Josiane Budni

INVESTIGAÇÃO DA AÇÃO ANTIDEPRESSIVA E NEUROPROTETORA DO ÁCIDO FÓLICO

Tese apresentada ao Programa de Pós-Graduação em Bioquímica do Centro de Ciências Biológicas da Universidade Federal de Santa Catarina, como requisito parcial para a obtenção do título de doutor.
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"Investigação da ação antidepressiva e neuroprotetora do ácido fólico"

por

Josiane Budni

Tese julgada e aprovada em sua forma final pelos membros titulares da Banca Examinadora (Port. 09/PPGBQA/2012) do Programa de Pós-Graduação em Bioquímica - UFSC, composta pelos Professores Doutores:

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Florianópolis, 24 de fevereiro de 2012.

“A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original”
(Albert Einstein)

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RESUMO

Ácido fólico é essencial para o funcionamento do sistema nervoso central, uma vez que desempenha um importante papel na neuroplasticidade e manutenção da integridade neuronal. O ácido fólico apresenta muitas propriedades importantes, como neuroprotetora, antidepressiva e cognitiva. O presente trabalho realizado através de estudos *in vivo* e *in vitro* avaliou os efeitos tipo-antidepressivo (teste do nado forçado, TNF, ou estresse agudo de contenção), cognitivo (TRO, teste do reconhecimento de objeto) e neuroprotetor (protocolos de morte celular induzidas por dexametasona ou glutamato) do ácido fólico. Os resultados dos estudos *in vivo* mostram que uma dose sub-ativa de ácido fólico (10 mg/Kg, p.o.) combinada com doses sub-ativas de inibidores da GSK-3β (glicogênio sintase cinase-3β) (AR-A014418 ou lítio), agonista PPARγ (receptor ativado por proliferador peroxissomal-γ) (rosiglitazona) ou inibidores de canais de potássio (K^+) (glibenclamida, caribdotoxina ou apamina), produziram um efeito tipo-antidepressivo sinérgico no TNF em camundongos. Por outro lado, o pré-tratamento dos animais com um inibidor da PI3K (fosfoinositol 3-cinase) (LY294002), um agonista PPARγ (GW-9662) ou um ativador de canais de K^+ (cromacalim) reverteu o efeito tipo-antidepressivo de uma dose ativa de ácido fólico (50 mg/Kg, p.o.). Além disso, outro estudo *in vivo* mostrou que o ácido fólico (50 mg/kg, p.o.) administrado 1 h antes do estresse de contenção foi capaz de proteger contra o aumento do tempo de imobilidade induzido pelo estresse no TNF, mas não foi efetivo contra o prejuízo de memória no TRO. Adicionalmente, o estresse agudo de contenção promoveu aumento dos níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) e das atividades da catalase (CAT), glutationa peroxidase (GPx) e glutationa redutase (GR) no córtex cerebral e hipocampo, e aumento da atividade da superóxido dismutase (SOD) somente no hipocampo. O tratamento com ácido fólico restaurou a atividade da SOD, CAT, GR e GPx e reduziu os níveis de TBARS no hipocampo. Contudo, glutationa (GSH), um antioxidante não-enzimático não foi alterada pelo estresse, nem mesmo pelo ácido fólico. Além disso, um estudo *in vitro* mostrou que o pré-tratamento com ácido fólico (10-300 μM) reduziu a toxicidade induzida por dexametasona (1mM), de maneira concentração-dependente, em linhagem de células neuroblastoma humano SH-SY5Y. Este efeito neuroprotetor foi revertido por um inibidor da PI3K/Akt (LY294002), proteína cinase dependente de Ca^{2+}/calmodulina II (CaMKII, KN-93) e proteína cinase A (PKA, H-89), mas não pelo inibidor da proteína cinase

ativada por mitógeno/cinase regulada por sinal extracelular (MEK 1/2, PD98059) e proteína cinase C (PKC, queleritrina).Um adicional estudo *in vitro*, também mostrou que o tratamento de fatias hipocampais de ratos com ácido fólico (100 μM) reduziu significativamente a morte celular e a liberação de D-[3H]aspartato induzidos pelo glutamato (1mM), os quais foram abolidos pela presença de LY294002. Além disso, as fatias hipocampais incubadas por 30 minutos com ácido fólico *per se* induziu fosforilação da GSK-3β. Adicionalmente, ácido fólico na presença de glutamato (decorridos 6 h de incubação das fatias em meio, depois da retirada do glutamato), induziu fosforilação da GSK-3β e expressão da β-catenina. O ácido fólico também reverteu o aumento da expressão da iNOS (óxido nítrico sintase induzida) promovido por glutamato. Estes resultados em conjunto, indicam que o efeito tipo-antidepressivo do ácido fólico no TNF pode ser dependente da modulação da via PI3K/Akt/GSK-3β, inibição de canais de K^+ e ativação do receptor PPARγ, bem como, desempenhar um específico perfil antidepressivo, pelo menos em parte, devido ao seu papel antioxidante. Além disso, o efeito neuroprotetor do ácido fólico contra os estímulos tóxicos, dexametasona ou glutamato, pode ser dependente PI3K/GSK-3β/β-catenina e iNOS, respectivamente.

Palavras-chave: ácido fólico, depressão, neuroproteção, teste do nado forçado, estresse de contenção, fatias de glutamato, estresse oxidativo.

ABSTRACT

Folate is essential for the functioning of the nervous system, since it plays an important role in neuroplasticity and in the maintenance of neuronal integrity. Many roles for folic acid have been reported, including neuroprotective, antidepressant and cognitive properties. The present work using an *in vivo* and *in vitro* approach evaluated the antidepressant-like (forced swimming test, FST or acute restraint stress), cognitive (ORT, object recognition test) and neuroprotective (dexamethasone or glutamate-induced cell death protocols) effects of folic acid. The results from *in vivo* studies showed that a sub-effective dose of folic acid (10 mg/Kg, p.o.) combined with sub-effective doses of GSK-3β (glycogen synthase kinase-3β) inhibitors (AR-A014418 or lithium), PPARγ (peroxisome proliferator-activated receptor-γ) agonist (rosiglitazone) or potassium (K^+) channel inhibitors (glibenclamide, charibdotoxin or apamin), elicited synergistic antidepressant-like effect in the mouse FST, while the pre-treatment of animals with a PI3K (phosphoinositide 3-kinase) inhibitor (LY294002), a PPARγ antagonist (GW-9662) or a K^+channel opener (cromakalim) reversed the antidepressant-like effect of an active dose of folic acid (50 mg/Kg, p.o.). Moreover, another *in vivo* study showed that folic acid (50 mg/kg, p.o.) administred 1 h before restraint stress was able to protect against the stress-induced depressive-like effect in the FST, but not the memory impairment in the ORT. Moreover, acute restraint stress increased thiobarbituric acid reactive substances (TBARS) levels and catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities in the cerebral cortex and hippocampus, and superoxide dismutase (SOD) activity in the hippocampus. Folic acid treatment restored the activity of SOD, CAT, GR and GPx and reduced TBARS levels in the hippocampus. Glutatione (GSH), a non-enzymatic antioxidant was not altered by stress and/or folic acid administration. Additionaly, an *in vitro* study found that folic acid pretreatment (10-300 μM) reduced dexamethasone (1mM)-induced toxicity in a concentration dependent manner in SH-SY5Y neuroblastoma cell line. This neuroprotective effect was reversed by the PI3K/Akt (LY294002), calcium/calmodulin-dependent protein kinase II (CaMKII, KN-93) and protein kinase A (PKA, H-89) inhibitors, but not the mitogen-activated protein/extracellular signal-regulated kinase (MEK 1/2, PD98059) and protein kinase C (PKC, Chelerythrine) inhibitors. A further *in vitro* study, also showed that the treatment of hippocampal slices with folic acid (100 μM) significantly reduced glutamate (1mM)-induced cell

death and D-[3H]aspartate release, which were abolished by LY294002. Moreover, hippocampal slices incubated with folic acid *per se* for 30 minutes induced GSK-3β phosphorylation. Furthermore, folic acid in presence of glutamate insult, in hippocampal slices maintained for an additional period of 6 h in fresh culture medium without glutamate and/or folic acid, induced phosphorylation of GSK-3β and β-catenin expression. In addition, folic acid was able to reverse the increase on iNOS expression induced by glutamate. Altogether, these results indicate that the antidepressant-like effect of folic acid in the FST might be dependent on the modulation of PI3K/Akt/GSK-3β pathway, inhibition of K^+ channels and activation of PPARγ. In addition, the antidepressant activity of folic acid in the restraint stress paradigm may be at least partly due to its antioxidant role. Moreover, the neuroprotective effect of folic acid against the toxic insults, dexametasone or glutamate, might be dependent on signaling pathway that involves PI3K/Akt, CaMKII and PKA or PI3K/GSK-3β/β-catenin and iNOS, respectively.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVEATURAS

5-HT	Serotonina
5-MTHF	5-metiltetrahidrofolato
AMPA	Alfa-amino 3-hidróxi 5-metilisoxazol ácido propiônico
AMPc	Adenosina monofosfato cíclico
BDNF	Fator neurotrófico derivado do cérebro
CaMKII	Cálcio calmodulina cinase II
CAT	Catalase
CREB	Proteína ligante ao elemento responsivo ao AMPc
DA	Dopamina
DSM-IV	Manual Estatístico e Diagnóstico de Transtornos Mentais-IV
ECS	Terapia eletroconvulsiva
ERNs	Espécies reativas de nitrogênio
EROs	Espécies reativas de oxigênio
$G_{i/o}$	Proteína G inibitória
GIRK	Canais de potássio retificadores interno ativados por proteína G
GMPc	Guanosina 3´5-monofosfato cíclico
GPx	Glutationa peroxidase
GR	Glutationa redutase
GSH	Glutationa
GSK-3β	Glicogênio sintase cinase-3β
GSSH	Glutationa oxidada
H_2O_2	Peróxido de hidrogênio
$HO^{\cdot}$	Radical hidroxila
HPA	Eixo hipotálamo-pituitária-adrenal
iNOS	Óxido nítrico sintase induzida
ISRS	Inibidores seletivos de recaptação de serotonina
Kir	Canais de potássio retificadores internos
MAO A	Monoamina oxidase A
mGluR	Receptores metabotrópicos de glutamato acoplados a proteína G
MTHFR	Metilenotetrahidrofolato redutase
NADPH	Nicotinamida adenina dinucleotídeo fosfato
NE	Noradrenalina
NMDA	N-metil D-aspartato
nNOS	Óxido nítrico sintase neuronal
NO	Óxido nítrico
NOS	Óxido nítrico sintase

NPY Y_1	Receptores de neuropeptídio Y
$O_2^{\cdot -}$	Ânion superóxido
PABA	Ácido p-aminobenzóico
PDK1	Cinase 1 dependente de fosfatidil inositol
PI3K	Fosfoinositol 3-cinase
PKA	Proteína cinase A
PKC	Proteína cinase C
PPARγ	Receptor ativado por proliferador peroxissomal-γ
SAM	S-adenosilmetionina
SERT	Transportador de 5-HT
SNC	Sistema nervoso central
SOD	Superóxido dismutase
TBARS	Substâncias reativas ao ácido tiobarbitúrico
TEA	Tetraetilamônio
THF	Tetrahidrofolato
TNF	Teste do nado forçado
TNF-α	Fator de necrose tumoral- α
TREK-1	Canal de K^+ de dois poros
TRO	Teste de reconhecimento de objetos
TSC	Teste de suspensão da cauda

SUMÁRIO

1. INTRODUÇÃO

DEPRESSÃO MAIOR

A depressão maior encontra-se entre os maiores problemas de saúde pública do mundo (Nestler e Carlezon, 2006; Nakajima et al., 2010). Estima-se que a taxa de prevalência da depressão e de transtornos de humor relacionados ao estresse na vida de um indivíduo seja em torno de 17 % resultando em enorme sofrimento pessoal, social e de alto custo financeiro (Duman e Voleti, 2012). Além disso, pacientes que sofrem de depressão severa apresentam altas taxas de comorbidades e mortalidade (Nemeroff, 2007).

O Manual Estatístico e Diagnóstico de Transtornos Mentais (DSM-IV) define a depressão como um episódio depressivo caracterizado por manifestações, por um período superior a duas semanas, de 5 ou mais sintomas, os quais incluem, humor deprimido (choro, sentimento de vazio, tristeza), anedonia, sentimentos de culpa, ideação suicida, mudanças no peso, apetite, sono, energia e função psicomotora e reduzida capacidade de pensar ou concentrar-se (DSM-IV-TR, 2000). O diagnóstico não é baseado na etiologia ou biologia da depressão. Além disso, acredita-se que múltiplos transtornos psiquiátricos, incluindo a depressão, provavelmente reflitam diversas patogêneses induzindo uma variada constelação de sintomas depressivos (Loftis et al., 2010), o que torna complexo o conhecimento dos seus mecanismos fisiopatológicos e em consequência disso, dificulta a descoberta de novos fármacos antidepressivos.

Em tese, não se conhece todos os mecanismos biológicos da depressão, mas já é possível identificar muito alvos. Portanto, a depressão pode resultar, pelo menos em parte, por uma deficiência na atividade monoaminérgica no cérebro (Elhwuegi, 2004). Além desses, vários outros sistemas de neurotransmissores e mecanismos de transdução de sinal estão envolvidos, como os receptores N-metil-D-aspartato (NMDA) e a via da L-arginina-óxido nítrico (NO) (Harkin et al., 1999; Sanacora et al., 2008; Ghasemi et al., 2009; Zomkowski et al., 2010), o sistema opióide (Brocardo et al., 2009; Negus et al., 2011), o sistema GABAérgico (Nakagawa et al., 1996; Cryan e Slattery, 2010), canais de cálcio (Galeotti et al., 2006), aumento dos níveis plasmáticos de glicocorticóides e desregulação do eixo hipotálamo-pituitária-adrenal (HPA) (Perera et al., 2007; Pittenger e Duman, 2008; Kunugi et al., 2010). A depressão pode ser desencadeada também por citocinas pró-inflamatórias como o fator de necrose tumoral- α (TNF-α), interleucina-

1, interleucina-6 e interfereron-α (Maes et al., 2009; Miller et al., 2009; Kaster et al., 2012).

Considerando a depressão como uma doença multifatorial e de etiologia pouco determinada, além dos sistemas acima envolvidos, a mesma pode ser decorrente da exposição a eventos estressantes na vida de um indivíduo (Henn e Vollmayre, 2005; Mann e Currier, 2010), que são estudados pré-clinicamente em modelos animais que induzem estresse, como o estresse de contenção em camundongos (Poleszak et al., 2006; Kumar e Goyal, 2008).

Outros mecanismos importantes envolvidos na fisiopatologia da depressão são as vias de sinalização que regulam a sobrevivência e neuroplasticidade celular, bem como a resposta a antidepressivos, como a enzima fosfoinositol 3-cinase (PI3K), a proteína serina/treonina cinase Akt, a enzima glicogênio sintase cinase-3β (GSK-3β) (Beaulieu et al., 2009) e receptor ativado por proliferadores peroxissomais-γ (PPARγ) (Rosa et al., 2008; Eissa Ahmed, 2009). Além disso, outras proteínas de sinalização estão envolvidas neste transtorno, como a cálcio calmodulina cinase II (CaMKII), proteína cinase C (PKC), proteína cinase A (PKA), proteína cinase ativada por mitógeno (MAPK)/cinase regulada por sinal extracelular (ERK), proteína ligante ao elemento responsivo ao AMPc (CREB), fator neurotrófico derivado do cérebro (BDNF), e também proteínas pró-apoptóticas como caspase 3 e 6 e proteína antiapoptótica Bcl2 (Picchini et al., 2004; Perera et al., 2007; Pittenger e Duman, 2007; Castrén e Rantamäki, 2010; Numakawa et al., 2010).

Muitos estudos pré-clínicos indicam também que a depressão pode ser desencadeada por uma interação com os canais de potássio, uma vez que diferentes inibidores destes canais, como tetraetilamônio, apamina, caribdotoxina e glibenclamida apresentam efeito tipo-antidepressivo no TNF em camundongos (Budni et al., 2007; Kaster et al., 2007; Jesse et al., 2009; Bortolatto et al., 2010).

Finalmente, além de todos os sistemas citados envolvidos na depressão, esta patologia também envolve um desbalanço entre espécies pró-oxidantes e antioxidantes, induzindo estresse oxidativo (Bilici et al., 2001; Maes et al., 2009).

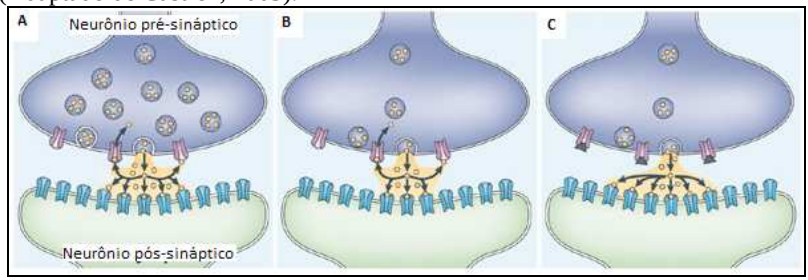
Para investigar a fisiopatologia da depressão e estudar novas drogas antidepressivas que possam ter como alvos alguns ou muitos dos mecanismos patológicos da depressão acima citados, são utilizados muitos modelos animais deste transtorno (Nestler e Hyman, 2010). Entre eles, encontram-se, o teste do nado forçado (TNF), descrito primeiramente por Porsolt et al. (1977) utilizado em ratos e

posteriormente em camundongos e o teste de suspensão da cauda (TSC), descrito primeiramente por Steru et al., (1985) utilizado em camundongos. Tanto o TNF quanto o TSC são modelos animais preditivos para a ação antidepressiva de um composto ou fármaco, uma vez que antidepressivos clássicos apresentam efeito tipo-antidepressivo nestes testes comportamentais.

Vários fármacos antidepressivos encontram-se no mercado farmacêutico, entre eles os antidepressivos tricíclicos e inibidores da enzima monoamina oxidase, os quais apresentam muitos efeitos colaterais que limitam o seu uso. Além desses, outros medicamentos foram desenvolvidos e estão disponíveis no mercado para a utilização no tratamento da depressão, como os inibidores seletivos de recaptação de serotonina e/ou noradrenalina e dopamina, que são tão efetivos quanto os tricíclicos, mas são mais seletivos e produzem menos efeitos colaterais. Além disso, recentemente sugiram os inibidores triplos da recaptação de monoaminas e, segundo estudos clínicos e pré-clínicos, apresentam um início de ação terapêutica mais rápida e maior eficácia do que os antidepressivos tradicionais (Berton e Nestler, 2006; Nemeroff, 2007; Chen e Skolnick, 2007; Millan, 2009; Prins et al., 2010).

Tipicamente os antidepressivos classicamente prescritos bloqueiam a recaptação de serotonina e/ou noradrenalina. Os fármacos mais prescritos para a depressão são os inibidores seletivos de recaptação de serotonina (ISRS). A resposta aguda dos antidepressivos baseia-se na hipótese monoaminérgica da depressão (Duman e Voleti, 2012). Segunda esta hipótese, pacientes fisiologicamente saudáveis apresentam níveis normais de monoaminas na fenda sináptica (**Figura 1A**), enquanto que pacientes deprimidos apresentam níveis reduzidos de neurotrasmissores (**Figura 1B**). Após o tratamento com antidepressivos ocorre um restabelecimento de monoaminas e receptores para monoaminas na fenda sináptica (**Figura 1C**) (Castrén, 2005).

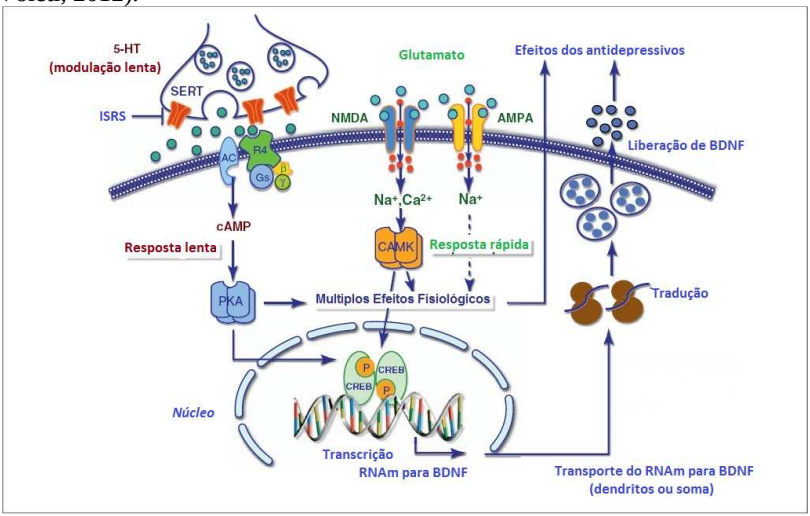
Figura 1. Hipótese monoaminérgica da depressão. A) Níveis de monoaminas em um paciente saudável. B) Níveis de monoaminas em um paciente deprimido. C) Níveis de monoaminas em um paciente após tratamento com antidepressivos. (Adaptado de Castrén, 2005).



Este mecanismo agudo da ação dos antidepressivos, não é suficiente para explicar a demora na remissão dos sintomas nos pacientes. Portanto, é compreensível que vias de sinalização e regulação de alvos gênicos (citados acima) estejam envolvidos em uma resposta tardia ao tratamento crônico com antidepressivos. Estas vias de sinalização e alvos gênicos, por sua vez resultam da adaptação dos sistemas e então regulam múltiplos processos fisiológicos, incluindo neuroplasticidade, neuroproteção e neurogênese no cérebro adulto (**Figura 2**) (Duman e Voleti, 2012).

Figura 2. Vias de sinalização reguladas pelo tratamento crônico com antidepressivos. Inibidores seletivos de recaptação de serotonina (ISRS) bloqueiam a recaptação de serotonina (5-HT) através da inibição do transportador de 5-HT (SERT). Em consequência, 5-HT liga-se a receptores serotoninérgicos pós-sinápticos e regula a ação desses receptores acoplados a proteína G, dos quais ativam sistemas de segundos mensageiros, como o AMPc (adenosina monofosfato cíclico), que por sua vez, ativa a proteína cinase A (PKA) e culmina na ativação da transcrição gênica para CREB (proteína ligante ao elemento responsivo ao AMPc). Estes efeitos exigem tratamento crônico com antidepressivos, em função da necessidade de dessensibilização de autoreceptores serotoninérgicos, uma vez que 5-HT produz resposta neuronal lenta. Em contraste, o glutamato produz um resposta rápida por excitação dos neurônios via estimulação de receptores ionotrópicos (AMPA [alfa-amino 3-hidróxi 5-metilisoxazol ácido propiônico] e NMDA [N-metil D-aspartato]), resultando em despolarização e rápida sinalização intracelular, através da proteína cinase dependente de Ca^{2+}-calmodulina (CAMK). A sinalização do glutamato e de 5-HT, indicados na figura, induzem a regulação de múltiplas respostas fisiológicas incluindo a regulação da plasticidade sináptica e expressão gênica. Um dos alvos do tratamento com antidepressivos e

sinalização para CREB é o fator neurotrófico derivado do cérebro (BDNF). A transcrição para BDNF pode permanecer no soma ou ser transportada para os dendritos, onde ocorre a sua tradução e liberação. A indução de BDNF e outros fatores neurotróficos contribuem para ações do tratamento por antidepressivos como a neuroproteção, neuroplasticidade e neurogênese. (Adaptado de Duman e Voleti, 2012).



O tratamento da depressão é geralmente seguro e efetivo, porém ainda está longe do ideal, pois o tempo de latência para obter benefícios clínicos é relativamente longo, dura semanas ou meses, e conforme mencionado anteriormente há grandes problemas ainda quanto aos efeitos colaterais. Além disso, menos de 50 % dos pacientes mostram remissão completa dos sintomas com a terapia com antidepressivos. Por isso, há uma grande necessidade de fármacos que apresentem ação rápida, e sejam seguros e efetivos para a depressão (Berton e Nestler, 2006; Nakajima et al., 2010).

ENVOLVIMENTO DOS CANAIS DE POTÁSSIO NA FISIOPATOLOGIA DA DEPRESSÃO

Os canais de potássio (K^+) são proteínas transmembrana, que formam poros iônicos seletivos a K^+, os quais estão constitutivamente abertos no repouso (Choe, 2002; Honoré, 2007). Estes canais apresentam um importante papel na função neuronal, já que neurônios frequentemente expressam múltiplos tipos de canais de K^+ que são

cruciais na sinalização de todos os tipos de células, estejam estas sob condições fisiológicas ou patológicas (Choe, 2002; Yuan e Chen, 2006; Honoré, 2007).

Entre os vários papéis fisiológicos desempenhados pelos canais de K^+ encontra-se a promoção da hiperpolarização para a restauração do potencial de membrana, uma vez que estes canais são conhecidos por regular a excitabilidade neuronal em diferentes populações de neurônios (Honoré, 2007). Além do papel bem estabelecido dos canais K^+ na excitabilidade neuronal, muitos estudos indicam que estes canais são importantes nos mecanismos de respostas a antidepressivos ou compostos dotados de propriedades antidepressivas (Kaster et al., 2005; Budni et al., 2007; Kaster et al., 2007; Su et al., 2007; Lodge e Li, 2008; Lockridge et al., 2010). De fato, diferentes tipos de inibidores de canais de K^+, como tetraetilamônio (TEA), apamina, caribdotoxina, gliquidona e glibenclamida apresentaram efeito tipo-antidepressivo no TNF em camundongos (Galeotti et al., 1999; Kaster et al., 2005), enquanto ativadores destes canais (Minoxidil ou cromacalim) induziram um aumento no tempo de imobilidade neste teste preditivo para a ação antidepressiva, indicando indução de um comportamento tipo-depressivo (Galeotti et al., 1999). Além disso, outros estudos pré-clínicos reportaram que doses sub-ativas de bloqueadores de canais de potássio (quinina ou gliburida) combinadas com doses sub-ativas de diferentes antidepressivos (citalopram, fluoxetina, paroxetina e imipramina) também produziram efeito tipo-antidepressivo no TNF (Guo et al., 1995; 1996).

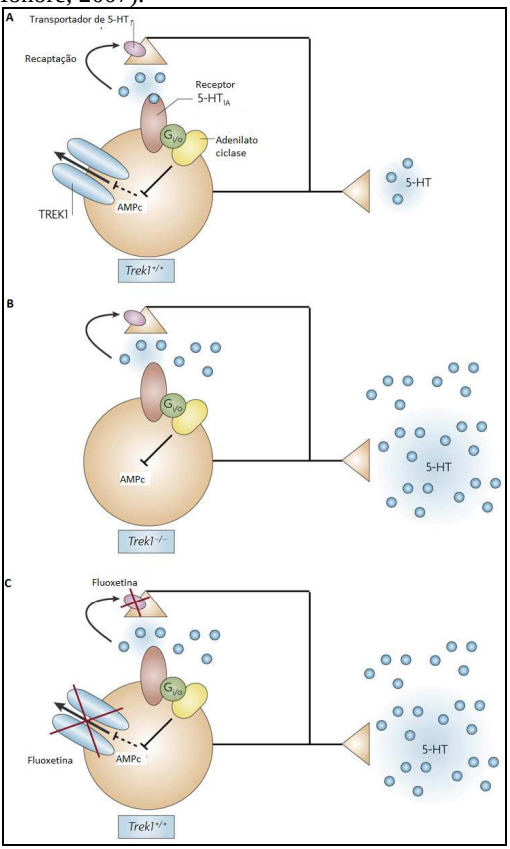
Muitos diferentes tipos de canais de K^+ estão envolvidos na depressão, entre eles encontram-se os canais de K^+ ativados por Ca^{2+} de alta e intermediária condutância, canais de K^+ ativados por Ca^{2+} de baixa condutância e canais de K^+ sensível ao ATP (Guo et al., 1995; 1996; Galeotti et al., 1999; Kaster et al., 2005). Os canais retificadores internos ativados por proteína G (GIRK), também denominados como canais Kir3, membros de uma grande sub-família de canais retificadores internos (Kir) de um único poro (Reimann e Ashcroft, 1999), também estão envolvidos na depressão. Esta suposição justifica-se, levando em conta estudos que mostram que o efeito antidepressivo da fluoxetina e paroxetina pode envolver a inibição destes canais (Kobayashi et al., 2003; 2004; 2006; Takahashi et al., 2006). Além disso, um recente estudo de Kobayashi et al. (2010) sugere que outros antidepressivos, como atomoxetina e reboxetina, inibem canais GIRK cardíaco e cerebral. Desta forma, antidepressivos também podem agir no SNC, inibindo canais GIRK.

Fortes evidências indicam que a fluoxetina também pode inibir outra classe de canais de K^+, canal de K^+ de dois poros, particularmente TREK-1, indicando uma associação muito importante destes canais com a depressão (Kennard et al., 2005; Heurteaux et al., 2006; Gordon e Hen, 2006; Honoré, 2007; Mathie e Veale, 2007). De fato, Kennard et al. (2005) mostrou que a fluoxetina e seu metabólito, norfluoxetina apresentaram inibição, concentração-dependente, da corrente de K^+ através de TREK-1. Corroborando com este estudo, Heurteaux et al. (2006) reportou que camundongos deficientes de TREK-1 apresentaram um aumento da neurotransmissão serotoninérgica, resistência à depressão em diferentes modelos animais de depressão (TNF, TSC, desamparo aprendido, supressão condicionada do teste de motilidade e teste da alimentação suprimida pela novidade) e reversão do aumento da corticosterona induzida por estresse. Por sua vez, animais tratados com fluoxetina exibiram comportamento similar àqueles deficientes de TREK-1, ou seja, efeito tipo-antidepressivo.

Portanto, a inibição de canais TREK-1 pode ser um possível mecanismo pelo qual os antidepressivos desempenham seu papel terapêutico na depressão como ilustrado na **Figura 3** (Kennard et al., 2005; Heurteaux et al., 2006; Gordon e Hen, 2006; Honoré, 2007; Mathie e Veale, 2007). Este esquema, adaptado de Honoré (2007) mostra a possível interação do sistema serotoninérgico com canais TREK-1. Este esquema é baseado no estudo de Heurteaux et al. (2006) que utilizou camundongos knockout para canais TREK-1, citado acima. Portanto, o esquema mostra que em neurônios do núcleo dorsal da rafe de camundongos selvagens *Trek*$^{+/+}$, a estimulação de autoreceptores 5-HT_{1A} reduz o disparo neuronal e, em função disso, a neurotransmissão serotoninérgica. Isso ocorre, pois a estimulação de autoreceptores 5-HT_{1A} inibe a enzima adenilato ciclase via proteína G inibitória ($G_{i/o}$). Como conseqüência, há redução na produção de AMPc, o qual contribui para a abertura dos canais TREK-1 devido a diminuição da fosforilação da proteína cinase A (PKA). Estes canais, por sua vez, induzem a hiperpolarização da célula, redução na taxa de disparo e diminuição da liberação de serotonina a partir dos neurônios do núcleo dorsal da rafe (**Figura 3A**). Neste sentido, em animais deficientes de TREK-1 ou camundongos knockout *Trek*$^{-/-}$, há redução no *feedback* negativo da serotonina nos neurônios pré-sinápticos resultando em aumento da neurotransmissão serotoninérgica (**Figura 3B**). ISRS, como a fluoxetina, em camundongos selvagens *Trek*$^{+/+}$ pode ter como alvo direto, a inibição dos canais TREK-1, efeito similar àqueles em camundongos knockout *Trek*$^{-/-}$, importante para o efeito terapêutico

deste antidepressivo na depressão e a consolidação da relação entre depressão e canais de K^+ (**Figura 3C**) (Honoré, 2007).

Figura 3. Envolvimento dos canais TREK-1 na fisiopatologia da depressão ilustrado em neurônios do núcleo dorsal da rafe. A) Interação entre os canais TREK-1 com o sistema serotoninérgico em camundongos selvagens *Trek-1*$^{+/+}$. B) Camundongos knockout *Trek-1*$^{-/-}$ e a estimulação de serotonina. C) Inibição de canais TREK-1 por fluoxetina (ISRS) em camundongos selvagens. (Adaptado de Honoré, 2007).



Outro mecanismo importante que associa a fisiopatologia da depressão aos canais de K^+ é a síntese de óxido nítrico (NO). Esta molécula é um importante mensageiro no SNC, produzido por ação catalítica da NO sintase (NOS). Fisiologicamente, as ações do NO podem ser mediadas pela sua produção local e também pela geração

subseqüente de um segundo mensageiro guanosina 3´5-monofosfato cíclico (GMPc). A produção desta molécula pode ativar diferentes tipos de canais de K^+ em vários tecidos, ou ainda, estes canais podem ser ativados por NO *per se* (Jeong et al., 2001; Shin et al., 1997). Estes achados corroboram com resultados de Inan et al. (2004), os quais sugerem que o efeito dual do NO (L-arginina, um precursor de NO, induz efeito tipo-antidepressivo, em altas doses, porém em baixas doses induz comportamento tipo-depressivo) no TNF em camundongos pode estar relacionado com a modulação da função dos canais de K^+. Confirmando esta hipótese, Kaster et al. (2005) mostraram que o efeito tipo-antidepressivo no TNF elicitado pela inibição de diferentes tipos de canais de K^+ poderia ser mediado, pelo menos em parte, por uma inibição da produção de NO e GMPc em camundongos (Kaster et al., 2005). Portanto, os canais de K^+ podem ser alvos fisiológicos do NO produzido centralmente (Jeong et al., 2001) e, consequentemente, a ativação destes canais pode desempenhar um papel muito importante na fisiopatologia da depressão.

Adicionalmente, deve ser considerado que muitos compostos com propriedades tipo-antidepressivas, como a adenosina, agmatina, tramadol e venlafaxina, além dos antidepressivos clássicos, mostraram um efeito sinérgico no TNF em camundongos quando combinados com inibidores de canais de K^+ (Budni et al., 2007; Kaster et al., 2007; Jesse et al., 2009; Bortolatto et al., 2010). Portanto, sugere-se que o efeito modulatório da excitabilidade neuronal induzidos por fármacos antidepressivos ou compostos com propriedades antidepressivas, via inibição de canais de K^+, pode representar uma via final comum desta ação farmacológica (Guo et al., 1995, 1996).

ENVOLVIMENTO DA VIA PI3K, GSK-3B E PPAR-Γ NA FISIOPATOLOGIA DA DEPRESSÃO

A enzima fosfatidil inositol-3 cinase (PI3K), uma cinase lipídica, é responsável por catalisar a fosforilação do lipídio fosfatidil inositol na posição 3 do anel inositol em resposta a receptores acoplados a proteína G ou receptores tirosina cinase ativados por fatores de crescimento, hormônios, citocinas ou neurotransmissores (Katso et al, 2001; Cantley, 2002; Beaulieu, 2012). Muitos estudos indicam que a PI3K está implicada na plasticidade sináptica, aprendizado, memória e depressão (Kelly e Lynch 2000; Dwivedi et al., 2008; Yang et al., 2008). Produtos lipídicos da PI3K agem como segundos mensageiros, uma vez que ativam proteínas como a Akt (Brazil et al., 2004, Hanada et al., 2004).

A Akt é uma serina/treonina cinase existente sob três isoformas codificadas por genes diferentes: *AKT1*, *AKT2* e *AKT3*. Todas as três isoformas são ativadas pela PI3K (Beaulieu, 2012). A ativação da Akt envolve seu recrutamento à membrana plasmática pela PI3K e subsequente fosforilação no seu resíduo regulatório (treonina 308) pela cinase 1 dependente de fosfatidil inositol (PDK1) seguido de fosforilação no resíduo de serina 473 pela PDK2 (Beaulieu et al., 2009). Quando ativada, a Akt é responsável por regular negativamente a atividade da enzima GSK-3β via fosforilação da serina 9 N-terminal (Cross et al., 1995; Hetman et al., 2000; Beaulieu et al., 2009).

