

Vanessa Valgas dos Santos

**INVESTIGAÇÃO DOS EFEITOS NEUROPROTETORES DA
GRELINA E DO NEUROPEPTÍDEO Y EM UM MODELO
EXPERIMENTAL DA DOENÇA
DE ALZHEIMER**

Tese apresentada ao programa de Pós-Graduação em Neurociências da Universidade Federal de Santa Catarina como requisito para a obtenção do título de Doutor em Neurociências.

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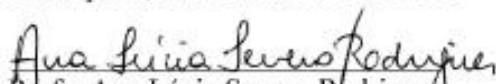
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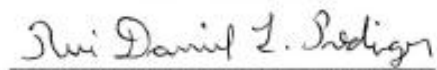
**“INVESTIGAÇÃO DOS EFEITOS NEUROPROTETORES
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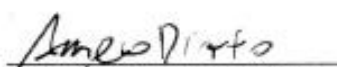
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**“A tarefa não é ver aquilo que ninguém viu, mas pensar o que
ninguém ainda pensou sobre algo que todo mundo vê...”**
Arthur Schopenhauer

“A gratidão é a memória do coração”

Dedico esta tese a minha
mãe, o alicerce de minha existência, por me [trans] formar em quem
sou...

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RESUMO

O acúmulo da proteína beta amiloide ($A\beta$) no sistema nervoso central (SNC) e os prejuízos cognitivos são sinais clássicos da doença de Alzheimer que estão fortemente associados ao estresse oxidativo e às alterações colinérgicas. Um número crescente de trabalhos tem relacionado à obesidade como um fator de risco para o desenvolvimento da doença de Alzheimer. Durante o ganho de peso existe uma redução nos níveis plasmáticos e no SNC dos hormônios orexígenos grelina (Ghr) e neuropeptídeo Y (NPY), que além de regularem a ingesta de alimentos, também participam da modulação de processos cognitivos, emocionais e neurodegenerativos. No presente estudo, foram investigados os efeitos do pré-tratamento com Ghr ou NPY sobre as alterações comportamentais e neuroquímicas induzidas pela infusão intracerebroventricular (i.c.v.) do peptídeo $A\beta$ 1-40, utilizado como um modelo experimental da doença de Alzheimer. Grupos independentes de camundongos albinos (3 meses de idade) receberam uma administração aguda de Ghr (3 nmol/ μ l, i.c.v.), NPY (0.0234 μ mol/ μ L, i.c.v.) ou PBS (i.c.v.) 15 min antes da infusão do $A\beta$ 1-40 (400 pmol/camundongo, i.c.v.). De 9 a 14 dias após os tratamentos, os animais foram avaliados em uma bateria de testes comportamentais para a investigação das funções cognitivas (realocação do objeto), emocionais (suspensão pela cauda e labirinto em cruz elevado) e locomotoras (campo aberto). Ao final dos testes comportamentais, os animais foram sacrificados para a avaliação da captação de glutamato no hipocampo. As análises de parâmetros bioquímicos relacionados ao estresse oxidativo e atividade da enzima acetilcolinesterase (AChE) foram realizadas 24 h após o tratamento com Ghr, NPY e $A\beta$ 1-40. O tratamento prévio com Ghr ou NPY preveniu os prejuízos na memória espacial e aumento no tempo de imobilidade no teste de suspensão pela cauda induzidos pela infusão i.c.v. de $A\beta$ 1-40. Além disso, o peptídeo $A\beta$ 1-40 induziu uma significativa peroxidação lipídica, redução na atividade da enzima glutatona redutase (GR) e na captação de glutamato, aumento na atividade da enzima AChE no córtex pré-frontal e/ou hipocampo de camundongos que foram prevenidos pelo pré-tratamento com Ghr ou NPY. Em conjunto, os resultados do presente estudo sugerem que a Ghr e o NPY são capazes de prevenir os déficits cognitivos induzidos pelo peptídeo $A\beta$ 1-40 e apresentar um comportamento tipo antidepressivo em camundongos, sendo estes efeitos protetores mediados, ao menos em

parte, pela inibição do estresse oxidativo e disfunção dos sistemas glutamatérgico e colinérgico.

Palavras chave: doença de Alzheimer; peptídeo beta amiloide ($A\beta_{1-40}$); grelina, neuropeptídeo Y, memória espacial, depressão, estresse oxidativo, glutamato, acetilcolinesterase

ABSTRACT

The accumulation of amyloid beta ($A\beta$) protein in the central nervous system (CNS) and the cognitive impairments are classic signs of Alzheimer's disease that are strongly associated with oxidative stress and changes in cholinergic system. A growing number of studies have related the obesity as a risk factor for the development of Alzheimer's disease. During the weight gain there is a reduction in CNS and plasmatic levels of orexigenic hormones ghrelin (GHR) and neuropeptide Y (NPY), which regulates the food intake and also participates in the modulation of cognitive, emotional and neurodegenerative disorders. In this study, we investigated the effects of pretreatment with Ghr or NPY on the behavioral and neurochemical changes induced by intracerebroventricular (i.c.v.) infusion of $A\beta$ 1-40 peptide, used as an experimental model of Alzheimer's disease. Independent groups of Swiss albino mice (3 months old) received a single acute administration of Ghr (3 nmol/ μ L, i.c.v.), NPY (0.0234 μ mol/ μ L, i.c.v.) or PBS (i.c.v.) 15 min before infusion of $A\beta$ 1-40 (400 pmol/mouse, i.c.v.). The animals were evaluated 9 to 14 days after the treatment on a battery of behavioral tests for the investigation of cognitive (object location), emotional (tail suspension and elevated plus-maze) and locomotion (open field) functions. At the end of behavioral tests, the animals were sacrificed for the evaluation of the glutamate uptake in hippocampus. The analysis of biochemical parameters related to oxidative stress and activity of the enzyme acetylcholinesterase (AChE) were performed 24 h after treatment with Ghr, NPY and $A\beta$ 1-40. Pretreatment with Ghr or NPY prevented the decline in spatial memory and the increased in immobility time in tail suspension test induced by the infusion icv of $A\beta$ 1-40. Moreover, the peptide $A\beta$ 1-40 induced a significant lipid peroxidation, reduction in glutathione reductase (GR) activity and glutamate uptake, also causes an increase in enzyme AChE activity in the prefrontal cortex and/or hippocampus of mice that were prevented by pretreatment with Ghr or NPY. Altogether, the result of this study suggest that Ghr and NPY are capable of preventing cognitive deficits induced by peptide $A\beta$ 1-40 and present an antidepressant like-effect in mice, and these protective effects are mediated, at least in part, by inhibition of stress oxidative and dysfunction of the glutamatergic and cholinergic system.

Keywords: Alzheimer's disease, amyloid-beta peptide (A β 1–40); ghrelin; neuropeptide Y; spatial memory; depression; oxidative stress, glutamate, acetylcholinesterase

LISTA DE ABREVIÇÕES

%	Porcentagem
6-OHDA	6-hidroxidopamina
AChE	Enzima acetilcolinesterase
aCSF	Líquido cefalorraquidiano artificial
AgRP	Proteína relacionada ao Agouti
apoE	Apolipoproteína E
APP	Proteína precursora amiloide
ARC	Núcleo arqueado do hipotálamo
Asp1	Aspartato 1
A β	Proteína Beta-amiloide
A β ₁₋₄₀	Peptídeo beta-amiloide com 40 aminoácidos
A β ₁₋₄₂	Peptídeo beta-amiloide com 42 aminoácidos
CaCl ₂	Cloreto de cálcio
CART	Transcrito regulado por cocaína e anfetamina
CAT	Enzima catalase
CO ₂	Gás carbônico
C-PON	Peptídeo flanqueador no terminal carboxílico
CTF β	Resíduo C- terminal (CTF β) do fragmento A β
DA	Doença de Alzheimer
DNA	Ácido desoxirribonucleico
DP	Doença de Parkinson
DPP4	Dipeptidil peptidase 4
DTNB	5,5-ditiobis 2-nitrobenzoato
GH	Hormônio do crescimento
GHS-R1a	Receptor secretagogo de homônio de crescimento do tipo 1 ^a
Glu 11	Glutamina 11
GOAT	Enzima grelina-O-aciltransferase
GPx	Enzima glutaciona peroxidase
GR	Enzima glutaciona redutase
GSH-R	Receptor secretagogo de homônio de crescimento
GSSH	Glutaciona oxidada
H ₂ O ₂	Peróxido de Hidrogênio
HEPES	Ácido 2-[4-(2-hidroxietil)1-piperazinil]-etanosulfônico
HFS	Estimulação de alta frequência
Hz	Hertz

i.c.v.	Intracerebroventricular
IBGE	Instituto Brasileiro de Geografia e Estatística
ICM	Índice de Massa Corporal
INRS	Receptores para insulina
KCl	Cloreto de potássio
LTD	Potenciação de Longa Depressão
LTP	Potenciação de Longa Duração
MgSO ₄	Sulfato de magnésio
MPP+	1-metil-4-fenilpiridínio
Na ₂ HPO ₄	Fosfato de sódio
NaCl	Cloreto de sódio
NaHCO ₃	Bicarbonato de sódio
NaOH	Hidróxido de sódio
NMDA	N-metil-D-aspartato
NPSH	Tióis não proteicos
NPY	Neuropeptídeo Y
O ₂	Oxigênio
ObR	Receptores para Leptina
PAM	Enzima peptidil-glicina α -amidante monoxigenase
PBS	Tampão fosfato salina
POMC	Pro-opiomelanocortina
PP	Peptídeo pancreático
PSEN1	Enzima Presenilina 1
PSEN2	Enzima Presenilina 2
PYY	Peptídeo YY
ROS	Espécies reativas de oxigênio
SDS	Dodecil sulfato de sódio
Ser3	Serina 3
SNC	Sistema nervoso central
TBA-RS	Substâncias reativas ao ácido tiobarbitúrico
TCA	Ácido tricloroacético
TNB	Ácido 2-nitro-5-mercapto –benzóico
UCP	Proteínas desacopladoras
α -MSH	α -melanotropina

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1. INTRODUÇÃO

1.1. Doença de Alzheimer, uma breve descrição

A história científica da doença de Alzheimer (DA) teve seu início em 25 de Novembro de 1901 quando o neurologista alemão Alois Alzheimer admitiu Auguste Deter como paciente no asilo de Frankfurt. Em 1906 a paciente veio a óbito e o grande *insight* do Dr. Alzheimer foi realizar análises histopatológicas do cérebro da paciente utilizando a técnica de impregnação argêntica, e associar os achados da morte neuronal, placas neuríticas e emaranhados neurofibrilares com as manifestações clínicas de desorientação, perda de memória, prejuízo cognitivo, afasia e apraxia que vinham sendo apresentadas pela paciente. Estes resultados foram apresentados em Novembro do mesmo ano na cidade de Tübingen (Alemanha), no XXXVII *Versammlung Südwestdeutscher Irrenärzte* (37º Encontro de Psiquiatria do Sudoeste Alemão) intitulado “Sobre uma enfermidade característica do córtex cerebral” e publicado no ano de 1907 (Graeber, Kösel *et al.*, 1997).

O segundo paciente, Johann F., aparentemente portador da mesma patologia, foi descrito novamente pelo Dr. Alzheimer em 1910. Convencido de que se tratava de uma nova patologia, Emil Kraepelin, o pioneiro da psiquiatria moderna, introduziu no mesmo ano o termo DA na edição do seu Tratado de Psiquiatria (Klünemann, Fronhöfer *et al.*, 2002).

Desde estas primeiras descrições sobre a DA, muitos avanços foram realizados no entendimento da doença e atualmente sabe-se que as características microscópicas visualizadas pelo Dr. Alzheimer tratam-se de agregados proteicos compostos em sua maioria pela proteína tau hiperfosforilada, uma proteína fisiologicamente envolvida na estabilização dos microtúbulos do citoesqueleto dos neurônios (Blennow, De Leon *et al.*, 2006). O componente majoritário das placas senis é o peptídeo beta-amilóide (A β), composto preferencialmente por 40 ou 42 aminoácidos e produzido pela clivagem da proteína precursora amiloide (APP) (Selkoe, 2011; Huang e Mucke, 2012).

A APP é uma proteína transmembranar processada pós-traducionalmente por proteases denominadas de α -, β - e γ -secretases (Ortega, Stott *et al.*, 2012). A APP pode sofrer duas clivagens distintas, a primeira (denominada não-amiloidogênica), através da ação da enzima α -secretase (entre os resíduos de Lis 16 e Leu 17) seguida pela enzima γ -secretase, com consequente formação do fragmento p3 solúvel e atóxico. E a segunda (denominada de via amiloidogênica), onde a APP é inicialmente clivada por uma protease aspártica, denominada β -secretase (Asp 1 e Glu 11), gerando as proteínas precursoras β amilóide

solúveis (APPs- β) e resíduos C-terminal (CTF β) do fragmento β . CTF β é então clivado pela γ -secretase produzindo os fragmentos A β com 40 ou 42 aminoácidos (Citron, Oltersdorf *et al.*, 1992; Haass e Selkoe, 2007; Winkler, Kamp *et al.*, 2012).

Com os avanços nos estudos a respeito desta patologia, observou-se que esta é a mais frequente doença neurodegenerativa e a causa mais comum de demência associada ao envelhecimento, sendo estimado que atualmente existam 36 milhões de pessoas acometidas pela DA no mundo (Alzheimer's Association, 2012). Acredita-se que somente nos Estados Unidos, os custos relacionados a esta patologia atinjam a cifra de US\$200 bilhões anualmente; estes valores estão relacionados aos gastos médicos diretos e a perda da produtividade do paciente (Alzheimer's Association, 2012).

Ao longo dos últimos 50 anos, a população brasileira quase triplicou: passou de 70 milhões, em 1960, para 190,7 milhões, em 2010. O crescimento do número de idosos, no entanto, foi ainda maior. Os idosos -pessoas com mais de 60 anos- somam 23,5 milhões dos brasileiros, mais que o dobro do registrado em 1991, quando esta faixa etária contabilizava 10,7 milhões de pessoas. Apesar deste processo ser considerado um grande triunfo em nosso país, é também considerado um grande desafio para nossa sociedade (IBGE, 2012). No Brasil não existem dados estatísticos precisos sobre a incidência da DA, entretanto, baseando-se nos dados estatísticos do IBGE, pode-se estimar que existam cerca de 1,2 milhões de brasileiros acometidos pela DA nos dias atuais (IBGE, 2012).

Os custos associados à DA, incluindo com cuidados médicos diretos e indiretos, gastos com cuidadores, e custos não médicos tais como a perda de produtividade no trabalho do paciente e do cuidador variam entre os estudos, mas os custos de demência em todo o mundo foram calculados em aproximadamente US\$160 bilhões (Alzheimer's Association, 2012).

Os avanços no entendimento dos mecanismos fisiopatológicos relacionados à DA apontam para novas estratégias no desenvolvimento de tratamentos mais eficazes, visto que esta é uma doença que ainda não tem cura e o principal objetivo das medicações atuais é minimizar os sintomas e a progressão da enfermidade (Barak e Aizenberg, 2010). Dentre os tratamentos utilizados estão às intervenções psicossociais e as estratégias farmacológicas, limitadas ao aumento da disponibilidade de acetilcolina na fenda sináptica através da inibição da enzima acetilcolinesterase (AChE) pelo uso de anticolinesterásicos (ex: donepezil e rivastigmina) e do bloqueio dos receptores glutamatérgicos

do tipo N-metil-D-aspartato (NMDA) (ex: memantina). Entretanto, estas terapias são incapazes de bloquear a progressão do processo neurodegenerativo e o declínio cognitivo. Por esta razão, existe um grande interesse médico-social pela busca de novas alternativas terapêuticas capazes de atuarem diretamente no processo neurodegenerativo na DA (Jahns, Kilimann *et al.*, 2012; Ritter, 2012)

A etiologia da DA é multifatorial, estando associada à interação entre fatores ambientais e genéticos, sendo a maioria dos casos esporádicos e apenas 5% dos casos associados a uma característica de herança dominante, apresentando uma instalação mais precoce do quadro clínico (Levy-Lahad, Wasco *et al.*, 1995; Sherrington, Rogaev *et al.*, 1995; Barak e Aizenberg, 2010).

Dentre os fatores genéticos, as mutações nos genes codificadores para a APP, PSEN1 (presenilina 1) e PSEN2 (presenilina 2) que influenciam significativamente na atividade da γ -secretase resultando em uma produção aumentada dos fragmentos $A\beta_{1-40}$ e $A\beta_{1-42}$ e a apoE (apolipoproteína E) também são associados à maiores riscos no desenvolvimento da DA (Saunders, Schmader *et al.*, 1993; Levy-Lahad, Wasco *et al.*, 1995; Rogaev, Sherrington *et al.*, 1995; Sherrington, Rogaev *et al.*, 1995; Huang e Mucke, 2012).

Mas o principal fator de risco para o aparecimento da DA é o envelhecimento (Nikaido, Austin *et al.*, 1971; Crapper e Deboni, 1978). Entretanto, a obesidade, baixa escolaridade, traumatismo craniano associado à perda de consciência, depressão, diabetes mellitus, hipertensão arterial, tabagismo, hiperinsulinemia e o sedentarismo estão também relacionados ao aparecimento da DA (Fratiglioni, Grut *et al.*, 1991; Palleschi, Vetta *et al.*, 1996; Craft, 2005; Elias-Sonnenschein, Bertram *et al.*, 2012).

1.2. Modelos animais para a investigação da doença de Alzheimer

A utilização de modelos animais auxilia no avanço do conhecimento acerca dos mecanismos etiopatogênicos e na busca de novos tratamentos para a DA (Götz e Ittner, 2008). Entre as espécies de animais de laboratório utilizadas, os camundongos são considerados ótimos modelos biológicos pela facilidade de manipulação e manutenção, além da elevada reprodutibilidade quando comparados a outros mamíferos (Hedrich H *et al.*, 2004).

Os modelos animais *in vivo* para pesquisa da DA emergiram lentamente no final da década de 1970 para a investigação da teoria colinérgica da patologia (Davies e Maloney, 1976). Estes modelos

foram concebidos na tentativa de reproduzir vários componentes bioquímicos e comportamentais característicos da doença, facilitando a compreensão das alterações que ocorrem com o progredir do processo patológico e possibilitando a investigação de possíveis fármacos (Philipson, Lord *et al.*, 2010; Kitazawa, Medeiros *et al.*, 2012).

Entre os diversos modelos utilizados pode-se citar o uso de animais não-transgênicos onde a infusão de peptídeos beta-amiloide (Geula e Asdourian, 1984) e lipopolissacarídeos (Hauss-Wegrzyniak, Dobrzanski *et al.*, 1998) conduzem a uma neuroinflamação com degeneração hipocampal, ocasionando déficits cognitivos, e o uso de animais senescentes, pois com o envelhecimento, estes animais passam a apresentar prejuízos mnemônicos semelhantes aqueles encontrados na DA (Hadlow, 1980; Ball, Macgregor *et al.*, 1983).

Devido à grande mudança na pesquisa decorrente dos avanços na biologia molecular, genômica e epidemiologia genética. A descoberta das mutações genéticas relacionadas ao início precoce de DA familiar levaram a criação de linhagens transgênicas específicas através da inserção de genes humanos no genoma de roedores (Crawford, Hardy *et al.*, 1991; Goate, Chartier-Harlin *et al.*, 1991; Murrell, Farlow *et al.*, 1991).

Mas apesar de serem considerados importantes ferramentas de pesquisa, tanto a utilização de animais transgênicos ou não-transgênicos, apresentam suas limitações que deverão ser ponderadas durante a investigação científica (Philipson, Lannfelt *et al.*, 2009).

1.3. Obesidade x doença de Alzheimer

A obesidade é definida como uma doença crônica, complexa e multifatorial que se desenvolve através da interação entre fatores ambientais e genéticos (Sabatti, Service *et al.*, 2009; Heitmann, Westerterp *et al.*, 2012). Nos últimos anos, o processo de modernização e reestruturação econômica influenciou profundamente no estilo de vida e na dieta da população. Com o avanço da genética epidemiológica conseguiu-se classificar mais de 250 genes relacionados ao ganho de peso e relacionar a influência da hereditariedade neste processo (Lindgren, Heid *et al.*, 2009; Sabatti, Service *et al.*, 2009; Willer, Speliotes *et al.*, 2009; Speliotes, Willer *et al.*, 2010; Speakman e O'rahilly, 2012)

A obesidade tem atingido proporções epidêmicas em todo mundo sendo considerado um importante problema de saúde pública. Atualmente estima-se que 1 bilhão de adultos estejam com sobrepeso e

pelo menos 300 milhões deles são considerados clinicamente obesos (WHO, 2012).

O diagnóstico da obesidade pode ser realizado pelo Índice de Massa Corporal (IMC) $>30 \text{ kg/m}^2$ e está fortemente associada a diversas co-morbidades como o diabetes tipo 2, doenças cardiovasculares, câncer e a demência senil (Satwanti, Singh *et al.*, 1980; Maurovich-Horvat, Massaro *et al.*, 2007; Gustafson, 2012).

Estudos recentes em humanos têm relacionado o índice de massa corporal e adiposidade a uma maior incidência de doenças neurodegenerativa (Abbott *et al.*, 2002; Hu *et al.*, 2006; Hu *et al.*, 2007).

A DA acomete aproximadamente 7-10% dos indivíduos com mais de 65 anos e à medida que o indivíduo envelhece, este percentual dobra a cada cinco anos (Amaducci, Rocca *et al.*, 1986; Rocca, Amaducci *et al.*, 1986). Entretanto, em indivíduos obesos de meia-idade, o risco do aparecimento desta doença é quatro vezes maior, sendo compreensível o crescente interesse no estudo desta relação entre a obesidade e a DA (Lee, 2011).

Embora as vias neurais relacionadas à obesidade não estejam totalmente elucidadas, sabe-se que hormônios como a insulina, a leptina, e a grelina estão alterados durante o aumento de peso (Speakman e O'rahilly, 2012). Estes hormônios são conhecidos por desempenhar um papel importante na regulação da ingestão e do peso corporal. Estes hormônios além de interagir uns com os outros, parecem inter-relacionar-se com outros fatores metabólicos/neuronais (NPY, AgRP, POMC, obestatina, somotostatin, adiponectina, FFAs) (Suzuki, Jayasena *et al.*, 2011)

1.4. Neuropeptídeos

O cérebro integra e coordena as informações com base, dentre outros fatores, no ambiente hormonal oriundo da circulação e, apesar de inicialmente ter se pensado que os hormônios agiriam apenas no hipotálamo para regulação das funções endócrinas, hoje é sabido que estas substâncias de fato exercem diversas ações em regiões cerebrais distintas, incluindo o hipocampo e córtex pré-frontal (Andrews, 2011).

Neste sentido, dentre os diversos hormônios que modulam o funcionamento do sistema nervoso central (SNC), recentemente as ações dos hormônios orexígenos grelina e neuropeptídeo Y (NPY) e suas possíveis ações terapêuticas tem recebido crescente atenção pela comunidade científica internacional.

1.4.1. Grelina

A grelina é um exemplo de ligante endógeno descoberto através da farmacologia reversa, pois primeiramente foi identificado o seu

receptor [receptor secretagogo de hormônio de crescimento GSH-R, assim denominado devido a sua habilidade de estimular a liberação de hormônio do crescimento (GH)], para posteriormente ocorrer à identificação do ligante grelina (Kojima, Hosoda *et al.*, 1999; Kojima, 2008).

Este é um hormônio multifuncional produzido por diversos tecidos, mas predominantemente secretado pelas células endócrinas do tipo X/A da camada de submucosa estomacal (Andrews, 2011).

A grelina é o primeiro exemplo de peptídeo que necessita de uma modificação por um ácido graxo (acilação na Ser3 do ácido n-octanóico através da enzima grelina-O-aciltransferase – GOAT) para que ocorra a interação com seu receptor. A forma desacilada da grelina também existe em níveis significativos em diferentes tecidos, mas é incapaz de se ligar aos receptores GSH-R ou estimular a liberação de GH (Kang, Zmuda *et al.*, 2011).

Têm sido atribuídas diversas funções para a grelina, como a regulação da ingesta, liberação de insulina e o controle do balanço energético. A ingesta alimentar é controlada pelos neurônios do centro homeostático hipotalâmico localizado no núcleo arqueado (ARC) (Gropp, Shanabrough *et al.*, 2005). Neste núcleo existem neurônios orexígenos [expressam neuropeptídeo Y (NPY) e a proteína relacionada ao Agouti (AgRP)] e neurônios anorexígenos [expressam POMC (Pro-opiomelanocortina), α -MSH (α -melanotropina) e o transcrito regulado por cocaína e anfetamina (CART)]. NPY/AgRP e POMC são neurônios quimiotáticos do controle da ingesta que respondem aos diferentes estados do metabolismo de glicose e ácidos graxos além de serem responsivos as variações hormonais devido a presença de receptores membranares para grelina (GHS-R), insulina (INRS) e leptina (ObR) (Cowley, Smith *et al.*, 2003; Luquet, Perez *et al.*, 2005; Claret, Smith *et al.*, 2007; Andrews, 2011).

O principal fator indutor da liberação da grelina é a fome, mas os mecanismos pelos quais a estimulação desta secreção hormonal acontece permanecem desconhecidos. Em um modelo simplificado, as flutuações de grelina no plasma apontam para um aumento significativo deste hormônio antes das refeições e uma redução drástica após as mesmas, demonstrando um ritmo ultradiano da produção de grelina (Kirsch e Zieba, 2011). Também foi demonstrado que em pacientes com anorexia nervosa, as concentrações plasmáticas da grelina estão elevadas (Otto, Cuntz *et al.*, 2001), enquanto que em indivíduos obesos os níveis plasmáticos estão reduzidos (Cummings, Weigle *et al.*, 2002; Le Roux, Patterson *et al.*, 2005) (Figura 1)

Como já citado, a obesidade vem sendo considerada um fator de risco importante para o desenvolvimento de diferentes doenças neurodegenerativas incluindo a DA (Sriram, Benkovic *et al.*, 2002). Foi observado que a grelina está inversamente relacionada à obesidade, onde os níveis são mais elevados durante o balanço energético negativo ou restrição calórica, e menores durante o balanço energético positivo ou obesidade (Tschop *et al.*, 2001).

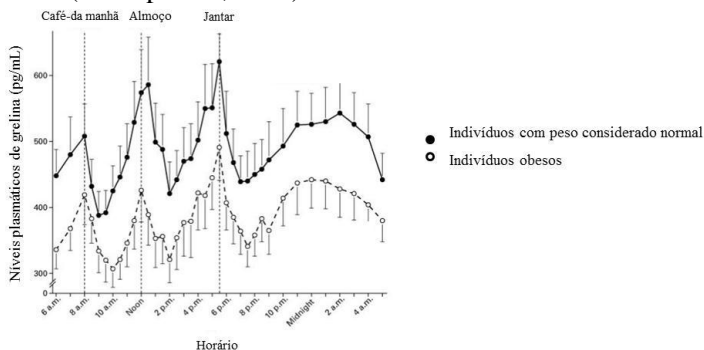


Figura 1: Ilustração das flutuações plasmáticas de grelina. As variações plasmáticas de grelina são relacionadas negativamente ao aumento de peso corporal. À medida que o indivíduo ganha peso, as concentrações do hormônio são gradativamente reduzidas. Além disso, a perda de massa gorda aumenta os níveis sanguíneos de grelina (Modificado de Cummings, Weigle *et al.*, 2002)

Foi também observado que a restrição calórica, onde os níveis de grelina estão aumentados, atenua a neurotoxicidade induzida pelo MPTP em primatas (Maswood *et al.*, 2004) e camundongos (Duan e Mattson, 1999), sendo esta toxina utilizada para a indução da degeneração de neurônios dopaminérgicos e, conseqüentemente, para modelar a DP em animais de laboratório (Prediger, Batista *et al.*, 2006)

Estudos *in vitro* demonstram que células tratadas com o soro de ratos com restrição calórica exibem biogênese mitocondrial e diminuição da produção de espécies reativas de oxigênio (ROS), indicando que os efeitos da restrição de calórica podem ser mediados por um fator hormonal que afeta o metabolismo mitocondrial, e que os níveis elevados de grelina poderiam ser os responsáveis pela neuroproteção observada nestes estudos (Lopez-Lluch *et al.*, 2006).

Dentre as ações neuroprotetoras relacionadas a este hormônio, foi demonstrado que tanto a grelina quanto agonistas de GSH-R, são

capazes de inibir o estresse oxidativo (Kheradmand, Alirezai *et al.*, 2010), a apoptose (Delhanty, Van Koetsveld *et al.*, 2007), a neuroinflamação (Erşahın, Toklu *et al.*, 2011), a disfunção mitocondrial (Zhang, Huang *et al.*, 2011) e a excitotoxicidade glutamatérgica (Matos, Augusto *et al.*, 2008) tanto *in vivo* quanto *in vitro*. Além disso, os efeitos neuroprotetores da grelina já foram descritos em modelos experimentais da DP (Jiang, Li *et al.*, 2008), esclerose múltipla (Theil, Miyake *et al.*, 2009), epilepsias (Obay, Taşdemir *et al.*, 2008) e recentemente na DA (Moon, Choi *et al.*, 2011).

Trabalhos recentes evidenciam as ações da grelina no controle das funções hipocampais. Foi demonstrado que a administração periférica de grelina estimula a proliferação e a diferenciação de neurônios hipocampais (Moon, Kim *et al.*, 2009). Corroborando estes achados, foi detectada a expressão de GHS-R1a em células progenitoras do hipocampo, confirmando o envolvimento de grelina na neurogênese desta região (Gahete, Córdoba-Chacón *et al.*, 2011). Com relação a DA, foi observado que a produção de grelina, assim como a expressão do seu receptor GHS-R1a e da enzima GOAT, encontram-se reduzidos no lobo temporal dos pacientes com a DA quando comparados com indivíduos controles de idades semelhantes (Gahete, Córdoba-Chacón *et al.*, 2010). Em conjunto, estes resultados sugerem que a redução nas concentrações deste neuropeptídeo no SNC poderia contribuir para a perda neuronal e déficits cognitivos observados na DA.

Reforçando estes achados clínicos, Moon e colaboradores (2011) demonstraram recentemente que a administração intraperitoneal (i.p.) de grelina (80 µg/kg) foi capaz de atenuar os prejuízos cognitivos e respostas inflamatórias induzidas pela infusão intra-hipocampal do peptídeo Aβ₁₋₄₂ em camundongos (Moon, Choi *et al.*, 2011).

1.4.2. NPY

O neuropeptídeo Y (NPY) é um dos neuropeptídeos mais abundantes no SNC de mamíferos. Evolutivamente bem conservado, pertence à família dos peptídeos pancreáticos, outros membros desta família são o hormônio intestinal peptídeo YY (PYY) e o peptídeo pancreático (PP) (Holzer, Reichmann *et al.*, 2012). Isolado pela primeira vez em 1982 do cérebro de porcos, foi assim denominado devido a grande proporção do aminoácido tirosina (designado também por Y), incluindo um resíduo nas porções C e N-terminais, na sua estrutura molecular com 36 aminoácidos (Tatemoto, Carlquist *et al.*, 1982).

