FACULDADE DE BIOCIÊNCIAS
Programa de Pós Graduação em Biologia Celular e Molecular

DOUTORADO

JULIANA PRESTI-TORRES

# Efeitos do Antagonismo dos Receptores de Peptídeos Semelhantes à Bombesina, o GRPR e o NMBR, durante o Desenvolvimento em Ratos

Porto Alegre, RS
Agosto 2011

PONTIFICIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
FACULDADE DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

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Orientadora: Prof.a Dr.a Nadja Schroder

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Aprovada em 23 de agosto de 2011.

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Porto Alegre

2011

*Aos meus pais, Clarice e Antonio Carlos;*

*Insubstituíveis, Incansáveis, Inigualáveis.*

*A Tiago Wisnevski, amor e apoio incondicional.*

## AGRADECIMENTOS

A Capes, CNPq e 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, que me disponibilizou uma formação de qualidade e respeito. Especialmente à Faculdade de Biociências e ao Programa de Pós-Graduação em Biologia Celular e Molecular, que me acolheram muito bem ao longo de minha graduação e pós-graduação. Será sempre com muito orgulho que representarei essa Universidade.

Ao professor Dr. Eduardo Eizirik que se demonstrou sempre atencioso, dedicado e disponível para resolver todos os percalços que apareceram ao longo desse trajeto.

Ao professor Dr. Maurício Bogo pelo apoio de sempre e pela confiança em minha capacidade de superação.

Aos meus colegas e amigos do laboratório brasileiros e canadenses pela ajuda prestada em todas as horas. A melhor parte de uma pesquisa é saber que podemos trabalhar em conjunto sempre. À Vanessa Garcia e Dr.a Arethuza Dornelles meus agradecimentos especiais. À Glaura e Betânia obrigado por me proporcionarem agradáveis horas de descontração e também pelo incentivo.

Ao Dr. Merali e à Dr.a Pâmela Kent pelo apoio, confiança, ensinamentos e por me proporcionarem uma experiência inesquecível na minha vida pessoal e científica.

À amiga Dr.a Maria Noêmia Martins de Lima que foi excepcional, não medindo esforços para me auxiliar em todo o meu trabalho, mesmo quando estávamos longe! Incentivadora em todas as horas, meticulosa, exigente, orientadora emprestada, minha professora particular de estatística. Minha amigona, que ao lado do Marcus atravessaram as Américas para me visitar. Obrigada de coração pelas palavras carinhosas de sempre: “Eu tenho CERTEZA” que tu vais conseguir!

Ao amigo Dr. Eduardo Rico, pesquisador perspicaz e inteligente, pelo incentivo, pelos ensinamentos moleculares e pelas agradáveis e

enriquecedoras conversas nos intervalos em uma das etapas mais difíceis deste doutorado.

Ao Dr. Rafael Roesler pelo apoio, cartas de recomendação, co-orientação, pelo cuidado, dedicação e auxílio ao longo de todo o doutorado. Obrigada professor!

À Dr.a Nadja Schroder, minha querida orientadora, a quem respeito imensamente e me inspiro, agradeço pela amizade, pela compreensão e interesse, pelo carinho nas horas alegre e tristes, confiança, mesmo diante das dificuldades, investimento e por me proporcionar um doutorado inesquecível! Com certeza levarei teus ensinamentos sempre comigo! Obrigada.

Aos meus pais, Clarice e Antonio. Dupla inseparável e inigualável. Meus alicerces, meus fãs, meus ídolos. Não me deixaram nunca desanimar e sempre compreenderam a importância da pesquisa em minha vida. Foi com eles que aprendi a estabelecer uma meta e persegui-la, ser teimosa, até conquistá-la, mesmo que ninguém mais ao redor acredite. A satisfação de alcançar um sonho, não tem preço. Obrigada. Vocês são os responsáveis por tudo isso!!!! Amo vocês.

A Tiago Wisnevski, homem que admiro e amo. Admirável pelo apoio incondicional, preocupação e ajuda em todas as horas. Muito Obrigada pela compreensão, amor e carinho sempre. Minha melhor escolha!

A Deus, por me mostrar de diversas formas que o caminho mais tortuoso e difícil é, com certeza, o mais bonito e melhor!  A receita é seguir em frente, buscando sempre o que sonhamos.

## Resumo

**INTRODUÇÃO:** Estudos anteriores de nosso grupo de pesquisa demonstraram que o bloqueio do receptor do peptídeo liberador de gastrina (GRPR) em ratos, durante o período neonatal, produz padrões comportamentais característicos de desordens neuropsiquiátricas do espectro do autismo, incluindo déficits em interação social e cognição. **OBJETIVOS:** O presente trabalho teve como objetivos avaliar os efeitos do antagonismo dos receptores de peptídeos semelhantes à bombesina, o GRPR e o NMBR, durante o desenvolvimento em ratos, em dois estudos relacionados. Sendo assim, no primeiro estudo, avaliamos a expressão de GRPR, NR1 e EGFR, três receptores neuronais potencialmente envolvidos na etiologia do Autismo, em córtex cerebral e hipocampo de ratos submetidos ao bloqueio neonatal do GRPR, pelo RC-3095. Adicionalmente, nosso segundo objetivo foi verificar se a Clozapina® seria capaz de reverter os prejuízos em comportamento social neste potencial modelo animal da síndrome do autismo. No segundo estudo, investigamos os efeitos do bloqueio de dois receptores de peptídeos semelhantes a bombesina, o GRPR e NMBR em comportamento de agregação e em interação social em ratos machos e fêmeas tratados no período neonatal com antagonistas seletivos RC-3095 e PD 176252. **MÉTODOS:** *Estudo 1*: os ratos receberam injeções de RC-3095 (antagonista do GRPR) ou de veículo do 1º ao 10º dias de vida pós natal, e foram testados em tarefas de comportamento social e memória de reconhecimento na fase adulta. Uma hora antes da realização da tarefa comportamental, os animais receberam injeções sistêmicas de Clozapina® ou salina. As expressões de mRNA da subunidade NR1 do receptor NMDA, do EGFR e GRPR foram medidas em hipocampo e córtex de um outro grupo de animais que receberam veículo ou RC-3095 no período neonatal. *Estudo 2*: Ratos Wistar machos e fêmeas receberam injeções do antagonista de GRPR, o RC-3095, ou antagonista do NMBR, o PD 176252, ou veículo do 1º ao 10º dias de vida pós natal, e foram testados em comportamento de agregação no 12° dia de vida pós-natal (PN 12) e na tarefa de interação social na fase jovem (PN 30). **RESULTADOS:** *Estudo 1*: Ratos tratados com RC-3095 na fase neonatal apresentaram decréscimos em

interação social e prejuízos em tarefa de memória de reconhecimento. A Clozapina® reverteu os prejuízos em interação social. O tratamento com RC-3095 resultou em decréscimos paralelos na expressão de GRPR, NR1 e EGFR em córtex e aumento de suas expressões em hipocampo. *Estudo 2*: ratos machos e fêmeas tratados com RC-3095 apresentaram decréscimo na manutenção do comportamento de agregação durante o período de desenvolvimento e em diferentes análises de interação social quando testados aos 30 dias de período pós-natal. **CONCLUSÃO:** Os resultados apresentados validam o modelo de autismo original, induzido pelo bloqueio neonatal de GRPR e demonstram pela primeira vez neste modelo, alterações na expressão de receptores neuronais associados a patogênese do autismo. Além disso, pela primeira vez demonstramos que o bloqueio neonatal do GRPR, em animais machos e fêmeas, está associado a prejuízos observados na agregação, um importante parâmetro de sociabilidade, e em interação social, que corresponde a um parâmetro importante na caracterização comportamental de modelos de roedores de autismo.

**Palavras-Chave**: Peptídeo liberador de gastrina - GRPR - Comportamento social - Memória de reconhecimento - Autismo - Receptor NMDA – NR1 - EGRF – Clozapina - Receptor Neuromedina B - NMBR - Agregação social - RC-3095 - PD 176252.

## Abstract

**INTRODUCTION:** We have previously shown that pharmacological blockade of the gastrin-releasing peptide receptor (GRPR) during the neonatal period in rats produces behavioral features of developmental neuropsychiatric disorders of the autism spectrum, including deficits in social interaction and cognition. **OBJECTIVES**: Assess the effects of the antagonism of bombesin-like peptides, the GRPR and NMBR, during development in rats, in two related studies. Thus, in the first study *we* analyzed the expression of GRPR, NR1, and EGFR, three neuronal receptors potentially involved in the etiology of autism, in cerebral cortex and hippocampus of rats submitted to neonatal GRPR blockade by RC-3095. In addition, our second goal was to verify whether clozapine could rescue social behavior impairment in this potential novel animal model of ASD. In the second study, we aimed to investigate the effects of the blockade of two bombesin-like petides; the GRPR and NMBR in aggregative behavior at postnatal day 12 and in social interaction at PN 30 of males and females rats neonatally treated with selective antagonists, RC-3095 and PD 176252. **METHODS:** *Study 1:* Rats were injected with the GRPR antagonist RC-3095 or vehicle from postnatal days 1 to 10, and tested for social behavior and recognition memory in the adulthood. One hour prior to the behavioral testing, rats were given a systemic injection of clozapine or saline. The mRNA expression of the NR1 subunit of the *N*-methyl-D-aspartate (NMDA) receptor, the epidermal growth factor receptor (EGFR), and GRPR was measured in the hippocampus and cortex of a separate set of rats given neonatal RC-3095 or vehicle. *Study 2:* Male and female Wistar rats were injected with the GRPR antagonist RC-3095 or NMB antagonist PD 176252 or vehicle from PN 1 to 10, and tested for aggregative behavior at PN 12 and social interaction in the young phase (at PN 30). **RESULTS:** *Study 1*: Rats given neonatal RC-3095 showed decreased social interaction and impaired object recognition memory. Clozapine rescued the social interaction impairment. Treatment with RC-3095 resulted in parallel decreases in the expression of GRPR, NR1, and EGFR in the cortex, and increased expression in the hippocampus. *Study 2:* Males and females rats given neonatal RC-3095 showed decreased in the maintenance of

aggregation behavior during the developmental phase (PN 12) and in different analyses of social interaction when tested at PN 30. **CONCLUSION:** The results further validate the novel rat model of autism induced by neonatal GRPR blockade, and show for the first time in this model alterations in the expression of neuronal receptors associated with the pathogenesis of autism. In addition, for the first time we showed that the GRPR neonatal blockade, in male and female animals, is associated with impairments observed in aggregation, an important pattern of sociability, and in social interaction, which corresponds to an important behavioral feature of rodent models of autism spectrum disorders.

**Key Words:** Gastrin releasing peptide receptor - Social behavior - Recognition memory – Autism- NMDA receptor – NR1 - EGFR - Clozapine - Neuromedin B receptor – NMBR - Aggregation - RC-3095 - PD 176252.

## LISTA DE ILUSTRAÇÕES

## LISTA DE SIGLAS

BB – Bombesina

BB1 – Receptor de bombesina do tipo 1

BB2 – Receptor de bombesina do tipo 2

cDNA - Ácido desoxirribonucléico complementar

DNA - Ácido desoxirribonucléico

EGF – Fator de crescimento da epiderme

EGFR – Receptor do fator de crescimento da epiderme

GRP – Peptídeo liberador de gastrina

GRPR – Receptor do peptídeo liberador de gastrina

LTM – Memória de longa duração

NMB - Neuromedina

NMBR – Receptor da Neuromedina B

NMDA - N- metil-D-aspartato

NR1 – Subunidade R1 do receptor NMDA

NSCLC - non-small cell lung cancer

NTS – Núcleo do trato solitário

PD 176252 – Antagonista do receptor de Neuromedina B - 3-(1H-indol-3-il)-N-[1-(5-metoxi-piridin-2-il)-ciclohexilmetil]-2-metil-2-[3-(nitrofenil)

PN – período pós-natal

RC-3095 – Antagonista seletivo de GRPR [D-Tpi6, Leu13 psi(CH2NH)-Leu14] bombesina (6-14)

RNA - Ácido ribonucléico

RNAm - Ácido ribonucléico mensageiro

RT-PCR - Reação em cadeia da polimerase - Transcrição reversa

SNC - Sistema nervoso central

SNP – Sistema nervoso periférico

SNPs - variações de polimorfismo único

FCS – fluido cerebroespinhal

**SUMÁRIO**

# 1 CAPITULO 1

## 1.1 INTRODUÇÃO

Um crescente número de evidências tem demonstrado que alterações em determinados peptídeos no Sistema Nervoso Central (SNC) estão relacionadas a patologias neuropsiquiátricas e neurodegenerativas. Neste contexto, a família da Bombesina, um peptídeo inicialmente identificado em anfíbios, vem sendo investigada em mamíferos como um peptídeo potencialmente envolvido na regulação de funções cerebrais normais, bem como em alterações comportamentais associadas a transtornos neuropsiquiátricos (Moody & Merali, 2004; Roesler et al., 2006a; Presti-Torres et al., 2007; Merali et al., 2006).

O peptídeo liberador de gastrina (GRP; do inglês, *Gastrin Releasing Peptide*), juntamente com o peptídeo Neuromedina B (NMB) correspondem a dois tipos de peptídeos identificados tanto no sistema gastrointestinal de mamíferos quanto em áreas do SNC (Moody *et al*., 1978, 1981; Wolf & Moody, 1985; Wada *et al*., 1990; Moody & Merali, 2004; Kamichi *et al*., 2005). Estudos recentes também demonstraram que o bloqueio de ambos os receptores correspondentes a esses peptídeos; o receptor do peptídeo liberador de gastrina (GRPR; do inglês *Gastrin Releasing Peptide Receptor*) e o receptor da Neuromedina B (NMBR) provocam prejuízos em comportamento social, memória de longa duração (Presti-Torres *et al*., 2007; Garcia *et al*., 2010) e em resposta ao estresse em ratos; características associadas a transtornos como autismo e esquizofrenia (Crawley *et al*., 2007).

Investigações envolvendo transtornos psiquiátricos associados a alterações em determinados peptídeos também sugerem o envolvimento do sistema glutamatérgico, especialmente receptores NMDA, na fisiopatologia da esquizofrenia e também do autismo (Mohn *et al*., 1999; Pereira *et al*., 2009). Além disso, o fator de crescimento epidermal (EGF) e seu receptor também apresentam níveis alterados em regiões cerebrais de pacientes com esquizofrenia e autismo, sugerindo que tal peptídeo corresponde a um fator adicional e importante no estudo de tais transtornos.

O presente estudo representa uma extensão de análises anteriores realizadas em nosso laboratório envolvendo o estudo do bloqueio do GRPR ainda na fase neonatal, (Presti-Torres et al., 2007; Garcia et al., 2010)

adicionadas a outros estudos realizados por Merali e *cols* (2004, 2006) demonstrando que o bloqueio do NMBR também pode estar relacionado a alterações associadas a transtornos psiquiátricos. Acreditamos que investigações adicionais envolvendo o modelo de autismo proposto anteriormente (Presti-Torres *et al.*, 2007; Garcia *et al.,* 2010) juntamente com a validação farmacológica e a avaliação de alterações em outros receptores, neste modelo, também associados ao autismo e esquizofrenia, podem contribuir para o melhor entendimentos das bases moleculares e comportamentais que medeiam tais transtornos.

## 1.2 REFERENCIAL TEÓRICO

### 1.2.1 BOMBESINA (BB)

O peptídeo Bombesina (BB) é um dos peptídeos ativos purificados da pele de anfíbios da espécie *Bombina bombina* (Anastasi *et al*., 1972). 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*., 1988).