GSK-3β, por sua vez, é uma enzima serina/treonina cinase multifuntional encontrada em todas as células eucarióticas, reconhecida recentemente como um componente chave de muitas vias de sinalização (Jope e Roh, 2006; Beaulieu, 2012). Em mamíferos são encontradas duas isoformas desta enzima: GSK-3α e GSK-3β. Estas cinases encontram-se constitutivamente ativas e podem ser inativadas por fosforilação de um simples resíduo de serina 21 (GSK-3α) ou serina 9 (GSK-3β) (Beaulieu et al., 2009; Beaulieu, 2012). GSK-3β é altamente expressa nos neurônios, além de ser regulada durante o desenvolvimento (Bhat et al., 2004). A fosforilação da GSK-3β no resíduo de serina-9 promove inibição desta enzima, envolvida, desta forma, na sobrevivência celular, enquanto que a fosforilação no resíduo de tirosina-216 aumenta a atividade da mesma (Hughes et al., 1993) e geralmente, está relacionada com a morte celular (Bhat et al., 2000).

Estudos indicam que esta enzima está implicada na fisiopatologia da depressão. Uma importante evidência encontra-se no fato de que estabilizadores de humor como o lítio e valproato, amplamente utilizados na clínica para o tratamento do transtorno bipolar (Jope, 1999), causam inibição direta da GSK-3β (Chen et al., 1999; Li et al., 2002). Adicionalmente, a terapia eletroconvulsiva (ECS), comumente utilizada na terapia para depressão resistente ao tratamento com antidepressivos clássicos, pode induzir mudanças bifásicas, com defosforilação imediata da GSK-3 e subseqüente hiperfosforilação da enzima e, portanto, inibição da mesma 3 h depois da ECS (Roh et al., 2003). Além disso, a atividade serotoninérgica aumentada após a administração de antidepressivos (fluoxetina e imipramina) inibe GSK-3β no cérebro (Li et al., 2004). Corroborando com o papel da GSK-3β como alvo para a ação de antidepressivos, alguns estudos mostram que inibidores da GSK-3β causam efeito tipo-antidepressivo, como por exemplo, o AR-A014418, um inibidor específico da GSK-3β, produz efeito tipo-antidepressivo no TNF em ratos (Gould et al., 2004) e

camundongos (Rosa et al., 2008). L803-mts (N-myristoyl-GKEAPPAPPQS(p)P), um novo inibidor da GSK-3 e NP031115, um novo composto da classe das tiadiazolidinonas que atua como inibidor da GSK3β, também produziram um efeito tipo-antidepressivo no TNF em camundongos (Kaidanovich-Beilin et al., 2004; Rosa et al., 2008), relacionando fortemente a enzima GSK-3β com a depressão.

Receptores ativados por proliferador peroxissomal (PPARs) são membros da super-família de receptores nucleares para hormônios que atuam como fatores de transcrição dependente de ligante e são responsáveis por regular a expressão gênica envolvida na reprodução, metabolismo, desenvolvimento e resposta imune (Desvergne e Wahli, 1999). PPARs apresentam três isoformas: α, β/δ e γ (Yessoufou e Wahli, 2010). PPARγ, bem como a GSK-3β, são alvos emergentes na farmacologia, com efeitos promissores na depressão (Rosa et al., 2008). A relação entre PPARγ e GSK-3β foi demonstrada em um estudo no qual o tratamento de neurônios hipocampais de ratos com agonista PPARγ (troglitazona) promoveu diminuição da atividade da GSK-3β (Inestrosa et al., 2005). Com relação ao envolvimento de PPARγ na depressão, nosso grupo mostrou que a administração de roziglitazona, um agonista PPARγ, produziu um efeito tipo-antidepressivo no TNF em camundongos, o qual foi prevenido pelo pré-tratamento dos animais com GW-9662, um antagonista PPARγ (Rosa et al., 2008). Além disso, neste estudo GW-9662 foi capaz de prevenir o efeito anti-imobilidade dos inibidores da enzima GSK-3β, NP031115 e ARA014418, no TNF, reforçando a interação entre GSK-3β e PPARγ na depressão (Rosa et al., 2008). Roziglitazona também mostrou efeito tipo-antidepressivo no TSC em camundongos e no TNF em ratos (Eissa Ahmed et al., 2009). Além disso, reforçando o papel do PPARγ na depressão, um recente estudo de Rasgon et al. (2010) mostrou que a administração de roziglitazona em pacientes não diabéticos com depressão maior ou depressão bipolar, promoveu melhora em relação a severidade da depressão e a impressão global clínica (Rasgon et al., 2010).

Portanto, agentes que modulam esta via PI3K/Akt, GSK-3β e PPARγ, podem tornar-se novos possíveis agentes para a depressão.

ESTRESSE SOCIAL, DEPRESSÃO E PREJUÍZO COGNITIVO

O estresse pode ser definido como um estado de distúrbio do equilíbrio fisiológico normal e homeostase ameaçada, o qual pode culminar em mudanças patológicas dependendo da severidade, tipo e duração do estímulo estressante (Munhoz et al., 2008; Chrousos, 2009;

Jaggi et al., 2011). Estas alterações (fisiológicas, psicológicas ou cognitivas) afetam diferentes órgãos e sistemas, principalmente o SNC (Linthorst e Reul, 2008; Munhoz et al., 2008).

Eventos estressantes em suas diferentes formas representam o maior componente ambiental para a susceptibilidade aumentada a diferentes doenças psiquiátricas. De fato, eventos estressantes da vida servem como um significativo preditor de doenças como a depressão (Calabrese et al., 2011), geralmente acompanhada por prejuízo cognitivo (Marazziti et al., 2010; Murrough et al., 2011), considerando que eventos estressantes tem uma considerável associação causal com a patologia deste transtorno, especialmente, em indivíduos predispostos geneticamente (Charney & Manji, 2004; Lanfumey et al., 2008; Kubera et al., 2011). O papel causal do estresse na depressão é suportado pela presença de disfunção ao nível do sistema de resposta ao estresse em indivíduos vulneráveis (Calabrese et al., 2011). Entre as disfunções do sistema de resposta ao estresse, encontra-se a hiperativação do eixo HPA, levando em consideração que pacientes deprimidos geralmente apresentam altos níveis de cortisol ou ACTH em resposta ao estresse psicosocial (Juruena et al., 2006; Rao et al., 2008; Chopra et al., 2009). A hiperatividade HPA é conseqüência de uma alterações no *feedback* inibitório realizado pelos glicocorticóides endógenos, também conhecido como “resistência aos glicocorticóides” o qual pode ocorrer devido uma *downregulation* dos receptores para este hormônio (Calabrese et al., 2011) .

É importante mencionar que o estresse e a depressão estão intimamente relacionados com o prejuízo cognitivo (Marazziti et al., 2010; Murrough et al., 2011). A disfunção cognitiva (aprendizado e memória) é uma das mais importantes características em pacientes com depressão e podem, muitas vezes, se manifestar mais precocemente do que os sinais de humor deprimido (Austin et al., 2001; Cohen et al., 2001; Porter et al., 2003). O tratamento com antidepressivos melhora o déficit cognitivo, em paralelo aos sintomas depressivos (Vermetten et al., 2003; Airaksinen et al 2004). Além disso, é bem documentado que a depresão está associada com a redução do volume hipocampal (Sheline et al., 2003), estrutura extremamente relacionada com função cognitiva (Gondi et al., 2010; Henson e Ganepain, 2010; Lagali et al., 2010). Animais expostos a estímulos estressantes também mostram alterações citogenéticas e estruturais no hipocampo (Czeh et al 2001; Czeh e Lucassen, 2007; Jayatissa et al 2008). Adicionalmente, modelos animais induzidos por estímulos estressantes no qual os roedores apresentam comportamento tipo-depressivo, induzem prejuízo cognitivo (Elizalde et

al., 2008; Dang et al., 2009), reforçando a hipótese de que há uma forte associação entre estresse, depressão e prejuízo cognitivo. Modelos de estresse que mimetizam comportamento tipo-depressivo e déficit cognitivo são importantes modelos para screening de fármacos antidepressivos.

Realmente muitos modelos animais de depressão induzida por estresse são amplamente utilizados para explorar mudanças induzidas por estresse no cérebro, para fazer *screening* de antidepressivos e estabelecer fenótipos comportamentais de animais transgênicos ou alvos gênicos que estejam relacionados à depressão e estresse (Kalueff et al. 2007). O estresse pode ser utilizado para induzir sentimento de perda de controle e prejuízo cognitivo, podendo resultar em um estado comportamental análogo a depressão bem como mudanças bioquímicas (Calabrese et al., 2011; Kubera et al., 2011; Marin et al., 2011). Já é bem conhecido que o cérebro adulto, durante a vida, tem a capacidade de regenerar novos neurônios e a redução da neurogênese hipocampal é uma possível causa da depressão (Fournier e Duman, 2012). Vários estudos mostram que modelos de estresse causam uma redução da neurogênese no hipocampo. Esta redução pode ocorrer após a exposição a diferentes agentes estressores, como o estresse de contenção (Rosenbrock et al., 2005; Yun et al., 2010), estresse induzido por isolamento social (Stranahan e Gould, 2006), derrota social (Lagace et al 2010), privação do sono (Mirescu et al., 2006), corticosterona (Cameron e Gould, 1994), estresse crônico moderado (Lee et al., 2006; Mineur et al., 2007), exposição aguda ao odor do predador (Tanapat et al., 2001) e estresse inescapável induzido por choque nas patas (Malberg e Duman, 2003). Além disso, a hiperativação do eixo HPA, o qual geralmente está associada com níveis elevados de corticosterona, em roedores, também está associado com os modelos de depressão induzida por estresse (Mao et al., 2009; Borsonelo et al., 2011; Naert et al., 2011; Snyder et al., 2011). Outra importante alteração que ocorre em animais submetidos a modelos de depressão induzida por estresse é o desequilíbrio oxidativo encontrado no cérebro de animais submetidos a estes modelos (Zafir et al., 2009; Moretti et al., 2012).

Estresse de contenção, objeto de estudo deste trabalho, é um modelo frequentemente utilizado para causar um estado comportamental depressivo envolvendo aplicação de estresse não controlado em estudos de estresse agudo ou crônico (Poleszak et al., 2006; Sevgi et al., 2006; Capra et al., 2010; O'Mahony et al., 2010; Christiansen et al., 2011; Huynh et al 2011) acompanhado com prejuízo cognitivo avaliado

através do teste de reconhecimento de objetos (TRO) (Baker e Kim, 2002; Walesiuk et al., 2005; Nagata et al., 2009; Li et al., 2012).

O estudo de fármacos em modelos de depressão, induzida por estresse, associada com prejuízo cognitivo é muito importante para definir a capacidade e a potência dos diferentes antidepressivos, bem como, dos compostos com potenciais antidepressivos, para reverter as alterações induzidas pelo estresse e que são centrais para a depressão. Além disso, este estudo pode levar a identificação de novos alvos terapêuticos, contribuindo para o desenvolvimento de novos agentes mais eficazes que podem eventualmente alcançar maiores índices de remissão terapêutica (Calabrese et al., 2011; Marin et al., 2011).

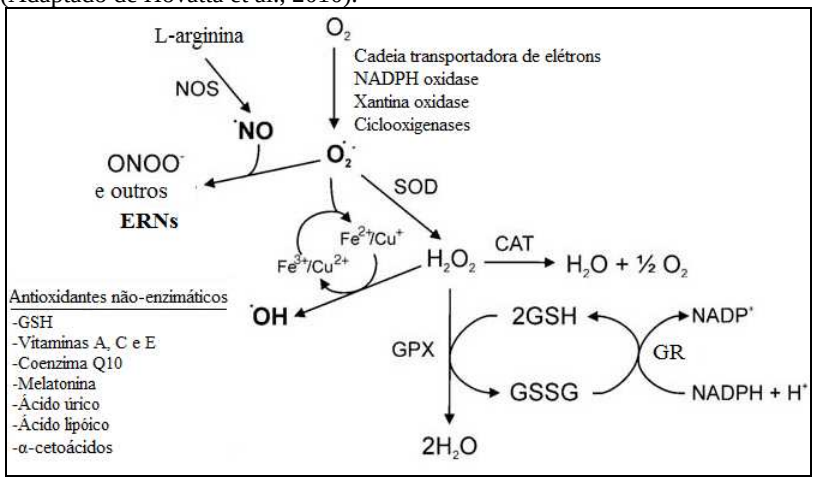
DANO OXIDATIVO, DEPRESSÃO E ESTRESSE SOCIAL

A mitocôndria é a principal fonte de geração de espécies reativas de oxigênio (EROs) e espécies reativas de nitrogênio (ERNs). Estes radicais livres (EROs e ERNs) são definidos como moléculas as quais possuem elétrons desemparelhados no seu orbital mais externo. A geração de radicais livres em baixas ou moderadas concentrações é uma característica fisiológica importante para a célula (Hovatta et al., 2010). Porém, quando ocorre um desbalanço entre a geração e a eliminação de EROs e ERNs, culmina em estresse oxidativo o qual induz dano oxidativo em importantes macromoléculas celulares, como lipídios, proteínas, carboidratos e ácidos nucléicos (Sies, 1986; Hovatta et al., 2010).

O tecido mais susceptível ao estresse oxidativo, sem dúvida, é o cérebro, uma vez que ele metaboliza 20% de todo o oxigênio corporal consumido e além disso, apresenta uma quantidade estritamente limitada de agentes antioxidantes. Portanto, quando sob estímulo, por exemplo, estresse aversivo, o cérebro reage, podendo produzir EROs como ânion superóxido ($O_2^{\cdot-}$), radical hidroxila ($HO^{\cdot}$) e peróxido de hidrogênio (H_2O_2), além das ERN ($ONOO^-$ e $NO^{\cdot}$). Quando as EROs e ERNs excedem a capacidade antioxidante do cérebro ocorre o estresse oxidativo e consequente dano aos neurônios (Floyd, 1999; Floyd e Carney, 1992; Kovacs et al., 1996; Halliwell, 2006). Importantes enzimas antioxidantes endógenas ou antioxidantes não-enzimáticos podem inibir a produção de EROs, promover a remoção ou eliminação dos mesmos e de seus precursores. Entre estas enzimas encontram-se: catalase (CAT), superóxido dismutase (SOD), glutationa peroxidase (GPx) e glutationa redutase (GR) (McCord e Fridovich, 1988; Hovatta et al., 2010). Entre os agentes antioxidantes não-enzimáticos endógenos

encontram-se: glutationa (GSH), vitaminas A, C, E, coenzima Q10, melatonina, ácido úrico, ácido lipóico e α-cetoácidos (**Figura 4**) (Hovatta et al., 2010).

Figura 4. Maiores vias de produção de radicais livres e defesas antioxidantes enzimáticas e não-enzimáticas. NOS: óxido nítrico sintase; ERNs: espécies reativas de nitrogênio; SOD: superóxido dismutase; CAT: catalase; GPx: glutationa peroxidase; GR: glutationa redutase; GSH: glutationa; GSSH: glutationa oxidada; NADPH: nicotinamida adenina dinucleotídeo fosfato (Adaptado de Hovatta et al., 2010).



Depressão e estresse oxidativo estão intimamente relacionados, levando em consideração o fato de que pacientes deprimidos apresentam prejuízos nas defesas antioxidantes plasmáticas e aumento na peroxidação lipídica (Bilici et al., 2001; Khanzode et al., 2003; Ozcan et al., 2004). Portanto, o dano oxidativo pode ser um importante mecanismo na fisiopatologia da depressão em humanos (Maes et al., 2011).

Similarmente, modelos de depressão induzida por estresse social, com o estresse de contenção em roedores, objeto de estudo do presente trabalho, precipita muitas disfunções neuroquímicas e neuroendócrinas que estão frequentemente associadas com um desequilíbrio do estado redox intracelular do cérebro. Muitos estudos mostram que o estresse de contenção induz aumento da peroxidação lipídica (García-Bueno et al., 2005; Kumari et al., 2007; Zafir e Banu, 2007; Kumar e Goyal, 2008; Zafir et al., 2009; Balk et al., 2010; Kumar et al., 2010) e aumento (Fontella et al., 2005; Kim et al., 2005; Balk et al., 2010) ou diminuição

(Pajović et al 2006; Kumari et al., 2007; Zafir e Banu, 2007; Kumar e Goyal, 2008; Zafir et al., 2009; Balk et al., 2010; Kumar et al., 2010) da atividade das enzimas antioxidantes em diferentes regiões do cérebro de roedores, dependendo da severidade e duração do protocolo de estresse de contenção. Estas alterações são passíveis de serem revertidas pelo tratamento dos animais com antidepressivos clássicos como a fluoxetina, imipramina e venlafaxina (Zafir et al., 2009).

Os mecanismos que levam ou regulam o estresse oxidativo, e que contribuem para doenças neuropsiquiátricas como a depressão são complexos. No entanto, algumas vias do estresse oxidativo são bem passíveis de manipulação farmacológica, o que torna esta via um alvo para terapias farmacológicas putativo para a depressão. Modelos animais de depressão induzidos por estresse social que promovam dano oxidativo pode ser um bom campo de estudo que relaciona depressão, estresse social e dano oxidativo, para uma melhor compreensão da mesma e estudo de possíveis alvos terapêuticos (Hovatta et al., 2010; Maes et al., 2011).

TOXICIDADE INDUZIDA POR GLICOCORTICÓIDES E NEUROPROTEÇÃO

Os glicocorticóides exercem muitos efeitos através dos receptores de glicocorticóides distribuídos pelo organismo. Estes hormônios exercem importantes efeitos no SNC, principalmente no hipocampo que apresenta altas densidades de receptores para os glicocorticóides e, portanto torna esta região bastante vulnerável a estes hormônios (Nichols et al., 2005; Czéh e Lucassen, 2007; Sorrells et al., 2009). Fisiologicamente, a ativação do eixo HPA e liberação de glicocorticóides constituem mudanças fisiológicas em “resposta ao estresse” e é essencial para a manutenção da homeostasia durante eventos estressantes (Aguilera, 2011). Enquanto que a função primária da secreção de glicocorticóides é a mobilização de energia em resposta a um estímulo estressante (Joëls, 2008), uma resposta exacerbada do eixo HPA induz uma elevação prolongada dos níveis de glicocorticóides, podendo desencadear processos patológicos como a depressão, que está relacionada com a redução do volume hipocampal e conseqüente morte de neurônios desta região (Lee et al., 2002; Kunugi et al., 2010) e de outras regiões do cérebro.

Dexametasona é um glicocorticóide sintético, muito utilizada em protocolos de morte celular *in vitro*, a fim de mimetizar a elevação de glicocorticóides, fenômeno que ocorre em muitos processos patológicos

como na depressão (Mitchell et al., 1998; Haynes et al., 2001; Jacobs et al., 2006; Zhu et al., 2006; Tazik et al., 2009). Muitos estudos demonstram que a dexametasona induz apoptose e reduz a proliferação celular em muitos tipos de células incluindo neurônios hipocampais (Haynes et al., 2001), estriatais (Mitchell et al., 1998), cerebelares (Jacobs et al., 2006), células da glia (Tazik et al., 2009) e também células de neuroblastoma humano, SHSY-5Y (Zhu et al., 2006; Tazik et al., 2009).

A dexametasona pode induzir morte celular por vários mecanismos. Entre eles encontra-se a indução do aumento na atividade da MAO A (Monoamina oxidase A, enzima que degrada as monoaminas) em células neuroblastomas e células glioblastomas através do seu papel como estressor celular (Ou et al., 2006). Além disso, este glicocorticóide sintético, pode induzir a expressão e atividade da MAO B em células neuronais (Tazik et al., 2009) e astrócitos (Carlo et al., 1996), bem como diminuir as células viáveis do cérebro (Yu et al., 2010).

Além disso, outros mecanismos estão implicados na ação deletéria da dexametasona, indicado por um estudo de Kumamaru et al. (2008), o qual demonstrou que a exposição de neurônios hipocampais imaturos à dexametasona, inibe o crescimento de dendritos e a formação sináptica dependente do fator neurotrófico BDNF. Portanto, o dano hipocampal induzido pela hiperatividade do eixo HPA, pode ocorrer principalmente pela disfunção do BDNF. Como o hipocampo regula o sistema de *feedback* do eixo HPA, seu dano aumenta a atividade deste eixo, o que pode formar um ciclo vicioso culminando em morte neuronal (Kunugi et al., 2010).

Um adicional estudo também mostrou, em células pancreáticas, que a dexametasona pode induzir morte celular apoptótica via inibição das vias de sinalização PI3K e PKA, assim como redução da expressão da proteína Bcl-2 (Wang et al., 2010).

Um recente estudo realizado por Kim et al. (2010) verificou que a dexametasona causa dano celular por induzir estresse oxidativo, uma vez que a administração deste glicocorticóide sintético promoveu aumento na geração de EROs em cultivos de neurônios hipocampais.

Apesar de a dexametasona induzir morte celular por diferentes mecanismos citados acima, diferentes agentes, dotados de propriedades neuroprotetoras são passíveis de proteção contra este dano celular (Zhu et al., 2006; Johnson et al., 2010; Kim et al., 2010). Um estudo realizado por Zhu et al. (2006), mostrou que a agmatina, uma amina catiônica endógena, apresentou efeito neuroprotetor contra a toxicidade induzida

por dexametasona em cultivo de neurônios hipocampais de ratos. Um recente estudo mostrou que antidepressivos inibidores da MAO A e/ou MAO B, M30 (inibidor da MAO A e MAO B de nova geração), rozagilina e seleginina (inibidorores da MAO B) reverteram a morte celular induzida por dexametasona (Johnson et al., 2010). Kim et al. (2010) mostrou que ginsenosídeos Rb1 e Rg3, uns dos principais compostos da raiz de ginseng, apresentaram efeito neuroprotetor contra o dano causado por dexametasona em células SH-SY5Y. Portanto, o protocolo de morte celular induzida por dexametasona pode ser um bom modelo para estudos relacionados com a neuroproteção.

Trabalhos realizados por Zhu et al. (2006), Tazik et al. (2009) e Kim et al. (2010), utilizaram protocolos de morte celular induzido por dexametasona em células SH-SY5Y o qual, será objeto de estudo do presente trabalho. Estas células são derivadas de uma linhagem celular de neuroblastoma humano as quais expressam muitas propriedades de células neuronais e são amplamente utilizadas como um modelo celular para investigar mecanismos intracelulares que medeiam as ações de fármacos ou compostos em neurônios humanos (Xie et al., 2010). Portanto, esta linha celular é muito útil para estudar mecanismos protetores de morte neuronal (Kim et al., 2008; Romero et al., 2010).

TOXICIDADE GLUTAMATÉRGICA E NEUROPROTEÇÃO

O glutamato é um aminoácido excitatório mais abundante do SNC, onde sua transmissão é mantida sob fino controle, uma vez que é extremamente importante para mediar a resposta sináptica excitatória (Mattson, 2008; Severino et al., 2011). Este aminoácido é importante para a neurogênese, crescimento de neuritos, sinaptogênese e sobrevivência neuronal (Mattson, 2008). Por isso, o sistema glutamatérgico desempenha importante papel em muitas funções fisiológicas neuronais, como aprendizado, memória, cognição, plasticidade neuronal e ações neurotróficas e neurotóxicas (Mattson, 2008; Verkhratsky e Kirchhoff, 2007; Popoli et al., 2011).

O glutamato exerce ações em níveis pré e pós-sinápticos através da estimulação de diferentes receptores glutamatérgicos, expressos praticamente em todas as células de origem neuronal. Os receptores glutamatérgicos podem ser classificados como receptores ionotrópicos, (incluindo os receptores NMDA [N-metil-D-aspartato], AMPA [ácido α-amino-3-hidroxi-5-metil-4-isoxazolpropionico] e cainato) e receptores metabotrópicos acoplados a proteína G (mGluR1 a mGluR8) (Verkhratsky e Kirchhoff, 2007). Os receptores de glutamato

ionotrópicos ativados induzem influxo de cátions, por meio dos canais, para as células pós-sinápticas. Esta resposta é fundamental para produzir sinais despolarizantes em numerosas sinapses centrais (Dingledine et al., 1999).

Quando o controle da excitação glutamatérgica é perdido, ocorre um ativação excessiva dos receptores glutamatérgicos, um processo chamado de excitotoxicidade, que pode resultar em disfunção e morte neuronal (Olney, 1969), consequência de um grande influxo de Ca^{2+} e Na^{+} nos neurônios (Orrenius et al., 2003). A excitotoxicidade glutamatérgica está implicada em uma grande variedade de condições neuropatológicas como a doenca de Huntington, de Alzheimer, Parkinson, esclerose amiotrófica lateral, esclerose múltipla, depressão, epilepsia e esquizofrenia (Sanacora et al., 2008; Dong et al., 2009). Embora os mecanismos envolvidos nos eventos excitotóxicos são muito complexos, estudos realizados em fatias hipocampais de ratos mostram que este processo pode envolver a liberação do citocromo c, ativação de caspase-3 e fragmentação do DNA, via ativação da sinalização mediada por p38MAPK (Molz et al., 2008a). A toxicidade glutamatérgica está também relacionada com a atividade reversa dos transportadores de glutamato, os quais induzem aumento na concentração de glutamato extracelular e então promovem a excitotoxicidade e morte neuronal (Molz et al., 2008b).

Além disso, um estudo de Molz et al. (2011) mostrou que o insulto com glutamato em fatias hipocampais induz ativação da enzima óxido nítrico sintase induzida (iNOS). Realmente, em condições patológicas, principalmente associadas a inflamação, iNOS é induzida na microglia e astrócitos, e uma vez expressa, produz concentrações nanomolares de NO, o qual apresenta-se em concetrações 100-1000 vezes maiores, em relação ao NO produzido pela óxido nítrico neuronal (nNOS) (Pannu e Singh, 2006; Saha e Pahan, 2006). A produção de NO pela iNOS a partir da microglia ativada culmina em morte neuronal (Brown, 2010), uma vez que o NO é considerado uma molécula que pode estar relacionada com a disfunção tecidual na inflamação, doenças neurológicas e no processo de envelhecimento (Carmeli et al., 2012). É importante enfatizar que os neurônios são capazes de produzir NO em pequenas quantidades através da nNOS e este NO age como um neurotransmissor nestas células (Gonchar e Burkhalter, 1997; Vruwink et al., 2001), enquanto as células da glia (astrócitos e microglia) expressam as três isoformas da NOS (nNOS, eNOS-isoforma endotelial- e iNOS) e a iNOS, por sua vez, é induzida por vários estímulos estressantes como a excitotoxicidade glutamatérgica (Nomura, 1998;

Licinio et al., 1999; Lopez-Figueroa et al., 2000; Olivenza et al., 2000; Moro et al., 2004; Molz et al., 2011).

Muitos protocolos que mimetizam um evento excitotóxico induzido por glutamato foram desenvolvidos para buscar estratégia neuroprotetoras, como por exemplo, o insulto com glutamato em fatias hipocampais de ratos (Molz et al., 2008a; 2008b; 2011) e em cultura de neurônios (Zacco et al., 2003; Yang et al., 2007; Liang e Chuang, 2007; Vest et al., 2010), entre outros protocolos. Apesar de intensas pesquisas a cerca dos mecanismos excitotóxicos, poucas estratégias farmacológicas foram encontradas e obtiveram sucesso no combate aos transtornos associados com excitotoxicidade glutamatérgica. Embora não existam intervenções farmacológicas capazes de fornecer significativa neuroproteção na clínica, muitas estratégias com grande potencial neuroprotetor podem ser investigados nestes protocolos de morte celular induzida por glutamato, como: antagonistas de receptores NMDA, antagonistas de receptores AMPA, antagonistas de receptors cainato, bloqueadores da liberação de glutamato, *scavengers* de radicais livres, antioxidantes e inibidores da NOS (Lau e Tymianski, 2010).

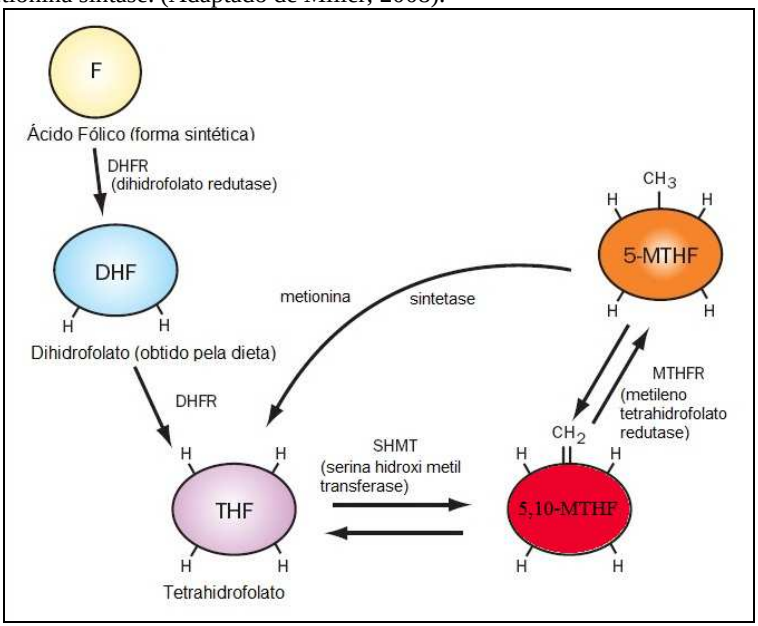
Levando em conta que (i) a excitotoxicidade glutamatérgica envolve vários mecanismos, (ii) e esta é um comum mecanismo para várias doenças, (iii) há muitos protocolos que mimetizam este evento; é importante pesquisar novas estratégias neuroprotetoras para o tratamento da excitotoxicidade glutamatérgica.

ÁCIDO FÓLICO

O ácido fólico (Folato) é uma vitamina do complexo B, uma das 13 vitaminas essenciais, obtido a partir da dieta ou suplementos. Geralmente os termos "ácido fólico" e "Folato" são usados como sinônimos. Folato é a forma obtida da dieta (forma desprotonada do ácido fólico), enquanto que o ácido fólico é a forma sintética desta vitamina utilizada para fortificar alimentos e suplementos nutricionais (Djukic, 2007; Miller, 2008). Folato é encontrado em vegetais de folhas verdes, legumes, feijões, frutas cítricas, fígados e grãos integrais (Mattson e Shea, 2003; Miller, 2008). Muitas reações bioquímicas são necessárias para converter o folato da dieta ou o ácido fólico sintético na sua forma biologicamente ativa (**Figura 5**). Vale ressaltar que o ácido fólico é altamente absorvido (85-95%) enquanto que o folato da dieta é absorvido em menor grau (50%) (Miller, 2009). A forma ativa co-enzimaticamente do ácido fólico é o tetrahidrofolato (THF), ao qual diferentes moléculas de carbono podem ser adicionadas. As formas

biologicamente ativas do ácido fólico apresentam diferentes níveis de redução e são susceptíveis a oxidação (Lamers, 2011). A enzima metilenotetrahidrofolato redutase (MTHFR), catalisa a conversão do 5,10-metilenotetrahidrofolato (5,10-MTHF) na forma 5-metiltetrahidrofolato (5-MTHF ou L-metilfolato). 5-MTHF é a forma predominante encontrada na circulação e tecidos (Mattson e Shea, 2003; Miller, 2008). (**Figura 5**).

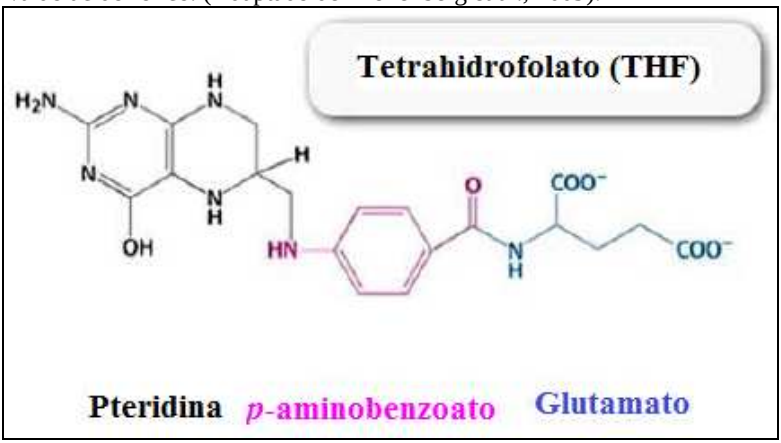
Figura 5. Formação do 5-MTHF a partir do ácido fólico e folato. O ácido fólico (forma sintética) ou o folato (dihidrofolato, DHF, obtido da dieta) sofrem ação da dihidrofolato redutase (DHFR) para converter à forma ativa, tetrahidrofolato (THF). Por sua vez, o THF sofre ação da serina hidroximetiltrasferase para formar o 5,10- metilenotetrahidrofolato (5,10-MTHF), que sofre a ação da metiltetrahidrofolato redutase (5-MTHF), forma predominante encontrada na circulação. 5-MTHF pode ser re-convertido a THF por ação da enzima metionina sintase. (Adaptado de Miller, 2008).



As três formas desta vitamina, ácido fólico, folato e 5-MTHF são formadas por um grupo de compostos heterocíclicos que possuem como características estruturais principais, e comuns as três formas, um grupo pteridina, um grupo ácido p-aminobenzóico (PABA) e uma cadeia de

ácido glutâmico (glutamato) de pesos variáveis como indicado na **figura 6** através da fórmula estrutural do THF (Djukic, 2007).

Figura 6. Fórmula estrutural do tetrahidrofolato (THF), forma biologicamente ativa do ácido fólico. (Adaptado de Kronenberg et al., 2009).

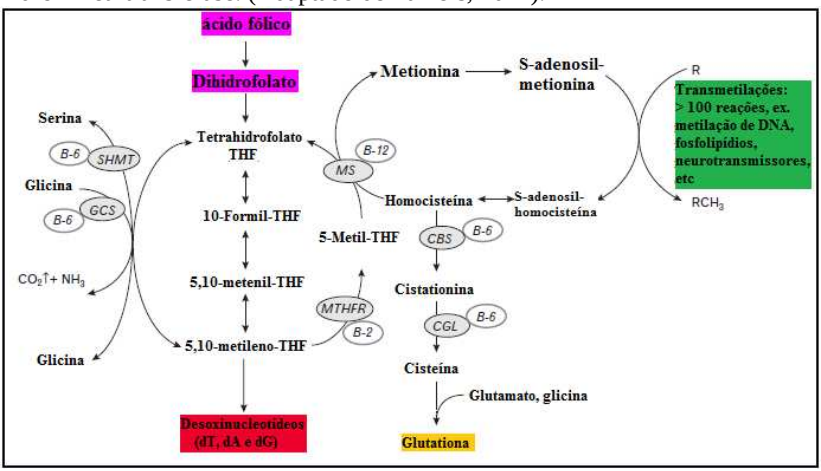


O 5-MTHF pode também ser obtido pela conversão do ácido folínico (5-formiltetrahidrofolato), um metabólito ativo do ácido fólico (**Figura 7**). Em função de não ocorrer síntese *de novo* de ácido fólico no SNC, a manutenção dos níveis adequados de ácido fólico e seu metabólito no cérebro depende do transporte adequado desta vitamina através da barreira hemato-encefálica, lembrando que a forma que atravessa a barreira hemato-encefálica é a 5-MTHF (Ramaekers e Blau, 2004).

