UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE - DEPARTAMENTO DE BIOQUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

PAPEL DOS RECEPTORES DO PEPTÍDEO LIBERADOR DE GASTRINA HIPOCAMPAIS NA MEMÓRIA MOTIVADA POR MEDO: POSSÍVEIS IMPLICAÇÕES PARA DOENÇAS DO SISTEMA NERVOSO CENTRAL

Tese de Doutorado

TATIANA LUFT

Orientador:

Prof. Dr. IVAN IZQUIERDO

Co-Orientador:

Prof. Dr. RAFAEL ROESLER

Porto Alegre, 2007.

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Tese de Doutorado apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul (UFRGS) como requisito parcial para obtenção do título de Doutor em Bioquímica.

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Dedico esta tese a todas as pessoas

que, de uma maneira ou outra,

me ajudaram a crescer

pessoal e intelectualmente.

Agradeço a Deus por mais essa conquista.

Só Ele sabe o que faz!

Of science and the human heart

*There is no limi*t

There is no failure here sweetheart

Just when you quit…

***Bono Vox* - U2**

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PARTE I

I. RESUMO

(**TATIANA LUFT,** ***Papel dos receptores do peptídeo liberador de gastrina hipocampais na memória motivada por medo: possíveis implicações para doenças do sistema nervoso central***) – O principal objetivo dos experimentos apresentados nesta tese foi investigar o envolvimento dos receptores do peptídeo liberador de gastrina (GRPRs) hipocampais nos processos de extinção e reconsolidação da memória e em um modelo de amnésia associado à doença de Alzheimer. No **Capítulo I** foram avaliados os efeitos do bloqueio do GRPR na extinção da memória aversiva. Ratos Wistar machos foram treinados na tarefa de esquiva inibitória e retornaram repetidamente ao contexto da sessão de treino sem choque durante três dias seguidos. A infusão no hipocampo dorsal de um antagonista dos GRPRs ou anisomicina, inibidor da síntese protéica, imediatamente após a primeira sessão de teste inibiram a extinção da memória. Estas drogas não tiveram o mesmo desempenho nas sessões subseqüentes quando a primeira sessão da extinção (primeiro dia após o treinamento) foi omitida. Os resultados indicam que os GRPRs estão envolvidos na extinção da memória motivada por medo no hipocampo. No **Capítulo II** foi avaliado o possível papel do GRPR e do receptor de glutamato do tipo *N*-metil-D-aspartato (NMDAR) em processos associados à reconsolidação da memória. Os resultados mostraram que a inativação de GRPR pelo antagonista RC-3095 ou de NMDARs hipocampais pelo antagonista ácido aminofosfonopentanoico (AP5), após a reativação da memória, prejudica temporariamente a retenção. Entretanto, o prejuízo da memória induzido por RC-3095 ou AP5 pós-reativação foi transitório e voltou aos níveis dos ratos-controle em um teste subseqüente 3 dias após o treino. O efeito das drogas se deu apenas após a reativação da memória, e não na ausência da mesma. Estes resultados fornecem a primeira evidência que a inativação de GRPR após a

reativação pode prejudicar a memória. No **Capítulo III** nós investigamos o efeito da ativação dos GRPRs em um modelo de amnésia associado à doença de Alzheimer. Os ratos receberam infusão bilateral de bombesina, agonista GRPR, ou salina 10 min antes do treino na tarefa de esquiva inibitória, e peptídeo β-amilóide (25-35) ou água destilada imediatamente após o treino. A infusão intrahipocampal pós-treino do peptídeo β-amilóide (25-35) induziu um prejuízo significativo na retenção da memória na tarefa de esquiva inibitória. A infusão pré-treino de bombesina previniu o prejuízo da retenção da memória induzido pelo peptídeo β-amilóide (25-35). O resultado indica que os agonistas de GRPR podem previnir os prejuízos da memória causados pelo peptídeo β-amilóide (25-35) no hipocampo.

II. ABSTRACT

(**TATIANA LUFT, *Role of hippocampal gastrin-releasing peptide receptors of aversive memory: possible implications for central nervous system disorders***) – The main purpose of the research presented in this thesis was to evaluate the involvement of hippocampal gastrin-releasing peptide receptors (GRPRs) in extinction and reconsolidation of fear memory, as well as the effects of GRPR activation in a rat model of memory dysfunction associated with Alzheimer's disease (AD). In **Chapter 1**, we evaluated the possible involvement of the GRPR in extinction of memory for aversive training. Male Wistar rats were trained in inhibitory avoidance (IA) conditioning and then returned repeatedly to the training context without shock on a daily basis for 3 days. Infusion of the GRPR antagonist RC-3095 or the protein synthesis inhibitor anisomycin into the CA1 area of the dorsal hippocampus immediately after the first extinction session blocked extinction. These drugs did not affect performance in subsequent sessions when the first extinction session (1 day after training) was omitted. The results provide the first evidence that hippocampal GRPRs are involved in memory extinction. In **Chapter 2**, we evaluated the possible role of hippocampal GRPRs and glutamate N-methyl-D-aspartate receptors (NMDARs) in reconsolidation-like processes. We show that inactivation of hippocampal GRPRs or NMDARs after memory reactivation temporarily disrupts retention of IA memory. Post-retrieval intra-hippocampal infusion of the GRPR antagonist RC-3095 or the NMDAR antagonist aminophosphonopentanoic acid (AP5) produced an impairment of IA performance tested 2 days after training in rats. However, the memory impairment induced by post-retrieval RC-3095 or AP5 was transient and recovered to levels of control rats in a subsequent test 3 days after training. The drug effects were only present after memory reactivation and not in the absence of reactivation. The findings provide the first

evidence that GRPR inactivation after memory retrieval can impair memory. In **Chapter 3**, we verified whether GRPR activation would affect IA memory retention in a rat model of memory dysfunction associated with AD. Rats were given bilateral infusions of the GRPR agonist bombesin or saline 10 min before IA training, and β-amyloid peptide (25-35) or distilled water immediately after training. Posttraining intrahippocampal infusion of β-amyloid peptide (25-35) induced a significant impairment of IA retention. Pretraining infusion of an otherwise ineffective dose of bombesin prevented the β-amyloid peptide (25-35)-induced retention impairment. The result indicates that GRPR agonists can prevent memory impairments elicited by β-amyloid peptide (25-35) in the hippocampus.

III. LISTA DE ABREVIATURAS

AC	Adenilil ciclase
Aβ	Beta-amilóide
BB	Bombesina
Ca^{2+}	Cálcio
DNA	Ácido desoxirribonucléico
EI	Esquiva inibitória
ERK	Proteína quinase regulada por sinal extracelular
GABA	Ácido gama-amino butírico (do inglês *gamma amino butyric acid*)
GRP	Peptídeo liberador de gastrina
GRPR	Receptor do peptídeo liberador de gastrina
MAPK	Proteína quinase ativada por mitógeno
NMB	Neuromedina B
PKC	Proteína quinase C
PLC	Fosfolipase C
RC-3095	Antagonista seletivo de GRPR [D-Tpi6, Leu13 psi (CH2NH)-Leu14] bombesina (6-14)
SNC	Sistema nervoso central
TEPT	Transtorno de estresse pós-traumático

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VI. INTRODUÇÃO

VI.1. Aprendizado e memória

Memória compreende a aquisição, formação, conservação e a evocação de informações. Podemos afirmar que '*somos aquilo que recordamos'*, pois não podemos fazer aquilo que não sabemos como fazer, nem comunicar nada que desconheçamos, isto é, nada que não esteja na nossa memória. O acervo de nossas memórias faz com que cada um de nós seja o que é, cada um um indivíduo, um ser para o qual não existe outro idêntico (Izquierdo, 2002).

A aprendizagem e a memória são propriedades fundamentais do sistema nervoso central, sendo que ambas estão intimamente relacionadas. Os indivíduos apresentam capacidade de adaptação e modificação de seu comportamento quando expostos a novas experiências, e a capacidade de aprender e recordar eventos depende de modificações induzidas no sistema nervoso pela percepção desses eventos (Ramon Y Cajal, 1911).

Sendo um processo dinâmico, a memória pode ser dividida em quatro etapas: aquisição, consolidação, armazenamento e evocação: **(1) Aquisição** da informação através da exposição a uma experiência, seja ela interna ou externa ao indivíduo. Tal processo se produz de forma mais ou menos automática e consta essencialmente da associação de estímulos e respostas entre si. Este processo associativo (ou não) inicial é intenso e se manifesta no fato de ser a memória de uma experiência recém-vivida, que geralmente é fiel e precisa ao estímulo que conduziu sua criação. Entretanto, com o passar do tempo, essa intensidade e claridade poderão sofrer um decréscimo

(Cammarota, 1998). Não recordamos tudo o que nos sucede e do que recordamos não possuímos todos os detalhes; só guardamos aquilo que, por determinadas circunstâncias, individuais e do contexto, parecem ser determinantes (necessárias e suficientes) para nos capacitarmos a recordar. **(2)** Esse processo de filtração e fixação progressiva da informação adquirida recebe o nome de **consolidação**, fase em que a informação é adquirida e processada. Esta é a fase do processamento da memória que a mesma se mostra mais lábil e mais suscetível a modificações (McGaugh, 2000). **(3)** Uma vez consolidadas, as memórias devem ser "guardadas" em algum lugar do cérebro, no qual sua preservação como tal permaneceria de maneira mais ou menos estável com o passar do tempo, ou seja, ocorre o **armazenamento** da informação (Izquierdo, 1989; McGaugh, 1996, 2000). Onde se armazenam no cérebro as informações já consolidadas, se existe um só lugar de "depósito de memórias", se não existe um lugar fixo mas as memórias se mantêm devido a novas interações neuronais que determinam mudanças na dinâmica comunicacional entre distintas estruturas cerebrais, são questões que ainda não possuem respostas definitivas mas, independente do lugar em que se conservam as memórias, indiscutivelmente certo é que estas só nos servem se podemos resgatá-las (Izquierdo, 2002). **(4)** A única maneira de estudar e avaliar o armazenamento da memória é através da **evocação** desta, quando observamos a mudança de comportamento do animal devido ao processo de memorização (Izquierdo et al., 1998, 2000; Vianna, 2000).

A conseqüência dos três primeiros processos envolvidos na memória seria uma aprendizagem que se manifesta por um novo comportamento ou a modificação de um pré-existente. Entretanto, a maioria dos estudiosos restringe o processo de aprendizagem somente à aquisição de novos conhecimentos, enquanto que a memória seria a retenção dos mesmos (Izquierdo et al., 1992; Kandel & Squire, 2000; Morgado,

1999). Por definição, não há aprendizagem sem memória e nem memória sem aprendizagem, pois ambos os processos encontram-se intimamente ligados e estão presentes em muitos processos cerebrais, como, por exemplo, o reconhecimento da percepção sensorial.

O aprendizado é quantificado experimentalmente como a probabilidade com que um organismo responderá, diferentemente, ao mesmo estímulo após sua repetição. Esta alteração está baseada na memória daquilo que foi aprendido pelo organismo após uma sessão de treino, que é sua exposição a uma novidade ou a um novo acontecimento (Agranoff, 1998). Devido à dificuldade (e muitas controvérsias) em se definir o que vem a ser literalmente aprendizagem, tem-se optado por um termo mais geral que é a plasticidade.

VI.1.1. Plasticidade sináptica e memória

As alterações observadas no processo de aprendizagem e memória ocorrem devido à plasticidade neural, fenômeno característico do sistema nervoso central (Ramón Y Cajal, 1911). O conceito de plasticidade é extremamente amplo, incluindo todas as formas de reorganização duradoura que ocorrem em um cérebro maduro. Essas reorganizações podem ser observadas sob o aspecto fisiológico (propriedades funcionais adquiridas pelos neurônios), morfológico (morfologia e ultraestrutura neuronal e glial) ou bioquímico (atividades enzimáticas, transdução de sinal e mudanças na expressão gênica). Refere-se a alterações estruturais e funcionais nas sinapses como resultado de processos adaptativos do organismo. Estas adaptações promovem alterações na eficiência sináptica e podem aumentar ou diminuir a

transmissão de impulsos com a conseqüente modulação do comportamento (Au Lois et al., 1997; McMahon & Barrionuevo, 2002).

O cérebro tem a extraordinária capacidade de desenvolver respostas plásticas durante longos períodos, podendo durar por toda a vida, sendo que a plasticidade funcional está acoplada a mudanças estruturais de longa duração (Au Lois et al., 1997). Estudos demonstraram que o SNC pode exibir plasticidade sináptica sutil e específica em resposta a uma dada atividade, como por exemplo, o aprendizado de uma nova tarefa (Cotman, 1998).

VI.1.2. Extinção ou inibição de memórias

Em paralelo à importância de formar e manter memórias associadas a situações aversivas, a inibição de respostas de medo apreendidas quando estas não são mais relevantes também é crucial. Dificuldades neste processo de inibição ou medo exagerado representam a base de desordens psiquiátricas relacionadas ao medo e à ansiedade, como fobias, pânico, ansiedade generalizada e estresse pós-traumático, patologias com repercussões sociais cada vez mais prevalentes (Jeffrey & Jay, 1998; Quirk & Gehler, 2003; Myers & Davis, 2002).

Paradigmas experimentais para a inibição dos comportamentos motivados pelo medo são conhecidos desde Pavlov (1927) e, embora suas bases neurais ainda não estejam adequadamente caracterizadas, seus princípios comportamentais são empregados no tratamento psiquiátrico em humanos.

O renovado interesse pelas bases biológicas do processo de extinção de memórias aversivas tem sido guiado pelo aumento da prevalência de desordens

relacionadas com medo e ansiedade na população mundial, e pela busca de mecanismos biológicos que constituam substratos para o tratamento efetivo destes transtornos (Myers & Davis, 2002).

Simultaneamente à descrição da mudança comportamental pelo condicionamento excitatório, Pavlov demonstrou o processo de condicionamento inibitório, ou seja, a inibição de uma memória previamente formada, chamada de inibição aprendida ou extinção. A extinção de um condicionamento se dá pela exposição sucessiva a um dos estímulos anteriormente associados, geralmente o estímulo condicionado, sem a repetição do estímulo incondicionado. Desta forma a evocação da memória previamente formada é seguida de um novo aprendizado, que representa a nova situação à qual o animal foi exposto: a extinção iniciada pela evocação sem reforço comportamental leva à formação de um novo conceito, que prevalece sobre o aprendizado inicial por representar de maneira mais adequada a resposta comportamental apropriada.

Quando descreveu o processo de extinção, Pavlov já sugerira que este constituía um novo aprendizado. Atualmente evidências experimentais de diferentes origens reforçam os preceitos de Pavlov (1927) e Konorski (1948) e sugerem que as memórias formadas para o aprendizado original e para o comportamento resultante do processo de extinção são armazenadas paralelamente e evocadas preferencialmente de acordo com a relação de hierarquia ultimamente estabelecida entre elas (Rescorla, 1988; Pearce & Bouton, 2001; Bouton, 2002).

Por constituir um novo aprendizado, o processo de extinção potencialmente envolve substratos neuroanatômicos, celulares e moleculares similares àqueles inicialmente recrutados para o condicionamento associativo. Demonstrações experimentais sugerem que diferentes tipos de paradigmas de aprendizado envolvem

diferentes estruturas cerebrais para o condicionamento inibitório que envolve o processo de extinção, alguns paralelos e coexistindo com aqueles envolvidos no condicionamento excitatório original (Chan et al., 2001; Dudai, 2003).

VI.1.3. O papel do hipocampo na formação e na extinção da memória

O hipocampo é apontado como estrutura central no processamento de informações contextuais e é crucial para a aquisição, consolidação e evocação de memórias aversivas baseadas no condicionamento associativo (Phillips & LeDoux, 1992; Eichenbaum, 1996, Lorenzini et al., 1996; Izquierdo & Medina, 1997).

O hipocampo processa a informação recentemente adquirida por um período de semanas ou meses e, após, transfere-a a áreas específicas do córtex cerebral para um armazenamento mais prolongado (Baddeley, 1997). Esta estrutura tem conexões com a amígdala e septo medial, córtex entorrinal, córtex pré-frontal e córtex parietal associativo (Hyman et al., 1990). Todas essas áreas são essenciais para a formação das memórias declarativas **(Figura 1).**

O papel central do hipocampo nos processos cognitivos estende-se ao fenômeno de extinção, como foi demonstrado há várias décadas por Douglas (1967) e Kimble (1968), utilizando lesões hipocampais que prejudicavam a inibição de comportamentos aprendidos previamente em roedores e replicadas recentemente por Benoit e colaboradores (1999).

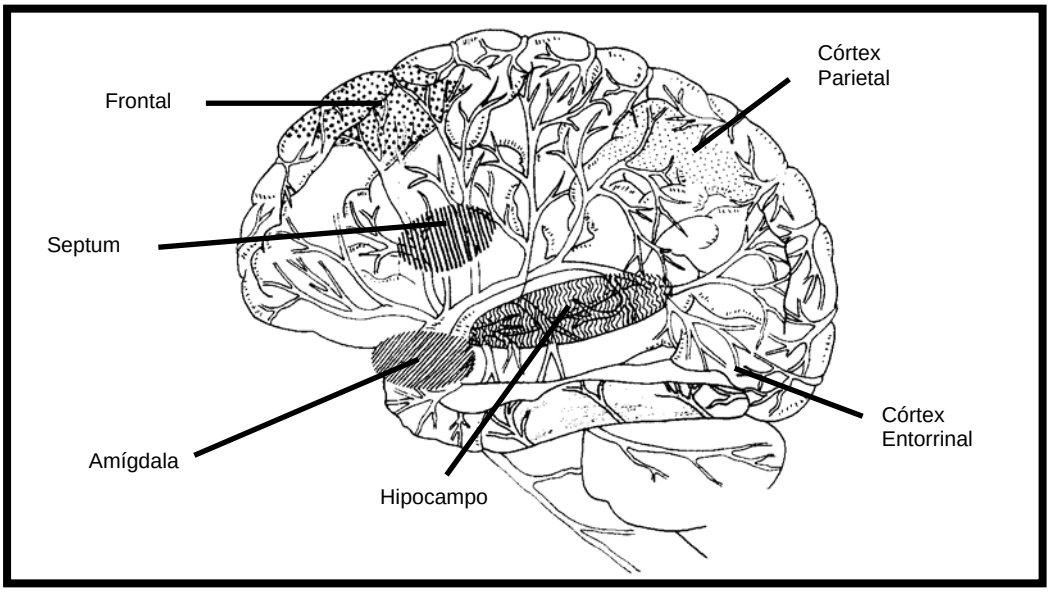

Figura 1. Mapeamento das principais áreas cerebrais envolvidas no processamento das memórias declarativas (Adaptado de Izquierdo, 2002).

VI.1.4. Reconsolidação da memória

A hipótese clássica da consolidação propõe que a memória é inicialmente lábil e gradualmente começa a estabilizar ou consolidar (McGaugh, 1966, 2000). A consolidação da memória pode ser melhorada ou prejudicada pela administração de tratamentos durante a fase lábil pós-treino (McGaugh, 2000; McIntyre et al., 2003). Durante muito tempo acreditou-se que o processo de consolidação acontecia uma única vez para cada traço mnemônico e, após consolidadas as memórias de longa duração, estas se tornavam incapazes de serem modificadas. Porém, nunca existiram provas conclusivas desta hipótese e a muitos resultava óbvio que um processo de armazenamento de informação que operasse dessa maneira não oferecia muitas vantagens adaptativas. Já na década de 1960 Misanin e colaboradores (1968)

apresentaram evidência experimental sugerindo que, como conseqüência de serem reativadas durante a sua expressão, as memórias de longa duração voltavam a ser suscetíveis à ação amnésica de diversos tratamentos, incluindo o eletrochoque e o trauma craniano. (Misanin et al., 1968; Schneider & Sherman, 1968; Dawson & McGaugh, 1969; DeVietti & Holliday, 1972; Lewis, 1979; Mactutus et al., 1979).

Embora a reconsolidação tenha sido definida usando inibidores de síntese protéica (Nader et al., 2000; Taubenfeld et al., 2001; Debiec et al., 2002; Milekic & Alberini, 2002; Suzuki et al., 2004), diversos estudos com outros candidatos envolvidos no processo de reconsolidação foram feitos com um número grande de receptores neuronais e vias de transdução de sinal. Estudos usando injeções sistêmicas ou intracerebrais em roedores têm mostrado que a memória em tarefas motivadas pelo medo pode ser prejudicada pela administração pós-reativação de uma variedade de outros agentes farmacológicos, incluindo antagonistas NMDAR (Suzuki et al., 2004; Lee et al., 2006), antagonistas dos receptores noradrenérgicos (Przybyslawski et al., 1999; Debiec & LeDoux, 2004), benzodiazepinas (Bustos et al., 2006), antagonistas dos receptores de glicocorticóides (Tronel & Alberini, 2007), inibidores de quinase regulada por sinalização extracelular/proteína quinase ativada por mitógeno (ERK/MAPK) (Cestari et al., 2005; Duvarci et al., 2005), inibidores de proteína quinase A (PKA) (Tronson et al., 2006), entre outros.

Os achados que indicam que a memória relacionada ao medo pode ser prejudicada por tratamentos farmacológicos após a reativação têm óbvias implicações clínicas para o desenvolvimento de intervenções terapêuticas para o tratamento de distúrbios psiquiátricos relacionados com o medo e as memórias traumáticas, tais como

transtorno de estresse pós-traumático (Debiec & LeDoux, 2006; Tronel & Alberini, 2007).

VI.2. Peptídeos da família da bombesina

Bombesina (BB) é um peptídeo de 14 aminoácidos inicialmente isolado da pele do sapo *Bombina bombina* (Anastasi et al., 1972). Os estudos feitos por Moody e colaboradores (1978, 2004) mostraram que a bombesina liga-se com afinidade às membranas cerebrais de ratos. O hipocampo possui uma grande densidade de peptídeos da bombesina em locais específicos (Moody et al., 1978).

Descreveu-se também que o principal peptídeo da família da bombesina presente nos mamíferos é o GRP (peptídeo liberador de gastrina), análogo funcional e estrutural da bombesina (McDonald et al., 1979; Cullen et al., 2000) (**Tabela 1**). Os 27 aminoácidos do GRP são sintetizados de um precursor de 148 aa (PreproGRP) no núcleo neuronal e subseqüentemente metabolizado pós-translacionalmente (Spindel et al., 1984; Lebacq-Verheyden et al., 1988; Spinde et al., 1990). Estudos de hibridização *in situ* mostraram a distribuição de GRP no cérebro de rato e encontraram altos níveis de mRNA GRP na área amigdalo-hipocampal, giro denteado e camadas II e III do isocórtex (Wada et al., 1990). Outro peptídeo da família da bombesina que ocorre em cérebros de mamíferos é a neuromedina (NM)B (Moody & Merali, 2004; Minamino et al., 1983, 1984; Battey & Wada, 1991). Um grande número de estudos usando técnicas ligantes ao receptor e seletivos ligantes à bombesina mostraram que GRP e NMB ligam-se a receptores distintos no cérebro do rato. NMB liga com grande afinidade ao receptor NMB, enquanto BB e GRP ligam com grande afinidade ao receptor GRP (BB2, GRPR) (Moody & Merali, 2004; Spindel et al., 1990; Battley & Wada, 1991).