Sintetizado na forma de um precursor inativo, o prepro-neuropeptídeo com 97 aminoácidos, contém dois fragmentos que são

clivados durante o processo de maturação postranslacional (Pedrazzini, Pralong *et al.*, 2003). Inicialmente, pela ação da peptidase sinal, ocorre a remoção do peptídeo sinal de 28 aminoácidos, resultando no pro-neuropeptídeo NPY. O pro-neuropeptídeo NPY com 69 aminoácidos é formado pelo NPY₁₋₃₉ e pelo peptídeo C-PON (C-terminal Flanking Peptide of NPY ou peptídeo flanqueador do NPY no terminal carboxílico). A seguir, a pro-hormônio converte-se e a enzima catepsina L clivam a ligação de aminoácidos Lys38-Arg39, liberando a porção carboxi-terminal C-PON. A cadeia peptídica NPY₁₋₃₉ poderá sofrer a ação da enzima carboxipeptidase originando o NPY₁₋₃₇, que por sua vez será o substrato da enzima peptidil-glicina α -amidante monoxigenase (PAM), resultando no peptídeo biologicamente ativo NPY₁₋₃₆, ou simplesmente NPY. O NPY poderá ser truncado a NPY₂₋₃₆ ou NPY₃₋₃₆ pelas enzimas amino peptidase P e dipeptidil peptidase 4 (DPP4), respectivamente (Pedrazzini, Pralong *et al.*, 2003) (Figura 2).

O NPY é expresso tanto no sistema nervoso periférico (SNP) quanto no SNC. No SNP, é encontrado principalmente nos nervos simpáticos onde é co-armazenado com a noradrenalina (Donoso, Delpiano *et al.*, 2006). Os neurônios simpáticos que expressam NPY inervam a musculatura lisa incluindo: vasos cerebrais, coração, glândula tireóide, trato respiratório, intestino, pâncreas, fígado e olhos, sendo também encontrado na medula adrenal e nas plaquetas (Malva, Xapelli *et al.*, 2012).

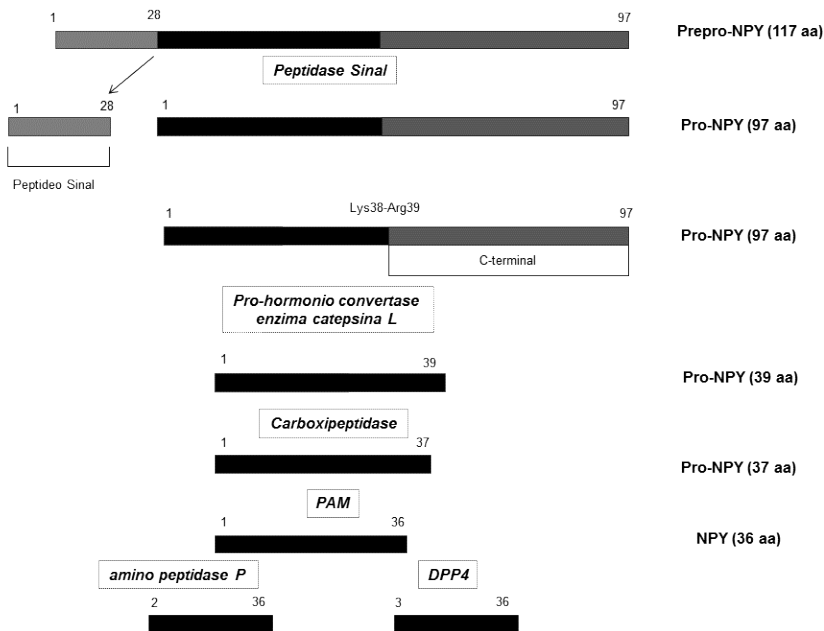


Figura 2: Processamento do NPY. Ver detalhes no texto.

Grande parte das funções periféricas do NPY é sinérgica ao sistema simpático. Este peptídeo não somente potencializa como também mimetiza as ações simpáticas, inibindo a liberação de acetilcolina das terminações vagais no coração, na traqueia e nos brônquios. Além disso, em qualquer condição em que ocorra um aumento da atividade adrenérgica, ocorre uma elevação nas concentrações plasmáticas de NPY (Donoso, Delpiano *et al.*, 2006).

Apesar do seu papel em doenças como hipertensão, doenças vasculares periféricas, aterosclerose e estresse crônico não estar inteiramente estabelecido, as concentrações plasmáticas deste peptídeo se encontram elevadas nestas condições, sendo verificada uma correlação positiva entre o NPY e estas doenças (Wahlestedt e Reis, 1993).

Já no SNC, o NPY age como um neurotransmissor e/ou modulador neuronal, podendo ser encontrado em co-localização com outros neuropeptídios como o AgRP, a somatostatina, a matostatina e a encefalina (Donoso, Delpiano *et al.*, 2006).

Concentrações elevadas de NPY são observadas no núcleo arqueado hipotalâmico, no tronco cerebral e na pituitária anterior. Nestas regiões, o NPY tem duas funções principais, estimular a ingesta

de alimentos e modular as respostas ao estresse. Além disso, o NPY está envolvido no comportamento sexual, na cognição, na neurogênese e no aprendizado e memória (Redrobe, Dumont *et al.*, 1999; Redrobe, Dumont e Quirion, 2002).

Os efeitos fisiológicos deste peptídeo são mediados através de seus receptores acoplados a proteína G. Estes receptores podem ser divididos em duas subfamílias: a subfamília Y1, que consiste nos subtipos Y1, Y4 e Y6, a subfamília Y2, que engloba os subtipos Y2 e o subtipo Y5 (Larhammar e Salaneck, 2004). Destes, os subtipos Y1 e Y2 são encontrados predominantemente no SNC, enquanto o subtipo Y4 é encontrado nos tecidos periféricos. Já o receptor Y6 não é funcional na maioria das espécies de primatas e mamíferos (Larhammar e Salaneck, 2004).

A lista de ações exercidas pelo NPY no SNC é longa e provavelmente ainda não está completa. A distribuição do peptídeo e seu receptor no cérebro ocasionam diversas ações e efeitos sobre a memória, a ansiedade, a nocicepção, o balanço eletrolítico, as regulações endócrinas e a homeostase energética (Pedrazzini, Pralong *et al.*, 2003).

Estudos demonstram que em ratos velhos, os baixos níveis de NPY no giro denteado e nas regiões CA1 e CA3 do hipocampo estão associados a uma diminuição na neurogênese e na função cognitiva (Hattiangady, Rao *et al.*, 2005; Rao, Hattiangady *et al.*, 2006). Além disso, que os efeitos ocasionados pelo NPY no aprendizado e memória são mediados via ativação do receptor pré-sináptico Y2 (Flood e Morley, 1989; Redrobe, Dumont *et al.*, 2004).

A estimulação da proliferação neuronal na fase adulta foi observada através da ativação de receptores Y1 no hipocampo (Decressac, Prestoz *et al.*, 2009). Além deste efeito proliferativo, o potencial neuroprotetor do NPY via receptores do tipo Y1, Y2 e Y5 foi demonstrado em modelos experimentais de neurodegeneração (Smialowska, Domin *et al.*, 2009).

O papel neuroprotetor do NPY foi evidenciado no modelo experimental da DP utilizando a 6-hidroxiopamina (6-OHDA), tanto *in vivo* quanto *in vitro* (Decressac, Pain *et al.*, 2012). O NPY, através da ativação dos receptores Y2 e Y5, está envolvido na neuroproteção em modelos experimentais de epilepsia induzidos pelo kainato e timelitina em ratos (Silva, Lourenço *et al.*, 2007; Xapelli, Silva *et al.*, 2007; Corvino, Marchese *et al.*, 2012). Já no modelo experimental da DA, foi demonstrado que animais transgênicos que expressam elevadas quantidades de proteína precursora amiloide (APP), o fragmento C-

terminal de NPY ocasionou uma redução nos efeitos neurotóxicos causados pela APP (Rose, Crews *et al.*, 2009).

Em paciente com a DA, tanto as concentrações plasmáticas, como o número de receptores para NPY encontram-se reduzidos. Foi demonstrado que a diminuição nos receptores torna-se extremamente significativa à medida que aumenta a severidade e a duração da doença (Martel, Alagar *et al.*, 1990; Nordberg, 1992).

2. JUSTIFICATIVA

Considerando as projeções de crescimento populacional, espera-se que o número de indivíduos com mais de 80 anos atinja os 300 milhões em 2050. Em consequência, estima-se que o número de pessoas acometidas pela DA sofra um crescimento significativo dos atuais 27 milhões para cerca de 107 milhões no ano de 2050 (Alzheimer's Association, 2012).

Embora mais de cem anos tenham se passado desde a primeira descrição da DA, muitas lacunas relacionadas aos mecanismos fisiopatológicos desta doença precisam ser preenchidas. Além disso, ainda não existem terapias capazes de curar ou impedir a progressão da DA.

Sendo assim, o desenvolvimento de novas estratégias terapêuticas para a DA é um tema de grande relevância científica, médica e social, existindo uma grande procura por substâncias capazes de exercer proteção contra a perda neuronal e que ao mesmo tempo sejam capazes de atenuar os prejuízos cognitivos observados pelos pacientes com a DA.

A grelina foi o último hormônio descoberto no século XX, desta maneira as evidências do potencial neuroprotetor deste peptídeo ainda são limitadas. Apesar disso, emerge um quadro encorajador do potencial deste neuropeptídeo no tratamento de patologias do SNC. A relação entre grelina e o NPY é estreita. Neurônios NPY são em sua maioria GHS-R positivos, e a interação da grelina com o receptor GHS-R tem a capacidade de modular a resposta destas células (Osterstock, Escobar *et al.*, 2010).

Já os neurônios GHS-R do núcleo arqueado apresentam receptores Y2, mas diferentemente dos neurônios NPY que são influenciados pela grelina, estudos demonstraram através da utilização do BIIE0246, um antagonista seletivo do receptor Y2, que o bloqueio destes receptores não altera a resposta dos neurônios GHS-R positivos (Osterstock, Escobar *et al.*, 2010).

Devido aos recentes trabalhos demonstrando o efeito protetor do NPY em modelos de neurodegeneração, e considerando as diversas funções benéficas da grelina em processos fisiopatológicos, existe um crescente interesse na identificação dos potenciais terapêuticos destes dois hormônios.

Desta maneira, diante do crescente aumento no número de indivíduos obesos e da possível relação entre obesidade e neurodegeneração, sabendo em tanto na obesidade como na DA às concentrações plasmáticas de grelina e NPY encontram-se alteradas e conhecendo os efeitos positivos da grelina e do NPY sobre processos de aprendizado e memória (Flood, Hernandez *et al.*, 1987; Sørensen, Kanter-Schlifke *et al.*, 2008; Carlini, Ghersi *et al.*, 2010) suas ações sobre a proliferação celular hipocampal (Diano, Farr *et al.*, 2006; Decressac, Prestoz *et al.*, 2009) e os diferentes mecanismos de neuroproteção (Smiałowska, Domin *et al.*, 2009; Gahete, Córdoba-Chacón *et al.*, 2011), no presente estudo buscou-se avaliar o efeito protetor dos hormônios grelina e NPY no modelo experimental da DA induzido pela administração i.c.v. do peptídeo $A\beta_{1-40}$ em camundongos (Medeiros, Prediger *et al.*, 2007; Prediger, Franco *et al.*, 2007; Prediger, Fernandes *et al.*, 2008).

3. OBJETIVOS

3.1. Objetivo Geral

O objetivo do presente estudo foi avaliar o efeito protetor dos hormônios grelina e NPY, nas alterações comportamentais e neuroquímicas induzidas pela administração intracerebroventricular (i.c.v.) do peptídeo $A\beta_{1-40}$ em camundongos.

3.1.1. Objetivos Específicos

- Investigar os efeitos da administração i.c.v. do peptídeo $A\beta_{1-40}$ (400 pmol/i.c.v.), da grelina (3,0 nmol, i.c.v.) e do NPY (0,0234 μ mol/i.c.v.) sobre as funções motoras, cognitivas e de emocionalidade em camundongos albinos avaliados em diferentes testes comportamentais: campo aberto, teste de suspensão pela cauda, labirinto em cruz elevado e realocação de objeto;
- Avaliar o efeito da administração i.c.v. do peptídeo $A\beta_{1-40}$ (400 pmol/i.c.v.), da grelina (3,0 nmol, i.c.v.) e do NPY (0,0234 μ mol/i.c.v.) sobre a captação de glutamato no hipocampo de camundongos.
- Investigar a atividade da enzima acetilcolinesterase (AChE) no córtex pré-frontal e hipocampo de camundongos infundidos com o peptídeo $A\beta_{1-40}$ (400 pmol/i.c.v.) e grelina (3,0 nmol, i.c.v.);

- Investigar as ações *in vitro* do peptídeo $A\beta_{1-40}$ (200 nM e 500 nM) e da grelina 1 nM e 1 μ M sobre a facilitação sináptica avaliada através da indução de LTP em fatias hipocâmpais de camundongos;
- Avaliar o efeito da administração i.c.v. da grelina (3,0 nmol, i.c.v.) e do NPY (0,0234 μ mol/i.c.v.) sobre marcadores de estresse oxidativo no córtex pré-frontal e hipocampo de camundongos infundidos com o peptídeo $A\beta_{1-40}$ (400 pmol/i.c.v.);
- Investigar a participação dos receptores Y2 na resposta protetora induzida pela grelina, através da administração do antagonista do receptor BIIE0246 (1 nmol/i.c.v.), no teste de realocação de objetos.

4. CAPITULO 1

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Title: Ghrelin as a neuroprotective and palliative agent in Alzheimer's and Parkinson's disease

Running title: Role of ghrelin in neurodegenerative diseases

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Abstract

Ghrelin is a gastric hormone that stimulates growth hormone (GH)
secretion and food intake to regulate energy homeostasis and body

weight by binding to its receptor, GH secretagogue receptor (GHSR1a), which is most highly expressed in the pituitary and hypothalamus. Nowadays there is considerable evidence showing that the GHSR1a is also expressed in numerous extra-hypothalamic neuronal populations and the physiological role of ghrelin is by far wider than considered before including learning and memory, anxiety, depression and neuroprotection. The present review attempts to provide a comprehensive picture of the role of ghrelin in the central nervous system and to highlight recent findings showing its potential as an innovative therapeutic agent in neurodegenerative diseases including Alzheimer's disease and Parkinson's disease.

Keywords: Ghrelin, neuroprotection, Alzheimer's disease, Parkinson's disease, learning and memory, anxiety, depression

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, accounting for 50–60% of all cases [1]. The prevalence of AD is below 1% in individuals aged less than 65 years, but it increases to approximately 25% of the individuals aged 85 years or older in the Western world [2]. This neurodegenerative disease is associated with progressive and permanent decline in memory and overall cognitive abilities, reducing the post-diagnosis lifespan to nearly half the duration of a nondemented elderly person [3]. The first cognitive function affected is the episodic memory [4], but during the progression of the disease, attention, executive functions, semantic memory, spatial orientation and even language are deteriorated [5]. The examination of post-mortem brains of AD patients indicated the main histopathological hallmarks of the disease: the formation of senile plaques and neurofibrillary tangles, which are mainly formed due to deposition of β -amyloid ($A\beta$) peptides and the hyperphosphorylated tau protein, respectively [6].

Parkinson's disease (PD) is the second most common neurodegenerative disorder that affects approximately 1% of the population older than 50 years [7], and it is characterized by a slow and progressive degeneration of neuromelanin-containing dopaminergic neurons in the substantia nigra pars compacta (SNpc) with presence of eosinophilic, intracytoplasmic, proteinaceous inclusions termed as Lewy bodies and dystrophic Lewy neurites in surviving neurons [8]. At the time of diagnosis, patients typically display an array of motor impairments including bradykinesia, resting tremor, rigidity, and postural instability. Although most of the typical motor impairments are due to the loss of nigrostriatal dopaminergic neurons, PD affects multiple neuronal systems both centrally and peripherally, leading to a constellation of non-motor symptoms including olfactory deficits, affective disorders (including depression and anxiety), memory impairments, as well as autonomic and digestive dysfunction [9]. These non-motor features of PD do not meaningfully respond to dopaminergic medication and are a challenge to the clinical management of PD [9].

The limitations of the current pharmacological treatments of AD and PD have led to extensive investigation of novel drugs that may provide alternative or adjunctive treatment for the relief of symptoms with a reduced profile of side-effects, as well as to the discovery of compounds to modify the course of these neurodegenerative diseases. The definition of neuroprotection is complex and involves the potential for preventing cell death and restoring function to damaged neurons, as

well as increasing neuronal number. The development of drugs to slow or prevent the progression of AD and PD might logically evolve from an improved understanding of the etiology and pathogenesis of such diseases. There have certainly been major advances in these areas over the past few years and the prospect for the introduction of "neuroprotective" therapies is much improved. However, despite extensive efforts and research, to date, there is no proven therapy to prevent cell death or to restore affected neurons to a normal state in AD and PD. Preclinical studies in laboratory animals have provided several candidate neuroprotective drugs, but clinical endpoints are readily confounded by any symptomatic effect of the study intervention and thus do not provide an unequivocal measure of disease progression that can be used to determine if a drug has a neuroprotective effect.

In this context emerges ghrelin, a gastric hormone that stimulates growth hormone (GH) secretion and food intake to regulate energy homeostasis and body weight by binding to its receptor, GH secretagogue receptor (GHS-R1a), which is most highly expressed in the pituitary and hypothalamus. Nowadays there is considerable evidence showing that the GHS-R1a is also expressed in numerous extra-hypothalamic neuronal populations and physiological role of ghrelin is by far wider than considered before including learning and memory, anxiety, depression and neuroprotection. The present review attempts to provide a comprehensive picture of the role of ghrelin in the central nervous system (CNS) and to highlight recent findings showing its potential as a new palliative and neuroprotective agent in neurodegenerative diseases.

GHRELIN: HISTORICAL BACKGROUND, RECEPTOR AND FUNCTIONS

The ghrelin history began in 1977, when Frank Momany and Cyril Bowers from Tulane University (New Orleans, USA) reported a series of synthetic peptide analogs of Leu- and Met-enkephalins that specifically released growth hormone from pituitary without any opioid activity [10-12]. Surprisingly, it was found that these molecules stimulate and amplify pulsatile GH secretion, independently from GH releasing hormone (GHRH) [13]. Then, some others peptidyl derivatives with similar properties were characterized. The family of these molecules, both peptidyl and non-peptidyl compounds, has been named growth hormone secretagogues (GHSs).

GHSs are synthetic compounds that are potent stimulators of GH release, working through a G-protein-coupled receptor (GPCR), the

GHS-receptor (GHS-R) [14]. Because GHSs are a group of artificial compounds and do not exist naturally, it was postulated that there must exist an endogenous ligand that binds to GHS-R and carries out similar functions to GHSs *in situ*. This makes the discovery of ghrelin an example of reverse pharmacology, starting with the synthesis of analogs and ending with the discovery of an endogenous ligand and its receptor.

A cultured cell line expressing the GHS-R was established and used to identify tissue extracts that could stimulate the GHS-R, as monitored by increases in intracellular Ca^{2+} levels. After screening several tissues, a very strong activity was unexpectedly founded in stomach [15], besides that, adenosine in hypothalamic extracts showed agonist activity on the GHS-R, and exhibited cross-desensitization with the synthetic ligands. However, in contrast to ghrelin and the synthetic GHS-R agonists, adenosine failed to stimulate GH release from pituitary cells [16]. Hence, ghrelin, as a new hormone and a closer mimetic of the synthetic GHS-R ligands, became the focus of subsequent research.

Ghrelin is a multifunctional 28-amino acid hormone produced in several tissues, with predominant source from the stomach in response to hunger and starvation by enteroendocrine X/A-like cells. However, other tissues such as ovary, placenta, kidney, pituitary gland and pancreas also produce ghrelin, although in lower amount when compared with the gastric source [17]. The ghrelin gene is expressed in the CNS, but just insignificant amounts of ghrelin can be found in rodents' neurons [18]. Nevertheless, circulating ghrelin gains access to the CNS and reached different structures as hippocampus and ventral tegmental area (VTA) [18]. The ghrelin gene was conserved throughout the evolution sharing 82.9% homology between rodents and humans, the functional 28 amino acid protein only differs by 2 amino acids between rats and humans. In humans, the ghrelin gene is located on chromosome 3p25–26 and the genomic structure of ghrelin is relatively simple and contains four prepro-ghrelin-coding exons (exon 1–4), and additional upstream exons, that codifies ghrelin and several bioactive molecules including desacyl-ghrelin and obestatin, a recent hormone with 23 amino acid ghrelin gene-derived peptide [19,20] (Fig. 1).

INSERT FIG. 1 ABOUT HERE

As illustrated in Fig. 1, during prepro-ghrelin processing, a 23 amino acid secretion-signal peptide is cleaved from the N-terminus of the 117 amino acid prepro-hormone, resulting in a 94 amino acid pro-ghrelin peptide. This 94 amino acid ghrelin pro-hormone is cleaved at

Arg28/Ala29 to yield the biologically active 28 amino acid N-terminal, the ghrelin peptide, and a 66 amino acid C-terminal propeptide, C-ghrelin [21]. The prepro-ghrelin signal peptide is encoded in exon 1, and the coding sequence of the 28 amino acid ghrelin peptide hormone is encoded by parts of exons 1 and 2, the C-terminal encoded by part of exon 2, plus exons 3 and 4 of the prepro-ghrelin gene and the exon 3 codes for obestatin [19,22].

Ghrelin can be post-translationally octanoylated (acylated) at its third residue, a serine (Ser3), by ghrelin O-acyltransferase (GOAT). This enzyme belongs to a family of the membrane-bound O-acyltransferases (MBOATs) that attach fatty acids to lipids and proteins, and octanoylates pro-ghrelin before it is transported to the Golgi apparatus, where it is cleaved by pro-hormone convertase (PC) to form the mature ghrelin, but both forms desacyl and acylated can be found in circulation [23] (Fig. 1). This n-octanoyl acylation on serine residues is unique to ghrelin and is crucial for binding, subsequent activation of the GHS-R1a and is essential for some of the hormone's bioactivity, including GH release and orexigenic effect [24].

The nonacylated form of ghrelin, des-acyl ghrelin, also exists at significant levels in stomach and blood, but the process underlying the production of des-acyl ghrelin remains unclear [25]. Des-acyl represents approximately 90% of the total ghrelin detected in serum and there is increasing evidence that the deacylation process rapidly occurs in the plasma been responsible for the reduction in the ghrelin's half-life. However, an alternative explanation for the production of nonacylated form suggests that des-acyl ghrelin is a result of an incomplete acylation of ghrelin [26,27]. Des-acyl ghrelin and the acylated form share many nonendocrine actions, such as the stimulation of food intake, modulation of cell proliferation, and minor effects on adipogenesis, but the local where the desacylated form binds is still an open question. Baldanzi and colleagues [28] suggested the existence of another ghrelin receptor distinct from GHSR-1a. They demonstrated that ghrelin and des-acyl ghrelin recognize sites on H9c2 cardiomyocytes, which do not express the ghrelin receptor. This new receptor probably is very similar to GHSR-1a, since it differs only in its lack of ability to discriminate between the esterified and unesterified ghrelin peptides [28].

The circulating level of ghrelin is determined by the balance among its secretion and degradation rate, and its clearance by the excretion in urine [29]. Ghrelin levels also are controlled by some hormones, such as insulin and glucagon. Recently it was demonstrated that the administration of insulin in the CNS reduces serum total ghrelin

concentration probably through a hypothalamus–stomach neuronal pathway [30]. Leptin is the most important signal which reflects peripheral energy balance and has opposite effects of ghrelin. While leptin decreases food intake by decreasing the neuronal activity of NPY/AGRP-containing neurons, ghrelin activates NPY/AGRP neurons stimulating food intake. Interestingly, leptin inhibits in a dose-dependent manner the ghrelin transcription *in vitro* and decreases ghrelin release from isolated rat stomach [31]. Therefore, these findings indicate that the anorexic effect of leptin may occur by decreasing ghrelin secretion.

There are two differently spliced variants of ghrelin receptor or GHS-R; the GHS-R1a and GHS-R1b. The first has features of a typical GPCR, including conserved cysteine residues in the first two extracellular loops, several potential sites for post-translational modifications (N-linked glycosylation and phosphorylation), and an aromatic triplet sequence (E/DRY) located immediately after TM-3 in the second intracellular loop. The last one is truncated and has been reported as an inactive form that fails to bind ghrelin and has no known signaling activity [32,33]. The GHS-R1a receptor is expressed in brain areas and peripheral organs including the hypothalamic arcuate nucleus (ARC), ventromedial nuclei (VMN), CA2, CA3 and dentate gyrus (DG) sub-fields of the hippocampal formation, vagal afferents, pancreas, spleen, myocardium, adipose tissue, thyroid gland, adrenal gland and gastric myenteric neurons [34]. In addition, the GHS-R1a forms heterodimers with other receptors such as the cannabinoid 1 (CB1) receptor (this interaction is crucial for the appetitive effects of ghrelin) [35] and the dopamine D1 receptor (ghrelin amplifies the dopamine signaling in neurons that co-express D1 receptors) [36].

The GHS-R1a belongs to a family of receptors operating via the Gq-phospholipase C signaling pathways (Fig. 2). The activation of the GHS-R1a receptor leads to generation of inositol triphosphate and Ca^{2+} release through the activation of the G protein $G_{\alpha q/11}$. Other signaling pathways involved with GHS-R1a activation are the extracellular signal-regulated kinase (ERK1/2), phospholipase C (PLC) and protein kinase C (PKC), and the protein kinase cascade Raf–MEK–MAPK (Fig. 2). The interaction of ghrelin with GHS-R1a modulates different functions such as glucose homeostasis, hormone secretion, gastrointestinal motility, cell proliferation, cardiovascular, pancreatic, pulmonary and immune functions, memory, reproduction and sleep (for review see [37-39]). Taken together, these recent findings indicate that ghrelin is more than simply a natural GHS.

GHRELIN AS A NEUROPROTECTIVE STRATEGY IN NEURODEGENERATIVE DISEASES

The previous section presents evidence suggesting that the physiological role of ghrelin is by far wider than considered before and the studies in the field should not continue restricted to the investigation of ghrelin effects on the stimulation of GH secretion and regulation of food intake. As an effort to illustrate the potential of ghrelin as an innovative target for future pharmacotherapies, the next sections attempt to review the results reported in clinical and animal studies to provide a comprehensive picture of the role of ghrelin in neurodegenerative diseases.

In 2002, Frago and colleagues [40] provided the first evidence of the neuroprotective effects of ghrelin, demonstrating that the systemic administration of the GH releasing peptide-6 (GHRP-6), a synthetic ligand for the ghrelin receptor, results in increased insulin-like growth factor (IGF-I) mRNA levels and increased expression of proteins involved in cell survival and neuroprotection in several brain areas of adult rats. One year later, the same group demonstrated that the treatment with GHRP-6 decreased cell death and inhibited caspase 3 and 9 activation in the cerebellum of aged rats [41].

As summarized in Table 1, the neuroprotective potential of ghrelin was further demonstrated by independent research groups in diverse experimental models of ischemia [42,43], traumatic brain injury (TBI) [44-46], spinal cord injury (SCI) [47,48], amyotrophic lateral sclerosis (ALS) [49,50], epilepsy [51-55], AD [56,57] and PD [58-61]. Therefore, ghrelin confers neuroprotection in diverse brain regions ranging from substantia nigra, striatum to hippocampus and cerebral cortex, and against a variety of brain noxious stimuli.

INSERT TABLE 1 ABOUT HERE

As illustrated in Fig. 2 and Fig. 3, several mechanisms have been implicated in the neuroprotective effects of ghrelin and a detailed review about this issue is beyond the scope of this article and can be found elsewhere [37,62]. At this moment, particular attention is paid to the role of ghrelin in modulating the activation of intracellular signaling cascades (such as Erk1/2, Akt1/2, PI3K and PKC pathways) that lead to the inhibition of apoptotic events, via the subsequent increase in the Bcl-2:Bax ratio, the prevention of cytochrome c release and the inhibition of caspase 3 activation [42,43,63,64]. Moreover, ghrelin prevents

activation of pro-apoptotic events, such as the activation of p38 and JNK. Furthermore, ghrelin prevents inflammatory microglial activation [60] and activates the mitochondrial protein uncoupling protein-2 (UCP2) [59]. This protein enhances neuroprotection by decreasing the generation of reactive oxygen species (ROS) and promoting mitochondrial biogenesis [59,63] (Fig. 2). Therefore, the common neuroprotective or neuromodulatory role of ghrelin in the brain could involve UCP2-dependent mitochondrial adaptation. Finally, these neuroprotective effects of ghrelin appear to be mediated through activation of GHSR-1a, as they were abolished by the pharmacological blockage or genetic deletion of this receptor.

INSERT FIG. 2 ABOUT HERE

These recent findings demonstrating that ghrelin is involved in neuroprotection, together with the wide distribution of ghrelin receptors in many brain areas, reinforce the idea that changes in this system could be involved in the development and/or progression of AD and PD.

Role of ghrelin in Alzheimer's disease

AD is the most prevalent age-related neurodegenerative disease that leads to cognitive impairments and dementia. The neuropathological hallmarks of AD are diffuse and neuritic plaques, which are predominantly composed of amyloid- β ($A\beta$) peptides, and neurofibrillary tangles composed of filamentous aggregates of hyperphosphorylated tau protein [65]. The classical $A\beta$ cascade hypothesis in AD pathogenesis postulates that the deposition of $A\beta$ peptides and the activation of glial cells surrounding senile plaques in brain areas involved in cognitive functions trigger marked neuronal alterations such as synaptic dysfunction, synaptic loss and neuronal death finally leading to cognitive impairments [65,66].

The first evidence showing a direct effect of ghrelin on AD-like alterations was reported in a mouse model widely used to examine the pathophysiology of early defects seen in AD. The senescence-accelerated mouse prone8 or SAMP8 mice develop early learning and memory impairments related to abnormalities in septo-hippocampal function, which are due to overproduction of β -amyloid peptides. Diano and colleagues [56] demonstrated that ghrelin improved retention of T-maze foot shock avoidance in 12 and 14 month-old SAMP8 mice. Of interest, ghrelin was found to promote both long-term potentiation (LTP) generation in hippocampal slices and the formation of spine

synapses in the stratum radiatum of the hippocampal CA1 subregion, which are considered to be basic mechanisms involved in spatial learning and memory [56] (Table 2).

More recently, Moon et al. [57] investigated the effects of ghrelin on memory impairments and neuropathological alterations induced by intra-hippocampal injection of $A\beta_{1-42}$ peptide in mice. The authors reported that repeated systemic injection of ghrelin rescued $A\beta_{1-42}$ -induced memory deficits evaluated in two behavioral paradigms (Y-maze and passive avoidance tasks). Moreover, ghrelin attenuated hippocampal microgliosis and neuronal loss induced by $A\beta_{1-42}$ administration [57] (Table 2).