Ácido fólico age como um cofator de enzimas que participam do metabolismo de transferência de moléculas de carbono (Mattson e Shea, 2003; Coppen e Boulander-Gouaille, 2005; Kronenberg et al., 2009) e, portanto, desempenha várias funções: a) remetila a homocisteína, um aminoácido citotóxico em concentrações elevadas, em metionina que por sua vez pode ser convertida a S-adenosilmetionina (SAM), composto responsável pela transferência de grupos metilas a muitas moléculas (Coppen e Bolander-Gouaille, 2005; Kronenberg et al., 2009); b) biossíntese de nucleotídeos como DNA, RNA e aminoácidos; c) regula a expressão gênica (Miller, 2008) d) previne defeitos no tubo neural durante o desenvolvimento do SNC (Mattson e Shea, 2003; Coppen e Boulander-Gouaille, 2005); e) aumenta a biossíntese de tetrahidrobiopterina, a qual é coenzima da enzima tirosina hidroxilase

para a hidroxilação de tirosina na biossíntese de dopamina e noradrenalina e da triptofânio hidroxilase para a síntese de serotonina (Coppen et al., 1989, Miller, 2008); f) possivelmente exerce um papel neuroprotetor em danos ao SNC, por promover reparo e crescimento neuronal (Iskandar et al., 2004). O esquema ilustrado na **figura 7** resume a importância do ácido fólico no metabolismo.

Figura 7: Esquema ilustrativo da transferência de moléculas de carbono no metabolismo do ácido fólico. O tetrahidrofolato (THF) é convertido em 5,10-metilenotetrahidrofolato (5,10-MTHF), importante para a síntese de nucleotídios, e em seguida reduzido a 5-metiltetrahidrofolato (5-MTHF) pela ação da enzima metileno tetrahidrofolato redutase (MTHFR), transferindo um grupo metil para a formação da metionina, etapa catalisada pela enzima metionina sintase (MS). A homocisteína, um aminoácido citotóxico, é metilada a metionina, um aminoácido importante para a síntese de S-adenosil-metionina, um agente importante nas mais de 100 reações de transmetilações (DNA, fosfolipídios e neurotransmissores) *in vivo*. CBS: cistationina β-sintetase; CGL:cistationina γ liase; CGS: Sistema da clivagem da glicina; MS: metionina sintase; MTFR: 5,10-metilenotetrahidrofolato redutase; R: compostos que recebem grupos metilas; RCH_3: composto metilado; SHMT: serina hidroximetiltransferase. (Adaptado de Lamers, 2011).



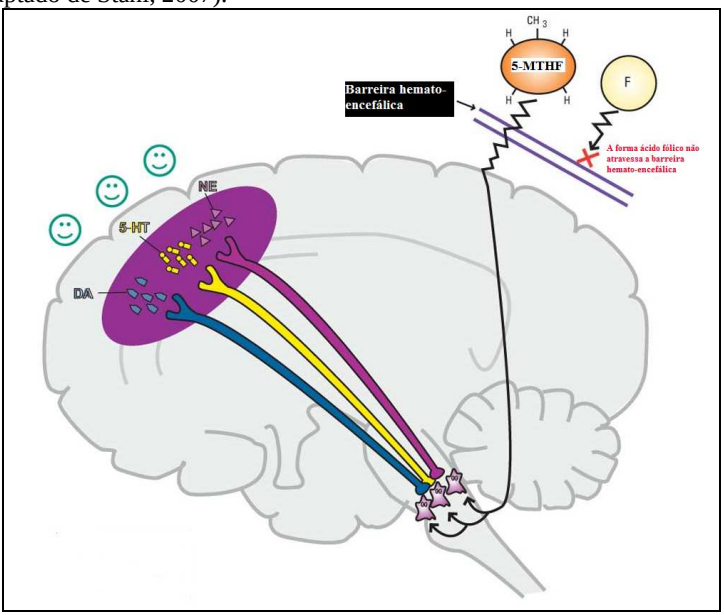
Em função destas inúmeras funções do ácido fólico, qualquer anormalidade que ocorra no metabolismo desta vitamina pode afetar o SNC. Esta visão é suportada pela alta incidência da deficiência do ácido fólico associadas a depressão, transtorno bipolar, esquizofrenia, doença de Parkinson, doença de Alzheimer, entre outras doenças (Mattson e

Shea, 2003; Coppen e Bolander-Gouaille, 2005; Stahl, 2007; Miller, 2008; Krebs et al., 2009).

ÁCIDO FÓLICO E DEPRESSÃO

Levando em consideração que o ácido fólico está envolvido no metabolismo e função de muitas substâncias que são essenciais ao SNC (purinas, pirimidinas, DNA, RNA, aminoácidos, compostos fosfatados, vitamina B-12, metionina, SAM e neurotransmissores) (Matson e Shea, 2003; Coppen e Bolander-Gouaille, 2005; Kronenberg et al., 2009; Lucock, 2011), é de se esperar que alterações no seu metabolismo possa resultar em doenças como a depressão. De fato, o ácido fólico, mais precisamente sua forma bioativa (5-MTHF) age como coenzima na síntese das três monoaminas: dopamina (DA), noradrenalina (NE) e serotonina (5-HT) (Stahl, 2007) (**Figura 8**).

Figura 8: Ácido fólico contribui para a síntese das três monoaminas: dopamina (DA), serotonina (5-HT) e noradrenalina (NE). A forma ácido fólico não ultrapassa a barreira hemato-encefálica, portanto necessita ser convertido a 5-MTHF para chegar ao SNC e participar da síntese de neurotransmissores. (Adaptado de Stahl, 2007).



A primeira descrição relacionando ácido fólico e depressão surgiu na década de 60, com o estudo de Herbert et al. (1962). Em seguida muitos outros trabalhos surgiram indicando que pacientes deprimidos e com prejuízos cognitivos apresentavam baixos níveis de ácido fólico no plasma, soro, eritrócito e líquor (Carney, 1967; Reynolds et al., 1970; Carney e Sheffield, 1978; Ghadirian et al., 1980; Abou-Saleh e Coppen, 1989; Bottiglieri et al., 2000; Lindeman et al., 2000). Outros estudos igualmente importantes mostraram uma forte correlação entre a deficiência do ácido fólico e a severidade da depressão (Abou-Saleh e Coppen, 1989; Levitt e Joffe,1989; Bottiglieri et al., 2000), bem como, indicaram que uma dieta pobre em ácido fólico aumenta muito o risco do desenvolvimento de sintomas depressivos (Tolmunen et al., 2003) e está associada à prevalência de depressão (Nanri et al., 2010). Em contraste, alguns estudos mostram ausência de correlação entre deficiência de ácido fólico e depressão (Williams et al., 2005; Ford et al., 2008; Walker et al., 2010; Skarupski et al., 2010). Portanto, novos estudos necessitam ser realizados para avaliar o papel do ácido fólico na depressão.

Além disso, há indícios na literatura de que a deficiência de ácido fólico está relacionada com duradouras recaídas depressivas durante o tratamento, como por exemplo, de fluoxetina (Papakostas et al., 2004b). Corroborando com este estudo, Astorg et al. (2008) mostraram que um dieta deficiente em ácido fólico está associada com risco de recorrência da depressão em homens de meia idade. Porém, ainda especula-se se a relação entre deficiência de ácido fólico e depressão possa ser meramente causal. Um estudo de Kendrick et al. (2008) sugere que a baixa concentração de ácido fólico pode ser mais uma consequência do que uma causa deste transtorno.

A grande relação entre deficiência de ácido fólico e depressão se sustenta ainda no fato de que a suplementação do ácido fólico em pacientes deprimidos promove melhora do quadro depressivo (Botez et al., 1982;1984; Godfrey et al., 1990; Alpert et al., 2002). Outro ponto importante é que a deficiência de ácido fólico está associada com uma fraca resposta à terapia com antidepressivos clássicos, como a fluoxetina, em pacientes deprimidos (Fava et al., 1997; Coppen e Bailey, 2000; Papakostas et al., 2004a; 2005). Além disso, o tratamento combinado com ácido fólico (5-MTHF) e o antidepressivo trazodona melhorou as escores na Escala Hamilton em pacientes deprimidos com níveis normais ou níveis baixos de ácido fólico (Passeri et al., 1993). O tratamento combinado com ácido fólico e fluoxetina, também resultou em melhora dos escores na Escala Hamilton (escala que estima a

severidade da depressão), em mulheres deprimidas (Coppen e Bailey, 2000). Outro estudo ainda mostrou que o ácido fólico pode ser um tratamento alternativo para pacientes com deficiência desta vitamina e resistentes ao tratamento com fluoxetina (Papakostas et al., 2004a). Portanto, pacientes deprimidos, hipofolatêmicos, mostram resposta tardia ao tratamento com fluoxetina quando comparado a paciente deprimidos, normofolatêmicos (Papakostas et al., 2005). Resler et al. (2008) mostrou que o tratamento combinado com ácido fólico e fluoxetina em pacientes deprimidos reduziu a homocisteína e induziu acúmulo de serotonina em linfócitos, provavelmente modificando a função destas células na depressão. Em contraste, um estudo mostrou que não há claras evidências de que o tratamento combinado com ácido fólico e antidepressivos melhora os sintomas da depressão em pacientes (Christensen et al., 2011).

Também é importante considerar que a suplementação com ácido fólico pode ser importante na prevenção do desenvolvimento da depressão após um acidente vascular cerebral (Almeida et al., 2010) e durante ou após o parto (Lewis et al., 2011).

Portanto, a literatura indica fortes evidências clínicas da associação entre deficiência de ácido fólico e depressão, mas muitas dúvidas ainda estão presentes, já que alguns estudos são contrários a esta hipótese. Tendo isso em vista, novos estudos clínicos, bem como estudos pré-clínicos necessitam ser realizados para elucidar o papel do ácido fólico na depressão.

Vários estudos pré-clínicos estão surgindo para elucidar o possível efeito tipo-antidepressivo do ácido fólico em modelos animais de depressão e futuramente estes estudos podem se tornar uma base para novos possíveis estudos clínicos. Nosso grupo de pesquisa demonstrou que a administração de ácido fólico (via oral e intracerebroventricular) produz efeito tipo-antidepressivo em modelos animais preditivos de atividade antidepressiva, TNF e TSC, em camundongos. Além disso, mostrou que o seu mecanismo de ação envolve, pelo menos em parte, uma interação com os sistemas monoaminérgico, incluindo os sistemas serotoninérgico (receptores5-HT_{1A} e 5-$HT_{2A/2C}$) e noradrenérgico (receptores α_1 e α_2) (Brocardo et al., 2008a). Adicionalmente, o efeito tipo-antidepressivo desta vitamina mostrou ser dependente da inibição de receptores NMDA ou redução da síntese de NO e guanosina monofosfato cíclico (GMPc) (Brocardo et al., 2008b), bem como de uma interação com o sistema opióide (receptores μ_1 e δ) (Brocardo et al., 2009). Além disso, nosso grupo demonstrou que o ácido fólico apresenta efeito anti-mania em um modelo animal de mania induzido

por ouabaína (Brocardo et al, 2010). Um estudo de Molina-Hernández et al. (2011), verificou que o ácido fólico sozinho ou em combinação com estradiol ou fluoxetina apresentou efeito tipo-antidepressivo em ratas fêmeas ovariectomizadas no TNF. O mesmo grupo, porém em outro estudo, mostrou que este efeito tipo-antidepressivo do ácido fólico pode ser em função de uma interação com os receptores de neuropeptídio Y (NPY Y_1). E mais recentemente, Molina-Hernández et al. (2012), mostrou que um infusão septal lateral de ácido fólico sozinho ou em combinação com doses sistêmicas de fluoxetina promoveu efeito tipo-antidepressivo no TNF em ratas, levando a confirmar que este efeito pode ser em função de uma interação, pelo menos em parte, com o sistema serotoninérgico.

Uma revisão mais detalhada da literatura referente ao papel do ácido fólico em doenças psiquiátricas, com ênfase na depressão, encontra-se no capítulo 6.

ÁCIDO FÓLICO E NEUROPROTEÇÃO

Como relatado anteriormente, o ácido fólico participa de muitas reações bioquímicas, portanto, é de se esperar que esta vitamina desempenhe um importante papel neuroprotetor (Matson e Shea, 2003; Coppen e Bolander-Gouaille, 2005; Miller, 2008; Kronenberg et al., 2009; Lucock, 2011). De fato, muitos estudos demonstram que o ácido fólico apresenta efeito neuroprotetor frente a vários estímulos tóxicos *in vivo* (Tagliari et al., 2006) e *in vitro* (Lin et al., 2004; Yu et al., 2009).

O ácido fólico apresenta um importante papel na prevenção da adição errônea de ribonucleotídios contendo a base uracila no DNA, bem como, a quebra da fita de DNA e a hipometilação do mesmo (Fenech, 2011). Além disso, foi reportado que depleção de ácido fólico induz aumento de Ca^{2+} citosólico, EROs e prejuízo da função mitocondrial (Ho et al., 2003; Tjiattas et al., 2004). O ácido fólico desempenha um importante papel protetor contra a citotoxicidade induzida por glutamato ou NMDA em cultivo de neurônios granulares cerebelares de camundongos (Lin et al., 2004). Um estudo de Yu et al. (2009) mostrou que o ácido fólico também protege neurônios do dano induzido pelo peptídeo β amilóide$_{31-35}$ por manter a função mitocondrial, integridade de DNA e regulação de genes associados à apoptose.

Esta vitamina apresenta propriedades antioxidantes intrínsecas, uma vez, que pode ser considerado um promissor agente modulador de Fe^{2+} (Patro et al., 2006) e/ou pode agir eliminando EROs (Joshi et al., 2001). Um estudo de Matté et al. (2009a) corrobora com esta hipótese,

já que a suplementação com ácido fólico reverteu o dano oxidativo, no sangue e córtex parietal de ratos, induzido por hiperhomocisteína crônica.

Além disso, o ácido fólico apresenta um importante papel cognitivo evidenciado por estudos pré-clínicos (Matté et al., 2007; Troen et al., 2008; Matté et al., 2009b) e clínicos (Kado et al., 2005; Ramos et al., 2005; Durga et al., 2006; De Lau et al., 2007).

Todos estes dados mostram o importante papel neuroprotetor desta vitamina. Portanto, o estudo neuroprotetor *in vitro* do ácido fólico torna-se promissor.

2. JUSTIFICATIVA

Estudos clínicos e epidemiológicos mostram que os transtornos mentais são altamente prevalentes (perfazem em torno de 28 % de todas as doenças), heterogêneos e apresentam etiologia multifatorial. Eles estão associados com sofrimento incalculável, prejuízos funcionais e alto custo financeiro (Mclntyre et al., 2006; Prince et al., 2007).

Os transtornos mentais são causas importantes de distúrbios funcionais à longo prazo na vida de um indivíduo, os quais, segundo a dados da OMS de 2005 perfazem 31,7 % da vida de um paciente. As doenças que mais contribuem para este prejuízo funcional são: depressão maior (11,8 %), transtornos relacionados ao uso do álcool (3,3 %), esquizofrenia (2,8 %), transtorno bipolar (2,4 %) e demência (1,6 %) (Prince et al., 2007).

A depressão é um transtorno altamente prevalente, complexo e muito heterogêneo. A complexidade e heterogeneidade dificultam o diagnóstico e o tratamento da mesma. Embora os antidepressivos clássicos sejam amplamente prescritos para a depressão, apresentam muitas limitações, como por exemplo, um longo período para a resposta terapêutica (semana a meses), baixa taxa de resposta terapêutica (um terço dos pacientes respondem ao primeiro fármaco prescrito e dois terços após muitas tentativas, o que frequentemente leva meses ou anos). Além disso, não podemos deixar de considerar que a depressão apresenta altas taxas de suicídio (Duman e Voleti, 2012), o que agrava e muito o problema da baixa eficácia dos antidepressivos. Portanto, há necessidade de novos agentes no mercado farmacêutico para a depressão.

Levando em consideração os seguintes achados: pacientes deprimidos frequentemente apresentam uma deficiência funcional de ácido fólico e desempenham pobre resposta à terapia com antidepressivos (Coppen e Bailey, 2000; Papakostas et al., 2004a; 2005); b) ácido fólico está associado a síntese de monoamines (Stahl, 2007); c) a suplementação com ácido fólico pode potenciar o efeito de fármacos antidepressivos (Resler et al., 2008; Almeida et al., 2010; Christensen et al., 2011); d) estudos pré-clínicos mostram que o ácido fólico apresenta efeito tipo-antidepressivo em ratos e camundongos (Brocardo et al., 2008a; Molina-Hernández et al., 2011); é razoável concluir que esta vitamina pode ser uma alternativa segura, simples e barata na terapia para a depressão (Roberts et al., 2007). Além disso, devemos levar em conta o potencial papel neuroprotetor (Tagliari et al., 2006; Lin et al., 2004; Yu et al., 2009; Matté et al., 2009a) e cognitivo

(Matté et al., 2007; Troen et al., 2008; Matté et al., 2009b) desta vitamina, os quais o torna um alvo importante de estudo para a depressão e neuroproteção em modelos animais de depressão, bem como protocolos de morte celular. Desta forma, o presente estudo poderá contribuir futuramente para que o ácido fólico possa ser utilizado na clínica sozinho ou em associação com antidepressivos, podendo tornar-se uma importante alternativa terapêutica no tratamento da depressão.

3. OBJETIVOS

3.1. OBJETIVO GERAL

Avaliar o efeito do ácido fólico em modelos animais de depressão em camundongos e em protocoloss *in vitro* de dano celular (glutamato e dexametasona) que possam estar envolvidos no mecanismo de ação desta vitamina nos referidos modelos, analisando, além dos aspectos comportamentais (estudos *in vivo*), os aspectos bioquímicos (estudos *in vivo e in vitro*) do efeito do ácido fólico.

3.2. OBJETIVOS ESPECÍFICOS

- Avaliar se o efeito antidepressivo do ácido fólico pode envolver a via da PI3K/Akt/GSK-3β e PPAR- γ no TNF e CA em camundongos;
- Avaliar se o efeito antidepressivo do ácido fólico pode envolver canais de potássio no TNF e CA em camundongos;
- Verificar o efeito da administração oral de ácido fólico no modelo animal de depressão induzida pelo estresse de contenção no TNF, TRO e CA em camundongos e avaliar parâmetros de estresse oxidativo em córtex cerebral e hipocampo;
- Avaliar o efeito do ácido fólico no protocolo de morte celular induzida pela dexametasona em células de neuroblastoma humano SH-SY5Y na viabilidade celular e investigar se este efeito envolve a ativação de proteínas de sinalização como PI3K/Akt, PKA, PKC, CAMKII e MEK.
- Avaliar o efeito do ácido fólico em um protocolo de morte celular induzida pela excitotoxicidade glutamatérgica em fatias de hipocampo, na viabilidade celular e na liberação de glutamato, bem como, investigar o imunoconteúdo de GSK-3β, β-catenina e iNOS;

4. MATERIAIS E MÉTODOS

Os materiais e métodos encontram-se, nos seus respectivos capítulos, descritos na sessão dos resultados.

5. RESULTADOS

Estudos in vivo

Capítulo 1

Involvement of PI3K, GSK-3β and PPARγ in the antidepressant-like effect of folic acid in the forced swimming test in mice. Budni J, Lobato KR, Binfaré RW, Freitas AE, Costa AP, Saavedra MD, Leal RB, Lopez MG, Rodrigues ALS. J Psychopharmacol. *In press*, 2012. (Em anexo)

Capítulo 2

Role of potassium channels in the antidepressant-like effect of folic acid in the forced swimming test in mice. Budni J, Freitas AE, Binfaré RW, Rodrigues ALS. Pharmacol Biochem Behav. 101(1):148-54, 2012. (Em anexo)

Capítulo 3

Folic acid prevents depressive-like behavior and hippocampal antioxidant imbalance induced by restraint stress in mice. Budni J, Zomkowski AD, Engel D, Santos DB, dos Santos AA, Moretti M, Valvassori SS, Ornell F, Quevedo J, Farina M, Rodrigues ALS. Artigo em preparação. (Em anexo)

Estudos *in vitro*

Capítulo 4

Neurotoxicity induced by dexamethasone in the human neuroblastoma SH-SY5Y cell line can be prevented by folic acid. Budni J, Romero A, Molz S, Martín-de-Saavedra MD; Egea J, Del Barrio L, Tasca CI, Rodrigues ALS, Lopéz MG. Neuroscience. 190:346-53, 2011. (Em anexo)

Capítulo 5

Excitotoxicity induced by glutamate in the hippocampus slices can be prevented by folic acid through the GSK-3β and iNOS inhibition. Budni J, Molz S, Dal-Cim T, Martín-de-Saavedra MD; Egea J, Lopéz MG, Tasca CI, Rodrigues ALS. Artigo em preparação. (Em anexo)

Capítulo de livro
Capítulo 6
The role of folic acid in psychiatric disorders. Budni J; Brocardo PS.; Rodrigues Ana LS. IN: In: Mária Szabó; Eve Varga. (Org.). Folic Acid: Properties, Medical Uses and Health Benefits. Hauppauge, New York: Nova Publishers, 2011, v. 0, p. 1-27. (Em anexo)

Estudos *in vivo*

CAPÍTULO 1

Involvement of PI3K, GSK-3β and PPARγ in the antidepressant-like effect of folic acid in the forced swimming test in mice. Budni J, Lobato KR, Binfaré RW, Freitas AE, Costa AP, Saavedra MD, Leal RB, Lopez MG, Rodrigues ALS. J Psychopharmacol. *In press*, 2012.

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Abstract

Preclinical and clinical studies indicate that deficiency in folic acid plays a role in the pathophysiology of depression. Considering that alterations in the signaling pathways that regulate neuroplasticity and cellular survival are implicated in depressive disorders, the present study investigated the involvement of the phosphoinositide 3-kinase (PI3K), glycogen synthase kinase-3 (GSK-3β), and peroxisome proliferator-activated receptor-γ (PPARγ) in the antidepressant-like effect of folic acid in the forced swimming test (FST). The intracerebroventricular (i.c.v.) pre-treatment of mice with LY294002 (10 nmol/site, a PI3K inhibitor) or GW-9662 (1 μg/site, a PPARγ antagonist) prevented the antidepressant-like effect of folic acid (50 mg/kg, p.o.) in the FST. In addition, the administration of subeffective doses of the selective GSK-3β inhibitor, AR-A014418 (3 mg/kg, i.p.), a non-selective GSK-3β inhibitor, lithium chloride (10 mg/kg, p.o) or a PPARγ agonist, rosiglitazone (1 μg/site, i.c.v.) in combination with a subeffective dose of folic acid (10 mg/kg, p.o.) significantly reduced the immobility time in the FST as compared with either drug alone, without altering the locomotor activity. These results indicate that the antidepressant-like effect of folic acid in the FST might be dependent on inhibition of GSK-3β and activation of PPARγ, reinforcing the notion that these are important targets for antidepressant activity.

Keywords

Antidepressant, folic acid, forced swimming test, GSK-3β, PI3K, PPARγ

Introduction

Although the underlying pathophysiological mechanisms of depression are not completely identified, novel targets have been identified for the development of new pharmacological treatments. There is increasing evidence that a disturbed one-carbon metabolism may be a significant factor contributing to depressive disorders (Coppen and Bolander-Gouaille, 2005; Sarris et al., 2009). Folic acid (folate) is a water-soluble vitamin that is essential for cell replication and plays an essential role in one-carbon metabolism that is crucial for neurological function (Kronenberg et al., 2009).

There are several clinical studies showing that folate deficiency is associated with a higher incidence of depression. Reduced plasma, serum, or red blood cell folate is commonly found in major depressive illnesses (Abou-Saleh and Coppen, 2006; Sarris et al., 2009). Moreover, a low folate status is associated with a poorer response to antidepressant medication, and supplementing antidepressant medication with folic acid improves the therapeutic effect (Godfrey et al., 1990; Coppen and Bailey, 2000; Alpert et al., 2002). Therefore, folate is proposed to have therapeutic potential as an augmentation strategy in the treatment of depressive disorder. However, it is not well established if this is the case both for people with normal folate levels, and for those with folate deficiency (Taylor et al., 2004).

Corroborating the role of folic acid in depression, preclinical studies from our group have shown that systemic and central administration of folic acid produces antidepressant-like effects in two predictive models of antidepressant activity, the forced swimming test (FST) and tail suspension test (TST) (Brocardo et al., 2008a). The mechanisms by which folic acid produces antidepressant-like effects are not fully established, but were shown to be dependent on the serotonergic and noradrenergic systems (Brocardo et al., 2008a), inhibition of N-methyl-D-aspartate (NMDA) receptors and nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) synthesis (Brocardo et al., 2008b), and interaction with the opioid system (μ1 and δ-opioid receptors) (Brocardo et al., 2009).

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Besides the well established involvement of the monoaminergic system in depression, the alterations in signaling pathways that regulate neuroplasticity and cellular survival, such as PI3K, the serine/threonine protein kinase Akt, GSK-3β enzyme (Beaulieu et al., 2009) and PPARγ are also implicated in the mechanisms underlying depression as well as antidepressant responses (Rosa et al., 2008; Eissa Ahmed et al., 2009).

The PI3K is one family of lipid kinases which catalyzes the phosphorylation of phosphatidylinositol lipids at the 3′ position of the inositol ring in response to cell stimulation by growth factors, hormones, and cytokines (Katso et al., 2001; Cantley, 2002). Several studies have implicated PI3K in synaptic plasticity, learning and memory, and major depression (Kelly and Lynch, 2000; Dwivedi et al., 2008; Yang et al., 2008). Lipid products of PI3K act as second messengers by recruiting proteins such as Akt and its activating kinases (Brazil et al., 2004, Hanada et al., 2004). It regulates negatively the activity of GSK-3β via phosphorylation at the N-terminal serine 9 (Cross et al., 1995; Hetman et al., 2000; Beaulieu et al., 2009).

GSK-3β is a multifunctional serine/threonine kinase found in all eukaryotes that is now recognized as a key component of multiple signaling pathways (Jope and Roh, 2006). In mammals, two closely related isoforms, GSK-3α and GSK-3β, are present. GSK-3β is highly expressed in neuronal tissue and it is regulated during development (Bhat et al., 2004). This enzyme has been suggested to be implicated in the pathogenesis of depression. For instance, the mood stabilizers lithium and valproate, largely used in the treatment of bipolar disorder (Jope, 1999), cause a direct inhibition of GSK-3β (Chen et al., 1999; Li et al., 2002). Moreover, the electroconvulsive shock (ECS) therapy, a common approach for the management of drug-resistant depression, may induce biphasic changes, with immediate dephosphorylation of GSK-3 and subsequent hyperphosphorylation of the enzyme within 3 h after ECS (Roh et al., 2003). In addition, increased serotonergic activity following the administration of antidepressants inhibits GSK-3β in the brain (Li et al., 2004). Further corroborating the role of GSK-3β as a target for antidepressant action, some studies have shown that GSK-3β inhibitors cause antidepressant-like effects. It was demonstrated that AR-A014418, a specific inhibitor of GSK-3β, has an antidepressant-like effect in the FST in rats (Gould et al., 2004a) and mice (Rosa et al., 2008). L803-mts (N-myristoyl-GKEAPPAPPQS[p]P), a novel GSK-3 peptide inhibitor, and NP031115, a novel thiadiazolidinone compound, also produce an antidepressant-like effect in the mouse FST (Kaidanovich-Beilin et al., 2004; Rosa et al., 2008).

PPARs are members of the nuclear hormone receptor superfamily of ligand-dependent transcription factors, and the three major PPAR isoforms are α, β/δ, and γ (Yessoufou and Wahli, 2010). PPARγ, besides GSK-3β, is an emerging target in pharmacology with promising effects in depression (Rosa et al., 2008). PPARγ are members of the nuclear receptor superfamily that function as ligand-activated transcription factors to regulate gene expression involved in reproduction, metabolism, development, and immune responses (Desvergne and Wahli, 1999). A relationship between PPARγ and GSK-3β was demonstrated by treatment of rat hippocampal neurons with a PPARγ agonist which decreased the GSK-3β activity (Inestrosa et al., 2005). Regarding the involvement of PPARγ in depression, our group has shown that the administration of the PPARγ agonist rosiglitazone causes an antidepressant-like effect in the mouse FST that was prevented by the pre-treatment of mice with the PPARγ receptor antagonist GW-9662 (Rosa et al., 2008). Moreover, in that study GW-9662 was able to prevent the anti-immobility effect of the GSK inhibitors NP031115 and AR-A014418 in the FST (Rosa et al., 2008). Recently, it was shown that rosiglitazone also produces antidepressant-like effects in the TST in mice and in the FST in rats (Eissa Ahmed et al., 2009). Moreover, reinforcing the role of PPARγ in depression, a recent study has shown that rosiglitazone administration to nondiabetic patients with unipolar or bipolar depression, who had surrogate blood markers suggestive of insulin resistance caused declines in depression severity scores (Rasgon et al., 2010).

Considering: (a) that reduced folic acid availability has been implicated in the pathophysiology of bipolar and major depression in humans; (b) the mechanisms underlying the antidepressant-like effects of folic acid are not completely elucidated; and (c) PI3K activation, GSK-3β inhibition, and PPARγ receptor activation have been associated with antidepressant activity; this study sought to investigate the participation of these molecular targets in the antidepressant-like effect of folic acid in the FST in order to contribute to the understanding of its mechanism of action.

Methods and materials

Animals

Swiss mice of either sex, weighing 30–40 g were maintained at 22–24°C with free access to water and food, under a 12:12 h light:dark cycle (lights on at 07:00). Animals (male and female mice were homogeneously distributed among groups) were acclimatized to the laboratory for at least 12 h before testing and were used only once throughout the experiments. Male and female mice were maintained in different cages. All manipulations were carried out between 09:00 and 16:00. All procedures in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The experiments were performed after approval of the protocol by the Ethics Committee of the institutions and all efforts were made to minimize animal suffering.

Drugs and treatment

The following drugs were used: folic acid, rosiglitazone, LY294002, AR-A014418 (Sigma Chemical Co., St Louis, USA), GW-9662 (Calbiochem, Darmstadt, Germany) and lithium chloride (MERCK, Darmstadt, Germany). Rosiglitazone and GW-9662 were dissolved in saline. LY294002 and AR-A014418 were dissolved in saline at a final concentration of 1% dimethyl sulfoxide (DMSO). Lithium chloride and folic acid were diluted in water. AR-A014418 was administered by intraperitoneal (i.p.) route. Lithium chloride and folic acid were administered by oral route (p.o.) through gavage. All drugs were administered in a constant volume of 10 ml/kg body weight, except LY294002, GW-9662 and rosiglitazone that were administered by intracerebroventricular (i.c.v.) route. I.c.v. administration was performed under ether anesthesia, directly into the lateral ventricle, in a volume of 5 µl per mouse, given over 30 s, and the cannula remained in place for another 30 s, as previously described (Kaster et al., 2007; Brocardo et al., 2008b). Appropriate vehicle-treated groups were also assessed simultaneously.

To investigate the involvement of PI3K in the antidepressant-like effect of folic acid, mice were pre-treated with LY294002, a PI3K inhibitor (10 nmol/site, i.c.v., a dose that produces no effect in the FST). After 15 min, folic acid (50 mg/kg, p.o.) or water was injected, and 60 min later the FST was carried out.

To test the hypothesis that the antidepressant-like effect of folic acid could be mediated by the inhibition of GSK-3β activity, mice were treated with folic acid (10 mg/kg, p.o., a sub-effective dose in the FST) or water (control group) and 30 min after, were injected with a sub-effective dose of AR-A014418 (3 mg/kg, i.p.) or vehicle. The FST or open-field test was carried out 30 min later. The dose of AR-A014418 was chosen based on a previous study from our group which shows that this is a sub-effective dose in the FST (Rosa et al., 2008). In another experiment, mice were treated with lithium chloride (10, 30, and 100 mg/kg, p.o., a mood stabilizer that acts as a non-selective GSK-3β inhibitor) or water (control group) and 60 min later the FST or open-field was carried out. This experiment was performed to choose a sub-effective dose of lithium chloride in the FST. In addition, mice received folic acid (10 mg/kg, p.o.) or water and immediately after, lithium chloride (10 mg/kg, p.o., a sub-effective dose in the FST) or water was administered. The FST or the open-field test was carried out 60 min later.

In order to investigate the involvement of PPARγ in the antidepressant-like effect of folic acid, mice were treated with folic acid (10 mg/kg, p.o) or water (control group) and 45 min after, were injected with a sub-effective dose of the PPARγ agonist rosiglitazone (1 μg/site, i.c.v.). After 15 min, the FST was carried out. In another set of experiments, mice were pre-treated with GW-9662, a PPARγ antagonist (10 μg/site, i.c.v., a dose that produces no effect in the FST). After 15 min, folic acid (50 mg/kg, p.o.) or water was administered, and 60 min later the FST was carried out. The doses of rosiglitazone and GW-9662 were selected based on a previous study from our group (Rosa et al., 2008).

The doses of folic acid (10 mg/kg, p.o., sub-effective dose in the FST and 50 mg/kg, p.o., effective dose in the FST) were chosen based on the dose-response curve in the mouse FST (Brocardo et al., 2008a).

Forced swimming test

Mice were individually forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water at 25 ± 1°C; the total duration of immobility during a 6 min test was scored as described previously (Rosa et al., 2003; Budni et al., 2007; Kaster et al., 2007; Rosa et al., 2008). Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. A decrease in the duration of immobility is indicative of an antidepressant-like effect (Porsolt et al., 1977).

Open-field test

The ambulatory behavior was assessed in an open-field test as previously described (Budni et al., 2007; Kaster et al., 2007). Briefly, the apparatus consisted of a wooden box measuring 40×60×50 cm with the floor of the arena divided into 12 equal squares. At the start of each trial a mouse was placed in the left corner of the field and was allowed to freely explore the arena. The number of squares crossed with all paws (crossing) was counted in a 6-min session. The arena floor was cleaned between the trials and the test was carried out in a temperature and light controlled room.

Statistical analysis

All experimental results are given as the mean ± SEM. Comparisons between experimental and control groups were performed by one-way ANOVA (dose-response curve of lithium chloride) or two-way ANOVA (interaction of folic acid with the pharmacological agents) followed by Newman–Keuls test when appropriate. A value of $P < 0.05$ was considered to be significant.

Results and discussion

The results presented in Figure 1A shows that the anti-immobility effect of folic acid (50 mg/kg, p.o.) was completely prevented by pre-treatment of animals with the PI3K inhibitor LY294002 (10 nmol/site, i.c.v.). Figure 1B shows that the administration of LY294002 alone or in combination with folic acid was devoid of effect in the open-field test.

The results illustrated in Figure 2A show that the administration of a sub-effective dose of AR-A014418 (3 mg/kg, i.p., a selective inhibitor of GSK-3β) in combination with a sub-effective dose of folic acid (10 mg/kg, p.o.) produced an antidepressant-like effect as compared with the administration of either drug alone. Figure 2B shows that the administration of AR-A014418 alone or in combination with folic acid did not affect locomotor activity in the open-field test.

In addition, lithium chloride, a non-selective GSK-3β inhibitor, was used as a pharmacological tool to reinforce the notion that folic acid inhibits GSK-3β. In order to investigate the effect of the acute administration of lithium chloride on the immobility time in the FST, a dose-response curve was performed. The results depicted in Figure 3 show that lithium chloride causes a reduction in the immobility time in the FST at doses of 30 and 100 mg/kg, p.o., but not at the dose of 10 mg/kg, p.o. Figure 3B shows that lithium chloride administration (10–100 mg/kg, p.o.) causes a reduction in the locomotor activity of mice in the open-field test. Drugs that cause a psychostimulant effect in the open-field test may give a false positive result in the FST. Therefore, the hypolocomotor effect elicited by lithium chloride administration does not account for the anti-immobility effect produced by this GSK-3β inhibitor. Figure 3C shows that the administration of lithium chloride at a sub-effective dose (10 mg/kg, p.o.) in combination with a sub-effective dose of folic acid caused an anti-immobility effect in the FST, as compared with either drug alone. Figure 3D shows that lithium chloride alone or in combination with folic acid decreased the locomotor activity of mice in the open-field test.