O antagonista seletivo de GRPR [D-Tpi6, Leu13 psi (CH2NH)-Leu14] bombesina (6-14) (RC-3095) foi usado como uma ferramenta farmacológica para investigar os efeitos comportamentais bloqueando GRPR em modelos de roedores. RC-3095 foi desenvolvido por Schally e colegas (1992) como uma droga antitumoral potencial (Pinski et al., 1992; Yano et al., 1992; Qin et al., 1994; Szepeshazi et al., 1997).

Tabela 1. Estrutura da bombesina e do peptídeo liberador de gastrina (GRP).

Bombesina: Pyr-Gln-Arg-Leu-Gly-Asn-Gln-**Trp-Ala-Val-Gly-His-Leu-Met-NH_2**
Peptídeo Liberador de Gastrina: Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-**Trp-Ala-Val-Gly-His-Leu-Met-NH_2**

(Adaptado de Roesler et al., 2006)

VI.3. Receptor do peptídeo liberador de gastrina (GRPR)

VI.3.1. Estrutura molecular e localização de GRPR no cérebro

O GRPR é um membro da família de receptores acoplados a proteínas G que contêm sete domínios transmembrana e 384aa **(Figura 2)**. Estudos usando técnicas *in vitro* indicaram que áreas cerebrais contendo altas densidades de GRPRs incluem o bulbo olfatório, amígdala central, formação dorsal hipocampal (área CA3 e giro denteado) **(Figura 3)**, assim como núcleo talâmico paraventricular, central medial e paracentral (Moody & Merali, 2004; Wolf et al., 1983; Zarbin et al., 1985).

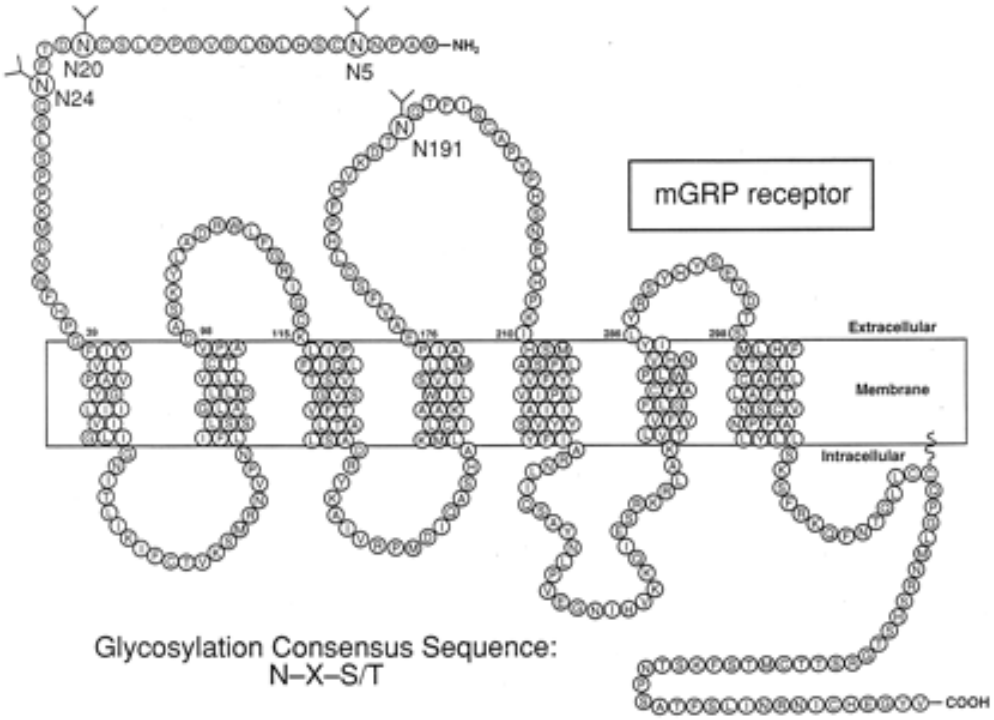

Figura 2. Estrutura molecular do GRPR (Benya et al., 2000).

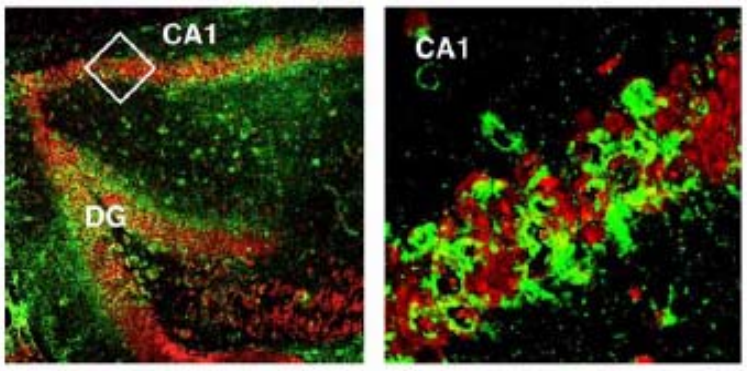

Figura 3. Localização de GRPR no cérebro de camundongos. Depois da marcação do anticorpo para GRPR (em verde) observado, as secções foram marcadas com propidio iodado (em vermelho) para visualizar os núcleos das células. Imunoreatividade específica para GRPR foi observado na região CA1 do hipocampo. Barra de escalas = 200 µm (coluna da esquerda) e 50 µm (coluna da direita). (Adaptado de Kamichi et al., 2005).

VI.3.2. Vias de sinalização celular para o GRPR

As respostas intracelulares à ativação do GRPR em células cancerosas e linhagens celulares neuroendócrinas envolvem cascatas de sinalização de proteínas quinases, particularmente proteína quinase C (PKC) e via de proteína quinase ativada por mitógeno/proteína quinse regulada por sinal extracelular (MAPK/ERK). A ativação de GRPR em células cancerosas duodenais estimulou a resposta de cAMP respondendo à fosforilação de CREB e transativação por uma via dependente de PKC e p38 MAPK (Qu et al., 2002). A despolarização da membrana neuronal induzida por GRP no hipocampo de ratos é bloqueada pelo inibidor de PLC (Lee et al., 1999). Nosso grupo mostrou recentemente que a modulação da função hipocampal de ratos pela bombesina depende de vias de PKC, MAPK e PKA (Roesler et al., 2006) **(Figura 4)**.

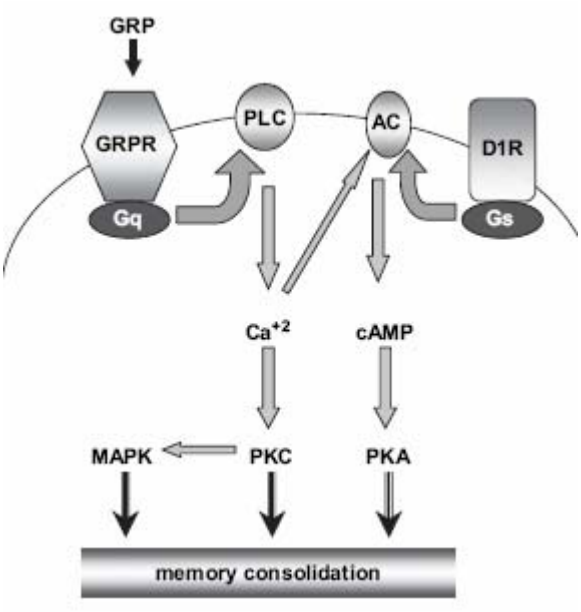

Figura 4. Diagrama esquemático de um modelo de mecanismos de sinalização celular mediando as ações regulatórias do GRPR na consolidação da memória no hipocampo. GRP (peptídeo liberador de gastrina) liberado pelo terminal sináptico liga-se à proteína Gq acoplada ao GRPR no terminal pós-sináptico. Ativação de GRPR induz o aumento de Ca^{2+}, o que leva à ativação das vias da fosfolipase C (PLC)/proteína quinase C (PKC) que, por sua vez, pode ativar MAPK. O receptor dopaminérgico D1R ligado à proteína Gs ativa adenilil ciclase (AC). A indução de cAMP pode ser potencializada sinergicamente pela estimulação de Cálcio, levando a um aumento na ativação da proteína quinase A (PKA). (Adaptado de Roesler et al., 2006).

VI.3.3. Efeitos dos agonistas e antagonistas de GRPR na memória

Estudos farmacológicos prévios investigando o papel dos peptídeos da família da bombesina e GRPRs na memória de aprendizagem avaliaram os efeitos dos agonistas de GRPR no desempenho de roedores em tarefas de memória. Administração sistêmica de bombesina ou GRP melhoram (Flood & Morley, 1988; Rashidy-Pour & Razvani, 1998), enquanto que injeções de antagonistas de GRPR prejudicam (Martins et al., 2005; Presti-Torres et al., 2007; Roesler et al., 2004; Santo-

Yamada et al., 2003) a retenção da memória em ratos e camundongos. Microinfusão de bombesina na região CA1 do hipocampo melhorou a consolidação da memória na esquiva inibitória. Nós investigamos também os mecanismos moleculares mediando o efeito de melhora da memória da administração intrahipocampal de bombesina: a modulação induzida pela bombesina na consolidação da memória foi prevenida pela infusão do antagonista dos receptores GRPR ou por inibidores das vias de sinalização PKC, MAPK e PKA. Estes achados indicam que a bombesina pode facilitar a função cognitiva pela ativação de GRPRs na membrana dos neurônios hipocampais, assim levando à ativação de sinais intracelulares de transdução conhecidos como mediadores na plasticidade sináptica e na formação da memória (Roesler et al., 2006). Consistente com o papel de GRPRs na regulação da plasticidade hipocampal, um estudo eletrofisiológico mostrou que GRP induz despolarização de membrana em neurônios hipocampais de ratos, um efeito que é bloqueado por antagonistas de GRP (Lee et al., 1999).

Inativação de GRPR tanto no hipocampo dorsal quanto na amígdala basolateral por infusão de antagonista seletivo GRPR [D-Tpi^{6}, Leu^{13} psi(CH_2NH)-Leu^{14}] bombesin (6-14) (RC-3095), impede a retenção da memória na tarefa de esquiva inibitória em ratos (Roesler et al., 2003, 2004; Venturella et al., 2005). Estes achados são suportados por evidências genéticas mostrando alteração tanto na formação da memória quanto na plasticidade sináptica em camundongos transgênicos deficientes de GRPR (Shumyatsky et al., 2002). Outros experimentos sugerem que o sistema de sinalização GRPR pode ter interações funcionais com receptores glicocorticóides (Venturella et al., 2005) e neurônios inibitórios liberando GABA (ácido gama-amino butírico) (Dantas et al., 2006) na regulação da formação da memória no hipocampo.

Juntos, os achados dos experimentos que examinam os efeitos dos agonistas ou antagonistas de GRPRs na memória dos roedores indicam que a ativação de GRPR no hipocampo e na amígdala (e possivelmente em outras áreas do cérebro) têm papel importante na modulação de aprendizagem e de memória motivada pela emoção. Embora mais evidências farmacológicas indicaram que a ativação de GRPR pelos agonistas melhoram, assim como antagonistas de GRP prejudicam plasticidade sináptica e memória, um importante estudo feito por Shumyatsky e colegas (2002) mostrou um efeito de antagonista de GRPR melhorando a potenciação de longa duração (LTP). Embora estudos adicionais sejam necessários para esclarecer o papel exato do GRPR na regulação da memória, atualmente há fortes evidências que coloca o GRPR entre diversos sistemas de receptores neuronais que participam da modulação da memória.

Evidências indicam que GRPRs podem ter um papel em doenças do SNC, incluindo disfunção de memória associada com a doença de Alzheimer e outras doenças neurodegenerativas. Assim, nosso grupo tem sugerido o GRPR como um novo alvo terapêutico para o desenvolvimento de terapias para tratar doenças neurológicas e psiquiátricas (Roesler et al., 2004, 2006).

VI.3.4. O GRPR como novo alvo terapêutico em transtornos neurológicos e psiquiátricos

Somados aos esforços para a compreensão do funcionamento do sistema nervoso central, as bases biológicas das memórias têm sido objeto de interesse também por sua relevância clínica. Déficits cognitivos acompanham patologias psiquiátricas e neurológicas fazendo a caracterização dos mecanismos biológicos

responsáveis pelo aprendizado e memória cruciais para a compreensão e tratamento destas doenças, cada vez mais prevalentes na população mundial.

Embora um papel conclusivo da disfunção de GRPR em doenças do SNC ainda não esteja estabelecido, alterações nos níveis de bombesina ou na função de GRPR são observadas em pacientes com doenças psiquiátricas e neurodegenerativas. Pacientes com doença do SNC mostraram alterações no número de neuropeptídeos (Gerner & Yamada, 1982; Bissette et al., 1985; Gerner et al., 1985; Nemeroff et al., 1989); entretanto, o significado dessas alterações para a patofisiologia do SNC ainda não é claro. É possível que uma redução nos níveis de peptídeos da bombesina em pacientes com a Doença de Parkinson (Bissete et al., 1985) ou com esquizofrenia (Gerner et al., 1985; Olincy et al., 1999) modifique sua atividade regulatória no SNC, assim contribuindo para manifestações clinicamente significantes. Alterações nos níveis de GRP no SNC humano também são observadas em pacientes com anorexia, bulimia nervosa e distúrbios alimentares (Merali et al., 1999; Frank et al., 2001).

O receptor de GRPR foi proposto por muitos anos como um alvo importante para o desenvolvimento de novas drogas anticâncer. Peptídeos da família da bombesina estimulam a proliferação celular e atuam como fatores de crescimento na progressão de diversos tipos de câncer humano pela estimulação de GRPR (Bologna et al., 1985; Moody & Cuttitta, 1993; Wang et al., 1996; Pansky et al., 2000; Kim et al., 2002), e tanto GRP e GRPR estão superexpressados em diferentes tipos de céulas tumorais (Carroll et al., 2000; Xiao et al., 2001; Matkowskyj et al., 2003; Waters et al., 2003; Fang et al., 2004; Scott et al., 2004). Antagonistas de GRPR vêm sendo desenvolvidos como um potencial agente antitumoral (Pinski et al., 1992; Yano et al., 1992; Qin et al., 1994; Szepeshazi et al., 1997; Chatzistamou et al., 2000, 2001). Embora o GRPR não estivesse diretamente implicado em doenças psiquiátricas e neurológicas, diversas evidências sugerem que GRPR poderiam ser considerados um alvo molecular para o

desenvolvimento de novidades terapêuticas para as doenças do SNC. Pacientes com a doença de Alzheimer podem ter alterações na sinalização celular de Cálcio mediado por GRPR; e modificações no gene GRPR podem estar presentes em pacientes com autismo. O sistema GRPR parece ter uma interação funcional com outros neurotransmissores e sistema de receptores (GABA, dopamina, e receptores glicocorticóides) implicados na patologia da doença de Parkinson, esquizofrenia, e mediando ansiedade e resposta ao estresse. Estudos futuros devem caracterizar melhor o papel neuromodulador dos GRPRs e verificar se drogas atuando nos GRPRs têm propriedades de melhora neuroprotetiva, antipsicótica e cognitiva.

VI.4. Doença de Alzheimer

Aloys Alzheimer, psiquiatra alemão, **(Figura 5)**, foi o primeiro a explorar os estudos histológicos no cérebro humano (Maurer et al., 1997, Maurer & Maurer, 1999, Spielmeyer, 1916). Em uma clínica psiquiátrica em Frankfurt, ele examinou Auguste D., uma mulher de 51 anos de idade com um histórico de quase 5 anos de progressiva perda de memória, alucinações, idéias paranóicas**,** apraxia, problemas com a fala e doenças sociais e comportamentais, que morreu no dia 8 de abril de 1906 **(Figura 6)**. Em seu cérebro, juntamente com várias atrofias, ele detectou emaranhados neurofibrilares em células nervosas e placas senis em todo o córtex cerebral (Graeber & Mehraein, 1999; Maurer & Maurer, 1999, 2003). Ele demonstrou essa “peculiar doença do córtex cerebral” na 37ª Reunião de psiquiatras alemães em Tübingen em Novembro de 1906. Sua rápida descrição (Alzheimer, 1907) foi apresentada com o nome de “Doença de Alzheimer” (Jellinger, 2006).

Muitos pesquisadores começaram a estudar a doença de Alzheimer, e enfatizaram que a doença seria idade-dependente (Malamud & Lowenberg, 1929). A nova era na pesquisa da demência foi iniciada nos anos 60 do século 20, com o avanço de técnicas de pesquisa como microscópio eletrônico, métodos quantitativos e histoquímicos, histológicos e bioquímicos.

Cem anos depois da primeira descrição, pelo psiquiatra alemão Aloys Alzheimer, a doença que leva seu nome hoje é a doença neurodegenerativa mais freqüente no mundo e em mais de 65% dos casos leva a prejuízos severos e, finalmente, à morte. É considerada a doença do século 21 (Jellinger, 2006).

O risco de uma pessoa com idade entre 65 e 100 anos ter a doença de Alzheimer é 33% para homem e 45% para mulher, com um aumento anual de 1-2% na sétima década para e 4% na oitava década. (van der Flier & Scheltens, 2005).

Figura 5. Aloys Alzheimer (1864-1915) (Jellinger, 2006).

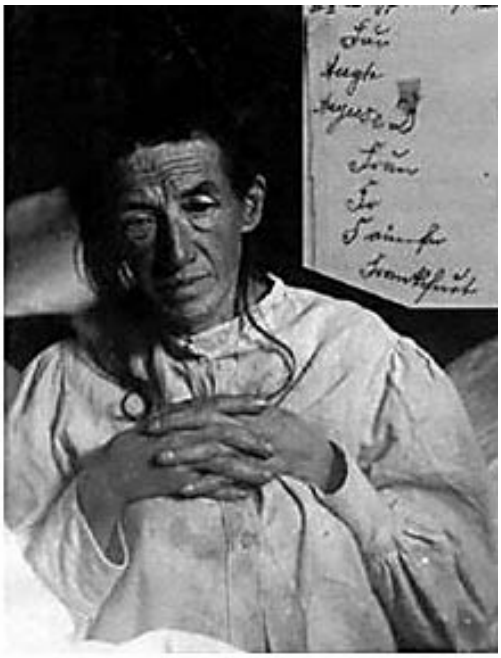

Figura 6. Fotografia de Auguste D. em 1902 com um exemplo de sua escrita. Ela esqueceu seu nome enquanto estava escrevendo. (Jellinger, 2006).

VI.4.1. Doença de Alzheimer e memória

A doença de Alzheimer é caracterizada tanto por modificações intracelulares (por exemplo, emaranhados neurofibrilares) quanto por modificações extracelulares, os depósitos de placas de β-amilóide (Aβ). É universalmente aceito que o acúmulo aberrante de Aβ se dá no início do desenvolvimento desta patologia e é crucial para o desenvolvimento da doença. O acúmulo gradual de Aβ inicia uma cascata de eventos complexos, que inclui gliose, mudanças inflamatórias, modificações nas sinapses e perda de transmissão sináptica (Tseng et al., 2004).

VI.4.2. Doença de Alzheimer e GRPR

Estudos em animais e humanos indicaram que anormalidades na sinalização celular estimulada pela bombesina e GRPRs podem estar associadas com a doença de Alzheimer. Desregulação na sinalização do cálcio está implicada tanto no cérebro idoso normal quanto na doença de Alzheimer. Exagerada liberação de cálcio intracelular induzida pela bombesina foi demonstrado em fibroblastos e neurônios de camundongos geneticamente modificados com uma mutação na presenilina 1 (PS-1) (Leissring et al., 2000). Estes camundongos transgênicos foram desenvolvidos para serem usados como modelo animal com mutações no gene PS-1 (presenilina-1) no cromossomo 14, os quais estão ligados em muitos casos nos primeiros estágios da doença de Alzheimer (Leissring et al., 2000; Guo et al., 1999). As alterações no aumento da sinalização de cálcio induzidas pela bombesina observada neste modelo de camundongo parece-se com os descritos em pacientes com a doença de Alzheimer. Assim, fibroblastos em pacientes com doença de Alzheimer familial e não-familial mostraram aumento na sinalização de cálcio induzido por bombesina se comparados

aos pacientes controles (Gibson & Huang, 2005; Gibson et al., 1996, 1997; Huang et al., 2005; Ito et al., 1994). Essa anormalidade na homeostase do cálcio regulada pela bombesina observada em fibroblastos na doença de Alzheimer têm sido proposto estar envolvida em alterações no estresse oxidativo (Gibson & Huang, 2005; Gibson et al., 1996, 1997; Huang et al., 2004, 2005). Juntos, esses achados em estudos com camundongos e humanos sugerem que a sinalização estimulada pela bombesina e a via de GRPR podem ter um papel importante na desregulação da homeostase do cálcio e no estresse oxidativo associado com disfunções neurodegenerativas e cognitivas em pacientes com a doença de Alzheimer.

Outras modificações celulares descritas em fibroblastos de pacientes com a doença de Alzheimer é a redução no número de receptores para bombesina (Ito et al., 1994). Estes achados interessantes aumentam a possibilidade que a diminuição neuronal da densidade de GRPR, levando a um prejuízo na função de GRP no cérebro de pacientes com a doença de Alzheimer, está relacionado à neurodegeneração e à perda de memória associada com esta doença.

VI.5. Transtorno de estresse pós-traumático (TEPT)

O risco de exposição a trauma tem feito parte da condição humana desde a evolução como espécie. Ataques de tigres de dentes de sabre ou de terroristas do século vinte provavelmente produziram seqüelas psicológicas semelhantes nos sobreviventes de tal violência.

O conceito do transtorno de estresse pós-traumático foi desenvolvido a partir de 1980, nas classificações internacionais (CID.10 e DSM.IV), que permitiu unificar uma série de categorias de transtornos emocionais reativos a acontecimentos traumáticos

anteriormente dispersos na classificação psiquiátrica. Em sua formulação inicial no DSM-III, um evento traumático foi conceitualizado como *estressor catastrófico fora do alcance da experiência habitual humana.* Os elaboradores do diagnóstico original de transtorno do estresse pós-traumático tinham em mente eventos tais como guerra, tortura, estupro, o holocausto nazista, o bombardeio atômico de Hiroshima e Nagasaki, catástrofes naturais (como terremotos, furacões e erupções vulcânicas), bem como catástrofes provocadas pelo homem (como explosões em indústrias, acidentes aéreos e acidentes com automóveis).

Estima-se que a prevalência do TEPT na população geral ao longo da vida é aproximadamente de 1 a 4% (Helzer, 1987; Kessler, 1995). Reinherz (1993), observou uma prevalência do 6,3% entre adolescentes, sem que tal prevalência se modificasse com o nível socioeconômico, uma vez que era similar tanto nas classes mais diferenciadas, quanto nas menos favorecidas.

Acredita-se que o transtorno de estresse pós-traumático envolva mecanismos moleculares, celulares e anatômicos similares aos implicados ao condicionamento ao medo (Elzinga & Bremner 2002; Rasmusson & Charney 1997; Rau et al., 2005; Shalev et al., 1992). Como não é possível reproduzir precisamente TEPT em modelos animais, o condicionamento ao medo em roedores pode ser usado para replicar e elucidar alguns aspectos da TEPT, incluindo o processamento do estímulo ao medo e o processamento da memória emocional (Miller & McEwen, 2006; Siegmund & Wotjak, 2006).