Corroborating these findings, unpublished results from our laboratory have indicated that the acute intracerebroventricular (i.c.v.) injection of ghrelin (3 nmol), 15 min before the infusion of $A\beta_{1-40}$ (400 pmol, i.c.v.), prevented the $A\beta_{1-40}$ -induced spatial memory impairments and depressive-like behaviors in adult Swiss mice evaluated in the object location and forced swimming task, respectively. Moreover, ghrelin mitigated a series of neurochemical changes induced by i.c.v. infusion of $A\beta_{1-40}$, including the increase of oxidative stress biomarkers and acetylcholinesterase (AChE) activity and the decrease of glutamate uptake in the hippocampus and frontal cortex of mice (Table 2). Finally, ghrelin (1 nM) was found to prevent the impairments on LTP generation induced by $A\beta_{1-40}$ (200 nM) in the CA1 subregion of hippocampal slices of mice (Santos et al., unpublished data) (Table 2).

Altogether, these results suggest that ghrelin may counteract neurotoxic effects of $A\beta$ peptides by reducing excitotoxicity, neuroinflammation, oxidative stress and activation of apoptotic cell death mechanisms. Moreover, the ghrelin's effects on AChE activity and LTP generation may represent potential mechanisms responsible for its cognitive enhancing properties (Fig. 3). A better understanding of how the multiple actions of ghrelin influence survival of neurons might further consolidate ghrelin as a potential neuroprotective agent for the treatment of AD.

INSERT TABLE 2 ABOUT HERE

INSERT FIG. 3 ABOUT HERE

At this moment, few clinical studies have attempted to comprehend the potential implication of the ghrelin system in human AD (Table 2). In 2002, it was reported that mean plasma ghrelin concentrations in older normal weight subjects were significantly lower

than those observed in young normal weight subjects, providing the first evidence for an age-related decline of peripheral ghrelin concentrations [67]. Nevertheless, Proto et al. [68] reported that ghrelin levels do not vary in the cerebrospinal fluid of AD patients when compared with age-matched controls. In a recent study, Castaño's group (University of Córdoba, Spain) analyzed the mRNA expression of the ghrelin system in three different regions of the temporal gyrus (inferior, medial and superior) of control and AD human brains, since it is one of the most affected memory-related regions in AD. This study showed, for the first time, that AD patients have a reduction in local brain ghrelin production, as compared with age-matched controls [69].

In addition, Shibata et al. [70] investigated whether single nucleotide polymorphisms (SNPs) of the ghrelin gene are associated with AD in a Japanese population. A total of 182 AD patients and 143 age-matched controls were included in this study and the SNPs were genotyped using TaqMan technology and were analyzed using a case-control study design. The authors observed that one SNP, rs4684677 (Leu90Gln), showed a marginal association with age of AD onset, but no additional association between other SNPs of the ghrelin gene and AD were detected [70] (Table 2). Moreover, Theodoropoulou et al. [71] investigated recently the potential relationship between serum ghrelin levels and weight loss in patients with AD. The authors reported that the area-under-the-curve (AUC) for serum ghrelin levels after 75 g of glucose load is lower in male patients with AD compared to control males, while no difference was observed between females AD and controls. Therefore, the disruption of the normal compensatory modulation of ghrelin secretion might contribute to the metabolic changes (e.g., lower lean mass content) observed in male patients with AD [71] (Table 2). However, it must be conceded that further multifactorial studies are needed to clarify the relationship between ghrelin and AD.

Role of ghrelin in Parkinson's disease

Classically, PD is considered to be a motor system disease and its diagnosis is based on the presence of a set of cardinal motor signs (e.g. rigidity, bradykinesia, rest tremor and postural reflex disturbance). These symptoms of PD mainly result from the progressive degeneration of dopamine neurons of the SNpc, which causes a consequent reduction of dopamine levels in the striatum [8]. Dopamine-replacement therapy has dominated the treatment of PD since the early 1960s and although the currently approved antiparkinsonian agents offer effective relief of

the motor deficits, especially in the early-moderate stages of the disease, they have not been found to alleviate the underlying dopaminergic neuron degeneration and drug efficacy is gradually lost [72]. Moreover, another major limitation of chronic dopaminergic therapy is the numerous adverse effects such as the development of abnormal involuntary movements (namely dyskinesia), psychosis and behavioral disturbance (e.g., compulsive gambling, hypersexuality) [73].

Dopamine replacement therapy is based on the importance of nigral dopaminergic cell loss, the ensuing striatal dopamine depletion, and onset of motor symptoms. However, the neurodegenerative processes that lead to sporadic PD begin many years before the appearance of the characteristic motor symptoms and additional neuronal fields and neurotransmitter systems are also involved in PD, including the anterior olfactory structures, dorsal motor nucleus of vagus, caudal raphe nuclei, locus coeruleus, the autonomic nervous system, hippocampus and the cerebral cortex [74]. Accordingly, cholinergic, adrenergic and serotonergic neurons are also lost which seems to be responsible for the non-motor symptoms of PD encompassing olfactory and memory impairments, sleep abnormalities and depression, as well as gastrointestinal disturbance, which precede the classical motor symptoms [9]. Non-motor features of PD invariably do not respond to dopaminergic medication and probably form the major current challenge faced in the clinical management of PD [9].

Therefore, the limitations of the current pharmacological treatment of PD have led to extensive investigation of novel non-dopaminergic drugs that may provide alternative or adjunctive treatment for both motor and non-motor symptoms relief with a reduced side-effect profile as well as the discovery of compounds to modify the course of PD. Over the last years, several lines of evidence have suggested the potential of ghrelin in the treatment of PD and an increasing number of studies have investigated the effects of ghrelin in different animal models and PD patients (Table 3).

Experimental models of PD have attempted to reproduce the pathogenic process and to involve areas of the brain pathologically affected in humans. Pathogenic modeling has been attempted using a range of toxins, as well as through the use of transgenic models of gene defects in familial PD and mutant rodent strains. However, there are still no accepted progressive models of PD that mimic the processes known to occur during cell death and that result in the motor and non-motor deficits, pathology and biochemistry features, and drug responsiveness as seen in humans (for recent review see [75]). Despite these limitations,

over the past couple of decades, the proneurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has become a widely used approach for modeling PD. In humans and non-human primates, MPTP causes a severe and irreversible PD-like syndrome [76]. Although rodents are less sensitive to MPTP toxicity, largely because of the economic, logistic, and ethical constraints related to experimental research in primates, the MPTP mouse model has become the most commonly used animal model of PD [77].

The MPTP toxicokinetics is complex and has several stages, and the pathogenic mechanisms involved in the neurodegeneration induced by MPTP include mitochondrial dysfunction, oxidative stress, activation of apoptotic cell death mechanisms and glutamatergic excitotoxicity (for review see [78]). Jiang and co-workers [58] published a pioneer study investigating whether ghrelin protects the dopaminergic neurons from MPTP insult *in vivo*. C57BL6 mice were pretreated with different doses of ghrelin (50, 100, 200 or 400 ng/mouse, i.c.v.) once per day for 8 consecutive days, and received MPTP (30 mg/kg, i.p.) for the last 5 days. The authors described that ghrelin, acting through GHS-R1a, inhibited MPTP-induced dopaminergic neuronal loss in the SNpc as well as dopamine depletion in the striatum [58]. Further *in vivo* [59,60] and *in vitro* [61] studies have confirmed the capability of ghrelin to protect dopaminergic neurons against the toxicity induced by MPTP (Table 3), suggesting its potential as a new neuroprotective agent in PD.

INSERT TABLE 3 ABOUT HERE

Although the sequence of events leading to the protective effects of ghrelin against the loss of dopaminergic neurons has not been fully elucidated, several mechanisms have been implicated in these effects. Ghrelin attenuates MPTP-induced caspase 3 activity by regulating intracellular apoptotic signaling molecules, such as Bcl-2 and Bax [58,61] (Table 3). Consistent with the known antiinflammatory effects of ghrelin in the periphery, inflammatory markers such as activated microglia, tumor necrosis factor- α (TNF- α) and interleukin 1 β (IL-1 β) were significantly inhibited by ghrelin in the substantia nigra and striatum of MPTP-treated mice [60] (Table 3).

In addition, the Andrews' group (Monash University, Australia) published a highlight study demonstrating that the systemic administration of ghrelin could reduce the MPTP-induced dopamine cell loss in the substantia nigra of mice by increasing mitochondrial respiration via uncoupling protein 2 (UCP2) [59]. The same group showed previously the crucial role of UCP2 for nigrostriatal

dopaminergic neuronal function and protection against MPTP-induced neuronal degeneration [83]. The crosstalk between ghrelin and dopamine was predictable based on interactions between the GHS-R and dopamine D1 receptor signaling pathways. Previous studies showed that ghrelin administration could increase extracellular concentration of dopamine in the nucleus accumbens [84]. GHS-R1a is abundantly expressed in dopaminergic neurons in the substantia nigra and ghrelin knockout mice were more susceptible to dopamine cell loss in the SNpc and dopamine depletion in the striatum after MPTP administration than wild type controls [59] (Table 3). Altogether, the data reviewed here reveal that peripheral ghrelin plays an important role in the maintenance and protection of normal nigrostriatal dopamine function and suggest that ghrelin may be a valuable therapeutic agent for neurodegenerative diseases such as PD.

Patients with PD frequently experience weight loss that may be related to different factors: gender, age, physical activity, gastrointestinal dysfunction, disease duration and pharmacological treatment (L-DOPA therapy) [85]. Subthalamic nucleus deep-brain stimulation (STN-DBS) is an alternative to L-DOPA therapy, improving both PD and motor fluctuations [86]. Interestingly, patients with PD gain weight after STN-DBS [87,88]. Considering that STN-DBS electrodes are located close to the hypothalamic centre regulating feeding behaviour, and ghrelin is secreted by the hypothalamic neurones [89], and among other functions, ghrelin is involved in the homeostatic regulation of appetite and energy balance, Corcuff et al. [79] investigated possible changes on serum ghrelin levels in PD patients treated with STN-DBS and/or L-DOPA. In this study, all patients were investigated before and after receiving dopamine treatment, and the group of patients with an implanted neurostimulator was investigated with and without ongoing neurostimulation. The results indicated that L-DOPA treatment did not have a significant acute effect on ghrelin levels either in L-DOPA-alone patients or in the STN-DBS patients off neurostimulation. Moreover, STN-DBS itself did not elicit a modification of ghrelin levels in STN-DBS patients off L-DOPA. Therefore, the authors concluded that total circulating ghrelin does not play an important role in the modification of weight homeostasis in PD patients treated with STN-DBS [79] (Table 3).

More recently, Markaki et al. [82] investigated a possible involvement of ghrelin in the weight gain of PD patients after STN-DBS. Twenty-three PD patients were submitted for body composition measurements and blood sampling 3 days before, and 3 and 6 months

after STN-DBS. Weight gain was significantly associated with the increase of peripheral concentrations of ghrelin at 6 months after STN-DBS. Therefore, contrasting with the previous observations by Corcuff et al. [79], STN-DBS seems to temporarily dysregulate the hypothalamic secretion of ghrelin that may be responsible for the weight gain of PD patients after STN-DBS [82]. Moreover, the authors of this study emphasize that a possible neuroprotective role of DBS, exerted through the increase of ghrelin levels, should be further investigated.

In another study, Fiszer et al. [80] measured the plasma active ghrelin concentration in 11 PD patients with unintentional weight loss, 16 PD patients without weight loss and 12 controls. The body mass index (BMI) was lower in all PD patients investigated (with and without the weight loss) and in PD patients with the weight loss in comparison to the control group. However, there was no difference between both groups of PD patients. BMI was positively correlated with plasma active ghrelin concentration. Interestingly, the lower BMI was, the lower plasma active ghrelin concentration was in PD patients with the weight loss [80] (Table 3).

As stated before, besides motor symptoms, PD patients frequently exhibit non-motor symptoms such as hyposmia, REM sleep behaviour disorder (RBD) and disturbed gastrointestinal motility [90-92] very early in the course of the disease. In relation to the gastrointestinal tract, the stomach has been proposed as one possible ignition point of PD related neuropathology. In respect to the sleep disorder iRBD, about two-thirds of patients with idiopathic RBD (iRBD) develop the alpha-synucleinopathy PD over time [93,94]. Therefore, iRBD is considered a putative pre-motor stage of PD. In this context, Unger et al. [81] measured fasting and post-prandial total ghrelin serum concentrations in 20 healthy controls, 39 (including 19 drug-naïve) PD patients and 11 iRBD patients. Controls showed a decrease of mean fasting ghrelin serum concentrations in the early postprandial phase, followed by a recuperation starting 60 min after the test meal and reaching a maximum at 300 min. The dynamic regulation of ghrelin in response to food intake is partially impaired in subjects at putative preclinical (iRBD) and clinical stages of PD (Table 3). The authors speculate that reduced ghrelin excretion might increase the vulnerability of nigrostriatal dopaminergic neurons in PD patients as suggested by animal studies. Finally, the impaired ghrelin excretion might qualify as a peripheral biomarker and be of diagnostic or therapeutic value [81].

GHRELIN AS A PALLIATIVE TREATMENT FOR THE MEMORY IMPAIRMENTS IN NEURODEGENERATIVE DISEASES

The increasing incidence of neurodegenerative diseases that involve the deterioration of cognitive function has led the scientific community to explore the underlying mechanisms of memory processes and possible novel therapeutic strategies to enhance learning and memory. In this context, ghrelin and GSH-R1a agonists emerge as potential palliative treatments for memory loss that accompanies aging as well AD and PD. Ghrelin and memory story starts silently in 1997 when Guan and colleagues [34] wrote about GHS receptors: *“In addition to the hypothalamus, mRNA encoding the GHS-R was also expressed in several other discrete regions of the rat brain. For example, specific signals were detected in the dentate gyrus, CA2 and CA3 regions of the hippocampal formation.... The functional significance of the GHS-R in these brain regions is not clear at this time... results described above revealed hypothalamus, hippocampal formation and pituitary as the regions with the most abundant expression of GHS-R mRNA.”*

Latter, this G-protein coupled receptor was deorphanized by Kojima et al. [15] that discovered the 28 amino acid octanoylated peptide ghrelin. Taking in mind the Guan’s results about the distribution of ghrelin receptors, and the pivotal role of hippocampus on learning and memory, de Barioglio’s group in the Universidad Nacional de Córdoba (Córdoba, Argentina) started the investigation of the putative role of ghrelin on memory in laboratory animals. Ghrelin was injected by i.c.v. route in rats and their performance in the open-field, plus-maze, and step-down inhibitory avoidance tasks was analyzed. The administration of ghrelin increased in a dose-dependent manner the latency to step-down in the test session, showing for the first time that ghrelin increases memory retention, possible through a hippocampal-dependent mechanism [95] (Table 4). Nevertheless, it is well known that the i.c.v. injection ensures that the peptide effects are centrally mediated but provide mere hints about their site of action.

In this context, localized and precise microinfusions in specific brain regions provide relevant information about “where” the processes under investigation occur. Then, in the next set of experiments the same research group identified extrahypothalamic targets for ghrelin that could justify the changes in the expression of anxiety-like behavior as well as the increase in memory retention induced by the peptide. Thus, ghrelin was injected into brain structures such as the hippocampus,

amygdala and dorsal raphe nucleus (DRN). The results suggested differential roles of the peptide in those structures in the regulation of memory, feeding, and anxiety-like behaviors [96]. Ghrelin administration in all these three regions clearly increased memory retention in a dose-dependent manner. Food intake increased in relation to control rats when ghrelin was injected in the hippocampus and DRN, but injections into the amygdala did not affect food intake [96]. The assumption that the increase in the latency time into the step-down could therefore be attributed to an anxiogenic effect of ghrelin was also clarified and the authors showed that the ghrelin's doses that improved memory retention of rats did not produce any anxiogenic-like behavior [96].

More recently, Carlini et al. [97] demonstrated that the memory enhancing properties of ghrelin can be also observed in a novel object recognition task in mice submitted to 28 days of 50% food restriction. This task differs from the step-down inhibitory avoidance task on the type of information that must be remembered since during the test session of the step-down task, the animals remember the footshock in association with the context. Thus, both paradigms evaluate memory retention but step-down evaluates a memory for aversive stimulus whereas the object recognition test evaluates just the ability to recognize objects. Likewise in mice, decreases in object recognition performance due to chronic food restriction were counteracted by ghrelin administration [97]. As recently reviewed by Gahete et al. [98], further studies have confirmed and extended the capability of ghrelin to improve learning and memory processes in laboratory animals (Table 4).

INSERT TABLE 4 ABOUT HERE

In line with the above mentioned findings, in 2006, the Horvath's group (Yale University School of Medicine, New Haven, USA) published very exciting results showing that circulating ghrelin crosses the blood-brain barrier, enters into the hippocampus and binds to neurons of the hippocampal formation, promoting dendritic spine synapse formation and generating LTP [56]. In this same study, the authors demonstrated that the subcutaneous (s.c.) administration of ghrelin or the ghrelin mimetic LY444711 led to a marked improvement in spatial memory retention in mice. In addition, ghrelin knockout mice presented a reduced number of spine synapses in the hippocampal brain region as well as displayed impaired performance in learning and memory paradigms [56] (Table 4). Moreover, Atcha et al. [100]

demonstrated that the oral or s.c. administration of two structurally non-peptide ghrelin receptor agonists (GSK894490A and CP-464709-18) readily cross the blood/brain barrier and elicit pro-cognitive effects in recognition and spatial learning and memory tasks in rats (Table 4).

Interestingly, Carlini et al. [101] showed that intra-hippocampal ghrelin administration prior the training session, but not prior the test session (performed 24 h after training), improved the long-term memory of rats in the step-down inhibitory avoidance task (Table 4). These findings suggest that ghrelin modulates molecular and/or cellular signaling events involved in memory acquisition and/or consolidation, but not in memory retrieval [101]. Moreover, recent electrophysiological studies provided evidence showing that *in vivo* ghrelin microinjection into the CA1 subregion of hippocampus of rats reduced the threshold values to generate LTP in the dentate gyrus, which is the first synaptic input arising the hippocampus from entorhinal cortex. Moreover, a significant negative correlation was established between this electrophysiological phenomena and the ghrelin effect on the step-down inhibitory avoidance task [102] (Table 4).

In relation to the signaling pathway, it was demonstrated that ghrelin increases nitric oxide synthase (NOS) activity in a dose-dependent manner in trained animals, suggesting the participation of the NOS/NO pathway in the ghrelin's effects on memory [102]. Moreover, it has been also postulated that GHS-R1a likely serves as a modifier of key neurotransmitters required for memory formation such as glutamate, dopamine and serotonin [104]. For instance, Albarran- Zeckler and co-workers [104] demonstrated that genetic deletion of GHS-R1a has opposing regulatory effects on learning and memory. While spatial memory was improved in the *ghsr*^{-/-} mice, contextual memory was impaired by the lack of this receptor (Table 4). One plausible explanation for these results is that ghrelin acts as a neuromodulator of other neurotransmitters such as dopamine. Of particular relevance, studies have shown that antagonism of dopamine D1 receptors in the hippocampus blocks formation of long-term memory [105] and dopamine D1 receptor knockout mice show deficits in contextual fear conditioning [106]. Interestingly, it was recently demonstrated by the Smith's group (The Scripps Research Institute, Florida, USA) that the ghrelin receptor (GHS-R1a) is co-expressed in neurons that express dopamine D1 and D2 receptors, and that a subset of GHS-R1a, which are not occupied by the agonist (apo-GHS-R1a), heterodimerize with these two receptors to regulate dopamine-induced feeding suppression in

mice [107]. It is thus very likely that there is similar importance of the GHS-R1a for effects of dopamine signaling on learning and memory.

In addition, it was demonstrated that the selective serotonin reuptake inhibitor (SSRI) fluoxetine, given i.p. 30 min prior to intra-hippocampal ghrelin injection, prevented the ghrelin-induced increase in food intake and short- and long-term memory retention in rats [99]. These findings suggest that the effects of ghrelin on both feeding and memory retention could depend on the availability of serotonin. Experiments using hippocampal slices demonstrated that few minutes after addition of ghrelin in the superfusion medium, serotonin release was inhibited [108]. In another set of experiments, ghrelin was injected into the hippocampus of rats and the animals were killed 24 h later for the measurement of serotonin release. Remarkably, ghrelin significantly inhibited the serotonin release 24 h after its *in vivo* administration, indicating that ghrelin-induced inhibitory effects on serotonin release starts immediately after injection and can persist for at least 24 h after its central administration [108].

Certainly additional brain systems and molecular mechanisms need to be studied to further clarify the role of ghrelin on learning and memory processes. However, from recent findings demonstrating the ability of ghrelin to improve the cognitive dysfunction in rats submitted to models of sepsis-associated encephalopathy [109] and diabetic encephalopathy [110], it appears that ghrelin might be particularly useful to restore impaired learning and memory processes associated to neurodegenerative diseases.

GHRELIN AS A PALLIATIVE TREATMENT FOR DEPRESSION IN NEURODEGENERATIVE DISEASES

Depression is a prevalent disease; 10-20% of people in the world's population will develop depression at least once in their lifetime, causing impairment in functioning and quality of life, with high medical and social costs [111,112]. According to World Health Organization major depression will be the world's second most debilitating disease by 2020, eclipsed only by heart disease [113]. This disease is characterized by apathy, indifference and anhedonia, behavioral sluggishness and increasing inactivity, feelings of guilt, pessimism, regret and low self-esteem, psychophysiological disturbances of sleep and appetite [111]. Major depression is frequently found coexisting with long-standing chronic medical conditions such as cardiovascular disease, diabetes mellitus, obesity and neurodegenerative diseases [114].

Depression is common and a clinically important feature of PD and can precede the onset of the motor symptoms. The prevalence of depression in patients with PD is approximately 40% [115,116]. Concomitant depression in PD is associated with greater healthcare system use, including medical hospitalizations [117]. Moreover, major depressive disorder is considered a risk factor for developing AD later in life [118]. Depressive symptoms are frequent and affect nearly 40% of AD patients [119]. Noteworthy, brains of patients with AD with comorbid depression showed higher levels of cortical neurofibrillary tangles than brains of patients with AD without comorbid depression, suggesting an interaction between depression and the neuropathologic processes in AD [120].

Drugs used in the treatment of depression cause several side effects and generally influence weight gain. Among hormones that act on weight regulation, ghrelin has been suggested to exert an antidepressant action. Regarding the preclinical studies, there are several pieces of evidence supporting a role for ghrelin in the modulation of mood (Table 5). The administration of antisense DNA for ghrelin into the lateral ventricle was reported to reduce the immobility time in the forced swimming test (FST) in rats, which is a result indicative of an antidepressant-like effect [121]. Further evidence of the possible antidepressant activity of ghrelin, it is a study by Lutter et al. [122] showing that the subcutaneous administration of ghrelin produced antidepressant-like responses in the FST. In addition, increasing ghrelin levels through a diet containing 60% of normal calories resulted in an antidepressant-like response in the FST. Moreover, mice submitted to chronic social defeat stress (CSDS) procedure, which induces behavioral deficits reminiscent of depression including social avoidance, had significantly elevated levels of acylated ghrelin that persisted for at least 4 weeks after the procedure [122]. Moreover, genetic deletion of GHSRs exacerbated depression-like behavior induced by CSDS, a finding also described by Chuang et al. [123]. The study by Lutter et al. [122] demonstrated that ghrelin's antidepressant-like effects in the FST were blocked in mice lacking orexin, suggesting that the antidepressant-like actions of this peptide may be dependent on a direct and/or indirect activation of orexin-containing neurons.

INSERT TABLE 5 ABOUT HERE

A recent evidence provided by our group [124] reinforced the notion of the ghrelin's antidepressant action, since it was shown that the

acute administration of ghrelin by i.c.v. route produced antidepressant-like effect in a predictive test of antidepressant activity, the tail suspension test (TST) in mice. In addition, this study also showed that ghrelin, administered acutely to mice by i.c.v. route, was able to abolish the depressive-like behavior induced by olfactory bulbectomy (OB) [124], an animal model of depression which produces behavioral, neurochemical and neuroendocrinological changes that resemble some of the symptoms observed in depressed patients [125]. Of note, these behavioral and neurochemical changes in OB rodents are reported to be normalized only by the chronic administration of antidepressants. Interestingly, there are few studies reporting that the acute administration of agents that inhibit the glutamatergic transmission such as zinc and riluzole produce a rapid reversal of the hyperlocomotion activity induced by OB [126,127], as opposed to the conventional antidepressants. Therefore, it remains to be established if an antiglutamatergic mechanism could be responsible for the antidepressant-like effect of ghrelin.

Clinical studies have also supported the idea that ghrelin exerts a possible beneficial role in depressive disorders. The serum ghrelin levels were lower before and after treatment in depressive patients as compared with non-depressed individuals [128,129]. Moreover, it was demonstrated that electroconvulsive therapy (ECT), an effective treatment for depression, decreased serum ghrelin levels in depressive patients as compared with the levels of this peptide before ECT [130]. Taking into account that ghrelin inhibits serotonin release [108,131], one of the hypothesis raised to explain these results is that the decreased circulating ghrelin levels may be a compensatory response to depression, potentially elevating serotonin levels [129]. Moreover, after a pulsatile administration of ghrelin to depressive patients, an improvement in the depressive symptoms assessed by a validated self-rating scale ('Befindlichkeits-Skala'), at trend level ($p=0.093$) in men, but not in women, was observed [132]. Furthermore, ghrelin gene polymorphism was previously associated with depression [133]. However, it must be conceded that other studies failed to show a correlation between ghrelin and depression [134,135]. For instance, nocturnal plasma ghrelin of depressed patients and matched healthy subjects did not differ when stratified for sex [134] and plasma ghrelin was not different between 83 depressed patients and 46 healthy controls [135].

Several studies have linked ghrelin with stress, which, in turn, is a risk factor for the development of depression. Humans subjected

acutely to psychosocial stress exhibit increased plasma ghrelin levels [136], and similar results were described in rats after acute psychological stress [137]. The increase in ghrelin levels could contribute to the mechanisms responsible for the development of stress-induced depression or may represent a protective mechanism to minimize manifestations of depression following stress [37,138,139]. This protective mechanism may be related to the activation of hedonic signalling pathway and stimulation of the intake of palatable caloric foods that, in turn, elicits central reward pathways and increases dopamine signaling [139]. However, the stress-induced food rewards behavior and hyperphagia of palatable caloric dense foods increases body weight. Following prolonged exposure to stress and palatable foods, a desensitization of reward signalling may be linked to the risk of depression and co-morbid obesity [139].

Another possible mechanism that links ghrelin to depressive disorders is that a ghrelin's action on mood may be mediated through the modulation of neuroinflammatory mechanisms [138], which has been demonstrated to play a role in the pathophysiology of depression [114,140,141]. Therefore, it remains to be established if ghrelin administration is able to ameliorate the depressive-like behavior elicited by neuroinflammatory conditions in rodents, taking into account that ghrelin or ghrelin mimetics are able to suppress the synthesis and release of pro-inflammatory cytokines [142].

GHRELIN AS A PALLIATIVE TREATMENT FOR OF STRESS AND ANXIETY DISORDERS IN NEURODEGENERATIVE DISEASES

The gut-brain connection has been implicated in brain disorders ranging from anxiety to depression and schizophrenia [143-145]. This connection between gastrointestinal tract and the hypothalamic-pituitary axis (HPA) is bidirectional and it is mediated through the release of peptides that exert responses within the brain as well as via neuroendocrine and sensory inputs from the gut. The exact mechanisms governing such communication are unclear and most studies focus on the impact of altered signaling from the brain to the gut although the reverse is now being studied (for review see [144,145]). Even though a complete discussion of the interrelationships between the gut and the brain in the control of stress and anxiety disorders is beyond the intended scope of this article, focus will be placed on briefly reviewing the current literature on ghrelin and its role in the mediation/modulation of stress/anxiety states.

There is increasing evidence that ghrelin has several physiological functions, especially in CNS, including a role in neuroprotection, learning and memory, reward and motivation, and depression as aforementioned, besides being important in anxiety and stressful conditions (for review see [37]), since plasmatic ghrelin is able to cross the blood–brain barrier, and to accumulate and bind to neurons in several brain areas underlying stress and anxiety responses. Actually, the orexigenic and pro-obesity targets of ghrelin’s actions are located in hypothalamic and mesolimbic circuits involved not only in energy balance, appetite and reward but also in regulating mood and cognition (for review see [146]).

Animal and human studies suggest that stressful conditions can result in low mood and increased energy intake, particularly from fatty acids and sugars, and potential changes in body weight, leading to obesity. These effects involve changes in neuroendocrine and peripheral metabolic substrates which alter feeding behavior [147]. Although it is well known the function of cortisol/corticosterone, as well as the role of HPA axis, in the stress-induced eating of caloric “comfort foods”, the molecular substrates and neuronal circuits controlling the complex behaviors responsible for these processes remain mostly unknown. However, one aspect has been established in recent years: stress-induced food reward is dependent on signaling by ghrelin [123], among other neuropeptides [148]. In view of the fact that stress-induced food reward is dependent on ghrelin [149], it is conceivable that ghrelin could be involved in the underlying mechanisms of stress responses and, therefore, of anxiety states.

Stress is known to modify circulating ghrelin and also ghrelin-O-acyltransferase (GOAT) levels with differential responses related to the type of stressors, including a reduction of ghrelin induced by physical stressors (abdominal surgery and immunological/endotoxin injection, exercise) and an elevation by metabolic (cold exposure, acute fasting and caloric restriction) and psychological stressors, which may contribute to the neuroendocrine and behavioral responses besides the energy requirement needed after repeated exposure to stressors (for review see [150]). Circulating levels of ghrelin are high during fasting in several species including man [151] and it is also increased by stressors, such as tail pinch [152], water immersion [153], social defeat [122], restraint stress [154] and chronic stress (caged filled with water) [155]. Of high importance, behavioral studies performed by independent research groups have shown that centrally administered ghrelin also

participates in the expression of anxiety-like behavior in rodents (Table 6).

INSERT TABLE 6 ABOUT HERE

Central or systemic administration of ghrelin promotes anxiogenic-like behavior in both rats and mice [95,96,152], as evaluated in the elevated plus maze and other behavioral tests. Ghrelin also promotes anxiogenesis in chicks evaluated in an open-field [159]. Moreover, ghrelin antisense oligonucleotides produced an anxiolytic-like effects in the elevated plus maze, black and white, and conditioned fear tests in rats [121]. These anxiogenic-like effects of ghrelin were inhibited by administration of a corticotropin-releasing hormone receptor antagonist [152]. Thus, they are probably due to the activation of paraventricular nucleus (PVN) where GHS-R1a mRNA is expressed at high levels [160], supporting the hypothesis of a direct activation of GHS-R1a on CRH-containing neurons. Ghrelin administration also stimulates adenocorticotrophic hormone (ACTH) cell hypertrophy and proliferation, and promotes ACTH and corticosterone/cortisol release in several species including humans [152,161,162]. Taken together, these findings suggest that ghrelin levels increases in response to psychological stress and that ghrelin may influence behavioral and neuroendocrine responses to stressors, possibly via the mobilization of ACTH. Furthermore, the stress-induced increase in plasma ghrelin was associated with the acute response of serum cortisol to stress [136].

