrição em regiões em desenvolvimento também vem diminuindo, mas ainda afeta 12,9% da população. No Brasil são 3,4 milhões de pessoas subalimentadas apesar da queda de 82% entre 2002 e 2014 (FAO, 2015).

Tanto em países desenvolvidos quanto naqueles em desenvolvimento a desnutrição materno-infantil tem repercussões evidentes sobre a saúde da população. A desnutrição não é causada apenas pela falta de alimentos, mas também pela dieta ocidental que prioriza gordura e carboidratos em detrimento de proteínas e vitaminas.

De acordo com dados de 2013 da Organização Mundial da Saúde (OMS), a desnutrição materna continua sendo um problema de saúde pública mundial e está relacionada ao baixo peso ao nascer (BPN). Anualmente nascem cerca de 20 milhões de crianças com BPN em todo mundo, representando cerca de 15,5% de todos nascimentos. O baixo peso ao nascer foi incluído no ultimo relatório da OMS que definiu uma meta global de reduzir o BPN em 30% até 2025.

O período pré-natal constitui uma convergência crítica dos fatores de curto e longo prazo que afetam a saúde ao longo da vida (Procter & Campbell, 2014). A quantidade inadequada de nutrientes essenciais durante os períodos cruciais de desenvolvimento fetal pode levar a reprogramação dos tecidos fetais, predispondo o individuo a doenças crônicas na vida adulta fundamentando o conceito da programação fetal (Ashton, 2000). O pesquisador inglês David Barker et al (1989) foi pioneiro em afirmar que a nutrição durante o período intrauterino e a infância estava associada à risco aumentado para o aparecimento de doença cardíaca no adulto e, alguns anos mais tarde também afirmou que crianças que nascem com baixo peso tem maior risco de desenvolver doença cardiovascular quando adultas, independente da exposição a outros fatores de risco clássicos como obesidade,

sedentarismo e tabagismo. Estas constatações levaram Barker, em 1989, a interpretar o ambiente fetal como um novo componente na etiologia das doenças cardiovasculares e fundamentaram aquela que ficou conhecida como hipótese de Barker. Posteriormente, Barker (1995; 1997; 1998) obteve novas evidências da associação entre o baixo peso ao nascer e o desenvolvimento de doenças cardiovasculares no adulto, fortalecendo a hipótese de que o retardo do crescimento intra-utero (RCIU) aumenta o risco de desenvolver doenças cardiovasculares na fase adulta.

Entretanto, os primeiros estudos são da década de 60, quando Rose (1964) mostrou que na família de pacientes com doença isquêmica cardíaca, havia o dobro de registros de irmãos natimortos e de mortes prematuras na infância em relação aos demais pacientes. Forsdahl, em 1967 demonstrou que os índices de mortalidade infantil em anos anteriores correlacionavam-se à prevalência de doença cardíaca aterosclerótica daquele ano na Noruega.

Roseboom e colaboradores, (2006) fizeram um levantamento importante de indivíduos que foram gestados durante o período da “Fome Holandesa”. Este foi um período (1944-45) de escassez intensa de alimentos imposta pelo exercito alemão durante a II Gerra Mundial. O suprimento foi cortado inclusive para crianças, lactantes e mulheres gravidas, que normalmente tinham direito a uma quantidade extra. Eles observaram que estes indivíduos apresentaram baixo peso ao nascer e redução da circunferência do crânio e quando adultos os mesmos apresentaram desequilíbrio metabólico. Mais tarde foram estabelecidas associações entre o surgimento de doenças em adultos e o período de exposição à fome durante a gestação e ficou evidente que aqueles expostos a fome logo no inicio da gestação apresentavam complicações maiores quando adultos (Painter et al, 2005; Rosebbom et al, 2001).

Porém, apenas no ano de 1991, o conceito de programação fetal foi definido por Alan Lucas, como sendo a “*resposta permanente do organismo a um estímulo ou insulto durante um período crítico do desenvolvimento embrionário e fetal”.* Portanto, o ambiente materno atua como uma previsão das condições que o

feto irá encontrar após o nascimento. O feto responde e adapta-se a esta previsão usando estratégias a fim de maximizar sua chance de sobrevivência (Hales, 2013).

Desde então vários modelos experimentais de subnutrição gestacional foram desenvolvidos, nos quais o baixo peso da prole ao nascer foi associado à elevação pressórica na idade adulta (Persson & Jansson, 1992; Woodall et al, 1996; Prentice 1991; Godfrey et al, 1996). Dentre os modelos experimentais têm sido bastante utilizada variações no conteúdo de proteína da dieta gestacional para produzir restrição leve (12% de caseína), moderada (9%) e severa (6%) (Langley-Evans et al, 1996). Esses experimentos resultaram em alterações variáveis no peso dos recém-natos e no tamanho das placentas. Os animais submetidos à restrição desenvolveram hipertensão arterial a partir da quarta semana de vida a qual foi mantida até a idade adulta (Langley-Evans et al, 1994; 1995).

As proteínas são formadas por agrupamentos de aminoácidos resultantes da ingestão de dietas e são necessárias para diversas funções do sistema nervoso central (SNC), dentre outros. Assim o déficit de proteína pode afetar diretamente o desenvolvimento cerebral (Lima et al, 1993; Morgane et al, 2002).

Diversos trabalhos demonstram que o baixo peso no nascimento é um fator de risco para o desenvolvimento de desordens psicológicas relacionadas ao desenvolvimento neural, tais como o autismo (Burd et al, 1999) e depressão podendo levar ao suicídio (Barker et al, 1995). Entretanto, Welberg & Seckl (2001) demonstraram que o baixo peso no nascimento não é um fator crucial *per se* servindo, provavelmente, como um marcador de possíveis efeitos deletérios durante o desenvolvimento do sistema nervoso.

Existem diversos fatores que podem estar envolvidos na gênese da programação fetal, no entanto, o mais estudado é a diminuição na concentração e atividade da enzima 11β-hidroxiesteróide desidrogenase tipo 2 (11β- HSD_2) placentária (Benediktsson et al, 1993; Stewart et al, 1995; Langley-Evans et al,

1996; Langley-Evans, 1997). A diminuição na11β-HSD_2 placentária, devida à restrição proteica gestacional, resulta no aumento da exposição fetal aos corticosteróides endógenos maternos (Langley-Evans et al., 1996). Normalmente o feto é exposto a 10%-20% de cortisol e este cortisol presente na circulação fetal é uma combinação do cortisol produzido pelo feto mais o cortisol materno derivado da placenta materna (Seth et al, 2015).

O cérebro é extremamente sensível à programação pré-natal e a exposição intra-uterina a glicocorticoides tem influência ampla na estrutura nervosa e formação de sinapses (Ding et al, 2016). Diversos agentes como fatores de crescimento, de transcrição e nutrientes podem afetar permanentemente o desenvolvimento neural (Welberg & Seckl, 2001). Particularmente os esteroides têm propriedades poderosas na programação neural (Matsumoto & Arai, 1997).

O fato da resposta ao estresse ocasionar liberação de grande quantidade de corticoides torna-o um candidato óbvio como fator programador no paradigma do estresse pré-natal (Welberg & Seckl, 2001).

Nos ratos o período que consiste no pico do desenvolvimento do SNC se da entre o 7º e 14º dia de vida, perdurando até 35º (Morgane et al, 2002). Durante esse período estão ocorrendo fenômenos como neurogênese, gliogênese e intensa mielinização em diferentes regiões do cérebro (Levitisky & Barnes, 1972; Morgane et al, 2002)

Existem evidências de que a função pós-natal do eixo hipocampo-hipotálamo-pituitária-adrenal (HHPA) pode ser alterada por eventos pré-natais. Tais alterações podem ocasionar, no adulto, exposição cronicamente aumentada a glicocorticoides (GC), bem como resposta exacerbada a estímulos estressantes. Essas alterações são geralmente atribuídas a modificações na capacidade de retroalimentação hipotálamo-hipofisária de esteroides, decorrente de modulações na expressão de genes codificantes de receptores de GC (incluindo receptores glicocorticóides – GR - e mineralocorticóide – MR - no hipocampo) que levam a redução destes receptores. Paralelamente, ocorrem alterações em diversos

neurotransmissores de varias outras regiões do encéfalo (para revisão ver Welberg & Seckl, 2001).

Alterações na expressão destes receptores podem levar, em longo prazo, à disfunção na regulação da concentração plasmática de adrenocorticotrofina (ACTH) e de GC. Em fetos de porcos, foi determinada a presença de RNAm para GR por todo o cérebro, sendo a maior concentração encontrada nos núcleos paraventriculares (PVN) hipotalâmicos. Já o RNAm para MR está presente exclusivamente no sistema límbico (hipocampo, amígdala, e giro dentado) ocorrendo maior concentração no hipocampo (Lingas, et al, 1999). Estes autores demonstraram, em porcos, que a restrição nutricional materna altera a expressão destes receptores em períodos gestacionais relacionados ao maior desenvolvimento neural.

Como os sistemas de neurotransmissores e GC neurais interagem para modular tanto o comportamento quanto a atividade do eixo HHPA (Mcewen, 1987) é possível que os efeitos do estresse pré-natal sejam mediados por alterações permanentes nestes sistemas (Welberg & Seckl, 2001). Devemos ainda considerar que a regulação do eixo HHPA pelo hipocampo e outras estruturas límbicas é mediada, em parte, por reles sinápticos no núcleo da estria terminal (BNST) (Cullinan et al, 1993; Feldman et al, 1990; Gray et al, 1993; Herman et al, 1994; Onaka & Yagi, 1998; Prewitt & Herman 1998; Zhu et al., 2001). O BNST está situado numa posição chave para regular não somente o comportamento de ansiedade, mas também as respostas ao estresse, implicadas em neuropatologias e distúrbios neuropsicológicos. Além disso, evidencias sugerem que o BNST tem alta plasticidade podendo esta ser influenciada pelo estresse (Walker et al, 2003).

Estudos desenvolvidos em nossos laboratórios, avaliando ratos machos adultos submetidos a restrição proteica gestacional, revelaram redução da arborização dendritica de neurônios do BNST paralelamente ao aumento significativo da concentração plasmática de corticosterona e da ansiedade. Desta forma, demostramos pela primeira vez que o BNST é programado pela restrição

proteica gestacional resultando em alterações na neuroquímica e na morfologia levando a alterações comportamentais (artigo submetido).

Sabe-se ainda que o comportamento de ansiedade é mediado pela amígdala, provavelmente via hormônio liberador de corticotrofina (CRF) (Schulkin et al, 1994) cuja transcrição é facilitada pelos corticosteróides (Makino et al, 1994; Hsu et al, 1998; Hatalski et al, 1998). A prole de ratas submetidas à carboxolano (que inativa a 11β-HSD) apresenta expressão aumentada de GR na amígdala, podendo aumentar a sensibilidade a concentrações elevadas de corticosteróides (Welberg et al, 2000) induzindo aumento do CRF.

Além disso, estudos demonstram que a exposição gestacional a glicocorticoides induz um fenótipo hiperemocional na vida adulta (Oliveira et al, 2006).

Como já está bem estabelecido o papel da amígdala e do BNST nos comportamentos de medo e ansiedade (Davis 1992, 2010) ambos relacionados à resposta ao estresse, estas estruturas constituem alvos de estudo importantes nos modelos de programação fetal.

1.1 Plasticidade do BNST e da amígdala

O núcleo da estria terminal (BNST) foi definido originalmente por Johnston, em 1923, como uma estrutura de massa cinzenta que envolve a estria terminal e se expande em suas extremidades caudal e rostral. A extremidade caudal descrita por Johnston (1923) é agora considerada como parte da amígdala. A região rostral, encontrando-se na zona ventral do septo lateral e dorsal para a área pré-óptica do hipotálamo, é denominada BNST. Johnston (1923) também observou que o BNST forma uma ligação contínua com estruturas da amígdala fato que originou o termo “amígdala estendida”.

O BNST é uma estrutura em posição privilegiada para integrar informações de estresse e regular tanto o estreses quanto os sistemas de recompensa. Tem sido demonstrado que tanto a exposição crônica a estressores como tratamentos farmacológicos (ex: corticosterona) influenciam na plasticidade do BNST (revisto em Hammack et al, 2010). Diversos estudos demonstram que a expressão de CRF no BNST foi aumentada após situação onde o indivíduo é subjugado socialmente por período prolongado (estresse social crônico), exposto a estresse crônico moderado ou ao tratamento crônico com corticosterona (Makino et al, 1994; Watts & Sanchez-Watts 1995; Schulkin et al, 1998). Adicionalmente, tem sido demonstrado que estes tratamentos aumentam os sinais de neuroplasticidade no BNST. A exposição ao modelo de estresse crônico inesperado foi associada ao aumento no volume do BNST e no comprimento dos dendritos neuronais (Pêgo et al, 2008).

A imobilização crônica aumentou o número de pontos de ramificações da arborização dendrítica de neurônios do BNST (Vyas et al, 2002; 2003) enquanto o modelo de estresse variado por uma semana aumentou o comprimento dendrítico de neurônios deste núcleo.

Têm sido observadas correlações funcionais relacionadas à neuroplasticidade do BNST já que a exposição crônica a drogas de abuso tem sido relacionada ao aumento das correntes excitatórias pós-sinápticas originadas no núcleo ventral tegmentar (VTA) que se associam a neurônios do BNST, bem como aumento na expressão de transportadores de norepinefrina no BNST (Macey et al, 2003).

Elevações na concentração de CRF e da neuroplasticidade do BNST tem sido associadas ao aumento do comportamento de ansiedade e anedonia (um sintoma de depressão, Stout et al, 2000). Consequentemente, a exposição crônica a estressores podem alterar a neuroquímica, a morfologia e a função do BNST levando ao aumento do medo e da ansiedade. Recentemente, Oliveira et al (2012) observaram que a administração de dexametasona em ratas prenhes leva a programação do BNST e da amígdala da prole paralelamente ao fenótipo de

hiperansiedade e alteração no comportamento de medo. Eles encontraram aumento no volume e no comprimento dendrítico no BNST e redução do volume e do comprimento dendrítico na amígdala.

Assim, podemos concluir que exposição crônica a estressores leva a modulações estruturais e funcionais no BNST, já que este apresenta grande plasticidade, que medeiam desordens de ansiedade.

1.2 Conexões entre a amígdala e o BNST

Alheid et al (1998) e Alheid & Heimer (1988) demonstraram que os núcleos central (CeA) e medial (CeM) da amigdala e o BNST são conectados por colunas de células localizadas por toda a estria terminal, pelas fibras do trato que conectam estes núcleos da amígdala ao BNST e também pela parte localizada ventralmente, do tronco cerebral basal. Estes autores também mostraram que o CeA emite projeções principalmente para a divisão lateral do BNST (BNSTL) e a amígdala medial (MeA) emite projeções principalmente para a divisão medial do BNST (BNSTM). Estas projeções foram denominadas como células de amígdala estendida. Além disso, o CeA e o BNSTL são muito similares anatomicamente em termos de “inputs”, “outputs”, tipos celulares, e conteúdo neuroquímico, especialmente no que diz respeito aos níveis elevados de peptídeos encontrados em ambas as estruturas (Alheid et al, 1995). O núcleo basolateral da amígdala (BLA) também emite projeções não só para o CeA, mas também para o BNSTL, especialmente partindo da região caudal do BLA (Dong et al, 2001; McDonald, 1983; Weller & Smith, 1982).

Duas áreas ricas em CRF que medeiam a resposta a estressores incluem o BNST e o CeA. Tem sido sugerido que a ativação do BNST ou do CeA coordenam a rede de respostas comportamentais apropriadas para a luta frente as ameaças percebidas e, ao mesmo tempo, engajando sistemas catabólicos periféricos que suportem estas alterações comportamentais. Consequentemente, tanto o CeA

quanto o BNST tem sido implicados na mediação da resposta ao estresse bem como tem sido considerados críticos na modulação de comportamentos afetivos (medo e ansiedade) (Walker et al, 2003, 2009.

1.3 Mediadores do medo e da ansiedade

É amplamente conhecido que as catecolaminas desempenham papel importante no sistema neuroquímico do cérebro, e estão envolvidas em uma série de funções cerebrais, dentre elas a resposta ao medo e a ansiedade, bem como distúrbios neuropsiquiátricos como esquizofrenia, dependência de drogas, transtorno de déficit de atenção e hiperatividade (TDAH) e doença de Parkinson (Genro et al. 2010 ; Howes & Kapur 2009 ; Melis et al. 2005 ; Oades et al. 2005 ; Piazza & Le Moal 1996). Além disso, neurotransmissores clássicos como dopamina e noradrenalina são responsáveis por controlar uma variedade de funções, incluindo a locomoção, função autonômica, secreção hormonal e os comportamentos complexos que estão associados com afeto, emoção e recompensa (Torres et al, 2003).

Em resposta ao estresse, o CRF regula a atividade do eixo HHPA através da ativação do receptor CRF1 (Davis, 1992; Merali et al., 2004; Muller , 2003). Adicionalmente à liberação de CRF no sangue, os neurônios do PVN apresentam projeções para outros locais do sistema nervoso central como BNST, CeA e VTA, resultando em uma variedade de respostas nestas regiões cerebrais (para revisão ver Corominas et al., 2010).

Além de regular a atividade do eixo HHPA, em resposta a estressores, a liberação de CRF desencadeia mudanças em outros sistemas de neurotransmissores, tais como a serotonina (5-HT) (Millan, 2005; Holsboer, 2003; Nestler et al., 2002; Leonard, 2005; Holmes et al, 2003).

Um substancial corpo de evidências na literatura tem implicado o sistema 5-HT na modulação do comportamento de medo e ansiedade (Graeff et al, 1996;

Handley et al 1993; Handley, 1995; Lowry et al, 2005; 2008). Os receptores de 5-HT têm sido classificados em sete famílias distintas (5-HT 1-7) que medeiam ações excitatórias e inibitórias quando ativados por 5-HT. Hammack et al, 2010 injetaram antagonistas seletivos do receptor 5-HT1A no BNST e observaram aumento dose-dependente no comportamento de ansiedade, isto é, um comportamento ansiolítico. Estes mesmos autores verificaram que a ativação de 5-HT_{2A}, 5-HT_{2c} e ou 5-HT_7 é ansiogênica.

