JULIANA PRESTI TORRES

EFEITOS COMPORTAMENTAIS DO TRATAMENTO NEONATAL COM UM ANTAGONISTA DO RECEPTOR DO PEPTÍDEO LIBERADOR DE GASTRINA: PERSPECTIVAS PARA UM MODELO ANIMAL DE TRANSTORNO DO DESENVOLVIMENTO NEUROLÓGICO

Dissertação apresentada como requisito para a obtenção do título de Mestre em Biologia Celular e Molecular, a ser apreciada pela Banca Examinadora do Programa de Pós Graduação em Biologia Celular e Molecular, da Faculdade de Biociências, da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientadora: Prof.a Dr.a Nadja Schröder

Porto Alegre
2006

JULIANA PRESTI TORRES

EFEITOS COMPORTAMENTAIS DO TRATAMENTO NEONATAL COM UM ANTAGONISTA DO RECEPTOR DO PEPTÍDEO LIBERADOR DE GASTRINA: PERSPECTIVAS PARA UM MODELO ANIMAL DE TRANSTORNO DO DESENVOLVIMENTO NEUROLÓGICO

Dissertação apresentada como requisito para a obtenção do título de Mestre em Biologia Celular e Molecular, a ser apreciada pela Banca Examinadora do Programa de Pós Graduação em Biologia Celular e Molecular, da Faculdade de Biociências, da Pontifícia Universidade Católica do Rio Grande do Sul.

Aprovada em 29 de junho de 2006.

BANCA EXAMINADORA:

Prof.a Dr.a Carla Denise Bonan - PUCRS

Prof. Dr. Iván Izquierdo - PUCRS

Prof. Dr. Flávio Pereira Kapczinski - UFRGS

Ao meu avô Prof. Dr. Sylvio Torres *(in memorian)* e ao meu tio Prof. Dr. Sylvio Thales Torres *(in memorian)*. Minhas fontes de inspiração para ser cada vez melhor.

AGRADECIMENTOS

Ao CNPq, a SOAD (South American Office for Anticancer Drug Development) e à Zentaris GmbH (Frankfurt, Alemanha) pelo apoio financeiro deste projeto.

À Pontifícia Universidade Católica do Rio Grande do Sul, especialmente à Faculdade de Biociências que me acolheu muito bem ao longo de minha graduação e pós-graduação.

Às meninas da secretaria do Programa de Pós Graduação em Biologia Celular e Molecular; Maria Luiza Moreira, Cátia Bonacina e Josilene Martins Rocha que com todo o carinho, paciência e competência me mantiveram sempre bem informada sobre os diversos aspectos do programa.

À professora Dr.a Clarice Alho que demonstrou-se sempre atenciosa e disponível para resolver todos os percalços que apareciam ao longo desse trajeto.

A minha amiga Clarissa Bergamaschi que sempre me ofereceu um grande apoio moral em todas as horas, e que mesmo longe nesse momento ainda o faz.

Ao Prof. Dr. Gilberto Schwartsmann pela disponibilidade e confiança tão carinhosamente cedida a mim e pelo entusiasmo com que analisava os resultados.

Aos meus colegas e amigos do laboratório Fábio Caldana, Felipe Scalco e Marcelo Guimarães pela ajuda prestada em todas as horas, a amiga Vanessa Athaíde Garcia que foi incansável em sua ajuda, sempre presente em qualquer hora e principalmente à amiga Maria Noêmia Martins de Lima que foi excepcional, não medindo esforços para me auxiliar em todo o meu trabalho; obrigada de coração.

À Glauramar Barbosa Martins, uma pessoa especial para mim, que desde a iniciação científica sempre me recebeu com o maior carinho, me ajudou em todos os momentos, foi confidente e amiga. Tenho certeza que essa amizade ficará para além desse trabalho.

À Prof.a Dr.a Elke Bromberg pelo auxílio em sua sala, por me permitir, nas horas mais impróprias, usar o computador, pela amizade e por me mostrar que às vezes é melhor encararmos as coisas com leveza e despreocupação, sem estresse.

Ao Prof. Dr. Rafael Roesler, meu co-orientador, sempre tão prestativo, entusiasmado, incentivador, perspicaz, obsessivo com a pesquisa e muito dedicado. Muito obrigado por tudo que fez por mim e ainda continua fazendo.

À minha querida amiga, mãe emprestada e orientadora Prof.a Dr.a Nadja Schröder, agradeço cada minuto que disponibilizou a mim, seja em sua sala, telefone ou mesmo em sua casa. Agradeço pelo apoio, por acreditar e investir em mim de uma forma tão singular e carinhosa e pela paciência em me ouvir falar ou chorar. Obrigada por ser mais que uma orientadora, ser uma amiga que, desde a iniciação científica até hoje me apoiou e me mostrou os melhores caminhos a seguir. Não tenho palavras para agradecer.

A minha querida Charlô, pela amizade, fidelidade e companheirismo canino e por inconscientemente me mostrar que as coisas boas da vida são as mais simples.

À mulher que mais admiro, minha mãe. Espetacular, que esteve sempre ao meu lado, me segurando pela mão, me oferecendo toda a sua energia, dedicação, incentivo, carinho e amor. Agradeço a ela pelo que sou hoje.

Ao meu pai, homem sem igual, obrigado pelos incentivos para procurar, ligar, perguntar, me informar, ser curiosa e nunca deixar de fazer as coisas. Cada palavra de carinho, de apoio foram e ainda são muito importantes para mim. A ele a minha eterna admiração.

Especialmente ao homem que amo, Tiago R. Wisnevski, pois sem ele esse mestrado não seria realizado. A ele agradeço pela dedicação oferecida a cada minuto, pela ajuda, estímulo diário, sinceridade, carinho, compreensão e amor. Obrigada por me fazer enxergar os fatos de uma forma mais simples e prática.

O melhor uso que podemos fazer de nossas vidas
é realizar algo que dure além da própria vida.

ÍNDICE

RESUMO

O receptor do peptídeo liberador de gastrina (GRPR) vem sendo relacionado a doenças do sistema nervoso central, incluindo desordens do desenvolvimento neurológico associadas ao autismo. No presente estudo, analisamos os efeitos do bloqueio do GRPR duramte o período neonatal em medidas comportamentais relevantes em modelos animais de desordens do desenvolvimento neurológico. Ratos Wistar machos receberam injeções intraperitoneais (i.p) de salina (SAL) ou do antagonista do GRPR [D-Tpi^{6}, Leu^{13} psi(CH_2NH)-Leu^{14}] bombesina (6-14) (RC-3095; 1 ou 10 mg/kg) duas vezes ao dia, do primeiro ao décimo dias de vida. Os animais tratados com RC-3095 demonstraram déficits pronunciados em interação social quando testados no período de 30-35 dias de idade. Prejuízos na retenção da memória 24h após o treino em ambas as tarefas; de reconhecimento do objeto novo (RON) e de esquiva inibitória, foram demonstrados quando os animais foram testados aos 60-71 dias de idade. O bloqueio neonatal do GRPR não afetou o comportamento em teste de memória de curta duração, 1,5h após o treino, tampouco o comportamento em campo aberto. As implicações desses achados em modelos animais de desordens do desenvolvimento neurológico são discutidas.

Palavras-chave: Peptídeo semelhante à Bombesina, Receptor do Peptídeo Liberador de Gastrina, Rc-3095, Comportamento Social, Memória, Desordens do Desenvolvimento neurológico.

ABSTRACT

The gastrin-releasing peptide receptor (GRPR) has been implicated in central nervous system diseases, including neurodevelopmental disorders associated with autism. In the present study we examined the effects of GRPR blockade during the neonatal period on behavioral measures relevant to animal models of neurodevelopmental disorders. Male Wistar rats were given an intraperitoneal (i.p.) injection of either saline (SAL) or the GRPR antagonist [D-Tpi^{6}, Leu^{13} psi(CH_2NH)-Leu^{14}] bombesin (6-14) (RC-3095; 1 or 10 mg/kg) twice daily for 10 days from postnatal days (PN) 1 to 10. Animals treated with RC-3095 showed pronounced deficits in social interaction when tested at PN 30-35 and impaired 24-h retention of memory for both novel object recognition (NOR) and inhibitory avoidance tasks tested at PN 60-71. Neither short-term memory tested 1.5 h posttraining nor open field behavior were affected by neonatal GRPR blockade. The implications of the findings for animal models of neurodevelopmental disorders are discussed.

Keywords: Bombesin-like peptides; Gastrin-releasing peptide receptor; RC-3095; Social behavior; Memory; Neurodevelopmental disorders

2. APRESENTAÇÃO DO TEMA

2.1 Introdução

O peptídeo liberador de gastrina (GRP) está presente no sistema digestório, sendo liberado por células G do antro gástrico. Recentemente, muitas linhas de evidência têm indicado que o peptídeo liberador de gastrina (GRP) e seu receptor (GRPR) estão distribuídos por todo o sistema nervoso central e periférico de mamíferos, estimulando a proliferação celular, revelando abrangência em atividades neuroendócrinas e agindo como um fator de crescimento na patogenia de muitos tipos de cânceres humanos. Além disso, esse peptídeo parece estar envolvido em alterações neuroquímicas associadas com algumas desordens psiquiátricas e neurológicas.

Estudos farmacológicos e genéticos em roedores têm demonstrado que GRPRs em áreas cerebrais, como hipocampo dorsal e amigdala, estão importantemente envolvidos na regulação da plasticidade sináptica e em aspectos do comportamento que devem estar alterados em desordens como ansiedade, esquizofrenia, depressão, autismo e demência.

Alterações no funcionamento ou nos níveis de GRP e de seu receptor podem ocorrer em diversas fases da vida. Entretanto, doenças associadas ao desenvolvimento, como o autismo, normalmente se desenvolvem nos primeiros três anos de vida, e podem estar relacionadas com alguma anormalidade do peptídeo e/ou de seu receptor nesse período ou, até mesmo, na fase neonatal.

Nesse contexto, propomos o estudo "**Efeitos do Tratamento Neonatal com o Antagonista do Receptor do Peptídeo Liberador de Bombesina, o RC-3095, sobre Parâmetros Comportamentais em Ratos Adultos".** Neste, procurou-se analisar se o

bloqueio deste receptor durante o desenvolvimento seria capaz de induzir alterações em parâmetros comportamentais incluindo comportamento social, locomoção, aprendizado e memória. Evidências da correlação entre tais alterações com desordens neurológicas, psiquiátricas e com doenças neurodegenerativas proporcionam o desenvolvimento de métodos possivelmente mais efetivos no tratamento de doenças. Além disso, pode ser possível estabelecer quais as causas que podem e devem ser consideradas na análise dessas patologias.

2.2. Peptídeo Bombesina: Caracterização

O peptídeo Bombesina (BB) é um dos peptídeos ativos purificados da pele de anfíbios espécie *Bombina bombina* (Anastasi et al., 1973). Esse peptídeo é composto por 14 aminoácidos, e muitos outros relacionados estruturalmente à bombesina foram isolados da pele de anfíbios e divididos em três grupos: família Bombesina; a qual inclui a Bombesina e Alitensina; família Ranatensina, que inclui Ranatensina, Litorina e seus derivados e a família Phyllolitorina (Erspamer et al., 1984). O primeiro peptídeo semelhante à Bombesina em mamíferos foi isolado de tecidos gástricos e denominado peptídeo liberador de gastrina ou GRP (Gastrin Releasing Peptide), devido a sua potente indução da liberação de gastrina (Mc Donald et al., 1979). Alguns estudos demonstraram que seu efeito farmacológico estende-se a vários aspectos fisiológicos: ação hipertensiva, efeitos contráteis em útero, cólon ou íleo, ação estimuladora na secreção gástrica, efeito hiperglicêmico ou aumento na secreção de insulina (Erspamer, et. al., 1970). Moody e cols (1978), em estudos embrionários, investigando a presença de sítios de ligação de BB no sistema nervoso central (SNC), demonstraram que a bombesina liga-se com alta afinidade em membrana cerebral de ratos e que as mais altas densidades de sítios de ligações específicos à bombesina localizavam-se no hipocampo, uma

área cerebral criticamente envolvida na plasticidade sináptica, memória e desordens neuropsiquiátricas tais como esquizofrenia e doença de Alzheimer. Além disso, através da utilização de técnicas de radioimunoensaio, foi evidenciada a ocorrência endógena de peptídeos semelhantes à bombesina em cérebros de ratos. Áreas cerebrais com altas concentrações de peptídeos bombesina incluíram o núcleo do trato solitário (NTS), amigdala e hipocampo (Moody & Pert, 1979; 1981). A distribuição de corpos neuronais e fibras nervosas contendo peptídeos bombesina em cérebro e medula espinhal vem sendo densamente descrita (Moody & Merali, 2004).

2.3. O GRP e sua Distribuição no SNC

Estudos anteriores descreveram que o peptídeo correspondente à bombesina em cérebro de mamíferos era o peptídeo liberador de gastrina (GRP). O GRP possui His-Leu-Met em sua região C-terminal e é funcionalmente e estruturalmente relacionado à bombesina (Mc Donald et al., 1979; Cullen et al., 2000). Tal peptídeo, composto por 27 aminoácidos, é sintetizado como um precursor com 148 aminoácidos (PreproGRP) no núcleo dos neurônios e, subsequentemente, metabolizado pós-tradução (Spindel et al., 1984; Leback-Verheyden et al., 1988; Spindel et al., 1990). Estudos de hibridização i*n situ* avaliando a distribuição do GRP em cérebro de ratos demonstraram altos níveis de mRNA para GRP na área amigdalo-hipocampal, giro denteado, núcleo do trato solitário, núcleo supraquiasmático e medial preóptico do hipotálamo e camadas II e III do isocórtex (Wada et al., 1990).

