
UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE CIÊNCIAS MÉDICAS

GABRIEL BOER GRIGOLETTI LIMA

# *PROGRAMAÇÃO FETAL: ANÁLISE COMPORTAMENTAL E DA CONSTITUIÇÃO CELULAR E PROTEICA HIPOCAMPAL DURANTE O ENVELHECIMENTO (ALZHEIMER SÍMILE) EM PROLE DE RATAS SUBMETIDAS A RESTRIÇÃO PROTEICA GESTACIONAL*

# *FETAL PROGRAMMING: BEHAVIORAL AND HIPPOCAMPAL PROTEIN AND CELLULAR CONSTITUTION ANALYSIS DURING AGING (ALZHEIMER SIMILE) IN OFFSPRING OF RATS SUBJECTED TO GESTATIONAL PROTEIN RESTRICTION*

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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 Doutor em Ciências.

ORIENTADOR: PROF. DR. JOSÉ ANTÔNIO ROCHA GONTIJO

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**GABRIEL BOER GRIGOLETTI LIMA**

**ORIENTADOR: DR. JOSÉ ANTÔNIO ROCHA GONTIJO**

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**5. PROF. DR. RAFAEL NAIME RUGGIERO**

Programa de Pós-Graduação em Fisiopatologia Médica da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

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## RESUMO

O período perinatal é responsável por modular diferentes aspectos que futuramente interferirão em parâmetros fisiológicos, anatômicos e comportamentais. Evidências sugerem que a etiologia da esquizofrenia, do autismo e da doença de Alzheimer, que são caracterizadas por distúrbios de aprendizado e memória, estão relacionadas a eventos ambientais no início da vida. O presente estudo avalia os efeitos da restrição protéica gestacional no hipocampo, na prole masculina, durante o envelhecimento por testes comportamentais e analise de proteínas e número de neurônios e gliócitos. Ratas foram alimentadas, durante a prenhes, com dietas com conteúdo normal (NP 17% caseína) ou baixo de proteína (LP 6% caseína). A prole masculina de LP apresentou baixo peso ao nascer, sem alteração significativa da massa cerebral e hipocampal na idade de 50 semanas. No entanto, os animais LP com 88 semanas de idade mostraram massa hipocampal aumentada quando corrigida pela massa corporal e cerebral. Além disso, após 49 semanas de vida, os animais LP apresentaram redução da memória de referência e, pelo teste do labirinto em cruz elevado, comportamento semelhante ao de ansiedade, quando comparados aos ratos NP. Em relação à via do IGF, no presente estudo, os níveis de BDNF foram significativamente elevados no hipocampo de LP em comparação aos animais NP. Do mesmo modo, as proteínas Tau e β-amilóide estavam aumentadas em animais LP, enquanto as proteínas relacionadas ao estresse HSP70 e HSP90 não foram alteradas. Levando em consideração esses achados, hipotetizamos que a restrição proteica gestacional, em ratos, pode estar relacionada ao desenvolvimento da doença de Alzheimer-símile na idade adulta. Se, relacionados à idade em

humanos, isto representa o desenvolvimento precoce de distúrbios relacionados a esta doença incapacitante. Em conjunto, esses dados refletem no maior impacto ao processo de envelhecimento, acelerando a perda da função hipocampal e desenvolvimento de doença de Alzheimer. Ressaltam ainda, a necessidade de políticas públicas que reduzam a porcentagem de crianças com baixo peso ao nascer, uma vez que a programação pode acarretar na perda precoce da capacidade produtiva bem como em doenças que demandam alto custo para seu tratamento.

*Palavras-chave:* Programação fetal, dieta com restrição de proteínas, envelhecimento, aprendizado e memória, ansiedade, hipocampo, doença de Alzheimer, peptídeo β-amiloide, proteínas Tau.

## ABSTRACT

The maternal undernutrition environment has been associated with psychiatric and neurological, which are characterized by learning and memory dysfunction. The current study aims to study the gestational protein restriction intake effects on learning and memory functions later in life, considering the lack of evidence. The current findings correlate the behavioral findings with the hippocampus cell number and protein contents related to Alzheimer's disease. The experiments were conducted in animals submitted to low protein diet (LP, 6% casein) or regular protein content (NP, 17% casein) throughout the pregnancy. Behavioral tests, isolated hippocampus isotropic fractionator cell study, blotting protein analysis, and survival lifetime were performed. The present findings also confirmed that the birthweight of the LP male progeny was significantly reduced relative to NP male offspring, accompanied by increased hippocampus mass in 88-week-old LP when compared to age-matched NP rats. The study also presents an elevated Morris water maze proximity measure and revealed anxiety-like behavior in LP compared to NP rats 48 and 86 weeks of life. Additionally, the estimated neuron number was unaltered in LP; however, glial and another cell number increase in LP when compared to NP. Here, we showed unprecedently and increased BDNF, β-amyloid peptide (Aβ), and Tau protein hippocampal deposition in elderly LP offspring. As far as we know, there are no predicted studies showing changes in the hippocampal cell number and morphological aspects of neurons in gestational protein-restricted elderly progeny. The current data suggest a high impact of maternal protein restriction on the aging process, accelerating the loss of hippocampal function, impacting learning/memory performance, and supposedly developing something like an

Alzheimer's syndrome in elderly offspring. Thus, we may carefully suppose that the maternal protein-restricted could be a probable elegant and new AD-simile model in adult male progeny.

*Keywords:* Fetal Programming, Alzheimer's Disease, Behavior and memory, Maternal protein restriction, Hippocampus, β-amyloid peptide, Tau protein.

LISTA DE ABREVIATURAS

OMS: Organização Mundial da Saúde

HPA: hipocampo-pituitária-adrenal

SNC: sistema nervosa central

SVZ: zona subventricular

SGZ: zona subgranular

DG: giro denteado

NPCs: células progenitoras neuronais

HV: hipocampo ventral

HD: hipocampo dorsal

DCX: *doublecortina*

DA: doença de Alzheimer

Aβ: β-amilóide

IGFR1: insulin-like growth factor 1

ERK2: Mitogen-activated protein kinase 1

PI3K: Phosphoinositide 3-kinase

BDNF: Brain derived neurotrophic factor

HSP70: 70 kilodalton heat shock protein

HSP90: 90 kilodalton heat shock protein

CEMIB: Animal Center of the State University of Campinas

NP: normal protein

LP: low protein

**SUMÁRIO**

## INTRODUÇÃO

Em 2014, a Organização Mundial da Saúde divulgou, de maneira inusitada, que em 2020 o número de pessoas com 60 anos ou mais deverá superar o número de crianças com menos de 5 anos de idade. Estima-se que em 2050 a população mundial com mais de 60 anos poderá totalizar 2 bilhões, sendo que oitenta por cento desses idosos estarão vivendo em países de baixa e média renda. Embora a longevidade venha aumentando as pessoas não são necessariamente mais saudáveis do que antes - quase um quarto (23%) do total de morte e de doenças ocorre em pessoas com idade acima de 60 anos, e grande parcela é atribuída a doenças causadas por doenças cardíacas e perturbações mentais e neurológicas dentre outras. Por exemplo, as estimativas mais recentes indicam que se espera que o número de pessoas com demência passe de 44 milhões hoje, para 135 milhões até 2050. Diante disso, torna-se fundamental a investigação de fatores que possam influenciar no aparecimento de distúrbios mentais.

Estudos sobre o período histórico conhecido como *Fome Holandesa* examinaram a relação entre a exposição pré-natal à fome e sua associação com desordens psiquiátricas e transtornos afetivos. Gerada pelo bloqueio nazista, no último ano da Segunda Guerra Mundial, a fome começou em outubro de 1944, alongando-se gradativamente nos meses seguintes. A fome atingiu, principalmente, cidades localizadas na Holanda ocidental. Por volta de fevereiro à abril de 1945, chegou ao seu auge, com alta mortalidade, baixa fertilidade e comprometimento de nascimentos viáveis (Stein *et al*., 1975). O curto período e o acometimento regional circunscrito da fome, paralelo à

extensa oferta de documentos sobre os hábitos alimentares e a grande base de dados clínicos sobre doenças psiquiátricas, permitiram uma inédita avaliação epidemiológica da subnutrição em associação com transtornos psiquiátricos (Brown *et al.,* 2000). Este foi o primeiro estudo que relacionou a exposição pré-natal à fome com o desenvolvimento de transtornos afetivos na idade adulta.

Um dos estudos mais relevantes implicando a programação fetal em distúrbios psiquiátricos é o trabalho de Susser (1998) e colegas sobre o acompanhamento de indivíduos concebidos durante a ocupação nazista da Holanda no final da Segunda Guerra Mundial. O chamado inverno holandês da fome fornece um experimento natural bastante horrível, no qual mulheres grávidas, juntamente com o restante da população civil, foram submetidas à extrema privação de alimentos (Susser *et al.,* 1998). Os pais de Susser conduziram estudos dos efeitos dessa fome, resultando no reconhecimento da importância do folato na gravidez para evitar defeitos no tubo neural (Stein *et al.,* 1972) . O jovem Susser comprometeu-se a determinar se efeitos, menos debilitantes no desenvolvimento do sistema nervoso resultantes da exposição à fome no útero, podem ter consequências para o risco psiquiátrico após o nascimento. Ele encontrou aumento significativo do risco de esquizofrenia e distúrbios relacionados entre aqueles cujas mães passaram pelo pico da fome durante o segundo trimestre da gravidez (Susser *et al.,* 1996). Trabalhos subsequentes com indivíduos nascidos durante a fome chinesa produziram resultados semelhantes (Susser *et al.,* 2008; Rong, 2019). Ainda não foi determinado se os mecanismos que medeiam esses efeitos são de natureza epigenética (de Rooij, 2006). Susser e colaboradores (1992), em um relatório sobre transtornos psiquiátricos neste mesmo grupo, demonstrou risco duas

vezes maior de desenvolver esquizofrenia no grupo exposto à fome severa no início da gestação.

Costello (2007) e colegas forneceram o segundo exemplo de influências pré-natais nos resultados psiquiátricos. Eles avaliaram uma amostra populacional de mais de 1400 meninos e meninas na Carolina do Norte entre as idades de 9 e 16 anos com sintomas psiquiátricos. Estes autores descobriram que a ocorrência de depressão dos adolescentes era quatro vezes mais alta (38,1%) nas meninas com baixo peso ao nascer do que nas meninas com peso normal ao nascer (8,4%), e sete vezes maior do que nos meninos independentemente do peso ao nascer (4,9%). A conhecida diferença de sexo em casos de depressão na adolescência foi, portanto, quase inteiramente explicada pelo maior risco em meninas com baixo peso ao nascer. No entanto, meninas com peso ao nascer baixo e normal que não apresentaram adversidades subsequentes, não apresentaram incidência de depressão. Porém, em circunstâncias adversas, a taxa de depressão em meninas com baixo peso ao nascer aumentou significativamente. Os autores sugerem que meninas com baixo peso ao nascer são mais sensíveis a circunstâncias adversas em termos de risco de depressão, resultado que sugere possível alteração das respostas fisiológicas ao estresse, podendo envolver o eixo hipocampo-pituitária-adrenal (HPA).