Figure 4A shows that the administration of a sub-effective dose of rosiglitazone (1 μg/site, i.c.v.) in combination with a sub-effective dose of folic acid (10 mg/kg, p.o.) produced a synergistic anti-immobility effect in the FST. Figure 4B shows that this effect is not due to a psychostimulant effect, since the administration of rosiglitazone alone or in combination with folic acid did not affect the locomotion of mice in the open-field test.

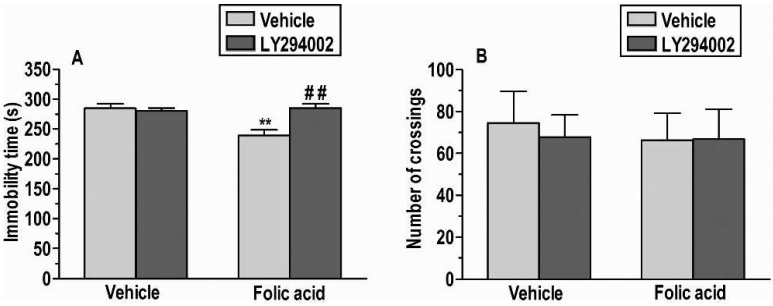

Figure 1. Effect of the pre-treatment of mice with LY294002 (10 nmol/site, i.c.v.) on the anti-immobility effect of folic acid (50 mg/kg, p.o.) in the forced swimming test (panel A) and on the number of crossings in the open-field test (panel B). Values are expressed as mean+SEM ($n = 6–10$). $^{**}p < 0.01$ as compared with the vehicle-treated control, $^{\#\#}p < 0.01$ as compared with the same group pretreated with vehicle. (A) Pre-treatment: ($F[1,28] = 6.76$, $p < 0.05$); folic acid treatment: ($F[1,28] = 6.71$, $p < 0.05$); LY294002 pre-treatment X folic acid treatment interaction: ($F[1,28] = 10.45$, $p < 0.01$); (B) LY294002 pre-treatment: ($F[1,20] = 0.05$, $p = 0.82$); folic acid treatment: ($F[1,20] = 0.12$, $p = 0.73$); LY294002 pre-treatment X folic acid treatment interaction: ($F[1,20] = 0.07$, $p = 0.79$).

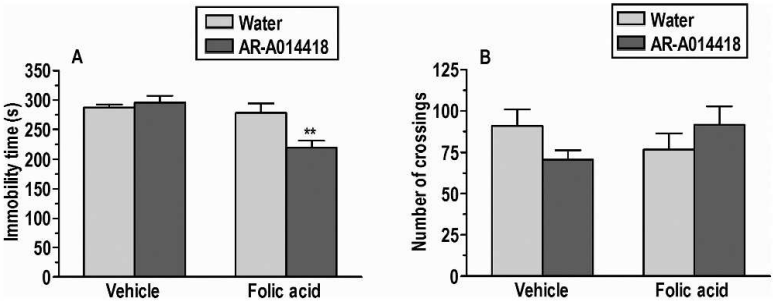

Figure 2. Effect of AR-A014418 (3 mg/kg, i.p.) in combination with a sub-effective dose of folic acid 10 mg/kg, p.o.) in the forced swimming test (panel A) and in the open-field test (panel B). Values are expressed as mean+SEM ($n = 6–8$). $^{**}p < 0.01$ as compared with the vehicle-treated control. (A) Folic acid pretreatment: ($F[1,25] = 13.18$, $p < 0.01$); AR-A014418 treatment: ($F[1,25] = 4.63$, $p < 0.05$); folic acid pre-treatment X AR-A014418 treatment interaction: ($F[1,25] = 8.00$, $p < 0.01$); (B) folic acid pretreatment: ($F[1,18] = 0.11$, $p = 0.74$); AR-A014418 treatment: ($F[1,18] = 0.07$, $p = 0.79$); folic acid pre-treatment X AR-A014418 treatment interaction: ($F[1,18] = 3.44$, $p = 0.08$).

The results presented in Figure 5A shows that the anti-immobility effect of folic acid (50 mg/kg, p.o.) was completely prevented by pre-treatment of animals with the PPARγ antagonist GW-9662 (10 μg/site, i.c.v.). Figure 5B shows that the administration of GW-9662 alone or in combination with folic acid was devoid of effect in the open-field test.

Several clinical studies have reported a reduction in folic acid levels in serum and erythrocytes of depressed patients and a therapeutical effect of this vitamin in combination with antidepressants for treatment of refractory depression has also been shown; two indications that support that folic acid plays an important role in the pathophysiology of depression (Coppen and Bailey, 2000; Coppen and Bolander-Gouaille, 2005).

Pre-clinical studies dealing with folic acid are not so abundant and the evidence that this vitamin administration produces an antidepressant-like effect in predictive models of antidepressant activity was given recently (Brocardo et al., 2008a, 2008b, 2009),

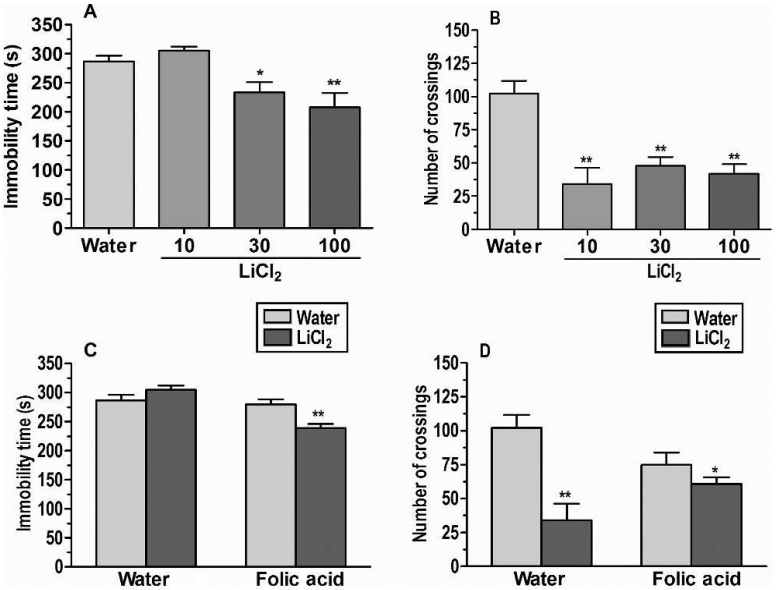

Figure 3. Dose-response curve of lithium chloride administration on the immobility time in the forced swimming test (FST) (panel A) and in the open-field test (panel B). Effect of lithium chloride (10 mg/kg, p.o.) in combination with a sub-effective dose of folic acid (10 mg/kg, p.o.) in the FST (panel C) and in the open-field test (panel D). Values are expressed as mean+SEM (n = 6–10). *p < 0.05, **p < 0.01 as compared with the vehicle-treated control (water, W). (A) lithium chloride treatment: (F[3,27] = 7.09, p < 0.01); (B) lithium chloride treatment: (F[3,27] = 10.78, p < 0.01); (C) folic acid pre-treatment: (F[1,30] = 1.80, p = 0.19); lithium chloride treatment: (F[1,30] = 18.43, p < 0.01); folic acid pre-treatment X lithium chloride treatment interaction: (F[1,30] = 12.07, p < 0.01); (D) folic acid pre-treatment: (F[1,25] = 17.19, p < 0.01); lithium chloride treatment: (F[1,25] = 0.002, p = 0.97); folic acid pre-treatment X lithium chloride treatment interaction: (F[1,25] = 7.51, p < 0.05).

but the mechanisms underlying its antidepressant-like action remain poorly understood.

In the present study we have shown, to our knowledge for the first time, that the administration of PI3K enzyme inhibitor, LY294002, abolished the antidepressant-like effect of folic acid in FST. Note worthily, we have recently shown that this enzyme inhibitor was able to prevent the neuroprotective effect of folic acid against dexamethasone-induced neurotoxicity in human neuroblastoma SH-SY5Y cell line (Budni et al., 2011). Furthermore, the acute administration of folic acid at a sub-effective dose in combination with sub-effective doses of the GSK-3β inhibitors (AR-A014418 and lithium chloride) or rosiglitazone, a PPARγ agonist, reduced the immobility time in the FST as compared with either drug alone, which is consistent with an antidepressant-like effect. Moreover, the administration of a PPARγ antagonist, GW-9662 was able to prevent the anti-immobility effect of folic acid, which was administered at a dose that was previously shown to be effective in the FST (Brocardo et al., 2008a). These results indicate that the antidepressant-like effect of folic acid in the FST may be mediated by PI3K enzyme activation, inhibition of GSK-3β activity and activation of PPARγ receptors. Our results extend the knowledge about the mechanisms underlying the antidepressant-like effects of folic acid in the FST, a widely used behavioral model that assess antidepressant potential of drugs, and reinforces the notion that PI3K/Akt/GSK-3β pathway as well as PPARγ receptor modulation may exert a significant role in the mechanism of action of antidepressant drugs.

GSK-3β is regulated upstream by another protein kinase, Akt-1, and together these two kinases are part of a signaling pathway regulated by PI3K (Cross et al., 1995; Grimes and Jope, 2001). The activation of PI3K induces the Akt activation, which is a primary target of PI3K. This protein is one of the key signaling enzymes that participates in many physiological functions in brain and is utilized by neurotrophins to mediate neuronal plasticity,

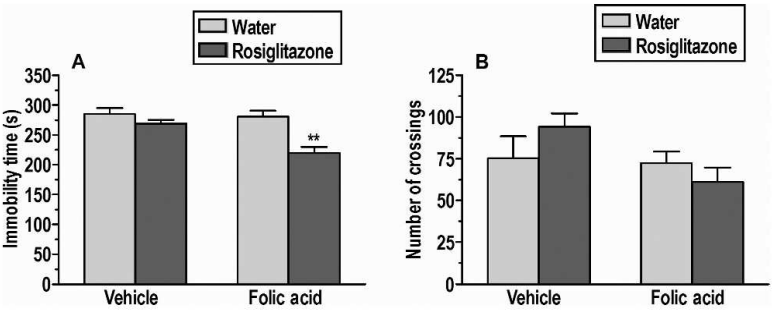

Figure 4. Effect of rosiglitazone (1 ug/site, i.c.v.) in combination with a sub-effective dose of folic acid (10 mg/kg, p.o.) in the forced swimming test (panel A) and in the open-field test (panel B). Values are expressed as mean+SEM ($n = 6–10$) $^{**}p < 0.01$ as compared with the vehicle-treated control. (A) folic acid pre-treatment: ($F[1,28] = 7.64$, $p < 0.01$); rosiglitazone treatment: ($F[1,28] = 15.49$, $p < 0.01$); folic acid pre-treatment X rosiglitazone treatment interaction: ($F[1,28] = 5.15$, $p < 0.05$); (B) folic acid pre-treatment: ($F[1,23] = 3.07$, $p = 0.09$); rosiglitazone treatment: ($F[1,23] = 0.14$, $p = 0.71$); folic acid pre-treatment X rosiglitazone treatment interaction: ($F[1,23] = 2.12$, $p = 0.16$).

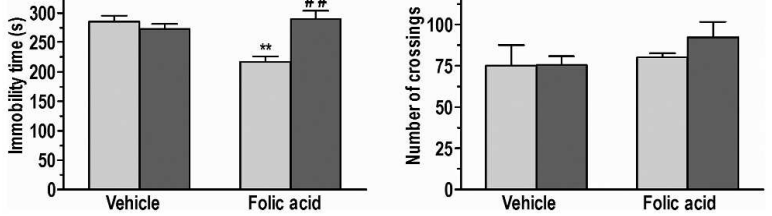

Figure 5. Effect of the pre-treatment of mice with GW-9662 (10 µg/site, i.c.v.) on the anti-immobility effect of folic acid (50 mg/kg, p.o.) in the forced swimming test (panel A) and on the number of crossings in the open-field test (panel B). Values are expressed as mean+SEM ($n = 6–8$). $^{**}p < 0.01$ as compared with the vehicle-treated control. $^{\#\#}p < 0.01$ as compared with the same group pre-treated with vehicle. (A) GW-9662 pre-treatment: ($F[1,22] = 7.68$, $p < 0.05$); folic acid treatment: ($F[1,22] = 5.48$, $p < 0.05$); GW-9662 pre-treatment X folic acid treatment interaction: ($F[1,22] = 15.41$, $p < 0.01$); (B) GW-9662 pre-treatment: ($F[1,25] = 1.45$, $p = 0.24$); folic acid treatment: ($F[1,25] = 0.48$, $p = 0.49$); GW-9662 pre-treatment X folic acid treatment interaction: ($F[1,25] = 0.43$, $p = 0.52$).

cell survival, and inhibition of apoptosis for several neuronal subtypes (Katso et al., 2001; Beaulieu et al., 2009). Abnormalities in activation and expression of PI3K may be involved in the mechanism of many psychiatric disorders as well as major depression. Dwivedi et al. (2008) showed, in a study performed in brain obtained from major depression subjects, that there was decreased activation of PI3K in the prefrontal cortex and hippocampus of these suicide subjects compared with normal controls, which implicates this enzyme in the pathophysiology of depression. Corroborating this notion, our results indicate that a mechanism related to the antidepressant-like effect of folic acid may involve the activation of PI3K, because pre-treatment of mice with PI3K inhibitor, LY294002, prevented the antidepressant-like effect of folic acid in FST. Our finding is somewhat in line with a previous study (Seto et al., 2010) which shows that oral folic acid supplementation restored the blunted acetylcholine-induced aortic relaxation in an animal model of diabetes-mellitus-associated hypertension, observed in mice, probably via enhancement of the activity of PI3K/Akt cascade. However, it is important to mention that GSK-3 and GSK-3 inhibitors have been more investigated in

the context of bipolar disorder than in the context of major depression (Gould et al., 2004b, 2006; Gould, 2006; Rowe et al., 2007; Kalinichev and Dawson, 2011). In addition, Brocardo et al. (2010) showed that folic acid produces antimanic action in an ouabain-induced animal model of mania. Hence, we cannot rule out the possibility that observed behavioral effects of folic acid are due to the fact that it acts similarly to mood stabilizers, like lithium. Indeed, lithium, valproate, and lamotrigine produce antidepressant-like effects in the FST (Redrobe and Bourin, 1999; Bourin et al., 2005; Ghasemi et al., 2010).

The PI3K/Akt pathway has been shown to act as an upstream mechanism of GSK-3β activity regulation, since this system might directly phosphorylate Ser9 of GSK-3β, leading to GSK-3β inactivation (Grimes and Jope, 2001; Bhat et al., 2004; Beaulieu et al., 2009). GSK-3 is a constitutively active enzyme, found as two isoforms (α and β) in mammals, and is highly expressed in brain and it has numerous cellular targets including transcription factors, cytoskeleton proteins, and molecules involved in cell division. Generally, active GSK-3 is proapoptotic and its inhibition, for example, by lithium or phosphorylation via Akt and other signaling molecules, is antiapoptotic (Picchini et al., 2004). A large amount of evidence has implicated GSK-3β as a drug target for certain brain disorders, including depression. Literature data report that GSK-3β inhibitors, including lithium and AR-A014418, and the thiazolidone NP031115 reduced immobility in the FST, thus mimicking the action of antidepressants (Gould et al., 2004a; Rosa et al., 2008). Our results show that the antidepressant-like effect of folic acid may involve the inhibition of GSK-3β, since treatment of mice with the GSK-3β inhibitor, AR-A014418, plus folic acid potentiated the antidepressant-like effect of folic acid in FST.

Besides the implication of GSK-3β in the pathophysiology of depression, our group has firstly shown that an activation of PPARγ by the i.c.v. administration of the PPARγ agonist rosiglitazone in mice produces an antidepressant-like effect in FST (Rosa et al., 2008). This notion was recently reinforced by a study that has shown that rosiglitazone administered orally produces an antidepressant-like effect in the FST in rats and in the TST in mice that was reversed by the PPARγ antagonist GW-9662 (Eissa Ahmed et al., 2009). We have also shown that the pre-treatment of mice with GW-9662 was effective in preventing the antidepressant-like effect elicited by NP031115, AR-A014418 (GSK-3β inhibitors) and rosiglitazone (Rosa et al., 2008). Moreover, we have shown that NP031115 and AR-A014418 activate PPARγ in CHO cells transfected with the reporter plasmid pPPRE-tk-luc containing three PPARγ consensus-binding sites (Rosa et al., 2008). Therefore, it seems that activation of PPARγ can inhibit GSK-3β. The inhibition of this enzyme may play a critical role in the antidepressant-like effect of some drugs in the FST. In the present study, the administration of folic acid at a sub-effective dose in combination with a sub-effective dose of rosiglitazone reduced the immobility time in the FST, suggesting that the antidepressant-like effect of folic acid is dependent on the activation of PPARγ. Further reinforcing this notion, our results show that the administration of GW-9662 was able to reverse the anti-immobility effect of folic acid.

Another interesting observation was that a sub-effective dose of lithium chloride produced a synergistic antidepressant-like effect in the FST when administered in combination with folic acid. Importantly, the therapeutic efficacy of lithium for the treatment of mood disorders may result from inhibition of GSK-3β. Although lithium acts on several molecular targets and may inhibit multiple enzymes, GSK-3β and GSK-3α are the only known protein kinases that are directly inhibited by lithium (Li et al., 2002; Gould and Manji, 2005).

The FST is sensitive to antidepressants from different pharmacological classes (Porsolt et al., 1977; Cryan et al., 2002, 2005). However, in the FST, drugs enhancing locomotor activity, such as caffeine may give a 'false' positive effect (Rodrigues et al., 2005). Therefore, in order to exclude the possibility that the synergistic effect of folic acid and GSK-3β inhibitors or rosiglitazone in the FST is a reflection of generalized increased locomotor activity, mice were also submitted to the open-field test for ambulation analysis. Our results, clearly show that the ability of the GSK-3β inhibitors or rosiglitazone to augment the behavioral response to folic acid in the FST is not due to a nonspecific locomotor stimulant effect of the drug combination because neither the GSK-3β inhibitors alone nor administered in combination with folic acid altered locomotor activity. Therefore, the synergistic antidepressant-like effect of folic acid combined with the GSK-3β inhibitors and PPARγ agonist rosiglitazone obtained in this study could not be attributed to general hyperactivity of mice. On the other hand, our results indicated that lithium chloride alone or in combination with folic acid decreased the locomotor activity of mice in the open-field test. However, this effect does not account for the anti-immobility effect elicited by the joint administration of folic acid and lithium chloride, since a hypolocomotor activity may cause a false depressant-like effect, but not a reduction in the immobility time in the FST. This effect in the open-field test underestimates the antidepressant-like effect of lithium chloride alone or in combination with folic acid.

It has been well established that drugs acting on serotonergic neurotransmission such as selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase (MAO) inhibitors, and tricyclic antidepressants cause GSK-3β inhibition in the frontal cortex, hippocampus, and striatum of mice (Li et al., 2004; Beaulieu et al., 2008). Moreover, functional *Tph2* gene mutation, that produces a severe reduction of brain serotonin synthesis in mice, leads to an increase in cortical GSK-3β activity and a depressant-like behavior in the TST (Beaulieu et al., 2008). These effects were reversed by the selective GSK-3β inhibitor TDZD-8. A previous study from our group has shown that activation of the serotonergic system is involved in the antidepressant-like effect of folic acid in the FST (Brocardo et al., 2008a). For that reason, activation of the serotonergic system elicited by folic acid administration might be implicated on the inhibition of GSK-3β observed in the present study. Hence, it remains to be established the relationship between the activation of the serotonergic system by folic acid and the results reported in the present study.

In conclusion, the present study significantly extends literature data regarding the mechanisms underlying the antidepressant-like action of folic acid by indicating that its anti-immobility effect in the FST may be dependent on the activation of PI3K/Akt pathway, inhibition of GSK-3β and activation of PPARγ (Figure 6). These findings reinforce the notion that these are important targets for antidepressant activity. Since the conventional pharmacotherapy for depression has several drawbacks, PI3K, GSK-3β, and PPARγ should be considered as novel molecular targets for the development of antidepressant drugs.

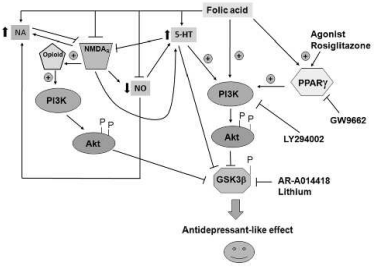

Figure 6. Proposal for mechanisms underlying the antidepressant-like effect of folic acid. The hypothetic diagram takes into account present results and previous findings on the involvement of noradrenergic, serotoninergic, glutamatergic (NMDA receptors), nitrergic and opioid systems in the antidepressant-like effect of folic acid (Brocardo et al., 2008a; 2008b; 2009) and the role of PI3K in its neuroprotective effect (Budni et al., 2011). An activation of the serotoninergic system elicited by folic acid (Brocardo et al., 2008a) probably stimulates PI3K, inhibiting GSK3β activity (Beaulieu et al., 2008; Beaulieu, 2011). An increase in serotonin and noradrenaline availability in the synaptic cleft induced by folic acid (Brocardo et al., 2008a) might inhibit NMDA receptors (Leão and Gersdorff, 2002; Masuko et al., 2004). Also, it is possible that folic acid directly inhibits NMDA receptors (Brocardo et al., 2008b) with the consequent increase in noradrenaline and serotonin availability (López-Gil et al., 2009; Dazzi et al., 2011). The inhibition of NMDA receptors elicited by folic acid also causes a decrease in nitric oxide synthesis (Brocardo et al., 2008b), but the possibility cannot be ruled out that folic acid directly inhibits nitric oxide synthase, also leading to an increase in monoamine availability (Johnson et al., 1989; Szabo et al., 1993; Segieth et al., 2001). Moreover, the inhibition of NMDA receptors probably causes an activation of the opioid system (Brocardo et al., 2009), which through δ-opioid receptors might activate PI3K (Heiss et al., 2009; Olianas et al., 2011). In addition, we have recently shown that folic acid exerts a neuroprotective effect against dexamethasone, at least in part, by activating PI3K (Budni et al., 2011). Noteworthy, the results of the present study extend previous data by suggesting that folic acid elicits an antidepressant-like effect via PI3K activation with the consequent GSK3β inhibition. In this study, PI3K inhibitor LY294002 and the PPARγ antagonist (GW9662) prevent the antidepressant-like effect of folic acid. In addition, the GSK3β inhibitors AR-A014418 and lithium, as well as the PPARγ agonist rosiglitazone (all administered at sub-effective doses) produce a synergistic effect with a sub-effective dose of folic acid. Therefore, folic acid could cause the activation of PI3K, which, in turn, leads to the inhibition of GSK3β through Akt-dependent phosphorylation mechanism. Moreover, folic acid might activate PPARγ receptors causing GSK3β inhibition by a mechanism likely dependent on PI3K activation.

5-HT, serotonin; GSK3β, glycogen synthase kinase 3-beta; NA, noradrenaline; NMDA, N-methyl-D-aspartate; NO, nitric oxide; PI3K, phosphoinositide 3-kinase; PPARγ, peroxisome proliferator-activated receptor-gamma.

Funding

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Conflict of interest

The authors declare that there is no conflict of interest.

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CAPÍTULO 2

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Role of potassium channels in the antidepressant-like effect of folic acid in the forced swimming test in mice

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ABSTRACT

Potassium (K^+) channels have been implicated in depressive disorders and in the mechanism of action of antidepressants. Considering that several studies have indicated that folic acid plays an important role in the pathophysiology of depression, the present study investigated the involvement of potassium channels in the antidepressant-like effect of this vitamin. For this aim, the effect of the combined administration of different types of K^+ channel blockers and folic acid in the forced swimming test (FST) was investigated. Treatment of mice by intracerebroventricular (i.c.v.) route with subactive doses of glibenclamide (an ATP-sensitive K^+ channels blocker, 0.5 pg/site), charybdotoxin (a large- and intermediate-conductance calcium-activated K^+ channel blocker, 25 pg/site) or apamin (a small-conductance calcium-activated K^+ channel blocker, 10 pg/site), augmented the effect of folic acid (10 mg/kg, p.o., subeffective dose) in the FST. Additionally, the administration of folic acid and the K^+ channel blockers, alone or in combination, did not affect locomotion in the open-field test. Moreover, the reduction in the immobility time in the FST elicited by folic acid administered at a higher dose (50 mg/kg, p.o.) was prevented by the pretreatment of mice with the K^+ channel opener cromakalim (10 μg/site, i.c.v.), without affecting locomotor activity. The results of this study indicate that the antidepressant-like effect of folic acid in the FST may be at least partly due to its modulatory effects on neuronal excitability, via inhibition of K^+ channels.

1. Introduction

Depression is a chronic, severe and debilitating mental illness that affects millions of people worldwide. Although the underlying pathophysiological mechanisms of depression are not completely established, novel targets have been identified for the development of new pharmacological treatments (Lee et al., 2010). There is increasing evidence that a folic acid status is an important factor that may contribute to depressive disorders and its treatment (Coppen and Bolander-Gouaille, 2005; Morris et al., 2008; Sarris et al., 2009). Folic acid (folate), one of the 13 essential vitamins which is obtained from dietary sources or supplements, is essential for the functioning of nervous system, since it displays an important role in neuroplasticity and maintenance of neuronal integrity (Fenech, 2010; Kronenberg et al., 2009).

Many important metabolic processes are dependent on folic acid availability, including the synthesis of norepinephrine, dopamine and serotonin, which are neurotransmitters implicated in the pathogenesis and treatment of depression (Fava and Mischoulon, 2009). There are several clinical studies regarding folic acid deficiency associated with a higher incidence of depression. These studies show that: a) reduced plasma, serum or red blood cell folic acid is commonly found in major depressive illnesses (Abou-Saleh and Coppen, 2006; Sarris et al., 2009); b) a low folic acid status is associated with poorer response to antidepressant medication; on the other hand, folic acid supplementation added to antidepressant medication improves its therapeutic effect (Alpert et al., 2002; Coppen and Bailey, 2000; Godfrey et al., 1990). Low folic acid status is associated with reduced serotonergic and/or neurotransmitter function; (c) preclinical studies from our group have shown that systemic and central administration of folic acid produces antidepressant-like effect in two predictive models of antidepressant activity, the forced swimming test (FST) and tail suspension test (TST) (Brocardo et al., 2008a). The mechanisms by which folic acid produces antidepressant-like effect are not fully established, but they were shown to be dependent on the serotonergic and noradrenergic systems (Brocardo et al., 2008a), inhibition of N-methyl-D-aspartic acid (NMDA) receptors and nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) synthesis (Brocardo et al., 2008b). Additionally, antidepressant-like effect of folic acid is also mediated by an interaction with the opioid system (μ_1- and δ-opioid receptors) (Brocardo et al., 2009), inhibition of glycogen synthase kinase-3 (GSK-3β) and activation of peroxisome proliferator-activated receptor-γ (PPARγ) (Budni et al., 2011b).

NO is an important messenger in the central nervous system. It is produced from L-arginine by the catalytic action of NO synthase

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(NOS). Physiologically, NO actions may be mediated by locally produced NO and in most instances by the subsequently generated second messenger molecule guanosine 3′5 cyclic monophosphate. Studies indicate that different types of K^+ channels in several tissues can be activated by NO *per se* or through cGMP production (Jeong et al., 2001; Shin et al., 1997). Additionally, a previous study of our group demonstrated that the antidepressant-like effect elicited by the inhibition of several subtypes of K^+ channels is dependent on the inhibition of NO–cGMP synthesis (Kaster et al., 2005). Thus, K^+ channels might be one of the physiological targets of NO in the brain (Jeong et al., 2001) and the inhibition of these channels might play an important role in the pathophysiology of depression.

Therefore, the aim of this study was to investigate whether the blockade of K^+ channels can contribute to the antidepressant-like effect of folic acid in the FST in mice.

2. Materials and methods

2.1. Animals

Adult Swiss mice of either sex (homogeneously distributed among groups), weighing 30–40 g were maintained at 20–22 °C with free access to water and food, under a 12:12 h light:dark cycle (lights on at 7:00 am). Male and female mice were maintained in different cages. All manipulations were carried out between 9:00 am and 4:00 pm, with each animal used only once. All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The experiments were performed after approval by the Ethics Committee of the Institution and all efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

2.2. Drugs and treatment

The following drugs were used: folic acid (Sigma Chemical Co., St. Louis, U.S.A.), charybdotoxin, cromakalim and glibenclamide (Tocris Cookson, Ballwin, MO, USA). Cromakalim was dissolved in saline with 10% Tween 80, whereas all the other drugs were dissolved in isotonic saline solution (NaCl 0.9%) immediately before use, except folic acid which was dissolved in distilled water. Appropriate vehicle-treated groups were also assessed simultaneously. All the drugs were administered by intracerebroventricular (i.c.v.) route, in a volume of 5 μl per mouse, except folic acid which was administered by oral route (p.o.) in a constant volume of 10 ml/kg body weight. I.c.v. injections were given under light ether anesthesia, directly into the lateral ventricle as described previously by Budni et al. (2007), with the bregma fissure as a reference. Vehicle, potassium channel blockers or potassium channel opener were injected in a volume of 5 μl, given over 30 s, and the cannula remained in place for another 30 s.

To test the hypothesis that the antidepressant-like effect of folic acid is mediated through the inhibition of K^+ channels, animals were pretreated with a subeffective dose of folic acid (10 mg/kg, p.o.), and 45 min later they received subeffective doses of glibenclamide (an ATP-sensitive K^+ channel blocker, 0.5 pg/site), charybdotoxin (a large- and intermediate-conductance calcium-activated K^+ channel blocker, 25 pg/site) or apamin (a small-conductance calcium-activated K^+ channel blocker, 10 pg/site) before being tested in the FST.

In order to rule out any psychostimulant effect of the interaction of K^+ channel blockers and folic acid, mice were pretreated by oral route with folic acid 45 min before the administration by i.c.v. route of glibenclamide (0.5 pg/site), charybdotoxin (25 pg/site) or apamin (10 pg/site). The open-field test was carried out 15 min later.

In another set of experiments, mice were pretreated with folic acid (50 mg/kg, p.o.), 45 min before the administration of cromakalim (a K^+ channel opener, 10 μg/site, i.c.v.). FST or the open-field test was carried out 15 min later.

The doses of folic acid were chosen based on previous studies from our group (Brocardo et al., 2008a, 2008b, 2009; Budni et al., 2011b).

The doses of glibenclamide, charybdotoxin, apamin and cromakalim were chosen on the basis of literature and are previously reported not to increase locomotor activity (Budni et al., 2007; Galeotti et al., 1999; Kaster et al., 2005, 2007).

2.3. Forced swimming test (FST)

Briefly, 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 duration of immobility was measured during 6-min period as described previously (Brocardo et al., 2008a; Budni et al., 2007; Kaster et al., 2005). 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).

2.4. Open-field test

To assess the possible effects of folic acid on locomotor activity, mice were evaluated in the open-field paradigm as previously described (Budni et al., 2007; Rodrigues et al., 1996). Animals were individually placed in a wooden box (40×60×50 cm) with the floor divided into 12 rectangles. The number of squares crossed with all paws (crossing) was counted in a 6 min session. The apparatus were cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.5. Statistical analysis

All experimental results are given as the mean ± SEM. Comparisons between experimental and control groups were performed by two-way ANOVA (interaction of folic acid with the pharmacological agents) followed by Newman–Keuls test when appropriate. A value of $p<0.05$ was considered to be significant.

3. Results

3.1. Effects of combined administration of subeffective doses of the K^+ channel blockers and folic acid in the FST

The results presented in Fig. 1A show the synergistic antidepressant-like effect of glibenclamide (an ATP-sensitive K^+ channel blocker, 0.5 pg/site, i.c.v.) combined with a subeffective dose of folic acid (10 mg/kg, p.o.) in the FST. The two-way ANOVA revealed a significant effect of glibenclamide treatment [$F_{1,28}=12.78$, $p<0.01$] and pretreatment × treatment interaction [$F_{1,28}=16.30$, $p<0.01$], but not of folic acid pretreatment [$F_{1,28}=2.49$, $p=0.12$].

Fig. 1B shows that apamin (a small-conductance calcium-activated K^+ channel blocker, 10 pg/site, i.c.v.) in combination with a subeffective dose of folic acid reduced the immobility time in the FST when compared with either drug alone. The two-way ANOVA revealed a significant effect of apamin treatment [$F_{1,28}=14.69$, $p<0.01$] and pretreatment × treatment interaction [$F_{1,28}=11.28$, $p<0.01$], but not of folic acid pretreatment [$F_{1,28}=2.67$, $p=0.11$].

As presented in Fig. 1C, the administration of charybdotoxin (a large- and intermediate-conductance calcium-activated K^+ channel blocker, 25 pg/site, i.c.v.) in combination with subeffective dose of folic acid also produced an antidepressant-like effect in the FST. The two-way ANOVA revealed a significant effect of folic acid pretreatment [$F_{1,28}=8.75$, $p<0.01$], charybdotoxin treatment [$F_{1,28}=6.72$,

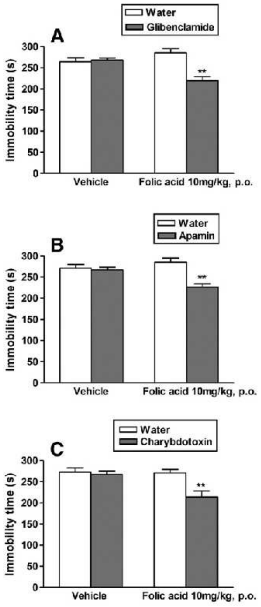

Fig. 1. Effect of treatment of animals with glibenclamide (0.5 pg/site, i.c.v., panel A), apamin (10 pg/site, i.c.v., panel B) or charybdotoxin (25 pg/site, i.c.v., panel C) in combination with a subeffective dose of folic acid (10 mg/kg, p.o.) on the immobility time in the FST. Values are expressed as mean + SEM (n = 8). $^{**}p<0.01$ compared with the vehicle-treated group.

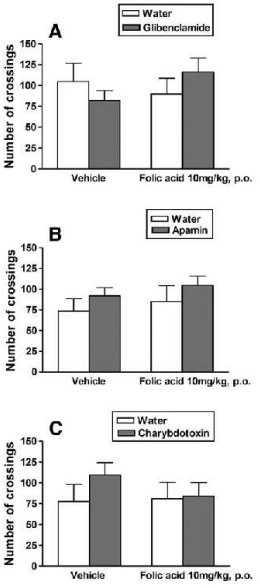

Fig. 2. Effect of treatment of mice with glibenclamide (0.5 pg/site, i.c.v., panel A), charybdotoxin (25 pg/site, i.c.v., panel B) or apamin (10 pg/site, i.c.v., panel C) combined with a subeffective dose of folic acid (10 mg/kg, p.o.) in the open-field test. Values are expressed as mean + SEM (n = 7).

$p<0.05$] and a significant interaction between pretreatment × treatment [$F_{1,28}=5.94$, $p<0.05$].

The post-hoc analyses revealed that the administration of the K^+ channel blockers (glibenclamide, apamin or charybdotoxin), at doses that do not produce an antidepressant-like effect in the FST, produced a synergistic antidepressant-like effect when combined with a subeffective dose of folic acid.

