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# AGMATINA, UM CANDIDATO À ADJUVANTE DA FARMACOTERAPIA DA DEPRESSÃO: ESTUDOS IN VITRO E IN VIVO

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*"O importante é não parar de questionar"*
*(Albert Einstein)*

## RESUMO

A agmatina é um neuromodulador, cuja atividade antidepressiva tem sido mostrada tanto em ensaios pré-clínicos como clínicos. Contudo, os mecanismos celulares e moleculares responsáveis por seu efeito não estão totalmente esclarecidos. Estudos dos últimos 40 anos têm demonstrado que episódios de estresse induzem anormalidades do eixo hipotálamo-pituitária-adrenal e hipersecreção de glicocorticóides os quais estão implicados na fisiopatologia da depressão. O presente estudo verificou a abilidade da agmatina em: i) produzir um efeito antidepressivo no teste da suspensão pela cauda (TSC) após sua administração sub-crônica em camundongos e o envolvimento de vias de sinalização intracelular hipocampal; ii) abolir o comportamento tipo-depressivo no teste do nado forçado (TNF) e o desequilíbrio oxidativo hipocampal induzidos pelo estresse de contenção (EC) em camundongos; iii) proteger células neuronais hipocampais murinas HT22 frente à citotoxicidade induzida pela corticosterona e o envolvimento do fator de transcrição Nrf2; iv) abolir o comportamento tipo-depressivo no TSC e anedônico no splash teste, induzidos pela corticosterona em camundongos e a participação dos sistemas monoaminérgicos, do fator neurotrófico derivado do encéfalo (BDNF), da proteína sináptica sinaptotagmina I (Syt I), das células neurogliais e do fator de transcrição Nrf2. O tratamento sub-crônico com agmatina (0,01 e 0,1 mg/kg, p.o.) produziu um efeito antidepressivo no TSC sem afetar a atividade locomotora dos animais. Adicionalmente, a agmatina (0,001-0,1 mg/kg, p.o.) aumentou a fosforilação de substratos da proteína cinase A (PKA), a fosforilação da proteína cinase B (PKB)/Akt ($Ser^{473}$), da enzima glicogênio sintase cinase-3β (GSK-3β) ($Ser^{9}$), da cinase regulada por sinal extracelular 1/2 (ERK1/2), da proteína de ligação responsiva ao AMP cíclico (AMPc) (CREB) ($Ser^{133}$) e o conteúdo de BDNF de uma maneira dose-dependente no hipocampo. Agmatina (0,001-0,1 mg/kg, p.o.) também reduziu a fosforilação da c-Jun N-terminal cinase 1/2 (JNK1/2). A fosforilação da proteína cinase C (PKC) e de $p38^{MAPK}$ não foi alterada em nenhuma condição experimental. Em relação ao modelo de EC em camundongos, este protocolo induziu um comportamento tipo-depressivo no TNF, peroxidação lipídica, aumento da atividade hipocampal das enzimas superóxido dismutase (SOD), glutationa peroxidase (GPx) e glutationa redutase (GR), reduziu a atividade da catalase (CAT) e aumentou a razão SOD/CAT, um índice de condições pró-oxidativas, mas não afetou os níveis de glutationa reduzida. Agmatina (10 mg/kg, p.o.)

aboliu o comportamento tipo-depressivo induzido pelo EC e preveniu a peroxidação lipídica e as alterações nas atividades de SOD, GR e CAT na razão SOD/CAT induzidas pelo EC. Na linhagem de células neuronais hipocampais murinas HT22, o tratamento com agmatina (10 μM) aboliu a produção de espécies reativas de oxigênio e a apoptose induzidas pela corticosterona, de uma maneira dose e tempo-dependentes. A combinação de concentrações sub-efetivas de agmatina e fluoxetina ou imipramina produziu um efeito citoprotetor sinérgico. O efeito neuroprotetor da agmatina foi abolido por ioimbina (antagonista de receptor $\alpha_2$-adrenérgico), cetancerina (antagonista de receptor 5-$HT_{2A}$), LY294002 (inibidor de PI3K), PD98059 (inibidor de MEK1/2), SnPP (inhibitor da enzima hemoxigenase-1, HO-1) e cicloheximida (inibidor da síntese protéica). Agmatina também aumentou a fosforilação de Akt e de ERK e induziu translocação nuclear de Nrf2 bem como a expressão de HO-1 e da subunidade catalítica da enzima glutamato cisteína ligase (GCLc); a indução dessas proteínas foi abolida por ioimbina, cetanserina, LY294002 e PD98059. Por último, utilizando camundongos Swiss, o tratamento por 21 dias com agmatina (0,1 mg/ kg, p.o) preveniu, de maneira similar ao antidepressivo clássico imipramina, o comportamento tipo-depressivo no TSC e anedônico induzidos pela corticosterona. Além disso, agmatina aumentou os níveis hipocampais de noradrenalina, serotonina (5-HT), e dopamina tanto nos animais controle como nos tratados com corticosterona; e ainda preveniu a diminuição de 5-HT e o aumento de glutamato induzidos pela corticosterona. A agmatina aumentou a fosforilação de CREB nos animais controle; o imunoconteúdo de BDNF maduro (BDNFm), reduziu o conteúdo de BDNF imaturo (pro-BDNF), aumentou a razão BDNFm/pro-BDNF e o conteúdo de Syt I, de HO-1 e de GCLc tanto nos animais controle como nos tratados com corticosterona. Adicionalmente, o tratamento com agmatina foi capaz de prevenir a redução do conteúdo de BDNFm e de Syt I, e a retração e diminuição da quantidade de células astrogliais e microgliais em CA1 hipocampal induzidas pela corticosterona. Agmatina produziu um efeito antidepressivo nos camundongos C57BL/6 Nrf2 (+/+) no TSC e splash teste, mas não nos animais Nrf2 (-/-). O presente estudo ampliou de maneira significativa o conhecimento dos mecanismos celulares e moleculares implicados no efeito antidepressivo da agmatina através de ensaios *in vitro* e *in vivo*. Nosso conjunto de resultados indica que o efeito antidepressivo da agmatina parece ser mediado pela sua atuação sobre as principais hipóteses que explicam a fisiopatologia da depressão e terapia antidepressiva: monoaminérgica, neurotrófica, oxidativa e

glutamatérgica, indicando seu potencial como adjuvante/monoterapia para o tratamento da depressão.

**Palavras chave:** agmatina, depressão, neuroproteção, estresse de contenção, corticosterona, estresse oxidativo, Nrf2, monoaminas, BDNF

## ABSTRACT

Agmatine is an endogenous neuromodulator, whose antidepressant activity has been shown in preclinical and clinical trials. However, the cellular and molecular mechanisms implicated in its effect are not entirely clarified. Studies over the last 40 years have shown that episodes of stress induce abnormalities in the hypothalamic-pituitary-adrenal axis and hypersecretion of glucocorticoids which are implicated in the pathophysiology of major depression. The present study verified the agmatine's ability to: i) produce an antidepressant-like effect in the tail suspension test (TST) followed its sub-chronic administration in mice and the involvement of hippocampal intracellular signaling pathways in such effect; ii) abolish the depressive-like behavior in the forced swimming test (TNF) and hippocampal antioxidant imbalance induced by acute restraint stress (ARS) in mice; iii) protect HT22 mouse hippocampal cells against the corticosterone-induced cytotoxicity and the involvement of the transcription factor Nrf2 in such effect; iv) abolish the depressive-like behavior in the TST, and anhedonic behavior induced by corticosterone and the involvement of the monoaminergic systems, brain-derived-neurotrophic factor (BDNF), synaptic protein synaptotagmin I (Syt I), neuroglial cells and the transcription factor Nrf2 in such effect. The sub-chronic agmatine (0.01 and 0.1 mg/kg, p.o.) administration produced a significant antidepressant-like effect in the TST and no locomotor effect in mice. Additionally, agmatine (0.001-0.1 mg/kg, p.o.) increased the phosphorylation of protein kinase A (PKA) substrates, protein kinase B (PKB)/Akt ($Ser^{473}$), glycogen synthase kinase-3β (GSK-3β) ($Ser^{9}$), extracellular signal-regulated kinases 1/2 (ERK1/2) and cAMP response elements (CREB) ($Ser^{133}$), and BDNF immunocontent in a dose-dependent manner in the hippocampus. Agmatine (0.001-0.1 mg/kg, p.o.) also reduced the c-jun N-terminal kinase 1/2 (JNK1/2) phosphorylation. Neither protein kinase C (PKC) nor $p38^{MAPK}$ phosphorylation was altered under any experimental conditions. Regarding ARS protocol, it caused a depressive-like behavior in the FST, hippocampal lipid peroxidation, and an increase in the activity of hippocampal superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activities, reduced catalase (CAT) activity and increased SOD/CAT ratio, an index of pro-oxidative conditions, but did not affect reduced glutathione levels. Agmatine (10 mg/kg, p.o.) was effective to abolish the depressive-like behavior induced by ARS and to prevent the ARS-induced lipid peroxidation and changes in SOD, GR and CAT activities and in

SOD/CAT activity ratio. In HT22 hippocampal neuronal cell line, corticosterone induced apoptotic cell death and increased reactive oxygen species production, effects that were abolished in a concentration- and time-dependent manner by agmatine (10 μM) treatment. The combination of sub-effective concentrations of agmatine with fluoxetine or imipramine afforded synergic protection. The neuroprotective effect of agmatine was abolished by yohimbine ($\alpha_2$-adrenoceptor antagonist), ketanserin (5-$HT_{2A}$ receptor antagonist), LY294002 (PI3K inhibitor), PD98059 (MEK1/2 inhibitor), SnPP (heme oxygenase-1 (HO-1) inhibitor), and cycloheximide (protein synthesis inhibitor). Agmatine increased Akt and ERK phosphorylation, and induced the transcription factor Nrf2 and the proteins HO-1 and glutamate cysteine ligase, catalytic subunit (GCLc); induction of these proteins was prevented by yohimbine, ketanserin, LY294002 and PD98059. Finally, in Swiss mice, the depressive-like behavior in the TST and the anhedonic behavior induced by corticosterone were prevented by agmatine (0.1 mg/kg, po) treatment for 21 days, similarly to the classical antidepressant imipramine. Additionally, agmatine increased the hippocampal levels of noradrenaline, serotonin (5-HT) and dopamine in the both control group and corticosterone-treated mice; and also prevented the decreased 5-HT and increased glutamate levels induced by corticosterone. Agmatine induced an increase in CREB phosphorylation in control mice as well as an increase in the mature BDNF (BDNFm), reduction in the immature BDNF (pro-BDNF) immunocontents, increase in the BDNFm/pro-BDNF ratio, and in the Syt I, HO-1 and GCLc immunocontents in the both control and corticosterone-treated mice. Additionally, agmatine treatment was able to prevent the reduction in the immunocontent of BDNFm and Syt I and the atrophy and reduction of astroglial and microglial cells in CA1 hippocampal induced by corticosterone. Agmatine produced an antidepressant-like effect in C57BL/6 Nrf2 (+/+) mice in the TST and splash test, but not in Nrf2 (-/-) mice. The present study extends the available data on the cellular and molecular mechanisms that underlie the antidepressant effect of agmatine by using *in vitro* and *in vivo* approachs. Taken together, our results show that the antidepressant effect of agmatine appears to be mediated by its effect on the key hypotheses that explain the pathophysiology of depression and antidepressant therapy: monoaminergic, neurotrophic, oxidative, and glutamatergic, indicating its potential to be used as adjuvant/monotherapy in the management of major depression.

**Keywords:** agmatine, depression, neuroprotection, restraint stress, corticosterone, oxidative stress, Nrf2, monoamines, BDNF

## LISTA DE FIGURAS

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## LISTA DE ABREVIATURAS

5-HT - Serotonina
ADTs - Antidepressivos tricíclicos
ANOVA - Análise de variância
ARE - Elementos de resposta antioxidante
CREB - Proteína de ligação responsiva ao AMP cíclico
BDNF - Fator neurotrófico derivado do encéfalo
CAT - Catalase
DA - Dopamina
DAT - Transportador de dopamina
EC - Estresse de contenção
ERK1/2 - cinase regulada por sinal extracelular 1/2
ERO - Espécies reativas de oxigênio
GCLc - Subunidade catalítica da enzima glutamato cisteína ligase
GFAP - Proteína glial fibrilar ácida
GPx - Glutationa peroxidase
GR - Glutationa redutase
GRs - Receptores de glicocorticóide
GSH - Glutationa reduzida
GSK-3β - Glicogênio sintase cinase-3β
HO-1 - Hemoxigenase-1
HPA - Hipotálamo-pituitária-adrenal
ISRS - Inibidores seletivos da recaptação de serotonina
JNK1/2 - c-Jun N-terminal cinase 1/2
LC-MS/MS - Cromatografia líquida acoplada a espectrometria de massa
L-DOPA - Levodopa
MAPKs - Proteínas cinases ativadas por mitógenos
MAO - Monoamina oxidase
NA - Noradrenalina
NET - Transportador de noradrenalina
NMDA - N-metil-D-aspartato
Nrf2 - Fator nuclear eritróide 2 relacionado ao fator 2
PCPA - p-clorofenilalanina
PI-3K - Fosfatidilinositol 3'-cinase
PKA - Proteína cinase A
PKB - Proteína cinase B
PKC - Proteína cinase C
PLC - Fosfolipase C
SAPKs - Proteínas cinases ativadas por estresse
SNC - Sistema nervoso central

SOD - Superóxido dismutase
Syt I - Sinaptotagmina I
TNF - Teste do nado forçado
TrkB - Tropomiosina cinase B
TSC - Teste da suspensão pela cauda
VEGF - Fator de crescimento endotelial vascular

**SUMÁRIO**

# 1. Introdução

## 1.1 Depressão maior

A depressão (depressão maior, denominação oficial) é um transtorno psiquiátrico crônico e comum que afeta 350 milhões de indivíduos de todas as idades ao redor do mundo (WHO, 2012a). Representa um sério problema de saúde, pois é a principal causa de incapacitação de indivíduos e é o terceiro contribuidor para a carga global de doença – projetada para ser o principal no ano de 2030 (WHO, 2008). Apresenta altos índices de suicídio (aproximadamente 1 milhão de vidas são perdidas anualmente devido à depressão, o que traduz 3000 mortes por suicídio todos os dias) (WHO, 2012b). Além do elevado índice de mortalidade associado ao suicídio, pacientes deprimidos são mais propensos a desenvolverem doença arterial coronariana, diabetes mellitus tipo 2 (Van der Kooy et al., 2007; Van der Feltz-Cornelis et al., 2010), hipertensão (Almas et al., 2014) e acidente vascular cerebral (Pan et al., 2011). Apesar de ser a principal causa de incapacitação de indivíduos de ambos os sexos, o impacto da depressão é 50% maior entre as mulheres (WHO, 2008). Pesquisas nos países em desenvolvimento mostraram que a depressão materna está associada a prejuízos para o crescimento e desenvolvimento normal de crianças em virtude de negligências de cuidado e falta de afetividade por parte da mãe em relação ao filho, o que pode chegar a afetar as gerações subsequentes (Rahman et al., 2008). A depressão possui uma importante carga hereditária, cerca de 40-50% dos casos são causados por um componente genético, acredita-se ainda, que os 50-60% dos casos remanescentes sejam causados por trauma na infância, estresse emocional, doença física, e ainda, infecções virais (Berton e Nestler, 2006).

A depressão maior é diagnosticada de acordo com critérios presentes no Manual de Diagnóstico e Estatístico dos Distúrbios Mentais (Associação Americana de Psiquiatria, 2000) que define que um episódio depressivo maior é caracterizado pela constatação de no mínimo cinco entre os nove sintomas citados na **Tabela 1** e exige a presença de pelo menos um dos dois primeiros sintomas mencionados (humor deprimido ou anedonia) presentes na maior parte do tempo, com uma duração mínima de duas semanas. A severidade da doença é julgada como leve, moderada ou grave com base no grau de prejuízo ocupacional e social. Alguns pacientes deprimidos podem apresentar sintomas de psicose ou perda de noção de realidade (como por exemplo, alucinações ou delírios) (Associação Americana de Psiquiatria, 2000). Destaca-se ainda que muitas vezes a depressão é acompanhada por sintomas de ansiedade (WHO, 2012a).

**Tabela 1.** Sintomas para diagnóstico de depressão maior

| |
|---|
| Humor deprimido |
| Anedonia (perda de interesse ou prazer) |
| Perda ou ganho de peso (>5% de alteração em um mês) |
| Insônia ou hipersonia |
| Retardo ou agitação psicomotora |
| Fadiga ou perda de energia |
| Sentimentos de culpa ou baixa auto-estima |
| Diminuição da capacidade de concentração |
| Pensamentos recorrentes de morte ou suicídio. |

Fonte: Manual de Diagnóstico e Estatístico dos Distúrbios Mentais (Associação Americana de Psiquiatria, 2000).

Em meados de 1960 surgiu a primeira hipótese para explicar a etiologia da depressão. A hipótese monoaminérgica postula que a depressão é causada por uma deficiência monoaminérgica na fenda sináptica, a qual é restabelecida pela farmacoterapia antidepressiva (**Figura 1**) (Schildkraut, 1965). Esta hipótese surgiu a partir de observações clínicas sobre o efeito antidepressivo de inibidores da receptação de monoaminas e de inibidores da enzima monoamina oxidase e pela descoberta de que pacientes que faziam uso do anti-hipertensivo reserpina (depletor das reservas de monoaminas dos neurônios) desenvolveram sintomas depressivos (Heninger et al., 1996; Berton e Nestler, 2006). Os antidepressivos atuais disponíveis apresentam melhor eficácia terapêutica e menores efeitos adversos em relação aos que surgiram em meados de 1960, mas ainda modulam a transmissão monoaminérgica de maneira aguda, seja pela inibição da recaptação pré-sináptica de monoaminas, como por exemplo os inibidores seletivos da recaptação de serotonina (ISRS) como a fluoxetina, ou através da inibição da sua degradação, como por exemplo os inibibores da enzima monoamina oxidase (MAO), como a tranilcipromina (Berton e Nestler, 2006; Krishnan e Nestler, 2008).

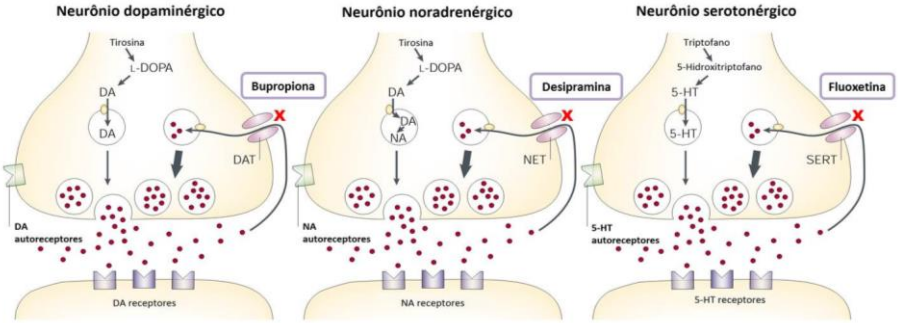


**Figura 1. Hipótese monoaminérgica da depressão.** Na depressão, a concentração de monoaminas (dopamina, noradrenalina e serotonina) na fenda sináptica está diminuída, acarretando o transtorno de humor. O bloqueio dos sítios de recaptação (em vermelho) aumenta a disponinibilidade dos neurotransmissores monoaminérgicos, os quais ativam seus receptores pós-sinápticos, sendo o humor restabelecido. Entre os antidepressivos que aumentam a disponibilidade de monoaminas, destacam-se a bupropiona (inibidor da recaptação de dopamina com atividade sutil sobre a recaptação de noradrenalina), a desipramina (inibidor preferencial da recaptação de noradrenalina) e a fluoxetina (inibidor seletivo da recaptação de serotonina). 5-HT, serotonina; DA, dopamina; DAT, transportador de dopamina; NE, noradrenalina; NET, transportador de noradrenalina; L-DOPA, levodopa. (Adaptado de Torres et al., 2003).

Apesar da eficácia dos antidepressivos monoaminérgicos e de que alterações na função monoaminérgica do sistema nervoso central (SNC) possam contribuir para a vulnerabilidade genética, a causa da depressão está longe de ser uma simples deficiência monoaminérgica no SNC (Ansorge et al, 2007; López-León et al., 2008). Os antidepressivos que modulam os sistemas monoaminérgicos, apesar de produzirem seus efeitos sobre a disponibilidade de monoaminas de maneira imediata, produzem seus efeitos benéficos sobre o humor somente após semanas do início do tratamento, indicando que outros sistemas neurais e mecanismos bioquímicos estejam envolvidos na etiologia e tratamento da depressão. Hoje em dia sabe-se, por exemplo, que o aumento agudo da disponibilidade sináptica de monoaminas produz uma rede de alterações neuroplásticas secundárias que ocorre a longo prazo e envolve alterações transcricionais e traducionais que regulam a plasticidade molecular e celular (hipótese neurotrófica da depressão) (Pittenger e Duman, 2008; Castrén e Rantamäki, 2010; Duman, 2014).

### 1.2. Envolvimento de vias de sinalização intracelular na fisiopatologia da depressão

O tratamento crônico, mas não agudo, com antidepressivos aumenta o nível de proliferação neuronal hipocampal em adultos (Sairanen et al., 2005). Estes achados corroboram com a hipótese neurotrófica da depressão que postula que o tratamento com antidepressivos estimula a atividade neuronal e promove aumento da expressão do fator neurotrófico derivado do encéfalo (BDNF) (Russo-Neustadt e Chen, 2005). Esta hipótese surgiu originalmente a partir da descoberta que eventos estressores agudos ou crônicos diminuem a expressão de BDNF hipocampal em roedores e que diversas classes de antidepressvivos revertem tal efeito, prevenindo os efeitos deletérios do estresse que foram produzidos a partir do excesso de glicorticóide (**Figura 2**) (Duman et al., 1997; Duman, 2004). Suportando tal hipótese, níveis reduzidos de BDNF hipocampal também foram encontrados em pacientes deprimidos e submetidos à autópsia – anormalidade que não foi verificada em pacientes deprimidos e que estavam sendo tratados com antidepressivos (Chen et al., 2001).

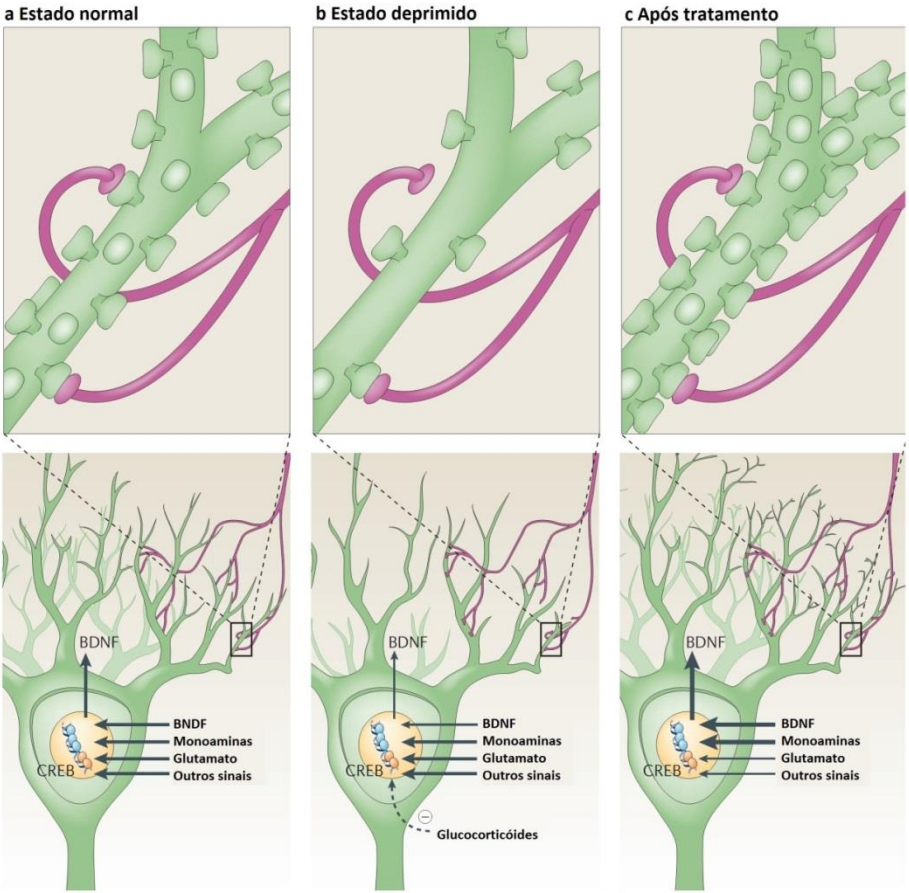


**Figura 2. Hipótese neurotrófica da depressão e ação antidepressiva. a.** Neurônio piramidal hipocampal: sua inervação por neurônios glutamatérgicos, monoaminérgicos, outros tipos de neurônios e regulação por BDNF. **b.** Episódios de estresse grave causam alterações nestes neurônios, reduzindo sua arborização dendrítica e expressão de BDNF. A diminuição de BDNF é mediada em parte pelo excesso de glicocorticóides, que por sua vez, pode afetar os mecanismos transcricionais normais – como por exemplo, a ativação do regulador trascricional proteína de ligação responsiva ao AMP cíclico (AMPc) (CREB). **c.** Os antidepressivos revertem os efeitos deletérios produzidos pelo estresse e excesso de glicocorticóides aumentando a arborização dendrítica e expressão de BDNF, efeitos que parecem ser mediados pela ativação de CREB. (Adaptado de Berton e Nestler, 2006).

A ligação de BDNF a seu receptor tropomiosina cinase B (TrkB) desencadeia a ativação de importantes vias de sinalização como a via Ras–Raf–ERK (cinase regulada por sinal extracelular) (Chen et al., 2007), fosfatidilinositol 3'-cinase (PI-3K)-Akt (Numakawa et al., 2010) e fosfolipase (PLC) γ (Reichardt, 2006). Essas vias de sinalização, assim como outras que promovem sobrevivência neuronal, convergem para a ativação de um regulador transcricional, a proteína de ligação ao elemento responsivo ao AMP cíclico (AMPc) (CREB). Uma variedade de fatores de crescimento e hormônios estimula a expressão de genes celulares quando CREB é ativado por fosforilação em $Ser^{133}$ (Tardito et al., 2006). Originalmente caracterizado como um alvo de fosforilação da proteína cinase A (PKA), atualmente sabe-se que outras cinases também são responsáveis pela ativação mediada por fosforilação de CREB, como a proteína cinase dependente de $Ca^{+2}$/calmodulina (CaM), proteína cinase C (PKC) e a via Ras-Raf-MAP cinase (MEK)-ERK que catalisa a transferência de um grupo fosfato do ATP para resíduos $Ser^{133}$ através de RSK2, um membro da família cinase ribossomal S6 (Nair e Vaidya, 2006). O CREB quando ativado liga-se ao elemento de resposta ao AMPc (CRE) no DNA para regular a expressão gênica de alvos como neurotrofinas e fatores tróficos como o BDNF que contribuem para a neuroplasticidade e modulação do humor mediadas pelo tratamento crônico com antidepressivos. A habilidade dos antidepressivos em ativar proteínas cinases intracelulares que fosforilam CREB indica que a ativação deste fator de transcrição faz parte do mecanismo de ação destes medicamentos. De fato, o tratamento crônico, e não agudo, com antidepressivos aumenta a fosforilação de CREB em várias regiões do encéfalo de roedeores, incluindo a amígdala, córtex, giro denteado, hipotálamo e hipocampo (Vinet et al., 2004; Nair e Vaidya, 2006; Gumuslu et al., 2013) (**Figura 3**).

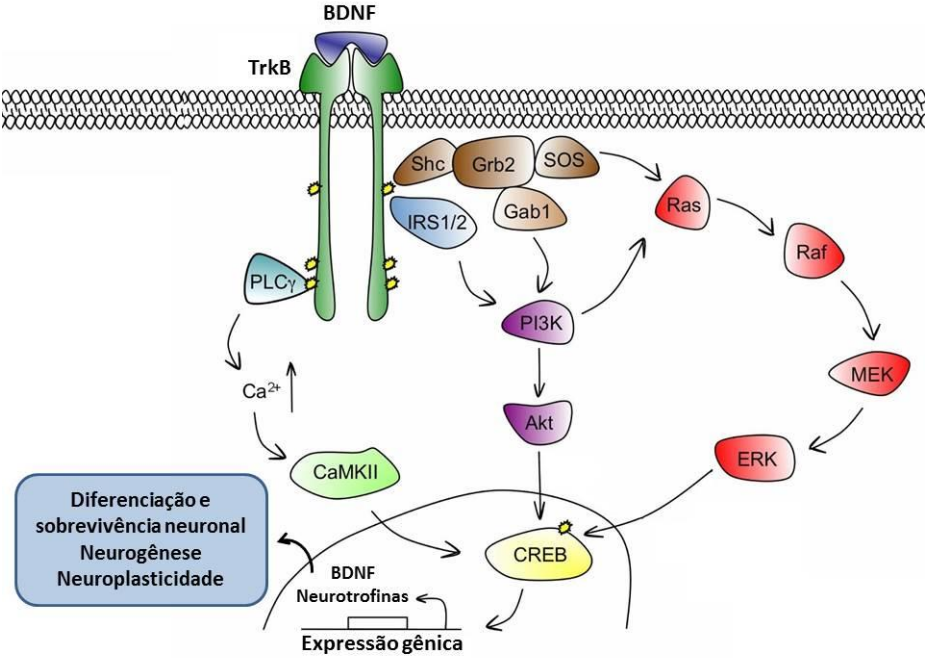


**Figura 3. Via de sinalização BDNF – TrkB.** A ligação de BDNF ao seu receptor TrkB induz sua dimerização e autofosforilação em resíduos de tirosina. A ativação de TrkB desencadeia a ativação de uma rede de vias de sinalização intracelular como PLCγ, PI3K e ERK. Estas vias, quando ativadas, induzem a fosforilação e ativação do fator de transcrição nuclear CREB que medeia a transcrição de genes que regulam a expressão de neurotrofinas como BDNF, que por sua vez, regulam a diferenciação e sobrevivência neuronal, neurogênese, neuroplasticidade e produzem efeitos positivos sobre o humor (Adaptado de Cunha et al., 2010).

A compreensão do envolvimento da família das proteínas cinases ativadas por mitógenos (MAPKs) nos mecanismos moleculares da depressão está crescendo. Entre as MAPKs, as cinases 1 e 2 reguladas por sinal extracelular (ERK1 e ERK2) são as mais bem caracterizadas. ERK é a principal via de convergência de todas as vias de sinalização, regulando apoptose, crescimento celular, diferenciação e neuroplasticidade (Johnson e Lapadat, 2002; Strnisková et al., 2002). Evidências têm mostrado que ERK está envolvida na modulação neuronal da depressão (Fumagalli et al., 2005; Todorovic et al., 2009). Níveis reduzidos de diferente efetores da via Ras–Raf–ERK foram verificados no hipocampo e córtex cerebral de pacientes deprimidos e de indivíduos que

cometeram suicídio (Dwivedi et al., 2001; Yuan et al., 2010). Corroborando com as evidências clínicas, estudos em animais mostraram que a inibição da via de sinalização ERK no hipocampo e córtex pré-frontal induz um comportamento tipo-depressivo (Qi et al., 2009). Adicionalmente, Shirayama et al. (2002) mostraram que a ativação de ERK hipocampal é um dos mecanismos implicados no efeito tipo-antidepressivo produzido por BDNF administrado via intra-hipocampal.

As proteínas cinases ativadas por estresse (SAPKs) formam um importante grupo dentro da família das MAPKs e são representadas pelas proteínas c-Jun N-terminal cinases (JNK) e $p38^{MAPK}$. A JNK é primariamente ativada por vários tipos de estresses ambientais, incluindo choque osmótico, choque térmico, estresse oxidativo, inibidores de síntese protéica, quimioterápicos e citocinas pro-inflamatórias, tais como o fator de necrose tumoral alfa (TNF-α) e interleucina-1 (IL-1) (Shen e Liu, 2006). Há uma quantidade crescente de evidências mostrando uma correlação positiva entre a ativação de JNK e a morte celular mediada por espécies reativas de oxigênio (ERO) e de nitrogênio (ERN) (Ventura et al., 2004; Papa et al., 2004, 2006; Ramiro-Cortés e Morán, 2009). Além disso, vários estudos têm demonstrado que inibidores de JNK1/2 e de $p38^{MAPK}$ possuem propriedades neuroprotetoras (Yeste-Velasco et al., 2009; Hu et al., 2012; Nijboer et al., 2013; Ord et al., 2013; Vázquez de la Torre, 2013; Zhou et al., 2014). Chuang (2004) mostrou que o estabilizador de humor lítio promove neuroproteção através do bloqueio da ativação de $p38^{MAPK}$ e da inibição de CREB induzidos por glutamato. Adicionalmente, Hwang et al. (2008) mostraram que antidepressivos tricíclicos são capazes de inibir a ativação inflamatória glial e neurotoxicidade através do bloqueio da via $p38^{MAPK}$. Considerando que a fisiopatologia da depressão envolve morte celular, fármacos capazes de bloquear as vias JNK e $p38^{MAPK}$ podem auxiliar a terapia farmacológica (Harper e LoGrasso, 2001; Borsello e Forloni, 2007; Yasuda et al., 2011).

Adicionalmente, importantes estudos têm demonstrado que inibidores da enzima glicogênio sintase cinase-3β (GSK-3β) têm potencial para serem usados como monoterapia ou adjuvantes da terapia antidepressiva (Beaulieu et al., 2009; Maes et al., 2012). GSK-3β é uma serina/treonina cinase multifuncional que recebeu este nome após a descoberta de seu envolvimento no metabolismo do glicogênio (Jope e Roh, 2006). GSK-3 (isoformas α e β) é um importante regulador da síntese de glicogênio, transcrição de genes, plasticidade sináptica e apoptose (morte celular) (Jope, 2003). Está bem estabelecido na literatura que a GSK-3β regula o comportamento, afetando os níveis de β-catenina, receptores glutamatérgicos, os ritmos circadianos e a neurotransmissão serotoninérgica (Beaulieu et al., 2008). Adicionalmente, GSK-3β é capaz de ativar o fator de transcrição CREB (Bullock e Habener, 1988; Grimes e Jope, 2001). Todos estes alvos têm sido implicados na fisiopatologia dos transtornos de humor (Gould, 2006; Rosa et al., 2008; Du et al., 2010). Várias linhas de evidências têm mostrado que transtornos depressivos estão relacionados com a ativação de GSK-3β, através da falha em seu mecanismo de inibição mediado por

fosforilação (Beaulieu et al., 2009). Várias cinases como a Akt/proteína cinase B (PKB), a PKA e a PKC regulam negativamente a atividade da enzima GSK-3β por fosforilação na região N-terminal de $Ser^{9}$ (Beaulieu et al., 2009). A via Akt/PKB é de particular interesse porque está relacionada ao mecanismo de ação de fármacos antidepressivos (Wada, 2009; Freyberg et al., 2010; Maes et al., 2012; Molteni et al., 2009; Vidal et al., 2011). Akt/PKB é uma serina/treonina cinase que fosforila e regula a função de muitas proteínas celulares envolvidas em processos relacionados ao metabolismo, apoptose, proliferação e neuroplasticidade (Song et al., 2005). Akt/PKB contém dois sítios regulatórios de fosforilação, o $Thr^{308}$ no domínio catalítico e $Ser^{473}$ no domínio regulatório C-terminal (Nicholson e Anderson, 2002).

Por último, importantes estudos têm demostrado que um alvo promissor para o desenvolvimento de novos e melhores tratamentos para depressão baseia-se nos ativadores do fator de transcrição nuclear (eritróide 2 relacionado ao fator 2) Nrf2 (Maes et al., 2012; Lee et al., 2013; Martín-de-Saavedra et al., 2013). Recentemente, Martín-de-Saavedra et al. (2013) mostraram que camundongos Nrf2 KO (Nrf2-/-) apresentam comportamento tipo-depressivo o qual está relacionado a: diminuição dos níveis de dopamina e serotonina e aumento de glutamato no córtex pre-frontal; expressão diminuída do fator neurotrófico VEGF (fator de crescimento endotelial vascular) e de sinaptofisina (marcador de plasticidade sináptica); e microgliose na região CA1 hipocampal. Nrf2 é um regulador central das defesas antioxidantes celulares e das respostas celulares ao estresse. Nrf2 ativa uma rede de genes regulatórios sensíveis aos sinais redox, refletindo seu papel na manutenção da homeostasia redox do cérebro e protegendo os neurônios de morte celular (Johnson et al., 2008). Em condições basais, Nrf2 está sequestrado no citoplasma formando um complexo inativo com sua proteína repressora Keap1 (Kelch-like-ECH-associated protein 1), levando Nrf2 à degradação proteossomal (Kobayashi et al., 2004; Uruno e Motohashi, 2011). Contudo, sob sinais químicos, Nrf2 é liberado de Keap1, translocado do citoplasma para o núcleo e ativa a expressão de importantes genes citoprotetores que promovem sobrevivência celular (Dinkova-Kostova et al., 2002; Niture et al., 2010). Importantes cinases tais como PI-3K/Akt, PKC e ERK são capazes de fosforilar Nrf2 em resíduos de serina e treonina induzindo sua dissociação de Keap1 e translocação nuclear (Huang et al., 2002; Surh, 2003; Nguyen et al., 2004; Turpaev, 2013). No núcleo, Nrf2 se liga a elementos de resposta antioxidante (ARE) e estimula genes que codificam enzimas detoxificantes de fase II e/ou enzimas antioxidantes como a glutationa-S-transferase α2 (GSTA2), NAD(P)H: quinona oxidorredutase 1 (NQO1), subunidade catalítica da glutamato cisteína ligase (GCLc) e heme oxigenase I (HO-1) (Surh, 2003; Jaiswal, 2004; Turpaev, 2013) (**Figura 4**).