O sistema 5-HT é suscetível ao estresse e ao cortisol. O estresse agudo causa liberação de 5-HT pelas células do núcleo da rafe, mas o estresse em longo prazo pode depletar estes estoques (Fontenot et al., 1995). Esta depleção pode ser permanente: em macacos que foram estressados por 14 meses e mantidos em recuperação por mais 14 meses as concentrações de 5-HT no córtex pré-frontal ventral nunca retornou aos valores observados antes do estresse (Fontenot et al., 1995). O estresse pode afetar as células da rafe através dos receptores de glicocorticóides, os quais estão presentes em células serotonérgicas (Laaris et al., 1995) e afetam o tipo e a quantidade de proteínas produzidas por estas células. No BNST GR (Kream et al, 1983) e MR (para revisão ver Gomez-Sanchez, 2011) também estão implicados no comportamento ansiogênico e ansiolítico, respectivamente.

Além disso, o fator neurotrófico derivado do cérebro (BDNF), uma neurotrofina extremamente responsiva ao estresse, a qual exerce papel central no desenvolvimento, na plasticidade, na fisiologia e nas doenças do sistema nervoso podendo estar implicado nos comportamentos de medo e ansiedade (Hammack et al, 2010).

2. JUSTIFICATIVA E OBJETIVOS

Tendo em vista a fundamentação apresentada acima o desenvolvimento do presente projeto se **JUSTIFICA** diante dos seguintes fatores:

- A prevalente desnutrição materno-infantil, com evidentes repercussões sobre a saúde e a necessidade de desenvolvimento de políticas de saúde publica que minimizem seus efeitos;
- A extrema sensibilidade do SNC à programação pré-natal;
- A programação fetal como fator de risco para o desenvolvimento de desordens psicológicas;
- A comprovada implicação da programação fetal em alteração do eixo HHPA podendo ocasionar, no adulto, exposição aumentada cronicamente a GC e/ou exacerbação na resposta ao estresse;
- O aumento da exposição fetal a corticosteroides em modelos de programação fetal;
- A já estabelecida ação moduladora neural dos corticosteroides podendo ocasionar alterações estruturais tanto no volume quanto na estrutura fina (por exemplo: número de sinapses e morfologia dendrítica) de neurônios;
- O BNST e a Amígdala medeiam respostas a estressores e o comportamento de ansiedade e medo estando implicados em distúrbios psicológicos;
- A exposição materna a restrição proteica gestacional leva, na idade adulta, a importantes mudanças neuroquímicas e morfológicas no BNST tornando estes animais mais ansiosos.
- A inexistência de trabalhos avaliando no inicio da vida a amígdala e o BNST neste modelo;

A ocorrência de desenvolvimento neural intenso entre o 7º e 14º dia de vida em ratos;

Foram **OBJETIVOS** do presente projeto estudar em ratos machos submetidos à restrição proteica *in útero*, comparativamente aos seus controles, no 7º e 14º dia de vida, a concentração de corticosterona basal e na amigdala e no BNST:

- O volume e número de neurônios e outros tipos celulares.
- A estrutura dendritica de neurônios;

- A concentração de MR, GR, CRF, CRF1, receptores de 5-OH-Triptamina (5HT1A e 5HT2A) e BDNF;
- A concentração de catecolaminas e serotonina;

3. MATERIAL E MÉTODOS

A primeira parte do estudo foi realizada na Universidade do Minho, Portugal durante o estágio de doutorado sanduiche.

3.1 Primeira parte

Os experimentos foram realizados nos laboratórios do Instituto de Ciências da Vida (ICVS), na Escola de Ciências da Saúde da Universidade do Minho, Braga, Portugal. Todo o estudo foi aprovado pela Direção Geral de Alimentação e Veterinária (DGAV, número 023432/2013-08-30).

Ratos Wistar Hannover machos e fêmeas foram obtidos do *Charles Rivers Laboratories* (Barcelona, Espanha) com nove semanas de vida. Após período de quarentena foram realocados no biotério do ICVS.

Os animais permaneceram com água e ração padrão para roedores *ad libitum*. Os mesmos foram mantidos no biotério com temperatura e umidade controlada, com sistema de luz 12h/12h.

Após o período de adaptação às condições ambientais de biotério, os animais foram submetidos ao acasalamento em sistema de harém durante todo o período noturno a partir da décima segunda semana de vida e, após constatação da presença de espermatozoides no lavado vaginal foram consideradas prenhas.

Então, a partir deste momento foram separadas individualmente de forma aleatoria em dois grupos experimentais. Um grupo passou a ser alimentado com ração normoproteica contendo 17% de caseína (n=20) sendo denominado grupo NP (*normal protein*). O outro grupo recebeu ração hipoproteica com 6% de

caseína (n=20) denominado grupo LP (*low protein*). Ambos os grupos receberam água e ração *ad libitum*. A ração (Tabela 1) foi produzida pela Ultragene (Equipamento para Experimentação e Investigação Cientifica Laboratorial de Portugal).

Tabela 1: Formulação da ração utilizada (g/kg).

INGREDIENTES	NORMOPROTEICA 17% (NP)	HIPOPROTEICA 6% (LP)
Amido de milho	410,10g	484,80g
Caseína	188,90g	66,70g
Amido dextrinizado	130,50g	159,00g
Sacarose	100,00g	121,00g
Óleo de soja	70,00g	70,00g
Celulose microcristalina	50,00g	50,00g
Mix mineral AIN 93	35,00g	35,00g
Mix vit AIN 93	10,00g	10,00g
L cistina	3,0g	3,0g
Bitartarato de colina	2,5g	2,5g
BHT	0,014g	0,014g

Semanalmente foi verificado o peso dos animais bem como seu consumo alimentar.

Ao nascimento as dietas foram retiradas e retornou-se a dieta padrão para roedores e água *ad libitum*.

Neste momento também foi mensurada a distância ano-genital (DAG) para verificar o sexo dos filhotes. Os animais com DAG inferior a 2 mm foram

consideradas fêmeas e aqueles com distancia superior a 2 mm machos. O peso ao nascer de cada filhote também foi verificado.

A ninhada foi reduzida a, no máximo, 8 filhotes por progenitora a fim de que todos os animais tivessem a mesma oferta alimentar e cuidado materno.

No sétimo e décimo quarto dia de vida pós-natal os grupos foram divididos da seguinte forma:

- ✓ *Normal Protein* sete dias (NP7D)
- ✓ *Normal Protein* quatorze dias (NP14D)
- ✓ Low Protein sete dias (LP7D)
- ✓ *Low protein* quatorze dias (LP14D)

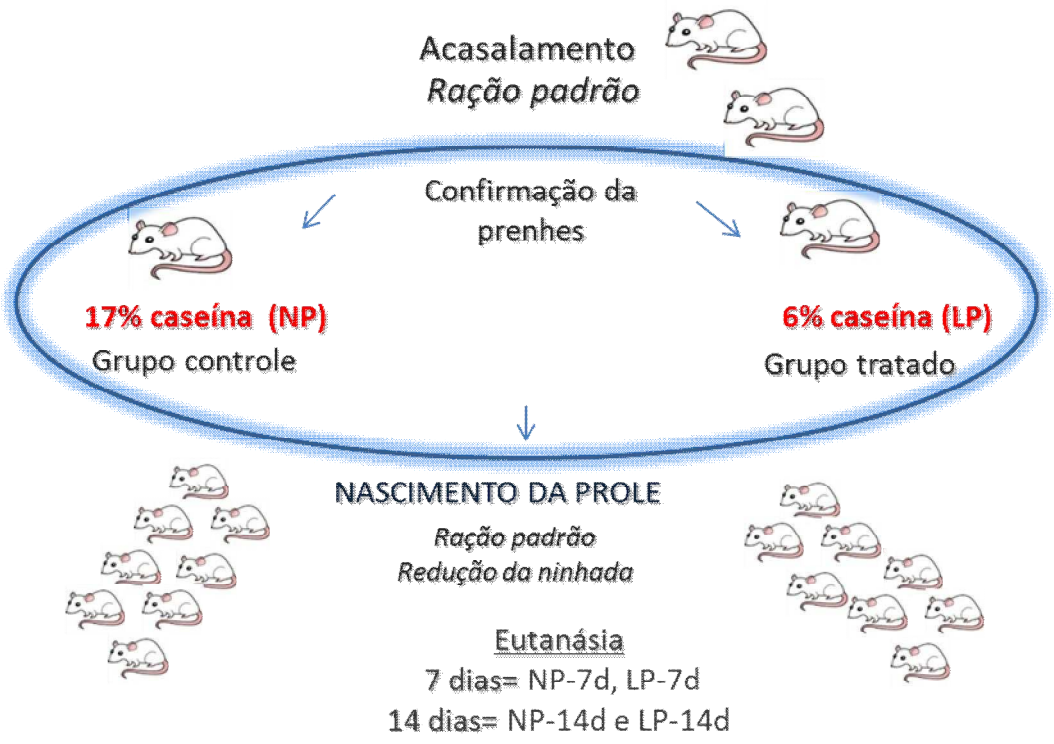

Figura 1. Desenho experimental

3.1.1 Reconstrução neuronal 3D

No 7º e 14º dia pós-natal, os animais (n= 4 NP e 4 LP de 4 diferentes progenitoras) foram perfundidos com solução salina 0,9%, sob anestesia profunda, com ketamina (75mg/kg) e xilasina (10mg/kg), e processada de acordo com o protocolo descrito por Gibb & Kolb (1998). Os cérebros foram removidos e imersos em solução de Golgi-Cox (solução 1:1 de dicromato de potássio 5% e cloreto de mercúrio 5% diluído 4: 10 com cromato de potássio 5% - Glaser & Van Der Loos, 1981) durante 14 dias; os cérebros foram então transferidos para uma solução de sacarose 30% (mínimo 3 dias), antes de serem cortados em um vibratomo. Secções coronais (200 µm de espessura) foram coletadas em sacarose 6% e secas em lâminas. Posteriormente alcalinizados em amônia 18,7%, em Dektol (Kodak), fixados em Kodak Rapid Fix (preparada conforme instruções da embalagem com omissão da solução B), desidratados em serie crescente de álcool, diafanizadas em xilol e montadas.

Para cada neurônio selecionado, todos os ramos da árvore dendrítica foram reconstruídos em ampliação de 600x usando um microscópio motorizado (Axioplan 2, Carl Zeiss, Alemanha), com objetivas de imersão, e ligado a uma câmara (DXC-390, Sony Corporation, Tóquio, Japão) e *software* Neurolucida (Microbrightfield, VT). A análise 3D dos neurônios reconstruídos foi realizada utilizando *software* Neurolucida (Microbrightfield). Foram comparadas as mudanças globais, o comprimento total das árvores e número de ramificações dendríticas entre os grupos, (Uylings & Van Pelt 2002). Para avaliar as diferenças na organização dendrítica, uma versão em 3D da análise de Sholl (Sholl 1956; Uylings & Van Pelt 2002) foi realizada. Para isso, contamos o número de intersecções com os dendritos de esferas concêntricas posicionados em intervalos radiais de 10 µm para o BNST e 20 µm para Amígdala, também foi avaliado o comprimento da árvore dendrítica localizada entre duas esferas consecutivas.

Neurônios completos do BNST e da Amígdala foram selecionados para análise de sholl.

3.1.2 Estereologia

Para estimativa do volume e número total de células utilizamos a estereologia. Então, no 7º e 14º dia pós-natal, os animais (n= 4 NP e 4 LP de 4 diferentes progenitoras) foram anestesiados com ketamina (75mg/kg) e xilasina (10mg/kg), e então perfundidos com solução salina 0,9% e paraformolaldeído (PFA) a 4%. Os cérebros foram removidos e imersos em PFA por mais 2 semanas e desidratados em uma bateria com concentração crescente de álcool, permanecendo em álcool 100% por 4 dias, sendo este trocado 2 vezes por dia. Após este período o material foi incluído em Tecnovit 7100 (Heraeus Kulzer, Gmbh). Secções de 30µm foram obtidas utilizando navalha de tungstênio, coletando apenas os cortes pares que foram corados com Giensa 20%. As estimativas de volume e número de células foram obtidas utilizando *software* Estereo Investigator ® (MicroBrightField, Williston, VT, EUA) e um microscópio motorizado (Axioplan 2, Carl Zeiss, Hamburgo, Alemanha) acoplado a uma câmara (DXC- 390, Sony Corporation, Tóquio, Japão). O princípio de Cavalieri foi usado para avaliar o volume de cada região. O número médio de células foi estimado utilizando método óptico. Coeficientes de erro foram calculados de acordo com as fórmulas publicadas anteriormente para números celulares e para volume estimado (Oliveira et al, 2012).

Foram analisados o volume e número médio de células do BNST anterodorsal e anteroventral.

3.1.3 Western Blotting

A expressão proteica foi avaliada pela técnica de *western blotting* (WB). Animais (n= 10 NP e 10 LP de 5 diferentes progenitoras) com 7 e 14 dias de vida pós-natal foram decapitados e seus crânios foram rapidamente congelados em nitrogênio líquido a fim de evitar degradação durante a macrodissecção. O BNST

e Amigdala foram cuidadosamente dissecados em uma placa de gelo e armazenados em *eppendorfs* em freezer -80ºC.

Extração de proteinas: O tecido foi homegeneizado em tampão de extração RIPA (Radio Immuno Preciptation Assay Buffer) com seringa 23G ou homegeneizador e, em seguida, adicionamos 10% de triton x 100 e 10% de SDS e incubamos em gelo por 1h.

As amostras foram centrifugadas por 10 minutos a 13.000 rpm (rotações por minuto) a 4ºc e foi coletado o sobrenadante.

Quantificação de proteínas: As proteínas totais foram quantificadas utilizando-se o método de Bradford e a leitura foi feita a 595nm.

As amostras foram diluidas em tampão de Laemmli, aquecido a 95°C por 5 minutos e a quantidade correspondente de proteína foi aplicados em gel SDS-PAGE e colocado em aparelho de eletroforese Bio-Rad (Mini-Protean, Bio-Rad). A eletroforese das proteínas no gel foi feita a 120V. Depois da separação eletroforética as proteínas foram transferidas para membrana de nitrocelulose e incubadas com anticorpo primário a 4°C durante a noite.

Posteriormente, as membranas foram lavadas com solução basal e incubadas com anticorpo secundário específico por 2 horas em temperatura ambiente. As bandas imunoreativas foram detectadas utilizando-se solução quimioluminescente. As imagens foram obtidas em fotodocumetador ChemiDoc XRS system (Biorad; 170870) e a intensidade das bandas quantificada por densidade ótica no *software* TINA. Os valores da densidade obtidos foram utilizados para análise estatística.

ANTICORPOS UTILIZADOS:

- ✓ GR (H-300- Santa Cruz 8992) anti-rabbit → 1:300
- ✓ MR (H-300- Santa Cruz) anti-rabbit → 1:300
- ✓ BDNF (Abcam; ab46176) anti-rabbit →1:4.000
- ✓ 5HT1A (ab101914) anti-goat → 1:1.000
- ✓ 5HT2A (ab160228) ani-rabbit → 1:1.000
- ✓ CRF (S-19- Snata Cruz 1761) anti-goat → 1:500
- ✓ CRF1 (ab 59023) anti-goat → 1:500
- ✓ Alfa tubulina (DSHB; AA4.3) anti-mouse→ 1:200
- ✓ Anti-goat (Santa Cruz biotechnology; sc-2020) →1:7.500
- ✓ Anti-rabbit (BioRad; 170-6515) →1:10.000
- ✓ Anti-mouse (BioRad 1721011) →1:10.000

3.1.4 Dosagem de neurotransmissores por HPLC

A concentração de catecolaminas e serotonina foram analisadas por cromatografia líquida de alto desempenho, combinada com detecção eletroquímica (HPLC / CE) usando um instrumento Gilson (Gilson, Middleton, WI, EUA), equipado com uma coluna analítica (Supleco 'Supelcosil LC-18, 3 mM, Bellefonte, PA, EUA; taxa de fluxo: 1,0 ml • min 1) (n= 6 NP e 6 LP de 5 diferentes progenitoras).

- ✓ *PREPARO DAS AMOSTRAS*

Foi adicionado 200ul de ácido perclorico (0,2) em cada amostra e, após 30 minutos de repouso em gelo, foram sonicadas e centrifugadas por 10 minutos a 13.000rpm a 4ºC.

O sobrenadante foi recolhido para um eppendorf com filtro e novamente centrifugado por 8 minutos a 10.000rpm a 4ºC e o pellet armazenado.

O sobrenadante resultante foi filtrado através de uma coluna de HPLC Spin-X (Costar, Lowell, MA, EUA) para remover os detritos e alíquotas de 150µl foram injetadas no sistema de HPLC, usando uma fase móvel de fosfato de potássio 0,7 M aquoso (pH 3,0) em 10% de metanol, ácido 1-heptano (222mgl 1) e Na-EDTA (40mgl 1). Uma curva padrão utilizando concentrações conhecidas de todas as catecolaminas foi corrida a cada dia.

A dosagem de proteina total foi feita a partir do pellet armazenado utilizando o método de Bradford a fim de normalizar a leitura realizada em HPLC.

Tabela 2: Padrões utilizados

NEUROTRANSMISSORES	REFERENCIAS
Serotonina	Sigma H-7752 1g 4ºC
5-HIAA	Sigma H-8876 500MG -20ºC
Dopamina	Sigma H-8502 5G 25ºC
DOPAC	Sigma D-9128 1G 25ºC
HVA	Sigma H-1252 250MG 25ºC
Epinefrina	Sigma E-4375 1g 4ºC
Norepinefrina	Sigma 74460 25ºC

3.2 Segunda parte.

Realizada na Universidade Estadual de Campinas (Unicamp)

Todo o estudo foi feito de acordo com os princípios éticos na Experimentação animal adotado pelo Colégio brasileiro de Experimentação Animal (COBEA) e foi aprovado pela Comissão de Ética na Experimentação Animal (CEEA) protocolo 3908-1 da Universidade Estadual de Campinas. Os estudos foram realizados no Laboratório de Metabolismo Hidrossalino no Núcleo de Medicina e Cirurgia Experimental da Unicamp.

