UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

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ESTUDO DO MECANISMO DE AÇÃO E DO EFEITO NEUROPROTETOR DE COMPOSTOS ANTIDEPRESSIVOS (DULOXETINA, ESCITALOPRAM)

Florianópolis/SC
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Tese apresentada ao Programa de Pós-Graduação em Bioquímica do Centro de Ciências Biológicas da Universidade Federal de Santa Catarina, como requisito parcial à obtenção do grau de Doutor em Bioquímica.

Orientador: Prof. Nelson Horácio Gabilan, Dr.

Co-orientadora: Profª. Ana Lúcia Severo Rodrigures, Drª.

Florianópolis/SC
2012

Ficha de identificação da obra elaborada pelo autor,
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Z83e Zomkowski, Andréa Dias Elpo

Estudo do Mecanismo de Ação e do Efeito Neuroprotetor de Compostos Antidepressivos (Duloxetina, Escitalopram) [tese] / Andréa Dias Elpo Zomkowski ; orientador, Nelson Horácio Gabilan ; co-orientadora, Ana Lúcia Severo Rodrigues. - Florianópolis, SC, 2012.
162 p. ; 21cm

Tese (doutorado) - Universidade Federal de Santa Catarina, Centro de Ciências Biológicas. Programa de Pós- Graduação em Bioquímica.

Inclui referências

1. Bioquímica. 2. Depressão. 3. Depressão e sistema glutamatérgico e via da L-arginina-NO. 4. Antidepressivos e vias de sinalização. 5. Depressão e neuroproteção. I. Gabilan, Nelson Horácio. II. Rodrigues, Ana Lúcia Severo . III. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Bioquímica. IV. Título.

"Estudo do mecanismo de ação antidepressivo e neuroprotetor dos compostos antidepressivos duloxetina e escitalopram"

por

Andrea Dias Elpo Zomkowski

Tese julgada e aprovada em sua forma final pelos membros titulares da Banca Examinadora (Port. 012/PPGBQA/2012) do Programa de Pós-Graduação em Bioquímica - UFSC, composta pelos Professores Doutores:

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Florianópolis, 25 de maio de 2012.

"As pessoas estão sempre a culpar as circunstâncias por aquilo que se tornam. Não acredito em circunstâncias. As pessoas que estão mais adiantadas neste mundo são as pessoas que se levantam e procuram as circunstancias que desejam, e se não as encontram, criam-nas."

George Bernard Shaw

AGRADECIMENTOS

Aos professores e amigos Nelson Horácio Gabilan e Ana Lúcia Severo Rodrigues pela amizade, dedicação e empenho dispensado na orientação deste trabalho.

Aos professores Marcelo Farina e Carla Inês Tasca, do departamento de Bioquímica da UFSC, pela permissão do uso do laboratório e pelos conhecimentos adquiridos.

Aos colegas de doutorado pelo companheirismo e amizade e aos professores do PG-Bioquímica pelos ensinamentos.

Aos amigos do lab de Neuroquímica I: Daiane,Vicente e Suelen, pela amizade e pela colaboração no trabalho. Á minha querida amiga e parceira de trabalho Daiane, pelos experimentos que realizamos juntas e por ter tornado este período tão agradável. Daiane, você é mais que uma amiga!!!! Obrigada por tudo!

Aos amigos do lab de Neurobiologia da Depressão: Josi, Dani, Andiara, Maurício, Luiz, André, pelo convívio. A Josi, pela colaboração direta dos experimentos e amizade!

As amigas do lab de Experimentação em Neuropatologias: Danúbia, Alessandra, Dirleise, Kety pela colaboração dos experimentos e pelos bons momentos!

As amigas Ana Lúcia Zeni e Fabiane Kaline, do lab de Neuroquímica II, pela amizade, carinho e colaboração no trabalho.

Ao Roberto, secretário da pós-graduação, por ser sempre atencioso e prestativo.

Ao meu marido e a minha filha, pelo carinho e compreensão nos momentos mais difíceis que passei. Obrigada pela força e pelo amor de vocês que me fizeram chegar até o final, e por mais esta conquistada! Eu devo este trabalho a vocês dois, amores da minha vida.

Aos meus pais pelo amor, carinho e ajuda nos momentos que mais precisei! Amo muito vocês.

ZOMKOWSKI, Andréa Dias Elpo. **Estudo do mecanismo de ação e do efeito neuroprotetor de compostos antidepressivos (duloxetina, escitalopram)**. 2012, 162f. Tese (Doutorado em Bioquímica) – Programa de Pós-Graduação em Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, 2012.

RESUMO

Os transtornos de humor são doenças comuns, severas, crônicas e muitas vezes, ameaçadoras de vida. A depressão é uma doença heterogenia com manifestações fisiológicas, comportamentais e psicológicas. Os sistemas de neurotransmissores que têm recebido maior atenção nos estudos da neurobiologia da depressão são os sistemas monoaminérgicos, outros sistemas neuronais e processos bioquímicos, como o estresse oxidativo, a neurogênese e a morte celular, podem estar envolvidos na patogênese da depressão. O escitalopram é um inibidor seletivo da recaptação de serotonina (ISRS) e a duloxetina é um inibidor da recaptação de serotonina e noradrenalina (IRSN), ambos usados no tratamento da depressão. Este estudo investigou o efeito antidepressivo do escitalopram e da duloxetina no teste do nado forçado (TNF) e no teste da suspensão da cauda (TSC) em camundongos e o seu mecanismo de ação. O escitalopram nas doses de 0,1-10 mg/kg, i.p. e a duloxetina nas doses de 1-30 mg/kg, i.p., causaram uma redução no tempo de imobilidade no TNF e no TSC, sem alterar a atividade locomotora no campo aberto. A administração oral de escitalopram (0,3-10 mg/kg) e de duloxetina (1-30 mg/kg) também reduziu o tempo de imobilidade no TNF. Foi avaliado o envolvimento do sistema glutamatérgico, via L-arginina-óxido nítrico e as vias de sinalização celular no efeito antidepressivo do escitalopram e da duloxetina no TNF. A ação antidepressiva do escitalopram (3 mg/kg, p.o.) e da duloxetina (10 mg/kg, p.o.) foi prevenida pelo pré-tratamento com NMDA, L-arginina, SNAP, sildenafil, H-89, GF109203X, LY294002, U0126, mas não com KN-62. O efeito antidepressivo da duloxetina foi também prevenido pelo pré-tratamento com SNAP. Além disso, quando administrado em doses sub-ativas, o escitalopram (0,1 mg/kg, p.o.) e a duloxetina (0,3 mg/kg, p.o.) produziram um efeito sinérgico com 7-nitroindazol, azul de metileno e ODQ, sendo que o MK-801 produziu um efeito sinérgico com a duloxetina. O escitalopram (1 µM) e da duloxetina (10 µM)

foram neuroprotetores contra a morte celular induzida pelo GLU (10 mM) em fatias hipocampais e este efeito foi bloqueado pelo LY294002 e SNAP. O escitalopram e a duloxetina diminuíram a liberação de GLU. Em conjunto os resultados sugerem que o efeito antidepressivo do escitalopram e da duloxetina é dependente do bloqueio dos receptores NMDA e da inibição da síntese de NO e GMPc. Nossos resultados sugerem que o efeito antidepressivo do escitalopram e da duloxetina é dependente da modulação das vias de sinalização como a PKA, PKC, PI3K e MAPK/ERK. O escitalopram e da duloxetina foram neuroprotetores contra a morte celular induzida pelo GLU por um mecanismo que envolve as vias NO e PI3K/AKT prevenindo a liberação de GLU. Estes antidepressivos podem ter um importante potencial neuroprotetor na depressão e nas doenças degenerativas do SNC.

Palavras-chave: Duloxetina. Depressão. Escitalopram. Glutamato. Vias de sinalização. Neuroproteção.

ABSTRACT

The mood disorders are common diseases, severe, chronic and often life threatening. Depression is a heterogeneous disease with physiological, behavioral and psychological manifestations. The brain systems that have received increasing attention in studies of the neurobiology of depression are the monoaminergic systems, however other neuronal systems and biochemical processes such as oxidative stress, neurogenesis and cell death may be involved in the pathogenesis of depression. Escitalopram is a selective serotonin reuptake inhibitor (SSRI) and duloxetine is a serotonin and noradrenaline reuptake inhibitor (SNRI), both used in the treatment of depression. This study investigated the effect of escitalopram and duloxetine in the mouse forced swimming test (FST) and in the tail suspension test (TST) and its mechanism of action. Escitalopram at doses of 0.1-10 mg/kg, i.p. and duloxetine at doses of 1-30 mg/kg, ip, caused a reduction in immobility time in the TNF and TSC, without changing locomotor activity in open-field. Oral administration of escitalopram (0.3-10 mg/ kg) and duloxetine (1-30 mg/kg) also reduced the immobility time in the TNF. We evaluated the involvement of the glutamatergic, system L-arginine-nitric oxide and cell signaling pathways in the antidepressant effect of escitalopram and duloxetine in TNF. The antidepressant action of escitalopram (3 mg/kg, p.o.) and duloxetine (10 mg/kg, p.o.) was prevented by pretreatment with NMDA, L-arginine, SNAP, sildenafil, H-89, GF109203X, LY294002, U0126, but not with KN-62. The antidepressant effect of duloxetine was also prevented by pretreatment with SNAP. Furthermore, when administered in a sub-active dose, escitalopram (0.1 mg/kg, p.o.) and duloxetine (0.3 mg/kg, p.o.) produced a synergistic effect with 7-nitroindazol, methylene blue and ODQ, and the MK-801 produced a synergistic effect with duloxetine. Escitalopram (1μM) and duloxetina (10 μM) were neuroprotective against hippocampal cell death induced by GLU and this effect was reversed by LY294002 and SNAP. The escitalopram and duloxetine decreased the release of GLU. Together the results suggest that the antidepressant effect of escitalopram and duloxetine is dependent on either a blockade of NMDA receptors or an inhibition of NO and cGMP synthesis. Our data suggest that the antidepressant effect of escitalopram

and duloxetine seems to be dependent on the cellular signaling modulated by PKA, PKC, PI3K and MAPK/ERK. Escitalopram and duloxetine were neuroprotective against GLU-induced cell death by a mechanism that involves NO and PI3K/Akt pathways preventing glutamate-induced GLU release. These antidepressants may be considered as an important neuroprotective strategies in depression and degenerative disorders of the CNS.

Key-words: Duloxetine. Depression. Escitalopram. Glutamate. Signaling Pathways. Neuroprotection.

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LISTA DE ABREVIATURA

Akt/PKB	A proteína cinase B
AMPA	Ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropiônico
AMPc	Adenosina monofosfato cíclico
BDNF	Fator neurotrófico derivado do cérebro
Ca^{2+}	Íon cálcio
CamkII	Proteina quinase II dependente de cálcio-calmodulina
CREB	Proteína ligante ao elemento de responsivo ao AMPc
ERK	Proteína cinase ativada por fator extracelular
GCs	Guanilato ciclase solúvel
GLU	Glutamato
GMPc	Guanosina 3'5'-monofosfato
GTP	Guanosina 5'-trifosfato
i.p.	Intraperitonealmente
iMAOs	Inibidores da monoamino oxidase
IRSN	Inibidores da recaptação de serotonina e noradrenalina
ISRNA	Inibidores seletivo da recaptação de noradrenalina
ISRS	Inibidores seletivo da recaptação de serotonina
KA	Ácido caínico
MAPK	Proteína cinase ativada por mitógeno
MK-801	Dizocilpina
NA	Noradrenalina
NMDA	N-metil D-aspartato
NO	Óxido nítrico
NOSn	Óxido nítrico sintase neuronal
p.o.	Oralmente
PI3K	Fosfatidilinositol-3 cinase
PIP2	Fosfatidilinositol-3,4-bifosfato
PIP3	Fosfatidilinositol-3,4,5-trifosfato
PKA	Proteína cinase A
PKC	Proteina cinase C
SE	Serotonina
SNC	Sistema nervoso central
TNF	Teste do nado forçado
TSC	Teste da suspensão pela cauda

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APRESENTAÇÃO

Esta Tese de Doutorado está organizada da seguinte forma: Introdução, Justificativa, Objetivos, Artigo científico publicado, submetido e em fase de submissão, Conclusões e Referências Bibliográficas.

A Introdução contém o embasamento teórico para a realização dessa Tese. Os Materiais e Métodos, os Resultados, assim como as Referências Bibliográficas específicas, encontram-se no corpo de cada trabalho, os quais estão apresentados na forma de Artigos Científicos em quatro capítulos.

A Discussão Geral contem a interpretação de todos os resultados obtidos.

A Conclusão descreve as conclusões gerais da Tese.

As Referências Bibliográficas apresentam as referências citadas na Introdução e na Discussão Geral da Tese.

Os capítulos 1, 2 e 3 dessa Tese foram desenvolvidos no Departamento de Bioquímica da UFSC, no laboratório de Neurobiologia da Depressão e Neuroquímica I sob a coordenação da Prof.a Dra Ana Lúcia Severo Rodrigues e do Prof.o Dro Nelson Horácio Gabilan.

O capítulo 4 dessa Tese foi desenvolvido no Departamento de Bioquímica da UFSC, no laboratório de Neuroquímica 4, sob coordenação da Profa. Dra Carla I. Tasca.

1 INTRODUÇÃO

1.1 Depressão

Os transtornos de humor são doenças comuns, severas, crônicas e muitas vezes, ameaçadoras de vida. Eles incluem transtornos unipolares (transtornos depressivos) e transtornos bipolares. Os transtornos unipolares (por exemplo, depressão maior e distimia) se distinguem dos transtornos bipolares, pelo fato de não terem históricos de episódios de mania ou hipomania (American Psychiatric Association, 1994). Os transtornos de humor são mudanças patológicas episódicas no estado emocional, associados a anormalidades na cognição e comportamento (American Psychiatric Association, 1994). A depressão é um transtorno psiquiátrico que afeta 20% da população (Berton; Nestler, 2006). O suicídio tem sido a causa de morte em aproximadamente 15% de indivíduos com depressão maior (Manji et al., 2001). Formas graves de depressão afetam 2% a 5% da população dos Estados Unidos (Nestler et al., 2002) e indivíduos que sofrem de depressão severa apresentam altas taxas de comorbidade e mortalidade (Nemeroff, 2007). A depressão é mais freqüente em mulheres (uma proporção de 5 mulheres para 2 homens) (Wong; Licinio, 2001).

A depressão é uma doença heterogênea com manifestações fisiológicas, comportamentais e psicológicas (American Psychiatric Association, 1994). Os critérios para o diagnóstico da depressão incluem cinco ou mais dos seguintes sintomas: humor depressivo; anedonia (perda de interesse ou satisfação em quase todas as atividades); perda ou ganho de peso ou de apetite; insônia ou hipersonia; retardo ou agitação psicomotora; fadiga ou perda de energia; sentimentos de desvalia ou culpa; diminuição da concentração ou indecisão e pensamentos ou tentativa de suicídio. Estes sintomas devem estar presentes por pelo menos duas semanas, sendo que pelo menos um deve ser humor depressivo ou anedonia quase todos os dias (American Psychiatric Association, 1994).

As bases biológicas da depressão e o preciso mecanismo de ação de antidepressivos não estão esclarecidos. Os sistemas que tem recebido maior atenção nos estudos da neurobiologia da depressão são os sistemas monoaminérgicos (D'Sa; Duman, 2002; Racagni; Popoli.,

2010). A hipótese monoaminérgica postula que a depressão resulta de uma deficiência de serotonina (SE), noradrenalina (NA) ou de receptores ineficientes (Wong; Licinio, 2001; Kiss, 2008). Assim, drogas com ação antidepressiva bloqueiam a recaptação de SE e/ou NA ou inibem a sua degradação, aumentando a concentração das mesmas na fenda sináptica (Brunello et al., 2002). Contudo, apesar do aumento nos níveis de monoaminas ocorrer quase que imediatamente após o início do tratamento, os efeitos terapêuticos dos antidepressivos se manifestam somente após algumas semanas de tratamento (Wong; Licinio, 2001). A razão para o atraso no efeito terapêutico ainda não é clara, mas sabe-se que o tratamento com antidepressivos aumenta os níveis de monoaminas no cérebro e ativa mecanismos de transdução de sinal envolvendo segundo mensageiros que resulta em alterações na expressão de genes (Lesch, 2001; Duman; Voleti, 2012).

Novas teorias complementam a hipótese monoaminérgica para melhor compreensão da fisiopatologia da depressão, como a hipótese neurotrófica que postula que a depressão pode ser desencadeada por alterações nas vias de sinalização que regulam a neuroplasticidade e a sobrevivência celular (Duman, 2002; Schmidt; Duman, 2007; Duman; Voleti, 2012). Além disso, estudos mostram que a depressão pode estar associada à neurodegeneração provocada por um aumento do estresse oxidativo (Forlenza; Miller, 2006; Ng et al., 2008). O envolvimento de outros sistemas de neurotransmissores na patologia da depressão tem sido demonstrado, como o sistema glutamatérgico (Palucha, 2005) e a via L-arginina-óxido nítrico **(**Kulkarni; Dhir, 2007; Brocardo et al., 2008).

A compreensão das vias e mecanismos responsáveis pela ação dos antidepressivos pode contribuir substancialmente para o entendimento dos transtornos depressivos e para o desenvolvimento de novas alternativas terapêuticas para o seu tratamento (Wong; Licinio, 2001).

1.2 Depressão e o sistema glutamatatérgico e via da L-arginina-óxido nítrico

O glutamato (GLU) é o principal neurotransmissor excitatório do sistema nervoso central (SNC) de mamíferos e é responsável por diversas funções cerebrais, incluindo plasticidade neuronal, aprendizagem e memória (Cotman et al., 1995; Platt, 2007). O GLU liberado para a fenda sináptica é recaptado pelos astrócitos onde é

novamente convertido à glutamina pela enzima glutamina sintetase, e liberado por intermédio de transportadores de glutamina para o meio extracelular. A glutamina liberada pelos astrócitos é captada pelas células neuronais e reconvertida a GLU fechando assim o ciclo GLU-glutamina (Schousboe; waagepetersen, 2005). Os receptores de GLU estão divididos em duas categorias: os ionotrópicos, que medeiam a abertura de canais iônicos e os metabotrópicos, os quais estão associados à proteína G. Os receptores ionotrópicos são subdivididos em três categorias: NMDA (ativados pelo N-metil D-aspartato), AMPA (sensíveis ao ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropiônico) e KA (respondem ao ácido caínico) (Pláteník et al., 2000). Os receptores ionotrópicos são os principais mediadores da excitotoxicidade provocada pelo excesso de GLU (Kornhber; Weller, 1997; Stone; Addae, 2002) (Ver Figura 1, abaixo).

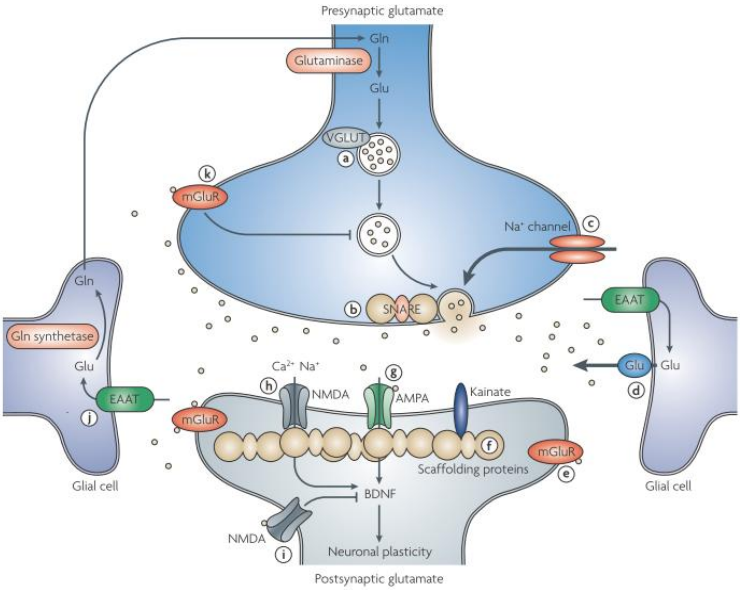

Figura 1. Neurotransmissão glutamatérgica.
O GLU liberado na fenda sináptica interage com seus receptores NMDA, AMPA, KA. A recaptação de GLU ocorre através da atividade de transportadores (EAAT) localizados nos astrócitos e nos neurônios. O GLU nos astrócitos é convertido em glutamina (GLn) pela enzima glutamina sintetase, a qual é liberada dos astrócitos e captada pelos neurônios, onde a GLn é novamente convertida em GLU pela enzima glutaminase. O GLU é armazenado em vesículas sinápticas sendo liberado mediante despolarização dependente de sódio (Na^+). Retirado de Sanacora et al., (2008).

Vários estudos têm demonstrado que o receptor NMDA pode ser o local de ação de antidepressivos. Tem sido demonstrado que antagonistas funcionais do complexo receptor NMDA, tais como o MK-801, exibem ações antidepressivas em modelos animais preditivos de ação antidepressiva (Skolnick, 1999; Petrie et al., 2000). Skolnick (1999) propôs que os inibidores da ativação do receptor NMDA podem se constituir em uma nova classe de antidepressivos, com efeito terapêutico mais breve que os antidepressivos clássicos. No entanto, os antagonistas clássicos do receptor NMDA possuem efeitos colaterais indesejados (Kornhuber; Weller, 1997).

O GLU tem sido implicado na patogênese dos transtornos depressivos (Skolnick, 1999; Sanacora et al., 2008). Estudo *postmortem* mostrou níveis aumentados de GLU no córtex frontal de pacientes com depressão (Hashimoto et al., 2007). Além disso, estudos *postmortem* demonstram mudanças no receptor NMDA no córtex frontal de vítimas suicidas (Nowak et al., 1995), bem como, uma redução na subunidade do receptor NMDA, NMDAR1, no hipocampo (Law; Deakin, 2001). Estudos recentes tem demonstrado um rápido efeito de antagonistas do receptor NMDA, particularmente a quetamina, em pacientes com depressão severa que são resistentes aos antidepressivos típicos (Berman et al., 2000; Zarate et al., 2006; Duman; Voleti, 2012). Além disso, a quetamina é também um tratamento rápido e eficaz para a depressão bipolar (Diazgranados et al., 2010) e pacientes com pensamentos suicidas com depresão resistentes ao tratamento (Price et al., 2009; Larkin e Beautrais, 2011). Outros estudos demonstram um efeito sinérgico de antagonistas do receptor NMDA com antidepressivos em um modelo animal preditivo de ação antidepressiva (Rogóz et al., 2004; Ghasemi et al., 2009). Além disso, estudos pré-clínicos e clínicos indicam que compostos que reduzem a transmissão dos receptores NMDA e antagonistas dos receptores NMDA produzem efeito semelhante a antidepressivos (Paul; Skolnick, 2003; Garcia et al., 2008; Sanacora et al., 2008). Desta forma, drogas que modulam a neurotransmissão glutamatérgica, particularmente em receptores NMDA, apresentam um eventual potencial terapêutico como antidepressivos.

Em resposta a ativação dos receptores NMDA, o óxido nítrico (NO) é sintetizado apartir da L-arginina pela óxido nítrico sintase neuronal (NOSn) (Contestabile, 2000; Esplugues, 2002). O NO é uma molécula sinalizadora do SNC e tem sido implicado na neurotransmissão, plasticidade sináptica, aprendizado, dor e depressão (Harkin et al., 1999; Da Silva et al., 2000; Esplugues, 2002; Heiberg et

al., 2002). Vários estudos têm demonstrado que inibidores da NOS possuem efeito semelhante a antidepressivo em modelos animais de depressão (Da Silva et al., 2000; Harkin et al., 1999, 2003; Heiberg et al., 2002; Volke et al., 2003). A administração de inibidores NOS foi também capaz de causar um aumento nos efeitos antidepressivos de inibidores seletivos da recaptação de SE (ISRS) em modelos animais (Harkin et al., 2004). Contudo, inibidor da NOSn 1-(2-trifluoromethilfenil)-imidazol aumentou o efeito comportamental de antidepressivos tricíclicos e ISRS, mas falhou em aumentar o efeito antidepressivo de inibidores seletivo da recaptação de NA (ISRN) em modelo animal de depressão (Ulak et al., 2008). Além disso, as concentrações plasmáticas de nitrito são significantemente maiores em pacientes deprimidos, sugerindo que a produção de NO está aumentada na depressão (Suzuki et al., 2001).

Algumas das ações fisiológicas do NO são mediadas através da enzima guanilato ciclase solúvel (GCs). Esta enzima converte a guanosina 5'-trifosfato (GTP) em guanosina 3'5'-monofosfato (GMPc), um mensageiro intracelular (Denninger; Marletta, 1999; Esplugues, 2002). Vários dados têm demonstrado que a inibição tanto da NOS e quanto da GCs pode, dependendo da dose, produzir efeito semelhante a antidepressivo em modelos animais de depressão (Eroglu; Caglayan, 1997; Heiberg et al., 2002; Kaster et al., 2005b, Joca; Guimarães, 2006).

1.3 Antidepressivos e vias de sinalização celular

Vários dados sugerem que alterações estruturais no cérebro, incluindo a plasticidade sináptica, sinaptogênese e a neurogênese, pode desempenhar um papel na patogênese dos transtornos de humor e no mecanismo de ação dos antidepressivos (Duman, 2004; Schmidt; Duman, 2007, Duman; Volet, 2012). Estudos com antidepressivos tem sido focalizados nas vias intracelulares, que são conhecidas por serem ativados por um número de sinais extracelulares, incluindo fatores de crescimento, hormônios e por neurotransmissores (Popoli et al., 2000; D'Sa; Duman et al, 2002; Gould; Manji, 2002). Além disso, os antidepressivos podem exercer seus efeitos terapêuticos por modularem as vias de sinalização intracelular, arborização dendríticas, plasticidade sináptica e aumentar a neurogênese (D'Sa; Duman et al, 2002; Hashimoto et al, 2004; Duman; Voleti, 2012) (ver Figura 2, abaixo).

A PKA e a MAPK/ERK estão envolvidas com a plasticidade sináptica e sobrevivência celular (D'Sa; Duman, 2002; Hashimoto et al.,

2004), sendo que a PKA também modula a síntese e a liberação de neurotransmissores (D'Sa; Duman, 2002; Gould; Manji, 2002). A PKC modula canais iônicos e a liberação de neurotransmissores (Popoli et al., 2000). A via PI3K está implicada na regulação do crescimento celular, sobrevivência, proliferação e neuroplasticidade (Kumar et al., 2005; Martin-Pena et al., 2006). A CaMKII tem sido implicada em processos diversos como a expressão gênica, homeostase do cálcio, regulação de receptores e de canais iônicos, síntese e liberação de neurotransmissores, transmissão e plasticidade sináptica (Popoli et al., 2000).