Modelos animais de má nutrição gestacional estão demonstrando que proles adultas apresentam déficit de aprendizado e memória (Tonkiss and Galler, 1990; Fukuda *et al.,* 2002). Estes animais acabam manifestando respostas excessivas a fatores estressantes (Trzctnska *et al.,*1999; Levay *et al.,* 2008) e uma predisposição à adição de drogas psicotrópicas (Laino *et al.,*

1993; Almeida *et al.,* 1996). Entretanto, os mecanismos celulares e moleculares implicados nestes distúrbios neuro-comportamentais ainda não são conhecidos. Sabemos que a organização morfológica e funcional do sistema nervoso central (SNC), particularmente em mamíferos, é estabelecida nos períodos pré- e pós-natais, e envolvem síntese de componentes celulares, gliogênese, neurogênese, migração e diferenciação celular. Os processos são afetados por uma diversidade de fatores ambientais, por exemplo, aspectos comportamentais e a nutrição materna, podendo causar, em longo prazo, alterações na função cerebral da prole (Matos *et al.,* 2011). Sabemos também que crianças, quando expostas à má nutrição em períodos perinatais, acabam apresentando déficits cognitivos (Galler and Ramsey, 1989; Walker *et al.,*2000) e aumentam o risco de desenvolverem doenças psiquiátricas como esquizofrenia (Wahlbeck *et al.,* 2001; Brown and Susser, 2008) e depressão (Brown *et al.,* 2000; Costello *et al.,* 2007).

A desnutrição materna é um problema de saúde pública que pode culminar em patologias ao longo da vida (Bryce et al, 2003; Bryce et al 2005). Barker foi o primeiro pesquisador a relacionar o baixo peso ao nascer com o desenvolvimento de doenças na idade adulta como diabetes, hipertensão, doenças cardiovasculares e psicopatologias (Barker et al., 1993; Eriksson et al., 1999; Law et al., 1993). Em 1991, outro pesquisador importante nesta área de conhecimento, Alan Lucas, utilizou pela primeira vez a expressão “programação fetal”, definida como reação permanente do organismo frente um estímulo ou agressão em períodoc críticos/sensíveis do desenvolvimento (Lucas, A. 1991).

A programação fetal resultante da desnutrição gestacional ocorre pela diminuição da quantidade e da atividade da enzima 11β-HSD2 placentária, aumentando a exposição fetal aos glicocorticoides maternos, que atravessam a barreira placentária e atingem o feto precocemente. Esta alteração materna promove, nos filhotes, modificações importantes como o aumento da ativação da via do estresse; diminuição da atividade enzimática no fígado; mau funcionamento das células beta no pâncreas; menor número de nefrons nos rins (Mesquita et al 2010; Langley-Evans, S.C.; Jackson, A.A 1994; Pêgo et al 2008).

O estresse nutricional e/ou psicológico no início da vida acarreta diminuição da arborização dendrítica e do número de sinápses, assim como diminui a plasticidade/capacidade de regeneração neural. Observa-se ainda diminuição da neurogênese e queda da função cognitiva (McEwen, B.S.. 2007). O hipocampo e a zona subventricular (SVZ) dos ventrículos laterais são regiões em que grandes números de novos neurônios continuam a ser produzidos na vida adulta. As células que originam do SVZ, migram para o bulbo olfatório diferenciando-se em interneurônios locais e, no hipocampo, novos neurônios nascem na zona subgranular (SGZ) do giro denteado (DG) a partir de uma população local de células radiais que atuam como células progenitoras neuronais (NPCs). Pelos processos de proliferação, diferenciação neuronal, migração, crescimento dos dendritos e do axônio e formação de sinapses, sabemos que em três semanas as NPCs apresentam-se integradas à rede neuronal do DG como células granulares e extendem projeções axonais ao longo das fibras de mossy para a região CA3 hipocampal (Stanfield and Trice, 1988; Hastings and Gould, 1999). Ainda que a importância da neurogênese

hipocampal no adulto não seja compreendida em sua totalidade, várias linhas evidenciam que ela contribui nos processos de formação da memória e pode estar envolvida no desenvolvimento de doenças psiquiátricas (David *et al.,* 2010; Deng *et al.,* 2010).

Neurogênese caracteriza-se pelo desenvolvimento e diferenciação de novos neurônios que ocorrem em diferentes fases independentes tais como: proliferação de células progenitoras, diferenciação destas células em neurônios, maturação de neurônios recém-diferenciados e, inclusão destes neurônios diferenciados nso circuitos neurais pré-existentes (Christie and Cameron, 2006; Kemppermann, 1997). Nestas etapas podem ocorrer morte celular por apoptose ou autofagocitose (Petreanu and Alvarez-Buylla, 2002). Os processos de morte celular e proliferação são independentemente autoregulados no entanto, o equilíbrio tênue entre os eventos que determina a origem destes novos neurônios e a renovação de todos os circuitos neurais (Christie and Cameron, 2006). Não existem dados na literatura avaliando o número de neurônios durante o envelhecimento de animais programados pela restrição proteica gestacional, mas, podemos supor que este número passa ser mais drasticamente reduzido.

O hipocampo, importante estrutura cerebral, está relacionado com as emoções, a memória e o aprendizado espacial, podendo ser dividido funcionalmente em hipocampo ventral (HV) e dorsal (HD) (Fanselow and Dong, 2010). Esta separação é amplamente discutida na literatura e se dá de acordo com sua funcionalidade. Durante o processamento de informações, o HV caracteriza-se por modular as respostas ao estresse e emoções, enquanto que o HD seria responsável pela memória espacial (Fanselow and Dong, 2010). O

hipocampo pode ser subdividido em Corno de *Ammon* (CA, de 1 a 4) e giro denteado (GD) (Campbell and Macqueen, 2004). O GD localiza-se ao longo de todo hipocampo, principal sítio de neurogênese, desta forma, qualquer segmento hipocampal pode ser utilizada para avaliar a neurogênese (Aimone *et al.,* 2010).

O hipocampo é um dos principais alvos de estudos relacionados à doença de Alzheimer (DA). Esta doença neurodegenerativa causa alterações patológicas no tecido cerebral através da deposição extracelular do peptídeo β-amilóide (Aβ) que gera placas neuríticas (Reitz, 2014). Outra característica da patologia é a fosforilação da TAU, uma proteína responsável pela polimerização de microtúbulos, que acaba gerando o desenvolvimento de emaranhados neurofibrilares intracelulares, como é possível ver na figura 1 *(*Falco, 2016). Outras mudanças podem ser observadas, como perda significativa de neurônios, sinapses e substância branca (Reitz, 2014). Isso leva a perda funcional significativa da memória, causando sofrimento considerável em outras atividades cotidianas em pessoas afetadas pela doença.

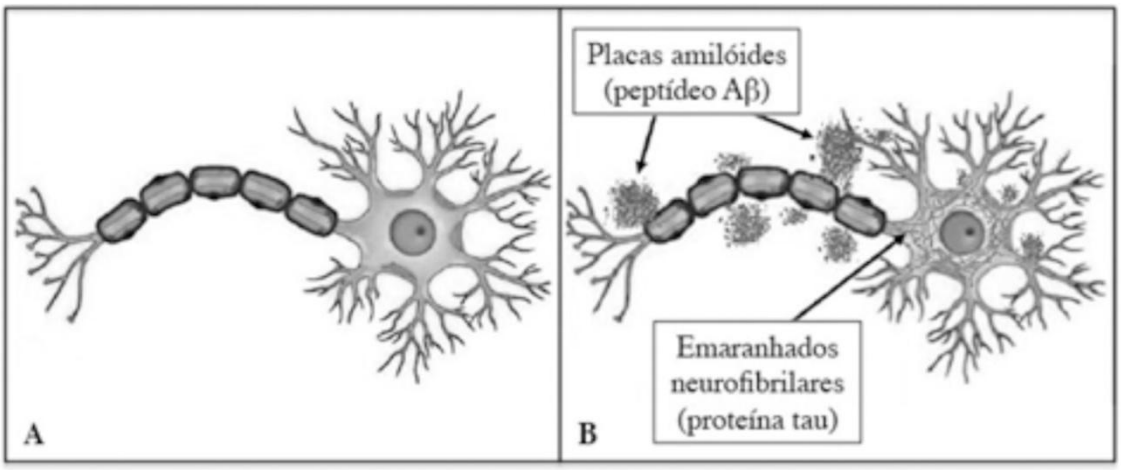

Figura 1. A: Neurônio saudável. B: Neurônio na doença de Alzheimer.

Tem sido proposta a conexão entre a atuação de fatores epigenéticos na gestação e a susceptibilidade ou resistência na vida adulta para o desenvolvimento de desordens neurodegenerativas como doença de Parkinson e Alzheimer (Faa, 2014). Dados recentes sugerem que a DA pode ter suas origens no início da vida (Borenstein, 2006). Provavelmente, a DA não é causada por um único fator etiológico, mas é o resultado da interação entre fatores genéticos e epigenéticos ao longo da vida, determinando o "fenótipo da DA". Tem sido demonstrado que quando o início da vida é afetado por estresse materno, infecções intra-uterinas e má nutrição materna e perinatal existe predispõe para o desenvolvimento da DA na vida adulta (Miller, 2008).

Assim, na presente tese investigamos se os machos da prole de ratas submetidas à restrição proteica gestacional apresentam alterações comportamentais durante o envelhecimento e se estas estão associadas a modificações na celularidade hipocampal. Além disso, avaliamos a expressão hipocampal de proteínas da via do IGF, relacionadas ao desenvolvimento da doença de Alzheimer e ao estresse tecidual.

Nossa hipótese era que a restrição proteica gestacional está envolvida em alterações cognitivas e desenvolvimento precoce de doença de Alzheimer durante o envelhecimento.

## JUSTIFICATIVA E OBJETIVOS

A partir da fundamentação teórica apresentada, a **JUSTIFICATIVA** para o desenvolvimento desta tese é pela:

- Aumento significativo da sobrevida de indivíduos com 60 anos ou mais em países de média e baixa renda;
- Estimativa da elevação dos casos de demência passando de 44 milhões hoje, para 135 milhões até 2050;
- A prevalência da desnutrição materno-infantil em países desenvolvidos e em desenvolvimento, alertando sobre necessárias intervenções em políticas públicas de saúde para essas populações;
- A constatação que a subnutrição maternal em períodos críticos do desenvolvimento embrio-fetal acarreta alterações no desenvolvimento da funcionalidade e da morfologia de órgãos e sistemas;
- Exacerbação na resposta ao estresse em animais programados;
- Comprovação que essas alterações possuem relação com modificações definitivas na modulação do sistema nervoso central.

## OBJETIVO GERAL

Estudar a formação hipocampal, durante o envelhecimento de ratos submetidos à restrição proteica gestacional através de parâmetros comportamentais, morfológicos e neuroquímicos.

## OBJETIVOS ESPECÍFICOS

Avaliar em animais com 48 e 88 semanas de vida os efeitos da desnutrição proteica materna e do envelhecimento sobre:

- O comportamento dos animais por testes específicos;
- O número de neurônios no hipocampo;
- A expressão proteica hipocampal de IGFR1, ERK2, pPI3K, BDNF, Tau, pTau, Aβ , HSP70 e HSP90.