O primeiro peptídeo semelhante à Bombesina em mamíferos foi isolado de tecidos gástricos e denominado peptídeo liberador de gastrina ou GRP (sigla do inglês, *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 BB liga-se com alta afinidade em membrana cerebral de ratos e que as mais altas densidades de sítios de ligações específicos à BB localizavam-se no hipocampo, uma área cerebral criticamente envolvida na plasticidade sináptica, memória e transtornos neuropsiquiátricos,

tais como esquizofrenia e doença de Alzheimer. Além disso, através da utilização de técnicas de radioimunoensaio, a ocorrência endógena de peptídeos semelhantes à bombesina foi evidenciada em cérebros de ratos. As áreas cerebrais com altas concentrações de peptídeos bombesina correspondem ao núcleo do trato solitário (NTS), amígdala e hipocampo (Moody *et al*., 1978; 1981). A distribuição de corpos neuronais e fibras nervosas contendo peptídeos bombesina em cérebro e medula espinhal vem sendo densamente descrita (Moody & Merali, 2004).

Em mamíferos, os receptores de bombesina pertencentes à família de receptores acoplados à proteína G são classificados em três grupos: o receptor do peptídeo liberador de gastrina - GRPR (sigla do inglês, *Gastrin Releasing Peptide Receptor*) ou receptor subtipo bombesina 2 (BB2), o receptor neuromedina B (NMB-R) ou receptor subtipo bombesina 1 (BB1) e o receptor bombesina "único" subtipo 3 – BRS-3 (*Bombesin Receptor Subtype 3)* (Jensen *et al*., 2008). As análise dos níveis de expressão de mRNA de receptores BB1 em humanos, camundongos, ratos e macacos foram descritas (Corjay *et al*., 1991; Wada *et al*., 1991; Ohki-Hamazaki *et al*., 1997; Sano *et al*., 2004). Em primatas não humanos, estudos revelaram que os mais altos índices de expressão de mRNA de BB1 estão localizados em áreas do sistema nervoso central como amígdala, núcleo caudado, hipocampo, hipotálamo, tálamo, tronco cerebral e medula espinhal (Sano *et al*., 2004). Da mesma forma, Battey & Wada (1991) reportaram os níveis de expressão de mRNA de BB2 em todas regiões cerebrais, demonstrando altas concentrações de expressão em hipotálamo e gânglios da base (Battey & Wada, 1991). Adicionalmente, técnicas de imunoreatividade detalharam que receptores do tipo BB2 estão amplamente distribuídos em isocórtex, formação hipocampal, córtex piriforme, amígdala e hipotálamo (Kamichi *et al*., 2005).

### 1.2.2 GRP E SEU RECEPTOR (GRPR)

Composto por 27 aminoácidos, o GRP é sintetizado como um precursor com 148 aminoácidos (PreproGRP) no núcleo de neurônios subseqüentemente sofre metabolização pós-traducional (Spindel *et al*., 1984, 1990). O GRPR é um membro da superfamília de receptores acoplados à proteína G, contendo sete

domínios transmembrana e 348 aminoácidos e corresponde ao receptor de peptídeo liberador de gastrina (GRP). Ambos estão distribuídos por todo o sistema nervoso central e periférico (Pert *et al*., 1980; Battey & Wada, 1991; Moody & Merali, 2004; Kamichi *et al*., 2005), estimulando a proliferação celular, revelando abrangência em atividades neuroendócrinas e agindo como um fator de crescimento na patogenia de diversos tipos de cânceres humanos (Yano *et al*., 1992).

Além disso, o GRP vem sendo investigado na regulação de funções cerebrais normais, bem como na patogênese de diversos transtornos psiquiátricos (Merali *et al*., 2002; Roesler *et al*., 2006). Estudos de hibridização *in situ*, avaliando a distribuição do GRP em cérebros de ratos, demonstraram altos níveis de mRNA de GRP na área amigdalohipocampal, 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 maioria das áreas cerebrais contendo mRNA de GRP também apresentam imunoreatividade ao GRP (Zoeller *et al*., 1989; Moody & Merali, 2004).

Estudos *in vitro* utilizando técnicas de auto-radiografia indicaram que as áreas que contêm altas densidades de receptores do GRP; o GRPR; incluem o bulbo olfatório, núcleo *accumbens*, caudado-putâmen, amígdala central, formação hipocampal dorsal (área CA3 e giro denteado), bem como os núcleos talâmicos paraventricular, central medial e paracentral (Wolf *et al*., 1983, Wolf & Moody, 1985, Zarbin *et al*., 1985; Moody & Merali, 2004).

Alguns estudos demonstraram alterações nos níveis de GRP ou na função do GRPR em pacientes com transtornos psiquiátricos, doenças neurodegenerativos e no desenvolvimento nervoso. É possível que uma redução dos níveis do peptídeo semelhante à bombesina em pacientes psiquiátricos altere a atividade regulatória daqueles peptídeos sobre funções do SNC, contribuindo assim para significantes manifestações clínicas (Bissette *et al*., 1985; Gerner *et al*., 1985; Olincy *et al*., 1999). A concentração de peptídeo semelhante à bombesina demonstrou estar significativamente reduzida no núcleo caudado e globo pálido de pacientes com doença de Parkinson (Bissette *et al*., 1985), assim como também está reduzida na urina (Olincy *et al*., 1999) e no fluido cerebroespinhal (FCS) (Gerner *et al*., 1985) de pacientes com esquizofrenia. Alterações nos níveis de GRPR no SNC humano também

podem estar envolvidas na anorexia, bulimia nervosa e transtornos de humor (Merali *et al*.,1999).

Algumas investigações farmacológicas e genéticas em roedores demonstraram que o GRPR em áreas cerebrais, como hipocampo e amígdala, está crucialmente envolvido na regulação da plasticidade sináptica (Wada *et al*., 1990; Sapolski, 2003), em aspectos do comportamento e no processamento emocional que podem estar alterados em transtornos como ansiedade, autismo, esquizofrenia, depressão e demência (Piggins & Merali*.,* 1989; Merali *et al*., 2002).

Alterações no funcionamento ou nos níveis de GRP, bem como de GRPR, podem ocorrer em diversas fases da vida. Entretanto, doenças relacionadas ao desenvolvimento como o autismo, normalmente ocorrem nos primeiros três anos de vida (Schneider & Przewlocki, 2005), podendo estar relacionadas com alguma anormalidade do peptídeo e/ou seu receptor nesse período ou até mesmo no período neonatal, uma etapa importante na maturação do sistema nervoso dos indivíduos.

Os efeitos comportamentais da administração de agonistas e antagonistas do GRPR vêm sendo descritos em alguns estudos. Dentre eles, a indução do comportamento de afagar a pele ou os pêlos de um membro da comunidade a fim de fortalecer os vínculos afetivos e manter a unidade do grupo em ratos (em inglês, *grooming*), através de infusões intracerebrais de BB. A indução de *grooming* nos animais por BB depende do GRPR (Piggins & Merali, 1989) e é atenuada por antagonistas de receptores de dopamina (Piggins & Merali, 1989, Merali *et al*., 1990). Sugere-se que esse comportamento induzido por BB deva estar relacionado à resposta ao estresse e, a administração de bombesina induza 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*., 1990, Moody & Merali, 2004).

Está bem estabelecido que administração de BB suprime a ingesta de alimento em numerosas espécies, incluindo ratos (Gibbs *et al*., 1979), camundongos (Cridland *et al*., 1992) e humanos (Muurahainen *et al*., 1993). Administrações sistêmicas do antagonista do receptor BB atenuam a redução na ingesta alimentar induzida por BB (Flynn, 1997).

### 1.2.3 NMB E SEU RECEPTOR (NMBR)

A Neuromedina do subtipo B (NMB) corresponde a um peptídeo semelhante à BB em mamíferos, exercendo sua função via receptor NMB (NMBR). Esse peptídeo foi originalmente purificado de medula espinhal de porcos (Minamino *et al*., 1983) e está amplamente distribuído tanto no sistema gastrintestinal quanto pelo SNC (Wada *et al*., 1990., Battey *et al*., 1991; Moody & Merali, 2004). Estudos baseados em psicofarmacologia e engenharia genética demonstraram que a NMB, em camundongos, está relacionada a uma ampla variedade de funções fisiológicas e comportamentais tais como a termorregulação, ansiedade, atividade espontânea e a ingesta alimentar (Ladenhein *et al*., 1994; Itoh *et al*., 1995; Rushing *et al*., 1996; Ohki-Hamazaki *et al*., 1999; Yamada *et al*., 2002). Além disso, alguns estudos sugerem que os mecanismos de resposta ao estresse devem ser controlados por peptídeos semelhantes à BB, incluindo a NMB (Kent *et al*., 1998; Yamada *et al*., 2002). Apesar da existência de diversas investigações a respeito do GRP/ GRPR, sabe-se pouco sobre as bases moleculares que medeiam a seletividade entre a NMB e seu receptor NMBR.

### 1.2.4 RC-3095: O ANTAGONISTA DO GRPR

O antagonista seletivo de GRPR [D-Tpi6, Leu13 psi(CH2NH)-Leu14] bombesina (6-14) (RC-3095) é utilizado como uma ferramenta na investigação dos efeitos comportamentais relacionados ao bloqueio do GRPR em modelos de roedores (Roesler *et al*., 2004; Fig.1). O RC-3095 foi desenvolvido por Schally e *cols*. (Pinski *et al.*, 1992) como um potente fármaco antitumoral (Szepeshazi *et al*., 1997, Schwartzmann, 2004).

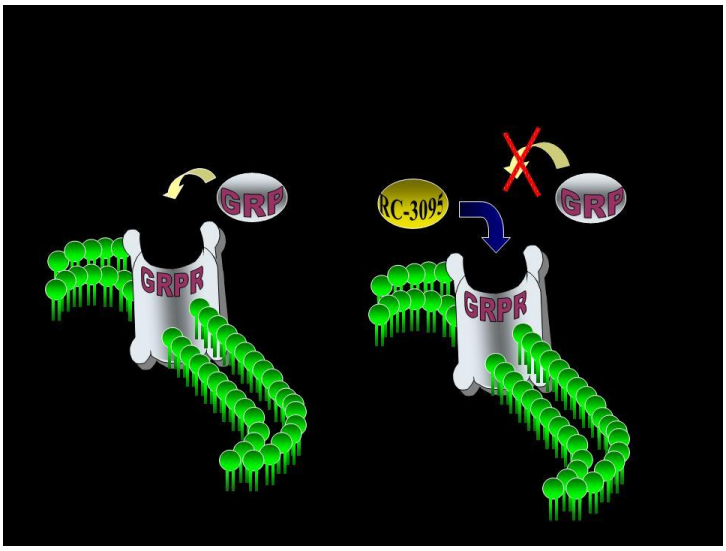


Fig. 1- Bloqueio do receptor GRPR pelo fármaco RC-3095.
A figura representa a ação do RC-3095, um fármaco que age como um antagonista seletivo do peptideo liberador de gastrina (GRP). Na presença deste fármaco, o peptideo liberador de gastrina fica impossibilitado de se acoplar ao seu receptor de membrana (GRPR). (Figura elaborada por Juliana Presti-Torres.)

Diante do fato de pacientes com esquizofrenia apresentarem níveis alterados do peptídeo semelhante à BB (Gerner *et al*., 1985, Olincy *et al*., 1999), foram investigados os efeitos da administração sistêmica de RC-3095 em comportamento de estereotipia induzidos pelo agonista do receptor dopaminérgico apomorfina ou o antagonista do receptor glutamatérgico NMDA, dizocilpina (MK-180) (Roesler *et al*., 2004; Meller *et al*., 2004). Comportamentos estereotípicos são padrões comportamentais observados em transtornos psiquiátricos, tais como a esquizofrenia, desordem obsessivo-compulsiva e autismo. Administrações sistêmicas de RC-3095 atenuaram significativamente a estereotipia induzida por apomorfina, dando suporte a hipótese de 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 antipsicótica (Meller *et al*., 2004).

Neste contexto, nosso grupo de pesquisa demonstrou em estudos anteriores, pela primeira vez, que a administração de um antagonista do GRPR, o RC-3095, em ratos em diferentes doses (1mg/kg e 10 mg/kg), durante os primeiros dez dias de vida, foi capaz de provocar alterações em diferentes

parâmetros comportamentais relacionados a alguns transtornos neuropsiquiátricos como autismo e esquizofrenia (Presti-Torres *et al*., 2007, Fig. 2). Durante a fase adulta, esses animais, ao serem submetidos à tarefa de interação social, de reconhecimento do objeto novo e esquiva inibitória, apresentaram um pronunciado isolamento social, além de importantes déficits cognitivos relacionados à memória de longa duração (LTM).

Recentemente, também demonstramos que o bloqueio do GRPR no período neonatal foi capaz de produzir uma redução no comportamento de preferência ao odor maternal em ratos machos no décimo primeiro dia de vida pós-natal (PN 11) (Garcia *et al*., 2010). A análise deste parâmetro comportamental, baseada na observação de vínculo entre filhote e mãe, e outros padrões observados em nossos estudos, podem representar indicadores de extrema importância no diagnóstico de algumas doenças neuropsiquiátricas como o autismo (Dawson *et al*., 2002, Bespalova & Buxbaum, 2003). Alguns efeitos do antagonismo de receptores do tipo BB1, também vêm sendo relacionados a alterações comportamentais relevantes como interação social (File *et al*., 1980), ansiedade (Merali *et al*., 2004) e desenvolvimento neurológico (Molewijk *et al*., 1996). Tais dados indicam a importância do GRPR nos primeiros anos de vida, pois nessa etapa ele parece ser fundamental para o desenvolvimento.

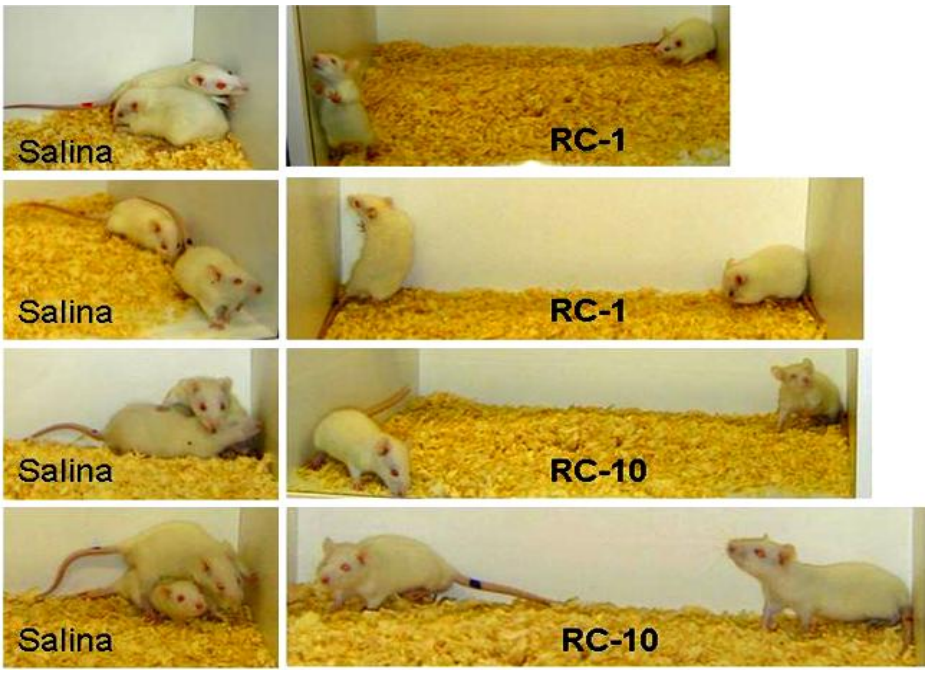


Fig. 2 Tarefa de Interação Social com animais jovens. Animais tratados no período neonatal com salina, RC-3095 1mg/kg (RC-1), RC-3095 10mg/kg (RC-10). A figura representa o aumento do isolamento social, durante a tarefa de comportamento social, em ratos Wistar machos que receberam o antagonista do GRPR (RC- 3095) durante o período neonatal. (Presti-Torres *et al*., 2007).