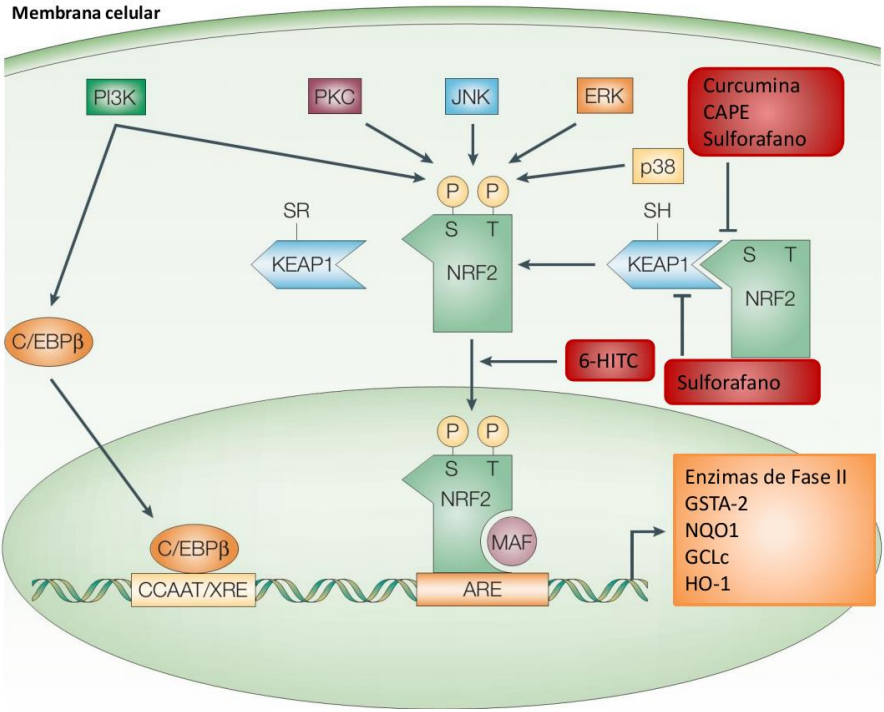


**Figura 4. Ativação do fator de transcrição nuclear Nrf2.** Nrf2 regula a expressão de enzimas detoxificantes e/ou antioxidantes. Em condições basais, Nrf2 está sequestrado no citoplasma formando um complexo inativo com sua proteína repressora Keap1 (Kelch-like-ECH-associated protein 1), levando Nrf2 à degradação proteossomal. Sob sinais químicos, Nrf2 é liberado de Keap1, translocado do citoplasma para o núcleo e ativa a expressão de importantes genes citoprotetores. Cinases como PI-3K/Akt, PKC, JNK e ERK são capazes de fosforilar Nrf2 em resíduos de serina (S) e treonina (T) induzindo sua dissociação de Keap1 e translocação nuclear. $p38^{MAPK}$ é capaz de ativar ou inibir a translocação nuclear de Nrf2. No núcleo, Nrf2 se associa ao fator de transcrição MAF (musculoaponeurotic-fibrosarcoma virus), formando um heterodímero que se liga a elementos de resposta antioxidante (ARE) e estimula genes que codificam enzimas detoxificantes de fase II e/ou enzimas antioxidantes como a glutationa-S-transferase α2 (GSTA2), NAD(P)H: quinona oxidorredutase 1 (NQO1), subunidade catalítica da glutamato cisteína ligase (GCLc) e heme oxigenase I (HO-1). PI-3K também fosforila C/EBPβ (proteína ligante ao amplificador CCAAT β), induzindo sua translocação nuclear e ligação a sequencia CCAAT/XRE (elementos de resposta xenobiótica). Curcumina e CAPE (éster fenólico do ácido caféico) são capazes de dissolver o complexo Nrf2-KEAP1, induzindo a ligação de Nrf2 a ARE. Sulforafano

interage diretamente com Keap1 por ligaçãoes covalentes com seus grupos tióis (SH), e 6-HITC (6-(metilsulfinil) hexil isotiocianato), um análago ao sulforafano derivado do "*wasabi*" (raiz-forte japonesa), também é um composto ativador de Nrf2 (Adaptado de Surh, 2003).

### 1.3. Estresse, Glicocorticóides e Depressão

Estudos dos últimos 40 anos têm demostrado que hiperatividade do eixo hipotálamo-pituitátia-adrenal (HPA) foi a descoberta biológica mais consistente para o estudo da fisiopatologia da depressão maior (Carroll et al., 1976; Pariante e Lightman, 2008; Frodl e O'Keane, 2013; Maric and Adzic, 2013). O eixo HPA não apenas controla as funções periféricas do corpo como o metabolismo e a imunidade, mas também desempenha um importante papel sobre o encéfalo. Os glicocorticóides (cortisol em humanos e corticosterona em roedores) regulam a sobrevivência neuronal, neurogênese, o tamanho e a complexidade de estruturas cerebrais como o hipocampo, a aquisição de novas memorias, e o julgamento emocional a eventos (Herbert et al., 2006; Pariante and Lightman, 2008). Considerando o seu papel na interface estresse e funcionamento cerebral, não é de se surpreender que anormalidades no eixo HPA sejam observadas em pacientes com transtornos neuropsiquiátricos, particularmente naqueles que sofrem de depressão maior. Níveis aumentados de cortisol na saliva, plasma e urina, bem como aumento de tamanho e atividade das glândulas pituitária e adrenal são verificados na maioria dos pacientes deprimidos (Nemeroff e Vale, 2005; Pariante e Lightman, 2008).

O eixo HPA corresponde ao sistema regulador primário de resposta ao estresse. Segundo dados epidemiológicos, episódios de estresse são um dos principais fatores ambientais que predispõem um indivíduo à depressão maior (Kessler, 1997; Mazure, 1998; Hammen, 2009; Slavich e Irwin, 2014). O eixo HPA integra as funções neurológicas e estímulos sensoriais à função endócrina e assim é um componente essencial que capacita o indivíduo a lidar com eventos estressantes (Berton e Nestler, 2006). Diante de um estímulo estressor, as células dos núcleos paraventriculares hipotalâmicos (PVN) secretam hormônio liberador de corticotrofina (CRH) e vasopressina (AVP) que atuam sinergicamente na ativação do eixo HPA. Na hipófise (pituitária) anterior, CRH e AVP estimulam a liberação do hormônio adrenocorticotrófico (ACTH) que por sua vez atua no córtex da glândula supra-renal (adrenal), promovendo a síntese e liberação de glicocorticóides como o cortisol em humanos e a corticosterona em roedores (Nemeroff, 1996; Cunha et al., 2010). Os glicocorticóides interagem com seus receptores em múltiplos tecidos-alvos, produzindo diversos tipos de respostas além de modular o próprio eixo HPA, onde são responsáveis pela inibição da secreção do ACTH pela pituitária e do CRH a partir do hipotálamo. Este controle inibitório além de contribuir para a regulação da resposta ao estresse auxilia na supressão dessa resposta quando o estímulo estressor cessa (Swaab et al., 2005; Aguilera et al., 2007). Além disso,

o hipocampo também auxilia no controle da ativação do eixo HPA, através da inibição dos neurônios hipotalâmicos secretores de CRH (Nestler et al., 2002; Cunha et al., 2010; Frodl e O'Keane, 2013) (**Figura 5**).

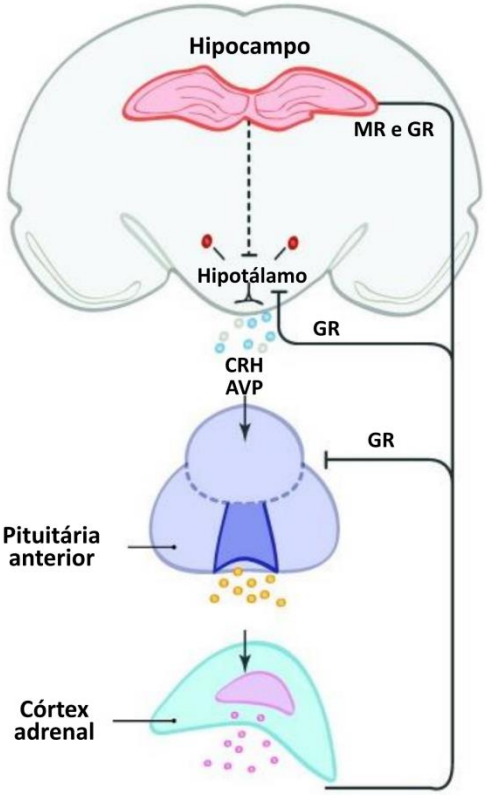


**Figura 5. Ativação do eixo hipotálamo-pituitátia-adrenal (HPA) em resposta ao estresse.** A ativação do eixo HPA induz a secreção de hormônio liberador de corticotrofina (CRH) e vasopressina (AVP) pelas células dos núcleos paraventriculares hipotalâmicos. Na hipófise (pituitária) anterior, CRH e AVP estimulam a liberação do hormônio adrenocorticotrófico (ACTH) que por sua vez atua no córtex da glândula supra-renal (adrenal), promovendo a síntese e liberação de glicocorticóides (cortisol em humanos e corticosterona em roedores). Os efeitos celulares dos glicocorticóides são mediados via ativação de receptores de glicocorticóide (GRs) e de mineralocorticóide (MRs). O controle regulatório sobre o eixo HPA é feito via *feedback* negativo sobre a pituitária e outras regiões cerebrais como o hipocampo (Adaptado de Cunha et al., 2010).

Adicionalmente, a hipersecreção de glicocorticóides está relacionada à morte neuronal, diminuição da neurogênese, do tamanho e número das arborizações dendríticas neuronais e à atrofia hipocampal observada na depressão maior (Lee et al., 2002; McKinnon et al., 2009). O excesso de glicocorticóides produz efeitos significativos sobre o hipocampo em virtude da alta concentração de receptores de glicocorticóide (GRs) presentes nesta estrutra cerebral (Duman, 2004b). O tratamento crônico com antidepressivos através da modulação inicial da concentração de monoaminas, ativação de cascatas de sinalização intracelular e expressão de genes-alvo, reverte os danos causados pelo excesso de glicocorticóides, aumentando a expressão de BDNF, abolindo a atrofia dos neurônios piramidais e restabelecendo a produção normal de novos neurônios no giro denteado (**Figura 6**). Adicionalmente, episódios de estresse causam aumento da liberação e transmissão glutamatérgica, efeito que parece ser crucial para as alterações estrutrais e funcionais dos neurônios hipocampais observadas nos pacientes deprimidos (Musazzi et al., 2011; Sanacora et al., 2012). Suportando estes achados, antagonistas de receptores glutamatérgicos do tipo N-metil-D-aspartato (NMDA) (subunidade NR2B), abolem os efeitos do estresse, indicando que a ativação deste receptor parece ser o gatilho inicial para a atrofia e morte neuronal (Sanacora et al., 2008; Calabrese et al., 2012).

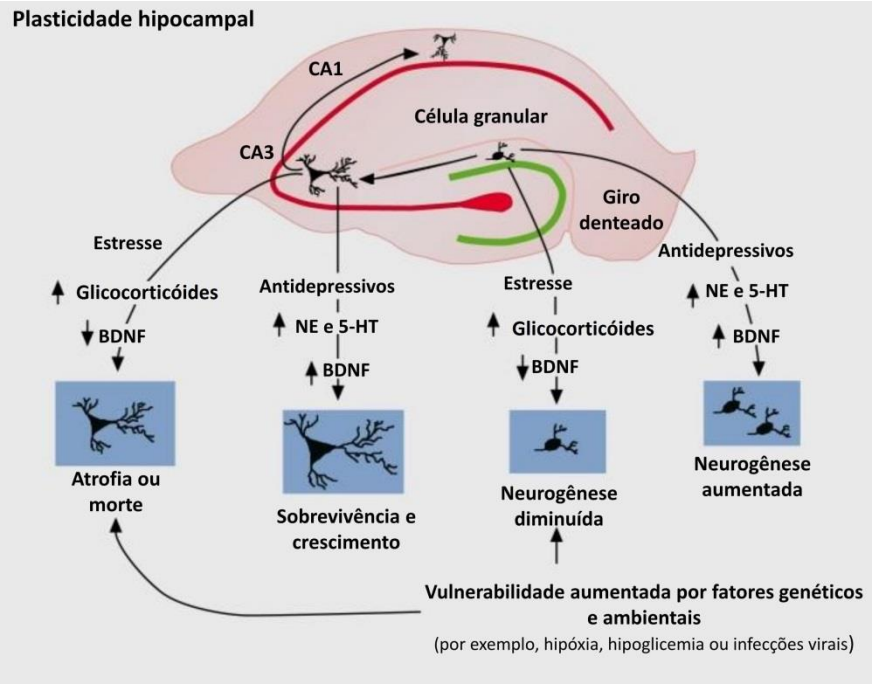


**Figura 6. Modelo de plasticidade hipocampal mostrando alterações estruturais em resposta ao estresse.** O estresse causa diminuição da neurogênese das células granulares do giro denteado, do tamanho e número das arborizações dendríticas e atrofia dos neurônios piramidais. O tratamento crônico com antidepressivos através da modulação inicial da concentração de monoaminas, ativação de cascatas de sinalização intracelular e expressão de genes-alvo, reverte os danos causados pelo excesso de glicocorticóides aumentando a expressão de BDNF, abolindo a atrofia dos neurônios piramidais e restabelecendo a produção normal de novos neurônios no giro denteado. NE, noradrenalina; 5-HT, serotonina (Adaptado de Duman, 2004b).

Os mecanimos envolvidos na morte neuronal induzida pelos glicocorticóides são explicados pela hipótese inflamatória e oxidativa da depressão, a qual postula que a liberação excessiva de glicocorticóides induz um estado oxidativo e inflamatório no cérebro acompanhado pela diminuição das defesas antioxidantes, peroxidação de lipídeos, danos ao DNA, disfunções mitocondriais, alterações nos sistemas monoaminérgicos e redução da neurogênese e plasticidade neuronal (Maes et al., 2009; Leonard e Maes, 2012). Comparado a outros tecidos, o SNC é particularmente vulnerável aos danos oxidativos uma vez que consome altas quantidades de oxigênio; é relativamente carente de defesas antioxidantes; é um tecido rico em lipídeos com ácidos

graxos insaturados, substratos passíveis de oxidação; contém metais como ferro e cobre, que catalizam reações de oxidação e neurotransmissores com potencial redutor (Dringen, 2005; Zafir et al., 2009). A ação central dos radicais livres leva à destruição oxidativa dos neurônios e à neurodegeneração que, por sua vez, está associada a transtornos psiquiátricos (Valko et al., 2007). Neste sentido, estudos têm demonstrado que os parâmetros de avaliação de estresse oxidativo estão alterados na depressão (Tsuboi et al., 2006; Sarandol et al., 2007). Pacientes com depressão apresentam menor atividade sérica das enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) (Bilici et al., 2001; Ozcan et al., 2004), bem como altos níveis séricos de produtos de peroxidação lipídica como malondialdeído e hidroperóxidos (Ozcan et al., 2004; Tsuboi et al., 2004). Além disso, estudos clínicos e pré-clínicos têm demonstrado que o tratamento com inibidores seletivos da recaptação de serotonina possui efeito antioxidante, revertendo o desequilíbrio oxidativo encontrado no estado depressivo (Bilici et al., 2001; Eren et al., 2007; Herken et al., 2007). Vários estudos têm demonstrado que diferentes tipos de estresse, incluindo o estresse de contenção, causam dano oxidativo cerebral (Enache et al., 2008; Kumar e Goyal, 2008; Balk et al., 2010; García-Fernández et al., 2012), que pode ser prevenido por fármacos antidepressivos de distintas classes como imipramina e clomipramina (antidepressivos tricíclicos, ADTs), fluoxetina e venlafaxina (inibidores seletivos da receptação de serotonina, ISRS) e trazodona (antidepressivo atípico) (Zafir e Banu, 2007; Kumar et al., 2009; Zafir et al., 2009; Balk et al., 2010; Kumar et al., 2010).

### 1.4. Envolvimento das células glias na patofisiologia da depressão

As células da glia do sistema nervoso central, chamadas de neuroglia, são as células mais numerosas do cérebro, encontrando-se na proporção 10:1 em relação aos neurônios (Kendel, 2000; Rajkowska e Miguel-Hidalgo, 2007). A neuroglia divide-se em três tipos celulares distintos: a) astrócitos; b) oligodendrócitos (ambos de origem ectodérmica, e que juntos são chamados de macroglia); e c) microglia (originada a partir da linhagem monocítica-macrocítica). Os três tipos de neuroglia desempenham papel crucial para o funcionamento adequado dos neurônios. Os oligodendrócitos são responsáveis pela produção de mielina para o isolamento axonal, já os astrócitos e a microglia participam nas respostas frente aos danos neuronais e na secreção de fatores neurotróficos (Kendel, 2000; Rajkowska e Miguel-Hidalgo, 2007).

Os astrócitos são as células mais numerosas e versáteis entre as células gliais. Desempenham importantes papéis no SNC tais como: controle do metabolismo da glicose e da nutrição neuronal, bem como das demais células gliais; modulação dos níveis sinápticos de neurotransmissores via transportadores específicos presentes nos processos astrocitários; regulação da disponibilidade e da recaptação de neurotransmissores como glutamato, GABA, serotonina, noradrenalina e dopamina; promovoção de uma sinalização eficiente

entre os neurônios pré-sinápticos (terminal axonal) e pós-sinápticos (espinhos dendríticos) (Hansson e Rönnbäck, 2003; Rajkowska e Miguel-Hidalgo, 2007); regulação da neurotransmissão glutamatérgica, pois desempenham papel chave na recaptação, metabolismo e reciclagem de glutamato, modulando seus níveis sinápticos através de transportadores específicos (Bezzi et al., 2004; Bergersen e Gundersen, 2009); controle dos processos inflamatórios e neurodegenerativos, juntamente com a micróglia e com os oligodendrócitos, os astrócitos tornam-se ativados em resposta a danos teciduais e à secreção de citocinas pró-inflamatórias (Laping et al., 1994; Rajkowska e Miguel-Hidalgo, 2007). Considerando que concentrações sinápticas elevadas de glutamato estão relacionadas à morte neuronal, os astrócitos parecem desempenhar um papel crucial para a manutenção neuronal, através da redução dos níveis sinápticos glutamatérgicos (Bezzi et al., 2004; Bergersen e Gundersen, 2009). O glutamato extracelular é captado pelos astrócitos, convertido à glutamina por ação da enzima glutamina sintetase (específica de astrócitos), a glutamina por sua vez, é liberada pelos astrócitos, captada pelos terminais neuronais, onde é reconvertida a glutamato, e a GABA (ciclo glutamina-glutamato) (Kendel, 2000; Rajkowska e Miguel-Hidalgo, 2007).

As células da micróglia, por sua vez, se relacionam com a linhagem fagocítica mononuclear e são as únicas células capazes de se ativarem de maneira rápida em resposta a qualquer alteração patológica, ao contrário dos outros tipos celulares do SNC. A microglia é, de fato, o macrófago do cérebro e ela responde rapidamente a qualquer alteração na integridade estrutural cerebral. Quando ativadas, as células da microglia proliferam e migram para o local da lesão onde se tornam altamente fagocíticas (Kendel, 2000; Brown e Neher, 2010).

A literatura tem demonstrado que não somente disfunções, morte e atrofia neuronal, mas também, degenerações, alterações morfológicas e disfunções das células gliais, especialmente, dos astrócitos, parecem desempenhar um papel importante na patofisiologia da depressão. Perda de células glias do córtex pré-frontal e hipocampo foram observadas em pacientes deprimidos e em modelos animais de estresse e depressão (Czéh et al., 2013; Popoli et al., 2011; Sanacora e Banasr, 2013). Níveis reduzidos da proteína glial fibrilar ácida (GFAP), marcador astrocitário, em regiões corticais e límbicas do cérebro de pacientes deprimidos foram verificados (Miguel-Hidalgo et al., 2000). Além disso, diminuição do imunoconteúdo de GFAP hipocampal foi observada em camundongos submetidos ao modelo de estresse crônico imprevisível, a qual foi abolida pelo tratamento com o antidepressivo clomipramina (Liu et al., 2009) (**Figura 7**).

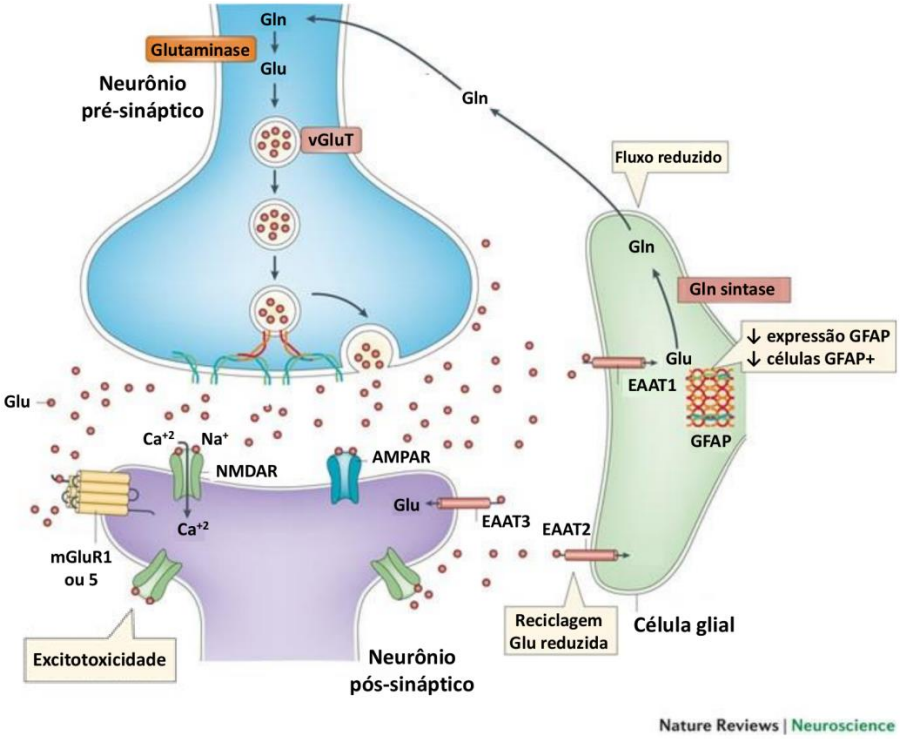


**Figura 7. Estresse crônico, especialmente os glicocorticóides, afetam o metabolismo glutamatérgico, e a função e morfologia glial.** Estudos têm demonstrado uma diminuição da expressão da proteína glial fibrilar ácida (GFAP) e do número de células GFAP-positivas (astrócitos) no hipocampo e córtex pré-frontal após episódios de estresse crônico e hipersecreção de glicocorticóides. O estresse crônico e os glicocorticóides afetam a reciclagem de glutamato (Glu) da fenda sináptica pelas células da glia através dos transportadores de aminoácidos excitatórios (EAATs), levando a um acúmulo de glutamato e aumento da ativação de receptores glutamatérgicos pós-sinápticos (excitotoxicidade). O metabolismo glutamatérgico também é afetado pela diminuição do fluxo de glutamato através do ciclo glutamato-glutamina (Gln). AMPAR, receptor AMPA; mGluR, receptor glutamatérgico metabotrófico; NMDAR, receptor NMDA; vGluT, transportador vesicular de glutamato. (Adaptado de Popoli et al., 2011).

Acredita-se que a degeneração astrocitária seja resultado da excitotoxicidade glutamatérgica e de um desbalanço entre os níveis de glutamato/GABA das estruturas cerebrais afetadas (Rajkowska e Miguel-Hidalgo, 2007; Smiałowska et al., 2013). Níveis reduzidos de interneurônios

GABAérgicos no córtex pré-frontal de pacientes deprimidos e em modelos de estresse em ratos também foram verificados (Smiałowska et al., 2013). Estes interneuronios fornecem o controle inibitório neuronal sobre os neurônios glutamatérgicos, desta maneira, acredita-se que a degeneração dos neurônios GABAérgicos pode estar envolvido neste desbalanço glutamato/GABA (Smiałowska et al., 2013).

### 1.5. Agmatina

A farmacoterapia atual utilizada para o tratamento da depressão maior possui baixa tolerabilidade em virtude dos efeitos adversos que produz, o que reflete baixa adesão ao tratamento e busca crescente por novas estratégias farmacológicas e/ou tratamentos combinados para esta enfermidade (Morilak e Frazer, 2004).

A agmatina é uma amina catiônica, conhecida como um produto intermediário no metabolismo de poliaminas, encontrada em plantas, bactérias e invertebrados. Ela foi descoberta em 1994 em mamíferos e é sintetizada pela descarboxilação de L-arginina em uma reação catalisada pela enzima arginina descarboxilase. A agmatina pode ser metabolizada a putrescina durante a biossíntese de poliaminas, por ação da enzima agmatinase ou metabolizada a ácido guanidino butírico, por ação da enzima diamina oxidase (Reis e Regunathan, 2000; Raasch et al., 2001; Moinard et al., 2005) (**Figura 8**).

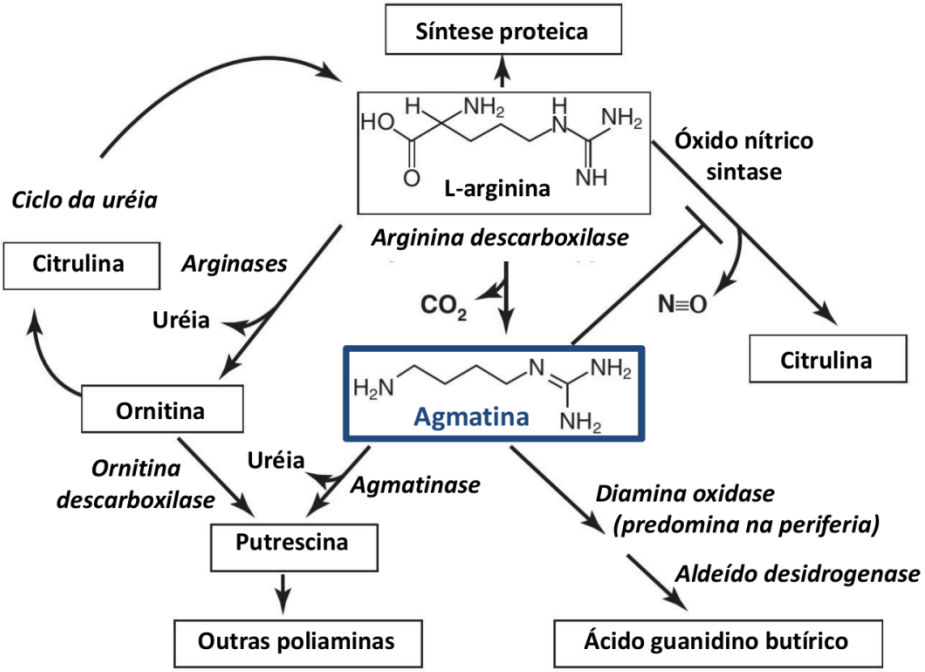


**Figura 8. Síntese e degradação da agmatina, destacando algumas das vias intercorrelacionadas ao metabolismo da L-arginina que regulam o ciclo da uréia, as poliaminas, o óxido nítrico (NO) e o metabolismo de proteínas.** A agmatina é sintetizada a partir da descarboxilação de L-arginina pela enzima arginina descarboxilase. É metabolizada a putrescina por ação da enzima agmatinase ou metabolizada a ácido guanidino butírico pela enzima diamina oxidase. (Adaptado de Piletz et al., 2013).

A concentração da agmatina em mamíferos varia regionalmente. No encéfalo representa aproximadamente 10% da concentração presente no estômago, órgão que apresenta altas concentrações desta amina. Ela está presente no córtex cerebral (principalmente lâmina VI e V), hipocampo, complexo amigdalóide, hipotálamo e tronco cerebral (regiões do encéfalo responsáveis pelo controle endócrino e visceral, processamento das emoções, dor, percepção e cognição). A nível sub-celular, agmatina está presente no corpo celular, dendritos, axônios e terminais axônicos neuronais (Reis e Regunnathan, 2000).

A agmatina foi proposta como um neuromodulador no SNC (Reis e Regunathan, 2000; Raasch et al., 2001): é sintetizada no encéfalo; estocada em vesículas sinápticas; acumulada por captação; liberada de maneira dependente

de cálcio por despolarização; metabolizada e degradada a putrescina pela agmatinase e captada por sinaptossomos (Reis e Regunathan, 2000).

Agmatina liga-se com moderada afinidade a receptores nicotínicos de aceticolina, $\alpha_2$-adrenérgicos e receptores imidazólicos tipo 1 ($I_1$) e 2 ($I_2$). A ativação de receptores $I_1$ promove redução da pressão sanguínea central, natriurese, fluxo urinário e analgesia. Por outro lado, a ativação de receptores $I_2$ causa redução da atividade da enzima monoamina oxidase (MAO) e crescimento celular. Além disso, agmatina é capaz de antagonizar receptores NMDA e de inibir todas as isoformas da enzima oxido nítrico sintase (NOS) no encéfalo (Reis e Regunathan, 2000; Raasch et al., 2001; Moinard et al., 2005), bem como ativar com baixa afinidade receptores $\alpha_1$ e β-adrenérgicos, 5-$HT_3$-serotoninérgicos, $D_2$-dopaminérgicos e κ-opióides (Raasch et al., 2001). Estudos têm demonstrado que a agmatina administrada por via oral é absorvida pelo trato gastro-intestinal e rapidamente distribuída pelo corpo (Haenisch et al., 2008), atravessa a barreira hemato-encefálica (Piletz et al., 2003) e apresenta um tempo de meia-vida de aproximadamente 2 h (Huisman et al., 2010). O sulfato de agmatina (500 mg – 1g) tem sido utilizado como suplemento nutricional por fisiculturistas no intuito de auxiliar o ganho de massa muscular e a perda de gordura. Apesar de tais efeitos não terem comprovação científica, a utilização de sulfato de agmatina nestas doses e por via oral não produz efeitos adversos severos, sendo que a via oral é uma rota de administração segura (Piletz et al., 2013).

Recentemente, Piletz et al. (2013) revisaram as principais funções biológicas da agmatina. Esta amina presenta propriedade neuroprotetora, pois protege culturas de células de hipocampo frente à excitotoxicidade glutamatérgica (Olmos et al., 1999; Wang et al., 2006) e à privação de glicose e oxigênio (Kim et al., 2004; Ahn et al., 2011), promove proteção em um modelo de neurotrauma (Gilad e Gilad, 2000) e de isquemia global transitória em ratos (Mun et al., 2010), e ainda possui a capacidade de seqüestrar radicais livres protegendo do estresse oxidativo induzido pelo aumento do volume mitocondrial, colapso do potencial de membrana, e apoptose (Arndt et al., 2010; Battaglia et al., 2010). Além disso, Matheus et al. (2012) mostraram que o tratamento sistêmico (via intraperitoneal) repetido com agmatina é capaz de prevenir danos motores e de memória e a perda de neurônios dopaminérgicos em um modelo experimental de Doença de Parkinson em camundongos envelhecidos.

A agmatina também apresenta função anti-amnésica. Estudos indicam que ela facilita a memória em um modelo de esquiva inibitória em ratos (Arteni et al., 2002). Ela também apresenta propriedade ansiolítica, uma vez que sua administração via oral em ratos e camundongos produz efeito ansiolítico em três modelos animais de ansiedade: teste de transição claro-escuro, teste do conflito de beber de Vogel e teste de interação social (Gong et al., 2006).

Estudos indicam que a agmatina possui propriedade anticonvulsivante em um modelo de convulsão induzida por eletrochoque (Bence et al., 2003; Su et al., 2004), sendo que este efeito pode ser mediado pelo antagonismo de

receptores NMDA (Su et al., 2004). Outros estudos mostram que agmatina protege frente à convulsão induzida por pentilenotetrazol (Demehri et al., 2003; Feng et al., 2005), um efeito provavelmente mediado por diminuição da liberação de glutamato (Feng et al., 2005). Santos et al. (2005) mostraram que a agmatina produz um efeito antinociceptivo dose-dependente em modelos animais de dor induzido por ácido acético, glutamato, capsaicina e formalina, sendo que este efeito parece ser mediado por um interação com o sistema opióide, serotoninérgico (5-$HT_{2A}$ e 5-$HT_3$), nitrérgico, bem como interação com $\alpha_2$-adrenoceptores e receptores imidazólicos $I_1$ .

Nosso grupo foi o primeiro a mostrar em 2002 que a administração aguda de agmatina por via sistêmica (intraperitoneal) e central (intracerebroventricular) em camundongos produz efeito antidepressivo no teste do nado forçado (TNF) e no teste da suspensão pela cauda (TSC) (Zomkowski et al., 2002). Um estudo posterior mostrou o efeito da agmatina (administrada pelas vias oral e subcutânea) no TNF em camundongos e em ratos (Li et al., 2003). O estudo dos mecanismos de ação responsáveis pela ação antidepressiva da agmatina no TNF mostrou que seu efeito é dependente do antagonismo de receptores NMDA e da inibição da síntese de óxido nítrico (NO) e de uma interação com os sistemas noradrenérgico (receptores $\alpha_2$-adrenérgicos), serotoninérgico (receptores 5-$HT_{1A/1B}$ e 5-$HT_2$), opióide (receptores δ- e μ-opióides) (Zomkowski et al., 2002, 2004, 2005; Jiang et al., 2008), com os receptores imidazólicos $I_1$ e $I_2$ (Zeidan et al., 2007) e pela inibição de canais de potássio (Budni et al., 2007). Recentemente, nosso grupo demonstrou que o tratamento sistêmico (via oral) de camundongos com agmatina é capaz de abolir o comportamento tipo-depressivo induzido por TNF-α, e que tal efeito parece estar associado com a ativação do sistema monoaminérgico, antagonismo de receptores NMDA e inibição da síntese de óxido nítrico (Neis et al., 2014). Além disso, Li et al. (2006) demonstraram que a agmatina promove aumento da proliferação de células progenitoras hipocampais *in vitro* e neurogênese de células do hipocampo *in vivo*, em camundongos estressados cronicamente. Tal efeito observado por Li et al. (2006) parece ser mediado pelo antagonismo de receptores NMDA. A participação da agmatina e de receptores imidazólicos no efeito antidepressivo da bupropiona (inibidor da recaptação de dopamina com atividade sutil sobre a recaptação de noradrenalina) (Kotagale et al., 2013a) e de inibidores seletivos da recaptação de serotonina (ISRS) (Taksande et al., 2009) também foi demonstrado em camundongos. Este mesmo grupo verificou um possível envolvimento do receptor do neuropeptídeo Y tipo Y1 (NPY-Y1) no efeito antidepressivo da agmatina no TNF em ratos (Kotagale et al., 2013b).

Considerando os estudos realizados em humanos, Bernstein et al. (2012) mostraram que indivíduos deprimidos apresentam aumento da expressão da enzima agmatinase – responsável pela degradação da agmatina neuronal hipocampal, sugerindo que esta enzima e/ou níveis neuronais hipocampais alterados de agmatina podem estar implicadas na fisipatologia da depressão maior. Além disso, Keynan et al. (2010) mostrou que a agmatina (2,670 g via oral, durante 14 dias, n=51) alivia a dor e melhora a qualidadede vida de

pacientes que sofrem de dor neuropática. É imprescindível citar o estudo de Shopsin (2013) que mostrou que a administração exógena de agmatina (2-3 mg/dia; 3-4 semanas; n=3 pacientes) é capaz de produzir efeitos antidepressivos em indivíduos diagnosticados com depressão maior. Os pacientes possuíam idades entre 29 a 52 anos (dois deles eram do sexo masculino e um do sexo feminino), apresentaram total remissão dos sintomas depressivos e não foram tratados com nenhum outro medicamento antidepressivo durante o estudo. Estes três indivíduos não apresentaram nenhum efeito adverso severo (apenas foi relatado desconforto gastrointestinal em um dos pacientes, e que tal efeito cessou em poucos dias sem que o tratamento com agmatina fosse descontinuado). Em seguida, os três pacientes receberam um co-tratamento com PCPA (p-clorofenilalanina; inibidor da síntese de serotonina; 250-1250 mg/dia; 1,5-2 semanas), o qual não reverteu os efeitos produzidos pelo tratamento com agmatina exógena. Estes achados sugerem que o sistema serotonérgico não participa do efeito antidepressivo produzido pela agmatina nestes pacientes. Shopsin (2013) sugere ainda que o efeito observado pode ser mediado pela inibição da excitotoxicidade gutamatérgica, mais especificamente, pela habilidade da agmatina em antagonizar receptores NMDA. Reconhece-se a importância deste trabalho, entretando suas limitações devem ser discutidas: o autor não realizou nenhum experimento que indicasse a participação dos receptores NMDA nos efeitos antidepressivos produzidos pela agmatina exógena; o trabalho foi realizado em um número muito reduzido de pacientes. De maneira geral, o estudo de Shopsin (2013) foi inovador ao mostrar, pela primeira vez, que a agmatina é capaz de produzir efeitos antidepressivos clínicos, entretando fica evidente a necessidade de novos estudos clínicos e pré-clínicos a fim de elucidar os mecanimos pelos quais a agmatina produz seus efeitos antidepressivos.

## 2. Justificativa

A depressão maior é um sério problema de saúde pública que afeta milhões de pessoas em todo o mundo, é acompanhada por sérios prejuízos funcionais e ocupacionais e altos custos para a sociedade (WHO, 2008; 2012a, 2012b).