Os animais foram obtidos no Centro Multidisciplinar para Investigações Biológicas da Unicamp (CEMIB – Unicamp). Foi utilizando o mesmo desenho experimental descrito anteriormente.

3.2.1 Corticosterona basal

Amostras de sangue foram coletadas de animais de 7 e 14 dias de vida (n= 14 NP e 15 LP animais de 5 progenitoras diferentes), no momento da eutanásia por decapitação, entre 8 e 9h. Após a coleta o sangue foi centrifugado 10 minutos a 13.000 rpm, o sobrenadante foi retirado e rapidamente estocado em freezer -80ºC.

A dosagem foi realizada pelo método de radioimunoensaio (RIA) usando um kit comercial de corticosterona da R&D Systems™ a biotechne brand seguindo instruções do fabricante:

- ✓ *PRÉ-TRATAMENTO DA AMOSTRA*

O pré-tratamento remove potenciais interferências de proteínas ligadas à proteína e de corticosterona ligada à proteína. As amostras foram analisadas dentro de 8h após o pré-tratamento.

Foi então adicionado 150µl de amostra e 150µl de pré-tratamento, agitado cuidadosamente e deixado em repouso por 15 minutos em temperatura ambiente. Após este período o material foi centrifugado por 4 minutos em centrifuga

previamente resfriada a 4ºC a 12.000 rpm e coletado o sobrenadante. O fator de diluição da curva padrão foi 2.

- ✓ *DOSAGEM EM MICROPLACA*

Em seguida ao pré-tratamento foi adicionado 50µl de anticorpo 1º anti corticosterona à microplaca excluindo o controle negativo. A placa foi coberta com película adesiva e incubada por 1h em temperatura ambiente sob agitação.

A seguir, após 4 lavagens consecutivas dos pocinhos foi adicionado 100µl de tratamento F, 50µl de amostra ou amostra padrão em duplicata e 50µl do conjugado de corticosterona em todos os pocinhos. A placa foi novamente coberta com película adesiva e incubada em temperatura ambiente por 2h sob agitação. Após mais 4 lavagens foi adicionado 200µl solução substrato a todos os pocinho e a placa foi incubada por 30 minutos em temperatura ambiente na bancada protegida da luz.

Adicionamos 100µl de solução *stop* em todos os pocinhos e determinamos a densidade ótica a 450nm e a 540nm.

- ✓ *ANALISE*

O Valor da leitura em 540nm foi descontado da leitura em 450nm para corrigir imperfeições ópticas da placa. A seguir foi feita a média de todas as medidas e descontado o valor do branco. A partir das leituras das amostras padrão foi construída a curva padrão e equação da curva que foi utilizada para calcular a concentração de corticosterona corrigida pelo fator de diluição.

A dose mínima detectada pelo kit foi de 0,028ng/ml.

3.2.2 Fracionamento isotrópico

Para estimar o número total de células e de neurônios aplicamos a técnica de fracionamento isotrópico (Herculano-Housel & Lent, 2005).

Os animais foram anestesiados no 7º ou 14º dia de vida (n= 7 NP e 7 LP animais de 3 progenitores diferentes) com ketamina (75mg/kg) e xilasina (10mg/kg) e perfundidos com solução salina 0,9% e paraformoldeído (PFA) a 4%. Depois os cérebros foram removidos e o BNST e a amigdala foram dissecados, pesados e imersos em PFA por 48 horas, em agitação e desidratados em uma bateria crescente de álcool, permanecendo em álcool 100%.

Cada amostra foi homogeneizada em um vidro homogeneizador com 3ml de solução de dissociação (40 Mm de citato de sódio e 1% de Triton X-100) e transferidos com pipetas de vidro para tubos de centrifugação graduados.

Após esta etapa homogeneizou-se manualmente 20 vezes a solução por tombamento, imediatamente uma alíquota de 1ml foi retirada e centrifugada durante 5' a 6000 rpm. O sobrenadante foi descartado e o pellet foi suspendido para 1ml com PBS sendo que esta lavagem (processo de centrifugação e re-suspenção do pellet) foi realizada 3x.

Após a última lavagem o pellet foi suspendido e incubado a temperatura ambiente por 24h com anti-NeuN igG (1:200 EM pbs; Chemicon, Temeluca, CA). No dia seguinte a alíquota foi lavada 3x e os núcleos foram incubados com anticorpo secundário, Goat Anti-Mouse conjugado a CY3 (1:200 em 40% PBS, 10g (normal Goat Serum e 50% DAPI) por 2h, sendo então lavados e suspendidos em PBS para contagem dos núcleos de neurônios em microscópio de fluorescência.

Após homogeneização mecânica uma alíquota de 10µl foi pipetada na câmara de Neubauer, aguardou-se por 5' e então fez-se a leitura, contando-se primeiramente os núcleos marcados com DAPI. Em realizou-se a contagem dos núcleos de neurônios NeuN+ pelo Cy3 .

Foi contado o número de células contidos em pelo menos 40µl e multiplicado pelo volume total da solução.

3.3 Análise Estatística

Quando foram avaliadas duas variáveis com dispersão paramétrica utilizando o teste t de Student. Quando avaliadas mais de duas variáveis com distribuição paramétrica foi realizada a Análise de Variância (ANOVA) com *post-hoc* de Bonferroni. Quando a distribuição dos dados não assumiu uma curva normal foi utilizado o teste estatístico de Kruskal-Wallis. O nível crítico definido foi de 5% ($p<0,05$). Os resultados estão expressos em Média ± Desvio Padrão da Média. *Outliers* foram identificados e eliminados da análise quando extrapolaram para mais ou para menos 2x o Desvio Padrão em relação à Média. O software para análise dos dados e confecção dos gráficos foi o GraphPad Prism v5.00

4. RESULTADOS

4.1 Artigo 1

EARLY MORPHOLOGICAL AND NEUROCHEMICAL CHANGES OF THE BED NUCLEUS OF STRIA TERMINALIS (BNST) IN GESTATIONAL PROTEIN-RESTRICTED OFFSPRING

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Abstract

The bed nucleus of the stria terminalis (BNST) is a structure involved in the stress-response and it is associated with the modulation of anxiety-like behavior and fear. Due to its plasticity, its behaviors can be affected by prenatal events as protein

restriction *in utero*, where the fetus is exposed to high maternal glucocorticoids levels, leading to a deregulation of HPA axis and exacerbated stress answer at adult life. Through severe maternal low protein diet with 6% casein we analyzed males offspring with 7 and 14 days post natal and found low birth weight until the 14th day. It was not significant effect on levels of basal corticosterone, however we found expressive changes on BNST morphology with reduction of length of dendrites, of volume of dorsal anterior region even as decreased at total numbers of cells, glial cells and BNST neurons. These changes may be involved with the expressed depletion of BDNF receptors that is essential for plasticity and neural survival. Beyond that, the gestational protein restriction led to reduction on GR and MR receptors expression. Considering that MR is involved in the basal activity maintenance, it's may be an indicative of HPA axis deregulation. Besides that, this axis has strong influence on the catecholaminergic and serotonergic activity, which was affected by the significant rise of norepinephrine and 5HIAA levels and reduction of dopamine turnover, showing that gestational protein restriction exerts a stressor effect on BNST trough the activation of these systems. Our results reveal that gestational protein restriction alters BNST morphology and neurochemistry in the beginning of postnatal life and may be involved with the anxiety processes in the adult life.

Keywords: BNST, Fetal programming, glucocorticoids, CRF, BDNF, Catecholamine.

Introduction

Maternal malnutrition is even nowadays one major public health issue worldwide (WHO 2013). Inadequate levels of nutrients during pregnancy can lead to permanent alterations in fetal tissues and to an increased risk of developing metabolic and endocrine dysfunctions and cardiovascular diseases in adulthood (Ashton, 2000). Researchers have shown that both nutritional and psychological stress during pregnancy can lead to a low weight at birth and are risk factors for the development of psychiatry disorders (Burd et al, 1999).

One of the mechanisms involved in the development of these disorders is the elevated exposure of the fetus to maternal glucocorticoids (GC) due to a low concentration and decreased activity of the placental enzyme 11beta-hydroxysteroid dehydrogenase (11β-HSD) type 2 (Benediktsson et al, 1993; Stewart et al, 1995; Langley-Evans et al, 1996; Langley-Evans, 1997). These prenatal alterations may lead to a chronic increase in GC as well as exacerbated response to stressful stimuli in adulthood (for review see Welberg & Seckl, 2001). There is a wide range of evidence that shows that the postnatal activity of the hypothalamic-pituitary-adrenal (HPA) axis, the main mechanism of stress-response in the brain, can be altered by prenatal events (Barker, 1995).

In particular, the brain is extremely sensitive to fetal programing and the intra-uterus exposure to GC can deeply influence the structure of the nervous system and synaptic formation (Ding et al, 2016). Among the different brain areas involved in the stress-response the bed nucleus of the stria terminalis (BNST) seems particularly sensitive to stress and early-life exposure to glucocorticoids (Oliveira et al, 2012) showing elevated plasticity (Walker et al, 2003; Pêgo et al, 2008). The BNST is positioned in a privileged location for the regulation of the stress-response and, in particular, the BNST has been strongly associated with the modulation of anxiety-like behavior. The sensitivity of the BNST to stress can be, at least in part, explained by the presence of corticosteroid receptors in this brain region. In fact, GC gluco- (GR) (Kream et al, 1983) and mineralocorticoid receptors (MR) (for review Gomez-Sanchez, 2011) within the BNST have been associated respectively with in anxiogenic and anxiolytic roles.

The BNST is rich in corticotrophin releasing factor (CRF) neurons. CRF is a neuropeptide that has long been associated with the stress-response and with anxiety-behavior. CRF can act through two different receptors: CRF_1 and CRF_2. While the role of CRF_2 is still not completely understood the activation CRF_1 has been strongly associated with an increase in anxiety (Davis, 1992; Merali et al, 2004; Muller, 2003). In the brain, in response to stress CRF is also released by neurons in the paraventricular nucleus of the hypothalamus (PVN) leading to the activation of the HPA axis. Considering the elevated connectivity of both BNST and

PVN neurons with projections to other areas in the central nervous system (CNS) such as the central nucleus of the amygdala (CeA), hippocampus, prefrontal cortex and ventral tegmental area (VTA), the release of CRF in these areas can influence the activity of many others regions (for review see Corominas et al, 2010).

As mentioned before in response to stressful stimuli CRF is able to regulate the activity of the HPA axis and this will also influence other neurotransmitter systems such as serotonin (5-HT) (Millan, 2005; Holsboer, 2003; Nestler et al, 2002; Leonard, 2005; Holmes et al, 2003). The serotonergic system is highly sensitive to both stress and corticosteroids; acute stress leads to the release of 5-HT by cells in the raphe nucleus but chronic stress can lead to the depletion of 5-HT in this area (Fontenot et al, 2005). Many other factors are also important for the stress response and among those the brain derived neurotropic factor (BDNF) seems to be extremely sensitive to stress. BDNF is a neurotropic factor that has a key role for physiology and pathology development in the CNS and for brain plasticity mechanisms and has been implicated in both fear and anxiety-behaviors (Hammack et al, 2009).

We found an impoverished dendritic arborization in BNST neurons, in parallel-enhanced anxiety-like behavior and elevated plasmatic corticosterone levels, in adult rats submitted to gestational protein restriction (submitted article). Thus, BNST is “programmed” by gestational protein restriction, resulting in fine structural changes and neurochemical adaptations of these brain regions that constitute potential underlying causes of the altered behavioral states.

In the current study, we aim to analyze the early effects of gestational protein restriction on BNST morphology and neurochemistry.

Materials and methods

Animals and treatments - The experiments were conducted on age-matched female offspring of sibling-mated Wistar Hannover rats (250-300g). The experiments were done in accordance with the general guidelines established by the Brazilian College of Animal Experimentation (COBEA) and approved by the

Institutional Ethics Committee (CEEA/UNICAMP #3908-1) and National Institutes of Health guidelines on animal care and experimentation and approved by Director General Veterinary (DGV; the Portuguese National Institute of Veterinary 023-432/08.30.2013). A part of our site colonies originated from the breeding stock supplied by Charles River Laboratories, Barcelona, Spain. Another part was originated from a breeding stock supplied by CEMIB/Unicamp, Campinas, SP, Brazil. The animals were housed in pairs under standard laboratory conditions (lights on from 8 a.m. to 8 p.m.) and had access to food and water *ad libitum* and followed up to 12 weeks of age. The rats were placed to mate and the day that sperm were seen in the vaginal smear was designated as day 1 of pregnancy. The dams were divided in two groups: one maintained on isocaloric standard rodent laboratory chow with normal protein content [NP] (17% protein) and other received a diet with low protein content [LP] (6% protein) *ad libitum* throughout the entire pregnancy. Food consumption was determined every day (subsequently normalized for body weight). All groups returned to the NP chow intake after delivery. On the day of birth the male pups were weighted and they were kept only 8 pups per female.

***Radioimmunoassay (RIA) method* -** Basal corticosterone was determined on blood samples that were collected from animals at postnatal day 7 and 14, between 8 and 9 am. After collection the blood was centrifuged (10-min at 13000 rpm) and the supernatant was quickly removed and stored in a freezer at -80°C. The dosage was performed by RIA using a commercial kit of R&D corticosterone SystemsTM Biotechne the brand following the manufacturer's instructions.

***Morphological analyses methods* -** At 7^{th} and 14^{th} postnatal day male rats from NP (n=27/per age from 10 different mothers) and LP (n=27/per age from 10 different mothers) groups were deeply anesthetized with a mixture of ketamine and xylasine (75 and 10mg/kg respectively, i.p.) and monitoring the corneal reflex controlled the level of anesthesia. For stereology and isotropic fractioning the rats were transcardially perfused with saline containing heparin (5%, for 15 min, under

constant pressure) followed by fixative solution: 0.1M phosphates buffer (PB; pH 7.4) containing 4% (w/v) paraformaldehyde. For 3D reconstruction we used only saline for perfusion.

Stereology - After perfusion the brains were removed and placed on the same fixative solution for 2 weeks, dehydrated and included in Tecnovit 7100 (Heraeus Kulzer. Gmbh). Coronal sections (30µm thick) were collected and stained with Giemsa 20%. The anterodorsal and anteroventral BNST volume and cell number were determined using the software Stereo Investigator (MicroBrightField. Williston VT USA) and a monotorized microscope (Axioplan 2 Carl Zeiss Hamburg Germany) attached to a camera (DXC- 390. Sony Corporation. Tokyo. Japan). Cavalier's principle was used to evaluate the volume of each region. Average cell number was estimated using the optical fractionator method. Coefficients of error were calculated based on previously published formulas for cell number and for volume estimates (Oliveira et al, 2012).

Isotropic fractionator method - For total cells and neuron quantification we used the technique described by Herculano-Houzel & Lent (2005). The brains of 14-day-old rats (3 NP and 3LP, from different mothers) were removed and BNST was dissected. A suspension of nuclei was obtained through mechanical dissociation in a standard solution (40mM sodium citrate and 1% Triton X-100), using a 40-ml glass Tenbroeck tissue homogenizer. After washed several times with dissociation solution and centrifuged (10-min at 4000 g) pelleted nuclei were suspended in phosphate-buffered saline (PBS) containing 1% 4'. 6-diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR, USA). DAPI-stained nuclei were counted under a fluorescence microscope at 400×objective. The nuclear density in the suspension was determined by averaging over at least eight samples the total number of cells in the original tissue was estimated by multiplying mean nuclear density by total suspension volume. Neuron number was estimated after immunohistochemistry using anti-NeuN antibody (1:300 in PBS; Chemicon Temecula, CA, USA) and CY3 goat anti-mouse secondary antibody (1:400 in 40%

PBS, 10% goat serum and 50% DAPI; Accurate Chemicals, Westbury, NY, USA) under the fluorescence microscope (Scabora et al., 2013). Total number of other BNST cells nuclei is calculated by subtracting the number of NeuN containing nuclei from the total number of nuclei.

Neuronal 3D reconstruction and dendritic tree analysis - Brain from 14 days old animals (4 NP and 4 LP from different mothers) were Golgi–Cox staining according to a published protocol (Gibb R & Kolb B, 1998). Briefly brains were removed and immersed in Golgi–Cox solution (Glaser & Van der Loos, 1981) for 14 days; brains were then transferred to a 30% sucrose solution (3-days) before being cut on a vibratome. Coronal sections (200 µm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia developed in Dektol (Kodak), fixed in Kodak Rapid Fix, dehydrated and cleared in xylene before being mounted and coverslipped. Slides were coded before morphometric analysis in both sets. For dendritic tree analyze the criteria used to select neurons for reconstruction were as follows: (i) full impregnation of the neurons along the entire length of the dendritic tree; (ii) dendrites without significant truncation of branches; (iii) relative isolation from neighboring impregnated neurons to avoid interference with the analysis; and (iv) no morphological changes attributable to incomplete dendritic impregnation of Golgi–Cox stain (Pêgo et al. 2008). Accordingly we chose neurons with bipolar conformation confined to the anteromedial area (BNSTam) for dendritic analysis using the following criteria: (i) presence of transverse anterior commissure; (ii) rostral location to the stria terminalis main bundle; and (iii) selection of neurons adjacent to the anterior commissure. These landmarks correspond to the rostral portion of the medial division described by McDonald (1983). For each selected neuron all branches of the dendritic tree were reconstructed at 600x magnification using a motorized microscope (Carl Zeiss Axioplan 2) attached to a camera (DXC-390; Sony Co, Japan) and Neurolucida software (Micro Bright Field, VT, USA). Three-dimensional analysis of the reconstructed neurons was performed using

NeuroExplorer software (MicroBrightField). For the dendritic analysis were reconstructed 91 neurons.