O condicionamento ao medo é induzido em animais de laboratório pela correlação contextual (estímulo condicionado) com o estímulo que induz o medo (estímulo incondicionado). Depois disso, a apresentação do contexto em que o condicionamento

foi realizado evoca uma resposta estereotipada no animal que inclui resposta ao medo, resposta a glicocorticóides e ativação do sistema nervoso autônomo. Similar aos animais condicionados ao medo, pacientes com TEPT manifestam anormalidades no sistema glicocorticóide (Yehuda, 2001), aumento na resposta ao susto (Grillon et al., 1996), e aumento na resposta do sistema nervoso autônomo (Orr et al., 2002; Yehuda, 2001).

IV.6. Tarefa comportamental

IV.6.1. Esquiva inibitória

Ivan Pavlov, em seu tratado "*Conditioned reflexes: an integrated investigation of the physiological activity of the cerebral cortex"* (1927), estabeleceu os preceitos do condicionamento associativo, também denominado condicionamento clássico ou Pavloviano. Baseado na associação de um estímulo condicionado, inicialmente sem significado evidente, ao um estímulo incondicionado, que elicita uma resposta comportamental característica, o condicionamento Pavloviano constitui o fundamento dos paradigmas comportamentais utilizados atualmente para o estudo das bases biológicas das memórias. Entre os modelos utilizados está a tarefa de esquiva inibitória (**Figura 7**) – utilizada como paradigma comportamental para os estudos compilados na presente tese, na qual o animal aprende a associar o contexto do aparato, inicialmente não aversivo, ao recebimento de um choque elétrico. A utilização de estímulos aversivos como estímulo incondicionado têm permitido, ao longo dos anos, a caracterização dos eventos cerebrais envolvidos em aprendizados de medo, contribuindo para a elucidação dos mecanismos biológicos do medo e da ansiedade e

suas repercussões comportamentais (LeDoux, 1996, 1998, Cahill & McGaugh, 1998) e ainda sendo a base para terapias utilizadas no tratamento de desordens psiquiátricas relacionadas ao medo (Fyer, 1998, Lang et al., 1998, Coplan & Lydiard, 1998).

A tarefa de esquiva inibitória envolve o aprendizado de uma tarefa aversiva onde, na sessão de treino, o animal recebe um choque de baixa intensidade ao descer da plataforma. Na sessão de teste, que pode ocorrer em vários tempos pós-treino, o animal é exposto novamente àquele ambiente, testando-se então sua memória. Para avaliar o quanto o animal aprendeu, mede-se o tempo de latência em que este permanece na plataforma e, conseqüentemente, a retenção da tarefa. Trata-se de um aprendizado adquirido em uma única tentativa, tornando-o ideal para o estudo de processos iniciados no treino (Izquierdo & Medina, 1997).

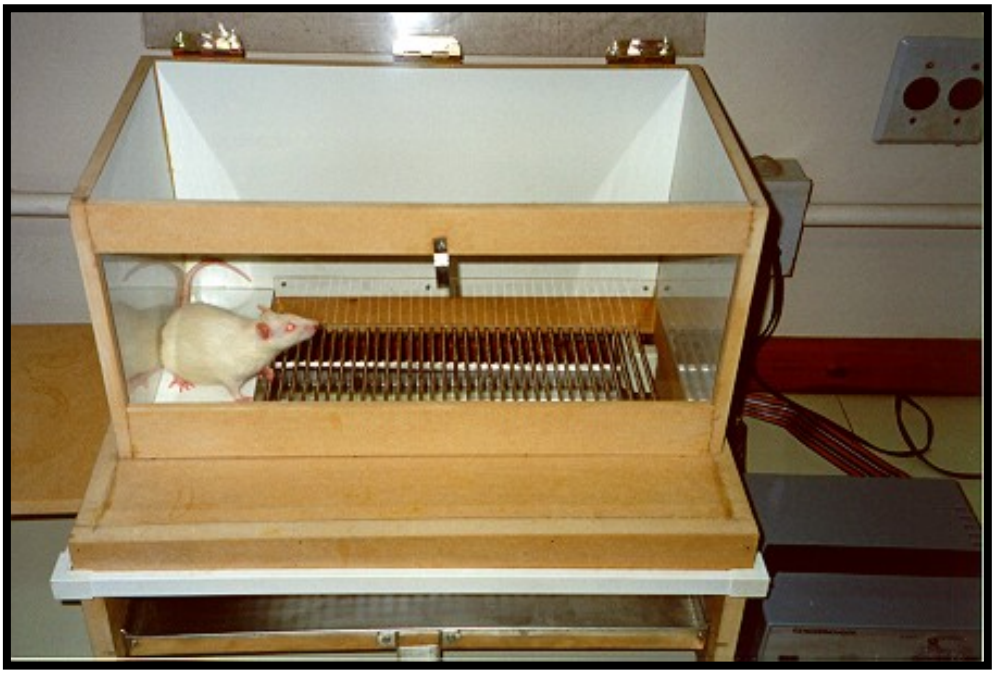

Figura 7. Aparato de esquiva inibitória.

VII. OBJETIVO GERAL

Os trabalhos compilados nesta tese dedicaram-se ao objetivo de investigar o papel dos receptores do peptídeo liberador de gastrina (GRPR) na consolidação, na extinção e na reconsolidação da memória aversiva em ratos. Para isso, utilizamos uma abordagem farmacológica associada ao paradigma comportamental de esquiva inibitória. Além disso, investigamos também o papel dos receptores do peptídeo liberador de gastrina (GRPR) em um modelo de amnésia associado à doença de Alzheimer.

PARTE II

CAPÍTULO I

Papel dos receptores do peptídeo liberador de gastrina (GRPR) na extinção da memória aversiva

A role for hippocampal gastrin-releasing peptide receptors in extinction of aversive memory

Neuroreport (2006) 17: 935-939

LEARNING AND MEMORY NEUROREPORT

A role for hippocampal gastrin-releasing peptide receptors in extinction of aversive memory

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Although the gastrin-releasing peptide receptor has been implicated in memory consolidation, previous studies have not examined whether it is involved in extinction. Here we show that gastrin-releasing peptide receptor blockade in the hippocampus disrupts extinction of aversive memory. Male rats were trained in inhibitory avoidance conditioning and then returned repeatedly to the training context without shock on a daily basis for 3 days. Infusion of a gastrin-releasing peptide receptor antagonist or the protein synthesis inhibitor anisomycin into the dorsal hippocampus immediately after the first extinction session blocked extinction. These drugs did not affect performance in subsequent sessions when the first extinction session (1 day after training) was omitted. The results indicate that hippocampal gastrin-releasing peptide receptors are involved in memory extinction. *NeuroReport* 17:935–939 © 2006 Lippincott Williams & Wilkins.

Keywords: bombesin-like receptors, extinction, gastrin-releasing peptide receptor, hippocampus, RC-3095

Introduction

Extinction is a process by which a conditioned fear response is reduced. In experimental animals, the extinction procedure involves exposure to repeated nonreinforced presentations of a conditioned stimulus that had been previously paired with an aversive unconditioned stimulus. As deficits in fear memory extinction in humans might contribute to posttraumatic stress disorder, and the experimental extinction procedure is analogous to clinical interventions used in the treatment of patients suffering from fear dysfunction, understanding the cellular mechanisms mediating extinction in animal models is likely to increase the efficacy of therapies for fear and anxiety disorders (for recent reviews, see [1–4]). Rodent studies have shown that the dorsal hippocampus is a brain area critically involved in extinction. Molecular mechanisms underlying extinction of memory for inhibitory avoidance (IA) training and other types of aversively motivated conditioning in the hippocampus include *N*-methyl-D-aspartate (NMDA) glutamate receptor activation, protein synthesis, gene expression, and intracellular signaling triggered by the protein kinase A (PKA), mitogen-activated protein kinase (MAPK), Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII), and phosphatidylinositol 3 kinase (PI3 K) pathways [3,5–12].

The gastrin-releasing peptide receptor (GRPR) has been increasingly implicated in aversively motivated memory [13–19]. The GRPR is member of the bombesin-like peptide receptor subfamily of G-protein coupled receptors that is activated by the amphibian peptide bombesin or its mammalian counterpart gastrin-releasing peptide (GRP) (for a recent review, see [20]). In the brain, GRP is proposed to be released from excitatory neurons in brain areas including the dorsal hippocampus and bind to GRPRs expressed on the postsynaptic membrane [14,21]. We have previously shown that systemic administration of the GRPR antagonist [D-Tpi^6, Leu^{13} ψ(CH_2NH)-Leu^{14}] bombesin (6–14) (RC-3095) impairs aversive memory without affecting nonaversive memory in rats [17]. In addition, posttraining infusions of RC-3095 into either the CA1 area of the dorsal hippocampus or the basolateral amygdala impair consolidation of IA memory in rats, indicating that the GRPR plays a role in consolidation of aversively motivated memory [15,18]. Previous studies, however, have not examined whether the GRPR is involved in memory extinction. The present study investigated the role of GRPRs in extinction by examining whether intrahippocampal infusion of a GRPR antagonist affects extinction of memory for IA in rats.

Methods

Animals

Adult male Wistar rats (220–310 g at time of surgery) from the State Health Research Foundation were housed five to a cage in a temperature-controlled colony room with food and water available *ad libitum*, and maintained on a 12-h light/dark cycle (lights on at 07:00 h). Behavioral procedures were conducted during the light phase of the cycle between 09:00 and 17:00 h. All experimental procedures were carried out in accordance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals (NIH publication No. 80–23 revised 1996). All efforts were made to minimize the number of animals and their suffering.

Surgery

Animals were implanted under thionembutal anesthesia (30 mg/kg, intraperitoneally) with bilateral 9.0-mm guide cannulae aimed 1.0 mm above the CA1 area of the dorsal hippocampus as described in previous studies [5,6,15,19]. Coordinates (anteroposterior, −4.3 mm from bregma, mediolateral, +3.0 mm from bregma, ventral, −1.4 mm from dura) were obtained from the atlas of Paxinos and Watson [22]. Animals were allowed to recover at least 6 days after surgery.

Inhibitory avoidance training and extinction procedures

Training and extinction procedures for IA in rats have been established in previous reports [5–7]. For IA training [5–7,15,17–19], rats were placed on a 2.5-cm-high, 8.0-cm-wide platform at the left of a 50.0 × 25.0 × 25.0-cm yellow acrylic training apparatus, whose floor was a series of parallel 0.2-cm-caliber bronze bars spaced 1.0 cm apart. Latency to step down onto the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, animals received a 0.5-mA, 2.0-s scrambled footshock. In all experiments, rats were given a single training trial. For IA extinction, rats were returned repeatedly to the IA training context without footshock on a daily basis for 3 days as previously described [5]. Rats' latencies to step down from the platform were recorded by an observer blind to drug treatment condition. This procedure has been previously shown to lead to extinction of IA memory without inducing reconsolidation [3,5–7]. In a second experiment (no retrieval controls), the second extinction session was omitted.

Drugs and infusion procedures

Intrahippocampal infusion procedures have been described elsewhere [5–7,15,19]. Briefly, at the time of infusion an infusion needle was fitted into the guide cannula. The tip of the infusion needle protruded 1.0 mm beyond the guide cannula and was aimed at the CA1 area of the dorsal hippocampus. Drugs were infused during a 30-s period. After the infusion of drug or vehicle, the infusion needle was left in place for an additional minute to allow diffusion of the drug away from the needle tip. Rats were given a bilateral 0.8 µl infusion of vehicle [2% dimethylsulfoxide in saline (0.9% NaCl)], the selective GRPR antagonist RC-3095 (0.1, 1.0, or 10.0 µg/side; Zentaris GmbH, Frankfurt, Germany), or the protein synthesis inhibitor anisomycin (80 µg/side; Sigma, St Louis, Missouri, USA) immediately after the first extinction session (first experiment) or 24 h after training in the absence of an extinction session (second experiment, no retrieval controls). Administration of anisomycin to the hippocampus blocks extinction of IA [5] and was used as a positive control. Drug doses were based on previous studies [5,15,19]. Drug solutions were prepared freshly before each experiment.

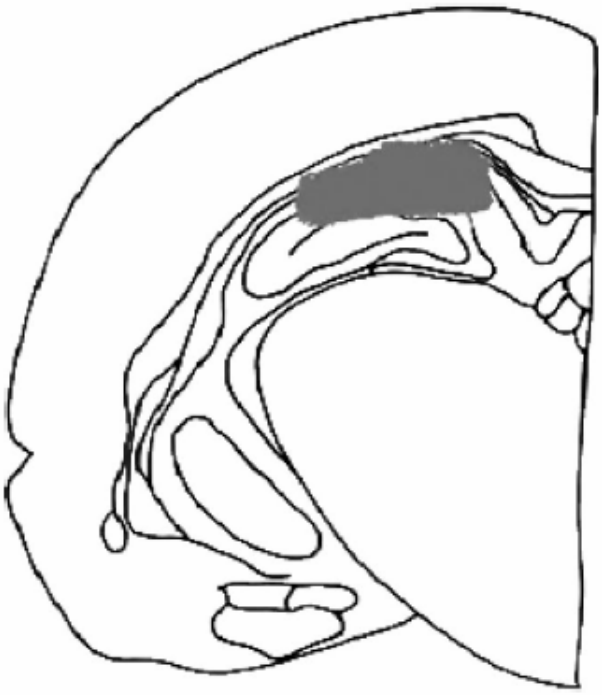

Fig. 1 Drawing of the plane A: −4.3 mm of the atlas of Paxinos and Watson [22] showing the area (hatched) where the infusion sites considered to be correct were placed.

Histology

Twenty-four to 72 h after behavioral testing, the animals were killed by decapitation and their brains were removed, stored in 5% formalin for at least 72 h and verified for infusion site placements in the dorsal hippocampus as described in previous reports [5–7,15,19]. Only data from animals with correct cannula placements (114 animals) were included in the final analysis (Fig. 1 [22]).

Statistics

Data are median (interquartile ranges) retention test latencies to step-down (s) [5–7,15,19]. Comparisons of training and retention test latencies between groups were performed using a Kruskal–Wallis analysis of variance followed by Mann–Whitney *U* tests, two-tailed, when necessary. Comparisons between behavioral sessions within individual groups were done with Wilcoxon tests [15,17–19]. In all comparisons, $P < 0.05$ was considered to indicate statistical significance.

Results

To examine whether hippocampal GRPRs are involved in extinction of aversive memory, the GRPR antagonist RC-3095 was infused into the CA1 area of the rat hippocampus immediately after the first session of extinction of IA

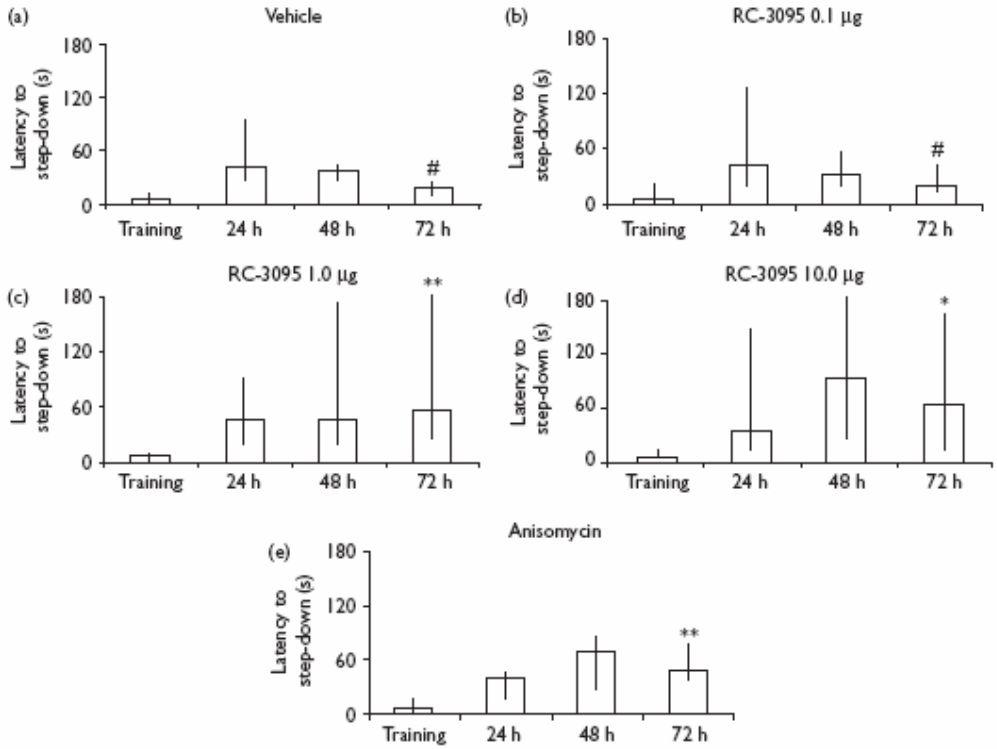

Fig. 2 Administration of a gastrin-releasing peptide receptor (GRPR) antagonist to the hippocampus disrupts extinction of aversive memory. Data are median (interquartile ranges) step-down latencies (s) in inhibitory avoidance (IA) training trial and extinction sessions carried out 24, 48, and 72 h after training. Rats were given bilateral 0.8 µl-infusions of vehicle (VEH), the GRPR antagonist RC-3095 (0.1, 1.0, or 10.0 µg), or the protein synthesis inhibitor anisomycin (80 µg) into the CA1 area of the dorsal hippocampus immediately after the first extinction session. n=10–13 animals per group. *$P<0.05$ and **$P<0.01$ compared with VEH-treated rats; $^{\#}P<0.01$ compared with the first extinction session within the same group.

conditioning. Animals given anisomycin were included as positive controls. Results are shown in Fig. 2. No significant difference was observed among groups in step-down latencies in the training trial (P=0.76). Vehicle-treated rats and rats given RC-3095 at 0.1 µg showed a significant decrease in step-down latency between the first (24 h after training) and the third (72 h after training) extinction sessions (both P values <0.01), indicating that repeated nonreinforced exposure to the IA training context produced significant extinction of IA memory [5–7]. In contrast, there was no significant decrease in latency along extinction sessions in rats given RC-3095 at 1.0 µg (P=0.44) or 10.0 µg (P=0.24) or in rats treated with anisomycin (P=0.21). In addition, rats given an intrahippocampal infusion of anisomycin or RC-3095 at 1.0 or 10.0 µg, but not the group given RC-3095 at 0.1 µg, showed significantly higher latencies in the third extinction session than the vehicle-treated group ($P<0.05$, group given RC-3095 at 10.0 µg versus vehicle; $P<0.01$, group given RC-3095 at 1.0 µg versus vehicle; $P<0.01$, group given anisomycin versus vehicle; P=0.15, group given RC-3095 at 10.0 µg versus vehicle). The results indicate that intrahippocampal administration of RC-3095 at 1.0 or 10.0 µg blocked consolidation of extinction of IA memory. When the first extinction session was omitted, that is, drug infusions were given 24 h after training but rats were not submitted to an extinction session, RC-3095 and anisomycin did not affect performance in subsequent sessions (second experiment, Fig. 3), indicating that the impairing effects of RC-3095 and anisomycin on extinction observed in the first experiment (Fig. 2) require retrieval of IA memory and are not attributable to nonspecific effects of the infusions.

Discussion

Extinction is proposed to form a new memory rather than erasing a previously formed conditioned association. In fact, consolidation of extinction in the hippocampus shares a number of mechanisms with consolidation of new memories. Thus, previous studies have implicated NMDA receptors, protein synthesis, gene expression, PKA, MAPK, CAMKII, and PI3 K in the dorsal hippocampus in extinction of memory for IA and other types of aversive conditioning [3,5–12]. As in most studies drug treatments were infused after animals were given nonreinforced exposure sessions to the training context (i.e., extinction sessions), drugs are likely to have affected the consolidation of memory extinction. Although recent pharmacological [13,15–19] and genetic [14] evidence has implicated the GRPR in regulating consolidation of aversively motivated memory, previous studies have not examined whether the GRPR plays a role in extinction. In the present study, we have found that GRPR antagonism in the hippocampus after nonreinforced exposure to the training context disrupts extinction of IA memory. We have previously shown that the impairing effects of intrahippocampal administration of the GRPR antagonist RC-3095 on memory consolidation are not attributable to neurotoxicity or long-lasting impairment

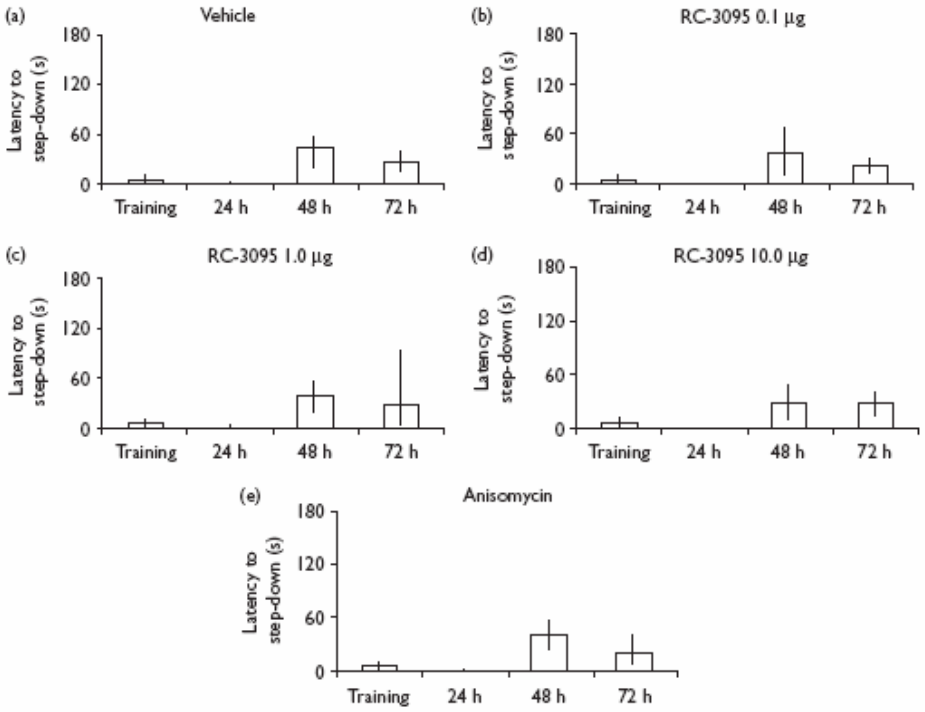

Fig. 3 Administration of a gastrin-releasing peptide receptor (GRPR) antagonist to the hippocampus 24 h after training does not affect subsequent performance when the extinction session is omitted. Data are median (interquartile ranges) step-down latencies (s) in an inhibitory avoidance (IA) training trial and extinction sessions carried out 48, and 72 h after training. Rats were given bilateral 0.8 µl-infusions of vehicle (VEH), the GRPR antagonist RC-3095 (0.1, 1.0, or 10.0 µg), or the protein synthesis inhibitor anisomycin (80 µg) into the CA1 area of the dorsal hippocampus 24 h after IA training. n=8–14 animals per group. No significant differences were observed between groups.

of memory retrieval [15]. In addition, our present observation that RC-3095 given 24 h after training did not affect subsequent performance when the first extinction session was omitted (Fig. 3) indicates that the drug-induced disruption of consolidation required retrieval of the previously learned memory and was not due to long-lasting impairment of neural function. To our knowledge, these findings provide the first evidence that the GRPR system is involved in memory extinction.