Ghrelin also controls anxiety-like behavior through the serotonergic system, since administration of ghrelin in specific rat brain regions showed that ghrelin promotes a more prominent anxiogenic-like behavior when injected into the dorsal raphe nuclei (DRN) [96], the primary site of serotonergic neurons in the brain, and this effect seems to be mediated by an inhibition of serotonin release. Actually, ghrelin appears to have an impact on the HPA response via a serotonergic pathway [163]. In this regard, 80% of DRN neurons were classified as putative serotonin-containing neurons and ghrelin depolarized 75% of them [164]. Ghersi et al. [108] also showed that ghrelin inhibited serotonin release in hippocampal slices. A chronic (4 weeks) central exposure to ghrelin promoted an increase in anxiety- and depression-like behavior in rats. Changes in expression of a number of genes representing key systems implicated in these behavioral effects were found as well as an inhibition of the electrophysiological response of DRN after a ghrelin challenge [156].

Additionally, anxiogenic and orexigenic effects of ghrelin seems to be mediated, at least in part, via endocannabinoid signaling since PVN injections of the ghrelin promoted anxiogenic-like profile and a significantly increase in food intake and these effects were blocked by AM251, a cannabinoid CB1 receptor antagonist [158].

On the other hand, caloric restriction for 10 days or acute administration of ghrelin promoted an increase in circulating ghrelin levels in *ad libitum*-fed C57BL6/J mice, producing anxiolytic-like responses in the elevated plus maze. However, when GHSR-null mice were calorie-restricted, these anxiolytic-like behavioral responses were no longer observed indicating a specific role of GHS-R1a receptors in this effect [122]. In addition, recently, it was shown that ghrelin knockout mice are more anxious after an acute restraint stress, compared with wild-type mice, as evaluated in three behavioral tests (elevated plus-maze, open-field and light/dark box). Acute restraint stress exacerbated neuronal activation in the hypothalamic PVN and medial nucleus of the amygdala in knockout mice compared with wild type mice, and the administration of exogenous ghrelin was able to reverse this effect. Spencer et al. [157] proposed that ghrelin is able to reduce anxiety after acute stress by stimulating the HPA axis at the anterior pituitary level, by involving urocortin 1 receptor on this effect. These observations showing an anxiolytic-like effect of ghrelin seem to be supported by findings that ghrelin levels were higher in the Sprague–Dawley rats (low-anxiety strain) than in the Wistar Kyoto rats (high-anxiety strain) after acute stress, whereas ACTH are equally enhanced in both strains [137,165]. Moreover, although psychological stress induces an increase in plasma ghrelin levels in humans, the post-stress induced urge for uncontrolled eating is not acutely modulated by stress related elevations in ghrelin levels [136]. In this regard, a recent study described that isolation stress resulted in a reduction of plasma ghrelin that seems to be dependent on CRF1-R, and MC4 receptor in PVN and 5-HT1B/2C receptors in the arcuate nucleus (ARN) [166]. The number of immunoreactive neurons in PVN of the hypothalamus was significantly increased after peripheral administration of ghrelin, an effect that seems to occur via NPY-positive projections from the ARN [167]. As the PVN is involved in a neuronal network mediating the autonomic, neuroendocrine, and skeletal-motor responses of fear and anxiety [168], an increased density of immunoreactive neurons in the PVN, after peripheral ghrelin administration, could also be the result of ghrelin transport into the brain and/or resultant of an activation of the hippocampal–amygdalic–hypothalamic network involved in the

regulation of fear and anxiety rather than an activation of hunger/satiety pathways along the ARN-PVN axis [167]. In addition, rates of anxiety and cognitive impairment were higher in the hypertensive elders, which were negatively correlated with plasma ghrelin levels, resulted from chronic cortisol response to long-term anxiety [169].

The reason for these contradictory findings are presently unknown, but it could be related in animals to the timing of the behavioral tests after ghrelin administration since the anxiogenic-like effect of ghrelin was seen when the behavioral evaluation was performed within 5-10 min [95,96,121,152,158], whereas the latter study conducted the evaluation after 45 min of the injection [122]. As ghrelin has a plasmatic half-life of about 30 min, it is conceivable that the dose which had significant effects on food intake in all previous studies, underlined the anxiogenic responses in the 5-10 min studies. These issues have to be clarified since not all studies show changes in ghrelin levels [134,135].

Several findings suggest that ghrelin was secreted as a result of alarm signals concerning physiological changes such as severe weight loss, which are potentially life threatening [155]. Altogether, evidence shows that ghrelin is involved in emotional reactivity in rodents, although no differences were found in patients with obsessive compulsive disorder [170]. Thus, ghrelin is a peptide hormone implicated in diverse biological functions such as the modulation of centrally controlled behaviors ranging from energy balance (food intake, body-weight regulation and glucose homeostasis) to stress, anxiety and memory processes. Some regions of the hypothalamus appear to be differentially sensitive and responsive to the feeding-stimulant, metabolic, and anxiogenic actions of ghrelin and that the ARN and PVN, in particular, exert a primary role in mediating these effects [158].

Therefore, the bidirectional effects of ghrelin on stress and anxiety behaviors seem to be stressor, test- and time-dependent, and may be partly mediated by ghrelin via the CRF, serotonin and the endocannabinoid systems.

CONCLUSION

This article reviews the recent evidence that the gastric hormone ghrelin plays an important role not only as a modulator of GH secretion and food intake, but fundamentally as an important factor for neuronal plasticity, growth and survival in the CNS. Possible therapeutic opportunities for neurological disorders through the modulation of ghrelin system are pointed out, in the expectancy that this review may

inspire clinical researchers and foster experimental approaches using ghrelin as the therapeutic target.

Two major conclusions should be drawn: first, the physiological role of ghrelin is by far wider than considered before including learning and memory, anxiety, depression and neuroprotection; second, changes in the GHS-R1a receptors seem to underlie these central actions of ghrelin. Establishing the function of ghrelin in physiological stress responses and whether control of its activity would be useful for prevention and/or treatment of stress-induced diseases, such as anxiety and mood disorders as well as psychiatric symptoms associated to neurodegenerative diseases, remain important research aims.

Since apoptotic cell death, mitochondrial dysfunction, oxidative stress and neuroinflammation have been identified as molecular processes associated with the neurological disorders here presented, manipulation of the ghrelin system beckons as an opportunity for neuroprotection, improving neuronal plasticity and counteracting the lost of brain functions in patients with neurodegenerative diseases, including AD and PD. Future research aiming the development of novel non-peptide ghrelin receptor antagonists, a growing number of ghrelin receptor selective ligands, and also inverse agonists, will help to elucidate the neurobiology and physiological role of ghrelin as well as its potential as novel palliative and neuroprotective agent in neurodegenerative diseases.

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FIGURE LEGENDS

Fig. 1. Schematic representation of the post-translational processing of ghrelin. A signal peptide peptidase cleaves the signal sequence. Acylation of pro-ghrelin occurs by means of ghrelin O-acyl transferase (GOAT), which is located in the ER compartment and mediates the translocation of octanoyl-CoA. Once the pro-ghrelin precursor reaches the trans-Golgi compartment, it is cleaved by PC1/3 pro-hormone convertase. Different forms of ghrelin are released to the circulation: acylated, unacylated, and other shorter forms.

Fig. 2. Schematic illustration of the possible molecular mechanisms associated with the neuroprotective effects of ghrelin observed in experimental models of ischemia, traumatic brain injury, spinal cord

injury, amyotrophic lateral sclerosis, epilepsy, Alzheimer's disease and Parkinson's disease. The interaction of ghrelin with GHS-R1a leads to activation of diverse signaling pathways including the extracellular signal-regulated kinase (ERK1/2), phospholipase C (PLC) and protein kinase C (PKC), and the protein kinase cascade Raf–MEK–MAPK. Activation of these kinase signaling pathways leads to the inhibition of apoptotic events, via the subsequent increase in the Bcl-2:Bax ratio, the prevention of Cyt release and the inhibition of caspase 3 activation. Furthermore, ghrelin prevents inflammatory microglial activation and activates the mitochondrial protein uncoupling protein-2 (UCP2). This protein enhances neuroprotection by suppressing reactive oxygen species (ROS) and promoting mitochondrial biogenesis.

Fig. 3. Schematic illustration of the possible molecular mechanisms associated with the neuroprotective and cognitive enhancing properties of ghrelin observed in experimental models of Alzheimer's disease. Ghrelin mitigates a series of neurochemical changes induced by infusion of amyloid-beta ($A\beta$) peptides including microgliosis, neuronal loss, increase of oxidative stress biomarkers, increase of acetylcholinesterase (AChE) activity, decrease of glutamate uptake and impairments on long-term potentiation (LTP) generation in the hippocampus and frontal cortex of mice.

FIGURA 1

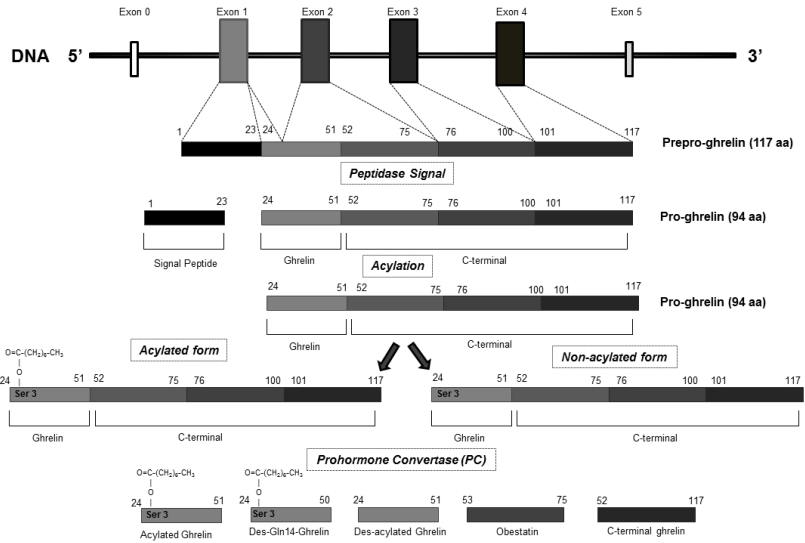


FIGURA 2

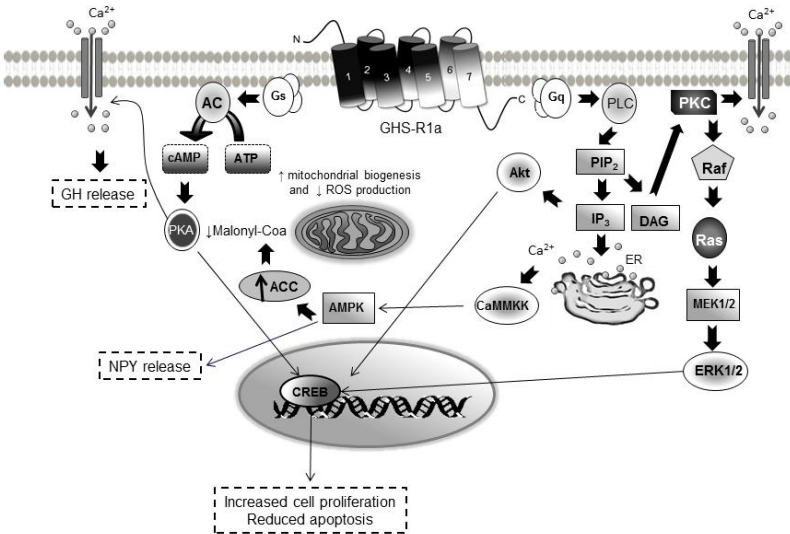


FIGURA 3

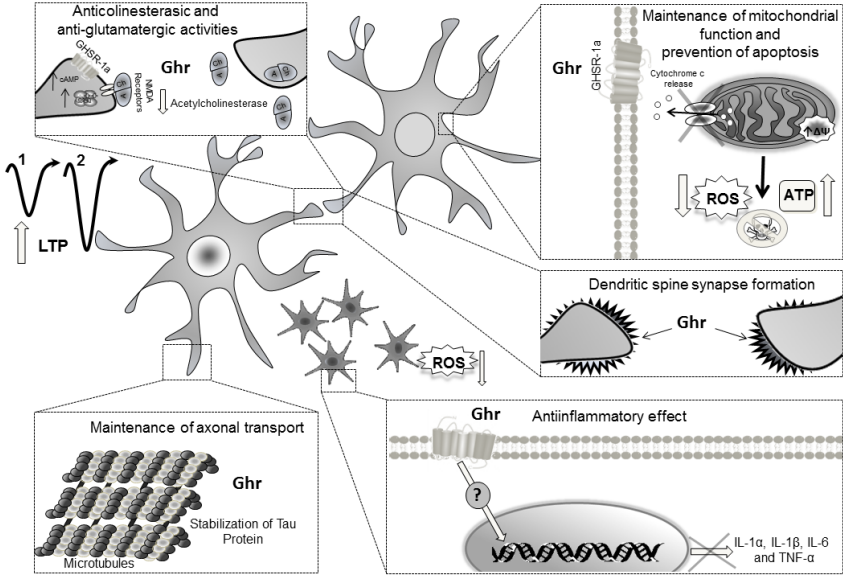


Table 1. Overview of ghrelin’s neuroprotective effects in different experimental models of brain injury.

Experimental model	Main findings	References
Treatment of adult rats with hormone-releasing peptide (GHRP)-6	GHRP-6, a synthetic ligand for the ghrelin receptor, increased insulin-like growth factor (IGF-I) mRNA levels in the hypothalamus, cerebellum, and hippocampus, with no effect in cerebral cortex. Phosphorylation of Akt and Bad was stimulated and the anti-apoptotic protein Bcl-2 was augmented in areas where IGF-I was increased	[40]
Treatment of aged rats with hormone-releasing peptide (GHRP)-6	GHRP-6 increased IGF-I mRNA levels, decreased cell death and inhibited caspase 3 and 9 activation in the cerebellum of aged rats	[41]
Cultured rat cortical neurons exposed to oxygen and glucose deprivation (OGD)	The neuroprotective effects of ghrelin was accompanied by an increased phosphorylation of extracellular signal-regulated kinase (ERK)1/2, Akt, and glycogen synthase kinase-3beta (GSK-3beta), suggesting the anti-apoptotic effects	[42]

	of ghrelin	
Ischemic injury induced by transient middle cerebral artery occlusion in rats	Ghrelin protected cortical neurons against ischemic injury through the inhibition of the pro-apoptotic gene Par-4 expression and apoptotic molecules in mitochondrial pathway	[43]
Traumatic brain injury (TBI)	TBI caused significant neuronal degeneration, increased vascular permeability and increased brain cytokines TNF- α and IL-6 levels. Treatment with ghrelin mitigated these effects	[44-46]
Spinal cord injury (SCI)	Ghrelin reduced the SCI-induced oxidative stress and exert antiinflammatory effects in the rat spinal cord following trauma. However, ghrelin failed to improve the impairment of the neurological functions due to SCI	[47]
Ischemia/reperfusion (I/R) injury in the spinal cord	Ghrelin inhibited spinal I/R injury via anti-apoptotic mechanisms and improved the neurologic function in	[48]

	rats	
Amyotrophic lateral sclerosis (ALS)	Ghrelin protected against chronic glutamate-induced cell death in organotypic spinal cord cultures (OSCC) by activating the MAPK and PI3K/Akt signaling pathways and preventing microglial activation	[49,50]
Epilepsy	Ghrelin delayed or prevented the development of seizures and hippocampal neurodegeneration in rodents induced by different compounds such as pentylenetetrazole, pilocarpine, kainic acid and penicillin	[51-55]

Table 2. Overview of the role of ghrelin in experimental models and human Alzheimer’s disease (AD).

Experimental model	Main findings	References
<p>Senescence-accelerated mouse prone8 (SAMP8 mice)</p>	<p>Ghrelin improved retention of T-maze foot shock avoidance in 12 and 14 month-old SAMP8 mice. Moreover, ghrelin promoted dendritic spine synapse formation and generation of long-term potentiation (LTP) in the hippocampus of mice. Disruption of the gene that encodes ghrelin resulted in decreased numbers of spine synapses in the CA1 region and impaired performance of mice in the T-maze foot shock avoidance task. Ghrelin administration reversed these alterations</p>	<p>[56]</p>
<p>Intra-hippocampal injection of Aβ₁₋₄₂ peptide in ICR mice</p>	<p>Intraperitoneal injection of ghrelin (80 μg/kg) improved Aβ₁₋₄₂ (10 μM, 3 μl)-induced memory deficits evaluated in the Y-maze and passive avoidance tasks. Moreover, ghrelin attenuated hippocampal</p>	<p>[57]</p>

	microgliosis and neuronal loss induced by $A\beta_{1-42}$ administration	
Intracerebroventricular injection of $A\beta_{1-40}$ peptide in Swiss mice	Ghrelin (3 nmol, i.c.v.) prevented the $A\beta_{1-40}$ (400 pmol, i.c.v.)-induced spatial memory impairments and depressive-like behaviors evaluated in the object location and forced swimming tasks. Moreover, ghrelin mitigated the increase of oxidative stress biomarkers and acetylcholinesterase (AChE) activity and the decrease of glutamate uptake in the hippocampus and frontal cortex of mice	Santos et al., unpublished data
Hippocampal slices of Swiss adult mice	Ghrelin (1 nM) prevented the impairments on LTP generation induced by $A\beta_{1-40}$ (200 nM) in the CA1 subfield of hippocampal slices of adult mice	Santos et al., unpublished data
Human studies	Main findings	References
12 young and 7 old normal weight subjects	Mean plasma ghrelin concentrations in older normal weight subjects were significantly lower than those present in young normal weight subjects	[67]

14 AD patients	Ghrelin levels in the cerebrospinal fluid of AD patients were similar to those of age-matched controls	[68]
Analysis of temporal lobe of 6 patients with AD and 6 non-demented controls obtained from the Netherlands Brain Bank	mRNA levels for ghrelin, ghrelin-O-acyltransferase (enzyme responsible for ghrelin acylation), and its receptor GHS-R1a were reduced, while expression of GHS-R1b increased, in the temporal lobe of AD patients	[69]
182 AD patients and 143 age-matched controls	One single nucleotide polymorphisms (SNP), rs4684677 (Leu90Gln), showed a marginal association with age of AD onset, but no additional association between other SNPs of the ghrelin gene and AD were detected	[70]
27 AD patients and 23 age-matched controls	The area-under-the-curve (AUC) for serum ghrelin levels after 75 g of glucose load was lower in male patients with AD compared to control males, while no difference was observed between females AD and controls	[71]

Table 3. Overview of the role of ghrelin in experimental models and human Parkinson’s disease (AD).

Experimental model	Main findings	Reference
<p>C57BL6 mice were pretreated with different doses of ghrelin (50, 100, 200 or 400 ng/mouse, i.c.v.) once per day for 8 consecutive days, and received MPTP (30 mg/kg, i.p.) for the last 5 days</p>	<p>Ghrelin, acting through GHS-R1a, inhibited MPTP-induced dopaminergic neuronal loss in the substantia nigra pars compacta (SNpc) as well as striatal dopamine depletion. Ghrelin also reversed the downregulation of Bcl-2, the upregulation of Bax, and caspase-3 activation caused by MPTP</p>	<p>[58]</p>
<p><i>In vitro</i> studies with MES23.5 cells treated with 1-methyl-4-phenylpyridinium (MPP⁺)</p>	<p>MES23.5 cells treated with 200 micromol/L of MPP⁺ showed decreased cell viability and mitochondrial transmembrane potential, an elevated level of reactive oxidative species production and activation of caspase-3 and apoptotic morphological changes. Pretreatment with ghrelin abolished the MPP⁺-induced apoptotic changes in a concentration-</p>	<p>[61]</p>

	dependent manner	
<p>C57BL6 received four intraperitoneal (i.p.) injections of MPTP (20 mg/kg) at 2-h intervals in a single day, and they were administered with ghrelin (20, 40, 80, or 160 µg/kg, i.p.) 2 h before the first MPTP injection and 30 min prior to each MPTP injection for a total of five injections</p>	<p>Ghrelin attenuated the loss of SNpc neurons and the striatal dopaminergic fibers through the activation of GHS-R1a. Ghrelin also reduced nitrotyrosine levels and improved the impairment of rotarod performance. Furthermore, ghrelin prevented MPTP-induced microglial activation in the SNpc and striatum, the expression of pro-inflammatory molecules TNF-α and IL-1β, and the activation of inducible nitric oxide synthase</p>	<p>[60]</p>
<p>C57/B6, UCP2 knockout and ghrelin knockout mice were injected with MPTP (40 mg/kg, i.p.)</p>	<p>Ghrelin knockout mice were more susceptible to dopamine cell loss in the SNpc and dopamine depletion in the striatum after MPTP administration than wild type controls. Ghrelin increased the firing rate of SNpc dopamine neurons, which enhances dopamine availability during the course of degeneration and</p>	<p>[59]</p>

	<p>lowers the loss of dopamine levels in the dorsal striatum.</p> <p>Moreover, ghrelin enhanced the uncoupling protein 2 (UCP2)-dependent mitochondrial respiration and proliferation, providing a bioenergetic status that makes these neurons more resistant to cellular stress</p>	
Human studies	Main findings	Reference
<p>12 PD patients taking chronic L-DOPA therapy and 12 PD patients with subthalamic nucleus deep-brain stimulation (STN-DBS) therapy associated with chronic L-DOPA treatment</p>	<p>L-DOPA treatment did not have a significant acute effect on ghrelin levels either in L-DOPA-alone patients or in the STN-DBS patients off neurostimulation. Moreover, STN-DBS itself did not elicit a modification of ghrelin levels in STN-DBS patients off L-DOPA</p>	<p>[79]</p>
<p>11 PD patients with unintentional weight loss, 16 PD patients without weight loss and 12 controls</p>	<p>The body mass index (BMI) was lower in all PD patients (with and without the weight loss) and in PD patients with the weight loss in comparison to the</p>	<p>[80]</p>

	<p>control group, however, there was no difference between both groups of PD patients. BMI was positively correlated with plasma active ghrelin concentration. The lower BMI was, the lower plasma active ghrelin concentration was in PD patients with the weight loss</p>	
<p>39 (including 19 drug-naïve) PD patients, 11 idiopathic REM sleep behaviour disorder (iRBD) patients and 20 healthy controls</p>	<p>Controls showed a decrease of mean fasting ghrelin serum concentrations in the early postprandial phase, followed by a recuperation starting 60 min after the test meal and reaching a maximum at 300 min.</p> <p>The dynamic regulation of ghrelin in response to food intake is partially impaired in subjects at putative preclinical (iRBD) and clinical stages of PD. Thus, the impaired ghrelin excretion might qualify as a peripheral biomarker and be of diagnostic or therapeutic value</p>	[81]
<p>23 PD patients were submitted for body</p>	<p>Weight gain was significantly</p>	[82]

composition measurements and blood sampling 3 days before, and 3 and 6 months after STN-DBS	associated with the increase of peripheral concentrations of ghrelin at 6 months after STN-DBS	
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Table 4. Summary of ghrelin's effects on learning and memory.

Experimental model	Main findings	References
Male Wistar rats were injected by intracerebroventricular (i.c.v.) route with ghrelin (0.3, 1.5, and 3 nmol/ μ l) and immediately later they were tested in the open field, elevated plus-maze and step-down inhibitory avoidance tasks	Ghrelin increased freezing in the open field and decreased the number of entries and the time spent on the open arms in the elevated plus-maze, indicating an anxiogenic effect. Moreover, ghrelin increased in a dose-dependent manner the latency time in the step-down test. A rapid and prolonged increase in food intake was also observed. These results indicate that ghrelin induces anxiogenesis and increases memory retention in rats	[95]
Male Wistar rats were microinjected bilaterally with ghrelin (0.3, 1.5, and 3 nmol/ μ l) into the hippocampus, amygdala or in dorsal raphe nucleus (DRN) and immediately later they were tested in the elevated plus-maze and step-down inhibitory avoidance tasks	The injection of ghrelin into the hippocampus and DRN, but not into the amygdala, increased food intake. Ghrelin dose dependently increased memory retention in the hippocampus, amygdala, and DRN. Moreover, ghrelin at different potencies induced anxiogenesis in these brain structures	[96]
Wild-type (C57BL6), ghrelin knockout and	Ghrelin enters the hippocampus and binds to neurons of the hippocampal formation,	

<p>CD-1 mice were subcutaneously (s.c.) injected with ghrelin (10 µg/kg) or the ghrelin mimetic LY444711 (5 mg/kg) and were tested in the spontaneous alternation plus-maze, T-maze footshock avoidance and step-down inhibitory avoidance tasks</p>	<p>where it promotes dendritic spine synapse formation and generation of long-term potentiation. These ghrelin-induced synaptic changes are paralleled by enhanced spatial learning and memory. Targeted disruption of the gene that encodes ghrelin resulted in decreased numbers of spine synapses in the CA1 region and impaired performance of mice in behavioral memory testing, both of which were rapidly reversed by ghrelin administration</p>	<p>[56]</p>
<p>Male Wistar rats were pretreated intraperitoneally (i.p.) with the selective serotonin reuptake inhibitors fluoxetine (5 mg/kg) or clomipramine (2.5 or 5 mg/kg) and 30 min later they were microinjected bilaterally with ghrelin (0.03, 0.3 and 3.0 nmol/µl) into the CA1 hippocampus or i.c.v. and immediately later they were tested in the step-down inhibitory avoidance</p>	<p>Ghrelin increased food intake and the short and long term memory retention and these effects were prevented by the pretreatment with fluoxetine. These results suggest that the effects of ghrelin on both feeding and memory retention in extrahypothalamic structures such as the hippocampus, could depend on the availability of serotonin</p>	<p>[99]</p>

task		
<p>Female Swiss-SWR/J mice submitted to 28 days of 50% food restriction were microinjected bilaterally with ghrelin (0.03, 0.3 and 3.0 nmol/μl, i.c.v.) and immediately later they were tested in the novel object recognition task</p>	<p>The animals with food restriction showed an increase in plasma ghrelin levels and a decrease in the percentage of novel object recognition time. The i.c.v. administration of ghrelin improved the memory impairments of food-restricted animals</p>	<p>[97]</p>
<p>Male Lister hooded rats were injected with the structurally non-peptide ghrelin receptor agonists GSK894490A (0.3 and 3.0 mg/kg p.o.) and CP-464709-18 (1 and 3 mg/kg s.c.) and were tested in the novel object recognition test, a modified water maze paradigm and a scopolamine-induced deficit in cued fear conditioning</p>	<p>Both compounds significantly improved performance in the novel object recognition and modified water maze tests but were unable to attenuate a scopolamine deficit in cued fear conditioning</p>	<p>[100]</p>
<p>Male Wistar rats were microinjected bilaterally with ghrelin (0.03, 0.3 and 3.0 nmol/μl) into the CA1 hippocampus or i.c.v. 15 min previous the training session or 15 min previous the</p>	<p>Intra-hippocampal ghrelin administration previous the training session improved the long-term memory in this task, but did not modify the short-term memory. Ghrelin administration previous</p>	<p>[101]</p>

<p>test session (performed 24 h after training) of the setp-down inhibitory avoidance task</p>	<p>the test session did not alter the memory performance. These results suggest that ghrelin may modulate specific molecular signaling involved in memory acquisition/consolidation but not in the retrieval</p>	
<p>Male Wistar rats were injected into the hippocampus with ACSF, L-NOArg (2 µg/µl), ghrelin (0.3 or 3.0 nmol/µl) or L-NOArg prior to ghrelin administration, immediately after training in the step-down. Thirty minutes later, the animals were sacrificed and the nitric oxide synthase (NOS) activity and electrophysiological paramateres were measured in the hippocampus</p>	<p>Intra-hippocampal ghrelin administration increased the NOS activity in a dose-dependent manner, and reduced the threshold for LTP generation in dentate gyrus of rat hippocampus. Moreover, pre-administration of L-NOArg in the hippocampus partially prevented the ghrelin-induced memory improvement, abolished the increase in NOS activity, and prevented the decreased threshold to generate LTP induced by ghrelin. These findings suggest that activation of the NOS/NO pathway in hippocampus participates in the effects of ghrelin on memory consolidation</p>	<p>[102]</p>
<p>Male Wistar rats were</p>	<p>Ghrelin infusion prolonged expression of LTP and induced long-</p>	

<p>bilaterally injected into the dorsal hippocampus with 1 nM of ghrelin (5 μL) and/or 1 μM of LY294002 (3 μL), a phosphoinositide 3-kinase (PI3K) inhibitor, once a day for 4 days and were later tested in the Morris water maze</p>	<p>lasting potentiation in the dentate gyrus (DG) <i>in vivo</i>. This potentiation was inhibited by PI3K antagonists. This dose of ghrelin time-dependently enhanced the phosphorylation of Akt-Ser473, a downstream molecule of PI3K.</p> <p>In addition, ghrelin infusion into the hippocampus improved water maze performance. These results suggest that ghrelin infusion into the hippocampus may activate the PI3K signaling pathway, and enhance synaptic plasticity and spatial memory</p>	<p>[103]</p>
<p>Congenic <i>ghsr</i>^{-/-} mice on the C57BL6/J background were subjected to a battery of behavioral tests including rota-rod, hot plate, open field, Morris water maze and fear conditioning tasks</p>	<p><i>ghsr</i>^{-/-} mice exhibited normal balance, movement, coordination, and pain sensation. Interestingly, the genetic deletion of GHS-R1a has opposing regulatory effects on learning and memory. While spatial memory was improved in the <i>ghsr</i>^{-/-} mice, contextual memory was impaired by the lack of this receptor</p>	<p>[104]</p>

Table 5. Summary of the ghrelin’s antidepressant-like effects observed in preclinical studies.

Animal model	Main findings	References
Forced swimming test	<p>Antidepressant-like effect following the administration of antisense DNA for ghrelin into the lateral ventricle, or subcutaneous administration of ghrelin in rats. Also, increasing ghrelin levels through a diet containing 60% of normal calories caused antidepressant profile.</p> <p>Antidepressant-like effects of ghrelin in the FST were blocked in mice lacking orexin</p>	[122]
Tail suspension test	Acute administration of ghrelin by i.c.v. route caused antidepressant-like effect in mice	[124]
Olfactory bulbectomy	Acute administration of ghrelin by i.c.v. route abolished the depressive-like behavior induced by olfactory bulbectomy in mice	[124]
Chronic social defeat stress (CSDS)	Genetic deletion of GHSR exacerbated depression-like behaviors induced by CSDS	[122,123]

Table 6. Summary of the ghrelin's effects on anxiety-like behavior observed in preclinical studies.

