Daiane Fátima Engel

# ESTUDO DO EFEITO NEUROPROTETOR DA DULOXETINA

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Orientador: Prof. Dr. Nelson Horácio Gabilan

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**DAIANE FÁTIMA ENGEL**

**"ESTUDO DO EFEITO NEUROPROTETOR DA DULOXETINA"**

Esta dissertação foi julgada e aprovada para a obtenção do Grau de Mestre em Neurociências na área de Neurobiologia Celular e Molecular no Programa de Pós-Graduação em Neurociências da Universidade Federal de Santa Catarina

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“O futuro pertence àqueles que acreditam na beleza de seus sonhos”.

(Elleanor Roosevelt)

## RESUMO

Alguns dos fatores envolvidos na patogênese da depressão são: níveis reduzidos de neurotrofinas, aumento do estresse oxidativo e morte de células neuronais; que levam a alterações estruturais no sistema límbico. O objetivo deste estudo foi investigar o mecanismo neuroprotetor do antidepressivo duloxetina, *in vivo* e *in vitro*. Inicialmente, foi avaliado o efeito da administração crônica (21 dias) de duloxetina (10mg/kg v.o.) sobre a expressão gênica de neurotrofinas e de proteínas apoptóticas, no córtex e no hipocampo de camundongos. A duloxetina aumentou o RNAm de BDNF e FGF-2 no córtex e no hipocampo; e de NT-3, Bcl-2 e Bcl-xL no córtex. Mais importante, o tratamento com duloxetina reduziu a expressão gênica das proteínas pró-apoptóticas Bax e p53 no hipocampo e de Bad no córtex. A duloxetina (2 µM) reduziu o estresse oxidativo (níveis de espécies reativas de oxigênio [ROS]) em células de neuroblastoma humano (SH-SY5Y) e preveniu da morte celular induzida pela rotenona. O efeito da duloxetina em reduzir os níveis de ROS e a morte celular foi prevenido pelo LY294002, um inibidor da via PI3K/Akt. Além disso, a duloxetina reverteu a inibição de Akt induzida por rotenona em células de neuroblastoma humano. Em conclusão, os dados *in vivo* e *in vitro* sugerem que estão envolvidos no efeito terapêutico da duloxetina: a ativação da via PI3K/Akt e aumento da expressão de neurotrofinas e de proteínas anti-apoptóticas e a redução das pró-apoptóticas e do estresse oxidativo. Estes fatores em conjunto atuariam na neuroplasticidade e na sobrevivência neuronal na depressão.



**Palavras-chave**: Duloxetina, neurotrofinas, proteínas apoptóticas, neuroproteção, via PI3K/Akt.

## ABSTRACT

Some of the factors involved in the pathogenesis of depression are: reduced levels of neurotrophins, increased oxidative stress and neuronal cell death; whose leads to structural changes in the limbic system. The aim of this study was to investigate the neuroprotective mechanism of the antidepressant duloxetine, *in vivo* and *in vitro*. Initially, was assessed the effect of duloxetine (10mg/kg p.o.) chronic administration (21 days) on the gene expression (mRNA) of neurotrophins and apoptotic proteins, in the cortex and hippocampus of mice. Duloxetine induced increased cortical and hippocampal mRNA levels of BDNF and FGF-2; cortical levels of NT-3, Bcl-2 and Bcl-xL. Most important, duloxetine reduced mRNA levels of the proapoptoic proteins Bax and p53 in the hippocampus, and Bad in the cortex. Duloxetine (2 μM) reduced oxidative stress (reactive oxygen species [ROS] levels) in human neuroblastoma cells (SH-SY5Y) and prevented rotenone-induced cell death. The duloxetine effect of reducing ROS and preventing cell death was abolished by LY294002, an inhibitor of PI3K/Akt pathway. Moreover, duloxetine reversed the inhibition of Akt induced by rotenone in human neuroblastoma cells. In conclusion, the *in vivo* and *in vitro* data suggest that are involved in the therapeutic effect of duloxetine: activation of the PI3K/Akt pathway and increased expression of neurotrophins and anti-apoptotic proteins and reduction of pro-apoptotic proteins and of the oxidative stress. These factors together would act on neuronal survival and neuroplasticity in depression.



**Keywords**: Duloxetine, neurotrophins, apoptotic proteins, neuroprotection, PI3K/Akt pathway.

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## LISTA DE ABREVIATURAS E SIGLAS

AIF: Fator indutor de apoptose
Akt : Serina treonina cinase ou proteína cinase B (PKB)
AMPc: Adenosina 3',5'-monofosfato cíclico
ASK1: Quinase reguladora do sinal de apoptose
BDNF: Fator neurotrófico derivado do cérebro
CaMKII: Proteína cinase II dependente de cálcio-calmodulina
CAT: Catalase
CREB: Proteína ligante ao elemento responsivo ao AMPc
DG: Giro denteado
ERK: Cinase regulada por sinal extracelular
FGF: Fator de crescimento de fibroblasto
FGFR: Receptor de FGF
FST: Teste do nado forçado
GDNF: Fator neurotrófico derivado da glia
GPx: Glutationa peroxidase
GR: Glutationa redutase
GSH: Glutationa
GSK-3β: Glicogênio sintetase cinase-3β
HO-1: Heme oxigenase-1
ISRS: Inibidor seletivo da recaptação de serotonina
JIP1: Proteína de interação com a JNK 1
JNK: Cinase c-Jun N-terminal
MAPK: proteína cinase ativada por mitógeno
Mcl-1: Proteína semelhante à Bcl-2
MDA: Malondialdeído
Mdm2: Proteína *murine doble minute 2*
NF-κB: Fator nuclear kapa B
NGF: Fator de crescimento do nervo
NO: Óxido nítrico
Nrf2: Fator nuclear (eritróide derivado do tipo 2)
NT-3: Neurotrofina 3
NT-4/5: Neurotrofina 4/5
$ONOO^-$: Peróxinitrito
PDK1: Cinase dependente de fosfatidilinositol 1
PIP2: Fosfolipídio de membrana fosfatidilinositol (4, 5)- difosfato
PIP3: Fosfatidilinositol (3, 4, 5)- trifosfato
PI3K: Fosfatidilinositol 3-cinase
PKC: Proteína cinase C
PLC: Fosfolipase C

ROS: Espécies reativas de oxigênio
SOD: Superóxido dismutase
Trk: Receptor tirosina cinase
VEGF: Fator de crescimento do endotélio vascular

**SUMÁRIO**

# 1. INTRODUÇÃO

## 1.1 Depressão e alterações em estruturas cerebrais

A depressão é uma doença crônica, recorrente, que reduz consideravelmente a qualidade de vida afetando mais de 14% da população e causa impacto significativo tanto para o indivíduo quanto para a sociedade (TANTI e BELZUNG, 2010). Apesar dos inúmeros estudos realizados, as bases biológicas da depressão e os possíveis mecanismos da eficácia dos compostos antidepressivos ainda não são bem conhecidos.

Por muitos anos, o sistema monoaminérgico foi considerado como a principal causa da depressão (D'SA e DUMAN, 2002; RACAGNI e POPOLI, 2010). Assim, a depressão resultaria da deficiência de serotonina, noradrenalina ou de seus receptores ineficientes, o que foi definido como *hipótese monoaminérgica* desta doença (KISS, 2008; WONG e LICINIO, 2001). Deste modo, drogas com ação antidepressiva bloqueiam a recaptação de serotonina e/ou noradrenalina, aumentando a concentração das mesmas na fenda sináptica. Contudo, o aumento nos níveis de monoaminas ocorre quase que imediatamente após o início do tratamento, mas os efeitos terapêuticos dos antidepressivos se manifestam somente após algumas semanas de tratamento (WONG e LICINIO, 2001).

Além da deficiência de monominas, algumas alterações cerebrais estruturais têm sido relacionadas com os transtornos depressivos e com a fisiopatologia da depressão (PITTENGER e DUMAN, 2008). Estudos de imagem *in vivo* demonstraram que pacientes depressivos apresentam uma redução no volume de estruturas límbicas, como o córtex pré-frontal e o hipocampo (BREMNER, 2002; CAMPBELL et al., 2004; VIDEBECH e RAVNKILDE, 2004). Vários autores sugerem que estas alterações resultam da atrofia e morte de neurônios e células gliais e de uma redução na plasticidade sináptica (neurogênese, ramificação axonal, dendritogênese e sinaptogênese) (KRISHNAN e NESTLER, 2008; MASI e BROVEDANI, 2011; PITTENGER e DUMAN, 2008). Diferentes estruturas cerebrais estão afetadas em pacientes depressivos. No córtex pré-frontal dorsolateral foi observada uma redução no tamanho dos neurônios e na densidade de células gliais (COTTER et al., 2002; PARADISE et al., 2012). Além disso, no córtex órbito-frontal lateral e no giro do cíngulo anterior foram encontradas alterações na densidade glial e neuronal (COTTER et al., 2002; ONGUR et al., 1998; RAJKOWSKA et al., 1999). No hipocampo ocorre uma redução no

tamanho do corpo celular dos neurônios, além da perda de arborização dendrítica e de processos gliais (HICKIE et al., 2005).

Estas alterações estruturais cerebrais possuem semelhança com a condição de "deprivação de fatores neurotróficos", o que foi chamado de *hipótese neurotrófica* da depressão. Assim, uma das possíveis causas para estas alterações parece ser a redução de fatores neurotróficos e a consequente ativação das vias de morte celular (CASTREN, 2004; NESTLER et al., 2002; SCHLOSS e HENN, 2004)

### 1.1.1 Depressão e neurotrofinas

As neurotrofinas ou fatores neurotróficos são uma família de proteínas que promovem a diferenciação, desenvolvimento e sobrevivência neuronal, além de modular a formação e a plasticidade das sinapses (POO, 2001; REICHARDT, 2006). Anteriormente, se acreditava que a atuação das neurotrofinas estaria restrita ao desenvolvimento embrionário, mas atualmente está estabelecido que elas possuem papel essencial na plasticidade de neurônios adultos (REICHARDT, 2006). Um neurônio pode ser responsável por sintetizar mais de uma neurotrofina em diferentes momentos do desenvolvimento. Assim, os efeitos dos fatores neurotróficos não estão restritos aos neurônios pós-mitóticos, podendo atuar tanto em neuroblastos, como em neurônios maduros, que já estabeleceram contatos sinápticos estáveis (REICHARDT, 2006; SKAPER, 2008).

A primeira neurotrofina identificada foi o fator de crescimento do nervo (NGF) que despertou o interesse na ação dos fatores neurotróficos (COHEN et al., 1954; LEVI-MONTALCINI e COHEN, 1956). Os dendritos secretam quantidades limitantes de fatores neurotróficos que atuam retrogradamente nos terminais pré-sinápticos. Uma das funções destes fatores é equilibrar o tamanho do tecido alvo com o número de neurônios que o inerva (POO, 2001; REICHARDT, 2006). Diversas populações de células não são responsivas ao NGF. Isto levou à caracterização da segunda neurotrofina designada fator neurotrófico derivado do cérebro (BDNF), caracterizada como a mais abundante do sistema nervoso central (BARDE et al., 1982; SKAPER, 2008). Em seguida, também foram identificados outros fatores como: as neurotrofinas 3 (NT-3) e 4/5 (NT-4/5), o fator neurotrófico derivado da glia (GDNF), fator de crescimento do fibroblasto (FGF) e o fator de crescimento vascular endotelial (VEGF) (POO, 2001; SKAPER, 2008).

A ação das neurotrofinas é mediada primariamente através da família de receptores de superfície de membrana tirosina cinase (Trk)

(Figura 1), que desempenham papel fundamental na comunicação intracelular. Estes receptores interagem com diversos sistemas de segundos mensageiros intracelulares induzindo respostas neuroquímicas geralmente de médio e longo prazo. Assim, o NGF ativa receptores TrkA, BDNF e NT-4/5 ativam receptores TrkB e NT-3 ativa TrkC (PATAPOUTIAN e REICHARDT, 2001).

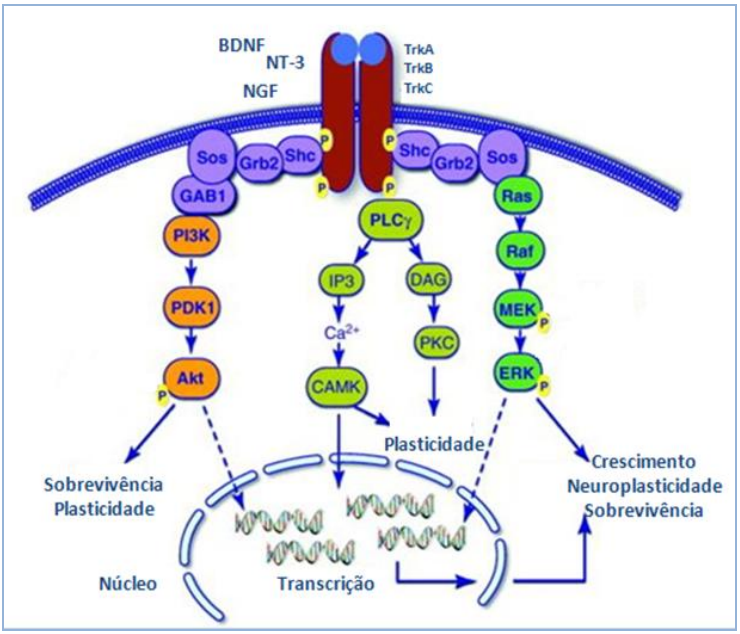


**Figura 1.** Efeitos biológicos das neurotrofinas e suas vias de sinalização celular. As neurotrofinas (BDNF, NT-3 e NGF) se ligam ao domínio extracelular do receptor Trk induzindo dimerização e ativação do domínio tirosina cinase intracelular. Isto resulta na autofosforilação dos resíduos de tirosina que então servem como sítio de interação com proteínas adaptadoras e ativação de cascatas de sinalização intracelular: PI3K-Akt (fosfatidilinositol 3-cinase) – (serina treonina cinase ou proteína cinase B), PLC (fosfolipase C) e Ras-MAPK (proteína cinase ativada por mitógenos). Estas vias modulam a plasticidade sináptica, sobrevivência, crescimento ou proliferação e diferenciação celular (DUMAN e VOLETI, 2012).

A ação das neurotrofinas diretamente nos terminais sinápticos ou indiretamente, no núcleo, resulta na modulação da sobrevivência, proliferação celular e função sináptica. No terminal sináptico os fatores neurotróficos atuam sobre o arranjo do citoesqueleto e sobre a atividade

de várias proteínas, canais iônicos e receptores (HUANG e REICHARDT, 2003). Além disso, no terminal pré-sináptico as neurotrofinas atuam sobre a síntese, o número de vesículas de armazenamento e a liberação de neurotransmissores (BIBEL e BARDE, 2000). No núcleo, as neurotrofinas controlam processos de efeito mais lento, como a transcrição gênica, a expressão de várias proteínas e a ativação ou repressão de genes responsáveis pelo desenvolvimento neuronal (BRONFMAN et al., 2007).

Estudos com pacientes depressivos demonstraram uma redução na expressão de vários genes associados com a família do FGF no córtex frontal (EVANS et al., 2004). Além disso, os níveis das neurotrofinas NT-3 e NT-4/5 estão significantemente diminuídos no hipocampo de vítimas de suicídio, quando comparados com cérebros de indivíduos normais (DWIVEDI et al., 2005). Análises *postmortem* do hipocampo e do córtex pré-frontal de pacientes suicidas apresentaram uma expressão reduzida (de RNAm e dos níveis de proteína) de BDNF e de seu receptor TrkB (DWIVEDI et al., 2005). Além do BDNF, os níveis de NGF também estão diminuídos no soro de pacientes depressivos (DINIZ et al., 2012; HASHIMOTO, 2010). Estes dados podem ser reproduzidos em animais submetidos a modelos de depressão e/ou estresse crônico, que apresentam níveis reduzidos de BDNF, NGF e NT-3 em diferentes áreas cerebrais (NIBUYA et al., 1995; REUS et al., 2011; SMITH et al., 1995).