**ARTIGO SUBMETIDO AO PERIÓDICO JOURNAL OF ALZHEIMER SYNDROME – SOB REVISÃO DOS AUTORES**

*Impact of maternal low-protein intake on hippocampus cellularity and behavioral test of male adult offspring: an Alzheimer-simile syndrome model?*

Gabriel Boer Grigoletti-Lima, Marcelo Gustavo Lopes, Ana Tereza Barufi Franco, Aparecida Marcela Damico, Patrícia Aline Boer, José Antonio Rocha Gontijo[1]

Fetal Programming and Hydroelectrolyte Metabolism Laboratory, Nucleus of Medicine and Experimental Surgery, Department of Internal Medicine, Faculty of Medical Sciences at State University of Campinas, Campinas, SP, Brazil

[1] Corresponding author:

José Antonio Rocha Gontijo

Department of Internal Medicine, School of Medicine, State University of Campinas, Campinas, SP, Brazil

Phone: +55 19 35217346, Fax: +55 19 3521-8925

E-mail: gontijo@fcm.unicamp.br

Patrícia Aline Boer, BSC, Ph.D.,

Department of Internal Medicine, School of Medicine, State University of Campinas, Campinas, SP, Brazil

Phone: +55 19 35217346, Fax: +55 19 3521-8925

E-mail: alineboer@yahoo.com.br

Gabriel Boer Grigoletti-Lima

Department of Internal Medicine, School of Medicine, State University of Campinas, Campinas, SP, Brazil

Phone: +55 19 35217346, Fax: +55 19 3521-8925

E-mail: gabrielboergrigoletti@gmail.com

Marcelo Gustavo Lopes

Department of Internal Medicine, School of Medicine, State University of Campinas, Campinas, SP, Brazil

Phone: +55 19 35217346, Fax: +55 19 3521-8925

E-mail: marcelolopes46@gmail.com

Ana-Tereza Barufi Franco

Department of Internal Medicine, School of Medicine, State University of Campinas, Campinas, SP, Brazil

Phone: +55 19 35217346, Fax: +55 19 3521-8925

E-mail: anatbfranco@yahoo.com.br

Aparecida Marcela Damico

Department of Internal Medicine, School of Medicine, State University of Campinas, Campinas, SP, Brazil

Phone: +55 19 35217346, Fax: +55 19 3521-8925

E-mail: marceladamico@hotmail.com

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ABSTRACT

The maternal undernutrition environment has been associated with psychiatric and neurological, which are characterized by learning and memory dysfunction. The current study aims to evaluate the effects of maternal protein restriction on learning and memory functions later in life, considering the lack of evidence. The study correlates the behavioral findings with the hippocampus cell number

and protein contents related to Alzheimer's disease. The experiments were conducted in animals submitted to low protein diet (LP, 6% casein) or regular protein content (NP, 17% casein) throughout the pregnancy. Behavioral tests, isolated hippocampus isotropic fractionator cell study, blotting protein analysis, and survival lifetime were performed. The results confirmed that the birthweight of the LP male pups was significantly reduced relative to NP male pups, accompanied by increased hippocampus mass in 88-week-old LP compared to age-matched NP offspring. The study also presents an elevated Morris water maze proximity measure and revealed anxiety-like behavior in LP compared to NP rats 48 and 86 weeks of life. Additionally, the estimated neuron number was unaltered in LP; however, glial and another cell number increase in LP compared to NP. Here, we showed unprecedently and increased BDNF, β-amyloid peptide (Aβ), and Tau protein hippocampal deposition in elderly LP offspring. As far as we know, there are no predicted studies showing changes in the hippocampal cell number and morphological aspects of neurons in maternal protein-restricted elderly offspring. The current data suggest a high impact of maternal protein restriction on the aging process, accelerating the loss of hippocampal function, impacting learning/memory performance, and supposedly developing something like an Alzheimer's syndrome in elderly offspring. Thus, we may carefully suppose that the maternal protein-restricted could be a probable elegant and new AD-simile model in adult male progeny.

*Keywords:* Fetal Programming, Alzheimer's Disease, Behavior and memory, Maternal protein restriction, Hippocampus, β-amyloid peptide, Tau protein.

## INTRODUCTION

Recent evidence shows that adverse events during critical periods of embryonic and fetal development can determine organs and structures of the system and subsequent functional disorders, predisposing individuals to metabolic, cardiovascular, and psychiatric diseases in adulthood [1-6]. Among these events, it can be suggested that maternal submission to psychological and nutritional stresses is involved in fetal programming, as observed in different experimental models [7-12]. The perinatal undernutrition is also a human severe worldwide health problem, especially in underdeveloped countries. It has been estimated that about 3.1 million children die annually due to fetal and childhood malnutrition, resulting in 45% of overall child mortality [13]. During World War II (1944-1945), the Dutch famine caused the twice-increased prevalence of schizophrenia in this population [14]. Another critical moment of recent undernutrition human exposure, known as the Chinese famine period, lasted more than three years (1959-1961) was responsible for extensive morbidity and mortality in China [15]. A recent study demonstrated that maternal Chinese famine promoted a significant incidence of lower cognitive performance, brain development, and mental disorders in adulthood [16]. In the embryonic phase, cell proliferation and meanly cell differentiation of vital organs and neural connections are successfully performed dependently on maternal nutrition. Thus, a poor intrauterine nutritional environment, has been associated with severe psychiatric and neurological disorders such as schizophrenia, autism, and Alzheimer's (AD), characterized by learning and memory dysfunction [17].

It is mostly known that the hippocampus is a central nervous system structure related to emotional behavior being crucial for specialized learning and

memorizes processes. Also, it has been shown that the synaptic plasticity of hippocampal neurons is involved in different functionalities, although the mechanisms of this complex modulation are not known in detail [18]. Many neurofunctional or structural studies evaluating the effects of maternal prenatal malnutrition on the fetus have shown decreased neuronal body size, loss of neurons, reduced apical dendrites, spinal branching, and density in the CA3 pyramidal cell layer [19-22 ]. WHO communications (2015) indicated that several people with dementia are rising steadily in developing countries [23]. Thus, it was estimated that 47.5 million people develop dementia in 2015, with a projection of 75.6 million and 135.5 million, respectively, in 2030 and 2050. AD presents specific pathological brain changes characterized by extracellular deposition of a β-amyloid (Aβ) peptide neurofibrillary tangles from TAU excessive phosphorylation. The significant loss of the number of neurons and synaptic extremities [24] leads to a progressive and significant functional loss of memory. The present study, considering the lack of evidence, aims to analyze the effects of maternal protein restriction [LP] on the offspring by comparing them with those observed in age-matched control rats [NP]. In both experimental groups, learning and memory functions in adulthood were evaluated. In addition to behavioral tests, the present study determines the profile and number of hippocampal cells and the protein content related to the genesis of AD in elderly male progeny, whose mothers were submitted to gestational protein restriction compared to the control offspring.

## MATERIAL AND METHODS

1. *Animals* - The tests were carried out on sibling-mated Wistar HanUnib age-matched rats (250-300g), with free access to tap water and standard rodent feed. The experiments followed the determinations established by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Institutional Ethics Committee (CEUA / UNICAMP # 3655-1). The animals originated from germ-free colonies provided by CEMIB / UNICAMP, Campinas, SP, Brazil. Three weeks after birth and weaning, the offspring were kept at a controlled temperature (25oC) and under light cycle conditions (8:00h to 18:00h), with free access to tap water and standard food for the rodent laboratory (Nuvital, Curitiba, PR, Brazil) up to 12 weeks of age. Mothers were maintained on ad libitum, isocaloric normal protein [NP], (17% protein, n = 20), or low protein [LP] (6% protein, n = 20) diet intake, during the whole gestational period. At the time, the rats were placed to mate, and the presence of sperm in the vaginal smear was designated day 1 of pregnancy. The dams were weighed during pregnancy, and food intake was measured daily. The body weight was recorded once a week. On the birthday, the puppies were weighed and kept only 8 puppies per mother, and all groups returned to NP intake after delivery. The study was performed in male LPs progeny between 48 and 88 weeks of age and compared to the age-matched NP offspring. The survival life of the animals in both groups was established for the progeny and kept in the house of the animals, under suitable environmental conditions previously defined.

*2. Behavioral analysis* - Male progeny of the NP (n=20), and LP (n=20) groups from each litter of different mothers, were submitted to behavioral tests. The test

room procedures were performed during the light cycle and under low-intensity white lighting (5-30 Lux).

*a. Morris water maze* – this test is widely used to evaluates spatial learning in rodents [25]. The cognitive performance test was accessed and analyzed in a blinded fashion by the same trained observer as previously described (Lopes et al., 2013) between the 48th to 50th weeks of age (n = 20) and between 86th to 88th weeks of age (n = 20). The studies were performed in a 170-cm diameter black tank with a depth of 31 cm (water filled at 22◦C) and placed in a dimly-lit test room. The tank was divided into four imaginary quadrants identified with extrinsic clues. A black 1.5 cm submerged hidden platform (12 cm diameter, 30 cm height) was placed in one of this quadrant.

*b. Working memory task* - This test, described by Kesner et al. (2000), was used as a prefrontal cortex (PFC) activity study to assess the ability of offspring to determine a hidden submerged platform position and to maintain this information during four consecutive trials [26]. The working memory tests were performed and data acquisition for 4 days (4 trials/day). For each experiment, the hidden positioning platform was changed, and on the four days, the platform passed through all quadrants. Experimental animals were placed at a different initial point north (N), east (E), south (S) or west (W) at one of each four trial days. A study was finalized when the rat escaped onto the hidden platform. In case of failed escape within 120 s, the rat was gently conducted to the submerged platform. The rats were maintained for 30 s on the escape platform before being undertaken at a new starting point. The latency time spent to reach the submerged platform was recorded in the consecutive trials (in seconds).

*c. Reference memory task* - Described by Morris (1984), the reference memory test assessed the hippocampus function to evaluate progeny ability to find the hidden platform position keeping the information throughout consecutive test days [25]. The analysis was performed, and data acquisition for 4 days (4 trials/day). On the fourth trial days, the hidden platform was kept in the same quadrant. Offspring were placed at a different initial point north (N), east (E), south (S) or west (W) of the each of the four trial days. A study was finalized when the rat escaped onto the hidden platform. In case of failed escape within 120 s, the rat was gently conducted to the submerged platform. The rats were maintained for 30 s on the escape platform before being undertaken at a new starting point. The latency time spent to reach the submerged platform was recorded in the consecutive trials (in seconds).

*d. The Morris water maze proximity measure* - Here, a new study methodology also using the Morris water maze, to assess the determination of the time (or latency) of the animals' proximity to the escape platform, allowed by digital tracking, identifying and establishing the distance and distance position of the animal, every ten measurements in mm, concerning the location of the hidden platform. This proximity measure was recently defined as a highly sensitive strategy for assessing age-related, learning, and memory dysfunction in elderly animals [Gallagher M et al. 2015]. For proximity measure determination, it was developed a collaborative program able to determine ten distances to target each second, for each animal was calculated the sum of the length per second. The averages of the NP (n=8) and LP (n=8) offspring distances were used to measure the animal's proximity to the quadrant where the platform should be (proximity to target in mm/sec).

*e. Elevated plus maze (EPM)* – NP and LP progeny from 48th to 50th (n = 20) and from 86th to 88th (n = 20) weeks of age were tested over 5-min in the EPM, a white polypropylene 'plus'-shaped maze at the height of 72 cm above the floor (ENV-560; Insight Equipment Ltd, Ribeirão Preto, SP, Brazil). The labyrinth consists of two facing open arms (50.8 x 10.2 cm) and two closed arms (50.8 x 10.2 x 40.6 cm). The times spent in the open arms, junction area, and closed arms, as well as the number of entrances and explorations in each section, were recorded using a system of infrared photo beams, the crossings of which were recorded by a computer. The times spent in each compartment of EPM are shown as percentages of the trial's total duration.