Estudos farmacológicos investigando o papel 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 BB ou GRP aumentaram o armazenamento de memória tanto em camundongos (Flood & Morley, 1988) quanto em ratos (Rashidy-Pour & Razvani, 1998). Além disso, administrações sistêmicas de GRP atenuaram déficits de memória em modelos amnésicos induzidos por escopolamina e hipóxia em camundongos (Santo-Yamada *et al*., 2001). Infusões de BB no NTS em ratos também foram capazes de melhorar o desempenho dos animais em tarefa de memória, indicando que esta região cerebral seria importante para o estabelecimento dos efeitos de agonistas de GRPR na memória de ratos (Williams *et al*.,1996), enquanto que inativações farmacológicas do NTS atenuaram os efeitos da administração sistêmica de BB na memória (Rashidy-Pour & Razvani, 1998).

Em conjunto, as análises de experimentos examinando os efeitos de agonistas ou antagonistas de GRPRs na memória em roedores, indicam que a ativação de GRPR em hipocampo e amígdala (e possivelmente em outras

áreas cerebrais) está importantemente envolvida na modulação do aprendizado emocional e memória. Diante desses estudos, pode-se concluir que há um forte aparato de evidências estabelecendo o GRPR entre os vários sistemas de neuroreceptores que participam da modulação da memória.

### 1.2.5 PD 176252: O ANTAGONISTA DA NMB

O PD 176252 [3-(1H-indol-3-il)-N-[1-(5-metoxi-piridin-2-il)-ciclohexilmetil]-2-metil-2-[3-(nitrofenil) corresponde a uma nova classe de antagonistas não peptídicos de alta afinidade a receptores de NMB (Ashwood *et al*., 1998). A ação deste antagonista vem sendo relacionada a disfunções na proliferação celular de alguns tipos de cânceres humanos (Moody *et al*., 2006), alterações comportamentais relacionados a transtornos como ansiedade (File *et al*., 1980; Merali *et al*., 2004) e a prejuízos no desenvolvimento neurológico em animais neonatos (Molewijk *et al*., 1996; Merali *et al*., 2006). Recentemente, Merali e *cols* (2006) demonstraram que animais tratados em diferentes doses com o antagonista PD 176252 apresentaram prejuízo na performance em tarefa de interação social e em resposta de sobressalto potencializada pelo medo. Além disso, a administração do antagonista diretamente em núcleo dorsal da rafe gerou um prejuízo em tarefa de interação social sob condições aversivas e suprimiu *in vivo* a liberação de serotonina (5-hidroxitriptamina ou 5-HT) em hipocampo ventral (Merali *et al*., 2006). Consistente com o provável papel dos peptídeos da família BB em parâmetros de ansiedade e comportamento, um estudo adicional demonstrou que neonatos de porquinhos-da-índia tratados com o antagonista de NMBR apresentaram prejuízos significativos em tarefa de vocalização ultra-sônica, quando separados temporariamente de suas progenitoras (Merali *et al*., 2006).

Diante dessas evidências e, ao mesmo tempo, da existência de poucas investigações envolvendo o PD 176252/NMB, estudos subseqüentes são necessários para o entendimento exato da ação do antagonismo deste receptor e quais alterações comportamentais e mnemônicas podem ser observadas.

### 1.2.6 RECEPTOR N-METIL D- ASPARTATO (NMDA)

O receptor N-metil D-aspartato (NMDA) representa uma subclasse de receptores glutamatérgicos que apresenta uma importância considerável devido a suas propriedades farmacológicas e eletrofisiológicas únicas e seu papel no refinamento sináptico, plasticidade neuronal e excitotoxidade (Nakanishi *et al*., 1998).

Pesquisas recentes têm sugerido que os receptores glutamatérgicos tipo NMDA estão envolvidos na fisiopatologia da esquizofrenia e podem ser alvo para tratamentos psicofarmacológicos (Seibt *et al*., 2011). O sistema glutamatérgico é o maior sistema excitatório do sistema nervoso central (SNC) humano. Ele se distribui na maior parte das estruturas do SNC e está envolvido em funções cognitivas fundamentais tais como memória e aprendizado. Os receptores glutamatérgicos tipo NMDA são sofisticados neuroreceptores ionotrópicos essenciais para a plasticidade neuronal, incluindo os mecanismos de LTP (LTP; sigla do inglês *Long Term Potentiation*), sinaptogênese e excitotoxicidade (Bliss *et al*., 1993). Alterações do sistema glutamatérgico estão envolvidas não apenas na esquizofrenia (Kim *et al*., 2011), mas também em doenças neurológicas como epilepsia, isquemias, doença de Alzheimer (Ikonomovic *et al*., 1999; Hynd *et al*., 2001), doença de Huntington (Cepeda *et al*., 2001) transtornos psiquiátricos como transtornos obsessivo-compulsivo (TOC) e afetivo bipolar, entre outros (Law & Deakin, 2001).

Existem diversos subtipos de receptores NMDA, diferindo-se em suas propriedades cinéticas, sensibilidade a vários ligantes, permeabilidade a íons divalentes e interações com proteínas intracelulares (Cull-Candy *et al*., 2001). Os NMDARs são compostos por duas subunidades NR1 e pelo menos, um tipo de subunidade NR2 com predominância das subunidades NR2A ou NR2B em hipocampos de ratos adultos (Wenzel *et al*., 1997). Durante o período pós natal, neurônios corticais exibem alterações na cinética de correntes excitatórias pós-sinápticas mediadas por NMDAR (Barth & Malenka, 2001; Lu *et al.*, 2001), correspondendo a mudanças desenvolvidas na composição de NMDARs, predominantemente nas subunidades NR1/NR2B a NR1/NR2A que induzem a um prejuízo na formação da potenciação de longa duração (Liu *et al*., 2004). Recentemente, tem sido proposto que os receptores NMDA contendo as subunidades NR2A e NR2B estão associados a diferentes cascatas intracelulares além de participarem de diferentes funções na

plasticidade sináptica e em determinadas patologias (Krapivinsky *et al*., 2003; Liu *et al*., 2004; Kim *et al*., 2005). Alterações que podem ocorrer na expressão destas subunidades do NMDAR, podem ocorrer durante as fases iniciais do desenvolvimento e como suas expressões se diferenciam também em diferentes áreas cerebrais como córtex e tálamo. Liu e *cols* (2004) demonstraram que, enquanto a subunidade NR2B apresenta níveis de expressão mais elevados na fase inicial do período pós-natal, o número de subunidades NR2A aumenta na fase adulta, estabelecendo-se um estreito equilíbrio na expressão de ambos tanto em córtex quanto em tálamo (Liu *et al*., 2004). Essas alterações na expressão das subunidades durante o desenvolvimento parecem, portanto, acontecerem em um período crítico na plasticidade durante o neurodesenvolvimento (Zhou & Baudry, 2006).

Mohn e *cols* (1999) desenvolveram uma espécie de camundongo que expressa somente 5% dos níveis normais da subunidade NR1, peça fundamental para que os receptores NMDA sejam funcionais. Os camundongos apresentaram alterações de comportamento tradicionalmente associadas a modelos animais de esquizofrenia, tais como aumento da atividade motora, estereotipias, déficit em interações sociais e sexuais. Interessantemente, estes comportamentos foram revertidos com tratamento antipsicótico (Haloperidol e Clozapina). Tanto os achados com antagonistas do receptor NMDA como os estudos com ratos transgênicos apontam para uma hipofunção de receptores NMDA na esquizofrenia (Mohn *et al*., 1999).

### 1.2.7 FATOR DE CRESCIMENTO DA EPIDERME (EGF) E SEU RECEPTOR (EGFR)

Diferentes representantes da família dos fatores de crescimento desempenham um papel chave na proliferação celular e diferenciação dos SNC e SNP. O fator de crescimento da epiderme ou EGF corresponde a um fator mitógeno que estimula a proliferação de diferentes tipos celulares (Wong & Guillaud, 2004) e está expresso em diversos tipos de cânceres, incluindo o câncer de células pulmonares não-pequenas (non-small cell lung cancer – NSCLC) (Sun *et al*., 2011). Além disso, esse fator está presente em todas as regiões cerebrais em desenvolvimento e em cérebro adulto (Yamada *et*

*al*.,1997; Xian & Zhou,1999), promovendo a diferenciação, maturação e sobrevivência de uma variedade de neurônios (Wong & Guillaud, 2004). Efeitos neurotróficos em culturas de neurônios corticais também foram observados em relação ao EGF, assim como a estimulação do crescimento neurítico em culturas de neurônios dopaminérgicos (Yamada *et al*.,1997) e a neurogênese (Cameron *et al*., 1998; Kuhn *et al*., 1996; Teramoto *et al*., 2003).

A relação entre o EGF e seu receptor (EGFR) com algumas desordens vem sendo descrita (Wong & Guillaud, 2003; Teramoto *et al*., 2003). Camundongos transgênicos para o receptor EGFR desenvolveram padrões de doenças neurodegenerativas durante o primeiro mês de vida (Wong, 2003). Recentemente, foram descritos níveis séricos de EGF significativamente alterados em pacientes com esquizofrenia e autismo (Futamura *et al*., 2002; Iseri *et al.*, 2011) e análises em variações de polimorfismo único (SNPs) revelaram significativa associação entre haplotipos de EGF com o autismo (Toyoda *et al*., 2007).

## 1.3 OBJETIVOS

### 1.3.1 OBJETIVOS GERAIS

Considerando o modelo de autismo proposto em nossos estudos anteriores (Presti-Torres *et al*., 2007; Garcia *et al*.; 2010), baseado no antagonismo do GRPR no período neonatal, e adicionando-se os resultados de nosso estudo subseqüente, onde demonstramos os déficits comportamentais gerados pelo antagonismo ainda no período neonatal (Garcia *et al*., 2010) o objetivo geral deste estudo foi, avaliar níveis de expressão gênica de receptores relevantes ao modelo e validar farmacologicamente o modelo proposto, bem como avaliar os efeitos do antagonismo do GRPR (RC-3095) e do receptor da NMB (PD 176252), durante a fase neonatal, sobre outros parâmetros sociais relacionados ao autismo, em ratos Wistar machos e fêmeas.

### 1.3.2 OBJETIVOS ESPECÍFICOS

- ✓ Avaliar a expressão gênica dos receptores GRPR, NR1 e EGFR em córtex cerebral e hipocampo de animais adultos tratados com RC-3095 no período neonatal.
- ✓ Avaliar a reversão dos déficits comportamentais na fase adulta, induzidos pelo tratamento com RC-3095 no período neonatal, através da administração do antipsicótico clozapina.
- ✓ Avaliar o padrão de agregação (sociabilidade) durante o desenvolvimento neurológico (PN 12) de ratos machos e fêmeas tratados no período neonatal (PN 1-PN 10) com RC-3095 e com PD 176252
- ✓ Avaliar os efeitos do RC-3095 e do PD 176252 em parâmetros de interação social em ratos machos e fêmeas com 30 dias de vida.

Os objetivos aqui apresentados originaram resultados apresentados sob forma de dois artigos científicos. As análises relativas aos dois primeiros objetivos específicos resultaram em estudo denominado "*Rescue of social behavior impairment by clozapine and alterations in the expression of neuronal receptors in a rat model of autism induced by GRPR blockade",* aceito para publicação no periódico *Journal of Neural Transmission.* Além disso, os objetivos seguintes, envolvendo o padrão de agregação e parâmetros de interação em machos e fêmeas, têm seus resultados expressos em "*Alterations in social behavior during development induced by neonatal blockade of bombesin receptors in rats",* manuscrito em fase de finalização para ser submetido*.* Esta etapa foi realizada em colaboração com o grupo de pesquisa do Prof. Dr. Zul Merali, no Institute of Mental Health Research, da University of Ottawa, Canada, durante o desenvolvimento de Programa de Doutorado com Estágio no Exterior (PDEE - Doutorado Sanduíche); CAPES outubro/2009 a março/2010.

# 2 CAPÍTULO 2

ARTIGO CIENTÍFICO ACEITO pela Revista Científica

***Journal of Neural Transmission***

# Rescue of Social Behavior Impairment by Clozapine and Alterations in the Expression of Neuronal Receptors in a Rat Model of Autism Induced by GRPR Blockade

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Number of words in the abstract: 243
Number of words in the article body: 3492
Number of figures: 03
Number of tables: 00
Supplemental information: 00

**Background:** We have previously shown that pharmacological blockade of the gastrin-releasing peptide receptor (GRPR) during the neonatal period in rats produces behavioral features of developmental neuropsychiatric disorders of the autism spectrum, including deficits in social interaction and cognition. Here we show that social interaction deficits in this model are reversed by the atypical antipsychotic clozapine given in the adulthood. In addition, we analyzed the mRNA expression of three neuronal receptors potentially involved in the etiology of autism.

**Methods:** Rats were injected with the GRPR antagonist RC-3095 or vehicle from postnatal days (PN) 1 to 10, and tested for social behavior and recognition memory in the adulthood. One hour prior to the behavioral testing, rats were given a systemic injection of clozapine or saline. The mRNA expression of the NR1 subunit of the *N*-methyl-D-aspartate (NMDA) receptor, the epidermal growth factor receptor (EGFR), and GRPR was measured in the hippocampus and cortex of a separate set of rats given neonatal RC-3095 or vehicle.

**Results:** Rats given neonatal RC-3095 showed decreased social interaction and impaired object recognition memory. Clozapine rescued the social interaction impairment. Treatment with RC-3095 resulted in parallel decreases in the expression of GRPR, NR1, and EGFR in the cortex, and increased expression in the hippocampus.

**Discussion:** The results further validate the novel rat model of autism induced by GRPR blockade, and show for the first time in this model alterations in the expression of neuronal receptors possibly associated with the pathogenesis of autism.

**Key Words:** Gastrin-releasing peptide receptor, social behavior, recognition memory, autism, NMDA receptor, EGFR.

Neuropeptides released as cotransmitters from nerve terminals regulate many aspects of behavior and have been increasingly implicated in neuropsychiatric disorders. Gastrin-releasing peptide (GRP), a neuropeptide that is the mammalian counterpart of the amphibian peptide bombesin, and its receptor (GRPR) are widely distributed in the brain and play an important neuromodulatory role in influencing several aspects of behavior. For example, we and others have shown that the GRPR in the brain is a molecular regulator of anxiety responses and fear memory (1-3). Based on this evidence, we have put forward the GRPR as a novel target for the development of therapeutic strategies for the treatment of central nervous system (CNS) disorders (3, 4).

The GRPR gene (*GRPR*) has emerged as a candidate locus for infantile autism (5, 6). We have recently shown that pharmacological GRPR blockade during CNS development in rats results in long-lasting behavioral deficits that are features of animal models of autism. Thus, rats given systemic injections of the GRPR antagonist RC-3095 during the neonatal period show long-lasting and profound deficits in social interaction and long-term memory (4). More recently, we also found that neonatal RC-3095 produces a reduction in maternal odor preference in juvenile rats (7). These findings indicate that neonatal GRPR blockade can have implications for the characterization of a novel rat model of autism spectrum disorders (ASD).