Animal model	Main findings	Reference
Elevated plus maze Open field Black and white box Social interaction	Increased anxiety-like behavior in ghrelin-treated rats	[156]
Elevated plus-maze Open-field	Anxiogenic-like effect in rats after central injection of ghrelin	[95]
Elevated plus-maze Black and white box Conditioned fear	Antisense oligonucleotides of ghrelin produced an anxiolytic-like effect in rats	[121]
Elevated plus-maze	Ghrelin promoted a more prominent anxiogenic-like behavior when injected into the dorsal raphe nuclei (DRN) in rats	[96]
Elevated plus-maze Open-field Light/dark box	Ghrelin knockout mice were more anxious than wild type mice after an acute restraint stress	[157]
Elevated plus-maze	Rats injected into the arcuate nucleus or paraventricular nucleus with ghrelin showed an anxiogenic-like behavior	[158]
Elevated-plus-maze	Anxiogenic-like effect in mic after intracerebroventricular and intraperitoneal administration of ghrelin	[152]

Elevated plus-maze	Acute administration of ghrelin promoted an anxiolytic-like responses in mice	[122]
Open-field	Anxiogenesis in chicks	[159]

5. CAPITULO 2

Investigação do efeito neuroprotetor da grelina no modelo experimental da doença de Alzheimer

5.1. Materiais e Métodos

5.1.1. Animais

Foram utilizados camundongos albinos Swiss machos com aproximadamente 3 meses de idade, procedentes do biotério central da Universidade Federal de Santa Catarina (UFSC), Florianópolis, Brasil. Os animais foram alojados em grupos de 15 animais por caixa (42 x 34 x 17 cm) e mantidos em câmaras ventiladas (Insight®), a uma temperatura de 22 ± 2 °C, umidade entre 60 – 80 % e ciclo claro/escuro de 12 horas, sendo alimentados com ração comercial e água ad libitum. Todos os procedimentos experimentais utilizados no presente estudo foram conduzidos cuidadosamente de acordo com as normas previstas pelo Comitê de Ética no Uso de Animais da UFSC (CEUA/UFSC; www.ceua.ufsc.br; protocolo 23080.003465/2010-55).

5.1.2. Drogas

5.1.2.1 Administração intracerebroventricular da β -amilóide

O fragmento $A\beta_{1-40}$ (Tocris, Ellisville, EUA) foi diluído em PBS (pH 7,4; 1 mg/ml) e incubado a 37° C durante 4 dias, como descrito previamente (Coraci, Husemann *et al.*, 2002). A forma agregada do peptídeo $A\beta_{1-40}$ (400 pmol/camundongo), grelina (Sigma-Aldrich), BIIE0246 (potente e seletivo antagonista do receptor Y2 do NPY -Tocris, Ellisville, EUA) e da solução veículo (PBS) foram administradas por via i.c.v. como descrito previamente (Laursen e Belknap, 1986).

Brevemente, os animais foram inicialmente anestesiados com isofluorano (1 ml/ml; Abbot Laboratórios do Brasil Ltda., RJ, Brazil) usando o sistema de vaporização (SurgiVet Inc., WI, USA). Após a perda do reflexo postural os animais tiveram os seus escalpos retirados, uma agulha 28 gauges e 2,7 mm de comprimento foi inserida a 1 mm equidistante do bregma. O volume de 1 μ L das soluções foram injetadas gradualmente e ao termino dos experimentos, os animais foram eutanasiados e a correta inserção da agulha foi avaliada através da análise histológica.

5.2. Desenho Experimental

5.2.1. Desenho Experimental 1: Sequencia experimental para investigação do estresse oxidativo, da atividade da acetilcolinesterase e da captação de glutamato em camundongos

O desenho experimental ilustrado na Figura 1 foi utilizado para analisar resposta ocasionada pela administração de grelina e $A\beta_{1-40}$ sobre o estresse oxidativo, a atividade da enzima acetilcolinesterase e a captação de glutamato. Dois grupos experimentais foram submetidos ao tratamento prévio com grelina seguidos da infusão com o fragmento tóxico $A\beta_{1-40}$. A análise do estresse oxidativo e da atividade da enzima acetilcolinesterase foi realizada 24 h após os tratamentos. A investigação da captação de glutamato em fatias hipocâmpais foi realizada 14 dias após o tratamento. Estes intervalos de tempo foram escolhidos baseados nos trabalhos realizados anteriormente pelo grupo (Prediger, Franco *et al.*, 2007; Piermartiri, Figueiredo *et al.*, 2010)

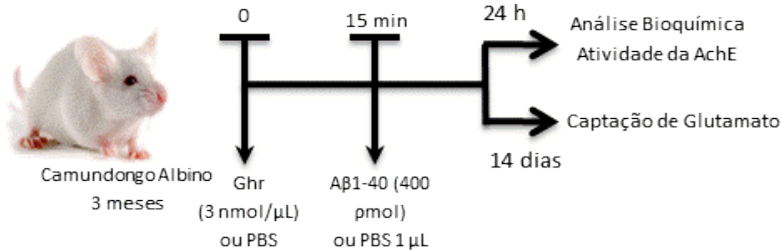


Figura 1: Sequência de experimentos utilizada para avaliar o papel do fragmento $A\beta_{1-40}$ e da grelina sobre os parâmetros de estresse oxidativo, atividade da enzima acetilcolinesterase e sobre a captação de glutamato em fatias hipocâmpais.

5.2.2. Desenho Experimental 2: Sequencia experimental utilizada para avaliação comportamental das funções cognitivas, motoras e de emocionalidade

O desenho experimental ilustrado na Figura 2 foi utilizado para avaliar as possíveis alterações cognitivas, de emocionalidade e motoras induzidas pela infusão do peptídeo $A\beta_{1-40}$ e os possíveis efeitos protetores do pré-tratamento com a grelina.

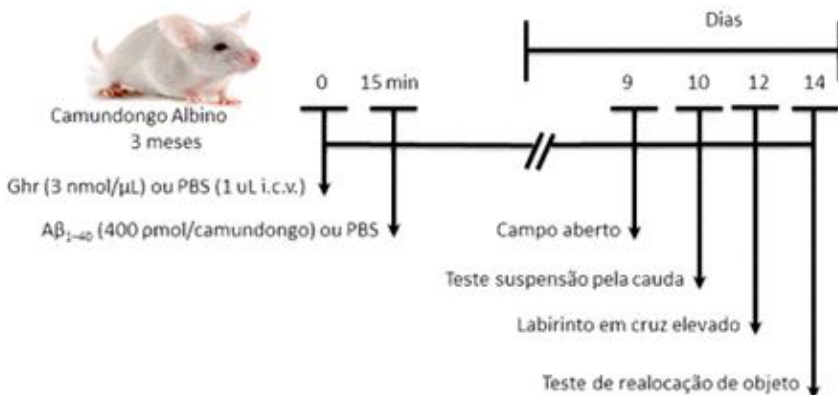


Figura 2: Sequência de experimentos utilizada para avaliação dos efeitos da administração do peptídeo Aβ₁₋₄₀ ou grelina nos testes comportamentais para avaliação das funções cognitivas, emocionalidade e motoras de camundongos.

5.2.3. Desenho Experimental 3: Sequencia experimental para investigação do efeito nootrópico da grelina

O desenho experimental ilustrado na Figura 3 foi utilizado para testar a hipótese do efeito nootrópico da grelina.

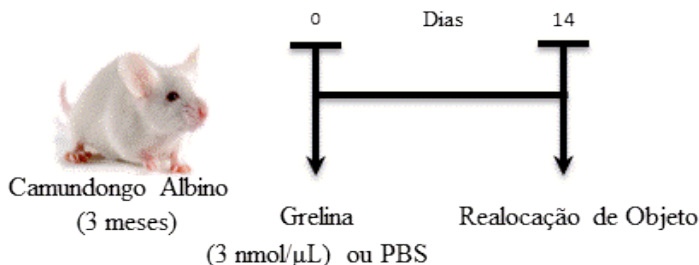


Figura 3: Sequência de experimentos foi utilizada para avaliar o possível efeito nootrópico da grelina no teste de realocação de objeto, utilizando-se um intervalo de tempo entre o treino e o teste de 360 min (ver detalhes do protocolo experimental na descrição do teste de realocação de objeto).

5.2.4. Desenho Experimental 4: Sequencia experimental para análise da influência dos receptores Y2 nos benefícios cognitivos induzidos pela grelina

O desenho experimental ilustrado na Figura 4 foi utilizado para investigar o envolvimento dos receptores Y2 na resposta ocasionada pela administração prévia de grelina seguida da infusão do peptídeo A β ₁₋₄₀.

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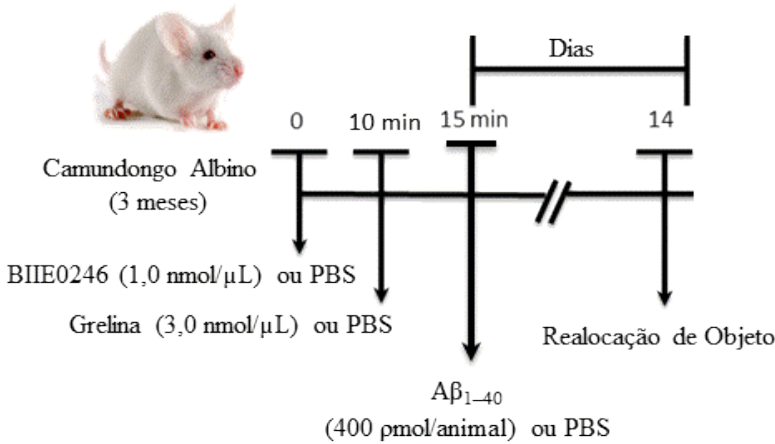


Figura 4: Sequência de experimentos foi utilizada para avaliar o envolvimento do NPY na resposta protetora da grelina através do bloqueio de receptores Y2 no teste de realocação de objeto.

5.3. Campo aberto

Para avaliar possíveis alterações locomotoras induzidas pelos diferentes tratamentos, 9 dias após o administração de A β ₁₋₄₀ ou grelina, os animais foram testados durante 5 min no campo aberto. O aparato feito de acrílico é formado por um chão de cor cinza (30 x 30 cm), com paredes transparentes de 15 cm de altura. Durante os experimentos, cada camundongo foi colocado no centro do campo aberto e as sessões experimentais foram gravadas por um sistema de câmera de vídeo. A distância percorrida foi analisada através da plataforma ANY-mazeTM (Prediger, Franco *et al.*, 2007).

5.4. Teste da suspensão pela cauda

Para investigar o tempo total de duração da imobilidade, foi utilizada a medida de acordo com o método determinado por Steru e colaboradores (1985). Nesta metodologia, os camundongos, acústica e visualmente isolados foram suspensos 50 cm acima do chão por fita adesiva e a imobilidade foi registrada durante 6 minutos, conforme descrito anteriormente (Steru, Chermat *et al.*, 1985).

5.5. Labirinto em cruz elevado

Um dos modelos mais amplamente utilizados na análise da ansiedade em ratos e camundongos é o labirinto em cruz elevado, que é baseado em respostas incondicionadas a ambientes potencialmente perigosos. O teste baseia-se na premissa que ambientes novos evocam curiosidade e medo, criando desta forma, um típico conflito de aproximação/esquiva (Lister, 1987b). Os padrões comportamentais observados representam uma análise da combinação de atividades exploratórias, movimentos de esquiva e comportamento motor de forma geral.

O aparato consistiu de um labirinto de madeira recoberto por fórmica preta composto de dois braços abertos (18 x 6 cm) unidos ortogonalmente a dois braços fechados com paredes forradas de preto, elevados a 60 cm do solo por um suporte de madeira (modificado de Lister, 1987). O animal é colocado no centro do aparato voltado para um dos braços fechados sendo permitido explorar livremente o aparato durante 5 min. Os experimentos foram gravados permitindo a leitura posterior dos comportamentos realizados pelo animal (Lister, 1987a). O número de entradas (definida pela colocação das quatro patas num braço) e o tempo de permanência gasto nos braços abertos e fechados foram registrados. A porcentagem do número de entradas nos braços abertos e o tempo de permanência nos braços abertos, foram calculados pelo número de entradas nos braços abertos dividido pelo número total de entradas nos braços. O número de entradas do braço fechado foi utilizado como uma medida da atividade locomotora (Cruz *et al.*, 1994).

5.6. Realocação de objeto

O aparelho utilizado para o teste da localização de objetos consistiu numa caixa quadrada de acrílico transparente (50,0 cm lados x 40,0 cm altura). Os objetos foram dois retângulos plásticos idênticos (3,0 cm lado x 4,5 cm lado x 4,5 cm altura) os quais foram colados ao chão de maneira equidistante 7 cm de dois lados da caixa. O teste da

localização de objetos foi proposto inicialmente para ratos (Ennaceur, Neave *et al.*, 1997) e posteriormente adaptado (Murai, Okuda *et al.*, 2007) e padronizado para camundongos Albinos Swiss (Assini, Duzzioni *et al.*, 2009). O procedimento consistiu em uma sessão de treino com duração de 3 min, na qual o animal foi colocado na caixa e explorava-a livremente. Após o treino, os animais foram colocados em outra caixa e aguardaram até o momento do teste. Os intervalos entre treino e teste foram de 90 ou 360 min para os diferentes grupos testados. Ou seja, dependendo do objetivo do experimento foi utilizado um ou outro intervalo entre treino e teste. De acordo com Assini e colaboradores (2009), quando os animais controle eram testados aos 360 min, estes não expressavam a memória de localização de objetos. Fato que acontecia apenas quando os animais eram testados com intervalos entre treino e teste de 90 min. Desta forma, para avaliar o efeito da administração de $A\beta_{1-40}$ e da grelina, utilizou-se o intervalo de tempo de 90 min, enquanto que para a investigação do possível efeito promnemônico da grelina, foi utilizado o tempo de 360 min.

No momento do teste os animais foram colocados novamente no aparato com os dois objetos, porém um dos objetos foi realocado para o lado oposto. Os comportamentos de cheirar, tocar ou observar o objeto a menos de 1 cm de distância foram considerados como exploração do objeto.

Vale ressaltar que foi realizado um controle motor dos animais através da análise locomotora utilizando-se para isto a plataforma ANY-mazeTM.

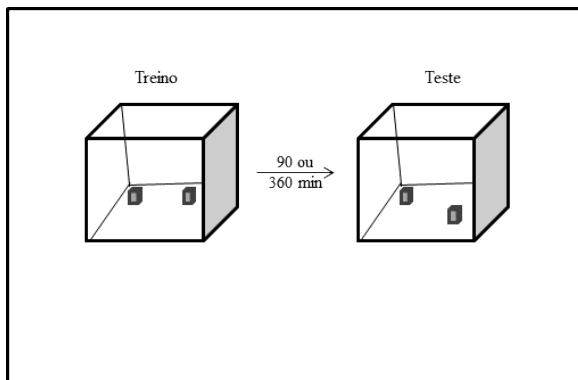


Figura 5: Representação esquemática do modelo de localização de objetos. Índice de localização do objeto = [tempo (s) objeto realocado x 100/ [tempo (s) objeto realocado + tempo (s) objeto encontrado no local do treino].

5.7. Estresse oxidativo

Grupos independentes de camundongos foram eutanasiados no período de 24 h após a administração dos peptídeos. Para cada animal, o córtex pré-frontal e os dois hipocampus foram dissecados separadamente para mensuração dos seguintes parâmetros relacionados a estresse oxidativo (Ellman, 1959; Ohkawa, Ohishi *et al.*, 1979; Wendel, 1981; Carlberg e Mannervik, 1985):

5.7.1. Avaliação da Glutathiona Reduzida

Os grupamentos tióis não proteicos (NPSH) nas amostras foram determinados usando reagente de Ellman, 5,5-ditiobis 2-nitrobenzoato (DTNB). As amostras de córtex pré-frontal e hipocampo foram homogeneizadas em tampão HEPES 20 mM, (pH 7,4) e centrifugadas a 20000 x g por 30 min a 4° C. 40 µL do sobrenadante foram misturada com 40 µL de ácido tricloroacético (TCA) a 10%. Em seguida, as amostras foram centrifugadas a 16.000 x g por 10 min. Os NPSH foram quantificados pela adição de 60 µL do sobrenadante ácido/desproteínizado em 125 µL de TFK 1M, pH 7,0 e 25 µL de DTNB 10 mM. O monitoramento da concentração dos grupos tióis foi avaliando através da medida da absorbância em 405 nm devido ao produto resultante da reação de NPSH (90% GSH) com DTNB, que gerou o TNB de cor amarela (Ellman, 1959).

5.7.2. Níveis de espécies reativas ao ácido tiobarbitúrico (TBARS)

Os níveis de peroxidação lipídica foram determinados por espectrofotometria no sobrenadante dos homogenatos centrifugados a 20000 x g por 30 min a 4° C contendo tampão de HEPES a 20 mM (pH 7,4). As TBARS foram mensuradas pelo método de Ohkawa *et al.* (1979), no qual o MDA, um produto da peroxidação de ácidos graxos, reage com ácido tiobarbitúrico. Os valores foram determinados pelo coeficiente de absorbância do complexo MDA-ácido tiobarbitúrico em 532 nm (Ohkawa, Ohishi *et al.*, 1979).

4.7.3. Avaliação da atividade Glutathiona Peroxidase (GPx)

A GPx catalisa a redução de H₂O₂, bem como outros lipoperóxidos, utilizando a glutathiona reduzida (GSH) como co-substrato e produzindo glutathiona oxidada (GSSG). A GSSG é reduzida pela glutathiona redutase com o consumo de NADPH, que foi mensurado através da leitura em espectrofotômetro em 340 nm (Wendel, 1981).

4.7.4. Avaliação da atividade Glutathiona Redutase (GR)

A GR catalisa a redução da GSSH através da oxidação do NADPH. Ao utilizar o substrato GSSG, a enzima leva ao consumo de NADPH, que é acompanhado em 340 nm. A velocidade de consumo de NADPH, em condições de saturação, expressa a atividade enzimática (Carlberg e Mannervik, 1985).

4.7.5. Dosagem de Proteínas

O conteúdo de proteínas foi quantificado pelo método de Bradford (1976), usando albumina de soro bovino como padrão (Bradford, 1976).

4.8. Atividade da enzima acetilcolinesterase (AChE)

A técnica espectrofotométrica mais utilizada para medir a atividade da acetilcolinesterase baseia-se na produção de tiocolina, a partir da hidrólise da acetiltiocolina. Isto é acompanhado por uma reação da tiocolina com o ácido 5',5 - ditiobis-2-nitrobenzóico (DTNB) para produzir o ânion ácido 2-nitro-5-mercapto -benzóico (TNB) com cor amarela avaliado através do uso de um espectrofotômetro (Ellman, Courtney *et al.*, 1961)

4.9. Captação de glutamato

Inicialmente, fatias de hipocampos oriundos de camundongos quatorze dias após o tratamento com os peptídeos foram utilizadas para a investigação. A captação L-[³H] glutamato foi realizada segundo descrito por Molz *et al.*, 2005. Após a incubação, o meio contendo tampão KRB foi retirado e as fatias foram incubadas com HBSS (CaCl₂ 1,29 mM, NaCl 136,9 mM, KCl 5,36 mM, MgSO₄ 0,65 mM, Na₂HPO₄ 0,27 mM, KH₂PO₄ 1,1 mM, Glicose 2 mM, Hepes 5 mM) ou HBSS colina (para retirar Na⁺, o NaCl e Na₂HPO₄ foram substituídos por cloreto de colina (137 mM) por 15 min. A captação foi iniciada após a adição de 0,33 µCi/ml L-[³H]glutamato e glutamato não marcado na concentração final de 100 µM, por 7 min. Após esse período o meio foi descartado e as fatias lavadas 2 vezes com HBSS ou HBSS-colina gelados. Em seguida as fatias foram solubilizadas em uma solução de 0,1 NaOH/0,01% SDS. A determinação do conteúdo intracelular de [³H]L-glutamato foi avaliada em um contador de cintilação líquida (Molz, Decker *et al.*, 2005).

As dosagens de proteínas para o experimento de captação de glutamato foram realizadas através da técnica de Lowry *et al.* 1951, utilizando a albumina de soro bovina como padrão (Lowry, Rosebrough *et al.*, 1951).

4.10. Procedimentos para eletrofisiologia extracelular hipocampal

Fatias de hipocampo foram obtidas de camundongos C57Bl/6 machos de 6 a 8 semanas de idade (Charles River, Barcelona, Espanha). Os animais foram anestesiados com halotano (Sigma-Aldrich, St Louis, MO, EUA) e decapitados. Os cérebros foram rapidamente removidos e imersos em líquido cefalorraquidiano artificial (aCSF) gelado contendo (em mM); 124 de NaCl, 4,5 de KCl, 2 de CaCl₂, 1 de MgCl₂, 26 de NaHCO₃, 1,2 de NaH₂PO₄ e 10 de D-glicose, gazeados com uma mistura de 95% O₂ / 5% CO₂. O tecido foi completamente submerso em aCSF gelado e seccionado no sentido coronal em fatias de 400 µm de espessura, utilizando um sistema de secção (Vibratome 1500, Leica, Wetzlar, Alemanha). As fatias permaneceram em repouso por no mínimo 60 minutos em temperatura ambiente, imersas em aCSF e continuamente gazeadas a 95% de O₂ / 5% de CO₂. As fatias foram então transferidas para a câmara de registro submerso. O posicionamento dos eletrodos obedece à organização do circuito CA3-CA1 do hipocampo. Os potenciais de campo extracelulares foram obtidos com micropipetas (2–4 MΩ) preenchidas com uma solução de 4 M de NaCl. Os estímulos elétricos foram gerados por um estimulador S44 (Grass Instruments, West Warwick, RI, EUA) em uma frequência de 0,05 Hz, através de um eletrodo bipolar. As leituras foram medidas como *fEPSP*, para estimativa da eficácia sináptica (Spitzer, 2006). O protocolo de estimulação de alta frequência (do inglês *High frequency stimulation, HFS*), consistiu de 1 trem de estímulo de 100 Hz durante 1 segundo. Os registros foram obtidos por um amplificador ISO-80 (World Precision Instruments, Hertfordshire, Inglaterra) e digitalizados por um sistema ADC-42 (Pico Technologies, Pelham, NY, EUA). As médias de 4 respostas consecutivas foram continuamente monitoradas em um computador com o software LTP 1.01 (Anderson and Collingridge, 2001). As fatias de hipocampo foram submetidas ao tratamento com grelina (1 nM e 1µM) por 20 minutos, e depois ao tratamento com o peptídeo beta-amilóide (200 nM e 500 nM) por 40 minutos. Após a definição das concentrações ideais tanto de grelina (1 nM) e de beta-amilóide (200 nM) foi realizado o protocolo de prevenção dos efeitos da beta-amilóide, onde por 20 minutos as fatias foram

tratadas com a grelina e na sequência tratadas com a beta amiloide durante 40 minutos.

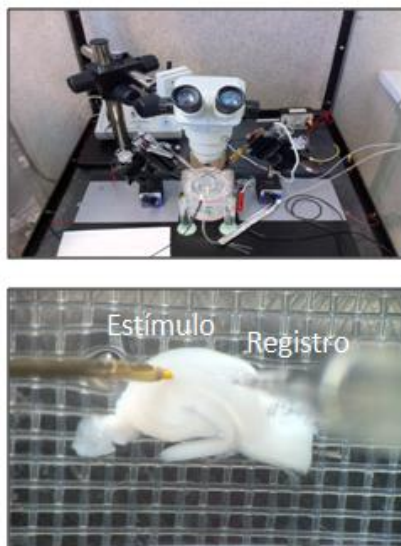


Figura 6: Sistema de eletrofisiologia extracelular (a). Fatia de hipocampo no sistema de eletrofisiologia extracelular do eletrodo e da pipeta de registro (Costenla, Diógenes *et al.*, 2011)

5. Resultados

5.1. A grelina na concentração de 3,0 nmol apresenta atividade protetora no estresse oxidativo e previne o aumento da enzima acetilcolinesterase

Inicialmente, dose de grelina utilizada na realização destes estudos foi selecionada com base nos trabalhos desenvolvidos pelo grupo da Professora Susana Rubiales e da Dr. Valeria Carlini (Carlini, Monzón *et al.*, 2002). Os efeitos protetores das concentrações de 0,03; 0,3 e 3,0 nmol/ μ L de grelina foram avaliadas no modelo experimental da DA através de ensaios bioquímicos avaliando o estresse oxidativo e a análise da atividade da enzima acetilcolinesterase nas regiões do córtex pré-frontal e do hipocampo de camundongos infundidos com o peptídeo $A\beta_{1-40}$.

Os resultados mostraram que o tratamento prévio com grelina na concentração de 3,0 nmol/ μ L foi capaz de prevenir a peroxidação

lipídica induzida pela administração de $A\beta_{1-40}$ em ambas as regiões [TBARS córtex: pretatamento $F_{3, 44}=3,4$; $P\leq 0,05$; tratamento: $F_{1,44}=3,4$ $P=0,07$; interação: $F_{3, 44}=2,3809$), [TBARS hipocampo: pretatamento: $F_{3, 44} = 3,38$, $P\leq 0,05$; tratamento: $F_{1,44} = 3,14$; $P=,083$; interação: $F_{3,44}=1,70$; $P = 0,17921$] (Figura 7A e B). Além disso, o aumento significativo na atividade da enzima acetilcolinesterase no córtex pré-frontal ocasionado pelo fragmento $A\beta_{1-40}$, foi prevenido pelo tratamento prévio com grelina $3,0 \text{ nmol/ } \mu\text{L}$ [AChE córtex: pretatamento $F_{3,48} = 3,60$, $P\leq 0,05$, tratamento: $F_{1, 48}=1,3440$, $P = 0,25$; interação: $F_{3,48} =3,60$, $P\leq 0,05$] (Figura 7C). As outras concentrações utilizadas não ocasionaram apresentaram efeitos protetores nos ensaios realizados. Além disso, a análise estatística não revelou alterações significativas nas concentrações de tióis não-proteicos (NPSH) e nos níveis de glutatona redutase, glutatona peroxidase e catalase nas regiões do córtex pré-frontal e hipocampo, bem como na atividade da AChE hipocampal (dados não mostrados).

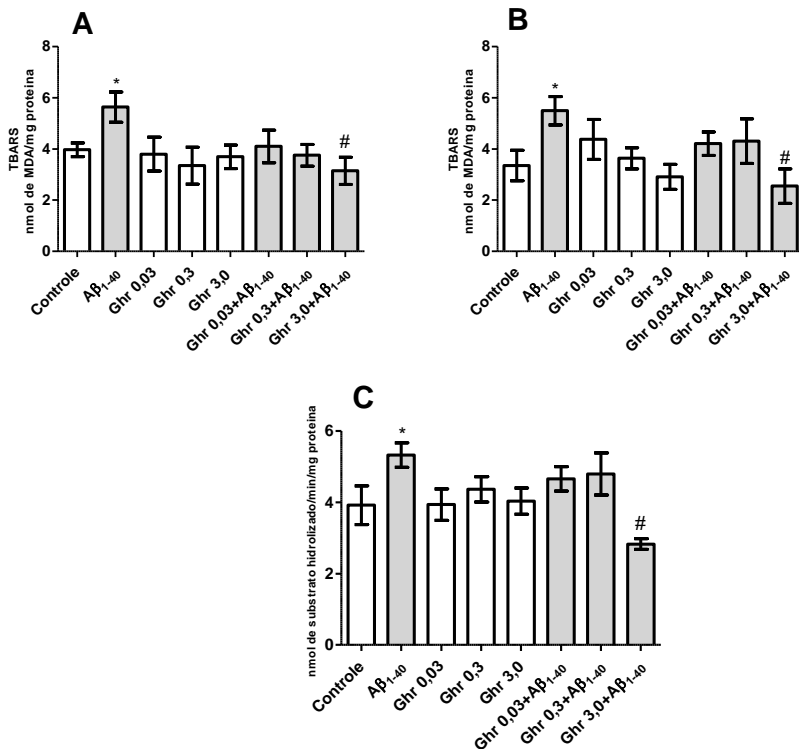


Figura 7: Análise do efeito protetor da administração de grelina nas concentrações de 0,03; 0,3 e 3,0 nmol/ μ L seguido da infusão de A β_{1-40} (400 nmol/camundongo). Peroxidação lipídica córtex pré-frontal (A) e hipocampo (B) e análise da atividade da enzima acetilcolinesterase no córtex pré-frontal (C). As colunas representam a média \pm S.E.M. * e # $p < 0,05$ quando comparados com os grupos controle através da análise de variância (ANOVA) de duas vias, seguidos pelo teste de Newman-Keuls. O símbolo asterisco (*) representa a análise entre os grupos PBS e A β_{1-40} , enquanto que o símbolo sustentado (#) representa a análise entre os grupos grelina + A β_{1-40} e A β_{1-40} .

5.2. A grelina previne o estresse oxidativo induzido pela administração i.c.v. do peptídeo A β_{1-40} em camundongos

Evidências experimentais sugerem que o peptídeo $A\beta_{1-40}$ é capaz de induzir estresse oxidativo no SNC de roedores (Medeiros, Prediger *et al.*, 2007; Matos, Augusto *et al.*, 2008). Sendo assim, foi investigado o possível dano oxidativo gerado pela infusão i.c.v. do peptídeo $A\beta_{1-40}$ através do teste de peroxidação lipídica utilizando como marcador a quantificação das substâncias reativas ao ácido tiobarbitúrico (TBA-RS). As figuras 8A e B ilustram que o peptídeo $A\beta_{1-40}$ induziu um aumento significativo na peroxidação lipídica tanto no córtex pré-frontal quanto no hipocampo de camundongos, observado 24 h após a sua administração. Já o tratamento prévio com a grelina foi capaz de prevenir os prejuízos induzidos pelo $A\beta_{1-40}$ em ambas as regiões [TBA-RS córtex: pretratamento: $F_{1,24}=3,19$; $P=0,087$; tratamento: $F_{1,24}=7,40$; $P\leq 0,05$, interação: $F_{1,24}=4,61$; $P\leq 0,05$] e [TBA-RS hipocampo: pretratamento: $F_{1,24}=8,00$; $P\leq 0,001$, tratamento: $F_{1,24}=5,38$, $P\leq 0,05$, interação: $F_{1,24}=4,61$, $P\leq 0,05$]

Existem relatos prévios na literatura dos efeitos antioxidantes da grelina (El Eter, Al Tuwaijiri *et al.*, 2007), para confirmar tal hipótese foram avaliadas as concentrações de tióis não proteicos (NPSH), glutatona redutase (GR), glutatona peroxidase (GPx) e catalase (CAT). As figuras 8C e D demonstram que nenhuma alteração significativa foi observada nas concentrações dos tióis não proteicos (NPSH) nas estruturas analisadas dos animais submetidos ao tratamento com $A\beta_{1-40}$. Por outro lado, observamos que o peptídeo $A\beta_{1-40}$ reduziu significativamente a atividade da enzima GR no córtex pré-frontal dos animais. Esta enzima é responsável pela manutenção da glutatona na sua forma reduzida, sendo considerado um sistema de defesa antioxidante não-enzimático. Já o tratamento prévio com a grelina preveniu a redução da atividade desta enzima [GR córtex: pretratamento $F_{1,23}=0,26$; $P=,60834$; tratamento $F_{1,23}=14,41$ $P< 0,05$; interação: $F_{1,23}=0,037$; $P=0,84$] (Figura 11A).