A redução dos níveis de fatores de crescimento prejudica a sobrevivência celular e a neuroplasticidade. O NGF, por exemplo, atua sobre o alongamento axonal durante o desenvolvimento (HUANG e REICHARDT, 2003). Além disso, o NGF é essencial aos neurônios colinérgicos do prosencéfalo basal, protegendo estas células da degeneração que ocorre com o envelhecimento. Na doença de Alzheimer o suporte de NGF às células está prejudicado ocorrendo uma maior perda de neurônios colinérgicos (CATTANEO e CALISSANO, 2012). Além disso, o NGF aumenta a sobrevivência de neurônios noradrenérgicos e de células PC12 (COWEN, 2007; CROWDER e FREEMAN, 1998; YAO e COOPER, 1995). O BDNF e seus receptor TrkB, aumentam a quantidade de neurotransmissor liberado na fenda sináptica, modulando a neurotransmissão (BIBEL e BARDE, 2000). Além deste efeito sobre a atividade sináptica, o BDNF também estimula a neurogênese e a sobrevivência neuronal. Johnson-Farley e colaboradores (2006) demonstraram que o BDNF inibe a apoptose em culturas hipocampais. O FGF-2 e seu receptor (FGFR) atuam na manutenção das interações recíprocas entre neurônios e glia no bulbo de

camundongos adultos e idosos (WANG et al., 2009). Além disso, o FGF-2 está envolvido na sobrevivência de neurônios dopaminérgicos *in vivo* e regula o desenvolvimento de células progenitoras dopaminérgicas *in vitro* (RATZKA et al., 2012). Em modelos animais da Doença de Huntington, o FGF-2 induziu efeito neuroprotetor e proliferativo (JIN et al., 2005). Pericitos (células associadas a vasos sanguíneos) submetidos à hipóxia produzem maior quantidade de NT-3. Em astrócitos, a NT-3 ativou a via ERK e induziu uma maior produção de NGF (ISHITSUKA et al., 2012). Além disso, a NT-3 aumentou a sobrevivência de neuroblastos noradrenérgicos em divisão e de neurônios noradrenérgicos. Estes neurônios posteriormente substituem sua dependência trófica por NGF (SKAPER, 2008). Esta substituição revela outra característica sobre a ação das neurotrofinas: o desenvolvimento de certas populações neuronais requer a ação sequencial de múltiplas neurotrofinas, além da expressão coordenada de seus receptores específicos (SKAPER, 2008).

### 1.1.2 Depressão e estresse oxidativo

Vários estudos demonstram que o estresse oxidativo está envolvido na morte neuronal associada a diversas doenças neurodegenerativas, como a Doença de Alzheimer, Doença de Parkinson, Esclerose lateral amiotrófica e a depressão (NAKAMURA et al., 2012). Em condições de estresse oxidativo espécies reativas de oxigênio (ROS) são geradas em grande quantidade induzindo dano e morte celular (VALENCIA e MORAN, 2004).

O ROS anion superóxido é gerado pela mitocôndria como subproduto da respiração celular e pode reagir com óxido nítrico produzindo uma forma ainda mais tóxica, o peróxinitrito ($ONOO^-$). Por outro lado, a enzima superóxido dismutase (SOD) converte radicais superóxido em outro tipo de ROS, o peróxido de hidrogênio, e este pode reagir com ferro (II) produzindo radicais hidroxila (reação de Fenton). Em condições fisiológicas, mais de 2% dos elétrons na cadeia transportadora de elétrons podem gerar anion superóxido que são, em grande parte, detoxificados pelos sistemas antioxidantes celulares (FUKUI e MORAES, 2008). Dentre as principais defesas antioxidantes enzimáticas podem ser citadas: a SOD, glutationa peroxidase (GPx), glutationa redutase (GR) e a catalase (CAT), além de defesas não enzimáticas como a glutationa (GSH). O equilíbrio da atividade e dos níveis intracelulares destas defesas antioxidantes é essencial para a homeostase celular (VALKO et al., 2007).

Entretanto, quando células como os neurônios estão sob uma ação estressora ou um sistema antioxidante insuficiente, a mitocôndria pode produzir altos níveis de ROS, contribuindo com o processo de neurodegeneração (FUKUI e MORAES, 2008). Nestas condições quantidades excessivas de ROS induzidas pelo estresse oxidativo podem atuar sobre proteínas, lipídeos e DNA, alterando sua função e ativando vias de sinalização levando a apoptose de células neuronais (KIM et al., 2005). O aumento dos níveis de ROS provoca a peroxidação lipídica de membranas biológicas, perda na fluidez da membrana celular, perda do potencial de membrana e eventual lise celular, levando a liberação do conteúdo intracelular (RAY et al., 2012). Além disso, níveis elevados de ROS podem causar aumento na permeabilidade da barreira hematoencefálica, alterações na tubulina e perturbação da transmissão sináptica (BAXTER et al., 2006). O peróxido de hidrogênio é uma das ROS encontradas fisiologicamente na circulação sanguínea. Pacientes depressivos apresentam níveis plasmáticos de peróxido de hidrogênio e também de óxido nítrico (NO) aumentados, quando comparados com indivíduos saudáveis (MAES et al., 2010; SELEK et al., 2008). Os pacientes depressivos apresentam altos níveis séricos de malondialdeído (MDA), um indicador de peroxidação lipídica, e da enzima SOD (KHANZODE et al., 2003).

Assim como foi observado em humanos depressivos, camundongos e ratos submetidos a modelos de estresse também apresentaram aumento dos níveis de substâncias reativas ao ácido tiobarbitúrico, como o MDA. Além disso, os animais apresentaram alterações na atividade e/ou na expressão das enzimas CAT, GPx, GR e SOD no hipocampo e no córtex cerebral (BUDNI et al., 2012; LEE et al., 2011; MORETTI et al., 2012; ZAFIR e BANU, 2009)

### 1.1.3 Depressão e apoptose

A apoptose, ou morte celular programada, é um mecanismo altamente regulado e geneticamente determinado. Neste processo são observadas alterações morfológicas celulares bem definidas: contração da célula, condensação da cromatina, fragmentação do DNA. A apoptose é um processo que pode ocorrer em todas as células nucleadas como resposta a estímulos fisiológicos, patológicos e oxidativos (DANIAL e KORSMEYER, 2004). A apoptose ocorre fisiologicamente na substituição programada das células durante a embriogênese e morfogênese, na eliminação de células anormais, desnecessárias ou em excesso, permitindo o controle do número de células e do tamanho dos

tecidos (KROEMER et al., 2009). Por outro lado, a apoptose também pode ocorrer de forma patológica. Por exemplo, uma apoptose em excesso pode contribuir para doenças degenerativas, enquanto que a falta dela pode facilitar o desenvolvimento de tumores (MEIER et al., 2000). A apoptose pode ser desencadeada por diversos fatores como: deprivação de fatores neurotróficos, ligação de moléculas a receptores de membrana (receptores de morte), agentes quimioterápicos, radiação ionizante, danos no DNA, choque térmico, baixa disponibilidade de nutrientes e níveis elevados de estresse oxidativo. A ativação da apoptose pode ser iniciada de duas maneiras: pela via intrínseca (mitocondrial) ou pela via extrínseca (citoplasmática) (HENGARTNER, 2000).

A regulação do processo apoptótico depende da família de proteínas Bcl-2, como Bcl-2 e Bcl-xL, que inibem a apoptose, pois previnem a liberação de citocromo *c* e são chamados de reguladores antiapoptóticos. Por outro lado, Bax, Bad Bid e Bak são proteínas pró-apoptóticas. A Bcl-2 tem função neurotrófica, atuando na ramificação dendrítica e regeneração axonal (SAPOLSKY, 2000). Além disso, esta proteína também apresentou uma ação neuroprotetora contra a isquemia-hipóxia e contra a excitotoxicidade glutamatérgica (HOWARD et al., 2002). A expressão de Bcl-2 é dependente da ativação da cascata de sinalização AMPc-MAPKs-CREB-BDNF (CHANG et al., 2003). A Bad pode ser fosforilada através da via MAPK e sua expressão ser inibida (CHARNEY et al., 2004). Após um estímulo de morte, a Bcl-2 inibe a permeabilização da membrana externa da mitocôndria, pelo sequestro de Bax ou por competir por sítios que seriam ocupados pela Bax na membrana externa mitocondrial (Figura 2). Bax e Bcl-2 são capazes de formar homodímeros (Bax-Bax e Bcl-2-Bcl-2) e heterodímeros (Bax-Bcl-2), sendo que o equilíbrio entre esses homodímeros e heterodímeros pode definir o balanço pró-apoptótico ou antiapoptótico na célula (PETROS et al., 2004). Além disso, a proteína antiapoptótica Bcl-2 é capaz de estabilizar o potencial de membrana mitocondrial (ESTAQUIER et al., 2012).

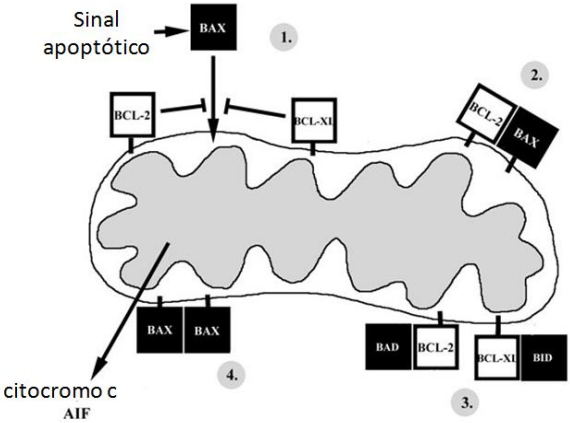


**Figura 2.** Interações entre proteínas anti-apoptóticas e pró-apoptóticas na membrana mitocondrial externa. Um sinal apoptótico estimula a translocação da Bax do citoplasma para a mitocôndria (1). A Bcl-2 e a Bcl-xL bloqueiam o efeito pró-apoptótico se ligando a Bax e formando heterodímeros (2). Porém, outras proteínas pró-apoptóticas como a Bad e Bid podem interagir com a Bcl-2 e a Bcl-xL prevenindo sua ação anti-apoptótica (3). Quando o número de proteínas pró-apoptóticas supera as anti-apoptóticas, as pró-apoptóticas ficam livres para atuar sobre a membrana mitocondrial. Ocorre então perda do potencial de membrana e liberação de moléculas promotoras de apoptose, como o citocromo c e o fator indutor de apoptose (AIF) (4) (VASKIVUO et al., 2002).

Estudos *postmortem* mostraram que pacientes depressivos apresentaram características de apoptose e atrofia de neurônios no córtex entorrinal, subículo e no giro denteado (DG) e nas regiões CA do hipocampo (BANASR et al., 2011; LUCASSEN et al., 2001). Eventos prévios como acidente vascular cerebral e lesões cerebrais por trauma mecânico, que levam a morte celular no cérebro, são fatores de risco para o desenvolvimento da depressão (PASCOE et al., 2011) (HART et al., 2011; OH et al., 2010; ROBINSON e SPALLETTA, 2010). Animais submetidos ao estresse imprevisível repetido, um modelo de depressão, apresentaram uma redução nos níveis de RNAm para Bcl-2 no córtex e de Bcl-xL em regiões do hipocampo (KOSTEN et al., 2008). Além disso, ratos submetidos a este modelo apresentaram no córtex cerebral um aumento no número de neurônios positivos para caspase-3, uma proteína indicadora de apoptose (BACHIS et al., 2008).

## 1.2 Mecanismo neuroprotetor dos antidepressivos

Diversas classes de antidepressivos bloqueiam ou revertem os efeitos causados pela depressão e/ou estresse, como a redução do número e do comprimento de espinhas dendríticas, neurogênese, gliogênese e morte celular (BANASR et al., 2011; CHEN et al., 2010). Por exemplo, o tratamento crônico de ratos com o antidepressivo tricíclico desipramina induziu o aumento do número de sinapses nas regiões CA1 e CA3 do hipocampo (HAJSZAN et al., 2009). Os mecanismos envolvidos nestes efeitos biológicos dos antidepressivos têm sido investigados nos últimos anos. Como se sabe, a neuroplasticidade é influenciada, dentre outros fatores, pelas neurotrofinas. Assim, recentemente surgiram evidências de que os antidepressivos induzem efeitos "tipo neurotróficos", auxiliando no reestabelecimento da comunicação neuronal por induzir a neurogênese, sinaptogênese e remodelagem das sinapses (DUMAN, 2002).

Vários trabalhos demonstram uma associação entre os antidepressivos e a expressão de fatores neurotróficos. Estudos clínicos mostraram que pacientes depressivos apresentam níveis reduzidos de FGF-2, BDNF e GDNF no cérebro e sangue e que o tratamento com antidepressivos atenuou esta diminuição (CHEN et al., 2001; EVANS et al., 2004; ZHANG et al., 2008). Estudos *in vitro* também demonstraram que a amitriptilina induziu a expressão de FGF-2, BDNF, VEGF e GDNF em culturas de astrócitos (KAJITANI et al., 2012). Os níveis de BDNF também aumentaram após tratamento de astrócitos com fluoxetina e paroxetina (ALLAMAN et al., 2011; ANGELUCCI et al., 2011) e de células PC12 com citalopram (TAN et al., 2010). Além disso, a expressão de GDNF foi aumentada pela imipramina e amitriptilina em culturas de astrócitos (HISAOKA et al., 2011; KIM et al., 2011).

O tratamento com os antidepressivos fluoxetina, tranilcipromina, sertralina, desipramina e mianserina induziu o aumento de FGF-2, BDNF, NGF e VEGF no cérebro de ratos e de camundogos (ABELAIRA et al., 2011; MALLEI et al., 2002; NIBUYA et al., 1995). As próprias neurotrofinas mimetizam os efeitos dos compostos antidepressivos em modelos de testes comportamentais. Microinjeções de FGF-2, VEGF e BDNF induzem efeito antidepressivo em modelos animais de depressão (TURNER et al., 2006).

Por outro lado, a presença ou deficiência de neurotrofinas ativa vias de sinalização que levam à sobrevivência ou morte celular. Assim, alguns antidepressivos afetam os níveis das proteínas da família Bcl-2, induzindo ou não neuroproteção em ensaios *in vitro* e *in vivo*.

Imipramina, desipramina, fluoxetina ou moclobemida induziram um aumento na expressão de Bcl-2 em células tronco neuronais (CHIOU et al., 2006a; CHIOU et al., 2006b; HUANG et al., 2007; PENG et al., 2008). Do mesmo modo, o tratamento crônico de ratos e camundongos com amitriptilina, imipramina, venlafaxina ou citalopram aumentou a expressão de proteínas anti-apoptóticas (Bcl-2 e Bcl-xL) e reduziu os níveis da pró-apoptótica Bax no cérebro dos animais (KOSTEN et al., 2008; MURRAY e HUTSON, 2007; WANG et al., 2011; XU et al., 2003). A administração de BDNF na região CA1 do hipocampo de ratos induziu um aumento da expressão de Bcl-xL, indicando como as neurotrofinas podem ter papel regulador da apoptose (CHAO et al., 2011).

A perda da capacidade de defesa contra o estresse oxidativo parece estar envolvida na depressão. Pacientes depressivos apresentam alterações na atividade de enzimas antioxidantes e altos níveis de lipoperoxidação; alterações estas que são revertidas pelo tratamento com antidepressivos (BILICI et al., 2001; MICHEL et al., 2007). Células PC12 tratadas com amitriptilina, bupropiona, doxepina ou venlafaxina, mostraram um aumento na expressão gênica da SOD (LI et al., 2000). O antioxidante ascorbato, que apresenta atividade antidepressiva em modelos animais, aumentou os níveis de BDNF e protegeu contra o estresse oxidativo em células de neuroblastoma (GRANT et al., 2005). Ratos submetidos ao estresse tratados com fluoxetina, imipramina e venlafaxina reverteram a redução dos níveis de glutationa e de enzimas antioxidantes (ZAFIR e BANU, 2009). Além disso, a venlafaxina reduziu a formação de malondialdeído, e aumentou os níveis de glutationa no hipocampo de camundongos (ABDEL-WAHAB e SALAMA, 2011). Também, o escitalopram aumentou os níveis de BDNF, reduziu os marcadores de estresse oxidativo e protegeu contra danos induzidos por isquemia (LEE et al., 2011).

### 1.2.1 Vias de sinalização envolvidas no efeito neuroprotetor de antidepressivos

As vias de sinalização celular envolvidas nos efeitos biológicos dos antidepressivos não estão bem elucidadas. Vários estudos sugerem que o aumento dos níveis sinápticos de serotonina e noradrenalina ativam os receptores de monoaminas e cascatas de transdução de sinal intracelulares que regulam a expressão de neurotrofinas (COWEN, 2007; D'SA e DUMAN, 2002). A ação da serotonina, por exemplo, seria mediada por alguns subtipos de receptores de serotonina que

poderiam ativar a via de sinalização ERK induzindo o aumento na síntese de fatores neurotróficos (COWEN, 2007).