*3. Isotropic fractionator* - The hippocampal cells were quantified by Herculano-Houzel and Lent (2005) technique [28]. The sacrificed 88-wk-old NP (n = 5), and age-matched LP (n = 5) offspring were perfused by cardiac puncture, with saline, followed by 4% buffered paraformaldehyde. After left hippocampus isolation, a suspension of nuclei was obtained through mechanical dissociation in a standard solution (40 mM sodium citrate and 1% Triton X-100) in a 40-ml glass Tenbroeck tissue homogenizer. Two ml of the homogenate is collected and centrifuged for 10 min at 6000 RPM. Pelleted nuclei are then washed three times in phosphate-buffered saline (PBS) and suspended in PBS containing 1% 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR, USA). DAPI-stained nuclei were counted in a hemocytometer under a fluorescence microscope at 400× magnification. The total number of cells in the original tissue was estimated by multiplying mean nuclear density by total suspension volume. The total neuron number was determined in a two µl of homogenate

washed in PBS, and incubated overnight at room temperature with anti-NeuN mouse antibody (1:300 in PBS; Chemicon, Temecula, CA, USA). The nuclei were washed in PBS, and incubated in 555-conjugated goat anti-mouse antibody (1:400 in PBS, 10% goat serum, and 50% DAPI; Accurate Chemicals, Westbury, NY, USA) for two hours, collected by centrifugation, washed in PBS, and then suspended in 200 µl of PBS for counting under the fluorescence microscope. The total number of non-neuronal nuclei was calculated by subtracting the number of NeuN containing nuclei from the total number of nuclei.

*4. Immunoblotting* - This technique was performed on how anteriorly described [11,12,29]. Briefly, the 88-wk old NP (n = 5) and age-matched LP (n = 5) offspring were anesthetized. The isolated hippocampus was homogenized in the freshly prepared ice-cold buffer. Bradford method was used for protein quantification in the tissue extracts. For immunoblotting of total protein, extracts were loaded onto the electrophoresis gel (SDS-PAGE). The hippocampal samples were treated with Laemmli buffer containing 100-mmol/l dithiothreitol (DTT), heated in a boiling water bath for 4 min. Then, subject to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Bio-Rad mini gel apparatus (Mini-Protean, Bio-Rad). The proteins transfer 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 blocking buffer (5% non-fat dry milk, 10 mmol/l Tris, 150 mmol/l NaCl, and 0.02% Tween 20). The nitrocellulose blots were incubated at 4°C overnight

with specific antibodies (IGFR1, ERK2, pPI3K, BDNF, Tau, pTau, β amyloid, HSP70, and HSP90). The membranes were subsequently incubated in peroxidase-conjugated secondary antibodies (1:10.000). The bands were detected by chemiluminescence method (RPN 2108 ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and density quantified by optical method (Scion Image software, ScionCorp, Frederick, MD, USA). Images of the developed radiographs were scanned (Epson Stylus 3500), and band intensities were quantified by optical densitometry (Scion Image Corporation). Membranes were stained with reversible Ponceau to discard possible inequalities in protein loading and transfer in western blots [32]. Only homogeneously stained membranes were employed in the study.

*5. Data presentation and statistical analysis* - All data are reported as mean ± SD. Data obtained over time were analyzed by appropriate one-way analysis of variance (ANOVA). Post-hoc comparisons between means used Bonferroni's contrast test when one-way ANOVA analysis indicated statistical differences between groups. Comparisons involving only two samples of independent studies, within or between groups, were evaluated by Student's t-test and the animal's survival lifetime, assessed by the Mantel-Cox and Gehanbreslow-Wilcoxon tests. GraphPad Prism 5.00 performed data analysis (1992-2007 - GraphPad Software, Inc., La Jolla, CA, USA). The level of significance was set at $p \leq 0.05$.

RESULTS

*Gestational low-protein diet affects birthweight and hippocampus masses* – the male LP pup's birth mass was significantly reduced when compared to NP male pups (NP: 6 ± 0.05 g, n = 20 vs. LP: 5.8 ± 0.05 g, n = 20, p = 0.006, Figure 1A). However, on the seventh day of life, the LP mass was similar to observed in the NP offspring (Figure 1B). The brain and hippocampus masses were not different in 50 week-old LP (n = 5) compared to the NP offspring (n = 5, Figure 2). However, in the 88 week-old LP the relative hippocampus, body (H/bd) or brain (H/br) mass ratio, was significantly enhanced relative to age-matched NP hippocampus mass (H/bd, LP: 0.03 ± 0.004, vs. NP: 0.01 ± 0.001 n = 5, p = 0.03 and, H/br, LP: 0.06 ± 0.005, vs. NP: 0.04 ± 0.006 n = 5, p = 0.01, Figure 2).

*Morris water maze*

*a. Working memory* - The working memory estimated by escape latency, was not statistically different in 46 and 86 week-old LP compared to age-matched NP offspring (49-wk-old, n = 20, p = 0.87) or (87-week-old, n = 20, p = 0.078) (Figure 3). These results suggest the same learning response in both experimental groups.

*b. Reference memory* - The hippocampus reference memory was significantly reduced in 49 week-old LP rats. These animals spent substantially more time finding the platform (latency) compared to age-matched NP offspring. When were compared 4º and 7º day trials, the reduced latency (learning capacity) was observed only in NP (NP, n = 20, p = 0.02) compared to LP offspring (n = 20, p = 0.08, Figure 3). However, to 87 week-old offspring study, no reference memory difference was observed for both groups.

*Proximity measure* - The present study showed an elevated sum of ten distance, by second, measured from each animal's position concerning the platform target location (proximity to target in mm/sec) to 87 week-old LP offspring compared to NP rats (LP: 51.6 x 104 ± 9.2 x 104 vs. NP: 48.0 x 104 ± 10.6 x 103, p = 0.05, n = 8 for each group, Fig. 4).

*Elevated plus maze (EPM)* – The current study shows that gestational protein-restricted diet triggers an anxiety-related behavior in adulthood compared to NP offspring in both, 48 and 86 weeks of life. The results show a significant reduction of time spent in open arms (in a sec) in 48 wk.-old LP compared with age-matched NP offspring (LP: 3 ± 1 vs. NP: 14 ± 5, p = 0.04, n = 9) and, in 86-wk-old progeny (LP: 7 ± 3 vs. NP: 37± 17, p = 0.03, n = 8, Fig. 5).

*Hippocampus total cells and neurons quant*ification - The total cell number was significantly enhanced in the hippocampus of LP when compared to NP (LP: 15.98 x 106 ± 17.69 x 105 vs. NP: 12.56 x 106 ± 33.85 x 104, p = 0.047, n = 5 for each group, Figure 6). The estimated neuron number was unaltered in LP compared to NP progeny. However, glial cells number show enhanced in LP comparatively to NP (LP: 13.91 x 106 ± 11.66 x 106 vs. NP: 10.58 x 106 ± 30.03 x 104, p = 0.04, Figure 6)

*Western blot analysis* - The whole hippocampus protein levels by immunoblotting comparative analysis was performed in 88 week-old NP compared to age-matched LP offspring. The current study revealed a 61% enhanced BDNF mature protein levels in LP progeny relative to NP offspring findings (n = 4, p = 0.04; Figure 7). In parallel, whole hippocampus extract also

show an increased Tau, Tau phosphorylated levels in LP offspring (48%, n = 4, p = 0.04) and, β-amyloid proteins level (77%, n = 4, p = 0.04) when compared to NP age-matched offspring. However, stress-related proteins (HSP70 and HSP90), IGFR1, pPI3K, and ERK2 were unchanged in both groups (Figure 7).

*Survival curve* - The animals' survival lifetime, evaluated by the Mantel-Cox and Gehanbreslow-Wilcoxon tests, demonstrated a significant reduction ($p < 0.0001$) in the life span to LP (n=10) offspring compared to NP rats (n=10). The estimated median survival time to NP was 120 weeks, while to LP was 108 weeks. These results showed a significant reduction in survival lifetime in LP when compared to PN offspring (Figure 8).

## DISCUSSION

Previous studies have shown that disturbances during crucial periods of fetal development can determine morphological and functional changes in organs and systems [1-6]. Consistently, the results have shown, in rodents, indicating that protein restriction during pregnancy is accompanied by a reduction in weight at birth, associated with changes in blood pressure related to sex, glucose metabolism and behavioral changes similar to anxiety disorders in male animals when compared to female offspring [30-32]. Sex hormones determine the sexual phenotype's dimorphism in the model of fetal disease programmed in adulthood by changes in long-term control of neural, cardiac, and endocrine functions. Thus, the present study was carried out on male rats, taking into account that the above findings and the reduction of interferences, mainly hormonal, due to gender differences. Previous studies in our laboratory sought

to elucidate the intimate mechanisms underlying fetal programming [11,12,21.33-38]. The present study confirms that the reduction in fetal weight at birth in the LP progeny may be related to inadequate protein intake during pregnancy, interfering in an adequate and healthy growth and development of embryos/fetuses. It was also demonstrated that the body mass of the LP offspring, assessed from the second week of extrauterine life, was similar to that observed in the control group (NP), a phenomenon known as rapid growth recovery or catch-up growth. Besides, the weight of the brains in LP rats at 50 weeks of age concerning the offspring of NP of the same age, were similar. However, in the 88-week-old LP animals, the hippocampus's ratio to body mass or relative to brain mass was significantly increased compared to the hippocampal masses of NP offspring of similar age. Thus, the present study showed by the isotropic fractionation technique, a significant increase in the total number of hippocampal cells, probably glia and others; otherwise, it demonstrated that the number of neurons in the hippocampus of LP offspring rats at 88 weeks of age, was unchanged compared to the offspring of mothers treated with normoprotein ration (NP) of the same age. Previous studies carried out to investigate the effects of maternal malnutrition on brain structure have already demonstrated a significant reduction in neurons [20], with a proportional decrease in the size of neuronal bodies, length of apical dendrites, reduction of ramifications and density of the pyramidal cell layer in different regions of the hippocampus [21,36,39]. Also, it was observed that the hippocampal subfield CA1 reduced the density of pyramidal cells in the molecular stratum [40]. These studies have shown that the dentate gyrus region has a reduced number of dendritic branches and terminations [21,36,41]. However, as far as we know, no

studies are showing these changes in the quantity and neuronal morphological aspects in maternal offspring after the restriction of gestational protein intake.

Previous studies have shown that degenerative neural changes result in a substantial proliferation of microglia cells, which occur at different intensities, and in specific brain regions [42]. Thus, as it has been shown that reactive gliosis is a characteristic of Alzheimer's disease (AD), in the present study, we have unprecedentedly suggested that a likely increased number of microglial cells may be related to pathological findings found in AD in human and brain experimental mouse models [43,44]. However, we cannot rule out a possible contribution from the infiltration of inflammatory cells from the peripheral blood when the blood-cerebrospinal barrier is compromised during the development and evolution of changes similar to those observed in Alzheimer's disease [45-48]. Thus, we can assume that the phenotypic changes in the cellular pattern of the hippocampus, may be related to a potential role in the spread of the pathology of tau [49], loss of synapses [50,51] and neuronal damage due to the release of pro-inflammatory factors [52,53].