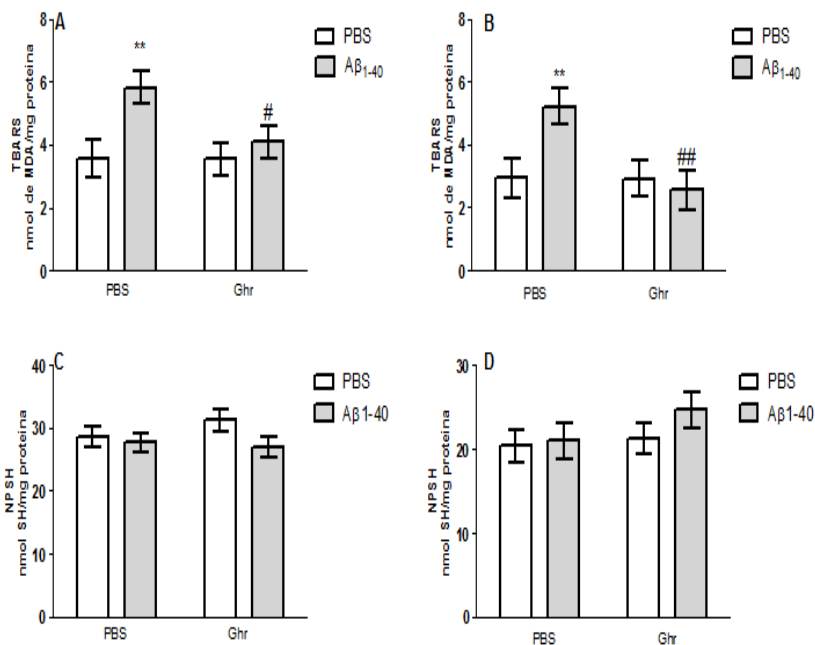


Figura 8: A grelina (3,0 nmol/ μ L , i.c.v.) previne o estresse oxidativo gerado pela administração i.c.v. do peptídeo A β ₁₋₄₀ (400 pmol/camundongo) no córtex pré-frontal (A e C) e no hipocampo (B e D) de camundongos. As barras representam as médias \pm SEM de 6-8 animais por grupo. As substâncias reativas ao ácido tiobarbitúrico (TBA-RS) e os tióis não proteicos (NPSH) foram expressos em nmol de MDA/mg proteína e nmol/mg proteína, respectivamente. * e # $p < 0,05$ quando comparados com os grupos controle através da análise de variância (ANOVA) de duas vias seguidos pelo teste de Newman-Keuls. O símbolo asterisco (*) representa a análise entre os grupos PBS e A β ₁₋₄₀, enquanto que o sustentado (#) representa a análise entre os grupos Ghr+ A β ₁₋₄₀ e A β ₁₋₄₀.

Ainda no córtex pré-frontal, foi observado que a administração de A β ₁₋₄₀ promoveu uma redução significativa na atividade da enzima CAT e que o tratamento prévio com grelina preveniu este efeito (pretratamento: $F_{1,24}=0,08$, $P=0,77$; tratamento: $F_{1,24}=11,162$, $P\leq 0,05$; interação: $P_{1,24}=0,16542$, $P=0,68$). Na região hipocampal não foram

observadas diferenças significativas na atividade (GR, GPx e CAT) entre os diferentes grupos experimentais (Figuras 9 B, D, F).

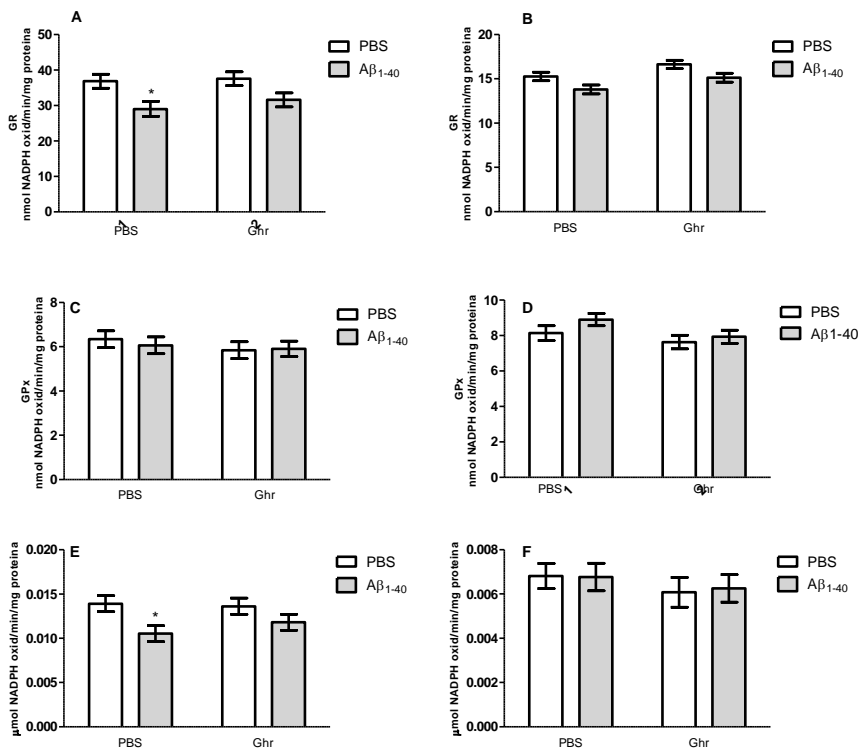


Figura 9: A grelina (3,0 nmol/μL) preserva as atividades enzimáticas no córtex pré-frontal induzido pela administração i.c.v. do peptídeo Aβ₁₋₄₀ (400 nmol/camundongo). As barras representam as médias ± SEM de 6-8 animais por grupo. Os resultados da glutationa redutase (GR) e a glutationa peroxidase (GPx) foram expressos em nmol de NADPH oxidado/mg proteína enquanto que a catalase foi expressa em μmol NADPH oxidado/mg proteína. Todos os grupos foram avaliados através da análise da variância (ANOVA). * e # p < 0,05 quando comparados com os grupos controle através da ANOVA de duas vias seguidos pelo teste de Newman-Keuls. O símbolo asterisco (*) representa a análise entre os grupos PBS e Aβ₁₋₄₀, enquanto que o sustenido (#) representa a análise entre os grupos Ghr+ Aβ₁₋₄₀ e Aβ₁₋₄₀.

5.3. A grelina previne o aumento significativo na atividade da enzima acetilcolinesterase e a redução na captação de glutamato nos animais tratados com o peptídeo $A\beta_{1-40}$

Sabendo que existe uma relação positiva entre os níveis do peptídeo $A\beta_{1-40}$ e a atividade da enzima AChE, neste estudo foi avaliada a atividade enzimática da AChE no córtex pré-frontal e hipocampo de camundongos 24 h após a administração i.c.v. do peptídeo $A\beta_{1-40}$ e da grelina. Como demonstrado na figura 12A, a administração do peptídeo $A\beta_{1-40}$ promoveu um aumento significativo na atividade da AChE no córtex pré-frontal [$F_{1,20} = 6,30$; $P \leq 0,05$]. Foi observado que estes valores foram reduzidos significativamente pelo tratamento prévio com a grelina ($F_{1,20} = 10,17$; $P \leq 0,05$). Por outro lado, a atividade da enzima AChE na região hipocampal permaneceu inalterada (Figura 10B).

O próximo passo foi avaliar os efeitos da grelina e do peptídeo $A\beta$ sobre a captação de glutamato em fatias de hipocampo. O glutamato é o principal neurotransmissor excitatório e está fortemente relacionado a processos mnemônicos (Reis, Guatimosim *et al.*, 2009). Há trabalhos relatando que a $A\beta$ é capaz de inibir a atividade dos receptores glutamatérgicos, interferindo com a captação de glutamato na fenda sináptica (Snyder, Nong *et al.*, 2005; Danysz e Parsons, 2012). As análises foram realizadas no 14º dia após os tratamentos, onde os animais foram eutanasiados e seus hipocampos dissecados para a quantificação da captação de glutamato. A Figura 10C mostrou que a infusão dos peptídeos $A\beta_{1-40}$ causou uma diminuição significativa na captação de glutamato com relação ao grupo-controle. O tratamento prévio com grelina preveniu os efeitos causados pelo peptídeo $A\beta_{1-40}$ (pretratamento: $F_{1,18} = 0,29$; $P = 0,59$; tratamento: $F_{1,18} = 2,2420$, $P = 0,15163$; interação: $F_{1,18} = 9,8$, $P \leq 0,05$). A grelina *per se* não alterou a captação basal de glutamato quando comparado com o grupo controle.

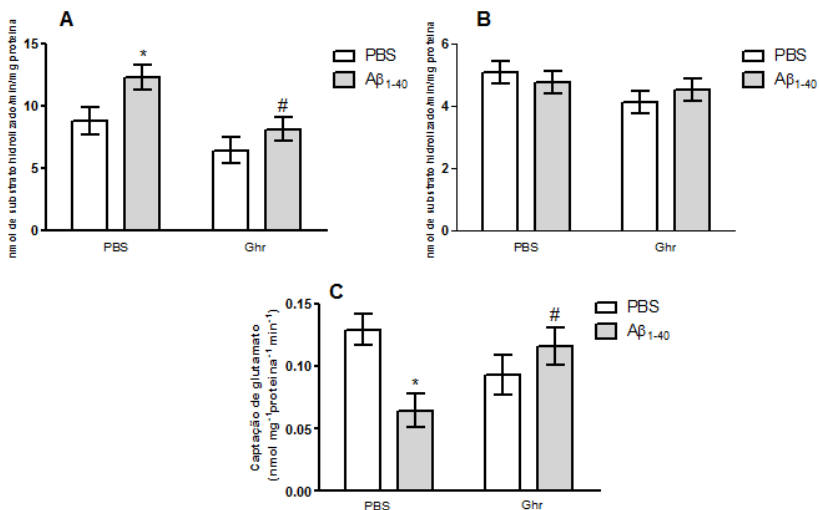


Figura10: A infusão i.c.v. do peptídeo Aβ₁₋₄₀ (400 pmol/camundongo) aumentou significativamente a atividade da enzima acetilcolinesterase, o tratamento prévio com a grelina (3,0 nmol/μL, i.c.v.) preveniu significativamente este aumento de atividade. As barras representam as médias ± SEM de 5-7 animais por grupo. Os gráficos A e B representam a atividade da enzima AchE no córtex e hipocampo, respectivamente. O gráfico C representa a captação de glutamato em fatias hipocâmpais. Os grupos foram avaliados através da análise da variância (ANOVA). O símbolo * representa a diferença entre os grupos PBS e Aβ₁₋₄₀, o símbolo # representa as diferenças entre os grupos Aβ₁₋₄₀ e Ghr+ Aβ₁₋₄₀. * e # p < 0,05 quando comparados através do teste ANOVA de duas vias seguidos pelo teste de Newman-Keuls.

5.4. Caracterização comportamental dos camundongos tratados com grelina e o peptídeo Aβ₁₋₄₀ pela via i.c.v.

Como próximo passo, investigamos os efeitos da administração i.c.v. da grelina e do peptídeo Aβ₁₋₄₀ avaliando a atividade locomotora e respostas de emocionalidade em camundongos avaliados nos testes do campo aberto, suspensão pela cauda e labirinto em cruz elevado.

Os resultados obtidos no teste do campo aberto demonstraram a inexistência de alterações na atividade exploratória dos animais que

receberam a infusão de grelina e/ou $A\beta_{1-40}$ (Figura 11A). No 10º dia após os tratamentos, observou-se que a administração do peptídeo $A\beta_{1-40}$ aumentou o tempo de imobilidade dos animais no teste da suspensão pela cauda. A administração prévia de grelina diminuiu significativamente o tempo de imobilidade dos animais infundidos com o peptídeo $A\beta_{1-40}$, sugerindo que a grelina apresenta um efeito antidepressivo no teste de suspensão pela cauda (Figura 11 B) (pretratamento: $F_{1, 30}=1,1635$, $P=0,28$; tratamento: $F_{1, 30}=2,6450$, $P=0,11$; interação: $F_{1, 30} = 28,05$; $P \leq 0,01$).

A avaliação no teste do labirinto em cruz elevado, 12 dias após a administração i.c.v. da grelina demonstrou que os animais tratados com o hormônio apresentavam uma diminuição significativamente o tempo de permanência nos braços abertos (Figura 11 C) (pretratamento: $F_{1, 36}=5,33$, $P<0,05$; tratamento: $F_{1, 36}=2,06$, $P=0,15$; interação: $F_{1, 36}=2,32$, $P=0,13$) caracterizando um comportamento tipo-ansiosgênico.

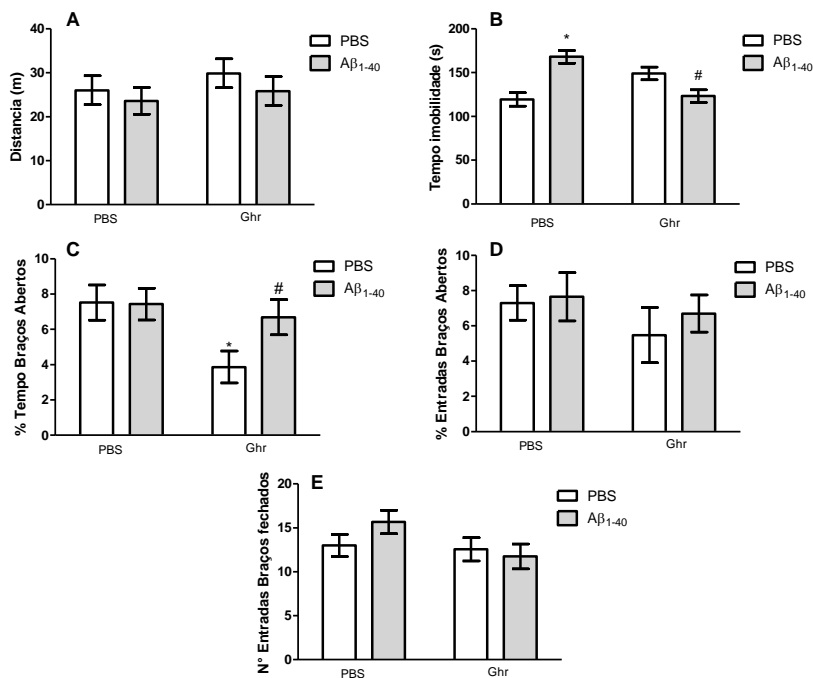


Figura 11: Os efeitos da administração i.c.v. da grelina (3,0 nmol/ μ L) e $A\beta_{1-40}$ (400 pmol/camundongo) sobre as funções motora e de emocionalidade foram avaliados nos testes comportamentais do campo aberto (A), suspensão pela cauda (B) e labirinto em cruz elevado (C, D, E). O tratamento prévio com grelina diminui significativamente o aumento no tempo de imobilidade induzido pela $A\beta_{1-40}$ (B), além disso, a grelina diminui significativamente o tempo de permanência nos braços abertos no teste do labirinto em cruz elevado quando comparado com o grupo PBS (C). As barras representam as médias \pm SEM de 10-12 animais por grupo. Todos os grupos foram avaliados através da análise da variância (ANOVA). * e # $p < 0,05$ quando comparados com os grupos controle através da ANOVA de duas vias seguidos pelo teste de Newman-Keuls. O símbolo asterisco (*) representa a análise entre os grupos PBS e $A\beta_{1-40}$, enquanto que o sustentado (#) representa a análise entre os grupos Ghr + $A\beta_{1-40}$ e $A\beta_{1-40}$.

5.5. A grelina previne o declínio cognitivo no modelo de realocação de objetos induzido pela $A\beta_{1-40}$

No presente estudo foi avaliada a capacidade mnemônica dos animais no teste de realocação de objetos. O intervalo de tempo utilizado entre o treino e o teste foi de 90 min. No treinamento, todos os grupos apresentam o mesmo desempenho na tarefa, despendendo 50% do tempo explorando cada um dos objetos, indicando o interesse destes animais pela novidade e ausência de preferência por um local específico (Figura 12A). No teste, como esperado a administração de $A\beta_{1-40}$ em camundongos resultou em um declínio da função cognitiva demonstrado pela incapacidade dos animais em discriminarem o objeto realocado na nova posição (Figura 14B) ($t = 0,3843$; $n=8$). O tratamento prévio com grelina foi capaz de prevenir este declínio cognitivo de maneira significativa ($t = 4,882$; $n=8$) (Figura 12B).

A investigação do possível efeito nootrópico da grelina foi realizado através da realocação de objeto com um intervalo de tempo de 360 min entre o treino e o teste. Não foi observada diferença significativa entre os grupos controle e grelina (Figura 12C).

A influencia de receptores Y2 em neurônios GHS-R foi realizada através da administração do antagonista do receptor BIIE0246, estes resultados demonstram que o bloqueio de Y2 não interfere na resposta desencadeada pelo hormônio grelina (Figura 12D).

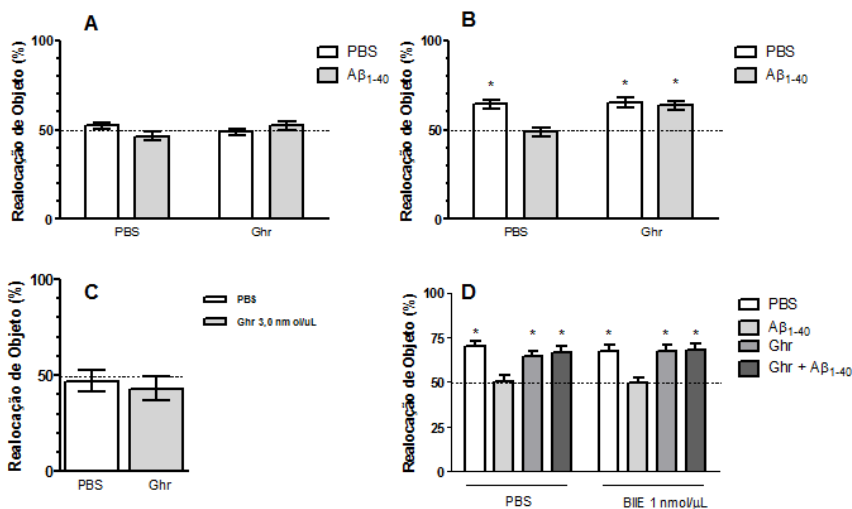


Figura 12: A grelina (3,0 nmol/ μ L) previne o declínio cognitivo induzido pela $A\beta_{1-40}$ (400 pmol/camundongo) no teste de realocação de objeto. As figuras A, B representam as avaliações no treino e no teste em 90 min. A figura C representa o teste 360 minutos após o treino, e a figura D representa a investigação do papel de receptores Y2 na resposta desencadeada pela grelina 90 minutos após o treino. As barras representam as médias \pm SEM de 10-12 animais. Os grupos foram avaliados através do teste t de student (%50 de investigação objeto realocado).

5.6. A grelina previne os prejuízos na LTP hipocampal induzidos pela administração i.c.v. do peptídeo $A\beta_{1-40}$.

O conjunto de resultados anteriores demonstrando as ações protetoras da grelina sobre as alterações mnemônicas, a atividade enzimática da acetilcolinesterase e a captação de glutamato, induzidas pela administração do peptídeo $A\beta_{1-40}$ nos conduziram a realização do experimento de avaliação de plasticidade sináptica.

O estudo foi realizado *in vitro* utilizando fatias hipocâmpais e mensurando a LTP. Os resultados demonstram que após a indução do estímulo de alta frequência, o percentual de *slope* das fatias incubadas com grelina (1,0 μ M) foi significativamente superior quando comparado com o grupo controle demonstrando seu papel facilitador sináptico (F_2 ,

$20 = 5,1 P \leq 0,05$) (Figura 13A e B). Já a incubação com 200 nM ou 500 nM de $A\beta_{1-40}$ promoveram uma redução significativa no *slope* em ambas as concentrações ($F_{2, 18} = 8,7 P \leq 0,05$) (Figura 13C e D). Avaliando o papel protetor da grelina, observamos que a incubação prévia com 1,0 nM de grelina durante 20 minutos, seguido da incubação com 200 nM de $A\beta_{1-40}$ durante 40 minutos, mantém os valores de *slope* semelhantes aos do grupo-controle, demonstrando que a grelina previne os prejuízos na plasticidade sináptica induzidos pelo peptídeo $A\beta_{1-40}$ ($F_{2, 16} = 4,6 P \leq 0,05$) (Figura 13E e F).

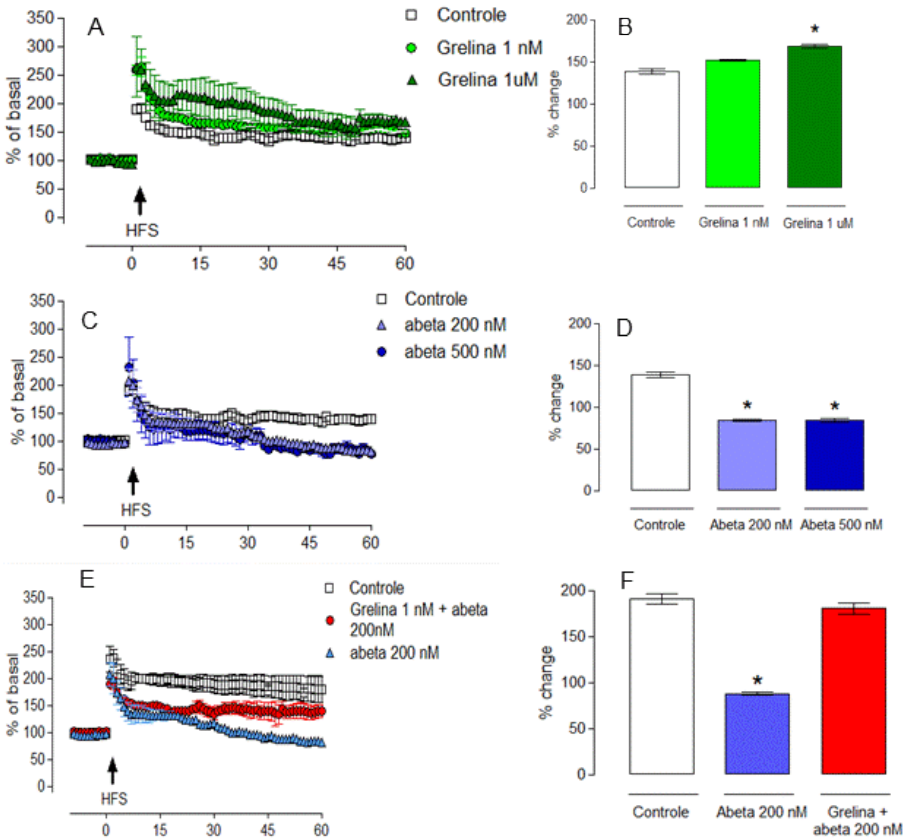


Figura 13: A incubação das fatias de hipocampo com 1,0 μ M grelina durante 20 minutos aumentam significativamente o *slope* dos potenciais excitatórios pós-sinápticos quando comparados com o controle (A e B).

A incubação com o fragmento $A\beta_{1-40}$ reduz significativamente o *slope* em ambas as doses (C e D). A incubação com grelina (1 nM) seguida da incubação com $A\beta_{1-40}$ (200 nM) previne a diminuição da LTP neste modelo experimental (E e F) * $p < 0,05$) quando comparados com os grupos controle através da ANOVA de duas vias seguidos pelo teste de Newman-Keuls.

6. CAPITULO 3

Artigo aceito para publicação na Behavioral Brain Research

Neuropeptide Y (NPY) prevents depressive-like behavior, spatial memory deficits and oxidative stress following amyloid- β ($A\beta_{1-40}$) administration in mice

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Abstract

Neuropeptide Y (NPY) is a 36-amino acid peptide widely distributed in the central nervous system (CNS) that has been associated with the modulation of several functions including food intake, learning and memory, mood and neuroprotection. There is great interest in understanding the role of NPY in the deleterious effects induced by the central accumulation of amyloid- β ($A\beta$) peptides, a pathological hallmark of Alzheimer's disease (AD). Herein, we evaluated the effects

of a single intracerebroventricular (i.c.v.) administration of NPY (0.0234 $\mu\text{mol}/\mu\text{l}$) 15 min prior to the i.c.v. injection of aggregated $\text{A}\beta_{1-40}$ peptide (400 pmol/mouse) in behavioral and neurochemical parameters related to oxidative stress in mice. Pretreatment with NPY prevented $\text{A}\beta_{1-40}$ -induced depressive-like responses and spatial memory impairments evaluated in the tail suspension and object location tasks, respectively. The protective effects of NPY on spatial memory of $\text{A}\beta_{1-40}$ -treated mice were abolished by the pretreatment with the selective Y2 receptor antagonist BIIIE0246. On the other hand, the administration of NPY and $\text{A}\beta_{1-40}$ did not alter the performance of the animals in the elevated plus-maze and open field arena, indicating lack of effects on anxiety state and locomotor function. Although $\text{A}\beta_{1-40}$ infusion did not change hippocampal and cortical glutathione peroxidase (GPx) activity and glutathione (GSH) levels, $\text{A}\beta_{1-40}$ -infused animals showed an increased lipid peroxidation in hippocampus and prefrontal cortex and decreased glutathione reductase (GR) activity in prefrontal cortex that were blunted by NPY administration. These findings indicate that central administration of NPY prevents $\text{A}\beta_{1-40}$ -induced depressive-like behavior and spatial memory deficits in mice and that this response is mediated, at least in part, by the activation of Y2 receptors and prevention of oxidative stress.

Keywords: Neuropeptide Y (NPY); amyloid- β protein; Alzheimer's disease; spatial memory; depression; oxidative stress; glutathione; mice.

1. Introduction

Neuropeptide Y (NPY) is a 36-amino acid peptide widely distributed in the central nervous system (CNS) being expressed preferentially in interneurons but it is also present in long projection neurons [1]. Intracerebroventricular (i.c.v.) administration of NPY stimulates food intake [2], modulates learning and memory [3-5], inhibits neuronal excitability [6] and has anticonvulsant effects [7,8]. In addition, NPY is thought to play a role in the pathophysiology of mood disorders and in the underlying mechanisms of antidepressant drugs [9-12].

In the rodent CNS, NPY acts mainly through three NPY receptors that belong to the family of seven-transmembrane domain receptors: NPY Y1, Y2 and Y5 receptors [13]. NPY Y1 receptor mRNA has been detected in the cerebral cortex, hippocampus, thalamus, hypothalamus and amygdala [14]. mRNA transcripts for NPY Y2 receptors are particularly abundant in the hippocampus, hypothalamus and amygdala [14,15], whereas NPY Y5 receptor mRNA is mainly

present in the dentate gyrus (DG) and CA3 sub-regions of the hippocampus, cingulate cortex, but also in thalamic and hypothalamic nuclei [14].

Noteworthy, changes in NPY levels in different brain structures, cerebrospinal fluid and plasma have been described in neurodegenerative diseases such as Parkinson's disease (PD) [16] and Alzheimer's disease (AD) [17-21]. Moreover, recent experimental studies have demonstrated the neuroprotective effects of exogenous NPY administration in animal models of PD [22] and AD [23-25]. The classical A β cascade hypothesis in AD pathogenesis postulates that the deposition of A β peptides and the activation of glial cells surrounding senile plaques in brain areas involved in cognitive functions trigger marked neuronal alterations such as synaptic dysfunction, synaptic loss and neuronal death finally leading to cognitive impairments [26,27].

There is increasing evidence indicating that NPY can modulate A β -induced neurotoxicity (for review, see [28]). For instance, A β peptide fragment 25–35 exposure to a neuroblastoma cell line causes cell loss, which is prevented by NPY [24]. The same authors observed that A β_{25-35} reduces intracellular nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), whereas NPY completely restores the levels of these growth factors [25]. Moreover, Rose et al. [23] demonstrated that the infusion of the amidated NPY C-terminal fragments into the brains of APP (amyloid precursor protein) tg mice ameliorated the neurodegenerative pathology in this genetic model of AD as well as protected human neuronal cultures from the neurotoxic effects of A β peptides.

The exact mechanisms of A β -induced neurotoxicity are not completely understood. In this context, in a previous series of studies, we described a mouse model consisting of a single i.c.v. injection of aggregated A β_{1-40} peptide (composed of oligomeric and monomeric forms) that resembles the early phases of AD [29-31]. Although unable to induce all pathological AD hallmarks, such as amyloid plaque and phospho-tau positive cells [32,33], the acute injection of A β_{1-40} peptide into the mouse brain can be an useful approach for the investigation of molecular mechanisms underlying A β toxicity, including the activity of mitochondrial complexes and oxidative stress, neuroinflammation, synaptic deficits and apoptotic neuronal cell death that lead to spatial learning and memory impairments in mice [29-31, 34-36]. However, as far as we know, there is no previous study addressing the role of NPY on A β_{1-40} -induced cognitive/emotional alterations in rodents. Therefore, in the current study we evaluated the effects of a single i.c.v.

administration of NPY (0.0234 $\mu\text{mol}/\mu\text{l}$) 15 min prior to the i.c.v. injection of aggregated $\text{A}\beta_{1-40}$ peptide (400 pmol/mouse) on behavioral performance of Swiss albino mice in tests of spatial memory (object location), emotionality (elevated plus-maze and tail suspension) and locomotion (open field). Moreover, biochemical parameters related to hippocampal and cerebro-cortical oxidative stress and glutathione (GSH)-dependent antioxidant status were evaluated in an attempt to elucidate potential events mediating the neuroprotective effects of NPY, since there is considerable evidence suggesting that oxidative stress is involved in the mechanisms of $\text{A}\beta$ -induced neurotoxicity [29, 31, 36] and AD pathogenesis [37].

2. Materials and methods

2.1. Animals

Experiments were conducted using 3-month-old male Swiss mice weighing 35-40 g purchased from our own breeding colony. The animals were kept in collective plastic cages (15 animals per cage) and maintained in a room under controlled temperature (22 ± 1 °C) and 12-h light cycle (lights on 7:00 AM), with free access to food and water. The animals were treated, manipulated and euthanized according to the “Principles of Laboratory Animal Care” (NIH publication no. 80-23, revised 1996) and approved by the Committee on the Ethics of Animal Experiments of the Federal University of Santa Catarina (CEUA/UFSC; www.ceua.ufsc.br; protocol number 23080.003465/2010-55). All efforts were made to minimize the number of animals used and their suffering.

2.2. Drugs and treatment

The animals were allocated to the following groups: (i) control + control ($n = 8$), (ii) NPY + control ($n = 8$), (iii) control + $\text{A}\beta_{1-40}$ ($n = 8$) or (iv) NPY + $\text{A}\beta_{1-40}$ ($n = 8$). The variation in animal’s body weight was considered and counterbalanced across the four groups. NPY (Bachem, AG, Switzerland) was dissolved in sterile 0.1 M sodium phosphate-buffered saline (PBS) (pH 7.4) at the concentration of 0.0234 $\mu\text{mol}/\mu\text{L}$ which was based in previous literature [38] and in pilot experiments (data not shown). Human $\text{A}\beta_{1-40}$ (Tocris, Ellisville, MO, USA) was prepared as stock solutions at a concentration of 1 mg/ml in sterile 0.1 M PBS (pH 7.4), and aliquots were stored at -20°C . $\text{A}\beta_{1-40}$ solutions were aggregated by incubation at 37°C for 4 days before use as described previously [29-31]. The aggregation and/or oligomerization state of $\text{A}\beta$ solutions was confirmed through Western blot analysis (data not shown).