The GRPR is a type of bombesin receptor expressed in several mammalian tissues. Within the brain, GRPR is highly expressed in the cell bodies and dendrites of neurons in areas including the dorsal hippocampus and lateral amygdala [23]. Hippocampal GRPRs might be activated by the mammalian bombesin-like peptide GRP released from excitatory neurons [14,21]. GRPR activation might in turn lead to activation of intracellular signaling cascades including the phospholipase C/protein kinase C and MAPK pathways [24]. Thus, the pattern of distribution of the GRPR in the brain, as well as the signaling mechanisms involved in mediating cellular responses to GRPR activation, is consistent with the behavioral data indicating that the GRPR plays an important role in regulating memory formation and extinction. Our present finding that GRPR antagonism impairs extinction, together with previous studies showing that RC-3095 impairs memory consolidation [15,18,19], indicates that GRPR activation plays a stimulatory role in synaptic plasticity and memory formation and extinction. Other studies, however, have proposed that the GRPR is located predominantly on inhibitory interneurons releasing γ-aminobutyric acid (GABA), and GRPR activation would lead to an increase in GABAergic transmission, which would in turn inhibit synaptic plasticity and memory [14,21]. Consistent with this view, bombesin induces depolarization of inhibitory interneurons in hippocampal slices [21] and GRPR-deficient mice show enhanced fear-motivated memory and amygdalar synaptic plasticity [14]. One possibility to reconcile these contrasting findings is that the GRPR is expressed on both inhibitory GABAergic neurons and non-GABAergic neurons releasing glutamate and other neurotransmitters. The recent finding that in the lateral amygdala only a subpopulation of cells expressing GRPRs are GABAergic neurons [23] is consistent with the possibility that GRPRs are expressed on non-GABAergic neurons releasing glutamate or other neurotransmitters. Thus, the impairing effects of RC-3095 on memory consolidation and extinction might be related to an inhibition of GRPRs located on excitatory neurons.

As animal studies aiming to clarify the neural mechanisms underlying extinction might have clinical relevance for the treatment of patients with fear-related disorders [1–4], our finding that the GRPR is involved in extinction supports the view that the GRPR is a therapeutic target for the treatment of psychiatric disorders including anxiety and fear disorders such as posttraumatic stress disorder [25]. Further experiments investigating the intracellular mechanisms mediating GRPR modulation of extinction and examining whether GRPR agonists can accelerate memory

extinction could have clinical implications for the treatment of fear-related disorders.

Conclusion

Using previously established behavioral training and hippocampal cannulation procedures, we have shown that administration of a GRPR antagonist to the CA1 hippocampal area disrupts consolidation of extinction of aversively motivated memory. This is the first evidence of a role for the GRPR in memory extinction. Moreover, the data support the view that the GRPR is a therapeutic target for the treatment of neuropsychiatric disorders.

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CAPÍTULO II

Prejuízo transitório da memória de medo induzido pelo bloqueio, após a reativação, de receptores do peptídeo liberador de gastrina (GRPR) ou dos receptores de N-Metil-D-Aspartato (NMDA) no hipocampo

Transient disruption of fear-related memory by post-retrieval inactivation of gastrin-releasing peptide or N-methyl-D-aspartate receptors in the hippocampus

Artigo submetido à *Neurobiology of Learning and Memory*

* **Manuscript**

Neurobiology of Learning and Memory

Research article

Transient disruption of fear-related memory by post-retrieval inactivation of gastrin-releasing peptide or *N*-methyl-D-aspartate receptors in the hippocampus

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Abstract

Molecular accounts of memory consolidation suggest that new learning generates persistent synaptic modifications through activation of an extensive set of neuronal receptors and intracellular signal transduction pathways, accompanied by RNA and protein synthesis. This traditional cellular consolidation theory has been challenged by evidence that reactivation of a previously consolidated memory might render this memory again susceptible to disruption by amnesic treatments, a process generally referred to as reconsolidation. Current evidence indicates that reconsolidation can be disrupted by administration of a variety of pharmacological agents after memory reactivation. Previous studies have indicated that the gastrin-releasing preferring type of bombesin receptor (GRPR) and the *N*-methyl-D-aspartate glutamate receptor (NMDAR) in the rat hippocampus are involved in consolidation of inhibitory avoidance (IA), a fear-related memory task. We show here that blockade of hippocampal GRPRs or NMDARs after memory reactivation temporarily disrupts memory retention. Post-retrieval intra-hippocampal infusion of the GRPR antagonist RC-3095 or the NMDAR antagonist aminophosphonopentanoic acid (AP5) produced an impairment of IA performance tested 2 days after training in rats. However, this impairment was transient and recovered to levels of control rats in a subsequent test 3 days after training. The drug effects were only present after memory reactivation and not in its absence. These findings provide the first evidence that GRPR inactivation after retrieval can impair memory.

Keywords: Gastrin-releasing peptide receptor; *N*-methyl-D-aspartate receptor; Hippocampus; Reconsolidation; Fear memory

1. Introduction

Memory consolidation is mediated and regulated by activation of an extensive set of neuronal receptors and intracellular signal transduction pathways accompanied by RNA and protein synthesis, resulting in persistent modifications of synaptic architecture and stabilization of the memory trace (reviewed in Dudai & Morris, 2000; McGaugh, 2000; Schaffe, Nader, Blair, & LeDoux, 2001; Izquierdo, Bevilaqua, Rossato, Bonini, Medina, & Cammarota, 2006). This traditional cellular consolidation theory, however, has been challenged by evidence that reactivation of a previously consolidated memory during retrieval might render this memory again susceptible to disruption by amnesic treatments, a process generally referred to as reconsolidation (Nader, Schafe, & LeDoux, 2000a; reviewed in Nader, Schafe, & LeDoux, 2000b; Sara, 2000; Dudai & Eisenberg, 2004; Alberini, 2005; Tronson & Taylor, 2007). Although reconsolidation has been defined using protein synthesis inhibitors (Nader et al. 2000a; Taubenfeld, Milekic, Monti, & Alberini, 2001; Debiec, LeDoux, & Nader, 2002; Milekic & Alberini, 2002; Suzuki, Josselyn, Frankland, Masushige, Silva, & Kida, 2004), several studies have extended the candidate mechanisms involved in reconsolidation-like processes to a number of neuronal receptors and signal transduction pathways. Thus, studies using systemic or intra-cerebral injections in rodents have shown that memory for fear-motivated tasks can be impaired by post-retrieval administration of a variety of pharmacological agents, including *N*-methyl-D-aspartate glutamate receptor (NMDAR) antagonists (Suzuki et al., 2004; Lee, Milton, & Everitt, 2006), a noradrenergic receptor antagonist (Przybyslawski, Roullet, & Sara, 1999; Debiec & LeDoux, 2004), a benzodiazepine (Bustos, Maldonado, & Molina, 2006), a glucocorticoid receptor antagonist (Tronel & Alberini, 2007), extracellular signal-regulated

kinase/mitogen-activated protein kinase (ERK/MAPK) inhibitors (Cestari, Costanzi, Castellano, & Rossi-Arnaud, 2005; Duvarci, Nader, & LeDoux, 2005), a protein kinase A (PKA) inhibitor (Tronson, Wiseman, Olausson, & Taylor, 2006), and a Zif268 antisense oligodeoxynucleotide (Lee, Everitt, & Thomas, 2004). These findings have obvious clinical implications for the development of therapeutic interventions to treat psychiatric disorders associated with fear and traumatic memories, such as post-traumatic stress disorder (PTSD) (Debiec & LeDoux, 2006; Tronel & Alberini, 2007).

Thus far, relatively few studies have examined whether inactivation of specific receptors known to play a role in memory consolidation can also produce memory deficits when induced after memory reactivation. We therefore decided to evaluate the effects of post-retrieval blockade of two types of neuronal receptors known to be involved in memory formation in the hippocampus on retention of a fear-conditioning based task in rats: gastrin-releasing peptide preferring type of bombesin receptor (GRPR) and the NMDAR.

The GRPR has emerged as a receptor importantly involved in regulating formation of memory for fear-related tasks in brain areas including the hippocampus and amygdala (Shumyatsky, Tsvetkov, Malleret, Vronskaya, Hatton, Hampton, Battey, Dulac, Kandel, & Bolshakov, 2002; Roesler, Meller, Kopschina, Souza, Henriques, & Schwartsmann, 2003a; Roesler, Kopschina, Rosa, Henriques, Souza, & Schwartsmann 2004b; Roesler, Lessa, Venturella, Vianna, Luft, Henriques, Izquierdo, & Schwartsmann 2004c; Mountney, Sillberg, Kent, Anisman, & Merali, 2006; Roesler, Luft, Oliveira, Farias, Almeida, Quevedo, Dal Pizzol, Schröder, Izquierdo, & Schwartsmann, 2006b; Bedard, Mountney, Kent, Anisman, & Merali, 2007). A series of studies from our laboratory has shown that post-training systemic, intra-amygdala, or intra-hippocampal infusion of the selective GRPR antagonist [D-Tpi^{6}, Leu^{13} psi(CH_2NH)-Leu^{14}] bombesin (6-14) (RC-3095) impairs

retention of fear-related memory in rats, indicating that GRPR blockade might disrupt fear memory consolidation (Roesler et al., 2003a; 2004c; Dantas, Luft, Henriques, Schwartsmann, & Roesler, 2006). Based on the increasing evidence for the involvement of the GRPR in fear conditioning and anxiety-related behaviors, we and others have put forward the GRPR as a potential molecular target in anxiety and fear-related disorders (Shumyatsky et al., 2002; Moody & Merali, 2004; Roesler, Henriques, & Schwartsmann, 2004a; Roesler et al., 2004c; Roesler, Henriques, & Schwartsmann, 2006a; Bedard et al., 2007).

The GRPR might have functional interactions with glutamatergic neurotransmission in regulating synaptic plasticity and memory. GRP has been proposed to be co-released with glutamate from glutamatergic neurons (Lee, Dixon, Gonzalez, Stevens, McNulty, Oles, Richardson, Pinnock, & Singh, 1999; Shumyatsky et al., 2002). Extensive evidence shows a critical role for glutamate-induced NMDAR activation in mediating formation of several types of memory (reviewed in Castellano, Cestari, & Ciamei, 2001; Riedel, Platt, & Micheau, 2003; Nakazawa, McHugh, Wilson, & Tonegawa, 2004). We have previously shown that post-training intra-hippocampal infusion of the NMDAR antagonist aminophosphonopentanoic acid (AP5) impairs consolidation of fear-related memory (Roesler, Vianna, Sant'Anna, Kuyven, Kruel, Quevedo, & Ferreira, 1998; Roesler, Schroder, Vianna, Quevedo, Bromberg, Kapczinski, & Ferreira, 2003b; Roesler, Reolon, Luft, Martins, Schroder, Vianna, & Quevedo, 2005). However, the effects of post-retrieval administration of NMDAR antagonists on memory are less understood. Systemic injections of NMDAR antagonists after retrieval have been shown to impair memory for fear conditioning (Suzuki et al., 2004; Lee et al., 2006). However, those studies have not examined the effects of NMDAR antagonists infused into specific brain areas. Transient

memory impairment after post-retrieval intra-hippocampal infusion of AP5 was cursorily observed in a previous study on extinction (Szapiro, Vianna, McGaugh, Medina, & Izquierdo, 2003) but to our knowledge no previous study has reported a more detailed assessment of the possible role of the NMDAR in the hippocampus in reconsolidation-like processes.

In the light of the evidence reviewed above, we asked whether inactivation of hippocampal GRPRs and NMDARs after retrieval could affect fear memory retention. In previous studies, we have used a single-trial inhibitory avoidance task (IA) as an experimental model to investigate the role of the GRPR and NMDAR in consolidation of a fear-conditioning based task (Roesler et al., 1998; 2003a; 2003b; 2004b; 2004c; 2005; Dantas et al., 2006; Roesler et al., 2006b). In the present study, we examined the effects of post-retrieval intra-hippocampal infusions of the GRPR antagonist RC-3095 and the NMDAR antagonist AP5 on IA memory in rats.

2. Materials and methods

2.1. Subjects

Adult male Wistar rats (230-307 g at time of surgery) from the State Health Research Foundation (FEPPS-RS, Porto Alegre, Brazil) were housed five to a cage in a temperature-controlled colony room with food and water available *ad libitum*, and maintained on a 12-h light/dark cycle (lights on at 7:00 A.M.). Behavioral procedures were conducted during the light phase of the cycle between 10:00 and 17:00. All experimental procedures were performed in accordance with the NIH Guide for Care and Use of

Laboratory Animals (NIH publication No. 80-23 revised 1996), and the protocols were approved by the institutional Research Ethics Committee. All efforts were made to minimize the number of animals and their suffering.

2.2. Surgery

Rats were implanted under thionembutal anesthesia (30 mg/kg, i.p.) with bilateral 9.0-mm, 23-gauge guide cannulae aimed 1.0 mm above the CA1 area of the dorsal hippocampus as described in previous studies (Roesler et al., 1998; 2003a; 2006b). Coordinates (anteroposterior, –4.3 mm from bregma, mediolateral, ±3.0 mm from bregma, ventral, –1.4 mm from dura) were obtained from the atlas of Paxinos & Watson (1997). Animals were allowed to recover at least 7 days after surgery.

2.3. Inhibitory avoidance

In IA training, animals learn to associate a location in the training apparatus with an aversive stimulus (footshock). The IA apparatus and general training and testing procedures have been described in previous studies (Roesler et al., 1998; 2003a; 2006b). Briefly, the IA apparatus was a 50 X 25 X 25-cm acrylic box (Albarsch, Porto Alegre, Brazil) whose floor consisted of parallel caliber stainless steel bars (1 mm diameter) spaced 1 cm apart. A 7-cm wide, 2.5-cm high platform was placed on the floor of the box against the left wall. On the training trial, rats were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, rats received a 0.8-mA, 2.0-s footshock and were removed from the

apparatus immediately after the footshock. Retention tests were procedurally identical to training, except that no footshock was presented and testing was terminated at 180 s if the rat did not step down. Step-down latencies on the retention tests were used as measures of IA memory retention.

In experiments 1 and 2, memory was tested 24 h after training (test-1d) by placing the rat back on the platform and measuring the latency to step down on the grid. Animals were retested for memory retention at 24 h (test-2d) and 48 h (test-3d) after test-1d. In experiment 3 (no reactivation control), test-1d was omitted (Amaral, Luft, Cammarota, Izquierdo, & Roesler, 2007).

2.4. Drugs and intra-hippocampal infusions

Intra-hippocampal infusion procedures were as described in previous studies (Roesler et al., 1998; 2003a; 2003b). At the time of infusion, a 30-gauge infusion needle was fitted into the guide cannula. The tip of the infusion needle protruded 1.0 mm beyond the guide cannula and was aimed at the CA1 area of the dorsal hippocampus. Drugs were infused during a 30-s period. After the infusion of drug or vehicle, the infusion needle was left in place for an additional minute to allow diffusion of the drug away from the needle tip.

Vehicle (VEH, 2% dimethylsulfoxide (DMSO) in saline; 0.5 μl), RC-3095 (1.0 μg in 0.5 μl; Zentaris GmbH, Frankfurt, Germany), or AP5 (5.0 μg in 0.5 μl; Sigma, St. Louis, MO, USA) dissolved in VEH were infused bilaterally into the hippocampus. Drug doses

were chosen on the basis of previous studies (Roesler et al., 2003a; 2003b). Drug solutions were freshly prepared before each experiment.

Drug or VEH infusions were given immediately after training (experiment 1), immediately after memory reactivation (test-1d; experiment 2), or 24 h after training in the absence of reactivation (experiment 3).

2.5. Histology

Twenty-four to 48 h after behavioral testing, animals were killed by decapitation and their brains were removed, stored in 5% formalin for at least 72 h and verified for infusion site placements as follows: 0.5 μl of a 4% methylene blue solution was infused as described above and the extension of the dye was taken as indicative of diffusion of the drugs previously given to each rat (Roesler et al., 2003a; 2003b; 2005; 2006b). Only data from animals with correct infusion sites were included in the final analysis.

2.6. Statistics

Data are shown as mean ± SEM retention test latencies to step-down (s). Comparisons of training and retention test step-down latencies among groups were performed using Kruskal-Wallis analysis of variance followed by two-tailed Mann-Whitney *U* tests when appropriate (Dantas et al., 2006; Roesler et al., 2006b). Comparisons across behavioral trials within individual groups were done with Friedman tests. In all comparisons, $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Experiment 1: Post-training inactivation of the GRPR and NMDAR in the hippocampus

To determine whether inactivation of hippocampal GRPRs and NMDARs would produce persistent impairment of consolidation of IA memory, rats were given a bilateral infusion of VEH, RC-3095 or AP5 into the dorsal hippocampus immediately after IA training. Results are shown in Fig. 1. There was no significant difference among groups in training trial latencies (Kruskal-Wallis test, H = 0.64, df = 2, $P = 0.88$). However, Kruskal-Wallis tests revealed significant differences among groups in all three retention tests (test-1d, H = 17.86, df = 2, $P < 0.01$; test-2d, H = 18.28, df = 2, $P < 0.01$; test-3d, H = 18.96, df = 2, $P < 0.01$). Further analysis with Mann-Whitney tests showed that both RC-3095 and AP5 induced a significant impairment of IA retention that persisted across all retention tests compared to the control group treated with VEH (all Ps < 0.01). In addition, the results showed that the behavioral training and testing protocol used induced no memory extinction (comparison of latencies across retention tests in the VEH-treated group using the Friedman test, H = 1.80, df = 2, $P = 0.41$).

Fig. 1 should be inserted here

3.2. Experiment 2: Post-retrieval inactivation of the GRPR and NMDAR in the hippocampus

The second experiment examined the effects of intra-hippocampal infusions of RC-3095 or AP5 after memory reactivation on IA retention tested in subsequent retention tests (Fig. 2). There were no significant differences among groups in training (Kruskal-Wallis test, H = 0.36, df = 2, $P = 0.84$) or memory reactivation (test-1d; Kruskal-Wallis test, H = 0.01, df = 2, $P = 0.99$) trials. Groups treated with intra-hippocampal RC-3095 or AP5 showed a significant retention impairment in the second retention test (test-2d) compared to controls (Kruskal-Wallis test, H = 10.37, df = 2, $P < 0.01$; Mann-Whitney test, VEH versus RC-3095, $P < 0.05$; Mann-Whitney test, VEH versus AP5, $P < 0.01$). However, the impairment induced by RC-3095 and AP5 was transient: rats treated with either drug showed retention latencies similar to those of the control group when retested 24 h after the second test (test-3d; Kruskal-Wallis test, H = 0.99, df = 2, $P = 0.61$). Again, no extinction was observed in the VEH-treated control group (comparison of latencies across retention tests in the VEH-treated group using the Friedman test, H = 0.63, df = 2, $P = 0.73$).

Fig. 2 should be inserted here

3.3. Experiment 3: No reactivation control

To determine whether the effect of post-retrieval infusion of RC-3095 and AP5 was specific to the reactivated memory rather than being related to nonspecific effects or to persistence of drug effects at the time of the second retrieval test, rats received a bilateral intra-hippocampal infusion of RC-3095 or AP5 24 h after training in the absence of test-1d (Fig. 3). There were no significant differences among groups in any behavioral trial (Kruskal-Wallis tests; training, H = 2.64, df = 2, $P = 0.27$; test-2d, H = 0.61, df = 2, $P = 0.74$; test-3d, H = 0.41, df = 2, $P = 0.81$).

Fig. 3 should be inserted here

3.4. Histology

All animals that were included in the statistical analysis (80 rats) had bilaterally placed cannula right above the CA1 area of the dorsal hippocampus. Fig. 4 shows a photomicrograph of a cannula placement and a schematic drawing of the spread of dye within the dorsal hippocampus.

Fig. 4 should be inserted here

4. Discussion

This study provides the first evidence that GRPR blockade after memory reactivation produces retention impairment. In addition, the findings extend previous studies from our laboratory indicating that post-training administration of RC-3095 or AP5 into the dorsal hippocampus blocks consolidation of IA memory (Roesler et al., 1998; 2003a; 2003b; 2005), and are consistent with previous evidence that NMDAR blockade after reactivation might impair fear memory (Szapiro et al., 2003; Suzuki et al., 2004; Lee et al., 2006). Moreover, the effects of RC-3095 and AP5, like those of anisomycin and other drugs proposed to block reconsolidation, is only present after memory reactivation and not in its absence. However, the retention impairment induced by post-retrieval administration of RC-3095 or AP5 was transient: animals treated with either drug showed normal memory retention in a third retention test carried out 48 h after memory reactivation. The pattern of results found in the present study is very similar to those reported in our previous study using post-retrieval muscimol inactivation of the dorsal hippocampus (Amaral et al., 2007).

Many studies, on the other hand, have found persistent memory impairment after administration of post-retrieval treatments, a fact which is more in line with the central tenet of the reconsolidation hypothesis (Debiec & LeDoux, 2004; Duvarci & Nader, 2004; Lee et al., 2004; Tronel & Alberini, 2005; Bustos et al., 2006). Still, the evidence that memory deficits after post-retrieval administration of amnesic agents might reverse spontaneously with time or after exposure to repeated testing or reminders constitutes an important caveat concerning the reconsolidation hypothesis and has been a matter of

intense debate (Mactutus, Riccio, & Ferek, 1979; Judge & Quartermain, 1982; Anokhin, Tiunova, & Rose, 2002; Szapiro et al., 2003; Lattal & Abel, 2004; Cai, Blundell, Han, Greene, & Powell, 2006; Power, Berlau, McGaugh, & Steward, 2006; Prado-Alcalá, Diaz del Guante, Garin-Aguilar, Diaz-Trujillo, Quirarte, & McGaugh, 2006; Amaral et al., 2007). Recovery of fear-related memory deficits induced by post-retrieval pharmacological interventions in rodents has been shown in studies using systemic or intra-hippocampal injections of anisomycin (Judge & Quartermain, 1982; Vianna, Szapiro, McGaugh, Medina, & Izquierdo, 2001; Lattal & Abel, 2004; Power et al., 2006), tetrodotoxin inactivation of the dorsal hippocampus or amygdala (Prado-Alcalá et al., 2006), muscimol inactivation of the dorsal hippocampus (Amaral et al., 2007), or systemic administration of glucocorticoids (Cai et al., 2006).

The implications of spontaneous recovery for the interpretation of the reconsolidation hypothesis have been a matter of debate (e.g., Lattal and Abel, 2004; Power et al., 2006; Prado-Alcalá et al., 2006; Amaral et al., 2007; Tronson & Taylor, 2007). Studies using different tasks, including IA, suggest that, at least under some conditions, retrieval can return memory to a labile state requiring protein-synthesis-dependent reconsolidation (although it is probably not identical to consolidation at the molecular level) (reviewed in Alberini, 2005). However, some authors argue that the findings of spontaneous or reminder-induced recovery from deficits induced by post-retrieval amnesic treatments support the view that the impairing effects of post-retrieval drug administration is best explained by temporary retrieval deficits than disruption of reconsolidation (e.g., Prado-Alcalá et al., 2006).