Neurochemical analyses methods - Male rats at 7 and 14 days were decapitated and the heads were snap-frozen in liquid nitrogen. The BNST was dissected and stored at -80°C until further analysis.

Western blot *(WB)* - The tissue was homogenized in extraction buffer RIPA (Radio Immune Precipitation Assay Buffer) and then 10% triton x 100 and 10% SDS were added to the homogenate. The tissue extracts were centrifuged (1300 *rpm* at 4°C for 40 min) and the supernatants used as a sample. Protein quantification was performed using the Bradford method. The samples were treated with a Laemmle buffer containing 100-mmol/l dithiothreitol (DTT) heated in a boiling water bath for 4 min and subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) in a Bio-Rad mini-gel apparatus (Mini-Protean Bio-Rad). Electrotransfer of proteins from the gel to the nitrocellulose membranes was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean). The non-specific protein binding to the nitrocellulose was reduced by preincubating the filter for 2 h at 22°C in a blocking buffer (BSA 5%, 10mmol/l Tris, 150mmol/l NaCl and 0.02% Tween 20). The nitrocellulose blots were incubated at 4°C overnight with antibodies against GR (H-300-Santa Cruz 8992), MR (H-300-Santa Cruz), BDNF (Abcam; ab46176), 5HT1A (ab101914), 5HT2A (ab160228), CRF (S-19- Santa Cruz 1761), CRF1 (ab 59023) and Alfa tubulin (DSHB; AA4.3) diluted in a blocking buffer (2.5% BSA, 10mmol/l Tris, 150mmol/l NaCl and 0.02% Tween 20). Immunoreactivity bands were detected using the enhanced chemiluminescence method (RPN 2108 ECL Western blotting analysis system; Amersham Biosciences) and were detected by a ChemiDoc XRS system (Biorad; 170870). The band intensities were quantitated by optical densitometry (*TINA* software) of the developed autoradiographs that were used at exposures in the linear range.

High performance liquid chromatography combined with electrochemical detection (HPLC/CE) - The level of catecholamine and serotonin in the BNST was assessed by HPLC/CE using a Gilson instrument (Golson, Middleton, WI, USA) equipped with an analytical column (Supleco Supelcosil LC-18, 3mM, Bellefonte, PA, USA, flow rate: 1.0ml/min). Percloric acid (200ul) was added to each sample that were incubated for 30 min in ice and then sonicated and centrifuged (13000rpm, 10min, 4ºC). Supernatant was collected to a 1.5ml tube and then centrifuged for 8min (10000rpm, 4ºC) and the resulting pellet was saved for later use. The supernatant was then filtered through an HPLC Spin-X column (Costar. Lowell, MA, USA) to remove debris and 150ul aliquots were injected into the HPLC system using a mobile phase of 0.7 M aqueous potassium phosphate (pH 3.0) in 10% methanol 1-heptanesulfonic acid (222mg l^{-1}) and Na-EDTA (40mg l^{-1}). A standard curve (Sigma H-7752) with known concentrations of each catecholamine was run each day for 5HIAA (Sigma H-8876), Dopamine (Sigma H-8502), DOPAC (Sigma D-9128), HVA (Sigma H-1252), Epinephrine (Sigma E-4375) and Norepinephrine (Sigma 74460).

Data presentation and statistical analysis - All data is reported as mean ± SD. Data obtained over time was analyzed using appropriate two-way analysis of variance (two-way ANOVA). Post hoc comparisons between selected means were made by Bonferroni's contrast test when initial two-way ANOVA indicated statistical differences between experimental groups. Comparison involving only two samples of independent observations tends within or between groups was made using a Student's test. The band intensities were quantitated by optical densitometry (*software TINA*). The Tukey–Kramer test for multiple comparisons was used for analysis. The level of significance was set at $P \leq 0.05$.

RESULTS

We have not observed differences in food intake and body weight when compared NP and LP dams during pregnancy. However, the male pups body weight was

significantly reduced from birth to the 14th day of life in LP offspring when compared to NP male pups (Fig 1).

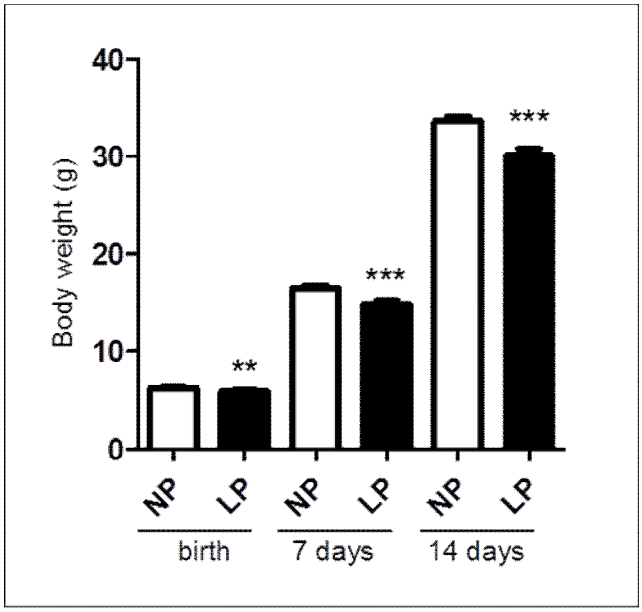


Fig. 1. Body weight at birth and with 7 and 14 days of life (**p =0.002, ***$p \leq 0.0001$).

Corticosterone serum levels

The corticosterone basal levels were not affected by gestational diet treatment in either postnatal day 7 (NP: 1.1 ± 0.02, n=7 *vs.* LP: 1.1 ± 0.03, n=7, p=0.8) or 14 (NP: 1.36 ± 0.01, n=7 *vs.* LP: 1.37 ± 0.01, n=8, p = 0.5) in both experimental groups.

Stereology

The volume of anterodorsal and anteroventral BNST divisions was not altered in 7 day-old offspring. Otherwise, 14 day-old LP rats showed 13% decreased anterodorsal BNST division volume when compared with NP offspring (Fig. 2). The total cells number was not altered in both divisions and time points.

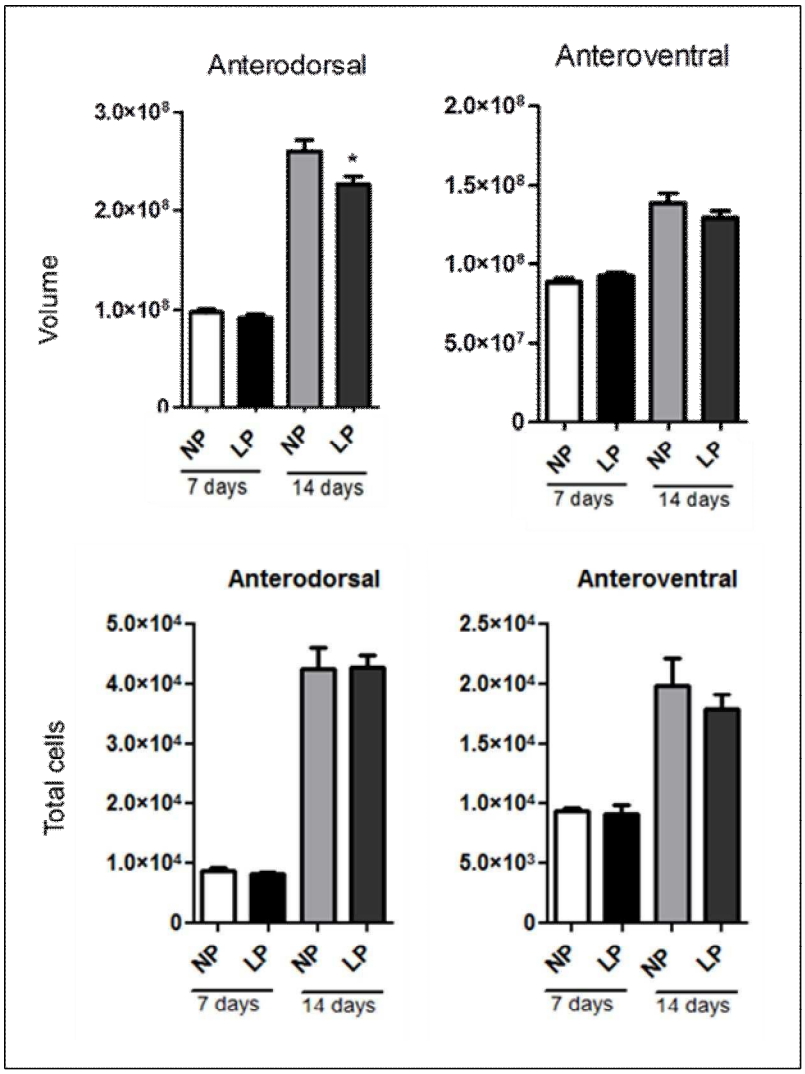


Fig 2. Volume and cells number in the anterodorsal and anteroventral divisions of the BNST (*p= 0.04).

BNST total cells and neurons quantification

On the 14th postnatal day the BNST total cells number was 61% reduced in LP when compared to that found in age-matched NP group (p = 0.03). The calculus of BNST neurons and non-neurons cells showed a reduction of 57,6% in the number of neurons (p=0.04) and 66% in other cells (p=0.02) (Fig.3).

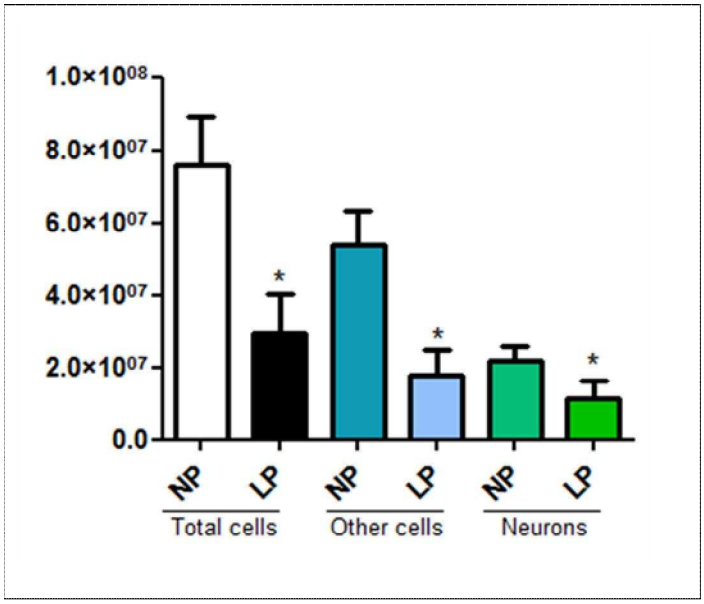


Fig 3. Number of neurons and other cells in the BNST of 14 days old animals.

Dendritic tree analysis

We found significant reduction of 13,5% in dendritic length of BNST neurons from 14 days-old LP rats when compared with age-matched NP (p=0.04). Sholl analysis revealed a reduction of 140µm at the length of dendrites and 13% reduced ramifications in the neurons of the LP offspring compared to NP rats (Fig.4).

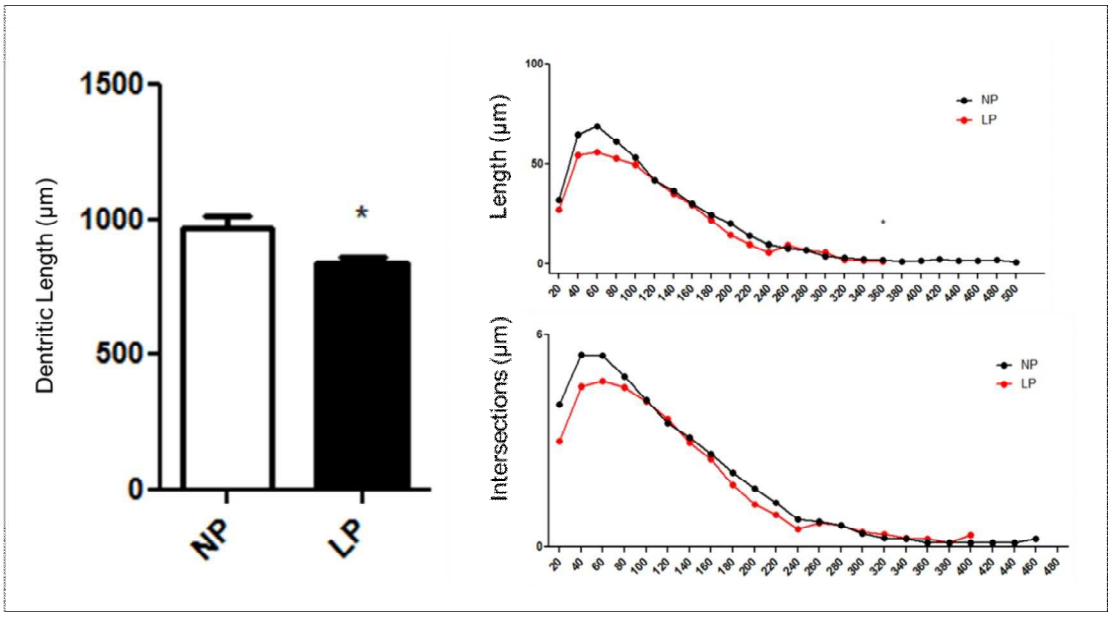
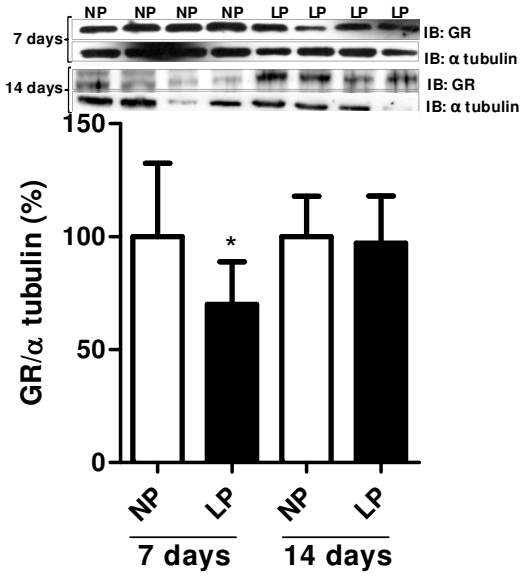


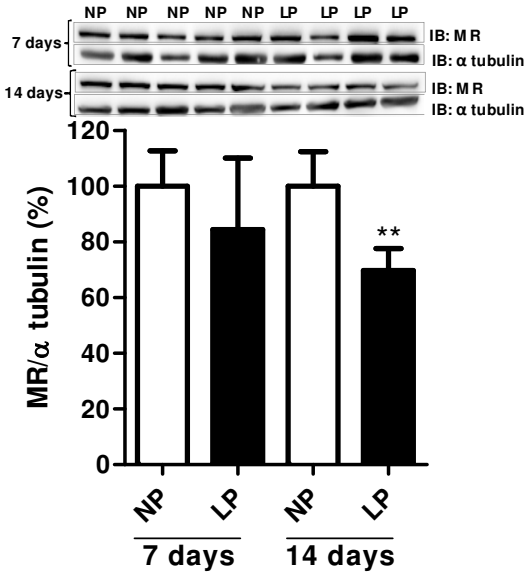
Fig. 4. The gestational protein restriction led to a decrease in the length of BNST dendrites after 14 days of life. By Sholl analysis we can observe de distance of dendrites from pericaria and dendrites bifurcations in LP and NP groups.

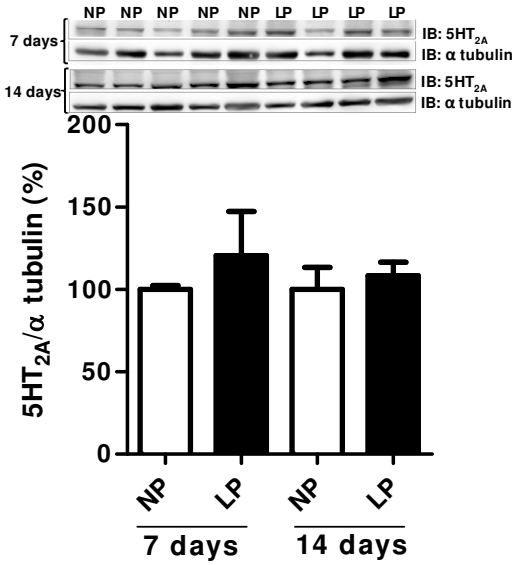
Western blot analysis

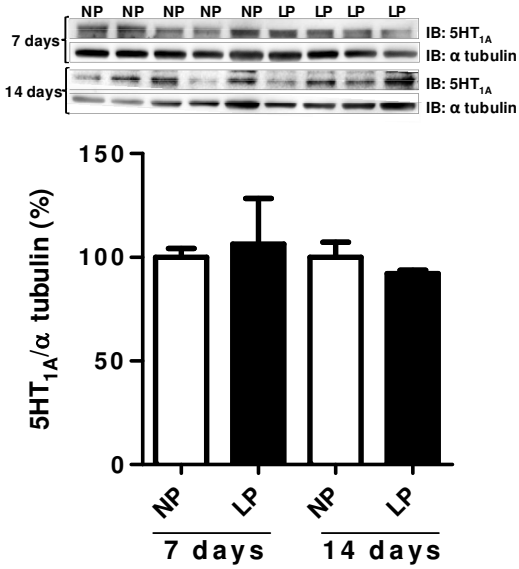
Western blot comparative analysis in the BNST of 7 day-old offspring from NP and LP groups revealed that the levels of GR and BDNF from LP group were significantly reduced in 31% (n=8, *p*=0.03) and 22% (n=5, *p*=0.01), respectively. The expression of CRF1 was significantly enhanced (7%, n=4, *p*=0.05). In BNST was observed a reduction of MR (16%, n=4, *p*=0.16) and CRF expression (19%, n=4, *p*=0.22) associated with an enhance of 5HT1A (6%, n=4, *p*=0.29) and 5HT2A (20%, n=4, *p*=0.09) in LP offspring when compared to NP age-matched group, although expressions has not achieved statistical significance.