A neuromedina-B (NMB) corresponde a um peptídeo similar ao GRP, contendo His-Phe-Met em sua porção C-terminal. Entretanto, ambos os peptídeos compartilham apenas 48% de identidade em níveis de nucleotídeos e estão localizados em diferentes cromossomos (GRP cromossomo 18; NM(B) cromossomo 15). A NM(B) também ocorre em cérebro de

mamíferos (Battey e Wada, 1991; Minamino et al., 1983;1984; Moody et al., 2004;), por outro lado, em cérebro de ratos o mRNA de GRP é mais abundante que o NM(B) mRNA, GRP e NM(B) apresentam diferentes padrões de distribuição (Chronwall et al., 1985; Wada et al., 1990; Moody & Merali, 2004).

2.4. Receptores GRP no SNC

O GRPR é um membro da superfamília de receptores acoplados à proteína G, contendo sete domínios transmembrana e 348 aminoácidos. Estudos utilizando técnicas de autoradiografia *in vitro* indicaram que aquelas áreas que contêm altas densidades de GRPRs incluem o bulbo olfatório, núcleo accumbens, caudado- putâmen, amigdala central, formação hipocampal dorsal (área CA3 e giro denteado) bem como os núcleos talâmicos paraventricular, central medial e paracentral (Wolf et al., 1983; Wolf e Moody,1985; Zarbin et al., 1985; Moody et al., 2004). Estudos de hibridização *in situ* revelaram a presença de mRNA para GRPR em áreas cerebrais incluindo o giro denteado, camada II do isocórtex, trato olfatório, gânglios da base e hipotálamo (Battey & Wada, 1991; Moody et al., 2004).

2.4.1 Alterações no Sistema GRP/GRPR em Pacientes com Transtornos Psiquiátricos, Neurodegenerativos e do Desenvolvimento Neurológico

Embora a causa da disfunção do receptor do peptídeo liberador de gastrina (GRPR) em desordens do SNC ainda não tenha sido estabelecida, alterações nos níveis do peptídeo liberador de gastrina ou na função do GRPR têm sido observadas em pacientes com desordens psiquiátricas, neurodegenerativas e no desenvolvimento do sistema nervoso. Pacientes com

doenças relacionadas ao SNC demonstraram níveis alterados no número de neuropeptídeos (Gerner & Yamada, 1982; 1985; Bissette et al., 1985; Nemeroff et al., 1989) Entretanto, ainda não está claro o significado daquelas alterações para a patofisiologia das doenças do SNC. É possível que uma redução dos níveis do GRP em pacientes psiquiátricos altere a atividade regulatória daqueles peptídeos em funções do SNC, contribuindo assim, para manifestações clínicas significativas. A concentração de GRP demonstrou estar significativamente reduzida no núcleo caudado e globo pálido de pacientes com doença de Parkinson (Bissette et al., 1985). Em contrapartida, Stoddard e cols (1991) não encontraram alterações em imunoreatividade de GRP em tecido adrenal medular de pacientes com doença de Parkinson, embora a concentração de outros neuropeptídeos (por exemplo; neuropeptídeo Y, substância P, peptídeo vasoativo intestinal e [Met]encefalina) estivessem diminuídas. Os níveis do peptídeo semelhante à bombesina também estão reduzidos na urina (Olincy et al., 1999) e no fluido cerebroespinhal (FCS) (Gerner et al., 1985) de pacientes com esquizofrenia. Além disso, têm-se proposto que alterações nos níveis de GRPR no SNC humano devem estar envolvidas na anorexia, bulimia nervosa e transtornos de humor (Merali et al., 1999; Frank et al., 2001). Em contrapartida, Gerner e Yamada (1982) demonstraram a existência de imunoreatividade normal de BB no fluido cerebroespinhal de pacientes com anorexia, depressão e mania. Níveis reduzidos de neurotensina, somatostatina, fator liberador de corticotropina, exceto de GRP ou hormônio liberador de tireotropina, foram encontrados em diferentes áreas cerebrais *post-morten* de pacientes com doença de Alzheimer (Nemeroff et al., 1989)

2.4.2 Efeitos Comportamentais e Eletrofisiológicos da Manipulação Farmacológica e Genética de GRPR em Roedores: Implicações para Desordens no SNC

Os efeitos comportamentais da administração de agonistas e antagonistas do receptor de peptídeo liberador de gastrina vêm sendo descritos em numerosos estudos. Infusões intracerebrais de bombesina (BB) induzem o comportamento de "grooming" em ratos e o núcleo do trato solitário parece ser particularmente sensível aos efeitos da administração de bombesina (BB) (Kulkossky et al., 1982; Gmerek & Cowan, 1983; Johnston e Merali., 1988). A indução de "grooming" induzida por BB depende do receptor de GRP (GRPR) e é atenuada por receptores antagonistas de dopamina (Piggins 7 Merali, 1989). Sugere-se que o comportamento de "grooming" induzido por bombesina deva estar relacionado à resposta ao estresse. A administração de bombesina poderia induzir a um efeito autônomo, endócrino e comportamental similar àqueles apresentados pela exposição a estressores, sugerindo um possível papel dos GRPRs na mediação de respostas ao estresse e a ansiedade (Merali et al., 2002; Moody & Merali, 2004).

Administrações de bombesina em ratos adultos demonstraram modular respostas nociceptivas. Injeções de BB intraventriculares produziram um aumento dose-dependente na atividade locomotora em ratos, bem como injeções na substância cinzenta periaquedural produziram uma reação antinociceptiva em testes de retirada da cauda a um estímulo térmico ("tail flick") e teste de placa quente ("hot-plate") (Pert et al., 1980). Cridland e Henry (1992) demonstraram que injeções subaracnóideas de bombesina na região vertebral lombar inferior produziram uma facilitação dose-dependente na resposta no teste "tail-flick".

Está bem estabelecido que administração de BB suprime a ingesta de alimento em numerosas espécies, incluindo ratos (Gibbs et al., 1979), camundongos (Taylor & Garcia, 1985) e humanos (Muurahainen et al., 1993). Administrações sistêmicas do antagonista do receptor bombesina atenuam a redução na ingesta alimentar induzida por bombesina (Flynn et al., 1997), e infusões de um antagonista seletivo de GRPR também no quarto ventrículo facilitam a ingesta de leite (Flynn et al., 1993). Evidências para o envolvimento do peptídeo

semelhante à bombesina e seus receptores (GRPRs) na regulação da ingesta alimentar são consistentes com a idéia de que disfunções desse sistema devem contribuir para desordens psiquiátricas, afetando a alimentação, incluindo a anorexia nervosa, bulimia e depressão (Flynn et al., 1994; Merali et al., 1999; Moody et al., 2004).

2.5 O RC-3095: Um Antagonista do GRPR

O antagonista seletivo de GRPR [D-Tpi6, Leu13 psi(CH2NH)-Leu14] bombesina (6-14) (RC-3095) vêm sendo utilizado como uma ferramenta na investigação dos efeitos comportamentais relacionados ao bloqueio do receptor de peptídeo liberador de gastrina em modelos roedores. O RC-3095 foi desenvolvido pelo grupo de Schally e cols. como um potente fármaco antitumoral (Pinski et al., 1992; Yano et al., 1992; Qin et al., 1994; Szepeshazi et al., 1997, Schwartsmann et al.,2004). Diante do fato de pacientes com esquizofrenia apresentarem níveis alterados do peptídeo semelhante à bombesina (Gerner et al., 1985; Olincy et al., 1999), Os efeitos da administração sistêmica de RC-3095 em comportamento de estereotipia induzidos pelo agonista de receptores dopaminérgicos, apomorfina por Roesler e cols (2004) ou pelo antagonista do receptor glutamatérgico N-metil-D-aspartato (NMDA), dizocilpina (MK-801) por Meller e cols. (2004). Comportamentos estereotípicos são padrões de desordens psiquiátricas, tais como a esquizofrenia, desordem obsessivo-compulsiva e autismo. Administrações sistêmicas de RC-3095 atenuaram significativamente a estereotipia induzida por apomorfina, suportando a hipótese que os receptores de GRP (GRPRs) estão envolvidos em padrões comportamentais de esquizofrenia e indicando que os antagonistas de GRPRs podem ser investigados como agentes com potencial atividade antipsocótica. Consistente com a interação funcional entre GRPRs e o sistema dopaminérgico no cérebro (Piggins et al., 1989; Merali & Piggins., 1990), o RC-3095

bloqueou a estereotipia induzida pela apomorfina, mas não apresentou nenhum efeito na estereotipia produzida por MK-801.

Recentemente, estudos farmacológicos investigando o papel do GRP e seus receptores em memória de aprendizado avaliaram os efeitos de agonistas de GRPR no desempenho de roedores em tarefas de memória. Administrações sistêmicas de bombesina (BB) ou GRP aumentaram o armazenamento de memória tanto em camundongos (Flood & Morley, 1988) quanto em ratos (Rashidy- Pour & Razvani, 1998).

Estudos em imunoreatividade do GRPR demonstraram que esse receptor está largamente distribuído em isocórtex, na formação hipocampal, córtex olfatório, amigdala, hipotálamo e tronco cerebral (Kamichi et al., 2005), em particular, no núcleo da amigdala basolateral (BLA) e central lateral (LA) e no NTS, importantes regiões no desempenho de memória. Subseqüentemente, a imunoreatividade de GRPR foi observada em neurônios GABAérgicos da região límbica. Esses resultados anatômicos suportam a idéia de que o sistema GRP/GRPR possui a função de mediadores no desempenho da memória por modular a liberação de neurotransmissores no sistema GABAérgico.

Roesler e cols (2004a) investigando os efeitos do bloqueio do GRPR na memória, examinaram que ratos tratados com infusões sistêmicas ou intracerebrais de RC-3095 demonstraram prejuízos na retenção a curto e longo prazo em tarefa de esquiva inibitória, um tipo de tarefa de memória emocional. Em contrapartida, o RC-3095 não apresentou alterações em tarefa de reconhecimento do objeto, um tipo de memória não emocional (Roesler et al., 2004b). Microinfusões de RC-3095 na área CA1 do hipocampo dorsal também prejudicaram a consolidação de memória de curta e longa duração em esquiva inibitória em ratos, indicando o papel de GRPRs em hipocampo, na consolidação de memória emocional (Roesler et al., 2003). Consistente com o papel dos receptores de GRP na regulação da plasticidade hipocampal, estudos eletrofisiológicos demonstraram que o peptídeo liberador de gastrina

induz a despolarização da membrana em neurônios hipocampais de ratos; um efeito que é bloqueado por um antagonista de GRP (Lee et al., 1999).

Os efeitos prejudiciais de infusões intrahipocampais de RC-3095 na memória podem ser revertidos por administrações sistêmicas de agonistas de receptor glicocorticóide, dexamethasona, indicando que o GRPR possui um papel regulatório na consolidação da memória emocional. Entretanto, esse não é um sistema importante requerido na formação de memória (Venturella et al., 2005). Diante de elucidações do papel dos glicocorticóides em mediar a morte neuronal e o prejuízo da plasticidade sináptica, aprendizado e memória induzidos por estresse severo ou prolongado (Lee et al., 2002; Sapolsky et al., 2003), a investigação de uma interação funcional entre o sistema glicocorticóide e o GRPR devem contribuir para o entendimento das bases neuroquímicas de desordens relacionadas ao estresse.

O complexo basolateral da amigdala (BLA, formado por basal, lateral e núcleo basal acessório) está criticamente envolvido na regulação da memória e da emoção, mediando, também, efeitos moduladores de drogas e hormônios em memória emocional (McGaugh et al., 1996; McGaugh, 2002). Propõe-se que anormalidades na amigdala desempenham um papel em desordens psiquiátricas, tais como esquizofrenia, ansiedade e autismo (Anand & Shekka., 2003; Zirlinger & Anderson, 2003; Niu et al., 2004). Infusões de RC-3095 na amigdala basolateral prejudicaram a retenção da memória em tarefa de esquiva inibitória em ratos (Roesler et al., 2004c). Conjuntamente a essas análises, Santo-Yamada e cols (2003) demonstraram efeitos prejudiciais da administração sistêmica de antagonistas de GRPR em tarefa de memória de esquiva inibitória em camundongos.

Em conjunto, os experimentos examinando os efeitos de agonistas ou antagonistas de GRPRs na memória em roedores indicam que a ativação de GRPR em hipocampo e

amigdala (e possivelmente em outras áreas cerebrais) está importantemente envolvida na modulação do aprendizado emocional e memória.

3. OBJETIVOS

Avaliar os efeitos da administração, no período neonatal do 1º ao 10º dia de vida, do RC-3095, um antagonista do peptídeo liberador de gastrina, sobre parâmetros neurocomportamentais em ratos.

3.1 OBJETIVOS ESPECÍFICOS

Avaliar os efeitos do RC-3095 (1mg/Kg e 10 mg/Kg) em parâmetros de interação social, no período entre 30-35 dias de vida dos animais.