Alternatively, the impairing effects of post-retrieval manipulations could be due to accelerated extinction (Koh & Bernstein, 2003; Fischer, Sananbenesi, Schrick, Spiess, &

Radulovic, 2004; Cai et al., 2006). As we and several other authors have argued, this interpretation is unlikely given that extinction of fear memory tasks, including IA, is a type of new learning that should not be facilitated by treatments such as protein synthesis inhibition and NMDAR blockade (Vianna et al., 2001; Amaral et al., 2007). Thus, the physiological meaning of temporary retrieval impairments induced by post-retrieval manipulations remains unclear, although it has been argued that the preservation of the original memory after post-reactivation treatments could be incorporated into the reconsolidation theory (Lattal and Abel, 2004, Tronson & Taylor, 2007). We have recently proposed that transient memory deficits induced by post-retrieval manipulations could also involve drug-induced temporary silencing of the original memory trace, learning of a short-lasting interfering conditioning, or parallel processing of multiple memory traces (Amaral et al., 2007).

It should be kept in mind that the present findings do not preclude the possibility that post-retrieval blockade of GRPRs and NMDARs could cause persistent disruption of reconsolidation under different conditions. Although we used doses of RC-3095 and AP5 that consistently induce a persistent blockade of IA consolidation, we cannot rule out the possibility that consolidation and reconsolidation of IA memory are sensitive to different doses of amnestic agents (Anokhin et al., 2002), and that higher doses or more prolonged blockade (Milekic, Brown, Castellini, & Alberini, 2006) could eventually cause permanent amnesia. It is also possible that longer reminder durations could potentially cause greater disruption of the memory trace (Suzuki et al., 2004) and lead to permanent amnesia; however, the nature of retrieval in the IA task precludes us from controlling this variable in the present study.

It is important to note that, in a previous report using an experimental procedure similar to the one used in the present study, we found that intra-hippocampal infusion of RC-3095 after the first retention test blocked extinction without inducing retention impairment in the subsequent retention test (Luft, Flores, Vianna, Schwartsmann, Roesler, & Izquierdo, 2006). These findings seem to contradict the present result that RC-3095 given after reactivation produced an impairment of performance in the second test. The only crucial difference in experimental conditions between these two studies was that a higher footshock intensity was used in the present study. Under this training condition, animals showed higher latencies in the first retention test and no memory extinction across tests was observed in control animals, whereas in the previous study repeated non-reinforced exposure to the training context induced significant extinction in control animals. These differences suggest that, in a condition in which extinction of fear memory occurs, blockade of hippocampal GRPRs can block extinction without inducing deficits in retrieval of the original fear memory, whereas in the absence of extinction GRPR blockade can induce a transient deficit of fear memory retention, similarly to what has been shown with protein synthesis inhibitors (Eisenberg, Kobilo, Berman & Dudai, 2003). Another possibility, however, is that a minimum reexposure duration is required to induce a reconsolidation process (Suzuki et al., 2004), and that it only occurs with the higher retrieval latencies induced by the stronger shock. These results illustrate that the production of memory impairment by post-retrieval manipulations is highly sensitive to details in experimental conditions. They are also consistent with previous reports that post-retrieval drug treatments can affect extinction, reconsolidation, or neither, depending on minor variations in experimental conditions, such as training intensity and test duration (Vianna et al., 2001; Szapiro et al., 2003; Cammarota, Bevilaqua, Medina, & Izquierdo, 2004; Suzuki

et al., 2004; Vianna et al., 2004; Lee et al., 2006; Power et al., 2006; Eisenhardt & Menzel, 2007).

We and others have previously shown that the GRPR is importantly involved in regulating consolidation and extinction of fear memory in brain areas such as the hippocampus and amygdala (Shumyatsky et al., 2002; Roesler et al., 2003a; 2004b; 2004c; Luft et al., 2006; Mountney et al., 2006; Roesler et al., 2006b; Bedard et al., 2007). Memory modulation by hippocampal GRPRs might depend on signal transduction cascades including the PKA, protein kinase C (PKC), and ERK/MAPK pathways (Roesler et al., 2006b). Also, our present findings that the effects of GRPR antagonism parallel those of NMDAR blockade in the hippocampus are consistent with the view that GRP interacts with glutamatergic transmission in influencing memory formation (Lee et al., 1999; Shumyatsky et al., 2002). The present study reveals for the first time another role for the GRPR in fear-related memory, namely, its involvement in post-reactivation memory deficits. This finding supports our view that GRPR-triggered signaling is an important system regulating several aspects of fear-related memory and could have clinical implications as a potential target in neuropsychiatric disorders including PTSD and other fear-related conditions (Roesler et al., 2004a; 2006a). Further research on the role of the GRPR, NMDAR, and other receptor systems in memory processing after retrieval could help us understand the biological basis and function of the memory reactivation process as well as its potential clinical implications.

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Legends for figures

Fig. 1. Post-training inactivation of gastrin-releasing peptide receptors (GRPR) or *N*-methyl-D-aspartate receptors (NMDAR) in the dorsal hippocampus impairs retention of an inhibitory avoidance (IA) task in rats. Data are mean ± SEM training and retention test step-down latencies (s) of rats given a bilateral 0.5 μl-infusion of vehicle (VEH; 2% dimethylsulfoxide (DMSO) in saline), the GRPR antagonist RC-3095 (1.0 μg), or the NMDAR antagonist aminophosphonopentanoic acid (AP5; 5.0 μg) into the dorsal hippocampus immediately after IA training. Rats were tested for retention at 24, 48, and 72h after training; $N = 8$-11 per group. ** $P < 0.01$ compared to the VEH-treated group.

Fig. 2. Post-retrieval inactivation of gastrin-releasing peptide receptors (GRPR) or *N*-methyl-D-aspartate receptors (NMDAR) in the dorsal hippocampus produces transient impairment of inhibitory avoidance (IA) performance in rats. Data are mean ± SEM training and retention test step-down latencies (s) of rats given a bilateral 0.5 μl-infusion of vehicle (VEH; 2% dimethylsulfoxide (DMSO) in saline), the GRPR antagonist RC-3095 (1.0 μg), or the NMDAR antagonist aminophosphonopentanoic acid (AP5; 5.0 μg) into the dorsal hippocampus immediately after the first retention test (test-1d). Rats were tested at 24, 48, and 72h after training; $N = 7$-11 per group. * $P < 0.05$ and ** $P < 0.01$ compared to the VEH-treated group.

Fig. 3. Inactivation of gastrin-releasing peptide receptors (GRPR) or *N*-methyl-D-aspartate receptors (NMDAR) in the dorsal hippocampus 24 h after training does not affect

inhibitory avoidance (IA) performance in the absence of memory reactivation. Data are mean ± SEM training and retention test step-down latencies (s) of rats given a bilateral 0.5 μl-infusion of vehicle (VEH; 2% dimethylsulfoxide (DMSO) in saline), the GRPR antagonist RC-3095 (1.0 μg), or the NMDAR antagonist aminophosphonopentanoic acid (AP5; 5.0 μg) into the dorsal hippocampus 24 h after IA training. Rats were tested for retention at 48 and 72h after training; *N* = 7-9 per group. There were no significant differences among groups.

Fig. 4. Photomicrograph of an example of intra-hippocampal cannula placement and drawing of the plane A -4.3 mm of the atlas of Paxinos and Watson (1997) showing the area (hatched) where the infusion sites considered to be correct were placed.

Figure 1
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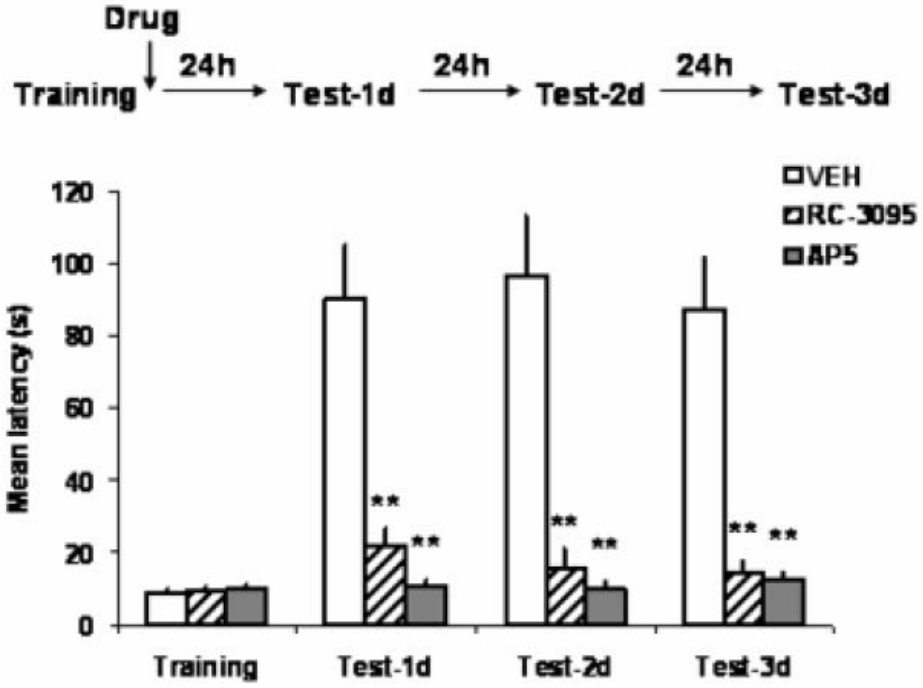

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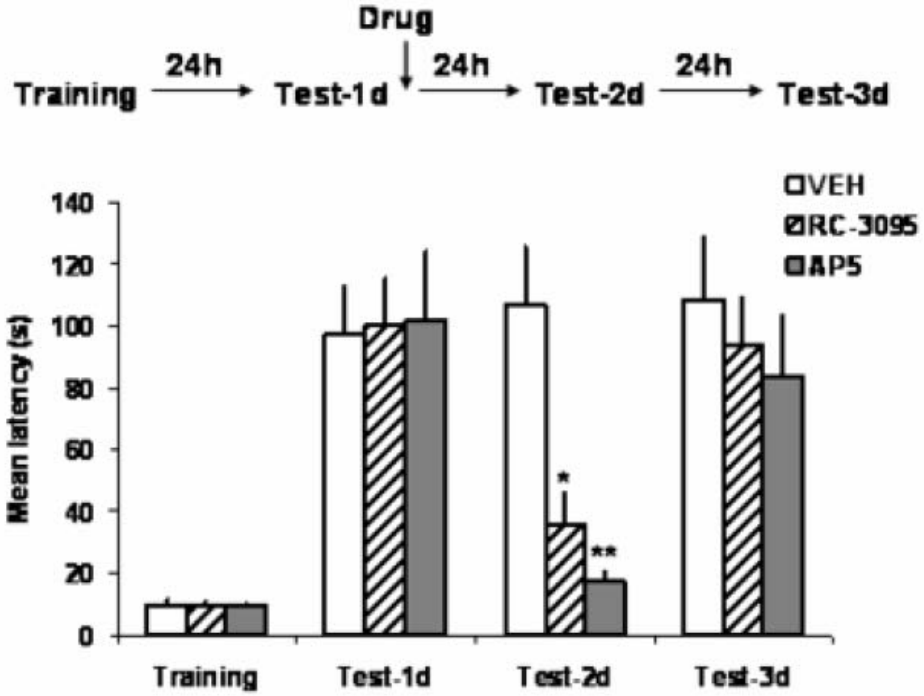

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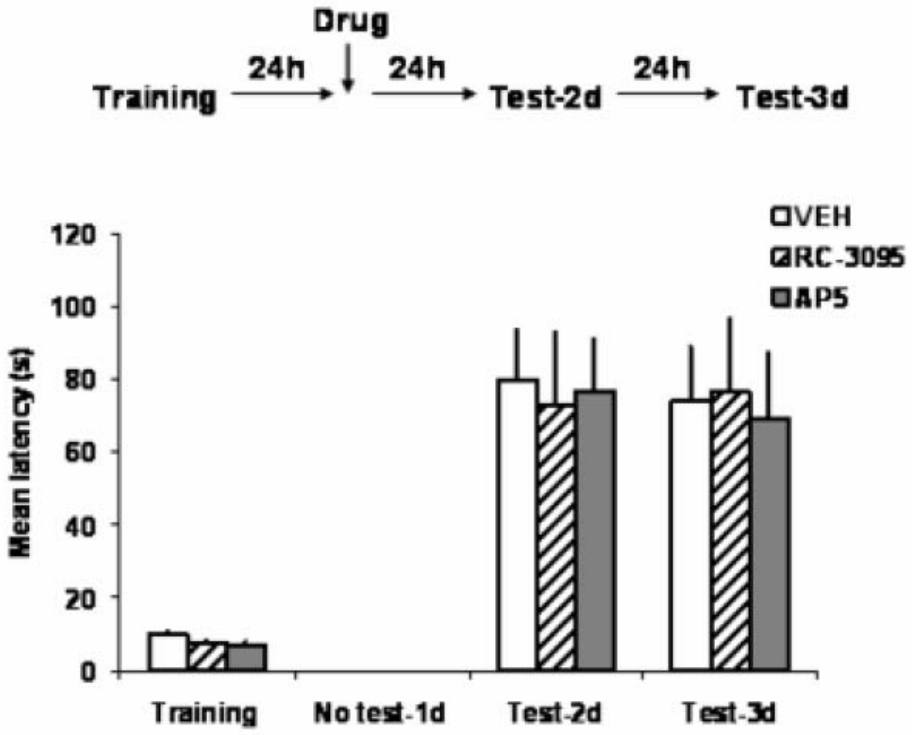

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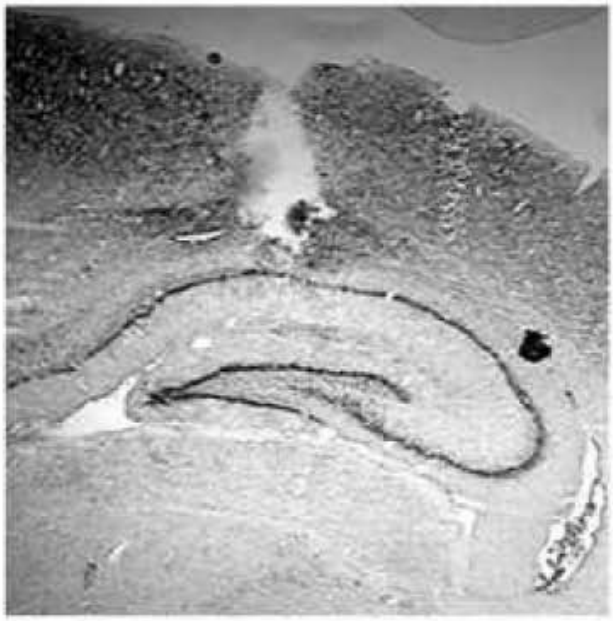

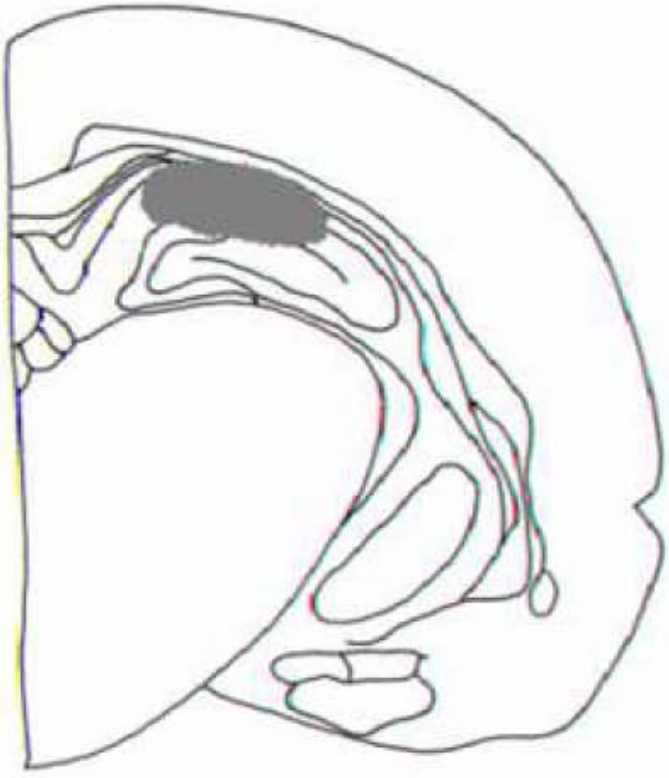

CAPÍTULO III

Efeito do agonista dos receptores do peptídeo liberador de gastrina (GRPR), bombesina, em um modelo de amnésia associado à doença de Alzheimer

Publicado em “Molecular mechanisms mediating gastrin-releasing peptide receptor modulation of memory consolidation in the hippocampus”

Neuropharmacology (2006) 51: 350-357

Materiais e Métodos

Animais

Ratos machos adultos (220 a 350g) obtidos da Fundação de Pesquisa de Saúde do Estado (FEPPS-RS) foram mantidos em número de cinco por caixa-moradia em uma sala com temperatura controlada, com alimento e água disponíveis, em ciclo claro-escuro de 12h (luzes acesas 7:00h). Os procedimentos comportamentais foram conduzidos durante a fase clara do ciclo, entre 10:00 e 17:00h. Todos os procedimentos experimentais foram executados de acordo com '*NIH Guide for Care and Use of Laboratory Animals'* (publicação da NIH Nº 80-23 revisada em 1996). Todos os esforços foram feitos minimizar o número dos animais e seu sofrimento.

Cirurgia

Os animais foram implantados sob anestesia com tionembutal (30/kg, i.p.) bilateralmente com cânula de 9,0 mm, 1,0 mm acima da região CA1 do hipocampo dorsal como já foi descrito em estudos prévios (Bevilaqua et al., 1997; Walz et al., 2000; Roesler et al., 2003; Quevedo et al., 2004; Venturella et al., 2005). As coordenadas (anteroposterior, 4.3 mm; mediolateral, 3.0 mm; ventral, 1.4 mm) foram obtidas do atlas de Paxinos e de Watson (1997). Os experimentos só iniciaram após recuperação dos animais da cirurgia de pelo menos 7 dias.

Tarefa comportamental

Nós utilizamos a tarefa de esquiva inibitória de uma via como modelo de memória dependente do hipocampo motivada pelo medo (Izquierdo & Medina, 1997; Taubenfeld et al., 1999; McGaugh, 2000). Na tarefa de esquiva inibitória, os animais aprendem a associar o local do treino com um estímulo aversivo (choque). A esquiva inibitória consiste em uma caixa de material plástico de 50 x 25 x 25cm, cujo assoalho é uma grade de barras de bronze paralelas, calibre 1 mm, separadas entre si por 1 cm. Os animais são colocados sobre uma plataforma de 2,5 cm de altura, 10 cm de largura e 15 cm de extensão, que ocupa o extremo esquerdo do assoalho. O tempo que os animais levam para descer da plataforma à grade (latência) é medido quando os mesmos colocam suas quatro patas na grade. Uma vez na grade, os ratos recebem um choque elétrico de 0,4 mA durante 2 segundos. Imediatamente após são retirados do aparato. Na sessão de teste, que é realizado 24h após o treino, para medir a memória de longa duração, o animal é colocado novamente no aparato de esquiva inibitória; porém, ao descer da plataforma, não recebe choque. Para avaliar o quanto o animal aprendeu, mede-se o tempo de latência (máximo 180s) em que este permanece na plataforma e, conseqüentemente, a retenção da tarefa.

Drogas e procedimentos de infusão

Os procedimentos de infusão intra-hipocampal foram descritos em estudos prévios (Bevilaqua et al., 1997; Walz et al., 2000; Quevedo et al., 1999, 2004; Roesler et al., 2003; Venturella et al., 2005). No momento da infusão, os animais receberam infusão da droga através da cânula-guia, localizada 1cm acima da região CA1 do

hipocampo. A agulha de infusão projeta-se 1 cm além da cânula-guia, atingindo a região CA1 do hipocampo dorsal. As drogas foram infundidas durante 30 segundos. Após a infusão da droga ou do veículo, a agulha de infusão permaneceu dentro da cânula-guia por 1min para permitir a difusão da droga para a região específica.

Bombesina (0,002 µg em 0,5 µg) ou salina (SAL) (0,5 µg) foram infundidos bilateralmente no hipocampo 10 minutos antes do treinamento na tarefa de esquiva inibitória; água destilada (DW, 0,5 µg) ou peptídeo β-amilóide$_{(25-35)}$ (0,02 µg em 0,5 µg; Sigma) foram infundidos imediatamente depois da sessão de treino. Experimentos prévios de nosso laboratório mostraram que uma única infusão intrahipocampal de 0,02 µg de peptídeo β-amilóide$_{(25-35)}$ induz prejuízo da memória na tarefa de esquiva inibitória sem causar morte neuronal significativa na área CA1 do hipocampo dorsal (Luft et al, resultados não publicados).

Histologia

Vinte e quatro à 48h após o final da tarefa comportamental, os animais foram decapitados e seus cérebros removidos, armazenados em 5% de formalina por no mínimo 72 h e foi verificado se o local de infusão estava correto como segue: 0,5 ml de uma solução de 4% de azul de metileno foi infundido como descrito acima e a extensão da tintura foi examinada como indicativo da difusão das drogas dadas previamente a cada rato (Bevilaqua et al., 1997; Walz et al., 2000; Quevedo et al., 1999, 2004; Roesler et al., 2003; Venturella et al., 2005). Somente os dados dos animais com o local de infusão correto foram incluídos na análise final.

Estatística

Os dados são mostrados com latências médias do teste de retenção da descida. As comparações entre as latências das sessões de treino entre os grupos foram feitas usando a análise de variância de Kruskal-Wallis seguida por testes de Mann-Whitney, quando necessário (Bevilaqua et al., 1997; Walz et al., 2000; Quevedo et al., 1999, 2004; Roesler et al., 2003; Venturella et al., 2005). Em todas as comparações, $P < 0.05$ indica diferença significativa.

Resultado

Bombesina previne o prejuízo na consolidação da memória no hipocampo induzido pelo peptídeo β-amilóide$_{(25-35)}$.