In recent studies determining the association of genes in humans, at specific loci, a relationship has been established between the risk of AD and genes highly expressed in microglia cells. Thus, we can dismiss an initial thought that the inflammatory infiltration was an incidental phenomenon triggered by amyloid deposits and in response to dystrophic neuritis. Therefore, the present findings may suggest that the presence of microglia infiltrate in specific brain regions may be a critical phenomenon in the stages that precede the development of the disease. In 1907, Alzheimer himself described the presence of reactive glial cells infiltrating neuritic plaques [54]. In subsequent further studies, the

presence of reactive astrocytes and microglia cells was confirmed in the vicinity of the Aβ amyloid deposits [55], which are currently evidently associated with neuroinflammation, which plays a critical role, both in the origin and evolution of Alzheimer's disease. Although the studies are not yet decisive concerning the role of glial cells in the pathogenesis of AD, in the present study, the high number of hippocampal non-neuronal cells strongly supports this hypothesis, inferring the causal involvement of these cells, despite still conflicting reports between the pathogenic or protective contribution of these cells during the disease. In some regions of the brain, these cells can act protectively by reducing bedsores and promoting debris phagocytosis. Simultaneously, in other situations, they may be functionally compromised with the neural structure contributing to neural degeneration [56]. As such, our understanding of the exact role of astrocytes in AD remains uncertain.

On the other hand, studies have shown a significant reduction in cells of the oligodendrocyte lineage in the gray brain matter associated with the deposition of amyloid plaques, presumably caused by oxidative stress, apoptosis, neuroinflammation or a combination of these factors [57-60]. Thus, in the present model, glial cells could play an essential role in dementia processes, probably by activating microglial cells. Therefore, it is assumed that the reduction of glial cell stimulation is a relevant strategy to counteract the disease's neurodegenerative progression. Thus, we can suggest that the relationship directly involving the volume/mass and cellularity of the hippocampus with manifestations of anxiety, maybe more complicated than expected. A higher number of studies on this topic may be necessary and

critical for a better understanding of the hippocampus's contributions to the understanding of normal or pathological behavioral manifestations.

The present study demonstrates that restricted protein intake during pregnancy may be associated with molecular changes in the hippocampus, which may explain how functional disorders in male rats descend from more treated with LP diet in adulthood. An increase in Tau and β-amyloid proteins' deposition has been consistently demonstrated throughout the 88-week-old LP hippocampus, compared to the age-matched progeny whose mothers were fed during pregnancy with NP feed. However, the semi-quantitative determination of stress-related proteins, such as HSP70 and HSP90, remained unchanged in both experimental groups. The alterations similar to Alzheimer's disease observed can be characterized by the appearance of dementia associated with cerebral astrogliosis, reduced number of neurons, and neuronal atrophy. The structural brain abnormalities typical of AD are also characterized by senile plaques and neuro-fibrillar tangles (NFT), bundles of helical filaments paired with the tau protein associated with the microtubules [61-65].

Currently, the pathological findings of AD are associated with the deposition of neuro-toxic Aβ oligomers formed from the self-association of Aβ monomers. According to current knowledge, the present study was the first to show a significant deposition of hippocampal Aβ in the adult offspring of mothers submitted to protein restriction during pregnancy. Previous research has shown that Aβ monomers are naturally produced and secreted in the synapse gap; Aβ monomers appear to be necessary for neuronal survival since inhibition of Aβ production is associated with the death of neuronal culture [66]. The present study results showed that, despite the increased deposition of β-amyloid in the

hippocampus of the adult progeny whose mothers were subjected to protein-restricted diets, this brain area, in the LP offspring, showed a significant increase in the expression of the neurotrophic factor brain-derived (BDNF). BDNF is one of the most critical factors in stimulating and maintaining the development and survival of neurons in the brain. Considering studies that show that astrocytes produce BDNF, we can assume that the increase in BDNF observed in the present study is compensatory related to the decrease in pro-inflammatory mediators. Thus, it is worth considering that BDNF is essential for the maintenance of adult cortical neurons, whose early dysfunction contributes to short-term memory loss in AD [67]. Therefore, this finding may suggest that this elevation of BDNF is a last attempt to keep the hippocampus functioning, protecting it from further damage. In this scenario, while high levels of BDNF in the hippocampus last, there may be a restriction in cognitive and functional hippocampal decline, at least temporarily.

Lines of evidence indicate that microglial cells are important and significant for establishing learning and memory, modulating neural synaptic structural plasticity dependent on experience [68,69]. However, it is not yet known whether and how microglia cell dysfunction is involved in changes in synaptic circuits, particularly in the fetus and its contribution to neurological, behavioral disorders related to learning and memory, as seen in the present study. In this study, we evaluated the Morris water labyrinth test (MWM), the learning and memory of offspring whose mothers were subjected to gestational protein restriction compared to NP rats [70]. The present study showed significant differences in working memory response, characterized by increased leak latency in LP at 49 weeks of life compared to NP progeny in the same age

group, but not at subsequently older ages. However, a new study methodology also using the Morris water maze, to assess the determination of the time (or latency) of the animals' proximity to the escape platform, allowed by digital tracking, identifying and establishing the distance and distance position of the animal, every ten measurements per second, concerning the location of the hidden platform. This proximity measure was recently defined as a highly sensitive strategy to assessment of age-related, learning, and memory dysfunction in elderly animals [27]. Thus, this measure of proximity between the position of the rat and the hidden platform (in cm) was significantly higher in the LP offspring at 86 weeks of age compared to NP animals of the same age. In the present study, the proximity measure was fundamental and sensitive for these correlational analyses. It took into account changes in the hippocampus neuro-cytology, abnormal protein deposition, and the functional decline in animals in the early aging process. Surprisingly, these data, in particular, may strongly suggest that the hippocampus operational referential memory process can be significantly altered early by restricting gestational protein restriction intake, particularly in elderly rats compared to their controls. The present study, in the experimental circumstances used, also revealed, in an unprecedented way, an association between the response of the referential learning/memory test, changes in the mass and cellularity of the hippocampus, as apparently a consequence of fetal programming in the offspring of aging animals. The present research also demonstrated anxiety-like behavior in LP offspring animals compared to NP offspring of the same age. Previous studies have shown that in patients with depressive conditions compared to healthy controls, a positive relationship between volume/mass of the hippocampus and the

development of anxiety [71]. The present study sought to explore the possible interrelationship between the weight of the hippocampus and anxiety in LP offspring. Interestingly, our study confirmed the hypothesis, showing a positive association between a high mass of the hippocampus and anxiety-like trait in LP animals; however, these data do not allow the rule out of possible confounds arising from the segregation of other behavioral disorders observed in the experiments above. The central nervous system activates the phosphatidyl-inositol-3-kinase pathway (PI3K/AKT), through the recruitment of insulin-like growth factor receptors (IGF-IRs), as well as by the elevation of element-binding proteins of cyclic AMP transcription (CREB) is stimulated by the Aβ monomer, a neuroprotective mediator, by sustaining transcription and release of BDNF [72,73]. In this study, we were unable to show any changes in the phosphorylation of IGF-IR and PI3K, suggesting that different pathways can express BDNF in addition to that comprising AKT kinase activation [56]. Recent studies also indicate that a crucial factor underlying the development and progression of Alzheimer's disease is the tau protein, not the Aβ complex. The present study demonstrated an increase in the expression of tau in the hippocampus associated with an increase of 28% of phosphorylated tau in rats in the LP aging process compared to NP, findings that support this idea. In rodent-like Alzheimer's-like syndrome, cytosolic tau protein is abnormally hyper-phosphorylated and polymerized in PHF.

The vast majority of experimental models of Alzheimer's-like disease consist almost exclusively of models of transgenic mice that express human genes that result in the formation of amyloid plaques and neuro-fibrillar tangles [74-78]. Other models include invertebrate animals like Drosophila melanogaster and

vertebrates like zebra-fish; however, these models presuppose a greater distance to human physiology, although they are extensively used [79-81]. Finding a model of AD that can occur in situations, although abnormal, naturally found in nature, is attractive because it would represent the changes seen in Alzheimer's disease more accurately. Previous studies, including from our laboratory, have shown that protein restriction during pregnancy leads to severe physiological and morphological changes in neurons [33,90-94], in addition to behavioral changes [82,83] and delays in cognitive and intellectual functions [84,85]. Thus, the present study, using the same experimental design of gestational protein restriction, proposes an original, innovative model for Alzheimer's-like syndrome, characterized by increased hippocampal deposition of Aβ and tau proteins. Also, confirming previous studies, LP offspring showed a significant reduction in life span (about 12 weeks) compared to NP offspring [86-89]. By the way, studies that investigated the relationship between early influence in the development phase, fetal programming, and shortening of telomeres were strongly associated with perinatal stressors, such as maternal stress due to dietary restriction or maternal overfeeding [90,94].

*In conclusion*, the results of the present study suggest a high impact of the restriction on protein intake during pregnancy in the aging process, accelerating the loss of hippocampal function, affecting learning/memory performance, and, supposedly, developing a syndrome very similar to Alzheimer's disease in the offspring. Thus, we may carefully suppose that the maternal protein-restricted could be a probable elegant and new AD-simile model in elderly male offspring.

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LEGEND OF FIGURES

Figure 1. Offspring body weight at birth (A) and (B) body weight (in grams) from 4-week to 88-week-old in NP (n = 20) compared to LP (n = 20) offspring. Data are reported as mean ± SD. Over-time results were analyzed by appropriate one-way analysis of variance (ANOVA), with posthoc comparisons between means using Bonferroni's contrast test. The level of significance was set at *$p < 0.05$.

Figure 2. Offspring brain and hippocampus weight (in grams) in 50-week to 88-week-old NP (n = 5) compared to LP (n = 5) progeny. The data are expressed as mean ± SD; Comparisons involving only two samples of independent studies, within or between groups, were evaluated by Student's t-test. The level of significance was set at *p < 0.05.

Figure 3. Graphic representation of the Morris water maze tests for the working memory and reference memory performed at 46 and 86-weeks-old LP (n = 20) compared to age-matched NP (n = 20) offspring. Data are reported as mean ± SD. Over-time results were analyzed by appropriate one-way analysis of variance (ANOVA), with posthoc comparisons between means using Bonferroni's contrast test. The level of significance was set at *p < 0.05.

Figure 4. Graphics representation of the Morris water maze proximity measure performed at 86-weeks-old LP (n = 8) compared to age-matched NP (n = 8) progeny. The data are expressed as mean ± SD; Comparisons involving only two samples of independent studies, within or between groups, were evaluated by Student's t-test. The level of significance was set at *p < 0.05.

Figure 5. Graphics representation of elevated plus maze (EPM) performed at 46 and 86-weeks-old LP (n = 9) compared to age-matched NP (n = 8) progeny. The data are expressed as mean ± SD; Comparisons involving only two samples of independent studies, within or between groups, were evaluated by Student's t-test. The level of significance was set at *p < 0.05.

Figure 6. Effects of maternal protein restriction on 88-week-old LP (n = 5) relative to age-matched NP (n = 5) on whole hippocampus cell number and neurons and non-neuron cell quantification. The data are expressed as mean ± SD; Comparisons involving only two samples of independent studies, within or between groups, were evaluated by Student's t-test. The level of significance was set at *p < 0.05.

Figure 7. Effect of maternal protein restriction on β-amyloid, TAU, BDNF HSP70, HSP90, IGF1R, pPI3K, and ERK2 proteins measured by western blot analysis in the isolated whole hippocampus to 88-week old LP (n = 4) compared to age-matched NP (n = 4) rats. Only one offspring of each litter was used for immunoblotting experiments. The data are expressed as mean ± SD; Comparisons involving only two samples of independent studies, within or between groups, were evaluated by Student's t-test. The level of significance was set at *p < 0.05.