3.2. Effects of K^+ channel blockers and folic acid in the open-field test

The result depicted in Fig. 2A shows that the administration of folic acid in combination with glibenclamide (0.5 pg/site, i.c.v.) did not affect the animal locomotor activity in the open-field test. The two-way ANOVA revealed no differences for folic acid pretreatment [$F_{1,24}=0.29$, $p=0.59$], glibenclamide treatment [$F_{1,24}=0.01$, $p=0.93$] and pretreatment × treatment interaction [$F_{1,24}=1.90$, $p=0.18$]. The results of Fig. 2B indicated that combined treatment with folic acid plus apamin (10 pg/site, i.c.v.) did not alter the locomotor profile in the open-field test. The two-way ANOVA revealed no differences for folic acid pretreatment [$F_{1,24}=0.70$, $p=0.41$], apamin treatment [$F_{1,24}=1.79$, $p=0.19$] and pretreatment × treatment interaction [$F_{1,24}=0.00$, $p=0.97$]. Fig. 2C shows that the treatment with folic acid combined to charybdotoxin (25 pg/site, i.c.v.) did not induce alterations of locomotor activity in the open-field test. The two-way ANOVA revealed no differences for folic acid pretreatment [$F_{1,24}=0.37$, $p=0.55$], charybdotoxin treatment [$F_{1,24}=0.95$, $p=0.34$] and pretreatment × treatment interaction [$F_{1,24}=0.62$, $p=0.44$]. These results indicate that the reduction in the immobility time observed in the FST when folic acid and K^+ channel blockers were administered in combination (Fig. 1A–C) was not due to a psychostimulant effect.

3.3. Effect of cromakalim on folic acid-induced anti-immobility effect in the FST and in the number of crossings in the open-field test

Fig. 3A shows that the pretreatment of mice with cromakalim (a K^+ channel opener, 10 μg/site, i.c.v.) was able to prevent the antidepressant-like effect of folic acid (50 mg/kg, p.o.) in the FST. The two-way ANOVA revealed significant differences for folic acid pretreatment [$F_{1,24}=19.48$, $p<0.01$], cromakalim treatment [$F_{1,24}=10.60$, $p<0.01$] and pretreatment × treatment interaction [$F_{1,24}=9.65$, $p<0.01$].

Moreover, the results depicted in Fig. 3B shows the effect of cromakalim (10 μg/site, i.c.v.) combined with folic acid (50 mg/kg, p.o.) in the open-field test. This co-treatment did not produce any change in the ambulatory behavior of mice, since the two-way ANOVA did not reveal significant differences for folic acid pretreatment [$F_{1,24}=0.10$, $p=0.76$], cromakalim treatment [$F_{1,24}=0.65$, $p=0.43$] and pretreatment × treatment interaction [$F_{1,24}=2.46$, $p=0.13$].

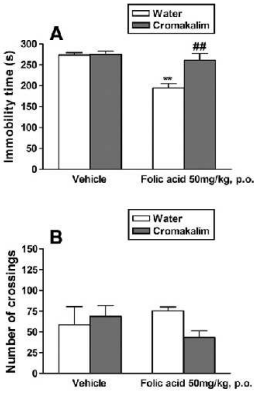

Fig. 3. Effect of pretreatment of mice with cromakalim (10 μg/site, i.c.v.) on the anti-immobility effect of folic acid (50 mg/kg, p.o.) in the FST (panel A) and open-field test (panel B). Values are expressed as mean + SEM (n = 7). **p < 0.01 compared with the vehicle treated group; ##p < 0.01 compared with the same group pretreated with vehicle.

4. Discussion

This study extends previous findings from our group that shows that folic acid administration produces antidepressant-like effect in the FST (Brocardo et al., 2008a, 2008b, 2009; Budni et al., 2011b). Herein, we provide evidence that the antidepressant-like effect of folic acid can be mediated by a block of different types of K^+ channels, since subeffective doses of different types of K^+ channel blockers combined with a subeffective dose of folic acid produce an antidepressant-like effect in the mouse FST. Additionally, to confirm our hypothesis, the pretreatment of mice with the K^+ channel opener cromakalim was able to prevent the antidepressant-like effect of folic acid in the FST. Noteworthy, FST is a test widely used due to its predictive validity, since it is sensitive to all classes of antidepressant drugs, including tricyclics, serotonin-specific reuptake inhibitors, monoamine oxidase inhibitors, and atypicals (Petit-Demouliere et al., 2005; Porsolt et al., 1977).

In this study, the open-field test was used to exclude the possibility that the synergistic effect of folic acid and K^+ channel blockers in the FST could be a consequence of an increased locomotor activity. Drugs that induced hyperlocomotion may give a "false" positive effect in the FST, whereas drugs decreasing locomotion may give a 'false' negative result (Borsini and Meli, 1988; Rodrigues et al., 2005). Our results indicate that the anti-immobility effect induced by folic acid in combination with K^+ channel blockers in the FST is not due to a nonspecific locomotor stimulant effect of the drugs combination, since drugs alone or in combination did not significantly alter locomotor activity.

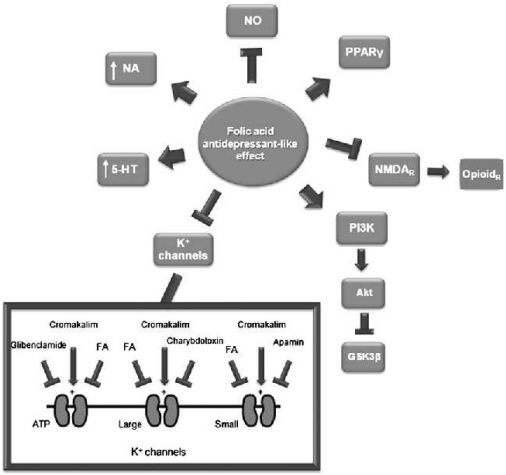

Fig. 4. Schematic illustration for the different targets or pathways involved in the antidepressant-like effect of folic acid taking into account present results and previous literature findings. Folic acid can exert an antidepressant-like effect through the increase 5-HT and NA availability (Brocardo et al., 2008a), inhibition of NO synthesis (Brocardo et al., 2009), activation of PPARγ receptors and PI3K and/or AKT with the consequent inhibition of GSK3β activity (Budni et al., 2011b), inhibition of $NMDA_R$ and activation of the opioid system (Brocardo et al., 2008b, 2009), and inhibition of different types of K^+ channels, as reported in the present study. In this study, subeffective doses of different types of K^+ channel blockers (glibenclamide, charybdotoxin and apamin) combined of a subeffective dose of folic acid produced an antidepressant-like effect. Moreover, cromakalim prevented the antidepressant-like effect of folic acid. Based on literature data regarding the neuroprotective effects of folic acid, we propose that hippocampus and pre-frontal cortex (Brocardo et al., 2010; Budni et al., 2011a; Chen et al., 2011; Figueiredo et al., 2011) are implicated in the molecular mechanisms involved in the antidepressant-like effect of folic acid, but the involvement of other brain regions, such as rafe nucleus, locus coeruleus and amygdala cannot be ruled out.5-HT, serotonin; GSK3β, glycogen synthase kinase 3-beta; K^+ channel, potassium channel; NA, noradrenaline; $NMDA_R$, N-methyl-D-aspartate receptor; NO, nitric oxide; PI3K, phosphoinositide 3-kinase; PPARγ, peroxisome proliferator-activated receptor-gamma.

Several clinical studies have reported a reduction in folic acid levels in serum and erythrocytes of depressed patients and a therapeutical effect of this vitamin in combination with antidepressants for treatment of refractory depression, two indicatives that folic acid plays an important role in the pathophysiology of depression (Coppen and Bailey, 2000; Coppen and Bolander-Gouaille, 2005). It is important to mention that pre-clinical studies performed with folic acid are not so abundant in the literature (Brocardo et al., 2008a, 2008b, 2009; Budni et al., 2011b). Therefore, the mechanisms underlying its antidepressant-like action deserve additional investigations.

In this study several compounds were used as pharmacological tools in the investigation of folic acid action mechanism in the FST: glibenclamide, charybdotoxin and apamin. The combined treatment with subeffective doses of these K^+ channel blockers in combination with folic acid provoked a robust reduction in immobility time, indicative of an antidepressant-like behavioral profile.

The K^+ channel blockers glibenclamide, charybdotoxin and apamin act through different mechanisms, since they are known to selectively block ATP-sensitive, large- and intermediate-conductance calcium-activated and small-conductance calcium-activated K^+ channels, respectively (Gehlert and Gackenheimer, 1993). Glibenclamide blocks K^+ channels from pancreatic β-cell type, cardiac, smooth muscle, skeletal muscle, and some brain neurons (Clapp, 1995; Proks et al., 2002). The ATP-regulated K^+ channels are known as the target for sulfonylureas, as glibenclamide, oral hypoglycemic agent widely used in the treatment of non-insulin-dependent diabetes mellitus to stimulate insulin release from pancreatic islet β cells. The mechanism of stimulation is through inhibition of ATP-regulated K^+ channels (Edwards and Weston, 1993; Ashcroft and Ashcroft, 1992). Charybdotoxin is a peptide contained in the venom of the scorpion *Leiurus quinquestriatus* (Nelson and Quayle, 1995) and it has been identified to cause potent selective block of Ca^{2+}-activated K^+ channels present in GH3 anterior pituitary cells and primary bovine aortic smooth muscle cells (Gimenez-Gallego et al., 1988). Apamin is a peptide contained in the venom of the honey bee *Apis mellifera* (Stocker, 2004). It selectivity blocks Ca^{2+}-dependent K^+ conductance, since voltage-clamp techniques showed that apamin has no effect on other ionic channels such as the fast Na^+ channel, the Et_4N^+-sensitive K^+ channel, or the slow Ca^{2+} channel (Hugues et al., 1982). These and other K^+ channel blockers (3,4-diaminopyridine [3,4-DAP] and gliquidone) were reported to exert an antidepressant-like effect in the FST at higher doses than those employed in the present study (Galeotti et al., 1999; Inan et al., 2004; Kaster et al., 2005). Furthermore, fluoxetine, desipramine, amitriptyline, nortriptyline, clomipramine, maprotiline, citalopram and paroxetine, also produce an inhibition of K^+ currents, which might underlie their therapeutic effects (Choi et al., 2004; Kobayashi et al., 2004, 2006; Nicholson et al., 2002; Tytgat et al., 1997; Yeung et al., 1999). Supporting the notion that the K^+ channels inhibition is related with the pathophysiology and treatment of depression, the results by Takahashi et al. (2006) demonstrated that continuous inhibition of wv GIRK2 channels (G protein-activated inwardly rectifying K^+ channels) by the antidepressants fluoxetine and desipramine caused a substantial suppression of the neuronal cell death and resulted in improvement of motor abilities in *weaver* mutant mice. Moreover, several studies have shown that the combined administration of antidepressants and K^+ channel blockers produced an antidepressant-like effect in the FST in mice (Bortolatto et al., 2010; Guo et al., 1995, 1996; Inan et al., 2004; Kaster et al., 2007). Other compounds with antidepressant properties such as agmatine, adenosine and tramadol combined with several types of K^+ channel blockers also produce an antidepressant-like effect in the FST (Budni et al., 2007; Jesse et al., 2009; Kaster et al., 2007). Furthermore, Heurteaux et al. (2006) demonstrated that the deletion of a gene coding for TREK-1, a class of two-pore domain K^+ channels, can cause resistance to depression, increased 5-HT neurotransmission, and reduced elevation of corticosterone levels under stress, suggesting that alterations in the function and regulation of these channels may alter mood. Therefore, these K^+ channels may be a potential target for developing new antidepressants. Moreover, it was shown that fluoxetine and norfluoxetine are blockers of TREK-1K^+ channels, consequently this blocking effect may occur in patients treated with these antidepressants (Kennard et al., 2005). Accordingly, our results are in line with literature, since the combined treatment with a subeffective dose of folic acid (a putative antidepressant agent) plus subeffective doses of K^+ channel blockers produced an antidepressant-like effect in the FST in mice.

Ca^{2+}-activated and voltage-dependent K^+ channel play a role in the modulation of immobility time in the FST in mice (Inan et al., 2004). In addition, large-conductance Ca^{2+}-activated K^+ channels have been suggested as one of the physiological targets of NO in the brain (Jeong et al., 2001). Moreover, blockers of different K^+ channels such as TEA, glibenclamide, apamin and charybdotoxin produced antidepressant-like effect in FST and this effect was prevented by the pretreatment of mice with L-arginine or sildenafil. Thus, this results suggest that NO and cGMP are important modulators of some K^+ channels (Kaster et al., 2005). Considering that the K^+ channels represent one of the major downstream targets regulated by the activation of NMDA receptors and L-arginine–NO pathway, it is believed that the inhibition of K^+ channels may be a consequence of inhibition of NMDA receptors and of NO production induced by folic acid, since a previous report indicates that the antidepressant-like effect of this vitamin may be mediated by, inhibition of NMDA receptors and NO synthesis (Brocardo et al., 2008b). Hence, an indirect modulation of the K^+ channels by folic acid via NMDA–L-arginine–NO pathway could account for the behavioral results reported in the present study. However, the possibility that folic acid causes a direct inhibition of these channels cannot be ruled out.

To further reinforce our hypothesis, we also show that the pretreatment of mice with a K^+ channel opener, cromakalim, prevented the decrease in the immobility time induced by an effective dose of folic acid in the FST, without changing the ambulatory behavior in the open-field test. Cromakalim is a K^+ channel opener that has high sensitivity to K_{ATP} channels (Clapp, 1995). Literature data report that the administration of cromakalim at higher doses than the one used in the present study increases the immobility time in the FST (Galeotti et al., 1999). In addition, Redrobe et al. (1996) demonstrated that the pretreatment of animals with cromakalim was able to reverse the anti-immobility effect of antidepressants such as imipramine, amitriptyline, desipramine and paroxetine.

Noteworthy, Kaster et al. (2007) showed that a subeffective dose of fluoxetine combined with subeffective doses of K^+ channel blockers produced an antidepressant-like effect in the FST and this effect was prevented by K^+ channel openers. These results are comparable with the results found in the present study with folic acid, which may likely exert antidepressant-like effect in the FST by a mechanism similar to either fluoxetine or K^+ channel blockers.

The most common cause for adverse cardiac events by antidepressants (mainly tricyclic antidepressants) is acquired long QT syndrome, which produces electrocardiographic abnormalities that have been associated with syncope, torsade de pointes arrhythmias, and sudden cardiac death. Acquired long QT syndrome is often caused by direct block of the cardiac potassium current I(Kr)/hERG, which is crucial for terminal repolarization in human heart (Dennis et al., 2011). In contrast to tricyclic antidepressants that are reported to cause adverse cardiac effects, folic acid has beneficial effects or no harm on the risk of cardiovascular diseases (Czeizel, 1996; Bazzano et al., 2006). In addition, a recent study showed that folic acid has protective properties against homocysteine-induced oxidative–nitrative stress in the heart of rats (Kolling et al., 2011). Therefore, it is feasible to suppose that folic acid does not significantly block cardiac K^+ channels implicated with cardiovascular risks and that the association of this vitamin with K^+ channel blockers such as

glibenclamide and/or antidepressants for the treatment of depression might be a therapeutic strategy for preventing or reducing cardiovascular risks associated with the blockade of cardiac K^+ channels. However, further studies regarding this issue are necessary.

5. Conclusions

The results of this study indicate that subeffective doses of different types of K^+ channel blockers (glibenclamide, charybdotoxin and apamin) combined of a subeffective dose of folic acid produce an antidepressant-like effect in the mouse FST. Moreover, cromakalim was able to reverse the antidepressant-like effect produced by a higher dose of folic acid. Altogether, the results shown herein suggest that the antidepressant-like effect of folic acid in the FST in mice may involve the modulation of neuronal excitability via inhibition of K^+ channels (Fig. 4).

Conflict of interest

The Authors declare that there is no conflict of interest.

Acknowledgments

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CAPÍTULO 3

Folic acid prevents depressive-like behavior and hippocampal antioxidant imbalance induced by restraint stress in mice. Budni J, Zomkowski AD, Engel D, Santos DB, dos Santos AA, Moretti M, Valvassori SS, Ornell F, Quevedo J, Farina M, Rodrigues ALS. Submetido ao Behav Brain Res. 2012.

Folic acid prevents depressive-like behavior and hippocampal antioxidant imbalance induced by restraint stress in mice

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Abstract

Experimental and epidemiological studies have shown the close relationship between stressful events, depression and cognitive impairment. Folic acid, which is essential for the proper functioning of the central nervous system, has been reported to present antidepressant-like effects in both experimental and clinical approaches; however, the mechanisms mediating such effects are not understood. In the present study, we evaluated if folic acid administration to mice could protect against acute restraint stress (ARS)-induced changes in parameters related to depressive (forced swimming test; FST), locomotor/exploratory (open-field test; OPT) and cognitive (object recognition test; ORT) behaviors, as well as if cerebrocortical and hippocampal oxidative stress could be involved in such events. ARS induced depressive-like behavior in the FST and memory impairment in the ORT, without altering locomotor activity of mice in the OFT. Folic acid (50 mg/kg, p.o.) administred 1 h before ARS was able to prevent the stress-induced increase on immobility time in the FST, but did not prevent memory impairment in the ORT. Moreover, ARS increased thiobarbituric acid reactive substances (TBARS) levels and catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities in the cerebral cortex and hippocampus, and superoxide dismutase (SOD) activity in the hippocampus. Folic acid treatment restored the activity of SOD, CAT, GR and GPx and reduced TBARS levels in the hippocampus. Glutatione (GSH), a non-enzymatic antioxidant was not altered by stress and/or folic acid administration. Together, the results of the present work indicate that the ARS-induced depressive-like behavior and cognitive deficit are accompanied by disturbances in the balance between pro- and anti-oxidative processes. Folic acid treatment improves the depressive behavior and restores the oxidative balance in the hippocampus. The results reinforce the notion that folic acid displays a specific antidepressant profile in the restraint stress paradigm that may be at least partly due to its antioxidant role.

Keywords: Folic acid, acute restraint stress, forced swimming test, object recognition test, oxidative stress, antioxidant.

Introduction

Stress may be defined as a state of threatened homeostasis that can produce adaptive physiologic and behavioral responses depending on severity, type and duration of stressful events in an attempt to reestablish body homeostasis (Chrousos, 2009; Jaggi et al, 2011; Munhoz et al, 2008). These physiological, psychological and cognitive alterations induced by stress affect different organs and systems, including central nervous system (CNS) (Linthorst & Reul, 2008; Munhoz et al, 2008). A consequence of stressful events is the increased susceptibility to different psychiatric disease, including depression (Calabrese et al, 2011), which frequently is accompanied by cognitive deficits (Marazziti et al, 2010; Murrough et al, 2011). Stressful life events have a considerable causal association with the pathophysiology of this disorder, especially, in genetically predisposed individuals (Charney & Manji, 2004; Kubera et al, 2011; Lanfumey et al, 2008).

Many animal models of depression induced by stress are widely used to explore stress-evoked brain abnormalities, screen antidepressant drugs, and establish the behavioral phenotypes of gene-targeted or transgenic animals (Kalueff et al, 2007). Using stress to induce a feeling of loss of control might result in a behavioral state analogous to depression (Calabrese et al, 2011; Kubera et al, 2011). Restrain stress is frequently employed to induce a depressive behavioral state in rodents. In particular, it has been widely used in acute and chronic stress studies (Capra et al, 2010; Christiansen et al, 2011; Huynh et al, 2011; O'Mahony et al, 2010; Poleszak et al, 2006; Sevgi et al, 2006). Moreover, another important issue is that acute stress may affect the memory of rodents. Studies have demonstrated that restraint stress impairs nonspatial recognition memory in the object-recognition test (ORT) (Baker & Kim, 2002; Li et al, 2012; Nagata et al, 2009; Walesiuk et al, 2005).

Stress exerts detrimental effects on several cellular functions, as evidenced by defective plasma antioxidant defenses in conjunction with enhanced lipid peroxidation in depressive patients (Bilici et al, 2001; Khanzode et al, 2003; Ozcan et al, 2004), indicating that oxidative damage is an important mechanism of pathophysiology of depression in humans (Maes et al, 2011). Similarly, restraint stress in rodents precipitates many neurochemical and hormonal abnormalities that are often associated with an imbalance in the brain's intracellular redox state. Many studies have shown that restraint stress induces increased lipid peroxidation (Balk et al, 2010; Garcia-Bueno et al, 2005; Kumar et al, 2010; Kumar & Goyal, 2008; Kumari et al, 2007; Zafir et al, 2009;

Zafir & Banu, 2007) and increase (Balk et al, 2010; Fontella et al, 2005; Kim et al, 2005) or decrease (Balk et al, 2010; Kumar et al, 2010; Kumar & Goyal, 2008; Kumari et al, 2007; Pajovic et al, 2006; Zafir et al, 2009; Zafir & Banu, 2007) antioxidant enzymes activities in different brain regions of rodents (mice or rats), depending on severity and duration of restraint stress protocol.

Indeed, brain is more susceptible to oxidative stress because it metabolizes 20% of total body oxygen and has a limited amount of antioxidant capacity. It is well known that aversive stimuli (like stress), especially in the brain, may result in the production of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$) and hydrogen peroxide (H_2O_2). When ROS production exceeds the antioxidant capacity, they could lead to lipid peroxidation, especially in membranes, which plays an important role in tissue injury (Floyd, 1999; Floyd & Carney, 1992; Halliwell, 2006; Kovacs et al, 1996). Important endogenous antioxidant enzymes, which inhibit the formation of ROS or promote the removal of free radicals and their precursors, include catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) (McCord & Fridovich, 1988).

Although the underlying pathophysiological mechanisms of stress-induced depression are not completely established, novel targets have been identified for the development of new pharmacological treatments (Lee et al, 2010). There is several evidence that folic acid is an important micronutrient that may contribute to depressive disorders and its treatment (Coppen & Bolander-Gouaille, 2005; Morris et al, 2008; Sarris et al, 2009). Besides its essential role to the synthesis of DNA, RNA, and proteins and for neurological function (Fenech, 2010; Kronenberg et al, 2009), folic acid has been postulated as a putative antidepressant agent. Indeed, several clinical studies have supported a strong association between folic acid deficiency and depressive symptoms (Abou-Saleh & Coppen, 1989; Astorg et al, 2008; Bottiglieri et al, 2000; Carney, 1967; Papakostas et al, 2004). Reinforcing the role of folic acid in depression, recent preclinical studies performed by our group indicated that folic acid has antidepressant-like effects (Brocardo et al, 2008a; Brocardo et al, 2008b; Brocardo et al, 2009; Budni et al, 2012a; Budni et al, 2012b). In addition, antimanic-like (Brocardo et al, 2010), cognitive (Matté et al, 2009b; Matté et al, 2007; Singh et al, 2011; Troen et al, 2008) and neuroprotective (Budni et al, 2011; Fenech, 2001; Fenech et al, 2005; Joshi et al, 2001; Lin et al, 2004; Matté et al, 2009a; Patro et al, 2006; Tagliari et al, 2006; Yu et al, 2009) properties

have been reported for this vitamin. Although the aforementioned studies point to antidepressant effects of folic acid in both experimental and clinical approaches, the potential involvement of anti- and/or pro-oxidative events in such effects is not clear.

Hence, considering the above considerations, the aim of present work was to study if folic acid administration to mice could protect against acute restraint stress (ARS)-induced changes in parameters related to depressive, locomotor/exploratory and cognitive behaviors, as well as if cerebrocortical and hippocampal oxidative stress could be involved in such events. Based on the potential antioxidant effects of folic acid, we hypothesized that it could prevent ARS-induced depressive behavior and cognitive decline by modulating oxidative stress-related events.

Materials and methods

Animals

Male Swiss mice weighing 40-45 g (4-month old) were maintained at 21-23°C with free access to water and food, under a 12:12 h light:dark cycle (lights on at 7:00 am). All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The experiments were performed after approval by the Ethics Committee of the Institution and all efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

Drugs and treatment

Folic acid (Sigma Chemical Co., St. Louis, U.S.A.) was dissolved in distilled water and administered orally (p.o.) by gavage at a dose of 50 mg/kg one hour before the ARS procedure. Folic acid solution was ***freshly*** prepared before administration and was administered in a volume of 1 ml/kg. To develop this study mice were divided into four groups, as follows: (1) vehicle + non-stressed; (2) folic acid + non-stressed; (3) vehicle + stressed; (4) folic acid + stressed. The dose of folic acid was chosen based on previous studies from our group (Brocardo et al, 2008a; Brocardo et al, 2008b; Brocardo et al, 2009; Budni et al, 2012a; Budni et al, 2012b).

Acute Restraint stress (ARS) procedure

ARS protocol was adapted from the previous procedure (Kumar & Goyal, 2008; Poleszak et al, 2006; Zafir et al, 2009). The animals were divided into four groups as mentioned above. Non-stressed groups were treated with vehicle or folic acid and were kept undisturbed in their home cages during the 8 hours receiving support of food and water. Stressed groups were administered with vehicle or folic acid and 1 h after the treatment they were submitted to stress. The immobilization was applied for a period of 7 h using an individual rodent restraint device made of plexiglas fenestrate. This restrained all physical movement without causing pain. The animals were deprived of food and water during the entire period of exposure to stress. After 7 hours, the animals were released from their enclosure and 40 min post-release the animals were submitted to the behavioral tests or sacrificed for the biochemical studies.

Behavioral tests

Forced swimming test (FST)

Briefly, 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 duration of immobility was measured during 6-min period as described previously (Brocardo et al, 2008b; Budni et al, 2007). 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 and an increase of immobility time, when compared to the control group, is considered a depressive-like effect (Kaster et al, 2012; Porsolt et al, 1977).

Open-field test

To assess the possible effects of folic acid on locomotor activity, mice were evaluated in the open-field paradigm as previously described (Budni et al, 2007; Rodrigues et al, 1996). Animals were individually placed in a wooden box (40×60×50 cm) with the floor divided into 12 rectangles. The number of squares crossed with all paws (crossing) was counted in a 6 min session. The apparatus were cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

Object recognition test (ORT)

The object recognition was performed as previously described (Réus et al, 2008). The

task took place in a 40x9x60 cm open-field surrounded by 50 cm high walls made of plywood with a frontal glass wall. The floor of the open-field was divided into 12 equal rectangles by black lines. The training session was conducted by placing individual mouse for 5 min in the apparatus, in which two identical objects (objects A1 and A2; both being cubes) were positioned in two adjacent corners, 10 cm from the walls. In a long-term recognition memory test given 24 h after training, the mice explored the open-field for 5 min in the presence of one familiar (A) and one novel (B, a pyramid with a square-shaped base) object. All objects had similar textures (smooth), colors (blue), and sizes (weight 150-200 g), but distinctive shapes. A recognition index calculated for each animal was calculated in the test session, and it reports the ratio TB/(TA + TB) (TA = time spent exploring the familiar object A; TB = time spent exploring the novel object B). Between trials, the objects were washed with 10% ethanol solution. Exploration was

defined as sniffing (exploring the object 3-5 cm away from it) or touching the object with the nose and/or forepaws.

Biochemical analysis

Tissue preparation

40 min after the ARS procedure, the animals were killed by decapitation and the cerebral cortices and hippocampi were removed and homogenized (1:10 w/v) in HEPES buffer (20 mM, pH 7.0). The tissue homogenates were centrifuged at 16,000 x *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 GSH and thiobarbituric acid reactive substances (TBARS).

Activity of antioxidant enzymes

Glutathione reductase (GR) activity was determined based on the protocol developed by (Carlberg & Mannervik, 1985). Briefly, GR reduces GSSG to GSH at the expense of NADPH, the disappearance of which can be followed at 340 nm. Glutathione peroxidase (GPx) activity was determined based on the protocol developed by (Wendel, 1981) by indirectly measuring the consumption of NADPH at 340 nm. The GPx uses GSH to reduce the *tert*-butyl hydroperoxide, producing GSSG, which is readily reduced to GSH by GR using NADPH as a reducing equivalent donor. Catalase 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 was measured spectrophotometrically at 240 nm. Superoxide dismutase activity was assayed spectrophotometrically as described by (Misra & Fridovich, 1972). 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 enzymatic activity was expressed as Units (U)/mg protein.

Glutathione (GSH) levels

GSH levels were measured as non-protein thiols based on the protocol developed by (Ellman, 1959). Hippocampal and cerebrocortical homogenates were precipitated in cooled trichloroacetic acid 10% and centrifuged at 5,000 x *g* for 10 min, and the supernatant was incubated with DTNB in a 1 M phosphate buffer, pH 7.0. Absorbances were measured at 412 nm. A standard curve of reduced glutathione was used to calculate GSH levels.

Thiobarbituric acid reactive species (TBARS) formation

TBARS levels, a measurement of lipid peroxidation, were determined in the hippocampal and cerebrocortical homogenates according to 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 minutes 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.

Determination of protein

The protein content was quantified according to the method described by (Lowry et al, 1951), using bovine serum albumin as a standard.

Statistical analysis

All data are presented as mean ± SEM. Data were analyzed by two-way ANOVA or two-way repeated measures ANOVA, followed by Duncan's post hoc test when the F value was significant. Differences were considered statistically significant if $p<0.05$.

Results

Behavioral tests

Forced swimming test (FST)

Fig. 1A shows the effect of treatment of mice with folic acid or vehicle on the depressive-like behavior elicited by ARS. The two-way ANOVA revealed significant differences for folic acid treatment [$F(1,26)=33.11$, $p<0.01$], restraint stress [$F(1,26)=13.28$, $p<0.01$] and ARS vs. folic acid treatment interaction [$F(1,26)=4.38$, $p<0.05$]. Post-hoc analyses indicated that stressful stimuli significantly increased the immobility time in the FST, as compared to control mice. Folic acid administration significantly reversed the increase in immobility time in stressed mice. Folic acid administration, in non-stressed mice, also decreased the immobility time in the FST as compared to non-stressed mice treated with vehicle.

Locomotor activity

The result depicted in Fig.1B shows that locomotor activity of mice submitted to 7 hours of ARS treated with folic acid or vehicle was not altered in the open-field test. The two-way ANOVA revealed no

significant differences for folic acid treatment [F(1,28)=0.05, p=0.82], restraint stress [F(1,28)=0.06, p=0.80] and treatment vs. restraint stress interaction [F(1,28)=0.05, p=0.82].

Object recognition test (ORT)

In order to evaluate if restraint stress induces impairment of cognitive function the ORT was carried out. In the training session all animal groups (non-stressed or stressed) showed no differences in the exploration between the two objects (means ± SEM of recognition index: vehicle/non-estressed=0.494 ± 0.010; folic acid/non-stressed=0.497 ± 0.005; vehicle/stressed=0.491 ± 0.015; folic acid/stressed=0.481 ± 0.015). If mice remember an object, they prefer to explore and/or sniff the novel object when the original object is replaced by a new one. In the test session (24 h after training session), mice from the vehicle/non-stressed group [0.685 ± 0.023] and folic acid/non-stressed group [0.473 ± 0.003]) preferred to explore the novel object, while the stressed mice group (0.474 ± 0.003) explored the novel and original objects similarly, which is an indicative of a cognitive impairment induced by restraint stress ($p<0.01$). Folic acid treatment (folic acid/stressed= 0.426 ± 0.027) was not capable of preventing the decline in the recognition of a novel object induced by restraint stress, since we observed a similar frequency of exploring and/or sniffing the novel object or the original one in the folic acid/stressed group. Also, it is important to mention that post hoc analysis indicated a significant difference between vehicle/non-stressed and folic acid/non-stressed group in the test session ($p<0.01$) (Fig. 2).

Measurement of biochemical parameters

Lipid peroxidation

Fig. 3 shows that TBARS levels were significantly increased in the cerebral cortex and hippocampus of stressed mice when compared to non-stressed mice. Treatment with folic acid (50 mg/kg, p.o.) significantly prevented the increase of TBARS levels in the hippocampus (Fig. 3B), but not in the cerebral cortex (Fig. 3A), when compared to stressed animals. The two-way ANOVA revealed significant differences for folic acid treatment [F(1,17)=8.79, $p<0.01$], folic acid treatment vs. ARS interaction [F(1,17)=5.51, $p<0.05$] and a great tendency of significance for ARS [F(1,17)=4.27, p=0.05] in the hippocampus. In the cerebral cortex, the two-way ANOVA showed

significant differences for folic acid treatment [$F(1,16)=64.96$, $p<0.01$] and folic acid treatment vs. ARS interaction [$F(1,16)=5.51$, $p<0.05$], but not for ARS [$F(1,16)=6.36$, $p=0.92$]. Folic acid administration to non-stressed mice did not produce any significant effect on TBARS levels.

GSH levels

Fig. 4 shows that no significant statistical difference was observed in GSH level in cerebral cortex (Fig. 4A) and hippocampus (Fig. 4B) of stressed mice compared to non-stressed animals treated with vehicle or folic acid.

Antioxidant enzyme activities

Restraint stress significantly increased CAT activity in cerebral cortex (Fig. 5A) and hippocampus (Fig. 5B) of stressed mice as compared to non-stressed mice. This increase induced by ARS was significantly blunted by the treatment with folic acid, but this effect was observed only in the hippocampus. In non-stressed mice group, the treatment with folic acid did not alter CAT activity in both cerebral structures. Two-way ANOVA revealed a significant folic acid vs. ARS interaction [$F(1,17)=8.91$, $p<0.01$] and a great tendency of significance for folic acid treatment [$F(1,17)=4.23$, $p=0.055$], but not for ARS [$F(1,17)=0.44$, $p=0.51$] in the hippocampus. In the cerebral cortex, two-way ANOVA revealed a significant main effect of folic acid treatment [$F(1,18)=26.74$, $p<0.01$], but not of ARS [$F(1,18)=1.92$, $p=0.18$], as well as a non significant folic acid treatment vs. ARS interaction [$F(1,18)=0.04$, $p=0.85$]).

As depicted in Fig 5D, ARS caused an increase on SOD activity in the hippocampus and this effect was reversed by folic acid treatment (folic acid treatment [$F(1,19)=6.90$, $p<0.05$], ARS [$F(1,19)=10.30$, $p<0.01$] and folic acid treatment vs. ARS interaction [$F(1,19)=8.32$, $p<0.01$]). Fig. 5C shows that no significant statistical alteration was observed in SOD activity in cerebral cortex of stressed mice compared to non-stressed animals (folic acid treatment [$F(1,19)=7.57$, $p<0.05$], ARS [$F(1,19)=0.004$, $p=0.95$] and folic acid treatment vs. ARS interaction [$F(1,19)=5.94$, $p<0.05$]). The treatment with folic acid alone did not alter CAT and SOD activity in the evaluated structures, independent on stress condition.

As can be observed in Fig. 6A (cerebral cortex) and Fig. 6B (hippocampus), stressed mice displayed increased GPx activity when compared with non-stressed control group. This effect was significantly reversed by folic acid treatment in the hippocampus (folic acid treatment

[F(1,17)=7.03, p<0.05], ARS [F(1,17)=0.29, p=0.59] and folic acid treatment vs. ARS interaction [F(1,17)=13.18, p<0.01]), but not in the cerebral cortex (folic acid treatment [F(1,18)=21.47, p<0.01], ARS [F(1,18)=1.06, p=0.31] and folic acid treatment vs. ARS interaction [F(1,18)=1.41, p<0.25]). The administration of folic acid alone did not affect the GPx activity in non-stressed group in both cerebral structures.

Finally, the results illustrated in the Fig. 6C (cerebral cortex) and 6D (hippocampus) show that the exposure of mice to ARS also resulted in a significant increase on the activity of the antioxidant enzyme GR in the hippocampus and cerebral cortex. This increase was significantly abolished by folic acid treatment in stressed mice in both brain regions. The administration of folic acid alone to non-stressed mice caused no significant alteration on GR activity. Two-way ANOVA revealed significant difference for folic acid treatment [F(1,19)=19.28, p<0.01], ARS [F(1,19)=4.78, p<0.05] and folic acid treatment vs. ARS interaction [F(1,19)=5.14, p<0.05] in the cerebral cortex. In the hippocampus, the two-way ANOVA revealed significant differences for folic acid treatment [F(1,19)=4.82, p<0.05], ARS [F(1,19)=6.03, p<0.05] and folic acid treatment vs. ARS interaction [F(1,19)=12.76, p<0.01].