A descoberta biológica mais consistente para o estudo da fisiopatologia da depressão maior foi a de que eventos estressores predispõem o desenvolvimento desta enfermidade através da hiperativação do eixo hipotálamo-pituitátia-adrenal (HPA) que induz uma liberação exacerbada de glicocorticóides (cortisol em humanos, corticosterona em roedores) (Carroll et al., 1976; Pariante e Lightman, 2008; Frodl e O'Keane, 2013; Maric and Adzic, 2013). O excesso de glicocorticóides causa danos ao cérebro principalmente ao hipocampo em virtude da alta densidade de receptores de glicocorticóide (GRs) presentes nesta estrutura cerebral (Duman, 2004b). Entre as alterações hipocampais induzidas pelos glicocorticóides destacam-se: diminuição da neurogênese das células granulares do giro denteado, do tamanho e número das arborizações dendríticas e atrofia dos neurônios piramidais e neuroglia, as quais são abolidas pelo

tratamento crônico com antidepressivos (Duman, 2004b; Popoli et al., 2011; Frodl e O'Keane, 2013). Sabe-se que as células neuronais são as primeiras a sofrerem os efeitos danosos causados pelos glicocorticóides (Lee et al., 2002; McKinnon et al., 2009), os quais são explicados pela hipótese inflamatória e oxidativa da depressão, que postula que o excesso de glicocorticóides induz um desequilíbrio oxidativo e inflamatório no cérebro acompanhado pela diminuição das defesas antioxidantes, peroxidação de lipídeos, alterações nos sistemas monoaminérgicos e redução da neurogênese e plasticidade neuronal (Maes et al., 2009; Leonard e Maes, 2012). Tem sido sugerido que uma nova estratégia farmacológica para o tratamento da depressão baseia-se nos ativadores do fator de transcrição Nrf2 (Maes et al., 2012; Lee et al., 2013; Martín-de-Saavedra et al., 2013). Sabe-se que o Nrf2 é um regulador central das defesas antioxidantes celulares e das respostas celulares ao estresse (Johnson et al., 2008).

Há uma necessidade crescente de novos compostos com atividade antidepressiva em virtude dos efeitos adversos causados e da baixa tolerabilidade da farmacoterapia atual disponível. A agmatina é um neuromodulador endógeno, que possui importantes propriedades biológicas (Piletz et al., 2013). Nosso grupo foi o pioneiro a demonstrar que sua administração sistêmica em camundongos produz efeitos antidepressivos em testes preditivos de atividade antidepressiva. Vários estudos subsequentes confirmaram o potencial antidepressivo da agmatina (Zomkowski et al., 2002, 2004, 2005). Além disso, um estudo recente verificou que a administração exógena deste neuromodular a pacientes deprimidos proporciona remissão dos sintomas sem causar efeitos adversos graves (Shopsin, 2013). Apesar dos estudos prévios que avaliaram os efeitos antidepressivos da agmatina, o efeito produzido pela sua administração crônica em camundongos, bem como os mecanismos moleculares implicados ainda não foram verificados. Adicionalmente, nenhum estudo avaliou a habilidade da agmatina em abolir um comportamento tipo-depressivo induzido por um modelo de depressão causado por estresse e acompanhado por um desequilíbrio oxidativo hipocampal.

Sabendo ainda que neuroproteção é um mecanismo de ação comum para os compostos antidepressivos (Drzyzga et al., 2009; Li et al., 2003), e que a linhagem de células neuronais hipocampais murinas HT22 é uma ferramenta válida para o estudo de enfermidades relacionadas ao hipocampo, particularmente a danos neuronais hipocampais como ocorre na depressão maior. Avaliar os efeitos da agmatina em um modelo de neurotoxicidade induzido por corticosterona em células neuronais hipocampais murinas HT22 é uma ferramenta válida para esclarecer os mecanismos moleculares e celulares envolvidos nos efeitos da agmatina. Em última análise, transpôr os resultados observados nas células neuronais hipocampais murinas HT22 para um organismo vivo (camundongo) tem sua relevância farmacológica, levando-se em conta a complexidade do sistema nervoso central. Considerando as afirmações anteriores, o estudo dos mecanismos de ação da agmatina em diferentes modelos de indução de depressão tem como objetivo ampliar as bases literárias acerca dos efeitos deste neuromodulador, bem como da patofisiologia

da depressão maior a fim de oferecer a longo prazo um uso clínico seguro para a agmatina como adjuvante/monoterapia.

# 3. Objetivos

## 3.1. Objetivo geral

Avaliar o efeito da agmatina em modelos animais de depressão induzidos por estresse/glicocorticóides em camundongos e em um modelo *in vitro* de neurotoxicidade em cultura de células hipocampais HT22, bem como os possíveis mecanismos bioquímicos envolvidos nos efeitos da agmatina.

## 3.2. Objetivos específicos

1. Verificar o efeito da administração crônica de agmatina durante 21 dias em camundongos no teste da suspensão pela cauda, teste do campo aberto e sobre vias de sinalização hipocampais relacionadas à sobrevivência neuronal PKA, PKC, ERK1/2, Akt, GSK-3β, JNK1/2, $p38^{MAPK}$, CREB e BDNF.
2. Verificar o efeito da administração oral de agmatina em um modelo animal de depressão induzida por estresse de contenção no teste do nado forçado, teste do campo aberto, e sobre parâmetros bioquímicos de avaliação de estresse oxidativo: níveis de glutationa total (GSH) e de TBARS (substâncias reativas ao ácido tiobarbitúrico), atividade de glutationa peroxidase (GPx), glutationa redutase (GR), catalase (CAT) e de superóxido dismutase (SOD) no hipocampo de camundongos.
3. Padronizar um modelo de neurotoxicidade *in vitro* induzido por glicocorticóides (corticosterona) utilizando a linhagem de células neuronais hipocampais murinas HT22.
4. Avaliar o efeito da agmatina no modelo de neurotoxicidade induzido por corticosterona em células neuronais hipocampais murinas HT22 sobre parâmetros de viabilidade celular, produção de espécies reativas de oxigênio (ERO), apoptose, participação do sistema monoaminérgico e da via de sinalização celular mediada pela ativação do fator de transcrição Nrf2.
5. Padronizar um modelo depressão induzido por glicocorticóides (corticosterona) em camundongos.
6. Avaliar o efeito da agmatina no modelo de depressão induzida por corticosterona em camundongos no teste da suspensão pela cauda, teste do campo aberto, splash teste e sobre parâmetros bioquímicos: expressão de fator neurotrófico derivado do encéfalo (BDNF) e da proteína sináptica (sinaptotagmina I); participação do fator de transcrição Nrf2; níveis hipocampais dos seguintes neurotransmissores: noradrenalina, serotonina, dopamina e glutamato; e morfologia hipocampal das células glias e astrocitárias.

## 4. Resultados

As metodologias e resultados estão divididos em capítulos:

**Capítulo I**

"Sub-chronic agmatine treatment modulates hippocampal neuroplasticity and cell survival signaling pathways in mice. Andiara E. Freitas, Luis E. B. Bettio, Vivian B. Neis, Morgana Moretti, Camille M. Ribeiro, Mark W. Lopes, Rodrigo B. Leal, Ana Lúcia S. Rodrigues".

Manuscrito completo referente ao objetivo 1 aceito para publicação no periódico *Journal of Psychiatric Research.*

**Capítulo II**

"Agmatine abolishes restraint stress-induced depressive-like behavior and hippocampal antioxidant imbalance in mice. Freitas AE, Bettio LE, Neis VB, Santos DB, Ribeiro CM, Rosa PB, Farina M, Rodrigues AL. Prog Neuropsychopharmacol Biol Psychiatry. 2014; 50:143-50".

Manuscrito completo referente ao objetivo 2 publicado no periódico *Progress in Neuro-Psychopharmacology & Biological Psychiatry*.

**Capítulo III**

"Agmatine protects against corticosterone-induced HT22 mouse hippocampal cell death through Nrf2 activation. Andiara E. Freitas, Javier Egea, Patricia Rada, Antonio Cuadrado, Ana Lúcia S. Rodrigues, Manuela G. López".

Manuscrito completo referente aos objetivos 3 e 4 aceito para publicação no periódico *Molecular Neurobiology*.

**Capítulo IV**

"Agmatina abole comportamento tipo-depressivo induzido por corticosterona em camundongos: Envolvimento do fator de transcrição Nrf2 e de vias de sinalização celular relacionadas à neuroplasticidade. Andiara E. Freitas, Javier Egea, Vanessa Gómez-Rangel, Izaskun Buendía, Esther Parada, Aneta Wojnicz, José Avendaño Ortiz, Antonio Cuadrado, Ana Ruiz-Nuño, Ana Lúcia S. Rodrigues, Manuela G Lopez".

Principais Materiais e Métodos e Resultados referentes aos objetivos 5 e 6 que irão compor o Manuscrito a ser submetido ao periódico *The International Journal of Neuropsychopharmacology*.

# Capítulo I

Sub-chronic agmatine treatment modulates hippocampal neuroplasticity and cell survival signaling pathways in mice

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**Abstract:** Agmatine is an endogenous neuromodulator which, based on animal and human studies, is a putative novel antidepressant drug. In this study, we investigated the ability of sub-chronic (21 days) p.o. agmatine administration to produce an antidepressant-like effect in the tail suspension test and examined the hippocampal cell signaling pathways implicated in such effect. Agmatine at doses of 0.01 and 0.1 mg/kg (p.o.) produced a significant antidepressant-like effect in the tail suspension test and no effect in the open-field test. Additionally, agmatine (0.001-0.1 mg/kg, p.o.) increased the phosphorylation of protein kinase A substrates (237-258% of control), protein kinase B/Akt ($Ser^{473}$) (116-127% of control), glycogen synthase kinase-3β ($Ser^{9}$) (110-113% of control), extracellular signal-regulated kinases 1/2 (119-137% and 121-138% of control, respectively) and cAMP response elements ($Ser^{133}$) (127-152% of control), and brain-derived-neurotrophic factor (137-175% of control) immunocontent in a dose-dependent manner in the hippocampus. Agmatine (0.001-0.1 mg/kg, p.o.) also reduced the c-jun N-terminal kinase 1/2 phosphorylation (77-71% and 65-51% of control, respectively). Neither protein kinase C nor $p38^{MAPK}$ phosphorylation was altered under any experimental conditions. Taken together, the present study extends the available data on the mechanisms that underlie the antidepressant action of agmatine by showing an antidepressant-like effect following sub-chronic administration. In addition, our results are the first to demonstrate the ability of agmatine to elicit the activation of cellular signaling pathways associated with neuroplasticity/cell survival and the inhibition of signaling pathways associated with cell death in the hippocampus.

**Keywords:** agmatine; PKA; Akt; GSK-3β; ERK1/2; JNK1/2; CREB; BDNF.

**Abbreviations:** ANOVA, analysis of variance; BDNF, brain-derived-neurotrophic factor; CaMK, Ca2+/calmodulin-dependent protein kinase; CRE, cAMP response elements; CREB, cyclic-AMP responsive-element binding protein; ERK, extracellular signal-regulated kinases; FST, forced swimming test; GSK-3β, glycogen synthase kinase-3β; JNK, c-jun N-terminal kinase; PI3K, phosphatidylinositol 3kinase; MAPK, mitogen-activated protein kinase; MDD, Major depressive disorder; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; RNS, reactive nitrogen species; TNF-α, tumor necrosis factor-α; TrkB, tropomyosin-related kinase B; TST, tail suspension test.

## 1. Introduction

Major depressive disorder (MDD) is a serious public health problem. It is the leading cause of disability in the U.S. for individuals aged 15-44 (WHO, 2008). It is well known that the pathophysiology of MDD involves a monoaminergic dysfunction (Elhwuegi, 2004; Heninger et al., 1996). Although most antidepressant drugs act acutely on the monoaminergic system by increasing its synaptic availability, the clinical effects of the antidepressant drugs are only observed 2-3 weeks after the onset of treatment (Gourion, 2008). This phenomenon is explained by the neurotrophic hypothesis of depression, which proposes that the long-term antidepressant treatment modulates signal transduction survival pathways. This modulation, in turn, induces the expression of neurotrophic factors, primarily the Brain-Derived Neurotrophic Factor (BDNF), promoting neurogenesis and restoring the neural networks altered in depressed subjects (Masi and Brovedani, 2011; Neto et al., 2011). Several kinases such as protein kinase A (PKA), phosphatidylinositol 3kinase (PI3K)-Akt, protein kinase C (PKC), and the extracellular signal-regulated kinases (ERK)/1-2 are able to activate by phosphorylating the transcriptional regulator cyclic-AMP responsive-element binding protein (CREB) (Lonze and Ginty, 2002; Shaywitz and Greenberg, 1999). Once phosphorylated, CREB binds to the BDNF promoter, up-regulates BDNF expression and lifts the depressive mood (Fišar and Hroudová, 2010; Numakawa et al., 2010).

There is a growing amount of evidence showing that the activation of the stress activated pathways c-jun N-terminal kinase (JNK) and $p38^{MAPK}$ play important roles in neuronal cell death, suggesting that JNK and $p38^{MAPK}$ inhibitors could constitute potential therapeutic drugs for neural diseases, including MDD (Borsello and Forloni, 2007; Harper and LoGrasso, 2001; Yasuda et al., 2011). Additionally, several studies have proposed that glycogen synthase kinase-3 β (GSK-3β) inhibitors have the potential to augment the efficacy of antidepressants or to be used as monotherapy (Beaulieu et al., 2009; Maes et al., 2012; Vidal et al., 2011).

Agmatine is a neuromodulator in the brain with antidepressant properties (Piletz et al., 2013). Our group was the first to demonstrate that agmatine is able to produce an antidepressant-like effect in the mouse forced swimming test (FST) and in the tail suspension test (TST), accompanied by modulation of the monoaminergic and opiod systems, NMDA receptors and the L-arginine-NO pathway (Zomkowski et al., 2002, 2004, 2005). More recently, we showed that agmatine produces an antidepressant-like effect that was paralleled by its capability to maintain the pro-/anti-oxidative homeostasis in the hippocampus (Freitas et al., 2013a), and we showed that agmatine is able to abrogate the depressive-like behavior induced by tumor necrosis factor-α (Neis et al., 2014). In addition, agmatine's ability to produce a clinical antidepressant effect was shown by Shopsin (2013). Despite agmatine's potential for use as a coadjuvant or monotherapy in the management of MDD, there is no study reporting its ability to produce an antidepressant-like effect following sub-chronic

administration as well as the molecular mechanisms underlying such an effect. Therefore, the aim of the present study was to investigate the effect of sub-chronic agmatine treatment on the regulation of hippocampal signaling targets associated with neuronal survival, namely PKA, PKC, ERK1/2, Akt, GSK-3β, JNK1/2, $p38^{MAPK}$, CREB and BDNF.

## 2. Materials and methods

### *2.1. Animals*

Female Swiss mice (3 months old, 40-45 g) were maintained at constant room temperature (20-22°C) with free access to water and food, under a 12:12 h light:dark cycle (lights on at 07:00 h). The cages were placed in the experimental room 24 h before the test for acclimatization. All manipulations were carried out between 9:00 and 17:00 h, with each animal used only once. The procedures in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the local Ethics Committee. All efforts were made to minimize animal suffering and the number of animals used in the experiments.

### *2.2. Drugs and treatment*

Agmatine (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in distilled water and administered once daily for 21 days via the oral route (p.o.) by gavage at doses of 0.001-0.1 mg/kg in a constant volume of 10 ml/kg body weight. A control group received distilled water as the vehicle. The number of mice per group was 8. To administer agmatine or vehicle, mice were first weighed to determine the dosing volume to be administered, following by the introduction of the gavage tube (feeding tubes approximately 31 mm in length with a rounded tip) in the diastema of the mouth by an experienced researcher. The tube was gently advanced along the upper palate, and the treatment was administered by a syringe attached to the end of the tube. After dosing, the tube was gently removed following the same angle as insertion. Finally, the animals were returned to their cage.

### *2.3. Tail suspension test (TST)*

The tail suspension test was performed 24 h after the last sub-chronic drug administration. The total duration of immobility induced by tail suspension was measured using the method described by Steru et al. (1985). Acoustically and visually isolated mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was registered during a 6-min period (Freitas et al., 2013b, 2013c, 2010).

### *2.4. Open-field test*

Five minutes after the tail suspension test, mice were evaluated in the open-field paradigm (Rodrigues et al., 1996) to assess the effects of agmatine on locomotor activity. The number of squares crossed with all paws (crossings)

counted in a 6-min session. The apparatus were cleaned with a solution of 10% ethanol between tests to hide animal clues.

*2.5. Western Blot*

Immediately after the behavioral observations, mice were decapitated. Brains were removed, and the hippocampus was rapidly dissected and placed in liquid nitrogen for storage at −80 °C until use. Western blot analysis was performed as previously described (Cordova et al., 2004; Freitas et al., 2013b, 2013d; Lopes et al., 2012, 2013). Briefly, hippocampal tissue was mechanically homogenized in 400 µl of Tris-base 50 mM pH 7.0, EDTA 1 mM, sodium fluoride 100 mM, PMSF 0.1 mM, sodium vanadate 2 mM, Triton X-100 1%, glycerol 10%, and protease inhibitor Cocktail; the tissue was then incubated for 30 min in ice. Lysates were centrifuged (10000 x g for 10 min, at 4 °C) to eliminate cellular debris; the supernatants were diluted 1/1 (v/v) in Tris-base 100 mM pH 6.8, EDTA 4 mM, and 8% SDS and then boiled for 5 min. Subsequently, the loading buffer (glycerol 40%, Tris-base 100 mM, bromophenol blue, pH 6.8) at a ratio of 25:100 (v/v) and β-mercaptoethanol (final concentration 8 %) were added to the samples. The protein content was estimated by the method described by Peterson (1977) using bovine serum albumin as protein standard. To compare the obtained signals, the same amount of protein (70 µg per lane) for each sample was electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) minigels and transferred to nitro-cellulose or polyvinylidene fluoride (PVDF) membranes using a semidry blotting apparatus (1.2 mA/cm2; 1.5 h). To verify the transfer efficiency process, gels were stained with Coomassie blue (Coomassie blue R-250 0.1%, methanol 50%, acetic acid 7%) and membranes with Ponceau S 0.5% in acetic acid 1%.

After this process, blots were incubated in a blocking solution (5% non-fat dry milk in Tris buffer saline solution (TBS) (Tris 10 mM, NaCl 150 mM, pH 7.5) for 1 h at room temperature, and targets were detected after overnight incubation (4 °C) with specific antibodies diluted in TBS with tween (TBS-T) that contained 2% BSA at the following dilutions: anti-phospho-PKA substrates (Cell Signaling Technology, Boston, MA, USA, 1:1000), anti-phospho-PKC substrates (Cell Signaling, 1:1000), anti-phospho-Akt (Sigma Chemical Co., 1:2000), anti-phospho-GSK-3β (Cell Signaling, 1:1000), anti-phospho-ERK1/2 (Sigma Chemical Co., 1:2000), anti-phospho-JNK1/2 (Cell Signaling, 1:5000), anti-phospho-$p38^{MAPK}$ (Millipore, Billerica, MA, USA, 1:10000), anti-phospho-CREB (Cell Signaling, 1:1000), anti-total-Akt (Cell Signaling, 1:1000), anti-total-GSK-3β (Cell Signaling, 1:1000), anti-total-ERK1/2 (Sigma Chemical Co., 1:40000), anti-total-JNK1/2 (Sigma Chemical Co., 1:5000), and anti-total-$p38^{MAPK}$ (Sigma Chemical Co., 1:10000), anti-total-CREB (Cell Signaling, 1:1000), anti-β actin (Santa Cruz, 1:2500) and anti-BDNF (Millipore, 1:1000). Then, the membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit (1:5000) or anti-mouse (1:2000) secondary antibodies for the detection of phosphorylated sites or the

total form of proteins. The reactions were developed by a chemioluminescence substrate (LumiGLO). All blocking and incubation steps were followed by washing three times (5 min) with TBS-T. β actin, the house-keeping protein, was evaluated to ascertain the same protein load for each experimental group. To detect the phosphorylated and total forms of Akt, GSK-3β, ERK1/2, JNK1/2 and $p38^{MAPK}$ in the same membrane, the immunocomplexes were stripped as previously described (Posser et al., 2007). Briefly, membranes were washed once with deionized water (5 min), followed by incubation with NaOH 0.2 M (5 min) and then washing with deionized water (5 min) and TBS-T (10 min). The stripped membranes were blocked and reprobed following the steps described above. PKA and PKC activities were evaluated by measuring the phosphorylation of their respective specific substrates using phospho-PKA and PKC substrate-specific antibodies.

The optical density (O.D.) of the bands was quantified using the Scion Image software®. The phosphorylation levels of Akt, GSK-3β, ERK1/2, JNK1/2 and $p38^{MAPK}$ were determined as a ratio of the O.D. of the phosphorylated band and the O.D. of the total band. The phosphorylation levels of the PKA and PKC substrates were determined as the ratio of the O.D. of the phosphorylated band and the O.D. of the β actin band. The BDNF immunocontent was determined from the relationship between the O.D of the BDNF band and the O.D. of the β actin band.

*2.6. Statistical analysis*

Comparisons between experimental and control groups were performed using a one-way ANOVA, followed by Duncan's multiple range test when appropriate. Pearson's correlation analysis was performed to investigate any possible relationship between behavioral and neurochemical data. A *p*-value of $p<0.05$ was considered to be significant.

## 3. Results

*3.1. Effect of a sub-chronic treatment with agmatine on the immobility time in the TST and locomotor activity in the open-field test*

As depicted in Fig. 1A, the sub-chronic administration of agmatine for 21 days decreased the immobility time in the TST, a behavioral profile that is indicative of an antidepressant-like effect. One-way ANOVA revealed a significant effect due to agmatine treatment [$F(3,28)=14.21$, $p<0.01$]. Post-hoc analysis indicated a significant decrease in the immobility time elicited by agmatine at doses of 0.01 and 0.1 mg/kg ($p<0.01$). The results depicted in Fig. 1B illustrate that the administration of agmatine (dose range 0.001-0.1 mg/kg, p.o.) did not affect the ambulation in the open-field test [$F(3,28)=0.21$, $p=0.89$].

*3.2. Cell signaling pathways*

Western blot analysis from hippocampal tissue homogenates showed that sub-chronic agmatine treatment produced a dose-dependent increase (237-258% of control) in the phosphorylation of PKA substrates (Fig. 2A and B). One-way ANOVA revealed a significant effect due to agmatine treatment [$F(3,20)=10.83$, $p<0.01$]. Post-hoc analysis indicated that agmatine at doses of 0.001, 0.01 and 0.1 mg/kg (p.o.) caused a significant increase in the phosphorylation of PKA substrates. Fig. 2C and D show that no significant change was observed in PKC substrates phosphorylation [$F(3,20)=0.74$, $p=0.54$].

The results depicted in Fig. 2E and F illustrate that the sub-chronic agmatine (0.001-0.1 mg/kg, p.o.) treatment induced a significant increase (change varied dose-dependently, ranging between 116-127% of control) in Akt ($Ser^{473}$) phosphorylation. One-way ANOVA revealed a significant effect due to agmatine treatment [$F(3,20)=8.34$, $p<0.01$]. Post-hoc analysis indicated a significant increase in Akt ($Ser^{473}$) phosphorylation produced by agmatine at doses of 0.001, 0.01 and 0.1 mg/kg (p.o.). Similarly, Fig. 2G and H show increased (110-113% of control) GSK-3β ($Ser^{9}$) phosphorylation elicited by agmatine (0.001-0.1 mg/kg, p.o.) treatment. One-way ANOVA revealed a significant effect due to agmatine treatment [$F(3,20)=15.04$, $p<0.01$]. Post-hoc analysis indicated a significant increase in GSK-3β ($Ser^{9}$) phosphorylation produced by agmatine at doses of 0.001, 0.01 and 0.1 mg/kg (p.o.).

Sub-chronic agmatine (0.001-0.1 mg/kg, p.o.) treatment induced a dose-dependent increase in both ERK1 and ERK2 phosphorylation (119-137% and 121-138% of control, respectively) (Fig. 3A, B and C). One-way ANOVA revealed a significant effect due to agmatine treatment [$F(3,20)=5.34$, $p<0.01$] with respect to ERK1 phosphorylation. Similarly, one-way ANOVA revealed a significant effect due to agmatine treatment [$F(3,20)=4.92$, $p<0.01$] for ERK2 phosphorylation. Post-hoc analysis indicated a significant increase in both ERK1 and ERK2 phosphorylation elicited by agmatine at doses of 0.001, 0.01 and 0.1 mg/kg (p.o.).

As depicted in Fig. 3D and E, agmatine (0.001-0.1 mg/kg, p.o.) treatment for 21 days reduced (77-71% of control) JNK1 phosphorylation in a dose-dependent manner. One-way ANOVA revealed a significant effect due to agmatine treatment [$F(3,20)=14.32$, $p<0.01$]. Similarly, Fig. 3D and F show that sub-chronic agmatine (0.001-0.1 mg/kg, p.o.) treatment resulted in a significant reduction (65-51% of control) in JNK2 phosphorylation. One-way ANOVA revealed a significant effect due to agmatine treatment [$F(3,20)=11.31$, $p<0.01$]. Post-hoc analysis indicated a significant reduction in both JNK1 and JNK2 phosphorylation produced by agmatine at doses of 0.001, 0.01 and 0.1 mg/kg (p.o.).

The effect of the sub-chronic agmatine treatment on $p38^{MAPK}$ phosphorylation was also investigated. Fig. 3G and H show that $p38^{MAPK}$ phosphorylation was not altered under any experimental conditions [$F(3,20)=0.83$, $p=0.49$].

Finally, sub-chronic agmatine (0.001-0.1 mg/kg, p.o.) treatment was able to increase (127-152% of control) both CREB ($Ser^{133}$) phosphorylation (Fig. 4A and B) and BDNF (137-175% of control) immunocontent in a dose-dependent manner (Fig. 4C and D). One-way ANOVA revealed a significant effect due to agmatine treatment [$F(3,20)=5.50$, $p<0.01$] with respect to CREB phosphorylation. Moreover, a one-way ANOVA revealed a significant effect due to agmatine treatment [$F(3,20)=9.68$, $p<0.01$] with respect to BDNF immunocontent. Post-hoc analysis indicated that agmatine elicited a significant increase in both CREB ($Ser^{133}$) phosphorylation and BDNF immunocontent at doses of 0.001, 0.01 and 0.1 mg/kg (p.o.).

Table 1 shows significant Pearson's correlations between immobility time, PKA, Akt, JNK2, CREB or BDNF and all signaling molecules studied. Additionally, GSK-3β correlated with PKA, Akt, JNK1, JNK2, CREB, and BDNF. A significant correlation was shown between ERK1 and PKA, Akt, ERK2, JNK1, JNK2, CREB or BDNF. ERK2 correlated with PKA, Akt, ERK1, CREB or BDNF. Additionally, JNK1 correlated with PKA, Akt, GSK-3β, ERK1, JNK2, CREB or BDNF. Finally, a significant correlation was found between JNK2 and PKA, Akt, GSK-3β, ERK1, JNK1, CREB or BDNF. The number of crossings and the PKC and $p38^{MAPK}$ levels did not correlate with any variable studied (data not shown).

## 4. Discussion

The present study clarified some mechanisms underlying the sub-chronic antidepressant-like action of agmatine. In contrast to the previous studies that investigated the acute antidepressant-like effect of agmatine, the present study was focused on behavioral and neurochemical alteration induced by sub-chronic agmatine treatment in mice. In the present study, female mice were chosen because more women than men suffer depression (Wong and Licinio, 2001). Agmatine, when administered for 21 days, produced a significant antidepressant-like effect in the TST, a commonly used behavioral test that predicts the efficacy of an antidepressant treatment (Steru et al., 1985). In addition, agmatine increased the phosphorylation of PKA substrates, Akt ($Ser^{473}$), GSK-3β ($Ser^{9}$), ERK1/2, and CREB ($Ser^{133}$), reduced JNK1/2 phosphorylation, and up-regulated the BDNF levels in the hippocampus. The phosphorylation of neither PKC substrates nor $p38^{MAPK}$ was altered under any experimental conditions.

It is well known that antidepressant drugs produce their beneficial effects on mood following chronic, not acute, treatment. The delayed effect elicited by the antidepressant drugs is explained by the fact that a long-term treatment is required to induce an up-regulation of brain neurotrophins, particularly BDNF. The up-regulation of BDNF promotes survival and differentiation, thereby increasing the branching of axons and dendrites and stabilizing synaptic contacts (Lee et al., 2001). Our results are in line with these assumptions because agmatine treatment for 21 days (0.01 and 0.1 mg/kg, p.o.) produced an

antidepressant-like effect in the TST, a predictive animal test that is widely used for screening the antidepressant activity of drugs (Cryan et al., 2005; Steru et al., 1985). This hypothesis is further supported by the positive correlation between immobility time and BDNF results. The TST is based on the observation that animals, after initial escape-oriented movements, develop an immobile posture when placed in an inescapable stressful situation. When antidepressant treatments are given prior to the tests, mice persist actively in an escape-directed behavior for longer periods of time compared with the control group (Cryan et al., 2005). In contrast to previous studies that showed an acute antidepressant-like effect of agmatine in the TST (Neis et al., 2014; Zomkowski et al., 2002), a critical contribution of the present study is to show that agmatine administered sub-chronically by oral route through gavage (0.01 and 0.1 mg/kg) is able to produce an antidepressant-like effect in the TST without causing any tolerance. Similarly, the chronic administration of antidepressant drugs, such as fluoxetine (Hodes et al., 2010) and venlafaxine (Abdel-Wahab and Salama, 2011), reduce the immobility time in the TST.

The possibility that a stressful effect associated with the sub-chronic administration of agmatine by gavage may have caused any interference in the behavioral results is not likely for the following reasons: (i) treatments were performed by professionals with extensive experience in the gavage procedure, and recently performed sub-chronic protocols (Freitas et al., 2013b; Freitas et al., 2013d; Moretti et al., 2012); (ii) all experimental groups were submitted to the same protocol, i.e., received the treatment by oral route for 21 day; (iii) no differences were observed when comparing the behavioral responses in the TST of an unhandled control and a handled control (data not shown). However, the absence of an unhandled control in the biochemical results is a limitation that should be noted.

A growing amount of evidence has implicated intracellular signal transduction pathways in the pathophysiology of depression. One key set of mechanisms involve phosphorylation enzymes such as protein kinase A (PKA) and C (PKC), which, upon activation, phosphorylates the transcription factor CREB (Lonze and Ginty, 2002; Shaywitz and Greenberg, 1999). The present study concurs with these assumptions because the sub-chronic agmatine treatment (0.001-0.1 mg/kg, p.o.) induced a significant phosphorylation of PKA substrates but not PKC. Further supporting this finding, a significant correlation between PKA and both immobility time and CREB results was found. A variety of studies have shown that PKA deficits could be associated with the pathophysiology of depression. A study by Perez et al. (2002) showed that depressed patients have significantly lower PKA levels than normal subjects. In addition, data in post-mortem human brains from depressive subjects indicate that reduced PKA activity may be associated with death by suicide (Dwivedi et al., 2003, 2004; Pandey et al., 2005).

A novel class of antidepressant drugs is based on GSK-3β inhibitors, and efforts have been made to find compounds that are able to modulate this pathway. GSK-3β is a serine/threonine (Ser/Thr) kinase that is widely

distributed in the brain. The GSK-3β pathway is classically involved in the regulation of cellular proliferation, primarily through the activation of transcription factors at the nuclear level (Grimes and Jope, 2001; Jacobs et al., 2012). Several signaling cascades are responsible for controlling the GSK-3β pathway. The Akt pathway is of particular interest regarding this issue because it has been linked to schizophrenia and the mechanism of action of both antipsychotic and antidepressant drugs (Freyberg et al., 2010; Maes et al., 2012; Molteni et al., 2009; Vidal et al., 2011; Wada, 2009). Akt negatively regulates GSK-3β activity through the phosphorylation of $Ser^{9}$ (Cross et al., 1995). Additionally, it is relevant that phosphorylated GSK-3β is able to activate CREB, a critical target of antidepressant drugs (Bullock and Habener, 1988; Grimes and Jope, 2001). The present study concurs with these assumptions because the sub-chronic agmatine treatment (0.001-0.1 mg/kg, p.o.) induced a significant inhibition of GSK-3β ($Ser^{9}$), most likely through Akt activation (Cross et al., 1995). This conclusion derives from the result that shows that the sub-chronic agmatine treatment (0.001-0.1 mg/kg, p.o.) induced a significant phosphorylation of Akt at $Ser^{473}$. Corroborating this data, a significant correlation between GSK-3β phosphorylation and immobility time and Akt or CREB phosphorylation was shown.

Mitogen-activated protein kinases (MAPKs) are evolutionary conserved enzymes connecting cell-surface receptors to critical regulatory cellular targets. One of the most studied MAPK signaling cascades is the ERK1/2 pathway, which plays a critical role in the regulation of cellular processes such as proliferation, differentiation, development, cell cycle and cell survival (Strnisková et al., 2002). A large number of nuclear, cytosolic and structural regulatory proteins can be phosphorylated by ERK, particularly transcription factors such as CREB (Johnson and Lapadat, 2002; Stork and Schmitt, 2002). In addition, PKA is able to activate ERK1/2 through its capacity to interact with the Raf-MEK pathway (Yamaguchi et al., 2003). Several lines of evidence have implicated ERK1/2 signaling in the pathophysiology of mood disorders. Post-mortem studies have shown decreased Raf-ERK1/2 signaling in the brain of suicide subjects (Duric et al., 2010; Dwivedi et al., 2001, 2006, 2009). Regarding pre-clinical findings, a growing amount of studies have shown that the effect of classical antidepressant drugs in animal models is paralleled by increased ERK1/2 phosphorylation (First et al., 2011; Gourley et al., 2008; Qi et al., 2006, 2008). Our results are in line with the literature data because agmatine treatment (0.001-0.1 mg/kg, p.o.) for 21 days was able to produce an antidepressant-like effect accompanied by ERK1/2 activation. Significant correlations between either ERK1 or ERK2 and immobility time or the CREB results were found. Additionally, PKA data correlated with both ERK1 and ERK2, suggesting that the activation of the ERK1/2 pathway could likely be mediated by an alternative mechanism that is dependent on PKA activation.

c-Jun N-terminal kinases (JNK), also known as stress-activated protein kinases, form an important subgroup of the MAPK superfamily. JNK1/2 are primarily activated by various environmental stresses, particularly oxidative

stress (Shen and Liu, 2006). There is a growing amount of evidence showing the importance of JNK activation in cell death mediated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Bubici et al., 2006; Papa et al., 2004, 2006; Ramiro-Cortés and Morán, 2009; Ventura et al., 2004). In addition, several studies have demonstrated that JNK1/2 inhibitors afford neuroprotection (Hu et al., 2012; Nijboer et al., 2013; Ord et al., 2013; Yeste-Velasco et al., 2009). Considering that the pathophysiology of MDD involves neuronal cell death, drugs capable of blocking the JNK1/2 pathway could be helpful as therapy coadjuvants. Our results concur with these findings because sub-chronic agmatine treatment (0.001-0.1 mg/kg, p.o.) reduced the phosphorylation of both JNK1 and JNK2. It is relevant to note that agmatine's ability to maintain redox homeostasis in the hippocampus was previously described by our group (Freitas et al., 2013a). Interestingly, a significant correlation between both JNK1 and JNK2 and immobility time was shown.

$p38^{MAPK}$ was originally identified as a protein that is activated in response to cellular stresses (Matsuzawa and Ichijo, 2008; Munshi and Ramesh, 2013). Studies have shown that blockade of the $p38^{MAPK}$ pathway promotes neuronal cell survival (Vázquez de la Torre, 2013; Zhou et al., 2014). In addition, Chuang (2004) has shown that the mood stabilizer lithium affords neuroprotection by abolishing the $p38^{MAPK}$ activation and CREB inhibition induced by glutamate. Furthermore, Hwang et al. (2008) showed that tricyclic antidepressants are able to inhibit glial inflammatory activation and neurotoxicity through a $p38^{MAPK}$ blockade. Despite the information mentioned above, our results showed that agmatine treatment (0.001-0.1 mg/kg, p.o.) for 21 days had no effect on $p38^{MAPK}$ phosphorylation.

In the present study, we found that cAMP response element-binding protein (CREB) was phosphorylated at $Ser^{133}$ to a significant level and was elicited by the sub-chronic agmatine treatment in all doses tested. CREB and CREB-dependent gene expression have critical roles in response to many signal transduction cascades that are activated by hormones, growth factors, synaptic activity, and other cellular stimuli implicated in neuronal plasticity (Lonze and Ginty, 2002). Sub-chronic, but not acute, antidepressant treatment activates and up-regulates CREB, particularly in the hippocampus (Nibuya et al., 1995, 1996; Vinet et al., 2004; Gumuslu et al., 2013). CREB is activated by phosphorylation at $Ser^{133}$ by kinases such as PKA, PKC, Akt/PKB, ERK, and $Ca^{2+}$/calmodulin-dependent protein kinase (CaMK) II and IV (Johannessen and Moens, 2007; Shaywitz and Greenberg, 1999). The primary gene promoter target induced by CREB is the neurotrophic factor BDNF (Nair and Vaidya, 2006; Tardito et al., 2006), which in turn plays a critical role in cell survival, neuroplasticity, neurogenesis and mood modulation (Gass and Riva, 2007; Pilar-Cuéllar et al., 2013; Masi and Brovedani, 2011). The primary role of BDNF regarding adult neurogenesis is not linked to proliferation but to an increase in cell survival, as described using BDNF and its receptor tropomyosin-related kinase B (TrkB) knock-out animals, which present reduced BDNF expression (Castrén and Rantamäki, 2010; Sairanen et al., 2005). BDNF is implicated in synaptic

plasticity, and proteins such as neuritin that are induced by BDNF are decreased in stress-induced animal models of depression (Son et al., 2012) and increased after chronic antidepressant treatment, contributing to the BDNF antidepressant effect (Larsen et al., 2010; Son et al., 2012). Corroborating these data, the present study showed that sub-chronic agmatine treatment (0.001-0.1 mg/kg, p.o.) up-regulated BDNF in the hippocampus, most likely through CREB activation. This hypothesis is supported by the significant correlation between BDNF and both CREB and immobility time results.

It is important to clarify that the ability of agmatine to modulate the signaling pathways studied in the present study most likely derives from its capability to activate α2-adrenergic and $5HT_3$ receptors, inhibit membrane $Ca^{(2+)}$ channels and block NMDA receptors (Piletz et al., 2013). It is well known that the modulation of such targets is implicated in neuronal survival, neurogenesis and behavioral effects (Castrén and Rantamäki, 2010; Elhwuegi, 2004; Masi and Brovedani, 2011; Neto et al., 2011).