Figure 8. The picture depicted the survival curve evaluated by the Mantel-Cox and Gehanbreslow-Wilcoxon tests to NP (n = 10) compared to LP (n = 10) offspring. The data are expressed as mean ± SD; the significance level was set at *p < 0.05.

FIGURES

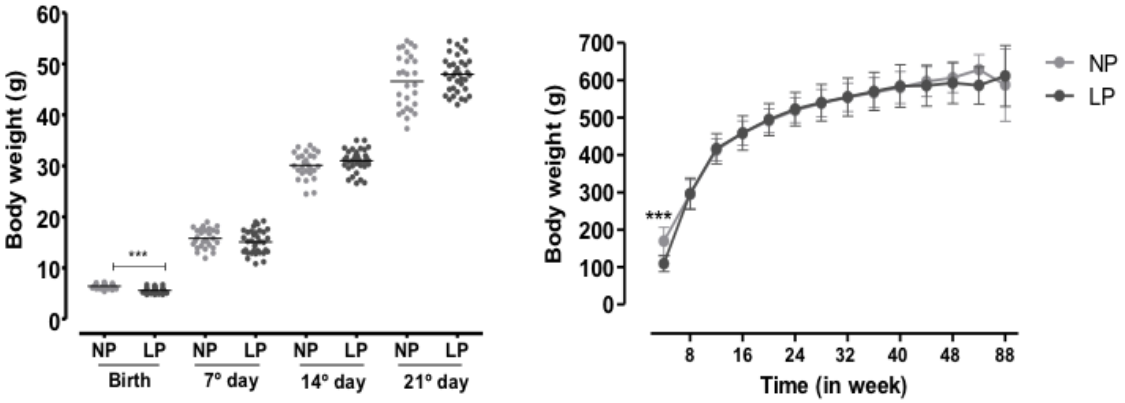


Figure 1

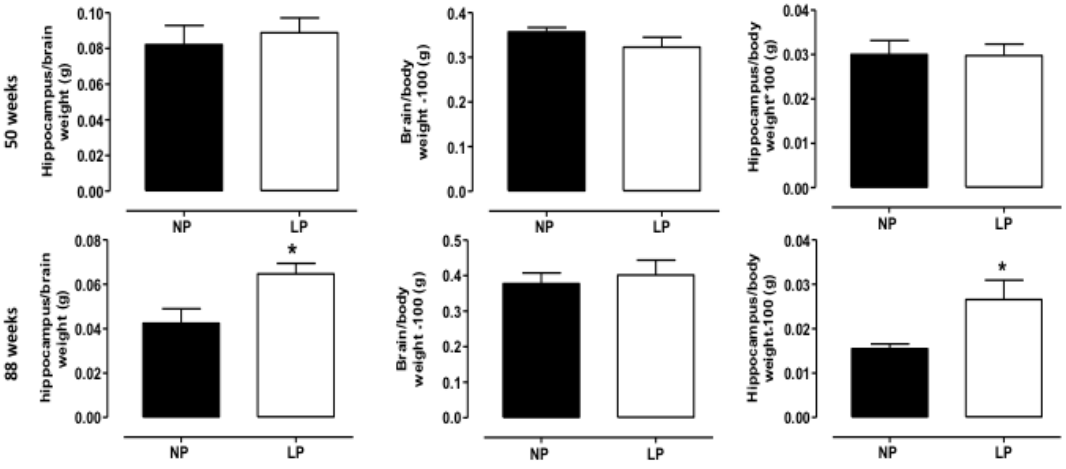


Figure 2

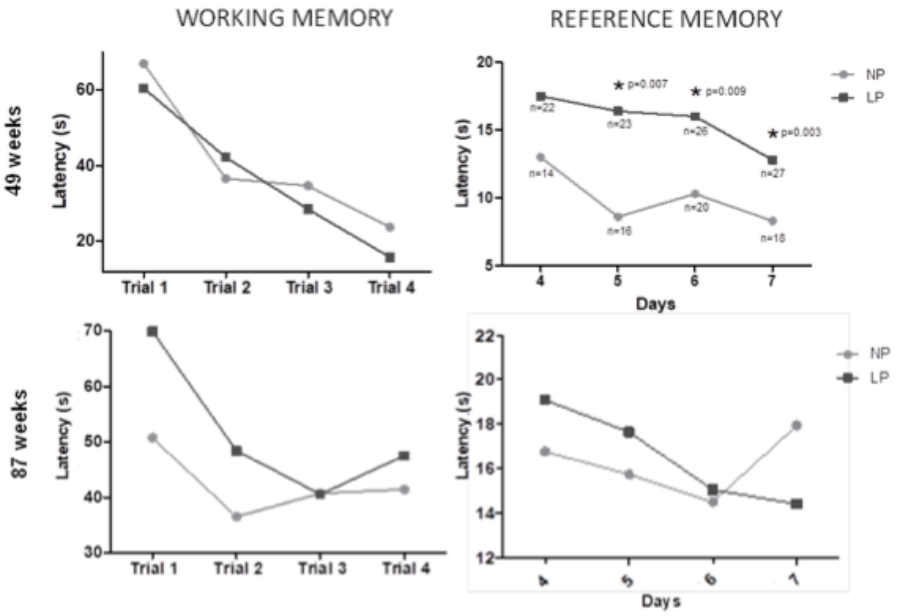


Figure 3

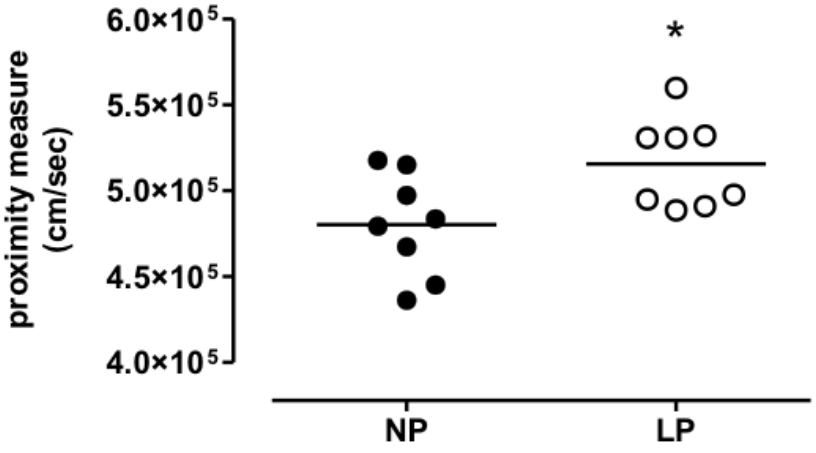


Figure 4

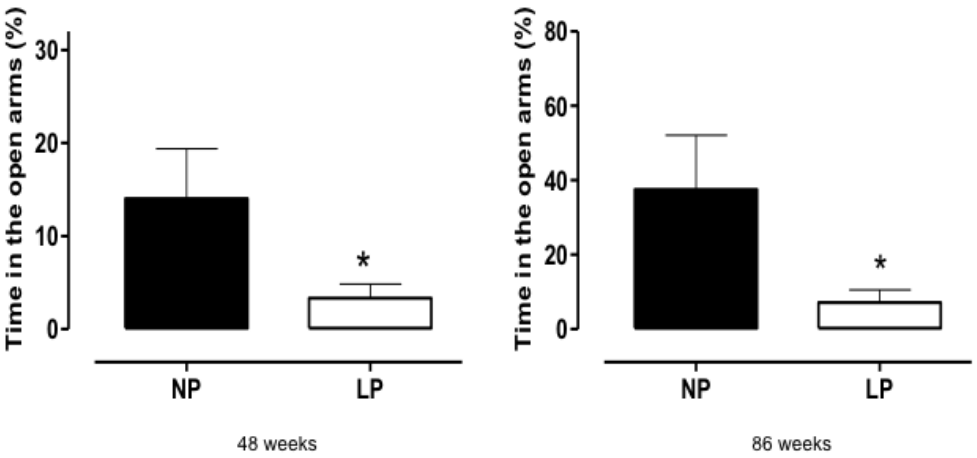


Figure 5

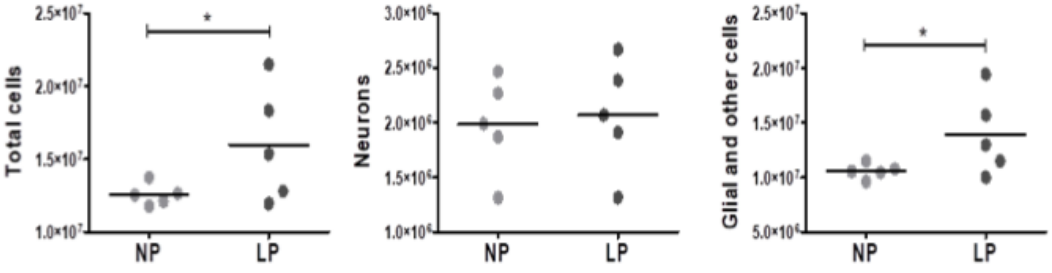


Figure 6

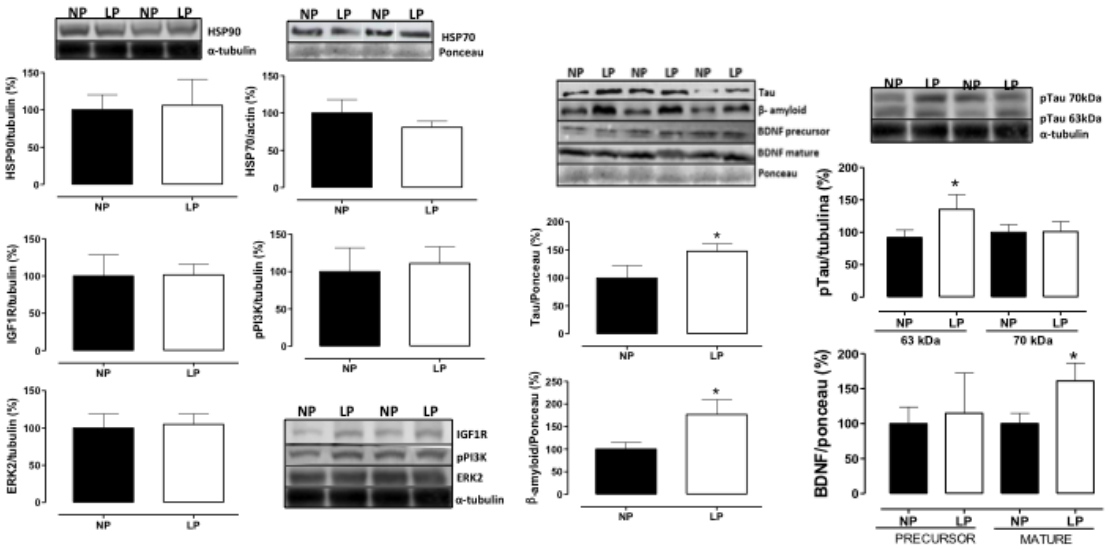


Figure 7

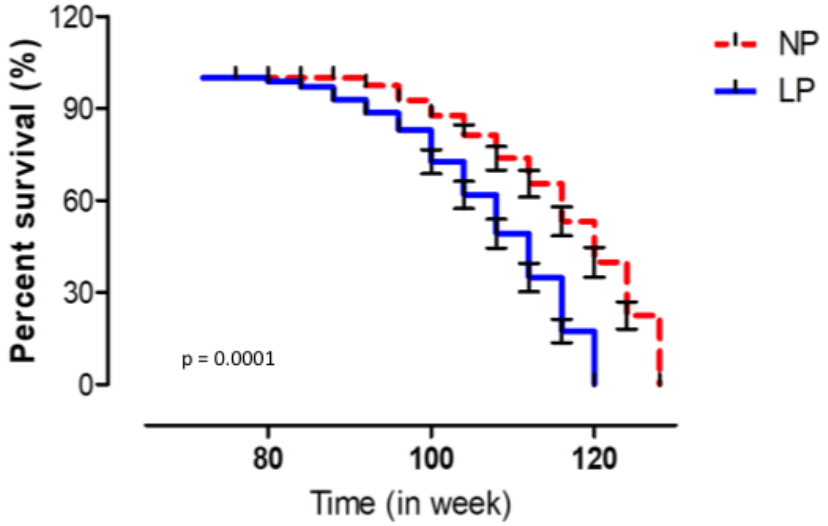


Figure 8

**Table 1.** Composition of standard rodent laboratory diet (standard normal-protein (NP) diet, 17% and low-protein (LP) diet, 6% (AIN 93G)