Tem sido relatado que a administração crônica de diferentes classes de antidepressivos atua fazendo uma "up regulation" da cascata AMPc-PKA-CREB (AMPc-Proteína cinase dependente de AMPc-CREB) (Popoli et al., 2000; D'Sa; Duman, 2002). Um efeito reconhecido do AMPc é a ativação da proteína cinase A (PKA). O tratamento com antidepressivo ativa a via da PKA (Gould; Manji, 2002), a proteina cinase II dependente de cálcio-calmodulina (CamkII) (Popoli et al., 2000; Du et al., 2004) e modula a proteína cinase ativada por mitógeno-proteína cinase ativada por fator extracelular (MAPK/ERK) (Einat et al., 2003). Além disso, estudos tem demonstrado que o tratamento com lítio (um estabilizador do humor) e antidepressivos modulam a via da proteina cinase C (PKC) (Chen et al., 1999; Einat et al., 2003; Taylor et al., 2005) e a via da fosfatidilinositol-3 cinase (PI3K) também têm sido envolvida nos efeitos comportamentais de antidepressivos (Beech; Duman, 2005). Estudos *postmorten* demonstram uma diminuição na atividade da PKA (Dwivedi et al., 2004) e da ERK-1/2 MAPK em vítimas suicidas (Dwivedi et al., 2009). Além disso, tem sido informado uma diminuição na atividade da PKC (Pandey et al., 2004) e da PI3K (Hsiung et al., 2003; Dwivedi et al., 2008) no córtex pré-frontal e hipocampo de vítimas de suicídio comparados com indivíduos saudáveis.

A administração crônica de antidepressivos superregula a via do AMPc em vários níveis, incluindo o aumento da expressão da proteína ligante ao elemento de responsivo ao AMPc (CREB) e do fator neurotrófico derivado do cérebro (BDNF) (Altar, 1999; D'Sa; Duman, 2002; Gur et al., 2007; Hayashi et al., 2008; Hashimoto, 2010). Além disso, a injeção de BDNF no hipocampo de ratos produziu efeito antidepressivo (Shirayama et al., 2002; Hashimoto, 2010). Estudos recentes mostraram que os níveis de BDNF estão diminuídos no plasma de pacientes com depressão e o tratamento com antidepressivos aumentou esses níveis (Brunoni et al., 2008; Molendijk et al., 2010; Wolkowitz et al., 2011). Em camundongos, o tratamento crônico com

fluoxetina aumentou os níveis de BDNF no hipocampo e córtex frontal (Hodes et al., 2010). Alguns antidepressivos atuam também como neuroprotetores, por modularem a via de sinalização celular da proteína ligante ao elemento responsivo ao AMPc (CREB) (D'Sa; Duman, 2002; Hayashi et al., 2008). Dentre os vários alvos gênicos regulados pelo CREB e que poderiam estar envolvido na ação neuroprotetora dos antidepressivos, estão o BDNF e a proteína antiapoptótica Bcl-2 (D'Sa; Duman, 2002; Hashimoto et al., 2004) (Ver Figura 2, abaixo) . Deste modo, o BDNF parece modular a atividade de sistemas neuroquímicos envolvidos na neuroproteção (Peng et al., 2008), exercendo um importante papel na patologia e no tratamento de doenças neurodegenerativas.

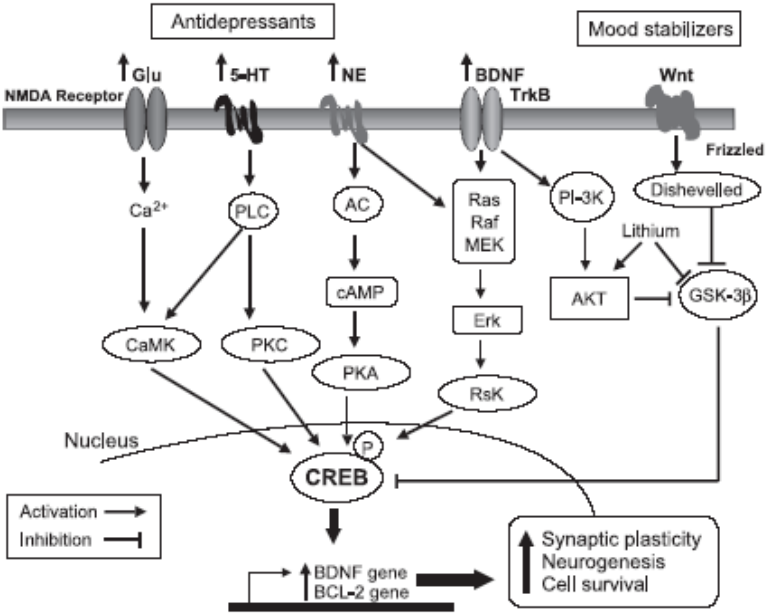

Figura 2. Vias de sinalização celular envolvidas na ação de antidepressivos e estabilizadores de humor.

A figura apresenta como os antidepressivos ativam as vias de sinalização intracelular da CaMKII, PKC, PKA e MAPK/ERK, que levaria à fosforilação de CREB e ativação de genes para a expressão de BDNF e da proteína antiapoptótica Bcl2. A ação dos antidepressivos resultaria na plasticidade sináptica, neurogênese e sobrevivência cellular. Retirado de Hashimoto et al., (2004).

1.4 Depressão e neuroproteção

O GLU tem um papel na plasticidade sináptica, mas em condições patológicas é conhecido por ser uma excitotoxina neuronal, provocando neurotoxicidade (Sanacora et al., 2008). A excitotoxicidade glutamatérgica refere-se à toxicidade causada pelo aumento da concentração de GLU durante a transmissão sináptica e consequente morte neuronal (Meldrum, 2000). A excitotoxicidade glutamatérgica também pode ser causada por uma falha em mecanismos de captação de GLU pelos astrocitos (Gagliardi, 2000). O excesso de GLU ativa principalmente receptores ionotrópicos como o receptor NMDA, canais iônicos modulados por ligantes e por voltagem, que promovem um grande influxo de íons Ca^{2+} para dentro da célula pós-sináptica (Mody; MacDonald, 1995; Stone; Addae, 2002). O aumento de íons Ca^{2+} ativa diferentes vias de sinalização intracelular que convergem para a morte celular e degradação da membrana plasmática (Platt, 2007) e o aumento de Ca^{2+} induz estresse oxidativo (Mattson, 2003) (Ver Figura 3, abaixo). A morte celular induzida por excitotoxicidade pode acontecer por apoptose, um fenômeno regulado, e também por necrose (Platt, 2007). Muitas doenças neurodegenerativas são causadas pela superestimulação dos receptores de GLU (Mattson; Magnus, 2006). Tem sido demonstrado que concentrações milimolares de GLU agudamente induzem a excitotoxicidade em fatias hipocampais e promovem uma redução na viabilidade celular (Molz et al., 2008a). Além disso, a toxicidade do GLU está relacionada com a atividade reversa dos transportadores de GLU, aumentando a concentração de GLU e excitoxicidade (Molz et al., 2008b).

O GLU tem sido implicado na fisiopatologia da depressão (Ferrero e Cereseto, 2004; Zarate et al., 2006; Sanacora et al., 2008). Segundo a teoria glutamatérgica da depressão, a exposição a um estímulo estressor aumenta a neurotransmissão glutamatérgica hipocampal e o aumento da concentração de GLU exerce um efeito tóxico nos neurônios hipocampais (Ferrero e Cereseto, 2004). Estudos de microdiálise mostram que o estresse aumenta os níveis extracelulares de GLU no hipocampo e que antagonistas do receptor NMDA atenuam a atrofia dos neurônios hipocampais induzido pelo estresse (Sapolsky, 2000; McEwen, 2003). A fluoxetina (ISRS) reduziu a liberação de GLU induzida agudamente pela 4-aminopiridina em sinaptosomas corticais *in vitro* (Wang et al., 2003). Os antidepressivos podem exercer seus efeitos terapêuticos por modularem as vias de sinalização celular, aumentando a neurogênese e a plasticidade neuronal (Tardito et al., 2006; Krystal et

al., 2009; Duman e Volet, 2012).

Outra consequência da ativação dos receptores NMDA é a ativação da enzima NOSn, que converte a L-arginina em NO (Contestabile, 2000; Esplugues, 2002). O aumento da produção de NO pela ativação da NOSn pode ser causada por processos como estresse crônico e hiperatividade glutamatérgica que está relacionada com doenças neurodegenerativas como a depressão (McLeod et al., 2001; Guix et al., 2005; Singh; Dikshit, 2007). Além disso, o excesso de NO pode ser causado pelo aumento do estresse oxidativo (Calabrese et al., 2007a).

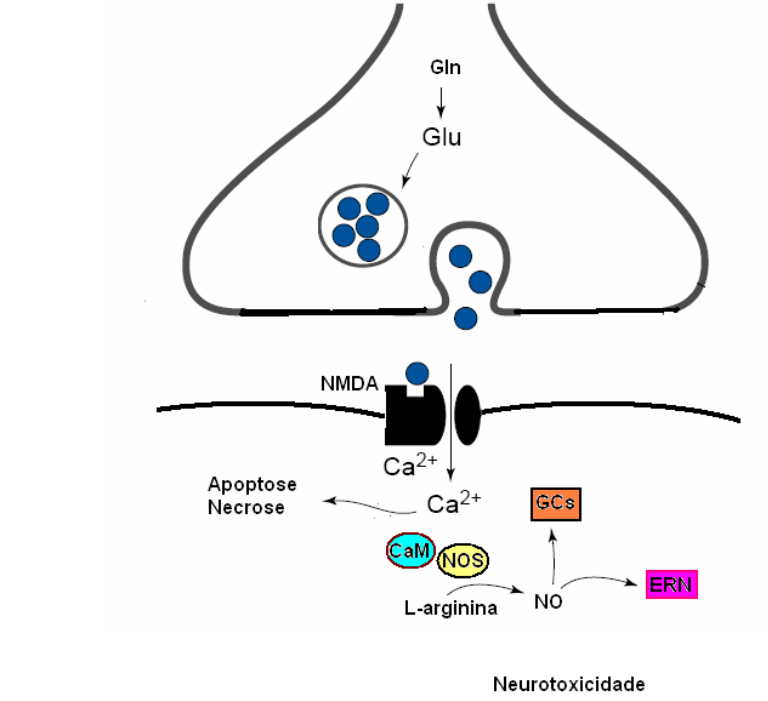

Figura 3. Excitoxicidade glutamatérgica.
A liberação excessiva de glutamato (Glu) sintetizado apartir da glutamina (Gln) pela ação da enzima glutaminase, leva á ativação exarcebada dos receptores NMDA. Estes receptores promovem a entrada excessiva de Ca^{2+} na célula, ativando vias e processos que podem culminar em morte celular por necrose ou apoptose. Outra consequência é a síntese de NO pela atiação da NOS pelo compelxo Ca^{2+}/calmodulina (CaM). O NO formado estimula a enzima guanilato ciclase solúvel (GSc), responsável pela produção de GMPc a partir do GTP, que modula vários alvos intracelulares. A produção excessiva de NO leva a formação de espécies reativas de nitrogênio (ERN), que podem levar ao estresse oxidative. Adaptado de Nicoletti et al., (1996).

A ativação da via de sinalização intracelular PI3K leva à formação de segundos mensageiros como o fosfatidilinositol-3,4,5-trifosfato (PIP3) e fosfatidilinositol-3,4-bifosfato (PIP2) e esses mensageiros ativam a proteína cinase B (Akt/PKB). Uma das funções mais importante da Akt é seu papel na sobrevivência celular. A exposição de culturas de neurônios corticais a concentrações excitotóxicas de GLU diminui a fosforilação da Akt e causa aumento da ativação de caspase-3 (Nishimoto et al., 2008). Além disso, em cultura de neurônios hipocampais submetidas à hipóxia, a ativação da via PI3K/Akt preveniu a apoptose através da inibição de genes e proteínas pró-apoptóticas (Yamaguchi et al., 2001)

1.5 Antidepressivos

Os antidepressivos têm sido amplamente usados nas últimas quatro décadas. A introdução dos inibidores da monoamino oxidase (iMAOs) e dos antidepressivos tricíclicos na década de 1950, seguido pelos ISRS na década de 1980, redefiniu o tratamento da depressão (Wong e Licinio, 2001; Haenisch e Bönisch, 2011). Os antidepressivos podem ser classificados de acordo com o seu mecanismo de ação em: iMAO (iproniazida, tranilcipromina), antidepressivos tricíclicos (amitriptilina, imipramina, desipramina), ISRS (fluoxetina, citalopram, escitalopram, sertralina), ISRN (reboxetina, atomoxetina), inibidores da recaptação de SE e NA (IRSN) (duloxetina, venlafaxina) e antidepressivos atípicos (mirtazapina, tianeptina) (Berton; Nestler, 2006; Haenisch; Bönisch, 2011).

Uma variedade de antidepressivos atua por diferentes mecanismos, incluindo os sistemas serotonérgico, noradrenérgico e/ou dopaminérgico (Wong; Licinio, 2001). Contudo, os antidepressivos convencionais produzem vários efeitos colaterais e requerem várias semanas de tratamento para produzir um efeito terapêutico (Nestler et al., 2002). Neste estudo utilizamos novos compostos antidepressivos, como o escitalopram e a duloxetina que apresentam vantagens em relação a tolerabilidade, segurança, eficácia, menores efeitos adversos e efeitos terapêuticos mais rápidos que os antidepressivos convencionais (Manning, 2004).

Os ISRS têm sido amplamente usados na clínica durante duas décadas e são as compostos de escolha para o tratamento da depressão e ansiedade. O escitalopram (Lexapro) é um ISRS, que tem pouca afinidade para os receptores α_1-adrenérgico, M_1-muscarínico e H_1-

histamínicos (Manning, 2004) e tem sido usado no tratamento da depressão, da ansiedade e transtorno do pânico (Stahlet al., 2002; Höschl et al., 2008; Lam et al., 2008). A dose diária definida (DDD) pela Who Collalaborating Centre for Drug Statistics Methodology de escitalopram, é de 10 mg. O escitalopram tem mostrado ser mais potente clinicamente que sua forma racêmica, o citalopram, no tratamento da depressão e tem efeito mais rápido quando comparado com doses do citalopram (Sánchez et al., 2004; Colonna et al., 2005; Moore et al., 2005). Além disso, o escitalopram apresenta melhor eficácia e tolerabilidade em relação aos ISRS e outros antidepressivos (Ali; Lam, 2011). O escitalopram é bem tolerado e apresenta poucos efeitos adversos como náusea (Manning, 2004), diminuição de libido em comparação com a fluoxetina (Sidi et al., 2011) e menores efeitos cardiovas culares em comparação com os antidepressivos tricíclicos (Tseng et al., 2012). As taxas de respostas e de remissão do escitalopram foram melhores que as do citalopram e da fluoxetina (Ali; Lam, 2011). Sánchez et al. (2003) demonstraram que o escitalopram reduziu o tempo de imobilidade de camundongos no teste do nado forçado (TNF). O tratamento crônico com escitalopram aumentou as defesas antioxidantes (glutationa peroxidase e glutationa) em ratos submetidos ao estresse crônico (Eren et al., 2007). Um estudo demonstrou que o escitalopram produziu efeito neuroprotetor contra a isquemia cerebral por aumentar os níveis de BDNF e diminuir o estresse oxidativo no hipocampo (Lee et al., 2011). Além disso, o tratamento crônico com escitalopram aumentou os níveis de RNAm de BDNF, bem como os níveis de BDNF no soro de pacientes deprimidos (Cattaneo et al., 2010).

A duloxetina (Cymbalta) é IRSN e tem baixa afinidade para receptores muscarínicos, histamínicos e β_1-adrenérgicos (Bymaster et al., 2001, Karpa et al., 2002) é utilizada no tratamento da depressão, da ansiedade e dor neuropática em diabéticos (Detke et al., 2004; Thase et al., 2007; Carter and McCormack, 2009). A dose diária definida (DDD) pela Who Collalaborating Centre for Drug Statistics Methodology de duloxetina, é de 60 mg. A duloxetina tem mais seletividade para bloquear o transporte de SE do que o transporte da NA (Stahl et al., 2005). Alguns dados clínicos sugerem que IRSN pode ser mais eficaz do que ISRS (Thase et al., 2001; Smith et al., 2002; Shelton et al., 2005). A duloxetina apresenta maior eficácia em relação aos ISRS como a fluoxetina e a paroxetina em pacientes com depressão moderada a severa (Thase et al., 2007). Além disso, a duloxetina apresenta melhor tolerabilidade do que a venlafaxina (Stahl et al., 2005). A duloxetina geralmente é bem tolerada e apresenta menores efeitos adversos como

boca seca, náuseas, fadiga (Hunziker et al., 2005), disfunção sexual (Stahl et al., 2005) e pouco ou nenhum efeito no sistema cardiovascular (Hunziker et al., 2005; Stahl et al., 2005). As taxas de respostas e de remissão da duloxetina foram melhor que a fluoxetina e a paroxetina (Hunziker et al., 2005). A duloxetina reduz o tempo de imobilidade de ratos no TNF (Rénéric e Lucki, 1998, Ciulla et al., 2007). O tratamento crônico com duloxetina aumentou a expressão do RNAm e da proteína BDNF no córtex frontal de ratos (Calabrese et al., 2007b). Além disso, o tratamento crônico, mas não agudo, com duloxetina aumentou os níveis de BDNF no córtex pré-frontal (Mannari et al., 2008). O tratamento crônico com duloxetina em ratos "knockout" para o transportador de SE, aumentou os níveis do RNAm de BDNF no córtex e hipocampol (Calabrese et al., 2010).

2 JUSTIFICATIVA

A depressão é um distúrbio que afeta milhões de pessoas no mundo todo e é a segunda condição crônica mais comum na prática clínica (Altar, 1999; Nestler et al., 2002). A Organização Mundial de Saúde estima que em 2020, a depressão seja a segunda causa de incapacitação no mundo e em 2030 perderá apenas para doenças cardiovasculares (Mathers; Loncar, 2006; Sanacora et al., 2008). O estudo do mecanismo neuroquímico da depressão, do mecanismo de ação de compostos antidepressivos e a pesquisa de novos compostos e suas ações para o controle dos sintomas associados com os transtornos depressivos é necessário devido a alta incidência desta doença na população. Apesar dos grandes avanços obtidos com o desenvolvimento de novos compostos antidepressivos nos últimos anos, o tratamento com antidepressivos não é totalmente eficaz, pois somente 60% dos pacientes respondem a esse tratamento (Gareri et al., 2000) e, além disso, em muitos casos o tratamento com antidepressivos apresenta efeitos adversos (Nestler et al., 2002).

Recentes evidências indicam que a depressão está relacionada, dentre outros fatores, com a morte neuronal, alterações na sinalização celular, diminuição da neuroplasticidade e estresse oxidativo. Além disso, vários compostos antidepressivos são também neuroprotetores por induzirem a expressão de proteínas envolvidas na sobrevivência celular.

Em vista disso, tem sido necessário que continuem a serem realizadas pesquisas para investigar "novos" compostos, a fim de se controlar os sinais e sintomas associados aos distúrbios depressivos e, se possível, minimizar os efeitos adversos do tratamento.

O escitalopram e a duloxetina são "novos" compostos antidepressivos utilizados no tratamento da depressão, no entanto, não se sabe por quais mecanismos de ação estes compostos induzem seus efeitos antidepressivos. Alguns trabalhos têm mostrado que: i) reduções regionais do número (morte celular) ou do tamanho de glias e neurônios no hipocampo têm sido associados à depressão (Manji et al., 2001), e ii) o tratamento com antidepressivos induz a expressão de BDNF **(**D'Sa; Duman, 2002; Hashimoto et al., 2004). Apesar destas evidências, não há trabalhos sobre o mecanismo de ação e as vias de sinalização celular envolvidos na ação antidepressiva da duloxetina e escitalopram; assim

como os seus efeitos na neuroproteção. Portanto, a compreensão das vias e mecanismos responsáveis pela ação dos antidepressivos pode contribuir substancialmente para o entendimento dos transtornos depressivos e para o desenvolvimento de novas alternativas terapêuticas para o seu tratamento. O conhecimento sobre os "alvos" de ação dos antidepressivos pode auxiliar a compreensão sobre como estes compostos afetam direta ou indiretamente a sobrevivência celular e a neuroplasticidade (Santarelli et al., 2003). Assim, este trabalho investigou o mecanismo de ação do efeito antidepressivo do escitalopram e da duloxetina.

3 OBJETIVOS

3.1 Objetivo geral

O objetivo deste estudo foi investigar o mecanismo de ação dos compostos antidepressivos, escitalopram e duloxetina, e seus efeitos na neuroproteção.

3.2 Objetivos específicos

- Investigar o efeito da administração aguda dos compostos antidepressivos, escitalopram e duloxetina em modelos animais preditivos de ação antidepressiva como o teste do nado forçado (TNF) e o teste da suspensão pela cauda em camundongos (TSC) (*Paper 1 e Manuscrito 2*);
- Verificar o envolvimento do sistema glutamatérgico e da via L-arginina-óxido nítrico-GMPc na ação antidepressiva aguda da escitalopram e duloxetina (*Paper 1 e Manuscrito 2*);
- Investigar a participação das vias de sinalização celular (PKA, PKC, PI3K, MAPK/ERK e CaMKII) na ação antidepressiva produzida pela administração aguda da escitalopram e duloxetina (*Manuscrito 3)*;
- Avaliar a neuroproteção exercida pelo escitalopram e pela duloxetina na excitotoxicidade induzida pelo GLU em fatias de hipocampo (*Manuscrito 4)*.

CAPÍTULO 1

Involvement of NMDA receptors and l-arginine-nitric oxide-cyclic guanosine monophosphate pathway in the antidepressant-like effects of escitalopram in the forced swimming test.

Eur Neuropsychopharmacol. 2010;20:793-801.

European Neuropsychopharmacology (2010) 20, 793–801

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Involvement of NMDA receptors and L-arginine-nitric oxide-cyclic guanosine monophosphate pathway in the antidepressant-like effects of escitalopram in the forced swimming test

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Received 1 April 2010; received in revised form 9 June 2010; accepted 23 July 2010

KEYWORDS
Depression;
Escitalopram;
Forced swimming test;
L-arginine;
NMDA;
Tail suspension test

Abstract

Escitalopram is a serotonin reuptake inhibitor used in the treatment of depression and anxiety disorders. This study investigated the effect of escitalopram in forced swimming test (FST) and in the tail suspension test (TST) in mice, and tested the hypothesis that the inhibition of NMDA receptors and NO-cGMP synthesis is implicated in its mechanism of action in the FST. Escitalopram administered by i.p. route reduced the immobility time both in the FST (0.3–10 mg/kg) and in the TST (0.1–10 mg/kg). Administration of escitalopram by p.o route (0.3–10 mg/kg) also reduced the immobility time in the FST. The antidepressant-like effect of escitalopram (3 mg/kg, p.o.) in the FST was prevented by the pretreatment of mice with NMDA (0.1 pmol/site, i.c.v.), L-arginine (750 mg/kg, i.p., a substrate for nitric oxide synthase) or sildenafil (5 mg/kg, i.p., a phosphodiesterase 5 inhibitor). The administration of 7-nitroindazole (50 mg/kg, i.p., a neuronal nitric oxide synthase inhibitor), methylene blue (20 mg/kg, i.p., an inhibitor of both nitric oxide synthase and soluble guanylate cyclase) or ODQ (30 pmol/site i.c.v., a soluble guanylate cyclase inhibitor) in combination with a subeffective dose of escitalopram (0.1 mg/kg, p.o.) reduced the immobility time in the FST as compared with either drug alone. None of the drugs produced significant effects on the locomotor activity in the open-field test. Altogether, our data suggest that the antidepressant-like effect of escitalopram is dependent on inhibition of either NMDA receptors or NO-cGMP synthesis. The results contribute to the understanding of the mechanisms underlying the antidepressant-like effect of escitalopram and reinforce the role of NMDA receptors and L-arginine-NO-GMP pathway in the mechanism of action of antidepressant agents.
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0924-977X/$ - see front matter © 2010 Elsevier B.V. and ECNP. All rights reserved.
doi:10.1016/j.euroneuro.2010.07.011

1. Introduction

The selective serotonin reuptake inhibitors have gained extensive clinical use during the last two decades and are drugs of choice for treatment of depressive and anxiety disorders. Escitalopram, the *S*-isomer of citalopram, is a specific serotonin reuptake inhibitor that has been used in the treatment of depression and anxiety disorders (Höschl and Svestka, 2008; Lam et al., 2008). It has been shown to be clinically more potent than its racemate citalopram in the treatment of depression and has faster onset of action than comparable doses of citalopram (Colonna et al., 2005; Moore et al., 2005; Sánchez et al., 2004). In vivo microdialysis studies of rat brain cortex also show a greater propensity for escitalopram to elevate serotonin levels than for citalopram (Hyttel et al., 1992). Moreover, escitalopram is effective in animal models predictive of antidepressant and anxiolytic activities (Sánchez et al., 2003a,b).

Glutamate has been implicated in the pathogenesis of depressive disorders (Sanacora et al., 2008; Skolnick, 1999). A post-mortem study found increased levels of glutamate in the frontal cortex of patients with major depression (Hashimoto et al., 2007). Postmortem studies report changes in the NMDA receptor complex in the frontal cortex of suicide victims (Nowak et al 1995), as well as a reduction in the subunit for the NMDA receptor, NMDAR1, in the hippocampus (Law and Deakin, 2001). Depressed patients also showed a significant improvement after the administration of an NMDA receptor antagonist, ketamine (Berman et al., 2000; Zarate et al., 2006). Pre-clinical data have suggested that compounds that reduce transmission at NMDA receptors exhibit antidepressant-like actions, and that chronic antidepressant treatment can, in turn, impact on NMDA receptor function (Sanacora et al., 2008; Skolnick, 1999).