## 5. Conclusion

This study represents novel findings on the mechanisms underlying the antidepressant-like effect of agmatine. Here, we showed for the first time that sub-chronic treatment with agmatine produces an antidepressant-like effect in the TST that is accompanied by a modulation of PKA/Akt/GSK-3β/ERK/JNK/CREB/BDNF but not the PKC and $p38^{MAPK}$ pathways in the hippocampus. The role of each of these pathways deserves further study to determine their direct relation to the antidepressant action of agmatine. However, the involvement of neuroplastic and neuroprotective targets on the sub-chronic antidepressant-like effect of agmatine suggests that it should be further investigated as an adjuvant drug or monotherapy in MDD management.

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**Legends to the figures:**

**Figure 1.** Effect of sub-chronic treatment (21 days) of mice with agmatine (0.001-0.1 mg/kg, p.o.) on the immobility time in the TST (panel A) and locomotor activity in the open-field test (panel B). Each column represents the mean + S.E.M. of 8 animals. Statistical analysis was performed by one-way ANOVA, followed by Duncan's test. $^{**}p<0.01$ compared with the control group (vehicle).

**Figure 2.** Effect of sub-chronic treatment (21 days) of mice with agmatine (0.001-0.1 mg/kg, p.o.) on PKA substrates (panels A and B), PKC substrates (panels C and D), Akt (panels E and F), and GSK-3β (panels G and H) phosphorylation. Panels A, C, E and G show a representative western blot. Quantitative analyses are illustrated in panels B, D, F and H. The data are expressed as the ratio between phosphorylated (p-PKA substrates, p-PKC substrates) and β-actin and as the ratio between phosphorylated (p-Akt, p-GSK-3β) and total (T-Akt, T-GSK-3β) forms of Akt and GSK-3β. Each column represents the mean + S.E.M. of 6 experiments. Statistical analysis was performed by one-way ANOVA, followed by Duncan's test. $^{**}p<0.01$ compared with the control group (vehicle).

**Figure 3.** Effect of sub-chronic treatment (21 days) of mice with agmatine (0.001-0.1 mg/kg, p.o.) on ERK1 (panels A and B), ERK2 (panels A and C), JNK1 (panels D and E), JNK2 (panels D and F) and $p38^{MAPK}$ (panels G and H) phosphorylation. Panels A, D and G show a representative western blot. Quantitative analyses are illustrated in panels B, C, E, F and H. The data are expressed as ratios between phosphorylated (p-ERK1 and p-ERK2) and total (T-ERK1 and T-ERK2) forms, as ratios between phosphorylated (p-JNK1 and p-JNK2) and total (T-JNK1 and T-JNK2) forms, and as a ratio between phosphorylated (p-$p38^{MAPK}$) and total (T-$p38^{MAPK}$) forms of $p38^{MAPK}$. Each column represents the mean + S.E.M. of 6 experiments. Statistical analysis was performed by one-way ANOVA, followed by Duncan's test. $^{*}p<0.05$ and $^{**}p<0.01$ compared with the control group (vehicle).

**Figure 4.** Effect of sub-chronic treatment (21days) of mice with agmatine (0.001-0.1 mg/kg, p.o.) on CREB (panels A and B) phosphorylation and BDNF (panels C and D) immunocontent. Panels A and C show a representative western blot. Quantitative analyses are illustrated in panels B and D. The data are expressed as a ratio between phosphorylated (p-CREB) and total (T-CREB) form of CREB and as a ratio between BDNF content and β-actin. Each column represents the mean + S.E.M. of 6 experiments. Statistical analysis was

performed by two-way ANOVA, followed by Duncan's test. **p*<0.05 and ***p*<0.01 compared with the control group (vehicle).

**Figure 5.** Schematic illustration for the different pathways implicated in the sub-chronic antidepressant-like effect of agmatine. The present study provides evidence that the antidepressant-like effect of agmatine following its sub-chronic administration is dependent, at least in part, on the phosphorylation of PKA, Akt, GSK-3β and ERK1/2, with the subsequent activation of the transcription factor CREB. Upon activation, CREB binds to the DNA sequence cAMP response elements (CRE), thereby inducing transcription of the neurotrophic factor BDNF. Moreover, the present study found that the effect of agmatine is paralleled by an inhibition of JNK1/2 pathway. The modulation of such targets promotes cell survival, neuroplasticity and positives effects on mood. Arrow heads indicate an increase in phosphorylation or transcription, and lines with perpendicular lines indicate inhibition.

**Figure 1.**

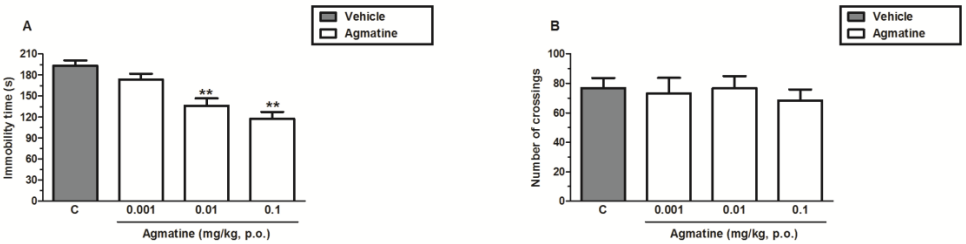
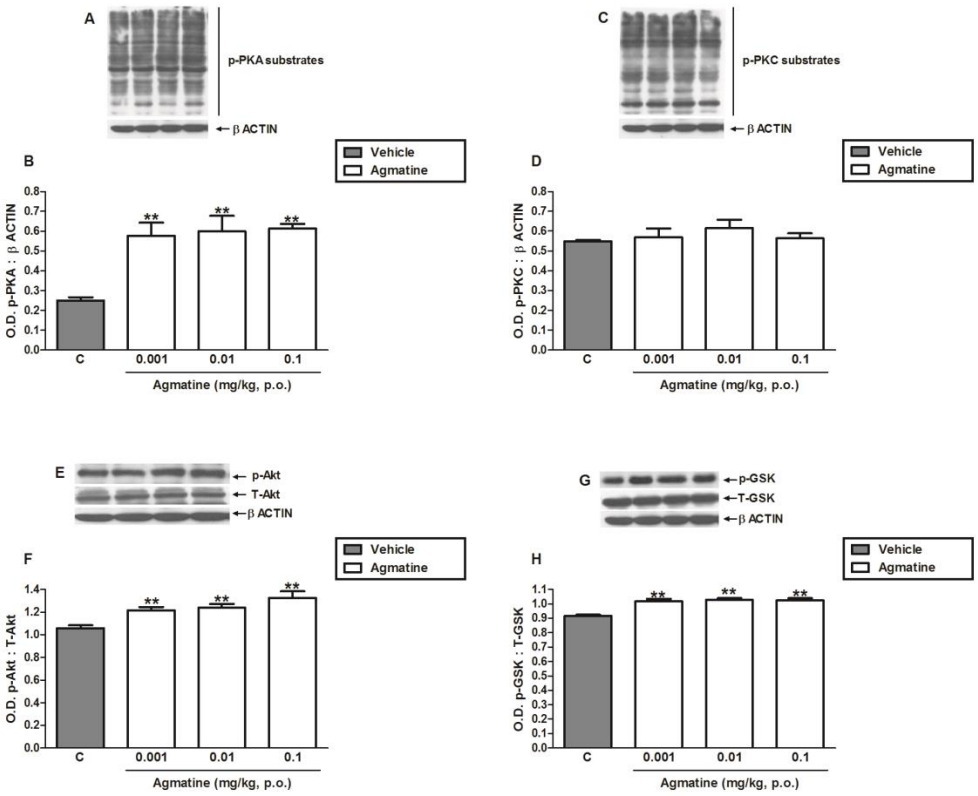


**Figure 2.**


A
p-PKA substrates
β ACTIN
C
p-PKC substrates
β ACTIN
B
Vehicle
Agmatine
O.D. p-PKA : β ACTIN
C
0.001
0.01
0.1
Agmatine (mg/kg, p.o.)
D
Vehicle
Agmatine
O.D. p-PKC : β ACTIN
C
0.001
0.01
0.1
Agmatine (mg/kg, p.o.)
E
p-Akt
T-Akt
β ACTIN
F
Vehicle
Agmatine
O.D. p-Akt : T-Akt
C
0.001
0.01
0.1
Agmatine (mg/kg, p.o.)
G
p-GSK
T-GSK
β ACTIN
H
Vehicle
Agmatine
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C
0.001
0.01
0.1
Agmatine (mg/kg, p.o.)

**Figure 3.**

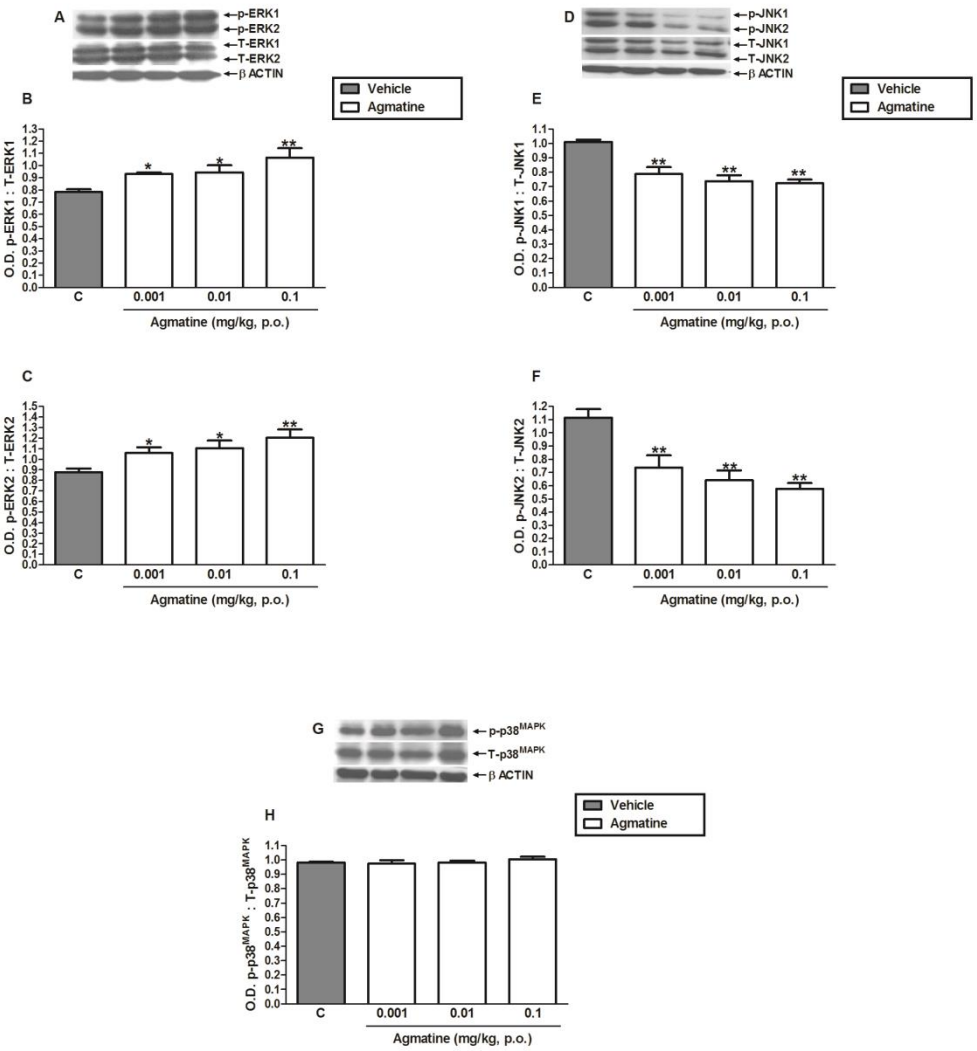
A
p-ERK1
p-ERK2
T-ERK1
T-ERK2
β ACTIN
B
Vehicle
Agmatine
O.D. p-ERK1 : T-ERK1
C
0.001
0.01
0.1
Agmatine (mg/kg, p.o.)
D
p-JNK1
p-JNK2
T-JNK1
T-JNK2
β ACTIN
E
O.D. p-JNK1 : T-JNK1
C
Agmatine
O.D. p-ERK2 : T-ERK2
F
O.D. p-JNK2 : T-JNK2
G
p-p38MAPK
T-p38MAPK
β ACTIN
H
O.D. p-p38MAPK : T-p38MAPK

**Figure 4.**

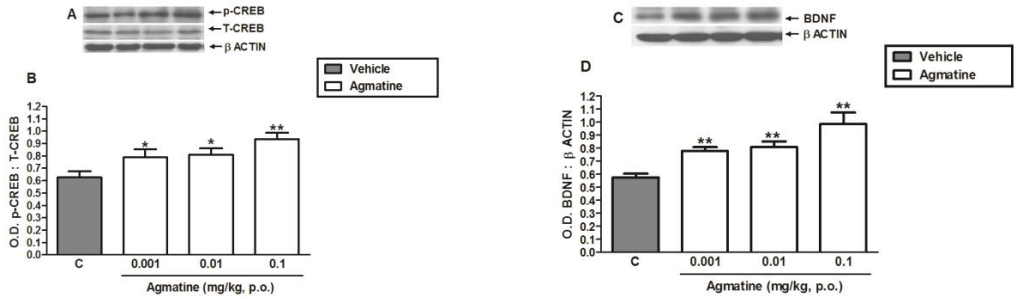


**Figure 5.**

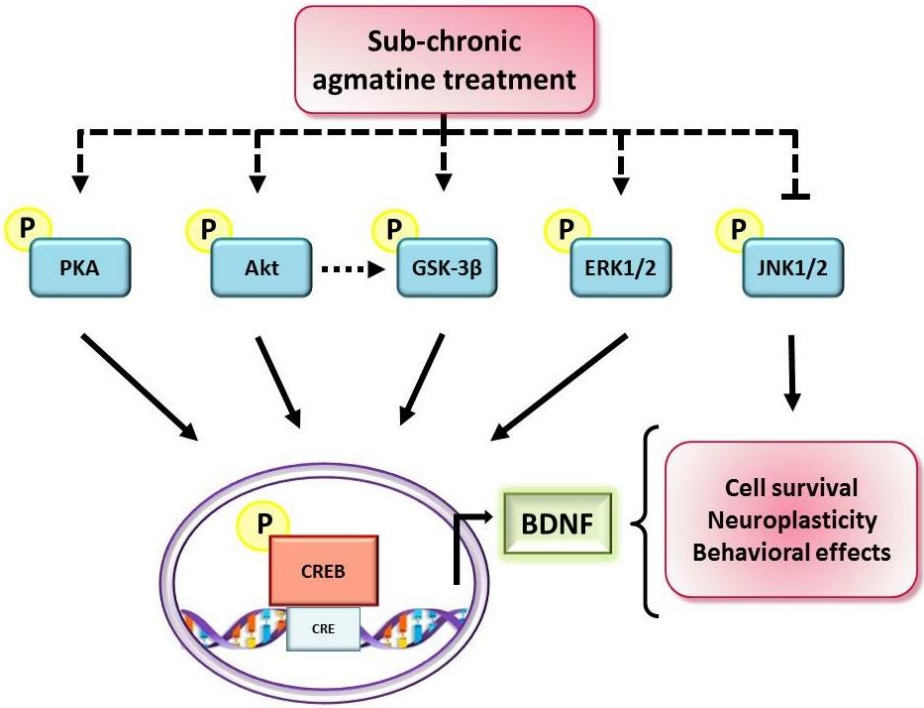

**Table 1.** Pearson's correlation among selected variables.

| ***Measures*** | Immobility time | PKA | Akt | GSK-3β | ERK1 | ERK2 | JNK1 | JNK2 | CREB |
|---|---|---|---|---|---|---|---|---|---|
| PKA | -0.58** | | | | | | | | |
| Akt | -0.47* | 0.44* | | | | | | | |
| GSK-3β | -0.47* | 0.63** | 0.41* | | | | | | |
| ERK1 | -0.67** | 0.55** | 0.67** | 0.29 | | | | | |
| ERK2 | -0.66** | 0.58** | 0.49* | 0.39 | 0.62** | | | | |
| JNK1 | 0.41* | -0.67** | -0.76** | -0.57** | -0.45* | -0.32 | | | |
| JNK2 | 0.43* | -0.62** | -0.79** | -0.54** | -0.45* | -0.28 | 0.92** | | |
| CREB | -0.41* | 0.66** | 0.67** | 0.51* | 0.44* | 0.47* | -0.64** | -0.55** | |
| BDNF | -0.49* | 0.41* | 0.73** | 0.50* | 0.41* | 0.42* | -0.60** | -0.64** | 0.53** |

Significant at *P< 0.05 or **P< 0.01.

# Capítulo II

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## Agmatine abolishes restraint stress-induced depressive-like behavior and hippocampal antioxidant imbalance in mice

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ABSTRACT

Agmatine has been recently emerged as a novel candidate to assist the conventional pharmacotherapy of depression. The acute restraint stress (ARS) is an unavoidable stress situation that may cause depressive-like behavior in rodents. In this study, we investigated the potential antidepressant-like effect of agmatine (10 mg/kg, administered acutely by oral route) in the forced swimming test (FST) in non-stressed mice, as well as its ability to abolish the depressive-like behavior and hippocampal antioxidant imbalance induced by ARS. Agmatine reduced the immobility time in the mouse FST (1–100 mg/kg) in non-stressed mice. ARS caused an increase in the immobility time in the FST, indicative of a depressive-like behavior, as well as hippocampal lipid peroxidation, and an increase in the activity of hippocampal superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activities, reduced catalase (CAT) activity and increased SOD/CAT ratio, an index of pro-oxidative conditions. Agmatine was effective to abolish the depressive-like behavior induced by ARS and to prevent the ARS-induced lipid peroxidation and changes in SOD, GR and CAT activities and in SOD/CAT activity ratio. Hippocampal levels of reduced glutathione (GSH) were not altered by any experimental condition. In conclusion, the present study shows that agmatine was able to abrogate the ARS-induced depressive-like behavior and the associated redox hippocampal imbalance observed in stressed restraint mice, suggesting that its antidepressant-like effect may be dependent on its ability to maintain the pro-/anti-oxidative homeostasis in the hippocampus.



### 1. Introduction

The relationship between onset of a major depressive episode and prior stressful life events has been extensively reported (Hammen et al., 2009; Kessler, 1997; Mazure, 1998). Abnormalities of the hypothalamic pituitary adrenal (HPA) axis have been shown to play a critical role in development of depressive symptoms, persistence of symptoms, and recurrence of depression (Gold et al., 1988; Morris et al., 2012; Plotsky et al., 1998). The progressive HPA abnormalities caused by traumatic stress or chronic stress not only trigger behaviors and emotions related to depression and anxiety, but also might result in prolonged cortisol hypersecretion that can account for neuronal death and hippocampal atrophy observed in depressed individuals (Lee et al., 2002; McKinnon et al., 2009).

The neurotoxic effects of glucocorticoids induced by stress could be partly explained by the inflammatory and neurodegenerative hypothesis of depression, which postulates that oxidative and nitrosative stresses contribute to neurodegenerative processes in depression (Anisman et al., 2002; Lang and Borgwardt, 2013; Maes et al., 2009, 2012). It is well recognized that the brain tissue is particularly vulnerable to oxidative damage, compared with other organs, due to its relatively high content of iron and peroxidizable fatty acids, besides its limited antioxidant capacity (Floyd, 1999; Herbert et al., 1994). There is now ample evidence that ROS produced in the brain are deleterious to neurons since they may overwhelm the brain antioxidant defenses, thereby causing oxidative damage to lipids, proteins and DNA, events that play a key role in neuronal cell death (Forlenza and Miller, 2006; Lang and Borgwardt, 2013; Sarandol et al., 2007).

Several studies have demonstrated that different kinds of stress, including restraint stress, cause an impairment in the antioxidant status in the brain (de Balk et al., 2010; Enache et al., 2008; García-Fernández et al., 2012; Kumar and Goyal, 2008), which could be prevented by antidepressant drugs from distinct classes, namely imipramine and clomipramine (tricyclic antidepressants, TCAs), fluoxetine and venlafaxine (selective serotonin reuptake inhibitors,

*Abbreviations:* ANOVA, analysis of variance; ARS, acute restraint stress; CAT, catalase; FST, forced swimming test; GSSG, glutathione disulfide; GPx, glutathione peroxidase; GR, glutathione reductase; $H^+$, hydrogen ion; $H_2O_2$, hydrogen peroxide; HO•, hydroxyl radical; HPA, hypothalamic pituitary adrenal; MDA, malondialdehyde; NMDA, N-methyl-D-aspartate; $O_2$, molecular oxygen; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NOS, nitric oxide synthase; NPSH, nonprotein thiols; GSH, reduced glutathione; NADPH, reduced nicotinamide adenine dinucleotide phosphate; SSRIs, selective serotonin reuptake inhibitor; $O_2^{\bullet-}$, superoxide anion radical; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TCAs, tricyclic antidepressants; TST, tail suspension test.

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SSRIs), and trazodone (atypical antidepressant) (de Balk et al., 2010; Kumar et al., 2009, 2010; Zafir and Banu, 2007; Zafir et al., 2009). However, most of the clinically available antidepressants have only marginal efficacy compared with placebo (Kirsch, 2009). Therefore, there is a clear need for novel drugs or augmentation therapies to increase the efficacy of antidepressants.

Also known as "decarboxylated arginine", agmatine is an endogenous metabolite situated between the critical pathways of nitric oxide production, polyamine metabolism, and the urea cycle (Reis and Regunathan, 2000). Agmatine has been proposed as a neuromodulator in the central nervous system, considering that it : a) is stored in a large number of neurons with selective distribution in the brain (Otake et al., 1998); b) is released from synaptosomes in a $Ca^{2+}$-dependent manner (Sastre et al., 1997); c) is enzymatically degraded by agmatinase in synaptosomes (Tabor and Tabor, 1984); d) is inactivated by selective reuptake (Sastre et al., 1997); and e) has several molecular targets in the brain, namely $I_1R$, and α2-adrenergic receptors, $5HT_3$ receptors; inhibits membrane $Ca^{(2+)}$ channels, all isoforms of nitric oxide synthase (NOS) in the brain, and blocks N-methyl-D-aspartate (NMDA) receptors (Reis and Regunathan, 1998, 1999, 2000). In addition, it is remarkable to note agmatine's ability to act as a free radical scavenger protecting from oxidative stress-induced mitochondrial swelling, membrane potential collapse, NF-κB translocation, and apoptosis (Arndt et al., 2009; Battaglia et al., 2010; Condello et al., 2011). In addition, agmatine has been emerging as a putative alternative therapeutic tool that could help the conventional pharmacotherapy of depression. Previous studies by our group have demonstrated that it is able to produce an antidepressant-like effect in the mouse forced swimming test (FST) and in the tail suspension test (TST), which was paralleled by modulation of monoaminergic and opioid systems, NMDA receptors and the L-arginine–NO pathway (Zomkowski et al., 2002, 2004, 2005). Of note, a recent study by Shopsin (2013) showed a clinical antidepressant effect of agmatine in depressed subjects. However, there is no study reporting the ability of this neuromodulator to abolish depressive-like behavior induced by a model that reproduces symptoms of depression accompanied by an oxidative imbalance. Therefore, the present study was aiming at investigating the ability of agmatine to abrogate alterations in behavior and in the hippocampal cellular redox status induced by acute restraint stress (ARS).

## 2. Materials and methods

### 2.1. Animals

Female Swiss mice (3 months old, 40–45 g) were maintained at constant room temperature (20–22 °C) with free access to water and food, under a 12:12 h light:dark cycle (lights on at 07:00 h). The cages were placed in the experimental room 24 h before the test for acclimatization. All manipulations were carried out between 9:00 and 17:00 h, with each animal used only once. The procedures in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the local Ethics Committee. All efforts were made to minimize animal suffering and the number of animals used in the experiments.

### 2.2. Drugs and treatment

Agmatine (Sigma Chemical Co., St. Louis, U.S.A.) was dissolved in distilled water and was administered by oral route (p.o.) by gavage at doses of 1–100 mg/kg. The dissolution of agmatine was freshly done immediately before its administration. A control group received distilled water as vehicle.

In the experiments designed to study the antidepressant-like effect of agmatine in the FST, the immobility time in the FST and the locomotor activity in the open-field were assessed in independent groups of mice 60 min after an acute administration of agmatine by gavage (1–100 mg/kg, p.o.). This experiment was carried out in order to choose the dose of agmatine to be used in subsequent experiments that investigated the ability of agmatine to abolish the depressive-like behavior and hippocampal redox impairment induced by ARS.

In the ARS protocol, agmatine (10 mg/kg, p.o.) was administered 1 h before the ARS. The animals were assigned to the following groups: (a) unstressed/vehicle, (b) unstressed/agmatine, as the control groups; (c) ARS/vehicle and (d) ARS/agmatine. Number of mice per group was 7–8. A diagram of all experimental schedules is given in Fig. 1.

### 2.3. Acute restraint stress procedure

After 1 h of the treatment, ARS procedure was performed by a method described previously (Kumar and Goyal, 2008; Poleszak et al., 2006; Zafir et al., 2009) and standardized in our laboratory (Budni et al., 2013; Moretti et al., 2013). The animals were maintained in their home cages with free access to water and food in the period (1 h) that elapsed between the treatment and ARS procedure. The immobilization was applied for a period of 7 h using an individual rodent restraint device made of Plexiglas fenestrate. This restrained all physical movements without causing pain. The animals were deprived of food and water during the entire period of exposure to stress. The unstressed-groups were treated with vehicle or agmatine and were kept without food and water during the entire period of exposure to stress. Forty min after ARS, the animals were released from their enclosure, submitted to the behavioral observations, and then to the neurochemical analysis.

### 2.4. Behavioral tests

#### 2.4.1. Open-field test

To assess the effects of agmatine on locomotor activity, mice were evaluated in the open-field paradigm as previously described by Rodrigues et al. (1996). The number of squares crossed with all paws (crossings) was counted in a 6 min session. The apparatus was cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

#### 2.4.2. Forced swimming test (FST)

Mice were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 25 ± 1 °C; the total amount of time each animal remained immobile during a 6-min session was recorded (in seconds) as immobility time, as described previously (Brocardo et al., 2008; Freitas et al., 2010). Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. A decrease in the duration of immobility is indicative of an antidepressant-like effect (Porsolt et al., 1977).


**Fig. 1.** Diagram of all experimental schedules. Agmatine at dose of 10 mg/kg (p.o.) was administered 1 h before the ARS procedure. The animals were submitted to immobilization for a period of 7 h. 40 min post-release from their enclosure, independent group of animals was submitted to the open-field, or FST, or hippocampi tissue was rapidly dissected and prepared for biochemical analysis.

*2.5. Biochemical analysis*

Considering that: a) the glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) are key endogenous antioxidant enzymes, which inhibit the formation of ROS and/or promote the removal of free radicals and their precursors (Bilici et al., 2001; Del Río et al., 1992; Floyd, 1999; Halliwell, 2007; Kodydková et al., 2009); b) impairments in these detoxification system have been implicated in the etiology of depression (Anisman et al., 2002; Lang and Borgwardt, 2013; Leonard and Maes, 2012; Maes et al., 2009); c) lipid peroxidation is one of the major consequences of free-radical-mediated injury to the brain (Halliwell and Gutteridge, 1999; Niki, 2012), and could be estimated by TBARS levels, the present study assessed these neurochemical targets.

*2.5.1. Tissue preparation*

After the behavioral observations, mice were decapitated and hippocampus was rapidly dissected on ice and homogenized (1:10 w/v) in HEPES buffer (20 mM, pH 7.0). The tissue homogenates were centrifuged at 16,000 ×*g*, at 4 °C for 20 min and the supernatants obtained were used for the determination of enzymatic activities and for the quantification of the levels of glutathione (GSH) and thiobarbituric acid reactive substances (TBARS). The protein content was quantified according to the method described by Lowry et al. (1951), using bovine serum albumin as a standard.

*2.5.2. Activity of antioxidant enzymes*

Hippocampal glutathione reductase (GR) activity was measured using an NADPH reduction assay following the protocol developed by Carlberg and Mannervik (1985) using glutathione disulfide (GSSG) as substrate. GR activity was monitored by decreases in NADPH absorbance at 340 nm at 37 °C in a TECAN Genios Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). Results were based on a molar extinction coefficient for NADPH of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$.

Hippocampal glutathione peroxidase (GPx) activity was measured using an NADPH reduction assay following the technique of Wendel (1981). Tissue supernatant (around 200 μg protein) was added to a reaction mixture containing GSH, GR and NADPH in phosphate buffer (pH 7.4). The reaction was initiated by adding *tert*-butyl hydroperoxide, and the absorbance decrease at 340 nm was recorded at 37 °C in a TECAN Genios Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). The activity in the absence of the samples was subtracted. Results were based on a molar extinction coefficient for NADPH of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$. Both GR and GPx activities were expressed as nmol NADPH oxidized/min/mg protein.

Catalase (CAT) activity was measured by the method of Aebi (1984). The reaction was started by the addition of freshly prepared 30 mM $H_2O_2$. The rate of $H_2O_2$ decomposition by catalase was measured spectrophotometrically at 240 nm and the enzyme activity was expressed as μmol $H_2O_2$ consumed/min/mg protein.

Superoxide dismutase (SOD) activity was assayed spectrophotometrically as described by Misra and Fridovich (1972). The spectrophotometer used for these assays was a TECAN Genios Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). This method is based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50%. The SOD enzymatic activity was expressed as units (U)/mg protein.

*2.5.3. Assessment of glutathione (GSH) content*

GSH content was measured as nonprotein thiols (NPSH) according to a method previously described (Ellman, 1959). Briefly, after the incubation, trichloroacetic acid 10% was added to the reaction medium. After centrifugation (5000 ×*g* at 4 °C for 10 min), the protein pellet was discarded and free thiol groups were determined in the clear supernatant (which was neutralized with 0.1 M NaOH). Absorbance was read in 405 nm, using GSH as standard. The values are expressed as nmol NPSH/mg protein.

*2.5.4. Determination of thiobarbituric acid reactive substance (TBARS) levels*

TBARS levels were determined in the hippocampal homogenates using the method described by Ohkawa et al. (1979), in which malondialdehyde (MDA), an end-product of lipid peroxidation, reacts with thiobarbituric acid to form a colored complex.

The samples were incubated at 100 °C for 60 min in acid medium containing 0.45% sodium dodecyl sulfate and 0.67% thiobarbituric acid. After centrifugation, the reaction product was determined at 532 nm using MDA as standard.

*2.5.5. Statistical analysis*

Comparisons between experimental and control groups were performed by one-way or two-way ANOVA followed by Duncan's multiple range test, when appropriate. Pearson's correlation analysis was performed to investigate any possible relationship between behavioral and neurochemical data. $P < 0.05$ was considered significant.

## 3. Results

*3.1. Effect of an acute treatment with agmatine on the immobility time in the FST and locomotor activity in the open-field test*

The results depicted in Fig. 2A show that agmatine given by oral route decreased the immobility time in the FST, a behavioral profile characteristic of an antidepressant-like effect. One-way ANOVA revealed a significant effect of agmatine [$F(3,24) = 8.72$, $P < 0.01$]. Post hoc analysis indicated a significant decrease in the immobility time elicited by agmatine at doses of 1, 10 and 100 mg/kg ($P < 0.01$). Fig. 2B shows that the administration of agmatine (dose range 1–100 mg/kg, p.o.) produced no effect in the locomotor activity in the open-field test [$F(3,24) = 0.50$, $P = 0.69$].

*3.2. Agmatine abolished the depressive-like behavior induced by ARS*

Fig. 3A shows that ARS caused a significant increase in the immobility time, which is in agreement with its ability to induce depressive-like behavior. The acute administration of agmatine (10 mg/kg, p.o.) was able to prevent the depressive-like behavior induced by ARS in the FST. Agmatine administration, in control mice, also significantly decreased the immobility time in the FST, as compared to unstressed mice treated with vehicle. A two-way ANOVA revealed significant effects of ARS [$F(1,28) = 3.92$, $P < 0.05$], treatment [$F(1,28) = 49.06$, $P < 0.01$] and ARS × treatment interaction [$F(1,28) = 6.84$, $P < 0.01$]. Post hoc analyses indicated that agmatine treatment to stressed mice prevented the depressive-like behavior caused by ARS ($P < 0.01$). Fig. 3B shows that the administration of agmatine produced no effect in the open-field test, indicating that a locomotor effect does not account for the antidepressant-like effect observed in the FST. The two-way ANOVA revealed no significant differences of ARS [$F(1,28) = 0.10$, $P = 0.75$], treatment [$F(1,28) = 0.49$, $P = 0.48$], and ARS × treatment interaction [$F(1,28) = 0.002$, $P = 0.96$].

*3.3. Agmatine abolished the ARS-induced oxidative imbalance*

The results depicted in Fig. 4 illustrate that ARS significantly increased the hippocampal levels of TBARS (an indicative of lipid peroxidation) and that this event was significantly blocked by agmatine (10 mg/kg, p.o.) treatment; however no changes were observed in the unstressed animals. The two-way ANOVA revealed significant differences of ARS [$F(1,28) = 4.34$, $P < 0.05$], treatment [$F(1,28) = 6.10$, $P < 0.01$] and ARS × treatment interaction [$F(1,28) = 7.84$,

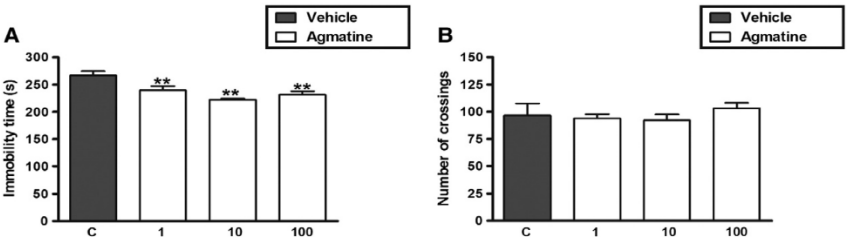


**Fig. 2.** Effect of an acute administration by oral route (p.o.) of agmatine (dose range 1–100 mg/kg, p.o.) in the FST (panel A) and open-field test (panel B). Each column represents the mean + S.E.M. (n = 7–8). Statistical analysis was performed by one-way ANOVA, followed by Duncan's test. $^{**}P < 0.01$ as compared with the vehicle-treated group (C).

$P < 0.01$]. Post hoc analyses indicated that agmatine treatment abolished the increase in hippocampal TBARS levels caused by ARS ($P < 0.01$).

Fig. 5 shows that neither ARS nor agmatine treatment altered hippocampal GSH (the most important non-enzymatic endogenous antioxidant) levels. A two-way ANOVA revealed no significant differences of ARS [$F(1,28) = 1.91$, $P = 0.18$], treatment [$F(1,28) = 0.20$, $P = 0.66$] and ARS × treatment interaction [$F(1,28) = 0.38$, $P = 0.54$] for GSH levels.

As depicted in Fig. 6A, ARS caused an increase on SOD (a class of enzymes that catalyzes the reduction of superoxide to hydrogen peroxide) activity and this effect was abrogated by agmatine (10 mg/kg, p.o.) treatment, however no changes were observed in the unstressed animals. The two-way ANOVA revealed significant differences of ARS [$F(1,28) = 8.64$, $P < 0.01$], treatment [$F(1,28) = 15.84$, $P < 0.01$] and ARS × treatment interaction [$F(1,28) = 4.97$, $P < 0.05$]. Also, stressed mice presented a decreased CAT (a relevant endogenous antioxidant enzyme responsible for hydrogen peroxide detoxification) activity, which was prevented by the treatment with agmatine (Fig. 6B), without causing significant effects in the unstressed animals. A two-way ANOVA revealed significant effects of ARS [$F(1,24) = 9.90$, $P < 0.01$] and ARS × treatment interaction [$F(1,24) = 4.96$, $P < 0.05$], but no significant main effect of treatment [$F(1,24) = 2.90$, $P = 0.10$]. Post hoc analyses indicated that agmatine treatment abolished the alteration in CAT and SOD activities induced by ARS ($P < 0.01$). Of note, ARS caused a huge increase in the hippocampal SOD/CAT ratio (Fig. 6C), which has been pointed as an index of pro-oxidative conditions (Halliwell, 2007). Agmatine prevented the ARS effects toward hippocampal SOD/CAT ratio (Fig. 6C). The two-way ANOVA revealed significant differences of ARS [$F(1,28) = 16.00$, $P < 0.01$], treatment [$F(1,28) = 19.40$, $P < 0.01$] and ARS × treatment interaction [$F(1,28) = 12.21$, $P < 0.01$]. Post hoc analyses indicated that agmatine treatment abolished the increase in hippocampal SOD/CAT ratio caused by ARS ($P < 0.01$).

Finally, the effect of the treatment of ARS-mice with agmatine on GPx and GR activities (important endogenous antioxidant enzymes) was verified. ARS, but not vehicle or agmatine in unstressed mice, produced a slight increase in GPx activity (Fig. 7A). The two-way ANOVA revealed a significant effect of ARS [$F(1,28) = 6.19$, $P < 0.05$], but no significant main effects of treatment [$F(1,28) = 0.004$, $P = 0.94$] and ARS × treatment interaction [$F(1,28) = 0.72$, $P = 0.40$]. Fig. 7B shows that the treatment of ARS-mice with agmatine (10 mg/kg, p.o.) was able to prevent the increase on GR activity caused by the stress procedure. It is remarkable that agmatine treatment did not induce these changes in unstressed animals. The two-way ANOVA revealed significant differences of ARS [$F(1,28) = 9.81$, $P < 0.01$], treatment [$F(1,28) = 6.44$, $P < 0.01$] and ARS × treatment interaction [$F(1,28) = 4.17$, $P < 0.05$]. Post hoc analyses indicated that agmatine treatment abolished the hippocampal increase on antioxidant activities of GPX and GR caused by ARS ($P < 0.01$).