The animals received a single i.c.v. administration of NPY (0.0234 $\mu\text{mol}/\mu\text{L}$) or PBS (1 μL) 15 min before the i.c.v. infusion of $\text{A}\beta_{1-40}$ (400 pmol/mouse) or PBS (1 μL). The i.c.v. microinjections were performed using a microsyringe (1 μL , Hamilton) connected to a 26-gauge stainless-steel needle that was inserted perpendicularly 3 mm deep through the skull according to the procedure originally described by Haley and McCormick [39] and modified by Maurice et al. [40]. Briefly, the animals were anesthetized with isoflurane (1 ml/ml; Abbot Laboratórios do Brasil Ltda., RJ, Brazil) using a vaporizer system (SurgiVet Inc., WI, USA) and then gently restrained by hand for i.c.v. injections. The sterilization of the injection site was carried out using a gaze embedded in 70% ethanol. Under light anesthesia (i.e. just that necessary for loss of the postural reflex), the needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and the ears and perpendicular to the plane of the skull. A volume of 1 μL of PBS, NPY or $\text{A}\beta_{1-40}$ was delivered gradually into the lateral ventricle. Mice exhibited normal behavior within 5 min after injection and the biochemical and behavioral experiments were performed 24 h and from 9 to 14 days, respectively, after i.c.v. administration (Fig. 1).

In addition, it was investigated the possible involvement of Y2 receptors through the use of the selective Y2 receptor antagonist BIIE0246 (Tocris, Ellisville, MO, USA). A volume of 1 μL of PBS (pH 7.4) or BIIE0246 (1 nmol/ μL ; dissolved in PBS) was injected directly into the lateral ventricle 10 min before the administration of NPY (0.0234 $\mu\text{mol}/\mu\text{L}$, i.c.v.) as described previously [41]. The accurate placement of the injection (needle track) was confirmed during dissection of the animals to conduct biochemistry assays. Results from mice presenting cannula misplacement or any sign of cerebral hemorrhage were discarded from the statistical analysis (overall less than 5% of the total animals used).

2.3. Behavioral tests

During a period of 9–14 days after the i.c.v. infusion of $\text{A}\beta_{1-40}$, the animals were submitted to a battery of behavioral paradigms that included the open field, tail suspension, elevated plus maze and object location tasks (Fig. 1). The time point for the performance of each behavioral task was chosen based on previous studies using the $\text{A}\beta_{1-40}$ model [29,35,36,42].

All tests were carried out between 9:00 and 14:00 h and they were scored by the same rater in an observation room where the mice

had been habituated for at least 1 h before the beginning of the tests. Behavior was monitored through a video camera positioned above the apparatuses and the images were later analyzed with the ANY Maze® video tracking (Stoelting Co., Wood Dale, IL, USA) by an experienced experimenter who was unaware of the experimental group of the animals tested.

Insert Figure 1 about here.

2.3.1. Open field

To examine the spontaneous locomotor activity, the animals were placed for 5 min in the open field arena. The apparatus, made of acrylic, had a grey floor of 50 cm x 50 cm and transparent walls, 40 cm high. The experiments were conducted in a sound-attenuated room under low-intensity light (7 lx). Each mouse was placed in the center of the open field and the total distance traveled (m) was registered with the ANY Maze® video tracking. The apparatus was cleaned with ethanol solution (10% v/v) and dried with paper towels after each trial in order to avoid odor impregnation.

2.3.2. Tail suspension

The tail suspension test has become one of the most widely used tests for assessing antidepressant-like activity in mice. It is based on the fact that animals subjected to the short-term inescapable stress of being suspended by their tail, will develop an immobile posture. The total duration of immobility induced by tail suspension test was measured according to the method described by Steru et al. [43]. Briefly, mice both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded during a 6 min period. Mice were considered immobile only when they hung passively and completely motionless. Antidepressant treatments reduce the time of immobility and increase active escape behaviors displayed during inescapable situation in the tail suspension test [43]. The immobility time was recorded by an observer blind to the drug treatment.

2.3.3. Elevated plus-maze

The elevated plus-maze was used to evaluate anxiety-like behaviors in mice [44]. The apparatus was made of wood covered with impermeable black Formica, elevated 60 cm from the floor, with four arms (18 cm long, 6 cm wide) arranged in the shape of a plus sign. Two opposite arms were surrounded by walls (6 cm high, enclosed arms),

while the other two were devoid of enclosing walls (open arms). The four arms were connected by a central platform (6 cm × 6 cm). The experiments were conducted in a sound-attenuated room under low intensity light (12 lx). The animals were individually placed in the central area of the maze facing an enclosed arm and were observed for 5 min. The apparatus was cleaned with ethanol solution (10% v/v) and dried with paper towels after each trial in order to avoid odor impregnation. The number of entries (defined by the placement of the four paws into an arm) and the time spent in open and enclosed arms were recorded. The percentage of open-arm entries and time were calculated by the number of open arm entries divided by the total number of arm entries. The number of enclosed-arm entries was used as a measure of locomotor activity [45].

2.3.4. Object location task

The spatial memory of mice was assessed with the object location task. The task is based on the spontaneous tendency of rodents, previously exposed to two identical objects, to later explore one of the objects (replaced in a novel location) for a longer time than they explore the non-displaced object [46], and has been used for the evaluation of hippocampal-dependent memories [47,48]. The experimental apparatus used in this study was an open-field box (50 cm wide × 50 cm deep × 40 cm high) made of transparent Plexiglas, placed in a dimly lit (7 lx) and sound-isolated room. Identical plastic rectangles (4 cm high × 4.5 cm wide) were used as objects. The protocol used was based on the previously described by Assini et al. [47]. The mice were placed in the center of the apparatus with two identical objects for 3 min. The objects were placed 7 cm away from the walls of the open field. Exploration of the objects was recorded by a stopwatch when mice sniffed, whisked, or looked at the objects from no more than 1 cm away. After the training phase, the mice were removed from the apparatus for an intertrial interval of 90 or 360 min depending on the purpose of the protocol. After the intertrial interval, one object was moved to a new location. The time spent by the animals exploring the objects in new (novel) and old (familiar) locations was recorded during 3 min. All locations of the objects were counterbalanced among the groups. In order to analyze the cognitive performance, a location index was calculated as previously described by [46]: $(T_{\text{novel}} \times 100) / (T_{\text{novel}} + T_{\text{familiar}})$, where T_{novel} is the time spent exploring the displaced object and T_{familiar} is the time spent exploring the non-displaced object.

2.4. Tissue preparation for biochemical analyses

For the investigation of possible neuroprotective effects of NPY against the oxidative stress and putative alterations in the antioxidant glutathione (GSH) system induced by the i.c.v. infusion of A β ₁₋₄₀, six animals of each group were decapitated 24 h after A β ₁₋₄₀ administration [31], and the prefrontal cortex and hippocampus were removed and homogenized (1:10 w/v) in HEPES buffer (20 mM, pH 7.0). The tissue homogenates were centrifuged at 16,000 \times g, at 4 °C for 20 min and the supernatants obtained were used for the determination of enzymatic activities and for the quantification of the levels of GSH and thiobarbituric acid reactive substances (TBARS).

2.5. Biochemical analyses

2.5.1. Determination of thiobarbituric acid reactive substances levels

TBARS were determined in the prefrontal cortex and hippocampal homogenates using the method described by [49] in which malondialdehyde (MDA), an end-product of lipid peroxidation, reacts with thiobarbituric acid to form a colored complex. The samples were incubated at 100 °C for 60 min in acid medium containing 0.45% sodium dodecyl sulfate and 0.67% thiobarbituric acid. After centrifugation, the reaction product was determined at 532 nm using MDA as standard.

2.5.2. Glutathione levels

Prefrontal cortex and hippocampal glutathione (GSH) levels were measured as non-protein thiols (NPSH) based on the protocol developed by Ellman [50]. Homogenates were precipitated in cooled trichloroacetic acid 10% and centrifuged at 10000 \times g for 5 min, and the supernatant was incubated with DTNB in a 1 M phosphate buffer, pH 7.0. Absorbance was measured at 412 nm. A standard curve of reduced glutathione was used to calculate GSH levels.

2.5.3. Antioxidant enzymes

Prefrontal cortex and hippocampal glutathione reductase (GR) activity was measured using an NADPH oxidation assay following the protocol developed by Carlberg and Mannervik [51] using glutathione disulfide (GSSG) as substrate. GR activity was monitored by decreases in NADPH absorbance at 340 nm at 37 °C in a TECAN Genios Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). Results were based on a molar extinction coefficient for NADPH of 6.22 \times 10³M⁻¹ cm⁻¹. Prefrontal cortex and hippocampal glutathione

peroxidase (GPx) activity was measured using an NADPH oxidation assay following the technique of Wendel [52]. Tissue supernatant (around 200 µg protein) was added to a reaction mixture containing reduced glutathione, glutathione reductase, and NADPH in phosphate buffer (pH 7.4). The reaction was initiated by adding *tert*-butyl hydroperoxide, the absorbance decrease at 340 nm was recorded at 37 °C in a TECAN Genios Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). The activity in the absence of the samples was subtracted. Results were based on a molar extinction coefficient for NADPH of $6.22 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$.

2.5.4. Protein determination

The protein content was quantified by the method of Bradford [53], using bovine serum albumin as a standard.

2.6. Statistical analysis

All values are expressed as means \pm SEM. The object location task was analyzed by one-sample *t* tests to determine whether the location index was different from chance performance (50%). The rest of statistical analyses were carried out using two-way analysis of variance (ANOVA) with pretreatment and treatment as the independent variables. Following significant ANOVAs, multiple *post hoc* comparisons were performed using the Newman-Keuls test. The accepted level of significance for the tests was $P \leq 0.05$. All tests were performed using the Statistica® software package (Stat Soft Inc., Tulsa, OK, USA).

3. Results

3.1. NPY prevents spatial memory deficits induced by A β_{1-40} peptide: role of Y2 receptors

The actions of A β peptide on laboratory rodents have been extensively studied, specifically regarding its effects on the learning and memory processes [29-36]. To investigate whether the pretreatment with NPY may prevent the A β_{1-40} -induced cognitive decline, the object location task was used.

Fig. 2 illustrates the effects of the i.c.v. administration of NPY (0.0234 µmol/µL) and/or A β_{1-40} (400 pmol/mouse) on location index of mice when they were trained and tested after a short intertrial interval (90 min). In the training phase, all animal groups showed similar performance on the task, spending around 50% of the time exploring each object, indicating the interest of these animals by the novelty and

lack of preference for a specific location of the objects (Fig. 2A). Corroborating previous findings [47], during the test phase, control-treated mice explored for a longer time the object replaced in a novel location than the non-displaced object as indicated by a significant increase in location index (Fig. 2B). On the other hand, $A\beta_{1-40}$ -treated mice were not able to identify the spatial alteration in the open field spending similar time exploring both objects. Notably, the animals that received NPY prior the infusion of $A\beta_{1-40}$ peptide had an exploratory index similar to control animals in the test phase, indicating that the pretreatment with NPY prevented the spatial memory deficits induced by $A\beta_{1-40}$ peptide (Fig. 2B).

Insert Fig. 2 about here.

To investigate the role of Y2 receptors in the protective effects of NPY on the spatial memory deficits induced by $A\beta_{1-40}$ in mice, the selective Y2 receptor antagonist BIIE0246 (1 nmol/ μ L) was injected directly into the lateral ventricle 10 min before the administration of NPY (0.0234 μ mol/ μ L, i.c.v.). There were no differences on total investigation time of both objects among groups in the training session (data not shows). As can be seen in Fig. 3A, the increase in location index induced by NPY in $A\beta_{1-40}$ -treated mice during the test phase (performed 90 min after the training phase) was abolished by the previous administration of the selective Y2 receptor antagonist BIIE0246, indicating that these animals were not able to identify the spatial alteration in the open field.

Insert Fig. 3 about here.

The object location task provides a qualitative index (i.e., the animals discriminate or not the object location change), and for this reason a putative increase in the performance of mice by NPY administration in comparison to control group could not be observed using a short (90 min) intertrial interval. Therefore, to rule out a putative *per se* effect of the dose of NPY tested in the spatial memory of mice, additional groups of Swiss albino mice were evaluated in the object location task 14 days after i.c.v. injection of NPY (0.0234 μ mol/ μ L) using a longer (360 min) intertrial interval between training and test phases. As illustrated in Fig. 3B, control- and NPY-treated mice were not able to identify the spatial alteration in the open field after a longer intertrial interval (360 min). Altogether, these results suggest that a

single i.c.v. administration of NPY does not interfere *per se* with the spatial memory of mice, at least at the dose here tested.

3.3. Effects of $A\beta_{1-40}$ and NPY administration on emotionality and locomotor activity of mice

Fig. 4 illustrates the effects of $A\beta_{1-40}$ and/or NPY administration on depressive-like responses of mice evaluated in the tail suspension test. Two-way ANOVA revealed a significant effect for the interaction factor between pretreatment and treatment [$F_{1,28} = 4.23$, $P \leq 0.05$] in the immobility time in the tail suspension test. Subsequent Newman-Keuls test indicated that central infusion of $A\beta_{1-40}$ induced a significant increase in the immobility time which was completely abolished by the pretreatment with NPY. These results suggest that NPY was able to prevent the depressive-like behavior induced by $A\beta_{1-40}$ infusion in mice.

Insert Fig. 4 about here.

Table 1 summarizes the effects of the i.c.v. administration of NPY (0.0234 $\mu\text{mol}/\mu\text{L}$) and/or $A\beta_{1-40}$ (400 pmol/mouse) on anxiety-related parameters and locomotor activity of mice evaluated in the elevated plus-maze and open field tests, respectively. Two-way ANOVA revealed no significant effects for the main factors and their interaction in the time spent on open arms [pretreatment: $F_{1,28} = 0.01$, $P=0.91$; treatment: $F_{1,28} = 1.96$, $P=0.17$; interaction: $F_{1,28} = 0.006$, $P=0.94$], open-arm entries [pretreatment: $F_{1,28} = 1.05$, $P=0.32$; treatment: $F_{1,28} = 0.29$, $P=0.60$; interaction: $F_{1,28} = 0.79$, $P=0.38$] and in the number of enclosed-arm entries [pretreatment: $F_{1,28} = 0.23$, $P=0.64$; treatment: $F_{1,28} = 0.86$, $P=0.36$; interaction: $F_{1,28} = 0.74$, $P=0.40$]. Moreover, no significant differences among groups was observed in the distance traveled in the open field arena [pretreatment: $F_{1,28} = 0.001$, $P=0.97$; treatment: $F_{1,28} = 2.43$, $P=0.13$; interaction: $F_{1,28} = 0.84$, $P=0.37$] (Table 1).

Insert Table 1 about here.

3.4. Effects of $A\beta_{1-40}$ and NPY administration on oxidative stress and glutathione-dependent antioxidant system

The antioxidant glutathione (GSH) system is essential to mediate protection against several (pro)-oxidant molecules in the CNS [54] and previous studies from our group [29,31,36] have demonstrated

that the i.c.v. infusion of A β ₁₋₄₀ peptide reduces the GSH-related antioxidant defenses, altering the cellular redox state. Corroborating these previous findings, in the present study we observed that the i.c.v. infusion of A β ₁₋₄₀ induced a marked increase of end products of lipid peroxidation (TBARS) in the prefrontal cortex [$F_{1, 24} = 4.43, P \leq 0.05$] (Fig. 5A) and hippocampus [$F_{1, 24} = 5.45, P \leq 0.05$] of mice (Fig. 5B). More importantly, the pretreatment with NPY blunted this A β ₁₋₄₀-induced increase on lipid peroxidation in both the prefrontal cortex [$F_{1, 24} = 6.30, P \leq 0.05$] (Fig. 5A) and hippocampus [$F_{1, 24} = 5.45, P \leq 0.05$] (Fig. 5B). On the other hand, the GSH content in the prefrontal cortex (Fig. 5C) and hippocampus (Fig. 5D) was not significantly altered by A β ₁₋₄₀ and/or NPY administration.

Insert Fig. 5 about here.

Moreover, i.c.v. administration of A β ₁₋₄₀ caused a significant decrease in glutathione reductase (GR) activity in the prefrontal cortex [$F_{1, 24} = 4.43, P \leq 0.05$] (Fig. 6A), but not in the hippocampus [$F_{1, 24} = 0.04, P = 0.83$] (Fig. 6B). Remarkably, the pretreatment with NPY prevented the reduction of GR activity in the prefrontal cortex induced by A β ₁₋₄₀ [$F_{1, 24} = 3.53, P \leq 0.05$] (Fig. 6A). On the other hand, the GPx activity in prefrontal cortex (Fig. 6C) and hippocampus (Fig. 6D) was not significantly altered by A β ₁₋₄₀ and/or NPY administration.

Insert Fig. 6 about here.

4. Discussion

There is increasing evidence of the involvement of NPY on AD pathophysiology. A significant reduction of NPY levels in the hippocampal regions of AD brains was observed [17] in association with an altered number of NPY receptors [55]. Moreover, a reduction of NPY levels on plasma [19] and cerebrospinal fluid [18] has been observed during AD progression. Interestingly, recent *in vivo* and *in vitro* studies have demonstrated the neuroprotective effects of NPY in transgenic [23] and A β [24,25] mouse models of AD. However, the exact role of NPY in the A β -induced neuronal impairments remains far from being elucidated.

Here we further addressed this issue through the evaluation of the potential neuroprotective effects of NPY on the behavioral and

neurochemical deficits induced by the central infusion of $A\beta_{1-40}$ in mice. The present results show that a single i.c.v. administration of NPY (0.0234 $\mu\text{mol}/\mu\text{l}$) prevented $A\beta_{1-40}$ -induced depressive-like responses and spatial memory impairments. The protective effects of NPY on spatial memory of $A\beta_{1-40}$ -treated mice were abolished by the pretreatment with the selective Y2 receptor antagonist BIIE0246. The observed behavioral benefits of exogenous NPY administration were accompanied by the prevention of $A\beta_{1-40}$ -induced increase in lipid peroxidation in the hippocampus and prefrontal cortex. To the best of our knowledge, this is the first study reporting the beneficial effects of NPY in depressive-like responses and spatial memory deficits in an experimental model of AD based on $A\beta$ -mediated neurotoxicity.

The earliest AD symptoms have been attributed to the soluble forms of $A\beta$ peptides, whereas the insoluble forms lead to extracellular plaques that are deposits of fibrils and amorphous aggregates [56]. Previous studies from our group [29-31,34-36] as well as a validation study by Takeda and co-workers [33] showed that a single i.c.v. injection of $A\beta_{1-40}$ causes a specific dysfunction of memory processes, which at least in part fulfils three validity criteria for AD [33].

In the present study, $A\beta_{1-40}$ -treated mice, as expected, displayed a poor performance in the object location task, which is highly dependent upon the hippocampal and cortical regions [47,48]. These findings extend previous results demonstrating spatial learning and memory impairments induced by i.c.v. $A\beta_{1-40}$ infusion in mice in different behavioral paradigms, such as the Morris water maze [29-31,34,35]. Of great interest, the current data show for the first time that a single i.c.v. administration of NPY prevents the cognitive deficits in $A\beta_{1-40}$ -treated mice. However, at 14 days after a single i.c.v. administration of NPY, no cognitive-enhancing properties of this peptide were observed in mice evaluated in the object location task (intertrial interval of 360 min). In fact, this is not a surprising result, since previous studies have demonstrated that the effects of NPY on learning and memory retention is time-dependent, with no effects being observed when NPY was administered 90 min after training [57]. Therefore, the observed ability of NPY to prevent the cognitive decline in $A\beta_{1-40}$ -treated mice seems to be directly associated to its protective effects against $A\beta_{1-40}$ toxicity rather than a pro-mnemonic effect.

The Y2 receptor appears to be one of the main targets underlying the neuroprotective effects of NPY, since the administration of selective Y2 receptor antagonists abolishes this response [58-60]. Moreover, the highest density of NPY Y2 receptors is found in the

hippocampus which is particularly involved in spatial learning/memory and the performance of the object location task [47]. In the present study, the protective effects of NPY on spatial memory of $A\beta_{1-40}$ -treated mice were abolished by the pretreatment with the selective Y2 receptor antagonist BIIIE0246, providing the first evidence of the involvement of these receptors in the protective effects triggered by NPY in a mouse model of AD.

In addition, since the i.c.v. infusion of $A\beta_{1-40}$ does not cause gross motor alterations (as evaluated in the open field) that would preclude the assessment of emotional functions, we also investigated whether such behaviors are affected in these animals. We observed that the cognitive impairments in $A\beta_{1-40}$ -treated mice occurred together with increased emotional responses in the tail suspension test, resembling the emotional disturbances observed in AD patients (for review see [61]). These results are in accordance with previous findings by Pamplona et al. [42] that reported increased immobility time of $A\beta_{1-40}$ -treated mice in the forced swimming test, another behavioral paradigm widely used for the screening of antidepressant drugs [62]. This is an important contribution to the field on its own, since emotionality has been seldom assessed in animal models of AD. Although it is known that the incidence of depression in AD patients is associated with increased $A\beta_{1-42}$ [63], we found no previous reference to it in animal models. A single study directly addressing this topic not only found no difference in $A\beta_{25-35}$ -treated mice in comparison to control group in the forced swimming test, but observed increased sensitivity to antidepressant treatments as well [64].

On the other hand, both pre-clinical and clinical studies have suggested that NPY, together with its receptors, may have a direct implication in several psychiatric disorders, including depression and related illnesses (for review see [10]). It has been shown that NPY displayed antidepressant-like activity in several behavioral paradigms including the forced swimming test [65, 66], olfactory bulbectomized rats [67] and maternal separation in rats [68]. Additionally, chronic antidepressant treatments with imipramine [9], fluoxetine [69] and electroconvulsive shock stimulation [11] have been shown to increase NPY immunoreactivity in the rodent brain. However, clinical studies have generated somewhat inconsistent findings and methodological issues including differences in experimental design, incorrect patient diagnosis, small sample size, difficulties with sample availability and comorbidity with other psychiatric disorders have been suggested to account for the differences in clinical data (for review, see [10]). Despite

these limitations, it appears that NPY may play a role in the pathophysiology of depression, and thus may represent a potential novel target for the treatment of this illness. Reinforcing this view, in the current study we provide the first evidence that a single i.c.v. NPY administration prevents depressive-like responses in mice induced by central infusion of A β ₁₋₄₀ peptide.

There is considerable evidence suggesting that oxidative stress is involved in the mechanisms of A β -induced neurotoxicity [70] and AD pathogenesis [37]. For example, increases in lipid peroxidation, protein carbonyls and oxidation of mitochondrial DNA have been observed in the brains of AD patients [71]. Similarly, exposure to A β increases lipid peroxidation, protein oxidation and the formation of hydrogen peroxide in cultured cells [72]. Despite this evidence indicating oxidative damage in AD patients and in rodent models, a causal link still needs to be established. Nevertheless, studies on the potential therapeutic effects of antioxidants for the treatment of AD have produced promising results [35,36,73].

In this context, it is important to note that the i.c.v. A β ₁₋₄₀ infusion increased the levels of lipid peroxidation in the hippocampus and prefrontal cortex and that NPY significantly blunted this phenomenon. These results suggest that oxidative stress played an important role in mediating the deleterious effects of A β ₁₋₄₀ and that the antioxidant properties of NPY were important in counteracting A β ₁₋₄₀ effects. This idea is supported by studies reporting the pro-oxidative properties of A β ₁₋₄₀ [29,31,35,36].

Glutathione (GSH) is the major non-protein thiol antioxidant in mammalian cells and it is considered to be the main intracellular redox buffer [54]. Previous studies have indicated that the GSH system may be activated as a response to oxidative stress in the brains of AD patients [74], and that the inhibition of GSH synthesis leads to an increase in A β -induced cell death and intracellular A β accumulation [75]. In the present study, A β ₁₋₄₀ decreased the GR activity in prefrontal cortex of mice, which could contribute to the toxic effect generated by this fragment. More importantly, once again the previous i.c.v. administration of NPY was able to prevent this reduction in GR activity induced by A β .

In conclusion, the present findings indicate that the central administration of NPY prevents A β ₁₋₄₀-induced depressive-like behavior and spatial memory deficits in mice and that this response is mediated, at least in part, by the activation of Y2 receptors and the protection against oxidative stress. Therefore, the present study provides novel *in*

vivo evidence suggesting that NPY and its receptors can represent important molecular targets to counteract the A β -induced neurotoxicity.

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Figure legends

Fig. 1 - Time course of behavioral and neurochemical tests following the pretreatment with control (PBS, i.c.v.) or NPY (0.0234 $\mu\text{mol}/\mu\text{L}$) and a single i.c.v. infusion of control (PBS) or $\text{A}\beta_{1-40}$ (400 pmol/mouse) in 3-month-old male Swiss albino mice.

Fig. 2 - NPY prevents the cognitive impairments induced by $\text{A}\beta_{1-40}$ in mice. The animals were treated i.c.v. with NPY (0.0234 $\mu\text{mol}/\mu\text{L}$) or PBS 15 min before the i.c.v. infusion $\text{A}\beta_{1-40}$ (400 pmol/mouse) or PBS administration. Graphs represent the discrimination ratio in training (A) and test session (B). * $P \leq 0.05$ versus chance level (50% of displaced object investigation in test trial) (Student *t* tests).

Fig. 3 – Role of Y2 receptors on the NPY-induced cognitive benefits on mice infused with $\text{A}\beta_{1-40}$. The graph A represents the performance of the animals treated with the selective Y2 receptor antagonist BIIE0246 (1 nmol/ μL) 10 min before the NPY (0.0234 $\mu\text{mol}/\mu\text{L}$) or PBS administration, followed by the challenge with $\text{A}\beta_{1-40}$ (400 pmol/mouse) or PBS administration using an intertrial interval of 90 min. The graph B indicates the absence of cognitive-enhancing properties of NPY (0.0234 $\mu\text{mol}/\mu\text{L}$) when the intertrial interval was extended to 360 min. * $P \leq 0.05$ versus chance level (50% of displaced object investigation in test trial) (Student *t* tests).

Fig. 4 - Effects of the $\text{A}\beta_{1-40}$ and NPY on immobility time of mice evaluated in the tail suspension test (for 6 min). The animals were treated i.c.v. with NPY (0.0234 $\mu\text{mol}/\mu\text{L}$) or PBS 15 min. before the $\text{A}\beta_{1-40}$ (400 pmol/mouse) or PBS administration. Data are expressed as the mean \pm S.E.M. * $P \leq 0.05$ versus chance level (50% of displaced object investigation in test trial) (Student *t* tests).

Fig. 5 - Effect of $\text{A}\beta_{1-40}$ and NPY on thiobarbituric acid-reactive substances (TBARS) levels and non-protein sulfhydryl (NPSH) levels. The animals were treated i.c.v. with NPY (0.0234 $\mu\text{mol}/\mu\text{L}$) or PBS 15 min before the $\text{A}\beta_{1-40}$ (400 pmol/mouse) or PBS administration. The graphs represent the levels of TBARS in prefrontal cortex (A) and (B) hippocampus. The NPSH are indicated in graphs (C) in prefrontal cortex and (D) hippocampus. Data are expressed as the mean \pm S.E.M. * $P \leq 0.05$ compared to control-control group (Two-way ANOVA followed by Newman-Keuls test).

Fig. 6 - Effect of $A\beta_{1-40}$ and NPY on glutathione reductase (GR) and glutathione peroxidase (GPx) activities. The animals were treated i.c.v. with NPY (0.0234 $\mu\text{mol}/\mu\text{L}$) or PBS 15 min before the $A\beta_{1-40}$ (400 pmol/mouse) or PBS administration. The graphs represent the activity of GR in prefrontal cortex (A) and (B) hippocampus. The GPx activity are represented in graphs (C) in prefrontal cortex and (D) hippocampus. Data are expressed as the mean \pm S.E.M. ANOVA followed by Newman-Keuls test. * $P \leq 0.05$ compared to control-control group (Two-way ANOVA followed by Newman-Keuls test).