In response to activation of the NMDA receptor, nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthase (NOS) (Contestabile, 2000; Esplugues, 2002). NO is a signalling molecule in the brain and has been implicated in neurotransmission, synaptic plasticity, learning, perception of pain and depression (Da Silva et al., 2000; Esplugues, 2002; Harkin et al, 1999; Heiberg et al., 2002). Several studies have demonstrated that NOS inhibitors exert antidepressant-like effects in animal models predictive of antidepressant activity (Da Silva et al., 2000; Harkin et al., 1999, 2003; Heiberg et al., 2002; Volke et al., 2003). The administration of NOS inhibitors was also reported to cause an increase in the effects of serotonin reuptake inhibitors in the forced swimming test (FST) (Harkin et al., 2004). Moreover, neuronal NOS inhibitor 1-(2-trifluoromethylphenyl)-imidazole augmented the behavioral effect of tricyclic antidepressants and selective serotonin reuptake inhibitors, but failed to augment the antidepressant effect of noradrenaline reuptake inhibitors in the FST (Ulak et al., 2008). In addition, plasma nitrate concentrations were significantly higher in depressed patients, suggesting that NO production is increased in depression (Suzuki et al., 2001).

NO has been suggested to act upon multiple targets, among which the soluble guanylate cyclase (sGC) is the most extensively characterized. This enzyme converts guanosine 5'-triphosphate (GTP) to the intracellular messenger cyclic guanosine 3'5'-monophosphate (cGMP) (Denninger and Marletta, 1999; Esplugues, 2002). Several data have demonstrated that the inhibition of both NOS and sGC may, depending on the dose, elicit antidepressant-like effects in the FST (Eroglu and Caglayan, 1997; Heiberg et al., 2002; Kaster et al., 2005b, Joca and Guimarães, 2006).

An animal behavioral study supports the antidepressant properties of escitalopram in the FST (Sánchez et al., 2003a). However, the exact mechanism of action of the antidepressant-like effect of escitalopram in this model is not fully understood. In the present study the antidepressant-like effect of escitalopram was examined in the FST and also in another model predictive of antidepressant activity, the TST (Cryan et al., 2005). Considering the involvement of NMDA receptors and of the NO-cGMP pathway in the pathogenesis of depression and the importance of these molecular targets for the efficacy of antidepressants (Harkin et al., 1999; Heiberg et al., 2002; Skolnick, 1999; Volke et al., 2003; Yildiz et al., 2000), we investigated whether NMDA receptor signalling and NO-cGMP pathway are involved in the antidepressant-like effect of escitalopram in the mouse FST.

2. Experimental procedures

2.1. Animals

Female Swiss mice (30–40 g) were maintained at 22–24 °C with free access to water and food, under a 12:12 h light/dark cycle (lights on at 07:00 h). All manipulations were carried out between 9:00 and 16:00 h, with each animal used only once. All procedures in this study were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). The experiments were performed after approval of the protocol by the Institutional Ethics Committee and all efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

2.2. Drugs

The following drugs were used: Escitalopram (H. Lundbeck, Denmark), L-arginine, methylene blue, NMDA (N-methyl-D-aspartate), (1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one) (ODQ), 7-nitroindazole (Sigma Chemical Co, USA), and sildenafil (Pfizer). All drugs were dissolved in saline, except ODQ which was dissolved in saline with 1% DMSO and 7-nitroindazole that was dissolved in saline with few drops of Tween 80. All drugs were administered by intraperitoneal (i.p.) route in a constant volume of 10 ml/kg body weight, except NMDA and ODQ which were administered by intracerebroventricular (i.c.v.) route.

I.c.v. administration was performed under ether anesthesia as previously described (Brocardo et al., 2008). Briefly, a 0.4 mm external diameter hypodermic needle attached to a cannula, which was linked to a 25 μl Hamilton syringe, was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse. A volume of 5 μl was then administered in the left lateral ventricle. The injection was given over 30 s, and the needle remained in place for another 30 s in order to avoid the reflux of the substances injected. The injection site was 1 mm to the right or left from the mid-point on a line drawn through to the anterior base of the ears.

Escitalopram was also administered by oral (p.o.) route by gavage in a volume of 10 ml/kg body weight.

2.3. Behavioral tests

2.3.1. Forced swimming test (FST)

The FST was carried out in mice individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water at 25 ± 1 °C; the total duration of immobility during the 6-min test was scored as described previously (Kaster et al., 2005a,b; Zomkowski et al., 2002). Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water.

2.3.2. Tail suspension test (TST)

The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al. (1985). Briefly, mice both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded during a 6 min period (Binfaré et al., 2009).

2.3.3. Open-field behavior

The ambulatory behavior was assessed in an open-field test as described previously (Zomkowski et al., 2002). The apparatus consisted of a wooden box measuring 40×60×50 cm high. The floor of the arena was divided into 12 equal squares. The number of squares crossed with all paws (crossings) was counted in a 6-min session. The light was maintained at minimum to avoid anxiety behavior. The apparatus were cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.4. Pharmacological treatments

In order to investigate the antidepressant-like effect of the escitalopram, it was administered (dose range: 0.1–10 mg/kg, i.p.) 30 min before the FST, TST or open-field test. Alternatively, escitalopram or vehicle was administered by oral (p.o.) route (dose range: 0.1–10 mg/kg, p.o.) 60 min before the FST or open-field test.

In a separate series of experiments, we investigated whether the antidepressant-like effect of escitalopram in the FST is mediated through the inhibition of NMDA receptors. To this end, mice were pretreated with NMDA (0.1 pmol/site, i.c.v.) and 15 min after, escitalopram (3 mg/kg, p.o.) or vehicle was administered. Sixty minutes later the FST was carried out.

To investigate the possible involvement of the L-arginine-nitric oxide pathway in the anti-immobility effects of escitalopram in the FST, mice were pre-treated L-arginine, a precursor of nitric oxide (750 mg/kg, i.p., a dose that produces no effect in the FST) or vehicle and after 30 min they received escitalopram (3 mg/kg, p.o.) or vehicle injection before being tested in the FST after 60 min.

In another set of experiments, we investigated the effect of the combined administration of a subeffective dose of escitalopram (0.1 mg/kg, p.o.) with subeffective doses of 7-nitroindazole (50 mg/kg, i.p., a neuronal NO synthase inhibitor), ODQ (30 pmol/site i.c.v., a sGC inhibitor) or methylene blue (20 mg/kg, i.p., an inhibitor of both NO synthase and soluble guanylate cyclase). Escitalopram or vehicle was administered 30 min before of 7-nitroindazole, methylene blue or 40 min before ODQ. A further 30 min (after i.p. administration of 7-nitroindazole or methylene blue) or 20 min (after i.c.v. ODQ administration) were allowed to elapse before the animals were tested in the FST.

In order to investigate the role of cyclic GMP (cGMP) in the antidepressant action of escitalopram, mice received an injection of sildenafil (5 mg/kg, i.p., a phosphodiesterase (PDE) 5 inhibitor) or vehicle 30 min before escitalopram (3 mg/kg, p.o.) and 60 min later the FST was carried out.

The doses of the drugs used were selected on the basis of literature data and on previous results from our laboratory (Almeida et al., 2006; Brocardo et al., 2008; Da Silva et al., 2000; Dhir and Kulkarni, 2007; Ghasemi et al., 2008; Kaster et al., 2005a,b; Kulkarni and Dhir, 2007; Rosa et al., 2003). The doses of escitalopram administered were chosen on the basis of experiments previously performed in our laboratory and literature data (Sánchez et al., 2003a).

2.5. Statistical analysis

Comparisons between treatment groups and control were performed by one-way or two-way ANOVA followed by Tukey's HSD test when appropriate. A value of $p<0.05$ was considered to be significant.

3. Results

3.1. Effect of escitalopram in the immobility time in the FST, in the TST and on the locomotor activity in the open-field test

Fig. 1A and B shows that the treatment of mice with escitalopram given by intraperitoneal route significantly decreased the immobility time both in the FST (dose range 0.3–10 mg/kg) ($F(5,30)=85.32$, $p<0.01$) and in the TST (dose range 0.1–10 mg/kg) ($F(5,35)=14.92$, $p<0.01$), respectively. However, escitalopram did not produce any change in ambulation in an open-field in a separate experiment as compared to the control group (data not shown). Fig. 1C shows that escitalopram also caused a reduction in the immobility time in the FST when administered by p.o. route (dose range: 0.3–10 mg/kg; $F(5,30)=48.00$, $p<0.01$), but did not produce any change in ambulation in mice tested in an open-field in a separate experiment (data not shown).

3.2. Involvement of NMDA receptors and L-arginine-NO-cGMP pathway on the antidepressant-like effect of escitalopram in the FST

Fig. 2A shows the influence of pre-treatment of mice with NMDA (0.1 pmol/site, i.c.v.) on the anti-immobility effect of escitalopram (3.0 mg/kg, p.o.) in the FST. The pre-treatment of mice with NMDA was able to reverse the antidepressant-like effect of escitalopram. A two-way ANOVA revealed significant differences for the NMDA pretreatment ($F(1,20)=87.97$, $p<0.01$), escitalopram treatment ($F(1,20)=122.06$, $p<0.01$) and NMDA×escitalopram interaction ($F(1,20)=51.44$, $p<0.01$). The administration of NMDA alone or in combination with escitalopram did not affect ($p>0.05$) the ambulation in the open-field (Fig. 2B).

The results depicted in Fig. 2C show that the pre-treatment with L-arginine (750 mg/kg i.p., a nitric oxide precursor) prevented the antidepressant-like effect of escitalopram (3.0 mg/kg, p.o.) in the FST. The two-way ANOVA showed significant differences for L-arginine pre-treatment ($F(1,20)=133.91$, $p<0.01$), escitalopram treatment ($F(1,20)=15.18$, $p<0.01$), L-argine×escitalopram interaction ($F(1, 20)=18.22$, $p<0.01$). The administration of L-arginine alone or in combination with escitalopram did not affect ($p>0.05$) the ambulation in the open-field (Fig. 2D).

The results illustrated in Fig. 3A show the administration of 7-nitroindazole (50 mg/kg i.p., a specific neuronal nitric oxide synthase inhibitor) in combination with escitalopram (0.1 mg/kg, p.o.) produced an antidepressant-like effect as compared with

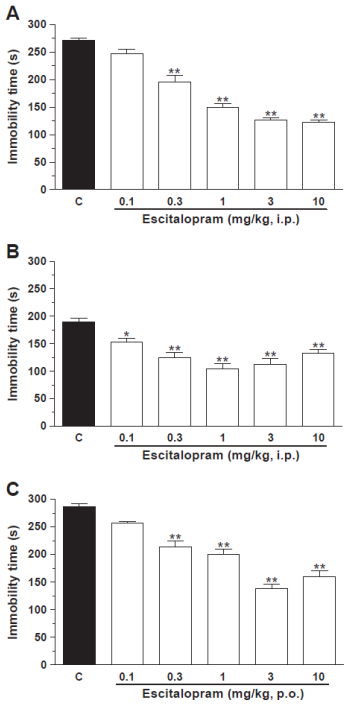

Figure 1 Effect of i.p. administration of escitalopram (0.1–10 mg/kg) in the immobility time in the FST (A), TST (B) and the effect of p.o. administration of escitalopram (0.1–10 mg/kg) in the immobility time in the FST (C) in mice. Values are expressed as mean ± S.E.M. ($n = 6$). $^{*}p<0.05$ and $^{**}p<0.01$ compared with the vehicle-treated control group (one-way ANOVA followed by Tukey's HSD test).

the administration of either drug alone. A two-way ANOVA revealed significant differences for the escitalopram pretreatment (F (1,20) = 10.86, $p<0.01$), 7-nitroindazole treatment ($F(1,20) = 19.80$, $p<0.01$) and escitalopram × 7-nitroindazole interaction ($F(1,20) = 8.03$, $p<0.05$). Fig. 3B shows that the administration of 7-nitroindazole alone or in combination with escitalopram did not affect ($p>0.05$) locomotor activity in the open-field test. Fig. 3C shows that methylene blue (20 mg/kg i.p., direct inhibitor of both nitric oxide synthase and soluble guanylate cyclase) in combination with escitalopram (0.1 mg/kg, p.o.) also produced an anti-immobility effect in the FST as compared with the administration of either drug alone. A two-way ANOVA revealed significant differences for the escitalopram pretreatment ($F(1,20) = 11.70$, $p<0.01$), methylene blue treatment ($F(1,20) = 18.24$, $P<0.01$) and escitalopram × methylene blue interaction ($F(1,20) = 7.97$, $p<0.05$). The administration of methylene blue (20 mg/kg i.p.) alone or in combination with escitalopram did not affect ($p>0.05$) the ambulation in the open-field (Fig. 3D). The results depicted in Fig. 3E shows that ODQ (30 pmol/site i.c.v., a specific inhibitor of soluble guanylate cyclase) in combination with escitalopram (0.1 mg/kg, p.o.) also produced an anti-immobility effect in the FST as compared with the administration of either drug alone. A two-way ANOVA revealed significant differences for the escitalopram pretreatment ($F(1,20) = 24.29$, $p<0.01$), ODQ treatment ($F(1,20) = 30.26$, $p<0.01$) and escitalopram × ODQ interaction ($F(1,20) = 18.62$, $p<0.01$). The administration of ODQ (30 pmol/site i.c.v.) alone or in combination with escitalopram did not affect ($p>0.05$) the ambulation in the open-field (Fig. 3F).

Fig. 4A shows that the anti-immobility effect of escitalopram (3 mg/kg, p.o.) was completely prevented by pretreatment of animals with sildenafil (5 mg/kg, i.p., a phosphodiesterase 5 inhibitor). A two-way ANOVA revealed significant differences for the sildenafil pretreatment ($F(1,20) = 33.44$, $p<0.01$), escitalopram treatment ($F(1,20) = 57.10$, $p<0.01$) and sildenafil × escitalopram interaction ($F(1,20) = 41.32$, $p<0.01$). The administration of sildenafil (5 mg/kg, i.p.) alone or in combination with escitalopram did not affect ($p>0.05$) the locomotor activity in the open-field (Fig. 4B).

4. Discussion

The results presented here show that the escitalopram given systemically (i.p. route) or orally (p.o. route) is effective in producing significant antidepressant-like effects, when assessed in the FST. The antidepressant-like action of escitalopram administered i.p. was confirmed in a second model, the TST.

Although the FST and TST are well established screening paradigms for antidepressants (Porsolt et al., 1977; Steru et al., 1985), false-positive results can be obtained with certain drugs, in particular psychomotor stimulants, which decrease immobility time by stimulating locomotor activity (Bourin et al., 2001). The anti-immobility effect of escitalopram seems not to be associated with any motor effects, since mice treated with escitalopram did not exhibit increased ambulation when tested in an open-field. This indicates that a psychostimulant effect is not responsible for the decrease in the immobility elicited by escitalopram in the FST. Similar to our results, it was demonstrated that escitalopram causes antidepressant-like effects in the FST in mice without affecting locomotor activity (Sánchez et al., 2003a).

In this study we investigated the possible involvement of NMDA receptors and L-arginine-NO-cGMP pathway in the antidepressant-like effect of escitalopram. For this aim, oral administration of escitalopram was selected because it is the most common route of administration for antidepressants in psychiatric patients. It is important to note that this study was performed in female mice, since several studies have shown that the prevalence of depression is about two fold higher in women than in men (Wong and Licinio, 2001). The FST was chosen

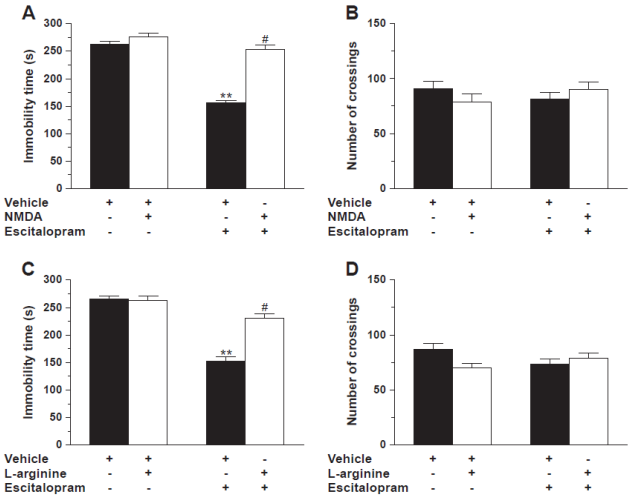

Figure 2 Effect of the pretreatment of mice with NMDA (0.1 pmol/site, i.c.v.) or L-arginine (750 mg/kg, i.p.) on the anti-immobility action of escitalopram (3 mg/kg, p.o.) in the FST (panels A and C, respectively) and on the number of crossings in the open-field test (panels B and D, respectively). Values are expressed as mean±S.E.M. ($n=6$). **$p<0.01$ compared with the vehicle-treated control group; # $p<0.01$ compared with the same group pretreated with vehicle (two-way ANOVA followed by Tukey's HSD test).

considering that the effects of the modulation of NMDA receptors and NOS-cGMP pathway on the antidepressant-like behavior in this test are well known, as compared to the TST (Almeida et al., 2006; Brocardo et al., 2008; Dhir and Kulkarni, 2007; Ghasemi et al., 2009,2010; Harkin et al., 1999, 2003, 2004; Kaster et al., 2005a,b; Kulkarni and Dhir, 2007; Rosa et al., 2003).

The reversal of the antidepressant-like effect of escitalopram in the FST by NMDA, suggests that the antidepressant-like effect of escitalopram is dependent on the inhibition of NMDA receptor activation, although our behavioral data does not allow us to conclude about the mechanism by which escitalopram interacts with the NMDA receptor. In fact, the reversal of the anti-immobility effect of some compounds in the FST by the pre-treatment of mice with NMDA has been shown (Brocardo et al., 2008, Ghasemi et al., 2010). Our data is consistent with the fact that NMDA receptor is closely implicated in the pathogenesis of depression, since several preclinical and clinical data have demonstrated that NMDA receptor antagonists produce antidepressant effects (Garcia et al., 2008; Paul and Skolnick, 2003; Skolnick, 1999). In addition, several studies have demonstrated a synergistic effect of NMDA receptor antagonists with antidepressants and lithium in the FST (Ghasemi et al., 2009, 2010; Rogóz et al., 2002, 2004). Further reinforcing the implication of NMDA in the mechanism of action of antidepressants, it has been reported that some of these agents reduce expression and function of NMDA receptor (Boyer et al., 1998, Szasz et al., 2007). It is noteworthy that the NMDA antagonist ketamine produces rapid and relatively sustained antidepressant effects in patients with treatment-resistant depression (Berman et al., 2000, Zarate et al., 2006). Moreover, it has been shown that NMDA receptor NR2A subunit knockout mice exhibit anxiolytic-like and antidepressant-like effects in the FST and TST in mice (Boyce-Rustay and Holmes, 2006). Considering that escitalopram is an antidepressant with anxiolytic properties and that produces a faster onset of action than citalopram (Colonna et al., 2005; Sánchez et al., 2003a,b, 2004), it remains to be investigated whether the likely inhibition of NMDA receptors elicited by this agent may contribute to its pharmacological properties.

A functional coupling of NMDA receptor with nitric oxide synthase and nitric oxide signalling pathways has been shown. It is considered that the activation of NMDA receptors in postsynaptic neurons of discrete brain regions leads to increased intracellular Ca^{2+}, which binds to calmodulin and activates neuronal nitric oxide synthase (nNOS), leading to increased formation of NO. The interaction of NMDA receptor with NOS via PSD-95 protein facilitates NO synthesis following Ca^{2+} influx (Ledo et al., 2004, Moncada and Bolaños, 2006). The relationship between the production of NO and the NMDA receptor as well as the antidepressant-like effects of functional NMDA antagonists have led to studies of the putative antidepressant action of NOS inhibitors (Da Silva et al., 2000; Harkin et al., 2003; Volke et al., 2003; Wegener et al., 2003; Yildiz et al., 2000). Indeed, it has been reported

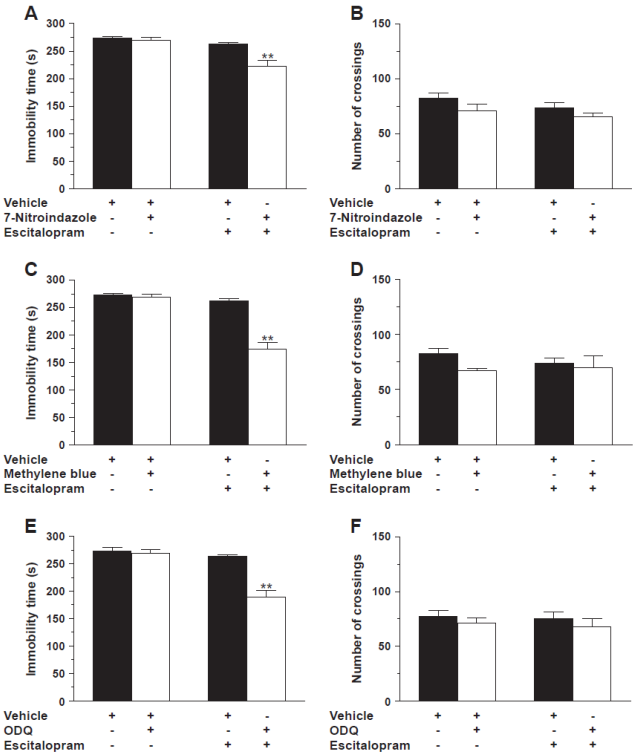

Figure 3 Effect of 7-nitroindazole (50 mg/kg, i.p.), methylene blue (20 mg/kg, i.p.) or ODQ (30 pmol/site i.c.v.) in combination with a subeffective dose of escitalopram (0.1 mg/kg, p.o.) in the FST in mice (panels A, C and E, respectively) and in the open-field test (panels B, D and F, respectively). Values are expressed as mean ±S.E.M. ($n=6$). $^{**}p<0.01$ compared with the vehicle-treated control group (two-way ANOVA followed by Tukey's HSD test).

that the L-arginine-NO-cGMP pathway is implicated in the pathophysiology of depression and in the mechanisms underlying the antidepressant like effects of several compounds that exhibit antidepressant effects in the FST (Almeida et al., 2006; Brocardo et al., 2008; Kaster et al., 2005a,b; Kulkarni and Dhir, 2007; Rosa et al., 2003).

In the present study we show that pretreatment with L-arginine (NOS substrate) was able to reverse the reduction of immobility time elicited by escitalopram in the FST. These results indicate that the effect of escitalopram may be dependent on, at least in part, the inhibition of NO synthesis. Similar to our results, the antidepressant-like effects of imipramine, venlafaxine and lithium were also blocked by pretreatment with L-arginine (Dhir and Kulkarni, 2007; Ghasemi et al., 2008; Harkin et al., 2004). Indeed, several studies have demonstrated that NOS inhibitors, depending on their concentration, exert antidepressant-like effects (Da Silva et al., 2000; Harkin et al., 2003; Volke et al., 2003; Wegener et al., 2003; Yildiz et al., 2000). Moreover, a reduction of NO levels within the hippocampus was shown to induce antidepressant-like effects, thus implicating endogenous hippocampal NO in the neurobiology of depression (Joca and Guimarães, 2006).

Further reinforcing our hypothesis that the anti-immobility effect of escitalopram in the FST is dependent on the decrease in NO synthesis, in this study, the pretreatment of

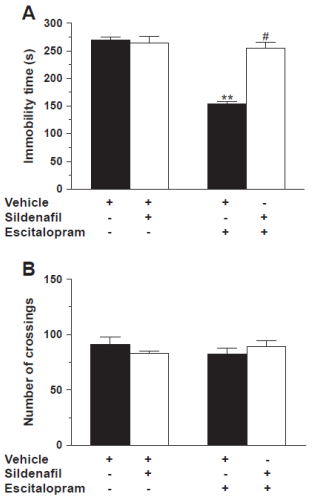

Figure 4 Effect of the pretreatment of mice with sildenafil (5 mg/kg, i.p.) on the anti-immobility effect of escitalopram (3 mg/kg, p.o.) in the FST (panel A) and on the number of crossings in the open-field test (panel B). Values are expressed as mean ± S.E.M. ($n=6$). $^{**}P<0.01$ compared with the vehicle-treated control group; $^{\#}p<0.01$ compared with the same group pretreated with vehicle (two-way ANOVA followed by Tukey's HSD test).

mice with a subeffective dose of 7-nitroindazole (a specific neuronal nitric oxide synthase inhibitor), methylene blue (direct inhibitor of both nitric oxide synthase and soluble guanylate cyclase) or ODQ (a specific inhibitor of soluble guanylate cyclase) produced a synergistic antidepressant-like effect with escitalopram. Taking into account that NO activates guanylate cyclase that generates cyclic guanosine monophosphate (cGMP), which mediates many of the effects of NO (Denninger and Marletta, 1999), our results also suggest that the antidepressant-like effect of escitalopram may be mediated through the reduction of cGMP, likely as a consequence of the reduction of NO synthesis. Our data are in accordance with the reported reduction in the immobility time in FST by 7-nitroindazole (Volke et al., 2003; Yildiz et al., 2000), methylene blue (Eroglu and Caglayan, 1997) and ODQ (Ergün and Ergün, 2007; Heiberg et al., 2002, Kaster et al., 2005b). Recently, a study has shown that the 7-nitroindazole and methylene blue potentiated the antidepressant-like effect of venlafaxine in mice (Dhir and Kulkarni, 2007). In addition, in another study, 7-nitroindazole augmented the behavioral effects of imipramine and fluoxetine (Harkin et al., 2003). ODQ was reported to produce an antidepressant-like effect in the FST, which was reversed by the NO precursor, L-arginine (Heiberg et al., 2002) and sildenafil (Kaster et al., 2005b). In a previous study, our group also demonstrated that ODQ potentiated the antidepressant-like effect of adenosine in the mouse FST (Kaster et al., 2005b). Similar to our results, the antidepressant-like effect of lithium was also potentiated by ODQ in the FST (Ghasemi et al., 2008).

Our results also showed the reversal of the antidepressant-like effect of escitalopram by the pretreatment of mice with sildenafil, a selective type 5 PDE inhibitor that increases the cGMP level in target tissues (Beavo, 1995). This finding reinforces the assumption that escitalopram administration produced a reduction in cGMP levels, and that this target may be important for its effect in the FST. Our results are somewhat in accordance with literature data that showed that sildenafil was able to prevent the reduction of immobility time elicited by venlafaxine (Dhir and Kulkarni, 2007) and lithium (Ghasemi et al., 2008).