Worth of note is the positive Pearson's correlation (Table 1) among immobility time and both TBARS ($P < 0.05$), GR ($P < 0.05$), and SOD ($P < 0.01$). Also, a negative correlation was found between immobility time and CAT ($P < 0.01$). It is interesting to note that a significant correlation was found between TBARS and GR ($P < 0.05$), and between GSH with either GR ($P < 0.05$) or GPx ($P < 0.05$). A negative correlation was

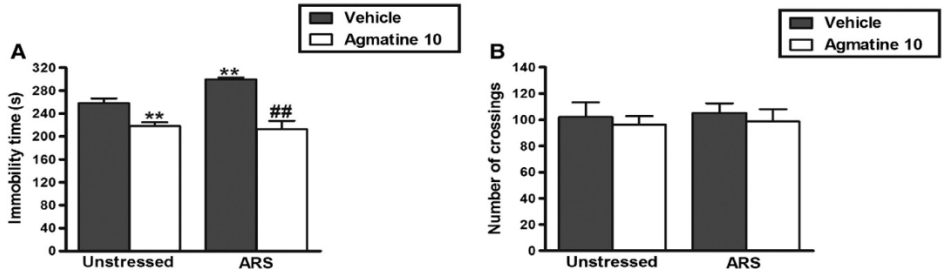


**Fig. 3.** Effect of the treatment of ARS-mice with agmatine (10 mg/kg, p.o.) in the FST (panel A) and open-field test (panel B). Each column represents the mean + S.E.M. (n = 7–8). Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test. $^{**}P < 0.01$ as compared with the control group (unstressed-vehicle); $^{\#\#}P < 0.01$ as compared with the ARS-vehicle group.

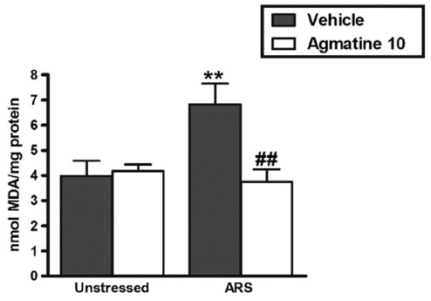


**Fig. 4.** Effect of the treatment of ARS-mice with agmatine (10 mg/kg, p.o.) on thiobarbituric acid reactive substances (TBARS) in the hippocampus. Each column represents the mean + S.E.M. (n = 7–8). Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test. $^{**}P < 0.01$ as compared with the control group (unstressed-vehicle); $^{\#\#}P < 0.01$ as compared with the ARS-vehicle group.

shown between GR and CAT ($P < 0.05$). Finally, GPx correlates with SOD ($P < 0.05$). Number of crossings did not correlate with any variable studied (data not shown).

## 4. Discussion

Agmatine has been proposed as a novel neuromodulator in the central nervous system and is emerging as a novel candidate to assist the conventional pharmacotherapy of depression. Previous findings from our group have shown that agmatine administration by intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) routes produces antidepressant-like effect in the FST and TST (Zomkowski et al., 2002, 2004, 2005). Nevertheless, in the present study we demonstrated that an acute agmatine administration by oral route (p.o.) produced a significant antidepressant-like effect in the FST, a commonly used behavioral test that predicts the efficacy of antidepressant treatment (Cryan and Holmes, 2005; Porsolt et al., 1977). Additionally, a consistent antidepressant-like activity of agmatine in mice submitted to the acute restrain stress, a procedure that has been extensively shown to cause behavioral alterations indicative of depressive-like behavior (Kumar and Goyal, 2008; Poleszak et al., 2006; Zafir et al., 2009), was shown. The previous studies reporting antidepressant-like effects of agmatine did not use animal models of depression. In this regard, the results presented herein, which evince the agmatine's ability to produce an antidepressant-like effect in a model of depression

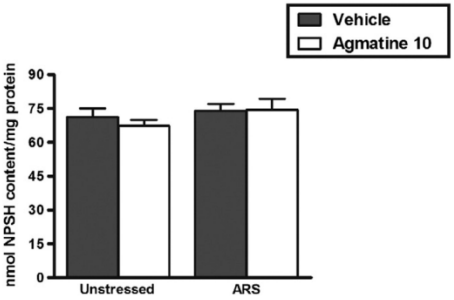


**Fig. 5.** Effect of the treatment of ARS-mice with agmatine (10 mg/kg, p.o.) on glutathione levels in the hippocampus. Each column represents the mean + S.E.M. (n = 7–8). Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test.

associated with an oxidative imbalance, represent a novelty on the theme of antidepressant strategies.

The ARS has been proposed as a model of depression induced by stress, which combines both emotional and physical components in addition to affect the brain's intra-cellular redox status (Buynitsky and Mostofsky, 2009; Glavin et al., 1994; Paré and Glavin, 1986). The main improvement of using restraint is that it produces an inescapable physical and mental stress which is not accompanied with a conditioned response (Jaggi et al., 2011). It is relevant to clarify that this study was performed in female mice because women are more susceptible to development of depressive disorder followed to lifetime stress events than men (Mazure and Maciejewski, 2003). Indeed, the ARS model of depression standardized previously in our laboratory (Budni et al., 2013; Moretti et al., 2013) was able to cause depressive-like behavior associated with impairment in the in vivo antioxidant defenses, providing validation for the model. Our results are in line with this assumption, since ARS caused emotional stress, evidenced by the depressive-related behavior in the FST observed in animals submitted to this protocol. Of note, agmatine administration by oral route (p.o.) was able to prevent this behavioral alteration, reinforcing the antidepressant-like effect of this neuromodulator.

It is well-known that the central nervous system is extremely sensitive to peroxidative damage due to its rich content of oxidizable substrates, high oxygen tension and low antioxidant capacity (Metodiewa and Koška, 2000; Zafir et al., 2009). Several studies have shown that stressful life events are associated with brain oxidative damage as a consequence of an increase in the production of ROS, such as superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), and hydrogen peroxide ($H_2O_2$) (Anisman et al., 2002; Leonard and Maes, 2012; Maes et al., 2009). The endogenous detoxification system involves the activity of several antioxidant enzymes. SOD, is the first line of defense against ROS and catalyzes the dismutation of superoxide anion radical into hydrogen peroxide (Singh, 1982). The $H_2O_2$ produced from $O2^{\bullet -}$ can be reduced to water and molecular oxygen by either catalase (CAT) (Del Río et al., 1992) or glutathione peroxidase (GPx) (Brigelius-Flohé, 1999). The $H_2O_2$ detoxification mediated by GPx expends reduced glutathione (GSH), which in turn is oxidized producing glutathione disulfide (GSSG) (Flohé, 1971). The GSSG produced in GPx mediated reaction can be restored to GSH through a reaction catalyzed by glutathione reductase (GR), at the expense of the reducing equivalents from NADPH (Kirsch and De Groot, 2001).

Lipid peroxidation is considered a critical mechanism of injury occurring in cells during oxidative stress (Halliwell and Gutteridge, 1999; Niki, 2012). An initial formation of large amounts of ROS during stress may also initiate lipid peroxidation as demonstrated to occur in brain (Hayashi et al., 2012). Emotional stress, which accompanies severe depression, may enhance lipid peroxidation (Goncharova et al., 2008) and clinical studies have directly demonstrated higher levels of MDA—an end-product of lipid peroxidation, in patients with affective disorders (Lang and Borgwardt, 2013; Ozcan et al., 2004). In addition, several studies have demonstrated that the restrained stress significantly elevated lipid peroxidation level in the hippocampus of rats (Abidin et al., 2004; Atif et al., 2008; de Balk et al., 2010; Fontella et al., 2005) and mice (Budni et al., 2013; García-Fernández et al., 2012; Moretti et al., 2013; Perez Nievas et al., 2011). In line with this, our results show that the ARS procedure caused a significant lipid peroxidation, as evidenced by increased amount of TBARS levels in ARS-mice, which was abolished by agmatine treatment. Thus, the beneficial effects of agmatine on behavior could be associated with its capacity to prevent the lipid peroxidative damage caused by immobilization stress. This hypothesis is supported by the positive correlation among immobility time and TBARS results.

The glutathione (GSH) system is an important tool mediating protection against several (pro)-oxidant molecules in the brain (Dringen and Hirrlinger, 2003) and alterations is this system are involved in several neuropathological conditions (Lovell et al., 1998). A

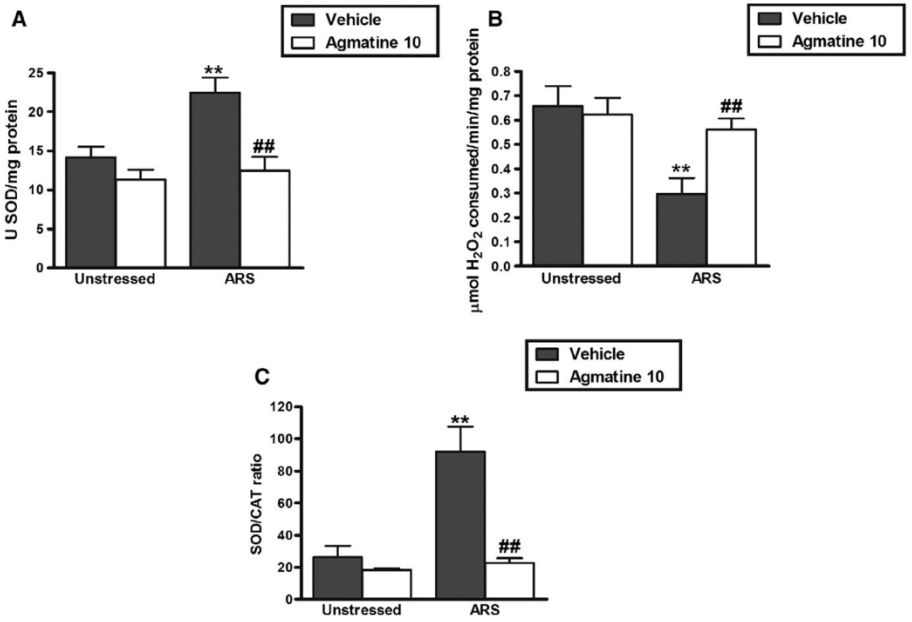


**Fig. 6.** Effect of the treatment of ARS-mice with agmatine (10 mg/kg, p.o.) on SOD activity (panel A), on CAT activity (panel B) and on SOD/CAT ratio in the hippocampus (panel C). Each column represents the mean + S.E.M. (n = 7–8). Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test. $^{**}P < 0.01$ as compared with the control group (unstressed-vehicle); $^{\#\#}P < 0.01$ as compared with the ARS-vehicle group.

recent study by Rosa et al. (2013) indicated that the central administration of GSH elicits an antidepressant-like response in the FST and TST in mice. Recent studies performed by Budni et al. (2013), Moretti et al. (2013) and Méndez-Cuesta et al. (2011), however, reported that restraint stress in rodents did not affect GSH levels in the brain. The present study is in agreement with these findings, since neither agmatine nor ARS caused alterations in GSH levels in the hippocampus of mice, suggesting that this molecular target is not directly underlying the antidepressant effect of agmatine in ARS-mice. Nevertheless, considering that a positive correlation among GSH results and both GR and GPx was shown, it could be speculated that GSH is somehow involved in the maintenance of pro/anti-oxidative homeostasis of ARS-mice hippocampus.

In the present study, we identified a significant imbalance in SOD, GPx, GR and CAT hippocampal activities after ARS procedure. Indeed, Sarandol et al. (2007) have demonstrated that patients suffering from

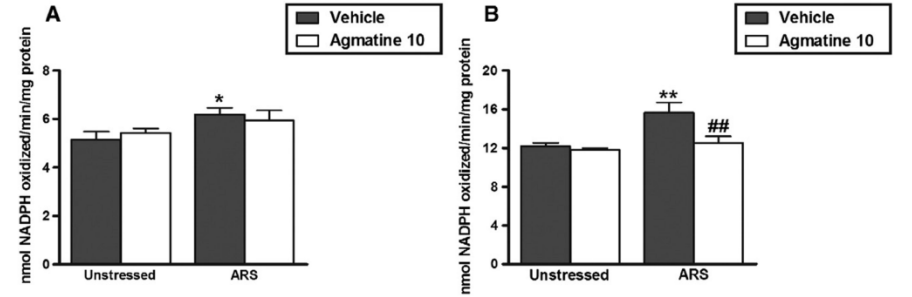


**Fig. 7.** Effect of the treatment of ARS-mice with agmatine (10 mg/kg, p.o.) on GPx activity (panel A) and on GR activity (panel B) in the hippocampus. Each column represents the mean + S.E.M. (n = 7–8). Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the control group (unstressed-vehicle); $^{\#\#}P < 0.01$ as compared with the ARS-vehicle group.

**Table 1**
Pearson's correlation among selected variables.

| Measures | Immobility time | TBARS | GSH | GR | GPx |
|---|---|---|---|---|---|
| TBARS | 0.43* | | | | |
| GSH | 0.03 | 0.11 | | | |
| GR | 0.40* | 0.45** | 0.37* | | |
| GPx | −0.02 | 0.21 | 0.40* | 0.30 | |
| CAT | −0.45** | −0.33 | −0.23 | −0.36* | −0.16 |
| SOD | 0.50** | 0.29 | 0.17 | 0.30 | 0.35* |

Significant at $^{*}P < 0.05$ or $^{**}P < 0.01$.

major depressive disorder (MDD) presented higher MDA levels and SOD activity, as compared to the control group. In addition, this study found a significant positive correlation among the severity of the disease and SOD activity. Similarly, Kotan et al. (2011) demonstrated that patients who fully met the fourth Diagnostic and Statistical Manual of Mental Disorder criteria for MDD presented increased levels of MDA and SOD activity. Furthermore, it was also demonstrated that MDD patients presented higher levels of plasma GR and MDA levels, and erythrocyte GPx and SOD than those of control (Bilici et al., 2001). Increased GR activity was further found in depressive women (Kodydková et al., 2009). Finally, Ozcan et al. (2004) have shown a lower CAT activity in MDD patients. The present results concur with these clinical evidence since the ARS-mice presented a depressive-like behavior accompanied by increased lipid peroxidation, SOD, GPx, GR activities and a lower activity of CAT in the hippocampus. Considering the pre-clinical studies, several authors have shown similar increased lipid peroxidation, SOD, GPx, and GR activities induced by immobilization stress in the hippocampus (Budni et al., 2013; de Balk et al., 2010; Fontella et al., 2005; García-Fernández et al., 2012; Moretti et al., 2013). Additionally, a lower activity of brain CAT caused by immobilization stress was also shown (Akpinar et al., 2008; Derin et al., 2006; Enache et al., 2008; Kumar et al., 2009). Further supporting this notion, a positive correlation was found among immobility time and both TBARS, GR, and SOD. In addition, TBARS correlates with GR, and GSH with both GR and GPx. Besides, a significant correlation between SOD and GPx was found. Finally, CAT correlates negatively with both GR and immobility time. Altogether, these results indicate a potential relationship among the several oxidative stress-related parameters in the hippocampus of animals subjected to ARS. Of note, ARS caused a significant increase in the hippocampal SOD/CAT ratio, which has been pointed as an index of pro-oxidative conditions; it favors the higher levels of hydrogen peroxide because SOD converts superoxide anion to hydrogen peroxide, but CAT is not able to metabolize hydrogen peroxide to water (Halliwell, 2007). In this context, the significant increase in hippocampal GR activity in ARS group might represent a potential secondary response to prevent ARS-induced pro-oxidative damage, as already observed in other studies (Budni et al., 2013; Farina et al., 2005). However, this response was not enough to prevent hippocampal oxidative damage, evidenced by the increased lipid peroxidation rate in animals from the ARS group. Of note, agmatine, which abolished ARS-induced changes on CAT and SOD activities, also prevented the increases in GR activity and lipid peroxidation, strongly reinforcing the potential relationship among the evaluated biochemical parameters.

## 5. Conclusion

The present study extends literature data by indicating some possible mechanisms underlying the antidepressant-like effect of agmatine in a model of depression induced by restraint stress. ARS induced a significant lipid peroxidation and alterations in the activity of the enzymes SOD, GPx, GR, CAT and in the SOD/CAT ratio in the hippocampus, indicating an oxidative imbalance. Furthermore, agmatine administered acutely by oral route produced a significant antidepressant-like effect in the FST and was effective to prevent the behavioral alterations induced by ARS. These effects of agmatine seem to be associated with a modulation of TBARS, SOD, GR, and CAT, but not directly with GSH and GPx. The role of each of these targets deserves further studies to determine their direct relation to the antidepressant action of agmatine. Nevertheless, the present study delves into one of the pleiotropic effects of agmatine by highlighting that its antidepressant-like effect in the ARS model of depression is, at least in part, accompanied by its capability of maintaining pro-/anti-oxidant homeostasis in the hippocampus.

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# Capítulo III

## Agmatine induces Nrf2 and protects against corticosterone effects in hippocampal neuronal cell line

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**Abbreviations:** 7-AAD, 7-aminoactinomycin D; ANOVA, analysis of variance; ARE, antioxidant response element; ERK, extracellular signal-regulated kinase; GCLc, glutamate cysteine ligase, catalytic subunit; GSTA2, glutathione S-transferase α2; HO-1, heme oxygenase-1; HPA, hypothalamic-pituitary-adrenal; JNK, c-Jun NH 2-terminal kinase; Keap1, Kelch-like-ECH-associated protein 1; NQO1, NAD(P)H:quinone oxidoreductase; Nrf2, (erythroid 2-derived)-like 2; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant.

**Abstract**

Hyperactivation of the hypothalamic-pituitary-adrenal axis is a common finding in major depression; this may lead to increased levels of cortisol, that are known to cause oxidative stress imbalance and apoptotic neuronal cell death, particularly in the hippocampus, a key region implicated in mood regulation. Agmatine, an endogenous metabolite of L-arginine, has been proposed for the treatment of major depression. Corticosterone induced apoptotic cell death and increased ROS production in cultured hippocampal neuronal cells, effects that were abolished in a concentration- and time-dependent manner by agmatine. Interestingly, the combination of sub-effective concentrations of agmatine with fluoxetine or imipramine afforded synergic protection. The neuroprotective effect of agmatine was abolished by yohimbine ($\alpha_2$-adrenoceptor antagonist), ketanserin (5-$HT_{2A}$ receptor antagonist), LY294002 (PI3K inhibitor), PD98059 (MEK1/2 inhibitor), SnPP (HO-1 inhibitor), and cycloheximide (protein synthesis inhibitor). Agmatine increased Akt and ERK phosphorylation, and induced the transcription factor Nrf2 and the proteins HO-1 and GCLc; induction of these proteins was prevented by yohimbine, ketanserin, LY294002 and PD98059. In conclusion, agmatine affords neuroprotection against corticosterone effects by a mechanism that implicates Nrf2 induction via $\alpha_2$-adrenergic and 5-$HT_{2A}$ receptors, Akt and ERK pathways, and HO-1 and GCLc expression.

**Keywords:** agmatine; neuroprotection; monoamines; Akt; ERK; Nrf2; antioxidant; HT22 cells, hemoxygenase-1.

## Introduction

Major depressive disorder is a chronic, recurrent, and potentially life-threatening mental condition. It is the leading cause of disability worldwide and the third contributor to the worldwide burden of disease; by 2030, it is expected to be the main contributor [1]. In the USA, the annual cost of depression is estimated at $ 104 billion [2], whereas in Europe it is estimated to be over € 120 billion [3]. Studies over the last 40 years have demonstrated that hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is one of the most consistent biological findings in major depression [4-7]. The HPA axis is a multifaceted regulatory system that integrates neuronal and endocrine function, and coordinates the production of the stress hormone cortisol in the adrenal glands. Cortisol (humans) and corticosterone (rodents) are glucocorticoids, so called because they alter the function of numerous tissues in order to mobilize or store energy to meet the demands when confronting a stressful situation [8, 9]. Nevertheless, overexposure to cortisol or corticosterone during prolonged periods of stress is harmful to the brain by inducing apoptotic neuronal cell death, particularly in the hippocampus – a key brain region for mood regulation [5, 6, 10]. The mechanisms underlying the glucocorticoid-mediated neuronal cell death are explained by the inflammatory and neurodegenerative hypothesis of depression which postulates that the hypersecretion of glucocorticoids induces an oxidative and inflammatory status in the brain accompanied by decreased antioxidant defenses, lipid peroxidation, DNA damage, mitochondrial dysfunction, abnormalities in the monoaminergic systems, and reduced neurogenesis and neuronal plasticity [11, 12]. It is well known, that: (i) neurons are the primary cells in the brain that suffer the cytotoxic effects elicited by glucocorticoids [5, 8, 10]; (ii) the hippocampus contains a high concentration of glucocorticoid receptors, and is the main brain structure affected by glucocorticoids in major depression [5, 6, 7, 9]; (iii) major depression is accompanied by hippocampal atrophy and neuronal loss [5, 6, 8, 10]. In this study we have used HT22 cells, from an immortalized mouse hippocampal cell line, that are widely used as an *in vitro* model to understand the cellular and molecular processes relevant to the hippocampus, and thereby, a good model to evaluate the mechanisms of action of potential antidepressant drugs.

The nuclear factor (erythroid 2-derived)-like 2 (Nrf2) is the master regulator of the antioxidant defense response. Nrf2 activates a redox-sensitive gene regulatory network, reflecting its role in maintaining redox homeostasis in the brain and protecting neurons against cell death [13]. Several lines of evidence have shown that a promising target for the development of new, improved treatments for major depression is based on Nrf2 activators [14-16]. In resting conditions, Nrf2 is sequestered in the cytoplasm as an inactive complex with its cytoplasmic repressor protein Kelch-like-ECH-associated protein 1 (Keap1), which allows Nrf2 to undergo proteasomal degradation [17, 18]. However, upon recognition of chemical signals afforded by oxidative and electrophilic

molecules, Nrf2 is released from Keap1, translocates from the cytoplasm to the nucleus, and induces the expression of several cytoprotective genes that enhance cell survival [19, 20]. Several kinases such as phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), c-Jun NH 2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) are able to phosphorylate Nrf2 at serine and threonine residues and induce its Nrf2 dissociation from Keap1 and its subsequent translocation to the nucleus [21-24]. In the nucleus, Nrf2 binds to the antioxidant response element (ARE) and stimulates genes encoding phase II detoxifying enzymes and/or antioxidant enzymes such as glutathione S-transferase α2 (GSTA2), NAD(P)H:quinone oxidoreductase (NQO1), glutamate cysteine ligase, catalytic subunit (GCLc), and heme oxygenase-1 (HO-1) [23-25].

Agmatine is an endogenous metabolite of L-arginine, synthesized by arginine decarboxylase and hydrolyzed to putrescine and urea by the enzyme agmatinase [26]. As reviewed recently by Piletz et al. [27], agmatine is considered as a neuromodulator in the brain for the following reasons: (i) it is taken up by presynaptic axon terminals, stored in synaptic vesicles, and released through membrane depolarization [28, 29]; (ii) it is inactivated by the enzyme agmatinase [30]; (iii) it has been co-localized with other neurotransmitters, particularly with glutamate in nerve cell bodies and synaptic terminals [31]; (iv) it activates several postsynaptic membrane receptors, namely $I_1R$, and $\alpha_2$-adrenergic receptors, and $5HT_3$ receptors [26, 28, 32]; (v) it inhibits membrane $Ca^{(2+)}$ channels, all isoforms of nitric oxide synthase (NOS) in the brain, and blocks N-methyl-D-aspartate (NMDA) receptors [32]. Therefore, agmatine is being considered a potential drug that could assist the conventional pharmacotherapy of major depression. We have previously demonstrated that agmatine produces an antidepressant-like effect in predictive antidepressant tests, which was accompanied by the modulation of monoaminergic and opioid systems, NMDA receptors and the L-arginine-NO pathway [33-35]. More recently, our group has shown that agmatine produces an antidepressant-like effect by controlling pro-/anti-oxidative homeostasis in the hippocampus [36], and the inflammatory response induced by tumor necrosis factor-α [37]. From a clinical point of view, Shopsin [38] has shown that agmatine has antidepressant effects in depressed subjects. Finally, neuroprotective effects of agmatine against cell damage caused by glucocorticoids in cultured hippocampal neurons through a possible blockade of the N-methyl-D-aspartate receptor channels or a potential anti-apoptotic property was demonstrated before by Zhu et al. [39]. To conclude this section, we have planned this study on the following observations: (i) hyperactivity of the HPA axis is a major etiological basis of major depression [4, 5]; (ii) overexposure to cortisol induces an oxidative imbalance, thereby causing hippocampal neuronal cell death [6]; (iii) agmatine produces an antidepressant-like effect accompanied by its ability to maintain the intra-cellular redox status in the hippocampus [36]; (iv) Nrf2 is a key regulator of antioxidant responses [25] and (v) there is compelling evidence showing the antidepressant-like effect of agmatine, however the molecular

mechanisms underlying its therapeutic activity remain unclear. Therefore, the objective of this study was to determine the ability of agmatine to afford protection against corticosterone-induced toxicity and the implication of the transcription factor Nrf2 in such effect.

**Materials and methods**

Drugs

Agmatine sulfate, corticosterone, cycloheximide, 11β-(4-dimethyl-amino)-phenyl-17β-hydroxy-17-(1-propynyl)-estra-4,9-dien-3-one (RU-486), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle Medium (DMEM), fluoxetine hydrochloride, imipramine hydrochloride, ketanserin tartarate, methyllycaconitine, pindolol, prazosin hydrochloride, and yohimbine hydrochloride were obtained from Sigma (Madrid, Spain). 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride (LY294002) were purchased from Tocris (Biogen Científica, Madrid, Spain). Tin (IV) protoporphyrin-IX dichloride (SnPP) was obtained from Frontier Scientific Europe (Lancashire, UK). 2′,7′-Dichlorofluorescein diacetate ($H_2$DCFDA) was purchased from Molecular Probes (Invitrogen, Madrid, Spain). Penicillin, pyruvate, and heat-inactivated fetal bovine serum (FBS) were purchase from Invitrogen (Madrid, Spain).

Culture of HT22 cells

HT22 cells (a gift from Dr. David Schubert, Salk Institute, La Jolla, CA, USA) were cultured in Dulbecco's Modified Eagle Medium, supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were seeded into flasks containing supplemented medium and kept at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. For assays, HT22 cells were sub-cultured in 48-well plates at a seeding density of $1\times10^5$ cells per well. Cells were treated with the drugs before confluence in DMEM with 10% FBS. Cells were used at passages below 13.

Cell viability assay

Cell viability, virtually the mitochondrial activity of living cells, was measured by a quantitative colorimetric assay with MTT (3-[4,5dimethylthiazol-2-yl]-2,5-diphenyltetrazolic bromide). Briefly, 50μl of the MTT labeling reagent, at a final concentration of 0.5 mg/ml, was added to the DMEM of each well at the end of the treatment period; then, the plate was placed in a humidified incubator at 37 °C with 5% $CO_2$ and 95% air (v/v) for 2-h. Mitochondrial dehydrogenases of viable cells cleave and reduce the soluble

yellow MTT dye into the insoluble purple formazan [40]. The insoluble formazan was dissolved by the addition of dimethyl sulfoxide, resulting in a colored compound whose optical density was spectrophotometrically assayed at 540 nm. Control cells treated with vehicle (DMEM/F-12) were taken as 100 % viable.

Western Blot

HT22 cells ($5\times10^6$) were washed once with cold phosphate-buffered saline and lysed in 100 µL ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mmol/L NaCl, 20 mmol/L Tris–HCl, pH 7.5, 1 µg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L NaF, 1 mmol/L sodium pyrophosphate, and 1 mmol/L $Na_3VO_4$). Protein (30 µg) from these cell lysates was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF membranes (Millipore Iberica SA, Madrid, Spain). Membranes were incubated with anti-Akt (1:1000), anti-phospho-Akt (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-extracellular regulated kinase (ERK; 1:1000), anti-phospho-ERK (1:1000) (Santa Cruz Biotechnology Inc., Heidelberg, Germany), anti-HO-1 (1:1000) (Chemicon, Hampshire, UK), anti-GCLc (1:1000) (Chemicon, Hamp-shire, UK), or anti-β-actin (1:100,000; Sigma). Appropriate peroxidase-conjugated secondary antibodies at 1:10,000 were used to detect proteins by enhanced chemiluminescence. The optical density (O.D.) of the bands was quantified using Scion Image software®. The phosphorylation level of Akt and ERK were determined as a ratio of O.D. of phosphorylated band/O.D. of total band. The immunocontents of HO-1 and GCLc were determined by the relationship between the O.D of the HO-1 or GCLc band/O.D of β actin band. Immunoblots shown in the Figures correspond to a representative experiment that was repeated four times with similar results.

Plasmids

The luciferase reporter gene assay was performed by using an expression vector for Renilla (Promega, Madison, WI, USA) and ARE-Luciferase (a gift from Dr. J. Alam, Department of Molecular Genetics, Ochsner Clinic Foundation, New Orleans, LA, USA).

Luciferase reporter gene assay

The cells were plated in 24-well plates ($5\times10^4$ cells per well), co-transfected with an ARE-luciferase plasmid [41] and a Renilla luciferase control plasmid (Promega), and cultured for 16 h. After recovery from transfection, cells were lysed and assayed for luciferase activity with the dual luciferase assay system (Promega), according to the manufacturer's instructions. Relative light units were measured in a GloMax 96 microplate luminometer with dual injectors (Promega).

Estimation of reactive oxygen species (ROS) production

Formation of ROS was estimated with the molecular probe $H_2$DCFDA, as described previously by Ha et al. [42]. HT22 cells were collected by centrifugation and resuspended in 100 μl of 1× binding buffer and loaded with 45 μl $H_2$DCFDA, which diffuses through the cell membrane and is hydrolyzed by intracellular esterases to the nonfluorescent form dichlorofluorescein (DCFH). In the presence of ROS, DCFH is rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF). After incubation at room temperature for 15 min in the dark, 200 μl of 1× binding buffer was added. Fluorescence was read using a FACScan and CELLQuest software (Beckman Coulter, Madrid, Spain). At least 20,000 events per assay well were included.

Flow cytometry analysis for cell apoptosis

The number of viable, early apoptotic, and late apoptotic cells was detected and analyzed by flow cytometry using an annexin V-PE and 7-AAD (7-aminoactinomycin D) double staining kit (BD Bioscience, Madrid, Spain) in accordance with the manufacturer's instructions. Briefly, cells were collected by centrifugation and resuspended in 100 μl of 1× binding buffer with 5 μl annexin V-PE and 5 μl 7-AAD. After incubation at room temperature for 15 min in the dark, 200 μl of 1× binding buffer was added. The cells were analyzed using a FACScan and CELLQuest software (Beckman Coulter, Madrid, Spain). At least 20,000 events per assay well were included and represented as dot plots. Annexin+/7-AAD− cells were considered early apoptotic cells, annexin+/7-AAD+ late apoptotic cells, and annexin−/7-AAD− viable cells. Percentage of apoptosis was calculated based on all the Annexin+/7-AAD− (early apoptotic) plus the annexin+/7-AAD+ (late apoptotic) cells and were represented as bar graphs.

Statistical analysis

Comparisons between experimental and control groups were performed by one-way ANOVA followed by Newman–Keuls test, when appropriate. $p<0.05$ was considered significant.

**Results**

Cell death induced by corticosterone

In order to validate the corticosterone-induced cell death model, HT22 cells were treated with corticosterone at increasing concentrations, ranging from 100-1000 μM, for 24h. After the treatment period, cell viability was measured by the MTT assay. The results depicted in Fig. 1a show that corticosterone induced significant cell death at the concentrations of 100, 500 and 1000 μM (cell death

percentages were approximately 30%, 40% and 60%, respectively). To ascertain that this cell death was specifically caused by the activation of glucocorticoid receptor (GR), the GR antagonist RU486 was used. Pre-incubation of RU486 (2.5 μM), 30 min before and during the 24-h exposure to corticosterone, prevented (64%, $p<0.001$) corticosterone's toxic effects (Fig. 1b). Altogether, these results indicate that the cytotoxicity elicited by corticosterone in HT22 cells appears to be mediated by GR.

We considered of interest, before evaluating the potential neuroprotective effects of agmatine, to determine whether certain classical antidepressants could protect against corticosterone-induced toxicity. The ability of these classical antidepressants to abrogate the cytotoxic effect of corticosterone would support our model in the context of depression. Specifically, we selected fluoxetine (a selective serotonin reuptake inhibitor, SSRI) and imipramine (a tricyclic antidepressant, TCA). For these studies, HT22 cells were incubated 24 h before the addition of corticosterone and during the 24 h exposure to corticosterone, with increasing concentrations of fluoxetine (0.01-1 μM) or imipramine (0.01-1 μM). Fluoxetine at 0.1 and 1 μM afforded significant protection (40%, $p<0.05$; and 65%, $p<0.001$, respectively) against corticosterone- induced toxicity (Fig. 1c); likewise, imipramine at 0.1 and 1 μM was also protective (protection of 50%, $p<0.01$; and 100%, $p<0.001$, respectively) (Fig. 1d).

Neuroprotective effect of agmatine on corticosterone-induced cell death

Firstly, to rule out that agmatine was not toxic *per se*, we exposed HT22 cells for 24 h to increasing concentrations of agmatine. As shown in Fig. 2a, agmatine *per se* (1–1000 μM) produced no effect on cellular viability at any of the concentrations tested.

Once the experimental conditions were standardized, the potential protective effect of agmatine on the cytotoxicity induced by corticosterone was evaluated. The experimental protocol consisted in pre-incubating HT22 cells with increasing concentrations of agmatine (0.01–100 μM) for 24h, and then co-incubating them with corticosterone plus agmatine for an additional 24-h period (See protocol on the top part of Fig. 2). Thereafter, cell viability was measured by the MTT assay. In Fig. 2b, the concentration-response protective curve for agmatine is represented; agmatine showed significant protection at the concentration of 0.01 μM and at 10 μM it caused maximum cytoprotection (75%, $p<0.001$). Therefore, for the following experiments, the concentration of 10 μM of agmatine was selected.

Next, we investigated the time interval in which agmatine (10 μM) initiates its cytoprotective effect. For this purpose, agmatine was pre-incubated for 1h, 6h, 10 or 24 h before the addition of corticosterone, and co-incubated for an additional 24-h period in the presence of corticosterone (100 μM). Agmatine initiated its cytoprotective effect (45%, $p<0.01$) after 6 h of pre-incubation and achieved its maximal effect (75%, $p<0.001$) after 24-h (Fig. 2c). Therefore, in

the following experiments we used the 24 h pre-incubation protocol with agmatine.

Agmatine reduces corticosterone-induced ROS generation and apoptosis

The inflammatory and neurodegenerative hypothesis of depression postulates that the neurotoxic effects of glucocorticoids are mediated by oxidative and nitrosative stress [43, 44]. The amounts of ROS generated by glucocorticoid exposure are deleterious to the brain by inducing apoptotic neuronal cell death, which appears to be a key mechanism for the development of depression [11, 45]. The present study assessed this issue by evaluating the effect of corticosterone on ROS production and apoptosis, as well as agmatine's ability to abrogate such effects. HT22 cells were pre-incubated (24 h before corticosterone) with agmatine (10 μM) and co-incubated with corticosterone (100 μM) for another 24 h. At the end of the experiment, cells were collected and ROS production and apoptosis were estimated by flow cytometry. The results depicted in Fig. 3a and b illustrate that corticosterone (100 μM) significantly increased ROS production (1.8-fold above basal, $p<0.01$) and that this event was significantly blocked by agmatine (10 μM) treatment (95%, $p<0.01$); agmatine *per se* had no effect on this parameter. Similarly, agmatine prevented the increase of apoptotic cell death induced by corticosterone. Basal apoptosis amounted to 13% (Figs. 3c and g), corticosterone (100 μM) treatment increased apoptosis to 30% ($p<0.001$) (Figs. 3e and g), and agmatine reduced the corticosterone-induced apoptosis to 16% ($p<0.001$; Figs. 3f and g). Of note, no significant apoptotic cell death was observed in the agmatine *per se* group (10%; Figs. 3d and g).

Monoaminergic systems contribute to the cytoprotective effect of agmatine

Previous studies have reported that agmatine produces an antidepressant-like effect by activation of the monoaminergic systems [34, 46]. In addition, a recent study by Parada et al. [47] showed that activation of the α-7 nicotinic acetylcholine receptor induces HO-1 expression via Nrf2. Thus, in order to assess the participation of the monoaminergic and cholinergic systems in the cytoprotective effect of agmatine, HT22 cells were pre-treated with different inhibitors for 30 min prior to the addition of agmatine and throughout the experiment (see protocol on top of Fig. 5a). The following inhibitors were used: prazosin (1 μM, an $\alpha_1$-adrenoceptor antagonist), yohimbine (0.1 μM, an $\alpha_2$-adrenoceptor antagonist), pindolol (10 μM, a 5-$HT_{1A/1B}$ receptor/β-adrenoceptor antagonist), ketanserin (1 μM, a preferential 5-$HT_{2A}$ receptor antagonist), and methyllycaconitine (0.1 μM, a selective α7 nicotinic acetylcholine receptor antagonist). As shown in Fig. 4a, only the pretreatment with yohimbine ($\alpha_2$-adrenoceptor antagonist) or ketanserin (a preferential 5-$HT_{2A}$ receptor antagonist) abrogated the neuroprotective effect of agmatine (80%, $p<0.001$ and 70%, $p<0.001$, respectively). HT22 cells treated with the drugs alone caused no

effect on cellular viability (data not shown). Altogether, these results suggest that the cytoprotective effect of agmatine could be dependent on the activation of both $\alpha_2$-adrenoceptors and 5-$HT_{2A}$ receptors.

Agmatine produces a synergic cytoprotective effect when combined with classical antidepressants

Considering the involvement of $\alpha_2$-adrenoceptors and 5-$HT_{2A}$ receptors in the protective effect of agmatine, in the following experiments we investigated the potential synergistic effect of agmatine with classical antidepressants. Therefore, we co-incubated the cells with sub-effective concentrations of agmatine (0.001 μM) and fluoxetine (0.01 μM) or imipramine (0.01 μM) for a 24-h period. After, the cells were co-incubated with corticosterone (100 μM) in the presence of sub-effective concentrations of agmatine (0.001 μM) in combination with fluoxetine (0.01 μM) or imipramine (0.01 μM) for an additional period of 24 h (see protocol on top of Fig. 4b). The results obtained with the combination of drugs is shown in Fig. 4b; we observed a synergic and significant cytoprotective effect when sub-effective concentrations of fluoxetine or imipramine were combined with agmatine (cytoprotection 70%, $p<0.001$; and 70%, $p<0.001$, respectively). Thus, our results reinforce the notion that noradrenergic and serotonergic neurotransmission could be critical targets implicated in the protective effect of agmatine.