The pathogenesis of ASD can involve alterations in specific mechanisms mediating synaptic transmission. Abnormalities possibly involved in ASD include altered expression of glutamate receptors and other molecules related

to glutamatergic transmission (8). Impaired social behavior in autism and other psychiatric disorders such as schizophrenia might be related to a reduction in the expression or function of *N*-methyl-D-aspartate (NMDA) glutamate receptors (9), and mice with NMDA receptor hypofunction have been developed as a potential animal model of autism (10). Epidermal growth factor (EGF) plays an important role in CNS development and might also be involved in the pathogenesis of autism. Increased and decreased levels of serum EGF have been found in autistic children and adults, respectively (11, 12). Single-nucleotide polymorphism (SNP) analyses have revealed a significant haplotypic association of EGF with autism (13). In experimental tumors in mice, reduced expression of the EGF receptor (EGFR) is one of the main consequences of treatment with RC-3095 (14, 15).

Atypical antipsychotics including clozapine, risperidone, and olanzapine have been prescribed to patients with ASD, based on evidence that they can ameliorate autistic symptoms with a lower incidence of adverse reactions compared to typical antipsychotics. Some studies have found improvements of social behavior in autistic patients treated with atypical antipsychotics (16, 17). In addition, clozapine rescues deficits in social behavior in mice with reduced expression of the NR1 NMDA receptor subunit (18).

In the present study, we used semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to examine the expression of GRPR, NR1, and EGFR in the brains of rats given neonatal GRPR blockade by RC-3095. In addition, we verified whether clozapine could rescue social behavior impairment in this potential novel animal model of ASD.

# Methods and Materials

## 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 maintained in groups of three to five in individually ventilated cages with sawdust bedding. For postnatal treatments, animals were given standardized pellet food and tap water *ad libitum*. All behavioral experiments were performed at light phase between 09:00 h and 16:30 h. All experimental procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals (NIH publication No. 80-23 revised 1996) and approved by the Institutional Ethics Committee of the Pontifical Catholic University (021/08-CEUA). All efforts were made to minimize the number of animals and their suffering.

## Drugs and pharmacological procedures

Neonatal treatment with the GRPR antagonist RC-3095 has been described in detail in previous studies (4, 7). Briefly, male Wistar rats were given an intraperitoneal (i.p.) injection of either saline (SAL) or RC-3095, [Leu(13) psi(CH(2)NH)-Leu(14)] bombesin (6-14); AEterna Zentaris GmbH, Frankfurt, Germany; 1 mg/kg or 10 mg/kg] dissolved in SAL twice daily (at 8 AM

and 6 PM) for 10 days from postnatal days (PN) 1 to 10. Each treatment group consisted of rats derived from, at least, ten different litters. We have previously shown that this treatment regimen is ideal for producing persistent behavioral deficits, and that the dose of 1 mg/kg produces deficits comparable to a dose 10 times higher (4). Accordingly, in the present study, behavioral experiments were conducted only in rats treated with RC-3095 1 mg/kg. Drug solutions were prepared immediately prior to administration.

In order to further characterize the behavioral impairments described previously, we sought to investigate the possibility of reversion of these deficits by using the atypical antipsychotic clozapine. For these experiments, groups of rats neonatally treated with SAL or RC-3095 (1 mg/kg) at PN 60 were further divided into groups that received an acute i.p injection of SAL or clozapine (1 mg/kg or 5 mg/kg) 1 hour prior to social interaction testing (18, 19). Ten days later, groups were semi-randomized, in order to guarantee that a rat would not receive the same previous treatment, and received an acute injection of SAL or clozapine (1 mg/kg or 5 mg/kg) 1 hour before the training session in the novel object recognition task.

**Social behavior**

Procedures for the social interaction test have been described in detail on previous study (4). Briefly, the animals were tested on PN 60 under dim/light and unfamiliar conditions, in a rectangular open field arena (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 3.5 h prior to the experiment. This

isolation period has been shown to produce a half maximal increase in the amount of social interaction. The test consisted of 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 apparatus for 10 min. Pairs were tested in a randomized order for groups, and animals did not differ by more than 15 g in body weight. The social behavior was assessed for a pair of animals, so behavior of individual animals was not analyzed (4, 20). The total time spent engaged in 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 number of social contacts, and the number of anogenital inspections were measured (20).

**Novel object recognition**

The natural preference for novel objects displayed by mice and rats is used in the novel object recognition (NOR) procedure to assess cognitive alterations in rodent models of neurodevelopmental and neurodegenerative disorders. Rats were trained at postnatal day 70 in an open field arena similar to the one described above. 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 a training trial in which they were exposed to two identical objects (A1 and A2). The rats were allowed to freely explore the objects until they had accumulated 30 s of total inspection time or for a maximum of 20 min. 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 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 novel object B. In long-term retention test trial, the novel object was placed in 50% trials in the right side and 50% trials in the left side of the open field. 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 for each animal was calculated as the ratio $T_N/(T_F + T_N)$, where $T_F$ = time spent exploring the familiar object (A), $T_N$ = time spent exploring the novel object (B). For the training trial, the index was the ratio of time spent exploring object A2 to time spent exploring both objects [$T_{A2}/(T_{A1} + T_{A2})$) (4, 21). The training protocol used in the present study, in which animals are trained to meet the criterion of 30 s exploring objects was chosen in order to overcome the possible motor and motivational/exploratory effects induced by pharmacological treatments.

**Reverse transcriptase-polymerase chain reaction (RT–PCR)**

TRIzol reagent, SuperScriptTM III First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT–PCR) kit, and Taq DNA polymerase were purchased from Invitrogen. The expression analysis of GRPR, NR1, and EGFR was carried out by a semi-quantitative reverse transcriptase polymerase chain reaction (RT–PCR) assay. Cerebral cortex and hippocampus

from 60-day-old rats with were isolated for total RNA extraction with TRIzol reagent in accordance with the manufacturer instructions. The cDNA species were synthesized with SuperScript First-Strand Synthesis System for RT–PCR from 2 µg of total RNA and oligo (dT) primer in accordance with the suppliers. RT reactions were performed for 50 min at 50°C. cDNA (1 µl) was used as a template for PCR with the specifics primers which were designed using the program Oligos 9.6. β-actin- PCR was carried out as an internal standard. The following set of primers were used: for GRPR: forward 5'- CTG TCA GCT GAC AGG TAC AAA GCC ATC G – 3' and reverse 5'-GCA CTC TGA ATC AGA TTT CGG GCA ATG-3'; for NR1: forward 5'- CAG GAA TGC GAC TCC CGC AGC AAT G – 3' and reverse 5'- CCC TCC TCC CTC TCA ATA GCG CGT C – 3'; for EGFR: forward 5'- GCC ACA TCT CCC AAA GCC AAC AAG G – 3' and reverse 5' - AGT CAC GGT GTA CCA AAC GCC GGT C – 3'; and for β-actin: forward 5'-TAT GCC AAC ACA GTG CTG TCT GG-3'; and reverse 5'- TAC TCC TGC TTC CTG ATC CAC AT-3'. PCR reactions were performed (total volume of 25 µl) using a concentration of 0.2 µM of each primer indicated and 200 µM and 1 U Taq polymerase in the supplied reaction buffer. RT–PCRs were optimized in order to determine the number of cycles that would allow product detection within the linear phase of mRNA transcripts amplification. Conditions for all reactions were as follows: initial 1 min denaturation step at 94°C, 1 min at 94°C, 1 min annealing step at 59.8° C (for GRPR) or 60.6° C (for NR1 and EGFR), 1 min extension step at 72°C for 34 cycles and a final 10 min extension at 72°C. Conditions for β-actin PCR were as follows: initial 1 min denaturation step at 94°C,1 min at 94°C, 1 min annealing step at 58.5°C, 1 min extension step at 72°C for 34 cycles and a final 10 min extension at 72°C. The

amplification products were: GRPR 323bp; NR1 395bp; EGFR 265bp; β-actin 210pb. The fragment length of PCR reactions was confirmed with Low DNA Mass Ladder (Invitrogen, USA). PCR products were submitted to electrophoresis using a 1% agarose gel and the relative abundance of mRNA versus β-actin was determined by densitometry using freeware ImageJ 1.37 for Windows (22).

### Statistics

Data for 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. 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 analysis of variance (ANOVA) followed by Tukey posthoc tests when necessary (4). The RT-PCR data were analyzed by one-way analysis of variance (ANOVA). Main effects were further analyzed by multiple comparisons of means using Tukey posthoc tests. In all comparisons, $p$ <.05 was considered to indicate statistical significance.

## Results

### Social behavior

Results for the social interaction test are shown in Figure 1. Comparisons using one-way ANOVAs revealed significant differences among groups in both the total amount of time spent engaged in social interaction ($F(5,31) = 2.51$, $p < .05$, Figure 1A) and frequency of anogenital inspections ($F(5,31) = 6.99$, $p < 0.0001$, Figure 1C), but not in the total number of social contacts ($F(5,31) = 2.08$, $p = 0.094$, Figure 1B). Further analysis with Tukey post-hoc tests showed that rats treated neonatally with RC-3095 given vehicle 1h prior to the social interaction task (RC-3095 plus SAL) showed a significant decrease in both the total amount of time spent engaged in social interaction ($p = 0.037$) and the frequency of anogenital inspections ($p < 0.0001$) when compared to controls given vehicle in the neonatal period plus vehicle SAL 1 h before the behavioral task. Clozapine rescued the social behavior impairment. Groups treated with RC-3095 plus clozapine (1 or 5 mg/kg) did not differ statistically from the control group ($p$s < 0.27 and 0.30, respectively) in the total time engaged in social interaction. In addition, RC-3095 groups given clozapine at 1 or 5 mg/kg showed a significantly higher frequency of anogenital inspections when compared to the group given RC-3095 plus SAL ($p$s < 0.014 and 0.005, respectively), and did not differ from the control group. There was no significant difference in the latency to start interacting (mean ± SEM latencies were 2.5 ± 0.8 in the SAL plus SAL-treated group; 4.7± 1.6 in the group given SAL plus clozapine 1 mg/kg; 6.2 ± 1.3 in the SAL plus clozapine 5 mg/kg; 5.6 ± 1.7 in the group given RC-30095 1 mg/kg plus SAL; 7.7 ± 2.7 in the RC-3095 plus clozapine 1 mg/kg; and 3.9 ± 1.7 in the RC-3095 plus clozapine 5 mg/kg; $F(5,31) = 0.88$; $p = 0.53$).

**Novel object recognition**

In order to exclude the possibility that the effects of RC-3095 or clozapine would alter general aspects of behavior that could interfere with the memory acquisition process, we used a training protocol in which rats had to accumulate 30 s of total exploration of both objects. Statistical comparison of latency to reach the criterion of 30 s exploring both objects during the object recognition training session was used as an index of motor and exploratory activity. Kruskal-Wallis analysis of variance revealed no significant differences in the latency to reach criterion among groups (df = 5, $p$ = 0.392, data not shown).

Results for novel object recognition memory are shown in Figure 2. Comparison of recognition indexes using Kruskal-Wallis analyses of variance showed a significant difference among groups in the long-term retention test (df = 5, $p$ = 0.003), but not in the training session (df = 5, $p$ = 0.607). Further analyses with Mann-Whitney U tests showed that rats treated neonatally with RC-3095 that received vehicle in adulthood present significantly lower recognition indexes in long-term retention test than the control group ($p$ = 0.001), indicating that RC-3095 given in the neonatal period induces recognition memory impairment. RC-3095-treated rats that received clozapine (1 and 5 mg/kg) were also memory-impaired when compared to controls ($p$s < 0.001 and 0.05, respectively). Groups that received SAL plus clozapine have also shown significantly lower recognition indexes than controls (SAL plus SAL; both $p$s < 0.05).

**Expression of GRPR, NR1, and EGFR**

Neonatal RC-3095 administration produced a significant difference among groups in GRPR mRNA levels in both the cortex ($F(2,9) = 21.76$, $p < 0.05$) and hippocampus ($F(2,9) = 167.91$, $p < 0.001$). Tukey posthoc tests showed a significant decrease in GRPR mRNA expression in the cortex of rats treated with either 1 or 10 mg/kg of RC-3095 in comparison to the control group ($p = 0.036$ and $p = 0.0001$ respectively; Figure 3A). In contrast, in the hippocampus, RC-3095 at 1 mg/kg produced a significant increase in GRPR mRNA levels when compared to the SAL group ($p < 0.0001$; Figure 3B).

Results for the expression of NR1 in the cortex are shown in Figure 3C. Comparisons using ANOVA showed significant differences among groups in NR1 mRNA levels in the cortex ($F(2,9) = 7.85$, $p < 0.01$) and hippocampus ($F(2,9) = 8.81$, $P < 0.01$). Tukey tests showed that, in the cortex, NR1 expression was significantly reduced by RC-3095 at 10 mg/kg when compared to controls ($p = 0.008$). In the hippocampus, NR1 mRNA expression was significantly increased by RC-3095 at 1 mg/kg ($p = 0.008$; Figure 3D).

There were significant differences in EGFR mRNA expression among groups in the cortex ($F(2,9) = 14.12$, $p < 0.01$) and hippocampus ($F(2,9) = 30.88$, $p < 0,001$). Tukey tests showed that, in the cortex, EGFR mRNA expression was reduced in the group given 10 mg/kg RC-3095 when compared to control animals ($p < 0.0001$; Figure 3E). In the hippocampus, RC-3095 at 1 mg/kg lead to a significant increased EGFR expression when compared to controls ($p < 0.0001$; Figure 3F).

## Discussion

In the present study, we examined alterations in the expression of relevant neuronal receptors in the cortex and hippocampus and the effects of clozapine on behavioral impairments produced by pharmacological blockade of the GRPR during CNS development in rats. Treatment with a GRPR antagonist resulted in impaired social interaction revealed by a significant decrease in the amount of time engaged in social interaction and frequency of anogenital inspections, as well as impaired cognitive function measured by novel object recognition. These behavioral alterations were accompanied by parallel long-term changes in the expression of GRPR, NR1, and EGFR in the cortex and hippocampus. Social behavior impairments were rescued by clozapine. Together with our previous findings that rats given neonatal RC-3095 display behavioral deficits that are features of ASD (4, 7), the present study indicates that neonatal GRPR blockade might be a novel animal model of autism.

Atypical antipsychotics, including clozapine, have emerged as the first line pharmacological treatment to improve several behavioral alterations associated with autism (23, 24). The beneficial effects of clozapine in autistic patients can include dramatic improvement in social behavior (25). In addition, clozapine has been shown to ameliorate deficits in social interaction in rodent models of neuropsychiatric disorders (18). Our finding that clozapine rescued the impairment in social interaction induced by neonatal GRPR blockade is thus consistent with our view that GRPR hypofunction during CNS development might result in behavioral alterations consistent with those expected from an experimental model of ASD. Clozapine did not improve the impairment in object

recognition memory induced by RC-3095. However, the interpretation of this experiment might be limited by the memory-impairing effect produced by clozapine alone in rats not given RC-3095, which indicates that the doses of clozapine used might not be ideal for the identification of memory-rescuing effects.

Alterations in the function of GRPR, NMDA receptors, and EGF/EGFR signaling have been proposed as possible mechanisms involved in ASD (5, 6, 8, 9, 11–13). The present results show that neonatal GRPR blockade led to parallel increases in the hippocampus, and reductions in the cortex, of mRNA levels for GRPR, NR1, and EGFR. The enhancement in hippocampal receptor expression was induced only by the lower dose of RC-3095, which was used for the behavioral experiments in the present study, whereas the decrease in receptor expression in the cortex was produced by a higher dose. Since we have previously shown that the two doses of RC-3095 produce similar behavioral effects (4), it is unlikely that these alterations in receptor expression alone mediated the behavioral deficits. However, the results suggest that the expression of GRPR, EGFR and NMDAR might be regulated in a common pattern in response to GRPR blockade during development, and all three receptor systems might present functional interactions and play a complementary role in regulating aspects of behavior altered in RC-3095-treated rats. In particular, the reduction in the levels of NMDAR in the cortex would be consistent with the hypoglutamatergic hypothesis of autism (8, 9).