Avaliar os efeitos do antagonista RC-3095 (1mg/Kg e 10 mg/Kg), administrado no período neonatal sobre a memória de reconhecimento em ratos adultos.

Avaliar os efeitos do antagonista RC-3095 (1mg/Kg e 10 mg/Kg), administrado no período neonatal sobre a memória aversiva em ratos adultos.

Avaliar o comportamento em campo aberto em ratos com 30-35 e 80-85 dias de vida, submetidos ao tratamento neonatal com RC-3095 (1mg/Kg e 10 mg/Kg) .

4. ARTIGO

Neuropharmacology

Research Paper

Impairments of social behavior and memory after neonatal gastrin-releasing peptide receptor blockade in rats: implications for an animal model of neurodevelopmental disorders

J. Presti-Torres[a], M.N. de Lima[a], F.S. Scalco[a], F. Caldana[a], V.A. Garcia[a], M.R. Guimarães[a], G. Schwartsmann[b,c], R. Roesler[c,d,*], N. Schröder[a]

[a] *Neurobiology and Developmental Biology Laboratory and Graduate Program in Cellular and Molecular Biology, Faculty of Biosciences, Pontifical Catholic University, 90619-900 Porto Alegre, RS, Brazil*

[b] *Department of Internal Medicine, Faculty of Medicine, Federal University of Rio Grande do Sul, 90035-003 Porto Alegre, RS, Brazil*

[c] *Cancer Research Laboratory, Academic Hospital Research Center, Federal University of Rio Grande do Sul, 90035-003 Porto Alegre, RS, Brazil*

[d] *Cellular and Molecular Neuropharmacology Research Group, Department of Pharmacology, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, 90046-900 Porto Alegre, RS, Brazil*

Running title: Behavioral deficits after neonatal GRPR blockade

* Corresponding author. Department of Pharmacology, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, Rua Sarmento Leite, 500, ICBS, Campus Centro,/UFRGS, 90046-900 Porto Alegre, RS, Brazil. Tel.: +55 51 3316 3183; fax: +55 51 3316 3121.

E-mail address: rroesler@terra.com.br (R. Roesler).

Abstract

The gastrin-releasing peptide receptor (GRPR) has been implicated in central nervous system (CNS) diseases, including neurodevelopmental disorders associated with autism. In the present study we examined the effects of GRPR blockade during the neonatal period on behavioral measures relevant to animal models of neurodevelopmental disorders. Male Wistar rats were given an intraperitoneal (i.p.) injection of either saline (SAL) or the GRPR antagonist [D-Tpi^{6}, Leu^{13} psi(CH_2NH)-Leu^{14}] bombesin (6-14) (RC-3095; 1 or 10 mg/kg) twice daily for 10 days from postnatal days (PN) 1 to 10. Animals treated with RC-3095 showed pronounced deficits in social interaction when tested at PN 30-35 and impaired 24-h retention of memory for both novel object recognition (NOR) and inhibitory avoidance tasks tested at PN 60-71. Neither short-term memory tested 1.5 h posttraining nor open field behavior were affected by neonatal GRPR blockade. The implications of the findings for animal models of neurodevelopmental disorders are discussed.

Keywords: Bombesin-like peptides; Gastrin-releasing peptide receptor; RC-3095; Social behavior; Memory; Neurodevelopmental disorders

1. Introduction

The gastrin-releasing peptide (GRP)-preferring type of bombesin (BB) receptor (GRPR, also known as BB2 receptor) has been increasingly implicated in regulating normal brain function as well as in the pathogenesis of neurological and psychiatric disorders (for recent reviews, see Moody and Merali, 2004; Roesler et al., 2006a). The GRPR is a G-protein coupled receptor expressed in the cell membranes of several tissues, including neuronal dendrites and cell bodies (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, GRP, affects a range of cellular and neuroendocrine functions (Moody and Merali, 2004; Ohki-Hamazaki et al., 2005; Roesler et al., 2006a).

Recent studies have indicated that GRP and the GRPR are implicated in regulating formation and extinction emotional memory in brain areas including the dorsal hippocampus and basolateral amygdala (Flood and Morley, 1988; Santo-Yamada et al., 2001; 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; Dantas et al., 2006; Luft et al., 2006; Roesler et al., 2006b). In addition, increasing evidence suggests that the GRPR is a molecular target for the development of novel therapeutics for the treatment of central nervous system (CNS) disorders including memory dysfunction, Alzheimer's disease (AD), schizophrenia, anxiety, and brain cancer (Ito et al., 1994; Kiaris et al., 1999; Santo-Yamada et al., 2001; Shumyatsky et al., 2002; Meller et al., 2004; Moody and Merali, 2004; Roesler et al., 2004a,b,c; Martins et al., 2005; Luft et al., 2006; Roesler et al., 2006a,b).

Central nervous system diseases possibly involving the GRPR also include neurodevelopmental disorders associated with autism (Ishikawa-Brush et al., 1997; Shumyatsky et al., 2002; Marui et al., 2004; Roesler et al., 2006a). An X;8 translocation

occurring in the first intron of the GRPR gene has been identified in a female patient with multiple exostoses and autism accompanied by mental retardation and epilepsy, indicating that the GRPR is a candidate gene in autism and changes in the function of brain GRPRs during development might play a role in producing behavioral features associated with neurodevelopmental disorders (Ishikawa-Brush et al., 1997). Behavioral alterations in rodents relevant to autism include social interaction deficits, stereotyped behavior, and impaired cognitive function (for a recent review, see Moy et al., 2006). The effects of pharmacological manipulation of the GRPR during the neonatal period on some aspects of adult behavior relevant for stress and anxiety have been described (Piggins and Merali, 1992; Piggins et al., 1993). However, previous studies have not verified the effects of GRPR blockade during development on other behaviors relevant for models of CNS disorders, such as social behavior and cognitive function. The present study describes behavioral alterations in rats after neonatal GRPR blockade.

2. Methods

2.1. Animals

Pregnant Wistar rats were obtained from the State Health Science Research Foundation (FEPPS-RS, Porto Alegre, Brazil). After birth each litter was adjusted within 48 h to eight rat pups, and to contain offspring of both genders in about equal proportions. Each pup was kept together with its mother in a plastic cage with sawdust bedding in a room temperature of 21± 1ºC and a 12/12 h light/dark cycle. At the age of 4 weeks, pups were weaned and the males were selected and raised in groups of three to five rats. For postnatal treatments, animals were given standardized pellet food and tap water *ad libitum*. Weight gain

was measured at postnatal days (PN) 1-10, 15, 30, 60, and 90. All behavioral experiments were performed at light phase between 09:00 h and 16:30 h. The same animals were used in different behavioral experiments. 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). The protocol for this research was approved by the Institutional Ethics Committee of the Pontifical Catholic University. All efforts were made to minimize the number of animals and their suffering.

2.2. Drugs and pharmacological procedures

Male pups were given two daily intraperitoneal (i.p.) 1 ml/kg injections of saline (SAL; NaCl 0.9%), or [D-Tpi^{6}, Leu^{13} psi(CH_2NH)-Leu^{14}] bombesin (6-14) (RC-3095; 1 or 10 mg/kg dissolved in SAL; Zentaris GmbH, Frankfurt, Germany), at PN 1-10. RC-3095, which is a selective GRPR antagonist developed by Schally and colleagues as an experimental anticancer drug (Radulovic et al., 1991; Szepeshazi et al., 1997; Schwartsmann et al., 2005,2006), has been consistently used in previous studies as a tool to investigate the role of GRPR in brain function (Roesler et al., 2003, Roesler et al., 2004b,c; Martins et al., 2005; Venturella et al., 2005; Dantas et al., 2006; Luft et al., 2006; Roesler et al., 2006b). Drug solutions were prepared immediately prior to administration. Drug doses and treatment regimen were chosen on the basis of previous studies (Piggins and Merali, 1992; Piggins et al., 1993; Roesler et al., 2004b,c).

2.3. Social interaction test

Impaired social behavior is a key behavioral feature of rodent models of autism spectrum disorders and schizophrenia (Mohn et al., 1999; Schneider and Prezewlocki, 2005; Moy et al., 2006). Social interaction was tested at PN 30-35. This age was chosen on the basis of previous studies on rat models of autism (Schneider and Prezewlocki, 2005). Animals were tested under dim/light and unfamiliar conditions, in a rectangular open field apparatus (45 x 40 x 60 cm). On the day of the experiment, the animals were socially isolated in plastic cages measuring 43 x 28 x 15 cm (l × w × h) for 3.5h prior to the experiment. This isolation period has been shown to produce a half maximal increase in the amount of social play (Niesink and Van Ree, 1989). The task consisted in placing two animals from the same experimental group but from different litters and cages (RC-3095 versus RC-3095, SAL versus SAL) into the test cage for 15 min. Pairs were tested in a randomized order for groups, and animals did not differ by more than 15 g in body weight. Animals were tested between 30 and 35 days of life. The social behavior was assessed for a pair of animals, so behavior of individual animals was not analyzed (Schneider and Prezewlocki, 2005). Latency to start social behavior (following or approaching the test partner, mounting or crawling over the test partner, sniffing or grooming any part of the body of the test partner), the total time spent engaged in social behavior, and the number of social contacts were measured (Niesink and Van Ree, 1989; Schneider and Prezewlocki, 2005).

2.4. Novel object recognition

The novel object recognition (NOR) procedure uses the natural preference for novel objects displayed by rats and mice to assess cognitive alterations in rodent models of neurodevelopmental and neurodegenerative disorders (Chen et al., 2000; Bourtchouladze et

al., 2003; Vaillend et al., 2004; Ventura et al., 2004; de Lima et al., 2005a,b). Rats were trained in the NOR task at postnatal day 60. A rectangular open field similar to the one described above with sawdust covering its floor was used for the NOR task. On the first day, rats were submitted to a habituation session during which they were placed in the empty open field for 5 min. On the following day, rats were given one 5-min training trial in which they were exposed to two identical objects (A1 and A2). All objects were made of plastic Duplo Lego Toys and had a height of about 10 cm. Objects presented similar textures, colors and sizes, but distinctive shapes. The objects were positioned in two adjacent corners, 9 cm from the walls. Between trials, the objects were washed with a 10% ethanol solution. On the short-term memory retention test trial (1.5 h min after training), rats were allowed to explore the open field for 5 min in the presence of two objects: the familiar object A and a novel object B. These were placed in the same locations as in the training session. On the long-term memory retention test trial (24 h after training), rats were allowed to explore the open field for 5 min in the presence of two objects: the familiar object A and a third novel object C. In both retention test trials, the novel object was placed in 50% trials in the right side and 50% trials in the left side of the open field. The same animals were used to evaluate 1.5 and 24-h retention. Object exploration was measured by two experimenters blind to group treatment assignments; using two stopwatches to record the time spent exploring the objects during the experimental trials. Exploration was defined as follows: sniffing or touching the object with the nose. Sitting on the object was not considered as exploration. A recognition index calculated for each animal was expressed by the ratio TB / (TA + TB) [TA = time spent exploring object A; TB = time spent exploring object B] (de Lima et al., 2005a,b; Schröder et al., 2003). The possibility that RC-3095 affected sensorimotor function inducing alterations in locomotion, anxiety or motivation was assessed by evaluating the total time exploring objects during the training trials (Schröder et al., 2003).

2.5. Inhibitory avoidance

The inhibitory avoidance (IA) conditioning is a well established model of emotionally motivated memory in rats (Izquierdo and Medina, 1997; Taubenfeld et al., 1999; McGaugh, 2000) which can be used in the characterization of cognitive deficits in rodent models of neuropsychiatric and neurodevelopmental disorders (DeLorey et al., 1998; Roesler et al., 1999; Moy et al., 2006; Reolon et al., 2006). We have previously shown that the GRPR plays an important role in regulating formation of IA memory (Roesler et al., 2003, 2004b,c; Venturella et al., 2005; Dantas et al., 2006; Roesler et al., 2006b). Rats were given IA training at postnatal day 70. The IA apparatus was a 50 × 25 × 25 cm acrylic box whose floor consisted of parallel 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. Animals were placed on the platform and their latency to step-down on the grid with all four paws was recorded with an automated device. In training sessions, immediately after stepping down on the grid, the animals were given a 0.6 mA, 1.0 s footshock. In two retention test sessions carried out 1.5 (short-term retention) and 24 h (long-term retention) after training, no footshock was given and the step-down latency (maximum 180 s) was used as a measure of retention, as previously described (Roesler et al., 1999; 2003, Quevedo et al., 2004; Roesler et al., 2004b).

2.6. Open field behavior

Behavioral measures relevant for rodent models of neurodevelopmental and neuropsychiatric disorders (e.g., locomotion, exploratory behavior, repetitive behavior,

stereotypies, self-injury, and anxiety) can be assessed during exploration of an open field (DeLorey et al., 1998; Roesler et al., 1999; Turner et al., 2001; Fernandez-Teruel et al., 2002; Henderson et al., 2004; Singer et al., 2005; Moy et al., 2006; Reolon et al., 2006). Open field behavior was evaluated at PN 33-38 and again at PN 80-85. The open field was similar to the one described above. The floor of the arena was divided into 12 equal squares by black lines. Animals were placed in the rear left corner and left to explore the field freely for 5 min. Latency to start locomotion, line crossings, rearings and the number of fecal pellets produced were counted. The number of crossings and rearings were used respectively as measures of locomotor activity and exploratory behavior, whereas the latency to start locomotion and the number of fecal pellets were used as measures of anxiety (DeLorey et al., 1998; Roesler et al., 1999; Fernandez-Teruel et al., 2002; Henderson et al., 2004; Singer et al., 2005; Reolon et al., 2006).