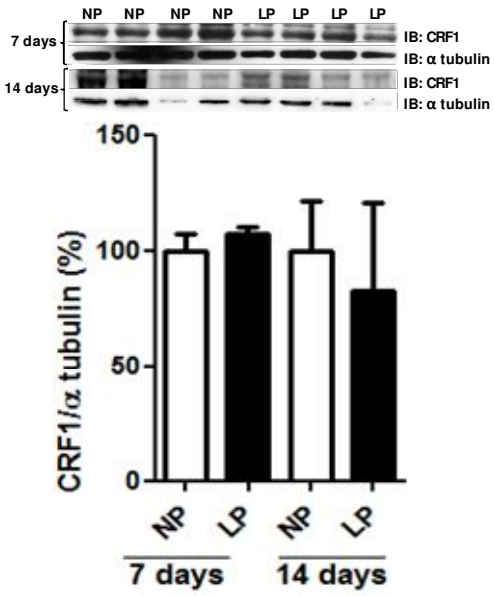
The comparative analysis in the 14 d-old offspring showed that the levels of MR and 5HT1A from LP group were significantly reduced in 31% (n=9, *p*=0.003) and 8% (n=4, *p*=0.03), respectively. Although do not statistically significant, the present study shows a reduced GR (3%, n=9, *p*=0.38) and CRF1 (18%, n=9, *p*=0.14) expressions and, enhanced 5HT2A (8%, n=8, *p*=0.77) and BDNF (29% (n=5, *p*=0.15) in LP when compared to NP age-matched group. (Fig.5).

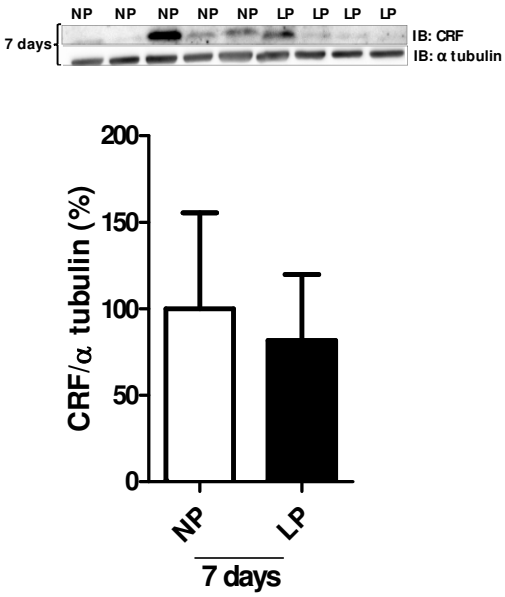

NP NP NP NP LP LP LP LP
7 days
IB: GR
IB: α tubulin
14 days
IB: GR
IB: α tubulin
GR/α tubulin (%)
150
100
50
0
*
NP LP NP LP
7 days
14 days


NP NP NP NP NP LP LP LP LP
7 days
IB: MR
IB: α tubulin
14 days
IB: MR
IB: α tubulin
MR/α tubulin (%)
120
100
80
60
40
20
0
**
NP LP NP LP
7 days
14 days


NP NP NP NP NP LP LP LP LP
7 days
IB: 5HT2A
IB: α tubulin
14 days
IB: 5HT2A
IB: α tubulin
5HT2A/α tubulin (%)
200
150
100
50
0
NP LP NP LP
7 days
14 days


NP NP NP NP NP LP LP LP LP
7 days
IB: 5HT1A
IB: α tubulin
14 days
IB: 5HT1A
IB: α tubulin
5HT1A/α tubulin (%)
150
100
50
0
NP LP NP LP
7 days
14 days


NP NP NP NP LP LP LP LP
7 days
IB: CRF1
IB: α tubulin
14 days
IB: CRF1
IB: α tubulin
CRF1/α tubulin (%)
150
100
50
0
NP LP NP LP
7 days
14 days


NP NP NP NP NP LP LP LP LP
7 days
IB: CRF
IB: α tubulin
CRF/α tubulin (%)
200
150
100
50
0
NP LP
7 days

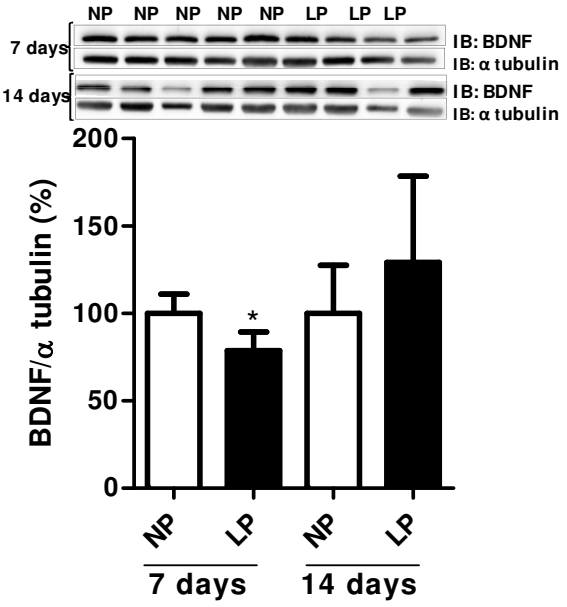


Fig 5. Effects of maternal protein restriction on expression of BNST proteins Protein levels for 5HT2A, 5HT1A, GR, MR, BDNF, CRF and CRF1 in the BNST of NP and LP animals at postnatal day 7 and 14. The results of scanning densitometry were expressed as relative to NP, assigning a value of 100% to the control rats. Columns and bars represent the mean ± SD. $^{*}P \leq 0.05$, NP versus LP.

High-Performance Liquid Chromatography (HPLC)

The HPLC analysis shows a significant increase in norepinephrine BNST concentration in 7 d-old and increased 5 HIAA BHST concentrations in 14 d-old LP offspring compared to NP age-matched group. Additionally, 14 day-old LP rats present a significant decreased of dopamine *turnover* when compared to control offspring (Table 1).

Table 1: Determination of the concentration of neurotransmitters by HPLC analysis of neurochemical or BNST: Values represent the mean ± standard deviation. Statistical comparisons were made with the Student t test and statistical significance for $p \leq 0.05$.

BNST						
	7 days			14 days		
	NP	LP	p	NP	LP	p
Norepinephrine	83.5 ± 38.7 N=4	244 ± 26.7 N=4	0.007**	123 ± 13 N=5	162.5 ± 22 N=3	0.07
Epinephrine	5± 0.9 N=4	5.5 ± 0.66 N=4	0.4	12± 1.4 N=5	13± 1.1 N=3	0.34
Dopamine	23.6 ± 6 N=3	22.4 ± 2.3 N=3	0.42	28± 5.5 N=5	40.6 ± 16N=3	0.204
DOPAC	14.9 ± 4 N=3	22.7 ± 4.6N=4	0.14	36± 4.431 N=5	21 ± 6 N=3	0.04*
5HIAA	6. ± 0.9 N=4	7± 0.9 N=4	0.21	8± 0.46 N=4	20 ± 2N=3	0.0006***
5HT	6.8 ± 0.87 N=4	7.7 ± 0.2 N=4	0.17	12± 1 N=3	12.7 ± 2 N=4	0.45
TURNOVER 5HT	1± 0.3 N=4	0.8 ± 0.03 N=3	0.31	1± 0.1 N=3	1.57 ± 0.36 N=3	0.1
TURNOVER DOPAMINE	1.14 ± 0.1 N=3	1.25 ± 0.1 N=4	0.27	1.47 ± 0.09 N=4	1.1 ± 0.07 N=4	0.01*

DISCUSSION

According to 2013 data from the WHO maternal malnutrition is still a worldwide public health issue and it is directly correlated with low weight at birth and to several health issues in adulthood. The concept of fetal programming defined by David Baker (Baker et al, 1989) establishes that insults occurring during gestation can lead to several diseases in adulthood such as metabolic and cardiovascular disorders. In the current study, we hypothesize an association between gestational protein restriction and BNST morphological and neurochemistry changes proposing that, the BNST is a CNS area in which permanent changes underlie, at least in part, the development of behavioral disorders in this experimental model. Nutritional undernutrition during critical periods of development may lead to several metabolic, morphological and neurochemical disorders. This study confirms previous studies of our laboratory (Mesquita et al, 2010; Lima et al, 2012; Scabora et al, 2013) showing a significant reduction of offspring birthweight in gestational protein-restricted group accompanied by renal, cardiac and neuronal dysfunctions when compared to NP offspring. These studies demonstrate that effect was

associated with a significant enhance in arterial blood pressure beyond the 7-week of age in LP offspring (Mesquita et al., 2010).

Otherwise, BNST cell quantification and stereology studies show a significant reduction of the total cells number, decreased ratio between neurons and non-neuron cells associated with reduced volume of the anterodorsal BNST division in 14 d-old LP offspring when compared to that found in age-matched NP group. Also, in the present study, Sholl analysis revealed a reduction of the length of dendrites and reduced ramifications in the neurons of the LP offspring. Several factors may be involved in fetal programming but the hypothesis more strongly considered involve a fetal high exposure to GC. Maternal undernutrition or protein restriction leads to increased and persistent fetal exposure to maternal GC, which promotes disruption of the HPA axis balance and consequently, faster fetal tissues and organs maturation (Drake, 2007). Recently, we and other authors have demonstrated that gestational protein malnutrition leads to CNS developmental changes particularly, by reducing dendritic arborization (Lopes et al, 2013; Kim Sung-Yon et al, 2013), the number of synaptic ends and, in the neural myelination (Lima & Voigt, 1999). Supporting the present data, we priory demonstrate that gestational protein restriction cause a significant reduction of the BNST dendritical length and in the anterodorsal BNST division volume in 16 wk-old protein-restricted offspring (submitted article). Since the BNST is highly plastic, studies have demonstrated that encephalic region during fetal development, is extremely vulnerable to environmental stresses and exposure to endogen and exogenous corticosterone high levels, which in turn lead to several morphological and functional disorders (for review see Hammack et al, 2010). In fact, exposure to unpredictable chronic psychological stress is associated with an increase in the volume and dendritic length in the BNST (Pêgo et al, 2008) and chronic immobilization paradigms increases dendritic arborization in BNST neurons (Vyas et al, 2002; 2003). The BNST is considered the main integrator nucleus of excitatory and inhibitory inputs that regulate the HPA axis (Forray & Gysling, 2004). Studies have shown that the gestational exposure to psychological stress (Weinstock et al, 1992) or by administration of stress hormones (Fameli et al,

1994) presents an increased corticosterone plasma level in the offspring. The activation of HPA axis promoted by stress stimulus ends with the release of corticosteroids by the adrenals (Ieraci et al, 2016). Corticosteroids are the main hormones for the maturation in the final days of gestation (Wood 2016) and the lack of balance in the expression of their receptors (MR and GR) can increase the vulnerability of the CNS to adverse effects (Sousa, et al 2008). In this work we observed a decrease in the expression of GR and MR both 7 and 14 postnatal days. Nevertheless the expression of MR was decreased in 28% in 14 days old animals. Endogenous GCs in basal conditions have higher affinity for MR (De Kloet et al, 1998) and the activation of MR seems to be involved with survival actions and is primarily involved in the maintenance of basal activity. Chronic stress can lead to the continuous activation of both receptors causing dendritic atrophy and deficits in synaptic plasticity (Sousa et al, 2008). Taking in account this results and once corticosteroids modulate structural alterations in the CNS that include changes in cellularity, structural volume and also, in synaptic and dendritic branching and morphology (Leão et al, 2007), we suppose that present finding may also be explained by this phenomenon.

Furthermore, the present work demonstrates by western blotting studies, a decreased expression of gluco- and mineralocorticoid receptors in 7 and 14-d old accompanied, by fall in BDNF and enhanced CRF1 receptor expression in the BNST of the 7-d old LP offspring when compared to NP control rats, despite of unchanged corticosterone plasma level. The basal levels of corticosterone may be used as an indication of higher or lower stress levels (Ventura-Silva et al, 2012). Several studies have demonstrated that the gestational exposure to stress (Weinstock et al, 1992) or the administration of stress hormones (Fameli et al, 1994) during gestation lead to an increase in the plasma concentration of corticosterone in the offspring. Prenatal stress is associated with alterations in the HPA axis in the offspring (for review see Charil et al, 2010). Previous studies from our lab (submitted article) have shown that animals that suffered gestational protein restriction have higher levels of plasmatic corticosterone at 16 weeks of age. In the current study, however, 7 and 14 d-old LP offspring compared to NP

group did not present any difference in the basal corticosterone levels. This results may be explained by well-characterized stress hyporresponsive period (SHRP) associated to decreased activity of HPA axis, that may last until the 14th postnatal-day (Sapolsky & Meane 1986). At this time, there is a reduced secretion of corticosterone, which remains low until the second postnatal week (Sapolsky & Meane 1986). The SHRP may assume as a protective mechanism ensuring low levels of glucocorticoids during early postnatal development (Mesquita et al, 2007). BDNF is strongly connected with the serotonergic system and both are involved with memory processes and mood (Van Donkelaar et al, 2009). The two systems may act together to regulate neuronal plasticity and survival of new neurons (Mattson, 2004). Our study shows a significant decrease in the expression of BDNF in 7-d old LP offspring. The current study shows a significant decrease in the neurons and non-nuron cells and in volume of anterodorsal portion of BNST of 14-d old LP offspring. We may state that this reduction may be associated with the decrease in the expression of BDNF since this factor acts directly in glial and neuronal progenitor cells (Rial, 2016). Also, sustained our study in this model, previous results have been showed that stress exposure decreases the level of BDNF in brain regions associated with depression (Duman, 2004; Barrientos et al, 2003).

In addition, the 14 d-old LP offspring BNST presents a reduced $5HT_{1A}$ receptor subtype levels, reciprocally accompanied, by increased $5HT_{2A}$ receptors compared to age-matched NP offspring. It is also known that different ways of exposure to stress can alter the serotonergic system which is involved in emotional behavior and anxiety disorders (Ressler & Nemeroff, 2000). Both chronic stress and treatment with anxiogenic drugs have been shown to activate a subset of serotonergic neurons in raphe nucleus that has main targets limbic areas such as the BNST (Grahn et al, 1999a; Lowry et al, 2000; Singewald et al, 2000). $5HT_{1A}$ and $5HT_{2A}$ are expressed widely throughout the central nervous system including neocortex, hippocampus, septum, amygdala, raphe nucleus, basal ganglia, thalamus and the olfactory tubercle. Especially high concentrations of these receptors on the apical dendrites of pyramidal cells in layer V of the cortex

may modulate cognitive processes, working memory and attention (Ciranna, 2006). The mammalian $5HT_{1A}$ and $5HT_{2A}$ are subtypes of the serotonin receptor G protein-coupled receptor (GPCR). 5-HT_{1A} receptor agonists are involved in neuromodulation of behavioral activity, learning and memory in rodents. Activation of central 5-HT_{1A} receptors triggers the inhibition of norepinephrine and enhances acetylcholine release, depending on species and areas of the brain. Also, 5-HT_{1A} receptor activation has been shown to increase dopamine release in the medial prefrontal cortex, striatum, and hippocampus, and may be useful for improving the symptoms of schizophrenia and Parkinson's disease. 5-HT_{1A} receptor agonists relieve the anxiety and depression meanly by synaptic serotonin increasing concentration. The $5HT_{2A}$ is the main excitatory receptor subtype among the GPCRs for serotonin (5-HT). Study has describes that overdensity of post-synaptic $5HT_{2A}$ receptor is involved in the pathogenesis of depression. Interestingly stressors that activate the BNST also activate central serotonergic systems (Dilts & Boadle-Biber, 1995. Grahn et al, 1999b. Takase al, 2004). Additionally, several studies have implicated the serotonergic system in the modulation of fear and anxiety-like behavior (Graeff et al, 1996; Handley et al, 1993; Handley, 1995; Lowry et al, 2005). In the present study, we may hypothesized that reduced BNST $5HT_{1A}$ and elevated $5HT_{2A}$ expression may be related to anxiogenic and fear behavior observed for us, in previous study (submitted article).

Additionally, the BNST of 7-d old LP rats presents an enhanced level of norepinephrine compared with the NP age-matched offspring. This phenomenon was accompanied by decreased BNST dopamine turnover and DOPAC level, a metabolite of the neurotransmitter dopamine in 14 day-old protein-restricted offspring. As mentioned above, it is widely known that catecholamine play an important role in the neurochemistry of the brain and are involved in a series of brain functions among them the response to fear and anxiety. Sympathetic nervous terminals as well as chromaffin cells in adrenals are the main sources of circulating catecholamines (Lymperopoulos et al, 2016). In the CNS catecholamines work as neurotransmitters in the synaptic cleft and are a crucial part of the maintenance of

homeostasis quickly responding to any stressor that threatens the homeostasis of the organism (Andreis, 2016; Riedemann et al, 2010).

Our findings show a significant increase in the BNST norepinephrine levels of as well as serotonin precursor (5HIAA) in 7-d old LP offspring. The BNST is a structure that receives projections from noradrenergic receptors from the brain stem and for this plays a series of responses to stress. Nociceptive stimuli or immobilization stress in rats cause an increase in the BNST norepinephrine suggesting that an aversive stimulus activates noradrenergic projections to the BNST (Onaka, 1998; Pacak et al, 1995).

Finally, the present study shows a reduced dopamine turnover and 3,4-Dihydroxyphenylacetic acid (DOPAC), a metabolite of the neurotransmitter dopamine, BNST concentration, just in 14 d-old LP offspring. Here, these results suggest that at 14 d-old LP animals present a decreased neuronal release and degradation of dopamine. Oliveira et al., (2012) have demonstrated that prenatal administration of dexamethasone does not alter the concentration of dopamine, DOPAC and HVA in the BNST of the adult offspring. Nevertheless there were alterations in the HPA axis (Oliveira et al, 2006), which can act as a modulator of dopaminergic circuits (Piazza & Le Moal. 1996).