In summary, the present study provides further behavioral and pharmacological evidence that GRPR blockade in the neonatal period might represent a novel rat model of autism, and suggest that alterations in the

expression and function of GRPR, NMDAR and EGFR might play a role in the behavioral alterations observed in this model.

## Acknowledgements

This research was supported by the National Council for Scientific and Technological Development (CNPq; grant number 305905/2009-0 to N.S.), and the National Institute for Translational Medicine (INCT-TM). R.R. is supported by CNPq grant number 303703/2009-1, and the South American Office for Anticancer Drug Development. J.P.T. is supported by a CAPES fellowship.

**Disclosure of biomedical financial interests and potential conflicts of interest**

All authors declare that they have no biomedical financial interests or potential conflicts of interest to disclose.

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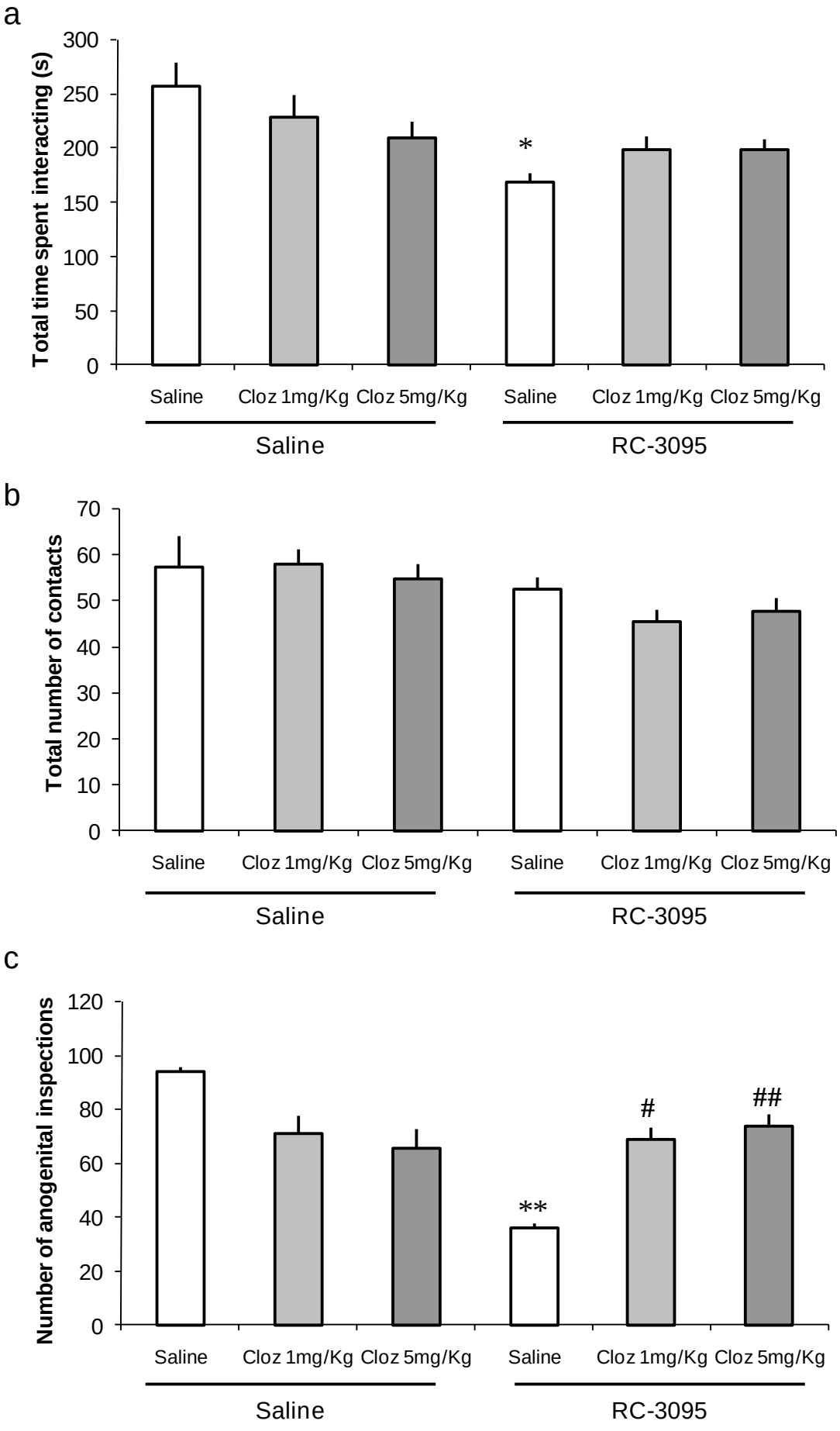
Figure 1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Total time spent interacting (s)
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Figure 2

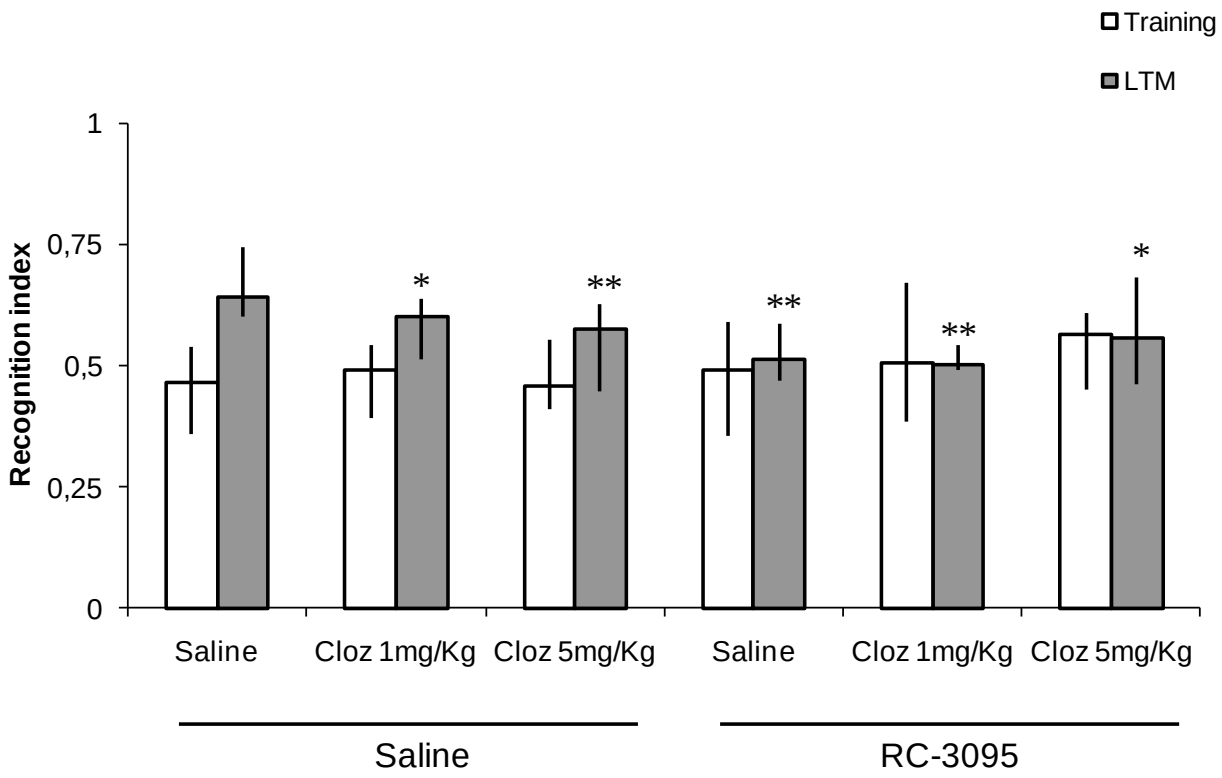
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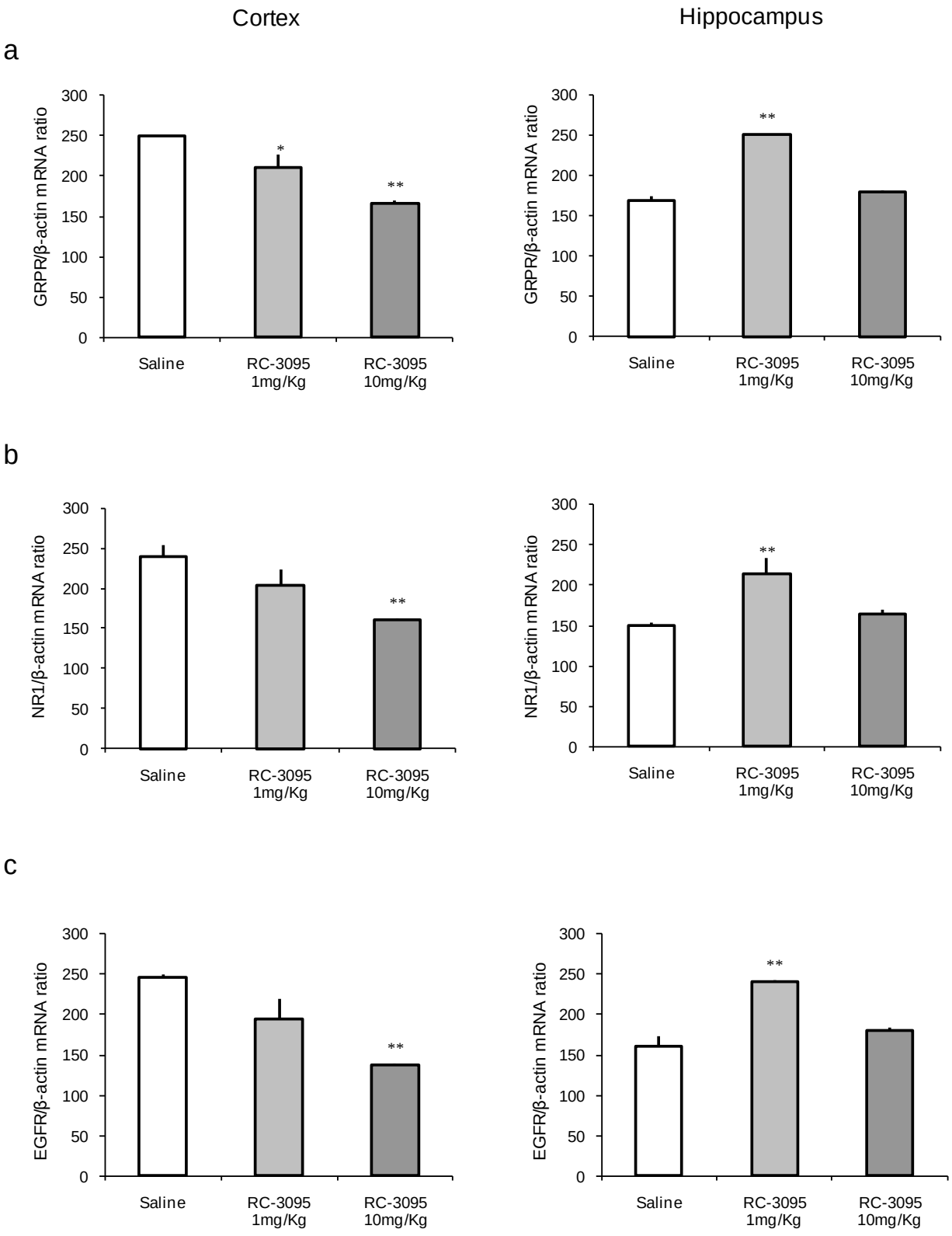
Figure 3


Cortex
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Saline
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RC-3095 10mg/Kg
b
NR1/β-actin mRNA ratio
c
EGFR/β-actin mRNA ratio

## Legends for figures

**Figure 1.** Impairments in social behavior produced by neonatal gastrin-releasing peptide receptor (GRPR) blockade are reversed by acute administration of clozapine. Rats were given an intraperitoneal (i.p.) injection of saline (SAL) or the GRPR antagonist RC-3095 (1 mg/kg) twice daily from postnatal days (PN) 1 to 10. Social behavior was tested at PN 60. An i.p. injection of SAL or clozapine (1 or 5 mg/kg) was administered 1h prior to behavioral testing (A) Mean ± SEM time spent engaged in social interaction (s); (B) Mean ± SEM total number of social contacts; (C) Mean ± SEM number of anogenital inspections. $n$ = 4-8 pairs of animals per group; * $p < 0.05$ compared to control rats given SAL plus SAL; ** $p < 0.01$ compared to the control group.

**Figure 2.** Neonatal gastrin-releasing peptide receptor (GRPR) blockade impairs retention of novel object recognition (NOR) memory in rats. Rats were given an intraperitoneal (i.p.) injection of saline (SAL) or the GRPR antagonist RC-3095 (1 mg/kg) twice daily from postnatal days (PN) 1 to 10, and an i.p. injection of SAL or clozapine (1 or 5 mg/kg) 1h prior to object recognition training. NOR training was carried out at PN 70. Data are median (interquartile ranges) exploratory preference during the training and 24-h retention test trials; $n$ = 12-15 animals per group, * $p < 0.05$ and ** $p < 0.01$ compared to controls.

**Figure 3.** Effects of neonatal gastrin-releasing peptide receptor (GRPR) blockade on mRNA levels of (A) GRPR, (B) *N*-methyl-D-aspartate (NMDA) NR1, and (C) epidermal growth factor receptor (EGFR) in the cortex and hippocampus. 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. mRNA levels were measured with semi-quantitative RT-PCR as described in Methods and Materials. Data are expressed as mean $\pm$ SEM receptor/β-actin mRNA ratio (expressed as arbitrary units) of 4 independent experiments; * $p < 0.05$ and ** $p < 0.01$ compared to controls.

# 3 CAPITULO 3

ARTIGO CIENTÍFICO EM PREPARAÇÃO

# Alterations in social behavior during development induced by neonatal blockade of bombesin receptors in rats

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Abstract

Previous studies have suggested that pharmacological blockade of the gastrin-releasing peptide receptor (GRPR), a type of bombesin (BB) receptor, during development produced long-lasting behavioral alterations in male rats. Here we examined several parameters of social behavior in male and female rats given systemic injections of BB receptor antagonists during the neonatal period. Rats were given an intraperitoneal (i.p.) injection of saline (SAL), the GRPR antagonist RC-3095 (1 mg/kg), or the GRPR/neuromedin B (NMBR) antagonist PD-176252 (0.5 mg/kg) twice daily from postnatal days 1 to 10. Both BB receptor antagonists reduced aggregative behavior in rat pups [tested at postnatal day (PND) 12] and produced gender-specific impairing effects on parameters of social behavior tested at PND 30. These findings provide further evidence that BB receptors are important in regulating social and emotional behavior during central nervous system (CNS) development, and support the view that BB receptor blockade in the neonatal period might represent a novel rat model relevant for some features of autistic spectrum disorders.

Keywords: bombesin receptor; gastrin-releasing peptide receptor, social behavior, neurodevelopmental disorders; autism spectrum disorders

## Introduction

Autism spectrum disorders (ASD) are characterized by marked impairments in communication, repetitive and stereotypic behaviors, as well as social impairment and deficits in attention and cognitive function. Clinical signs of ASD are frequently present at 3 years of age and recent prospective studies in toddlers indicate that behavioral abnormalities that may represent early indicators of autism can be detected as early as 14 months of age (Landa et al., 2007). Males are at higher risk for ASD than females, with a gender ratio of approximately 4:1 (Bespalova et al., 2003, Dawson, 2010).