2.7. Statistics

Data for IA and exploratory preferences in the NOR task are shown as median (interquartile ranges). Comparisons between groups were performed using a Kruskal-Wallis analysis of variance followed by Mann-Whitney *U*-tests, two-tailed when necessary. Comparisons within the same group were done with Wilcoxon tests. Using nonparametric statistics is preferred for the analysis of data for memory tasks because data often do not show a normal distribution and a ceiling is imposed in retention test trials in tasks such as IA (Roesler et al., 1999; 2003; Quevedo et al., 2004; Roesler et al., 2004b,c; de Lima et al., 2005a,b; Dantas et al., 2006; Luft et al., 2006; Reolon et al., 2006; Roesler et al., 2006b). Data for the social interaction test, total exploration time in the NOR task, and open field behavior

are shown as mean ± SEM. Comparisons between groups were performed using an one-way anaysis of variance (ANOVA) followed by Tukey posthoc tests when necessary (Roesler et al., 1999; Reolon et al., 2006). In all comparisons, $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Body weight

Neonatal treatment with RC-3095 did not affect body weight measured at PD 10, 15, 30, 60, and 90 (Table 1). There were no significant differences among groups (PD 10, $F = 0.85$, $p = 0.43$; PD 15, $F = 0.80$, $p = 0.92$; PD 30, $F = 2.48$, $p = 0.10$; PD 60, $F = 0.10$, $p = 0.91$; PD 90, $F = 3.13$, $p = 0.06$).

Table 1 should be inserted here

3.2. Social interaction

Neonatal administration of RC-3095 induced significant social deficits (Fig. 1). Rats given either 1 or 10 mg/kg RC-3095 showed decreases in both the frequency of contacts (Fig. 1B) and the amount of time spent engaged in social interaction (Fig. 1C) in comparison with controls (all ps < 0.01). There was no significant difference in the latency to start interacting (mean ± SEM latencies were 6.80 ± 3.09 in the SAL-treated group; 6.93 ± 2.55 in the group

given 1 mg/kg RC-3095; and 6.86 $\pm$ 1.81 in the group given 10 mg/kg RC-3095; F = 0.001; p = 0.10).

Fig. 1 should be inserted here

3.3. Novel object recognition

There was no significant difference among groups in the total time spent exploring both objects during the training trial (F = 1.8; p = 0.19; Table 2), indicating that RC-3095 did not affect locomotion, exploration, motivation, or other sensorimotor parameters that could affect NOR performance. Results for exploratory preferences are shown in Fig. 2. There were no significat differences among groups in the training trial (p = 0.14), or in 1.5-h memory retention (p = 0.08). Rats treated with RC-3095 at either 1 or 10 mg/kg showed a significant impairment of 24-h retention when compared to control animals (both ps < 0.01). All groups showed a significant preference for the novel object in the 1.5-h retention test trial compared to exploration of any object during training (ps < 0.01 in the control group given SAL, and the group given 1 mg/kg RC-3095; p < 0.02 in the group given 10 mg/kg RC-3095). Animals given SAL, but not animals treated with any dose of RC-3095, showed significant preference for the novel object in the 24-h retention test trial in comparison with exploration of any object during training (p < 0.01 in the SAL group; ps = 0.51 in the groups given 1 or 10 mg/kg RC-3095). The results indicate that neonatal GRPR blockade impairs 24 h retention of NOR.

Table 2 should be inserted here

Fig. 2 should be inserted here

3.4. Inhibitory avoidance

Results for IA are shown in Fig. 3. There was no significant difference among groups in training trial step-down latencies ($p = 0.17$) or 1.5-h IA retention ($p = 0.13$). However, rats treated with RC-3095 at either 1 or 10 mg/kg showed a significant decrease in 24-h retention test latencies in comparison to the control group treated with SAL ($p < 0.01$, RC-3095 1 mg/kg versus control; $p < 0.05$, RC-3095 10 mg/kg versus control). The results indicate that neonatal treatment with RC-3095 produced an impairment of 24-h retention of IA.

Fig. 3 should be inserted here

3.5. Open field behavior

Neonatal administration of RC-3095 did not affect open field behavior (Table 3). When animals were tested at PN 33-38 and again at PN 80-85, there were no significant differences among groups in the latency to start locomotion (PN 33-38, $F = 0.72$, $p = 0.50$; PN 80-85, $F = 0.10$, $p = 0.91$), number of crossings (PN 33-38, $F = 0.002$, $p = 0.10$; PN 80-85, $F = 0.90$, $p = 0.42$) or rearings (PN 33-38, $F = 0.05$, $p = 0.95$; PN 80-85, $F = 1.01$, $p = 0.37$) performed, or defecation (PN 33-38, $F = 0.35$, $p = 0.71$; PN 80-85, $F = 0.85$, $p = 0.43$). The results indicate that neonatal treatment with RC-3095 did not affect locomotion, exploration, or anxiety. Moreover, no spontaneous stereotyped or repetitive behaviors or self-injury were observed during open field exploration or any other behavioral experiment.

Table 3 should be inserted here

---

4. Discussion

Previous studies investigating the effects of neonatal manipulation of GRPRs have shown that neonatal treatment with a GRPR antagonist altered behavior in an elevated plus maze without affecting other behavioral measures or adult sensitivity to BB (Piggins et al., 1993), whereas administration of BB to rat pups had no pronounced effect on adult behavior but elicited grooming in pups and resulted in an increased sensitivity to central administration of BB (Piggins and Merali, 1992). The present study shows that rats given a neonatal treatment with the GRPR antagonist RC-3095 from PN 1 to 10 show impairments in social interaction and long-term memory assessed in both the IA and NOR tasks, but normal short-term memory and open field behavior. Importantly, although the GRPR has been implicated

in feeding behavior, satiety, anorexia, and bulimia (McCoy and Avery, 1990; Moody and Merali, 2004), rats given RC-3095 showed no drug-induced alterations in body weight.

Although memory for both aversive training assessed in the IA task and object recognition assessed with the NOR procedure were impaired in RC-3095-treated rats when memory retention was tested 24 h after training, short-term memory tested 1.5 h after training was not signficantly affected by GRPR blockade. The differential effects of neonatal RC-3095 on retention measured at 1.5 and 24-h delays might be related to the differential mechanisms mediating short- and long-term memory. For instance, IA memory measured 24 h after training requires protein synthesis in the hippocampus and related brain areas, whereas 1.5-h memory retention does not, and short- and long-term memory are differentially regulated by protein kinase-mediated signaling pathways (Quevedo et al., 2004). The interactions of neuronal GRPRs with signaling pathways relevant for memory formation are briefly addressed below and discussed in detail in a previous report (Roesler et al., 2006b).

It has been proposed that neurodevelopmental disorders such as autism and Rett syndrome result at least partially from postnatal disruption of synaptic mechanisms (Zoghbi, 2003). Neuromodulatory pathways implicated in autism include neuropeptide systems such as oxytocin and vasopressin and their receptors. Thus, it has been suggested that postnatal manipulation of neuropeptide receptors might be relevant for the development of novel rodent models of autism (Young et al., 2002; Lim et al., 2005).

Previous studies have implicated the GRPR in psychiatric, neurodegenerative, and neurodevelopmental disorders (for a review, see Roesler et al., 2006a). Regarding neurodevelopmental disorders, evidence indicates that the GRPR regulates emotional processing and cognitive function in the hippocampus and amygdala (Shumyatsky et al., 2002; Roesler et al., 2003; 2004c), which are brain areas importantly involved in the pathogenesis of autism (Aylward et al., 1999), and a translocation in the GRPR gene in an

autistic patient has been reported (Ishikawa-Brush et al., 1997). Aspects of rodent behavior relevant to the autism phenotype include social interaction deficits assessed in social interaction tests; alterations in locomotion, motivation, and anxiety, stereotypy, self-injury, and repetitive behavior, which can be assessed during open field exploration; and memory dysfunction, which is relevant to symptoms of autism related to mental retardation and can be measured in rodent behavioral tasks such as IA and NOR (Rodier et al., 1997; DeLorey et al., 1998; Turner et al., 2001; Bourtchouladze et al., 2003; Murcia et al., 2005; Schmitz et al., 2005; Schneider and Przewlocki, 2005; Moy et al., 2006). In the present study, animals submitted to neonatal GRPR blockade by systemic administration of a GRPR antagonist showed a pronounced impairment of social interaction and deficits in long-term memory for both IA and NOR, but normal locomotion, exploration, and anxiety during open field exploration. Also, we did not observe spontaneous stereotypy or self-injury in RC-3095-treated rats. Thus, although neonatal administration of a GRPR antagonist produced some key behavioral features of rodent models of neurodevelopmental disorders (i.e., impaired social behavior and cognition), it did not affect other aspects of behavior considered to be relevant in for those models. Because reduced social interaction is also a key feature of rodent models of schizophrenia and alterations in synaptic function might be involved in the pathogenesis of schizophrenic disorder, it is possible that the pronounced impairment in social behavior observed in rats given neonatal RC-3095 is relevant for schizophrenia models (Mohn et al., 1999; Gainetdinov et al., 2001). However, RC-3095-treated rats in our study lack key features of rodent models of schizophrenia, such as a spontaneous increase in locomotion and stereotyped behaviors (Mohn et al., 1999).

A seminal genetic study has shown enhanced fear-motivated memory in GRPR-deficient mice, whereas no alterations in spatial memory, locomotion, stereotypy, or social behavior were reported (Shumyatsky et al., 2002). One would expect that, if GRPR

dysfunction during development plays a role in autism spectrum disorders, mice lacking the GRPR would show behavioral features of models of autism, including pronounced impairments of social behavior. Thus, the findings by Shumyatsky et al. (2002) seem to contradict the present findings and our view that GRPR blockade during development might produce features relevant for models of neurodevelopmental disorders. It is possible that the discrepancies between pharmacological and genetic models are related to compensatory changes such as upregulation of alternative signaling pathways in knockout mice (Routtenberg, 1995).

The purpose of the present study was to provide an initial investigation of some aspects of behavior relevant for models of neurodevelopmental and psychiatric disorders in animals submitted to neonatal GRPR blockade. Further experiments are required to characterize the effects of GRPR blockade using neurochemical and neuropathological approaches, as well as to validate it as a model with potential to be used in the testing of candidate therapeutic compounds. For instance, future experiments should: (1) verify whether the neonatal RC-3095 treatment regimen induces histopathological features, such as altered dendritic spine morphology, similar to those observed in some mouse models of neurodevelopmental disorders (Moy et al., 2006); (2) verify whether rats given RC-3095 are more susceptible than control animals to apomorphine-induced stereotypy and dizocilpine (MK-801)-induced hyperlocomotion, since stereotypy induced by dopamine agonists such as apomorphine and hyperlocomotion induced by the *N*-methyl-D-aspartate (NMDA) receptor blocker MK-801 in rodents are established pharmacological models of psychiatric disorders (Hitri et al., 1993; Andine et al., 1999), and (3) examine biochemical and molecular alterations in protein kinase pathways downstream of the GRPR in the brains of rats given neonatal RC-3095. Molecular mechanisms mediating the neuronal actions of GRPR might include the protein kinase C (PKC), mitogen-activated protein kinase (MAPK)/extracellular

signal-regulated protein kinase (ERK), and dopamine D1/D5 receptor (D1R)/cAMP/ protein kinase A signaling pathways (Roesler et al., 2006b).

In summary, the present study indicates that administration of a GRPR antagonist during the neonatal period induces deficits in social interaction and formation of long-term memory without affecting other aspects of behavior in rats. The findings that neonatal GRPR blockade resulted in impaired social behavior and cognitive function might have implications for the development of novel animal models of neurodevelopmental disorders.

Acknowledgements

This research was supported by CNPq-MCT (grants 306413/2003-5 and 474700/2004-6 to R.R.), the South American Office for Anticancer Drug Development, and Zentaris GmbH (Frankfurt, Germany).

References

Andine, P., Widermark, N., Axelsson, R., Nyberg, G., Olofsson, U., Martensson, E., Sandberg, M., 1999. Characterization of MK-801-induced behavior as a putative rat model of psychosis. Journal of Pharmacology and Experimental Therapeutics 290, 1393-408.

Aylward, E.H., Minshew, N.J., Goldstein, G., Honeycutt, N.A., Augustine, A.M., Yates, K.O., Barta, P.E., Pearlson, G.D., 1999. MRI volumes of amygdala and hippocampus in non-mentally retarded autistic adolescents and adults. Neurology 53, 2145-2150.

Battey, J., Wada, E., 1991. Two distinct receptor subtypes for mammalian bombesin-like peptides. Trends in Neurosciences 14, 524-528.

Bourtchouladze, R., Lidge, R., Catapano, R., Stanley, J., Gossweiler, S., Romashko, D., Scott, R., Tully, T., 2003. A mouse model of Rubinstein-Taybi syndrome: defective long-term memory is ameliorated by inhibitors of phosphodiesterase 4. Proceedings of the National Academy of Sciences of the United States of America 100, 10518-10522.