As vias de sinalização comumente ativadas por antidepressivos são: a via do AMPc, a proteína cinase C (PKC), a proteína cinase II dependente de cálcio-calmodulina (CaMKII), a via da fosfatidilinositol-3-cinase/proteína cinase B (PI3K/Akt) e a das proteínas cinases ativadas por mitógenos/cinase regulada por sinal extracelular (MAPK/ERK) (Figura 1) (DUMAN e VOLETI, 2012). Dentre estas, as vias ERK, MAPK e Akt estão geralmente associadas à plasticidade neuronal (D'SA e DUMAN, 2002). Estas vias convergem fosforilando a proteína ligante ao elemento responsivo ao AMPc (CREB) e finalmente esta ativa a expressão de BDNF e da proteína anti-apoptótica Bcl-2. Além disso, o aumento de BDNF leva a uma ativação dos receptores TrkB, que potencializam a ativação destas vias intracelulares (D'SA e DUMAN, 2002). Um bloqueio da via de sinalização da proteína ERK prejudica a atividade de células piramidais e granulares do hipocampo (GREWAL et al., 1999). Estudos *postmortem* demonstram que o hipocampo de pacientes depressivos apresenta uma redução na atividade e expressão de ERK e MAPK (HSIUNG et al., 2003). Os antidepressivos desipramina e sertralina ativam a via MAPK/ERK. O bloqueio farmacológico desta via resulta em comportamento tipo depressivo e inibe o efeito dos antidepressivos em modelos animais de depressão (DUMAN et al., 2007).

A ativação de receptores de fatores neurotróficos está diretamente ligada à ativação destas vias de sinalização intracelulares (TANIS e DUMAN, 2007). O BDNF e o NGF, através de receptores Trk, ativam as vias de sinalização MAPK e PI3K/Akt e protegem células tronco neuronais frente a estímulos apoptóticos (NGUYEN et al., 2009; QIAO et al., 2004; SHELTON et al., 2004). A ativação de ERK e Akt promove neuroproteção em vários modelos *in vitro* de morte celular por apoptose (CROWDER e FREEMAN, 1998; YAO e COOPER, 1995; ZHENG e QUIRION, 2004). Além disso, a ativação de Akt e ERK possui papel na regulação da neurogênese hipocampal (ABERG et al., 2003). A fluoxetina aumenta a expressão de BDNF e GDNF em astrócitos de ratos ativando a via de sinalização de ERK (MERCIER et al., 2004). Além disso, o tratamento combinado de fluoxetina e olanzapina aumentou os níveis de FGF-2 e a fosforilação da Akt no córtex pré-frontal de ratos (MARAGNOLI et al., 2004).

### 1.2.2 Papel da via PI3K/Akt na neuroproteção

A Akt, também conhecida como proteína cinase B, é uma proteína serina-treonina cinase amplamente expressa no citoplasma (HEMMINGS e RESTUCCIA, 2012). A ativação de receptores acoplados a proteína G ou tirosina cinase por diferentes fatores de crescimento leva ao recrutamento de PI3K para a membrana (Figura 3). A Ras, uma cinase de membrana, ativa PI3K que fosforila o PIP2 (fosfolipídio de membrana fosfatidilinositol (4, 5)-difosfato), convertendo-o em PIP3 (fosfatidilinositol (3, 4, 5)-trifosfato). O acúmulo de PIP3 promove a translocação da Akt inativa do citosol para a membrana plasmática, onde esta se liga ao PIP3 pelo seu domínio PH. Na membrana, a PDK1 (cinase dependente de fosfatidilinositol-1), quando ligada ao PIP3, ativa a Akt por fosforilação do resíduo Thr308 (ALESSI et al., 1996; FILIPPA et al., 2000; RESTUCCIA e HEMMINGS, 2009). A atividade máxima da Akt requer a fosforilação adicional do sítio Ser473 (SARBASSOV et al., 2005). A Akt ativada atua sobre diversas proteínas "alvo", envolvidas na morte e na sobrevivência celular (Figura 3).

Além de modular as proteínas da família Bcl-2, a Akt também modula proteínas nucleares envolvidas na morte celular (Figura 4) (GARDAI et al., 2004). A Akt fosforila Mdm2, o que leva à ubiquitinilação da proteína p53 marcando-a para a sua degradação. Assim, a Akt reduz a expressão gênica da proteína pró-poptótica p53, induzindo a sobrevivência celular (GOTTLIEB et al., 2002).

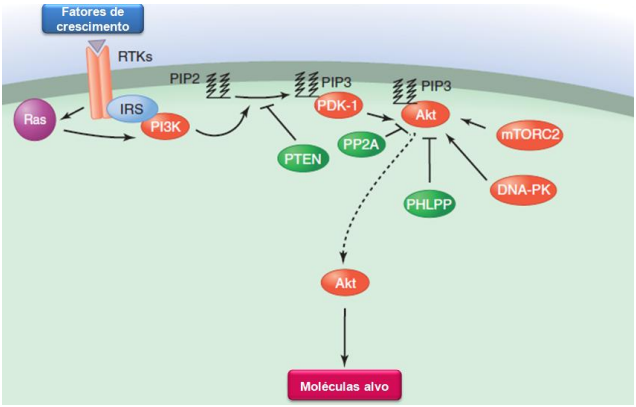


**Figura 3.** Neurotrofinas e via de sinalização celular Ras-PI3K-Akt. A PI3K fosforila o PIP2 (fosfolipídio de membrana fosfatidilinositol (4, 5)- difosfato),

convertendo-o em PIP3 (fosfatidilinositol (3, 4, 5)- trifosfato). O acúmulo de PIP3 promove a translocação da Akt para a membrana plasmática onde esta se liga ao PIP3. A PDK1 (cinase dependente de fosfatidilinositol 1) ativa a Akt por fosforilação do resíduo Thr308. A Akt ativada pode então fosforilar várias proteínas "alvo" intracelulares (HEMMINGS e RESTUCCIA, 2012).

A Akt modula por fosforilação a atividade de proteínas pró e anti-apoptóticas citosólicas (Figura 4). A proteína Mcl-1 é uma proteína anti-apoptótica que forma dímero com a Bax e assim inibe a liberação do citocromo *c* e a morte celular. A proteína GSK-3β (glicogênio sintetase cinase 3β) fosforila a Mcl-1, que causa a sua ubiquitinilação, para posterior degradação e consequente inativação. A Akt ativada fosforila a GSK-3β, inibindo sua atividade de fosforilação da Mcl-1, inibindo a morte celular (DUDEK et al., 1997; MAURER et al., 2006; PARCELLIER et al., 2008). A proteína JNK (*c-Jun N-terminal kinase*) fosforila Bcl-2 e Bcl-xL, antagonizando a atividade anti-apoptótica destas proteínas (DHANASEKARAN e REDDY, 2008). A Akt regula negativamente a fosforilação e ativação da JNK por fosforilação direta da proteína ASK1 (*apoptosis signal-regulating kinase 1*) e JIP1 (*JNK-interacting protein 1*) (KIM et al., 2001; KIM et al., 2002). A Akt também fosforila a Bad formando um sítio de ancoramento para a proteína 14-3-3; esta interação causa a retirada da Bad da mitocôndria para o citosol, prevenindo a apoptose (DATTA et al., 1997; ZHA et al., 1996). Além disso, a Akt pode fosforilar a Bax em Ser184, mantendo a proteína no citoplasma. Ali, a Bax fica associada com proteínas anti-apoptóticas da família Bcl-2, impedindo sua translocação para a mitocôndria e bloqueando a morte celular (GARDAI et al., 2004).

Outra proteína "alvo" da Akt é o fator de transcrição Nrf2. A fosforilação de Nrf2 provoca a sua translocação do citosol para o núcleo. No núcleo, Nrf2 induz a transcrição de diversas enzimas antioxidantes, como a HO-1 (heme oxigenase-1) e a Cu/Zn-SOD, conferindo uma defesa frente ao estresse oxidativo (DU et al., 2012)

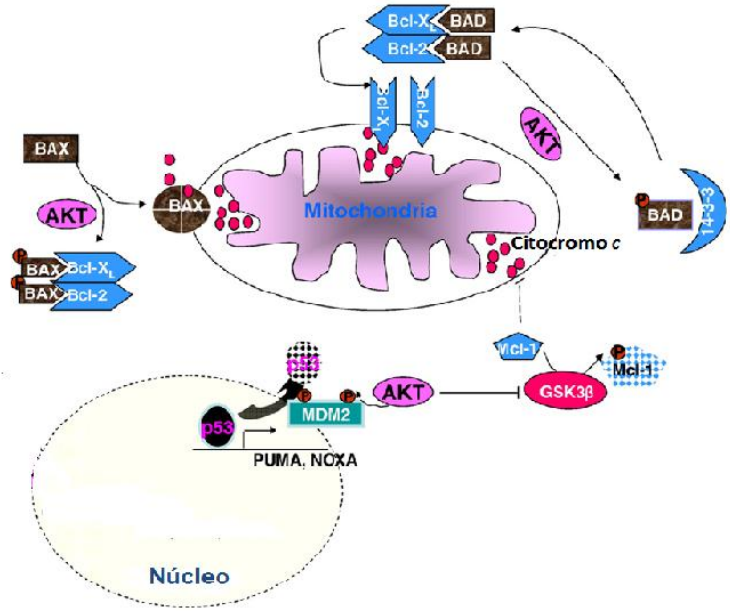


**Figura 4.** Regulação de fatores apoptóticos pela Akt**.** A Akt fosforila Bax e Bad, inibe a GSK-3β e fosforila Mdm2, que leva a degradação da p53. (STILES, 2009).

O conjunto destes dados mostra o porquê desta via PI3K/Akt ser considerada uma via de sinalização neuroprotetora. Assim, como esperado, a atividade da Akt está diminuída no córtex occipital de vítimas suicidas (HSIUNG et al., 2003) e no córtex pre-frontral ventral de pacientes depressivos (KAREGE et al., 2007). De modo contrário, pacientes depressivos que foram tratados cronicamente com antidepressivos apresentaram níveis aumentados de Akt fosforilada na área tegmental ventral (KRISHNAN et al., 2008). O tratamento crônico de animais com antidepressivos reproduziu os dados. O tratamento com fluoxetina reverteu a redução na fosforilação da Akt induzida por estresse em camundongos (KRISHNAN et al., 2008). Além disso, o tratamento agudo de ratos com fluoxetina aumentou os níveis totais de Akt (REUS et al., 2012). Estudos *in vitro* também demonstraram que a fluoxetina aumenta os níveis de Akt fosforilada em células tronco neuronais de ratos e a fluvoxamina em células PC12 (HUANG et al., 2012; NAKANO et al., 2010).

## 1.3 Duloxetina

Os antidepressivos inibidores seletivos da recaptação de serotonina (ISRS), como a fluoxetina, são os fármacos mais vendidos e utilizados na terapêutica. Contudo, vários pacientes são refratários aos ISRS e como alternativa, os antidepressivos de 2ª geração têm sido utilizados (RUHE et al., 2006). Estes novos fármacos apresentam maior eficácia, menor tempo de ação e melhores taxas de remissão quando comparados aos ISRS, além de causarem menos efeitos colaterais do que os antidepressivos tricíclicos (PAPAKOSTAS et al., 2007; TRAN et al., 2003).

A duloxetina é um antidepressivo de 2ª geração, que atua como inibidor seletivo da recaptação de serotonina e de noradrenalina. A dose efetiva para terapia antidepressiva está entre 40 a 120 mg/dia e a dose de manutenção é de 60mg/dia (HUNZIKER et al., 2005). Estudos pré-clínicos comprovam que a duloxetina liga-se com alta afinidade aos transportadores de serotonina e de noradrenalina. Ela não possui afinidade pelos receptores de dopamina, serotonina, adrenérgicos, histamínicos ou opióides (BAUER et al., 2006).

Assim como outros antidepressivos, o tratamento crônico com duloxetina induziu um aumento na expressão de BDNF no cérebro de ratos (CALABRESE et al., 2010; CALABRESE et al., 2007; MANNARI et al., 2008; MOLTENI et al., 2009). A proteína Arc (ou Arg 3.1), relacionada com a plasticidade sináptica, também foi aumentada no córtex frontal (MOLTENI et al., 2008). De modo semelhante, a duloxetina aumentou os níveis de BDNF em culturas de células de astrocitoma (C6) de ratos (PRICKAERTS et al., 2012). Recentemente, nosso grupo demonstrou que o efeito tipo antidepressivo da duloxetina em camundongos é devido ao bloqueio de receptores NMDA e que esta ação depende da via de sinalização L-arginina-óxido nítrico-guanosina monofosfato cíclico (ZOMKOWSKI et al., 2012).

## 2. JUSTIFICATIVA

Dentre outros fatores, pacientes depressivos apresentam uma redução nos níveis de neurotrofinas, alterações na sinalização celular e redução das defesas antioxidantes que resultam em morte neuronal. Estes fatores contribuiriam para as alterações estruturais observadas no córtex e no hipocampo de indivíduos depressivos (BANASR et al., 2011; HOVATTA et al., 2010). O tratamento com antidepressivos é capaz de induzir a expressão de neurotrofinas, de modular vias de sobrevivência celular (BANASR et al., 2011; LAUTERBACH et al., 2010) e de atenuar os efeitos do estresse oxidativo (BEHR et al., 2012). Entretanto, os mecanismos envolvidos nestes efeitos dos compostos antidepressivos ainda não estão bem esclarecidos.

Este trabalho foi realizado com a duloxetina, um antidepressivo utilizado na terapêutica, mas cujo mecanismo de ação também é pouco conhecido. Um dado conhecido sobre a ação da duloxetina é de que ela aumenta a expressão de BDNF *in vivo* e *in vitro* (CALABRESE et al., 2010; CALABRESE et al., 2007; PRICKAERTS et al., 2012).

Entretanto, apesar de ser bem conhecido que o BDNF pode ativar vias de sinalização celular como a PI3K/Akt e esta modular a atividade e a expressão de proteínas de sobrevivência celular, não há nenhum estudo sobre a ação neuroprotetora da duloxetina. Estes dados podem contribuir para a melhor compreensão da neurobiologia da depressão e auxiliar a definir estratégias terapêuticas no tratamento de pacientes depressivos.

# 3. OBJETIVOS

## 3.1 Objetivo geral

O objetivo deste trabalho foi investigar os mecanismos envolvidos no efeito neuroprotetor da duloxetina em modelos experimentais *in vivo* e *in vitro.*

.

## 3.2 Objetivos específicos

1. Verificar se o tratamento crônico de camundongos com duloxetina induz efeito antidepressivo no teste do nado forçado;

2. Analisar o córtex e o hipocampo de animais tratados cronicamente com duloxetina para verificar a expressão gênica (RNAm) de proteínas envolvidas na neuroplasticidade e sobrevivência celular: neurotrofinas BDNF, NGF, FGF-2 e NT-3; proteínas anti-apoptóticas Bcl-2 e Bcl-xL e pró-apoptóticas Bax, Bad e p53;

3. Avaliar a citotoxicidade da duloxetina em culturas de células de neuroblastoma humano (SH-SY5Y);

4. Analisar se o pré-tratamento de neuroblastoma humano com duloxetina tem efeito protetor contra o estresse oxidativo induzido pela rotenona;

5. Investigar a participação da via de sinalização celular PI3K/Akt no efeito neuroprotetor da duloxetina em células de neuroblastoma humano

# 4. RESULTADOS

Os resultados que fazem parte desta Dissertação de Mestrado estão apresentados sob a forma de manuscritos. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, encontram-se nos manuscritos.

4.1 Os resultados referentes aos objetivos 1 e 2 estão contemplados no manuscrito "A administração crônica de duloxetina reduz a expressão gênica de proteínas pró-apoptóticas e aumenta a de neurotrofinas no córtex cerebral e hipocampo de camundongos".

4.2 Os resultados referentes aos objetivos 3, 4 e 5 estão contemplados no manuscrito "Duloxetina ativa a via PI3K/Akt e reduz o estresse oxidativo em células de neuroblastoma humano (SH-SY5Y)".

## 4.1 Manuscrito 1

**“Chronic administration of duloxetine and mirtazapine downregulates proapoptotic proteins and upregulates neurotrophin gene expression in the hippocampus and cerebral cortex of mice”,** publicado no “*Journal of Psychiatric Research*”.