To our knowledge there is no data concerning pharmacokinetic interaction between escitalopram and nitric oxide modulators in literature. Considering that all the experiments point to the same conclusion, i.e., the involvement of NMDA receptors and L-arginine-nitric oxide-cyclic guanosine monophosphate pathway in the antidepressant-like effects of escitalopram not correlated with changes in locomotor activity, it is unlikely that any pharmacokinetic interaction accounts for the results.

In conclusion, our results show that escitalopram exerts an antidepressant-like effect in the mouse FST when administered by i.p. and oral route, confirming the results obtained when it was administered by s.c. route (Sánchez et al., 2003a). Moreover, its antidepressant-like effect in the TST in mice was shown. The results suggest that the antidepressant-like effect of escitalopram in the FST is mediated by an inhibition of either the NMDA receptor activation or NO-cGMP synthesis, supporting the notion that these targets may be critical to the antidepressant action of escitalopram.

Role of the funding source

Funding for this study was provided by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Instituto Brasileiro de Neurociência (IBN-Net) and CAPES. They had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Contributors

A.D.E. Zomkowski and A.L.S. Rodrigues designed the study, wrote the protocol, and wrote the manuscript. A.D.E. Zomkowski and D. Engel performed the experiments. A.D.E. Zomkowski undertook the statistical analysis. A.L.S. Rodrigues and N. Gabilan managed the literature searches and analysis. All authors contributed to and have approved the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP) Research Grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" #01.06.0842-00, and CAPES. ALSR and NHG are recipients of CNPq fellowship.

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CAPÍTULO 2

Evidence for the involvement of NMDA receptors and l-arginine-nitric oxide-cyclic guanosine monophosphate pathway in the antidepressant-like effects of duloxetine in the forced swimming test

(Manuscrito submetido ao ***Psychopharmacology, Biochemistry and Behavior***)

Elsevier Editorial System(tm) for Pharmacology, Biochemistry and Behavior
Manuscript Draft

Manuscript Number: PBB-D-11-00529

Title: The role of the NMDA receptors and L-arginine-nitric oxide-cyclic guanosine monophosphate pathway in the antidepressant-like effect of duloxetine in the forced swimming test

Article Type: Research Article

Keywords: depression; duloxetine; forced swimming test; nitric oxide; NMDA; tail suspension test.

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Abstract: Duloxetine is a selective serotonin and noradrenaline reuptake inhibitor used as antidepressant. However, its mechanisms of action are not fully understood. This study investigated the effect of duloxetine in the mouse forced swimming test (FST) and in the tail suspension test (TST) and the involvement of the NMDA receptors and the L-arginine-NO-cGMP pathway in its effect in the FST. Duloxetine reduced the immobility time both in the FST and in the TST (dose range of 1-30 mg/kg, i.p.), without changing locomotion in an open-field. Duloxetine administered orally (1-30 mg/kg) also reduced the immobility time in the FST. The effect of duloxetine (10 mg/kg, p.o.) in the FST was prevented by pre-treatment with NMDA (0.1 pmol/site, i.c.v.), L-arginine (750 mg/kg, i.p.), S-nitroso-N-acetyl-penicillamine (SNAP, 25 µg/site, i.c.v) or sildenafil (5 mg/kg, i.p.). The administration of MK-801 (0.001 mg/kg, i.p.), 7-nitroindazole (50 mg/kg, i.p.), methylene blue (20 mg/kg, i.p.) or 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) (30 pmol/site i.c.v.) in combination with a sub-effective dose of duloxetine (0.3 mg/kg, p.o.) reduced the immobility time in the FST. Altogether the results suggest that the effect of duloxetine in the FST is dependent on either a blockade of NMDA receptors or an inhibition of NO and cGMP synthesis. In addition, our results further reinforce the role of NMDA receptors and L-arginine-NO-cGMP pathway, besides the monoaminergic systems, in the mechanism of action of current prescribed antidepressant agents.

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Opposed Reviewers:

***Cover Letter**

22th December, 2011.

Dear Dr. George F. Koob, Editor-in-Chief

Please find enclosed a file of our manuscript "The role of the NMDA receptors and L-arginine-nitric oxide-cyclic guanosine monophosphate pathway in the antidepressant-like effect of duloxetine in the forced swimming test" by Andréa D. E. Zomkowski, Daiane Engel, Nelson H. Gabilan and Ana Lúcia S. Rodrigues to be considered for publication in Pharmacology Biochemistry & Behavior.

Our manuscript was formatted as a research report to Pharmacology Biochemistry & Behavior. We inform that all authors have agreed to the submission and that the article is not currently being considered for publication by any other print or electronic journal. Moreover, all the authors have contributed substantially to the scientific process leading up to the writing of the paper, including the conception and design of the project, the performance of experiments and the analysis and interpretation of data. We also declare that all the authors are entirely responsible for the scientific content of the paper. It is important to emphasize that all procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The experiments were performed after approval by the Ethics Committee of the Institution and all efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

Hoping to hear from you in due course.

Yours sincerely,

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*Highlights

Research highlights

- ✓ This study confirms the antidepressant-like effect of duloxetine in the FST.
- ✓ The mechanisms underlying antidepressant action of duloxetine was investigated.
- ✓ The inhibition NMDA receptors and NO and cGMP synthesis is implicated in duloxetine effect.
- ✓ NMDA receptors and L-arginine-NO-cGMP pathway are important targets for antidepressant activity.

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The role of the NMDA receptors and L-arginine-nitric oxide-cyclic guanosine monophosphate pathway in the antidepressant-like effect of duloxetine in the forced swimming test

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Abstract

Duloxetine is a selective serotonin and noradrenaline reuptake inhibitor used as antidepressant. However, its mechanisms of action are not fully understood. This study investigated the effect of duloxetine in the mouse forced swimming test (FST) and in the tail suspension test (TST) and the involvement of the NMDA receptors and the L-arginine-NO-cGMP pathway in its effect in the FST. Duloxetine reduced the immobility time both in the FST and in the TST (dose range of 1-30 mg/kg, i.p.), without changing locomotion in an open-field. Duloxetine administered orally (1-30 mg/kg) also reduced the immobility time in the FST. The effect of duloxetine (10 mg/kg, p.o.) in the FST was prevented by pre-treatment with NMDA (0.1 pmol/site, i.c.v.), L-arginine (750 mg/kg, i.p.), S-nitroso-N-acetyl-penicillamine (SNAP, 25 µg/site, i.c.v) or sildenafil (5 mg/kg, i.p.). The administration of MK-801 (0.001 mg/kg, i.p.), 7-nitroindazole (50 mg/kg, i.p.), methylene blue (20 mg/kg, i.p.) or 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) (30 pmol/site i.c.v.) in combination with a sub-effective dose of duloxetine (0.3 mg/kg, p.o.) reduced the immobility time in the FST. Altogether the results suggest that the effect of duloxetine in the FST is dependent on either a blockade of NMDA receptors or an inhibition of NO and cGMP synthesis. In addition, our results further reinforce the role of NMDA receptors and L-arginine-NO-cGMP pathway, besides the monoaminergic systems, in the mechanism of action of current prescribed antidepressant agents.

Keywords: depression; duloxetine; forced swimming test; nitric oxide; NMDA; tail suspension test.

1. Introduction

Depression is a disorder that has a high lifetime prevalence (around 16 %,) and is often manifested with symptoms at the psychological, behavioral and physiological levels. Suicide is usually a consequence of depression and other deleterious health-related effects have been reported (Wong and Licinio, 2001). Despite the devastating impact of depression, its etiology or pathophysiology is not well established. The monoaminergic systems (serotonergic, noradrenergic and dopaminergic) have received a great attention in neurobiological studies of depression (Wong and Licinio, 2001). Some clinical reports suggest that the combination of selective serotonin (5-HT) reuptake inhibitors with selective noradrenaline (NE) reuptake inhibitors may present a therapeutic advantage, resulting in a more effective or rapid antidepressant effect in comparison to each drug given alone (Shelton et al., 2005; Thase et al., 2007).

Duloxetine is a drug that inhibit 5-HT and NE reuptake, with weak effects on dopamine reuptake (Hunziker et al., 2005). It is used in the treatment of depression (Detke et al., 2004), anxiety (Carter and McCormack, 2009) and pain (Bellingham and Peng, 2010). Regarding preclinical studies, it was shown that duloxetine reduced the immobility time in the forced swimming test (FST) in rats (Ciulla et al., 2007; Rénéric and Lucki, 1998).

Several clinical studies have demonstrated abnormalities in glutamate function in mood disorders. Some studies have indicated that compounds that reduce transmission at N-methyl-D-aspartate (NMDA) receptors and NMDA receptor antagonists exhibit antidepressant-like effects (Sanacora et al., 2008; Skolnick, 1999; Zarate et al., 2006). Depressed patients also showed a significant improvement after the acute administration of ketamine, a non-competitive NMDA receptor antagonist (Berman et al., 2000; Zarate et al., 2006). Moreover, it is also noteworthy that NMDA

receptor antagonists exert synergistic antidepressant-like effects with classical antidepressants in the FST (Rogóz et al., 2002). Also, the use of MK801, a non-competitive NMDA antagonist, during imipramine withdrawal following chronic imipramine treatment resulted in significant suppression of swim stress immobility (Harvey et al., 2002).

The NMDA receptor stimulation induces the activation of nitric oxide (NO) synthase (NOS). The activated NOS then converts L-arginine to NO and L-citrulline (Esplugues, 2002). NO is a signaling molecule in the brain and has been implicated in depression (Da Silva et al., 2000; Harkin et al., 1999, 2004; Heiberg et al., 2002). It has been demonstrated that the reduction of NO levels within the hippocampus can induce antidepressant-like effects, thus implicating endogenous hippocampal NO in the neurobiology of depression (Joca and Guimarães, 2006). In addition, plasma nitrate concentrations were significantly increased in depressed patients, suggesting that NO production is enhanced in depression (Suzuki et al., 2001). Several studies have demonstrated that NOS inhibitors, depending on their concentration, exert antidepressant-like effects (Da Silva et al., 2000; Harkin et al., 2003, 2004; Volke et al., 2003). Some of the physiological actions of NO are mediated through the enzyme guanylate cyclase (sGC) with the consequent increase in cyclic guanosine monophosphate (cGMP) levels. It is currently accepted that the NO-cGMP pathway is the main effector of NO actions in the brain (Esplugues 2002).

Although the behavioral studies using duloxetine in the FST support its antidepressant properties (Ciulla et al., 2007; Rénéric and Lucki 1998), its mechanisms of action besides the monoaminergic system are not fully understood. The present study examined whether duloxetine produces antidepressant-like effect in the FST and also in another model predictive of antidepressant activity, the TST, in mice. These behavioral

tests have good predictive value for antidepressant potency in man (Cryan et al., 2002). Considering that the blockade of NMDA receptor is associated with reduced levels of NO and cGMP (Snyder, 1992) and the inhibition of both NOS and guanylate cyclase may, depending on the dose, elicit antidepressant-like effects in the FST (Eroglu and Caglayan, 1997; Da Silva et al., 2000; Harkin et al., 2003; Heiberg et al., 2002; Volke et al., 2003; Yildiz et al., 2000), we investigated whether NMDA receptors and L-arginine-NO-cGMP pathway may be involved in the effect of acute duloxetine administration in mouse FST.

2. Materials and methods

2.1. Animals

Swiss albino female mice (30-40 g, 55-70 days old) were maintained at 21-23°C with free access to water and food, under a 12:12 h light/dark cycle (lights on at 7:00 h). Animals were obtained from the Central Biothery of Universidade Federal de Santa Catarina (UFSC). All manipulations were carried out between 9:00 a.m. and 4:00 p.m., with each animal used only once. All procedures in this study were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). The experiments were performed after approval of the protocol by the Institutional Ethics Committee and all efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

2.2. Drugs and pharmacological treatments

Duloxetine HCl (Eli Lilly & Co.), L-arginine, methylene blue, N-methyl-D-aspartate (NMDA), 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ), 7-

nitroindazole (Sigma Chemical Co, USA), S-nitroso-N-acetyl-penicillamine (SNAP), sildenafil (Pfizer) and MK-801 (Research Biochemicals International, USA) were used. All drugs were dissolved in saline, except ODQ which was dissolved in saline with 1% dimethylsulfoxide (DMSO) and 7-nitroindazole that was dissolved in 5% Tween 80. All drugs were administered by intraperitoneal (i.p.) route in a constant volume of 10 ml/kg body weight, except NMDA, ODQ and SNAP which were administered by intracerebroventricular (i.c.v.) route (5 µl/site).

I.c.v. administration was performed under ether anesthesia as previously described (Brocardo et al. 2008). Briefly, mice were placed inside a glass jar containing ether. The animals remained inside the glass jar for 30 s and immediately after, the i.c.v. administration was performed. Briefly, a 0.4 mm external diameter hypodermic needle attached to a cannula, which was linked to a 25 µl Hamilton syringe, was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse. A volume of 5 µl was then administered in the left lateral ventricle. The injection was given over 30 s, and the needle remained in place for another 30 s in order to avoid the reflux of the substances injected. The injection site was 1 mm to the right or left from the mid-point on a line drawn through to the anterior base of the ears.

Duloxetine was also administered by oral (p.o.) route by gavage in a volume of 10 ml/kg body weight.

In order to investigate the antidepressant-like effect of the duloxetine, it was administered (dose range: 0.3-30 mg/kg, i.p.) 30 min before the FST, TST or open-field test. Alternatively, duloxetine or vehicle was administered by oral (p.o.) route (dose range: 0.3-30 mg/kg, p.o.) 60 min before the FST or open-field test.

In a separate series of experiments, we investigated whether the antidepressant-like effect of duloxetine in the FST is mediated through the blockade of NMDA

receptors. To this end, mice were pretreated with NMDA (0.1 pmol/site, i.c.v.) and 15 min after, duloxetine (10 mg/kg, p.o.) or vehicle was administered. Sixty min later the FST was carried out.

In another set of experiments, we investigated the synergistic effect of a sub-effective dose of duloxetine (0.3 mg/kg, p.o.) with a sub-effective dose of MK-801 (0.001 mg/kg, i.p., a non-competitive NMDA receptor antagonist). Duloxetine or vehicle was administered 30 min before MK-801. A further 30 min were allowed to elapse before the animals were tested in the FST.

To investigate the possible involvement of the L-arginine-nitric oxide pathway in the anti-immobility effects of duloxetine in the FST, mice were pre-treated with L-arginine, a precursor of nitric oxide (750 mg/kg, i.p., a dose that produces no effect in the FST) or with the NO donor SNAP (25 µg/site, i.c.v., a dose that produces no effect in the FST) or vehicle. Thirty minutes after L-arginine or 15 min after SNAP administration, duloxetine (10 mg/kg, p.o.) or vehicle was injected, and 60 min later the FST was carried out.

In another set of experiments, we investigated the effect of the combined administration of a sub-effective dose of duloxetine (0.3 mg/kg, p.o.) with sub-effective doses of 7-nitroindazole (50 mg/kg, i.p., a specific neuronal NO synthase inhibitor), ODQ (30 pmol/site i.c.v., a specific sGC inhibitor) or methylene blue (20 mg/kg, i.p., an inhibitor of both NO synthase and soluble guanylate cyclase). Duloxetine or vehicle was administered 30 min before of 7-nitroindazole, methylene blue or 40 min before ODQ. A further 30 min (after i.p. administration of 7-nitroindazole or methylene blue) or 20 min (after i.c.v. ODQ administration) were allowed to elapse before the animals were tested in the FST.

In order to investigate the role of cGMP in the antidepressant action of duloxetine, mice received an injection of sildenafil (5 mg/kg, i.p., a phosphodiesterase 5 inhibitor) or vehicle 30 min before duloxetine (10 mg/kg, p.o.) and 60 min later the FST was carried out.

The doses of the drugs used were selected on the basis of literature data and on previous results from our laboratory (Almeida et al., 2006; Brocardo et al., 2008; Da Silva et al., 2000; Dhir and Kulkarni, 2007; Kaster et al., 2005a, 2005b; Rosa et al., 2003; Zomkowski et al., 2010). The doses of duloxetine administered were chosen on the basis of experiments previously performed in our laboratory and literature data (Bomholt et al., 2005; Rénéric and Lucki 1998).

2.3. Behavioral tests

2.3.1. Forced swimming test

The FST was carried out in mice individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water at 25 ± 1°C; the total duration of immobility during the 6-min test was scored as described previously (Brocardo et al., 2008). Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water.

2.3.2. Tail suspension test

The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al. (1985). Briefly, mice both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed

approximately 1 cm from the tip of the tail. Immobility time was recorded during a 6 min period (Binfaré et al., 2009).

2.3.3. Open-field behavior

The ambulatory behavior was assessed in an open-field test as described previously (Brocardo et al., 2008). The apparatus consisted of a wooden box measuring 40 x 60 x 50 cm high. The floor of the arena was divided into 12 equal squares. The number of squares crossed with all paws (crossings) was counted in a 6-min session. The light was maintained at minimum to avoid anxiety behavior. The apparatus was cleaned with a solution of 10% ethanol between tests in order to hide animal cues.

2.4. Statistical analysis

Comparisons between treatment groups and control were performed by one-way (dose-response experiments) or two-way (experiments dealing with the role of NMDA receptors and L-arginine-NO-cGMP pathway in the antidepressant effect of duloxetine) analysis of variance (ANOVA) followed by Tukey's HSD test when appropriate. A value of $p<0.05$ was considered to be significant.

3. Results

3.1. Effect of duloxetine in the immobility time in the FST and TST and on the locomotor activity in the open-field test

The treatment of mice with duloxetine at the dose range 1-30 mg/kg given by intraperitoneal route significantly decreased the immobility time both in the FST [$F(5,44)=83.01$, $p<0.01$; Fig. 1A] and in the TST [$F(5,30)=70.47$, $p<0.01$; Fig. 1B]. The doses of duloxetine that produced an anti-immobility effect in the FST and TST (1-30 mg/kg) did not cause any change in ambulation in an open-field in a separate

experiment as compared to the control group [$F(5,32)=1.56$, $p=0.20$; Fig. 1C]. Fig. 1D shows that duloxetine also caused a reduction in the immobility time in the FST when administered by p.o. route at the dose range 1-30 mg/kg [$F(5,30)=51.91$, $p<0.01$], but did not produce any change in ambulation in mice tested in an open-field in a separate experiment [$F(5,30)=2.15$, $p=0.09$; Fig. 1E].

[Figure 1 near here]

3.2. Involvement of NMDA receptors on the antidepressant-like effect of duloxetine in the FST

Fig. 2A shows that the pre-treatment of mice with NMDA (0.1 pmol/site, i.c.v.) was able to reverse the antidepressant-like effect of duloxetine (10 mg/kg, p.o.) in the FST. A two-way ANOVA revealed significant differences for the NMDA pre-treatment [$F(1,20)=102.15$, $p<0.01$], duloxetine treatment [$F(1,20)=95.78$, $p<0.01$] and NMDA X duloxetine interaction [$F(1,20)=64.39$, $p<0.01$]. The administration of NMDA alone or in combination with duloxetine did not affect the ambulation in the open-field (Fig. 2B). A two-way ANOVA did not reveal significant differences for the NMDA pre-treatment [$F(1,20)=1.26$, $p=0.27$], duloxetine treatment [$F(1,20)=2.00$, $p=0.17$] and NMDA X duloxetine interaction [$F(1,20)=0.25$, $p=0.62$]. The results illustrated in Fig. 2C show that the administration of MK-801 (0.001 mg/kg, i.p., non-competitive NMDA receptor antagonist) in combination with duloxetine (0.3 mg/kg, p.o.) produced an antidepressant-like effect as compared with the administration of either drug alone. A two-way ANOVA revealed significant differences for the duloxetine pre-treatment [$F(1,20)=23.62$, $p<0.01$], MK-801 treatment [$F(1,20)=31.62$, $p<0.01$] and duloxetine X MK-801 interaction [$F(1,20)=12.48$, $p<0.01$]. Fig. 2D shows that the administration of MK-801 alone or in combination with duloxetine did not affect locomotion in the open-

field test. A two-way ANOVA did not reveal significant differences for the duloxetine pre-treatment [$F(1,20)=3.72$, $p=0.07$], MK-801 treatment [$F(1,20)=1.18$, $p=0.29$] and duloxetine X MK-801 interaction [$F(1,20)=0.35$, $p=0.56$].

[Figure 2 near here]

3.3. Involvement of L-arginine-NO-cGMP pathway in the anti-immobility effect of duloxetine in the FST

The results depicted in Fig. 3A show that pre-treatment with L-arginine (750 mg/kg i.p., a nitric oxide precursor) prevented the antidepressant-like effect of duloxetine (10 mg/kg, p.o.) in the FST. The two-way ANOVA showed significant differences for L-arginine pre-treatment [$F(1,20)=22.13$, $p<0.01$], duloxetine treatment [$F(1,20)= 43.96$, $p<0.01$] and L-arginine X duloxetine interaction [$F(1,20)=24.75$, $p<0.01$]. The administration of L-arginine alone or in combination with duloxetine did not affect the ambulation in the open-field (Fig. 3B). A two-way ANOVA did not reveal significant differences for the L-arginine pre-treatment [$F(1,20)=1.29$, $p=0.27$], duloxetine treatment [$F(1,20)=0.08$, $p=0.77$] and L-arginine X duloxetine interaction [$F(1,20)=3.25$, $p=0.86$]. Fig. 3C shows that SNAP (25 µg/site, i.c.v., a NO donor) was able to prevent the reduction in the immobility time elicited by duloxetine (10 mg/kg, p.o.) in the FST. The two-way ANOVA showed significant differences for SNAP pre-treatment [$F(1,20)=53.68$, $p<0.01$], duloxetine treatment [$F(1,20)= 65.99$, $p<0.01$] and SNAP X duloxetine interaction [$F(1,20)=109.42$, $p<0.01$].The administration of SNAP alone or in combination with duloxetine did not affect the ambulation in the open-field test (Fig. 3D). A two-way ANOVA did not reveal significant differences for SNAP pre-treatment [$F(1,20)=0.79$, $p=0.38$], duloxetine treatment [$F(1,20)=0.55$, $p=0.47$] and SNAP X duloxetine interaction [$F(1,20)=0.61$, $p=0.44$].

[Figure 3 near here]

The results illustrated in Fig. 4A show the administration of 7-nitroindazole (50 mg/kg i.p., a specific neuronal nitric oxide synthase inhibitor) in combination with duloxetine (0.3 mg/kg, p.o.) produced an antidepressant-like effect as compared with the administration of either drug alone. A two-way ANOVA revealed significant differences for the duloxetine pre-treatment [$F(1,20)=34.36$, $p<0.01$], 7-nitroindazole treatment [$F(1,20)=60.45$, $p<0.01$] and duloxetine X 7-nitroindozole interaction [$F(1,20)=26.31$, $p<0.01$]. Fig. 4B shows that the administration of 7-nitroindazole alone or in combination with duloxetine did not affect locomotor activity in the open-field test. A two-way ANOVA did not reveal significant differences for the duloxetine pre-treatment [$F(1,20)=2.27$, $p=0.15$], 7-nitroindazole treatment [$F(1,20)=1.28$, $p=0.27$] and 7-nitroindazole X duloxetine interaction [$F(1,20)=0.32$, $p=0.58$]. Fig. 4C shows that methylene blue (20 mg/kg i.p., direct inhibitor of both nitric oxide synthase and soluble guanylate cyclase) in combination with duloxetine (0.3 mg/kg, p.o.) also produced an anti-immobility effect in the FST as compared with the administration of either drug alone. A two-way ANOVA revealed significant differences for the duloxetine pre-treatment [$F(1,20)=16.48$, $p<0.01$], methylene blue treatment [$F(1,20)=28.53$, $p<0.01$] and duloxetine X methylene blue interaction [$F(1,20)=10.27$, $p<0.01$]. The administration of methylene blue (20 mg/kg i.p.) alone or in combination with duloxetine did not affect the ambulation in the open-field (Fig. 4D). A two-way ANOVA did not reveal significant differences for the duloxetine pre-treatment [$F(1,20)=4.02$, $p=0.06$], methylene blue treatment [$F(1,20)=0.86$, $p=0.36$] and duloxetine X methylene blue interaction [$F(1,20)=1.16$, $P=0.29$]. The results depicted in Fig. 4E shows that ODQ (30 pmol/site i.c.v., a specific inhibitor of soluble guanylate

cyclase) in combination with duloxetine (0.3 mg/kg, p.o.) also produced an anti-immobility effect in the FST as compared with the administration of either drug alone. A two-way ANOVA revealed significant differences for the duloxetine pre-treatment [$F(1,20)=19.33$, $p<0.01$], ODQ treatment [$F(1,20)=21.47$, $p<0.01$] and duloxetine X ODQ interaction [$F(1,20)=15.11$, $p<0.01$]. The administration of ODQ (30 pmol/site i.c.v.) alone or in combination with duloxetine did not affect the ambulation in the open-field (Fig. 4F). A two-way ANOVA did not reveal significant differences for the duloxetine pretreatment [$F(1,20)=1.20$, $p=0.28$], ODQ treatment [$F(1,20)=0.01$, $p=0.90$] and duloxetine X ODQ interaction [$F(1,20)=0.04$, $p=0.85$].

[Figure 4 near here]

Fig. 5A shows that the anti-immobility effect of duloxetine (10 mg/kg, p.o.) was completely prevented by pre-treatment of animals with sildenafil (5 mg/kg, i.p., a phosphodiesterase 5 inhibitor inhibitor). A two-way ANOVA revealed significant differences for the sildenafil pre-treatment [$F(1,20)=47.54$, $p<0.01$], duloxetine treatment [$F(1,20)=66.88$, $p<0.01$] and sildenafil X duloxetine interaction [$F(1,20)=57.44$, $p<0.01$]. The administration of sildenafil (5 mg/kg, i.p.) alone or in combination with duloxetine did not affect the locomotor activity in the open-field (Fig. 5B). A two-way ANOVA did not reveal significant differences for the sildenafil pre-treatment [$F(1,20)=0.05$, $p=0.83$], duloxetine treatment [$F(1,20)=1.72$, $p=0.20$] and sildenafil X duloxetine interaction [$F(1,20)=3.56$, $p=0.07$].

[Figure 5 near here]

4. Discussion

The FST and the TST are quite sensitive and relatively specific for all major classes of antidepressant drugs including tricyclics, serotonin-specific reuptake inhibitors, monoamine oxidase inhibitors, and atypicals (Porsolt et al., 1977; Steru et al., 1985). Antidepressant drugs are reported to reduce the immobility time of mice in both tests (Porsolt et al., 1977; Steru et al., 1985). The results presented here show that duloxetine, given systemically (i.p. route) or orally (p.o. route), is effective in producing significant antidepressant-like effects, when assessed in the FST, which is the most widely used tool for assessing antidepressant activity preclinically (Cryan et al., 2002). The antidepressant-like action of duloxetine administered i.p. was confirmed in the TST.