Discussion

The present study shows that ARS induced depressive-like behavior, cognitive deficits and oxidative imbalance (increased hippocampal and cerebrocortical TBARS levels and SOD, CAT, GPx and GR activities). Noteworthy, the depressive-like behavior and all the neurochemical changes observed in the hippocampus were restored by folic acid treatment. On the other hand, folic acid was not able to protect against cognitive impairment induced by restraint stress. Moreover, glutathione, a non-enzymatic antioxidant was not altered by stress and/or folic acid administration.

Taking into account that stressful life events have been reported to facilitate the evolution of depressive disorders (Calabrese et al, 2011), chronic or ARS in rodents has been widely used as a model of depression (Capra et al, 2010; Christiansen et al, 2011; Huynh et al, 2011; O'Mahony et al, 2010; Poleszak et al, 2006; Sevgi et al, 2006). Because ARS represents the most severe type of stress which causes emotional stress in rodents and has a comparative effect in humans (Calabrese et al, 2011; Kubera et al, 2011) it was used in the present study in an attempt to induce a depressive-like behavior. Moreover, the major advantage of using restraint stress as a stress-induced model of depression is that it produces an inescapable physical and mental stress to which adaptation is seldom exhibited (Jaggi et al, 2011).

Numerous studies have reported that rodents (mice and/or rats) exposed to emotional stress, such as restraint stress, in different duration of stressful events, exhibit depressive like-behavior, evidenced by increased immobility time, particularly in the FST (Capra et al, 2010; Naert et al, 2011; Park et al, 2010; Poleszak et al, 2006; Zafir et al, 2009) and tail suspension test (Hayase, 2011; Park et al, 2010). This behavioral alteration in mice is comparable to depressed mood in humans (Wong & Licinio, 2004). Corroborating these studies, our results indicate that ARS for 7 h induced an increase of immobility time in the FST in mice, without causing changes in the locomotor activity. Further corroborating several recent studies that have suggested an antidepressant potential of folic acid (Brocardo et al, 2008a; Brocardo et al, 2008b; Brocardo et al, 2009; Budni et al, 2012a; Budni et al, 2012b), the present study clearly shows that this depressive-like behavior was reversed by folic acid treatment. Moreover, folic acid in non-stressed mice induced antidepressant-like effect in the FST, 8 h after its administration, indicating a prolonged effect of this vitamin. Folic acid alone did not affect the locomotion of non-stressed or stressed mice in the OFT.

It has been shown that folic acid (administered by oral, intracerebroventricular, or intraperitoneal routes) displays antidepressant-like effect in mice submitted to the FST and the TST, two behavioral tests predictive of antidepressant activity (Brocardo et al, 2008b). Several mechanisms may be involved in the antidepressant-like effect of this vitamin. Brocardo et al. (2008b) showed that serotonergic (5-HT1A and 5-HT2A/2C receptors) and noradrenergic (α1- and α2-adrenoceptors) systems are implicated in the antidepressant-like effect of folic acid. Furthermore, the antidepressant-like effects of this vitamin were shown to be dependent on the inhibition of either N-methyl-D-aspartate (NMDA) receptors or nitric oxide (NO) and cyclic guonosine monophosphate (cGMP) synthesis (Brocardo et al, 2008a), as well as through an interaction with the opioid system (μ1 and δ receptors) (Brocardo et al, 2009). More recently, we showed that the antidepressant effect of folic acid might also be dependent, at least in part, on the inhibition of glycogen synthase kinase-3 (GSK-3β), activation of peroxisome proliferator-activated receptor-γ (PPARγ) (Budni et al, 2012b) and inhibition of different types of potassium channels (Budni et al, 2012a). However, there were no studies in the literature investigating the potential relationship between the antidepressant-like effects of folic acid and its properties to modulate the anti-/pro-oxidant status in the central nervous system.

Taking into account that drugs which induce hiperlocomotion may give a ‘‘false’’ positive effect in the FST, and drugs decreasing locomotion may give a “false” negative result (Borsini & Meli, 1988; Rodrigues et al, 2005), in the present study the effects of folic acid or vehicle in non-stressed mice as well as in mice submitted to ARS were tested in the OFT. Folic acid administered to stressed or non-stressed mice did not significantly alter locomotor activity in the OFT, a result that indicates that the depressive-like effect induced by ARS and the reversal of this effect elicited by folic acid are not due to any locomotor effect.

Noteworthy, the restraint stress applied in this study induced considerable cognitive impairment in the ORT, a result that is in agreement with several literature data (Baker & Kim, 2002; Nagata et al, 2009). Moreover, a study by Li et al. (2012) showed that restraint stress can affect different memory components since it impaired the memory retrieval and interrupted the consolidation of short-term memory into long-term memory in the ORT. Interestingly, our results show that folic acid treatment was not able to abolish the restraint stress-induced cognitive deficits in the ORT, indicating that the folic acid effect in the

restraint stress protocol employed in the present study is specific for depression. Thus, folic acid provides a specific antidepressant-like effect in this animal model. However, it is interesting to note that mice treated with folic acid *per se* (non-stressed group), in the session test, preferred to explore the novel object more than the original object, as compared vehicle/non-stressed group, indicating a role of folic acid *per se* in the cognition. Indeed, several studies have indicated that folic acid has a cognitive improvement property, since it caused an improvement on memory status in elderly rats (Singh et al, 2011) and in cognitive deficits induced by hyperhomocysteine (Matté et al, 2009b; Matté et al, 2007). Additionally, a deficiency of this vitamin is associated with cognitive impairment in rodents (Troen et al, 2008) and many clinical studies have indicated that folic acid is important on cognitive performance, contributing to the notion that low folic acid itself might be a risk factor for cognitive impairment (de Lau et al, 2007; Durga et al, 2006; Kado et al, 2005; Ramos et al, 2005). However, in spite of all this literature evidence, in the present study, although folic acid treatment caused a cognitive improvement in non-stressed mice, it was not able to protect against restraint stress-induced cognitive deficits. However, it cannot be ruled out that a higher dose of folic acid or a repeated treatment with this vitamin would be able to reverse the cognitive deficit induced by restraint stress.

The results of the present work also indicate oxidative imbalance, alteration of markers of oxidative damage to lipids and antioxidant defense in mice submitted to restraint stress for 7 h. The results show that depressive-like behavior and the cognitive impairment induced by restraint stress was accompanied by a significant lipid peroxidation, as evidenced by increased amount of TBARS levels in cerebral cortex and hippocampus of mice. Ours results are in accordance with findings reported by Kumar et al. (2010) which shows that 6 h of acute immobilization stress significantly increased MDA in brain of mice. Moreover, a significant increase in the production of brain MDA was shown in rats submitted to ARS for 4 h (Zafir et al, 2009). Other studies support the idea that ARS induces significant lipid peroxidation (Garcia-Bueno et al, 2005; Kumar et al, 2010; Kumar & Goyal, 2008; Kumari et al, 2007; Zafir & Banu, 2007). Also, supporting our study, clinical studies indicating raised levels of MDA in depressed patients, established the co-existence of increased oxidative stress with symptoms of depression (Bilici et al, 2001; Khanzode et al, 2003). Therefore, in line with literature data, our results suggest that restrain stress for 7 h causes lipid peroxidation in the cerebral cortex and hippocampus, two

structures closely related to the pathophysiology of depression (Bennett, 2011; Duman & Voleti, 2012; Yu & Chen, 2011). Moreover, taking into account that lipid peroxidation is one of the major consequences of free-radical-mediated injury to the brain (Dotan et al, 2004), the present study suggests that behavioral alterations caused by restraint stress may be associated with oxidative damage in the cerebral cortex and hippocampus.

It is possible to observe that the ARS-induced raise on lipid peroxidation was reversed by folic acid treatment in the hippocampus, but not in the cerebral cortex. This finding is somewhat similar to the one reported by Brocardo et al. (2010), which showed that folic acid treatment prevented ouabain-induced increase on lipid peroxidation in the hippocampus of rats. However, in this study, folic acid neuroprotective effect was also observed in the cerebral cortex (Brocardo et al, 2010). Moreover, Singh et al. (2011) showed that 6-, 11-, and 16-month-old rats treated with folic acid at a dose of 5 mg/kg for a period of 8 weeks displayed reduction on MDA levels, indicating the prevention of lipid peroxidation in cerebral cortex, mid brain, and cerebellar regions of rat brain. Another study reported that rats submitted to a folate deprivation for four weeks had elevated TBARS hippocampal levels compared with vehicle control rats (Chen et al, 2011). Besides these data, other investigations showed that folic acid deficiency promotes lipid peroxidation in human cells and rodent tissues (Chern et al, 2001; Huang et al, 2001). Furthermore, a clinical study performed by Racek et al. (2005) indicated that folic acid supplementation in patients with hyperhomocysteinemia induced partial prevention of plasmatic lipid peroxidation (Racek et al, 2005).

The present study found the restraint stress caused an increase on the activity of the antioxidant enzymes directly involved in the neutralization of ROS, namely SOD (hippocampus), CAT, GR and GPx (cerebral cortex and hippocampus), indicating an alteration in antioxidant brain defenses in mice exposed to ARS that present a depressive-like behavior. It is important to emphasize that SOD is the first line defense against ROS and catalyzes the dismutation of superoxide anion radical (O_2) into hydrogen peroxide (H_2O_2) (McCord & Fridovich, 1988). This molecule, H_2O_2, can be reduced to water and molecular oxygen by either CAT (Chelikani et al, 2004) or GPx (Flohe, 1971). Besides detoxifying H_2O_2, GPx can also reduce lipid and non-lipid hydroperoxides at the expense of reduced glutathione (GSH), which is in turn oxidized, forming glutathione disulfide (GSSG) (Flohe, 1971). The increased activity of these antioxidant enzymes in response

to restraint stress, found in this study, is in agreement with the results of several clinical studies. Bilici et al. (2001) showed that depressed patients, especially melancholic patients, had higher activities on plasma GR and erythrocyte GPx and SOD than those of healthy controls. Moreover, a recent study reported that patients during acute depressive episodes, had significantly higher activity of SOD and CAT on erythrocytes, as compared to healthy controls (Galecki et al, 2009). Other human studies show increased SOD activity in prefrontal cortex of postmortem patients (Michel et al, 2007) and in erythrocyte of patients with depressive disorder (Kotan et al, 2011). Similarly, a pre-clinical study by Fontella et al. (2005) showed that chronic restraint stress (1h/day during 40 days) caused an increase on GPx activity in the rat hippocampus. Other study by Kim et al. (2005) indicated an increase on SOD and CAT activities in the brain of mice submitted to ARS for 2 h every day for 3 days. More recently, Balk et al. (2010) found increased CAT activity in striatum of rats submitted to chronic restraint stress (1h/day during 40 days).

Regarding the use of stress protocols in animal models, it is important to mention that the available studies have observed different effects on antioxidant enzymes. An increase on antioxidant enzyme activities has been reported in animals submitted to repeated or chronic restraint stress conditions (Balk et al, 2010; Fontella et al, 2005; Kim et al, 2005), whereas ARS (only one exposition) was shown to decrease CAT and GR activity (Kumar et al, 2010; Kumar & Goyal, 2008; Kumari et al, 2007), as opposed to the results of the present work. Also, studies performed with chronic restraint stress (4h/21 days) showed reduction on SOD, CAT and GR activity (Zafir et al, 2009; Zafir & Banu, 2007). These inconsistencies may be a consequence of a number of variations of the procedures or animals used, including age and gender of animals, intensity, duration, frequency and type of stressor (Buynitsky & Mostofsky, 2009).

Therefore, increased SOD, CAT, GPx and GR activities (specially in the hippocampus) found in this study are in accordance with some preclinical (Balk et al, 2010; Fontella et al, 2005; Kim et al, 2005) and clinical (Bilici et al, 2001; Galecki et al, 2009; Kotan et al, 2011; Michel et al, 2007) literature data and might be due to a compensatory response to increased free radical formation induced by ARS in mice. In this regard, some lines of evidence have reported that the occurrence of pro-oxidative stimulus is necessary to trigger an increase in the levels (and consequently, activities) of antioxidant enzymes (Bea et al, 2009; Maher et al, 2008; Suzuki et al, 2008). From a

mechanistic point of view, it is important to mention the crucial role of the transcription factor Nrf2 (NFE2-related factor 2), which orchestrates the synthesis of antioxidant and phase-2 enzymes in an attempt to compensate the deleterious effects of pro-oxidative agents (de Vries et al, 2008). Thus, based on these evidences and on our results, one could suppose that the ARS was able to stimulate hippocampal and cerebrocortical pro-oxidative events, which were able to trigger the observed compensatory increased of SOD, CAT, GPx and GR activities. The fact that folic acid pretreatment prevented such increase indicates that this vitamin blunted primary pro-oxidative stimulus induced by ARS. Such data seems to be of great significance since indicates that folic acid administration could prevent the occurrence of oxidative damage in important structures of the central nervous system, even when such structures are submitted to a pro-oxidative challenge. This idea is reinforced by the fact that folic acid prevented ARS-induced hippocampal and cerebrocortical lipid peroxidation. Of particular importance, such events were likely correlated to the observed antidepressant-like effects of the vitamin.

Although the modulatory effects of folic acid toward anti- and/or pro-oxidant events might be important (as previously discussed), additional mechanisms might underlie its protective effects observed in the present study. Indeed, folic acid deprivation induces increase on cytosolic Ca^{2+} and ROS, and impairs mitochondrial function (Ho et al, 2003; Tjiattas et al, 2004). Folic acid also plays a protective role against glutamate and NMDA-induced cytotoxicity in cultured mouse cerebellar granule neurons (Lin et al, 2004) and protects neurons from the damage caused by $A\beta_{25-35}$ peptide by maintaining mitochondrial function and DNA integrity and regulating apoptosis-related genes (Yu et al, 2009).

Our results show no alterations in glutathione (GSH) levels in cerebral cortex and hippocampus of mice, independent on stress condition or folic acid treatment. Our results are in agreement with a recent study, which observed no changes in GSH level in the brain of rats submitted to restraint stress for 24 h (Méndez-Cuesta et al, 2011). GSH is the most important non-enzymatic endogenous antioxidant and can be regenerated by GR with the consumption of nicotinamide adenosine dinucleotide phosphate (NADPH) (Krohne-Ehrich et al, 1977). This non-enzymatic antioxidant play a role in detoxifying a variety of electrophilic xenobiotics, producing less toxic compounds (Jakoby, 1978). The absence of changes in GSH levels as a result of stress and folic acid treatment suggests that this non-enzymatic

antioxidant does not play a significant role in this model upon the conditions employed in this study.

Our results are in line with evidence that oxidative stress has been implicated in the pathology of depression and antioxidants may offer resistance against oxidative stress by scavenging free radicals, inhibiting the lipid peroxidation, and by many other mechanisms that may contribute to prevent this illness (Maes et al, 2011). Folic acid was able to protect against restraint stress-induced depressive-like behavior and oxidative stress, particularly in the hippocampus. Similar to folic acid, classical antidepressants treatment also attenuate the increase of antioxidant enzymes activities in depressed patients (Kotan et al, 2011). A study performed by Bilici et al. (2001) showed that depressive patients, especially melancholic patients had higher MDA levels and plasma GR and erythrocyte GPx and SOD activities than those of healthy controls. After treatment for 3 months with fluoxetine, sertraline, fluvoxamine and citolapram, the enzymatic activities and MDA levels of the patients were significantly decreased to normal levels. Based on our findings and on literature data, it is tempting to suggest that folic acid might act similarly to antidepressants. In line with this hypothesis, the co-administration of sub-effective doses of folic acid and fluoxetine in mice produced antidepressant-like effects in the FST (Brocardo et al, 2008b). Moreover, in another study it was possible to verify that folic acid alone or combined with estradiol or fluoxetine also produced antidepressant-like effects in ovariectomized female rats in the FST (Molina-Hernandez et al, 2011).

It is important to mention that the results of present study show that folic acid treatment in mice submitted to ARS affects differently hippocampal and cerebrocortical oxidative status, since folic acid protected against restraint stress-induced hippocampal oxidative stress, but was only effective to reverse the stress-induced GR activity in the cerebral cortex, without affecting TBARS levels and SOD, CAT, GPx activities in this brain structure. This suggests that these brain areas have specific differences in defense mechanisms when submitted to stressful stimuli. These differences may underlie the different susceptibilities of distinct brain areas to folic acid treatment under stressful stimuli. Therefore, a region- and stressor-specific response induced by folic acid treatment might occur in these brain regions.

Finally, this investigation revealed that the restorative action of folic acid, as evidenced by the normalization of depressive-like behavior may be associated with a balance of endogenous antioxidant defenses in the hippocampus, since this vitamin was capable of mitigating

hippocampal oxidative damage induced by mouse ARS. Thus, taking into account that the effects of ARS is associated with depression in humans and many patients do not tolerate or respond adequately to the available classical antidepressant, the present findings warrant further studies to evaluate the therapeutic relevance of folic acid for the treatment of depression and as a co-adjuvant treatment with antidepressants.

Legends

Figure 1. Effect of treatment with folic acid on immobility time in the FST (panel A) and on locomotor activity (panel B) in mice submitted to restraint stress. Values are expressed as mean ± SEM (n=7-8). **p<0.01 and *p<0.05 compared with non-stressed group treated with vehicle, ##p<0.01 compared with stressed group treated with vehicle, according to two-way ANOVA followed by the Duncan's post hoc test.

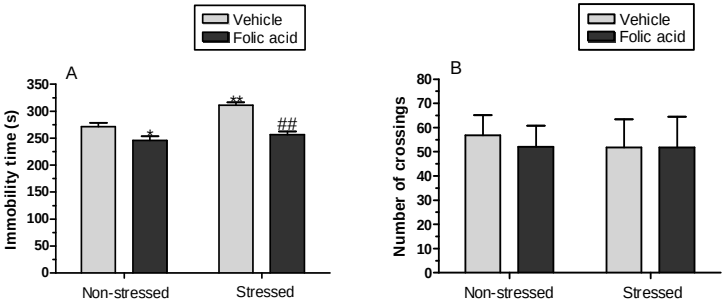


Figure 2. Effect of treatment with folic acid in the object recognition test (ORT) in mice submitted to restraint stress. Recognition index for the objects in the training and test sessions for non-stressed and stressed groups of mice. Results are presented as means ±SEM of the recognition index (n=6-8). The test session was performed 24 h after the training session. **p<0.001 compared with the same group of training session. ##p<0.01 indicates difference from the recognition index between vehicle/non-stressed and folic acid/non-stressed group of test session, according to two-way repeated measures ANOVA followed by the Duncan's post hoc test.

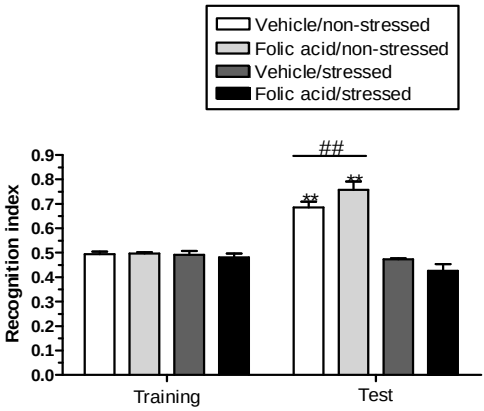


Figure 3. Effect of treatment with folic acid on thiobarbituric acid reactive substances (TBARS) in cerebral cortex (panel A) and hippocampus (panel B) of mice submitted to restraint stress. Values are expressed as mean ± SEM (n=5-6). **p<0.01 compared with non-stressed group treated with vehicle, ##p<0.01 compared with stressed group treated with vehicle, according to two-way ANOVA followed by the Duncan's post hoc test.

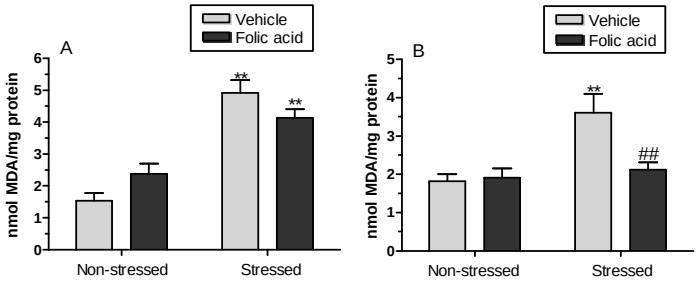


Figure 4. Effect of treatment with folic acid on glutathione levels in cerebral cortex (panel A) and hippocampus (panel B) of mice submitted to restraint stress. Values are expressed as mean ± SEM (n=6). Two-way ANOVA showed no significant effect.

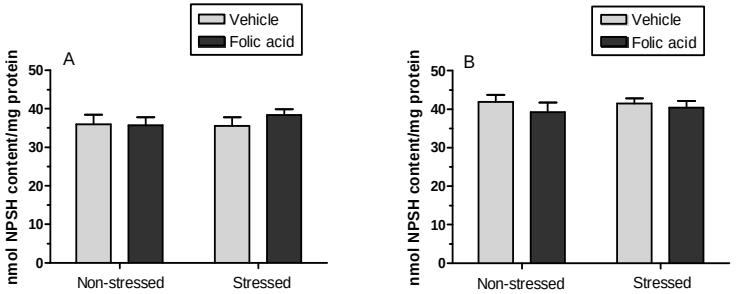


Figure 5. Effect of treatment with folic acid on SOD activity (panel A: cerebral cortex; panel B: hippocampus) and on CAT activity (panel C: cerebral cortex; panel D: hippocampus) of mice submitted to restraint stress. Values are expressed as mean ± SEM (n=5-6). **p<0.01 compared with non-stressed group treated with vehicle, ##p<0.01 and #p<0.05 compared with stressed group treated with vehicle, according to two-way ANOVA followed by the Duncan's post hoc test.

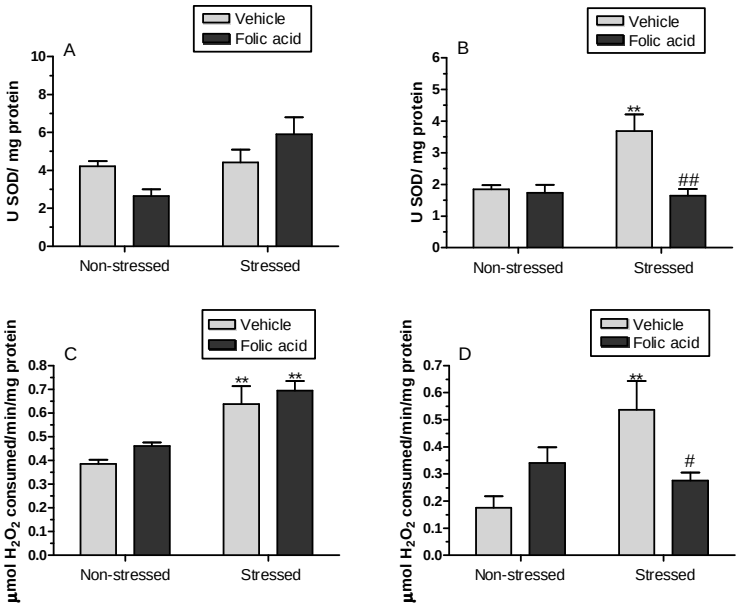
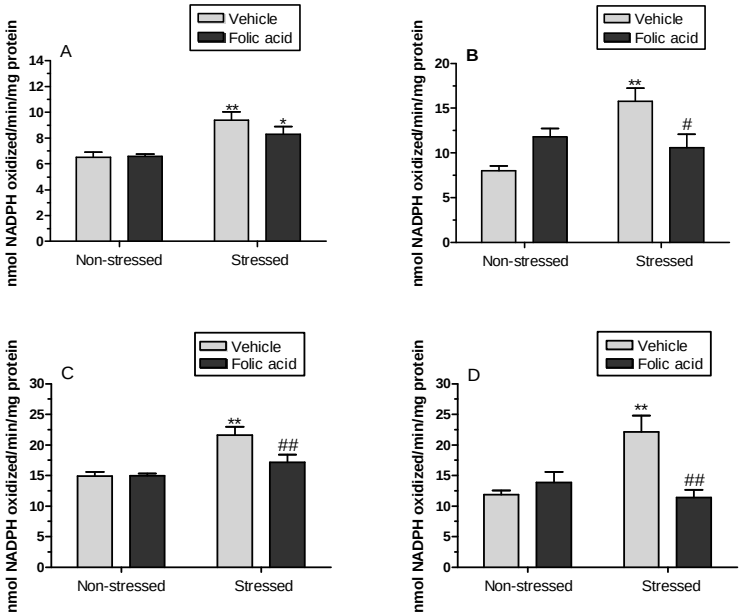


Figure 6. Effect of treatment with folic acid on GPx activity (panel A: cerebral cortex; panel B: hippocampus) and on GR activity (panel C: cerebral cortex; panel D: hippocampus) of mice submitted to restraint stress. Values are expressed as mean ± SEM (n=5-6). **p<0.01 and *p<0.05 compared with non-stressed group treated with vehicle, ##p<0.01 and #p<0.05 compared with stressed group treated with vehicle, according to two-way ANOVA followed by the Duncan's post hoc test.



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Estudos *in vitro*

CAPÍTULO 4

Neurotoxicity induced by dexamethasone in the human neuroblastoma SH-SY5Y cell line can be prevented by folic acid. Budni J, Romero A, Molz S, Martín-de-Saavedra MD; Egea J, Del Barrio L, Tasca CI, Rodrigues ALS, Lopéz MG. (Em anexo). Neuroscience. 190:346-53, 2011.

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NEUROTOXICITY INDUCED BY DEXAMETHASONE IN THE HUMAN NEUROBLASTOMA SH-SY5Y CELL LINE CAN BE PREVENTED BY FOLIC ACID

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Abstract—Folic acid (folate) is a vitamin of the B-complex group that is essential for cell replication. Folate is a major determinant of one-carbon metabolism, in which S-adenosylmethionine donates methyl groups that are crucial for neurological function. Many roles for folic acid have been reported, including neuroprotective and antidepressant properties. On the other hand, increased concentrations of corticoids have proven neurotoxic effects and hypersecretion of glucocorticoids has been linked to different mood disorders. The purpose of this study was to investigate the potential protective effect of folic acid on dexamethasone-induced cellular death in SH-SY5Y neuroblastoma cell line and the possible intracellular signaling pathway involved in such effect. Exposure to 1 mM dexamethasone for 48 h caused a significant reduction of cell viability measured as 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) reduction. Exposure of SH-SY5Y cells for 72 h to increasing concentrations of folate (1–300 μM) was not cytotoxic. However, pretreatment with folate (10–300 μM) reduced dexamethasone-induced toxicity in a significant manner. To explore the putative intracellular signaling pathways implicated in the protective effect of folate we used different protein kinase inhibitors. The protective effect of folic acid on dexamethasone-induced neurotoxicity was reversed by the phosphatidylinositol-3 kinase/Akt (PI3K/Akt, LY294002), Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII, KN-93), and protein kinase A (PKA, H-89) inhibitors, but not the mitogen-activated protein/extracellular signal-regulated kinase (MEK1/2, PD98059) and protein kinase C (PKC, chelerythrine) inhibitors. In conclusion, the results of this study show that folic acid can protect against dexamethasone-induced neurotoxicity and its protective mechanism is related to a signaling pathway that involves PI3K/Akt, CaMKII, and PKA. © 2011 Published by Elsevier Ltd on behalf of IBRO.

Key words: folic acid, dexamethasone, neuroprotection, PI3K, CaMKII, PKA.

Folic acid (folate) is a water-soluble vitamin whose biologically active form is tetrahydrofolic acid (THF), which participates in the transfer of one-carbon units (such as methyl, methylene, and formyl groups) to the essential substrates involved in the synthesis of DNA, RNA, and proteins, crucial for neurological function (Kronenberg et al., 2009; Fenech, 2010). The metabolism of folic acid in the cell is initiated by dihydrofolate reductase in a two-step reaction; the first step, conversion to dihydrofolate (DHF) is a slow and rate-limiting step. In the second step dihydrofolate is further reduced to THF. THF can then be converted into additional physiological folates including 5-methyl-THF, the form that is normally found in the circulation and in tissues. 5-methyl-THF is also replenished by the conversion of folinic acid (5-formyltetrahydrofolate), an active metabolite of folic acid. Because *de novo* folate synthesis is not present in the CNS, it depends on adequate folate transport across the blood–brain barrier. Within neurons, part of the folate pool will be catabolized by oxidation to dihydrofolates and folic acid, which can be reconverted to THF by dihydrofolate reductase (Ramaekers and Blau, 2004). A growing number of epidemiological studies have linked folate deficiency with an increased risk of neurodegenerative and neuropsychiatric diseases, including Alzheimer's, depression, and schizophrenia (Reynolds, 2002; Mattson and Shea, 2003; Coppen and Gouaille-Bolander, 2005; Farah, 2009). Recent preclinical studies by our group indicate that folic acid has antidepressant-like (Brocardo et al., 2008a,b, 2009) and antimanic-like properties (Brocardo et al., 2010).

Glucocorticoids are adrenal steroids secreted during stress and their numerous actions are essential for the stress response (Lee et al., 2002). However, excess of glucocorticoids, through the activation of glucocorticoid receptors, has been implicated in the reduction of the hip-

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Abbreviations: BDNF, brain-derived neurotrophic factor; CaMKII, Ca^{2+}/Calmodulin-dependent protein kinase II; CREB, cAMP response element-binding; CRH, corticotropin-releasing hormone; EMEM, Eagle's minimum essential medium; HPA, hypothalamic-pituitary-adrenal; H-89, N-[2-[[3-(4-bromophenyl)-2-propenyl] amino]ethyl]-5-isoquinolinesulfonamide hydrochloride; KN-93, N-[2-[[3-(4-chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulphonamide; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride; MEK1/2, mitogen-activated protein/extracellular signal-regulated kinase kinase; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NMDA, N-methyl-D-aspartate; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; PI3K, phosphatidylinositol-3 kinase; PKA, protein kinase A; PKC, protein kinase C; THF, tetrahydrofolic acid.

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pocampal volume observed in depression (Bremner et al., 2000; Sheline et al., 2003; Sterner and Kalynchuk, 2010). While the primary function of glucocorticoids is the mobilization of energy to respond to the stressor, they can also favor the cells to undergo apoptosis (Joëls, 2008). Specifically, dexamethasone, a synthetic glucocorticoid, has been reported to induce cellular death in different kinds of cells, namely dexamethasone induces apoptosis in striatal (Mitchell et al., 1998), hippocampal (Hassan et al., 1996; Haynes et al., 2001), cerebellar granule neurons (Jacobs et al., 2006), glial cells (Tazik et al., 2009), and SH-SY5Y cells (Tazik et al., 2009).

The human neuroblastoma cell line SH-SY5Y expresses several properties of neuronal cells and has been widely used as a cellular model to investigate the intracellular mechanisms mediating the actions of drugs on human neurons (Xie et al., 2010). Therefore, this cell line is useful to study mechanisms of neural death and protection (Kim et al., 2008; Romero et al., 2010).

Folic acid has previously been shown to exert neuroprotective actions by preventing the damage caused by acute hyperhomocysteinemia *in vivo* (Tagliari et al., 2006). It has also been reported to prevent neurotoxicity produced either by glutamate and N-methyl-D-aspartate (NMDA) in cultured mouse cerebellar granule neurons (Lin et al., 2004) or by beta-amyloid peptide in cultured cortical neurons (Yu et al., 2009). Moreover, other studies have reported that deprivation of folic acid can compromise the health of cultured dorsal root ganglion neurons (Tjiattas et al., 2004), embryonic cortical neurons, and differentiated SH-SY5Y human neuroblastoma cells (Ho et al., 2003). However, more compelling evidence is needed for elucidating its neuroprotective role on dexamethasone-induced cell death in SH-SY5Y cells. Therefore, in this study, we investigated the effect of folic acid against neuronal damage produced by dexamethasone in SH-SY5Y cultures and the signaling transduction pathways regulating its effect. Further elucidation of the neuroprotective effects of folic acid on SH-SY5Y may lead to novel therapeutic strategies for some diseases, especially depression, taking into account that hypersecretion of glucocorticoids is linked to mood disorders (Bremner et al., 2000; Kunugi et al., 2010; Sterner and Kalynchuk, 2010).

EXPERIMENTAL PROCEDURES

Drugs and chemicals

Folic acid was obtained from Sigma (Madrid, Spain). Chelerythrine, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride (LY294002), N-[2-[[[3-(4-chlorophenyl)-2-propenyl] methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulphonamide (KN-93), and N-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide hydrochloride (H-89) were purchased from Tocris (Biogen Científica, Madrid, Spain). Eagle's minimum essential medium (EMEM), fetal bovine serum, and penicillin/streptomycin were purchased from GIBCO (Madrid, Spain).

Culture of SH-SY5Y cells

SH-SY5Y cells were maintained in a 1:1 mixture of F-12 Nutrient Mixture (Ham12) (Sigma Aldrich, Madrid, Spain), and EMEM supplemented with 15 nonessential amino acids, 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (reagents from Invitrogen, Madrid, Spain). Cultures were seeded into flasks containing supplemented medium and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were seeded into 48-well culture plates at a seeding density 1×10^5 cells per well. All treatments were performed when cells were grown to about 65% confluence and at the end of treatment, cells reached about 80–90% confluence. All cells in this study were used at a low passage number (<13) and were maintained in 10% serum medium until the dexamethasone treatment.

SH-SY5Y cells treatment

Firstly, the cells were treated with F-12/EMEM alone (serum-free medium, 0 mM dexamethasone) or F-12/EMEM (serum-free medium) containing dexamethasone in concentrations ranging from 0.05 to 1 mM. MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) reduction was analyzed 48 h after dexamethasone addition (Fig. 1).

Additionally, to study the effect of folic acid in this cell death protocol, SH-SY5Y cells were preincubated (24 h before dexamethasone) and co-incubated (48 h during dexamethasone incubation) with folic acid in concentrations ranging from 1 to 300 μM, diluted in serum-free medium. MTT reduction was analyzed 72 h after folic acid addition (Fig. 2).

In order to investigate the mechanisms underlying the neuroprotective effect of folic acid against dexamethasone-induced cell death, the cells were preincubated with phosphatidylinositol-3 kinase/Akt (PI3K/Akt) inhibitor (LY294002, 10 μM), CaMKII inhibitor (KN-93, 1 μM), protein kinase A (PKA) inhibitor (H-89, 2 μM), mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK1/2) inhibitor (PD98059, 10 μM), or protein kinase C (PKC) inhibitor (chelerythrine, 0.1 μM), 1 h prior to the addition of folic acid. The inhibitors were present throughout folic acid incubation period (Fig. 3).

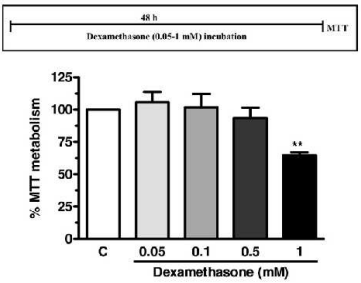

Fig. 1. Dose-dependent cell death induced by dexamethasone in SH-SY5Y neuroblastoma cells. Cells were treated with F-12/EMEM alone (0 mM dex) or F-12/EMEM containing four different concentrations of dexamethasone. MTT reduction was analyzed 48 h after dexamethasone addition. Data are shown as the mean±SEM of five different cell batches. ** $P<0.01$ with respect to control.