# Chronic administration of duloxetine and mirtazapine downregulates proapoptotic proteins and upregulates neurotrophin gene expression in the hippocampus and cerebral cortex of mice

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**Abstract**

Structural alterations in the limbic system, neuronal cell loss, and low levels of neurotrophins have been implicated in the pathogenesis of depression. While it is generally accepted that increasing monoamine levels in the brain can effectively alleviate depression, the precise neurobiological mechanisms involved are unclear. In the present study, we examined the effects of two antidepressants, duloxetine and mirtazapine, on the expression of apoptotic and neurotrophic proteins in the cerebral cortex and hippocampus of mice. Duloxetine (10 mg/kg) and mirtazapine (3 mg/kg) were chronically administered for 21 days, and qRT-PCR analysis was carried for the following: neurotrophins (BDNF, NGF, FGF-2, and NT-3); anti-apoptotic proteins (Bcl-2 and Bcl-xL) and pro-apoptotic proteins (Bax, Bad, and p53). Both duloxetine and mirtazapine produced antidepressant activity in the forced swimming test and induced increased cortical and hippocampal mRNA expression of BDNF. Duloxetine also increased Bcl-2, Bcl-xL, FGF-2, and NT-3 expression in the cerebral cortex, and FGF-2 expression in the hippocampus. Moreover, duloxetine reduced Bax and p53 expression in the hippocampus, and Bad expression in the cerebral cortex. Mirtazapine decreased Bcl-xL and Bax expression in the hippocampus, and Bad and p53 expression in both the hippocampus and cerebral cortex. Mirtazapine also increased the expression of neurotrophins, NGF and NT-3, in the cerebral cortex. These results suggest that duloxetine and mirtazapine could elicit their therapeutic effect by modulating the activity of apoptotic and neurotrophic pathways, thus enhancing plasticity and cell survival in depressive patients.
**Keywords:** Duloxetine; Mirtazapine; Antidepressants; Neurotrophins; Apoptotic proteins; Neuroprotection.

## 1. Introduction

Depression is the most common psychiatric disorder, usually associated with acute and persistent symptoms leading to life quality impairment. Despite extensive studies, its exact pathophysiology is not completely understood, though both genetic and environmental factors have been proposed, as well as the mediating role of monoamines (Pittenger and Duman, 2008).

Targeting the monoaminergic system for the treatment of depression has been the focus of several reports. Monoaminergic pathways play a crucial role in the control of cognition, affect, endocrine secretion, appetite, and motor function, all of which are profoundly disrupted in depressive states. For this reason, a perturbation of monoaminergic transmission is implicated in the etiology of depressive disorders. Indeed, almost every compound that inhibits monoamine reuptake, thus increasing monoamine concentration in the synaptic cleft, has proved to be a clinically effective antidepressant. However, while it is generally accepted that increasing monoamine levels in the brain can effectively alleviate depression, the precise neurobiological mechanisms involved are unclear (Pittenger and Duman, 2008).

It has been demonstrated by *in vivo* imaging studies that patients with major depressive disorder display reduced hippocampal and prefrontal cortex volume (Sheline, 2000; Bremmer, 2002). In addition, it has been proposed that these structural alterations result from atrophy and loss of neurons and glia (Krishnan and Nestler, 2008; Pittenger and Duman, 2008) by several mechanisms, including glutamate excitotoxicity, decreased neurotrophic factor expression, and activation of apoptotic pathways (Banasr et al., 2011). Further evidence that cell loss plays a role in depression comes from the finding that conditions associated with cell loss, like stroke and traumatic brain injury, can lead to the development of depression (Robinson and Spalletta, 2010; Hart et al., 2011).

Besides cells loss, it has been suggested that dysregulation of adult neurogenesis might also contribute to the pathogenesis of depressive disorders, and it has been hypothesized that therapies targeted at enhancing neurogenesis could provide neuroprotection by inducing neuroplasticity (Krishnan and Nestler, 2008; Banasr et al., 2011). In recent year, brain-derived neurotrophic factor (BDNF) has received much attention regarding its effect on neurogenesis. Moreover, it has been reported that BDNF by itself has antidepressant effects in animal behavioral models of depression (Shirayama et al., 2002).

Interestingly, depressive-like behaviors and reduced neurogenesis are both reported to be associated with a decrease in BDNF levels. Moreover, deprivation of other neurotrophic factors, such as nerve growth factor (NGF), has been shown to trigger the apoptotic pathway, altering the balance of activity between pro-apoptotic proteins, like Bax, and anti-apoptotic proteins, like Bcl-xL and Bcl-2 (Putcha et al., 2002; Lindsten et al., 2005). Further, there is some evidence to suggest that BDNF activates signaling pathways involved in upregulating the expression of the anti-apoptotic protein, Bcl-2 (Krishnan and Nestler, 2008; McKernan et al., 2009).

Thus, it follows that therapies aimed at reducing cell loss and enhancing neurogenesis could be therapeutically beneficial. Indeed, it has been proposed that antidepressants elicit their effect by inducing the expression of neurotrophins while targeting apoptotic proteins at the same time, blocking or even reversing apoptotic-induced structural alterations in limbic structures as a result (McKernan et al., 2009; Banasr et al., 2011). Other studies have suggested that their efficacy is directly related to increased levels of the monoamine, serotonin, which can alter the activity of specific growth factor receptors, and kinases that are regulated by growth factors (Cowen, 2007). For example, it has been reported that some subtypes of serotonin receptors can activate extracellular signal-regulated kinases (ERK) and Akt signaling pathways; these in turn are reported to mediate neuroplasticity changes induced by neurotrophic growth factors (Cowen, 2007).

Duloxetine and mirtazapine are second-generation antidepressant drugs, both with a combined serotoninergic-noradrenergic mechanism of action. Duloxetine acts by inhibiting the pre-synaptic serotonin and noradrenaline transporters, while mirtazapine increases the activity of noradrenergic and serotoninergic neurons (Karpa et al., 2002; Peña et al., 2005). Clinical data demonstrate that these new drugs can be an advantageous alternative for patients with insufficient response to selective serotonin reuptake inhibitors (Papakostas et al., 2007). Moreover, it has been reported that they have improved efficacy, and possess a faster time of action and greater remission rates when compared with selective serotonin reuptake inhibitors; moreover, they have improved side effects compared with tricyclic antidepressants (Papakostas et al., 2007). Regarding their effect on neuroplasticity, it has been demonstrated that chronic treatment with duloxetine produces an increase in BDNF mRNA levels in the frontal cortex and hippocampus of rats (Calabrese et al., 2007; Molteni et al., 2009; Calabrese et al., 2010). Similarly, repeated treatment with mirtazapine has also been

reported to increase BDNF mRNA expression in the hippocampus and cerebral cortex of rats (Rogóz et al., 2005).

However, other than their effect on BDNF expression, little information is available regarding the action of duloxetine and mirtazapine on neuroplasticity and cell survival. Considering that BDNF can upset the balance between pro-apoptotic and anti-apoptotic proteins, thus modulating the apoptotic pathway, the aim of this study was to assess if chronic administration of duloxetine and mirtazapine could modulate other proteins in the apoptotic pathway, as well as induce the expression of neurotrophic proteins. In order to do this, mice were chronically treated with duloxetine and mirtazapine, and the mRNA expression of the following proteins, were assessed in the hippocampus and cerebral cortex: anti-apoptotic proteins (Bcl-2 and Bcl-xL); pro-apoptotic proteins (Bax, Bad, and p53); nerve growth factor (NGF), fibroblast growth factor-2 (FGF-2), and neurotrophin-3 (NT-3).

## 2. Material and Methods

### *2.1. Animals*

Swiss female mice (30-40 g) were 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). All manipulations were carried out between 9:00 a.m. and 4:00 p.m. 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 protocol and experiments were approved by the local Ethical Committee of Animal Research (CEUA/UFSC).

### *2.2. Drugs and treatment*

The animals were treated once daily for 21 consecutive days with mirtazapine 3 mg/kg (Cima Laboratories Inc., USA) and duloxetine 10 mg/kg (Eli Lilly & Co., USA), administered by oral (p.o.) route by gavage in a volume of 10 ml/kg body weight. The administered doses were chosen based on previous experiments of our group (Zomkowski et al., 2012).

*2.3. Behavioral tests*

*2.3.1. Forced swimming test*

After 24 h from the last treatment, mice were submitted to the FST. They were 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., 2012). 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. Open-field test*

The ambulatory behavior was assessed in an open-field test as described previously (Zomkowski et al., 2012). 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.

*2.4. RNA extraction, cDNA synthesis and quantitative Real Time-PCR*

Immediately after behavioral tests, mice were decapitated, the brains were removed and the hippocampus and cerebral cortex were separated from brain samples and frozen. Total RNA was extracted using SV Total RNA Isolation Kit (Promega, USA) and 0.4 µg of each sample was reverse transcribed to cDNA using TaqMan RT reagents (Applied Biosystems, USA). The final concentrations of reagents were: 1 x TaqMan RT buffer, 5.5 mM $MgCl_2$, 2 mM dNTP mixture, 2.5 µM Random Hexamers, 0.4 U/µl RNase inhibitor and 1.25 U/µl reverse transcriptase in RNase-free water to a total volume of 20 µl. The reaction mix was incubated at 25°C for 10 min, 48°C for 1 h and 95°C for 5 min. Quantitative real-time PCR (qRT-PCR), using 7900HT System Standard and SYBR-Green Master Mix (Applied Biosystems, USA) was performed according to the manufacture's protocol. 2 µl of cDNA was amplified in a total volume of 10 µl. PCR reactions were conducted using primers, designed with the Primer Express version 3.0 software (Applied Biosystems, USA) for BDNF forward (5'-CATAGACAAAAGGCACTGGAACTC -3') and reverse (5'-TAAGGGCCCGAACATACGAT-3'), Bcl-2 forward (5'-

AAGGGCTTCACACCCAAATCT-3') and reverse (5'-TTCTACGTCTGCTTGGCT TTGA-3'), Bcl-xL forward (5'-CCCCCCACATCTCAGTTCTCT-3') and reverse (5'-GCCTCCAAGGAGCTGGTTTAG-3'), Bax forward (5'-AGGATGCGTCCACCAAGAAG-3') and reverse (5'-CCATATTGCTGTCCAGTTCATCTC-3'), Bad forward (5'-CAGGGAGAAGAGCTGACGTACA-3') and reverse (5'-CCCCTCCGTGGCTATTGC-3'), p53 forward (5'-GCTCACCCTGGCTAA AGTTCTG-3') and reverse (5'-AGTCGCTACCTACAGCCAGGAT-3'), NGF forward (5'-TACAGGCAGAACCGTACACAGATAG-3') and reverse (5'-CAGTGGGCTTCAGGGACAGA-3'), FGF-2 forward (5'-TGGTATGTGGCACTGAAACGA-3') and reverse (5'-TCCAGGTCCCGTTTTGGAT-3'), NT-3 forward (5'-GGTGGGCGAGACTGAATGA-3') and reverse (5'-AGGGACGTCGACATGAAGAGAA-3') and β-actin forward (5'-AAATCGTGCGTGACATCAAAGA-3') and reverse (5'-GCCATCTCCT GCTCGAAGTC-3'). These primers were used in a final concentration of 0.3 μM and reaction conditions were 50°C for 2 min, 90°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The results were analyzed in the provided software Sequence detection systems (SDS) version 2.4 software (Applied Biosystems, USA). A dissociation step was added for SYBR-Green runs. For each sample, gene expression was quantified using a standard curve and normalized against the expression of β-actin gene.

### *2.5. Statistical analysis*

The statistic analysis was performed by unpaired Student *t* test for the behavioral tests and paired test for the qRT-PCR results. A value of $p<0.05$ was considered to be significant.

## 3. Results

### *3.1. Antidepressant-like effect of chronic administration of duloxetine and mirtazapine*

The chronic treatment of mice (21 days) with duloxetine (10 mg/kg, p.o.) and mirtazapine (3 mg/kg, p.o.) significantly decreased the immobility time in the FST (Figure 1). Moreover, both antidepressants

did not produce any change in the ambulation in the open-field in a separate experiment as compared to the control group (data not shown).

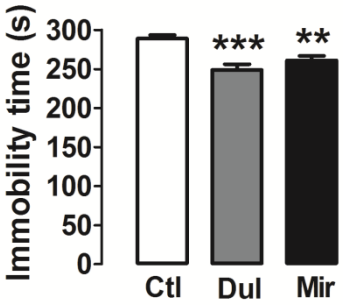


**Fig 1.** Effect of chronic administration (21 days) of duloxetine (Dul, 10 mg/kg, p.o.) and mirtazapina (Mir, 3 mg/kg, p.o.) in the immobility time in the FST in mice. Values are expressed as mean ± S.E.M. (n = 8). **p<0.01 and ***p<0,001 compared with the vehicle-treated control group (Ctl).

*3.2. Antidepressants and the expression of neuroprotective factors*

The effect of duloxetine and mirtazapine on the mRNA expressions of the BDNF and of the anti-apoptotic proteins Bcl-2 and Bcl-xL were evaluated in the cerebral cortex and hippocampus of mice (Figure 2A and 2B). The treatment with duloxetine and mirtazapine significantly increased hippocampal and cortical BDNF mRNA expression compared with vehicle treated controls. Duloxetine treatment led to a marked up-regulation in BDNF mRNA levels in the cerebral cortex and hippocampus by 31 and 19%, respectively, whereas mirtazapine increased its expression by 32 and 52%, in these tissues. Duloxetine also upregulated Bcl-2 cerebro-cortical mRNA expression by 52% and Bcl-xL was increased by 28%. Conversely, a decrease of 52% on Bcl-xL hippocampal mRNA expression was observed after mirtazapine treatment.

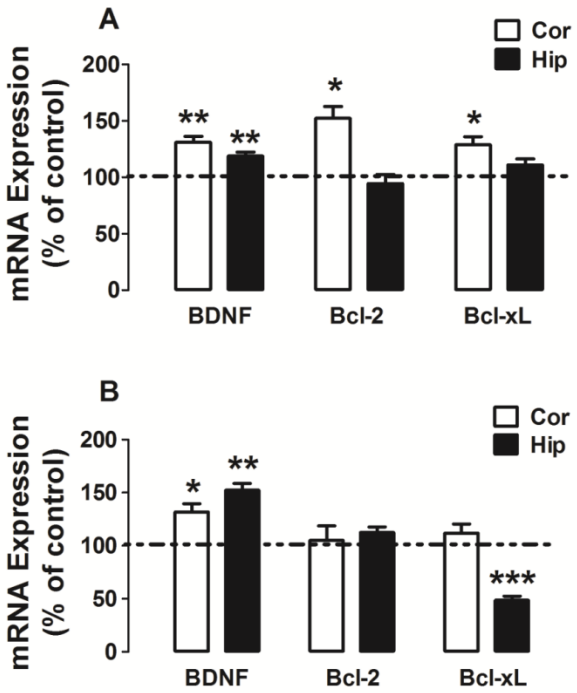


**Fig 2**. Effect of chronic administration of duloxetine (10 mg/kg, p.o) (A) or mirtazapine (3 mg/kg, p.o) (B) on the BDNF, Bcl-2 and Bcl-xL mRNA expression in the mice cerebral cortex (Cor) and hippocampus (Hip). Data are expressed as means ± S.E.M. (n = 3-4). Results were compared with the housekeeping gene β-actin and normalized to control group (vehicle-treated animals) as 100%. *p<0.05, **p<0.01 and ***p<0.001.

### *3.3. Antidepressants and the expression of cell death factors*

The apoptotic inducers Bax, Bad and p53 were downregulated by the chronic antidepressant treatment (Figure 3A and 3B). Duloxetine decreased hippocampal Bax and p53 mRNA expression by 32 and 67%, respectively, when compared with the control group. Also, Bad mRNA expression decreased 19% in the cerebral cortex. In addition, mirtazapine reduced the expression of Bax (30%) and of p53 (26%) in the hippocampus. Mirtazapine also decreased cerebro-cortical p53 mRNA expression by 30% and Bax by 16%. Moreover, Bad mRNA

expression was decreased 43% in the cerebral cortex and 41% in the hippocampus.

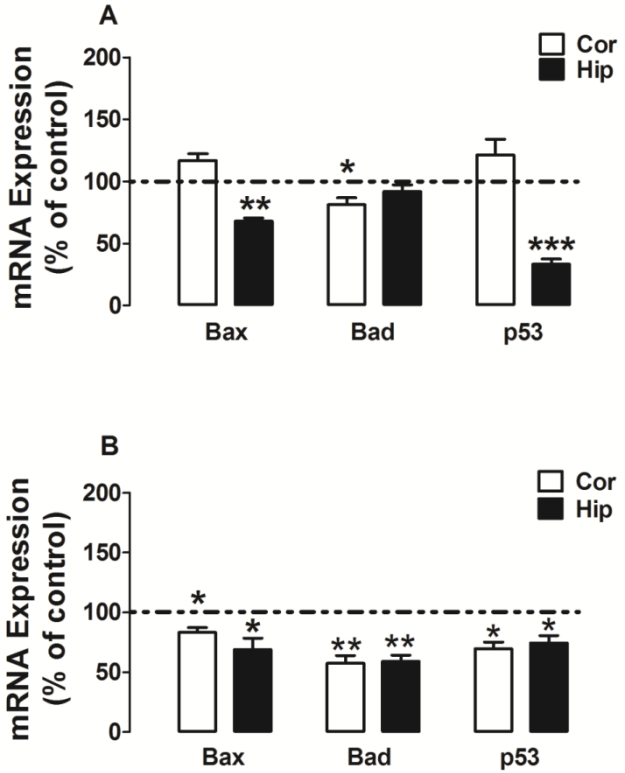


**Fig 3.** Effect of chronic administration of duloxetine (10 mg/kg, p.o) (A) or mirtazapine (3 mg/kg, p.o) (B) on the Bax, Bad and p53 mRNA expression in the mice cerebral cortex (Cor) and hippocampus (Hip). Data are expressed as means ± S.E.M. (n = 3-4). Results were compared with the housekeeping gene β-actin and normalized to control group (vehicle-treated animals) as 100%. *p<0.05, **p<0.01 and ***p<0.001.