| **Diet composition** | AIN-93G **diet 6% (in %)** | AIN-93G diet 17**% (in %)** |
|---|---|---|
| Cornstarch | 48.0% | 39.67% |
| **Dextrinizade** Starch (90-94%) | 15.9% | 13.05% |
| **Sucrose** | 12.1% | 10.0% |
| **Carbohydrate** | **76.0%** | **62.72%** |
| **Casein** (84%) | 7.15% | 20.23% |
| **L-cysteine** | 0.1% | 0.3% |
| **Cholin bitartrate** | 0.25% | 0.25% |
| **Protein** | **7.5%** | **20.78%** |
| **Soybean oil** | 7.0% | 7.0% |
| **Total fats** | **7.0%** | **7.0%** |
| **Cellulose microfine (fiber)** | 5.0% | 5.0% |
| **Fiber** | **5.0%** | **5.0%** |
| **Mineral mix** | 3.5% | 3.5% |
| **Vitamin mix** | 1.0% | 1.0% |

| | | |
|---|---|---|
| | 100% | 100% |
| **BHT (Butylhydroxitoluol)** | 0.0014% | 0.0014% |
| **Energy content** | 3.88 kcal.g$^{-1}$ of chow | 3.80 kcal g$^{-1}$ of chow |

## DISCUSSÃO GERAL

(Esta Discussão insere os achados do trabalho de Tese no contexto do conhecimento sobre o tema desenvolvido).

Estudos anteriores demonstraram que distúrbios durante períodos cruciais do desenvolvimento fetal podem determinar alterações morfológicas e funcionais nos órgãos e sistemas [Ashton N, 2000; Barker DJ, 1998; O'Regan et al, 2004; Plagemann A, 2004; Seckl JR and Meaney MJ, 2004; Lesage J, et al, 2006]. De maneira consistente, os resultados têm demonstrado, em roedores, mostrando que a restrição protéica durante a gestação é acompanhada pela redução da massa ponderal ao nascer, associada a alterações na pressão arterial relacionadas ao sexo, no metabolismo da glicose e comportamentais com alterações similares a distúrbios de ansiedade em animais machos quando comparados à prole de fêmeas [Kwong et al. 2000; Ozaki T, 2001; Gillette R, et al. 2017]. Os hormônios sexuais determinam o dimorfismo do fenótipo sexual no modelo de doença fetal programada na idade adulta por alterações no controle a longo prazo das funções neurais, cardíacas e endócrinas. Assim, o presente estudo foi realizado em ratos machos, levando em consideração que os achados acima e a redução das interferências, principalmente hormonais, em decorrência das diferenças de gênero. Estudos anteriores em nosso laboratório buscaram elucidar os íntimos mecanismos subjacentes da programação fetal [Mesquita FF, et al. 2010[a]; Mesquita et al. 2010b; Lopes A, et al. 2013; 33. de Lima MC, et al. 2012; Vaccari B, et al. 2015; Scabora JE, et al. 2015; Torres DB, et al. 2018; Custódio AH, et al. 2017; Assalin HB, et al. 2019]. O presente estudo confirma que a redução do peso

fetal ao nascer, na progênie de LP pode estar relacionada à ingestão inadequada de proteínas durante o período gestacional, interferindo em um crescimento e desenvolvimento saudável e adequado de embriões/fetos. Também foi demonstrado que a massa corporal da prole LP, avaliada a partir da segunda semana de vida extrauterina, foi similar à observada no grupo controle (NP); um fenômeno conhecido como recuperação rápida do crescimento ou ***catch-up growth***, em língua inglesa. Adicionalmente, o peso dos cérebros em ratos LP com 50 semanas de idade em relação à prole de NP de mesma idade, foram semelhante. Entretanto, nos animais LP de 88 semanas de vida, a proporção de hipocampo em relação à massa corporal ou relativa à massa cerebral foram significativamente aumentadas em comparação com as massas hipocampais da prole NP de idade similar. Assim, o presente estudo mostrou pela técnica de fracionados isotrópico, uma elevação significativa do número total de células hipocampais, provavelmente glias e outras; da mesma forma, demonstrou que o número de neurônios no hipocampo de ratos da prole LP com 88 semanas de idade, foi inalterado em comparação com os filhotes de mães tratadas com ração normoprotéica (NP) de mesma idade. Estudos anteriores realizados para investigar os efeitos da desnutrição materna sobre a estrutura cerebral já demonstraram uma redução expressiva de neurônios [Lister JP, et al. 2005], com proporcional diminuição da dimensão dos corpos neuronais, comprimento dos dendritos apicais, redução das ramificação e densidade da camada celular piramidal em diferentes regiões do hipocampo [Lopes A, et al. 2013; Torres DB, et al. 2018; Díaz-Cintra S, et al. 1994]. Além disso, observou-se que o subcampo hipocampal CA1 apresenta uma redução na densidade das células piramidais

no estrato molecular [40]. Esses estudos têm também mostrado, que a região do giro dentado, apresenta um número reduzido de ramificações e terminações dendríticas [Lopes A, et al. 2013; Torres DB et al. 2018; Cintra A, et al. 1990]. No entanto, até onde sabemos, não existem estudos mostrando estas mudanças na quantidade e aspectos neuronais morfológicos na prole materna após a restrição da ingestão proteica gestacional.

Estudos anteriores mostraram que alterações neurais degenerativas resultam em uma proliferação substancial de células da micróglia, que ocorre em diferentes intensidades, e em regiões cerebrais específicas [Kamphuis W, et al. 2012]. Assim, como tem sido demonstrada que a gliose reativa é uma característica da doença de Alzheimer (DA), no presente estudo, sugerimos de forma inédita, que um provável número aumentado de células da micróglias, pode estar relacionado com achados patológicos encontrados na DA em humanos e no cérebro modelos experimentais de camundongos [Olmos-Alonso A, et al. 2016; Fan Z, et al. 2017]. No entanto, não podemos descartar uma possível contribuição da infiltração de células inflamatórias do sangue periférico quando do comprometimento da barreira hemato-liquórica durante o desenvolvimento e evolução de alterações similares aquelas observadas na doença de Alzheimer [Liebner S, et al. 2018; Carrano A, et al. 2011; Erickson MA and Banks WA, 2013; Montagne A, et al. 2015]. Desta forma, nós podemos supor que as alterações fenotípicas no padrão celular do hipocampo, possam estar relacionadas a um potencial papel na disseminação da patologia da tau [49], perda de sinapses [Spangenberg EE and Green KN, 2017; Hong S, et al. 2016] e a danos neuronais pela liberação de fatores pró-inflamatórios [Liddelow SA, et al. 2017; Heneka MT, et al. 2013].

Em recentes estudos determinando a associação de genes em seres humanos, em loci específicos, tem estabelecido uma relação entre o risco para o desenvolvimento de DA e genes altamente expressos em células da micróglia. Desse modo, podemos afastar uma ideia inicial achando que a infiltração inflamatória se tratava de um fenômeno incidental desencadeado por depósitos amiloides e como resposta à neurite distrófica. Portanto, os presentes achados podem sugerir que a presença de infiltrado de micróglias em regiões cerebrais específicas, podem se tratar de fenômenos críticos, nas etapas que precedem o desenvolvimento da doença. Em 1907, o próprio Alzheimer descreveu a presença de células reativas da glia infiltrando as placas neuríticas [Alzheimer A, 1907]. Em estudos adicionais posteriores, foram confirmadas a presença de astrócitos reacionais e de células da micróglia, nas proximidades dos depósitos de amiloide Aβ [Verkhratsky A,  et al. 2016], que atualmente estão evidentemente associados à neuro-inflamação que desempenha papel crítico, tanto na origem como na evolução das doenças de Alzheimer. Embora os estudos não sejam ainda decisivos em relação ao papel da participação das células da glia na patogênese da DA, no presente estudo, o alto número de células não-neuronais do hipocampo apoia fortemente esta hipótese, inferindo o envolvimento causal dessas células, a despeito de relatos ainda conflitantes entre a contribuição patogênica ou protetora destas células durante a evolução da doença. Em algumas regiões do cérebro, estas células podem agir de forma protetora reduzindo escaras e promovendo a fagocitose de debris, enquanto em outras situações podem estar comprometidas funcionalmente com a estrutura neural contribuindo para a degeneração neural [Alibhai JD,  et al. 2018]. Como tal, nossa compreensão sobre o papel exato do

astrócitos na DA permanece incerta. Por outro lado, estudos tem demonstrado uma significativa redução de células da linhagem de oligodendrócitos na substância cerebral cinzenta associada à deposição de placas amiloides, presumivelmente, causadas por estresse oxidativo, apoptose, neuro-inflamação ou uma combinação deste fatores [Mitew S, et al. 2010; Behrendt G, et al. 2013; Nasrabady SE, et al. 2018; Cai Z and Xiao M, 2016]. Assim, parece que no presente modelo, as células da glia desempenham um papel essencial nos processos demenciais, provavelmente pela ativação das células da micróglia. Portanto, presume-se que a redução da estimulação das células da glia, seja uma estratégia relevante para contrabalançar a progressão neuro-degenerativa da doença. Desta forma podemos sugerir que a relação envolvendo diretamente o volume/massa e celularidade do hipocampo com manifestações de ansiedade, pode ser mais complicada do que se espera. Um numero maior de estudos sobre esse tema podem ser necessários e críticos para a melhor compreensão das contribuições do hipocampo para o entendimento de manifestações comportamentais normais ou patológicas.

O presente estudo demonstra que a ingestão restrita de proteínas na gestação pode estar associada a alterações moleculares no hipocampo, o que pode explicar os distúrbios funcionais em ratos machos descendentes de mais tratadas com dieta LP em idade adulta. Um aumento da deposição de proteínas Tau e β-amiloide foi consistentemente demonstrado em todo o hipocampo de LP com 88 semanas de idade, em comparação com a progênie pareada por idade cujas mães foram alimentadas durante a gestação com ração NP. Entretanto, a determinação semi-quantitativa de proteínas relacionadas ao estresse, tais como HSP70 e HSP90, permaneceram

inalteradas nos dois grupos experimentais. As alterações similares a doença de Alzheimer observadas, podem ser caracterizadas pelo aparecimento de demência associada à astrogliose cerebral, número reduzido de neurônios e atrofia neuronal. As anormalidades estruturais cerebrais típicas da DA são também caracterizadas pela presença de placas senis e emaranhados neuro-fibrilares (NFT), feixes de filamentos helicoidais emparelhados da proteína tau associada aos micro-túbulos [Brion JP, et al. 1985; Ihara Y, et al. 1986; Kosik KS, et al 1989; Wischik CM, et al. 1988; Goedert M, et al, 1992a].