Different approaches, including clinical assessment, neuroimaging (Happé et al., 1996, Schultz et al., 2000) and neuropathological studies have been used to assess the structural and morphological brain abnormalities in ASDs. The use of animal models is an important tool for investigating the mechanisms underlying this disorder as they afford the ability to tightly control experimental variables and to measure outcome factors (such as neurochemical) that we do not yet have the technology to measure in vivo in humans (Crawley et al., 2007). Points of concern in animal research, including the validity of animal models to human conditions and the understanding of the links between animal and human physiology, are constantly improving.

The bombesin (BB) peptide family, initially implicated in the mediation of peripheral satiety signals (Gibbs et al., 1979; Kulkosky et al., 1982; Merali et al., 1999), has been increasingly implicated in regulating normal brain function and might play a role in behavioral alterations associated with neuropsychiatric disorders (Moody & Merali, 2004; Roesler et al., 2006a; Presti-Torres et al., 2007; Merali et al, 2006). The amphibian peptide BB and its mammalian counterparts, the BB-like peptides neuromedin B (NMB) and gastrin-releasing peptide (GRP), elicit their effects by binding to different subtypes of BB receptors (Battey and Wada, 1991; Moody & Merali, 2004; Jensen et al., 2008). Whereas NMB binds preferentially to BB1 receptors (also known as NMBR), GRP [GRP(1–27)] or neuromedin C [NMC, GRP(18–27)] have a greater affinity for BB2 receptors (GRPR). The GRPR is a G-protein coupled receptor expressed in the cell membranes of several tissues, including neuronal

dendrites and cell bodies (Moody & Merali, 2004; Kamichi et al., 2005; Roesler et al., 2006a; Jensen et al., 2008).

Several animal studies have indicated that the GRPR in brain areas including the dorsal hippocampus and basolateral amygdala is implicated in regulating cognitive function and emotional responses (Shumyatsky et al., 2002; Roesler et al., 2003; Santo-Yamada et al., 2003; Roesler et al., 2003; 2004a; 2004b; Mountney et al., 2008; Merali et al., 2011). 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, raising the possibility that 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 like autism (Ishikawa-Brush et al., 1997). Pharmacological manipulation of the GRPR during the neonatal period has been shown to affect aspects of adult behavior relevant for stress, anxiety responses and social behavior (Piggins and Merali, 1992a; 1992b; Piggins et al., 1993; Presti-Torres et al., 2007; Mackay et al., 2009; Garcia et al., 2010). For example, we have previously described that rats treated with the GRPR antagonist RC-3095 showed pronounced deficits in social interaction when tested at postnatal days (PND) 30-35 and impaired retention of memory for both novel object recognition (NOR) and inhibitory avoidance (IA) tasks tested at PND 60-71 (Presti-Torres et al., 2007; submitted manuscript). Mackay et al. (2009), using the same treatment, showed pronounced communication deficits on rats treated with RC-3095. Extending on these findings, Merali and collleagues have shown that neonatal treatment with RC-3095 also produce impairments in sociability as assessed in the social approach task, as well as communication deficits and increased expression of learned fear (Merali et al., 2006).

As with the GRPR, the NMBR acts as a regulator of emotional responses and social behavior. Infusion of NMB into the dorsal raphe nucleus suppresses social interaction in rats (Merali et al., 2006), and intracerebroventricular (i.c.v.) administration of the NMBR antagonist BIM 23127 elicited elicited anxiolytic effects and reduced the fear potentiated startle response (Bédard et al., 2007). In addition, NMBR-deficient knockout mice show reduced anxiety-related behavior (Yamada et al., 2002).

In the light of these observations, the primary objective of the present study was to investigate the effects of neonatal BB receptor blockade in rats on behavioral measures relevant for animal models of ASD. We examined behavioral parameters related to ASD, including aggregative behavior and social play behavior in male and female rats given systemic injections of BB receptor antagonists during the neonatal period.

## Materials and methods

### Animals

Pregnant Wistar were obtained from the Charles River Laboratory, St-Constant, Canada. 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°C and a 12/12 h light/dark cycle. For postnatal treatments, animals were given standardized pellet food and tap water ad libitum. 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) and efforts were made to minimize the number of animals and their suffering.

### Drugs and pharmacological procedures

Female and male pups received two daily intraperitoneal (i.p.) injections of saline solution (SAL; NaCl 0.9%), the peptidergic selective GRPR antagonist RC-3095 (1 mg/kg, Zentaris GmbH, Frankfurt, Germany) or the nonpeptide GRPR/NMBR antagonist PD-176252 (0.5 mg/kg, Parke Davis, UK, 0.621 nmol) 3-(1H-indol-3-yl)-N-[1-(5-methoxy-pyridin-2-yl)-cyclohexylmethyl]-2-methyl-2-[3-(nitrophenyl) ureido] propionamide (Ashwood et al., 1998) dissolved in SAL at PND 1 to 10. RC-3095 and PD-176252 have been consistently used in previous studies (Merali et al., 2006; Roesler et al., 2003; 2004a; 2004b; Bédard et al.,

2007; Presti-Torres et al., 2007, Garcia et al., 2010; Merali et al., 2011) as tools to investigate the role of BB receptors in brain function and their possible implications for neuropsychiatric disorders. Drug solutions were prepared immediately prior to administration. Drug doses were chosen on the basis of previous studies (Merali et al., 2006; Presti-Torres et al, 2007; Garcia et al, 2010). The treatment regimen (i.e., two daily injections of SAL, RC-3095 or PD-176252 from PN 1 to 10) was chosen on the basis of previous studies (Piggins and Merali, 1992a; 1992b; Piggins et al., 1993; Mackay et al., 2009; Presti-Torres et al., 2007).

Aggregative behavior

Starting at infancy and continuing throughout adult life, huddling or aggregation is a major component of the behavioral repertoire of rats (Schank and Alberts, 1997). Huddling behavior maintains the cohesion of litters throughout early life and in adulthood it remains a consistent feature of social behavior of Rattus norvegicus (Alberts et al., 1978).

The pups were tested on PN 12. At this age, it has been described that the pups show a good cohesiveness, indicating also an important sociability pattern (Schank and Alberts, 1997). The infant rats were placed in a clean polycarbonate rectangular box (18 cm x 21 cm x 20 cm) with a thermo regulated heating pad, and were kept together in a small tube before the test. At the beginning of the test, the pups were allowed to follow the group cohesiveness by themselves. The pup's behavior was assessed for 15 min and recorded using a camera placed directly above the arena. The videos were analyzed and the different levels of cohesion were represented numerically, called Aggregon Index, recorded at every 5 seconds (Schank and Alberts, 1997). This index is based on rat pups orienting themselves in different group formations which can be represented numerically. This measure was used to assess group behavior in the present study. Based on this index, the variations in group formations were each assigned to different numerical index, called aggregons (a number to represent a specific formation: singles, pairs and clusters) (Schank and Alberts, 1997). The "singles" pattern represented the cumulated number of time blocks during which a pup was alone, and out of the nest. The "pairs" represented the

frequency that two pups had separated from the litter, showing a pair cohesion. In addition, the “clusters” pattern represented the cumulated number of independent clusters that were formed for each time block (singles were included as a cluster).

Social play behavior

Qualitative and quantitative impairments in social interaction are the first defining feature of autism and a critical component of rodent models of ASD (Moy et al., 2006). Social interaction was tested at PN 30 and 35, as previously described (Presti-Torres et al 2007). Animals were tested under dim light and unfamiliar conditions, in a rectangular open field apparatus (45 cm x 40 cm x 60 cm). On the day of the experiment, males and females were socially isolated in plastic cages measuring 43 cm x 28 cm x 15cm (l x w x h) for 3.5 h 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 procedure consisted in placing two animals from the same experimental group but from different litters and cages (PD-176252 versus PD-176252, 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. The social behavior of individual animals was analyzed using the ODLog v.2.5.2 program. For each animal the frequency (number of contacts) and the duration of sniffing and grooming any part of the body of the test partner, allogrooming, mounting or crawling over the test partner – over and under, following, sniffing the anogenital part of the partner, pinning, aggression and play fighting were measured (Schneider and Przewlocki, 2005, Presti-Torres et al., 2007).

Statistics

Data for aggregative behavior and social play behavior are shown as mean ± SEM. Comparisons between groups were performed using an one-way analysis of variance (ANOVA) followed by Tukey or Games-Howell post hoc tests when necessary (Merali et al., 2006; Presti-Torres et al., 2007; Mackay et

al., 2009). In all comparisons, $p < 0.05$ was considered to indicate statistical significance.

## Results

### Aggregative behavior

Results for the aggregative behavior test are shown in Fig. 1. There were no significant differences among groups for Aggregon Index [$F(2, 1083) = 2.674$, $p= 0.069$] (Fig. 1A). However, ANOVA comparison among groups regarding the pattern of aggregation has indicated a significant difference in the frequency of singles [$F(2, 1083) = 15.485$, $p= 0.000$], pairs [$F(2, 1083) = 4.722$, $p= 0.009$] and clusters [$F(2, 1083) = 13.027$, $p= 0.000$]. Further analyses with Games-Howell post hoc test indicated that rats treated with RC-3095 showed an increased frequency of the singles, pairs and clusters patterns ($P < 0.05$, $P = 0.038$ and $P < 0.05$ respectively; Fig. 1), when compared with the group treated with SAL. In addition, neonatal PD 176252 administration produced a significant increase in the frequency of the singles and clusters pattern when compared with the SAL group ($p < 0.05$ , Fig. 1B and $p= 0.040$, Fig. 1D respectively). These results suggest that treatment with the BB receptor antagonists induced a reduction in aggregative behavior.

### Social behavior

Results for the social behavior in males are shown in Fig. 2. Comparisons using one-way ANOVA revealed significant differences among groups in the frequency of sniffing [$F(2,29) = 4.89$, $p = 0.015$], over and under [$F(2,29) = 43.38$, $p = 0.000$], allogrooming [$F(2,29) = 7.80$, $p = 0.002$] and aggression [$F(2,29) = 11.00$, $p = 0.000$], but not in the frequency of following the partner [$F(2,29) = 0.091$, $p = 0.913$], grooming [$F(2,29) = 2.69$, $p = 0.085$], pinning [$F(2,29) = 1.04$, $p = 0.365$], play-fighting [$F(2,29) = 2.19$, $p = 0.13$], or anogenital inspections [$F(2,29) = 1.83$, $p = 0.179$) (Fig. 2A). Further analysis with Tukey post-hoc test showed that rats treated neonatally with PD-176252, but not RC-3095, showed a significant increase in the frequency of sniffing ($p =$

0.012) when compared to control rats given SAL. Groups treated neonatally with RC-3095 and PD-176252 showed a significant decrease in the frequency of over and under behavior (both p's = 0.000) and of allogrooming (p = 0.002 and p = 0.024, respectively) when compared to the control group. Tukey post-hoc test has also revealed that RC-3095 treatment, but not PD-176252, induced a significant increase in the frequency of aggressive behavior (p= 0.000, Fig. 2A).

Comparisons using one-way ANOVA revealed significant differences among groups in the total time spent sniffing [$F(2,29) = 4.91$, $p = 0.015$], over and under [$F(2,29) = 20.22$, $p = 0.000$], allogrooming [$F(2,29) = 4.78$, $p = 0.016$] and in aggressive behavior [$F(2,29) = 5.08$, $p = 0.013$], but not in the duration of following the partner [$F(2,29) = 0.296$, $p = 0.746$], grooming [$F(2,29) = 1.42$, $p = 0.258$], pinning [$F(2,29) = 2.36$, $p = 0.113$], play-fighting [$F(2,29) = 2.65$, $p = 0.088$], or in anogenital inspections [$F(2,29) = 0.32$, $p = 0.725$) (Fig. 2B). Further analysis with Tukey post-hoc test showed that rats treated neonatally with PD-176252, but not RC-3095, showed a significant increase in the duration of sniffing (p = 0.011) when compared to control rats given SAL. Groups treated neonatally with RC-3095 and PD-176252 showed a significant decrease in the total time spent in over and under behavior (both p's = 0.000). RC-3095 has also induced a significant reduction in time spent allogrooming (p = 0.019) when compared to the control group. Tukey post-hoc test has also revealed that RC-3095 treatment, but not PD-176252, induced a significant increase in the total time spent in aggressive behavior (p= 0.010, Fig. 2B).

Results for the social behavior in females are shown in Fig. 3. Comparisons using one-way ANOVA revealed significant differences among groups in the frequency of sniffing [$F(2,31) = 21.47$, $p = 0.000$], over and under [$F(2,31) = 32.45$, $p = 0.000$], allogrooming [$F(2,31) = 3.39$, $p = 0.047$], aggression [$F(2,31) = 12.91$, $p = 0.000$], and play-fighting [$F(2,31) = 4.42$, $p = 0.020$], but not in the frequency of following the partner [$F(2,31) = 0.401$, $p = 0.673$], grooming [$F(2,31) = 2.10$, $p = 0.139$], pinning [$F(2,31) = 2.03$, $p = 0.148$], or anogenital inspections [$F(2,31) = 1.35$, $p = 0.274$) (Fig. 3A). Further analysis with Tukey post-hoc test showed that female rats treated neonatally with RC-3095 or PD-176252 showed a significant decrease in the frequency of sniffing (both p's = 0.000) when compared to control rats given SAL. As observed in

males, female rats treated neonatally with RC-3095 or PD-176252 showed a significant decrease in the frequency of over and under behavior (both p's = 0.000). RC-3095, but not PD-176252 has also induced a significant reduction in the frequency of allogrooming (p = 0.037) when compared to the control group. Tukey post-hoc test has also revealed that both RC-3095 and PD-176252, induced a significant increase in the frequency of aggressive behavior (p= 0.000 and p = 0.050, respectively, Fig. 3A) as well. In females, both RC-3095 and PD-176252 given neonatally decreased the frequency of play fighting (p= 0.036 and p = 0.047, respectively, Fig. 3A).

Comparisons using one-way ANOVA revealed significant differences among groups in the total time spent sniffing [$F(2,31) = 30.15$, $p = 0.000$], over and under [$F(2,31) = 74.12$, $p = 0.000$], allogrooming [$F(2,31) = 5.30$, $p = 0.010$], in aggressive behavior [$F(2,31) = 6.32$, $p = 0.005$], and play-fighting [$F(2,31) = 3.65$, $p = 0.038$]. In females, ANOVA has also indicated a significant difference in the total time spent following the partner [$F(2,31) = 3.98$, $p = 0.029$], but not in the duration grooming [$F(2,31) = 1.08$, $p = 0.351$], pinning [$F(2,31) = 2.10$, $p = 0.139$], or in anogenital inspections [$F(2,31) = 0.212$, $p = 0.811$) (Fig. 3B). Further analysis with Tukey post-hoc test showed that female rats treated neonatally with PD-176252 or RC-3095, showed a significant decrease in the duration of sniffing (both p's = 0.000) and in the total time spent in over and under behavior (both p's = 0.000) when compared to control rats. In females, RC-3095 has also induced a significant reduction in time spent allogrooming (p = 0.008) and following the partner (p = 0.039) when compared to the control group. Tukey post-hoc test has also revealed that RC-3095 treatment, but not PD-176252, induced a significant increase in the total time spent in aggressive behavior (p= 0.004).