### *3.4. Antidepressants and the expression of neurotrophins*

Figure 4A and 4B shows the effect of chronic administration of antidepressant on the NGF, FGF-2 and NT-3 expression. Duloxetine increased the FGF-2 expression in the cerebral cortex and hippocampus by 38 and 97%, respectively. Also, the cerebro-cortical NT-3 mRNA

expression was increased by 43%. On the contrary, duloxetine decreased the NGF expression by 41% in the hippocampus. The treatment with mirtazapine increased the cerebro-cortical NGF and NT-3 expression by 24 and 75%, respectively.

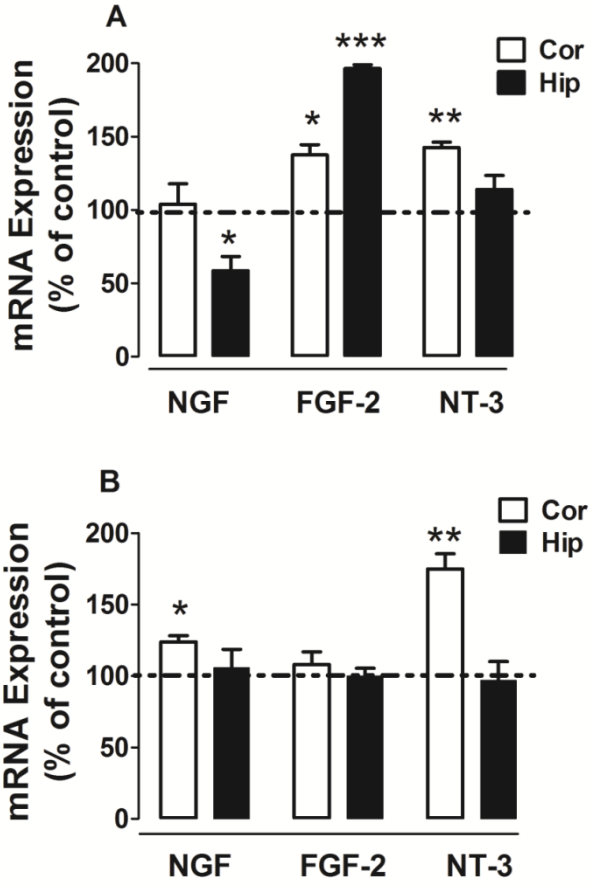


**Fig 4**. Effect of chronic antidepressant administration of duloxetine (10 mg/kg, p.o) (A) or mirtazapine (3 mg/kg, p.o) (B) on the NGF, FGF-2 and NT-3 mRNA expression in the mice cerebral cortex (Cor) and hippocampus (Hip)**.** Data are expressed as means ± S.E.M. (n = 3-4). Results were compared with the housekeeping gene β-actin and normalized to control group (vehicle-treated animals) as 100%. *p<0.05, **p<0.01 and ***p<0.001.

## 4. Discussion

The aim of this study was to investigate the potential neuroprotective role of duloxetine and mirtazapine in the hippocampus and cerebral cortex of mice. In order to do this, mice were chronically treated with these antidepressants, and the gene expression of neurotrophins and proteins involved in the apoptotic pathway were assessed.

Chronic treatment with duloxetine and mirtazapine was effective in producing antidepressant-like effects in the FST. This result coincides with previously reports regarding acute treatment with duloxetine (Zomkowski et al., 2012) and chronic treatment with mirtazapine (Nowarowska et al., 1999). The anti-immobility effect of both antidepressants did not appear to be associated with any motor effects, since treated mice did not exhibit increased ambulation when tested in an open field.

In this study, hippocampal and cortical BDNF mRNA expression was significantly increased by chronic treatment with both duloxetine and mirtazapine, which is in agreement with previous findings (Rogóz et al., 2005; Calabrese et al., 2007; Molteni et al., 2009; Calabrese et al., 2010). Interestingly, chronic treatment with mirtazapine has been shown to alleviate frontal and hippocampal BDNF deficits in rats submitted to chronic unpredictable mild stress (Zhang et al., 2010). Moreover, it has been demonstrated that duloxetine is able to restore the physiological levels of Npas4, a transcription factor that regulates BDNF, in the hippocampus and frontal cortex of serotonin transporter knockout rats (Guidotti et al., 2012). Additionally, duloxetine reportedly increases BDNF levels in rat astrocytoma C6 cells after 24 and 48 h of treatment (Prickaerts et al., 2012). These results highlight the possibility that duloxetine and mirtazapine elicit their protective effects by enhancing BDNF expression.

In addition to BDNF, we also investigated the effect of duloxetine and mirtazapine on the expression of the anti-apoptotic proteins, Bcl-2 and Bcl-xL, and the pro-apoptotic proteins, Bax, Bad, and p53; this was to assess the role of these proteins in the mechanistic action of duloxetine and mirtazapine. We found that duloxetine administration for 21 days resulted in an up-regulation in the expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, in the cerebral cortex, but not in the hippocampus, of mice. On the other hand, mirtazapine did not alter the expression of Bcl-2 in either the cerebral cortex or hippocampus, but it decreased hippocampal Bcl-xL expression. Bcl-2 is a membrane-bound

protein that has been shown to inhibit apoptosis and enhance neuronal survival by blocking cytochrome c release through the heterodimerization with the pro-apoptotic protein, Bax (Xu et al., 2003; Lindsten et al., 2005). Bcl-xL acts by sequestering the proapoptotic members of the Bcl-2 family and BH3-only proteins in the mitochondrial membrane, thereby preventing their release into the cytosol. In agreement with our results for duloxetine, Bcl-2 has been known to be upregulated by chronic treatment with fluoxetine, reboxetine, and tranylcypromine, which selectively increase cortical, but not hippocampal, Bcl-2 gene expression (Kosten et al., 2008). Furthermore, venlafaxine, an antidepressant from the same therapeutic class as duloxetine, reportedly increases hippocampal Bcl-2 and Bcl-xL of rats after 21 days of treatment (Xu et al., 2003; Wang et al., 2011). Additionally, chronic treatment with tranylcypromine has been demonstrated to increase Bcl-xL mRNA expression in the hippocampus and dentate gyrus of rats (Kosten et al., 2008). In another study, the mRNA and protein levels of Bcl-xL were increased by direct administration of BDNF into the rat hippocampal CA1 area, thus suggesting that the neuroprotective effect of BDNF in hippocampal neurons could have a direct effect on Bcl-2 family proteins (Chao et al., 2010). Hence, it is possible that the duloxetine-induced increase in BDNF expression observed in this study, was in turn responsible for the increased expression of Bcl-2 and Bcl-xL.

In this study, duloxetine decreased hippocampal, but not cortical, Bax mRNA expression, whereas mirtazapine decreased its expression in both brain structures. The apoptotic process is programmed and critically controlled by the balance between anti- and pro-apoptotic proteins within the cell. The apoptotic suppressors, Bcl-2 and Bcl-xL, interact with the death-promoting pro-apoptotic protein, Bax, via homo and heterodimerization in the outer mitochondrial membrane (Lindsten et al., 2005). When the levels of Bcl-2 and Bcl-xL are reduced, it lowers the threshold for apoptosis to occur (McKernan et al., 2009). Bax and Bak are the two major proapoptotic family members, and their apoptotic effect occurs by compromising mitochondrial membrane integrity leading to leakage of apoptogenic factors, such as cytochrome c release into the cytosol, caspase activation, and cell death. With regard to this study, similar findings for fluoxetine (Kosten et al., 2008) and venlafaxine (Wang et al., 2011) have been reported. Thus, antidepressants that modulate the expression and activity of Bax may play a role in preventing the neuronal loss associated with depressive individuals.

Our results demonstrate that chronic treatment with duloxetine reduces Bad mRNA expression in the cerebral cortex, whereas mirtazapine decreased both hippocampal and cortical Bad mRNA expression. However, Bax and Bad expression were not changed when neural stem cells were treated with antidepressants desipramine (Huang et al., 2007) or imipramine (Peng et al., 2008).

We also assessed the effect of duloxetine and mirtazapine on the pro-apoptotic protein p53; we found that p53 expression was downregulated by both duloxetine and mirtazapine. Duloxetine decreased hippocampal p53 mRNA expression, while mirtazapine reduced its cortical and hippocampal expression. It has been suggested that the regulation of Bax is the most probable link between p53-mediated activation and apoptosis in neurons (Culmsee and Mattson, 2005). Recent studies have demonstrated that p53 mediates mitochondrial permeabilization through direct physical interaction with Bax. Moreover, p53 interacts with Bcl-2 family members and acts as a pseudo-BH3-only family member. Furthermore, it has been shown to promote apoptosis by liberating Bax repression from Bcl-xL (Chipuk et al., 2005). In the absence of cellular stress, cytosolic p53 forms a complex with Bcl-xL. In response to cellular stress, p53 is then activated, leading to increased nuclear levels of p53, which in turn leads to the transcriptional activation of Puma. Puma then binds to Bcl-xL, displacing it from p53, and thereby releasing p53 to directly activate Bax (Chipuk et al., 2005).

It has been suggested that p53 may mediate apoptosis induced by a range of insults, including DNA damage, hypoxia, withdrawal of trophic support, and oxidative stress (Culmsee and Mattson, 2005). It is interesting to mention that the mood stabilizer, lithium, as well as magnesium, a metal that exerts antidepressant activity, are also known to decrease p53 expression (Chen and Chuang, 1999; Muir et al., 1999). Its expression is elevated in damaged neurons in brain tissue samples derived from animal models and patients with chronic neurodegenerative diseases; this could explain the increased neuronal death observed in these individuals, since the p53 provides a key survival checkpoint in neurons, acting as a sensor responsible for integrating multiple pro-apoptotic and pro-survival signals (Culmsee and Mattson, 2005; Jacobs et al., 2006).

As mentioned above, it is possible that the changes in the expression of apoptotic proteins observed in this study are related to the up-regulation of BDNF. It has previously been demonstrated that neurotrophic factors can activate the MAPK/CREB signaling pathway,

inhibiting the cell death cascade and increasing Bcl-2 protein expression (Cowen, 2007). Moreover, antidepressants that increase BDNF, activate the PI3K/Akt pathway; Akt phosphorylates and inactivates the glycogen synthase kinase-3β (GSK-3β), which in turn decreases CREB's affinity to DNA and blocks the transcription of BDNF and Bcl-2, leading to cell death. Akt can also phosphorylate Bad (Culmsee and Mattson, 2005) inhibiting its complex formation with Bcl-2 and Bcl-xL, and thus preventing cell death. Furthermore, Akt activates Mdm2 (Gottlieb et al., 2002), which prevents p53 from binding to DNA, which also prevents cell death. These data demonstrate the intricate relationship between BDNF and proteins involved in the apoptotic pathway. In our study, it is possible that the duloxetine- and mirtazapine-induced increase in BDNF expression, which was accompanied by an up-regulation in anti-apoptotic proteins and a down-regulation in pro-apoptotic proteins, reflects the ability of these antidepressants to modulate BDNF expression, which in turn modulates the activity of apoptotic proteins.

It has been suggested the reduction in brain structures volume and morphological atrophy observed in depressed patients may be due to neural loss or apoptosis, as well as deficits in neurotrophic factor levels (Krishnan & Nestler, 2008; Banasr et al., 2011). Taking this information into account, we also investigated whether chronic treatment with duloxetine and mirtazapine had an impact on neurotrophin gene expression. We found that treatment with mirtazapine increased cortical NGF mRNA expression, while duloxetine decreased hippocampal NGF expression. It has previously been demonstrated that chronic treatment with lamotrigine results in increased NGF levels in the prefrontal cortex of rats (Abelaira et al., 2011). Moreover, NGF levels are reportedly reduced in chronically stressed mice, a phenomenon that is reversed by treatment with tianeptine (Alfonso et al., 2005). Interestingly, NGF has antidepressant-like effects, though it does not appear to have the typical biochemical action of most antidepressants (Overstreet et al., 2010).

In this study, we also found that chronic treatment with duloxetine was able to upregulate FGF-2 mRNA expression in the cerebral cortex and hippocampus, as well as increase cortical NT-3 mRNA expression. In the adult brain, FGF promotes neuronal survival and axonal branching, and it has been demonstrated that its expression is decreased in the hippocampus and frontal cortex of depressed patients (Evans et al., 2004). Furthermore, other antidepressants have been implicated in reversing these changes. For example, chronic treatment with desipramine, fluoxetine, and mianserin all increase FGF-2 levels in the cerebral cortex and hippocampus of rats (Mallei et al., 2002; Bachis

et al., 2008). In addition, the combined chronic treatment of rats with fluoxetine and olanzapine led to increased FGF-2 levels in different brain regions, as well as the phosphorylation of Akt in the prefrontal cortex, thus suggesting the involvement of this signaling pathway in the mechanism of action of such agents (Maragnoli et al., 2004).

The combination of drugs, fluoxetine and olanzapine, also reportedly increases NT-3 levels in the prefrontal cortex, but not in the hippocampus or amygdala, of rats (Agostinho et al., 2011). In agreement, our results demonstrate that duloxetine and mirtazapine treatments increases NT-3 mRNA expression only in the cerebral cortex. Previously, it was demonstrated that NT-3 infusion increases BDNF levels in the cerebral cortex of rats, suggesting a connection between the effects of antidepressants and the expression of these two neurotrophins (Schütte et al., 2000). Both NT-3 and BDNF appear to share common intracellular protective pathways in response to several forms of antidepressant drugs; the combination of fluoxetine and olanzapine increases BDNF, NT-3, CREB, Akt, and Bcl-2 levels (Agostinho et al., 2011; Réus et al., 2012).

It is important to note that we observed different responses in the cerebral cortex and hippocampus with regard to apoptotic protein and neurotrophin mRNA expression; similar findings have been reported for other antidepressants (Kosten et al., 2008; Agostinho et al., 2011). A previous study reported different results when assessing BDNF levels in distinct regions of the same structure, or different structures, after antidepressant treatment in mice (Calcagno et al., 2007). Interestingly, duloxetine produced distinct responses in a 21-day treatment trial in rats, showing increased BDNF expression only in the cerebral cortex (Calabrese et al., 2007). Therefore, when we compare different brain structures, the responses to the antidepressants seems to be diverse, which may be clarified in posterior investigations.

In conclusion, our results demonstrate that chronic treatment with duloxetine and mirtazapine, in addition to enhancing BDNF gene expression, are able to modulate the gene expression of other neurotrophins and proteins involved in apoptotic pathway. We also observed differential responses when comparing the expressions of these proteins in the cerebral cortex and hippocampus. These results enforce the proposal that the antidepressant activity of some drugs could be partially due to the modulation of apoptotic and neurotrophic pathways, involved in plasticity and cell survival.

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## 4.2 Manuscrito 2

**“Duloxetine activates PI3K/Akt pathway and reduces oxidative stress in human neuroblastoma cells”** a ser submetido ao “*Journal of Molecular Neuroscience*”.

# Duloxetine activates PI3K/Akt pathway and reduces oxidative stress in human neuroblastoma cells

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**Abstract**

Oxidative stress, induced by mitochondrial disfunction and increased reactive oxygen species, can lead to neuronal cell death in cerebral structures of depressive pacients. In this study, we investigated the neuroprotective effect of the antidepressant duloxetine against rotenone-induced oxidative stress in human neuroblastoma cells (SH-SY5Y). SH-SY5Y cells were pretreated with duloxetine (1, 2 and 5 µM) for 24 h, followed by a 24 h rotenone (10 µM) exposure. 2 µM duloxetine pretreatment antagonized rotenone-induced overproduction of ROS and decreased rotenone-induced death of SH-SY5Y cells. Further, the PI3K/Akt pathway inhibitor, LY294002 (10 µM), blocked duloxetine neuroprotection. Moreover, duloxetine prevented downregulation of Akt phosphorilated immunoreactivity induced by rotenone. Taken together, these results indicate that the mechanisms of duloxetine neuroprotection might involve reduction of oxidative stress and activation of PI3K/Akt pathway. These data suggest that this signaling pathway could be a potential therapeutic target for neurodegenerative conditions associated with oxidative stress and cell death.