Estudos prévios indicam que os peptídeos da família da bombesina e GRPR podem estar envolvidos na patogênese da doença de Alzheimer (Ito et al., 1994; Gibson & Huang, 2005; Roesler et al., 2006). Além disso, a administração sistêmica de GRP melhora déficits na consolidação da memória em um modelo de amnésia induzida por escopolamina em camundongos (Santo-Yamada et al., 2001). Aplicação de peptídeo β-amilóide$_{(25-35)}$ na área CA1 hipocampal *in vivo* e *in vitro* foi usada como modelo para investigar o prejuízo na plasticidade sináptica associada à doença de Alzheimer (Saleshando & O' Connor, 2000; Freir et al., 2001; Costello & Herron, 2004). Infusões intrahipocampal e intracerebroventricular de peptídeo β-amilóide$_{(25-35)}$ em ratos têm sido usadas como modelo de prejuízo cognitivo associado à Doença de Alzheimer (Chen et al., 1996; Stepanichev et al., 2005). Examinamos, no presente trabalho, se a

bombesina poderia previnir o déficit de memória na tarefa de esquiva inibitória induzido pela administração aguda pós-treino de peptídeo β-amilóide$_{(25-35)}$ no hipocampo. Os resultados estão mostrados na **Figura 8**. A infusão intrahipocampal pós-treino de peptídeo β-amilóide$_{(25-35)}$ induziu um prejuízo significativo na retenção da memória em esquiva inibitória ($P < 0,01$ comparado ao grupo controle SAL e DW). A infusão pré-treino de uma dose sem efeito de bombesina preveniu o prejuízo da retenção da memória induzido pelo peptídeo β-amilóide$_{(25-35)}$. Não houve nenhuma diferença significativa entre grupos nas latências do treino. O resultado indica que os agonistas de GRPR podem prevenir os prejuízos da memória causados pelo peptídeo β-amilóide$_{(25-35)}$ no hipocampo.

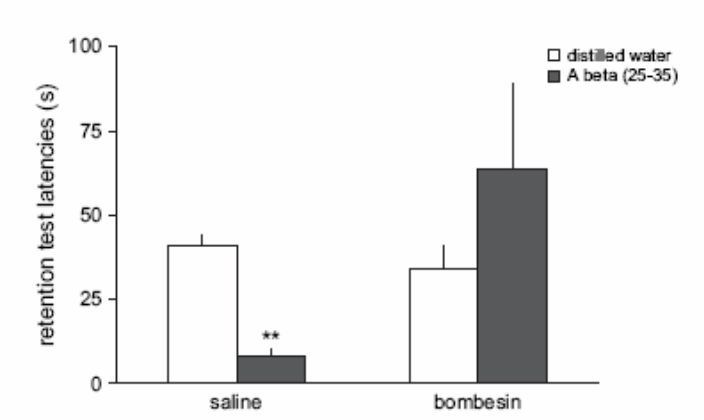

Figura 8. Bombesina previne o prejuízo na consolidação da memória no hipocampo induzido pelo peptídeo β-amilóide$_{(25-35)}$. Os dados são as latências de descida da plataforma de ratos que receberam infusão bilateral de 0.5 µl de bombesina (0,002 µg) ou salina 10min antes do treino na tarefa de esquiva inibitória, e peptídeo β-amilóide$_{(25-35)}$ ou água destilada imediatamente após o treino (N = 8 a 14 animais por grupo). ** $P < 0,01$ comparado com o grupo tratado com salina e água destilada.

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PARTE III

DISCUSSÃO

1. Administração de um antagonista de GRPR no hipocampo dorsal prejudica a extinção da memória motivada por medo.

Extinção é proposta como uma forma de memória nova que desfaz ou apaga uma associação condicionada previamente formada. A consolidação da extinção no hipocampo compartilha de um grande número de mecanismos semelhantes aos da consolidação de novas memórias. Estudos prévios têm mostrado que a extinção da memória no hipocampo dorsal em esquiva inibitória e outras tarefas de condicionamento aversivo requer receptores NMDA, síntese protéica, expressão gênica, PKA, MAPK, CAMKII, e PI3K (Cammarota et al., 2005; Vianna et al., 2001, 2003, 2004; Szapiro et al., 2003; Fischer et al., 2004, Chen et al., 2005; Corcoran et al., 2005, Power et al., 2006). Como na maioria dos estudos de tratamentos com drogas, estas são infundidas após o animal receber a exposição não-reforçada à sessão do treino (isto é, sessões de extinção), afetando assim, a consolidação da extinção da memória. Embora evidências farmacológicas (Flood et al., 1988, Roesler et al., 2003, 2004; Santo-Yamada et al., 2003; Venturella et al., 2005) e genéticas (Shumyatsky et al., 2002) sugerem um papel importante do GRPR na regulação da consolidação da memória aversiva, estudos anteriores não examinaram se os GRPR têm algum papel na extinção. No presente estudo, nós encontramos que o antagonista GRPR (RC-3095) no hipocampo, depois da exposição sem reforço ao contexto do treino, prejudica a extinção da memória na tarefa de esquiva inibitória. Nós previamente mostramos que

os efeitos prejudiciais da administração hipocampal do antagonista dos GRPR (RC-3095) na consolidação da memória não estava atribuído à neurotoxicidade ou a um prejuízo de longa duração na recuperação da memória (Roesler et al., 2003). Além disso, nossos resultados mostram que RC-3095 dado 24h depois do treino não afetou desempenho subseqüente quando a primeira sessão da extinção foi omitida, o que indica que o prejuízo induzido pela droga requer recuperação da memória previamente aprendida e não se deu por prejuízo de longa duração da função neural. Esses achados comprovam, pela primeira vez, que o sistema GRPR está envolvido na extinção da memória.

O GRPR é um tipo de receptor dos peptídeos da família da bombesina expresso em diversos tecidos de mamíferos. No cérebro, o GRPR está expresso em grande quantidade nos corpos celulares e dendritos dos neurônios em áreas que incluem o hipocampo dorsal e a amígdala lateral (Kamichi et al., 2005). Em mamíferos, GRPRs hipocampais podem ser ativados pelo GRP liberado por neurônios excitatórios (Shumyatsky et al., 2002; Lee et al., 1999). A ativação de GRPR pode, por sua vez, conduzir à ativação de cascatas de sinalização intracelular incluindo as fosfolipases proteína quinase C (PKC) e MAPK (Hellmich et al., 1999). O padrão de distribuição do GRPR no cérebro, assim como os mecanismos de sinalização envolvidos mediando respostas celulares à ativação de GRPR, são consistentes com os dados comportamentais que indicam que GRPR tem um papel importante na memória regulando tanto a formação quanto a extinção. Nosso resultado de que o antagonista GRPR RC-3095 prejudica a extinção, juntamente com estudos prévios mostrando que RC-3095 prejudica a consolidação da memória (Roesler et al., 2003, 2004; Venturella et al., 2005), indicam que a ativação de GRPR tem um papel estimulatório na plasticidade sináptica, na formação da memória e na extinção. Outros estudos,

entretanto, propuseram que o GRPR está localizado predominantemente nos interneurônios inibitórios que liberam ácido gama-amino butírico (neurônios GABAérgicos), e que a ativação de GRPR poderia levar a um aumento na transmissão GABAérgica, o que poderia vir a inibir a plasticidade sináptica e a memória (Shumyatsky et al., 2002; Lee et al., 1999). Consistente com esse ponto de vista, a bombesina induz despolarização nos interneurônios inibitórios em fatias hipocampais (Lee et al., 1999), e os camundongos deficientes de GRPR mostram um aumento na memória motivada pelo medo e plasticidade sináptica na amígdala (Shumyatsky et al., 2002). Uma possibilidade para harmonizar estes achados contrários é que os receptores GRPR estão expressos tanto em neurônios inibitórios GABAérgicos quanto em neurônios não-GABAérgicos que liberam glutamato e outros neurotransmissores. O recente achado que na amígdala lateral somente uma subpopulação de células expressando GRPRs são neurônios GABAérgicos (Kamichi et al., 2005) é consistente com a possibilidade de que GRPRs estão expressados em neurônios não-GABAérgicos que liberam glutamato e outros neurotransmissores. Assim, os efeitos prejudiciais de RC-3095 na consolidação da memória e na extinção podem estar relacionados com a inibição de GRPRs em neurônios excitatórios.

Como os estudos com animais têm como objetivo esclarecer os mecanismos neurais da extinção, estes podem ter relevância clínica para o tratamento de pacientes com doenças relacionadas ao medo, ou fobias (Myers et al., 2002, Barad et al., 2005, Cammarota et al., 2005, Milad et al., 2006), nossos achados que os GRPRs estão envolvidos na extinção suporta que o sistema GRPR é um importante alvo terapêutico para tratamento de doenças psiquiátricas incluindo ansiedade e fobias como transtorno de estresse pós-traumático (Roesler et al., 2004). Experimentos futuros investigando os mecanismos intracelulares mediados pela modulação dos GRPRs na extinção e

examinando se os agonistas dos GRPRs podem acelerar a extinção da memória podem trazer implicações clínicas para o tratamento de doenças psiquiátricas relacionadas ao medo.

2. Inativação dos receptores do peptídeo liberador da gastrina (GRPR) ou dos receptores de N-Metil-D-Aspartato (NMDA) produz prejuízo na retenção da memória pós-reativação.

Este estudo fornece a primeira evidência de que a inativação de GRPR após a reativação da memória produz prejuízo na retenção. Além disso, achados de estudos prévios do nosso laboratório indicam que a administração de RC-3095 ou AP5 no hipocampo dorsal bloqueia a consolidação da memória na tarefa de esquiva inibitória (Roesler et al., 1998, 2003, 2003, 2005; Venturella et al., 2005; Dantas et al., 2006; Preissler et al., 2007), e estão consistentes com evidências prévias que o bloqueio de NMDAR após a reativação pode prejudicar a memória de medo (Szapiro et al., 2003; Suzuki et al., 2004; Lee et al., 2006).

Além disso, os efeitos de RC-3095 e de AP5, assim como aqueles com anisomicina e outras drogas propostas a bloquear a reconsolidação, estão somente presentes após a reativação da memória, e não na sua ausência. Entretanto, o prejuízo na retenção induzido pela administração pós-reativação de RC-3095 ou AP5 foi transitório: animais tratados com cada uma das drogas mostraram retenção normal da memória no terceiro teste de retenção 48h após a reativação da memória. O padrão dos resultados encontrados no presente estudo é muito similar àqueles relatados em

nossos estudos prévios usando inativação pós-reativação com muscimol no hipocampo dorsal (Amaral et al., 2007).

A evidência que déficits de memória após a reativação pela administração de agentes amnésicos podem reverter espontaneamente com o tempo ou após a exposição a testes ou lembranças repetidas constituem uma importante informação à hipótese da reconsolidação (Mactutus et al., 1979; Judge & Quartermain, 1982; Anokhin et al., 2002; Szapiro et al., 2003; Lattal & Abel, 2004; Cai et al., 2006; Power et al., 2006; Prado-Alcalá et al., 2006; Amaral et al., 2007). Recuperação de prejuízo de memória relacionada ao medo por intervenções farmacológicas pós-reativação em roedores foi mostrada em estudos usando injeção sistêmica ou intrahipocampal de anisomicina (Judge & Quartermain, 1982; Vianna et al., 2001; Anokhin et al., 2002; Latal & Abel, 2004; Powers et al., 2006), inativação com tetrodoxina no hipocampo dorsal ou amígdala (Prado-Alcalá et al., 2006), inativação com muscimol no hipocampo dorsal (Amaral et al., 2007), ou administração sistêmica de glicocorticóides (Cai et al., 2006). As implicações da recuperação espontânea para a interpretação da hipótese da reconsolidação têm sido matéria para extensos debates (Lattal & Abel, 2004; Powers et al., 2006; Prado-Alcalá et al., 2006; Amaral et al., 2007). Estudos usando diferentes tarefas, incluindo esquiva inibitória, sugerem que, pelo menos sob algumas circunstâncias, a evocação pode retornar a memória à um estado lábil que requer reconsolidação dependente de síntese protéica (Alberini, 2005). Nós propusemos recentemente que os déficits transitórios da memória induzidos por manipulações pós-reativação poderiam também envolver um ‘silêncio temporário’ do traço da memória original, ou um processamento paralelo de múltiplos traços da memória (Amaral et al., 2007).

É importante notar que, em um estudo prévio usando procedimento experimental similar ao utilizado no presente estudo para examinar o envolvimento dos GRPRs

hipocampais na extinção, nós observamos que a infusão intrahipocampal de RC-3095 após o primeiro teste de retenção bloqueou a extinção, mas não induziu um prejuízo na retenção em um teste subseqüente (Luft et al., 2006). Os resultados desse estudo prévio parecem, portanto, contradizer os presentes resultados que o RC-3095 dado depois da reativação produz um prejuízo na performance no segundo teste. A única diferença crucial entre os dois estudos foi a condição experimental, onde foi dado um choque de maior intensidade no presente estudo. Nesta condição de treinamento, os animais mostraram latências mais elevadas no primeiro teste da retenção e nenhuma extinção da memória foi observada nos testes subseqüentes nos animais-controle, uma vez que no estudo prévio, a reexposição sem esforço induziu uma extinção significativa dos animais-controle. Estas diferenças entre os dois estudos sugerem que, em uma circunstância em que a extinção da memória do medo ocorre, a inativação de GRPRs hipocampais pode bloquear a extinção, mas na ausência do bloqueio da extinção GRPR pode induzir um déficit transitório da retenção da memória do medo. Estes resultados ilustram que o prejuízo da memória por manipulações pós-reativação é altamente sensível aos detalhes das circunstâncias experimentais. Além disso, os dados são consistentes com achados prévios onde tratamentos com drogas pós-recuperação podem afetar extinção, reconsolidação, ou nenhum dos dois, dependendo das variações mínimas das condições experimentais, como intensidade do treino e a duração do teste (Vianna et al., 2001, 2004; Szapiro et al., 2003; Cammarota et al., 2004; Suzuki et al., 2004; Lee et al., 2006; Power et al., 2006; Eisenhardt & Menzel, 2007).

Nós e outros grupos mostramos previamente que o GRPR está envolvido na consolidação e na extinção da memória de medo em áreas cerebrais como hipocampo e amígdala (Shumyatsky et al., 2002; Roesler et al., 2003, 2004, 2006; Luft et al., 2006; Mountney et al., 2006; Bedard et al., 2007). Os achados atuais revelam pela primeira

vez um outro papel para o GRPR em memória relacionada ao medo, sua participação na memória pós-evocação.

Assim, os resultados mostram que o GRPR é um importante sistema que regula vários aspectos da memória relacionada ao medo e, assim, pode ter implicações clínicas como um alvo potencial em doenças neuropsiquiátricas, como transtorno por estresse pós-traumático e outras condições clínicas relacionadas ao medo (Roesler et al., 2004, 2006). Futuras pesquisas do papel do GRPR, NMDAR e outros sistemas de receptores no processamento da memória após a evocação podem nos auxiliar a compreender melhor as bases biológicas e a função do processo de reativação da memória, assim como suas implicações clínicas potenciais.

3. Administração pré-treino de bombesina preveniu o prejuízo da memória induzido pelo peptídeo β-amilóide$_{(25-35)}$

Diversas linhas de evidências indicaram que o GRPR pode estar envolvido em disfunções cognitivas associadas com a Doença de Alzheimer e outras doenças neurodegenerativas e psiquiátricas (Roesler et al., 2006). As alterações na densidade de GRPR e as disfunções na sinalização de $Ca2^{+}$ elicidada pela bombesina foram descritas em fibroblastos e leucócitos de pacientes com a Doença de Alzheimer (Ito et al., 1994; Gibson & Huang, 2005). Estes dados mostram que a bombesina pode melhorar a retenção da memória estimulando vias de proteína quinase criticamente envolvidas na mediação da plasticidade sináptica, e sugerem que o GRPR pode ser considerado um alvo molecular para o desenvolvimento de novas terapias cognitivas. Baseados em experimentos prévios eletrofisiológicos (Saleshando & O' Connor, 2000;

Freire et al., 2001; Costello & Herron, 2004) e comportamentais (Chen et al., 1996; Stepanichev et al., 2005), no presente estudo nós usamos uma infusão única intrahipocampal de uma dose baixa do peptídeo β-amilóide$_{(25-35)}$ em ratos como um modelo de disfunção de memória associada com a Doença de Alzheimer. Administração do peptídeo β-amilóide$_{(25-35)}$ no hipocampo *in vitro* ou *in vivo* pode prejudicar a plasticidade sináptica por mecanismos envolvendo a via da MAPK (Saleshando & O' Connor, 2000; Freir et al., 2001; Costello & Herron, 2004). Nossos achados que a administração pré-treino de uma dose inefetiva de bombesina preveniu o prejuízo da memória induzido pelo peptídeo β-amilóide$_{(25-35)}$ suporta a idéia de que o GRPR é um alvo molecular no desenvolvimento de melhoras cognitivas no tratamento de disfunção de memória associada com a doença de Alzheimer e outras doenças neuropsiquiátricas.

CONCLUSÕES

1. Usando um modelo de treinamento comportamental previamente estabelecido e procedimentos de canulação hipocampal, nós demonstramos que a administração de um antagonista de GRPR (RC-3095) na área CA1 do hipocampo prejudica a consolidação da extinção da memória aversiva. Esta é a primeira evidência do papel do GRPR na extinção da memória. Além disso, os dados indicam que GRPR é um novo alvo terapêutico para o tratamento de doenças neuropsiquiátricas.

2. A inativação de GRPRs ou de NMDARs hipocampais após a reativação da memória prejudica temporariamente a retenção da memória. A infusão intrahipocampal do antagonista de GRPR - RC-3095 ou do antagonista de NMDAR - AP5 produziu um prejuízo na tarefa de esquiva inibitória quando testado 48h após o treino. Entretanto, este prejuízo pós-reativação foi transitório e recuperado aos níveis dos ratos-controle em um teste subseqüente 72h dias após o treino. O efeito das drogas se deu apenas após a reativação da memória, e não na ausência da mesma. Estes achados fornecem a primeira evidência que a inativação de GRPR após a reativação da memória pode prejudicar a memória.

3. Administração do agonista de GRPR bombesina preveniu o prejuízo da memória induzido pelo peptídeo β-amilóide$_{(25-35)}$ no hipocampo. Esse resultado sugere que agonistas GRPR podem apresentar efeitos de facilitação cognitiva e potencial atividade terapêutica no tratamento da doença de Alzheimer.

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ANEXOS

ANEXO I

Molecular mechanisms mediating gastrin-releasing peptide receptor modulation of memory consolidation in the hippocampus

Roesler R, **Luft T**, Oliveira SHS, Farias CB, Almeida VR, Quevedo J, Dal Pizzol F, Schröder N, Izquierdo I, Schwartsmann G.

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Observação: Os dados do artigo em anexo apresentados nesta Tese de Doutorado são os mostrados na Figura 5. Os dados mostrados nas demais figuras foram ou poderão ser apresentados em outras dissertações ou teses.

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Molecular mechanisms mediating gastrin-releasing peptide receptor modulation of memory consolidation in the hippocampus

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Abstract

Although the gastrin-releasing peptide-preferring bombesin receptor (GRPR) has been implicated in memory formation, the underlying molecular events are poorly understood. In the present study, we examined interactions between the GRPR and cellular signaling pathways in influencing memory consolidation in the hippocampus. Male Wistar rats received bilateral infusions of bombesin (BB) into the dorsal hippocampus immediately after inhibitory avoidance (IA) training. Intermediate doses of BB enhanced, whereas a higher dose impaired, 24-h IA memory retention. The BB-induced memory enhancement was prevented by pretraining infusions of a GRPR antagonist or inhibitors of protein kinase C (PKC), mitogen-activated protein kinase (MAPK) kinase and protein kinase A (PKA), but not by a neuromedin B receptor (NMBR) antagonist. We next further investigated the interactions between the GRPR and the PKA pathway. BB-induced enhancement of consolidation was potentiated by coinfusion of activators of the dopamine D1/D5 receptor (D1R)/cAMP/PKA pathway and prevented by a PKA inhibitor. We conclude that memory modulation by hippocampal GRPRs is mediated by the PKC, MAPK, and PKA pathways. Furthermore, pretraining infusion of BB prevented beta-amyloid peptide (25–35)-induced memory impairment, supporting the view that the GRPR is a target for the development of cognitive enhancers for dementia.
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Keywords: Bombesin-like peptides; Gastrin-releasing peptide receptor; Beta-amyloid peptide; Cellular signaling; Hippocampus; Memory consolidation

1. Introduction

When memory is formed, new information acquired by the nervous system is at first labile and becomes subsequently stable through a process of consolidation involving long-lasting synaptic modifications. Extensive pharmacological and genetic evidence indicates that consolidation of long-term

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memory for spatial and contextual tasks in rats involves a number of neurotransmitter receptors and intracellular signal transduction pathways, as well as protein synthesis and gene expression in the CA1 area of the dorsal hippocampus (for reviews see Izquierdo and Medina, 1997; Alberini, 1999; McGaugh, 2000; Silva, 2003; Tonegawa et al., 2003).

The gastrin-releasing peptide-preferring receptor (GRPR, also known as BB2 receptor) has recently emerged as a system importantly involved in regulating memory formation (Williams and McGaugh, 1994; Rashidy-Pour and Razvani, 1998; Shumyatsky et al., 2002; Roesler et al., 2003; Santo-Yamada et al., 2003; Roesler et al., 2004b,c; Martins et al., 2005; Venturella et al., 2005). The GRPR is a member of the bombesin (BB)-like peptide receptor subfamily of G-protein coupled receptors, and is expressed in the cell surfaces of several mammalian tissues. Other subtypes of mammalian BB receptors include the neuromedin B receptor (NMBR). Within the brain, the GRPR occurs on dendrites and cell bodies of neurons in regions including the dorsal hippocampus and lateral amygdala (Wolf and Moody, 1985; Zarbin et al., 1985; Battey and Wada, 1991; Kamichi et al., 2005). GRPR activation by the amphibian peptide BB or its mammalian counterpart gastrin-releasing peptide (GRP) affects a range of cellular and neuroendocrine functions, including cell proliferation and differentiation, cancer growth, feeding behavior, and stress responses (for recent reviews, see Moody and Merali, 2004; Ohki-Hamazaki et al., 2005; Roesler et al., 2006). Recent evidence has also implicated the GRPR in neurodegenerative and neuropsychiatric disorders including Alzheimer's disease (AD), autism, and anxiety (Ito et al., 1994; Ishikawa-Brush et al., 1997; Meller et al., 2004; Roesler et al., 2004a; Gibson and Huang, 2005; for a review, see Roesler et al., 2006).

Findings from early pharmacological studies have indicated that systemic administration of GRPR agonists can improve memory retention in rodent models (Flood and Morley, 1988; Rashidy-Pour and Razvani, 1998). In addition, previous studies from our laboratory have indicated that infusions of a GRPR antagonist into either the CA1 hippocampal area or the basolateral amygdala impair consolidation of memory for aversive conditioning (Roesler et al., 2003, 2004c; Venturella et al., 2005). Furthermore, GRPR-deficient mice show altered fear conditioning and synaptic plasticity in the amygdala (Shumyatsky et al., 2002). Together, these findings indicate that GRPRs in brain areas including the dorsal hippocampus and amygdala are involved in memory formation.

The signal transduction mechanisms underlying the actions of the GRPR in the brain are poorly understood. Studies using cancer and neuroendocrine cell lines have indicated that cellular responses to GRPR activation require the protein kinase C (PKC) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) signaling pathways (Hellmich et al., 1999; Kim et al., 2000; Qu et al., 2002; Xiao et al., 2003; Chen and Kroog, 2004; Schwartsmann et al., 2005; Stangelberger et al., 2005; Thomas et al., 2005). The involvement of the cAMP/protein kinase A (PKA) pathway in mediating GRPR actions remains to be clarified (Kim et al., 2000; Qu et al., 2002). Previous studies have not examined the intracellular signaling mechanisms mediating the modulatory effects of GRPR activation on memory. In the present study, we used previously established behavioral training and hippocampal infusion procedures to investigate interactions between the GRPR and the PKC, MAPK, and PKA signaling pathways in memory consolidation in the dorsal hippocampus. We have also examined whether GRPR activation in the hippocampus alters memory impairment induced by intrahippocampal infusion of beta-amyloid peptide (Abeta) (25–35).