Chen, G., Chen, K.S., Knox, J., Inglis, J., Bernard, A., Martin, S.J., Justice, A., McConlogue, L., Games, D., Freedman, S.B., Morris, R.G., 2000. A learning deficit related to age and beta-amyloid plaques in a mouse model of Alzheimer's disease. Nature 408, 975-979.

Dantas, A.D., Luft, T., Henriques, J.A., Schwartsmann, G., Roesler, R., 2006. Opposite effects of low and high doses of the gastrin-releasing peptide receptor antagonist RC-3095 on memory consolidation in the hippocampus: Possible involvement of the GABAergic system. Peptides Apr 27, Epub ahead of print.

de Lima, M.N., Laranja, D.C., Caldana, F., Grazziotin, M.M., Garcia, V.A., Dal-Pizzol, F., Bromberg, E., Schroder, N., 2005a. Selegiline protects against recognition memory impairment induced by neonatal iron treatment. Experimental Neurology 196, 177-183.

de Lima, M.N., Polydoro, M., Laranja, D.C., Bonatto, F., Bromberg, E., Moreira, J.C., Dal-Pizzol, F., Schroder, N., 2005b. Recognition memory impairment and brain oxidative stress induced by postnatal iron administration. European Journal of Neuroscience 21, 2521-2528.

DeLorey, T.M., Handforth, A., Anagnostaras, S.G., Homanics, G.E., Minassian, B.A., Asatourian, A., Fanselow, M.S., Delgado-Escueta, A., Ellison, G.D., Olsen, R.W., 1998. Mice lacking the beta3 subunit of the GABAA receptor have the epilepsy phenotype and many of the behavioral characteristics of Angelman syndrome. Journal of Neuroscience 18, 8505-8514.

Fernandez-Teruel, A., Escorihuela, R.M., Gray, J.A., Aguilar, R., Gil, L., Gimenez-Llort, L., Tobena,, A., Bhomra, A., Nicod, A., Mott, R., Driscoll, P., Dawson, G.R., Flint, J., 2002. A quantitative trait locus influencing anxiety in the laboratory rat. Genome Research 12, 618-626.

Flood, J.F., Morley, J.E., 1988. Effects of bombesin and gastrin-releasing peptide on memory processing. Brain Research 460, 314-322.

Gainetdinov, R.R., Mohn, A.R., Caron, M.G., 2001. Genetic animal models: focus on schizophrenia. Trends in Neurosciences 24, 527-533.

Henderson, N.D., Turri, M.G., DeFries, J.C., Flint, J., 2004. QTL analysis of multiple behavioral measures of anxiety in mice. Behavior Genetics 34, 267-293.

Hitri, A., O'Connor, D.A., Cohen, J.M., Keuler, D.J., Deutsch, S.I., 1993. Differentiation between MK-801- and apomorphine-induced stereotyped behaviors in mice. Clinical Neuropharmacology 16, 220-236.

Ishikawa-Brush, Y., Powell, J.F., Bolton, P., Miller, A.P., Francis, F., Willard, H.F., Lehrach, H., Monaco, A.P., 1997. Autism and multiple exostoses associated with an X;8

translocation occurring within the GRPR gene and 3' to the SDC2 gene. Human Molecular Genetics 6, 1241-1250.

Ito, E., Oka, K., Etcheberrigaray, R., Nelson, T.J., McPhie, D.L., Tofel-Grehl, B., Gibson, G.E., Alkon, D.L., 1994. Internal Ca^{2+} mobilization is altered in fibroblasts from patients with Alzheimer disease. Proceedings of the National Academy of Sciences of the United States of America 91, 534-538.

Izquierdo, I., Medina, J.H., 1997. Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. Neurobiology of Learning and Memory 68, 285-316.

Kamichi, S., Wada, E., Aoki, S., Sekiguchi, M., Kimura, I., Wada, K., 2005. Immunohistochemical localization of gastrin-releasing peptide receptor in the mouse brain. Brain Research 1032, 162-170.

Kiaris, H., Schally, A.V., Sun. B., Armatis, P., Groot, K., 1999. Inhibition of growth of human malignant glioblastoma in nude mice by antagonists of bombesin/gastrin-releasing peptide. Oncogene 18, 7168-7173.

Lim, M.M., Bielsky, I.F., Young, L.J., 2005. Neuropeptides and the social brain: potential rodent models of autism. International Journal of Developmental Neuroscience 23, 235-243.

Luft, T., Flores, D.G., Vianna, M.R., Schwartsmann, G., Roesler, R., Izquierdo, I., 2006. A role for hippocampal gastrin-releasing peptide receptors in extinction of aversive memory. NeuroReport 17, 935-939.

Martins, M.R., Reinke, A., Valvassori, S.S., Machado, R.A., Quevedo, J., Schwartsmann, G., Roesler, R., 2005. Non-associative learning and anxiety in rats treated with a single systemic administration of the gastrin-releasing peptide receptor antagonist RC-3095. Peptides 26, 2525-2529.

Marui, T., Hashimoto, O., Nanba, E., Kato, C., Tochigi, M., Umekage, T., Kato, N., Sasaki, T., 2004. Gastrin-releasing peptide receptor (GRPR) locus in Japanese subjects with autism. Brain and Development 26, 5-7.

McCoy, J.G., Avery, D.D., 1990. Bombesin: potential integrative peptide for feeding and satiety. Peptides 11, 595-607.

McGaugh, J.L., 2000. Memory - a century of consolidation. Science 287, 248-251.

Meller, C.A., Henriques, J.A.P., Schwartsmann, G., Roesler, R., 2004. The bombesin/gastrin releasing peptide receptor antagonist RC-3095 blocks apomorphine but not MK-801-induced stereotypy in mice. Peptides 25, 585-588.

Mohn, A.R., Gainetdinov, R.R., Caron, M.G., Koller, B.H., 1999. Mice with reduced NMDA receptor expression display behaviors related to schizophrenia. Cell 98, 427-436.

Moody, T.W., Merali, Z., 2004. Bombesin-like peptides and associated receptors within the brain: distribution and behavioral implications. Peptides 25, 511-520.

Moy, S.S., Nadler, J.J., Magnuson, T.R., Crawley, J.N., 2006. Mouse models of autism spectrum disorders: the challenge for behavioral genetics. American Journal of Medical Genetics Part C: Seminars in Medical Genetics 142, 40-51.

Murcia, C.L., Gulden, F., Herrup, K., 2005. A question of balance: a proposal for new mouse models of autism. International Journal of Developmental Neuroscience 23, 265-275.

Niesink, R.J.M., Van Ree, J.M., 1989. Involvement of opioid and dopaminergic systems in isolation-induced pinning and social grooming of young rats. Neuropharmacology 28, 411-418.

Ohki-Hamazaki, H., Iwabuchi, M., Maekawa, F., 2005. Development and function of bombesin-like peptides and their receptors. International Journal of Developmental Biology 49, 293-300.

Piggins, H.D., Merali, Z., 1992. Short- and long-term behavioral effects of neonatal exposure to bombesin. Behavioral and Neural Biology 57, 213-225.

Piggins, H.D., Moody, T.W., Merali, Z., 1993. Effects of neonatal blockade of bombesin (BN) receptors with [D-Phe6, phi Leu13-Cpa14]BN(6-14) on adult behavior and sensitivity to BN. Peptides 14, 845-848.

Quevedo, J., Vianna, M.R., Martins, M.R., Barichello, T., Medina, J.H., Roesler, R., Izquierdo, I., 2004. Protein synthesis, PKA, and MAP kinase are differentially involved in short- and long-term memory in rats. Behavioural Brain Research 154, 339-343.

Radulovic, S., Cai, R.Z., Serfozo, P., Groot, K., Redding, T.W., Pinski, J., Schally, A.V., 1991. Biological effects and receptor binding affinities of new pseudononapeptide bombesin/GRP receptor antagonists with N-terminal D-Trp or D-Tpi. International Journal of Peptide and Protein Research 38, 593-600.

Reolon, G.K., Braga, L.M., Camassola, M., Luft, T., Henriques, J.A.P., Nardi, N.B., Roesler, R., 2006. Long-term memory for aversive training is impaired in Idua(-/-) mice, a genetic model of mucopolysaccharidosis type I. Brain Research 1076, 225-230.

Rodier, P.M., Ingram, J.L., Tisdale, B., Croog, V.J., 1997. Linking etiologies in humans and animal models: studies of autism. Reproductive Toxicology 11, 417-422.

Roesler, R., Walz, R., Quevedo, J., de Paris, F., Zanata, S.M., Graner, E., Izquierdo, I., Martins, V.R., Brentani, R.R., 1999. Normal inhibitory avoidance learning and anxiety, but increased locomotor activity in mice devoid of PrPc. Molecular Brain Research 71, 349-353.

Roesler, R., Meller, C.A., Kopschina, M.I., Souza, D.O., Henriques, J.A., Schwartsmann, G., 2003. Intrahippocampal infusion of the bombesin/gastrin-releasing peptide antagonist RC-3095 impairs inhibitory avoidance retention. Peptides 24, 1069-1074.

Roesler, R., Henriques, J.A.P., Schwartsmann, G., 2004a. Neuropeptides and anxiety disorders: bombesin receptors as novel therapeutic targets. Trends in Pharmacological Sciences 25, 241-242.

Roesler, R., Kopschina, M.I., Rosa, R.M., Henriques, J.A., Souza, D.O., Schwartsmann, G., 2004b. RC-3095, a bombesin/gastrin-releasing peptide antagonist, impairs aversive but not recognition memory in rats. European Journal of Pharmacology 486, 35-41.

Roesler, R., Lessa, D., Venturella, R., Vianna, M.R., Luft, T., Henriques, J.A., Izquierdo, I., Schwartsmann, G., 2004c. Bombesin/gastrin-releasing peptide receptors in the basolateral amygdala regulate memory consolidation. European Journal of Neuroscience 19, 1041-1045.

Roesler, R., Henriques, J.A.P., Schwartsmann, G., 2006a. Gastrin-releasing peptide receptor as a molecular target for psychiatric and neurological disorders. CNS and Neurological Disorders - Drug Targets 5, 197-204.

Roesler, R., Luft, T., Oliveira, S.H., Farias, C.B., Almeida, V.R., Quevedo, J., Dal-Pizzol F., Schroder, N., Izquierdo, I, Schwartsmann G., 2006b. Molecular mechanisms mediating gastrin-releasing peptide receptor modulation of memory consolidation in the hippocampus. Neuropharmacology May 28, Epub ahead of print.

Routtenberg, A., 1995. Knockout mouse fault lines. Nature 374, 314-315.

Santo-Yamada, Y., Yamada, K., Wada, K., 2001. Post-training administration of gastrin-releasing peptide (GRP) improves memory loss in scopolamine- and hypoxia-induced amnesic mice. Physiology and Behavior 74, 139-143.

Santo-Yamada, Y., Yamada, K., Wada, E., Goto, Y., Wada, K., 2003. Blockade of bombesin-like peptide receptors impairs inhibitory avoidance learning in mice. Neuroscience Letters 340, 65-68.

Schmitz, C., van Kooten, I.A., Hof, P.R., van Engeland, H., Patterson, P.H., Steinbusch, H.W., 2005. Autism: neuropathology, alterations of the GABAergic system, and animal models. International Reviews of Neurobiology 71, 1-26.

Schneider, T., Przewlocki, R., 2005. Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology 30, 80-89.

Schröder, N., O'Dell, S.J., Marshall, J.F., 2003. Neurotoxic methamphetamine regimen severely impairs recognition memory in rats. Synapse 49, 89–96.

Schwartsmann, G., Di Leone, L.P., Dal Pizzol, F., Roesler, R., 2005. MAPK pathway activation in colorectal cancer: a therapeutic opportunity for GRP receptor antagonists. Lancet Oncology 6, 444-445.

Schwartsmann, G., Di Leone, L.P., Horowitz, M., Schunemann, D., Cancella, A., Pereira, A.S., Richter, M., Souza, F., da Rocha, A.B., Souza, F.H., Pohlmann, P., De Nucci, G., 2006. A phase I trial of the bombesin/gastrin-releasing peptide (BN/GRP) antagonist RC3095 in patients with advanced solid malignancies. Investigative New Drugs 24, 403-412.

Shumyatsky, G.P., Tsvetkov, E., Malleret, G., Vronskaya, S., Hatton, M., Hampton, L., Battey, J.F., Dulac, C., Kandel, E.R., Bolshakov, E.Y., 2002. Identification of a signaling network in lateral nucleus of amygdala important for inhibiting memory specifically related to learned fear. Cell 111, 905-918.

Singer, J.B., Hill, A.E., Nadeau, J.H., Lander, E.S., 2005. Mapping quantitative trait loci for anxiety in chromosome substitution strains of mice. Genetics 169, 855-862.

Szepeshazi, K., Schally, A.V., Halmos, G., Lamharzi, N., Groot, K., Horvath, J.E., 1997. A single in vivo administration of bombesin antagonist RC-3095 reduces the levels and mRNA expression of epidermal growth factor receptors in MXT mouse mammary

cancers. Proceedings of the National Academy of Sciences of the United States of America 94, 10913-10918.

Taubenfeld, S.M., Wiig, K.A., Bear, M.F., Alberini, C.M., 1999. A molecular correlate of memory and amnesia in the hippocampus. Nature Neuroscience 2, 309-310.