Thus, the current study, as far as we know is the first description of the modulation of dendritic plasticity, morphology and neurochemistry of the BNST in the early life, by protein restriction during developmental period. These interesting findings may represent the adaptation during embryonic development to exposure to elevated maternal corticosteroids as a consequence of nutritional stress. It is important to consider that different stimuli as well as intensities and time can lead to different responses in the CNS morphology (Oliveira et al, 2012). Also, additional studies must be done, but the present study suggests strongly that morphological and neurochemical results may be associated with the development of psychiatric disorders in adulthood.

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4.2 Artigo 2

GESTATIONAL PROTEIN RESTRICTION ALTERS EARLY AMYGDALA NEUROCHEMISTRY IN MALE OFFSPRING

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Abstract

The amygdala's cytology and neurochemical composition has been poorly documented in gestational protein-restricted offspring, therefore, in the present

study we investigate the effects of gestational protein restriction on whole amygdala neurochemical compound in parallel with cytological content and neuron structure (basolateral amygdala) in male offspring, in key moments of post-natal neural development. The current study shows a significant decrease in body birthweigh that remain up to 14 day of age, in gestational protein-restricted offspring . Additionally, this study also confirms several previous that have shown that protein restriction intake did not alter the brain weight in youth or adults offspring. In the current study, the amygdala neuronal 3D dendritic analysis of dendrites length and dendritic ramifications by Sholl analysis, was not altered in 14 d-old NP compared with age-matched gestational protein-restricted offspring. Also, the amygdala neurons and non-neuronal cells number was similar in 14 day-old LP and NP offspring. In the present study we also investigate the effects of gestational protein restriction on whole amygdala neurochemical compound in male offspring, in key moments of post-natal neural development. Here, we demonstrate significant decrease in the amygdala content of norepinephrine, epinephrine, dopamine, CRF and BDNF in 7 day-old rats, as well as reduction in the expression GR and MR and CRF in 14 day-old LP offspring. In conclusion, the amygdala neurochemical changes observed in this study may contribute to behavioral alterations induced by gestational protein restriction and may also be a primer for alterations in other brain regions. Also, these findings may represent the adaptation during embryonic development to exposure to elevated maternal corticosteroids as a consequence of nutritional stress.

Keywords: Amygdala, gestational protein-restricted intake, neuron morphology, neurochemistry, dendritic analysis.

Introduction

The concept of “fetal programming” suggests that the fetus may be programmed during intrauterine development (Barker, 1995; Lucas, 1991). Stressful stimuli during this period may be risk factors for the development of

neuropsychiatric disorders in adulthood (Borges, 2014). Gestational protein restriction promotes an increased fetal exposure to maternal glucocorticoids by decreased placental concentration and activity of the enzyme type-2 11 β-HSD2, which promotes faster maturation of fetal tissues and organs leading to a decreased birthweight offspring and, increasing risk of behavioral disorders (Lopes, 2013; Mesquita, 2010; Scabora, 2012, Burd et al., 1999).

One of the mechanisms involved in the development of these disorders is the elevated exposure of the fetus to maternal glucocorticoids (GC) due to a low concentration and decreased activity of the placental enzyme 11beta-hydroxysteroid dehydrogenase (11β-HSD) type 2 (Benediktsson et al, 1993; Stewart et al, 1995; Langley-Evans et al, 1996; Langley-Evans, 1997). These prenatal alterations may lead to a chronic increase in GC as well as exacerbated response to stressful stimuli in adulthood (for review see Welberg & Seckl, 2001). There is a wide range of evidence that shows that the postnatal activity of the hypothalamic-pituitary-adrenal (HPA) axis, the main mechanism of stress-response in the brain, can be altered by prenatal events (Barker, 1995). Recently, we have demonstrated hyperanxious phenotype associated with atrophy of the dendritic neurons arborization from bed nucleus of the stria terminalis (BNST) in gestational protein-restricted adult offspring (unpublished data). No work has shed light on the amygdala morphology and neurochemical compounds of gestational protein restriction programed animals.

The amygdala plays a crucial role coordinating behavioral, autonomic, and neuroendocrine stress responses, via mostly excitatory influences on the hypothalamus and brainstem (Aggleton, 2000). Anxiety-like behavior and fear-enhancing effect are enhanced by amygdala steroids stimulation (Venkova et al 2010) through local activation of high density of MR and GR (Herman et al 1989; Oitzl et al 2001). In the central amygdaloid nucleus (CeA), MR and GR are expressed in corticotrophin-releasing factor (CRF) neurons, a key modulator of stress-related anxiety and the role of the amygdala in the modulation of the HPA axis, suggesting a regulatory effect of glucocorticoid on this nucleus (Honkaniemi et al., 1992; Davis, 1992; Merali et al., 2004; Muller et al., 2003). Also, studies have

demonstrate that type 1 CRF receptor could modulates a number of neurotransmitter systems such as serotonin (5HT) and dopamine (Millan, 2005; Holsboer, 2003; Nestler et al., 2002; Leonard, 2005; Holmes et al, 2003). In addition, they may be interacting with brain derived neurotrophic factor (BDNF). This network is deeply involved in fear and anxiety (Hammack et al, 2009; Duman, 2004; Kumari, et al 2016).

The amygdala's cytology and neurochemical composition has been poorly documented in gestational protein-restricted offspring, therefore, in ten present study we investigate the effects of gestational protein restriction on whole amygdala neurochemical compound in parallel with cytological content and neuron structure (basolateral amygdala) in male offspring, in key moments of post-natal neural development.

Materials and methods

Animals and treatments - The experiments were conducted on age-matched female offspring of sibling-mated Wistar Hannover rats (250-300g). The experiments were done in accordance with the general guidelines established by the Brazilian College of Animal Experimentation (COBEA) and approved by the Institutional Ethics Committee (CEEA/UNICAMP #3908-1) and National Institutes of Health guidelines on animal care and experimentation and approved by Director General Veterinary (DGV; the Portuguese National Institute of Veterinary 023-432/08.30.2013). A part of our site colonies originated from the breeding stock supplied by Charles River Laboratories, Barcelona, Spain. Another part was originated from a breeding stock supplied by CEMIB/Unicamp, Campinas, SP, Brazil. The animals were housed in pairs under standard laboratory conditions (lights on from 8 a.m. to 8 p.m.) and had access to food and water ad libitum and followed up to 12 weeks of age. The rats were placed to mate and the day that sperm were seen in the vaginal smear was designated as day 1 of pregnancy. The dams were divided in two groups: one maintained on isocaloric standard rodent laboratory chow with normal protein content [NP] (17% protein) and other received a diet with low protein content [LP] (6% protein) ad libitum throughout the entire pregnancy. Food consumption was determined every day (subsequently

normalized for body weight). All groups returned to the NP chow intake after delivery. On the day of birth the male pups were weighted and they were kept only 8 pups per female. At 7th and 14th postnatal day the brain, thymus and adrenals were weighted.

Morphological analyses methods - At 7th and 14th postnatal day male rats from NP (n=27/per age from 10 different mothers) and LP (n=27/per age from 10 different mothers) groups were deeply anesthetized with a mixture of ketamine and xylasine (75 and 10mg/kg respectively, i.p.) and monitoring the corneal reflex controlled the level of anesthesia. For isotropic fractioning the rats were transcardially perfused with saline containing heparin (5%, for 15 min, under constant pressure) followed by fixative solution: 0.1M phosphates buffer (PB; pH 7.4) containing 4% (w/v) paraformaldehyde. For 3D reconstruction we used only saline for perfusion.

Isotropic fractionator method - For total cells and neuron quantification we used the technique described by Herculano-Houzel & Lent (2005). The brains of 14-day-old rats (3 NP and 3 LP, from different mothers) were removed and amygdala was dissected. A suspension of nuclei was obtained through mechanical dissociation in a standard solution (40mM sodium citrate and 1% Triton X-100), using a 40-ml glass Tenbroeck tissue homogenizer. After washed several times with dissociation solution and centrifuged (10-min at 4000 g) pelleted nuclei were suspended in phosphate-buffered saline (PBS) containing 1% 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR, USA). DAPI-stained nuclei were counted under a fluorescence microscope at 400×objective. The nuclear density in the suspension was determined by averaging over at least eight samples the total number of cells in the original tissue was estimated by multiplying mean nuclear density by total suspension volume. Neuron number was estimated after immunohistochemistry using anti-NeuN antibody (1:300 in PBS; Chemicon Temecula, CA, USA) and CY3 goat anti-mouse secondary antibody (1:400 in 40% PBS, 10% goat serum and 50% DAPI; Accurate Chemicals, Westbury, NY, USA) under the fluorescence microscope (Scabora et al., 2013). Total number of other

amygdala cells nuclei is calculated by subtracting the number of NeuN containing nuclei from the total number of nuclei.

Neuronal 3D reconstruction and dendritic tree analysis - The amygdalafrom 14 days old offspring (4 NP and 4 LP from different mothers) were Golgi–Cox staining according to a published protocol (Gibb R & Kolb B, 1998). Briefly brains were removed and immersed in Golgi–Cox solution (Glaser & Van der Loos, 1981) for 14 days; brains were then transferred to a 30% sucrose solution (3-days) before being cut on a vibratome. Coronal sections (200 µm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia developed in Dektol (Kodak), fixed in Kodak Rapid Fix, dehydrated and cleared in xylene before being mounted and coverslipped. Slides were coded before morphometric analysis in both sets. The basolateral amygdala, strongly associated with fear and anxiety behavior was selected for neuron reconstruction. For dendritic tree analyze the criteria used to select neurons for reconstruction were as follows: (i) full impregnation of the neurons along the entire length of the dendritic tree; (ii) dendrites without significant truncation of branches; (iii) relative isolation from neighboring impregnated neurons to avoid interference with the analysis; and (iv) no morphological changes attributable to incomplete dendritic impregnation of Golgi–Cox stain (Pêgo et al. 2008). For each selected neuron all branches of the dendritic tree were reconstructed at 600x magnification using a motorized microscope (Carl Zeiss Axioplan 2) attached to a camera (DXC-390; Sony Co, Japan) and Neurolucida software (Micro Bright Field, VT, USA). Three-dimensional analysis of the reconstructed neurons was performed using NeuroExplorer software (MicroBrightField). For the dendritic analysis were reconstructed 91 neurons.

Neurochemical analyses methods - Male rats at 7 and 14 days were decapitated and the heads were snap-frozen in liquid nitrogen. The amygdala was dissected and stored at -80 °C until further analysis.

***Western blot** (WB)* - The tissue was homogenized in extraction buffer RIPA (Radio Immune Precipitation Assay Buffer) and then 10% triton x 100 and 10% SDS were added to the homogenate. The tissue extracts were centrifuged (1300 *rpm* at 4°C for 40 min) and the supernatants used as a sample. Protein quantification was performed using the Bradford method. The samples were treated with a Laemmle buffer containing 100-mmol/l dithiothreitol (DTT) heated in a boiling water bath for 4 min and subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) in a Bio-Rad mini-gel apparatus (Mini-Protean Bio-Rad). Electrotransfer of proteins from the gel to the nitrocellulose membranes was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean). The non-specific protein binding to the nitrocellulose was reduced by preincubating the filter for 2 h at 22°C in a blocking buffer (BSA 5%, 10mmol/l Tris, 150mmol/l NaCl and 0.02% Tween 20). The nitrocellulose blots were incubated at 4°C overnight with antibodies against GR (H-300-Santa Cruz 8992), MR (H-300-Santa Cruz), BDNF (Abcam; ab46176), 5HT1A (ab101914), 5HT2A (ab160228), CRF (S-19- Santa Cruz 1761), CRF1 (ab 59023) and Alfa tubulin (DSHB; AA4.3) diluted in a blocking buffer (2.5% BSA, 10mmol/l Tris, 150mmol/l NaCl and 0.02% Tween 20). Immunoreactivity bands were detected using the enhanced chemiluminescence method (RPN 2108 ECL Western blotting analysis system; Amersham Biosciences) and were detected by a ChemiDoc XRS system (Biorad; 170870). The band intensities were quantitated by optical densitometry (*TINA* software) of the developed autoradiographs that were used at exposures in the linear range.

High performance liquid chromatography combined with electrochemical detection (HPLC/CE) - The level of catecholamine and serotonin in the amygdala was assessed by HPLC/CE using a Gilson instrument (Golson, Middleton, WI, USA) equipped with an analytical column (Supleco Supelcosil LC-18, 3mM, Bellefonte, PA, USA, flow rate: 1.0ml/min). Percloric acid (200ul) was added to each sample that were incubated for 30 min in ice and then sonicated and centrifuged (13000rpm, 10min, 4ºC). Supernatant was collected to a 1.5ml tube

and then centrifuged for 8min (10000rpm, 4ºC) and the resulting pellet was saved for later use. The supernatant was then filtered through an HPLC Spin-X column (Costar. Lowell, MA, USA) to remove debris and 150ul aliquots were injected into the HPLC system using a mobile phase of 0.7 M aqueous potassium phosphate (pH 3.0) in 10% methanol 1-heptanesulfonic acid (222mg l^{-1}) and Na-EDTA (40mg l^{-1}). A standard curve (Sigma H-7752) with known concentrations of each catecholamine was run each day for 5HIAA (Sigma H-8876), Dopamine (Sigma H-8502), DOPAC (Sigma D-9128), HVA (Sigma H-1252), Epinephrine (Sigma E-4375) and Norepinephrine (Sigma 74460).

Data presentation and statistical analysis - All data is reported as mean ± SD. Data obtained over time was analyzed using appropriate two-way analysis of variance (two-way ANOVA). Post hoc comparisons between selected means were made by Bonferroni's contrast test when initial two-way ANOVA indicated statistical differences between experimental groups. Comparison involving only two samples of independent observations tends within or between groups was made using a Student's test. The band intensities were quantitated by optical densitometry (*software TINA*). The Tukey–Kramer test for multiple comparisons was used for analysis. The level of significance was set at $P \leq 0.05$.

Results

The birthweight of LP offspring was significantly decreased when compared with that obtained in NP group. This reduction was also verified at LP 7 and 14 day-old offspring when compared to age-matched NP rats. The brain, thymus and adrenals weight were similar in both groups, when normalized for body weight (Table 1).

Table 1. Body birthweight, brain, thymus and adrenal weight of offspring from NP and LP groups. Mean ± SEM. Statistically difference accepted when $p < 0.05$.

	Weight (g)			Organ/body weight (g/g)		
	NP (n=20)	LP (n=20)	p	NP (n=20)	LP (n=20)	p
Birth weigth	6.251 ± 0.06074	5.910 ± 0.09273	0.0023**			
Body weight 7º day	16.52 ± 0.2652	14.93 ± 0.3020	0.0002***			
Body weight 14º day	33.64 ± 0.4805	30.12 ± 0.6916	0.0001***			
Brain 7º day	0.7231 ± 0.005922	0.6841 ± 0.008642	0.0004***	14.22 ± 1.747	15.63 ± 1.478	0.5366
Brain 14º day	1.270 ± 0.01091	1.206 ± 0.01471	0.0008***	13.10 ± 2.530	9.318 ± 2.529	0.2979
Adrenal 7º day	0.003396 ± 0.0001811	0.003359 ± 0.0001936	0.8916	0.0002064 ± 0.00001017	0.0002338 ± 0.00001362	0.1211
Adrenal 14º day	0.008333 ± 0.0005236	0.008433 ± 0.0003930	0.8944	0.0002529 ± 0.00001533	0.0002905 ± 0.00001900	0.1413
Thymus 7º day	0.04669 ± 0.002280	0.04754 ± 0.001781	0.7714	0.002816 ± 0.0001341	0.003260 ± 0.0001065	0.0125*
Thymus 14º day	0.1105 ± 0.005273	0.09426 ± 0.01102	0.1484	0.003354 ± 0.0001451	0.003084 ± 0.0002097	0.2889

Neuronal 3D reconstruction and dendritic tree analysis

The neuronal 3D dendritic analysis studying dendrites length and dendritic ramifications by Sholl analysis, in neurons from amygdala was not different in 14 d-old NP and LP offspring (Fig. 1).

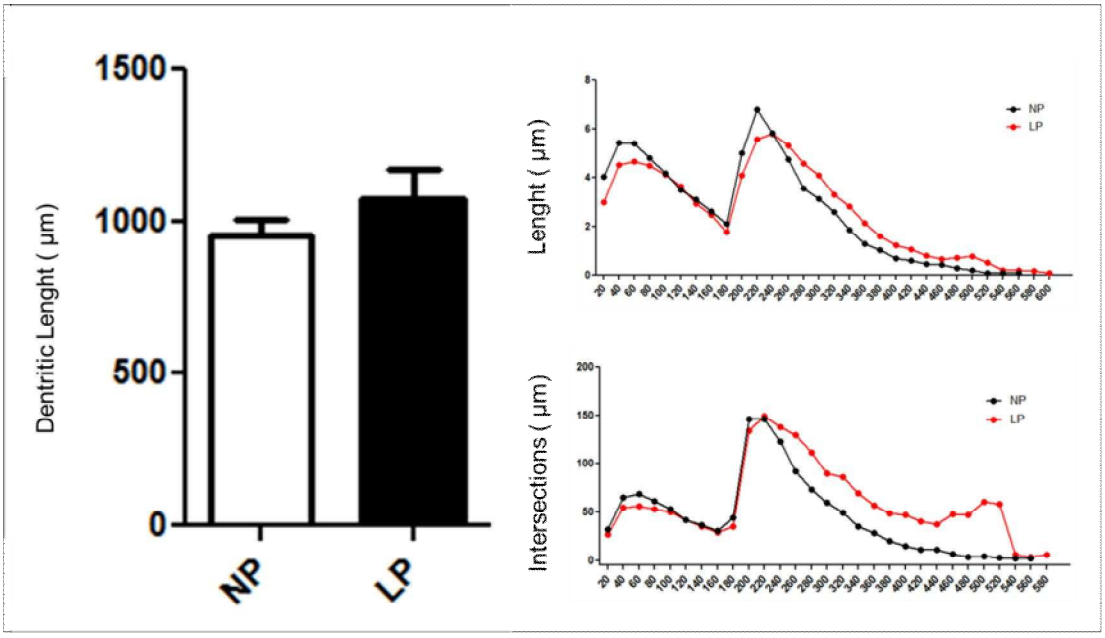


Fig 1. The gestational protein restriction has not effects in the length and ramification of amygdala neuronal dendrites.