2. Methods

2.1. Animals

Adult male Wistar rats (220–315 g at time of surgery) from the State Health Research Foundation (FEPPS-RS) were housed five to a cage in a temperature-controlled colony room with food and water available ad libitum, and maintained on a 12-h light/dark cycle (lights on at 07:00 h). Behavioral procedures were conducted during the light phase of the cycle between 10:00 and 17:00 h. All experimental procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals (NIH publication No. 80-23 revised 1996). All efforts were made to minimize the number of animals and their suffering.

2.2. Surgery

Animals were implanted under thionembutal anesthesia (30 /kg, i.p.) with bilateral 9.0-mm, 23-gauge guide cannulae aimed 1.0 mm above the CA1 area of the dorsal hippocampus as described in previous studies (Bevilaqua et al., 1997; Walz et al., 2000; Roesler et al., 2003; Quevedo et al., 2004; Venturella et al., 2005). Coordinates (anteroposterior, −4.3 mm from bregma, mediolateral, ±3.0 mm from bregma, ventral, −1.4 mm from dura) were obtained from the atlas of Paxinos and Watson (1997). Animals were allowed to recover at least 7 days after surgery.

2.3. Behavioral training

We used the single-trial step-down inhibitory avoidance (IA) conditioning as an established model of aversively motivated, hippocampus-dependent memory (Izquierdo and Medina, 1997; Taubenfeld et al., 1999; McGaugh, 2000). In IA training, animals learn to associate a location in the training apparatus with an aversive stimulus (footshock). Consolidation of long-term memory for IA in rats has been previously shown to depend on activation of a number of neurotransmitter receptors and protein kinase pathways as well as protein synthesis and gene expression in the dorsal hippocampus (Bevilaqua et al., 1997; Izquierdo and Medina, 1997; Quevedo et al., 1999; Taubenfeld et al., 1999; McGaugh, 2000; Walz et al., 2000; Quevedo et al., 2004).

The IA behavioral training and retention test procedures were described in previous reports (Bevilaqua et al., 1997; Quevedo et al., 1999, 2004; Walz et al., 2000; Roesler et al., 2003; Roesler et al., 2004b,c; Venturella et al., 2005). The IA apparatus was a 50 × 25 × 25-cm acrylic box (Albarsch, Porto Alegre, Brazil) whose floor consisted of parallel caliber stainless steel bars (1 mm diameter) spaced 1 cm apart. A 7-cm wide, 2.5-cm high platform was placed on the floor of the box against the left wall. On the training trial, rats were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, rats received a 0.4-mA, 2.0-s footshock and were removed from the apparatus immediately after the footshock. A retention test trial was carried out 24 h after training. The retention test trial was procedurally identical to training, except that no footshock was presented. Step-down latencies on the retention test trial (maximum 180 s) were used as a measure of IA retention.

2.4. Drugs and infusion procedures

Intrahippocampal infusion procedures have been described in previous reports (Bevilaqua et al., 1997; Walz et al., 2000; Quevedo et al., 1999, 2004; Roesler et al., 2003; Venturella et al., 2005). At the time of infusion, a 30-gauge infusion needle was fitted into the guide cannula. The tip of the infusion needle protruded 1.0 mm beyond the guide cannula and was aimed at the CA1 area of the dorsal hippocampus. Drugs were infused during a 30-s period. After the infusion of drug or vehicle, the infusion needle was left in place for an additional minute to allow diffusion of the drug away from the needle tip.

For the first experiment, BB (0.002, 0.01, 0.05, or 0.25 μg in 0.5 μl; Sigma, St. Louis, MO, USA) was dissolved in saline (SAL, 0.9% NaCl) and infused bilaterally into the hippocampus immediately after IA training. Control animals were given a 0.5-μl bilateral infusion of SAL. For the second experiment, the GRPR antagonist [D-Tpi^{6}, Leu^{13} psi(CH_2NH)-Leu^{14}] bombesin (6–14) (RC-3095, 0.2 μg in 0.5 μl; Zentaris GmbH, Frankfurt, Germany), the NMBR antagonist BIM 23127 (0.1 μg in 0.5 μl; Sigma), the PKC inhibitor Gö 7874 (0.5 pg in 0.5 μl; Calbiochem, San Diego, USA), the MAPK kinase inhibitor PD098059 (5.0 ng in 0.5 μl; Calbiochem), the PKA inhibitor Rp-cAMPs (0.02 μg in 0.5 μl; Sigma), or vehicle (VEH, 2% dimethylsulfoxide (DMSO) in SAL; 0.5 μl) were infused bilaterally into the hippocampus 10 min prior to IA training, and BB (0.01 μg in 0.5 μl) or SAL were infused immediately after training. For the third experiment, RC-3095 (0.2 μg in 0.5 μl), the dopamine D1/D5 receptor (D1R) agonist SKF 38393 (7.5 μg in 0.5 μl; Sigma), the adenylyl cyclase (AC) stimulator forskolin (0.5 μg in 0.5 μl; Sigma), the cAMP analog 8-Br-cAMP (1.25 μg in 0.5 μl; Sigma), or the PKA inhibitor Rp-cAMPS (0.02 μg in 0.5 μl; Sigma), were infused alone or in combination with BB (0.01 μg in 0.5 μl) immediately after IA training. All drugs were dissolved in VEH. Control animals were given a bilateral 0.5-μl infusion of VEH. For the fourth experiment, BB (0.002 μg in 0.5 μl) or SAL (0.5 μl) were bilaterally infused into the hippocampus 10 min prior to IA training, and distilled water (DW, 0.5 μl) or Abeta (25–35) (0.02 μg in 0.5 μl; Sigma) were infused immediately after training. Previous experiments from our laboratory have indicated that a single intrahippocampal infusion of Abeta (25–35) at 0.02 μg induces IA memory impairment without causing significant neuronal death in the CA1 area (Luft et al., unpublished results). For all experiments, drug doses were chosen on the basis of previous studies (Bevilaqua et al., 1997; Vianna et al., 2000a,b; Walz et al., 2000; Freir et al., 2001; Roesler et al., 2003; Costello and Herron, 2004; Quevedo et al., 2004; Tsushima and Mori, 2005) and pilot experiments. Although higher doses of Gö 7874 and Rp-cAMPS have been shown to impair memory retention when infused immediately posttraining into the hippocampus in previous studies from our group (Vianna et al., 2000a,b; Quevedo et al., 2004), lower doses of those drugs were used in the present study which did not affect IA memory. Drug solutions were freshly prepared before each experiment.

2.5. Histology

Twenty-four to 48 h after behavioral testing, the animals were killed by decapitation and their brains were removed, stored in 5% formalin for at least 72 h and verified for infusion site placements as follows: 0.5 μl of a 4% methylene blue solution was infused as described above and the extension of the dye was taken as indicative of diffusion of the drugs previously given to each rat (Bevilaqua et al., 1997; Walz et al., 2000; Quevedo et al., 1999, 2004; Roesler et al., 2003; Venturella et al., 2005). Only data from animals with correct infusion sites (314 rats) were included in the final analysis (Fig. 1).

2.6. Statistics

Data are mean ± SEM retention test latencies to step-down (s). Comparisons of training and retention test step-down latencies among groups were performed using Kruskal–Wallis analysis of variance followed by Mann–Whitney *U*-tests, two-tailed, when necessary (Bevilaqua et al., 1997; Walz et al., 2000; Quevedo et al., 1999, 2004; Roesler et al., 2003; Venturella et al., 2005). In all comparisons, $P < 0.05$ was considered to indicate statistical significance.

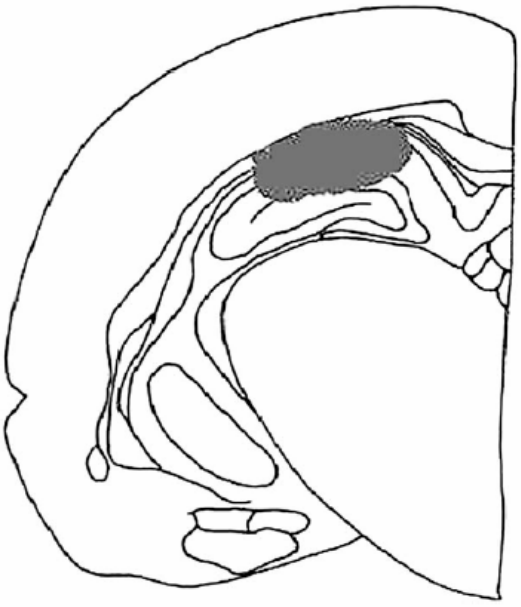

Fig. 1. Drawing of the plane A −4.3 mm of the atlas of Paxinos and Watson (1997) showing the area (hatched) where the infusion sites considered to be correct were placed.

3. Results

3.1. Bombesin modulation of memory consolidation in the hippocampus

The first experiment examined the effects of posttraining intrahippocampal infusions of BB on IA memory retention. BB at the doses of 0.01 and 0.05 μg induced a significant

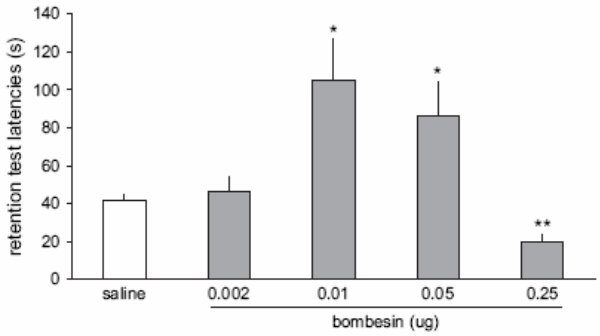

Fig. 2. Bombesin (BB)-induced modulation of memory consolidation in the hippocampus. Data are mean ± SEM 24-h retention step-down latencies (s) of rats given bilateral 0.5 μl-infusions of BB (0.002, 0.01, 0.05, or 0.25 μg) or saline (SAL) into the dorsal hippocampus immediately after inhibitory avoidance (IA) training ($n = 7–12$ animals per group). *$P < 0.05$ and ** $P < 0.01$ compared to the SAL-treated group.

enhancement of IA memory retention (both *P*s <0.05 compared to the SAL-treated group), whereas BB at 0.25 μg impaired retention ($P < 0.01$ compared to the SAL-treated group) (Fig. 2). There was no significant difference among groups in training trial latencies ($P = 0.50$, overall mean ± SEM training trial step-down latency (s) was 9.14 ± 0.51). The results indicate that low and high doses of BB induce opposite effects on IA memory consolidation in the dorsal hippocampus.

3.2. Bombesin-induced enhancement of memory consolidation in the hippocampus depends on GRPRs, PKC, MAPK, and PKA

The second experiment examined the mechanisms underlying the effect of a memory-enhancing dose of BB into the hippocampus. Results are shown in Fig. 3. Posttraining infusion of BB at 0.01 μg induced significant IA retention enhancement ($P < 0.01$ compared to the control group treated with SAL and VEH). Pretraining infusions of the GRPR antagonist RC-3095, the PKC inhibitor Gö 7874, the MAPK kinase inhibitor PD 098059, or the PKA inhibitor Rp-cAMPS did not affect retention, but prevented the retention enhancement induced by posttraining BB. Animals treated with a pretraining infusion of the NMBR antagonist BIM 23127 and a posttraining infusion of BB showed a significantly higher retention than the control group treated with SAL and VEH ($P < 0.05$). There was no significant difference among groups in training trial latencies ($P = 0.27$, overall mean ± SEM training trial step-down latency (s) was 8.88 ± 0.51). The results suggest that BB-induced enhancement of memory retention in the hippocampus depends on GRPRs, PKC, MAPK and PKA, but not NMBRs.

3.3. Bombesin-induced enhancement of memory consolidation in the hippocampus is potentiated by stimulators of the D1R/cAMP/PKA pathway and prevented by a PKA inhibitor

The finding that PKA inhibition prevented BB-induced memory enhancement was somewhat unexpected because previous studies in cancer cells have indicated that PKA inhibitors do not affect the cellular effects of GRPR activation (Kim et al., 2000; Qu et al., 2002). We thus decided to further evaluate the interactions between the GRPR and the PKA pathway in the hippocampus. The third experiment examined the effects of a memory-enhancing dose of BB coinfused with stimulators of the D1R/cAMP/PKA pathway or a PKA inhibitor after IA training. Results are shown in Fig. 4. The group treated with BB alone showed a significant enhancement of IA retention ($P < 0.01$ compared with the VEH group). Coinfusion with RC-3095 prevented the BB-induced retention enhancement, indicating that the BB effect was mediated by GRPR activation. The D1R receptor agonist SKF 38393, the AC activator forskolin, and the cAMP analog 8-Br-cAMP did not affect retention when infused alone, but potentiated the memory-enhancing effect of BB. The groups treated with BB combined with SKF 38393, forskolin, or 8-Br-cAMP showed significantly higher retention latencies than the group treated with BB alone (all *P*s <0.05). Infusion of an otherwise ineffective dose of the PKA inhibitor Rp-cAMPS prevented the memory-enhancing effect of BB. There was no significant difference among groups in training trial latencies ($P = 0.49$, overall mean ± SEM training trial step-down latency (s) was 7.77 ± 0.48). The results suggest that the enhancing effect of BB on IA memory retention in the hippocampus requires PKA and is potentiated by stimulation of the D1R/cAMP/PKA pathway.

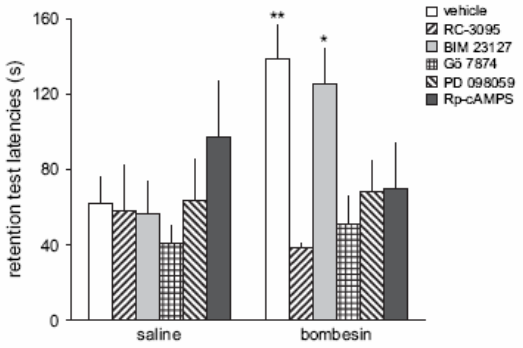

Fig. 3. Bombesin (BB)-induced enhancement of memory consolidation in the hippocampus requires gastrin-releasing peptide receptors (GRPR), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and protein kinase A (PKA). Data are mean ± SEM 24-h retention step-down latencies (s) of rats given bilateral 0.5 μl-infusions of the GRPR antagonist RC-3095 (0.2 μg), the neuromedin B receptor (NMBR) antagonist BIM 23127 (0.1 μg), the PKC inhibitor Gö 7874 (0.5 pg), the MAPK kinase inhibitor PD098059 (5.0 ng), the PKA inhibitor Rp-cAMPs (0.02 μg), or vehicle (VEH, 2% dimethylsulfoxide (DMSO) in saline (SAL)) 10 min before inhibitory avoidance (IA) training, and BB (0.01 μg in 0.5 μl) or SAL immediately after training ($n = 7–13$ animals per group). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the group treated with VEH and SAL.

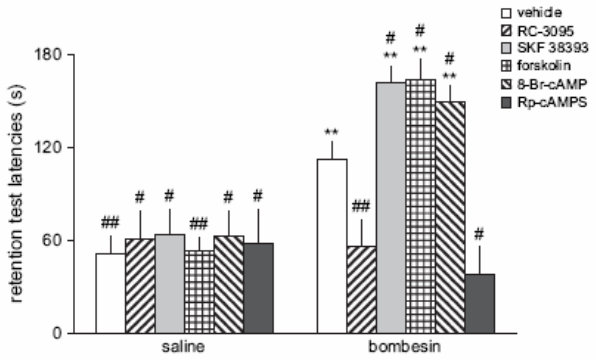

Fig. 4. Bombesin (BB)-induced enhancement of memory consolidation in the hippocampus is potentiated by activators of the dopamine D1/D5 receptor (D1R)/cAMP/protein kinase A (PKA) pathway and prevented by PKA inhibition. Data are mean ± SEM 24-h retention step-down latencies (s) of rats given bilateral 0.5 μl-infusions of the gastrin-releasing peptide receptor (GRPR) antagonist RC-3095 (0.2 μg), the D1R agonist SKF 38393 (7.5 μg), the adenylyl cyclase (AC) stimulator forskolin (0.5 μg), the cAMP analog 8-Br-cAMP (1.25 μg), the PKA inhibitor Rp-cAMPS (0.02 μg), or vehicle (VEH, 2% dimethylsulfoxide (DMSO) in saline (SAL)), alone or combined with BB (0.01 μg), immediately after inhibitory avoidance (IA) training ($n = 7–12$ animals per group). $^{**}P < 0.01$ compared to the VEH-treated group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to the group treated with BB in VEH.

3.4. Bombesin prevents beta-amyloid peptide (25–35)-induced impairment of memory consolidation in the hippocampus

Previous studies have indicated that BB-like peptides and the GRPR might be involved in the pathogenesis of AD (Ito et al., 1994; Gibson and Huang, 2005; Roesler et al., 2006). In addition, systemic administration of GRP has been shown to improve memory deficits in the scopolamine-induced amnesia model in mice (Santo-Yamada et al., 2001). Application of Abeta (25–35) to the CA1 hippocampal area in vivo and in vitro has been used as a model to investigate the impairment of synaptic plasticity associated with AD (Saleshando and O'Connor, 2000; Freir et al., 2001; Costello and Herron, 2004). Intrahippocampal and intracerebroventricular infusions of Abeta (25–35) in rats have also been used as models of cognitive impairment associated with AD (Chen et al., 1996; Stepanichev et al., 2005). The fourth experiment examined whether BB could prevent IA memory deficit induced by a single posttraining administration of Abeta (25–35) into the hippocampus. Results are shown in Fig. 5. Posttraining intrahippocampal infusion of Abeta (25–35) induced a significant impairment of IA retention ($P < 0.01$ compared to the control group given SAL and DW). Pretraining infusion of an otherwise ineffective dose of BB prevented the Abeta (25–35)-induced retention impairment. There was no significant difference among groups in training trial latencies ($P = 0.16$, overall mean ± SEM training trial step-down latency (s) was 8.20 ± 0.78). The result indicates that GRPR agonists can prevent memory impairments elicited by Abeta (25–35) in the hippocampus.

4. Discussion

The present experiments used IA behavioral training and hippocampal infusions to examine the cellular signaling mechanisms mediating the effects of BB on memory consolidation in the hippocampus. Our results can be summarized as follows: (1) lower doses of BB enhance, whereas a higher dose of BB impair consolidation of IA memory when infused posttraining into the CA1 hippocampal area; (2) the BB-induced memory enhancement in the hippocampus requires GRPRs, PKC, MAPK, and PKA, but not NMBRs; (3) the memory-enhancing effect of BB in the hippocampus is potentiated by stimulators of the D1R/cAMP/PKA pathway and prevented by PKA inhibition; and (4) BB prevents the impairment of memory consolidation induced by administration of Abeta (25–35) into the hippocampus. The use of posttraining infusions of BB rules out the possibility that the effects were due to drug-induced alterations in attentional, motivational, motor, or sensory-perceptual mechanisms at training.

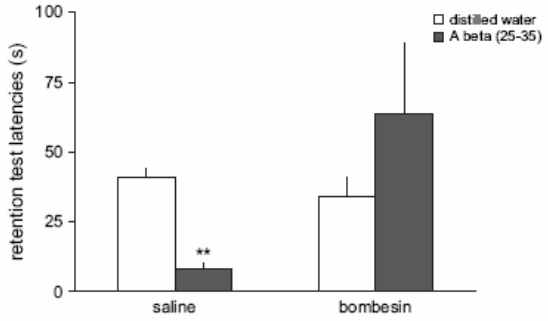

Fig. 5. Bombesin (BB) prevents beta-amyloid peptide (Abeta) (25–35)-induced impairment of memory consolidation in the hippocampus. Data are mean ± SEM 24-h retention step-down latencies (s) of rats given bilateral 0.5 μl-infusions of BB (0.002 μg) or saline (SAL) 10 min before inhibitory avoidance (IA) training, and Abeta (25–35) or distilled water (DW) immediately after training ($n = 8$–14 animals per group). $^{**}P < 0.01$ compared to the group treated with SAL and DW.

The mammalian counterpart of BB, GRP, has been proposed to be co-released with glutamate from glutamatergic neurons, and act by binding to GRPRs on postsynaptic sites (Lee et al., 1999; Shumyatsky et al., 2002). The GRPR is expressed in neurons throughout the mammalian central nervous system, including the CA1 area of the dorsal hippocampus (Kamichi et al., 2005). Previous studies have shown that systemic administration of the GRPR agonists BB and GRP induce memory enhancement in rats and mice (Flood and Morley, 1988; Rashidy-Pour and Razvani, 1998; Santo-Yamada et al., 2001). Conversely, GRPR antagonists induce memory impairment when given systemically (Santo-Yamada et al., 2003; Roesler et al., 2004b; Martins et al., 2005) or into brain areas including the dorsal hippocampus and basolateral amygdala (Roesler et al., 2003, 2004c; Venturella et al., 2005). These findings suggest that GRPR activation plays a stimulatory role in memory formation. However, other studies have proposed that the GRPR is located predominantly on inhibitory interneurons releasing gamma-aminobutyric acid (GABA), and GRPR activation would lead to an increase in GABAergic transmission, which would in turn inhibit synaptic plasticity and memory (Lee et al., 1999; Shumyatsky et al., 2002). Consistent with this view, BB induces depolarization of inhibitory interneurons in hippocampal slices (Lee et al., 1999) and GRPR-deficient mice show enhanced fear-motivated conditioning and synaptic plasticity in the amygdala, but normal hippocampal memory (Shumyatsky et al., 2002). Thus, the role of the GRPR in hippocampal function and memory formation remains controversial. The results of the present study clearly indicate that the GRPR in the dorsal hippocampus modulates memory consolidation of an emotionally motivated, hippocampus-dependent task, and that BB at lower doses induces memory enhancement through stimulation of GRPRs in the dorsal hippocampus. How could one reconcile the present findings, together with those from other studies indicating that GRPR activation stimulates synaptic plasticity and memory, with studies suggesting that the GRPR acts as an inhibitory system? One possibility is that the GRPR is expressed on both inhibitory GABAergic neurons and excitatory glutamatergic neurons, as well as on neurons releasing other neurotransmitters such as serotonin and dopamine. Although to our knowledge there is no direct evidence for the expression of GRPRs on excitatory neurons, the recent finding

by Kamichi et al. (2005) that in the lateral amygdala only a subpopulation of cells expressing GRPRs are GABAergic neurons is consistent with the possibility that GRPRs are expressed on non-GABAergic neurons releasing glutamate or other neurotransmitters. Thus, different doses of GRPR agonists could induce differential effects on excitatory and inhibitory transmission, either stimulating or inhibiting synaptic plasticity and memory. Our finding that low and high doses of BB induced opposite effects on memory consolidation, as well as our recent observation that high doses of the GRPR antagonist RC-3095 can enhance IA memory consolidation (Dantas et al., in press) support this possibility.