Implication of PI3K/Akt, ERK and HO-1 in the protective effect of agmatine

We next decided to study the intracellular signaling pathway activated by agmatine to provide protection. There is a growing body of evidence that indicates that Nrf2 activators may be advanced to phase 2 clinical trials in depression because these drugs are able to maintain the cerebral pro-/anti-oxidative homeostasis in the mitochondria, and possess anti-inflammatory and neuroprotective effects [14, 15]. Activation of several upstream kinases including MAPKs and PI3K/Akt has been reported to induce nuclear localization of Nrf2 and subsequent expression of HO-1 and GCLc [23, 48], which can be transcribed by Nrf2. Despite that agmatine's antioxidant effect has been previously reported [36], the molecular mechanisms underlying this effect remain to be established. To address this issue we have initially used the inhibitors LY294002 (3 μM, a PI3K inhibitor), PD98059 (5 μM, a MEK1/2 inhibitor), SnPP (3 μM, HO-1 activity inhibitor) and cycloheximide (1 μM, a protein synthesis inhibitor). Our results show that LY294002 (45%, $p<0.01$), PD98059 (73%, $p<0.001$), SnPP (78%, $p<0.001$) or cycloheximide (60%, $p<0.01$) abolished the cytoprotective effect of agmatine (Fig. 5a and b). Therefore, PI3K/Akt, MEK1/2 and HO-1 seem to participate in the protective mechanism of agmatine against corticosterone-induced cell death. Noteworthy, the treatment of HT22 cells with the inhibitors alone did not affect the cellular viability (data not shown).

To further support the contribution of Akt and ERK on the cytoprotection afforded by agmatine, HT22 cells were treated with agmatine (10 μM) for 15, 30 or 60 min, and phosphorylation levels of Akt and ERK were determined by western blot assay. As depicted in Fig. 6a, 15 min incubation with agmatine produced a significant increase of Akt phosphorylation (4.6-fold above basal, $p<0.001$). Furthermore agmatine incubated for 15 and 30 min also caused a significant augmentation of ERK phosphorylation (Fig. 6b; 2.5-fold above basal, $p<0.01$ and 2.4-fold above basal, $p<0.01$, respectively). Altogether, these results support the participation of Akt and ERK phosphorylation in the protective effect of agmatine.

Agmatine induces HO-1 and GCLc by a mechanism that involves $\alpha_2$-adrenoceptor receptors, $5HT_{2A}$ receptors, PI3K/Akt and ERK

To ascertain that agmatine causes the induction of Nrf2 signaling via modulation of $\alpha_2$-adrenergic and 5-$HT_{2A}$ receptors, Akt and ERK activation in terms of HO-1 and GCLc expression, cells were pretreated with yohimbine (0.1 μM, an $\alpha_2$-adrenoceptor antagonist), ketanserin (1 μM, a preferential 5-$HT_{2A}$ receptor antagonist), LY294002 (3 μM, a PI3K inhibitor), or PD98059 (5 μM, a MEK1/2inhibitor) for 30 min, and co-incubated with agmatine (10 μM) for 24 h. After the different treatments, the immunocontents of HO-1 and GCLc were measured by western blot. Agmatine treatment for 24 h caused a significant increase of HO-1 and GCLc immunocontent (3.5-fold above basal, $p<0.001$ and 6.2-fold above basal, $p<0.001$, respectively). On the other hand, yohimbine (77%, $p<0.001$), ketanserin (82%, $p<0.001$), LY294002 (100%, $p<0.001$) and PD98059 (100%, $p<0.001$) abolished the increase on HO-1 immunocontent (Figs. 6d and e). Similarly, yohimbine (86%, $p<0.001$), ketanserin (79%, $p<0.001$), LY294002 (56%, $p<0.001$) and PD98059 (79%, $p<0.001$) reduced the immunocontent of GCLc (Figs. 6f and g). Therefore, the results indicate that the cytoprotective effect of agmatine appears to be related to activation of $\alpha_2$-adrenergic and 5-$HT_{2A}$ receptors, Akt and ERK phosphorylation, that in turn, could participate in Nrf2 activation with the consequent expression of HO-1 and GCLc.

Agmatine induces activation of the transcription factor Nrf2

In order to confirm that agmatine could activate the transcription factor Nrf2, HT22 cells were co-transfected with a luciferase reporter construct carrying three tandem sequences of the Nrf2-dependent antioxidant response element (ARE-LUC) and a Renilla vector for normalization. After transfection, cells were treated with agmatine (0.001-10 μM) for 16 h and the luciferase activity was measured. As shown in Fig. 7a, agmatine caused a significant augmentation in luciferase activity at the concentrations of 0.1 μM (1.8-fold increase, $p<0.05$), 1 μM (2.6-fold increase, $p<0.001$), and 10 μM (2.7-fold increase, $p<0.001$). In a separate set of experiments, the participation of $\alpha_2$-

adrenergic and 5-$HT_{2A}$ receptors, as well as the Akt and ERK signaling pathways on the Nrf2 activation by agmatine was evaluated. For this purpose, HT22 cells were transfected with the ARE-LUC and Renilla vectors. After transfection, cells were pretreated with yohimbine (0.1 μM, an $\alpha_2$-adrenoceptor antagonist), ketanserin (1 μM, a preferential 5-$HT_{2A}$ receptor antagonist), LY294002 (3 μM, a PI3K inhibitor), or PD98059 (5 μM, a MEK1/2 inhibitor) for 30 min. Then, cells were exposed to agmatine (10 μM) treatment for 16 h and the luciferase activity was measured. Figs. 7b and c show that the presence of the inhibitors abolished Nrf2 induction afforded by agmatine (77%, $p<0.001$; 65%, $p<0.001$; 95%, $p<0.001$; and 80%, $p<0.001$, respectively). Taken together, our results support the notion that the cytoprotective effect of agmatine on the corticosterone-induced cell death could be accompanied by its capability to induce nuclear translocation of Nrf2 via activation of $\alpha_2$-adrenergic and 5-$HT_{2A}$ receptors, Akt and ERK pathways, and subsequent expression of phase II enzymes, namely HO-1 and GCLc.

**Discussion**

The present study provides new evidence regarding the mechanism underlying the neuroprotective effect of agmatine in an *in vitro* model related to one of the main pathomechanisms implicated in major depression, i.e. increased levels of glucocorticoids as a consequence of alterations in the HPA axis [11, 12]. We have identified the induction of the transcription factor Nrf2 as a critical target for agmatine to afford neuroprotection and, most probably, its antidepressant effect. In line with our results, Zhu et al. [39] demonstrated a neuroprotective effect of agmatine against cell damage caused by glucocorticoids in cultured hippocampal neurons by a mechanism that seems to implicate the blockade of the N-methyl-D-aspartate receptor channels or a potential anti-apoptotic property. Considering that the glutamatergic neurotransmission has been related to major depression [49, 50], particularly in the neurotoxic effects induced by glucocorticoids [5, 51], it could be speculated that an additional mechanism implicated in the neuroprotective effect of agmatine could be the inhibition of the excitatory glutamatergic transmission.

The ability of agmatine to provide neuroprotection has been described firstly by Gilad et al. [52], followed by a variety of subsequent studies in experimental models such as brain ischemia [53-55], spinal cord injury [56-58], 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity [59, 60], glutamate and NMDA excitotoxicity [61, 62], beta-amyloid(25–35)-induced memory impairments [63] or rotenone [64]. However, taken into account that agmatine has been reported by our group and others to produce antidepressant effects in mice [33-37] and humans [38], we were interested in identifying its mechanism of action in an *in vitro* model related to depression. We have focused on studying the neuroprotective actions of agmatine because it is well documented that a critical etiologic component for the development of major depression is the chronic activation of the HPA axis, which leads to increased

levels of glucocorticoids in the brain [4-7]. Cortisol in humans and corticosterone in rodents induce an oxidative imbalance followed by neuronal cell death [5, 6, 10]. Furthermore, we have used HT22 hippocampal neuronal cell line for the following reasons: (i) the hippocampus, a region that plays a key role in mood regulation, contains a high concentration of glucocorticoids receptors and, consequently is the main region affected by the deleterious effects of cortisol [5, 10], (ii) HT22 cells are widely used to investigate novel mechanisms of drugs [65-68], iii) the neurons are the primary cell in the brain that suffer the cytotoxic effects elicited by glucocorticoids in major depression [5, 8, 10], and iv) the cell death induced by corticosterone in the HT22 hippocampal neuronal cell line mimics, at least in part, the cellular and molecular alterations observed in the hippocampus of depressive patients.

Cytotoxicity induced by corticosterone caused morphologic changes indicative of cell death, increased ROS production and apoptosis in the HT22 hippocampal neuronal cell line. The cytotoxic effect of corticosterone was abrogated by the glucocorticoid receptor antagonist RU486, supporting the notion that the cell death induced by corticosterone was mediated via glucocorticoid receptors. Corticosterone-induced cell death was prevented by classical antidepressants like fluoxetine or imipramine, which indicates that this cytotoxicity model could predict antidepressant effects. Corroborating these findings, studies from other groups have shown that antidepressants drugs, such as fluoxetine [69], venlafaxine [70], desipramine, and moclobemide [69] are able to afford protection from the toxicity induced by corticosterone, further supporting the experimental model used.

Regarding the membrane receptors targeted by agmatine, we have identified $\alpha_2$-adrenergic and 5-$HT_{2A}$ receptors as the ones implicated in its neuroprotective effect against corticosterone-induced toxicity. As to $\alpha_2$-adrenergic receptors, there is growing evidence supporting that agmatine can activate these receptors [27, 32]. However, regarding 5-$HT_{2A}$ receptors, literature data concerning their participation in the antidepressant-like effect of agmatine is contradictory. Krass et al. [71] demonstrated that the antidepressant-like effect of agmatine is not mediated by serotonin. Additionally, Shopsin's clinical case report indicated that serotonin is not involved in agmatine's antidepressant effect [38]. Despite the importance of demonstrating a clinical antidepressant effect for agmatine, the reduced number of depressive patients (n=3) who participated in the study is a limitation, and indicates that further confirmatory studies are required. In contrast, Zomkowski et al. [34] showed the involvement of 5-$HT_{1A/1B}$ and 5-$HT_{2A}$ receptors in the antidepressant like-effect of agmatine. In addition, Jiang et al. [46] showed that agmatine produces an antidepressant-like action by modulating $\alpha_2$-adrenergic and 5-$HT_{1A/1B}$ receptors. Further supporting these findings, a study by Taksande et al. [72] showed an antidepressant like effect of SSRIs involving the modulation of imidazoline receptors by agmatine. Our results are compatible with an effect of agmatine on both $\alpha_2$-adrenergic and 5-$HT_{2A}$ receptors.

We have also assessed an interesting synergic cytoprotective effect of agmatine with classical antidepressants which modulate the monoaminergic neurotransmission. We verified that co-incubation of a sub-effective concentration of agmatine with sub-effective concentrations of fluoxetine or imipramine was able to produce a significant neuroprotective effect against the toxicity induced by corticosterone. These results indicate that neuroprotection seems to be a critical mechanism of action for antidepressant drugs [69, 73, 74] and, from a clinical point of view, they suggest that combination of agmatine with sub-effective doses of classical antidepressants would reduce their side effects, maintain drug adherence and, probably, improve their clinical efficacy.

Several reports point at the existence of a redox imbalance in major depressive patients [12, 14, 15]. In this context, induction of the transcription factor Nrf2, the master regulator of inducible antioxidant response, is being proposed as a novel therapeutic strategy for the treatment of major depression. Our group has recently reported that Nrf2 knockout mice show a depressive-like behavior, increased oxidative parameters, neuroinflammation and altered neurotransmitter levels in their brains. We also showed that induction of Nrf2 with sulforaphane was able to restore the depressive-like behavior and reduce neuroinflammatory markers in mice treated with LPS [16]. In line with these observations, Lin et al. [75] described that the tricyclic antidepressant drug desipramine protects from neuronal cell death and induces HO-1 expression mediated by Nrf2 activation through the ERK and JNK signaling pathways. Therefore, drugs capable of inducing Nrf2 may increase antioxidant defenses, protect mitochondria, have anti-inflammatory actions and afford neuroprotection [14-16]. Considering these issues, the main contribution of the present study is that agmatine exerts its neuroprotective effect through the nuclear translocation of the transcription factor Nrf2 via activation of $\alpha_2$-adrenergic and 5-$HT_{2A}$ receptors, Akt and ERK signaling pathways, and subsequent expression of the phase II detoxifying enzymes HO-1 and GCLc. Such conclusion derives from the following findings: (i) the PI3K inhibitor LY294002, as well as the MEK1/2 inhibitor PD98059, the HO-1 inhibitor SnPP, and the protein synthesis inhibitor cycloheximide abrogated the cytoprotective effect of agmatine; (ii) agmatine induced a significant augmentation of Akt and ERK phosphorylation; (iii) agmatine induced a significant increase in the immunocontent of HO-1 and GCLc, which were abolished by yohimbine (an $\alpha_2$-adrenoceptor antagonist), ketanserin (a preferential 5-$HT_{2A}$ receptor antagonist), LY294002 (PI3K inhibitor), and PD98059 (MEK1/2 inhibitor); (iv) agmatine increased Nrf2 transcriptional activity, which was abrogated by yohimbine, ketanserin, LY294002, and PD98059.

In conclusion, this study reveals a new mechanism of action for agmatine in the context of the pathophysiology of major depression. Agmatine afforded neuroprotection and reduction of oxidative stress in the corticosterone-induced model of toxicity standardized in our laboratory. Like agmatine, the antidepressants fluoxetine and imipramine also afforded a similar

cytoprotective effect. Interestingly, agmatine produced a synergic neuroprotective effect with fluoxetine and imipramine. The positive effects observed with agmatine were related to its ability to induce the transcription factor Nrf2 and the consequent expression of HO-1 and GCLc; these actions were mediated by $\alpha_2$-adrenergic and 5-$HT_{2A}$ receptors as well as Akt and ERK signaling pathways. Thereby, agmatine could be a useful strategy for major depression management since it combines antidepressant, neuroprotective and antioxidant properties.

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**Conflict of interest**

All authors have no conflict of interest.

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**Figure legends**

**Fig. 1.** Cell death induced by corticosterone is abolished by the GR antagonist RU486 and by the classical antidepressants fluoxetine and imipramine. (**a**) HT22 cells were incubated with increasing concentrations of corticosterone for 24 h. (**b**) In HT22 cells pre-incubated with 2.5 µM RU486 for 30 min and co-incubated with 100 µM corticosterone for 24 h, reduction in cell viability induced by corticosterone was prevented. (**c**-**d**) HT22 cells were pre-incubated with increasing concentrations of the antidepressants (ATD) fluoxetine or imipramine for 24 h, and co-incubated with 100 µM corticosterone for 24 h. Both fluoxetine and imipramine afforded a concentration-dependent protection measured as MTT reduction. Each column represents the mean + S.E.M. of six different cell batches. Statistical analysis was performed by one-way ANOVA, followed by Newman–Keuls test. ***$p$<0.001 as compared with the basal group; $^{\#}p$<0.05, $^{\#\#}p$<0.01, and $^{\#\#\#}p$<0.001 as compared with the corticosterone group. ATD, antidepressant; CORT, corticosterone; FLU, fluoxetine; IMI, imipramine.

**Fig. 2.** Agmatine does not modify basal cell viability and protects against cell death induced by corticosterone in a concentration- and time-dependent manner. (**a**) HT22 cells incubated with increasing concentrations of agmatine (1-1000 µM) for 24 h, showed no significant reduction in cell viability. (**b**) Following the protocol described on the top part of the figure, agmatine afforded a concentration-dependent protection against corticosterone-induced toxicity in HT22 cells; significant protection was achieved at 0.01 □M and was maximal at 10 □M. (**c**) HT22 cells were pre-incubated with 10 µM agmatine for 1, 6, 10 or 24 h, and co-incubated with 100 µM corticosterone for 24 h; agmatine showed significant protection after 6 h pre-incubation and maximum protection was achieved after 24 h pre-incubation. Viability was measured as MTT reduction, basal conditions was considered as 100%. Each column represents the mean + S.E.M. of six different cell batches. Statistical analysis was performed by one-way ANOVA, followed by Newman–Keuls test. ***$p$<0.001 as compared with the basal group; ##$p$<0.01, ###$p$<0.001 as compared with the corticosterone group. AGM, agmatine; CORT, corticosterone.

**Fig. 3.** Agmatine abolishes corticosterone-induced ROS generation and apoptosis. (**a**) HT22 basal cells, cells treated with 10 µM agmatine for 24 h, cells treated with 100 µM corticosterone for 24 h, and cells pre-incubated with 10 µM agmatine for 24 h followed by a co-incubation with 100 µM corticosterone for 24 h were collected and ROS production was estimated by flow cytometry using $H_2$DCFDA. (**b**) DCF-fluorescence curves showing overlay of basal; agmatine; corticosterone; and agmatine + corticosterone

treated cells. Representative scatter diagrams of (**c**) basal cells, (**d**) cells incubated with 10 µM agmatine for 24 h, (**e**) cells incubated with 100 µM corticosterone for 24 h, and (**f**) cells incubated with 10 µM agmatine for 24 h followed by a co-incubation with 100 µM corticosterone for 24 h. Cells were collected, stained with annexin V-PE and 7-AAD, and analyzed by flow cytometry. (**g**) Averaged data of the apoptotic population (annexin+/7-AAD− and annexin+/7-AAD+) for each group is represented. Each column represents the mean + S.E.M. of six different cell batches. Statistical analysis was performed by one-way ANOVA, followed by Newman–Keuls test. **$p<0.01$, ***$p<0.001$ as compared with the basal group; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ as compared with the corticosterone group. AGM, agmatine; CORT, corticosterone.

**Fig. 4.** The monoaminergic system contributes to the cytoprotective effect of agmatine. (**a**) HT22 cells were pre-incubated with 10 µM agmatine for 24 h, and co-incubated with 100 µM corticosterone for 24 h in the presence or absence of 1 µM prazosin (PRA), 0.1 µM yohimbine (YOH), 10 µM pindolol (PIN), 1 µM ketanserin (KET) or 0.1 µM methyllycaconitine (MLA); only yohimbine and ketanserine blocked the protective effect of agmatine. (**b**) HT22 cells were pre-incubated with sub-effective concentrations of agmatine (0.001 µM) for 24 h, and co-incubated with 100 µM corticosterone for 24 h in the presence or absence of sub-effective concentrations of fluoxetine (FLU; 0.01 µM) or imipramine (IMI, 0.01 µM). Combination of agmatine with the classical antidepressants (ATD) showed a synergic protective effect. Viability was measured as MTT reduction and basal absorbance was considered as 100%. Each column represents the mean + S.E.M. of six different cell batches. Statistical analysis was performed by one-way ANOVA, followed by Newman–Keuls test. ***$p<0.001$ as compared with the basal group; ###$p<0.001$ as compared with the corticosterone group; \$\$$p<0.01$, \$\$\$$p<0.001$ as compared with the agmatine group. ATD, antidepressant; AGM, agmatine; CORT, corticosterone; FLU, fluoxetine; IMI, imipramine; KET, ketanserin; MLA, methyllycaconitine; PIN, pindolol; PRA, prazosin; YOH, yohimbine.

**Fig. 5.** The cytoprotective effect of agmatine is accompanied by Akt and ERK phosphorylation and HO-1 induction. (**a**) The protective effects of agmatine were partially abolished by the PI3K/Akt and ERK inhibitors: LY294002 (3 µM) and PD9805 (5 µM). (**b**) Both the HO-1 (SnPP; 3 µM) and the protein synthesis inhibitor (cycloheximide; 1 µM) prevented the protective effect of agmatine. Cell viability was measured as MTT reduction and basal absorbance was considered as 100%. Each column represents the mean + S.E.M. of six different cell batches. Statistical analysis was performed by one-way ANOVA, followed by Newman–Keuls test. ***$p<0.001$ as compared with the basal group; ##$p<0.01$, ###$p<0.001$ as compared with the corticosterone group;

$$$p<0.01, $$$$p<0.001 as compared with the agmatine group. AGM, agmatine; CORT, corticosterone.

**Fig. 6.** Agmatine induces HO-1 and GCLc expression via $\alpha_2$-adrenergic and 5-$HT_{2A}$ receptors, and Akt/ERK signaling pathways. (**a**) HT22 cells were incubated for 5, 15, 30, and 60 min with 10 μM agmatine. The top part of the figure illustrates representative immunoblots of total and p-Akt and the histogram represents the densitometric quantification of p-Akt/T-Akt. (**b**) HT22 cells were incubated for 5, 15, 30, and 60 min with 10 μM agmatine. The top part shows representative immunoblots of total and p-ERK and histogram represents the densitometric quantification of the ratio of p-ERK/T-ERK. (**c**) Representative immunoblots and densitometric quantification of HO-1 induction by agmatine (10 μM) after 24 h incubation in the presence or absence of 0.1 μM yohimbine (YOH) or 1 μM ketanserin (KET). (**d**) Representative immunoblots and densitometric quantification of HO-1 induction by agmatine (10 μM) after 24 h incubation in the presence or absence of 3 μM LY294002 or 5 μM PD98059. The histograms represent the densitometric quantification of HO-1 induction/β actin. (**e**) Representative immunoblots and densitometric quantification of GCLc induction by agmatine (10 μM) after 24 h incubation in the presence or absence of 0.1 μM yohimbine (YOH) or 1 μM ketanserin (KET). (**f**) Representative immunoblots and densitometric quantification of GCLc induction by agmatine (10 μM) after 24 h incubation in the presence or absence of 3 μM LY294002 or 5 μM PD98059. Densitometric bands of HO-1 and GCLc were normalized with respect to β-actin density. Each column represents the mean + S.E.M. of four different cell batches. Statistical analysis was performed by one-way ANOVA, followed by Newman–Keuls test. *$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared with the basal group; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ as compared with the agmatine group. AGM, agmatine; CORT, corticosterone; KET, ketanserin; YOH, yohimbine.

**Fig. 7.** Agmatine activates the transcription factor Nrf2 via $\alpha_2$-adrenergic and 5-$HT_{2A}$ receptors, and Akt/ERK signaling pathways. To measure Nrf2 induction, HT22 cells were transfected with the ARE-LUC and Renilla vectors. (**a**) After transfection, cells were incubated with increasing concentrations of agmatine for 16 h, and the luciferase activity was measured. Agmatine (0.001-10 μM) increased Nrf2 induction in a concentration-dependent manner, being significant at 0.1, a1 and 10 μM. (**b**) 0.1 μM yohimbine (YOH) or 1 μM ketanserin (KET) prevented Nrf2 induction caused by agmatine treatment (10 □M for 16 h). (**c**) 3 μM LY294002 or 5 μM PD98059 significantly reduced Nrf2 induction caused by agmatine treatment (10 □M for 16 h). Data correspond to the mean + S.E.M. of six different cell batches. Statistical analysis was performed by one-way ANOVA, followed by Newman–Keuls test. *$p<0.05$, ***$p<0.001$ as compared with the basal group; $^{\#\#\#}p<0.001$ as

compared with the agmatine group. AGM, agmatine; CORT, corticosterone; KET, ketanserin; YOH, yohimbine.

**Fig. 8.** Schematic illustration for the proposed signaling pathway involved in the neuroprotective effect of agmatine. The present study provides evidence that agmatine's ability to afford cytoprotection against corticosterone-induced cell death is dependent, at least in part, on the activation of $\alpha_2$-adrenergic and 5-$HT_{2A}$ receptors, PI3K/Akt, and MEK/ERK signaling pathways, which induce Nrf2 dissociation from Keap1, Nrf2 nuclear translocation and binding to ARE, induction of HO-1 and GCLc expression, and finally alleviation of ROS production and apoptosis to afford neuroprotection.

**Fig. 1.**

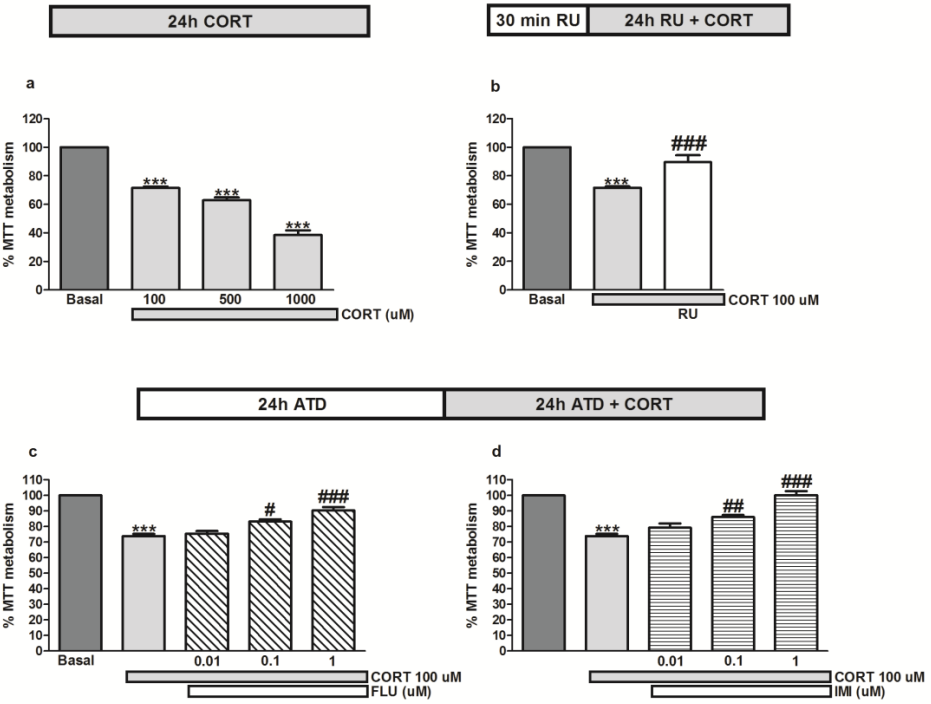
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**Fig. 2.**

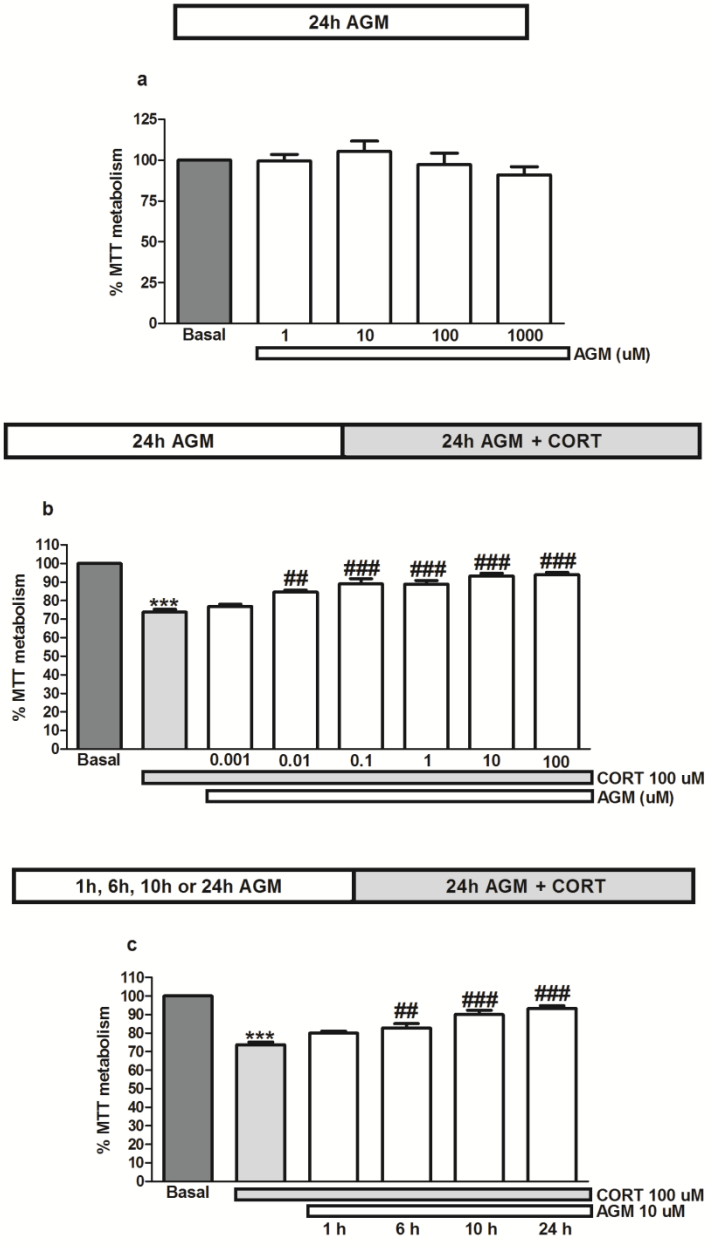
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**Fig.3.**

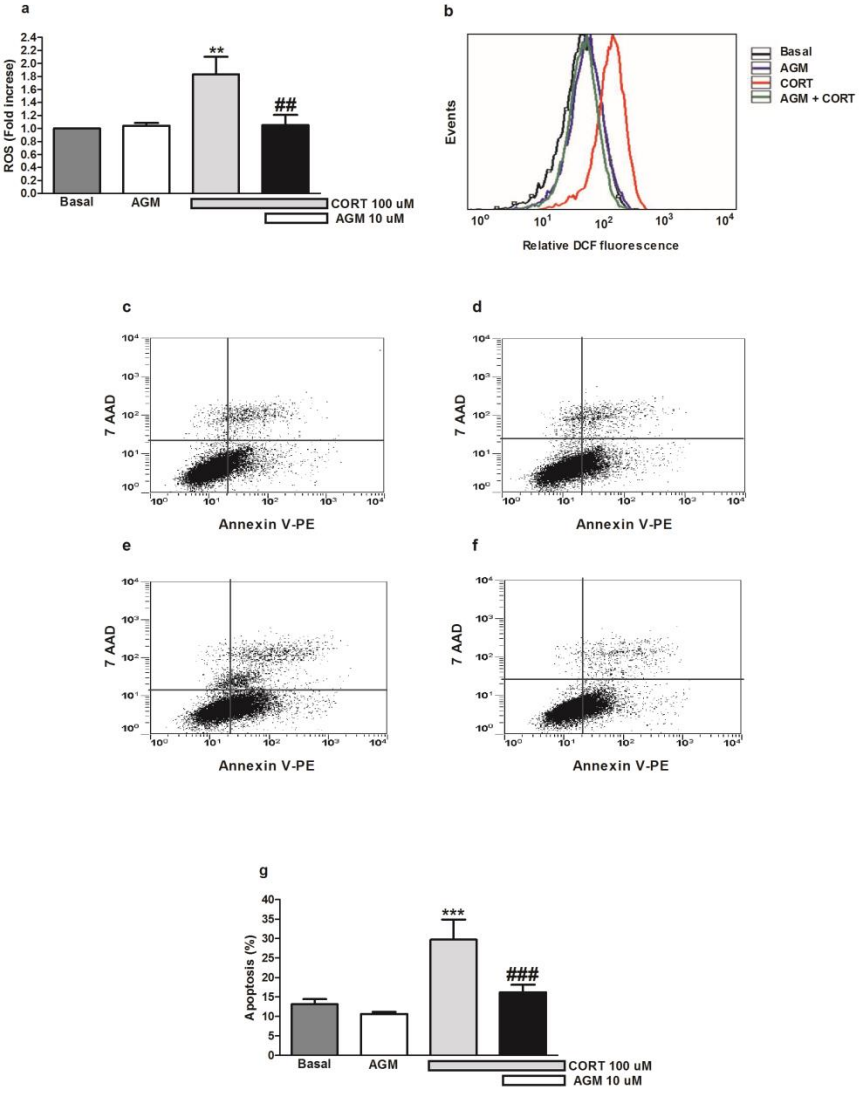
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**Fig. 4.**

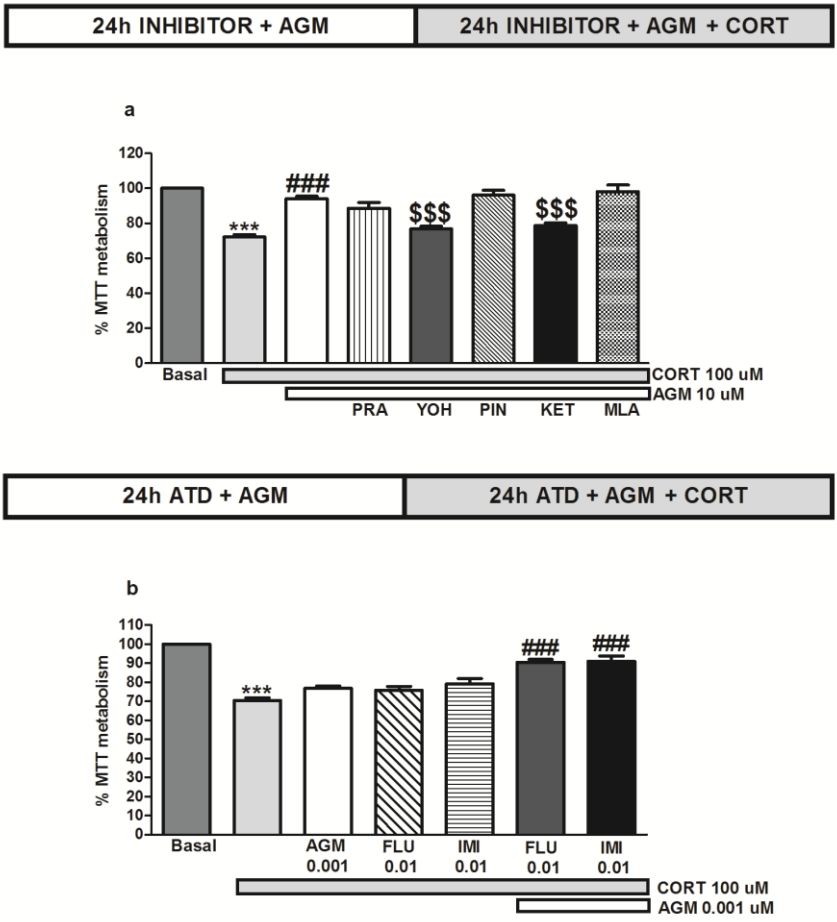
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**Fig. 5.**

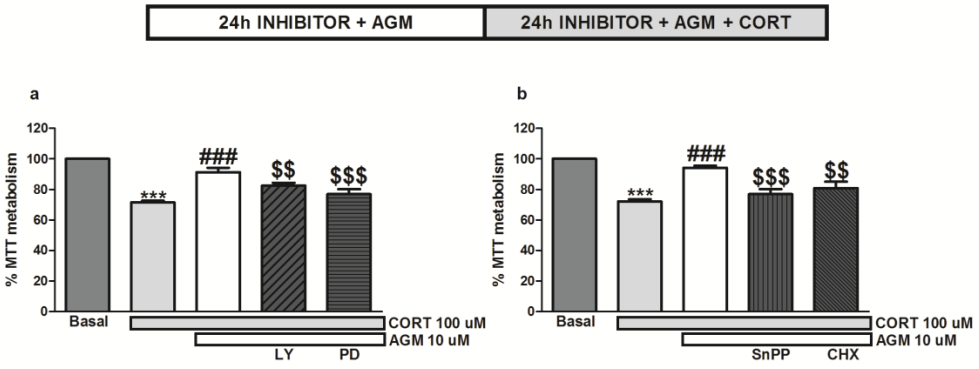
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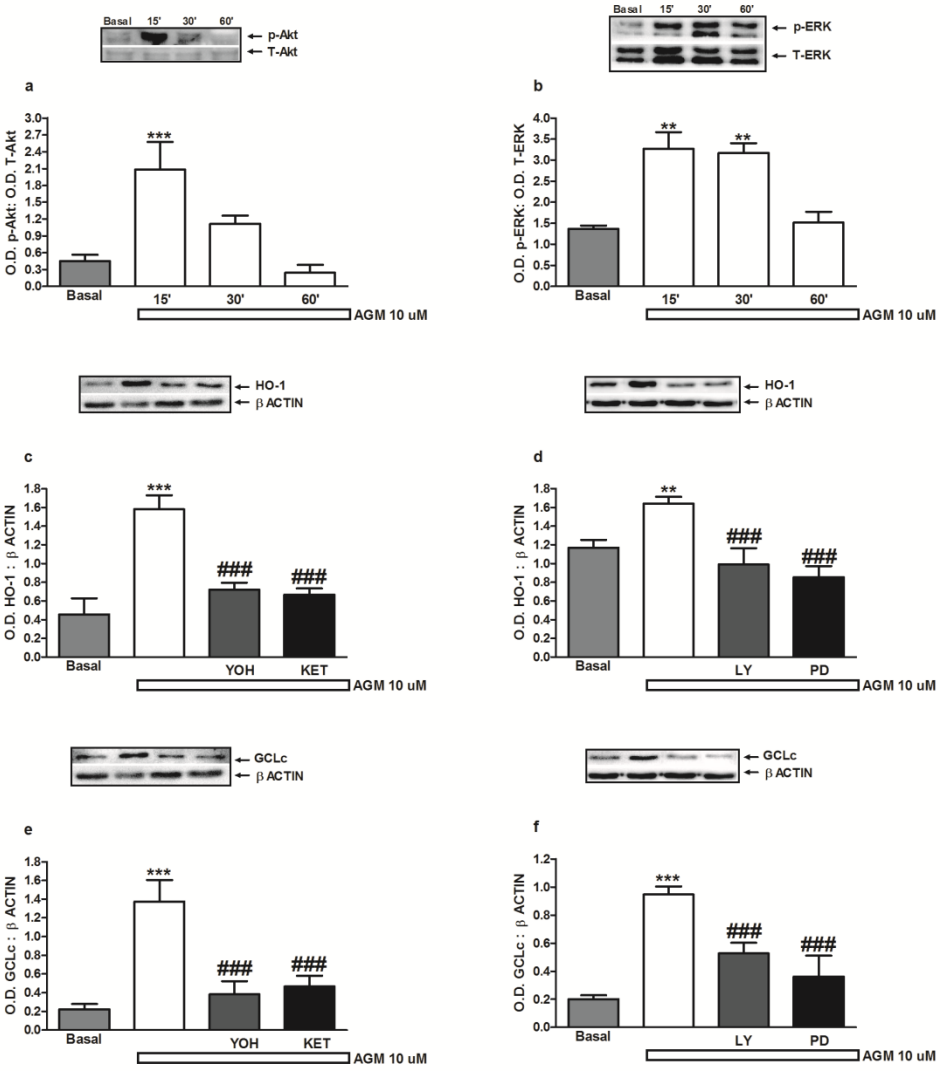
**Fig. 6.**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15'
30'
60'
p-Akt
T-Akt
a
O.D. p-Akt: O.D. T-Akt
***
Basal
15'
30'
60'
AGM 10 uM
b
p-ERK
T-ERK
O.D. p-ERK: O.D. T-ERK
**
**
HO-1
β ACTIN
c
O.D. HO-1 : β ACTIN
***
###
###
YOH
KET
d
LY
PD
GCLc
e
O.D. GCLc : β ACTIN
f