Amygdala *total cells and neurons quantification*:

The results show a statistically non-significant reduction of neurons and non-neurons cells number in the amygdala of LP offspring when compared to NP rats (Fig. 2).

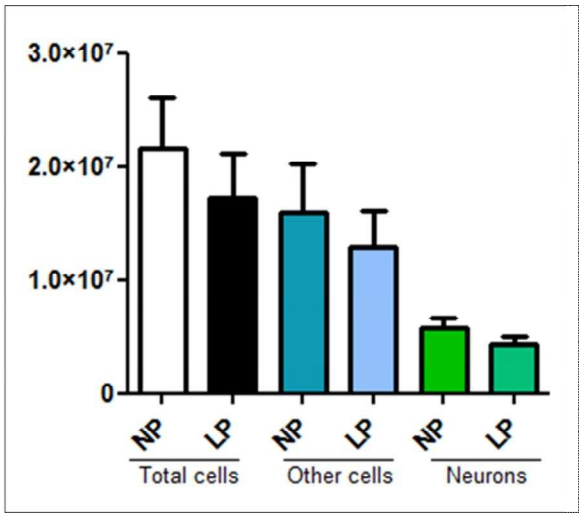
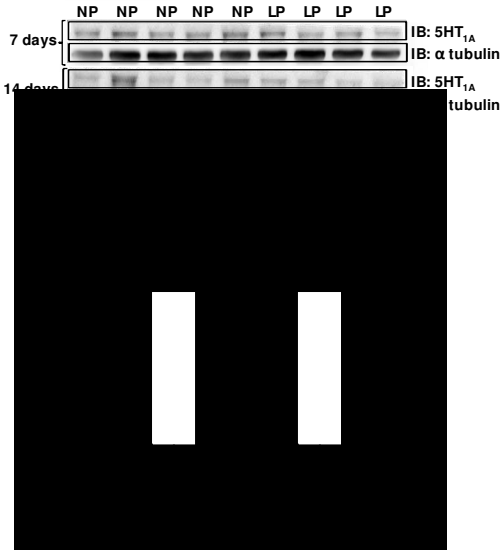
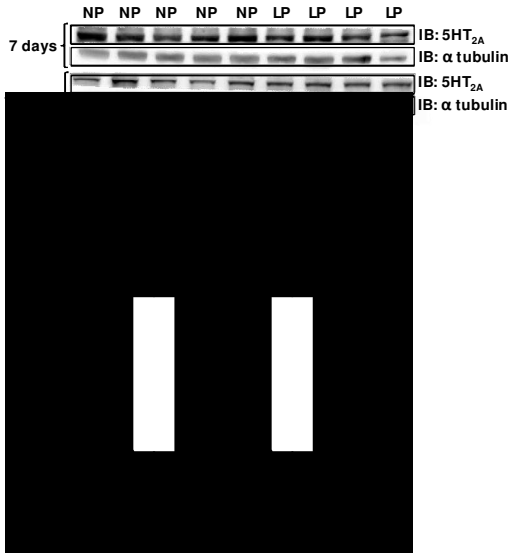


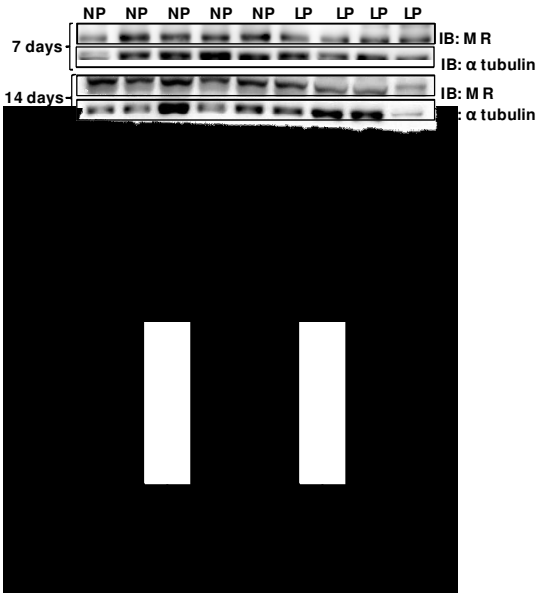
Fig 2. Neurons and other cells number in the amygdala of 14 day-old animals.

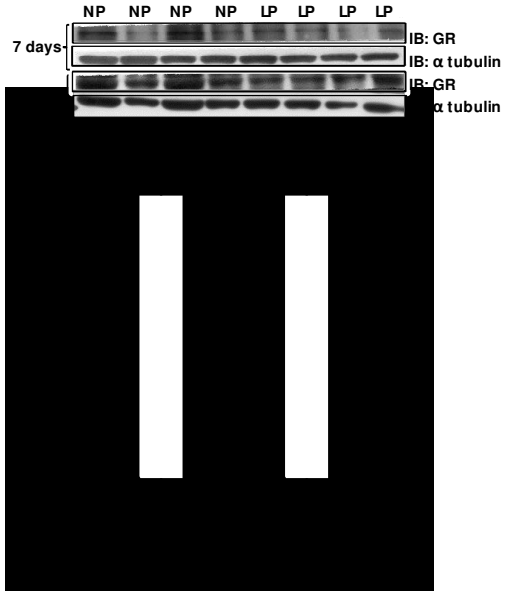
Western blot analysis

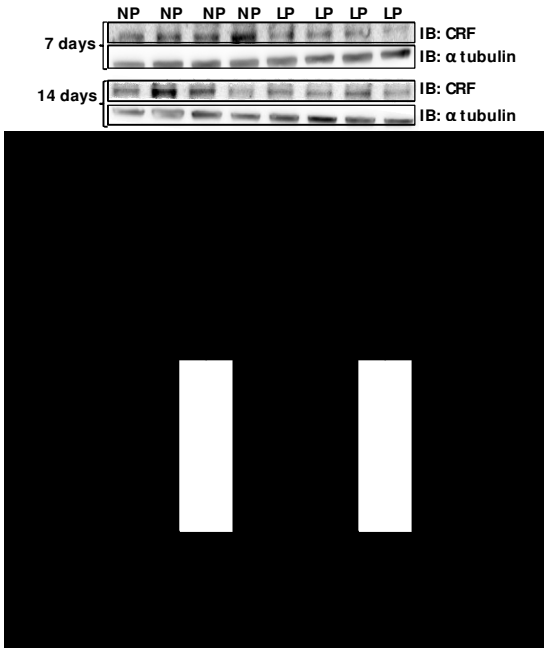
The western-blotting analyses show a significant reduction of GR expression (24%) in 14 day-old LP when we compared to NP offspring. Also, MR expression was reduced at 7 and 14 day-old LP respectively, 20% (p=0.07) and 45% (p=0.01) compared to age-matched NP animals. The CRF expression was significantly lower in both 7th (36,5%) and 14th (69%) day of life in LP group. Additionally, BDNF expression was 12,5% (p=0.03) and 25% (p=0.08) reduced in LP group respectively at 7 and 14 day-old offspring. The study did not show statistical difference in both experimental groups to $5HT_{1A}$, $5HT_{2A}$ and CRF1 receptors expression (Fig. 3).

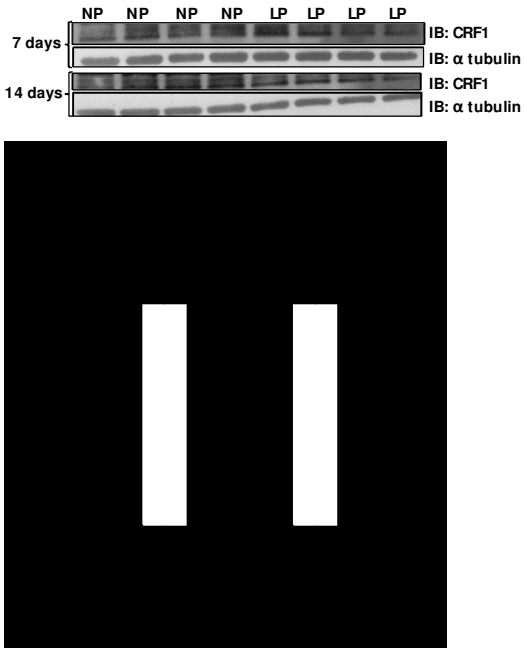

NP NP NP NP NP LP LP LP LP
7 days
IB: 5HT1A
IB: α tubulin
IB: 5HT1A
tubulin


NP NP NP NP NP LP LP LP LP
7 days
IB: 5HT2A
IB: α tubulin
IB: 5HT2A
IB: α tubulin


NP NP NP NP NP LP LP LP LP
7 days
IB: M R
IB: α tubulin
14 days
IB: M R
: α tubulin


NP NP NP NP LP LP LP LP
7 days
IB: GR
IB: α tubulin
IB: GR
α tubulin


NP NP NP NP LP LP LP LP
7 days
IB: CRF
IB: α tubulin
14 days
IB: CRF
IB: α tubulin


NP NP NP NP LP LP LP LP
7 days
IB: CRF1
IB: α tubulin
14 days
IB: CRF1
IB: α tubulin

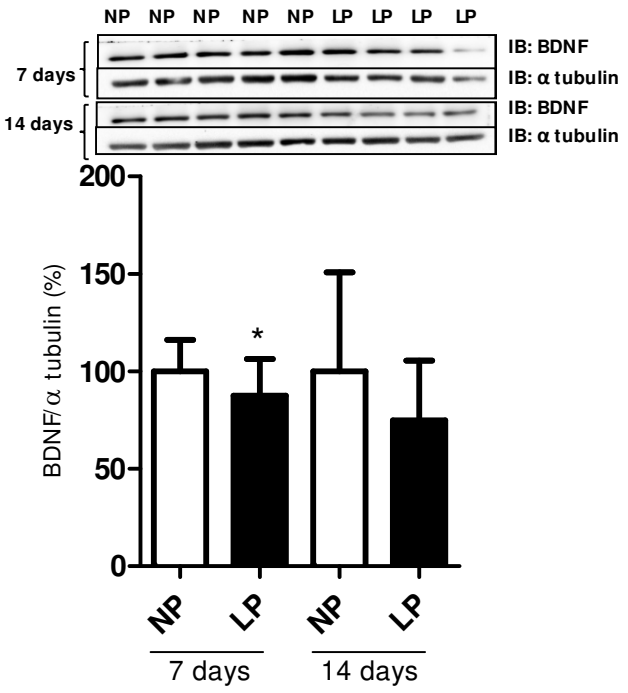


Fig 3. Western blot analysis of the 5HT2A, 5HT1A, GR, MR, BDNF, CRF and CRF1 expression in the amygdala of animals from NP and LP groups with either 7 or 14 days old. Results of scanning densitometry are presented as relative to NP, assigning a value of 100% to NP rats. Columns and bars represent the mean ± SD. $^{*}P < 0.05$, NP versus LP.

High-Performance Liquid Chromatography (HPLC)

The concentrations of norepinephrine and DOPA, assessed by HPLC were significantly decreased in 7 day-old LP animals when compared to age-matched NP offspring. No differences were found in the level of other neurotransmitters analyzed.

Table 2: Determination of the concentration of neurotransmitters by HPLC: Values represent the mean ± standard deviation. Statistical comparisons were made with the Student t test and statistical significance was accepted for p≤0.05.

	Amygdala					
	7 days			14 days		
	NP	LP	p	NP	LP	p
Norepinephrine	35.50 ± 4.41 N=3	17.95 ± 1.06 N=3	0.009**	44.63 ± 13.32 N=5	20.91 ± 4.54 N=4	0.09
Epinephrine	6.53 ± 0.95 N=3	4.22 ± 0.42 N=3	0.04*	9.24 ± 2.12 N=5	7.46± 0.8 N=3	0.28
Dopamine	14.89 ± 2.69 N=4	7.02 ± 1.59 N=4	0.02 *	5.21 ± 1.18 N=4	3.8 ± 0.75 N=4	0.18
DOPAC	10.39 ± 1.55 N=4	6.89 ± 1.83 N=3	0.1	8.2 ± 2.05 N=5	4.72 ± 1.16 N=4	0.11
5HIAA	8.9 ± 2.76 N=3	6.65 ± 1.73 N=4	0.25	8.94 ± 2.04 N=5	5.74 ± 1.71 N=4	0.14
5HT	11.72 ± 1.25 N=4	11.90 ± 2.24N=3	0.47	24.12 ± 7.8 N=5	9.33 ± 2.81 N=3	0.11
TURNOVER 5HT	0.42 ± 0.09 N=4	0.61 ± 0.08 N=3	0.09	0.43 ± 0.09 N=6	0.35 ± 0.12 N=4	0.3
TURNOVER DOPAMINE	1.16 ± 0.1365 N=5	1.25 ± 0.13 N=4	0.32	1.33 ± 0.07N=5	1.2 ± 0.09 N=4	0.14

Discussion

In accordance with previous reports, in the current study, we also observe a significant decrease in body birth weight that remains up to 14 day of age, in gestational protein-restricted offspring. Additionally, this study also confirms several previous that have shown that protein restriction intake did not alters the brain weight in adults offspring (Lingas et al, 1999). Recent study in our laboratory has demonstrated that 16 week-old male offspring from gestational protein-restricted dam, also, did not present any changes in the brain mass. In this study, we also do not show significant difference in the brain mass at 7 and 14 day-old LP rats. In this sense, our data add support to the “selfish brain” theory, which proposes that intrauterine adverse events such as emotional stress, either undernutrition, restriction of specific nutrients or hypoxia, may program the brain to maintain stability in its own energy sources. Particularly, alterations in early life, program the developing neuronal substrate to decrease neuroendocrine activity to, in turn, decrease the somatic growing rate, guarantying an adequate nutrition to the developing brain (Lumbers, et al, 2001). This theory can explain the reduced body weight of LP animals without affecting the brain mass.

The present study also evaluated the post-mortem adrenal and thymus weight. At 7 day-old LP offspring the results show an increased thymus weight that not remain in 14 day-old offspring. This thymus change may be related to steroidal plasma levels that could be associated with alteration in lymphoid tissues proliferation of protein-restricted animals. In fact, it has been strongly suggested that neuronal plasticity may be modulated by the immune system (Li et al; 2016). Here surprisingly, the adrenal mass was unchanged in both age-matched groups. This finding, despite fact that stress in adults rats induces an increased adrenal mass (Ventura-Silva et al., 2012), in the current study, it seems that the gestational protein restriction does not affect this organ in the times studied.

In the current study, the amygdala neuronal 3D dendritic analysis of dendrites length and dendritic ramifications by Sholl analysis, not show alteration in 14 d-old NP compared with age-matched gestational protein-restricted offspring. Also, the amygdala neurons and non-neuronal cells number was similar in 14 day-old LP

and NP offspring. It is noteworthy that both dendrites' volume and length was evaluated in specific region on basolateral amygdala while the cells' number was determined on the whole amygdala. The dendritical length and arborization was determined in the basolateral amygdala, once this nucleus is the main responsible to modulate signals coming from several regions of CNS and, furthermore, is strongly associated with fear and anxiety behavior (Izquierdo et al, 2016; Li et al, 2016).

By the way, Pêgo et al., (2008) did not report any morphological alterations in the amygdala in animals that were exposed to chronic unpredictable stress while chronic immobilization stress leads to an hypertrophy of dendrites in this brain structure (Mitra & Sapolsky , 2008). The intensity and duration of stress exposure are therefore, seems to be key factors for the development of morphological and neurochemical alterations in the amygdala (Wilson et al, 2015). Thus, studies showing that chronic immobilization stress increases dendritic length, the number of ramifications and the number of spines in neurons of the basolateral amygdala (Mitra et al, 2005; Vyas et al, 2002). Additionally, these morphological changes are related with anxiety-like behavior in chronic stressed-animals (Vyas & Chattarji, 2004).

It is broadly confirmed that gestational protein restriction may induce fetal programming leading to modification in the brain structure and behavior of offspring that remain up to adulthood (Ashton, 2000; Procter & Campbell, 2014, Barker, 1995; Forsdahl, 1967). Animal studies have shown that early-life undernutrition has a deep impact on fetal tissues and organs development, causing permanent changes in a wide range of morphological and physiological functions, including brain function (Alamy & Bengelloun, 2012). However, the amygdala's functional neurochemical modulation has been poorly documented in gestational protein-restricted offspring, therefore, in the present study we investigate the effects of gestational protein restriction on whole amygdala neurochemical compound in male offspring, in key moments of post-natal neural development. Here, we demonstrate significant decrease in the norepinephrine, epinephrine and dopamine levels, as well as reduction in the expression GR and MR steroid

receptors and CRF in the amygdala in 7 and 14 day-old LP offspring compared to control group.

The amygdala plays a crucial role coordinating behavioral, autonomic, and neuroendocrine stress responses, via mostly excitatory influences on the hypothalamus and brainstem (Aggleton, 2000). It stimulates the HPA axis through indirect projections to the hypothalamic paraventricular nucleus (PVN), including a disinhibitory pathway via the bed nucleus of the stria terminalis (Feldman et al., 1990; Herman et al., 2003, Freese & Amaral, 2009). PVN stimulation releases corticotrophin-releasing factor (CRF) into the portal vasculature, which binds to CRF receptors in the anterior pituitary stimulating the release of adrenocorticotrophic hormone (ACTH), which stimulates the synthesis and release of glucocorticoids by the adrenals (Ulrich-Lai and Herman, 2009). Glucocorticoids inhibit their own release via negative feedback through binding to glucocorticoid receptors (GRs) in the pituitary, PVN, and extrahypothalamic brain regions (Myers et al., 2012). In the present study, the reduced expression of amygdala GR and MR may have a key role on excitatory HPA axis response, priory recorded, in LP offspring.