## Discussion

We have previously shown that pharmacological blockade of the GRPR during the neonatal period results in long-lasting deficits in some measures social behavior, attachment, and memory (Presti-Torres et al., 2007; Mackay et al., 2009; Garcia et al., 2010; Presti-Torres et al., submitted manuscript). Some of these alterations were ameliorated by treatment with the atypical

antipsychotic clozapine, which have been prescribed to patients with ASD (Presti-Torres et al., submitted manuscript). In the present study, we extended those findings by including additional detailed measures of social behavior during the neonatal phase, examining possible sex-related differences in the effects of BB receptor antagonists, and comparing the effects of selective GRPR blockade with those of an antagonist that also blocks the NMBR. Overall, the results indicate that 1) both BB receptor antagonists reduce aggregative behavior in pups; 2) there were sex-related differences for the effects of BB receptor antagonists in some measures (i.e., sniffing and following behaviors); and 3) the selective GRPR antagonist, but not the GRPR/NMBR antagonist, produced an increase in aggressive behavior.

BB receptors in neurons play a role in regulating emotional responses and cognitive function (Moody & Merali, 2004; Merali et al., 2006; Roesler et al., 2006a; Jensen et al., 2008). At the cellular signaling level, BB receptor activation is associated with protein kinase pathways, such as the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) cascades (Roesler et al., 2006b; 2009), that are known to regulate synaptic plasticity and behavior and may also be involved in neuropsychiatric and neurodevelopmental disorders (Belmonte et al., 2004). We have recently shown that neonatal GRPR blockade in rats results in impaired social interaction, as well as impaired cognitive function measured by novel object recognition. These behavioral alterations were accompanied by parallel long-term changes in the expression of mRNAs for the GRPR, N-methyl-D-aspartate (NMDA) and epidermal growth factor (EGF) receptors in the cortex and hippocampus (Presti-Torres et al., 2011). The expression of GRPR, EGFR and NMDAR might be regulated in a common pattern in response to GRPR blockade during development, and all three receptor systems might present functional interactions and play a complementary role in regulating aspects of behavior altered in RC-3095-treated rats. Alterations in the function of these receptors might be associated with the pathogenesis of ASD (Carlsson, 1998; Toyoda et al., 2007). Moreover, the possibility that the GRPR gene is a candidate gene in autism has been raised by genetic studies in autistic patients, although additional studies are

required to validate it as a candidate gene (Ishikawa-Brush et al., 1997; Marui et al., 1997; Seidita et al., 2008).

In addition to the GRPR antagonist RC-3095, which was used in previous studies as a pharmacological tool to block the GRPR in the neonatal period, the present study included the use of a small-molecule BB receptor antagonist. The non-selective GRPR/NMBR antagonist PD-176252 (Ashwood et al., 1998) has been shown to affect anxiety-like behaviors in ethologically relevant tests, including social interaction (File, 1980), approach to a familiar palatable snack in a novel (anxiogenic) environment (Merali et al., 2004), and in the guinea pig pup vocalization test (Molewijk et al., 1996). Although the BB peptide family was initially implicated in the mediation of peripheral satiety signals (Gibbs et al., 1979; Kulkosky et al., 1982; Merali et al., 1999), more recent studies suggested that these peptides may be active neuromodulators/neuromediators affecting several brain mechanisms and altering behaviors associated with stress and anxiety (Merali et al., 1998, 2002). Effects of PD-176252 on fear-potentiated startle were also assessed to determine its impact on learned fear responses (Bédard et al., 2007).

Together with our previous findings, the present results support the view that the antagonism of BB receptors in rats might be used as a tool for the development of novel animal models of neurodeveopmental disorders, including ASD. The use of several different behavioral measures and the similarities and differences between the sexes are crucial to increase the validity of models of such complex human disorders. ASD is one of the most common neurodevelopmental conditions, affecting approximately 0.6 to 1.57% of the general population (Volkmar *et al*., 2005) and the diagnostic criteria for autism are aberrant reciprocal social interactions, impaired communication, stereotyped repetitive behaviors with narrow restricted interests, aggression and idiosyncratic responses to sensory stimuli. Also, within ASD there is asymmetry in sex ratio, males outnumbering females with a sex ratio of 4.3 : 1 (Volkmar *et al*., 2005). It is important that animal models of ASD aim to reproduce these behavioral deficits (Crawley et al., 2004; Moy et al., 2006; Crawley et al., 2007). Thus, animal models of ASD should include differences between sexes and measures of several aspects of social behavior, including aggression, as well as parameters of cognitive function and emotional responses.

In summary, the present findings provide further pharmacological evidence that BB receptors are important in regulating emotional and social behavior during CNS development of male and female, and BB receptor blockade in the neonatal period might represent a novel rat model relevant for some features of ASD.

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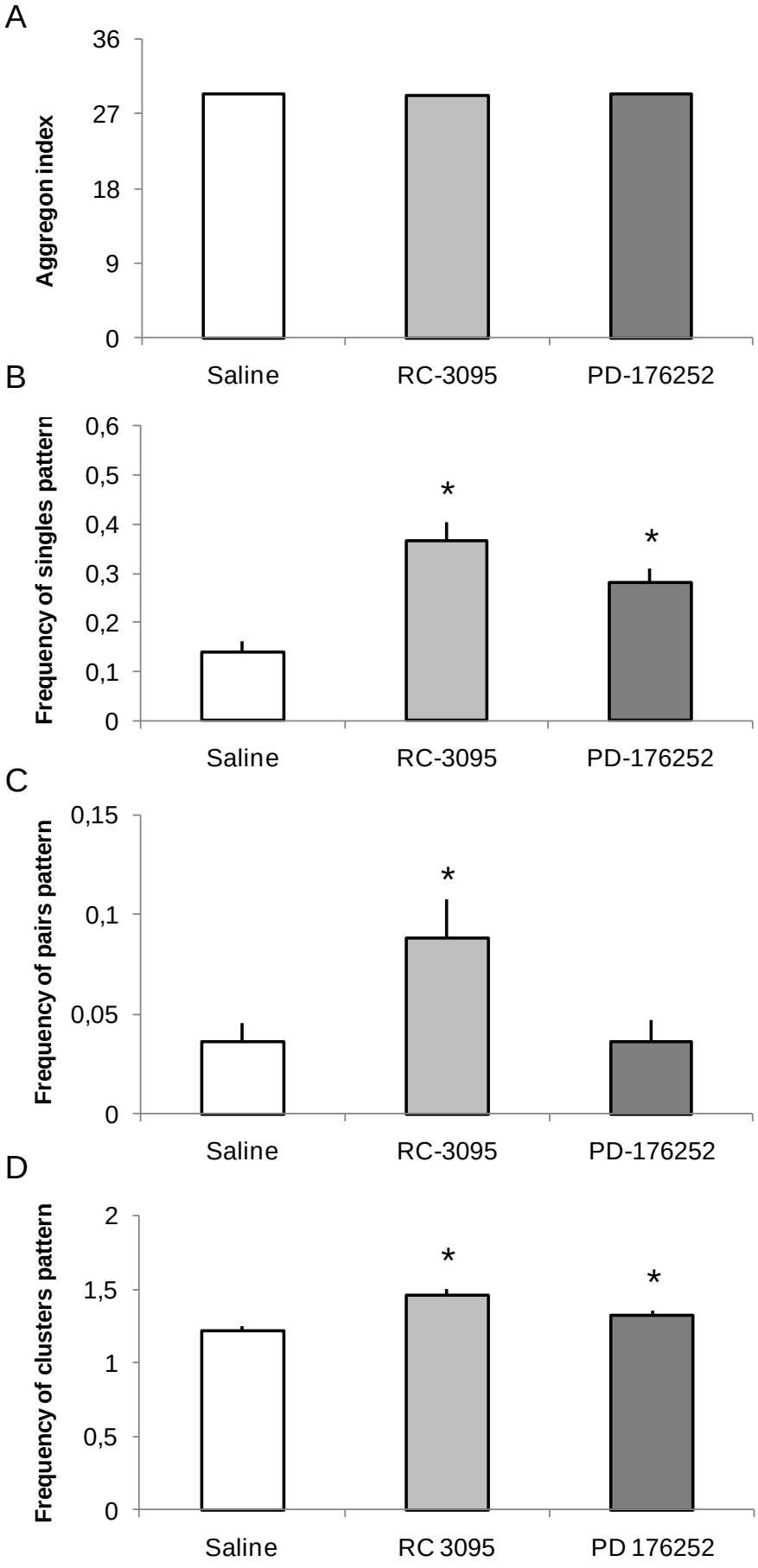
Figure 1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B
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Saline
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C
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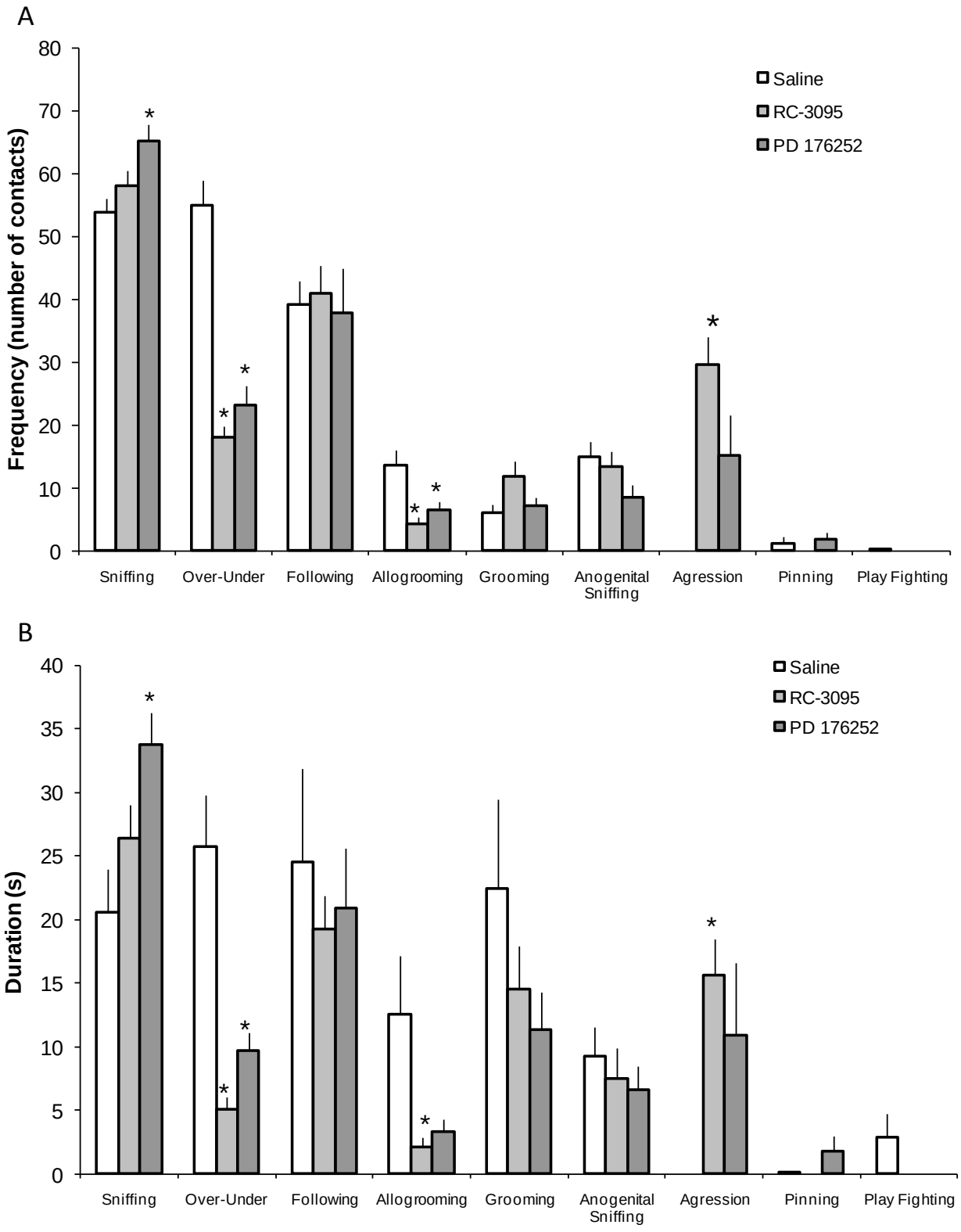
Figure 2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60
50
40
30
20
10
0
Saline
RC-3095
PD 176252
Sniffing
Over-Under
Following
Allogrooming
Grooming
Anogenital Sniffing
Agression
Pinning
Play Fighting
B
Duration (s)
40
35
30
25
20
15
10
5
0
Saline
RC-3095
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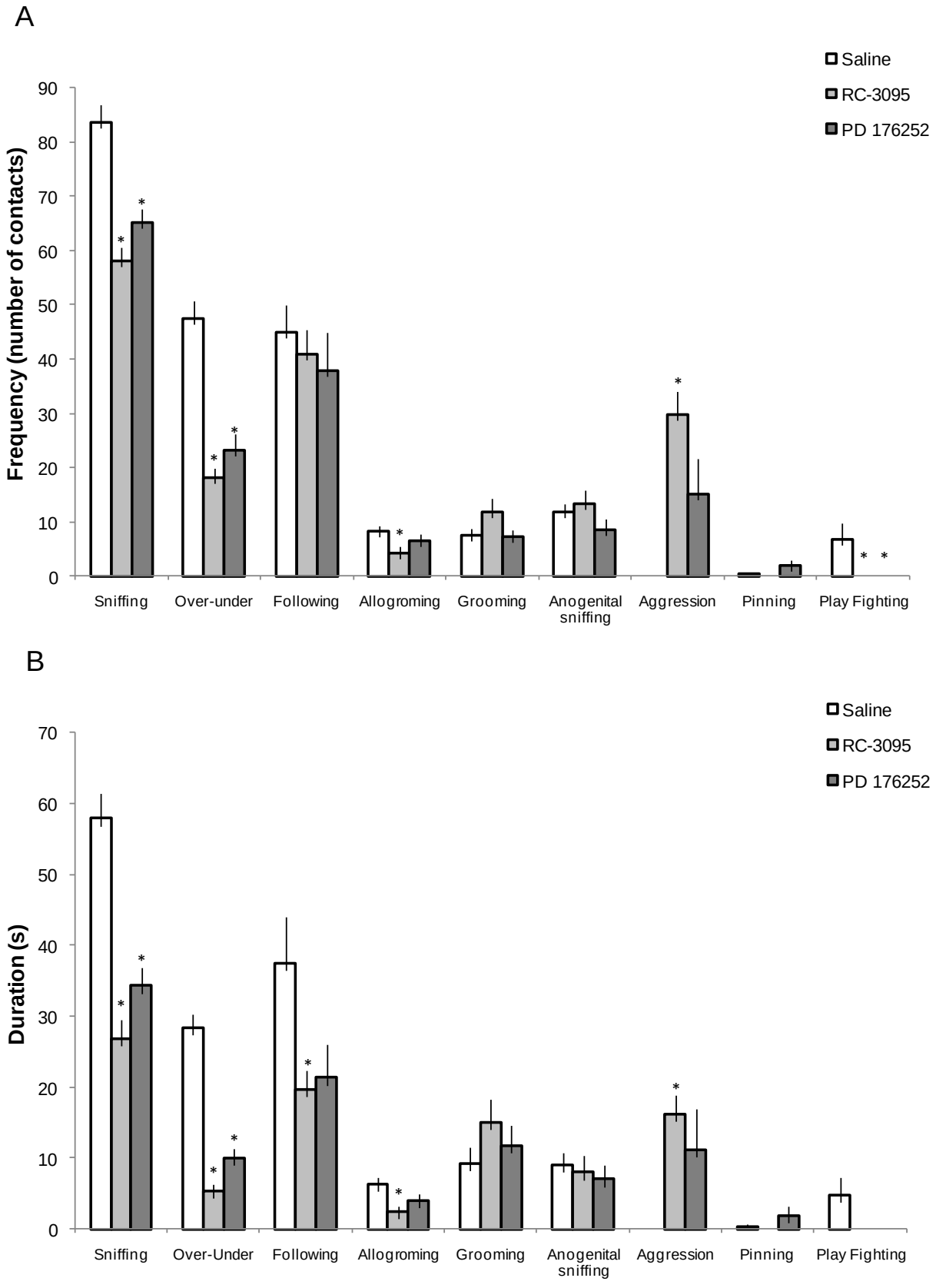
Sniffing
Over-Under
Following
Allogrooming
Grooming
Anogenital Sniffing
Agression
Pinning
Play Fighting

Figure 3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PD 176252
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Over-under
Following
Allogroming
Grooming
Anogenital sniffing
Aggression
Pinning
Play Fighting
B
Saline
RC-3095
PD 176252
Duration (s)
70
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Grooming
Anogenital sniffing
Aggression
Pinning
Play Fighting

Legends for Figures

Fig. 1. Neonatal blockade of bombesin (BB) receptors impairs aggregation behavior in rat pups. Animals were given an intraperitoneal (i.p.) injection of saline (SAL), the GRPR antagonist RC-3095 (1 mg/kg), or the GRPR/NMBR antagonist PD-176252 (0.5 mg/kg) twice daily from postnatal days 1 to 10. Aggregation behavior was tested at PN 12. (A) mean ± SEM Aggregon Index; (B) mean ± SEM frequency (number of singles separated from the group); (C) mean ± SEM (number of pairs separated from the group); (D) mean ± SEM Frequency (number of clusters separated from the group); n = 18 animals per group; * $p < 0.05$ compared to the SAL-treated group.