Although BB-like peptides and the GRPR have been previously implicated in memory formation, previous studies have not investigated the underlying molecular mechanisms. Extensive evidence indicates that the PKC, MAPK and PKA pathways are critical in mediating memory consolidation in the hippocampus (Bevilaqua et al., 1997; Izquierdo and Medina, 1997; McGaugh, 2000; Quevedo et al., 2004). Previous studies using cancer and neuroendocrine cells have suggested that intracellular responses to GRPR activation involve a GRPR-elicited [Ca^{2+}] increase and activation of the phospholipase C (PLC)/PKC pathway, which, in turn activates the MAPK/ERK pathway. Thus, cellular responses to GRPR agonists are blocked by PKC and MAPK inhibitors (Hellmich et al., 1999; Kim et al., 2000; Qu et al., 2002; Xiao et al., 2003; Chen and Kroog, 2004; Stangelberger et al., 2005; Thomas et al., 2005). Consistent with these findings, our results clearly indicate that memory modulation by the GRPR in the hippocampus requires both PKC and MAPK. In addition, our findings indicate that memory modulation by BB was blocked by an otherwise ineffective dose of a PKA inhibitor and potentiated by activators of the PKA pathway. These findings were somewhat unexpected because the GRPR is coupled to the G_q family of G proteins, which directly activates the PKC but not the PKA pathway (Chan and Wong, 2005). In addition, previous studies have indicated that PKA inhibition does not prevent GRPR-elicited cellular responses (Kim et al., 2000; Qu et al., 2002). However, a possible role for cAMP signaling in the effects of GRPR antagonists in human pancreatic adenocarcinoma has been suggested by Qin et al. (1995), and a recent study has described a complex interaction between the GRPR and the D1R/cAMP/PKA pathway in COS-7 cells, in which co-stimulation of the GRPR and D1R inhibits GRPR-triggered protein kinase activity (Chan and Wong, 2005). Several mechanisms involved in cross-talk among the PKC, MAPK and PKA pathways could explain the requirement of PKA for BB modulation of memory consolidation. For instance, MAPK/ERK activity is synergistically enhanced by Ca^{2+} and activators of the cAMP/PKA pathway in hippocampal neurons (Impey et al., 1998). One possibility is that a GRPR-triggered increase in [Ca^{2+}] leads to stimulation of Ca^{2+}-responsive adenylyl cyclase (AC), thus further enhancing the raise in cAMP levels induced by stimulators of the D1R/cAMP/PKA pathway. This would be consistent with the model recently proposed by Chan and Wong (2005), in which a rise in [Ca^{2+}] elicited by GRPR stimulation leads to increased AC activity and cAMP levels in COS-7 cells, and also with the finding that Ca^{2+}-stimulated AC in the dorsal hippocampus plays a critical role in synaptic plasticity and long-lasting memory (Wong et al., 1999). Fig. 6 shows a schematic for a proposed model of GRPR interactions with the PKC, MAPK, and PKA pathways in regulating memory consolidation in the hippocampus.

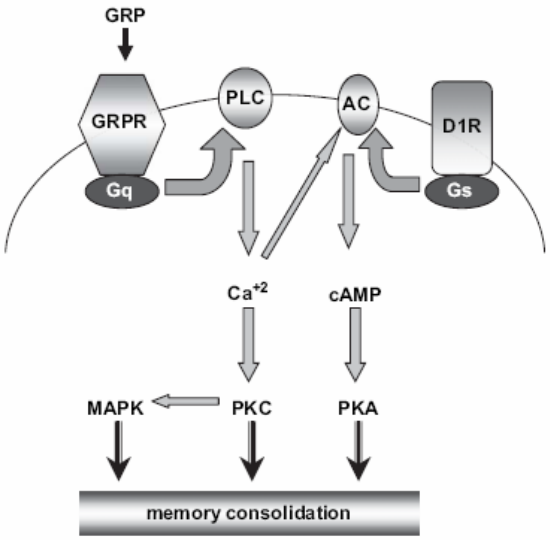

Fig. 6. Schematic diagram for a model of cellular signaling mechanisms mediating the regulatory actions of GRPR on memory consolidation in the hippocampus. Gastrin-releasing peptide (GRP) released from synaptic terminals binds to the G_q protein (G_q)-coupled GRP receptor (GRPR) at postsynaptic sites. GRPR activation induces an increase in [Ca^{2+}] and triggers activation of the phospholipase C (PLC)/protein kinase C (PKC) pathway, which, in turn, can activate mitogen-activated protein kinase (MAPK) (Hellmich et al., 1999). The dopamine D1/D5 receptor (D1R) is coupled to G_s protein (G_s) and adenylyl cyclase (AC) activation. The D1R-induced cAMP signal might be synergistically potentiated by [Ca^{2+}]-induced stimulation of [Ca^{2+}]-responsive types of AC (Wong et al., 1999; Chan and Wong, 2005), leading to increased activation of protein kinase A (PKA).

Several lines of evidence have indicated that the GRPR might be involved in cognitive dysfunctions associated with AD and other neurodegenerative and psychiatric disorders (for a review, see Roesler et al., 2006). For instance, alterations in GRPR density and dysfunctions in BB-elicited Ca^{2+} signaling have been described in fibroblasts and leucocytes from patients with AD (Ito et al., 1994; Gibson and Huang, 2005). These data, together with the present finding that BB might enhance memory retention by stimulating protein kinase pathways critically involved in mediating synaptic plasticity suggest that the GRPR could be considered a molecular target for the development of novel cognitive enhancers. Consistent with the view that GRPR agonists can display cognitive-enhancing properties in models of amnesia, Santo-Yamada et al. (2001) have shown that systemic administration of GRP attenuated scopolamine-induced memory impairment in mice. Based on previous electrophysiological (Saleshando and O'Connor, 2000; Freir et al., 2001; Costello and Herron,

2004) and behavioral (Chen et al., 1996; Stepanichev et al., 2005) experiments, in the present study we used a single intrahippocampal infusion of a low dose of Abeta (25–35) in rats as a model of memory dysfunction associated with AD. Administration of Abeta (25–35) to the hippocampus in vitro or in vivo can impair synaptic plasticity through a mechanism involving the MAPK pathway (Saleshando and O'Connor, 2000; Freir et al., 2001; Costello and Herron, 2004). Our finding that pretraining administration of an otherwise ineffective dose of BB prevented the Abeta (25–35)-induced impairment of IA retention supports the view that the GRPR is a molecular target for the development of cognitive enhancers for treatment of memory dysfunction associated with AD and other neuropsychiatric disorders.

In summary, the present results suggest that the GRPR regulates memory consolidation in the hippocampus through a mechanism involving the PKC, MAPK and PKA signaling pathways. In addition, administration of the GRPR agonist BB prevented memory impairment induced by Abeta (25–35) in the hippocampus. This is the first study investigating the molecular mechanisms mediating memory modulation by the GRPR.

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ANEXO II

Targeting the gastrin-releasing peptide receptor pathway to treat cognitive dysfunction associated with Alzheimer's disease

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Targeting the gastrin-releasing peptide receptor pathway to treat cognitive dysfunction associated with Alzheimer's Disease

Rafael Roesler[1,2], Tatiana Luft[1,3], Gilberto Schwartsmann[2,4]

Abstract – Increasing evidence indicates that bombesin (BB)-like peptides (BLPs), such as the gastrin-releasing peptide (GRP) and its receptor (GRPR), might play a role in neurological and psychiatric disorders. The present study reviews findings from animal and human studies suggesting that the GRPR should be considered a target for the treatment of cognitive dysfunction in patients with Alzheimer's disease (AD). Abnormalities in GRPR-triggered signaling have been described in both fibroblasts from patients with AD, and in transgenic mouse models of AD. Pharmacological and genetic preclinical studies have indicated that BLPs and the GRPR are importantly involved in regulating cognitive function. Moreover, drugs acting at the GRPR have been shown to enhance memory and ameliorate cognitive dysfunction in experimental models of amnesia associated with AD. Taken together, these findings support the view that the GRPR is a novel therapeutic target for the treatment of memory deficits associated with AD.

Key words: bombesin-like peptides, gastrin-releasing peptide, gastrin-releasing peptide receptor, cognitive enhancers, memory disorders, Alzheimer disease.

O receptor do peptídeo liberador de gastrina como novo alvo terapêutico para o tratamento da disfunção cognitiva associada à Doença de Alzheimer

Resumo – Estudos recentes indicam que os peptídeos da família da bombesina (BB), como o peptídeo liberador de gastrina (GRP) e seu receptor (GRPR), podem estar envolvidos em doenças neurológicas e psiquiátricas. Este artigo apresenta uma revisão de estudos tanto em humanos como em modelos animais que sugerem que o GRPR deve ser considerado um alvo molecular para o desenvolvimento de novas terapias para o tratamento de déficits cognitivos em pacientes com doença de Alzheimer (DA). Anormalidades na sinalização celular dependente do GRPR têm sido descritas tanto em fibroblastos de pacientes com DA como em modelos de DA em camundongos transgênicos. Além disso, estudos pré-clínicos utilizando estratégias farmacológicas e genéticas indicam que os peptídeos da família da BB e o GRPR estão envolvidos de forma importante na regulação da função cognitiva. Finalmente, resultados recentes mostram que drogas que agem como ligantes do GRPR podem melhorar a memória e prevenir disfunções cognitivas em modelos experimentais de amnésia asssociada à DA. Em conjunto, os dados indicam que o GRPR é um novo alvo terapêutico para o tratamento de déficits de memória associadas à DA.

Palavras-chave: bombesina, peptídeo liberador de gastrina, receptor do peptídeo liberador de gastrina, facilitadores cognitivos, disfunções de memória, doença de Alzheimer.

Bombesin-like peptides and their receptors in the brain

Bombesin (BB) is a 14 amino acid initially isolated from the skin of frogs *Bombina bombina*. It was later described that gastrin-releasing peptide (GRP), a 27 amino acid peptide functionally and structurally related to BB, is a mammalian counterpart of BB (Table 1). BB and GRP, as well as other related peptides such as neuromedin (NM) B (NMB), constitute a family of BB-like peptides (BLPs). BLPs have been described to affect a range of cellular and

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Table 1. Structures of bombesin (BB) and gastrin-releasing peptide (GRP).

Bombesin
Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
Gastrin-releasing peptide
Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

Adapted from [1,4,7].

neuroendocrine functions, including cell proliferation and differentiation, cancer growth, feeding behavior, and stress responses (for recent reviews, see[1-4]).

Early studies investigating the presence of BB binding sites in the mammalian central nervous system (CNS) showed that BB bound with high affinity to rat brain membranes. The hippocampus, a brain area critically involved in cognitive function and neurodegenerative and neuropsychiatric disorders, including Alzheimer's disease (AD), had the highest density of specific BB binding sites.[5] Subsequent studies identified the occurrence of endogenous BLPs as neuropeptides in the rat CNS. It is now well established that GRP, the main mammalian BLP, is like a co-transmitter released from both central and peripheral neurons that regulates aspects of brain function including memory and emotional processing (for reviews, see[1,4]) (Table 1).

The gastrin-releasing peptide (GRPR) receptor and associated signal transduction pathways

BB and GRP exert most of their biological actions by binding at the GRP receptor (GRPR, also known as BB2 receptor). GRPR is a member of the G-protein coupled receptor superfamily containing seven transmembrane domains and 384 amino acids.[6-8] GRPR is highly expressed in the brain. Studies using in vitro autoradiographic techniques have indicated that brain areas containing high densities of GRPRs include the olfactory bulb, nucleus accumbens, caudate putamen, central amygdala, dorsal hippocampal formation, as well as the paraventricular, central medial, and paracentral thalamic nuclei.[1,4,9,10] A recent seminal immunohistochemical study has used affinity-purified GRPR antibodies to examine the precise distribution of GRPR in the mouse brain. GRPR immunoreactivity was widely distributed in the isocortex, hippocampus, piriform cortex, amygdala, hypothalamus, and brain stem, with high concentrations in the dorsal hippocampus and lateral amygdala. In addition, GRPR expression was specific for the cell membranes of neuronal dendrites and cell bodies.[11]

Intracellular responses to GRPR activation were initially examined in cancer and neuroendocrine cell lines. Cellular signaling pathways for the GRPR have been shown to include protein kinase signaling cascades, particularly the protein kinase C (PKC) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) pathways.[12-14] In the brain, GRP-induced neuronal membrane depolarization in the rat hippocampus is blocked by a PLC inhibitor,[15] and we have recently shown that modulation of the rat hippocampal function by BB depends on the PKC, MAPK and PKA pathways (Figure).[16]

An increasing body of evidence indicates that BLPs

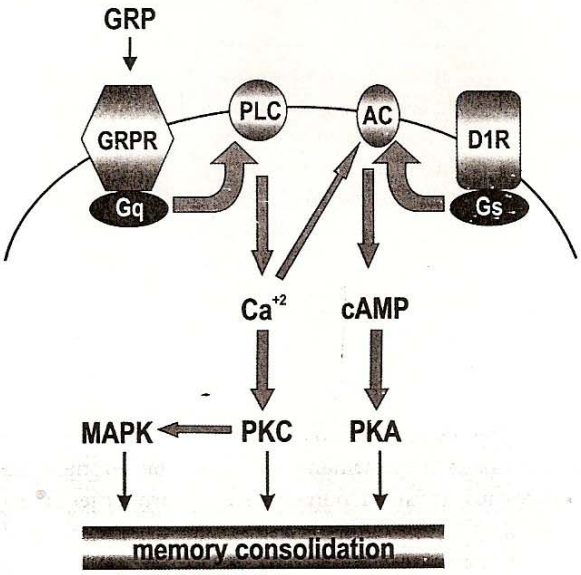

Figure. *Proposed signaling pathways associated with the gastrin-releasing peptide receptor (GRPR) in the central nervous system. Gastrin-releasing peptide (GRP) released from synaptic terminals binds to the G_q protein-coupled GRPR at postsynaptic sites. GRPR activation induces an increase in $[Ca^{2+}]$ and triggers activation of the phospholipase C (PLC)/protein kinase C (PKC) pathway, which, in turn, can activate mitogen-activated protein kinase (MAPK). The dopamine D1/D5 receptor (D1R) is coupled to G_s protein (G_s) and adenylyl cyclase (AC) activation. The D1R-induced cAMP signal might be synergistically potentiated by $[Ca^{2+}]$-induced stimulation of $[Ca^{2+}]$-responsive types of AC, leading to increased activation of protein kinase A (PKA). Reproduced from [16], with permission.*

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and the GRPR might play a role in CNS disease, including memory disorders associated with AD and other neurodegenerative disorders. Thus, our group has put forward the GRPR as a novel therapeutic target for the development of therapies to treat neurological and psychiatric disorders.[4,17] The present study reviews current evidence suggesting the GRPR should be considered a target for the treatment of cognitive dysfunction in patients with AD.

Abnormalities in GRPR function in Alzheimer's disease: evidence from mice and human studies

Increasing evidence from animal and human studies has indicated that abnormalities in BLPs- and GRPR-triggered cellular signaling might be associated with AD. Dysregulation of calcium signaling has been causally implicated in both normal brain aging and AD. BB stimulates calcium release from BB-releasable calcium stores in the endoplasmic reticulum (ER). Exaggerated BB-induced intracellular calcium release has been demonstrated in fibroblasts and neurons from genetically modified mice bearing a mutation in the presenilin-1 (PS-1) mutation.[18] These transgenic mice have been developed as a useful animal model since mutations in the presenilin-1 (PS1) gene on chromosome 14 are causally linked to many cases of early-onset inherited AD.[18,19] Importantly, the alterations in BB-induced enhancement of calcium signaling observed in this mouse model resemble those described in patients with AD. Both increased and reduced calcium signals have been described in AD patients. Thus, fibroblasts from familial and non-familial AD cases have shown enhanced calcium signals induced by BB compared to controls.[20-24] In contrast, in fibroblasts from patients with familial Alzheimer's disease presenting the Swedish APP670/671 mutation, BB-induced elevations in calcium were found to be reduced by 40%.[21] These abnormalities in BB-regulated calcium homeostasis observed in AD fibroblasts have been proposed to involve alterations in oxidative stress.[20-23,25] Since alterations in calcium signaling and oxidative stress might be involved in neurodegeneration and cognitive impairment in AD patients, these findings from mouse and human studies support the view that BLP-triggered signaling and the GRPR pathway might play a role in the pathogenesis of AD.

Another cellular change related to BLP- and GRPR-elicited signaling described in fibroblasts from patients with AD, is a reduced number of BB receptors.[24] This interesting finding raises the possibility that decreased neuronal GRPR density, leading to impaired BLP function in the brain of AD patients, is related to neurodegeneration and memory loss associated with the disease. Table 2 summarizes relevant alterations in the GRPR pathway observed in patients with AD (Table 2).

Effects of drugs acting at the GRPR on cognitive function: preclinical findings

The present and other authors have used rodent models of learning and memory to investigate the role of brain BLPs and the effects of drugs acting at the GRPR in cognitive function. Systemic administration of BB or GRP enhances memory retention in rats and mice,[26,27] whereas injections of GRPR antagonists cause impairment.[28-32] GRPR agonists and antagonists also modulate memory formation and extinction when infused intracranially into specific brain areas.[16,31,33-38] For instance, GRPR inactivation in either the dorsal hippocampus or basolateral amygdala by infusions of the selective GRPR antagonist [D-Tpi,[6] Leu[13] psi(CH_2NH)-Leu[14]] bombesin (6-14) (RC-3095), a synthetic BB analog, hinders retention of memory for inhibitory avoidance, a type of fear conditioning-based task, in rats.[31,36,37] Moreover, the findings from pharmacological studies are supported by genetic evidence showing altered memory formation and synaptic plasticity in GRPR-deficient knockout mice.[39]

Our group has shown that the dorsal hippocampus is a brain area crucially involved in mediating the regulatory actions of BLPs on memory.[16,33,34,36,37] Importantly, microinfusion of BB into the rat CA1 hippocampal area has enhanced inhibitory avoidance consolidation. We went on to investigate the molecular mechanisms mediating the memory-enhancing effect of intrahippocampal BB administration. BB-induced modulation of memory consolidation was prevented by infusion of a GRPR antagonist or

Table 2. Abnormalities in the gastrin-releasing peptide receptor (GRPR) pathway in patients with Alzheimer's disease (AD).

Finding	References
Enhanced bombesin (BB)-induced calcium release in fibrobasts	[23, 24]
Reduced BB-induced calcium mobilization in fibrobasts in patients with the Swedish APP670/671 mutation	[21]
Increased response of BB-induced calcium release to oxidant agents in patients with presenilin-1 (PS-1) mutation	[23]
Reduced number of gastrin-releasing peptide receptors (GRPRs) in fibroblasts	[24]

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Table 3. Findings from preclinical studies indicating that drugs acting at the gastrin-releasing peptide receptor (GRPR) can display cognitive-enhancing properties.

Species	Finding	References
Rat	Memory enhancement by systemic administration of bombesin (BB) or gastrin-releasing peptide (GRP)	[26, 27]
Rat	Enhancement of fear memory by intrahippocampal infusion of BB	[16]
Rat	Memory enhancement by infusion of BB into the nucleus tractus solitarius (NTS)	[38]
Rat	Enhancement of fear memory by intrahippocampal infusion of an administration of a GRP receptor (GRPR) antagonist	[33]
Rat	Enhancement of fear memory by intraamygdala infusion of a GRPR antagonist	[35]
Mouse	Enhancement of fear memory and synaptic plasticity in GRPR-deficient knockout mice	[39]
Mouse	Improvement of scopolamine and hypoxia-induced amnesia by systemic administration of GRP	[40]
Rat	Prevention of memory impairment induced by beta-amyloid peptide (25-35) by intrahippocampal infusion of BB	[16]

inhibitors of the PKC, MAPK, and PKA signaling pathways. These findings indicated that BB (and presumably other BLPs) might facilitate cognitive function by activating GRPRs in hippocampal neuronal membranes, thus leading to activation of intracellular signal transduction pathways known to mediate synaptic plasticity and memory formation.[16] Other experiments have suggested that the GRPR signaling system might have functional interactions with glucocorticoid receptors[37] and inhibitory neurons releasing gamma-aminobutyric acid (GABA)[33] in regulating memory formation in the hippocampus.

Prevention of memory impairment induced by the Alzheimer peptide through a GRPR agonist in a rat model

Our findings described above, that BB can stimulate cellular signaling mechanisms that mediate synaptic plasticity and enhance memory formation, suggest that BLPs should be further evaluated as potential cognitive enhancers in experimental amnesia. In fact, systemic injection of GRP has been shown to attenuate memory deficits in the scopolamine- and hypoxia-induced models of memory impairment in mice.[40] We thus decided to examine the effects of GRPR activation by BLPs in an experimental model of memory disorders associated with AD. Rats were given an infusion of a low dose of the neurotoxic fragment of beta-amyloid peptide (Abeta 25-35) into the CA1 hippocampal area. Intrahippocampal administration of Abeta (25-35) produced an impairment of retention of memory for inhibitory avoidance conditioning. GRPR activation by administration of BB to the hippocampus before avoidance training prevented the Abeta (25-35)-induced memory impairment.[16] This finding indicates that BB and other GRPR agonists might prevent cognitive deficits associated with AD. Table 3 summarizes findings from animal studies supporting the view that drugs acting on the GRPR might display cognitive-enhancing properties.

Perspectives on the clinical use of drugs acting at the GRPR as cognitive enhancers in patients with Alzheimer's disease

The data reviewed above can be summarized as follows: (1) the human BLP, GRP, and its receptor, GRPR, are expressed in neurons, and particularly high densities of GRP and GRPR occur in brain areas importantly involved in cognitive function and dementia, such as the hippocampus; (2) evidence from mouse and human studies suggest that abnormalities in GRPR expression and aspects of GRPR signaling relevant for neurodegeneration and cognitive function (i.e., cellular calcium homeostasis, oxidative stress) might be associated with AD; (3) preclinical studies show that GRP and the GRPR are importantly involved in regulating synaptic plasticity and memory formation in the hippocampus and other brain areas; and (4) GRPR agonists can prevent memory disorders in a rat model of amnesia associated with AD. Together, these findings constitute a consistent body of evidence supporting the view that drugs acting at the GRPR should be further evaluated as potential cognitive enhancers to treat memory disorders associated with AD and other neurodegenerative and psychiatric disorders. In addition to the amphibian and mammalian BLPs that act as GRPR agonists, namely BB and GRP, we have recently shown that the BB analog and GRPR antagonist RC-3095 can also enhance memory when given at high doses to rats.[33] Thus, both naturally-occurring BLPs and synthetic BB analogs, might display cognitive-enhancing properties and could be considered candidate drugs for the treatment of memory disorders. In addition, our recent findings that the GRPR modulates inflammatory responses,[41] raises the possibility that GRPR ligands could

display neuroprotective actions in addition to facilitating memory in AD patients. Since previous clinical studies in the fields of gastroenterology and oncology have indicated that BLPs and RC-3095 do not induce overt side effects when administered intravenously in humans,[42,43] clinical trials evaluating the effects of drugs acting at the GRPR on cognitive function in patients with AD and other neurodegenerative and psychiatric disorders are warranted.

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APÊNDICE

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