The amygdala's excitatory influence on the HPA axis stress response has been primarily demonstrated in adult animals, with electrical stimulation increasing secretion of glucocorticoids (Redgate & Fahringer, 1973; Ehle et al., 1977) and lesions resulting in blunted HPA axis stress responses (Kalin et al., 2004; Machado & Bachevalier, 2008). Recent study has demonstrated administration of corticosteroid into amygdala nucleus promotes an anxiety-like behavior (Venkova et al 2010) by MR and GR stimuli (Herman et al 1989). Also, reporters suggested that both receptors are implicated in glucocorticoids fear-enhancing effect (Oitzl et al 2001). In the central amygdala nucleus (CeA) MR and GR are expressed in CRF neurons, suggesting direct glucocorticoid regulation of CRF expression in this nucleus (Honkaniemi et al., 1992). The amygdala is also rich in corticotrophin-releasing factor (CRF), a specific modulator of stress-related anxiety and the role of the amygdala in the modulation of the HPA axis may be through the activation of CRF receptor (CRF1) (Davis, 1992; Merali et al., 2004; Muller et al., 2003).

However, recent data from Raper et al (2014) has demonstrate that early amygdala damage (decreasing GR/MR and CRF amygdala levels) alters the typical development of the primate HPA axis resulting in increased rather than decreased activity, presumably via alterations in central CRF and GR systems in neural structures that control its activity. Thus, in contrast to evidence that the amygdala stimulates both CRF and HPA axis systems in the adult, our data suggest an opposite inhibitory role of the amygdala on the HPA axis during early development, which fits with decreased GR, MR and CRF levels observed in the present study. The CRF receptors activation may also induces alterations in other neurotransmitter systems such as serotonin (5HT), dopamine and norepinephrine (Millan, 2005; Holsboer, 2003; Nestler et al., 2002; Leonard, 2005; Holmes et al, 2003) and the amygdala CRF reduction in protein-restricted offspring, may affect this neurotransmitter release. In addition, they may be interacting with brain derived neurotrophic factor (BDNF), network deeply involved in fear and anxiety (Hammack et al, 2009; Duman, 2004; Kumari, et al 2016).

In the current work, the animals submitted to gestational nutritional restrictions presented a decrease in amygdala norepinephrine, epinephrine and dopamine levels. It is well known that stress exposure leads to a cascade of neuroendocrine response that promotes release of corticosteroids and catecholamines (Wolf, 2015). Nevertheless, authors report a decrease in dopaminergic and/or noradrenergic release in the depression pathophysiology (Guiard et al, 2008). *In vitro* studies suggest that the excitability of neurons in the amygdala is modulated by dopamine (Kroner et al, 2005) and that there is a strong relation between catecholamines and HPA axis alterations (Leonard, 2001). On the other hand, norepinephrine activates CRF, which, in turn, can induce the release of norepinephrine, leading to a cycle (Koob, 1999). This modulatory system may be blunted in the current study. These results may be explained by well-characterized stress hyporresponsive period (SHRP) associated to decreased activity of HPA axis, that may last until the 14th postnatal-day (Sapolsky & Meane 1986). At this time, there is a reduced secretion of corticosterone, which remains low until the second postnatal week (Sapolsky & Meane 1986). The SHRP may assume as a

protective mechanism ensuring low levels of glucocorticoids during early postnatal development (Sapolsky et al, 1986; Mesquita et al, 2007). The decrease in the expression of catecholamines in gestational protein-restricted model, may be one of mechanisms that contribute to the lower activity of the HPA axis during this period of life.

In conclusion, the amygdala's role on behavioral, autonomic, and neuroendocrine stress responses has been poorly studied in programmed models, with scarce studies reporting the morphological and neurochemistry characteristics and the relationship with the key role of the amygdala in the stress response (Herman & Cullinan 1997; Van de Kar & Blair, 1999). The amygdala neurochemical changes observed in this study may contribute to behavioral alterations induced by gestational protein restriction and may also be a primer for alterations in other brain regions. Also, these findings may represent the adaptation during embryonic development to exposure to elevated maternal corticosteroids as a consequence of nutritional stress. Additional studies must be done, but the present study suggests strongly that amygdala morphological and neurochemical disorders may be associated with the development of psychiatric disorders in adulthood.

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5. CONSIDERAÇÔES GERAIS

A amígdala estimula o eixo HPA através de projeções indiretas para o PVN, incluindo uma via excitatória através do BNST (Feldman et al, 1990; Herman et al, 2003 Freese & Amaral, 2009). O estimulo ao PVN promove a liberação de CRF nos vasos portais, que se liga aos receptores de CRF da hipófise estimulando a secreção de ACTH, que por sua vez estimula a síntese e liberação de GC pelas glândulas suprarrenais (Ulrich-Lai e Herman, 2009). Os GC inibem a sua própria libertação através de alças de retroalimentação negativas, pela ligação a GR na hipófise, no PVN e em regiões extra-hipotalâmicas do cérebro, tais como hipocampo e núcleos da amigdala (Myers et al., 2012).

A influência excitatória da amígdala sobre a resposta do eixo HPA foi demonstrada primeiramente em animais adultos, pela estimulação eléctrica desta causando um aumento na secreção de GC (Mason, 1959; Redgate & Fahringer, 1973; Ehle et al, 1977) e, através de lesões eletrolíticas em núcleos amigdaloides que resultaram na redução na atividade do eixo HPA associada a um estado de estresse (Beaulieu et ai, 1986; Feldman et al., 1994; Kalin et al, 2004).

Estudos em roedores mostram que a amígdala encontra-se funcionalmente em estado latente antes do desmame, quando os filhotes são mais dependentes de cuidados maternos, e acredita-se que este estado seja para inibir reações medo da mãe. Posteriormente, ocorre a ativação dos núcleos da amígdala para que seja possível a ocorrência de reações de medo/defesa coincidindo com o aumento dos níveis séricos e liquóricos de corticosterona durante fase de transição do desenvolvimento (pós-natal imediato) para fase de independência do filhote, após 16º. dia de vida (Rincón-Cortés & Sullivan, 2014).

Raper e colaboradores (2014) sugerem que durante o desenvolvimento, a amigdala tem influência inibitória na atividade basal do eixo HPA, tanto em primatas quanto em roedores, mudando mais tarde, para um estado excitatório na adolescência e idade adulta. Várias doenças psiquiátricas (p.e. autismo, esquizofrenia dentre outras) envolvem alterações no desenvolvimento da amigdala

e desregulação do controle da síntese e dos níveis liquóricos e séricos de CRF e de suas ações sobre o eixo HPA (Schumann et al., 2011; Tottenham, 2014)

Desta forma, a redução do CRF (36,6 e 69% respectivamente no 7º e 14º dia de vida) observada e, dos receptores de GC na amígdala e BNST de animais oriundos de mães submetidas á restrição proteica gestacional, pode atenuar a ação inibitória destas estruturas sobre o eixo HPA (Figura 1). Assim, nossos resultados confirmam prévios estudos mostrando que modificações morfológicas e/ou neuroquímicas da amigdala durante o desenvolvimento podem comprometer a ação destes núcleos cerebrais na modulação de respostas ao estresse e ao medo (Rincón-Cortés & Sullivan, 2014; Raper et al, 2014).

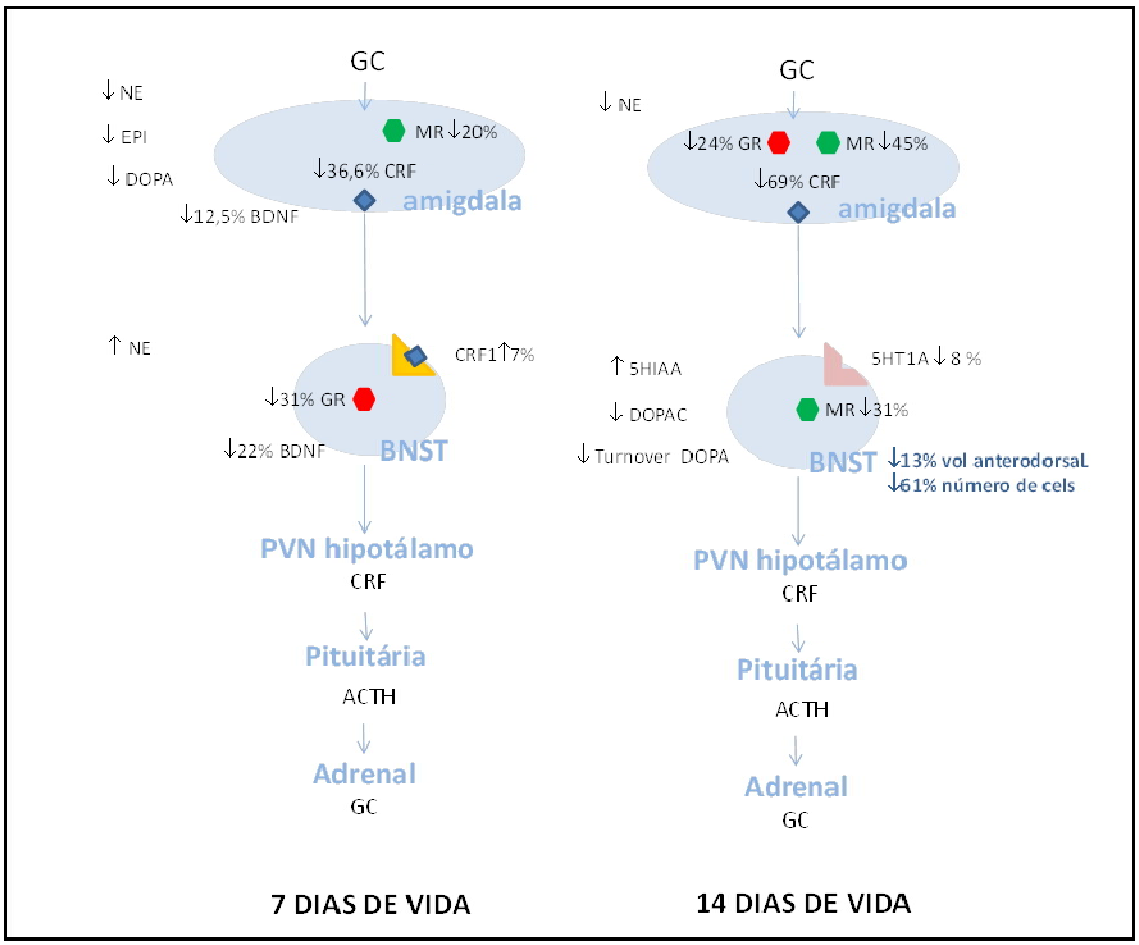

Figura 2. Compilação das alterações neuroquímicas e estruturais observadas em animais do grupo LP.

6. CONCLUSÃO

Os resultados do presente estudo mostram que a restrição proteica gestacional leva a alterações neuroquímicas na amígdala e neuroquímicas e morfológicas no BNST do sétimo ao décimo quarto dias de vida da prole. Estes achados sugerem a interferências do estresse nutricional durante a gestação, sobre o desenvolvimento de vias de sinalização dos glicocorticoides e do CRF que implica diferentes estruturas do sistema nervoso central resultando em hiperatividade destas e, consequentemente do eixo HPA, associada à elevação do corticosteroide plasmático e do estado de ansiedade, já observado na idade adulta nestes animais.

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8. ANEXO

8.1 Certificado Comitê de Ética- Unicamp

UNICAMP

CEUA/UNICAMP

CERTIFICADO

Certificamos que o projeto intitulado **"Análise dos efeitos da restrição proteica in utero no BNST e na amígdala de ratos: Estudo da estrutura dendrítica neural, de parâmetros funcionais e moleculares"**, protocolo nº **3908-1**, sob a responsabilidade de **Dra. Patricia Aline Boer / Daniele Braz Torres**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica ou ensino, encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais e do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal - CONCEA**, e foi aprovado pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em **31 de julho de 2015**.

Vigência do projeto: 08/2015-02/2017
Espécie/Linhagem: Rato heterogênico Wistar
No. de animais: 48
Peso/Idade: 04 semanas / 250gr
Sexo: 24 machos / 24 fêmeas
Origem: CEMIB/UNICAMP

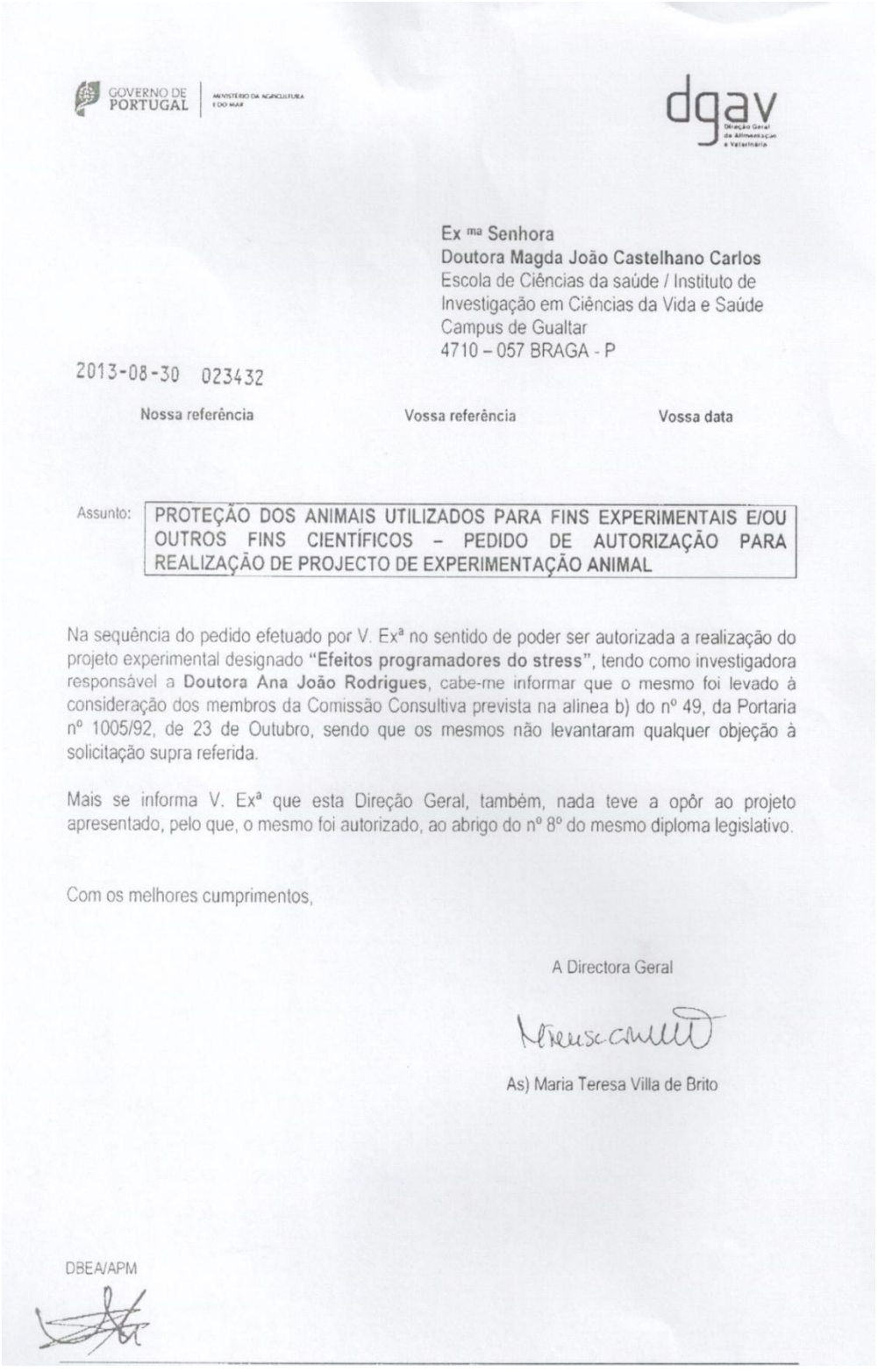
A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

Campinas, **31 de julho de 2015**.

Profa. Dra. Liana Maria Cardoso Verinaud
Presidente

Fátima Alonso
Secretária Executiva

8.2 Certificado Comitê de Ética-ICVS



GOVERNO DE PORTUGAL | MINISTÉRIO DA AGRICULTURA E DO MAR

dgav Direção Geral de Alimentação e Veterinária

Ex ma Senhora
Doutora Magda João Castelhano Carlos
Escola de Ciências da saúde / Instituto de Investigação em Ciências da Vida e Saúde
Campus de Gualtar
4710 – 057 BRAGA - P

2013-08-30 023432

Nossa referência | Vossa referência | Vossa data

Assunto: **PROTEÇÃO DOS ANIMAIS UTILIZADOS PARA FINS EXPERIMENTAIS E/OU OUTROS FINS CIENTÍFICOS – PEDIDO DE AUTORIZAÇÃO PARA REALIZAÇÃO DE PROJECTO DE EXPERIMENTAÇÃO ANIMAL**

Na sequência do pedido efetuado por V. Exª no sentido de poder ser autorizada a realização do projeto experimental designado **"Efeitos programadores do stress"**, tendo como investigadora responsável a **Doutora Ana João Rodrigues**, cabe-me informar que o mesmo foi levado à consideração dos membros da Comissão Consultiva prevista na alinea b) do nº 49, da Portaria nº 1005/92, de 23 de Outubro, sendo que os mesmos não levantaram qualquer objeção à solicitação supra referida.

Mais se informa V. Exª que esta Direção Geral, também, nada teve a opôr ao projeto apresentado, pelo que, o mesmo foi autorizado, ao abrigo do nº 8º do mesmo diploma legislativo.

Com os melhores cumprimentos,

A Directora Geral

As) Maria Teresa Villa de Brito

DBEA/APM