**Fig. 7.**

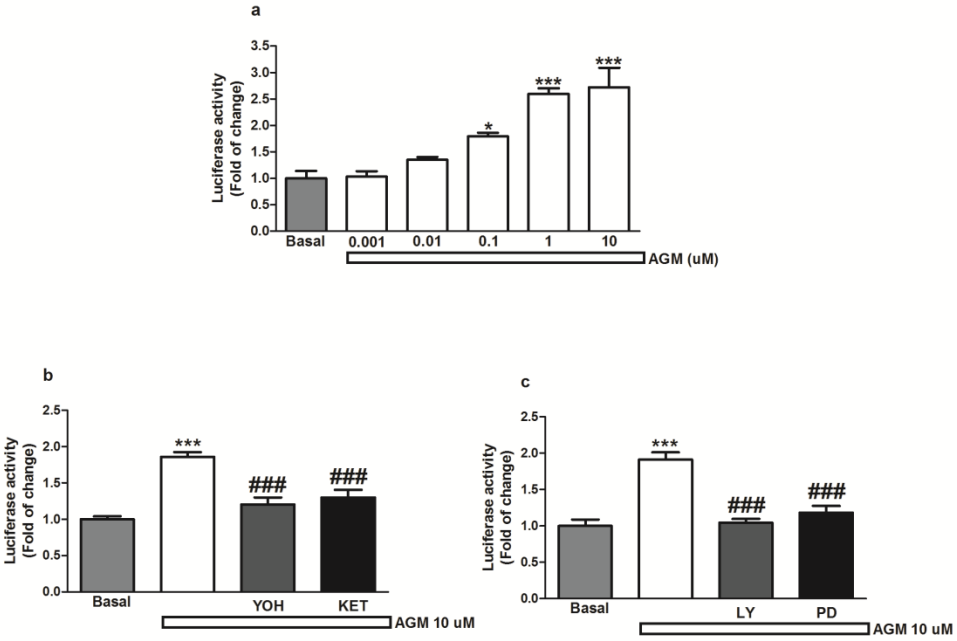
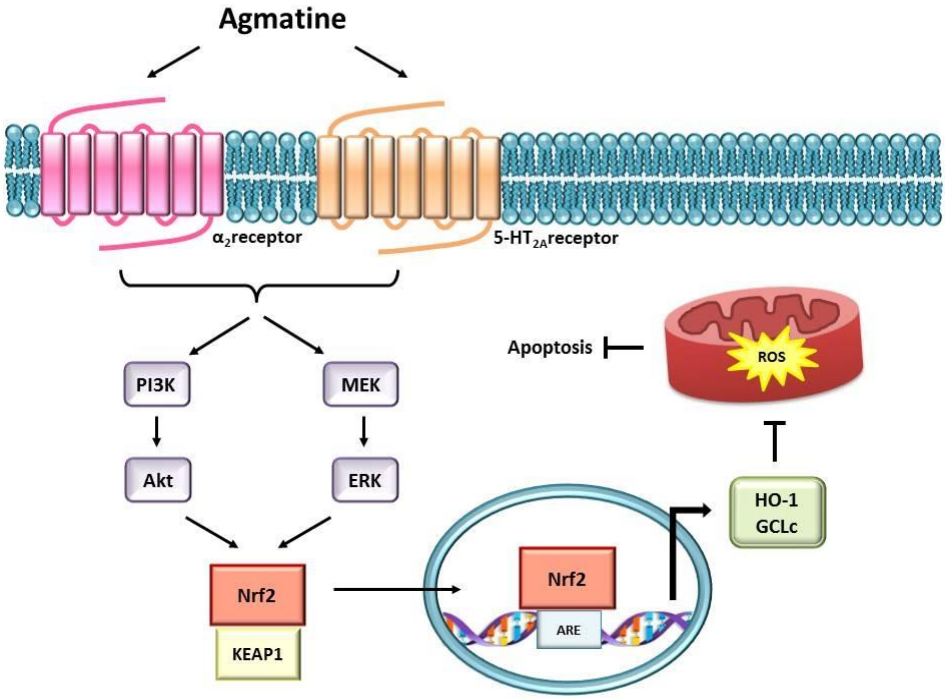

a
Luciferase activity
(Fold of change)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
***
***
Basal
0.001
0.01
0.1
1
10
AGM (uM)
b
Luciferase activity
(Fold of change)
2.5
2.0
1.5
1.0
0.5
0.0
***
###
###
Basal
YOH
KET
AGM 10 uM
c
Luciferase activity
(Fold of change)
2.5
2.0
1.5
1.0
0.5
0.0
***
###
###
Basal
LY
PD
AGM 10 uM

**Fig. 8.**


Agmatine
α2receptor
5-HT2Areceptor
PI3K
MEK
Akt
ERK
Nrf2
KEAP1
Apoptosis
ROS
HO-1
GCLc
Nrf2
ARE

# Capítulo IV

## Agmatina abole comportamento tipo-depressivo induzido por corticosterona em camundongos: Envolvimento do fator de transcrição Nrf2 e de vias de sinalização celular relacionadas à neuroplasticidade

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**Objetivo**

Ampliar os resultados obtidos com a linhagem de células neuronais hipocampais murinas HT22 utilizando o modelo animal de depressão induzida por corticosterona em camundongos no teste da suspensão pela cauda, teste do campo aberto, splash teste e sobre parâmetros bioquímicos: expressão de fator neurotrófico derivado do encéfalo (BDNF) e da proteína sináptica (sinaptotagmina I); participação do fator de transcrição Nrf2; níveis hipocampais dos seguintes neurotransmissores: noradrenalina, serotonina, dopamina e glutamato; e morfologia hipocampal das células glias e astrocitárias.

**Materiais e métodos**

**Animais**

Camundongos Swiss, C57BL/6 selvagem (Nrf2 (+/+)) e Nrf2 KO (Nrf2 (-/-)) fêmeas foram mantidos à temperatura ambiente constante (20-22°C) com livre acesso à água e comida, sob ciclo claro:escuro 12:12 h (luzes acendidas às 07:00 h). Os animais foram colocados na sala experimental 24 h antes dos testes experimentais para ambientação. Todas as manipulações foram realizadas entre 9:00 e 17:00 h. Os procedimentos do presente estudo foram realizados de acordo com o Comitê de Ética de uso de animais em pesquisa do Instituto de Pesquisa em Saúde do Hospital La Paz (IdiPAZ), Diretrizes da Comunidade Européia de 24 de novembro de 1986 (86/609/EEC), e com o Decreto Real Espanhol (RD-1201/2005). Todos os esforços foram feitos para minimizar o sofrimento dos animais e o número de animais utilizados nos experimentos. Nrf2 KO e Nrf2 selvagem foram cordialmente cedidos pelo Dr. Masayuki Yamamoto (Tohoku University, Graduate School of Medicine, Sendai, Japan) (Itoh et al., 1997).

**Drogas e tratamentos**

As seguintes drogas foram utilizadas: agmatina (0,1 mg/kg, Sigma Chemical Co., St. Louis, U.S.A.), corticosterona (20 mg/kg, Sigma Chemical Co., St. Louis, U.S.A.) e imipramina (0,1 mg/kg, Sigma Chemical Co., St. Louis, U.S.A.). Agmatina e imipramina foram dissolvidas em água destilada. Corticosterona foi dissolvida em água destilada contendo 2% Tween 80 e 0,2% DMSO. Todas as drogas foram administradas por um pesquisador experiente pela via oral (p.o.) por gavagem em um volume constante de 10 ml/kg. Veículos apropiados foram utilizados para os diferentes grupos experimentais. As doses das drogas utilizadas foram baseadas na literatura e não causam efeito locomotor (Ago et al., 2013; Freitas et al., 2014; Kong et al., 2014; Lussier et al., 2011; Neis et al., 2014).

**Modelo de depressão induzido por corticosterona**

Camundongos Swiss foram tratados durante 21 dias com agmatina ou imipramina (controle positivo) na dose de 0,1 mg/kg (p.o.) imediatamente antes do tratamento com corticosterona (20 mg/kg, p.o.). Decorridas 24 h da última administração das drogas, os camundongos foram submetidos aos testes comportamentais. Os animais foram divididos nos seguintes grupos: (a) veículo/veículo, (b) imipramina/veículo, (c) agmatina/veículo como grupos controle; (d) veículo/corticosterona, (e) imipramina/corticosterona e (f) agmatina/corticosterona. O número de animais por grupos foi 8-9.

**Envolvimento do fator de transcrição Nrf2 no efeito antidepressivo da agmatina**

Camundongos C57BL/6 Nrf2 selvagem (Nrf2 (+/+)) e Nrf2 KO (Nrf2 (-/-)) foram tratados durante 21 dias com agmatina (0,1 mg/kg, p.o.) ou veículo. Após 24 h da última administração de agmatina ou veículo, os camundongos foram submetidos aos testes comportamentais. Os animais foram divididos nos seguintes grupos: (a) Nrf2 (+/+)/veículo, (b) Nrf2 (+/+)/agmatina como grupos controle; (c) Nrf2 (-/-)/veículo e (d) Nrf2 (-/-)/agmatina. O número de animais por grupos foi 6-7.

**Testes comportamentais**

**Teste da Suspensão pela Cauda (TSC)**

Após 24 h da última administração das drogas, o tempo total de imobilidade em animais submetidos à suspensão pela cauda foi mensurado de acordo com o método descrito por Steru et al. (1985). O tempo de imobilidade foi avaliado durante um período de 6 minutos em camundongos acústica e visualmente isolados, suspensos pelo menos 50 cm acima do chão e presos pela extremidade da cauda com fita adesiva (Freitas et al., 2010, 2013a, 2013b, 2013c, 2014).

**Teste do Campo Aberto**

A fim de excluir a possibilidade de que a diminuição do tempo de imobilidade no TSC seja devido a uma estimulação motora, 60 min após o TSC, os animais foram submetidos ao teste do campo aberto durante 6 minutos (Rodrigues et al., 1996). Este teste foi realizado em uma caixa de madeira medindo 40x60x50 cm, com o chão dividido em 12 quadrados iguais. O número de quadrados cruzados com todas as patas corresponde ao cruzamento e foi o parâmetro utilizado para avaliação da atividade locomotora. O aparato foi limpo com uma solução de 10% etanol entre os testes a fim de remover vestígios do animal anterior.

**Splash teste (teste da borrifagem com sacarose)**

O Splash teste (teste da borrifagem com sacarose) foi realizado após 1 h do teste do compo aberto como descrito previamente por Isingrini et al. (2010). O teste consite na aplicação de uma solução de 10 % de sacarose no dorso de um animal colocado individualmente dentro de uma caixa de acrílico (9 x 7 x 11 cm). A solução de sacarose é palatável e viscosa fazendo com que o animal inicie um comportamento de auto-limpeza. Após a aplicação da solução, o tempo de latência para auto-limpeza e o tempo total de auto-limpeza foram mensurados durante 5 min como um índice de auto-cuidado e comportamento motivacional (Freitas et al., 2013a,c). O aparato foi limpo com uma solução de 10% etanol entre os testes a fim de remover vestígios do animal anterior.

**Análises Bioquímicas**

**Western Blot**

Após os testes comportamentais, os camundongos foram mortos, os encéfalos removidos e os hipocampos rapidamente dissecados, colocados em nitrogênio líquido e armazenados em -80 ºC até serem utilizados. Western blot foi realizado através de método previamante descrito (Freitas et al., 2013a, 2013c, 2014). Os sobrenadantes das amostras foram diluídos a 1/1 (v/v) em Tris 100 mM pH 6,8, EDTA 4 mM, SDS a 8% e aquecidas a 100 °C durante 5 min. Após foi adicionado a amostra uma solução de glicerol a 40%, Tris 100 mM, azul de bromofenol, pH 6,8 na proporção de 25:100 (v/v) e adicionado β-mercaptoetanol (concentração final de 8%). A dosagem de proteína foi realizada pelo método descrito por Peterson (1977) e a concentração protéica estimada a partir de uma curva padrão de albumina bovina. Para a comparação dos sinais obtidos, a mesma quantidade proteica (60 μg por poço) para cada amostra foi eletrotransferida ao minigel de SDS-PAGE 10% e transferidos para as membranas de nitro-celulose ou fluoreto de polivinilideno (PVDF). Para controle da eficiência do processo de transferência, os géis foram corados com Coomassie blue (azul Coomassie R-250 0,1%, metanol 50%, ácido acético 7%) e as membranas com Ponceau 0,5% em ácido acético 1%. As membranas foram bloqueadas com 5% de leite desnatado em TBS (Tris 10 mM, NaCl 150 mM, pH 7,5). O imunoconteúdo de BDNF, de Sinaptotagina I, de HO-1 e de GCLc e das formas fosforilada e total de CREB foram detectadas após incubação com anticorpos específicos diluído em TBS-T (Tris 10 mM, NaCl 150 mM, Tween-20 0,1%, pH 7,5) contendo BSA a 2% em diluições de 1:1000: anti-BDNF (Millipore, Madrid, Spain 1:1000), anti-Sinaptotagmina I (Millipore, Madrid, Spain 1:1000), anti-HO-1 (Chemicon, Hampshire, UK), anti-GCLc (Chemicon, Hampshire, UK), anti-fosfo-CREB ($Ser^{133}$) (Cell Signaling, Madrid, Espanha) e anti-total-CREB (Cell Signaling, Madrid, Espanha). Em seguida, as membranas

foram incubadas com anticorpo secundário, anti-coelho ou anti-camundongo conjugados a peroxidase (HRP) (1:5.000) durante 1 h e a imunorreatividade das bandas foi detectada por quimioluminescência (LumiGLO®). Todas as etapas de bloqueio e incubação foram seguidas por três lavagens (5 min) das membranas com TBS-T. A fim de se assegurar que a mesma quantidade protéica foi adicionada em cada grupo experimental, a quantidade de β-actina foi avaliada usando um anticorpo anti-β-actina (Santa Cruz, 1:1000). A densidade óptica (DO) das bandas foi quantificada usando Scion Image software®. O nível de fosforilação de CREB foi determinado pela razão entre a DO da forma fosforilada/DO da forma total. O imunoconteúdo de BDNF, Sinaptotagmina I, HO-1 e GCLc foi determinado pela relação entr e a DO da banda/DO de β - actina.

**Imunofluorescência para astrócitos e micróglia**

Os camundongos foram anestesiados com isofluorano e transcardiamente perfundidos: primeiro com uma solução salina, seguida de uma solução fixadora (4 % paraformaldeído diluído em 0,1 M de tampão fostato, pH 7,4). Os encéfalos foram então removidos e crioprotegidos durante 2 dias em uma solução 30 % sacarose diluída em 0,1 M tampão fosfato, e cortados em fatias de 30-µm em um micrótomo rotatório (RM2255; Leica, Wetzlar, Alemanha). As fatias foram tratadas com 1 % $H_2O_2$ para inativar a peroxidase endógena, incubadas com uma solução de bloqueio (10 % albumina sérica bovina e 10 % soro normal de cabra diluídos em PBS) durante 1 h. Em seguida, as fatias foram incubadas *overnight* com anticorpos primários anti-GFAP (mascador astrocitário; 1:500; Chemicon International, Temecula, CA) ou anti-Iba-1 (marcador microglial; 1:200; Wako, Richmond, VA), e subsequentemente com anticorpo secundário (anti-mouse) conjugado com fluorocromo (Alexa 488; Invitrogen, Carlsbad, CA) e os núcleos marcados com Hoechst 33342 (Invitrogen, Carlsbad, CA). As lâminas foram montadas e analizadas em microscópio confocal (TCS SPE; Leica, Wetzlar, Alemanha).

**Determinação de neurotransmissores**

Os níveis hipocampais dos seguintes neurotransmissores: noradrenalina (NE), serotonina (5-HT), dopamina (DA) e glutamato (Glu) foram determinadas por cromatografia líquida acoplada a espectrometria de massa (LC-MS/MS) através de método descrito previamente (Gonzalez et al, 2011; Su et al, 2009; Zhu et al, 2011).

**Análise Estatística**

Os resultados foram avaliados através da análise de variância (ANOVA) de duas vias, seguido pelo teste de Duncan quando apropriado. Os resultados foram considerados significativos para $p<0,05$.

## Resultados

### Comportamento depressivo e anedônico induzidos pela corticosterona

Os resultados presentes na Fig. 1a mostram que a administração de corticosterona (20 mg/kg, p.o.) por 21 dias causou um aumento significativo do tempo de imobilidade dos animais no TSC, indicativo de comportamento tipo-depressivo. A administração sub-crônica dos animais com o antidepressivo clássico imipramina (0,1 mg/kg, p.o.) foi capaz de prevenir o comportamento tipo-depressivo induzido pela corticosterona no TSC. O tratamento com imipramina, nos animais controle, também diminuiu significativamente o tempo de imobilidade no TST, quando comparado aos animais tratados com veículo. A ANOVA de duas vias revelou efeitos significativos produzidos pela corticosterona [$F(1,29)=4,20$, $p<0,05$], tratamento [$F(1,29)=69,39$, $p<0,01$] e interação corticosterona x tratamento [$F(1,29)=8,08$, $p<0,01$]. A análise de post-hoc indicou que o tratamento com imipramina preveniu o comportamento tipo-depressivo produzido pela corticosterona ($p<0,01$). Fig. 1b mostra que o tratamento sub-crônico com imipramina (0.1 mg/kg, p.o.) não produziu nenhum efeito sobre a atividade locomotora dos animais no teste do campo aberto. A ANOVA de duas vias revelou nenhuma diferença significativa produzida pela corticosterona [$F(1,29)=0,06$, $p=0,81$], tratamento [$F(1,29)=0,01$, $p=0,92$] e interação corticosterona x tratmento [$F(1,29)=0,02$, $p=0,88$].

Os resultados apresentados na Fig. 1c mostram que o aumento do tempo de latência para auto-limpeza, um indicativo de perda de auto-cuidado e de comportamento motivacional, produzido pela corticosterona (20 mg/kg, p.o.) foi significativamente prevenido pela imipramina (0.1 mg/kg, p.o.). Além disso, o tratamento com imipramina, nos animais controle, também reduziu o tempo de latência, quando comparado aos animais controle tratados com veículo. A ANOVA de duas vias revelou efeitos significativos produzidos pela corticosterona [$F(1,28)=7,38$, $p<0,05$], tratamento [$F(1,28)=29,03$, $p<0,01$] e interação corticosterona x tratamento [$F(1,28)=5,09$, $p<0,05$]. A Fig. 1d mostra que a diminuição do tempo de auto-limpeza causado pela corticosterona, um outro parâmetro indicativo de perda de auto-cuidado e comportamento motivacional foi prevenido pela imipramina. Além disso, o tratamento com imipramina, nos animais controle, também aumentou o tempo de auto-limpeza. A ANOVA de duas vias revelou efeitos significativos produzidos pela corticosterona [$F(1,29)=9,80$, $p<0,01$], tratamento [$F(1,29)=43,67$, $p<0,01$] e interação corticosterona x tratamento [$F(1,29)=4,64$, $p<0,05$]. A análise post-hoc indicou que o tratamento com imipramina preveniu o comportamento anedônico produzido pela corticosterona ($p<0,01$).

**Agmatina abole o comportamento depressivo e anedônico produzido pela corticosterona**

Fig. 2a ilustra que a administração sub-crônica de agmatina (0.1 mg/kg, p.o.) aboliu o comportamento tipo-depressivo induzido pela corticosterona no TSC. Adicionalmente, o tratamento com agmatina, nos animais controle, diminuiu o tempo de imobilidade no TSC, quando comparado aos animais controle tratados com veículo. A ANOVA de duas vias revelou efeitos significativos produzidos pela corticosterona [$F(1,28)=8,66$, $p<0,01$], tratamento [$F(1,28)=236,30$, $p<0,01$] e interação corticosterona x tratamento [$F(1,28)=48,55$, $p<0,01$]. A análise de post-hoc indicou que o tratamento com agmatina preveniu o comportamento tipo-depressivo produzido pela corticosterona ($p<0,01$). A Fig. 2b mostra que o tratamento com agmatina não produziu nenhum efeito no teste do campo aberto. A ANOVA de duas vias revelou nenhuma diferença significativa produzida pela corticosterona [$F(1,28)=0,34$, $p=0,56$], tratamento [$F(1,28)=0,08$, $p=0,78$], e interação corticosterona x tratmento [$F(1,28)=0,0001$, $p=0,99$].

A Fig. 2c mostra que o aumento do tempo de latência para auto-limpeza produzido pela corticosterona (20 mg/kg, p.o.) foi significativamente abolido pelo tratamento sub-crônico com agmatina (0,1 mg/kg, p.o.). O tratamento com agmatina, nos animais controle, também reduziu o tempo de latência, quando comparado aos animais controle tratados com veículo. A ANOVA de duas vias revelou efeitos significativos produzidos pela corticosterona [$F(1,28)=4,17$, $p<0.05$], tratamento [$F(1,28)=35,22$, $p<0,01$] e interação corticosterona x tratamento [$F(1,28)=8,39$, $p<0,01$]. Fig. 2d mostra que a diminuição do tempo de auto-limpeza produzido pela corticosterona foi prevenido pela agmatina. Adicionalmente, o tratamento com agmatina, nos animais controle foi capaz de aumentar o tempo de auto-limpeza. A ANOVA de duas vias revelou efeitos significativos produzidos pela corticosterona [$F(1,29)=7,82$, $p<0,01$], tratamento [$F(1,29)=34,39$, $p<0,01$] e interação corticosterona x tratamento [$F(1,29)=6,26$, $p<0,01$]. A análise post-hoc indicou que o tratamento com agmatina aboliu de maneira significativa o comportamento tipo-depressivo produzido pela corticosterona ($p<0,01$).

**Vias de sinalização envolvidas no efeito antidepressivo da agmatina**

Os resultados presentes na Fig. 3a mostram que o tratamento sub-crônico com agmatina (0.1 mg/kg, p.o.) induziu um aumento significativo da fosforilação de CREB no grupo controle, e ainda causou um aumento sutil, porém não siginificativo no grupo tratado com agmatina e com corticosterona. A ANOVA de duas vias revelou um efeito significativo produzido pelo tratamento [$F(1,16)=11,67$, $p<0,01$], mas não pela corticosterona [$F(1,16)=1,59$, $p=0,23$] e interação corticosterona x tratamento [$F(1,16)=2,06$, $p=0,17$]. A Fig. 3b mostra que o tratamento com agmatina (0,1 mg/kg, p.o.) foi capaz de abolir a redução da expressão de BDNF maduro (BDNFm) produzida pela

corticosterona. Além disso, o tratamento com agmatina causou um aumento significativo da expressão de BDNFm no grupo controle tratado com veículo. A ANOVA de duas vias revelou efeitos significativos produzidos pela corticosterona [$F(1,16)=5,75$, $p<0,05$], tratamento [$F(1,16)=40,77$, $p<0,01$] e interação corticosterona x tratamento [$F(1,16)=5,05$, $p<0,05$]. Adicionalmente, o tratamento sub-crônico com agmatina (0,1 mg/kg, p.o.) reduziu a expressão de BDNF imaturo (pro-BDNF) tanto no grupo tratado com veículo como no grupo tratado com corticosterona. Por outro lado, o tratamento com corticosterona não causou nenhuma alteração significativa sobre a expressão de pro-BDNF (Fig. 3c). A ANOVA de duas vias revelou um efeito significativo produzido pelo tratamento [$F(1,16)=33,94$, $p<0,01$], mas não pela corticosterona [$F(1,16)=0,03$, $p=0,87$] e interação corticosterona x tratamento [$F(1,16)=1,38$, $p=0,26$]. Fig. 3d mostra que o tratamento com agmatina produziu um aumento significativo da razão mBDNF/pro-BDNF em ambos os grupos tratados com veículo e com corticosterona. O tratamento com corticosterona, entretanto, não causou alteração na razão mBDNF/pro-BDNF. A ANOVA de duas vias revelou um efeito significativo produzido pelo tratamento [$F(1,16)=56,64$, $p<0.01$], mas não pela corticosterona [$F(1,16)=0,85$, $p=0,37$] e interação corticosterona x tratamento [$F(1,16)=3,24$, $p=0,09$]. Fig. 3e ilustra que a redução da expressão da proteína de vesícula sináptica, Sinaptotagmina I (Syt I) causada pela corticosterona foi abolida de maneira significativa pela agmatina. Além disso, o tratamento com agmatina nos animais controle também aumentou a expressão de Syt I. A ANOVA de duas vias revelou efeitos significativos produzidos pela corticosterona [$F(1,16)=6,91$, $p<0,05$], tratamento [$F(1,16)=62,75$, $p<0.01$] e interação corticosterona x tratamento [$F(1,16)=4,39$, $p<0,05$].

O presente estudo também verificou a habilidade da agmatina em induzir a expressão de HO-1 e de GCLc hipocampal. O tratamento com agmatina induziu um aumento significativo da expressão de HO-1 e de GCLc tanto no grupo tratado com veículo como no grupo tratado com corticosterona (Fig. 4a e b, respectivamente). A ANOVA de duas vias revelou um efeito significativo produzido pelo tratamento [$F(1,16)=19,27$, $p<0,01$], mas não pela corticosterona [$F(1,16)=0,04$, $p=0,84$] e interação corticosterona x tratamento [$F(1,16)=0,47$, $p=0,50$] para a HO-1 e também um efeito significativo produzido pelo tratamento [$F(1,16)=14,81$, $p<0,01$], mas não pela corticosterona [$F(1,16)=0,18$, $p=0,89$] e interação corticosterona x tratamento [$F(1,16)=2,00$, $p=0,18$] para a GCLc.

**Agmatina abole as alterações das células neurogliais induzidas pela corticosterona**

A imunofluorescência para GFAP, marcador astrocitário, analizada na região CA1 hipocampal revelou uma retração significativa dos processos astrocitários bem como perda de células astrogliais induzidas pela corticosterona (Fig. 5c). O tratamento sub-crônico com agmatina foi capaz de

restabelecer a morfologia e o número de células (Fig. 5d). De maneira similar, a imunofluorescência para Iba-1, marcador microglial, analisada na região CA1 hipocampal revelou que o tratamento com corticosterona induziu uma retração significativa dos processos microgliais bem como perda de células (Fig. 6c). Fig. 6d mostra que o tratamento com agmatina foi capaz de prevenir as alterações na morfologia e no número de células microgliais induzidos pela corticosterona. Nenhuma alteração astrocitária (Fig. 5b) ou microgial (Fig. 6b) foi observada no grupo veículo tratado com agmatina.

**Níveis de noradrenalina, serotonina, dopamina e glutamato hipocampais**

Através de cromatografia líquida acoplada a espectrometria de massa (LC-MS/MS) (Tabela 1) foi verificado que o tratamento sub-crônico com agmatina induziu um aumento significativo dos níveis hipocampais de noradrenalina e de dopamina tanto nos animais tratados com veículo ($p<0,01$) como naqueles tratados com corticosterona ($p<0,01$). Além disso, o tratamento com agmatina aboliu a diminuição dos níveis de serotonina induzido pela corticosterona ($p<0,05$), e ainda, causou um aumento dos níveis de serotonina no grupo controle tratado com veículo ($p<0,01$). Adicionalmente, o tratamento com agmatina foi capaz de diminuir o aumento dos níveis de glutamato produzido pela corticosterona ($p<0,01$).

**Efeito antidepressivo da agmatina é dependente da ativação de Nrf2**

Os resultados presentes na Fig. 7a mostram que o tratamento sub-crônico com agmatina (0,1 mg/kg, p.o.) dos animais C57BL/6 Nrf2 (+/+) produziu um efeito antidepressivo no TSC. Entretando, a habilidade da agmatina em produzir seu efeito antidepressivo no TSC foi abolida nos animais Nrf2 (-/-). Adicionalmente, os animais Nrf2 (-/-) tratados com veículo apresentaram um comportamento tipo-depressivo. A ANOVA de duas vias revelou efeitos significativos produzidos pelo genótipo [$F(1,21)=80,92$, $p<0,01$], tratamento [$F(1,21)=17,66$, $p<0,01$] e interação genótipo x tratamento [$F(1,21)=5,00$, $p<0,05$]. Fig. 7b mostra que a locomoção dos animais não foi alterada em nenhuma condição exprimental. A ANOVA de duas vias não revelou nenhuma diferença significativa produzida pelo genótipo [$F(1,21)=0,34$, $p=0,56$], tratamento [$F(1,21)=0,001$, $p=0,99$] e interação genótipo x tratamento [$F(1,21)=0,94$, $p=0,34$].

Adicionalmente, os animais Nrf2 (-/-) tratados com veículo apresentaram um comportamento tipo-anedônico no splash teste verificado pelo aumento do tempo de latência para auto-limpeza e redução do tempo total de auto-limpeza (Fig. 7c e d, respectivamente). O tratamento dos animais Nrf2 (+/+) com agmatina reduziu o tempo de latência e aumentou o tempo total de auto-limpeza no splash teste. A abilidade da agmatina em abolir um comportamento tipo-anedônico foi abolida nos animais Nrf2 (-/-). A ANOVA de duas vias revelou efeitos significativos produzidos pelo genótipo [$F(1,21)=80,40$, $p<0,01$],

tratamento [F(1,21)=5,96, p<0,01] e interação genótipo x tratamento [F(1,21)=4,95, p<0,05] para a latência para auto-limpeza e efeitos significativos produzidos pelo genótipo [F(1,21)=33.69, p<0,01], tratamento [F(1,21)=4.72, p<0,05] e interação genótipo x tratamento [F(1,21)=6.06, p<0,02] para o tempo total de auto-limpeza.

**Considerações finais**

O presente estudo além de reproduzir muitos dos efeitos observados nas células neuronais hipocampais HT22 em um modelo mais fisiológico (camundongos), ampliou de maneira significativa a literatura sobre os mecanismos implicados no efeito antidepressivo da agmatina. Nosso conjunto de resultados mostrou que a agmatina é capaz de abolir o efeito tipo-depressivo e anedônico produzido pela corticosterona, e que tais efeitos parecem ser mediados pelo menos em parte pelo: i) aumento de monoaminas e diminuição de glutamato sináptico; ii) restabelecimento da quantidade de células e morfologia astrocitária e microglial; iii) ativação do fator de transcrição nuclear CREB, aumento do fator neurotrófico BDNF (maduro) e da proteína de vesícula sináptica Sinaptotagmina I; iv) indução do fator de transcrição Nrf2 e aumento da expressão de HO-1 e de GCLc.

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**Legenda das figuras**

Fig. 1. Efeito do tratamento sub-crônico (21 dias) com o clássico antidepressivo imipramina (0,1 mg/kg, p.o.) nos animais tratados com corticosterona sobre o tempo de imobilidade no TSC (painel a), atividade locomotora no teste do campo aberto (painel b), latência para auto-limpeza (painel c) e tempo de auto-limpeza (painel d) no splash teste. Resultados avaliados por ANOVA de duas vias seguida pelo post-hoc de Duncan. Os valores estão expressos como média + E.P.M. (n = 8-9), *p<0,05 e **p<0,01 quando comparado com o grupo controle (veículo-veículo); ##p<0.01 quando comparado ao grupo veículo-corticosterona.

Fig. 2. Efeito do tratamento sub-crônico (21 dias) com o agmatina (0.1 mg/kg, p.o.) nos animais tratados com corticosterona sobre o tempo de imobilidade no TSC (painel a), atividade locomotora no teste do campo aberto (painel b), latência para auto-limpeza (painel c) e tempo de auto-limpeza (painel d) no splash teste. Resultados avaliados por ANOVA de duas vias seguida pelo post-hoc de Duncan. Os valores estão expressos como média + E.P.M. (n = 8-9), *p<0,05 e **p<0,01 quando comparado com o grupo controle (veículo-veículo); ##p<0.01 quando comparado ao grupo veículo-corticosterona.

Fig. 3. Efeito do tratamento sub-crônico (21 dias) com o agmatina (0.1 mg/kg, p.o.) nos animais tratados com corticosterona sobre a fosforilação de CREB (painéis a e b), imunoconteúdo de mBDNF (painéis c e d), imunoconteúdo de pro-BDNF (painéis e e f), razão mBDNF/proBDNF (painéis g e h) e imunoconteúdo de Syt I (painéis i e j) no hipocampo. Painéis a, c, e, g e i mostram um representativo western blot. Análise quantitativa está ilustrada nos painéis b, d, f, h e j. Os dados estão expressos como a razão entre a forma fosforilada (p-CREB) e total (T-CREB) de CREB, como a razão entre o

conteúdo de mBDNF e pro-BDNF e β-actina, como a a razão entre os valores de mBDNF/proBDNF, e como a razão entre a conteúdo de Syt I e β-actina. Resultados avaliados por ANOVA de duas vias seguida pelo post-hoc de Duncan. Os valores estão expressos como média + E.P.M. (n = 5), **p<0,01 quando comparado com o grupo controle (veículo-veículo); ##p<0.01 quando comparado ao grupo veículo-corticosterona.

Fig. 4. Efeito do tratamento sub-crônico (21 dias) com o agmatina (0.1 mg/kg, p.o.) nos animais tratados com corticosterona sobre o imunoconteúdo de HO-1 (painéis a e b) e de GCLc (painéis c e d) hipocampais. Painéis a e c mostram um representativo western blot. Análise quantitativa está ilustrada nos painéis b e d. Os dados estão expressos como a razão entre o conteúdo de HO-1 e de GCLc e β-actina. Resultados avaliados por ANOVA de duas vias seguida pelo post-hoc de Duncan. Os valores estão expressos como média + E.P.M. (n = 5), *p<0,05 e **p<0,01 quando comparado com o grupo controle (veículo-veículo); ##p<0.01 quando comparado ao grupo veículo-corticosterona.

Fig. 5. Efeito do tratamento sub-crônico (21 dias) com o agmatina (0.1 mg/kg, p.o.) nos animais tratados com corticosterona sobre a morfologia astrocitária da região CA1 hipocampal. Painéis a (veículo-veículo), b (agmatina-veículo), c (veículo-corticosterona) e d (agmatina/corticosterona) mostram uma representativa imunofluorescência. Aumento: 200x.

Fig. 6. Efeito do tratamento sub-crônico (21 dias) com o agmatina (0.1 mg/kg, p.o.) nos animais tratados com corticosterona sobre a morfologia microglial da região CA1 hipocampal. Painéis a (veículo-veículo), b (agmatina-veículo), c (veículo-corticosterona) e d (agmatina/corticosterona) mostram uma representativa imunofluorescência. Aumento: 200x.

Fig. 7. Efeito do tratamento sub-crônico (21 dias) com o agmatina (0,1 mg/kg, p.o.) nos animais Nrf2 selvagem (Nrf2 (+/+)) e Nrf2 KO (Nrf2 (-/-)) sobre o tempo de imobilidade no TSC (painel a), atividade locomotora no teste do campo aberto (painel b), latência para auto-limpeza (painel c) e tempo de auto-limpeza (painel d) no splash teste. Resultados avaliados por ANOVA de duas vias seguida pelo post-hoc de Duncan. Os valores estão expressos como média + E.P.M. (n = 6-7), *p<0,05 e **p<0,01 quando comparado com o grupo Nrf2 (+/+) tratado com veículo (Nrf2 (+/+)-veículo); ##p<0.01 quando comparado ao grupo Nrf2 (+/+) tratado com agmatina (Nrf2 (+/+)-agmatina).

Fig. 1.

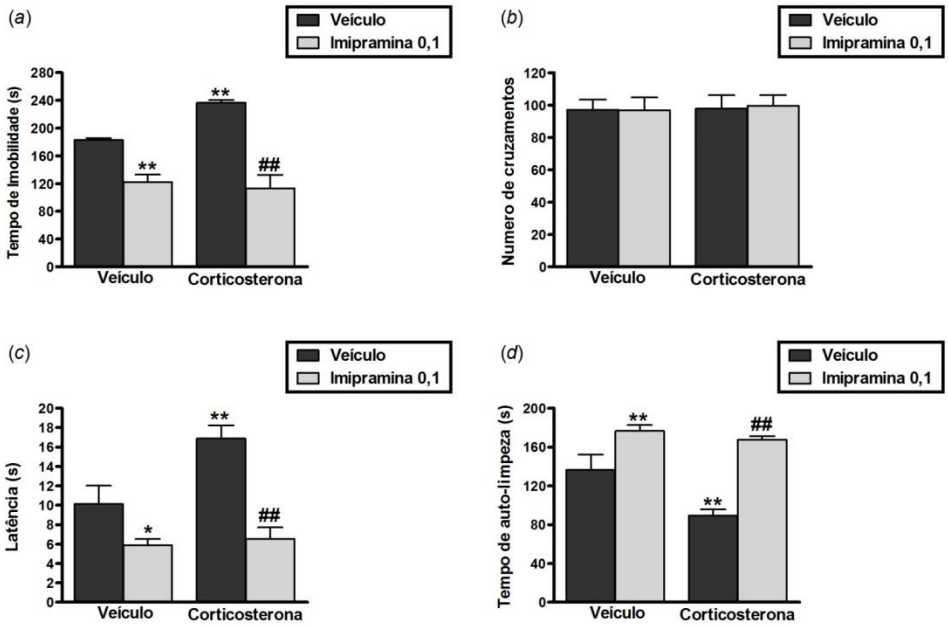
(a)
Veículo
Imipramina 0,1
Tempo de Imobilidade (s)
280
240
200
160
120
80
40
0
**
**
##
Veículo
Corticosterona
(b)
Veículo
Imipramina 0,1
Numero de cruzamentos
120
100
80
60
40
20
0
Veículo
Corticosterona
(c)
Veículo
Imipramina 0,1
Latência (s)
20
18
16
14
12
10
8
6
4
2
0
*
**
##
Veículo
Corticosterona
(d)
Veículo
Imipramina 0,1
Tempo de auto-limpeza (s)
200
160
120
80
40
0
**
**
##
Veículo
Corticosterona

Fig. 2.