Turner, C.A., Presti, M.F., Newman, H.A., Bugenhagen, P., Crnic, L., Lewis, M.H., 2001. Spontaneous stereotypy in an animal model of Down syndrome: Ts65Dn mice. Behavior Genetics 31, 393-400.

Vaillend, C., Billard, J.M., Laroche, S., 2004. Impaired long-term spatial and recognition memory and enhanced CA1 hippocampal LTP in the dystrophin-deficient Dmd(mdx) mouse. Neurobiology of Disease 17, 10-20.

Ventura, R., Pascucci, T., Catania, M.V., Musumeci, S.A., Puglisi-Allegra, S., 2004. Object recognition impairment in Fmr1 knockout mice is reversed by amphetamine: involvement of dopamine in the medial prefrontal cortex. Behavioural Pharmacology 2004, 15, 433-442.

Venturella, R., Lessa, D., Luft, T., Roozendaal, B., Schwartsmann, G., Roesler, R., 2005. Dexamethasone reverses the memory impairment induced by antagonism of hippocampal gastrin-releasing peptide receptors. Peptides 26, 821-825.

Wolf, S.S., Moody, T.W., 1985. Receptors for GRP/bombesin-like peptides in the rat forebrain. Peptides Supplement 1, 111-114.

Young, L.J., Pitkow, L.J., Ferguson, J.N., 2002. Neuropeptides and social behavior: animal models relevant to autism. Molecular Psychiatry 7 Supplement 2, S38-S39.

Zarbin, M.A., Kuhar, M.J., O'Donohue, T.L., Wolf, S.S., Moody, T.W., 1985. Autoradiographic localization of (125I-Tyr4)bombesin-binding sites in rat brain. Journal of Neuroscience 5, 429-437.

Zoghbi, H.Y., 2003. Postnatal neurodevelopmental disorders: meeting at the synapse? Science 302, 826-830.

Table 1

Mean ± SEM body weights in rats given neonatal treatment with a gastrin-releasing peptide receptor (GRPR) antagonist

Group	N	***Mean ± SEM body weight (g)***				
		PN 10	PN 15	PN 30	PN 60	PN 90
Saline	12	21.07±0.64	30.85±0.92	86.91±5.48	244.02+7.45	354.07+8.60
RC-3095 1 mg/kg	17	20.28±0.29	30.61±0.26	95.29±1.72	239.43+9.55	327.38+6.00
RC-3095 10 mg/kg	14	20.79±0.44	31.06±1.23	95.68±1.37	244.56+10.01	333.75+8.70

Rats were given an intraperitoneal (i.p.) injection of saline (SAL) or GRPR antagonist RC-3095 (1 or 10 mg/kg) twice daily from postnatal days (PN) 1 to 10. There were no significant differences among groups.

Table 2

Mean ± SEM total time (s) spent exploring both objects during training in a novel object recognition (NOR) task in rats given neonatal treatment with a gastrin-releasing peptide receptor (GRPR) antagonist

Group	***Mean ± SEM exploration time (s)***
Saline	60.95 ± 2.72
RC-3095 1 mg/kg	63.53 ± 5.43
RC-3095 10 mg/kg	52.98 ± 3.77

Rats were given an intraperitoneal (i.p.) injection of saline (SAL) or GRPR antagonist RC-3095 (1 or 10 mg/kg) twice daily from postnatal days (PN) 1 to 10. NOR training was given at PN 60; n = 10 animals per group. There was no significant difference among groups.

Table 3

Open field behavior after neonatal gastrin-releasing peptide receptor (GRPR) blockade in rats

		Postnatal days 33-38		
Group	**Mean ± SEM latency (s)**	**Mean ± SEM # of crossings**	**Mean ± SEM # of rearings**	**Mean ± SEM # of fecal pellets**
Saline	6.02 ± 3.91	82.75 ± 10.72	25.50 ± 3.92	6.12 ± 1.24
RC-3095 1 mg/kg	2.59 ± 0.60	81.91 ± 8.21	26.45 ± 3.03	5.90 ± 0.92
RC-3095 10 mg/kg	4.29 ± 1.15	82.25 ± 5.92	25.17 ± 2.36	6.92 ± 0.74
		Postnatal days 80-85		
Group	**Mean ± SEM latency (s)**	**Mean ± SEM # of crossings**	**Mean ± SEM # of rearings**	**Mean ± SEM # of fecal pellets**
Saline	2.97 ± 1.41	102.14 ± 10.14	24.78 ± 1.96	4.00 ± 0.86
RC-3095 1 mg/kg	2.69 ± 0.92	90.41 ± 5.57	20.53 ± 2.01	4.59 ± 0.74
RC-3095 10 mg/kg	3.36 ± 0.87	87.78 ± 8.06	22.79 ± 2.47	3.14 ± 0.78

Rats were given an intraperitoneal (i.p.) injection of saline (SAL) or the GRPR antagonist RC-3095 (1 or 10 mg/kg) twice daily from postnatal days (PN) 1 to 10. Open field behavior was tested at both PN 33-35 and 80-85; *n* = 12-17 animals per group. There were no significant differences among groups.

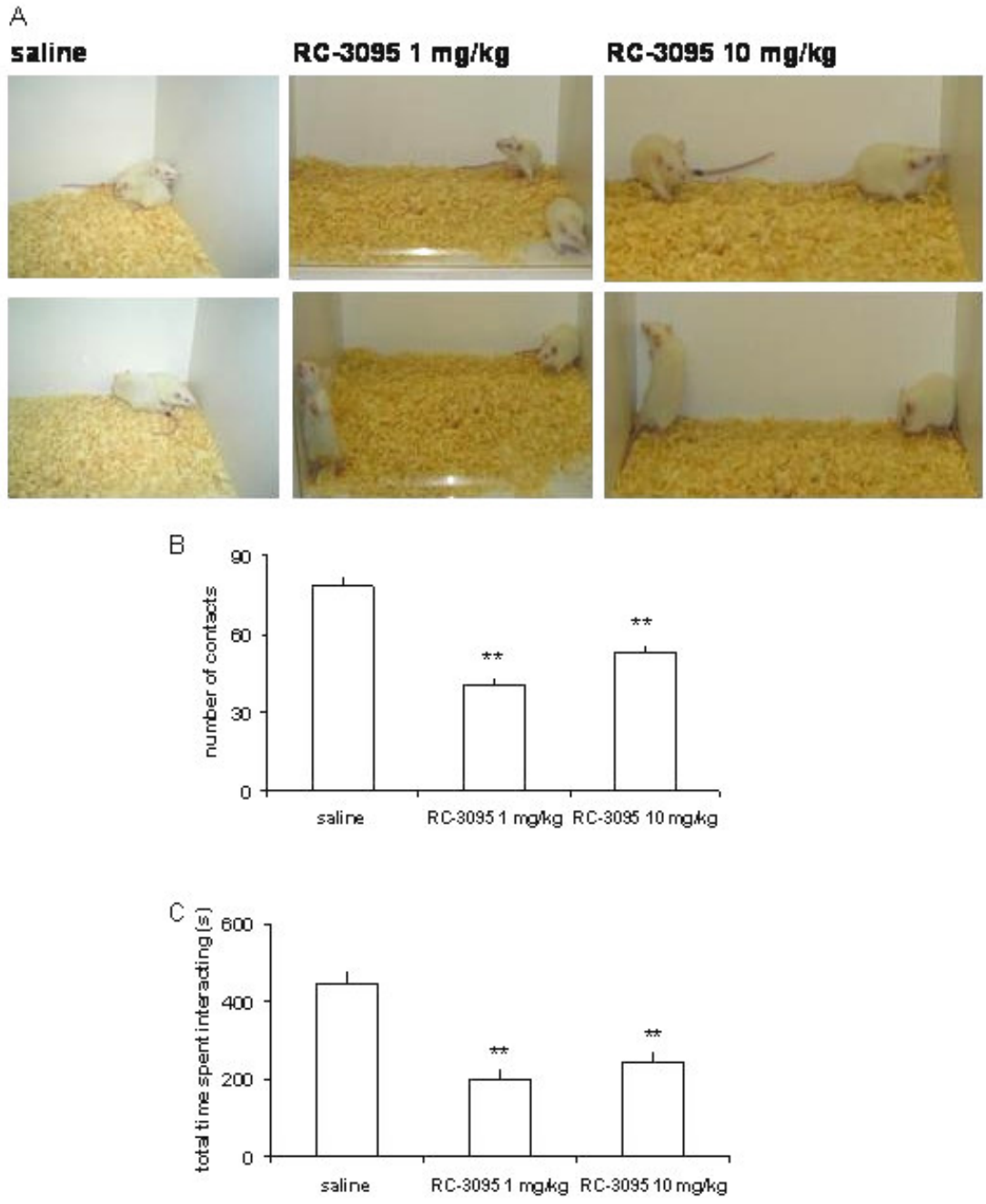

Fig. 1. Neonatal gastrin-releasing peptide receptor (GRPR) blockade impairs social behavior in rats. Animals were given an intraperitoneal (i.p.) injection of saline (SAL) or the GRPR antagonist RC-3095 (1 and 10 mg/kg) twice daily from postnatal days (PN) 1 to 10. Social behavior was tested at PN 30. (A) Photographs of rats given SAL or RC-3095 (1 or 10 mg/kg) during the social interaction test. (B) Mean $\pm$ SEM number of social contacts. (C) Mean $\pm$ SEM time spent engaged in social interaction (s); n = 6-7 animals per group; ** $p < 0.01$ compared to the SAL-treated group.

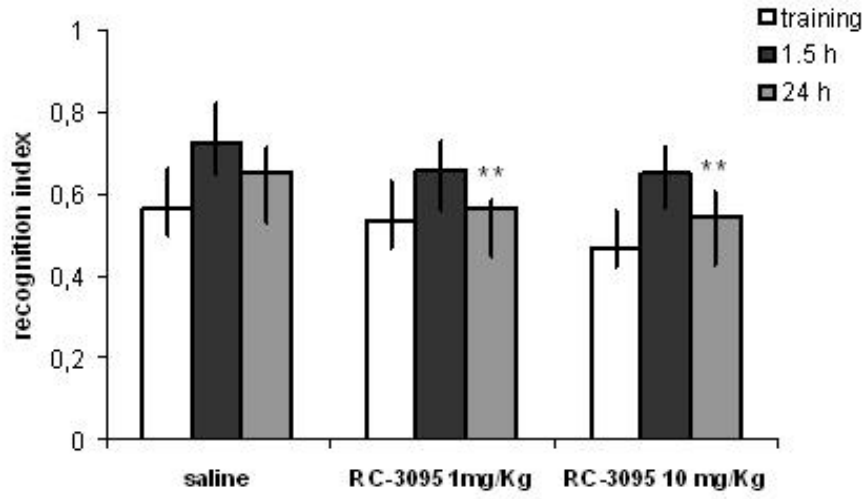

Fig. 2. Neonatal gastrin-releasing peptide receptor (GRPR) blockade impairs 24-h retention of novel object recognition (NOR) memory in rats. Animals were given an intraperitoneal (i.p.) injection of saline (SAL) or the GRPR antagonist RC-3095 (1 or 10 mg/kg) twice daily from postnatal days (PN) 1 to 10. NOR training was carried out at PN 60. Data are median (interquartile ranges) exploratory preference during the training, 1.5-h retention, and 24-h retention trials; $n = 10$ animals per group, ** $p < 0.01$ compared to the SAL-treated group.

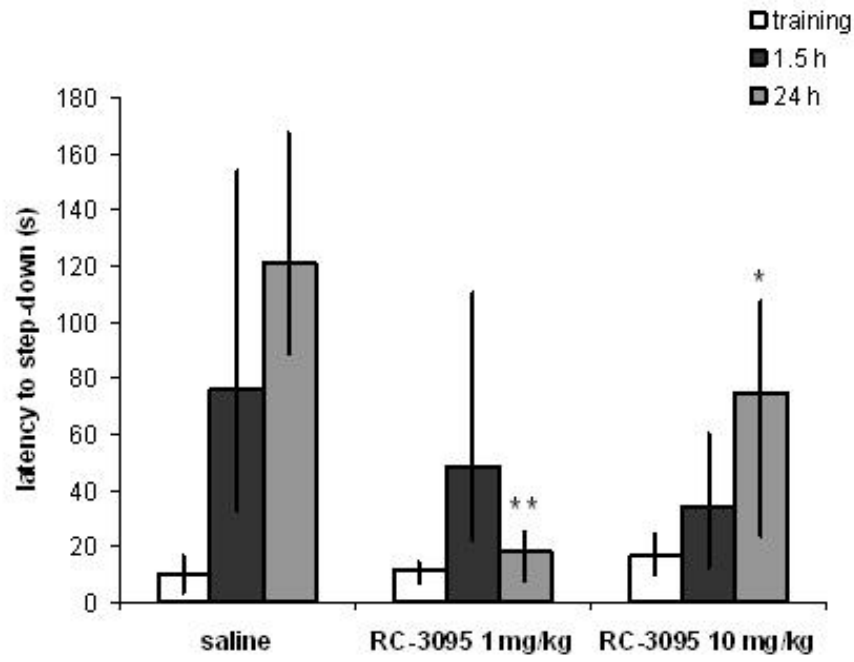

Fig. 3. Neonatal gastrin-releasing peptide receptor (GRPR) blockade impairs 24-h retention of inhibitory avoidance (IA) memory in rats. Animals were given an intraperitoneal (i.p.) injection of saline (SAL) or the gastrin-releasing peptide receptor (GRPR) antagonist RC-3095 (1 or 10 mg/kg) twice daily from postnatal days (PN) 1 to 10. IA training was carried out at PN 70. Data are median (interquartile ranges) Latencies to step-down during the training, 1.5-h retention, and 24-h retention trials; n = 12-14 animals per group, * $p < 0.05$ and ** $p < 0.01$ compared to the SAL-treated group.