The results indicate that anti-immobility effect of duloxetine is not associated with any motor effects, since mice treated with duloxetine did not exhibit increased ambulation when tested in an open-field. This indicates that a psychostimulant effect is not responsible for the decrease in the immobility elicited in the FST. A number of studies have demonstrated that several antidepressants administered by i.p. route produced antidepressant-like effect in the FST in rodents (Ciulla et al., 2007; Dhir and Kulkarni, 2007; Ghasemi et al., 2009; Zomkowski et al., 2010). Duloxetine was shown to produce antidepressant effect after its administration by the i.p. (40 mg/kg) and subcutaneous routes (10-40 mg/kg) to rats in the FST (Ciulla et al., 2007; Rénéric and Lucki, 1998, respectively). In the present study, we confirm that duloxetine administered by the i.p. route produces an antidepressant-like effect in the FST in mice, but at a lower dose-response range (1-30 mg/kg). In order to further reinforce this antidepressant effect, we tested duloxetine in the TST.

Although the FST and TST are widely used for assessing antidepressant activity preclinically, we should take into account that they have some limitations and their validity as simulations of the psychiatric condition is questionable. Some of the limitations are related to the fact that many symptoms of depression are not measurable in preclinical models; they are stressor-based and although there is evidence for a link between depression and stress, this does not always follow. In addition, they respond to the acute antidepressant treatment (Cryan et al., 2002; Cryan and Slattery, 2007). In spite of these limitations, these tests are useful for studying the mechanisms underlying the antidepressant-like effects of several compounds.

We investigated the participation of NMDA receptors and L-arginine–NO–cGMP pathway in the antidepressant-like effect of duloxetine in the FST. This test was chosen considering that the effects of the modulation of NMDA receptors and NOS-cGMP pathway on the antidepressant-like behavior in this test are well known, as compared to the TST (Almeida et al., 2006; Brocardo et al., 2008; Dhir and Kulkarni, 2007; Ghasemi et al., 2009, 2010; Harkin et al., 1999, 2003, 2004; Kaster et al., 2005a, 2005b; Rosa et al., 2003). For this aim, oral administration of duloxetine was selected because it is the most common route of administration for antidepressants in psychiatric patients. In this study, we showed that the reduction of the immobility time elicited by duloxetine in the FST was prevented by NMDA. Similar to our results, the antidepressant-like effect of lithium and escitalopram in the FST was prevented by the pre-treatment with NMDA (Ghasemi et al., 2010; Zomkowski et al., 2010). Moreover, it was shown that NMDA receptor NR2A subunit knockout mice exhibit anxiolytic-like effects in elevated plus-maze, light-dark exploration, novel and familiar open field tests and home cage locomotor activity and antidepressant-like effects in the FST and TST in mice (Boyce-Rustay and Holmes, 2006). In addition, antidepressants have been reported

to reduce binding, expression and function of NMDA receptors (Boyer et al., 1998; Szasz et al., 2007). An evidence of a direct action of fluoxetine and desipramine inhibiting NMDA-evoked currents in rat cortical cell cultures was reported, suggesting that direct inhibition of NMDA receptors may contribute to the clinical effects of antidepressants (Szasz et al., 2007). Therefore, our results are in line with literature data reinforcing the role of the NMDA receptors in the mechanism of action of antidepressant agents and in the pathophysiology of depression, since they clearly indicate that the antidepressant-like effect of duloxetine is mediated through a blockade of NMDA receptor. However, our behavioral results do not allow us to conclude about the mechanism by which duloxetine interacts with the NMDA receptor and further studies are necessary to better clarify this issue.

The synergistic antidepressant-like effect observed when mice were treated with the non-competitive NMDA receptor antagonist MK-801 combined with duloxetine reinforce the assumption that duloxetine exerts its antidepressant-like effect by blocking NMDA receptor activation. It is known that MK-801 preferentially binds to the activated NMDA receptor complex (Javitt et al., 1989) interacting with a site located within the NMDA channel pore. In concordance with our results, a study recently demonstrated that the combined treatment of sub-effective doses of paroxetine and MK-801 exerted antidepressant-like effect in the FST in mice (Ghasemi et al., 2009). Moreover, some studies have demonstrated a synergistic effect of NMDA receptor antagonists with antidepressants and lithium in the FST (Ghasemi et al., 2010; Rogóz et al. 2002). However, there are little data on the mechanisms by which NMDA antagonists enhance the effects of antidepressants in the FST. A microdialysis study has demonstrated that acute administration of noneffective doses of the NMDA antagonist amantadine combined with noneffective doses of several antidepressants (paroxetine, reboxetine,

budipine and clomipramine) increases the cortical release of 5-HT in freely moving rats (Owen and Whitton, 2005). Hence, we cannot rule out the possibility that the antidepressant-like effects elicited by treatment of mice with sub-effective doses of duloxetine and NMDA antagonists may be due to their effects on the central 5-HT release.

The L-arginine–NO–cGMP pathway has been implicated in the pathogenesis of depression (Kaster et al., 2005a, 2005b; Mantovani et al., 2003). NO plays a significant role in the central nervous system and pharmacological manipulation of the NO pathway may constitute a novel therapeutic approach for the treatment of depression (Harkin et al., 2003, 2004). In this study, we showed that the reduction of immobility time elicited by duloxetine in the FST was reversed by pre-treatment of mice with L-arginine (NOS substrate) or SNAP, a nitric oxide donor. Similarly to our results, the antidepressant-like effects of imipramine, escitalopram, paroxetine and venlafaxine were also blocked by pre-treatment with L-arginine (Dhir and Kulkarni, 2007; Ghasemi et al., 2009; Harkin et al., 2004; Zomkowski et al., 2010). In previous studies, our group demonstrated that L-arginine and SNAP reversed the antidepressant-like effects of putative antidepressant agents such as zinc chloride (Rosa et al., 2003), adenosine (Kaster et al., 2005b), memantine (Almeida et al., 2006), folic acid (Brocardo et al., 2008) and ascorbic acid (Moretti et al., 2011). Therefore, a great amount of literature data point to a significant role of nitrergic system in the mechanism of action of antidepressant agents. Corroborating this notion, our results indicate that the effect of duloxetine may be dependent on the inhibition of NO synthesis. Further reinforcing this assumption, in this study, the pre-treatment of mice with a sub-effective dose of 7-nitroindazole (a specific neuronal nitric oxide synthase inhibitor), methylene blue (direct inhibitor of both nitric oxide synthase and soluble guanylate cyclase) or ODQ (a

specific inhibitor of soluble guanylate cyclase) produced a synergistic antidepressant-like effect with duloxetine. Together this set of results suggest that the inhibition of NO synthesis is implicated in the anti-immobility effect of duloxetine in the FST and are in accordance with the fact that NOS inhibitors exert antidepressant-like effect in animal models of depression (Eroglu and Caglayan, 1997; Volke et al., 2003; Yildiz et al., 2000). In addition, in line with our results, studies have demonstrated that 7-nitroindazole potentiated the antidepressant-like effect of venlafaxine (Dhir and Kulkarni, 2007), imipramine and fluoxetine (Harkin et al. 2004) in the FST. Similarly to our results, a study showed that methylene blue potentiated the antidepressant-like effect of venlafaxine in the FST (Dhir and Kulkarni, 2007). Moreover, it has been reported that the antidepressant-like effect produced by ODQ in the FST in rats was reversed by pre-treatment with L-arginine (Heiberg et al. 2002). In line with our results, ODQ potentiated the antidepressant-like effects of lithium (Ghasemi et al., 2008), adenosine (Kaster et al., 2005b) and memantine in the mouse FST (Almeida et al. 2006). These results reinforce our hypothesis that the anti-immobility effect of duloxetine in the FST is dependent on the decrease in NO synthesis and also that it may be mediated through the reduction of cGMP, likely as a consequence of the reduction of NO synthesis. Although this study does not allow us to know exactly the mechanism responsible for the observed behavioral effects, one possibility is that the effects are indirectly dependent on an increase in the release of 5-HT. It is interesting to note that the effects of NOS inhibitors in the FST are reported to be dependent on endogenous 5-HT, since the depletion of 5-HT caused by the treatment of rats with PCPA prevented the effects of NOS inhibitors in the FST (Harkin et al., 2003). Furthermore, some studies have demonstrated that inhibition of NOS can modulate the release of central 5-HT (Kiss, 2000; Smith and Whitton, 2000; Wegener et al., 2000).

Of note, we also showed that the pre-treatment of mice with sildenafil (selective type 5 phosphodiesterase inhibitor) prevented the antidepressant-like effect of duloxetine in the FST. Sildenafil increases the cGMP level in target tissues (Beavo, 1995) and was previously reported to reverse the anti-immobility effect of ODQ in the mouse FST (Kaster et al., 2005b). Our results are somewhat in accordance with literature data that showed that the reduction in the immobility time elicited by the antidepressants venlafaxine (Dhir and Kulkarni, 2007) and escitalopram (Zomkowski et al., 2010) was also prevented by the pre-treatment with sildenafil. This finding reported here reinforces the notion that duloxetine exerts its effect in the FST by decreasing cGMP levels.

In conclusion, our study demonstrated that duloxetine exerts an antidepressant-like effect in the FST in mice when administered intraperitoneally and orally, confirming the results obtained previously (Ciulla et al., 2007; Rénéric and Lucki, 1998) in the FST in rats. In addition, its antidepressant-like effect also was shown in the TST in mouse. Moreover, our results significantly extend literature data by indicating for the first time the involvement of the NMDA receptors and L-arginine-NO-cGMP pathway in the antidepressant-like action of duloxetine in the FST. It supports the notion that the blockade of NMDA receptor, with the consequent inhibition of NO and cGMP production may be critical to the antidepressant action of duloxetine and further reinforces the role of NMDA receptors and L-arginine-NO-cGMP pathway, besides the monoaminergic systems, in the mechanism of action of current prescribed antidepressant agents.

Acknowledgements

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP) Research Grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" #01.06.0842-00, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). ALS Rodrigues and NH Gabilan are recipients of CNPq fellowship.

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Legend to the Figures

Fig. 1- Effect of intraperitoneal administration of duloxetine (0.3-30 mg/kg) in the immobility time in the FST (A), TST (B) and on the number of crossings in the open-field test (C) and the effect of oral administration of duloxetine (0.3-30 mg/kg) in the immobility time in the FST (D) and on the number of crossings in the open-field test (E) in mice. Values are expressed as mean ± SEM. Values are expressed as mean ± SEM, n=6-11. *P <0.05 and **P <0.01 compared with the vehicle-treated control group.

Fig. 2- Effect of the pre-treatment of mice with NMDA (0.1 pmol/site, i.c.v.) on the anti-immobility action of duloxetine (10 mg/kg, p.o.) in the FST (A) and on the number of crossings in the open-field test (B). Effect of MK-801 (0.001 mg/kg, i.p.) in combination with a sub-effective dose of duloxetine (0.3 mg/kg, p.o.) in the FST (C) and in the open-field test (D). Values are expressed as mean ± SEM, n=6. **p<0.01 compared with the vehicle-treated control group; $^{\#}$p< 0.01 compared with the same group pretreated with vehicle.

Fig. 3- Effect of the pre-treatment of mice with L-arginine (750 mg/kg, i.p.) on the anti-immobility action of duloxetine (10 mg/kg, p.o.) in the FST (A) and on the number of crossings in the open-field test (B). Effect of the pre-treatment of mice with SNAP (25 µg/site, i.c.v) on the anti-immobility action of duloxetine (10 mg/kg, p.o.) in the FST (C) and on the number of crossings in the open-field test (D). Values are expressed as mean ± SEM, n=6. **p< 0.01 compared with the vehicle-treated control group; $^{\#}$p< 0.01 compared with the same group pretreated with vehicle.

Fig. 4- Effect of 7-nitroindazole (50 mg/kg, i.p.), methylene blue (20 mg/kg, i.p.) or ODQ (30 pmol/site i.c.v.) in combination with a sub-effective dose of duloxetine (0.3 mg/kg, p.o.) in the FST (panels A, C and E, respectively) and in the open-field test (panels B, D and F, respectively). Values are expressed as mean ± SEM, n=6. **$p < 0.01$ compared with the vehicle-treated control group; $^{\#}p < 0.01$ compared with the same group pretreated with vehicle.

Fig. 5- Effect of the pre-treatment of mice with sildenafil (5 mg/kg, i.p.) on the anti-immobility effect of duloxetine (10 mg/kg, p.o.) in the FST (A) and on the number of crossings in the open-field test (B). Values are expressed as mean ± SEM, n=6. **$p <$ 0.01 compared with the vehicle-treated control group; $^{\#}p < 0.01$ compared with the same group pretreated with vehicle.

Figure 1
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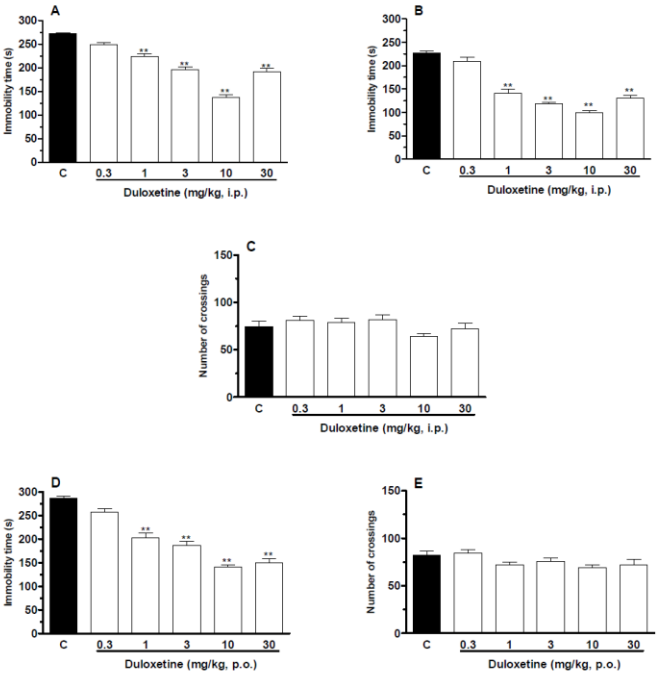

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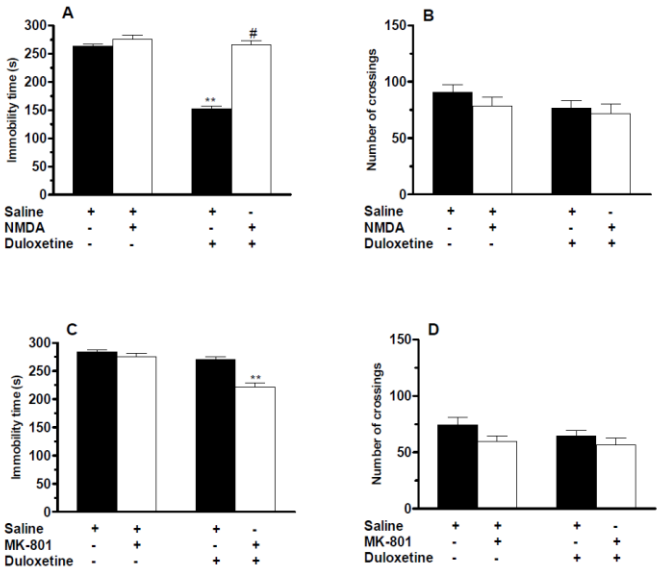

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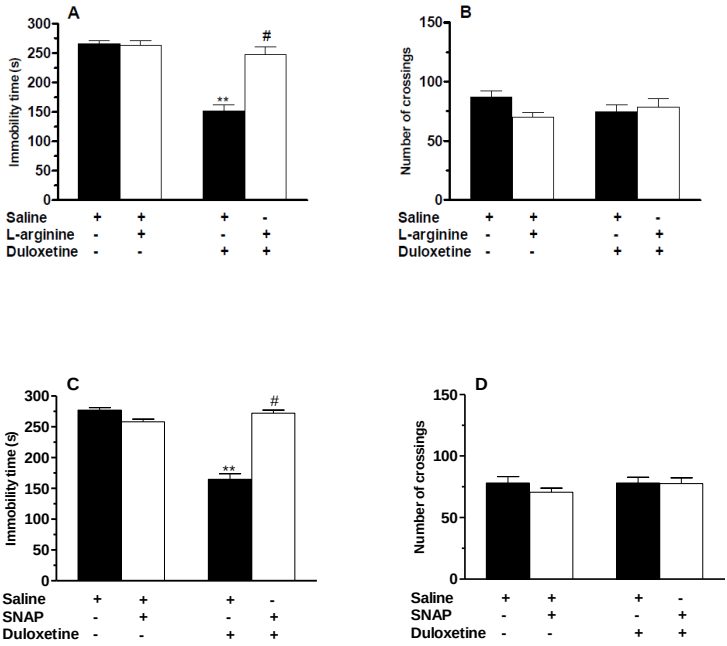

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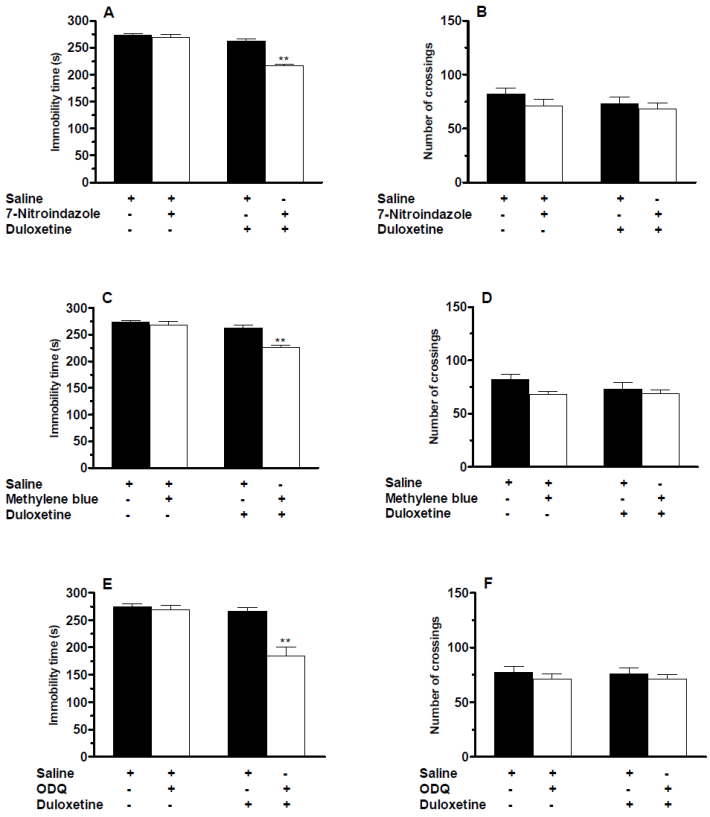

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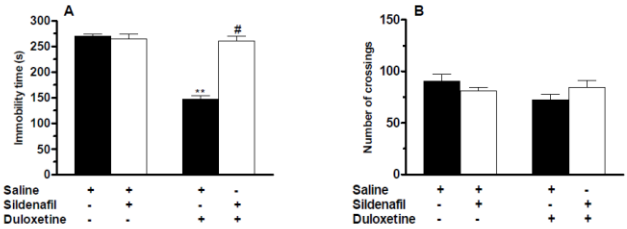

CAPÍTULO 3

Involvement of PKA, PKC, PI3K and MAPK/ERK, but not CAMKII pathways in the antidepressant-like effects of escitalopram and duloxetine in the forced swimming test

(Manuscrito em preparação a ser submetido ao ***Behavioural Brain Research***)

Involvement of intracellular signaling pathways in the antidepressant-like effects of escitalopram and duloxetine in the forced swimming test

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Abstract

Escitalopram is a serotonin reuptake inhibitor and duloxetine is a serotonin and noradrenaline reuptake inhibitor, both are used in the treatment of depression. This study investigated the cellular signaling pathways involved in the antidepressant-like effect of these antidepressants in the forced swimming test (FST) in mice. The anti-immobility effect of escitalopram (3 mg/kg, p.o.) and duloxetine (10 mg/kg, p.o.) was prevented by pretreatment of mice with H-89 (1 µg/site, i.c.v., an inhibitor of protein kinase A, PKA), GF109203X (5 ng/site, i.c.v., an inhibitor of protein kinase C, PKC), LY294002 (10 nmol/site, i.c.v., an inhibitor of phosphatidyl inositol-3 kinase, PI3K), U0126 (5 µg/site, i.c.v., an inhibitor of Mitogen Activated Protein Kinase 1and 2, MEK1,2), but not by KN-62 (1 µg/site, i.c.v., an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II, CaMKII). None of the drugs produced significant effects on the locomotor activity in the open-field test. Altogether, our data suggest that the antidepressant-like effects of escitalopram and duloxetine are dependent on the cellular signaling modulated by PKA, PKC, PI3K and MAPK, but not by CaMKII. The results contribute to the understanding of the mechanisms underlying the antidepressant-like effect of these antidepressants and reinforce the role of cellular signaling in the mechanism of action of antidepressant agents.

Keywords: Depression; Duloxetine; Escitalopram; Forced swimming test; Signalling pathways.

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1. Introduction

Depression is a common, recurring disorder that is considered as the leading cause of disability. It has been proposed that this disease may be due to a deficiency in the neurotransmission mediated by biogenic amines (serotonin, norepinephrine and dopamine), and the treatment with antidepressants increase the availability of these neurotransmitters in the brain (D'Sa and Duman, 2002, Lanni et al., 2009, Malberg and Blendy, 2005).

The selective serotonin reuptake inhibitors have been widely used in clinical practice for two decades and are the compounds of choice for the treatment of depression and anxiety. Newer antidepressants, including selective serotonin reuptake inhibitors, serotonin noradrenaline reuptake inhibitors, and novel mechanism agents offer fewer side effects, improved tolerability and safety and are safer in overdose compared with tricyclic antidepressants and monoamine oxidase inhibitors (Ali et al., 2011). Some clinical data suggest that serotonin noradrenaline reuptake inhibitors may be more effective than serotonin reuptake inhibitors (Shelton et al., 2005; Thase et al., 2001).

Escitalopram, the *S*-isomer of citalopram, is a selective serotonin reuptake inhibitor that has been used in the treatment of depression and anxiety disorders (Höschl et al., 2008, Lam et al., 2008). It has been shown to be clinically more potent than its racemate citalopram in the treatment of depression and it has faster onset of action than comparable doses of citalopram (Colonna et al., 2005, Moore et al., 2005, Sánchez et al., 2004). In vivo microdialysis studies of rat brain cortex also show a greater propensity for escitalopram to elevate serotonin levels than for citalopram (Hyttel et al., 1992). Moreover, escitalopram is effective in animal models predictive of antidepressant and anxiolytic activities (Sánchez et al., 2003 a,b, Zomkowski et al., 2010).

Duloxetine is a drug that inhibit serotonin and noradrenaline reuptake, with weak effects on dopamine reuptake (Bymaster et al., 2001, Hunziker et al., 2005, Karpa et al., 2002). It is used in the treatment of depression and anxiety (Carter and McCormack, 2009, Detke et al., 2004). Several data have demonstrated that the duloxetine reduced the immobility time in the forced swimming test (FST) in rats (Ciulla et al., 2007, Menezes et al., 2008, Rénéric and Lucki, 1998).

Research on depression and antidepressants has focused on intracellular pathways, which are known to be activated by a number of extracellular signals, including growth factors, stress and neurotransmitters (D'Sa and Duman, 2002, Malberg and Blendy, 2005,

Tanis et al., 2007). Dysfunction of neurotrophic and neuroplastic pathways is involved in the pathology of depression. Antidepressants tend to converge around promotion of neurotrophic and neuroplastic pathways (Pettinger and Duman, 2008) modulating intracellular signaling pathways, increasing neurogenesis and neural plasticity (Krystal et al., 2009, Tardito et al., 2006). Studies have shown that the administration of antidepressants up-regulates the cAMP pathway (Gur et al., 2007, Nair and Vaidya, 2006), activates protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and modulates some pathways in brain like mitogen-activated protein kinase-extracellular signal-regulated kinase (MAPK/ERK1/2) and protein kinase C (PKC) pathways (Einat et al., 2003, Malberg and Blendy, 2005, Popoli et al., 2000, Taylor et al., 2005, Tardito et al., 2006, Abrial, et al., 2011). Previous studies have implicated the phosphatidyl inositol-3 kinase (PI3K)-Akt pathways in the neurogenesis-promoting and behavioral properties of antidepressants (Beech and Duman, 2005).

Considering that escitalopram and duloxetine are clinically used antidepressants with different mechanisms of action (Carter and McCormack, 2009, Detke et al., 2004, Höschl et al., 2008, Lam et al., 2008) and that other antidepressants, such as desipramine, fluoxetine, imipramine, sertraline modulate intracellular signaling pathways (D'Sa and Duman, 2002, Hashimoto et al., 2004, Tardito et al., 2006), this study therefore investigated whether the PKA, PKC, PI3K, MAPK/ERK and CaMKII pathways may be involved in the antidepressant-like effect of these antidepressants in order to better understand the mechanisms underlying their antidepressant activities.

2. Materials and methods

2.1. Animals

Female Swiss mice (30-40 g) were maintained at 22-24°C with free access to water and food, under a 12:12 h light/dark cycle (lights on at 07:00 h). All manipulations were carried out between 9:00 and 16:00 h, with each animal used only once. All procedures in this study were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). The experiments were performed after approval of the protocol by the Institutional Ethics Committee and all efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

2.2. Drugs and treatment

The following drugs were used: duloxetine (Eli Lilly & Co.), escitalopram (H. Lundbeck, Denmark), GF109203X, H-89, LY294002, KN-62 and U0126 (Sigma Chemical Co., USA). All drugs were dissolved in saline with 1% DMSO, except antidepressants which were dissolved in saline. All drugs were administered by intracerebroventricular (i.c.v.) route (5 µl/site), except antidepressants which were administered by oral (p.o.) route by gavage in a volume of 10 ml/kg body weight. I.c.v. administration was performed under ether anesthesia as previously described (Zomkowski et al., 2010). Briefly, a 0.4 mm external diameter hypodermic needle attached to a cannula, which was linked to a 25 µl Hamilton syringe, was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse. A volume of 5 µl was then administered in the left lateral ventricle. The injection was given over 30 s, and the needle remained in place for another 30 s in order to avoid the reflux of the substances injected. The injection site was 1 mm to the right or left from the mid-point on a line drawn through to the anterior base of the ears.