**Keywords:** Duloxetine, neuroprotection, rotenone, oxidative stress, Akt, SH-SY5Y cells.

## Introduction

Oxidative stress, mitochondrial functional disruption, and impairment of protein degradation, have been considered as three interrelated molecular pathways that execute neuron death related to mood disorders (Nakamura et al. 2012). Several neuropsychiatric diseases have as initial stage injury and synaptic damage, followed by neuronal loss in specific regions of the brain. In depressive disorders the hippocampus and the prefrontal cortex are the most susceptible areas to undergo neurodegeneration (Bremner 2002; Campbell et al. 2004). On the other hand in Alzheimer's disease and Parkinson's disease the hippocampus and pars compacta of the substantia nigra, are target to degeneration, respectively (Beal 2001; Marella et al. 2007).

In situations that cells such as neurons are stressed with degenerative insults, mitochondria may produce higher levels of reactive oxygen species (ROS) (Fukui and Moraes 2008). Consequently the lipid peroxidation, damage to the cell membrane and DNA, loss of membrane potential and cell lysis is also increased (Ray et al. 2012). However, these ROS can be detoxified by antioxidant systems resident in cells, such as enzymatic: superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) and non enzymatic antioxidant defences: glutathione (Valko et al. 2007).

Recent findings suggest that the oxidative stress is implicated in the pathophysiology of depression (Behr et al. 2012). Serum levels of hydrogen peroxide and nitric oxide are increased in depressive patients (Selek et al. 2008; Maes et al. 2010). In addition, lipid peroxidation is increased too (Khanzode et al. 2003). Such as in humans, analysis of hippocampus and cerebral cortex of rodents submitted to experimental models of depression revealed changes in activity and levels of enzymes of the antioxidant system, such as CAT, GPx, GR and SOD (Zafir and Banu 2009; Lee et al. 2011; Budni et al. 2012; Moretti et al. 2012). On the other hand, recent findings in clinical studies report that antidepressants are able of attenuate those changes, protecting neuronal cells from oxidative damages (Bilici et al. 2001; Michel et al. 2007). Antidepressants can reestablish enzymatic levels, reduce formation of oxidized products and increase antioxidant systems (Zafir and Banu 2009; Abdel-Wahab and Salama 2011). In addition, in *in vitro* models antidepressants have demonstrated induction of the brain derived neurotrophic factor (BDNF) and reduction of oxidative stress, protecting from neuronal death (Grant et al. 2005; Lee et al. 2011).

The BDNF is the most abundant neurotrophin of the brain, with essential role on release of neurotransmitter, neurogenesis and neuronal survival and plasticity (Bibel and Barde 2000). When BDNF binds to the tyrosine kinase receptor B (TrkB), it can lead to activation of intracellular signaling pathways such as extra-cellular signal regulated kinase (ERK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) (Qiao et al. 2004; Shelton et al. 2004). The Akt protein negatively regulates several enzymes and pro-apoptotic proteins, among other: glycogen synthase kinase-3β (GSK-3β), Bad, Bax and Caspase-9. Therefore it has been demonstrated that the PI3K/Akt is a neuroprotetive pathway (Parcellier et al. 2008).

Clinical findings demonstrated that Akt activity decreases in the different brain regions of depressed patients and suicide victims (Hsiung et al. 2003; Karege et al. 2007) and that patients treated chronically with antidepressants displayed a significant increase in the p-Akt levels (Krishnan et al. 2008). The Akt has been implicated in the protective effect of BDNF against apoptotic insults in neural stem cells (Nguyen et al. 2009). In addition, it has been reported that antidepressant induces increased BDNF levels activating survival pathways such as PI3K/Akt (Kitagishi et al. 2012). The duloxetine is a selective serotonin (5-HT) norepinephrine (NE) reuptake inhibitor, indicated for the treatment of major depressive disorder (Hunziker et al. 2005). Recent findings demonstrated that chronic treatment with duloxetine produces an increase in BDNF levels in the different brain regions of rats (Calabrese et al. 2007; Mannari et al. 2008; Molteni et al. 2009; Calabrese et al. 2010). Furthermore, *in vitro* treatment of rat astrocytoma C6 cells also induced increased BDNF levels (Prickaerts et al. 2012).

Firstly was evaluated if duloxetine could protect neuroblastoma cells against rotenone induced citotoxicity, a widely used model to induce oxidative stress and search neuroprotective compounds (Condello et al. 2011; Sapkota et al. 2011). Rotenone is an inhibitor with high affinity to the Complex I of the mitochondrial electron transport chain, which result in massive production of anion superoxide and other ROS leading to cell death (Isenberg and Klaunig 2000; Panov et al. 2005). Although it has been demonstrated that duloxetine induces BDNF and this neurotrophin has neuroprotective action, there are no data demonstrating duloxetine neuroprotective effects, as well as the related mechanisms by which it could lead to increased neuronal cell survival.

## Materials and Methods

### Materials

Dulbecco's modified Eagle's medium: Nutrient mixture F-12 (DMEM/F12), Antibiotic-Antimycotic, Fetal Bovine Serum (FCS) purchased from GIBCO (Grand Island, NY, USA). Duloxetine (Eli Lilly and Company, Indianapolis-Indiana, USA). Rotenone, dichlorodihydrofluorescein diacetate ($H_2$DCF-DA), 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride (LY294002), dimethylsulfoxide (DMSO) and MTT purchased from Sigma (St. Louis, MO, USA). DAPI (4, 6-Diamidino-2- phenylindole) purchased from Invitrogen. Antibody against phosphorylated form of Akt (pSer473) purchased from Sigma. Alexa Fluor-568 anti-rabbit IgG purchased from Invitrogen.

### Cell culture and treatment

Human neuroblastoma SH-SY5Y cells, gently donated by Dr. Marcelo Farina, were maintained in DMEM/F12 supplemented with 10% of FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and kept at 37°C in 5% $CO_2$/humidified air. SH-SY5Y cells, at passages between 3 and 20, were subcultured in 96-well plates (20.000/well) or in 24-well plates (50.000/well) in DMEM/F12 with 1% of FBS. To study the neuroprotective action of duloxetine against rotenone-induced cell death, duloxetine was added to the culture medium 24 h before rotenone treatment. Rotenone (0.5-10 μM) was then added to the same culture medium (pretreated with duloxetine). Twenty-four hours after rotenone challenge, cell viability; measurement of reactive oxygen species or immunofluorescent staining were assessed as described further. In order to investigate the mechanisms underlying the neuroprotective effect of duloxetine against rotenone-induced oxidative stress, the cells were preincubated with phosphatidylinositol-3 kinase/Akt (PI3K/Akt) inhibitor (LY294002, 10 μM), 1 h prior to the addition of duloxetine.

### Cell viability

MTT assay was used to analyze cell viability as previously described (Peng et al. 2008). MTT solution was prepared in Krebs-HEPES buffer (144 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl2, 2 mM $CaCl_2$, 10 mM HEPES and 11 mM Glucose). After treatment, the medium was

removed and 100 µl of MTT solution (0.5 mg/ml, final concentration) was added to each well and plates were incubated at 37°C for 1 h. Then, the solution was removed and 100 µl of DMSO was added. The absorbance of colored reaction was measured at a 540 nm wavelength in a microplate reader. The control cells, without treatment, were considered 100% of viability**.**

Measurements of reactive oxygen species

Levels of intracellular ROS were measured using the fluorescent probe 2, 7- dichlorodihydrofluorescein diacetate ($H_2$DCF-DA). Briefly, cells were incubated for 20 min at 37ºC in a HBSS buffer whose composition was in mM: NaCl 136, KCl 5.4, MgCl 1.4, $NaH_2PO_4$ 1, $CaCl_2$ 1.4, HEPES 10, Glucose 9, pH 7.4, in the presence of 3 µM of $H_2$DCFDA. $H_2$DCFDA diffuses across neuronal membranes, where acetates migrate via intracellular esterases. Oxidation of $H_2$DCFDA occurs almost exclusively in the cytosol, generating a fluorescent response proportional to ROS generation. After loading the dye, cells were washed in HBSS buffer and fluorescence was measured at a 485 nm excitation wavelength and an emission wavelength of 520 nm, using a fluorimetric microplate reader (Tecan, Grödig/Salzburg, Austria). After the fluorescence measurement cells were harvested in 1% Triton X-100. Cell lysates were analyzed for protein content using the Lowry method, and DCF fluorescence was normalized for total protein content.

Immunofluorescence staining

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized for 10 min in PBS containing 0.2% Triton X-100. To reduce nonspecific background staining, the fixed cells were then blocked for 60 min with PBS containing 5% bovine serum albumin. Blocked were incubated overnight at 4ºC in primary antibody rabbit anti-p-Akt (1:100) diluted in PBS-Tween 20 0.1%. After rinsing, SH-SY5Y cells were incubated with the secondary antibody Alexa Fluor-568 anti-rabbit IgG (1:400) for 1h at room temperature. Finally, cells were counterstained with DAPI to color the nuclei. The fluorescence images were acquired using a microscopy (Olympus Bx41, Japan), and analyzed by software (Image J).

Statistical analysis

Statistically significant differences between groups were determined by one-way ANOVA followed by Tukey's post hoc analysis. The level of statistical significance was taken at $p<0.05$.

## Results

Cytotoxicity of duloxetine and rotenone on human neuroblastoma cells

The addition of duloxetine during 24 or 48 h alone did not significantly affect the cell viability (1-10 μM) as shown by colorimetric measurements of MTT reduction. However, from 20 μM it was cytotoxic (Figure 1A). Since rotenone exposure is able to induce apoptosis in human neuroblastoma SH-SY5Y cells (Watabe and Nakaki 2007), we observed that treatment of SH-SY5Y neuroblastoma cells for 24 h with different rotenone concentrations (0.5-10 μM) caused reduction of cell viability (Figure 1B). In particular, 10μM rotenone strongly reduced cell viability by 28 ± 4% ($p \leq 0.001$) when compared with control. Based on these results, this concentration was chosen for use in subsequent experiments.

Duloxetine prevents rotenone-induced ROS and cell death

Rotenone exposure was used to induce exogenous oxidative stress because pro-oxidative events have been reported in depressive patients (Behr et al. 2012). Since rotenone causes oxidative stress (Watabe and Nakaki 2007), ROS generation was measured with the fluorescent probe $H_2$DCFDA (Figure 2A). The cells exposed to rotenone had 1.5 ± 0.11 ($p \leq 0.001$) fold increased ROS production. The pretreatment with 2 μM duloxetine reduced the amount of ROS produced in the rotenone treated cells ($p \leq 0.001$, compared with cells treated with rotenone only) (0.92 ± 0.10). 5 μM duloxetine reduced the amount of ROS to 1.18 ± 0.04 ($p < 0.05$, compared with cells treated with rotenone only). We then evaluated the effect of duloxetine pretreatment on rotenone-induced cell death. Duloxetine was added to cell cultures 24 h before rotenone exposure. As illustrated in Figure 2B, 2 μM duloxetine demonstrated a protective effect against rotenone-induced injury, with cell viability

increasing to 90 ± 2% of control in the presence of 2 µM duloxetine ($p < 0.05$, compared with cells treated with rotenone only).

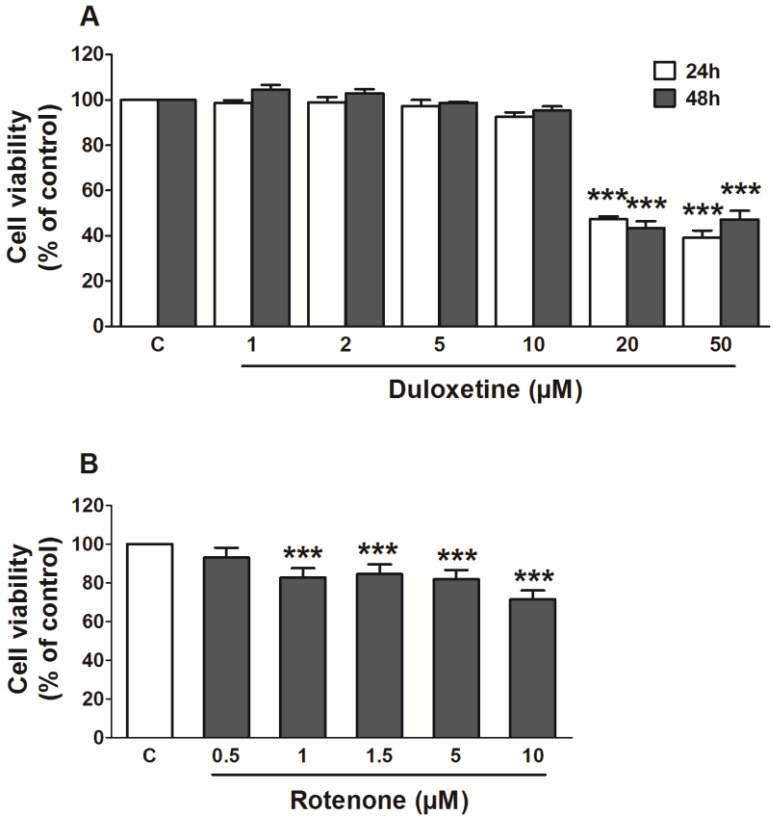


**Figure 1**. Cytotoxicity of duloxetine and rotenone on human neuroblastoma cells**.** (A) SH-SY5Y cells were incubated with duloxetine (1-50 µM) for 24 and 48 h. (B) Effect of rotenone (0.5-10 µM, 24 h) on human neuroblastoma cell viability. Data are expressed as means ± SEM and compared with control cells (considered as 100%). ***$p \leq 0.001$, when compared with control (n=3). C, control (one-way ANOVA analysis followed by Tukey post hoc test).

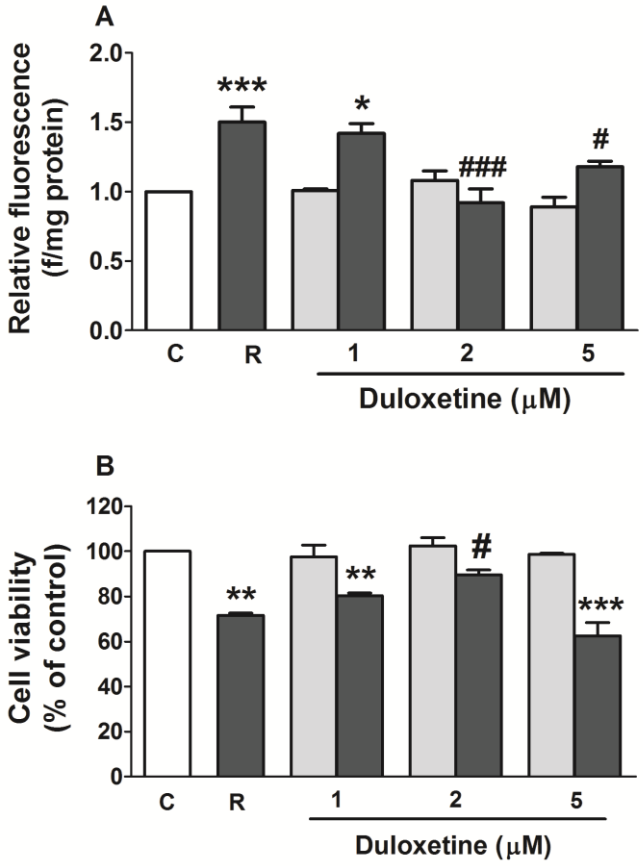


**Figure 2**. Duloxetine protects from rotenone-induced ROS production and cell death in SH-SY5Y cells. Cells were preincubated with 1-5 μM duloxetine for 24 h, followed by 10 μM rotenone (R) treatment for 24 h. (A) Intracellular ROS production was determined by DCF fluorescence. Results are expressed as fold increase of the fluorescence intensity over control cells (considered as 1.0) (B) Cell viability was evaluated by MTT assay. The treated groups were compared with control cells (considered as 100%). Data are expressed as mean ± SEM. For statistical evaluations, one-way ANOVA analysis followed by Tukey post hoc test was performed. *$p < 0.05$; **$p \leq 0.01$ *** and ***$p \leq 0.001$ when compared with control; #$p < 0.05$; ###$p \leq 0.001$ compared with cells treated with rotenone only (n=3). C, control; R, rotenone.