5. CONSIDERAÇÕES FINAIS

O fármaco RC-3095 consiste em um antagonista do peptídeo liberador de gastrina/ bombesina e uma droga anti-câncer experimental que inibe o crescimento de células tumorais em diversos modelos experimentais (Chatzistamou et al., 2001; Qin et al., 1994). Assim, diante da ampla utilização desse antagonista no tratamento de diversos tipos de cânceres humanos e do fato de peptídeos semelhantes à bombesina regularem diversos aspectos de funções neurais e comportamentais (Flood et. al., 1988; McCoy et al., 1990; Santo-Yamada et al., 2001; Wada et al., 1998; Williams et al., 1994; Yamada et al., 2002), torna-se clinicamente relevante a investigação dos efeitos das funções dos antagonistas de GRP e seu receptor no cérebro. Embora recentes estudos investiguem os efeitos da atividade antitumoral desse fármaco, além de seus efeitos cognitivos quando aplicados em áreas cerebrais específicas (Roesler et al., 2003) e efeitos de estereotipia (Meller et al., 2004), até o presente momento não foram relatados os efeitos, sob diversos aspectos comportamentais, na fase adulta do bloqueio do GRPR precocemente no período neonatal.

Em nossos estudos, partindo-se de um bloqueio do receptor do peptídeo liberador de gastrina apenas no período neonatal; foi possível acompanhar o desenvolvimento neurológico dos animais, utilizando-se tarefas capazes de explicitar efetivamente quais atividades estavam prejudicadas ou não na fase adulta.

Com isso, observou-se que o tratamento com o RC-3095, nos primeiros dez dias de vida dos animais foi capaz de provocar alterações significativas, na fase adulta, no comportamento social, memória e aprendizado, sem afetar outros aspectos comportamentais. Em animais tratados com RC-3095 foram analisados significativos parâmetros de isolamento social; característica mais distintiva e qualitativa observada no diagnóstico de algumas patologias neuropsiquiátricas como o autismo,

Tal correlação, associada a estudos prévios em roedores e humanos, sugere que o sistema GRP/GRPR também deve ser considerado como alvo terapêutico na investigação de novos agentes potenciais para o tratamento de desordens neuropsiquiátricas. Nesse contexto, o tratamento com o antagonista RC-3095 demonstrado no presente estudo, pode servir, futuramente, para o desenvolvimento de um novo modelo de autismo.

6. REFERÊNCIAS

Anand, A.; Shekhar, A. Brain imaging studies in mood and anxiety disorders: special emphasis on the amygdala. *Ann. N. Y. Acad. Sci., 2003, 985, 370*.

Anastasi, A.; Erspamer, V.; Bucci, M. Isolation and amino acid sequences of alytesin and bombesin, two analogous active tetradecapeptides from the skin of European discoglossid frogs *Arch. Biochem. Biophys., 1973, 148, 443.*

Battey, J.; Wada, E. Two distinct receptor subtypes for mammalian bombesin-like peptides. *Trends Neurosci., 1991, 14, 524*

Bissette, G.; Nemeroff, C.B.; Decker, M.W.; Kizer, J.S.; Agid, Y.; Javiy-Agid, F. Alterations in regional brain concentrations of neurotensin and bombesin in Parkinson's disease. *Ann. Neurol., 1985, 17, 324*.

Chronwall, B.M.; Pisano, J.J.; Bishop, J.F.; Moody, T.W.; O'Donohue, T.L. Biochemical and histochemical characterization of ranatensin immunoreactive peptides in rat brain: lack of coexistence with bombesin/GRP *Brain Res., 1985, 338, 97*.

Cridland, R.A.; Henry, J.L. Bombesin, neuromedin C and neuromedin B given intrathecally facilitate the tail flick reflex in the rat. *Brain Res, 1992, 584, 163*.

Cullen, A.; Emanuel, R.L.; Torday, J.S.; Asokananthan, N.; Sikorski, K.A.; Sunday, M.E. Bombesin-like peptide and receptors in lung injury models: diverse gene expression, similar function. *Peptides, 2000, 21, 1627.*

Erspamer V, Erpamer GF, Inselvini M. Some pharmacological actions of alytesin and bombesin. *J Pharm Pharmacol. 1970 Nov;22(11):875-6.*

Erspamer V, Erspamer GF, Mazzanti G, Endean R.Active peptides in the skins of one hundred amphibian species from Australia and Papua New Guinea. *Comp Biochem Physiol C. 1984; 77(1):99-108.*

Flood, J.F.; Morley, J.E. Effects of bombesin and gastrin-releasing peptide on memory processing. *Brain Res., 1988, 460, 314*.

Flynn, F.W. Bombesin receptor antagonists block the effects of exogenous bombesin but not of nutrients on food intake. *Physiol. Behav., 1997, 62, 791*.

Flynn., F.W. Am. *J.* Fourth ventricular injection of selective bombesin receptor antagonists facilitates feeding in rats.*Physiol., 1993, 264, R218*

Flynn., F.W. Bombesin-like peptides in the regulation of ingestive behavior. *Ann. N. Y. Acad. Sci., 1994, 739, 120*.

Frank, G.K.; Kaye, W.H.; Ladenheim, E.E.; McConaha, C. Reduced gastrin releasing peptide in cerebrospinal fluid after recovery from bulimia nervosa. *Appetite, 2001, 37, 9*.

Gerner, R.H.; van Kammen, D.P.; Ninan, P.T. Cerebrospinal fluid cholecystokinin, bombesin and somatostatin in schizophrenia and normals. *Prog. Neuropsychopharmacol. Biol. Psychiatry, 1985, 9, 73*.

Gerner, R.H.; Yamada, T. Altered neuropeptide concentrations in cerebrospinal fluid of psychiatric patients *Brain Res., 1982, 238, 298*.

Gibbs, J.; Fauser, D.J.; Rowe, E.A; Rolls, B.J.; Rolls, E.T.; Maddison, S.P. Bombesin suppresses feeding in rats. *Nature, 1979, 282, 208*.

Gmerek, D.E.; Cowan, A. Studies on bombesin-induced grooming in rats.*Peptides, 1983, 4, 907*.

Johnston, S.A.; Merali, Z.. Specific neuroanatomical and neurochemical correlates of grooming and satiety effects of bombesin. *Peptides. 1988, 9 Suppl 1, 245.*

Kamichi, S.; Wada, E.; Aokib, S.; Sekiguchib, M.; Kimuraa, I.; Wadab, K. Immunohistochemical localization of gastrin-releasing peptide receptor in the mouse brain. *Brain Res.,* 2005, 1032: 162 -170

Kulkosky, P.J.; Gibbs, J.; Smith, G.P. Feeding suppression and grooming repeatedly elicited by intraventricular bombesin.*Physiol Behav., 1982, 28, 505*

Leback-Verheyden, A.M.; Krystal, G.; Sartor, O.; Way, J.; Battey, J.F. The rat prepro gastrin releasing peptide gene is transcribed from two initiation sites in the brain*Mol. Endocrinol., 1988, 2, 556*.

Lee, A.L.; Ogle, W.O.; Sapolsky, R.M. Stress and depression: possible links to neuron death in the hippocampus. *Bipolar Disord., 2002, 4, 117*.

Lee, K.; Dixon, A.K.; Gonzalez, I.; Stevens, E.B.; McNulty, S.; Oles, R.; Richardson, P.J.; Pinnock, R.D.; Singh. L. Bombesin-like peptides depolarize rat hippocampal interneurones through interaction with subtype 2 bombesin receptors*J. Physiol., 1999, 518, 791*.

McDonald, T.J.; Jornvall, H.; Nilsson, G.; Vagne, M.; Ghatei, M.; Bloom, S.R.; Mutt, V. Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue*Biochem. Biophys. Res. Commun., 1979, 90, 227*.

McGaugh, J.L. Memory consolidation and the amygdala: a systems perspective. *Trends Neurosci., 2002, 25, 456*.

McGaugh, J.L.; Cahill, L.; Roozendaal, B. *Proc.* Involvement of the amygdala in memory storage: interaction with other brain systems. *Natl. Acad. Sci. U.S.A., 1996, 93, 13508*.

Meller, C.A.; Henriques, J.A.; Schwartsmann, G.; Roesler, R. The bombesin/gastrin releasing peptide receptor antagonist RC-3095 blocks apomorphine but not MK-801-induced stereotypy in mice. *Peptides, 2004, 25, 585*

Merali, Z.; Kent, P.; Anisman, H. Role of bombesin-related peptides in the mediation or integration of the stress response. Cell Mol Life Sci. 2002 Feb;59(2):272-87. Review. *Cell. Mol. Life Sci., 2002, 59, 272*

Merali, Z.; McIntosh, J; Anisman, H. Role of bombesin-related peptides in the control of food intake. *Neuropeptides, 1999, 33, 376*.

Merali, Z.; Piggins, H. Effects of dopamine D1 and D2 receptor agonists and antagonists on bombesin-induced behaviors. *Eur. J. Pharmacol., 1990, 191, 281*.

Minamino, N.; Kangawa, K.; Matsuo, H. Neuromedin B is a major bombesin-like peptide in rat brain: regional distribution of neuromedin B and neuromedin C in rat brain, pituitary and spinal cord *Biochem. Biophys. Res. Commun., 1984, 124, 925*.

Minamino, N.; Kangawa, K.; Matsuo, H. Neuromedin B: a novel bombesin-like peptide identified in porcine spinal cord.*Biochem. Biophys. Res. Commun., 1983, 114, 541*.

Moody, T.W.; Merali, Z. Bombesin-like peptides and associated receptors within the brain: distribution and behavioral implications *Peptides, 2004, 25, 511*.

Moody, T.W.; O'Donohue, T.L.; Jacobowitz, D.M. Biochemical localization and characterization of bombesin-like peptides in discrete regions of rat brain.*Peptides, 1981, 2, 75.*

Moody, T.W.; Pert, C.B. Bombesin-like peptides in rat brain: quantitation and biochemical characterization. *Biochem. Biophys. Res. Commun., 1979, 90, 7-14*.

Moody, T.W.; Pert, C.B.; Rivier, J.; Brown, M.R. Bombesin: specific binding to rat brain membranes. *Proc. Natl. Acad. Sci. U.S.A., 1978, 75, 5372.*

Muurahainen, N.E.; Kissileff, H.R.; Pi-Sunyer, F.X. Intravenous infusion of bombesin reduces food intake in humans. *Am. J. Physiol., 1993, 264, R350*.

Nemeroff, C.B.; Kizer, J.S.; Reynolds, G.P.; Bissette, G. Neuropeptides in Alzheimer's disease: a postmortem study.*Regul. Pept., 1989, 25:123*.

Niu, L.; Matsui, M.; Zhou, S.Y.; Hagino, H.; Takahashi, T.; Yoneyama, E.; Kawasaki, Y.; Suzuki, M.; Seto, H.; Ono, T.; Kurachi, M. Volume reduction of the amygdala in

patients with schizophrenia: a magnetic resonance imaging study. *Psychiatry Res., 2004, 132, 41*.

Olincy, A.; Leonard, S.; Young, D.A.; Sullivan, B.; Freedman, R. Decreased Bombesin Peptide Response in Schizophrenia. *Neuropsychopharmacology, 1999, 20, 52.*

Pert, A.; Moody, T.W.; Pert, C.B.; Dewald, L.A.; Rivier, J. Bombesin: receptor distribution in brain and effects on nociception and locomotor activity. *Brain Res., 1980, 193, 209*.

Piggins, H.; Merali, Z. The effects of concurrent D-1 and D-2 dopamine receptor blockade with SCH 23390 and eticlopride, on bombesin-induced behaviours.*Prog. Neuropsychopharmacol. Biol. Psychiatry., 1989, 13, 583*.

Pinski, J.; Yano, T.; Rekasi, Z.; Cai, R.Z.; Radulovic, S.; Schally, A.V. High potency of a new bombesin antagonist (RC-3095) in inhibiting serum gastrin levels; comparison of different routes of administration. *Regul. Pept., 1992, 41, 185.*

Qin, Y.; Ertl, T.; Cai, R.Z.; Halmos, G.; Schally, A.V. Inhibitory effect of bombesin receptor antagonist RC-3095 on the growth of human pancreatic cancer cells in vivo and in vitro.*Cancer Res, 1994, 54, 1035*.

Rashidy-Pour, A.; Razvani, M.E. Unilateral reversible inactivations of the nucleus tractus solitarius and amygdala attenuate the effects of bombesin on memory storage.*Brain Res., 1998, 814, 127*.

Roesler, R.; Kopschina, M.I.; Rosa, R.M.; Henriques, J.A.; Souza, D.O.; Schwartsmann, G. RC-3095, a bombesin/gastrin-releasing peptide receptor antagonist, impairs aversive but not recognition memory in rats.*Eur. J. Pharmacol., 2004a, 486, 35*.