Atualmente, os achados patológicos da DA estão associados à deposição de oligômeros neuro-tóxicos de Aβ, formados a partir da auto-associação de monômeros de Aβ. De acordo com o conhecimento atual, o presente estudo foi o primeiro a mostrar uma deposição significativa de Aβ hipocampal na prole adulta de mães submetidas à restrição proteica durante a gestação. Pesquisas anteriores demonstraram que os monômeros de Aβ são naturalmente produzidos e secretados na fenda sinapses; os monômeros de Aβ parecem ser necessários para a sobrevivência neuronal, uma vez que a inibição da produção de Aβ está associada à morte de neurônios em cultura [Abramov E, 2009]. Os resultados do presente estudo demonstraram que, apesar do aumento da deposição de β-amiloide no hipocampo da progênie adulta cujas mães foram submetidas a dietas com restrição de proteínas, essa área cerebral, na prole LP, apresentou um aumento significativo na expressão do fator neurotrófico derivado do cérebro (BDNF). O BDNF é um dos fatores mais importantes no estimulo e na manutenção manutenção do desenvolvimento e sobrevivência dos neurônios no cérebro. Considerando estudos que mostram que os astrócitos produzem BDNF, podemos supor que o

aumento do BDNF observado no presente estudo esteja relacionado compensatoriamente, à diminuição nos mediadores pró-inflamatórios. Assim, vale pressupor que o BDNF seja essencial para a manutenção dos neurônios corticais adultos, cuja disfunção precoce contribui para a perda de memória de curto prazo na DA [Nagahara AH, 2009]. Portanto, esse achado pode ser sugerir que esta elevação de BDNF, trata-se de uma última tentativa de manter o hipocampo funcionando, protegendo-o de danos adicionais. Nesse cenário, enquanto durar os níveis elevados de BDNF no hipocampo, pode estar havendo uma restrição no declínio cognitivo e funcional hipocampal, pelo menos temporariamente.

Linhas de evidências indicam que células da micróglia sejam importantes e de maneira significativa para o estabelecimento da aprendizagem e da memória, modulando a plasticidade estrutural sináptica neural dependente da experiência [Grutzendler J, et al. 2002; Yang G, et al. 2009]. No entanto, ainda não se sabe se e como a disfunção de células da micróglia está envolvida nas alterações dos circuitos sinápticos, particularmente, no feto e sua contribuição para distúrbios neurológicos, comportamentais relacionados ao aprendizado e à memória, como visto no presente estudo. Neste estudo, avaliamos pelo teste de labirinto aquático de Morris (MWM), o aprendizado e a memória de filhotes cujas mães foram submetidas à restrição de proteína gestacional em comparação com ratos NP [Stewart CA and Morris RGM, 1993]. O presente estudo mostrou diferenças significativas na resposta da memória de trabalho, caracterizada pelo aumento da latência de escape no LP com 49 semanas de vida em comparação com os filhos NP na mesma faixa etária mas, não em idades subsequentemente

maiores. No entanto, a utilização de nova metodologia de estudo utilizando o labirinto aquático de Morris, para avaliar a determinação do tempo (ou latência) de proximidade dos animais em relação a plataforma de escape, permitiram o rastreamento digital, identificando e estabelecendo a distancia e a posição do animal, a cada dez medidas por segundo, em relação à localização da plataforma encoberta. Esta medida de proximidade foi recentemente definida como estratégia de alta sensibilidade para a definição de disfunção relacionada a idade, do aprendizado e da memoria em animais idosos [Gallagher M, et al. 2015]. Assim, esta medida de proximidade entre a posição do rato e a plataforma oculta (em cm) foi significativamente maior na prole LP com 86 semanas de idade comparada aos animais NP de mesma idade. No presente estudo, a medida de proximidade foi fundamental e sensível para estas análises correlacionais, levando em consideração alterações na neuro-citologia do hipocampo, a deposição anormal de proteínas e, o declínio funcional nos animais em processo precoce de envelhecimento. Surpreendentemente, esses dados em particular podem sugerir fortemente que o processo de memória referencial operacional do hipocampo pode ser significativamente alterado precocemente pela restrição ingestão de restrição protéica gestacional, particularmente em ratos idosos comparados com seus controles. O presente estudo, nas circunstancias experimentais utilizadas, também revelou de maneira inédita, uma associação entre a resposta do teste de aprendizado/memória referencial, alterações na massa e na celularidade do hipocampo, como aparentemente, consequência de programação fetal em prole de animais em envelhecimento precoce. A presente pesquisa também demonstrou um comportamento ansiedade-símile em animais da prole LP

comparados aos animais da prole NP de mesma idade. Estudos anteriores demonstraram que em pacientes com quadro depressivos comparados a controles saudáveis, uma relação positiva entre volume/massa do hipocampo e o desenvolvimento de ansiedade [Kalisch R, et al. 2006]. No presente estudo procurou-se explorar a possível inter-relação entre a massa do hipocampo e a ansiedade na prole de LP. Curiosamente, nosso estudo confirmou a hipótese, mostrando uma associação positiva entre elevada massa do hipocampo e característica semelhante à ansiedade nos animais LP; no entanto, esses dados não permitem descartar possíveis interferências decorrentes da segregação de outros transtornos comportamentais observados nos experimentos acima.

O sistema nervoso central ativa a via da fosfatidil-inositol-3-cinase (PI3K/AKT), através do recrutamento de receptores de fatores de crescimento semelhante à insulina (IGF-IRs), bem como, pela elevação de proteínas de ligação ao elemento de transcrição de AMP cíclico (CREB) estimulada pelo Aβ monômero, um mediador neuro-protetor que em ultima instancia, sustenta a transcrição e produção de BDNF [Zimbone S, et al. 2018; Giuffrida ML et al 2009]. Neste estudo nós não fomos capazes de mostrar nenhuma alteração na fosforilação de IGF-IR e PI3K, sugerindo que o BDNF pode ser expresso por diferentes vias adicionais àquela que compreende a ativação da AKT quinase [Alibhai JD et al 2018)]. Estudos recentes também indicam que um fator crucial subjacente ao desenvolvimento e progressão da doença de Alzheimer é a proteína tau, e não o complexo Aβ. O presente estudo demonstrou um aumento da expressão da tau no hipocampo associado a elevação de 28% de tau fosforilada em ratos em processo de envelhecimento LP comparados com

NP, achados que apoiam a referida ideia. Na síndrome Alzheimer-símile de roedores, a proteína tau citosólica é anormalmente hiper-fosforilada e, polimerizada em PHF.

A grande maioria dos modelos experimentais de doença de Alzheimer-símile consiste quase exclusivamente, de modelos de camundongos transgênicos que expressam genes humanos que resultam na formação de placas amiloides e emaranhados neuro-fibrilares [Boutajangout A and Wisniewski T, 2014; Dujardin S, et al. 2015; Puzzo D, et al; 2015; Wisniewski T and Goni F, 2015; Wisniewski T and Sigurdsson EM, 2010]. Outros modelos, incluem animais invertebrados como *Drosophila melanogaster* e vertebrados como *zebra-fish*; no entanto, estes modelos pressupõe a uma maior distância á fisiologia humana, embora extensivamente usados [Bouleau S and Tricoire H, 2015; Hannan SB, et al. 2016; Newman M, et al. 2014]. Encontrar um modelo de AD que possa ocorrer em situações, embora anormais, naturalmente encontradas na natureza é atraente, porque representaria com mais precisão as alterações encontradas na doença de Alzheimer. Estudos anteriores, incluindo de nosso laboratório, demonstraram que a restrição de proteínas durante a gestação leva a graves alterações fisiológicas e morfológicas de neurônios [de Lima MC, et al. 2012; Huang LT, et al. 2003 Jennings BJ, et al. 1999; Ozanne SE and Hales CN, 2004; Martin-Gronert MS, et al. 2008; Langley-Evans SC and Sculley DV, 2006], além de alterações comportamentais [de Oliveira LM, 1985; Riul TR, et al. 1999] e atrasos nas funções cognitivas e intelectuais [Barnes R.H, 1976; Wainwright PE and Colombo J, 2006]. Assim, o presente estudo utilizando o mesmo desenho experimental de restrição proteica gestacional, propõe um modelo experimental original para a síndrome

Alzheimer-símile, caracterizada pelo aumento da deposição hipocampal das proteínas Aβ e tau. Além disso, confirmando estudos anteriores, a prole LP apresentou uma redução significativa no tempo de vida (cerca de 12 semanas) em relação à prole NP [Hermel et al 2001; Feoli AM, et al 2006; Sayer AA, et al 2001]. A propósito, estudos que investigaram a relação entre a influência no início da fase de desenvolvimento, programação fetal e encurtamento de telômeros foram fortemente associados à presença de estressores perinatais, tais como estresse materno pela restrição alimentar ou hiper-alimentação materna [Huang LT, et al. 2003; Jennings BJ, et al. 1999; Ozanne SE and Hales CN, 2004; Martin-Gronert MS, et al. 2008; Langley-Evans SC and Sculley DV, 2006]. Em conclusão, os resultados do presente estudo confirmam resultados anteriores e sugerem um alto impacto da restrição a ingestão de proteínas durante a gestação no processo de envelhecimento, acelerando a perda da função hipocampal, afetando o desempenho da aprendizagem/memória e, supostamente, desenvolvendo uma síndrome muito similar a doença de Alzheimer na prole. Podemos portanto supor com cuidado devido, que a restrição materna de proteínas durante a gestação, pode ser um possível e mais próximo e elegante modelo desta incidente doença humana – um novo modelo de desenvolvimento relativamente precoce de Alzheimer-símile em roedores machos.

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**ANEXO 1**

Manuscript Receipt Confirmation

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para gontijo, gabrielboergrigoletti, marcelolopes46, anatbfranco, marceladamico, boer

Dear Dr. Gontijo,

Thank you for your submission of the manuscript entitled "Impact of maternal low-protein intake on hippocampus cellularity and behavioral test of male adult offspring: an Alzheimer-simile syndrome model?."

We will review your submission and assign a tracking number as soon as possible.

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**C E R T I F I C A D O**

Certificamos que o projeto "**NEUROGÊNESE HIPOCAMPAL EM RATOS SUBMETIDOS À RESTRIÇÃO PROTEICA DURANTE A ONTOGÊNESE ENCEFÁLICA: ESTUDO COMPORTAMENTAL E INFLUÊNCIAS DO AMBIENTE ENRIQUECIDO**" (protocolo nº **3634-1**), sob a responsabilidade de **Prof. Dr. José Antonio Rocha Gontijo / Gabriel Boer Grigoletti Lima**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

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

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **1º. de dezembro de 2014**.

Campinas, 1º. de dezembro de 2014.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

CEUA/UNICAMP
Caixa Postal 6109
13083-970 Campinas, SP – Brasil

Telefone: (19) 3521-6359
E-mail: comisib@unicamp.br
http://www.ib.unicamp.br/ceea/