The involvement of PI3K/Akt pathway in the neuroprotective effect of duloxetine against oxidative stress

Next, we investigated whether the Akt signaling pathway is associated with these protection effects of duloxetine 2 μM treatment, since at 2 μM concentration maximum protection was oberseved. Pretreatment with LY294002, a specific inhibitor of PI3K/Akt signaling pathway, abolished duloxetine protection against rotenone-induced ROS production (1.51 ± 0.15) ($p \leq 0.01$, compared with cells treated with duloxetine plus rotenone) (Figure 3A). The duloxetine effect of discharge the cells from death, was lost with addition of LY294002 (69 ± 4%) ($p < 0.05$, compared with cells treated with duloxetine plus rotenone) (Figure 3B).

Duloxetine reverses rotenone-induced downregulation of phosphorylated-Akt

We further investigated the involvement of PI3K/Akt activation using immunofluorescence analysis. Here we used 4 h of 100 μM zinc treatment as a positive control, since it was demonstrated to induce Akt phosphorylation (An et al. 2005). Figure 4 illustrates the reduced phosphorylated Akt immunoreactivity induced by rotenone (Rot). Pretreatment with 2 μM duloxetine prevented this reduction (Dul+Rot) and LY294002 abolished duloxetine effect (LY+Dul+Rot). Taken together, these results indicate that duloxetine protects SH-SY5Y cells against oxidative stress by a mechanism that implicates PI3K/Akt pathway.

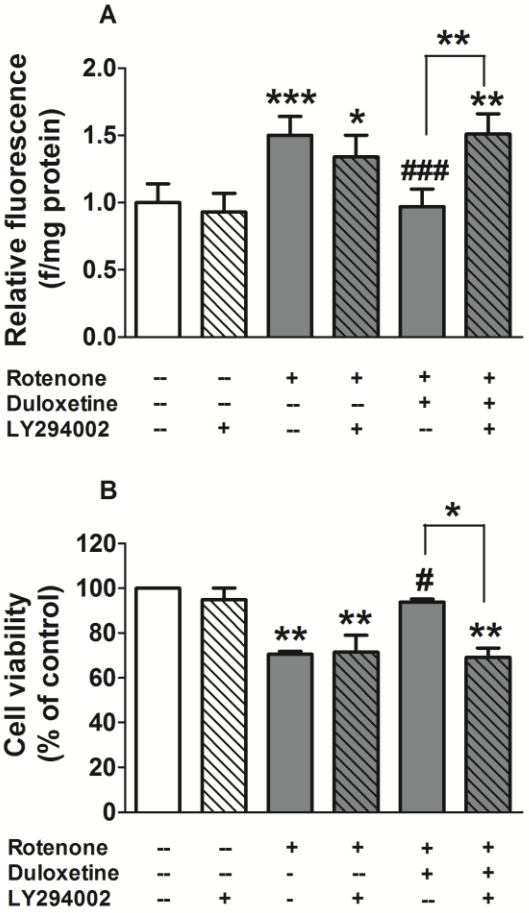


**Figure 3**. Effect of PI3K-Akt inhibition on duloxetine effect on rotenone induced ROS production and cell death. Cells were treated with LY204002 1h prior to duloxetine 2 μM, then incubated for 24 h, followed by 10 μM rotenone (R) treatment for more 24 h. (A) Intracellular ROS production was determined by DCF fluorescence. Results are expressed as fold increase of the fluorescence intensity over control cells (considered as 1.0). (B) Cell viability was evaluated by MTT assay. The treated groups were compared with control cells (considered as 100%). Data are expressed as mean ± SEM. For statistical evaluations, one-way ANOVA analysis followed by Tukey post hoc test was performed. *p <0.05; **p ≤ 0.01; ***p ≤ 0.001 when compared with control; #p ≤ 0.05; ###p ≤ 0.001 compared with cells treated with rotenone only (n=3).

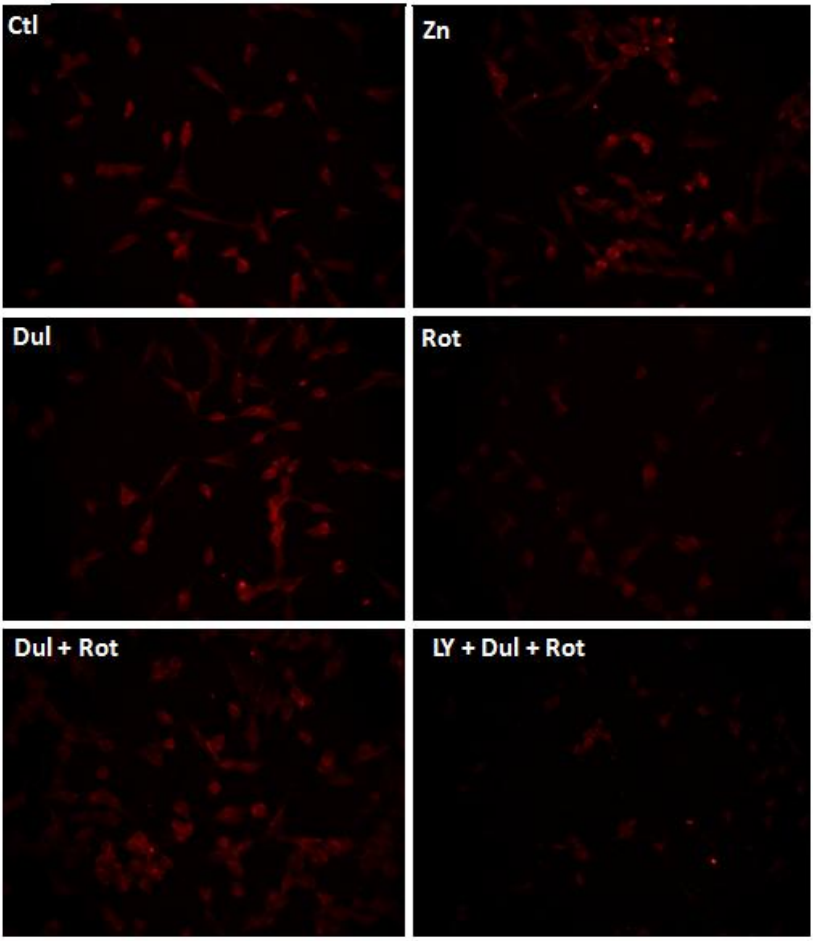


**Figure 4**. Representative image of immunofluorescence staining of p-Akt in SH-SY5Y cells. Cells were preincubated with 2 μM duloxetine (Dul) for 48 h, or 100 μM zinc (Zn) for 4 h, or 10 μM rotenone for 24 h, or 2 μM duloxetine for 24 h followed by 10 μM rotenone treatment for 24 h (Dul+Rot), or 1 h with LY294402 prior 2 μM duloxetine for 24 h followed by 10 μM rotenone treatment for 24 h (LY+Dul+Rot). A clear down regulation p-Akt immunoreactivity occurs following exposure of SH-SY5Y cells to rotenone.

## Discussion

Oxidative stress is involved in the pathofisiology of most of the neurodegenerative disorders. In the degeneration process neurons exhibit several common pathological features including increased oxidative stress, mitochondrial dysfunction and activation of cell death pathway (Nakamura et al. 2012). Since increased neuronal death has been reported by *postmortem* studies in depressed patients (Lucassen et al. 2001; Banasr et al. 2011) and increased ROS production can lead to apoptotic cell death (Danial and Korsmeyer 2004), we established the rotenone-induced cell death as a model to study potential neuroprotective action of the antidepressant duloxetine. This model has been used to evaluate neuroprotective action of several compounds in SH-SY5Y cells against oxidative stress and apoptosis (Chen et al. 2008; Xiong et al. 2009; Sapkota et al. 2011; Filomeni et al. 2012). The mechanism of rotenone-induced cell death involves the overproduction of reactive oxygen species (Marella et al. 2007), imbalance of cellular antioxidant systems (Ding et al. 2008), mitochondrial membrane depolarization (Moon et al. 2005), the opening and of the mitochondrial permeability transition pore (Isenberg and Klaunig 2000), release of cytochrome c (Doran and Halestrap 2000) that leads to cell death..

Previously, it was demonstrated that treatment with 10 μM rotenone for 24 h resulted in apoptotic cell death in SH-SY5Y cells (Pan et al. 2005; Wu et al. 2011). In our study, rotenone induced toxicity in different concentrations, and we chose 10 μM for next assays. We then determined if pretreatment with duloxetine could protect from rotenone-induced injury. The increased production of ROS induced by rotenone was reduced by duloxetine pretreatment (2 and 5 μM). Moreover, the loss on cell viability induced by 10 μM rotenone was blocked by the pretreatment of 2 μM duloxetine.

These data corroborate with other studies demonstrating the antioxidant effect of antidepressants. Pretreatment with amitriptyline and fluoxetine induced increased SOD activity and reduced cell death induced by hydrogen peroxide in PC12 cells (Kolla et al. 2005). Moreover, fluoxetine significantly inhibited LPS-induced activation of microglia and ROS production (Zhang et al. 2012). Furthermore, pretreatment with imipramine, fluvoxamine, or reboxetine inhibited nitric oxide production in a concentration-dependent manner in interferon-gamma-induced microglial activation (Hashioka et al. 2007). Schmidt et al. (2008) reported that treatment with desipramine, imipramine, maprotiline and mirtazapine was able to modulate mRNA

levels of SOD, GST, and GR enzymes in human monocytic U-937 cells. In *in vivo* assays venlafaxine, an antidepressant from the same therapeutic group that duloxetine, reversed oxidative stress (Kumar et al. 2010; Abdel-Wahab and Salama 2011; Khanam et al. 2012). However, despite many studies, the molecular mechanisms that lead to antidepressant neuroprotective effects remain unknown. We than explored the mechanism involved in duloxetine neuroprotection.

Among others, the PI3K/Akt is an important neuroprotetive pathway (Parcellier et al. 2008). Akt, also known as protein kinase B, is a widely expressed cytoplasmic serine-threonine protein kinase. Akt is normally inactive in the cell cytosol and can be activated by a number of growth factors, through a phosphatidylinositol 3-kinase (PI3-K)–dependent mechanism. Once activated, PI3K phosphorylates the membrane phospholipid phosphatidylinositol (4, 5)-diphosphate (PIP2), converting it to phosphatidylinositol (3,4,5)-triphosphate (PIP3). The accumulation of PIP3 promotes the translocation of Akt to the plasma membrane, where Akt binds to PIP3 via its PH domain, allowing phosphorylation of Thr308 on Akt by phosphoinositide-dependent kinase 1(PDK1) (Hemmings and Restuccia 2012). Active Akt is present in the cytosol and nucleus (Meier et al. 1997; Bijur and Jope 2003). Akt inhibits GSK-3β (a major substrate of Akt) activity by enhancing phosphorylation at Ser9. Inactivation of Akt triggers GSK-3β activity through decreasing phosphorylation, which plays a key role in neuronal loss occurring in neurodegenerative diseases (Parcellier et al. 2008).) (Kim et al. 2001; Kim et al. 2002). Moreover, Akt phosphorylates Bad at Ser136 producing a docking site for 14-3-3 protein and this interaction leads Bad to be keeping in the cytosol away from mitochondria, preventing apoptosis (Parcellier et al. 2008). Akt has also been shown to phosphorylate Bax at Ser 184. This phosphorylation affects Bax translocation to mitochondria: the phosphorylated Bax form keeps it heterodimerized with Bcl-2 and Bcl-xL avoindig its proapoptotic activity (Gardai et al. 2004).

The Akt role in several biological events has been evaluated using as experimental tool the specific inhibitor of the PI3K/Akt pathway LY294002 (Yamaguchi et al. 2004; Dhandapani et al. 2005; Shinohara et al. 2006). In this study when we used the inhibitor of the PI3K/Akt pathway, we found the effect of duloxetine on rotenone-induced ROS production and decrease on cell viability was blocked. Moreover, in the immunofluorescence staining we observed that rotenone reduced phosphorylated Akt immunoreactivity and the pretreatment with duloxetine was able to restore it. However, duloxetine

loses its effect in the presence of the LY294002. The decreased Akt phosphorylation can be related to the rotenone-induced increase on ROS levels. It has been reported that high ROS levels induce Akt dephosphorylation leading to caspase-dependent and independent degradation pathways (Martin et al. 2002; Mann et al. 2008; Kim et al. 2011).

In agreement with our data other antidepressants activate Akt in *in vitro* models. It was demonstrated that fluoxetine significantly upregulated expression of the phosphorylated-Akt in rat neuronal stem cells and fluvoxamine in PC12 cells (Nakano et al. 2010; Huang et al. 2012). Phenelzine, a monoamineoxidase inhibitor, also reversed the decrease in p-Akt induced by formaldehyde in cortical neurons (Song et al. 2010).

A possible mechanism underling the antidepressant activation of Akt pathway could be related to the increased BDNF levels induced by antidepressants (Duman and Monteggia 2006; Kitagishi et al. 2012). Enforcing this hypothesis it was demonstrated that Akt is implicated in the protective effect of BDNF against apoptotic insults in neural stem cells (Nguyen et al. 2009). Furthermore, the cell death induced by serum withdrawal in cortical neurons is reverted by BDNF through PI-3K-Akt pathway activation (Hetman et al. 1999). Recently, *in vivo* studies also reported such effects of antidepressants supporting this idea. Fluoxetine chronic treatment could markedly reverse the decrease in p-Akt levels of stress-induced depression mouse (Krishnan et al. 2008). Furthermore, acute administration of fluoxetine increased Akt, CREB, BDNF and Bcl-2 total protein levels in the prefrontal cortex, hippocampus and striatum of rats (Reus et al. 2012). Since duloxetine was able to induce BDNF expression *in vitro* and *in vivo* studies (Calabrese et al. 2007; Mannari et al. 2008; Molteni et al. 2009; Calabrese et al. 2010; Prickaerts et al. 2012), this BDNF-Akt survival pathway could have essential role in the neuroprotective effect we observed.

Taking in account that duloxetine was able promote neuroprotective effect against ROS induction and cell death in SH-SY5Y cells; and that the neuroprotective effect was dependent on Akt activation; we could speculate that the mechanism underling this effect could be related to the downstream Akt targets on survival pathways (Figure 5). The duloxetine induced Akt activation could be leading to Nrf2 nuclear translocation which increases antioxidant enzymes expression like HO-1, SOD and GPx whose could reduce cellular ROS levels (Dringen et al. 2005; McCord and Edeas 2005; Osburn and Kensler 2008). In addition, Nrf2 activators have been pointed as

promising therapeutic strategies to depression treatment (Maes et al. 2012). A previous study shown that pretreatment with desipramine induced increased HO-1 expression mediated by Nrf2, thus protecting neuronal Mes23.5 cells against rotenone- and 6-hydroxydopamine-induced neuronal death (Lin et al. 2012). Moreover activating the antioxidant, Akt could directly act blocking the apoptotic pathways such as Bad, Bax and GSK-3β (Parcellier et al. 2008) (Figure 5). On the other hand, further assays are necessary to confirm and clearly understand this mechanism. This is the first study demonstrating that duloxetine activates Akt, reduces ROS and protect from neuronal cell death. In addition we corroborate with other studies that report antidepressant potential of promote neuroprotection through an Akt dependent manner.

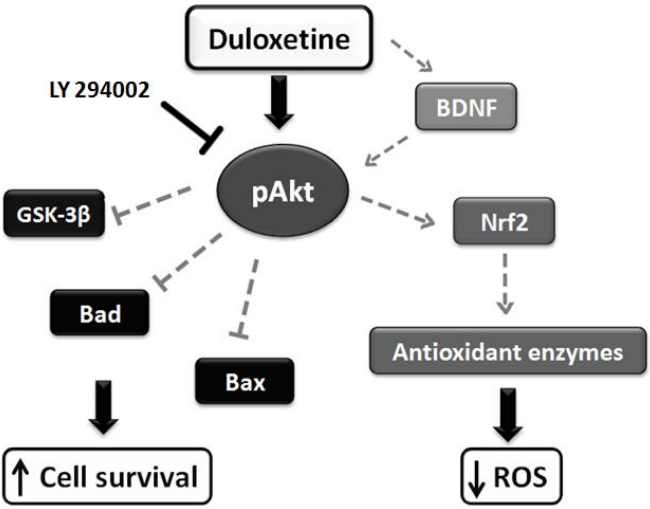


**Figure 5**. Schematic illustration of the neuroprotective effect of duloxetine. The filled arrows represent observations from our study, while the dotted arrows indicate the results from literature findings.

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# 5. DISCUSSÃO GERAL

Vários compostos antidepressivos compartilham o mesmo mecanismo de ação: aumentam rapidamente os níveis de monoaminas na fenda sináptica (BERTON e NESTLER, 2006).