In order to investigate the antidepressant-like effect of the escitalopram and duloxetine in the FST is mediated through the activation of PKA, PKC, PI3K, ERK and CaMKII, mice were pretreated with vehicle or with H-89 (1 µg/site), GF109203X (5 ng/site), LY294002 (10 nmol/site, i.c.v.), U0126 (5 µg/site) or KN-62 (1 µg/site) and after 15 min they received escitalopram (3 mg/kg, p.o.), duloxetine (10 mg/kg, p.o.) or vehicle injection before being tested in the FST after 60 min. The same protocol of the administration of the kinase inhibitors alone or with the antidepressants was used to evaluate their influence on the locomotor activity in the open-field test.

The doses of the drugs used were selected on the basis of literature data and on previous results from our laboratory (Stemmelin et al., 1999, Vianna et al., 2000, Narita et al., 2002, Sato et al., 2004, Almeida et al., 2006, Ueno et al., 2006). The doses of escitalopram and duloxetine administered were chosen on the basis of experiments previously performed in our laboratory and literature data (Rénéric e Lucki, 1998, Sánchez et al., 2003a, Zomkowski et al., 2010).

2.3.. Forced swimming test (FST)

The FST was carried out in mice individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water at 25 ± 1°C; the total duration of immobility during the 6-min test was scored as described previously (Zomkowski et al., 2010). Each mouse was judged to be immobile when it ceased

struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water.

2.4. Open-field behavior

The ambulatory behavior was assessed in an open-field test as described previously (Zomkowski et al., 2010). The apparatus consisted of a wooden box measuring 40 x 60 x 50 cm high. The floor of the arena was divided into 12 equal squares. The number of squares crossed with all paws (crossings) was counted in a 6-min session. The light was maintained at minimum to avoid anxiety behavior. The apparatus were cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.5. Statistical analysis

Comparisons between treatment groups and control were performed by one-way or two-way ANOVA followed by Tukey's HSD test when appropriate. A value of $p<0.05$ was considered to be significant.

3. Results

3.1. Involvement of cellular signaling in the antidepressant-like effect of escitalopram in the FST

The results depicted in Fig. 1A show that the pre-treatment of mice with H-89 (1 µg/site, an inhibitor of PKA) on the anti-immobility effect of escitalopram (3 mg/kg, p.o.) in the FST. The pre-treatment of mice with H-89 was able to reverse the antidepressant-like effect of escitalopram. A two-way ANOVA revealed significant differences for the H-89 pre-treatment ($F(1,24)=65.85$, $p<0.01$), escitalopram treatment ($F(1,24)=66.59$, $p<0.01$) and H-89 X escitalopram interaction ($F(1,24)=91.69$, $p<0.01$). Fig. 1B shows the influence of pre-treatment with GF109203X (5 ng/site, i.c.v., an inhibitor of PKC) prevented the antidepressant-like effect of escitalopram (3 mg/kg, p.o.) in the FST. The two-way ANOVA showed significant differences for GF109203X pre-treatment ($F(1,24)=44.42$, $p<0.01$), escitalopram treatment ($F(1,24)=94.10$, $p<0.01$), GF109203X X escitalopram interaction ($F(1,24)=78.49$, $p<0.01$). Fig. 1C shows that the pre-treatment with LY294002 (10 nmol/site, i.c.v., an inhibitor of PI3K) prevented the antidepressant-like effect of escitalopram (3.0 mg/kg, p.o.) in the FST. The two-way ANOVA showed significant differences for LY294002

pre-treatment ($F(1,24)=25.95$, $p<0.01$), escitalopram treatment ($F(1,24)=36.00$, $p<0.01$), LY294002 X escitalopram interaction ($F(1,24)=51.11$, $p<0.01$). The results illustrated in Fig. 1D show that the pre-treatment with U0126 (5 µg/site, i.c.v., an inhibitor of ERK) prevented the antidepressant-like effect of escitalopram (3 mg/kg, p.o.) in the FST. The two-way ANOVA showed significant differences for U0126 pre-treatment ($F(1,24)=81.75$, $p<0.01$), escitalopram treatment ($F(1,24)=161.75$, $p<0.01$), U0126 X escitalopram interaction ($F(1, 24)=134.16$, $p<0.01$). The results depicted in Fig. 1E show that the pre-treatment with KN-62 (1 µg/site, i.c.v., an inhibitor of CaMKII) did not significantly alter the antidepressant-like effect of escitalopram (3 mg/kg, p.o.) in the FST. The two-way ANOVA reveal a main effect of escitalopram treatment ($F(1,24)=88.76$, $p<0.01$), but not of KN-62 pre-treatment ($F(1,24)=0.36$, $p=0,55$), nor KN-62 X escitalopram interaction ($F(1, 24)=3.25$, $p=0.08$).

3.2. Involvement of cellular signaling in the antidepressant-like effect of duloxetine in the FST

Fig. 2A shows the influence of pre-treatment of mice with H-89 (1 µg/site, an inhibitor of PKA) on the anti-immobility effect of duloxetine (10 mg/kg, p.o.) in the FST. The pre-treatment of mice with H-89 was able to reverse the antidepressant-like effect of duloxetine. A two-way ANOVA revealed significant differences for the H-89 pretreatment ($F(1,24)=51.87$, $p<0.01$), duloxetine treatment ($F(1,24)=55.59$, $p<0.01$) and H-89 X duloxetine interaction ($F(1,24)=71.45$, $p<0.01$). The results illustrated in Fig. 2B show that the pre-treatment with GF109203X (5 ng/site, i.c.v., an inhibitor of PKC) prevented the antidepressant-like effect of duloxetine (10 mg/kg, p.o.) in the FST. The two-way ANOVA showed significant differences for GF109203X pre-treatment ($F(1,24)=75.19$, $p<0.01$), duloxetine treatment ($F(1,24)=153.08$, $p<0.01$), GF109203X X duloxetine interaction ($F(1, 24)=124.59$, $p<0.01$). The results depicted in Fig. 2C show that the pre-treatment with LY294002 (10 nmol/site, i.c.v., an inhibitor of PI3K) prevented the antidepressant-like effect of duoxetine (10 mg/kg, p.o.) in the FST. The two-way ANOVA showed significant differences for LY294002 pre-treatment ($F(1,24)=27.59$, $p<0.01$), duloxetine treatment ($F(1,24)=76.88$, $p<0.01$), LY294002 X duloxetine interaction ($F(1,24)=58.02$, $p<0.01$). Fig. 2D shows that the pre-treatment with U0126 (5 µg/site, i.c.v., an inhibitor of ERK) prevented the antidepressant-like effect of duloxetine (10.0 mg/kg, p.o.) in the FST. The two-way ANOVA showed significant differences for U0126

pre-treatment ($F(1,24)=66.52$, $p<0.01$), duloxetine treatment ($F(1,24)=150.89$, $p<0.01$), U0126 X duloxetine interaction ($F(1,24)=117.73$, $p<0.01$). The results depicted in Fig. 2E show that the pre-treatment with KN-62 (1 µg/site, i.c.v., an inhibitor of CaMKII) did not significantly alter the antidepressant-like effect of duloxetine (10 mg/kg, p.o.) in the FST. The two-way ANOVA reveal a main effect of duloxetine treatment ($F(1,24)=154.21$, $p<0.01$), but not of KN-62 pre-treatment ($F(1,24)=0.11$, $p=0.73$), nor KN-62 X duloxetine interaction ($F(1, 24)=3.93$, $p=0.06$).

3.3. Effects of antidepressants and inhibitors on the locomotor activity in the open-field test

The results illustrated in Fig. 3A show that the administration H-89 (1 µg/site, an inhibitor of PKA), GF109203X (5 ng/site, i.c.v., an inhibitor of PKC), LY294002 (10 nmol/site, i.c.v., an inhibitor of PI3K), U0126 (5 µg/site, i.c.v., an inhibitor of ERK) or KN-62 (1 µg/site, i.c.v., an inhibitor of CaMKII) alone or in combination with escitalopram did not affect the ambulation in the open-field. A two-way ANOVA did not reveal significant differences for the pretreatment ($F(5,60)=1.94$, $p=0.10$), treatment ($F(1,60)=0.14$, $p=0.71$) and interaction ($F(5,60)=0.44$, $p=0.82$). Fig. 3B show that the administration H-89 (1 µg/site, an inhibitor of PKA), GF1090203X (5 ng/site, i.c.v., an inhibitor of PKC), LY294002 (10 nmol/site, i.c.v., an inhibitor of PI3K), U0126 (5 µg/site, i.c.v., an inhibitor of MAPK/ERK) or KN-62 (1 µg/site, i.c.v., an inhibitor of CaMKII) alone or in combination with duloxetine did not affect the ambulation in the open-field. A two-way ANOVA did not reveal significant differences for the pretreatment ($F(5,60)=2.35$, $p=0.05$), treatment ($F(1,60)=0.27$, $p=0.60$) and interaction ($F(5,60)=0.21$, $p=0.96$).

4. Discussion

Several evidence suggest that structural alterations in the brain, including neurogenesis, may play a role in both the pathogenesis of mood disorders and the mechanism of antidepressants action (Castrén and Rantamäki, 2010, Duman, 2004, Schmidt and Duman, 2007). Depression is associated with impairment of structural plasticity and cellular resilience. Studies have demonstrated that signaling pathway involved in regulating cell survival and cell death are long-term targets for the action of antidepressants (D'Sa and Duman, 2002, Tanis et al., 2007). In this study we investigated the possible involvement of cellular

signaling pathways (PKA, PKC, PI3K, MAPK/ERK, CaKMII) in the antidepressant-like effect of escitalopram and duloxetine in the FST. For this aim, oral administration of escitalopram and duloxetine was selected because it is the most common route of administration for antidepressants in psychiatric patients.

PKA signaling pathway is involved with synaptic plasticity, cell survival, neurotransmitter synthesis and release (D'Sa and Duman, 2002, Malberg and Blendy, 2005, Dwivedi and Pandey; 2010). It has been reported that the treatment with fluoxetine and desipramine activate PKA (Popoli et al., 2000, Tardito et al., 2006). In addition, a study demonstrated that the combined treatment with ketamine (uncompetitive NMDA receptor antagonist) and imipramine increased the PKA phosphorylation in hippocampus and amygdala (Réus et al., 2011). Several evidence has demonstrated an involvement of the cAMP-dependent signaling pathway in the mechanism of action of antidepressants (Nair and Vaidya, 2006, Tardito et al., 2006). Previous research using post-mortem brain suggests that depressed patient exhibit reduced PKA activity (Dwivedi et al., 2004, Shelton et al., 2009). The mechanism of PKA-mediated function is through the phosphorylation of specific substrates, which include cAMP response element binding protein (CREB) (D'Sa and Duman, 2002, Gould and Manji, 2002). Studies have demonstrated that different classes of antidepressants, including a selective serotonin reuptake inhibitor (fluoxetine, sertraline) and a selective noradrenaline reuptake inhibitor (desipramine) increases expression of CREB mRNA and CREB protein in the hippocampus (D'Sa and Duman, 2002; Gould and Manji, 2002). It has been demonstrated that the increased expression of CREB by different classes of antidepressants could explain how this transcription factor is a common post-receptor target of antidepressant induced neural plasticity (Shaywitz et al., 1999). In addition, studies *in vitro* show that CREB can be activated directly by the cAMP–PKA pathway via stimulation of the serotonin ($5\text{-}HT_{4,7}$) and noradrenaline (α_1 and β-adrenergic) receptors (D'Sa and Duman, 2002, Duman, 1998). In this study, we showed that the reduction of the immobility time elicited by escitalopram and duloxetine in the FST was prevented by H-89, an inhibitor of PKA, suggesting that the antidepressant-like effect of escitalopram and duloxetine could be due to the activation of PKA pathway. In concordance with our results, the antidepressant-like effects of memantine in the FST was prevented by the pretreatment with H-89 (Almeida et al., 2006).

An important role for PKC in the pathophysiology of depression

has been demonstrated by the PKC decreased activity in suicide victims (Pandey et al., 2004, 2007). PKC signaling pathway is involved with synaptic plasticity, cell growth, differentiation and proliferation (Sun and Alkon, 2009). It has been shown that CREB activity can also be directly induced by PKC which is activated by stimulation of the noradrenaline (α_1-adrenergic) and serotonin (5-HT_2) receptors (D'Sa and Duman, 2002, Shaywitz and Greenberg, 1999). Our data demonstrated that the antidepressant-like effects of escitalopram and duloxetine were abolished by the pretreatment of mice with GF109203X, an inhibitor of PKC. These results indicate that the effect of escitalopram and duloxetine may be dependent on, at least in part, of activation of the PKC pathway. Studies have showed that the treatment with lithium and antidepressants modulate PKC pathway (Einat et al., 2003, DiazGranados and Zarate 2008, Taylor et al., 2005). In addition, a study demonstrated that the sertraline, paroxetine, citalopram and fluoxetine increased PKC activity in rat cortical synaptoneurosomes (Giambalvo and Price, 2003). The acute administration of fluoxetine resulted in an up-regulation of PKC (Rausch et al., 2002). Recently, a study showed that the co-administration of imipramine with ketamine increased the PKC phosphorylation in prefrontal cortex (Réus et al., 2011).

The PI3K pathway is classically implicated in the regulation of cell growth, survival, proliferation and neuroplasticity (Kumar et al., 2005, Martin-Pena et al., 2006, Peltier et al., 2007). Recently, a study demonstrated that the PI3K activity was decreased in suicide victims (Dwivedi et al., 2008). Moreover, it has been reported that the PI3K pathway is involved behavioral properties of antidepressants (Beech and Duman, 2005). The mood stabilizers with lithium influence signal transduction cascades including the PI-3K/AKT pathway (Hashimoto et al., 2004). Recently, a study demonstrated that treatment with fluvoxamine (a selective serotonin reuptake inhibitor) increased phosphorylation of Akt-1 in PC12 cells and this effect was blocked by LY294002, an inhibitor of PI3K (Nakano et al., 2010). In addition, the mood stabilizers (lithium and valproate) activate intracellular signaling pathway, including the PI3-K/AKT (Hashimoto et al., 2004). In our study LY294002 was able to reverse the anti-immobility effect elicited by treatment with escitalopram and duloxetine, which implicates that this signaling pathway is activated after treatment with these antidepressants.

The MAPK/ERK pathway is involved with synaptic plasticity and cell survival. (Hashimoto et al., 2004). Studies have observed that activation of ERK1/2 is significantly reduced in post-mortem brain of

suicide victims (Dwivedi et al., 2001, 2009). Research indicates that BDNF can ameliorate depression via activating the ERK pathway (Shirayama et al., 2002) and the 5-hydroxytraptamine treatment can induce ERK phosphorylation (Cipriani et al., 2005, Mattson et al., 2004). In addition, it has been shown that the MAPK/ERK pathway can be activated via stimulation of the serotonin ($5\text{-}HT_{1A}$) receptors. The reversal of the antidepressant-like effect of escitalopram and duloxetine in the FST by U0126, a MEK inhibitor suggests that the antidepressant-like effect of escitalopram and duloxetine could be due to the activation of the ERK pathway. Our results are in accordance with the reported that the PD098059, a MEK inhibitor, inhibited the anti-immobility effect of memantine in mice (Almeida et al., 2006). In addition, it has been demonstrated that the PD184161, a MEK inhibitor, reversed the anti-immobility effect elicited by desipramine in the FST, but not fluoxetine (Duman et al., 2007). A study showed that the inhibition of the ERK pathway in hippocampus by administration of U0126 resulted in anhedonia and anxiety-like behavior. In this study, the phosphorylation of the CREB was decreased following the ERK pathway inhibition (Qi et al., 2009). Moreover, it has been demonstrated that fluoxetine stimulated phosphorylation of ERK1/2 in cultured rat astrocytes (Mercier et al., 2004). The chronic treatment with fluoxetine induced the activation of the MAPK/ERK pathway in the prefrontal cortex and frontal in rats (Tiraboschi et al., 2004). Furthermore, it has been shown that the chronic treatment with fluoxetine reversed the reduction in the activities of the ERK and CREB in hippocampus and cortex prefrontal in stressed rats (Qi et al., 2008).

The CaMKII pathway has been implicated in processes as diverse as gene expression, calcium homeostasis, receptor and ion-channel regulation, synthesis and release of neurotransmitters, synaptic transmission and synaptic plasticity (Okamoto et al., 2009, Popoli et al., 2000). CREB activity can also be induced by CaKMII which is activated by stimulation of the serotonin ($5\text{-}HT_2$) and noradrenaline (α_1-adrenergic) receptors (D'Sa and Duman, 2002, Shaywitz e Greenberg, 1999). Studies are reported that the treatment with antidepressants activates CaMKII (Du et al., 2004, Nair and Vaidya, 2006). In addition, it has been shown that chronic treatment with paroxetine, fluvoxamine and venlafaxine to increase autophosphorylation and activity of CaMKII in the hippocampus and that only the chronic treatment (but not acute) with fluoxetine, desipramine and reboxetine, increased the enzymatic activity of CaMKII in the prefrontal cortex in rats (Popoli et al., 2002). However, our data showed that the acute antidepressant-like effect of

escitalopram and duloxetine was not prevented by the pretreatment of mice with KN-62, an inhibitor of CaMKII. This result indicates that this signaling pathway seems not to be involved in antidepressant-like action of escitalopram and duloxetine in the FST.

The reversal of the antidepressant-like effect of escitalopram and duloxetine in the FST by H-89, GF109203X, LY294002 and U0126 is not due to any locomotor action, since the administration of H-89, GF109203X, LY294002, U0126 and KN-62 alone or previously to escitalopram and duloxetine did not significantly alter the locomotor activity of mice in the open field test.

It has been showed that antidepressants increase levels of serotonin and noradrenaline in the brain, resulting in activation of intracellular signal transduction cascades that couple to receptors serotoninergic and noradrenergic (Hashimoto et al., 2004, Nair and Vaidya, 2006, Tardito et al., 2006). The antidepressants can also activate Ca^{2+}-dependent kinases via stimulation of NMDA receptors (Hashimoto et al., 2004). Furthermore, NMDA receptor antagonists have been shown to increase the release of serotonin in the brain (Callado et al. 2000; Gaikwad et al., 2005, Tso et al., 2004). All these pathways, AMPc, PKA, MAPK/ERK, CaMKII, activate CREB and the activation of CREB causes the expression of proteins such as BDNF, which has been implicated in the maintenance of neurons, cell survival and neuronal plasticity (Hashimoto et al., 2004, Nair and Vaidya, 2006, Tardito et al., 2006). Although this study does not allow us to know exactly the mechanism responsible for behavioral effects by which escitalopram and duloxetine affects PKA, PKC, MAPK/ERK and PI3K pathways, one possibility to account for this action is that these antidepressants, increased levels of serotonin and noradrenaline in the synaptic cleft and may thus activate cell signaling pathways by binding to their receptors. Another possibility for the mechanism of action of antidepressants may be because they act as NMDA receptor antagonists and thus increase serotonin release.

5. Conclusions

Our results suggest that the antidepressant-like effect of escitalopram and duloxetine in the FST may is mediated by activation PKA, PKC, PI3K and MAPK/ERK pathways, supporting the notion that these targets may be critical to the action of escitalopram and duloxetine. Moreover, our results significantly extend literature data by indicating that antidepressants may intracellular pathways affect and a

downstream consequence is activation of CREB and the increase expression of BDNF, which may contribute to the antidepressants mediated changes in neuronal plasticity and behavior (Carlson et al., 2006, D'Sa and Duman, 2002, Nair and Vaidya, 2006).

Acknowledgements

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (**FINEP) Research Grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" #01.06.0842-00,** PROCAD-CAPES (167/2007), CAPES. ALSR and NHG are recipients of CNPq fellowship.

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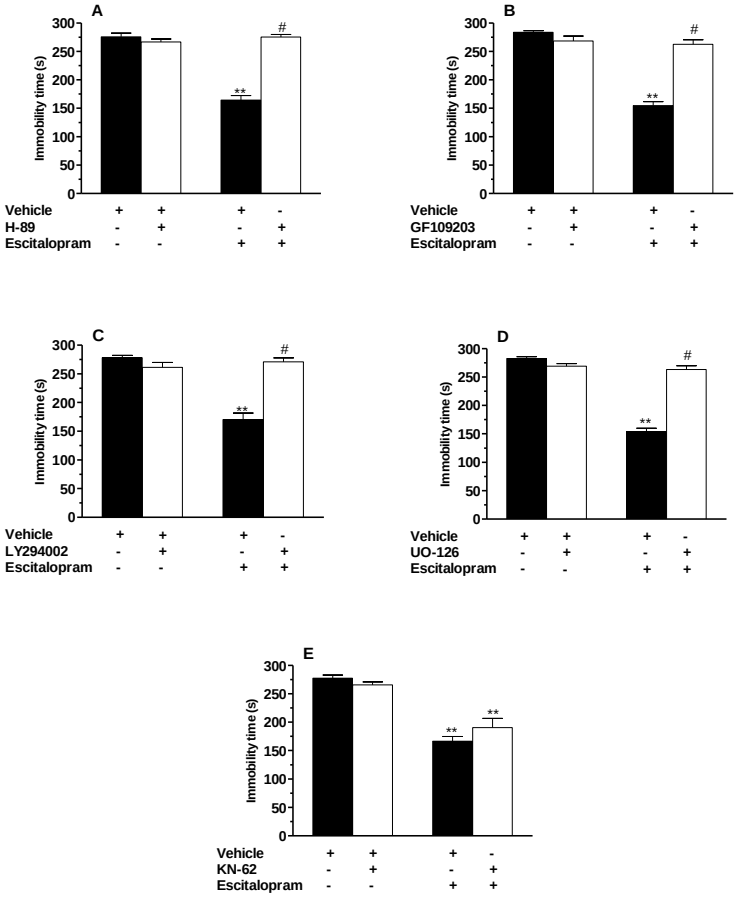

Fig. 1. Effect of the pretreatment of mice with H-89 (1 µg/site, i.c.v.), GF1090203X (5 ng/site, i.c.v.), LY294002 (10 nmol/site, i.c.v.), U0126 (5 µg/site, i.c.v.) or KN-62 (1 µg/site, i.c.v.) on the anti-immobility action of escitalopram (3 mg/kg, p.o.) (panel A-E, respectively) in the FST. Values are expressed as mean±S.E.M. (n=7). **p< 0.01 compared with the vehicle-treated control group; $^{\#}p$< 0.01 compared with the same group pretreated with vehicle.

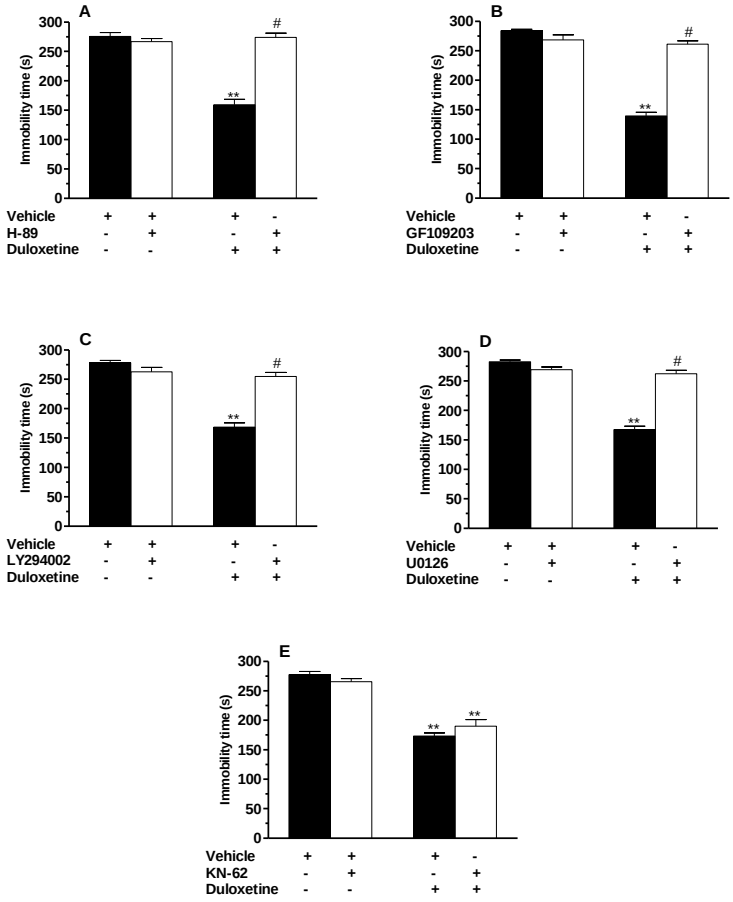

Fig. 2. Effect of the pretreatment of mice with H-89 (1 µg/site, i.c.v.), GF1090203X (5 ng/site, i.c.v.), LY294002 (10 nmol/site, i.c.v.), U0126 (5 µg/site, i.c.v.) or KN-62 (1 µg/site, i.c.v.) on the anti-immobility action of duloxetine (10 mg/kg, p.o.) (panel A-E, respectively) in the FST. Values are expressed as mean±S.E.M. (n=7). **p< 0.01 compared with the vehicle-treated control group; $^{\#}p$< 0.01 compared with the same group pretreated with vehicle.