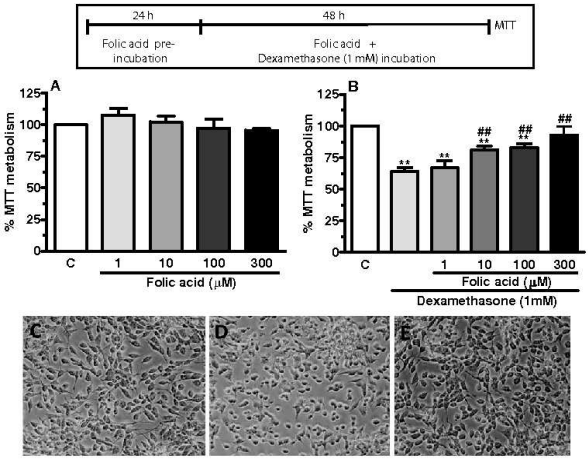

Fig. 2. Cytoprotection afforded by folic acid against the dexamethasone-induced neurotoxicity in SH-SY5Y neuroblastoma cells. The experimental protocol consisted of preincubation (24 h before dexamethasone) and co-incubation (48 h during dexamethasone incubation) of the SH-SY5Y cells with folic acid (1, 10, 100, and 300 μM, diluted in serum-free medium). MTT reduction was analyzed 72 h after folic acid addition. Folic acid at increasing concentrations (10–300 μM) was not cytotoxic "per se" (A). When it was present preceding and during the dexamethasone period, reduced cell death in a concentration-dependent manner and maximum protection was achieved at the concentration of 300 μM folic acid (B). The microphotographs show phase-contrast images taken at 40× magnification of control cells (C); cells exposed to 1 mM dexamethasone for 48 h (D) and cells incubated with 300 μM folic acid for 24 h before and during the 48-h exposure to dexamethasone (E). Data are shown as the mean±SEM of four to seven different cell batches. ** $P<0.01$ with respect to control. ## $P<0.01$ when compared to cells exposed to 1 mM dexamethasone (B). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Quantification of cell viability by MTT in SH-SY5Y cells

Cell viability was measured by quantitative colorimetric assay with MTT (Sigma Aldrich), as described previously (Denizot and Lang, 1986). Briefly, 50 μl of the MTT-labeling reagent, at a final concentration of 0.5 mg/ml, was added to each well at the end of the dexamethasone/folic acid period and the plate was placed in a humidified incubator at 37 °C with 5% CO_2 and 95% air (v/v) for an additional 2-h period. Then, the insoluble formazan was dissolved with dimethylsulfoxide; colorimetric determination of MTT reduction was measured at 540 nm. Control cells treated with vehicle (F-12/EMEM) were taken as 100% viability.

Data analysis

Data are represented as means±SEM. Comparisons between experimental and control groups were performed by one-way ANOVA followed by the Duncan post hoc test. Statistical difference was accepted when P values were <0.05.

RESULTS

We first examined the effect of dexamethasone on cultured SH-SY5Y neuroblastoma cells. The results of Fig. 1 show the effect of the incubation of cells with F-12/EMEM (control) or dexamethasone (diluted in serum-free medium) at the concentrations of 0.05, 0.1, 0.5, and 1 mM for 48 h. The cellular viability was evaluated by the MTT reduction assay and was expressed as percentage of control cells which represents cells incubated for 48 h in culture medium (100% cellular viability). Dexamethasone induced significant cell death at the dose of 1 mM. It decreased around 40% of the cellular viability, when compared to cultures incubated under basal conditions.

Next, we examined the possible neuroprotective effects of folic acid on dexamethasone-induced cell death. Folic acid was preincubated (24 h before of dexamethasone) and co-incubated (during 48-h exposure to 1 mM dexamethasone), in the same protocol, with the cells at the concentrations 1, 10, 100, and 300 μM (diluted in serum-free medium) (Fig. 2). The results of Fig. 2A indicate that folic acid at the concentrations of 1–300 μM was not cytotoxic "per se". Fig. 2B shows that folic acid (10–300 μM) was able to protect these cells against dexamethasone-induced cell death with a maximum protection at 300 μM. As shown in Fig. 2E, the cells, in the presence of 300 μM folic acid before and during exposition to dexamethasone (1 mM), recovered their initial density and exhibited an absence of cell shrinkage, when compared with cells

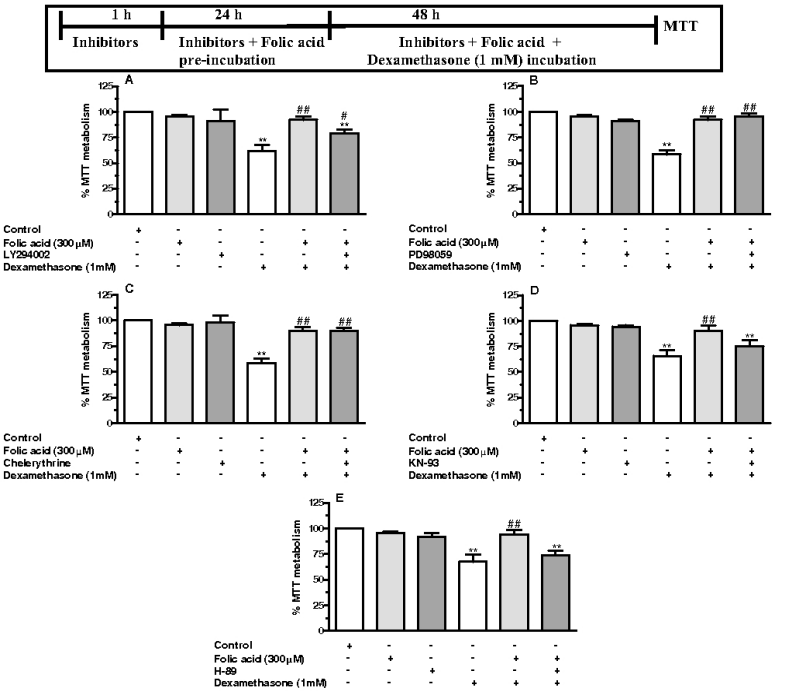

Fig. 3. Protein kinase inhibitors used to investigate the mechanism underlying the neuroprotective effect of folic acid. Protection afforded by folic acid was partially prevented by PI3K inhibitor (LY294002, 10 μM, panel A) and totally prevented by CaMKII inhibitor (KN-93, 1 μM, panel D) and PKA inhibitor (H-89, 2 μM, panel E), but not by MEK1/2 inhibitor (PD98059, 10 μM, panel B) or PKC inhibitor (chelerythrine, 0.1 μM, panel C). Data are represented as the mean±SEM of three to seven experimental determinations. ** $P<0.01$, when compared with control. # $P<0.05$ or ## $P<0.01$ is in comparison with 1 mM dexamethasone group.

treated with dexamethasone alone (Fig. 2D) and control (F-12/EMEM, Fig. 2C).

In order to analyze the signaling pathway that could participate in the neuroprotective mechanism of folic acid against dexamethasone-induced cell damage in SH-SY5Y cells, we performed experiments using LY294002, a PI3K inhibitor, PD98059, an MEK1/2 inhibitor, chelerythrine, a PKC inhibitor, KN-93, a CaMKII inhibitor, and H-89, a PKA inhibitor. Under these experimental conditions, as shown in Fig. 3A, the treatment with 10 μM LY294002 (PI3K/Akt inhibitor) partially blocked the neuroprotective effect of folic acid. Fig. 3B, C show that the MEK1/2 inhibitor (10 μM PD98059) and PKC inhibitor (0.1 μM chelerythrine), respectively, were not able to reverse the neuroprotective effect of folic acid. However, the treatment with 1 μM KN-93 (CaMKII inhibitor) and 2 μM H-89 (PKA inhibitor) reversed the neuroprotective effect of folic acid against dexamethasone-induced cell damage (Fig. 3D, E).

DISCUSSION

The results of this study show that 1 mM dexamethasone causes neurotoxicity in SH-SY5Y neuroblastoma cells and that 300 μM folic acid can protect them against this damage. Additionally, we investigated the signal transduction pathways regulating the neuroprotective response of folic acid in this toxicity model. Dexamethasone-induced cell death could be blocked by the protein kinase inhibitors

LY294002, KN-93, and H-89, but not by PD98059 or chelerythrine. Therefore, we showed that the protection afforded by folic acid was likely mediated through the activation of PI3K/Akt, CaMKII, and PKA signaling pathway rather than MEK1/2 and PKC pathways.

Physical and psychological stressors stimuli activate the hypothalamic-pituitary-adrenal (HPA) axis by increasing the production and release of corticotropin-releasing hormone (CRH) and arginine vasopressin from the paraventricular nucleus of the hypothalamus. CRH stimulates the pituitary to produce adrenocorticotropic hormone (ACTH), which enters the bloodstream and activates the adrenal glands to release glucocorticoids, including cortisol in humans and corticosterone in rodents. Glucocorticoids, in turn, exert inhibitory feedback effects mainly at the hypothalamus and pituitary glands to inhibit the synthesis and secretion of CRH and ACTH, respectively. The hippocampus also confers an inhibitory effect on the HPA axis (Kunugi et al., 2010). The sites in the brain where glucocorticoids act are determined by the distribution of glucocorticoid receptors. The hippocampus is a region with high levels of these receptors (Conrad, 2008; Joëls, 2011). Depending on the intensity or duration of the stress, as well as individual qualities (genetics, psychological state, etc.), the endocrine response to stress (which is supposed to be adaptive) becomes pathological because it involves hypersecretion of glucocorticoids by HPA axis overstimulation, triggering disorders such as Cushing's disease, posttraumatic stress disorder, bipolar disorder, and depression (Bremner et al., 2000; Conrad, 2008; Kunugi et al., 2010). Corroborating the relationship between the HPA axis overstimulation and depression, a study from Haynes et al. (2004) showed that chronic treatment with different antidepressants, such as tranylcypromine (a monoamine oxidase inhibitor), fluoxetine (a selective serotonin reuptake inhibitor), and desipramine (a tricyclic antidepressant) resulted in marked protection from dexamethasone-induced neuronal damage in the striatum and the hippocampus of rats.

In this study we used a model of neurotoxicity that mimics the hypersecretion of glucocorticoids by incubating SH-SY5Y cells with dexamethasone, a synthetic glucocorticoid (Mitchell et al., 1998; Haynes et al., 2001; Jacobs et al., 2006; Zhu et al., 2006; Tazik et al., 2009), taking into account that a glucocorticoid hypersecretion occurs in most of depressive patients (Wolkowitz et al., 2009). The hippocampal neuronal damage caused by glucocorticoids has been well documented *in vivo* and *in vitro* (Woolley et al., 1990; Virgin et al., 1991; Hassan et al., 1996; Mitchell et al., 1998; Ahlbom et al., 2000; Joëls, 2001; Lu et al., 2003; Crochemore et al., 2005; Zhu et al., 2006). As previously reported in the literature, our results show that dexamethasone induces neuronal damage at the concentration of 1 mM. Literature data indicate that several putative mechanisms may be implicated in this damage. One is that the inhibition of the PI3K/Akt signaling pathway enhances dexamethasone-induced cell death (Nuutinen et al., 2006). Another possibility is that glucocorticoid induces the increase of extracellular glutamate in the hippocampus (Sapolsky, 2000) which can be prevented by blocking NMDA receptors (Armanini et al., 1990). Moreover, excessive glucocorticoid reduces the expression and impairs brain-derived neurotrophic factor (BDNF) function, which damages the hippocampus and other brain areas (Kumamaru et al., 2008; Kunugi et al., 2010). It is interesting to mention that glucocorticoids exert antiproliferative effects in many cell types (Crochemore et al., 2002; Hong et al., 2011) by inhibition of cell cycle progression or induction of cell death (Crochemore et al., 2002). Our results indicated that dexamethasone induces morphological alteration in SH-SY5Y cells which is indicative of cell death rather than reduced proliferation, but additional studies are necessary for confirm these results.

Under the experimental conditions of this study, 24-h preincubation with folic acid, at increasing concentrations (10–300 μM) preceding the dexamethasone period (see protocol on top of Fig. 2), reduced neurotoxicity in a concentration-dependent manner (Fig. 2B). Maximum protection was achieved at the concentration of 300 μM of folic acid (29% protection; Fig. 2B, E). Fig. 2D shows that treatment with 1 mM dexamethasone for 48 h changed healthy birefringence cells (Fig. 2C) into cells without birefringence and with granular cytoplasm. The literature reports that folic acid deficiency induces neurotoxicity by multiple routes. It was reported that folic acid deprivation increased cytosolic Ca^{2+} and reactive oxygen species (ROS) and impaired mitochondrial function (Ho et al., 2003; Tjiattas et al., 2004). Folic acid plays a protective role against glutamate and NMDA-induced cytotoxicity in cultured mouse cerebellar granule neurons (Lin et al., 2004). Moreover, a previous study of Yu et al. (2009) showed that folic acid protects neurons from the damage caused by $A\beta_{25-35}$ peptide by maintaining mitochondrial function and DNA integrity and regulating apoptosis-related genes.

The neuroprotective actions of folic acid described in this study are relevant in the context of previous results: (a) the dexamethasone-induced cell death protocol mimics the hypersecretion of glucocorticoids *in vitro* (Joëls, 2001, 2008; Jacobs et al., 2006; Tazik et al., 2009); (b) chronic treatment with antidepressants resulted in protection from dexamethasone-induced neuronal damage in hippocampal and striatal neuronal populations of rats (Haynes et al., 2004); (c) preclinical studies of our group indicate that folic acid has antidepressant-like properties (Brocardo et al., 2008a,b, 2009); (d) folic acid was reported to have neuroprotective properties (Ho et al., 2003; Lin et al., 2004; Tjiattas et al., 2004; Yu et al., 2009).

It can be hypothesized that folic acid-induced cell survival was dependent on different signaling pathways, which are key elements of signal transduction involved on cell proliferation, differentiation, and stress response such as PI3K/Akt, PKA, MAPK/ERK, CaMKII, or PKC pathways (Cantley, 2002; Vivanco and Sawyers, 2002; Einat et al., 2003; Cañas et al., 2007; Gokce et al., 2009; Romero et al., 2010).

Our results indicate that exposure of SH-SY5Y cells with LY294002, a PI3K inhibitor, partially suppressed the

neuroprotective effects of folic acid in dexamethasone-exposed cells. A large body of genetic and pharmacological evidence indicates that the PI3K/Akt signaling pathway is critical for cell growth and survival (Hennessy et al., 2005). The PI3K/Akt pathway is particularly important for mediating neuronal survival under a wide variety of circumstances (Brunet et al., 2001; Shao et al., 2011) and has been found to be sufficient and, in some cases, necessary for the protection mediated by trophic factors such as insulin-like growth factor-1 (IGF-1) on neuronal cell death induced by corticosterone in hippocampal neurons (Nitta et al., 2004). Moreover, dexamethasone can decrease proliferation and increase apoptosis in chondrocyte cell cultures through inhibition of Akt phosphorylation and therefore inhibition of the PI3K/Akt signaling pathway (Chrysis et al., 2005). To reinforce our results, a previous study of Seto et al. (2010) showed that in an animal model of diabetes mellitus-associated hypertension, oral folic acid supplementation restored the blunted acetylcholine-induced aortic relaxation observed in mice, probably via enhancement of the activity of PI3K/Akt cascade. In line with this finding, our results indicate that LY294002 partially blocked the neuroprotective effect of folic acid against dexamethasone-induced cell injury in SH-SY5Y cells. This result reinforces that the PI3K/Akt signaling pathway is implicated in the neuroprotective effect of folic acid.

Additionally, our results show that PD98059 and chelerythrine were not able to block the protective action of folic acid against dexamethasone-induced damage. These results suggest that this signaling pathway does not participate in the neuroprotective effect of folic acid in this protocol.

Moreover, CaMKII is the most abundant protein kinase in the brain involved in neuronal plasticity (Popoli et al., 2000; Cammarota et al., 2002). Downstream calcium-dependent responses to BDNF involve the activation of calcium/calmodulin-dependent kinases (CaMKs) (Spencer et al., 2008). It involves downstream calcium-dependent mechanisms and the CaMK-dependent phosphorylation induces activation of cAMP response element-binding (CREB) transcription factor. Our results show that KN-93 suppressed the neuroprotective effect of folic acid, therefore this signaling pathway seems to be involved in the neuroprotective mechanism related to folic acid in this cell death protocol.

We also found that H-89 suppressed the neuroprotective effect of folic acid against dexamethasone-induced neurotoxicity. H-89 is an isoquinolinesulfonamide that acts as a competitive inhibitor against ATP binding to the catalytic subunit of PKA (Chijiwa et al., 1990). This enzyme is involved in several physiologic functions in the brain, including neurotransmitter synthesis and release, gene expression, synaptic plasticity, memory, cell growth and differentiation, and cell survival. The major mechanism of PKA-mediated function is through the phosphorylation of specific substrates, which include CREB (D'Sa and Duman, 2002; Gould and Manji, 2002; Blendy, 2006). The activation of CREB causes the expression of proteins such as BDNF, which has been implicated in the maintenance of neurons, cell survival, and neuronal plasticity (D'Sa and Duman, 2002; Blendy, 2006). In our study H-89 was able to reverse the dexamethasone-induced SH-SY5Y neurotoxicity, suggesting that the activation of this signaling pathway by folic acid is also likely implicated in its neuroprotective effect.

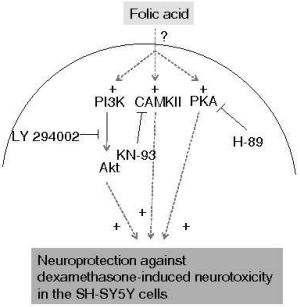

Fig. 4. Schematic diagram illustrating the putative intracellular survival pathways activated by folic acid to prevent dexamethasone-induced neurotoxicity in the SH-SY5Y cell line. Neuroprotection afforded by folic acid in this neurotoxicity protocol involves molecular targets such as PI3K/Akt, CaMKII, and PKA activation. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

As depicted in Fig. 4 this study presents clear evidence that folic acid can protect the human neuroblastoma SH-SY5Y cell line against dexamethasone-induced cell damage and that PI3K/Akt, CaMKII, and PKA are molecular targets implicated in the neuroprotective effect of folic acid.

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Contributors—Josiane Budni projected the study, performed the experiments, analyzed the data, and wrote the paper; Alejandro Romero performed the human neuroblastoma SH-SY5Y cells culture and reviewed the manuscript; Simone Molz performed the experiments and reviewed the manuscript; María Dolores Martín-de-Saavedra projected the study and reviewed the manuscript; Javier Egea, projected the study; Laura Del Barrio reviewed the manuscript; Carla Inês Tasca analyzed the data and reviewed the manuscript; Ana Lúcia Severo Rodrigues analyzed the data and reviewed the manuscript; Manuela García López provided financial support for the conduct of the research, analyzed the data and reviewed the manuscript.

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CAPÍTULO 5

Excitotoxicity induced by glutamate in the hippocampus slices can be prevented by folic acid through the GSK-3β and iNOS inhibition. Budni J, Molz S, Dal-Cim T, Martín-de-Saavedra MD; Egea J, Lopéz MG, Tasca CI, Rodrigues ALS.

Excitotoxicity induced by glutamate in the hippocampal slices can be prevented by folic acid through GSK-3β and iNOS inhibition

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Abstract

Folic acid (folate) is a vitamin of the B-complex group crucial for neurological function. Many roles for folic acid have been reported, including neuroprotective properties. On the other hand, the excitotoxicity and cell death induced by glutamate is involved in many disorders. The purpose of this study was to investigate the potential neuroprotective effect of folic acid on glutamate-induced cell death in rat hippocampal slices and the possible intracellular signaling pathway involved in such effect. The treatment of hippocampal slices with folic acid (100 µM) significantly reduced glutamate (1mM)-induced cell death measured by MTT and inhibited D-[^{3}H]aspartate release induced by glutamate. To investigate the putative intracellular signaling pathways implicated in the neuroprotective effect of folic acid we used a PI3K inhibitor, LY294002, which abolished the neuroprotective effects of folic acid against glutamate-induced cell death and D-[^{3}H]aspartate release. Moreover, hippocampal slices incubated with folic acid alone

for 30 minutes induced the phosphorylation of gycogen synthase kinase-3β (GSK-3β) at Ser9. Furthermore, folic acid in presence of glutamate insult in hippocampal slices maintained for an additional period of 6 h in fresh culture medium without glutamate and/or folic acid, induced phosphorylation of GSK-3β and β-catenin expression. In addition, glutamate treated hippocampal slices showed increased iNOS expression that was reversed by folic acid. In conclusion, the results of this study show that folic acid can protect against glutamate-induced excitotoxicity and its neuroprotective mechanism may be related to a signaling pathway that involves PI3K/GSK-3β/β-catenin and iNOS.

Key words: glutamate, folic acid, neuroprotection, PI3K, GSK3β, β-catenin, iNOS, hippocampal slices.

Abbreviations: Foli acid, FA; Glutamate, Glu; glycogen synthase kinase 3β, GSK3β; [2-(4-morpholinyl)-8-phenyl-4H-1benzo-pyran-4-one, LY 294002; inducible nitric oxide synthase, iNOS; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; NO, nitric oxide; phosphoinisitide-3 kinase, PI3K;

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Introduction

L-Glutamate is one of twenty essential amino acids and it is the most important excitatory neurotransmitter in the mammalian central nervous system (CNS) mediating excitatory synaptic responses (Mattson, 2008; Severino et al, 2011). This amino acid is important for neurogenesis, neurite outgrowth, synaptogenesis and neuron survival (Mattson, 2008). Hence, the glutamatergic system plays important roles in a wide range of neuronal physiological functions, including learning, memory, cognition, neuronal plasticity and neurotrophic actions (Popoli et al, 2011; Severino et al, 2011; Verkhratsky & Kirchhoff, 2007).

Glutamate exerts its action at the presynaptic and postsynaptic level through the stimulation of different glutamate receptors, expressed in virtually all cells of neural origin, and that can be classified as ionotropic receptors (including NMDA [N-methyl-D-aspartate], AMPA [α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid] and Kainate receptors) and G-protein-coupled metabotropic receptors (mGluR1 to mGluR8) (Verkhratsky & Kirchhoff, 2007). The ionotropic glutamate receptor activation allows an influx of cations into the postsynaptic cell, which is a fundamental response required to produce the depolarizing signal at numerous central synapses (Dingledine et al, 1999).

Excessive activation of glutamate receptors, a process called glutamate excitotoxicity, can result in neuronal dysfunction and death (Olney, 1969), consequence of a large inflow of Ca^{2+} and Na^{+} into neurons (Orrenius et al, 2003). Glutamate excitotoxicity is implicated in a variety of neuropathological conditions such as Huntington's disease, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, depression, epilepsy and schizophrenia (Dong et al, 2009; Sanacora et al, 2008). Although the mechanisms underlying glutamate excitotoxicity are complex, studies performed in hippocampal slices showed that this process may involve cytochrome c release, caspase-3 activation and DNA fragmentation, via p38MAPK signalling activation (Molz et al, 2008a). Glutamatergic excitotoxicity may be also related to the reversal activity of glutamate transporters, which causes an increase in extracellular glutamate concentration (Molz et al, 2008b).

Moreover, a previous study by Molz et al. (2011) showed that glutamate induces activation of inducible nitric oxide synthase (iNOS) in hippocampal slices. Indeed, in the diseased brain, mainly under inflammatory conditions, iNOS is induced in microglia and astrocytes, and once expressed produces nanomolar concentrations of NO which is 100-1000 times those produced by neuronal nitric oxide synthase

(nNOS) (Pannu & Singh, 2006; Saha & Pahan, 2006). The iNOS-induced production of NO from activated microglia culminates in neuron death (Brown, 2010). It is important to emphasize that neurons are capable of producing NO in small amounts through the catalytic action of the enzyme nNOS (Gonchar & Burkhalter, 1997; Vruwink et al, 2001), whereas glial cells (astrocytes and microglia) express all three NOS isoforms (nNOS, eNOS- endothelial form- and iNOS). The induction of iNOS is due to several stress stimuli, including glutamate excitotoxicity (Licinio et al, 1999; Lopez-Figueroa et al, 2000; Molz et al, 2011; Moro et al, 2004; Nomura, 1998; Olivenza et al, 2000).

Taking into account that the glutamate-induced excitotoxic damage involves several mechanisms and it is a common pathological event implicated in different diseases, it is important to research new neuroprotective strategies for treating glutamatergic excitotoxic.

Folic acid is a vitamin of the B-complex group crucial for many reactions important to nervous system function (Mattson & Shea, 2003), since it is involved in the metabolism and functioning of many substances, such as purines and pyrimidines, DNA, RNA, aminoacids, phosphorous compounds, vitamin B12, methionine, S-adenosyl-methionine, dopamine, epinephrine, norepinephrine, and serotonin (Matson and Shea, 2003; Coppen and Bolander-Gouaille, 2005; Kronenberg et al., 2009; Lucock, 2011). Moreover, it is important to mention that this vitamin exerts neuroprotective actions by preventing the damage caused by several toxic stimuli (Budni et al, 2011; Tagliari et al, 2006; Yu et al, 2009) including glutamate and NMDA (Lin et al, 2004).

Considering that recent studies have shown that: a) folic acid exerts antidepressant and neuroprotective effects through the activation of phosphoinositide-3 kinase (PI3K)/Akt/ synthase kinase 3β (GSK-3β) pathway (Budni et al, 2012b; Budni et al, 2011; Seto et al, 2010) and b) a promising strategy to combat glutamate excitotoxicity-related brain disorders could be attained through agents that activate this pathway (Chuang et al, 2011), the aim this study was to investigate the neuroprotective role of folic acid against glutamate-induced hippocampal slice injury and the role of the PI3K/GSK3/β-catenin pathway and iNOS in the possible folic acid's neuroprotective effect.

Materials and methods

Animals

Male Wistar rats (23-25 days of postnatal age) maintained on a 12-hour light-12 hour dark schedule at 22 ± 1º C, with food and water

ad libitum, were obtained from our local breeding colony. Experiments followed the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) and were approved by the local Ethical Committee of Animal Research (CEUA/UFSC).

Preparation and Incubation of Hippocampal Slices

Rats were killed by decapitation and the hippocampi were rapidly removed and placed in ice-cold Krebs-Ringer bicarbonate buffer (KRB) of the following composition: 122 mM NaCl, 3 mM KCl, 1.2 mM MgSO4, 1.3 mM CaCl2, 0.4 mM KH2PO4, 25 mM NaHCO3 and 10 mM D-glucose. The buffer was bubbled with 95% O2-5% CO2 up to pH 7.4. Slices (0.4 mm thick) were rapidly prepared using a McIlwain Tissue Chopper, separated in KRB at 4^{o} C and allowed to recover for 30 minutes in KRB at 37^{o}C to afford stabilization (Oliveira et al, 2002).

Hippocampal slice treatments

After slice stabilization, the slices were incubated with glutamate (Sigma) (1 mM) for 1 h in KRB buffer. After this period, the medium was withdrawn and replaced by a nutritive culture medium composed of 50 % of KRB, 50 % of Dulbecco's modified Eagle's medium (DMEM, Gibco), 20 mM of HEPES and 100 μg/ml of gentamicine. Slices were maintained for additional 6 h in a humidified atmosphere 95% air and 5% CO_2 at 37^{o}C to evaluate cell viability (Molz et al, 2008a).

When present, folic acid was added to the incubation medium 30 min before glutamate and maintained during the 1 h of incubation with glutamate. LY294002 (30 μM) was added to the incubation medium 15 minutes before folic acid and maintained during the folic acid preincubation period.

Evaluation of cell viability

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

Immunobloting

In order to evaluate GSK3β (Ser 9) phosphorylation (p-GSK-3β), slices were incubated for 60 min under control conditions, or treated with folic acid (100 μM) for 30 or 60 min. In another experiment, p-GSK-3β, β-catenin and iNOS levels were evaluated 6 h after glutamate exposure. Then, slices were homogenized in ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na3VO4). Proteins (30 μg) from these cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, Chalfont St. Giles, UK). Membranes were incubated with anti-p-GSK-3β (1:1,000); anti-total GSK-3β (1:1,000); anti-β-actin (1:100,000); anti-β-catenin (1:10,000); anti-iNOS (1:10,000) (Sigma). Appropriate peroxidase-conjugated secondary antibodies (1:10,000) were used to detect proteins by enhanced chemiluminescence. Different band intensities corresponding to immunoblot detection of protein samples were quantified using the Scion Image program (Scion Corporation, Frederick, MD, USA).

Glutamate release

Following preincubation period to allow slice recovery (30 min), hippocampal slices were incubated in Hank's balanced salt solution (HBSS), composition in mM: 1.29 $CaCl_2$, 136.9 NaCl, 5.36 KCl, 0.65 $MgSO_4$, 0.27 Na_2HPO_4, 1.1 KH_2PO_4, and 5 Hepes. When present, folic acid was incubated for 30 min and maintained during glutamate exposure. LY204002 (30 μM) was preincubated for 15 min before folic acid. Glutamate (1 mM) was incubated for 15 min and glutamate uptake was assessed by adding 0.33 μCi/ml D-[3H]aspartate with 100 μM unlabeled aspartate for 7 min and stopped by three ice-cold washes with HBSS. D-[3H]aspartate instead of L-[3H]glutamate was used in order to avoid glutamate metabolization in intracellular compartments, although similar results were obtained by using D-[^{3}H]aspartate or L-[3H]glutamate. The slices were then further incubated for 15 min in HBSS and the supernatant was collected in order to measure the amount of released D-[^{3}H]aspartate. Slices were disrupted by overnight incubation with 0.1% NaOH/0.01% SDS and aliquots of lysates were taken for determination of intracellular D-[^{3}H]aspartate content (Molz et al, 2008b). Intracellular and extracellular D-[^{3}H]aspartate content were determined through scintillation counting, calculated as nmol of

aspartate and the amount of released aspartate was expressed as percentage of total D-[^{3}H]aspartate.

Statistical analysis

Comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by Duncan's test if necessary, with $p<0.05$ considered to be statistically significant.

Results

Folic acid protects against glutamate-induced cell death by activation of PI3K pathway

Exposure of hippocampal slices to 1 mM glutamate resulted in a significant decrease in cell viability measured as reduction of MTT. Slices were treated with folic acid (1, 10 or 100µM). Folic acid (100 µM) significantly prevented the reduction in cell viability induced by glutamate, but this neuroprotective effect was not observed at lower doses of this vitamin (1 or 10 µM) (Fig. 1A).

In order to evaluate the involvement of PI3K in the neuroprotective effect of folic acid, a PI3K inhibitor, LY294002 was used. This inhibitor prevented the neuroprotective effect of folic acid against glutamate excitotoxicity. It is important to mention that folic acid (100 µM) and LY294002 (30 µM) alone did not cause reduction in cell viability as compared to control group (Fig. 1B).

Folic acid prevents glutamate release through PI3K activation

The presence of glutamate (1 mM) induced excessive D-[3H]aspartate release (glutamate release) in hippocampal slices. Folic acid (100 µM) prevented glutamate-induced D-[3H]aspartate release in the hippocampal slices. The presence of PI3K inhibitor, LY294002, was able to reverse the neuroprotective effect of folic acid on glutamate release (Fig. 2).

Folic acid protects against glutamate-induced excitotoxicity through GSK3β/β-catenin pathway modulation

Considering that: i) folic acid was able to protect against glutamate-induced cell death; ii) the effects of this vitamin were reversed by a PI3K inhibitor, which indicate involvement of PI3K pathway in the mechanism of neuroprotection afforded by folic acid, we decided to investigate downstream targets of PI3K, such as GSK3β and β-catenin.

For these experiments, hippocampal slices were incubated with 100 µM of folic acid for 30 and 60 minutes and cell lysates were then analyzed by immunoblotting. Fig. 3A shows a representative immunoblotting and quantitative analysis of GSK-3β phosphorilated at Ser9 (p-GSK-3β) and total-GSK-3β (t-GSK-3β). Our results show that incubation of hippocampal slices with folic acid increased p-GSK-3β after 30 minutes of exposition. The increase on p-GSK-3β induced by folic acid was not sustained. After 60 min incubation with folic acid a reduction on GSK-3β phosphorylation at control group level was observed (Fig. 3A).

Subsequently, we evaluated the GSK-3β phosphorylation in all experimental groups (Control; folic acid alone; LY294002 alone; glutamate alone; folic acid /glutamate; LY294002/folic acid/glutamate) 6 h after glutamate exposure. Our results indicated that folic acid, LY294002 or glutamate alone did not alter the GSK-3β phosphorylation. However, when hippocampal slices were subject to glutamate toxicity in the presence of folic acid an increase on GSK-3β phosphorylation was observed, an effect that was reversed by the PI3K inhibitor LY294002 (Fig. 3B).

To confirm that GSK-3β phosphorylation is implicated in the neuroprotective effect of folic acid against excitotoxicity induced by glutamate, the expression of β-catenin in all experimental groups was analyzed by immunoblotting (Control; folic acid alone; LY294002 alone; glutamate alone; folic acid/glutamate; LY294002/folic acid/glutamate) 6 h after glutamate exposure. We observed an increased β-catenin expression in the folic acid/glutamate group. LY294002 was able to reverse this alteration. LY294002, folic acid or glutamate alone did not alter β-catenin expression (Fig. 3C).

Folic acid prevents glutamate-induced iNOS expression

In order to investigate the role of iNOS on the neuroprotective effect of folic acid against the glutamate-induced excitotoxicity, we also evaluated the expression of iNOS in all experimental groups (Control; folic acid alone; LY294002 alone; glutamate alone; folic acid /glutamate; LY294002/folic acid/glutamate) after 6 h of incubation. We have found increased levels of iNOS after exposure of hippocampal slices to 1 mM glutamate. This effect was completely prevented when slices were preincubated with 100 µM of folic acid. The neuroprotective effect of folic acid was reversed by LY294002 (Fig. 4).

Discussion

The rat hippocampal slice preparation exposed to glutamate *in vitro* has been widely used to mimic a glutamatergic excitotoxicity condition (Molz et al, 2011; Molz et al, 2008a; Molz et al, 2008b). The present study is the first to demonstrate that folic acid can protect rat brain slices against glutamate toxicity. This study provides evidence that the folic acid neuroprotective effect is mediated by PI3K/GSK-3β/β-catenin pathway and inhibition of iNOS.

There are many advantages to use *in vitro* experiments such as maintenance of anatomic relation and natural synaptic connectivity and elimination of *in vivo* variables (blood flow, temperature and ionic environment). Therefore, an increasing number of brain slice models have been used to study brain function and neuroprotection (Lipton & Raley-Susman, 1999; Molz et al, 2011; Molz et al, 2008a; Molz et al, 2008b; Wang et al, 2007; Wang et al, 2006). In this study we used an *in vitro* protocol of cell death induced by glutamate in rat hippocampal slices. Glutamate receptor-mediated excitotoxicity is closely associated with a number of pathological conditions and neurological disorders (Dong et al, 2009; Lau & Tymianski, 2010; Sanacora et al, 2008). In certain pathological conditions, for instance brain ischemia or injury, extracellular glutamate is rapidly elevated, which results in excessive activation of glutamate receptors and consequent neuronal injury (Lau & Tymianski, 2010). Excitotoxicity may be modified by the efficiency of glutamate clearance from synaptic clefts by glutamate transporters, coupled to Na+/K+-ATPase activity, present in astrocytes and, to a lesser extent, in neurons (Anderson & Swanson, 2000; Beart & O'Shea, 2007; Danbolt, 2001; Markowitz et al, 2007; Sheldon & Robinson, 2007). Therefore, these transporters remove the excessive glutamate from brain extracellular space, thus preventing excitotoxicity (Choi et al, 1987; Danbolt, 2001) and modulating synaptic transmission(Huang & Bergles, 2004). Nevertheless, damage of transporters induced by redox modulation of reactive cysteine amino acid residues or depletion of ATP can cause reversal of glutamate transport and accumulation of this amino acid in the extracellular space (Camacho & Massieu, 2006; Rossi et al, 2000; Struzynska, 2009). A study by Molz et al. (2008b) showed that glutamate-induced slice damage might occur via reversal of plasma membrane glutamate transporters, since DL-TBOA, a glutamate transport inhibitor, reversed the loss of cell viability induced by glutamate and glutamate-induced L-[^{3}H]glutamate release.