Roesler, R.; Lessa, D.; Venturella, R.; Vianna, M.R.; Luft, T.; Henriques, J.A.; Izquierdo, I.; Schwartsmann, G. Bombesin/gastrin-releasing peptide receptors in the basolateral amygdala regulate memory consolidation.*Eur. J. Neurosci., 2004b, 19, 1041*.

Roesler, R.; Meller, C.A.; Kopschina, M.I.; Souza, D.O.; Henriques, J.A.; Schwartsmann, G. Intrahippocampal infusion of the bombesin/gastrin-releasing peptide antagonist RC-3095 impairs inhibitory avoidance retention. *Peptides, 2003, 24, 1069*.

Santo-Yamada, Y.; Yamada, K.; Wada, E.; Goto, Y.; Wada, K. Blockade of bombesin-like peptide receptors impairs inhibitory avoidance learning in mice. *Neurosci. Lett., 2003, 340, 65*.

Sapolsky, R.M. Stress and plasticity in the limbic system. *Neurochem Res., 2003, 28, 1735*.

Schwartsmann. G. Dexamethasone and gastrin-releasing peptide receptors in human lung cells. *Lung Cancer, 2004, 46, 129*.

Szepeshazi, K.; Schally, A.V.; Halmos, G.; Lamharzi, N.; Groot, K.; Horvath, J.E. A single in vivo administration of bombesin antagonist RC-3095 reduces the levels and mRNA expression of epidermal growth factor receptors in MXT mouse mammary cancers. *Proc. Natl. Acad. Sci. U.S.A., 1997, 94, 10913*.

Spindel, E.R.; Chin, W.W.; Price, J.; Besser, L.H.; Habener, J.F. Cloning and characterization of cDNAs encoding human gastrin-releasing peptide*Proc. Natl. Acad. Sci. U.S.A., 1984, 81, 5699*.

Spindel, E.R.; Giladi, E.; Brehm, J.; Goodman, R.H.; Segerson, T.P. Cloning and functional characterization of a complementary DNA encoding the murine fibroblast bombesin/gastrin releasing peptide receptor. *Mol. Endocrinol., 1990, 4, 1956*.

Stoddard, S.L.; Tyce, G.M.; Ahlskog, J.E.; Zinsmeister, A.R.; Nelson, D.K.; Carmichael, S.W. Decreased levels of [Met]enkephalin, neuropeptide Y, substance P, and vasoactive intestinal peptide in parkinsonian adrenal medulla.*Exp. Neurol., 1991, 114:23.*

Taylor, I.L.; Garcia, R. Effects of pancreatic polypeptide, caerulein, and bombesin on satiety in obese mice. *Am. J. Physiol., 1985, 248(3 Pt 1), G277.*

Venturella, R.; Lessa, D.; Luft, T.; Roozendaal, B.; Schwartsmann, G.; Roesler, R. Dexamethasone reverses the memory impairment induced by antagonism of hippocampal gastrin-releasing peptide receptors. *Peptides, 2005, in press*.

Wada, E.; Way, J.; Lebacq-Verheyden, A.M.; Battey, J.F. Neuromedin B and gastrin-releasing peptide mRNAs are differentially distributed in the rat nervous system.*J. Neurosci., 1990, 10, 2917*.

Wolf, S.S.; Moody, T.W. Receptors for GRP/bombesin-like peptides in the rat forebrain.*Peptides, 1985, 6 Suppl 1, 111*.

Wolf, S.S.; Moody, T.W.; O'Donohue, T.L.; Zarbin, M.A.; Kuhar, M.J. Autoradiographic visualization of rat brain binding sites for bombesin-like peptides. *Eur. J. Pharmacol., 1983, 87, 163*.

Yano, T.; Pinski, J.; Groot, K.; Schally, A.V. *Stimulation by bombesin and inhibition by bombesin/gastrin-releasing peptide antagonist RC-3095 of growth of human breast cancer cell lines* Cancer Res., 1992, 52, 4545.

Zarbin, M.A.; Kuhar, M.J.; O'Donohue, T.L.; Wolf, S.S.; Moody, T.W. Autoradiographic localization of (125-I-Tyr4)bombesin binding sites in the rat brain *J. Neurosci., 1985, 5, 429*.

Zirlinger, M.; Anderson, D. Molecular dissection of the amygdala and its relevance to autism *Genes Brain Behav., 2003, 2, 282*.

7. ANEXOS

O artigo resultante do trabalho de dissertação apresentado está submetido a revista científica *Neuropharmacology*. A seguir, encontram-se as normas para submissão do artigo desta revista.

Guide for Authors

Type of manuscript

Research Papers. Full papers can be of any length. However, authors should be as succinct as possible and excessively verbose manuscripts may be returned without review.

Mini-Reviews. Short, timely reviews will be by invitation. However suggestions for reviews are most welcome, and should be sent to the Chief Editor.

Online submission of papers

It is now possible to submit your manuscript to the *Neuropharmacology* Editorial office electronically.

Before submitting, it is essential that you refer to the Elsevier Artwork Guidelines:
http://www.elsevier.com/locate/authorartwork

Once you are ready to submit:
1. Select 'Submit online to this journal' option from the 'Journal Services' on the 'Author Gateway'.
2. Follow prompts online. Please note that at each stage of the submission process it is possible to go back a step, save the submission to continue later or remove/change any information already entered.
3. The submission tool will generate a PDF file to be used for the reviewing process.
4. You will receive confirmation of your submission, and further progress of your paper at every stage of its review period thereafter, via e-mail.

Hardcopy Submission of papers

The original and three high quality copies of the manuscript should be sent to: *Neuropharmacology*, Editorial Office, University of Bristol, Bristol, BS8 1TD, U.K. [Tel. +44 (0) 117 928 8085; Fax +44 (0) 117 928 7405; E-mail editor-neuropharmacology@bristol.ac.uk.].
Do not include a disk with the first submission.
An accompanying letter should indicate the number of pages, figures and tables in the manuscript, and may suggest an appropriate Editor for handling the paper. Manuscripts and figures will not be returned unless specifically requested, and will be discarded one month after publication.

Submission of final accepted paper

Authors are requested to submit a computer disk containing the **final** version of the papers along with the final manuscript to the editorial office. Please observe the following criteria:
1. When your paper has been refereed, revised if necessary and accepted, send a disk containing the final version with the final hard copy. Make sure that the disk and the hard copy match exactly.
2. Specify what software was used, including which release, e.g. WordPerfect 5.1.
3. Specify what computer was used (either IBM-compatible PC or Apple Macintosh).
4. Include the text file and separate table and illustration files, if available.
5. The file should follow the general instructions on style/arrangement and, in particular, the reference style of this journal as given in the Instructions to Authors.
6. The file should be single-spaced and should use the wrap-around end-of-line feature, i.e. no returns at the end of each line. All textual elements should begin flush left; no paragraph indents. Place two returns

after every element such as title, headings, paragraphs, figure and table call-outs.
7. Keep a back-up disk for reference and safety.

Transfer of Copyright
Elsevier will mail the corresponding author a "Transfer of Copyright" agreement once the paper has been received in production. All authors must sign the agreement before the article can be published. This transfer agreement enables Elsevier Ltd to protect the copyrighted material for the authors, but does not relinquish the author's proprietary rights. The copyright transfer covers the exclusive rights to reproduce and distribute the article, including reprints, photographic reproductions, microform or any other reproductions of similar nature and translations, and includes the right to adapt the article for use in conjunction with computer systems and programs, including reproduction or publication in machine-readable form and incorporation in retrieval systems. Authors are responsible for obtaining from the copyright holder permission to reproduce any figures for which copyright exists.

Experimental procedures
All animal experiments should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European Communities Council Directive of 24 November 1986 (86/609/EEC) or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed.

Manuscripts should be accompanied by a statement that all efforts were made to minimise animal suffering, to reduce the number of animals used, and to utilise alternatives to in vivo techniques, if available.

Authors are advised to consult A fair press for animals [New Scientist (1992) 1816: 1830] before preparing their manuscript. The Editors reserve the right to reject papers if there is doubt whether suitable procedures have been used.

Style

Manuscripts, including references and figure legends, should be typed double-spaced on one side of A4 paper (206x294 cm) or equivalent, with margins no less than 2.5 cm. Type should be no smaller than 12 point. All typed pages should be numbered consecutively, starting with the title page . Either U.K. or U.S. spelling may be used, but must be consistent throughout.

Abbreviations. Abbreviations should be kept to a minimum. All abbreviations should be written in full when first used and the abbreviation given in parentheses.

Title page. This should contain a brief, but informative, title, a running title (not exceeding 40 characters), the names and addresses of the authors and a list of keywords or phrases. The author for correspondence, with telephone, **Fax numbers and E-mail address**, should be clearly indicated on this page. The manuscript should normally be produced using the following headings. One additional level of sub-headings may also be used.

Summary. The second page should consist of a short summary (not exceeding 200 words) which should be readily accessible to the non-specialist and contain the important points of the paper.

Keywords. Authors should provide up to six keywords, to appear just underneath the summary section. The keywords will be used for indexing purposes.

Introduction. The third page should start with a succinct account of why the work was performed. Long historical introductions should not be used. A statement accessible to a lay audience indicating the potential benefit of the work to man or animals should be included. If any of the work contained in the manuscript has been published previously in the form of an abstract this must be referenced.

Methods. Sufficient detail is required to enable others to repeat the experiments. Where animals are involved full descriptions of all analgesic, anaesthetic and surgical procedures must be stated. Where abbreviations are used in place of long chemical names the full chemical name should be provided in this section. Details of any statistical analyses performed should be given. SI units should be used.

Results. The results should be fully illustrated. Negative findings should also be noted to avoid unnecessary replication by others.

Discussion. This should be as concise as possible. Its main function should be to discuss the results in context with the current state of the field.

Acknowledgements. These should be as brief as courtesy allows.

References. These should not normally exceed 40. Davies, J., Francis, A. A., Jones, A. W., Watkins, J. C., 1981. 2-Amino-5-phosphonovalerate (2APV), a potent and selective antagonist of amino acid-induced and synaptic excitation. Neuroscience Letters 21, 77-81.
Ascher, P., Johnson, J. W. 1989., The NMDA receptor, its channel, and its modulation by glycine. In: Watkins, J. C., Collingridge, G. L., (Eds), The NMDA Receptor. IRL Press at Oxford University Press, Oxford, pp. 109-121.

Papers that have been accepted for publication may be cited as 'In press', and a photocopy of the manuscript must be provided. Papers submitted or in preparation should be referred to as (unpublished observations/personal communications) within the text, if absolutely necessary.

In the text, references should be given as: Smith (1964) or (Smith, 1964). In the case of multiple authorship, et al. should be used throughout, i.e. Smith et al. (1964). If works published by the same author(s) in the same year are cited, they should be distinguished by the letters a, b, c, etc.

The reference list must be arranged alphabetically according to the surname of the first author, and chronologically if several papers by the same author(s) are referenced.

Illustrations. For hard-copy submission manuscripts, two sets of figures in the form of high quality photographs, line drawings or laser prints should be sent to the Editorial Office together with the figure legends typed double-spaced on separate sheets. The figures should be lettered and clearly identified on the reverse with authors, figure numbers and orientation. Additional sets of figures should be included in the copies of the manuscript. These may be high quality photocopies and, where possible, the legend should be included with each figure (single-spaced if necessary) to aid the refereeing process.

All colour figures in accepted manuscripts will be charged at the rate of USD 300 for the first page of color per manuscript, followed by USD 200 for each subsequent page. *Interactive Reports* will be published on the web and in the print journal free of charge.

Tables. These should be kept to a minimum and should be self explanatory without reference to the text. Data presented in tables should not reproduce that presented in figures or the text.

A suitable location for the placement of tables and figures should be indicated in the text.

Multimedia files. *Neuropharmacology* is now able to accept electronic supplementary material to support and enhance your scientific research. Supplementary files offer additional possibilities to publish supporting applications, movies, animation sequences, high-resolution images, background datasets and sound clips. Any files supplied will be published online alongside the electronic version of your article in Elsevier web products, including ScienceDirect. In order to ensure that your submitted material is directly usable, please ensure that data files are provided in one of our recommended file formats. Full details can be accessed on Elsevier's Author Gateway (www.AuthorGateway.com).

When supplementary files are supplied, an additional 'supplementary' figure list should also be submitted. Any supplementary material that is not directly referred to from within the text of your manuscript should be referred to via use of a footnote to the article title. In addition, it is also recommended that a short description is provided for each supplementary file supplied. When published online, the descriptive texts will appear as captions alongside links to the relevant supplementary files, an example layout of online supplementary material can be viewed via the following link: http://authors.elsevier.com/ArtworkInstructions.html.

Please note that any supplementary material supplied is subject to the normal peer review process.

Proofs

For all accepted manuscripts, page proofs will be sent to the corresponding author (or the first-named author) for checking. **Corrections to the proofs must be restricted to printer's errors**. Any substantial alterations other than these may be charged to the author. Authors are particularly requested to return their corrected proofs as quickly as possible in order to facilitate rapid publication. Please note that **authors are urged to check their proofs carefully before return, since late corrections cannot be guaranteed for inclusion in the printed journal**. Reprints and copies of the issue (at a

specially reduced rate) can be ordered on the form which will accompany the proofs. These should be returned to: Elsevier Ltd, Bampfylde Street, Exeter, EX1 2AH, U.K.