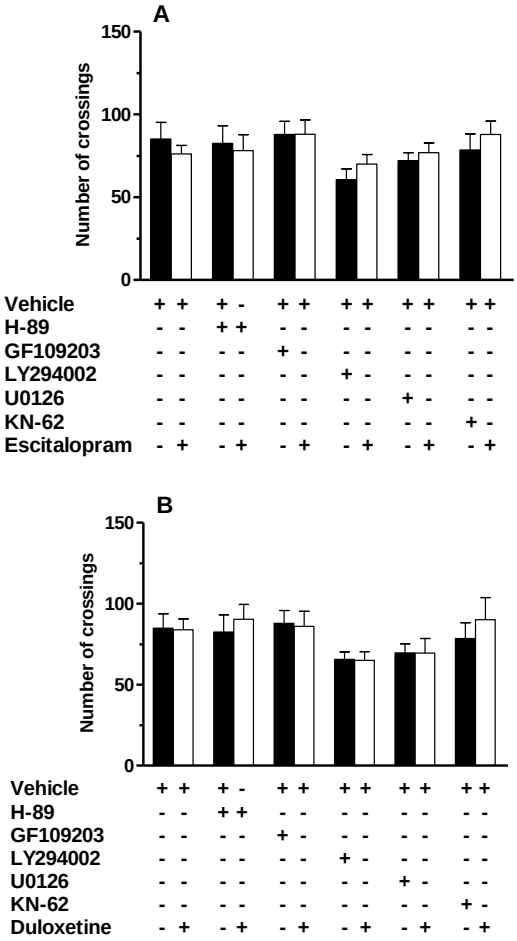

Fig. 3. Effect of the pretreatment of mice with H-89 (1 µg/site, i.c.v.), GF1090203X (5 ng/site, i.c.v.), LY294002 (10 nmol/site, i.c.v.), U0126 (5 µg/site, i.c.v.) or KN-62 (1 µg/site, i.c.v.) on the anti-immobility action of escitalopram (3 mg/kg, p.o.) or duloxetine (10 mg/kg, p.o.) on the number of crossings in the open-field test (panel A-B, respectively). Values are expressed as mean±S.E.M. (*n*=6).

CAPÍTULO 4

Neuroprotective effect of escitalopram and duloxetine against glutamate-induced cell damage in mice hippocampal slices: involvement of nitric oxide and phosphatidylinositol-3 kinase/akt pathways

(Manuscrito em preparação a ser submetido ao ***Neurochemical Research***)

Neuroprotective effect of escitalopram and duloxetine against glutamate-induced cell damage in mice hippocampal slices: Involvement of nitric oxide and phosphatidylinositol-3 kinase/Akt pathways

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Abstract

Glutamatergic excitotoxicity is related with neurodegenerative disorders. It has been demonstrated that millimolar concentrations of glutamate induce acute excitotoxicity in hippocampal slices and promote a reduction of cell viability. Glutamate has been also implicated in the pathogenesis of depression. Studies have demonstrated that treatment with antidepressants affects glutamate neurotransmission. Escitalopram is a serotonin reuptake inhibitor and duloxetine is a serotonin and noradrenaline reuptake inhibitor. This study investigated the neuroprotective effect of these antidepressants against glutamate-induced toxicity in hippocampal slices and the mechanism involved in such effect. We found that escitalopram (1 μM) and duloxetine (10 μM) were neuroprotective against 10 mM glutamate-induced cell death. SNAP (a NO donor) or LY294002 (PI3K/Akt inhibitor) inhibited the protective effect of escitalopram and duloxetine. Moreover, escitalopram and duloxetine were able to prevent glutamate release from hippocampal slices. Altogether, our data indicate that escitalopram and duloxetine protect hippocampal slices against glutamate toxicity by a mechanism that involves NO and PI3K/Akt pathways preventing glutamate-induced glutamate release.

Keywords: Escitalopram; duloxetine; glutamate; neuroprotection, hippocampus, PI3K, NO.

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INTRODUCTION

A great amount of evidence has suggested the involvement of serotonin (5-HT) and noradrenaline (NE) in the etiology and therapy of affective disorders (Lanni et al. 2009). Escitalopram, the *S*-isomer of citalopram, is a selective 5-HT reuptake inhibitor (Höschl and Svestka, 2008), whereas duloxetine inhibits 5-HT and NE reuptake (Hunziker et al., 2005). A relationship between the 5-HT system and the ionotropic glutamate receptors of the subtype NMDA is shown by the fact that classical antidepressants that inhibit NE and/or 5-HT uptake can impact on NMDA receptor function by causing a down-regulation of its subunits (Zarate et al. 2006). Moreover, antidepressants that enhance serotonergic neurotransmission decrease hippocampal nitric oxide synthase (NOS) activity induced by NMDA receptor activation (Wegener et al., 2003). We have previously reported that the antidepressant-like effect of escitalopram in the forced swimming test (FST) is mediated by an inhibition of either the NMDA receptor activation or NO-cyclic guanosine 3'5'-monophosphate (cGMP) synthesis (Zomkowski et al., 2010).

Glutamate is the major excitatory neurotransmitter in the nervous system. It has a role in synaptic plasticity, learning and memory, but under pathological conditions it is known to be a potent neuronal excitotoxin, triggering either rapid or delayed neurotoxicity (Sanacora et al., 2008). It has been shown that excitotoxicity is caused by overstimulation of synaptic glutamate receptors and consequently neuronal death (Lipton and Rosemberg, 1994). Many neurodegenerative diseases are caused by glutamate receptor overstimulation (Mattson and Magnus, 2006). It has been demonstrated that millimolar concentrations of glutamate induce excitotoxicity in acute hippocampal slices and promote a reduction of cell viability (Molz et al., 2008a). Furthermore, it has been shown that glutamate toxicity is related to the reversal activity of glutamate transporters, increasing extracellular glutamate concentration and excitotoxicty (Molz et al., 2008b).

Some clinical studies have shown that glutamate is involved in depressive disorders (Sanacora et al., 2008; Zarate et al., 2006). It has been shown that stress increases extracellular levels of glutamate in the hippocampus and that NMDA receptor antagonists attenuate the atrophy of hippocampal neurons induced by stress (Sapolsky, 2000). Post-mortem studies reported increased levels of glutamate in the frontal cortex of patients with depression (Hashimoto et al., 2007). In addition, a study reported that fluoxetine, a selective inhibitor of 5-HT reuptake,

reduces 4-aminopyridine-evoked glutamate release from cerebrocortical synaptosomes on acute exposure *in vitro* (Wang et al., 2003). It was also reported that the acute treatment with ketamine, a non-competitive NMDA receptor antagonist, elicited a significant improvement in depressed patients (Zarate et al., 2006). Furthermore, several preclinical studies have demonstrated that compounds that reduce transmission at NMDA receptors and NMDA receptor antagonists exhibit antidepressant-like effects (Zeni et al., 2011; Sanacora et al. 2008; Zarate et al. 2006).

The activation of NMDA receptors by glutamate can cause the increase of NO by activating the calcium-calmodulin dependent neuronal NOS enzyme (Esplugues 2002). NO, induced by NMDA receptor stimulation has been implicated in neurotransmission, synaptic plasticity, learning and depression (Esplugues, 2002; Heiberg et al., 2002; Da Silva et al., 2000). In addition, plasma nitrate concentrations were significantly higher in depressed patients, suggesting that NO production is increased in depression (Suzuki et al., 2001). It was demonstrated that the hippocampal nNOS expression is increased in postmortem brain from patients with depression (Oliveira et al., 2008). Furthermore, several studies have demonstrated that NOS inhibitors exert antidepressant-like effects (Da Silva et al. 2000; Harkin et al., 2004; Volke et al. 2003).

It is well established that depression is associated with hippocampal atrophy and decreased cellular survival (Tanis and Duman, 2007; D'Sa and Duman, 2002). Antidepressants may exert their therapeutic effects by modulating intracellular signaling pathways, counteracting cellular death cascades, increasing neurogenesis and mediating neural plasticity (Tardito et al., 2006). The phosphatidyl inositol-3 kinase (PI3K)-Akt pathway have been implicated in the neurogenesis-promoting and behavioral properties of antidepressants (Beech and Duman, 2005).

Considering that depression is associated with regional reductions in the number or the size of glial cells and neurons in the hippocampus, and the treatment with antidepressants induces the modulation of intracellular signaling, neural plasticity and cell survival (Tardito et al., 2006; Hashimoto et al., 2004; D'Sa and Duman, 2002) and also that the glutamatergic excitotoxicity is related with neurodegenerative disorders, we investigated, in a first set of experiments, the neuroprotective role of escitalopram and duloxetine against glutamate-induced hippocampal slice injury. Considering that escitalopram and duloxetine afforded neuroprotection, in a second set of experiments we investigated whether

NO and PI3K/Akt pathways may be involved in their neuroprotective effect. .

MATERIALS AND METHODS

Animals

Swiss female mice (30-40 g) maintained at 21-23°C with free access to water and food, under a 12:12 h light/dark cycle (lights on at 07:00 h) were used. The animals were obtained from our local breeding colony. The experiments followed the principles of laboratory animal care (NIH publication No. 85-23, revised 1985) and were approved by the local Ethical Committee of Animal Research (CEUA/UFSC).

Preparation and incubation of hippocampal slices

Mice were killed by decapitation and the hippocampi were rapidly removed and placed in ice-cold Krebs-Ringer bicarbonate buffer (KRB) of the following composition: 122 mM NaCl, 3 mM KCl, 1.2 mM MgSO4, 1.3 mM CaCl2, 0.4 mM KH2PO4, 25 mM NaHCO3, and 10 mM D-glucose. The buffer was bubbled with 95% O_2-5% CO2 up to pH 7.4. Slices (0.4 mm thick) were rapidly prepared using a McIlwain tissue chopper, separated in KRB at 4ºC, and allowed to recover for 30 min in KRB at 37ºC to slice stabilization (Oliveira et al., 2002).

Hippocampal slice treatments

In order to access the cell viability in a glutamate excitotoxicity protocol, after the preincubation of 30 min with escitalopram or duloxetine (0.1-100 μM), the slices were co-incubated with glutamate (Sigma, St. Louis, MO; 10 mM) and the antidepressants for 1 h in KRB buffer. After this period, the medium was withdrawn and replaced by a nutritive culture medium composed of 50% KRB, 50% Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY), 20 mM HEPES, and 100 μg/ml gentamicin, and slices were maintained for additional 6 h in a humidified atmosphere of 95% air and 5% CO2 at 37ºC to evaluate cell viability (Molz et al., 2008a).

When evaluating the mechanism of neuroprotective action of escitalopram (1 μM) or duloxetine (10 μM), they were added to the incubation medium 30 min before glutamate and maintained during the 1 h of incubation with glutamate. The NO donor S-nitroso-N-acetyl-l,l-penicillamine (SNAP; 1 mM) or the PI3K inhibitor 2-(4- morpholinyl)-

8-phenyl-4H-1benzo-pyran-4-one (LY294002; 30 μM) was added to the incubation medium 15 min before escitalopram or duloxetine and maintained during the escitalopram or duloxetine preincubation period.

Evaluation of cell viability

Hippocampal cell viability was evaluated 6 h after glutamate exposure. Cell viability was determined through the ability of cells to reduce 3-(4,5-dimethylthiazol-2-yl-diphenyltetrazolium bromide (MTT; Sigma; Mosmann, 1983). Hippocampal slices were incubated with MTT (0.5 mg/ml) in KRB for 30 min at 37^0C. The tetrazolium ring of MTT is cleaved by active dehydrogenases in order to produce a precipitated formazan. The formazan produced was solubilized by adding 200 μl of dimethylsulfoxide (DMSO), resulting in a colored compound whose optical density was measured in an ELISA reader (550 nm).

Glutamate Release

After the preincubation period to slices recovery (30 min), hippocampal slices were incubated for 15 min in a Hank's balanced salt solution (HBSS; composition in mM 1.29 CaCl2, 136.9 NaCl, 5.36 KCl, 0.65 MgSO4, 0.27 Na2HPO4, 1.1 KH2PO4, 2 glucose, and 5 HEPES). When present, escitalopram (1 μM) or duloxetine (10 μM) was incubated for 30 min and maintained during glutamate exposure. Glutamate (10 mM) was incubated for 15 min, and glutamate release was assessed by adding 0.33 μCi/ml D-[^{3}H]aspartate with 100 μM unlabeled aspartate for 7 min and stopped by three ice-cold washes with 1 ml HBSS. D-[^{3}H]aspartate was used in order to avoid aminoacid metabolization in intracellular compartments, although a previous study from our group has shown similar results by using D-[^{3}H]aspartate or L-[^{3}H]glutamate (Molz et al., 2011) The slices were then further incubated for 15 min in HBSS, and the supernatant was collected to measure the amount of released D-[^{3}H]aspartate. Slices were disrupted by overnight incubation with 0.1% NaOH/0.01% SDS, and aliquots of lysates were taken for determination of intracellular D-[^{3}H]aspartate content (Molz et al., 2008b). Intracellular and extracellular D-[^{3}H]aspartate content were determined through scintillation counting, calculated as nmol of aspartate and the amount of released aminoacid was expressed as a percentage of total released aminoacid.

Statistical analysis

Comparisons among groups were performed by one way ANOVA, followed by Duncan's test if necessary, with $P < 0.05$

considered to be statistically significant.

RESULTS

Escitalopram and duloxetine protects hippocampal slices against glutamate-induced cell death

Exposure of hippocampal slices to 10 mM glutamate resulted in a significant decrease in cell viability measured as reduction of MTT. Treatment of the slices with escitalopram (1 μM) or duloxetine (10 μM) significantly prevented the reduction in cell viability induced by glutamate. However, the neuroprotective effect was not observed when slices were preincubated with the concentrations of 0.1, 10 or 100 μM of escitalopram and in the concentrations of 1 or 100 μM of duloxetine (Fig. 1A and B). None of the escitalopram or duloxetine concentrations used was able to alter cellular viability *per se*. Therefore, we have shown that escitalopran and duloxetine afforded neuroprotection in a specific concentration, 1 μM and 10 μM, respectively.

The results depicted in Fig. 2 show that the preincubation of hippocampal slices with SNAP (1 mM, a NO donor), prior to escitalopram (1 μM, panel 2A) or duloxetine (10 μM, panel 2B) treatment significantly abolished the protective effect of escitalopram and duloxetine against glutamate-induced cell damage. Previous data, shown that the combination of SNAP plus glutamate did not further reduce cell viability of hippocampal slices in comparison with glutamate alone (Molz et al., 2011). These data point out to a neuroprotective mechanism of action of escitalopram and duloxetine against glutamate excitotoxicity, likely involving decrease of NO production in hippocampal slices.

The results illustrated in Fig. 3 show that the, preincubation of hippocampal slices with LY294002 (30 μM, a PI3K/Akt inhibitor), prior to escitalopram (1 μM, panel 3A) or duloxetine (10 μM, panel 3B) treatment significantly abolished the protective effect of escitalopram and duloxetine against glutamate-induced cell damage Thus, LY294002 prevented the neuroprotective effect of escitalopram and duloxetine and did not alter cell viability *per se*. These data suggest that the neuroprotective mechanism of action of escitalopram and duloxetine against glutamate excitotoxicity depend on the activation of the PI3K/Akt signaling pathway

Escitalopram and duloxetine prevented glutamate-induced glutamate release

Glutamate release was evaluated in the presence of escitalopram (1 μM) or duloxetine (10 μM). As previously shown, glutamate-induced cell damage in hippocampal slices promotes an increased glutamate release (as evaluated by its non-metabolized analog D-[^{3}H]aspartate) which contributes to the decreased cell viability induced by an excitotoxic concentration of glutamate (Molz et al., 2008b). Fig. 4 shows that escitalopram or duloxetine was able to prevent the increased glutamate-induced D-[^{3}H]aspartate release from hippocampal slices. Therefore, the neuroprotective mechanism of action of escitalopran and duloxetine involves the modulation of glutamate transport in hippocampal slices.

DISCUSSION

Several studies indicate that abnormalities in the glutamatergic transmission play an important role in the pathophysiology of depression and that a common mechanism of various antidepressant therapies may involve modifications in the function of the glutamatergic system (Kugaya and Sanacora, 2005; Palucha and Pilc, 2005). Furthermore, it has been demonstrated that antidepressants reduce binding, expression and function of NMDA receptors (Szasz et al., 2007; Boyer et al. 1998). A study reported a direct action of fluoxetine and desipramine inhibiting NMDA-evoked currents in rat cortical cell cultures, suggesting that direct inhibition of NMDA receptors may contribute to the effects of antidepressants (Szasz et al., 2007).

The results of the present study indicate that escitalopram and duloxetine were effective in preventing the decreased cell viability in hippocampal slices of mice subjected to an *in vitro* glutamatergic excitotoxicity model. It was shown that the precursors of 5-HT, L-tryptophan and 5-hydroxy-L-tryptophan, prevent glutamate-induced excitotoxicity (Kamei et al., 1991) and 5-HT_{1A} receptor activation protects against NMDA-induced cell death (Madhavan et al., 2003). In addition, lithium (a mood stabilizer) protects cultured rat brain neurons from glutamate-induced excitotoxicity mediated by NMDA receptors (Chuang et al., 2002). It has also been shown that the analogs with the (1S, 2R, 1'S)-configuration of minalcipran (a 5-HT/NE reuptake inhibitor) protect cultured cortical neurons against NMDA-induced neurotoxicity (Noguchi e al., 1999). Furthermore, it has demonstrated that the medicinal herbs *Mangifera indica* (*in vitro*) and *Aloysia gratissima* (*ex vivo*) provide neuroprotection against glutamate-induced

excitoxicity (Zeni et al., 2011; Lemus-Molina et al., 2009). Taken togheter, these studies point to an association between the antidepressant and neuroprotective effects of diverse compounds via the modulation of glutamatergic excitotoxicity.

The NO pathway has also been shown to be required for the therapeutic effects of selective serotonin reuptake inhibitors (Krass et al., 2011; Kumar et al., 2010) and NMDA receptor antagonists (Dhir and Kulkarni, 2008). In neurons, NO signaling is coupled to the glutamate system via Ca^{2+} influx through NMDA receptors, which activates nNOS. Post-mortem studies with brain collections have shown that depressed patients have increased hippocampal nNOS expression (Oliveira et al., 2008) and increased NO production (Suzuki et al., 2001). In order to investigate the role of NO signaling to the neuroprotective effect of duloxetine and escitalopram a NO donor, SNAP, at a concentration that did not reduce cellular viability, was used. NO produced by SNAP prevented the protective effect of escitalopram or duloxetine against glutamate-induced cell damage. Similar to our results, it has been shown that the preincubation of rat hippocampal slices with SNAP was able to inhibit the protective effect of guanosine against glutamate toxicity (Molz et al., 2011). In addition, we recently reported that the antidepressant-like effect of escitalopram in the forced swimming test was reversed by pretreatment of mice with L-arginine, a precursor of NO (Zomkowski et al., 2010). Furthermore, the antidepressant-like effects of imipramine and venlafaxine were also blocked by pretreatment with L-arginine (Dhir and Kulkarni 2007; Harkin et al., 2004). Several studies have shown that L-arginine and SNAP reversed the antidepressant-like effects of compounds with antidepressant action such as adenosine (Kaster et al., 2005) and folic acid (Brocardo et al., 2008). In addition, studies have demonstrated that 7-nitroindazole (a specific nNOS inhibitor) potentiated the antidepressant-like effect of escitalopram (Zomkowski et al., 2010), venlafaxine (Dhir and Kulkarni 2007), imipramine and fluoxetine (Harkin et al., 2004). Therefore, our results indicate that the neuroprotective effects of escitalopram and duloxetine involves its ability to modulate the production of NO in order to prevent cell damage. The exact mechanism by which these antidepressants produce neuroprotective effect is not completely elucidated, but it is possible that they mediated a blockade of NMDA receptor, with the consequent inhibition of NO production. Other possibility is that the effect would be indirectly dependent on an increase in the release of 5-HT, with the consequent reduction in NO levels.

Dysfunction of neurotrophic and neuroplastic pathways has emerged as a major pathological feature in depression (Pittenger and Duman, 2008). Many studies have demonstrated alterations in neuroplastic-related signal transduction and gene transcription cascades (Marsden, 2011). The glutamatergic system is linked to these pathways and this is also consistent with glutamatergic dysfunction in depression. The antidepressants also tend to converge to neurotrophic and neuroplastic pathways (Pittenger and Duman, 2008, Schmidt et al., 2008). It has been reported that PI3K/Akt pathway is involved in behavioral properties of antidepressants (Polter et al., 2012; Beech and Duman, 2005). In addition, it was shown that PI3K activity was decreased in suicide victims (Dwivedi et al., 2008). Here we show that the protective effect of escitalopram and duloxetine against the glutamate-induced cell damage was prevented by LY294002, an inhibitor of PI3K. These results suggest that the neuroprotective effect of these antidepressants could be due to the activation of PI3K pathway and are consistent with the fact that excitotoxicity induced by overactivation of glutamate receptors induce the inactivation of Akt (Chuang, 2005; Luo et al., 2003). Similar to our results, LY294002 was reported to prevent the neuroprotective effect of guanosine in hippocampal slices submitted to glutamate-induced toxicity in rats (Molz et al., 2011). In addition, a study demonstrated that *Aloysia gratissima*, a medicinal herb, protected hippocampal slices against glutamate excitotoxicity through activation of Akt pathway and decrease of iNOS expression (Zeni et al., 2011). Also, LY294002 blocked the increased phosphorylation of Akt-1 in PC12 cells induced by fluvoxamine (Nakano et al., 2010).

It is reported that acute and chronic treatment with antidepressants causes a reduction of glutamate release under basal (Bonanno et al., 2005; Michael-Titus et al., 2000) and depolarizing conditions (Michael-Titus et al., 2000). Furthermore, chronic treatment with desipramine, fluoxetine or venlafaxine completely abolished the stress-induced up-regulation of glutamate release (Musazzi et al., 2010). In addition, a microdialysis study showed that the acute administration of desipramine, imipramine and citalopram in prefrontal cortex of rats and the administration of α_2-adrenergic or 5-HT_{1B} receptor agonists, significantly decreased veratridine-stimulated glutamate and aspartate release. These results indicate that the regulation of glutamate release by antidepressants may be mediated through a direct effect on sodium channels or indirectly by involvement of α-2 or 5-HT_{1B} heteroceptors activated by the increased level of monoamines in response to the

blockade of respective transporters (Golembiowska and Zylewska, 1999). It was shown that the glutamate release in cerebral cortex synaptosomes is inhibited by 5-HT acting at 5-HT_{1D} receptors (Maura et al., 1998). Furthermore, a study reported that NE inhibits veratridine-induced glutamate release in cortical slices (Crowder and Bradford, 1987). Glutamate-induced cell death in hippocampal slices can occur due to activation of NMDA receptors (Molz et al., 2008b). Moreover, glutamate-induced toxicity promotes an increased glutamate release due to the reversed operation of glutamate transporters (Molz et al., 2008b), thus elevating extracellular glutamate levels. Our results showed that the hippocampal slices incubation with escitalopram and duloxetine prevented glutamate-induced glutamate release from hippocampal slices. Thus, the neuroprotective effect of these antidepressants may be due to its ability to reduce the extracellular levels of glutamate and prevent excitotoxicity, or indirectly by the involvement of 5-HT or NE receptors activation by the increased levels of these monoamines. Our results together with literature data suggest that the ability of antidepressant drugs to modulate glutamate neurotransmission may be an essential component of the therapeutical action of these drugs.

Impairment of glutamate transport, excitotoxiciy and activation of cell death cascades resulting in neuronal damage are processes involved in neurodegenerative disorders in the CNS and in the pathogenesis of depression (Sanacora et al., 2008; Tanis and Duman, 2007; Mattson and Magnus, 2006). Therefore, our results provide evidence that escitalopram and duloxetine exert a neuroprotective action possibly acting through the modulation of NMDA receptors and glutamate transport in mice hippocampal slices. In addition, our results also point to the involvement of NO and PI3K pathways in the neuroprotective effect of escitalopram and duloxetine. These antidepressants can be considered as potential neuroprotective strategies for depression and associated neurodegenerative disorders.

Acknowledgements

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (**FINEP) Research Grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" #01.06.0842-00** and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). ALS Rodrigues, CI Tasca and NH Gabilan are recipients of CNPq fellowship.

Conflict of interest
The authors declare that they have no conflicts of interest.

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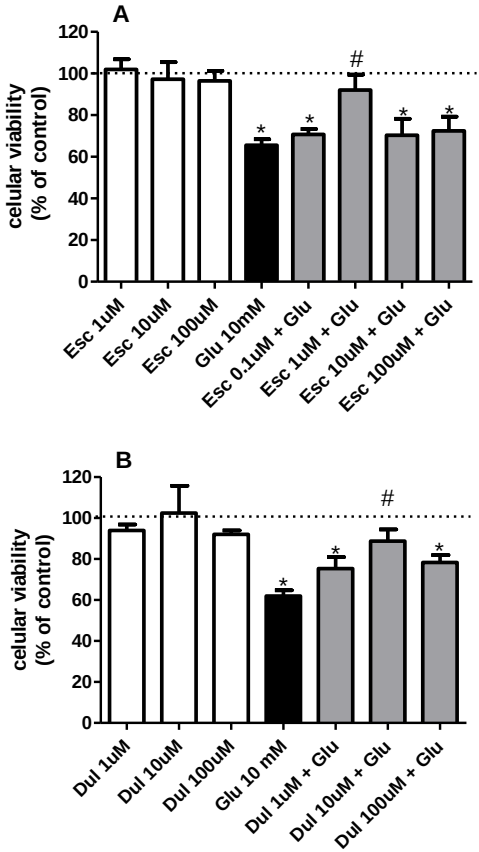

Fig.1. Cell viability of hippocampal slices subjected to glutamate in the presence of (A) escitalopram or (B) duloxetine. Hippocampal slices were incubated for 1 h with 10 mM glutamate (Glu). After this period, incubation medium was withdrawn and replaced with fresh culture medium without glutamate, and cells were maintained for an additional 6 h. When present, escitalopram (Esc) (0.1, 1, 10 or 100 μM) or duloxetine (Dul) (1, 10 or a 100 μM) was preincubated for 30 min before the addition of glutamate and maintained during glutamate exposure. The control group was considered as 100% viable and represents cell viability of slices incubated only in culture medium. MTT (0.5 mg/ml) was incubated for 20 min at 37°C, and cell viability was accessed at 550 nm. The values are expressed in % of cellular viability and represent mean ± SEM of five experiments carried out in triplicates. * represents means significantly different from control groups, $P<0.05$; # represents mean significantly different from Glu and all other groups in the presence of Glu, $P<0.05$ (ANOVA followed by Duncan's test).