Fig. 1

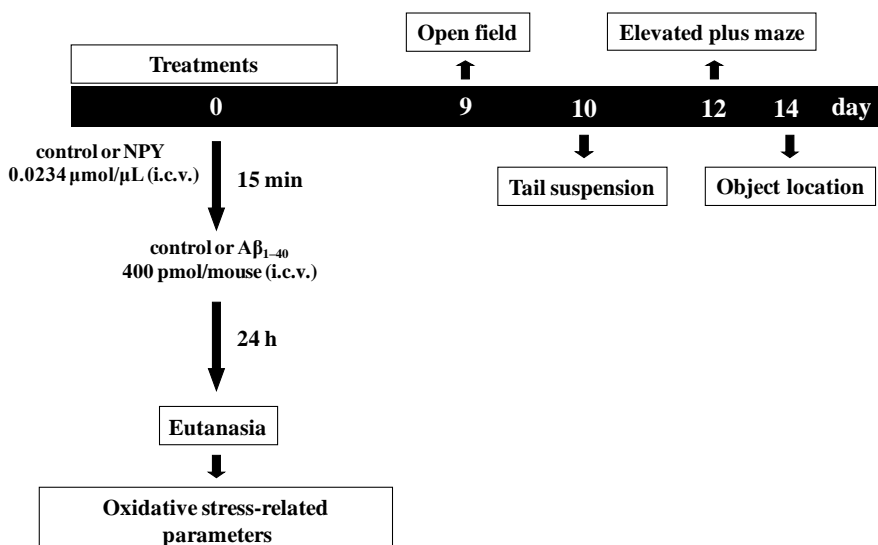


Fig. 2

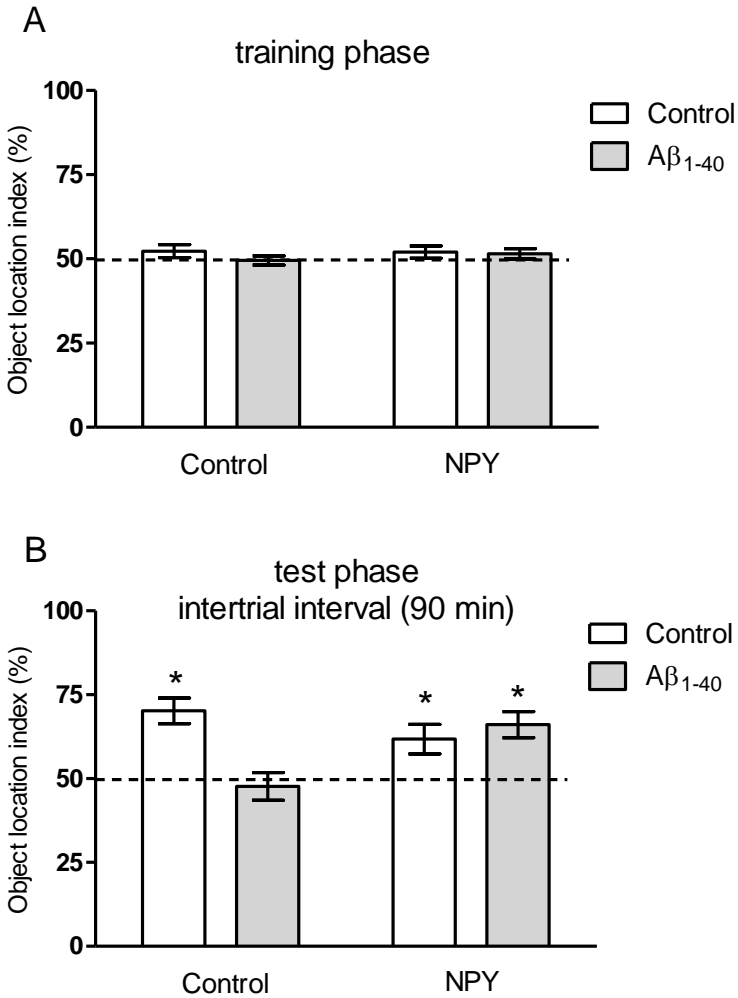


Fig. 3

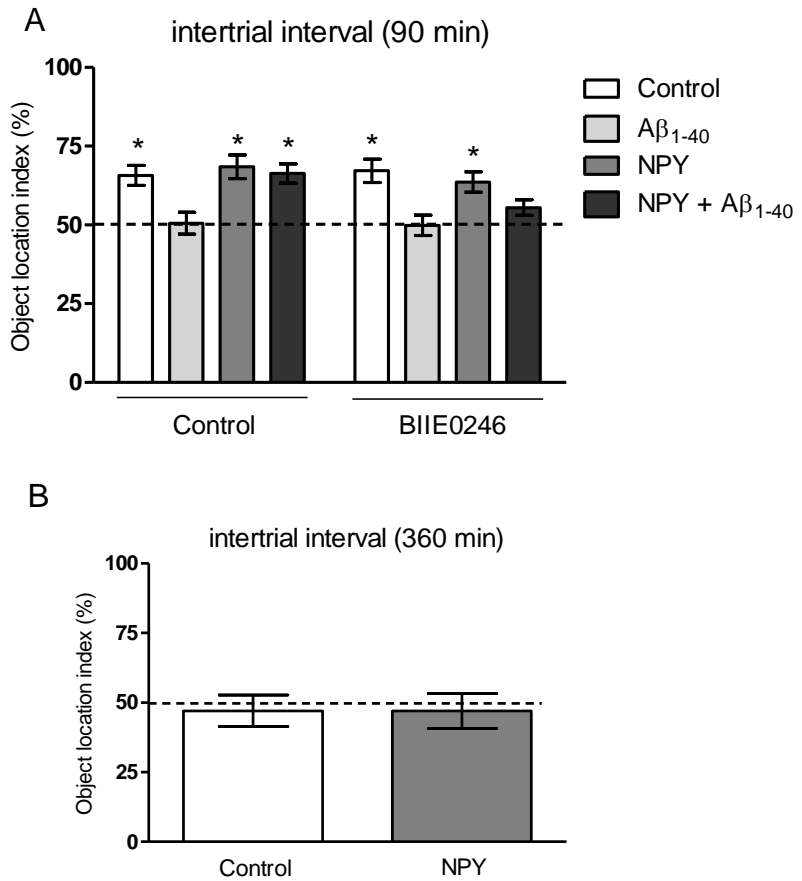


Fig. 4

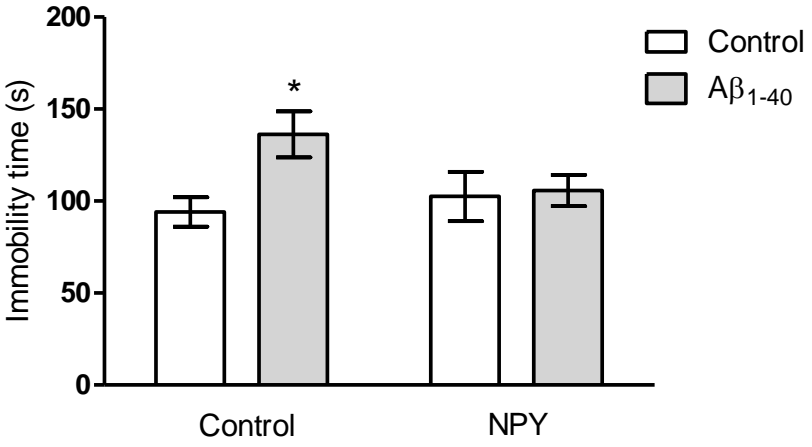


Fig. 5

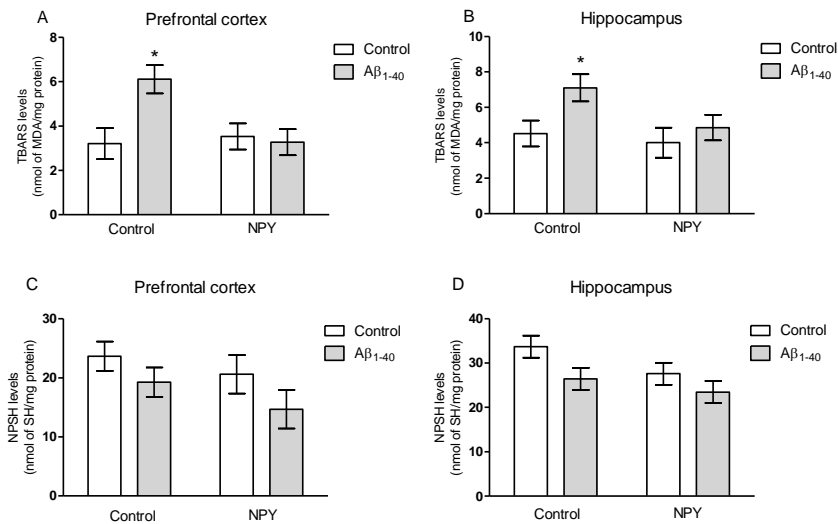


Fig. 6

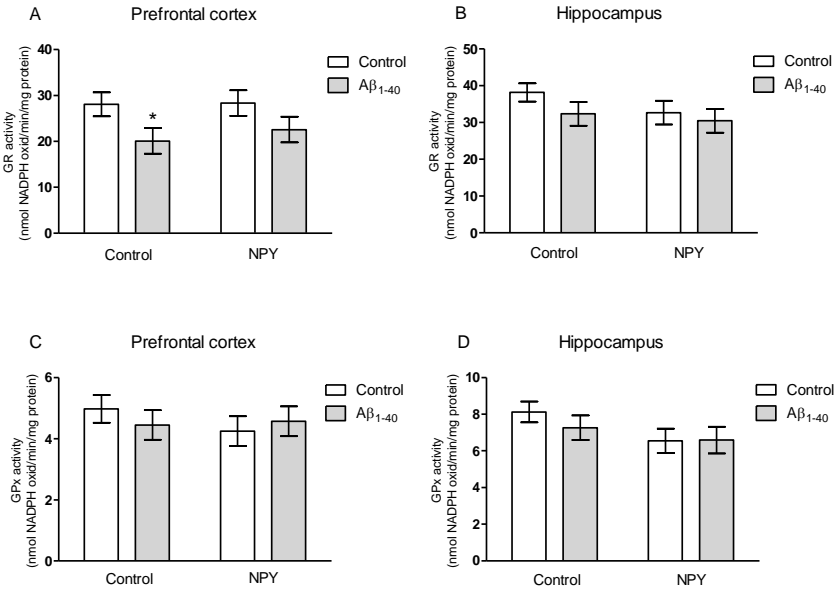


Table 1 - Effects of Aβ₁₋₄₀ and NPY on anxiety-like responses and locomotor activity of mice evaluated for 5 min in the elevated plus-maze and open field tests, respectively.

Behavioral test	Parameter	Control/Control	Control/A β ₁₋₄₀	NPY/Control	NPY/A β ₁₋₄₀
<i>Elevated plus-maze</i>	% Open arm time	7.5 ± 2.1	11.4 ± 2.9	8.0 ± 2.9	11.5 ± 2.6
	% Open arm entries	11.8 ± 1.7	11.1 ± 2.0	12.1 ± 2.2	15.0 ± 2.0
	Enclosed arm entries	13.0 ± 1.2	14.2 ± 1.5	13.2 ± 1.7	12.3 ± 1.5
<i>Open field</i>	Distance (m)	28.1 ± 4.0	27.8 ± 4.5	30.3 ± 3.1	25.7 ± 3.5

The animals were treated with NPY (0.0234 μ mol/ μ L, i.c.v.) or PBS (i.c.v.) 15 min before the i.c.v. infusion of A β ₁₋₄₀ (400 pmol/mouse) or PBS. Data are expressed as mean \pm S.E.M. of 8 animals per group. Two-way ANOVA revealed no significant effects for the main factors (pretreatment vs. treatment) in any of the parameters evaluated.

7. DISCUSSÃO

A doença de Alzheimer (DA) é a forma mais comum de demência sendo os seus marcadores histopatológicos os emaranhados neurofibrilares e as placas senis (Murrell, Farlow *et al.*, 1991). A fisiopatologia da DA está associada a dois eventos principais: a hiperfosforilação da proteína tau, resultando em um acúmulo de emaranhados neurofibrilares e a formação de agregados insolúveis da proteína beta-amiloide (Perry, Perry *et al.*, 1977; Götz, Eckert *et al.*, 2011; Reiniger, Lukic *et al.*, 2011)

Dentre os fatores etiológicos podem-se citar o envelhecimento, o diabetes, a depressão, os processos infecciosos que afetam o SNC e a obesidade (Chintamaneni e Bhaskar, 2012). A obesidade é um fator de risco para uma variedade de patologias. Enquanto seu envolvimento em doenças como o diabetes, doenças cardíacas e câncer está bem caracterizado, seu papel deletério no SNC começa a ser desvendado (Sriram, Benkovic *et al.*, 2002). A obesidade ocasiona um desequilíbrio nas concentrações plasmáticas dos hormônios leptina, insulina, estrogênio, testosterona, hormônio do crescimento, grelina, NPY, dentre outros (Lee, 2011). As alterações nas concentrações de leptina, grelina e NPY são consideradas um importante mecanismo capaz de modificar a ingestão alimentar (Suzuki, Jayasena *et al.*, 2012).

Estudos indicam um padrão rítmico entre a secreção de grelina e a liberação de NPY. Esta relação funcional entre estes dois hormônios foi demonstrada a partir da administração intravenosa (i.v.) e central (i.c.v.) de grelina e o aumento na expressão imediata do gene c-fos (um marcador da atividade neuronal) em neurônios NPY positivos, no núcleo arqueado e no núcleo paraventricular (Cowley, Smith *et al.*, 2003). Além disso, a administração exógena de grelina aumenta os níveis de RNAm de NPY no núcleo arqueado estimulando a ingestão de alimentos através de receptores hipotalâmicos do subtipo Y1 (Cummings, Weigle *et al.*, 2002; Osterstock, Escobar *et al.*, 2010).

Contraditoriamente, indivíduos obesos apresentam baixas concentrações de hormônios orexígenos (Cummings, Weigle *et al.*, 2002). As baixas concentrações de grelina e NPY também são observadas em portadores da DA (Silva, Xapelli *et al.*, 2005; Proto, Romualdi *et al.*, 2006; Adam e Epel, 2007).

Neste contexto, investigamos o papel protetor do pré-tratamento com os hormônios grelina e NPY em um modelo experimental da DA consistindo na administração central do peptídeo A β ₁₋₄₀, previamente padronizado em nosso laboratório (Medeiros, Prediger *et al.*, 2007; Prediger, Franco *et al.*, 2007; Prediger, Medeiros *et al.*, 2008)

Existem diversos mecanismos propostos para a neurotoxicidade induzida pelo peptídeo A β , incluindo neuroinflamação (Medeiros, Prediger *et al.*, 2007; Verri, Pastoris *et al.*, 2012), estresse oxidativo (Matos, Augusto *et al.*, 2008) e excitotoxicidade glutamatérgica (Snyder, Nong *et al.*, 2005). Foi reportado que a A β pode gerar radicais livres e uma produção excessiva de espécies reativas de oxigênio (EROs) causando dano celular e subsequente morte neuronal, não somente porque EROs oxida componentes vitais da maquinaria celular, mas também porque altera as vias de sinalização do neurônio (Zhao, Long *et al.*, 2012). Nossos resultados demonstram uma significativa peroxidação lipídica gerada por uma única administração de A β ₁₋₄₀ tanto no córtex pré-frontal quanto na região hipocampal dos camundongos e que tanto a grelina quanto o NPY são capazes de prevenir estes danos oxidativos.

Esta diminuição na peroxidação lipídica observada pelo tratamento prévio com a grelina em nossos experimentos pode estar relacionada ao fato de que a grelina diminui a geração de ROS. Relatos na literatura evidenciam que em camundongos tratados com MPTP, usado como um modelo experimental da DP, a grelina é capaz de reduzir as quantidades de EROs através de mecanismos que envolvem a ativação de proteínas desacopladoras mitocondriais (UCP-2) suprimindo

a produção destas espécies reativas e mantendo estável o potencial de membrana mitocondrial (Andrews, Erion *et al.*, 2009). Este aumento na atividade da UCP-2 pela grelina também foi observado no modelo de isquemia e reperfusão, aonde o hormônio reduziu os níveis de EROs e preveniu a morte de neurônios da região CA1 do hipocampo (Liu, Wang *et al.*, 2006). Estas mesmas observações também foram visualizadas em estudos *in vitro*, aonde a grelina foi capaz de reduzir o estresse oxidativo e a apoptose, em cultura de células H9c2 tratadas com peróxido de hidrogênio (Zhang, Huang *et al.*, 2011).

A prevenção da peroxidação lipídica encontrada em nossos trabalhos pela administração de NPY é o primeiro relato demonstrando o efeito protetor deste peptídeo frente ao estresse oxidativo gerado pela A β . A pesquisa realizada por Carpio e colaboradores também demonstrou que a administração de NPY em tilápias, apresenta uma ação antioxidante (Carpio, Acosta *et al.*, 2006). Apesar de não termos encontrado outros trabalhos demonstrando os efeitos protetores do NPY sobre a lipoperoxidação, nossos resultados são consistentes em demonstrar sua ação antioxidante.

Além da peroxidação lipídica, em nosso experimento foi observado que os sistemas enzimáticos de defesa antioxidante GR e CAT, a atividade das enzimas encontravam-se significativamente reduzidos pela administração A β_{1-40} e que a grelina demonstra um potencial antioxidante prevenindo os danos observados pela ação do peptídeo A β_{1-40} . Estes achados corroboram com as pesquisas realizadas por Liu e colaboradores que observaram que o tratamento prévio com a grelina em cultura de células dopaminérgicas H9c2 é capaz de prevenir a redução nos níveis de Cu/Zn-SOD e CAT induzida pelo tratamento com 1-metil-4-fenilpiridínio (MPP+) (Liu, Xu *et al.*, 2010).

Sabendo que o SNC é altamente vulnerável aos ataques oxidantes devido ao alto consumo de oxigênio, abundante conteúdo lipídico e quantidades de ferro, nossos resultados demonstram que A β_{1-40} induz a geração de estresse oxidativo bem como a diminuição das defesas enzimáticas no SNC, e que a grelina demonstrou um papel protetor quando administrada previamente a A β_{1-40} . Estas ações comprovam sua capacidade antioxidante e estes achados demonstram pela primeira vez que no modelo experimental da DA, a grelina foi capaz de suprimir a peroxidação lipídica, mantendo inalterados os sistemas de defesa enzimáticos da célula.

As pesquisas científicas realizadas com o objetivo de buscar avanços no tratamento farmacológico da DA obtiveram importante progresso com a caracterização das alterações neuropatológicas da DA

no início dos anos 1980. Os estudos revelaram que nos pacientes com DA existe uma grande deficiência colinérgica devido a dois fatores principais: a perda de neurônios colinérgicos e o aumento significativo na atividade da enzima AChE, associado à presença das placas amiloides (Dinamarca, Sagal *et al.*, 2010). Esta hipótese colinérgica da DA permitiu o desenvolvimento de inibidores da AChE. Desta maneira, os inibidores da AChE como a tacrina, donepezil, rivastigmina e galantamina, tornaram-se as primeiras drogas aprovadas pelo FDA e amplamente prescritas no tratamento da DA (Francis, Palmer *et al.*, 1999). Nossos resultados confirmaram que a A β ₁₋₄₀ aumenta significativamente a atividade da AChE no córtex pré-frontal de camundongos e que o tratamento prévio com grelina preveniu este efeito. Esta observação ficou restrita a região cortical (visto que alterações hipocâmpais não foram observadas) provavelmente porque os neurônios colinérgicos originam-se do núcleo basal e são projetados para o córtex cerebral (Hut e Van Der Zee, 2011).

A excitotoxicidade glutamatérgica contribui para a neurodegeneração induzida pela A β e o aumento de glutamato combinado com a presença de altas concentrações de A β pode ocasionar inúmeras respostas de inibição desse sistema glutamatérgico (Lauderback, Hackett *et al.*, 2001). Para prevenir a neurotoxicidade glutamatérgica, através de transportadores presentes na membrana plasmática, dá-se a remoção de glutamato do espaço extracelular via transportadores presentes na membrana plasmática de astrócitos e neurônios. Estes transportadores são capazes de encerrar a transmissão glutamatérgica e manter baixas as concentrações de glutamato na fenda sináptica (Perry, Gibson *et al.*, 1977; De Boni e McLachlan, 1985; Lauderback, Hackett *et al.*, 2001).

Os dados obtidos neste estudo confirmam os achados prévios demonstrando que o peptídeo A β promove uma diminuição significativa na captação de glutamato no hipocampo, estes resultados provavelmente são uma consequência da redução dos níveis e da atividade dos transportadores de glutamato (GLT-1 e GLAST) ocasionados pela presença da A β (Matos, Augusto *et al.*, 2008; Piermartiri, Figueiredo *et al.*, 2010).

É interessante notar que a grelina foi capaz de aumentar de maneira significativa a captação de glutamato no presente modelo, estes resultados sugerem que o bloqueio da excitotoxicidade glutamatérgica pode representar um dos mecanismos pelos quais a grelina previne os prejuízos cognitivos induzidos pelo peptídeo A β ₁₋₄₀. Trabalhos utilizando GHRP-6, um agonista sintético do receptor da grelina,

indicam que este é capaz de prevenir a excitotoxicidade glutamatérgica e consequente morte neuronal em ratos tratados subcutaneamente com glutamato monossódico (1 g/dia/7dias) (Delgado-Rubín De Célix, Chowen *et al.*, 2006). Além disso, foi demonstrado que a grelina pode atenuar a morte de neurônios hipocampais induzida por cainato em camundongos (Lee, Lim *et al.*, 2010). Desta maneira, nossos resultados demonstram de maneira pioneira que a grelina é capaz de modular a captação de glutamato no modelo experimental da $A\beta_{1-40}$.

Apesar de relatos na literatura demonstrarem o envolvimento do NPY inibindo a liberação de glutamato (Silva, Carvalho *et al.*, 2001; Malva, Xapelli *et al.*, 2012), em nossas pesquisas observamos que a administração de NPY não apresenta um efeito protetor sobre a captação de glutamato neste modelo experimental. Observamos que a infusão i.c.v. de $A\beta_{1-40}$ diminui significativamente a captação deste neurotransmissor excitatório em fatias hipocampais e que o NPY não é capaz de alterar este parâmetro de neurotoxicidade (dados não mostrados).

Os transtornos de humor (incluindo a depressão maior) afetam aproximadamente 40% dos pacientes com DA (Arbus, Gardette *et al.*, 2011). Em nossos experimentos observamos que o tratamento prévio com a grelina reduziu significativamente o tempo de imobilidade de camundongos infundidos com o peptídeo $A\beta_{1-40}$ avaliados no teste de suspensão pela cauda. Este efeito do tipo antidepressivo da grelina já havia sido observado em trabalhos utilizando o teste do nado forçado em animais submetidos ao estresse crônico através da restrição calórica, além disso, neste mesmo trabalho, a administração subcutânea de grelina (2 $\mu\text{g/g}$) também confirmou que os animais tratados com o hormônio apresentavam uma redução do comportamento tipo-depressivo quando comparados com animais controle (Lutter, Sakata *et al.*, 2008).

Relatos na literatura demonstram o envolvimento do NPY na modulação de processos emocionais, bem como, na patogênese da depressão (Heilig, 2004). Evidências demonstram que pacientes depressivos apresentam baixas concentrações de NPY no líquido, (Heilig, 2004; Heilig, Zachrisson *et al.*, 2004) sugerindo que os níveis reduzidos deste neuropeptídeo estariam relacionados com os processos depressivos.

Relatos na literatura demonstram que terapias antidepressivas são capazes de elevar as concentrações de NPY no SNC (Pacher e Kecskemeti, 2004). No presente estudo, a administração previa deste peptídeo foi capaz de reduzir o tempo de imobilidade gerado pela

infusão de $A\beta_{1-40}$ no teste de suspensão pela cauda avaliado 10 dias após o tratamento. Nossos resultados vão ao encontro dos estudos realizados com administrações agudas e crônicas de NPY que relatam o perfil antidepressivo induzido pelo NPY (Redrobe, Dumont, Fournier *et al.*, 2002; Ishida, Shirayama *et al.*, 2007; Gelfo, Tirassa *et al.*, 2012).

No teste do labirinto em cruz elevado, observamos que a administração de grelina induz um comportamento do tipo ansiogênico nos animais visualizado pela redução no tempo de permanência nos braços abertos. Nossos resultados são semelhantes àqueles encontrados por Carlini e colaboradores, que demonstraram que a grelina administrada agudamente no ventrículo de ratos desencadeava um comportamento do tipo ansiogênico nos animais (Carlini, Monzón *et al.*, 2002). A infusão com $A\beta_{1-40}$ ou NPY não altera este parâmetro comportamental.

Os resultados do presente estudo demonstram que uma única administração do peptídeo $A\beta_{1-40}$ resultou em prejuízos significativos na memória espacial de camundongos avaliados no teste da realocação de objetos, sendo que a administração prévia da grelina preveniu o declínio cognitivo nos animais.

São escassos os estudos investigando os efeitos da grelina em modelos experimentais da DA. Recentemente, Moon e colaboradores relataram que o tratamento durante 7 dias consecutivos com a grelina (80 $\mu\text{g}/\text{kg}$, i.p.) após a infusão de $A\beta_{1-42}$ (10 μM), foi capaz de atenuar os prejuízos cognitivos apresentados por camundongos no teste do labirinto em Y (Moon, Choi *et al.*, 2011)

Além disso, no presente estudo, o declínio cognitivo induzido pela administração de $A\beta_{1-40}$ também foi prevenido pela infusão prévia do peptídeo NPY. O papel do NPY na DA foi investigado pela primeira vez por Kowall e colaboradores em 1988. Neste relato pioneiro, a análise *post-mortem* do cérebro portadores da DA revelou a existência de uma redução significativa de neurônios com marcação positiva para NPY na região hipocampal (Kowall e Beal, 1988).

Posteriormente, estudos *in vitro* realizados em linhagens de neuroblastoma SH-SY5Y por Croce e colaboradores, demonstraram que a incubação prévia de NPY seguida da incubação com concentrações tóxicas do fragmento $A\beta_{25-35}$, prevenia a morte destas células (Croce, Dinallo *et al.*, 2011). Recentemente, este mesmo grupo apresentou resultados similares de neuroproteção em cultura primária de células corticais, relatando que o NPY é capaz de aumentar a sobrevida celular bem como reestabelecer os níveis de neurotrofinas em culturas incubadas com $A\beta_{25-35}$ (Croce, Ciotti *et al.*, 2012)

Nossos resultados demonstram pela primeira vez que a administração exógena de NPY previamente a infusão do peptídeo $A\beta_{1-40}$, apresenta um efeito protetor neste modelo animal, impedindo o declínio cognitivo ocasionado pelo fragmento tóxico.

Evidências da literatura demonstram que tanto a grelina quanto o NPY apresentam efeitos marcantes sobre a função hipocampal, sendo capazes de estimular a proliferação das células desta região. Estes trabalhos também demonstram que tanto a administração de grelina quanto de NPY melhoram o aprendizado e a memória em roedores (Redrobe, Dumont *et al.*, 1999; Redrobe, Dumont *et al.*, 2004).

Entretanto, apesar da grelina e do NPY estarem intimamente relacionados a uma melhora cognitiva, nossos resultados revelaram que no protocolo experimental utilizado para a realização deste trabalho, estes peptídeos não apresentaram um efeito pró-mnemônico. Esta conclusão foi possível a partir do ensaio realizado no teste de realocação de objeto utilizando-se um intervalo de tempo superior a 240 min entre o treino e o teste. Dados da literatura revelam que no teste de realocação de objeto, as administrações de drogas nootrópicas são capazes de aumentar significativamente o índice de reconhecimento quando utilizado em um intervalo de tempo de 360 min entre o treino e o teste (Assini, Duzzioni *et al.*, 2009). Neste experimento, observamos que não existiu diferença significativa entre os grupos controles e tratados com os neuropeptídeos. Deve ser ressaltado que estes experimentos foram realizados 14 dias após uma única administração de grelina ou NPY. Trabalhos demonstrando o efeito pró-mnemônico destes neuropeptídeos tem sido realizados poucos minutos após a administração aguda ou depois de tratamentos repetidos com os hormônios (Flood, Hernandez *et al.*, 1987; Carlini, Monzón *et al.*, 2002; Carlini, Perez *et al.*, 2010)

Desta maneira, concluímos que os efeitos observados pela administração dos hormônios são o resultado de uma resposta protetora reduzindo os déficits cognitivos induzidos $A\beta_{1-40}$. Portanto, o potencial da grelina e do NPY em prevenir o declínio cognitivo induzido pelo peptídeo $A\beta_{1-40}$ está provavelmente relacionado à prevenção dos mecanismos de neurotoxicidade induzidos pela $A\beta_{1-40}$.

Mais de 90% dos neurônios positivos para NPY apresentam receptores membranares para GHS-R (Osterstock, Escobar *et al.*, 2010). Sendo assim, a grelina pode influenciar na resposta destas células. Além disso, foi relatado que neurônios GHS-R podem apresentar receptores do subtipo Y2 em sua membrana. Desta maneira, para dissociar as respostas oriundas da administração de grelina e NPY, utilizamos o antagonista seletivo do receptor Y2 BIIE0246. Nossos resultados

demonstraram que a presença de receptores Y2 não interfere nas respostas desencadeadas pela grelina.

Estes achados corroboram o estudo realizado por Osterstock e colaboradores que demonstram que o bloqueio de receptores Y2 pelo antagonista BIIE0246, não modificam a atividade de neurônios positivos para GHS-R (Osterstock, Escobar *et al.*, 2010).

Uma elevada densidade de neurônios que expressam Y2 é encontrada no hipocampo (Ishida, Shirayama *et al.*, 2007). Modelos experimentais de isquemia (Ilebekk, Eriksen *et al.*, 2006), epilepsia (Woldbye, Nanobashvili *et al.*, 2005), e de excitotoxicidade glutamatérgica tem demonstrado o envolvimento de receptores Y2 na neuroproteção (Silva, Pinheiro *et al.*, 2003), e acredita-se que estes receptores são relevantes nas respostas de neuroproteção desencadeadas pelo NPY. Desta forma, outro aspecto importante observado neste estudo foi à relação deste subtipo de receptores com a proteção desencadeada pelo neuropeptídeo no modelo experimental de DA. Demonstramos que a o efeito protetor do NPY é resultante da ativação de receptores Y2, uma vez que a infusão de BIIE0246 previamente ao NPY bloqueia as respostas observadas no teste de realocação de objeto.

Diversos estudos têm demonstrado o envolvimento do glutamato e de seus receptores [N-metil-D-aspartato (NMDA), cainato, e ácido-amino-3-hidroxi-5-metil-4-isoxazolepropionico (AMPA)] em funções fisiológicas de desenvolvimento neural e plasticidade sináptica (aprendizado e memória). É sabido que a interação da A β com receptores pós-sinápticos do tipo AMPA e NMDA promove a internalização dos mesmos, induzindo a excitotoxicidade glutamatérgica e abolindo a potenciação de longa duração (LTP), um fenômeno relevante na formação da memória (Snyder, Nong *et al.*, 2005; Jürgensen, Antonio *et al.*, 2011; Rammes, Hasenjäger *et al.*, 2011). Sendo assim, através de uma colaboração com o laboratório do Dr. Rodrigo Cunha (Universidade de Coimbra, Portugal), avaliamos os efeitos da grelina e do peptídeo A β_{1-40} sobre a LTP hipocampal em camundongos.

Observamos que a LTP foi significativamente inibida em fatias incubadas com A β_{1-40} . A inibição da LTP é considerada uma das mais significativas e robustas medidas do efeito agudo de interrupção sináptica induzida pelo A β (Kim, Anwyl *et al.*, 2001). Um achado importante do presente estudo é de que a grelina foi capaz de reestabelecer a capacidade de sustentação da LTP em fatias hipocampais tratadas com o peptídeo A β_{1-40} . Estes resultados revelam um efeito

neuroprotetor frente ações inibitórias da $A\beta_{1-40}$, demonstrando que a grelina tem a capacidade de manter a plasticidade sináptica frente ao desafio com $A\beta$. Estes resultados devem-se, provavelmente, ao fato da existência de receptores para grelina em neurônios hipocâmpais, resultando em uma promoção da formação de espinhas sinápticas na região do *stratum radiatum* de CA1 e aumento no número de sinapses em neurônios piramidais gerando um acréscimo da LTP induzido pela grelina (Diano, Farr *et al.*, 2006; Chen, Xing *et al.*, 2011). No presente estudo observamos que a grelina é capaz de induzir um aumento no *slope* da LTP. Sabendo que plasticidade sináptica está associada ao incremento das funções cerebrais, podemos sugerir que agudamente este efeito nootrópico estaria acontecendo. Diano e colaboradores demonstraram que após a administração aguda de grelina ocorre um incremento cognitivo em testes de memória espacial e sugerem que a suplementação de grelina poderia atenuar o desenvolvimento de doenças neurodegenerativas (Diano, Farr *et al.*, 2006). Nossos resultados demonstraram que uma única administração de grelina previne os déficits gerados pela $A\beta$, mas que a grelina *per se* não promoveu uma melhora da função cognitiva quando avaliada 14 dias após a sua administração no teste de realocação de objetos. Sendo assim, estes resultados sugerem que para um potencial efeito nootrópico da grelina seria necessária a suplementação diária deste homônio.

8. CONCLUSÃO:

O limitado número de terapias existentes para a DA, dentre outros fatores, impulsiona as pesquisas na descoberta de novos e mais eficazes fármacos capazes de atuar o processo neurodegenerativo verificado nesta doença. Embora estudos adicionais sejam necessários para compreensão dos mecanismos protetores exercidos pelos hormônios grelina e NPY na DA, demonstramos no presente estudo que a administração exógena de grelina e NPY no SNC de roedores é capaz de prevenir alterações comportamentais e neuroquímicas induzidas pela infusão i.c.v. de $A\beta_{1-40}$.

Nossos resultados indicam que a grelina é capaz de desempenhar sua ação de protetora através da inibição dos mecanismos de excitotoxicidade e de estresse oxidativos, além de preservar a plasticidade sináptica e a atividade da enzima AChE, prevenindo os déficits cognitivos e comportamentos do tipo depressivo em animais submetidos ao protocolo experimental da DA.

Também demonstramos que o NPY, é capaz de prevenir o prejuízo na memória espacial, comportamentos do tipo-depressivo e estresse oxidativo induzidos pela $A\beta_{1-40}$.

Entretanto, deve ser enfatizado que o tratamento com a grelina não é isento de efeitos colaterais, visto que observamos um efeito ansiogênico nos animais tratados com este hormônio.

Sabendo que é crescente o numero de indivíduos obesos e que nesta patologia às concentrações plasmáticas dos hormônios grelina e NPY estão alteradas, sugerimos que este talvez seja um dos motivos que tornam a obesidade um fator de risco para o aparecimento da DA.

Por fim, o efeito protetor da grelina e do NPY podem levar a uma compreensão de estratégias farmacológicas neuroprotetoras nas doenças neurodegenerativas.

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