Fig. 2. Neonatal blockade of bombesin (BB) receptors impairs social behavior in male rats. Animals were given an intraperitoneal (i.p.) injection of saline (SAL), the GRPR antagonist RC-3095 (1 mg/kg), or the GRPR/NMBR antagonist PD-176252 (0.5 mg/kg) twice daily from postnatal days 1 to 10. Social behavior was tested at PN 30. (A) mean ± SEM frequency (number of contacts); (B) mean ± SEM time spent engaged in social interaction (s); n = 10-12 animals per group; * $p < 0.01$ compared to the SAL-treated group.

Fig. 3. Neonatal blockade of bombesin (BB) receptors impairs social behavior in female rats. Animals were given an intraperitoneal (i.p.) injection of saline (SAL), the GRPR antagonist RC-3095 (1 mg/kg), or the GRPR/NMBR antagonist PD-176252 (0.5 mg/kg) twice daily from postnatal days 1 to 10. Social behavior was tested at PN 30. (A) mean ± SEM frequency (number of contacts); (B) mean ± SEM time spent engaged in social interaction (s); n = 10-12 animals per group; * $p < 0.01$ compared to the SAL-treated group.

## 4 CONSIDERAÇÕES FINAIS

Após propormos o modelo de autismo baseado no bloqueio do receptor do peptídeo liberador de gastrina, e diante de poucos estudos relacionando o GRPR com neurodesordens como o autismo, nosso grupo de pesquisa demonstrou, pela primeira vez, a validação farmacológica deste modelo, bem como a análise da expressão de alguns receptores comumente relacionados a neuropatogenias (EGFR e NR1), além do GRPR em hipocampo e córtex cerebral. Partindo-se deste estudo, demonstramos que a clozapina foi capaz de reverter as alterações comportamentais, resultantes do tratamento neonatal com RC-3095, em interação social de animais adultos. Algumas análises, consistentes com nossas observações, têm demonstrado que a clozapina, considerada um agente antipsicótico atípico, é capaz de reverter déficits associados ao autismo (McDougle *et al*., 2008), como isolamento social em camundongos (Dixon *et al*., 1994) e atenuar a hiperlocomoção e estereotipia em camundongos com expressão reduzida do receptor NMDA (Mohn *et al*., 1999).

A análise da expressão dos receptores EGFR e NR1, bem como o GRPR, em hipocampo e córtex cerebral de animais adultos, previamente submetidos ao bloqueio do GRPR no período neonatal, indicou diferentes níveis desses receptores nessas áreas cerebrais. Investigações prévias têm identificado que os efeitos do antagonista do GRPR, o RC-3095, podem estar relacionados também a alterações na expressão de EGFR (Halmos & Schally, 1997; Szepeshazi *et al*., 1997) ou mesmo interações com o sistema de receptores de glicocorticóides (Venturella *et al.*, 2005). Halmos & Schally (1997) também demonstraram que o tratamento com antagonistas do GRP produziu uma redução nos níveis de EGFR, concomitante a inibição de crescimento tumoral. Ao mesmo tempo, alterações em alguns receptores como NR1 e EGFR têm sido relacionadas a alguns transtornos como autismo e esquizofrenia (Mohn *et al*., 1999, Suzuki *et al*., 2007, Iseri *et al*., 2010). Nesse aspecto, partindo-se da análise da expressão dos receptores NR1 (subunidade NR1 do NMDAR), EGFR e GRPR, em córtex e hipocampo de animais submetidos ao tratamento neonatal com RC-3095, nosso grupo de pesquisa

observou que os déficits em interação social eram acompanhados por diferentes padrões de alteração na expressão desses receptores. Enquanto em hipocampo, foi demonstrado aumento na expressão desses receptores, os mesmos apresentaram decréscimo em córtex cerebral, indicando que os receptores EGFR, NR1 e GRPR parecem estabelecer padrões similares em resposta ao antagonismo do GRPR durante o desenvolvimento. Os receptores em questão devem interagir funcionalmente e desempenhar um papel complementar na regulação de padrões comportamentais alterados em ratos tratados com o RC-3095.

Adicionalmente, nosso grupo de pesquisa, em parceria com Merali e *cols*., pela primeira vez, sugeriu a investigação do antagonismo neonatal do GRPR e também de outro tipo de receptor de bombesina, a Neuromedina B, em ratos Wistar fêmeas e machos. A NMB, assim como o GRP, vem sendo investigada na regulação de alguns padrões comportamentais como as respostas mediadas por estresse, medo e relacionados à memória (Shumyatsky *et al*., 2002, Luft *et al*., 2008). Nesta etapa de nosso estudo, o PD 176252, uma nova classe de antagonistas não peptídicos de alta afinidade a receptores de NMB (Ashwood *et al*., 1998), foi também administrado durante os primeiros 10 dias de vida a ratos Wistar, e igualmente na fase adulta dos animais, demonstrou o mesmo padrão de alterações comportamentais, sugerindo um padrão de regulação durante o desenvolvimento neurológico similar ao GRP/GRPR.

O bloqueio do receptor de NMB por tal fármaco vem sendo relacionado a prejuízos no desempenho em tarefa de interação social, em resposta de sobressalto potencializada pelo medo em ratos e em tarefa de vocalização ultra-sônica após separação materna em porquinhos-da-índia (Merali *et al*., 2006), alterações comportamentais relacionados a transtornos como ansiedade (File *et al*., 1980; Merali *et al*., 2004) e a prejuízos no desenvolvimento neurológico em animais neonatos (Molewijk *et al*., 1996; Merali *et al*., 2006).

O tratamento com o RC-3095 e com o PD 176252, nos primeiros dez dias de vida de ratos Wistar machos e fêmeas, foi capaz de provocar alterações significativas em parâmetros de agregação da ninhada, indicando prejuízo em um fator de sociabilidade, ou seja, em sua capacidade inerente de estabelecer um padrão único de coesão ainda nos primeiros dias de vida. Está

bem demonstrado que este parâmetro está alterado em animais com características de autismo, sendo observado um isolamento social ainda nos 15 primeiros dias de vida (Schank & Alberts, 1997). Também, na fase adulta, ratos machos e fêmeas apresentaram um comportamento de agressão em tarefa de interação social, fato observado pela primeira vez e que também está relacionado a alterações comportamentais observadas em transtornos como o autismo (Crawley *et al*., 2007).

Da mesma forma, prejuízos significativos em padrões de comportamento social ("*over and under*" e "*allogrooming*") foram analisados em ratos machos e fêmeas em tarefa de interação social, características mais distintivas e qualitativas observadas no diagnóstico do autismo. Mais precisamente, as análises comportamentais em fêmeas indicaram algumas diferenças em padrões de "cheirar o parceiro" (s*niffing*) e "seguir o parceiro" (*following*), em tarefa de interação social, quando comparadas aos machos, sugerindo que o bloqueio do receptor apresenta padrões diferentes relacionados ao gênero. Tais diferenças corroboram com o fato de que o autismo apresenta uma expressão mais significativa em indivíduos do sexo masculino (proporção de 4:1), durante os três primeiros anos de vida e indicam a necessidade de observações mais detalhadas acerca de parâmetros comportamentais e moleculares envolvidos nessa patologia em ambos os sexos.

Alterações associadas ao autismo descritas anteriormente em nossos estudos (Presti-Torres *et al*., 2007; Garcia *et al*., 2010) seguiram-se a duas investigações realizadas primeiramente em nosso laboratório. Inicialmente, observamos que ratos Wistar machos, tratados com RC-3095 do 1º ao 10º dias de vida, durante a fase jovem apresentaram alterações significativas em tarefa de interação social, indicando prejuízos comportamentais característicos observados no diagnóstico do autismo. Além disso, os mesmos animais exibiram déficits de memória de longa duração, em tarefa de reconhecimento do objeto novo, identificando que o GRPR deve estar relacionado ao neurodesenvolvimento, e seu bloqueio pode gerar prejuízos também mnemônicos na fase adulta.

Neste contexto, recentes investigações farmacológicas a respeito do 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 *et al*., 1988) quanto em ratos (Rashidy- Pour & Razvani, 1998). Roesler e cols (2004), investigando os efeitos do bloqueio do GRPR na memória, examinaram que ratos tratados com infusões sistêmicas ou intracerebrais de RC-3095 apresentaram prejuízos na retenção a curto e longo prazo em tarefa de esquiva inibitória, um tipo de tarefa de memória emocional. 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 na consolidação de memória emocional em hipocampo (Roesler *et al*., 2003). Consistente com o papel dos receptores de GRP na regulação da plasticidade hipocampal, análises eletrofisiológicas indicaram 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).

Em continuidade aos trabalhos anteriores, realizamos um segundo estudo, onde analisamos a preferência pelo odor maternal e o condicionamento aversivo ao odor ainda nas primeiras etapas do desenvolvimento, de ratos Wistar machos, tratados nos dez primeiros dias de vida, com o RC-3095 (Garcia *et al*., 2010). O período neonatal consiste em uma etapa importante na maturação do sistema nervoso e estudos envolvendo alterações no início do desenvolvimento permitem a observação de quais parâmetros comportamentais, emocionais, e de função cognitiva serão afetados tanto na fase inicial, quanto na vida adulta. Dentro deste contexto, Garcia e *cols* (2010) evidenciaram prejuízos de comportamento relacionado à preferência pelo odor da mãe em animais tratados com RC-3095 (PN1-PN10), sugerindo que o bloqueio do receptor GRPR reduz o vínculo entre os filhotes e a mãe. Este prejuízo é provavelmente devido a uma disfunção específica no comportamento de apego, mais do que um prejuízo no aprendizado do odor, pois, em relação à tarefa de condicionamento aversivo ao odor, foi observado que tanto os filhotes que receberam o tratamento com o antagonista do GRPR quanto àqueles que receberam solução salina, apresentaram níveis similares de *freezing* (comportamento de “paralização”). Esses resultados sugerem que o bloqueio do GRPR no período neonatal não interfere na memória de

condicionamento ao odor, mas influencia em um comportamento inerente de filhotes, a interação social com a mãe. Tal resultado parece estar relacionado àqueles inicialmente descritos em nossos trabalhos, em que animais jovens e adultos, também submetidos ao antagonismo do GRPR no período pós-natal, manifestam déficits em interação social.

Doenças associadas ao neurodesenvolvimento, como o autismo, normalmente se desenvolvem fase inicial de vida, até os três primeiros anos, podendo estar relacionadas com alguma anormalidade em sistemas de neuropeptideos ao longo deste período. Também, diversas investigações sugerem componentes genéticos fortemente relacionados a essa neurodesordem (Wassink & Piven., 2000; Cook *et al*., 2001; Folstein & Rosen-Sheidley, 2001; Shastry, 2003; Muhle *et al*., 2004). Entretanto, apesar de a etiologia desta desordem ainda não estar bem estabelecida, o diagnóstico baseia-se na análise de diversos padrões comportamentais, incluindo déficits em interação social, tanto na infância quanto na fase adulta, prejuízos na comunicação, ansiedade, distúrbios de sono, comportamento repetitivo, resistência em alterar a rotina, inabilidade na interpretação de emoções através de expressões faciais, atenção reduzida, hipersensibilidade a estímulos sensoriais e em alguns graus, a agressão (Kanner *et al*., 1943; Piven *et al*., 1997; Lord *et al*., 2000; Bodfish *et al*., 2000; Folstein & Rosen-Sheidley*.,* 2001; Volkmar *et al*., 2003; DSM – V; American Psychiatric Association 2011). A utilização de modelos em camundongos e/ou ratos para o estudo de neurotranstornos como a esquizofrenia e o autismo corresponde a um recurso importante, ao passo que tais modelos, quando bem delineados e estabelecidos, podem facilitar o acesso a sintomas semelhantes aos manifestados em humanos e a validação farmacológica, baseada em tratamentos pré-existentes e efetivos em humanos (Crawley *et al*., 2007).

Evidências da correlação entre os peptídeos Bombesina e seus receptores com transtornos neurológicos e psiquiátricos e com doenças neurodegenerativas proporcionam o desenvolvimento de métodos possivelmente mais efetivos no tratamento de tais doenças. Nossos estudos vêm demonstrando que o modelo de autismo em ratos baseado no bloqueio do GRPR incorpora componentes relevantes no diagnóstico do autismo, ao longo de diversas etapas do desenvolvimento como: déficits em interação social da

ninhada (agregação) e em comportamento de apego entre mãe e filhote, agressão e alterações em interação social em machos e fêmeas jovens, prejuízos em comportamento social durante a fase adulta revertidos com antipsicótico atípico, diferentes padrões de expressão de receptores relacionados a neurodesordens e prejuízos em memória de longa duração. Tais evidências comportamentais e moleculares sugerem que o bloqueio de receptores Bombesina, durante o período neonatal pode representar um novo modelo animal de autismo, podendo ser utilizado como ferramenta para o entendimento e para o desenvolvimento de novas terapias para essa desordem.

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# 6 ANEXOS

Comprovante de Aceite do Artigo Cientifico

***Rescue by social behavior impairment by clozapine and alterations in the expression of neural receptors in a rat model of neurodevelopmental impairment induced by GRPR blockade.***

**De:** em.jnt.0.24941b.b6faeb15@editorialmanager.com em nome de JNT Editorial Office
**Enviada:** dom 24/7/2011 04:01
**Para:** Nadja Schroder
**Assunto:** Your Submission

Ref.: Ms. No. JNT-D-11-00115R1
Rescue of social behavior impairment by clozapine and alterations in the expression of neuronal receptors in a rat model of neurodevelopmental impairment induced by GRPR blockade
Journal of Neural Transmission

Dear Dr Schroder,

I am pleased to tell you that your work has now been accepted for publication in Journal of Neural Transmission.

It was accepted on 24-07-2011

Thank you for submitting your work to this journal.

With kind regards,

Editorial Office
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