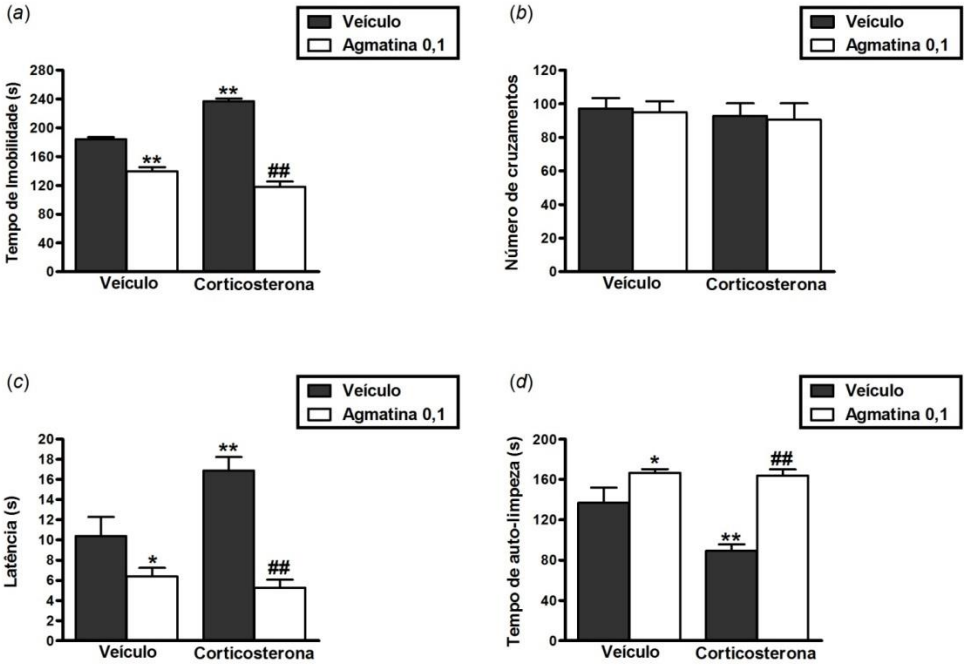
(a)
Veículo
Agmatina 0,1
Tempo de Imobilidade (s)
280
240
200
160
120
80
40
0
**
**
##
Veículo
Corticosterona
(b)
Veículo
Agmatina 0,1
Número de cruzamentos
120
100
80
60
40
20
0
Veículo
Corticosterona
(c)
Veículo
Agmatina 0,1
Latência (s)
20
18
16
14
12
10
8
6
4
2
0
*
**
##
Veículo
Corticosterona
(d)
Veículo
Agmatina 0,1
Tempo de auto-limpeza (s)
200
160
120
80
40
0
*
**
##
Veículo
Corticosterona

Fig. 3.

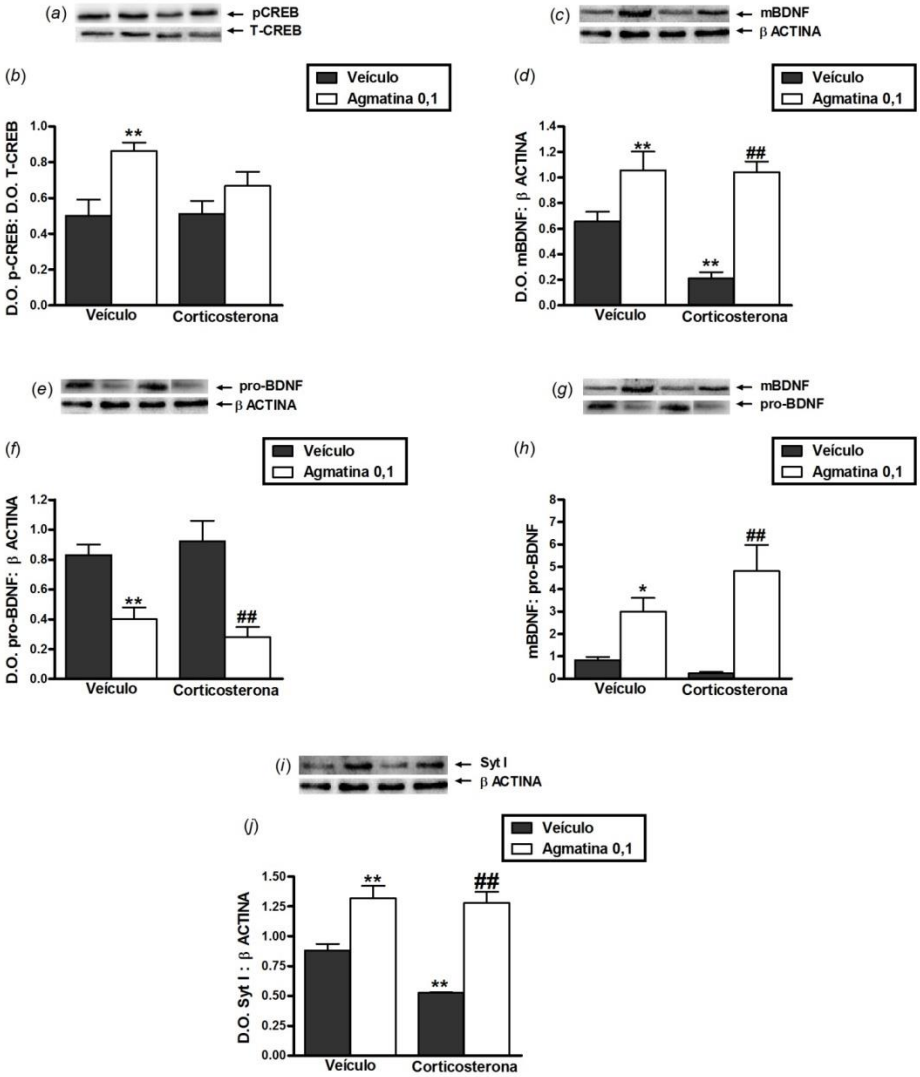
(a)
pCREB
T-CREB
(b)
Veículo
Agmatina 0,1
D.O. p-CREB: D.O. T-CREB
Veículo
Corticosterona
(c)
mBDNF
β ACTINA
(d)
D.O. mBDNF: β ACTINA
(e)
pro-BDNF
β ACTINA
(f)
D.O. pro-BDNF: β ACTINA
(g)
mBDNF
pro-BDNF
(h)
mBDNF: pro-BDNF
(i)
Syt I
β ACTINA
(j)
D.O. Syt I : β ACTINA

Fig. 4.

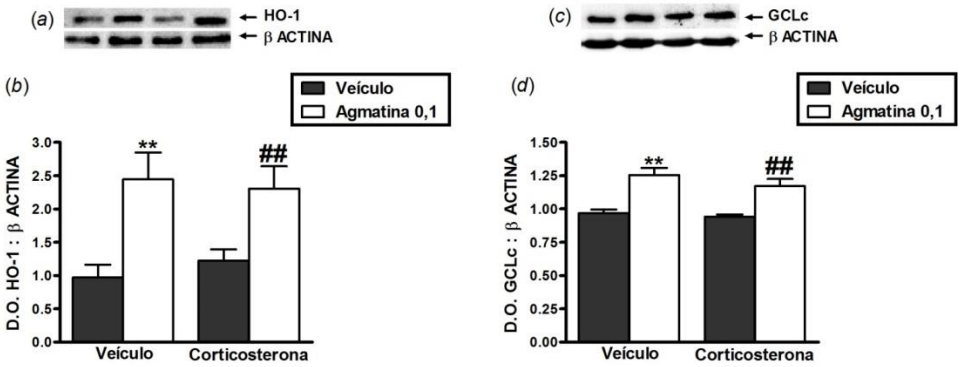
(a)
HO-1
β ACTINA
(c)
GCLc
β ACTINA
(b)
Veículo
Agmatina 0,1
D.O. HO-1 : β ACTINA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
##
Veículo
Corticosterona
(d)
Veículo
Agmatina 0,1
D.O. GCLc : β ACTINA
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
##
Veículo
Corticosterona

Fig. 5.

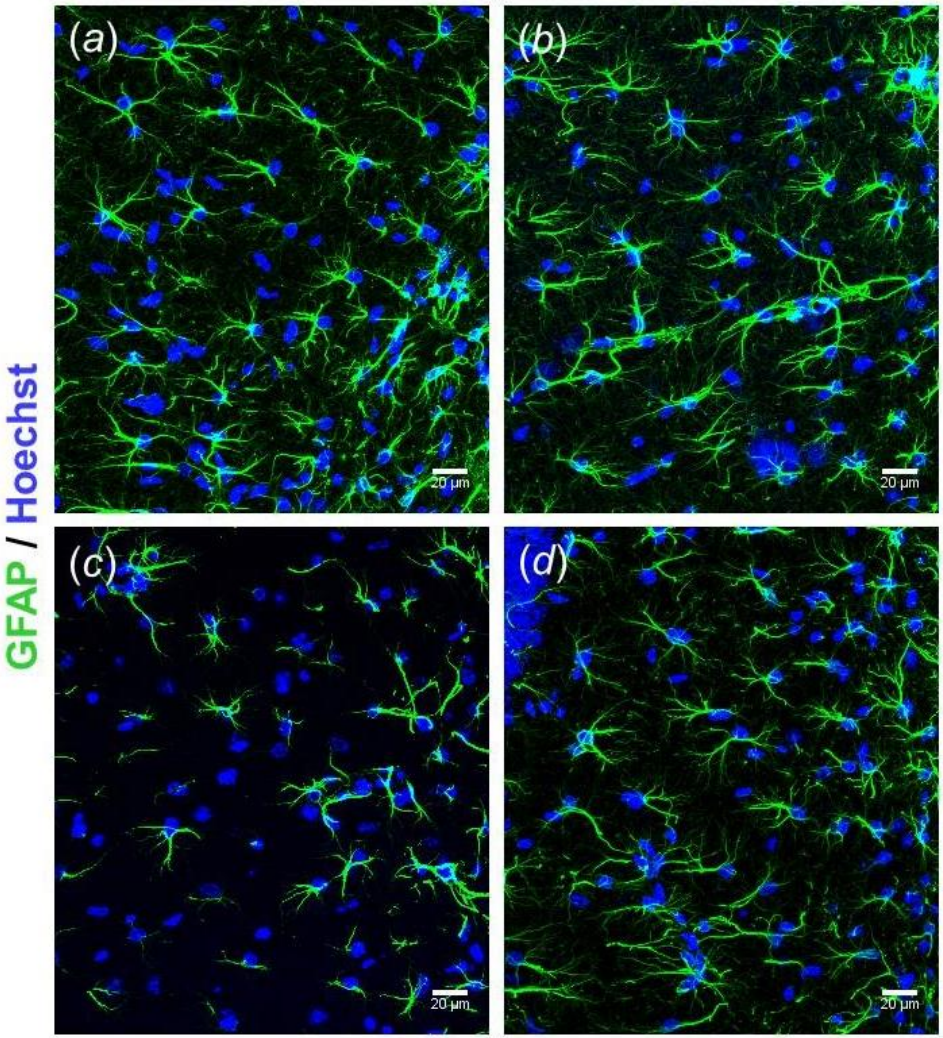

Fig. 6.

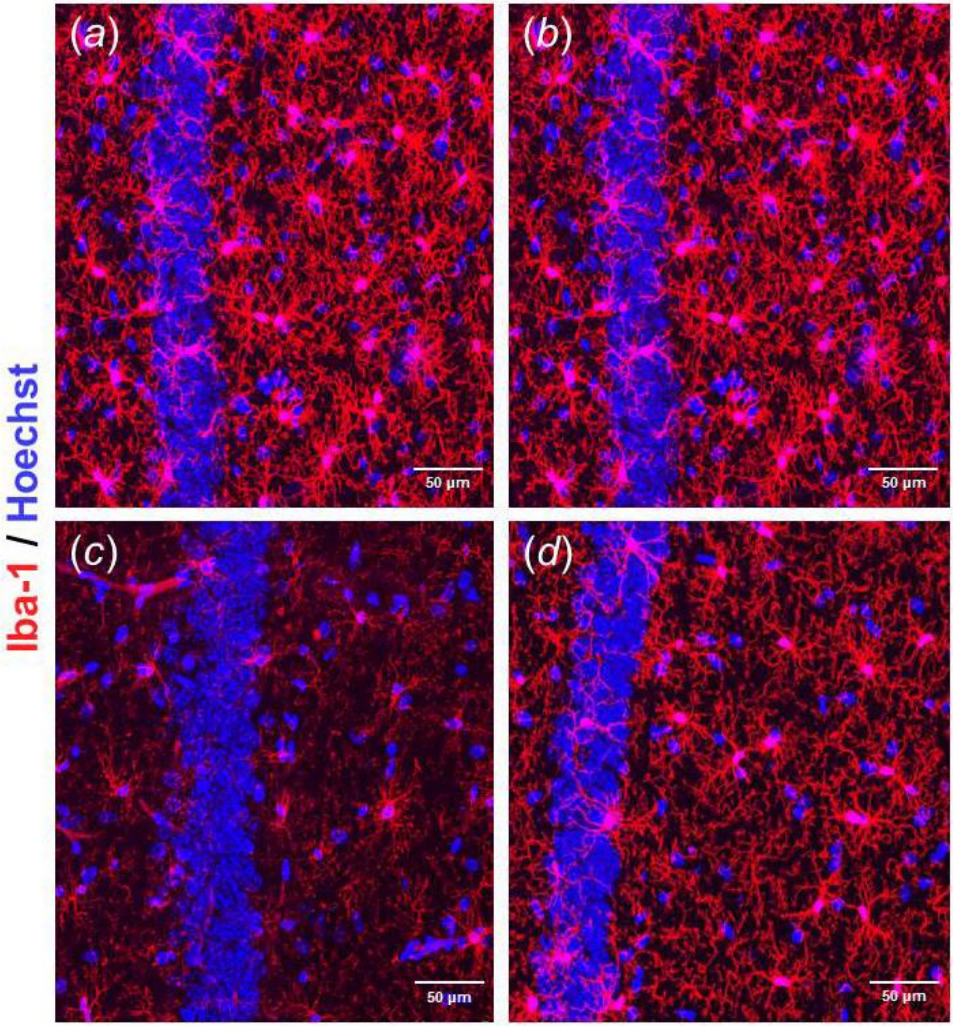

Fig. 7.

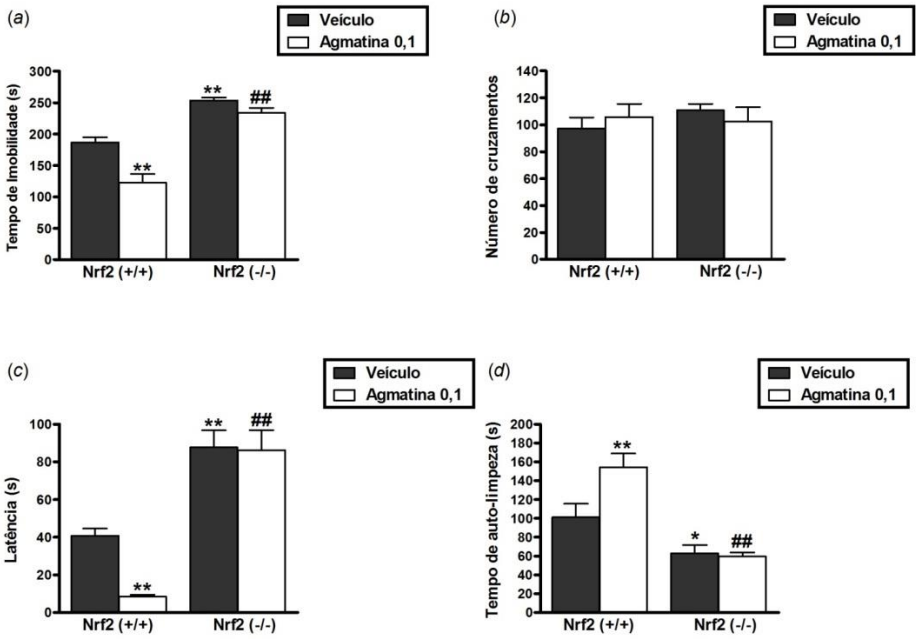


Tabela 1. Efeito do tratamento sub-crônico (21 dias) com o agmatina (0,1 mg/kg, p,o,) nos animais tratados com corticosterona sobre os níveis de transmissores hipocampais medidos por cromatografia líquida acoplada a espectrometria de massa (LC-MS/MS).

| | NA (ng/g tecido) | 5-HT (ng/g tecido) | DA (ng/g tecido) | Glutamato (mg/g tecido) |
|---|---|---|---|---|
| veículo/veículo | 44,9 ± 3,2 | 24,7 ± 2,5 | 1,5 ± 0,1 | 2,6 ± 0,4 |
| agmatina/veículo | 96,5 ± 8,8** | 41,7 ± 6,3** | 14,6 ± 3,9** | 1,7 ± 0,2 |
| veículo/corticosterona | 41,7 ± 4,7 | 11,3 ± 0,9* | 1,4 ± 0,06 | 4,1 ± 0,3** |
| agmatine/corticosterona | 83,6 ± 11,3## | 35,8 ± 5,5## | 11,2 ± 2,1## | 2,4 ± 0,4## |

Resultados avaliados por ANOVA de duas vias seguida pelo post-hoc de Duncan. Os valores estão expressos como média + E.P.M. (n = 5), *p<0,05 e **p<0,01 quando comparado com o grupo controle (veículo/veículo); #*p*<0,05 e ##p<0.01 quando comparado ao grupo veículo/corticosterona.

## 5. Discussão

O presente estudo ampliou significativamente a literatura relacionada aos mecanimos celulares e moleculares implicados no efeito antidepressivo da agmatina em um modelo de neurotoxicidade *in vitro* e em modelos animais de depressão *in vivo*.

Está bem descrito que a primeira hipótese que surgiu para explicar a etiologia da depressão foi a monoaminérgica e que a maioria dos antidepressivos atuais atuam aumentando a transmissão sináptica de monoaminas no cérebro (Schildkraut, 1965; Krishnan e Nestler, 2008). O presente estudo mostrou que os sistemas monoaminérgicos participam do efeito neuroprotetor e antidepressivo da agmatina no modelo de neurotoxicidade induzida por corticosterona nas células neuronais hipocampais HT22, e no modelo de indução de depressão induzido por corticosterona em camundongos, respectivamente. O pré-tratamento das células HT22 com ioimbina (antagonista de receptor $\alpha_2$-adrenérgico), ou com cetancerina (antagonista de receptor 5-$HT_{2A}$) aboliu o efeito citoprotetor produzido pela agmatina frente à toxicidade induzida por corticosterona. Além disso, a agmatina protegeu de maneira sinérgica as células HT22 dos efeitos citotóxicos da corticosterona quando combinada aos antidepressivos clássicos fluoxetina (ISRS) e imipramina (ADT). Por último, no modelo de indução de depressão induzido por corticosterona em camundongos, o tratamento por 21 dias com agmatina induziu um aumento significativo dos níveis hipocampais de noradrenalina, serotonina e dopamina, e ainda, aboliu a diminuição dos níveis de serotonina causada pelo modelo de depressão. Esse conjunto de resultados indica que os efeitos antidepressivos produzidos pela agmatina parecem ser mediados pelo aumento da transmissão noradrenérgica e serotonérgica e subsequente ativação de receptores $\alpha_2$-adrenérgicos e 5-$HT_{2A}$. Além disso, a transmissão dopaminérgica também parece estar envolvida. Nosso conjunto de dados está de acordo com estudos prévios que mostraram a abilidade da agmatina em ativar receptores $\alpha_2$-adrenérgicos (Reis e Regunathan, 1998; 1999; 2000; Piletz et al., 2013). Em relação à participação dos receptors 5-$HT_{2A}$ no efeito antidepressivo produzido pela agmatina, os estudos são um pouco controversos. Krass et al. (2008) mostraram que a administração aguda por via oral de agmatina produziu um efeito tipo-antidepressivo no teste do nado forçado (TNF) em camundongos C57Bl/6/Bkl, e que tal efeito não foi abolido pelo pré-tratamento dos animais durante quatro dias com PCPA (p-clorofenilalanina; inibidor da síntese de serotonina). Apesar dos resultados obtidos por Krass et al. (2008), a participação do sistema serotonérgico não pode ser totalmente descartada, pois o pré-tratamento dos animais com PCPA não havia abolido completamente as reservas de serotonina presentes no córtex pré-frontal dos camundongos (aboliu apenas 70%, restando 30%), medidas por HPLC. Além disso, Shopsin (2013) demonstrou um efeito antidepressivo clínio produzido por agmatina o qual não foi abolido pelo pré-tratamento dos pacientes com PCPA. Vale resultar, que o estudo de Shopsin (2013) foi realizado em apenas três pacientes e que a

completa depleção das reservas de serotonina não foi assegurada. Por outro lado, o estudo de Zomkowski et al. (2004) mostrou a participação dos receptors 5-$HT_{1A/1B}$ e 5-$HT_{2A}$ no efeito tipo-antidepressivo produzido pela agmatina no TNF em camundongos Swiss, e que tal efeito foi abolido pelo pré-tratamento dos animais com PCPA. Além disso, Jiang et al. (2008) mostraram um efeito tipo-antidepressivo produzido pela agmatina no TNF em camundongos através da modulação de receptores $\alpha_2$-adrenérgicos e 5-$HT_{1A/1B}$. Por último, Taksande et al. (2009) reportaram que o a modulação de receptores imidazólicos pela agmatina está implicada no efeito tipo-antidepressivo produzido pelos inibidores seletivos da receptação de serotonina (ISRS), paroxetina e fluoxetina, no TNF em camundongos. De maneira geral, nosso conjunto de dados, juntamente com a literatura, oferece mais evidencias para indicar do que descartar a participação do sistema serotonérgico no efeito antidepressivo produzido pela agmatina. Em relação ao sistema dopaminérgico, Li et al. (2003) mostraram que agmatina protege as PC12 células frente à toxicidade produzida por NMDA, pelo menos em parte, através do restabelecimento dos níveis de dopamina e de noradrenalina diminuídos pelo tratamento com NMDA. Além disso, Neis et al. (2014) mostraram que agmatina produz um efeito sinérgico no teste da suspensão pela cauda (TSC) e abole o comportamento tipo-depressivo induzido por TNF-α, quando combinada aos antidepressivos classicos fluoxetina (ISRS), imipramina (antidepressivo tricíclico, ADT) e bupropiona (inibidor da recaptação de dopamina com atividade sutil sobre a recaptação de noradrenalina) em camundongos.

A hipótese neurotrófica da depressão afirma que o tratamento crônico, mas não agudo, com antidepressivos produz uma rede de alterações neuroplásticas secundárias à modulação monoaminérgica inicial que regulam plasticidade molecular e celular através da ativação de cascatas de sinalização intracelular que ativam o fator de transcrição nuclear CREB, e aumentam expressão do fator neurotrófico BDNF (Pittenger e Duman, 2008; Castrén e Rantamäki, 2010; Duman, 2014). O presente estudo, diferentemente dos estudos anteriores que avaliaram os efeitos produzidos pela agmatina através de sua administração aguda, demonstrou pela primeira vez, que o tratamento sub-crônico com agmatina durante 21 dias modula uma série de vias de sinalização intracelular que regulam sobrevivência celular e neuroplasticidade: PKA, Akt ($Ser^{473}$), GSK-3β ($Ser^{9}$), ERK1/2, CREB ($Ser^{133}$) e JNK1/2, e aumenta o imunoconteúdo de BDNF hipocampal. Além disso, no modelo de indução de depressão induzido por corticosterona foi observado que o tratamento durante 21 dias com agmatina induziu um aumento da fosforilação de CREB e do imunoconteúdo de BDNF hipocampal, e ainda, aboliu a diminuição do imunoconteúdo de BDNF induzida pela corticosterona. Está bem descrito na literatura que a ativação das vias PKA, Akt, ERK1/2, e a inibição de GSK-3β é capaz de ativar CREB por fosforilação em $Ser^{133}$, que por sua vez, induz aumento da expressão de BDNF (Bullock e Habener, 1988; Grimes e Jope, 2001; Nair e Vaidya, 2006; Tardito et al., 2006). Além disso a literatura mostra que a modulação das vias PKA (Dwivedi et al., 2003; Pandey et al., 2005), Akt (Wada, 2009; Freyberg et al.,

2010; Maes et al., 2012; Molteni et al., 2009; Vidal et al., 2011), GSK-3β (Beaulieu et al., 2009; Maes et al., 2012), ERK1/2 (Fumagalli et al., 2005; Todorovic et al., 2009), CREB (Vinet et al., 2004; Nair e Vaidya, 2006; Gumuslu et al., 2013) e JNK1/2 (Harper e LoGrasso, 2001; Borsello e Forloni, 2007; Yasuda et al., 2011) parecem ser alvos promissores para a farmacoterapia antidepressiva. Sabe-se que o aumento da expressão de neurotrofinas como BDNF, mediado pelo tratamento crônico com antidepressivos, regula diferenciação e sobrevivência neuronal, neurogênese, neuroplasticidade e produz efeitos positivos sobre o humor (Pittenger e Duman, 2008; Castrén e Rantamäki, 2010; Cunha et al., 2010; Duman, 2014). Por último, é importante citar que o tratamento com agmatina durante 21 dias aumentou o imunoconteúdo hipocampal da proteína sináptica sinaptotagmina I, e ainda, aboliu a diminuição do imunoconteudo desta proteína induzida pelo tratamento dos animais com corticosterona. Este conjunto de resultados confirma o papel da agmatina na manutenção das sinapses, indução de neuroplasticidade, e de sobrevivência neuronal, que por sua vez, estão implicados na produção de efeitos antidepressivos.

Sabe-se que episódios de estresse induzem hiperatividade do eixo HPA, a qual foi a descoberta biológica mais consistente para o estudo da fisiopatologia da depressão maior (Carroll et al., 1976; Pariante e Lightman, 2008; Frodl e O'Keane, 2013; Maric and Adzic, 2013). Os efeitos deletérios do estresse são explicados pela hipótese inflamatória e oxidativa da depressão, a qual postula que a liberação excessiva de glicocorticóides induz um estado oxidativo e inflamatório no cérebro acompanhado pela produção excessiva de espécies reativas de oxigênio (ERO), diminuição das defesas antioxidantes, peroxidação de lipídeos, morte neuronal hipocampal e redução da neurogênese (Maes et al., 2009; Leonard e Maes, 2012). O presente estudo mostrou que a agmatina é capaz de abolir o comportamento tipo-depressivo induzido por um modelo de depressão induzido por estresse (estresse de contenção), através da manutenção do equilíbrio pró/anti-oxidante hipocampal bem como prevenção da peroxidação lipídica induzidos pelo modelo. Além disso, no modelo de neurotoxicidade induzido por corticosterona, o tratamento das células HT22 com agmatina preveniu os efeitos citotóxicos produzidos, através da diminuição da produção de ERO, da diminuição da apoptose, e pela indução de Nrf2. Já no modelo de indução de depressão induzido por corticosterona em camundongos, foi verificado que o tratamento durante 21 dias com agmatina aboliu o comportamento tipo-depressivo, e que tal efeito parece ser mediado, pelo menos em parte, pela diminuição dos níveis de glutamato hipocampal aumentado pela corticosterona. Sabe-se que o gatilho inicial para a atrofia e morte neuronal induzidas pelos glicocorticóides parece ser o aumento da liberação e da transmissão glutamatérgica (Musazzi et al., 2011; Sanacora et al., 2012). A habilidade da agmatina em inibir a excitotoxicidade glutamatérgica (Yang e Reis, 1999; Zhu et al., 2003; Wang et al., 2006), bem como proteger neurônios hipocampais dos danos causados por glicocorticóides (Zhu et al., 2006), já foi havia sido descrita anteriormente. Por último, sabe-se que o fator

de transcrição nuclear Nrf2 é um regulador central das defesas antioxidantes celulares e das respostas celulares ao estresse (Johnson et al., 2008), e que ativadores deste fator parecem ser um alvo promissor para o desenvolvimento de novos e melhores tratamentos para a depressão (Maes et al., 2012; Lee et al., 2013; Martín-de-Saavedra et al., 2013). Neste sentido, a principal contribuição do presente trabalho foi mostrar, pela primeira vez, a participação de Nrf2 no mecanismo de ação antidepressiva e neuroprotetora da agmatina. Tal evidência foi verificada inicialmente utilizando as células HT22, e os camundongos Swiss Nrf2 (+/+), e que posteriormente, foi confirmada utilizando os camundongos C57BL/6 Nrf2 (-/-).

Adicionalmente, a literatura mostra que não são apenas os neurônios os afetados pelos glicocorticóides, e que disfunções e alterações morfológicas das células gliais também são observadas (Czéh et al., 2013; Popoli et al., 2011; Sanacora e Banasr, 2013). Nesse sentido, o presente estudo também avaliou possíveis alterações na morfologia neuroglial, mais especificamente astrocitária e microglial, induzidas pelo modelo de indução de depressão por corticosterona em camundongos, e a abilidade da agmatina em abolir tais alterações. O modelo de depressão induziu uma retração significativa dos processos astrocitários e microgliais e redução do número celular na região CA1 hipocampal, as quais foram prevenidas pelo tratamento com agmatina. Estes resultados estão de acordo com a literatura que relata que os glicocorticóides causam disfunção, atrofia e redução do número de células gliais hipocampais e que o tratamento antidepressivo abole tais alterações (Czéh et al., 2013; Popoli et al., 2011; Sanacora e Banasr, 2013). A atrofia das células gliais causada pelos glicocorticóides diminui a reciclagem de glutamato sináptico, coordenado pelas células glias através do ciclo glutamato-glutamina. Desta meneira, o excesso de glutamato induz excitotoxicidade, a qual está intimamente relacionada à patofisiologia de depressão (Popoli et al., 2011). Considerando que um dos mecanismos da agmatina é justamente inibir a excitotoxicidade glutamatérgica (Yang e Reis, 1999; Zhu et al., 2003; Wang et al., 2006), pode-se especular que a inibição da excitotoxicidade produzida pelo excesso de glutamato parece ser um dos mecanismos pelos quais a agmatina atue no sentido de restabelecer a morfologia e função glial.

Com base nos resultados obtidos no presente estudo, podemos concluir que a agmatina parece produzir seus efeitos antidepressivos atuando sobre as principais hipóteses que explicam a fisiopatologia da depressão e o mecanismo de ação da terapia antidepressiva. A agmatina atua através do: i) aumento dos níveis de monoaminas sinápticas (hipótese monoaminérgica); ii) aumento da expressão do fator neurotrófico BDNF, através modulação de vias de sinalização intracelular, induzindo neuroplasticidade e aumento da proteína Sinaptotagmina I (hipótese neurotrófica); iii) e diminuição da produção de ERO, de apoptose, inibição da peroxidação lipídica, manutenção do equilíbrio pró/antioxidante, destacando-se ainda sua habilidade em ativar o fator de transcrição Nrf2 (hipótese oxidativa); e iv) inibição da excitotoxicidade

glutamatérgica, e da atrofia neuroglial (hipótese glutamatérgica) (**Figura 9)**. Esse conjunto de resultados oferece evidencias para sugerir que a agmatina tem potencial para atuar como adjuvante/monoterapia para o tratamento da depressão, entretando, estudos posteriores especialmente em humanos, ainda são necessários.

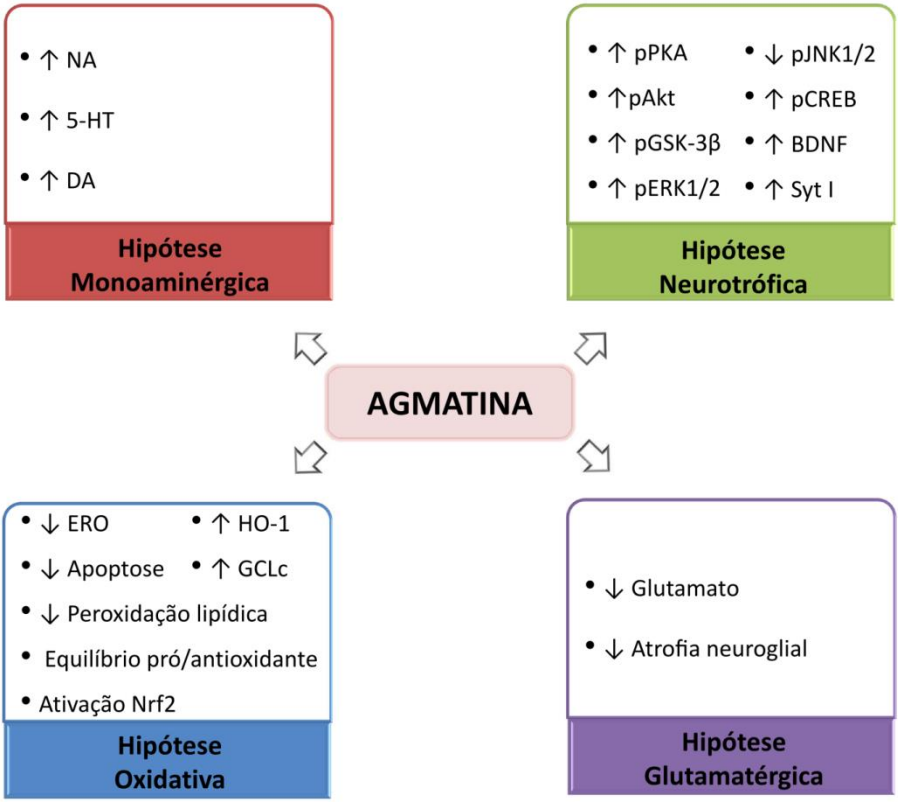


**Figura 9. Principais alvos mocelulares e celulares implicados no efeito antidepressivo da agmatina verificados a partir de estudos *in vitro* e *in vivo*.** Com base nos resultados obtidos no presente estudo, podemos concluir que a agmatina parece produzir seus efeitos antidepressivos atuando sobre as principais hipóteses que explicam a fisiopatologia da depressão e o mecanismo de ação da terapia antidepressiva. A agmatina atua através do: i) aumento dos níveis de monoaminas sinápticos (hipótese monoaminérgica); ii) aumento da expressão do fator neurotrófico BDNF, através modulação de vias de sinalização intracelular PKA/Akt/GSK-3β/ ERK1/2/JNK1/2/CREB induzindo neuroplasticidade e aumento da proteína Sinaptotagmina I (hipótese

neurotrófica); iii) e diminuição da produção de ERO, de apoptose, inibição da peroxidação lipídica, manutenção do equilíbrio pró/antioxidante, destacando-se ainda sua habilidade em ativar o fator de transcrição Nrf2, e induzir a expressão de HO-1 e GCLc (hipótese oxidativa); e iv) inibição da excitotoxicidade glutamatérgica e da atrofia neuroglial (hipótese glutamatérgica). 5-HT, serotonina; BDNF, fator neurotrófico derivado do encéfalo; CREB, proteína de ligação responsiva ao AMP cíclico; DA, dopamina; ERK1/2, cinase regulada por sinal extracelular 1/2; ERO, espécies reativas de oxigênio; GCLc, subunidade catalítica da glutamato cisteína ligase; GSK-3β, glicogênio sintase cinase-3β; HO-1, heme oxigenase I; JNK1/2, c-Jun N-terminal cinase 1/2; NA, noradrenalina; Nrf2, fator nuclear eritróide 2 relacionado ao fator 2; PKA, proteína cinase A; Syt I, sinaptotagmina I.

## 6. Conclusão

Com base nos resultados obtidos *in vitro* e *in vivo* descritos nos capítulos I, II, III e IV, podemos concluir que:

- O tratamento sub-crônico (21 dias) com agmatina em camundongos produziu um efeito antidepressivo no TSC acompanhado pela modulação de vias de sinalização intracelular envolvidas em sobrevivênvia celular e neuroplasticidade PKA/Akt/GSK-3β/ERK/JNK/CREB/BDNF hipocampal;
- O tratamento agudo com agmatina em camundongos foi capaz de abolir o comportamento tipo-depressivo induzido pelo estresse de contenção, e ainda, preveniu a peroxidação lipídica e as alterações nas atividades das anzimas SOD, GR, CAT e no índice SOD/CAT hipocampais induzidas pelo modelo de estresse;
- O tratamento das células neuronais hipocampais murinas HT22 com agmatina aboliu a citotoxicidade, produção de ERO e apoptose induzidos por corticosterona através da ativação dos receptores $\alpha_2$-adrenérgicos e 5-$HT_{2A}$, sinalização mediada por Akt e ERK1/2, ativação do fator de transcrição nuclear Nrf2 e indução da expressão de HO-1 e GCLc;
- O tratamento durante 21 dias com agmatina em camundongos aboliu o comportamento tipo-depressivo induzido por corticosterona através do aumento de monoaminas, redução de glutamato, restabelecimento da morfologia neuroglial, expressão de BDNF e de Sinaptotagmina I, ativação de Nrf2 e indução da expressão de HO-1 e GCLc hipocampais.

## 7. Perspectivas

- Investigar a participação do sistema dopaminérgico no efeito antidepressivo produzido pela agmatina no TSC utilizando antagonistas específicos e ensaios bioquímicos;
- Avaliar a habilidade do tratamento crônico com agmatina em induzir neurogênse utilizando ferramentas histológicas;
- Expandir os estudos sobre o efeito antidepressivo clínico produzido pela agmatina em pacientes deprimidos bem como os mecanismos bioquímicos envolvidos.

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