Contudo, os efeitos terapêuticos dos antidepressivos se manifestam somente após algumas semanas de tratamento. Este efeito "tardio" dos antidepressivos estaria relacionado à ativação de vias de transdução de sinal, transcrição gênica e expressão de proteínas envolvidas na neuroplasticidade (MASI e BROVEDANI, 2011). Os antidepressivos atenuam e/ou retardam as alterações estruturais observadas no cérebro de pacientes depressivos (CASTREN, 2004; NESTLER et al., 2002; SCHLOSS e HENN, 2004). Uma das possíveis causas destas alterações seria a redução nos níveis de neurotrofinas (BREMNER, 2002). De fato, as neurotrofinas que estão reduzidas em pacientes depressivos são: primeiramente o BDNF, além de NGF, FGF-2 e NT-3 (DINIZ et al., 2012; DWIVEDI et al., 2005; EVANS et al., 2004; HASHIMOTO, 2010). Portanto, uma deficiência destas proteínas afetariam a neurogênese, diferenciação e proliferação celular, expressão de proteínas sinápticas e a síntese e a liberação de neurotransmissores (SKAPER, 2008). Além disso, as neurotrofinas ativam vias de sinalização celular que regulam a expressão de proteínas envolvidas na sobrevivência e na morte celular (D'SA e DUMAN, 2002).

Neste sentido, os resultados apresentados na primeira parte desta Dissertação, se referem aos efeitos do tratamento crônico com duloxetina na expressão gênica de neurotrofinas e de proteínas apoptóticas em tecidos cerebrais de camundongos. O córtex e o hipocampo foram escolhidos para avaliar a expressão gênica, pois estas estruturas límbicas são as mais afetadas em pacientes depressivos (BREMNER, 2002; CAMPBELL et al., 2004; VIDEBECH e RAVNKILDE, 2004).

Os resultados do Manuscrito 1 mostram que a administração crônica de duloxetina aumentou a expressão gênica de BDNF no córtex e no hipocampo. Estes dados estão de acordo com estudos prévios demonstrando que a duloxetina (CALABRESE et al., 2010; CALABRESE et al., 2007) e vários outros antidepressivos (DURIC e MCCARSON, 2006; MOLTENI et al., 2006; ROGOZ e LEGUTKO, 2005; ROGOZ et al., 2005; XU et al., 2006) induzem aumento nos níveis de BDNF. O BDNF é um dos principais candidatos a mediador

dos efeitos dos antidepressivos na neuroplasticidade (CASTREN e RANTAMAKI, 2010). De fato, animais deficientes para o BDNF e/ou seu receptor TrkB não respondem ao tratamento com alguns antidepressivos (ADACHI et al., 2008; SHIRAYAMA et al., 2002).

Como mostrado na Figura 1 desta Dissertação, as neurotrofinas se ligam a receptores Trk, ativando vias de sinalização celular como a Akt, PLC e ERK. Estas atuam diretamente sobre "alvos" no citosol ou no núcleo, ativando a transcrição de proteínas reguladoras da sobrevivência celular, como a Bcl-2 e a Bcl-xL (D'SA e DUMAN, 2002). Como a duloxetina induziu BDNF, foi investigado se esta afetaria a expressão das proteínas reguladoras da apoptose. A administração crônica de duloxetina induziu um aumento na expressão gênica das proteínas anti-apoptóticas Bcl-2 e Bcl-xL no córtex de camundongos. Dados similares foram obtidos quando ratos submetidos a modelos de depressão tiveram diminuição nos níveis de Bcl-2 e Bcl-xL no córtex, e estes efeitos foram revertidos pelo tratamento crônico com antidepressivos (KOSTEN et al., 2008). A injeção de BDNF na região CA1 do hipocampo induziu um aumento no RNAm de Bcl-xL (CHAO et al., 2011). Nos animais tratados com duloxetina, o aumento de BDNF foi mais expressivo no córtex do que hipocampo; o aumento de Bcl-2 e Bcl-xL foi observado apenas no córtex. Este resultado pode indicar que o aumento significativo de BDNF no córtex regulou positivamente a expressão de Bcl-2 e de Bcl-xL. Estes dados estão de acordo com os resultados do tratamento crônico com venlafaxina (da mesma classe da duloxetina) que induziu um aumento significativo nos níveis das proteínas BDNF e Bcl-2 e do RNAm de Bcl-xL em ratos (WANG et al., 2011; XU et al., 2003). Um dado importante deste estudo foi que a duloxetina afetou a expressão gênica de proteínas pró-apoptóticas: reduziu Bax e p53 no hipocampo e de Bad no córtex. De modo diferente, o tratamento com venlafaxina também reduziu a expressão de Bax, mas no hipocampo de ratos (WANG et al., 2011). É importante salientar que este é o primeiro trabalho demonstrando que um antidepressivo reduz a expressão das proteínas indutoras de morte celular p53 e Bad.

O conjunto destes dados demonstra que a duloxetina foi capaz de aumentar a expressão gênica das neurotrofinas BNDF, FGF-2 e NT-3. Assim, através destes fatores neurotróficos, a duloxetina pode afetar vários processos celulares e a neuroplasticidade (REICHARDT, 2006). Uma possível explicação sobre como os antidepressivos poderiam induzir a expressão das neurotrofinas pode ser encontrada na revisão de COWEN (2007). Este autor sugere que os antidepressivos induzem o aumento de monoaminas na fenda. Estas se ligam aos seus receptores de

membrana pós-sinápticos que ativam vias de sinalização intracelular que aumentam a transcrição e expressão de neurotrofinas. Estas podem atuar sobre fatores de transcrição para aumentar a expressão de proteínas envolvidas na sobrevivência celular (D'SA e DUMAN, 2002).

Dentre as vias de sinalização implicadas na modulação de proteínas anti e pró-apoptóticas pode ser citada a via PI3K/Akt. A Akt fosforila e bloqueia a atividade pró-apoptótica das proteínas Bad e Bax (PARCELLIER et al., 2008). A Akt também atua sobre fatores de transcrição nucleares, regulando a expressão de proteínas anti e pró-apoptóticas. A Akt modula a ativação de NF-κB, fator de trancrição para a Bcl-xL e de CREB, fator de transcrição para a Bcl-2 (LAUDER et al., 2001). Além disso, a Akt fosforila e ativa a enzima Mdm2, uma ubiquitina ligase citosólica. Quando fosforilada, a Msm2 faz a ubiquitinilação do fator de transcrição da proteína p53 nuclear, o que causa a sua degradação, inibindo a morte celular. A p53 também induz o gene promotor para a transcrição da Bax, assim uma redução da p53 estaria relacionada também a uma redução de Bax (MIYASHITA e REED, 1995; OGAWARA et al., 2002). Os dados dos experimentos *in vivo* (Manuscrito 1) permitiram verificar quais fatores são aumentados ou reduzidos pelo tratamento com duloxetina. Porém o estudo dos mecanismos envolvidos no efeito neuroprotetor da duloxetina poderia ser mais facilmente elucidado em condições i*n vitro*.

A morte neuronal observada no cérebro de pacientes depressivos pode ser causada por vários fatores, tais como estresse oxidativo, excitotoxicidade glutamatérgica e níveis elevados de glicocorticoides (CZEH e LUCASSEN, 2007; DRZYZGA et al., 2009; MAES et al., 2012). Alguns antidepressivos como o escitalopram, venlafaxina, bupropiona, doxepina e amitriptilina mostraram uma ação antioxidante. Elas aumentam a expressão de enzimas antioxidantes como a SOD e GPx, ou de moléculas antioxidantes como a glutationa (ABDEL-WAHAB e SALAMA, 2011; LEE et al., 2011; LI et al., 2000; ZAFIR e BANU, 2009). Entretanto, os mecanismos pelos quais os antidepressivos atuam sobre estas proteínas não estão ainda elucidados (ABDEL-WAHAB e SALAMA, 2011; LEE et al., 2011; LI et al., 2000; ZAFIR e BANU, 2009).

Neste sentido, foi questionado se a duloxetina teria um possível efeito neuroprotetor contra o estresse oxidativo. Para realizar os experimentos foi utilizado o modelo de citotoxidade *in vitro* da rotenona (CHEN et al., 2008; FILOMENI et al., 2012; SAPKOTA et al., 2011; XIONG et al., 2009). A rotenona é um inibidor do complexo I da cadeia transportadora de elétrons e induz um aumento massivo de ROS. A

grande quantidade de ROS intracelular, um "sinal apoptótico" causa danos celulares e a abertura do poro de transição de permeabilidade mitocondrial (ISENBERG e KLAUNIG, 2000) levando a morte celular (ISENBERG e KLAUNIG, 2000; MARELLA et al., 2007)

No Manuscrito 2, células de neuroblastoma humano (SH-SY5Y) pré-tratadas com duloxetina apresentaram níveis reduzidos de ROS intracelular na presença de rotenona. Como consequência, nestas células a duloxetina protegeu da morte celular induzida pela rotenona. O mecanismo de indução de morte celular pela rotenona em células SH-SY5Y envolve a redução de Bcl-2 e o aumento de Bax (KIM et al., 2009). Entretanto, o tratamento *in vivo* com duloxetina aumenta Bcl-2 e reduz Bax (Manuscrito 1) e isto poderia estar bloqueando os efeitos da rotenona. Além disso, o efeito citotóxico da rotenona é dependente de Bad (WATABE e NAKAKI, 2004). Mas, a expressão desta proteína também foi reduzida pelo tratamento crônico de camundongos com a duloxetina (Manuscrito 1).

A GSK-3β é outra proteína envolvida na indução de morte pela rotenona, mas esta também sofre inibição quando a Akt é ativada, por exemplo, por neurotrofinas (GIMENEZ-CASSINA et al., 2012; KIM et al., 2001; KIM et al., 2002). Portanto, a pergunta seguinte foi se a neuroproteção induzida pela duloxetina envolve a via PI3K/Akt. O efeito da duloxetina em inibir a produção de ROS e proteger contra a morte celular foi revertido pelo inibidor da via PI3K/Akt, LY294002. A participação desta via foi também confirmada nos experimentos de imunofluorescência, onde a duloxetina aumentou os níveis de Akt-fosforilada. Além disso, a rotenona reduziu a expressão de Akt, mas a duloxetina também reverteu essa inibição. Os dados sugerem que a duloxetina ativa a via PI3K/Akt para reduzir os níveis de ROS e consequentemente induz um efeito neuroprotetor contra o estresse oxidativo. A ativação desta via protege as células da morte celular induzida por estresse oxidativo e deprivação de fatores tróficos e de nutrientes (TASAKI et al., 2010).

Porém, o mecanismo pelo qual a duloxetina atua reduzindo o estresse oxidativo não está apresentado. LIN e colaboradores (2012) demonstraram que a desipramina induz a ativação de Nrf2, levando a um aumento na expressão de HO-1 e protegendo células neuronais da morte induzida por rotenona e 6-hidroxidopamina. Deve ser ressaltado que um dos "alvos" da Akt ativada é a fosforilação de Nrf2, que provoca a sua translocação para o núcleo, onde ativa a transcrição gênica (ROJO et al., 2008). O Nrf2 é responsável por induzir a expressão de várias enzimas do sistema antioxidante como SOD, CAT, GPx e GR

(DRINGEN et al., 2005; MCCORD e EDEAS, 2005; OSBURN e KENSLER, 2008), que então reduzem os níveis de ROS. Assim, um possível mecanismo para explicar o efeito neuroprotetor pela duloxetina, seria que o antidepressivo ativa a via PI3K/Akt e esta ativa o Nrf2 a aumentar a expressão de enzimas antioxidantes, reduzindo o estresse oxidativo induzido pela rotenona.

O conjunto dos experimentos *in vivo* e *in vitro* apresentados nos Manuscritos 1 e 2 auxiliam na compreensão de como a duloxetina afeta a neuroplasticidade e a sobrevivência celular (Figura 5). A duloxetina aumenta os níveis de serotonina e de noradranalina nas sinapses, estas atuam sobre seus receptores de membrana ativando vias de sinalização celular como a PI3K/Akt, PLC ou MAPK/ERK. Estas ativam fatores de transcrição no núcleo, aumentando a expressão de neurotrofinas e de proteínas anti-apoptóticas (Bcl-2 e Bcl-xL) e/ou reduzindo as pró-apoptóticas (Bax, Bad e p53). As neurotrofinas são liberadas no meio extracelular e se ligam a receptores Trk na membrana que ativam a via PI3K/Akt. A Akt atua sobre alvos citosólicos potenciando os efeitos inicialmente desencadeados pelas monoaminas. Ela também pode diretamente inativar as proteínas pró-apoptóticas (Bax e Bad) ou ativar o fator de transcrição Nrf2 a aumentar a expressão de enzimas antioxidantes, reduzindo os níveis de ROS e protegendo as células da morte celular.

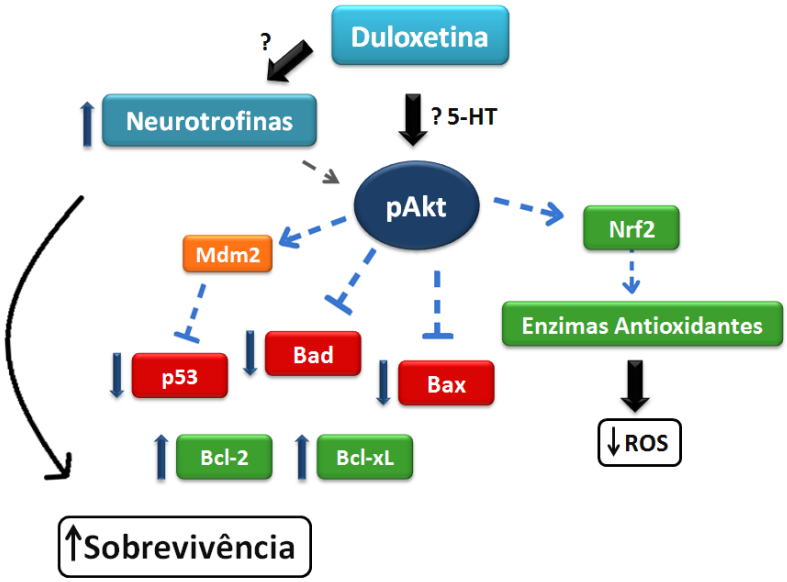


**Figura 5.** Proposta do mecanismo neuroprotetor da duloxetina.As linhas contínuas representam resultados observados neste trabalho, enquanto as linhas pontilhadas representam observações da literatura.

# 6. CONCLUSÕES

Os resultados obtidos neste trabalho indicam que:

- A administração crônica de duloxetina (21 dias, por via oral) produziu efeito antidepressivo no teste do nado forçado (TNF) em camundongos;
- O tratamento crônico de camundongos com duloxetina induziu um aumento na expressão gênica (RNAm) de BDNF e FGF-2 no córtex e no hipocampo, e de Bcl-2, Bcl-xL e NT-3 no córtex; além de reduzir Bax e p53 no hipocampo e de Bad no córtex;
- O pré-tratamento com duloxetina protegeu células de neuroblastoma humano contra o estresse oxidativo e a morte celular induzidos pela rotenona;
- O efeito neuroprotetor da duloxetina em células de neuroblastoma humano envolve a ativação da via PI3K/Akt.

## 7. PERSPECTIVAS

Alguns aspectos relacionados a este trabalho ainda precisam ser melhor elucidados e estas são algumas perspectivas:

- Investigar o mecanismo de ação do efeito antioxidante da duloxetina, determinando o papel das defesas antioxidantes: níveis de glutationa e superóxido dismutase, catalase, glutationa peroxidase, heme oxigenase-1;
- Avaliar o efeito *in vitro* da duloxetina na expressão gênica e de proteínas anti-apoptóticas (BDNF, NGF, FGF, NT-3, Bcl-2, Bcl-xL) e pró-apoptóticas (Bax, Bad e p53) e do fator Nrf-2;
- Confirmar os resultados utilizando RNAs de interferência para TrkB, Akt e Bax para estudar o papel destas proteínas no efeito neuroprotetor induzido pela duloxetina.

Durante a realização do Mestrado foram publicados os seguintes trabalhos em colaboração:

Zomkowski AD, Engel D, Cunha MP, Gabilan NH, Rodrigues AL. **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.** Pharmacol Biochem Behav. 2012

Budni J, Zomkowski AD, Engel D, Santos DB, Dos Santos AA, Moretti M, Valvassori SS, Ornell F, Quevedo J, Farina M, Rodrigues AL. **Folic acid prevents depressive-like behavior and hippocampal antioxidant imbalance induced by restraint stress in mice.** Exp Neurol. 2013

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