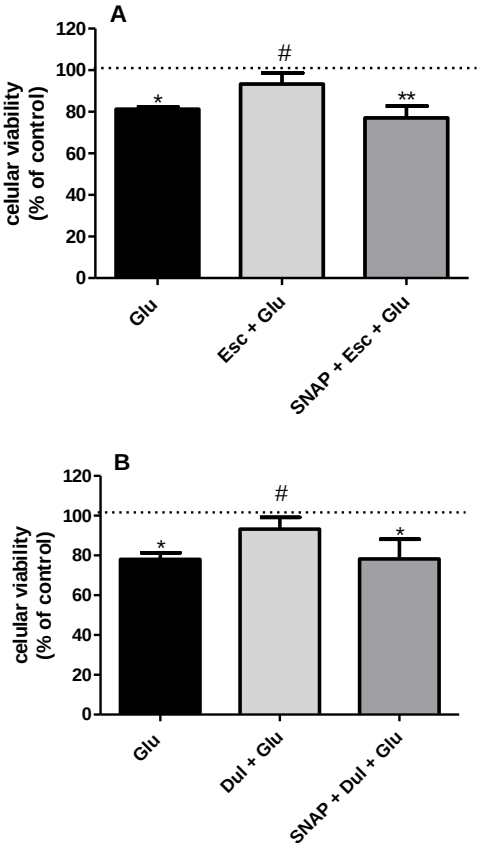

Fig. 2. The neuroprotective effect of escitalopram (Esc) (A) or duloxetine (Dul) (B) against glutamate-induced cell damage is prevented by a NO donor. Hippocampal slices were incubated for 1 h with 10 mM glutamate (Glu) in the presence or absence of Esc (1 µM) or Dul (10 µM). When present, Esc or Dul was preincubated for 30 min and SNAP (1 mM) was added to incubation medium 15 min before Esc, Dulox and/or Glu and maintained during the 1 h incubation period. After that, incubation medium was withdrawn and replaced by fresh culture medium without glutamate, and cells were maintained for an additional 6 h. Control group was considered as 100% and represents cell viability of slices incubated only in culture medium. The values are expressed in % of cellular viability, as measured by the MTT reduction assay and represent mean ± SEM of five experiments carried out in triplicates. * represents means significantly different from control groups, $P<0.05$ and ** represents means significantly different from control groups, $P<0.01$; # represents mean significantly different from Glu and all the other Glu groups, $P<0.05$.

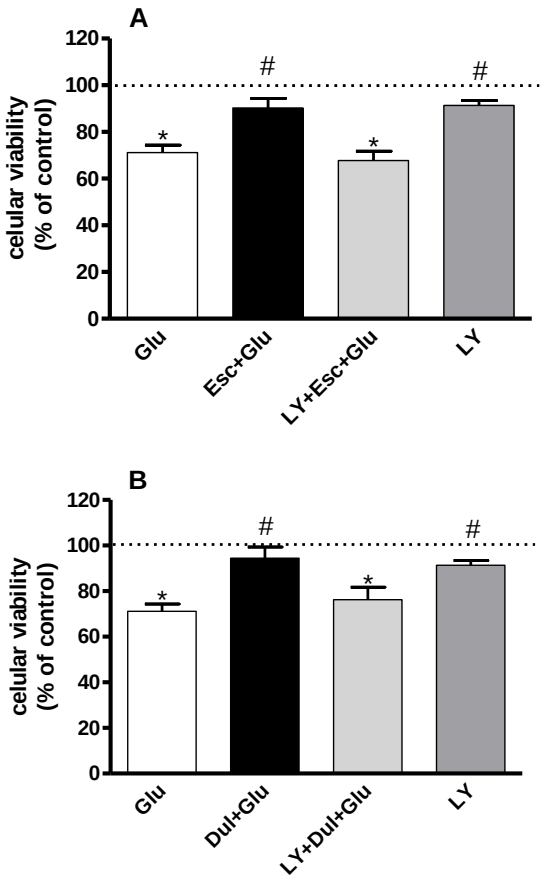

Fig. 3. The inhibition of PI3K pathway with LY294002 prevents the neuroprotective effect of Escitalopram (Esc) (A) or Duloxetine (Dul) (B) against glutamate-induced cell death. Hippocampal slices were incubated for 1 h with 10 mM glutamate (Glu) in the presence or absence of Esc (1 µM) or Dul (10 µM), preincubated for 30 min before the addition of Glu. LY294002 (30µM) was added to the incubation medium 15 min before Esc or Dul and maintained during the preincubation period. After this period, incubation medium was withdrawn and replaced with fresh culture medium without glutamate, and cells were maintained for additional 6 h. The control group was considered as 100% viable and represents cell viability of slices incubated only in culture medium. The values are expressed in % of cellular viability, as measured by the MTT reduction assay and represent mean ± SEM of five experiments carried out in triplicates. * represents mean significantly different from control groups, $P<0.05$; # represents mean significantly different from Glu and all the other Glu groups, $P<0.05$.

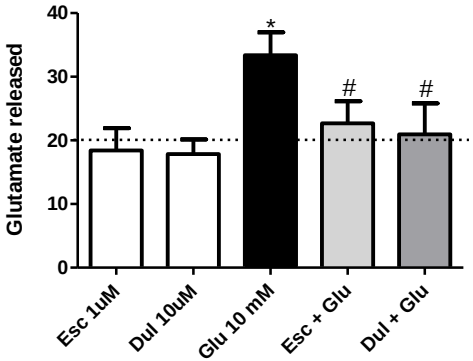

Fig. 4 Glutamate release from hippocampal slices challenged with glutamate (Glu) in the presence or not of Escitalopram (Esc) or Duloxetine (Dul). Hippocampal slices were incubated for 15 min with 10 mM Glu in the presence or absence of Esc or Dul. When present Esc (1 µM) or Dul (10 µM) was preincubated for 15 min. Glutamate release was assessed as described in Experimental procedures. Results were expressed as percentage of total D-[^{3}H] aspartate and represent means ± SEM of five experiments carried out in triplicates. * represents mean significantly different from control groups, $P<0.05$; # represents mean significantly different from Glu group, $P<0.05$.

4 DISCUSSÃO

A depressão é um transtorno de humor comum associado com altas taxas de recorrência, recidiva, comprometimento psicossocial e suicídio (Wong; Licinio, 2001). Existem várias classes de antidepressivos usados para o tratamento da depressão como os tricíclicos, os iMAO, os ISRS e os IRSN. No entanto, estes fármacos proporcionam uma completa remissão para apenas cerca de 50% dos indivíduos, sendo que mais de 80% dos pacientes exibam respostas parciais (Nestler, 2002), além de causarem efeitos adversos (Brunello et al., 2002). Além disto, a resposta terapêutica destes fármacos só ocorre 3-5 semanas após o início do tratamento (Brunello et al., 2002). Desta forma, existe uma grande necessidade do desenvolvimento de terapias antidepressivas alternativas ou de substâncias que possam aumentar a eficácia clínica no tratamento da depressão. Além da hipótese monoaminérgica, outros sistemas estão envolvidos na patologia da depresssão como o sistema glutamatérgio, a via da L-arginina-NO, alterações nas vias de sinalização celular, diminuição da neuroplasticidade e morte neuronal (Skolnick, 1999; Duman, 2002; Brocardo et al., 2008; Ng et al., 2008). Vários compostos antidepressivos são também neuroprotetores por induzirem a expressão de proteínas envolvidas na sobrevivência celular. Dessa forma, considerando estes fatores envolvidos na patogênese da depressão, neste estudo foram investigados i) o envolvimento dos receptores NMDA e da via L-arginin-NO-GMPc no efeito antidepressivo do escitalopram e da duloxetina no TNF; ii) o envolvimento das vias de sinalização celular (PKA, PKC, PI3K, MAPK/ERK, CaMKII no TNF; iii) o efeito neuroprotetor do escitalopram e da duloxetina frente a excitotoxicidade induzida pelo GLU em fatias hipocampais.

Tanto o TNF como o TSC, são aceitos como modelos animais de depressão amplamente usados para a triagem de novas fármacos antidepressivas. O TNF e o TSC são bastante sensíveis e relativamente específicos para a maioria das classes de medicamentos antidepressivos, incluindo antidepressivos tricíclicos, ISRS, iMAO, e atípicos (Porsolt et al, 1977; Steru et al, 1985). Os medicamentos antidepressivos reduzem o tempo de imobilidade de ratos em ambos os testes (Porsolt et al, 1977; Steru et al, 1985).

Os resultados apresentados mostram que o escitalopram (ISRS) e a duloxetina (IRSN) administrados intraperitonealmente (i.p.) ou oralmente (p.o.), produziu efeito antidepressivo em camundongos quando avaliados no TNF, que é a ferramenta mais utilizada para avaliar a atividade antidepressiva em estudos pré-clínicos (Cryan et al., 2002). A ação antidepressiva destes compostos administrados i.p., foi confirmada no TSC. Nossos resultados, confirmam os resultados obtidos previamente com o escitalopram (Sánchez et al.,2003) e com a duloxetina (Rénéric e Lucki, 1998; Ciulla et al., 2007) no TNF em ratos.

O papel dos receptores NMDA na fisiopatologia da depressão tem sido demonstrado. Estudos pré-clínicos e clínicos sugerem que antagonistas do receptor NMDA e compostos que bloqueiam os receptores NMDA apresentam atividade antidepressiva (Paul; Skolnick, 2003; Zarate et al., 2006; Garcia et al., 2008). Além disso, os antidepressivos reduzem o binding, a expressão e a função do receptor NMDA (Paul et al., 1994; Boyer et al., 1998; Golembiowska e DziubinA, 2000; Paul; Skolnick, 2003). Devido á importância desses receptores na fisiopatologia da depressão e no mecanismo de ação de antidepressivos, este estudo investigou primeiramente o envolvimento dos receptores NMDA no efeito antidepressivo do escitalopram e da duloxetina no TNF em camundongos.

Os resultados deste trabalho mostram que o pré-tratamento com NMDA (agonista do receptor NMDA) previniu completamente o efeito antidepressivo do escitalopram e da duloxetina no TNF. Portanto, nossos dados sugerem que a inibição da ativação do receptor NMDA é importante para o efeito destes antidepressivos. Um estudo demonstrou que antidepressivos inibem o receptor NMDA (Szasz et al., 2007). Além disso, Poleszak et al. (2007) mostraram que a ativação de receptores NMDA por NMDA e D-serina reverte o efeito anti-imobilidade de antagonistas NMDA no TNF. O efeito antidepressivo da fluoxetina, imipramina e reboxetina foi revertido pela D-serina (Poleszak et al., 2011) e do líto foi revertido pelo NMDA (Ghasemi et al., 2010). Além disso, o NMDA reverteu o efeito de compostos com ação antidepressiva (Brocardo et al., 2008; Moretti et al., 2011; Zeni et al., 2011). Nossos resultados também mostram um efeio sinérgico do tratamento combinado de MK-801 (um antagonista não competitivo do receptor NMDA) com duloxetina no TNF. É bem estabelecido na literatura que antagonistas de receptores NMDA possuem propriedades antidepressivas (Skolnick, 1999; Pual; Skolnick, 2003) e são capazes de potencializar a atividade de antidepressivos (Rogoz et al., 2002; Rosa et al., 2003; Ghasemi et al., 2009).

Várias evidências mostram que os sistemas serotoninérgico, noradrenérgico e glutamatérgico interagem no mecanismo de ação de antidepressivos (Bonanno et al., 2005; Pittaluga et al., 2007). De fato, a SE e a NA podem inibir a liberação de GLU (Travagli; Willians, 1996; Forray et al., 1999; Maura et al., 2000). Além disso, tem sido demonstrado que tratamento combinado de antidepressivos com amantadina (um antagonista NMDA) aumenta a liberação cortical de SE em ratos (Owen; Whitton, 2005). Como foi demonstrado por nossos resultados, o efeito sinérgico da duloxetina com o MK-801, pode ser uma consequencia de seus efeitos sobre a liberação de central de SE.

NO, é uma molécula sinalizadora no SNC, sendo sintetizado a partir de L-arginina pela NOS seguindo a ativação do receptor NMDA. NO pode, por sua vez, ativar a GCs que gera GMPc, que medeia muitos dos efeitos do NO (Denninger; Marletta, 1999). A via L-arginina-NO-GMPc está implicada na fisiopatologia da depressão e nos mecanismos de ação de vários compostos que apresentam efeitos semelhantes á antidepressivos no TNF (Kaster et al., 2005a; Almeida et al., 2006; Kulkarni; Dhir, 2007). Diante destas informações, investigamos o envolvimento da via da L-aginina-NO-GMPc no efeito tipo antidepressivo do escitalopram e da duloxetina no TNF.

Nossos resultados demonstram que o pré-tratamento com o precursor de NO, L-arginina, preveniu significativamente o efeito anti-imobilidade do escitalopram e da duloxetina no TNF. Além disso, o pré-tratamento dos camundongos com SNAP, um dador de NO, reverteu o efeito antidepressivo da duloxetina no TNF. Alguns estudos têm mostrado que a L-argina bloqueou o efeito de antidepressivos (Dhir; Kulkarni, 2007; Ghasemi et al., 2008) e de compostos com ação antidepressiva (Brocardo et al., 2008; Moretti et al., 2011; Zeni et al., 2011). Além disso, o SNAP bloqueou o efeito tipo a antidepressivo de alguns compostos com ação antidepressiva (Almeida et al., 2006; Moretti et al., 2011). Joca e Guimarães (2006), mostraram que a redução dos níveis de NO no hipocampo pode induzir a efeitos semelhantes a antidepressivos. Além disso, tem sido sugerido que a inibição da NOS poderia ser utilizada como uma estratégia para melhorar a eficácia clínica de antidepressivos serotoninérgicos (Harkin et al., 2004). Portanto, nossos resultados indicam que o efeito do escitalopram e da duloxetina pode ser dependente, pelo menos em parte, pela inibição da síntese de NO.

Os dados deste trabalho também mostram que o pré-tratamento com uma dose sub-efetiva de 7-nitroindazol (inibidor específico NOS), azul de metileno (inibidor de ambas NOS e GCs) ou ODQ (um inibidor

específico da GCs) produziu um efeito antidepressivo sinérgico com o escitalopram e com a duloxetina. Estes conjuntos de resultados indicam que a inibição da síntese de NO está envolvida no efeito anti-imobilidade do escitalopram e da duloxetina na TNF. De fato, inibidores NOS exercem efeitos semelhantes a antidepressivos e potencializam a ação de antidepressivos (Harkin et al., 2004) e do MK-801 no TNF (Dhir; Kulkarni, 2008), demonstrando uma relação direta entre a inibição dos receptores NMDA e da síntese de NO no efeito destes compostos. Além disso, alguns trabalhos mostram que a inibição da NOS pode modular a liberação de SE (Kiss, 2000; Wegener et al., 2000). Portanto, não podemos descartar a possibilidade que o efeito destes antidepressivos possa ser devido a um aumento na liberação de SE.

Os resultados do presente estudo mostram que o pré-tratamento dos animais com sildenafil (um inibidor seletivo da PDE5) reverteu o efeito antidepressivo do escitalopram e da duloxetina no TNF. Estes dados corroboram com a idéia que o escitalopram e a duloxetina exercem seu efeito através de uma diminuição dos níveis de GMPc. Kaster et al. (2005b) mostrou que o sildenafil é capaz de reverter o efeito tipo antidepressivo do ODQ no TNF. Além disso, o efeito da venlafaxina (Dhir; Kulkarni, 2007) e de compostos com ação antidepressiva foi revertido pelo sildenafil (Brocardo et al., 2008; Zeni et al., 2011). Nossos resultados indicam que o escitalopram e a duloxetina exercem seus efeitos antidepressivos por reduzirem os níveis de GMPc.

Além da participação do sistema glutamatérgico e da via L-arginina-NO-GMPc, tem sido focalizado o papel para as vias de sinalização celular na etiologia da depressão e na ação de antidepressivos (Castrén; Rantamäki, 2010). O tratamento com antidepressivos ativa PKA, a CaMKII e modula as vias da PKC e a via da MAPK/ERK (Einat et al., 2003; Giambalvo; Price, 2003; Hashimoto et al., 2004; Taylor et al., 2005; Tardito et al., 2006). A PI3K também está envolvida na ação de antidepressivos (Beech; Duman, 2005). A administração crônica de antidepressivos superregula a via do AMPc em vários níveis, incluindo o aumento da expressão do CREB e do BDNF (Altar, 1999; D'Sa; Duman, 2002; Gur et al., 2007; Hayashi et al., 2008; Hashimoto, 2010). Considerando que a depressão está associada com prejuízo na plasticidade sináptica e na sobrevivência celular e que os antidepressivos exercem seus efeitos por ativarem as vias de sinalização celular e regularem a morte celular (D'Sa; Duman, 2002, Hashimoto et al., 2004), nesse estudo foram investigadas as vias de sinalização celular

envolvidas na ação antidepressiva do escitalopram e da duloxetina no TNF.

O pré-tratamento dos camundongos com H-89 (um inibidor da PKA), GF109203X (um inibidor da PKC), LY294002 (um inibidor da PI3K) ou U0126 (um inibidor da ERK) reverteu o efeito antidepressivo do escitalopram e da duloxetina no TNF. Porem, o pré-tratamento com KN-62 (um inibidor da CaMKII) não reverteu o efeito anti-imobilidade destes antidepressivos no TNF. Estudos tem mostrado que o aumento dos níveis de SE e NA induzido pelos antidepressivos, podem ativar as vias de sinalização celular (Hashimoto et al. 2004, Duman; Vloleti, 2012). As vias de sinalização ativam o CREB e este induz a expressão do BDNF, que está envolvido na sobrevivência celuar e plasticidade neuronal (Hashimoto et al., 2004; Duman; Voleti, 2012). Portanto, uma possibilidade para os efeitos do escitalopram e da duloxetina pode ser devido ao aumento dos níveis de SE e NA causado pelo tratamento com estes antidepressivos e desta forma estas monoaminas se ligam nos seus receptores específicos e ativam as vias se sinalização. Outra possibilidade para explicar o efeito destes antidepressivos, pode ser por atuarem como antagonistas do receptor NMDA e desta forma aumentam a liberação de SE. Corroborando com esta hipótese, nós mostramos neste estudo que o efeito antidepressivo do escitalopram e a da duloxetina é dependente do bloqueio do receptor NMDA. Tem sido demonstrado que antagonistas do receptor NMDA e inibidores NOS aumentam a liberação de SE em regiões do cérebro como o hipocampo e o córtex frontal (Callado et al., 2000; Smith; Whitton, 2000). Em conjunto, estes dados demonstram que o efeito antidepressivo do escitalopram e da duloxetina no TNF envolve a ativação das vias de sinalização celular como a PKA, PKC, PI3K e ERK.

O GLU é considerado o principal neurotransmissor excitatório do SNC de mamíferos. O GLU tem um papel na cognição, aprendizagem, memória e plasticidade sináptica. Contudo, quando ocorre algum desequilíbrio da transmissão glutamatérgica, a concentração de GLU pode se elevar na fenda sináptica e causar a superestimulação dos receptores de GLU, culminando com a morte neuronal excitotóxica. Muitas doenças neurodegenerativas, incluindo a depressão, são causadas pela superestimulação dos receptores de GLU. O hipocampo é uma das estruturas cerebrais mais susceptíveis à excitotoxicidade. Dessa forma, considerando o envolvimento do GLU na morte celular excitotóxica e o envolvimento nas doenças neurodegenerativas, neste trabalho estudamos os efeitos neuroprotetores da duloxetina e do escitalopram na excitotoxicidade induzida pelo GLU utilizando-se fatias de hipocampo

de camundongos, bem como o envolvimento das vias da NO e PI3K/AKT no efeito neuroprotetor destes compostos antidepressivos.

Nossos resultados demonstram que a pré-incubação das fatias hipocampais de camundongos com escitalopram e duloxetina preveniu a morte celular induzida pelo GLU. Molz et al. (2008b) mostrou que o GLU diminui significativamente a viabilidade celular em fatias de hipocampo de ratos, mostrando um padrão de morte celular apoptótica, prevenida pelo MK-801. Similar aos nossos resultados, a guanosina protegeu as fatias hipocampais de ratos da morte celular induzida pelo GLU (Molz et al., 2011). Consistentes com nossos resultados, Kamei et al (1991) demonstrou que o L-triptofano (um precursor de SE) previniu a excitotoxicidade induzida pelo GLU.

Nossos resultados demonstram que SNAP (um doador de NO) reverteu o efeito neuroprotetor do escitalopram e da duloxetina contra a toxicidade induzida pelo GLU. O NO tem um efeito significativo no SNC e a manipulação desta via constitui-se em novas possibilidades terapêuticas nas doenças neurodegenerativas, inclusive na depressão (Kulkarni; Dhir, 2007; Ulak et al., 2008). Nós demonstramos que o efeito antidepressivo do escitalopram foi revertido pela L-arginina no TNF (Zomkowski et al., 2010) e também demonstramos nesta Tese que o efeito antidepressivo da duloxetina foi revertido pelo SNAP e pela L-arginina no TNF. Vários dados da literatura demonstram um papel importante do sistema nitrérgico no mecanismo de ação de antidepressivos e compostos com ação antidepressiva (Dhir; Kulkarni 2007; Brocardo et al., 2008). Alguns trabalhos mostram que a liberação central de SE pode ser modulada pela inibição da NOS (Kiss, 2000; Smith; Whitton, 2000). Além disso, Szasz et al. (2007) demonstram que antidepressivos se ligam no receptor NMDA e atuam diretamente como antagonistas. Portanto, nossos dados sugerem que o efeito neuroprotetor do escitalopram e da duloxetina envolve sua capacidade de modular a quantidade de NO, e desta forma, evita a morte celular por um mecanismo que pode ser dependente da inibição da síntese de NO. Além disso, não podemos descartar a hipótese que o efeito neuroprotetor possa ser indiretamente dependente de um aumento na liberação de SE.

Nossos dados mostram que o efeito neuroprotetor do escitalopram e da duloxetina foi inibido pelo LY294002, um inibidor da PI3K. Estes resultados sugerem que o efeito protetor destes antidepressivos pode ser devido à ativação da via PI3K. Esta via está envolvida nos efeitos de agentes neuroprotetores contra a excitotoxicidade induzida pelo GLU (Tolosa et al., 2008; Herculano et al., 2010). Na patologia da depressão as vias neurotróficas estão

alteradas. (Duman; Voleti, 2012). Tem sido demonstrado que o tratamento com estabilizadores do humor ativa a via da PI3K (Hashimoto et al., 2004). Além disso, a ativação desta via tem um importante papel na captação de GLU, bem como, no trafico dos transportadores de GLU e expressão na membrana celular (Guillet et al., 2005). A excitotoxicidade induzida pela superestimulação dos receptores de GLU induz a inativação da Akt (Luo et al., 2003).

No presente estudo, a pré-incubação das fatias hipocampais com escitalopram e duloxetina previniu a liberação de GLU. É bem conhecido que o estresse agudo está associado com aumento da neurotransmissão glutamatérgica no hipocampo e na amígdala (Lowy et al., 1995; Reznikov et al., 2007), e o tratamento crônico com antidepressivos causa uma redução na liberação de GLU (Bonanno et al., 2005). Alguns trabalhos mostram que a liberação de glu é inibida pela SE (Maura et al., 1998) e pela NA (Crowder; Bradford, 1987). A morte celular induzida pelo GLU nas fatias hipocampais pode ocorrer por atuar nos receptores NMDA (Molz et al., 2008b). Portanto, nossos resultados demonstram que o escitalopram e a duloxetina previnem a liberação de GLU, sugerindo que o efeito neuroprotetor destes antidepressivos pode ser devido a redução dos níveis extracelulares de GLU. Além disso, o efeito neuroprotetor destes antidepressivos pode ser indiretamente devido a ativação dos receptores de SE ou NA.

Em conjunto, estes dados mostram que o efeito neuroprotetor dos antidepressivos envolve possivelmente a modulação dos receptores NMDA, bem como o envolvimento das vias do NO e PI3K.

Finalmente, podemos propor através dos dados apresentados nessa Tese, que o efeito antidepressivo do escitalopram e da duloxetina é dependente do bloqueio do receptor NMDA, da inibição da síntese de NO e envolve a ativação das vias de sinalização celular (PKA, PKC, PI3K e MAPK/ERK). Em situação de excitotoxicidade estes antidepressivos apresentam um efeito neuroprotetor e envolve as vias NO e PI3K. Portanto, estes resultados podem contribuir para o entendimento dos transtornos depressivos e para o desenvolvimento de novas alternativas terapêuticas para o seu tratamento (Figura 4).

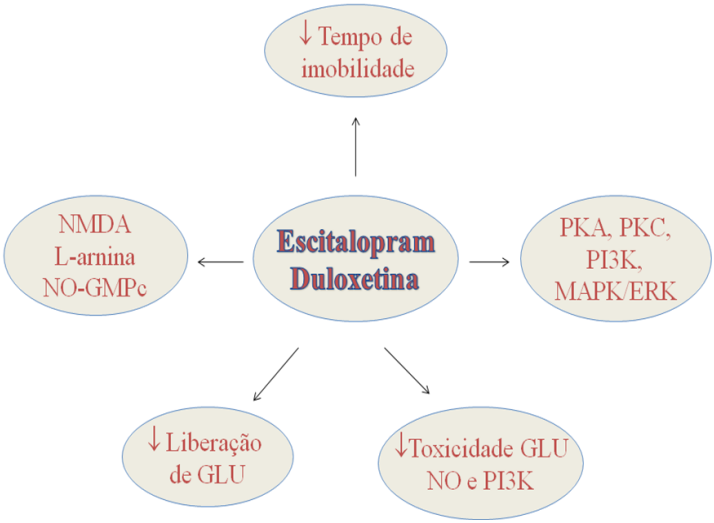

Figura 4. Efeito antidepressivo do escitalopram e da duloxetina. Envolvimento do sistema glutamatérgico, da via L-arginina-NO, das vias de sinalização celular e efeito neuroprotetor.

5 CONCLUSÕES

- O escitalopram (ISRS) e a duloxetina (IRSN), produziram um efeito antidepressivo no TNF em camundongos, quando administrados por via i.p. e p.o., confirmando dados obtidos previamente com o escitalopram (Sanches et al., 1998) e com a duloxetina (Rénéric; Lucki, 1998; Ciulla et al., 2007) no TNF em ratos. Além disso, a redução do tempo de imobilidade induzida por estes antidepressivos também foi mostrada no TSC.
- Nossos resultados indicam que o efeito antidepressivo do escitalopram e da duloxetina no TNF é dependente do bloqueio dos receptores NMDA e da inibição da NOS e da síntese de GMPc.
- Os resultados também sugerem que o efeito antidepressivo do escitalopram e da duloxetina no TNF parece envolver a modulação das vias de sinalização celular como a PKA, PKC, PI3K e MAPK/ERK, mas não com a CaMKII.
- A neuroproteção promovida pelo escitalopram e pela duloxetina frente ao dano celular induzido por glutamato em fatias hipocampais de camundongos, envolve diminuição da liberação de glutamato, ativação da via PI3K e modulação dos receptores NMDA.

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