The results of the present work demonstrate that the treatment of hippocampal slices with folic acid (100 μM) protected these cells

against glutamate-induced damage. Moreover, our results also indicated that glutamate-induced D-[3H]aspartate release from hippocampal slices was reversed by folic acid treatment. These findings indicate that folic acid can prevent the loss of glutamate-induced cell viability and inhibit synaptic glutamate release. Therefore, these results suggest that folic acid reduces hyperexcitability by inhibiting excessive glutamate release, acting as an endogenous neuroprotective agent, preventing excitotoxicity. Indeed, a study by Lin et al. (2004) demonstrated that folic acid was able to protect murine cerebellar granule cells against glutamate/NMDA-induced toxicity. Moreover, *in vivo* studies showed that the antidepressant-like effect of folic acid in the forced swimming test may involve the inhibition of NMDA receptors, implicating the glutamatergic system in its effect (Brocardo et al, 2008a). It is important to be mentioned that there are few *in vitro* studies dealing with the ability of folic acid to prevent glutamate-induced injury. The present work clearly indicates the ability of folic acid to modulate the glutamatergic system reducing glutamate excicitotoxicity in the hippocampus, a finding with therapeutic applications, considering the involvement of excitotoxicity in a large array of SNC diseases (Dong et al, 2009; Lau & Tymianski, 2010; Sanacora et al, 2008).

In the present study we show that the preincubation with PI3K enzyme inhibitor, LY294002, significantly abolished the protective effect of folic acid against glutamate-induced cell viability reduction. Additionally, the ability of folic acid to reduce glutamate release also was completely inhibited by LY294002. This evidence suggests that folic acid can protect against glutamate excitotoxicity (cell death and glutamate release) by activating PI3K-mediated signalling pathway.

The PI3K is a lipid kinase which targets the Akt, a protein threonine/kinase. PI3K is an enzyme that participates in many physiological functions in brain and is utilized by neurotrophins to mediate neuronal plasticity, cell survival, cell proliferation, cell migration, vesicle trafficking and inhibition of apoptosis for several neuronal subtypes (Beaulieu, 2012; Beaulieu et al, 2009; Katso et al, 2001; Marone et al, 2008; Mellor et al, 2012).

To reinforce the results found in this study, literature data have indicated the association between folic acid and PI3K activation. Seto et al. (2010) showed that in an animal model of diabetes mellitus-associated hypertension, oral folic acid supplementation restored the blunted acetylcholine-induced aortic relaxation observed in mice, probably via enhancement of the activity of PI3K/Akt cascade. Moreover, a previous study from our group showed clear evidence that

folic acid can protect the human neuroblastoma SH-SY5Y cell line against dexamethasone-induced cell damage, at least in part, by causing PI3K activation. This conclusion derives from the fact that LY294002 reversed the folic acid-induced neuroprotection against dexamethasone effect in this cell line (Budni et al, 2011). A further *in vivo* study demonstrated that the antidepressant-like effect of folic acid might be mediated by PI3K activation (Budni et al, 2012b). Therefore, the results of present study corroborate literature data regarding the implication of PI3K in the effects of folic acid.

In order to investigate the downstream mechanisms to PI3K involved in the folic acid neuroprotective effect against glutamate excitotoxicity (cell death and glutamate release), we evaluated the participation of GSK-3β in the effect of this vitamin. GSK-3 is a serine/threonine protein kinase highly expressed in the brain (Perez-Costas et al, 2010; Woodgett, 1990; Yao et al, 2002), both in neurons and glia (Ferrer et al, 2002). GSK-3 displays important roles in processes such as metabolism, cellular architecture, gene expression, neurobiological processes, synaptogenesis, neurodevelopment, axonal growth and polarity, immune response, circadian rhythms, and neuronal/cellular survival (Kaidanovich-Beilin & Woodgett, 2011; Sutherland, 2011). There are two isoforms of GSK-3 (GSK-3α and GSK-3β) and its isoforms are among the most extensively studied substrates of Akt (Beaulieu, 2012; Beaulieu et al, 2009). Akt regulates negatively GSK-3 through phosphorylation of serine residues on their amino-terminal domain (Ser21 for GSK-3α and Ser9 for GSK-3β) (Beaulieu, 2012; Kaidanovich-Beilin & Woodgett, 2011).

Here we observed that preincubation of hippocampal slices with folic acid (30 minutes) was sufficient to increase GSK-3β phosphorylation at Ser9. However, after 60 minutes this effect was not observed (GSK-3β phosphorylation at Ser9 returned to basal levels). To reinforce this hypothesis, we analyzed the GSK-3β phosphorylation at Ser9 after 6 hours of hippocampal slices incubation in fresh culture medium without glutamate and/or folic acid. Only the group incubated with folic acid plus glutamate presented an increased GSK-3β phosphorylation at Ser 9, which is an indicative of the inhibition of this enzyme. Corroborating our data, a recent *in vivo* study demonstrated that the antidepressant-like effect of folic acid might involve GSK-3β inhibition (Budni et al, 2012b), suggesting that GSK-3β can be an *in vivo* and *in vitro* signaling target involved in the effect of folic acid.

To confirm the participation of GSK-3β inhibition in the neuroprotective effect of folic acid against glutamate damage, we analysed the β-catenin expression after 6 hours of incubation. β-catenin is a cytoplasmic protein downstream mediator of the Wnt signaling pathway, which regulates the expression of a number of genes essential for cell proliferation and differentiation (Bauer & Willert, 2012; Takahashi-Yanaga & Sasaguri, 2007). In the cytoplasm, the β-catenin level is kept low through continuous ubiquitin-proteasome mediated degradation regulated by adenomtous polyposis coli (APC), casein kinase 1α (CK1α) and GSK-3β (Takahashi-Yanaga & Sasaguri, 2007). The activation of GSK-3β induces β-catenin degradation through phosphorylation of this protein. In contrast, the inhibition of GSK-3β culminates in dephosphorylation and stabilization of β-catenin, resulting in accumulation of β-catenin, which translocates to the nucleus to induce transcription in cooperation with transcription factors, inducing antiapoptotic effects and stimulating axonogenesis (Coyle & Duman, 2003). The results showed that only the group incubated with folic acid plus glutamate presented an increased β-catenin expression, similar to the results of GSK-3β phosphorylation at Ser 9. Therefore, the results of the present study suggest that folic acid may promote cell survival against glutamate damage through β-catenin accumulation, perhaps a consequence of the inhibition of GSK-3β. Hence, further studies involving folic acid and GSK-3β/β-catenin pathway are welcome.

Moreover, our results also demonstrate that in the presence of LY294002, the increase on GSK-3β phosphorylation and β-catenin expression in hippocampal slices incubated with folic acid plus glutamate was abolished.

Altogether, the results indicate that the neuroprotective effect of folic acid might involve Akt activation, since PI3K, GSK-β and β-catenin are shown to be involved in the effect of folic acid. Thus, Akt may be a link between the PI3K and GSK-β/β-catenin in the neuroprotective effect of folic acid observed in this study. This protein, also termed protein kinase B (PKB) is a downstream target of PI3K. Activated Akt, in turn, inhibits GSK-3β and consequently promotes β-catenin accumulation which induces neuronal survival (Beaulieu, 2012; Beaulieu et al, 2009; Sutherland, 2011; Toledo et al, 2008). Therefore, folic acid might act direct or indirectly through PI3K/Akt/ GSK-3β/β-catenin pathway modulation.

It is recognized that glutamate plays an important role in the neuroinflammation, since this excitatory amino acid can induce the

release of proinflamatory cytokines (interleukine 1β, IL-1β, and tumor necrosis factor, TNF-α), which can trigger iNOS expression (Cárdenas et al, 2000; Moro et al, 2004). A study performed by Molz et al. (2011) showed that glutamate induces increase on iNOS expression in rat hippocampal slices. Similarly, our results indicate that glutamate provokes iNOS induction in hippocampal slices of rats. It is well known that iNOS is induced mainly in microglia and astrocytes, causing neuronal death (Bal-Price & Brown, 2001; Brown, 2010; Brown & Bal-Price, 2003). There are several mechanisms by which activated glia kill neurons. The primary mechanism is the increased iNOS expression by glia, which produces NO. In turn NO (i) causes calcium mobilization from the endoplasmic reticulum, (ii) inhibits mitochondrial respiration in neurons and consequently depolarizes the neuron, causing release of glutamate. Moreover the activated microglia may also release glutamate, that contributes to the activation of NMDA receptor and production of reactive oxygen species, which react with superoxide to produce peroxynitrite, that in turn, induces neuronal death (Brown, 2010).

In this study we observed that folic acid was capable of preventing glutamate-induced iNOS expression. Indeed, literature data have shown that folic acid induces neuroprotection under different toxic stimuli through iNOS inhibition and consequently reducing NO level (Figueiredo et al, 2011; Majumdar et al, 2010). Moreover, a study showed that folic acid treatment significantly attenuated the plasma homocysteine level, suppressed the activation of microglia and astrocytes, and inhibited the expression of iNOS and TNF-α in spinal cord of mice deficient of superoxide diemutase 1 (SOD1) with high level of homocysteine, an animal model of amyotrophic lateral sclerosis (Zhang et al, 2008). Additionally, a recent study demonstrated that folic acid inhibited lipopolysaccharide (LPS)-induced production of NO, TNF-α and IL-1β and decreased iNOS, TNF-α and IL-1β mRNAs in RAW264.7 cells (Feng et al, 2011).

Finally, we also found that LY294002 was able to reverse the protective effect of folic acid against glutamate-induced iNOS expression in hippocampal slices. Similarly to the results of the present study, a PI3K inhibitor increased iNOS expression in response to LPS or cytokines in astrocytes or C6 glioma cells, indicating that the activity of PI3K pathway can modulate iNOS expression (Pahan et al, 1999). Moreover, Molz et al. (2011) showed that the neuroprotective effect of guanosine, a guanine nucleoside, in a glutamate-induced protocol of cell death, was also abolished by LY294002, another evidence linking PI3K pathway to iNOS expression.

Taken together, these results demonstrate that folic acid can protect hippocampal slices against injury induced by glutamate. Its neuroprotective effect is possibly mediated through of PI3K/GSK-3β/β-catenin pathway modulation and inhibition of iNOS in the hippocampus. However, additional studies should be conducted to provide a deeper understanding of the mechanisms underlying the neuroprotection elicited by folic acid against glutamate-induced excitotoxic.

Legends

Figure 1. Cell viability in hippocampal slices subjected to glutamate in the presence of folic acid**. (A)** Hippocampal slices were incubated for 1 h with 1mM glutamate (Glu). When present, folic acid (FA-1, 10 and 100µM) was preincubated for 30 min. After this period, incubation medium was withdrawn and replaced for fresh culture medium without glutamate and maintained for additional 6 h. **(B)** When present, LY294002 (30µM) was added to incubation medium 15 min before FA and maintained during the FA preincubation period. Control group was considered as 100 % and represents cell viability of slices incubated only in culture medium. MTT (0.5 mg/ml) was incubated for 20 min at 37º C and cell viability was accessed at 550 nm. The values represent means ± standard error of at least 4-6 experiments carried out in triplicates. *p<0.05 or **p<0.01, indicates means significantly different from control group (100 %) and # p<0.05 or ## p< 0.01, indicate mean different from Glu.

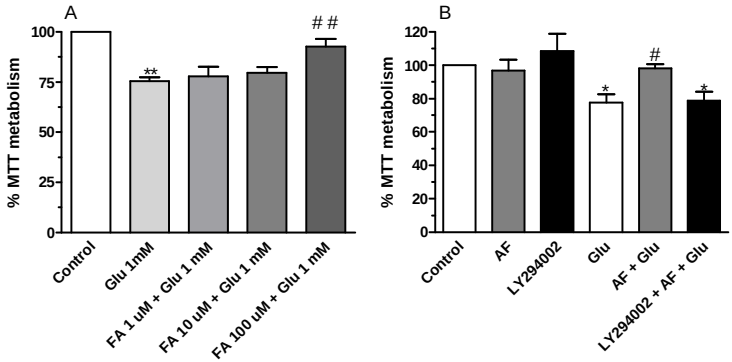


Figure 2. Glutamate release from hippocampal slices challenged with glutamate in the presence or not of folic acid. Hippocampal slices were incubated for 15 min with 1 mM glutamate (Glu) in the presence or absence of 100 µM of folic acid (FA). When present, FA was preincubated for 30 min before the addition of glutamate. LY294002 (30µM) was added to incubation medium 15 min before FA and maintained during the FA preincubation period. Control group was considered as 100 % and represents glutamate released from slices incubated only in HBSS. Glutamate release was assessed as described in Experimental procedures. Results were expressed as percentage of total D-[^{3}H] aspartate and represent mean ± standard error of four experiments carried out in triplicates. *P<0.05, represents mean significantly different from control groups; #P<0.05, represents mean significantly different from Glu group.

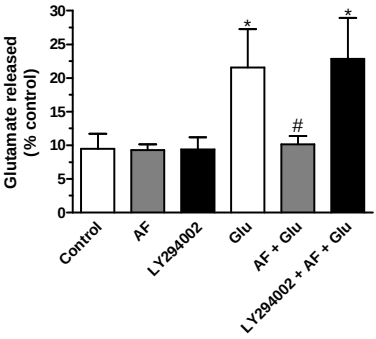


Figure 3. Immunodetection of GSK-3β or β-catenin in hippocampal slices submitted or not to glutamate in the presence of folic acid. **(A)** Representative western blot of phosphorylated GSK-3β at Ser9 (p-GSK-3β) related to total GSK-3β (t-GSK-3β) e quantitative analysis of p-GSK-3β related to t- GSK-3β in optical density of hippocampal slices exposed to folic acid (FA) for 30 and 60 min. **(B)** Representative western blot of p-GSK-3β related to t-GSK-3β e quantitative analysis of p-GSK-3β related to t- GSK-3β in optical density of hippocampal slices untreated (Control) or treated with glutamate (Glu-1 mM) incubated for 1 h in culture medium and maintained for an additional 6 h in fresh culture medium without glutamate. When present, FA (100 µM) was preincubated for 30 min. LY294002 (30µM) was added to incubation medium 15 min before FA and maintained during the FA preincubation period. **(C)** Representative western blot of β-catenin expression related to β-actine e quantitative analysis of β-catenin expression related to β-actine. Whole cells lysates were subjected to Western blot analysis to p-GSK-3β or β-catenin detection as described in Methods. The values represent means ± standard error of 3-6 independent experiments. * $p < 0.05$ or ** $p<0.001$, indicates means significantly different from control group;. ## $p<0.01$, represents mean significantly different from Glu group.

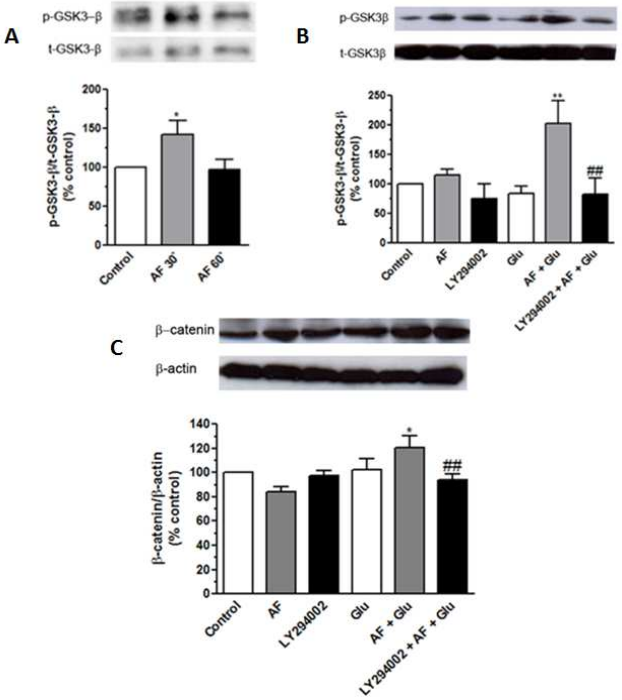
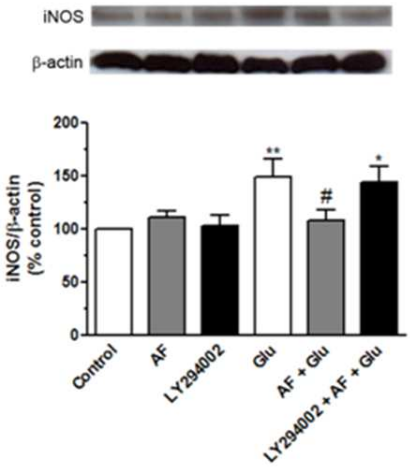


Figure 4. Immunodetection of iNOs in hippocampal slices submitted submitted or not to glutamate in the presence of folic acid. Representative western blot of iNOS in hippocampal slices submitted to Glu or FA/Glu e quantitative analysis by optical density of iNOS expression related to β-actine. Untreated (Control) or 1 mM glutamate (Glu)-treated hippocampal slices were incubated for 1 h in culture medium. Slices were maintained for an additional 6 h in fresh culture medium without glutamate. When present, folic acid (FA 100 µM) was preincubated for 30 min. LY294002 (30µM) was added to incubation medium 15 min before FA and maintained during the FA preincubation period. Whole cells lysates were subjected to Western blot analysis to iNOS detection. The control values were considered as 100 % (represents iNOS expression from slices incubated only in HBSS) and other treatments were expressed in relation to this value. The values represent means ± standard error of 6-5 independent experiments.* $p < 0.05$ or ** $p<0.001$, indicates means significantly different from control group;. # $p<0.05$, represents mean significantly different from Glu group.



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CAPÍTULO 6

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Chapter

The Role of Folic Acid in Psychiatric Disorders

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Abstract

Folic acid is one of 13 essential vitamins that can be obtained from dietary sources or supplements. Despite a relative abundance of folic acid in food, its deficiency is frequent and can arise as a consequence of poor diet, chronic illness, drugs, malabsorption, and an increased demand. Noteworthy, folic acid is essential for the functioning of the nervous system, since it plays an important role in neuroplasticity and in the maintenance of neuronal integrity. Several studies have indicated that folic acid plays a role in the pathophysiology and/or treatment of neuropsychiatric disorders such as depression, bipolar disorder, and schizophrenia. Individuals with low blood levels of folic acid seem more prone to the development of these neuropsychiatric disorders. Moreover, this vitamin deficiency has been correlated with a reduced effectiveness or a poor response to medication such as antidepressants. Although several preclinical and clinical studies have indicated a link between folic acid deficiency and neuropsychiatric disorders, the mechanisms underlying the beneficial effects of folic acid against these diseases have not been fully elucidated. This chapter presents an overview of the preclinical and clinical studies that have pointed to the link between folic acid and psychiatric disorders highlighting the evidence for the use of folic acid supplementation as a potential therapeutic strategy for the treatment of these diseases.

List of Abbreviations

CSF	cerebrospinal fluid

cGMP	cyclic guonosine monophosphate
DHF	dihydrofolate
FST	forced swimming test
GSK-3β	glycogen synthase kinase-3
HRS	Hamilton Depression Rating Scale
5-HT1A receptor	5-hydroxytryptamine 1A receptor
5-HT2A/2C receptors	5-hydroxytryptamine 2A/2C receptors
MTHF	5-methyl-THF
MTHFR	methylenetetrahydrofolate reductase
NMDA	N-Methyl-D-aspartic acid
NO	nitric oxide
PPARγ	peroxisome proliferator-activated receptor-γ
RBC	red blood cell
THF	tetrahydrofolate
TST	tail suspension test
TNF-α	tumor necrosis factor alpha

Introduction

Depression, bipolar disorder and schizophrenia are neuropsychiatric conditions that contribute the most disability-adjusted life-years. It is estimated that around 14% of the global burden of disease has been attributed to these neuropsychiatric disorders. The incidence of these psychiatric disorders is estimated to be about 16% for major depression, 1% for bipolar disorder, and 2.6% for schizophrenia (Kessler et al., 2005; Prince et al., 2007). Therefore, it is of paramount importance to develop and test new therapeutic agents for the treatment of these disorders.

Folic acid is involved in the metabolism and functioning of many substances that are essential to nervous system function. These include purines and pyrimidines, DNA, RNA, aminoacids, phosphorous compounds, vitamin B12, methionine, S-adenosyl-methionine, dopamine, epinephrine, norepinephrine, and serotonin (Matson and Shea, 2003; Coppen and Bolander-Gouaille, 2005; Kronenberg et al., 2009; Lucock, 2011). Abnormalities in the metabolism of folic acid may affect nervous system functioning by altering the metabolism of these compounds. This notion is supported by the high incidence of folic acid deficiency associated with multiple psychiatric conditions, including depression, bipolar disorder and schizophrenia (Coppen and Bolander-Gouaille, 2005; Stahl, 2007; Miller, 2008; Krebs et al., 2009).

In this chapter, we provide an overview of the literature supporting a relationship between folic acid and depression, bipolar disorder and schizophrenia. We then discuss the potential implications of these findings for the prevention and treatment of these neuropsychiatric disorders.

Folic Acid and Depression

Depression is one of the most common psychiatric conditions seen in the general medical setting, affecting millions of individuals in the world. The efficacy of the current pharmacotherapy has not improved since the 1950s, with only 30–40% of the patients reaching complete remission of symptoms. Since complete remission followed by sustained recovery is the best therapeutic strategy (Lee et al., 2010), new therapeutic agents are currently been studied for the treatment of depression, including folic acid.

Folic acid (folate) plays an important role for the functioning of the nervous system (Coppen and Bolander-Gouaille, 2005; Reynolds, 2006; Stahl, 2007). The metabolism of folic acid in the cell is initiated by dihydrofolate reductase in a two-step reaction; in the first step, folic acid is converted into dihydrofolate (DHF); whereas in the second step dihydrofolate is further reduced to tetrahydrofolate (THF). THF can then be converted into additional physiological folates including 5-methyl-THF (MTHF), the form that is normally found in the circulation and in tissues. MTHF is also replenished by the conversion of folinic acid (5-formyltetrahydrofolate), an active metabolite of folic acid. Because de novo folic acid synthesis does not occur in the central nervous system, the maintenance of appropriate levels of folic acid and its metabolites in the brain depends on the adequate transport of this vitamin across the blood-brain barrier (Ramaekers and Blau, 2004). MTHF acts as a critical co-factor for the synthesis of the three monoamines (dopamine, norepinephrine, and serotonin) (Stahl, 2007). Moreover, folic acid is required to re-methylate homocysteine to methionine. Homocysteine is a non-protein-forming sulfur amino acid that in high concentrations can be neurotoxic (Lipton et al., 1997; Kruman et al., 2000).

A large body of clinical evidence has supported a link between folic acid deficiency and depression (Lazarou and Kapsou, 2010; Nahas and Sheikh, 2011). The main findings from these studies are summarized in Table 1and discussed below.

The relationship between folic acid and depression was first described in a study from Herbert et al. (1962) that found mental

changes in a previously healthy man who was placed on a folic acid deficient diet. In support of this hypothesis, several studies have indicated that individuals showing psychiatric symptoms, such as depression and impaired cognitive functioning, also present low levels of folic acid in the plasma, serum, red blood cells (RBC), or cerebrospinal fluid (CSF) (Carney, 1967; Reynolds et al., 1970; Carney and Sheffield, 1978; Ghadirian et al., 1980; Abou-Saleh and Coppen, 1989; Bottiglieri et al., 2000; Lindeman et al., 2000). Importantly, patients with folic acid deficiency show a more severe psychiatric symptomatology than those reported in individuals with normal levels of this vitamin (Abou-Saleh and Coppen, 1989). Consistent with this notion, a study by Levitt and Joffe (1989) found that the duration of depressive episode was significantly correlated with low folic acid levels. Also, Bottiglieri et al. (2000) showed that around one third of severely depressed patients presented folic acid levels in RBC that are below the normal value (<150 μg/l). A different study indicated that patients with low dietary folic acid intake had a 67% increase in the risk of developing severe depressive symptoms when compared to patients with high dietary folic acid intake. Furthermore, this study also showed that in a Finish population of depressive male patients a lower consumption of folic acid was associated with the severity of their symptoms (Tolmunen et al., 2003). A recent study performed in Japanese adults (113 men and 79 women) with depressive symptoms, showed that low levels of folic acid in the serum were associated with an increased prevalence of depressive symptoms in men (Nanri et al., 2010). In contrast, some studies have reported no significant alterations in the Hamilton Depression Rating Scale (HRS) scores upon folic acid treatment (Williams et al., 2005; Ford et al., 2008; Walker et al., 2010). Moreover, a community-based population study found that only B6 and B12 vitamins, but not folic acid, can be predictive of depressive symptoms among older adults (Skarupski et al., 2010). Therefore, further studies are warranted in order to further evaluate the role of folic acid in depression.

It is noteworthy that folic acid deficiency leads to the accumulation of homocysteine. High levels of homocysteine in the central nervous system are associated with depression, dementia, Parkinson's disease, stroke, as well as negative symptoms of schizophrenia (Khanna, 2011). Moreover, it has been shown that folic acid deficiency can result in low levels of monoamines (serotonin, norepinephrine and dopamine) (Morris et al., 2008; Farah, 2009), which could contribute to the development of depressive symptoms (Krishnan

and Nestler, 2010). It is also important to mention that genetic factors can also affect folic acid levels. One example is the inborn error in methionine synthase (an enzyme that converts folic acid to L-methylfolate) caused by a C677T-polymorphism (Arinami et al., 1997; Kelly et al., 2004; Lewis et al., 2006; Fathy et al., 2011).

Moreover, various studies indicate that deficiency of folic acid is linked to severe and longer lasting depressive relapses. A study conducted among 71 depressive patients with low serum folate status indicated an association between folic acid deficiency and depressive relapse during treatment with fluoxetine, indicating that depressive patients with low serum levels of folic acid can be at a higher risk of depressive relapse during the treatment of this disorder (Papakostas et al., 2004b). In agreement with this study, Astorg et al. (2008) showed that a low folic acid intake was associated with an increased risk of recurrent depression in middle-aged men. By contrast, it has been speculated whether this relationship between low levels of folic acid and depression is merely causal. A cross-sectional study found no correlation between low blood levels of folic acid and incidence of depressive symptoms, suggesting that low blood folic acid content may be a consequence rather than a cause of depressive symptoms (Kendrick et al., 2008).

Several studies have investigated the potential beneficial effects of folic acid supplementation in depressive patients. An early study from Botez et al. (1982) indicated that folic acid supplementation was beneficial in a neuropsychiatric syndrome characterized by mental changes, polyneuropathy, and depression. Folic acid supplementation (10 mg daily for a period of 7-11 months) significantly improved neurological symptoms of depression (Botez et al., 1984). Moreover, a study conducted by Godfrey et al. (1990) in depressive patients showed that supplementation of the current antidepressant therapy with methylfolate (bioactive form of folic acid; 15 mg daily for a period of 6 months) was associated with, enhanced clinical and social recovery of these subjects. Additionally, a study by Alpert et al. (2002) showed that an active form of folic acid, folinic acid (15 to 30 mg/day for eight weeks), enhanced the effects of selective serotonin reuptake inhibitors in refractory depressed patients.

Importantly, deficiency of folic acid is associated with a poorer response to classical antidepressants, such as fluoxetine, in depressive patients (Coppen and Bailey, 2000; Papakostas et al., 2004a; 2005). A study showed that hypofolatemia (i.e., low levels of folic acid) predisposes subjects to melancholic depression and poor response to

fluoxetine (Fava et al., 1997). Moreover, the combined administration of 5'-methyltetrahydrofolic acid (50 mg) and a standard antidepressant (trazodone, 50 mg), twice daily significantly improved the clinical response of depressed patients with borderline or deficiency of folic acid. Indeed, in normofolatemic subjects the HRS scores were reduced following 3 weeks of treatment (Passeri et al., 1993). Further indicating that folic acid levels may be associated with the response to antidepressants, a study by Wesson et al. (1994) found that along with a significant correlation between red cell folic acid levels and severity of illness, there were significantly more responders (to a 5-week trial of desmethylimipramine) than non-responders who had an increase in RBC folic acid levels. In addition, a randomized, placebo controlled trial showed that folic acid (500µg daily, 10 week) plus fluoxetine (20 mg), improved the HRS scores, especially in women (Coppen and Bailey, 2000). Other similar study also found that folic acid can be an additional treatment for depressive hypofolatemic patients resistant to therapy with fluoxetine (Papakostas et al., 2004a). However, these patients show a delayed response to folic acid treatment when compared to eufolatemic subjects (Papakostas et al., 2005). A randomized study in twenty-seven patients that received fluoxetine (20 mg) and folic acid (10 mg/day) or fluoxetine and placebo alone for a period of 6 weeks, was also performed. The HRS scores were reduced in these patients. Furthermore, considering that folic acid is an important component of the endogenous defense system and it may elevate antidepressant responses, Resler et al. (2008) investigated the participation of lymphocyte serotonergic system in folic acid-supplemented depressed patients. The authors observed that treatment with a combination of folic acid plus fluoxetine reduced the homocysteine status and induced accumulation of serotonin in the lymphocytes, probably modifying the functioning of these cells in depression. Nevertheless, a community-based study that involved older adults with depressive symptoms provided no clear evidence for the potentiation of the effects of antidepressant medication by combining it with folic acid plus vitamin B12 (Christensen et al., 2011).

It is also important to consider that dietary supplementation or consumption of these B-vitamins (folic acid, B-6 and B-12) has been reported to prevent depression after stroke. In a controlled trial performed in 273 individuals, daily treatment with folic acid (2 mg), vitamin B6 (25 mg), and vitamin B12 (0.5 mg) for approximately 7 years suggested that this combination of vitamins might be an effective,

safe, and affordable intervention to reduce the risk of depression after stroke (Almeida et al., 2010).

A recent study found no strong evidence that folic acid supplementation reduced the risk of depression during pregnancy and up to 8 months after pregnancy. However, this work found evidence to suggest that folic acid supplements during pregnancy protected against depression 21 months postpartum (Lewis et al., 2011).

Considering the following findings: a) depressive patients frequently have a functional folic acid deficiency (which can be related to high homocysteine levels) and poor response to antidepressant therapy (Coppen and Bailey, 2000; Papakostas et al., 2004a; 2005); b) folic acid is associated with the synthesis of monoamines (Stahl, 2007); c) folic acid supplementation can potentiate the effects of antidepressant medication (Resler et al., 2008; Almeida et al., 2010; Christensen et al., 2011); it is reasonable to conclude that this vitamin can be a safe, simple and cheap alternative for the treatment of depression (Roberts et al., 2007). In support of this hypothesis, it has been shown that folic acid supplementation increases medication response in depressive patients (Fava, 2010; Fava and Mischoulon, 2010; Nahas and Sheikh, 2011), probably due to an increase in the availability of monoamines (Stahl, 2007).

Table 1. Summary of findings from clinical studies evaluating the role of folic acid in depression

Study	Major Findings
Carney, 1967	Patients suffering from various psychiatric syndromes had low serum folic acid concentrations.
Reynolds et al., 1970	24% of depressed patients had low serum folic acid concentrations.
Carney and Sheffield, 1978	21.3% of 272 psychiatric patients had low serum folic acid levels.
Ghadirian et al., 1980	Low folic acid content in depressive patients was correlated with higher scores on the Hamilton Rating Scale (HDRS) for depression.
Botez et al., 1982	Folic acid supplementation enhanced 5-hydroxyindoleacetic in the cerebrospinal fluid (CSF) of psychiatric patients with folic acid

Study	Major Findings
	deficiency.
Botez et al., 1984	Folic acid supplementation (10 mg daily) improved the scores on Ottawa-Wechsler Scale in psychiatric patients with serum and CSF folic acid deficiency.
Abou-Saleh and Coppen, 1989	Depressive patients had low serum and red blood cell (RBC) folic acid content.
Levitt and Joffe, 1989	Duration of depressive episode was significantly correlated with low folic acid levels.
Carney, 1990	Depressive patients had RBC folic acid deficiency (below 200 μg/l).
Godfrey et al., 1990	33% of 123 patients with psychiatric disorders including depression had low RBC folic acid status and the supplementation with methylfolate (15 mg/day, for 6 months) improved clinical and social recovery.
Passeri et al., 1993	5'-Methyltetrahydrofolic acid (5'-MTHF) supplementation reduced the HDRS score in depressed patients with borderline or definite folic acid deficiency and in normofolatemic patients after 3 weeks of treatment.
Wesson et al., 1994	Increased RBC folic acid levels was associated with a lower severity of depression and a better response to desmethylimipramine in depressive patients.
Arinami et al., 1997	The T677 allele of the methylenetetrahydrofolate reductase (MTHFR) gene was associated with schizophrenia and depression.
Fava et al., 1997	Depressive patients with low folic acid level were more susceptible to developing melancholic depression and less prone to respond to treatment with fluoxetine.
Herran et al., 1999	Depressive patients had serum folic acid deficiency but normal RBC folic acid.
Bottiglieri et al., 2000	52% of depressed patients had significant low serum, RBC, and CSF folic acid content.
Lindema	Folic acid deficiency was associated with

Study	Major Findings
n et al., 2000	cognitive dysfunction and depression.
Coppen and Bailey, 2000	Folic acid supplementation (500 µg/day) combined with fluoxetine (20 mg/day) improved the antidepressant effect of fluoxetine, particularly in women.
Alpert et al., 2002	An eight-week open study showed that folinic acid (an active form of folic acid, 15 to 30 mg/day for eight weeks) enhanced the effects of selective serotonin reuptake inhibitor-refractory depression.
Tolmune n et al., 2003	Patients with low intake of folic acid had elevated depressive symptoms.
Kelly et al., 2004	Patients with MTHFR C677T genotype had an increased risk for developing depressive episodes.
Papakost as et al., 2004a	Depressive patients with low folic acid status were associated with poorer response to treatment with fluoxetine.
Papakost as et al., 2004b	Depressive patients with low folic acid status were associated with relapse during continuation treatment with fluoxetine.
Papakost as et al., 2005	Depressive patients with low folic acid status were associated with delay to start the improvement of psychiatric symptoms during treatment with fluoxetine.
Williams et al., 2005	Folic acid supplementation (100 mg for 6 weeks followed by 200 mg for 6 weeks) did not improve psychiatric symptoms associated with depression.
Lewis et al., 2006	A meta-analysis showed that polymorphism (C677T) in MTHFR was associated with the risk of depression.
Roberts et al., 2007	Folic acid supplementation augmented antidepressant response.
Astorg et al., 2008	Low intake of folic acid predisposed recurrent depressive episodes in men.
Kendrick et al., 2008	Low RBC folic acid content did not predict the incidence of depressive symptoms.
Ford et	Folic acid supplementation was not effective

Study	Major Findings
al., 2008	in reducing depressive symptoms.
Resler et al., 2008	Folic acid supplementation combined with fluoxetine significantly reduced the HDRS scores in depressive patients.
Almeida et al., 2010	Supplementation with folic acid, as well as vitamins B6 and B12 in post-stroke survivors reduced the risk to depressive episodes.
Nanri et al., 2010	A higher serum folic acid status decreased the prevalence of depressive symptoms in men.
Skarupski et al., 2010	Folic acid supplementation was not effective in reducing depressive symptoms in depressed patients.
Walker et al., 2010	Folic acid supplementation was not effective in reducing depressive symptoms.
Christensen et al., 2011	Folic acid plus vitamin B12 supplementation improved depressive symptoms.
Fathy et al., 2011	33% of patients with depressive disorder or anxiety had MTFR C677T polymorphism. Additionally, there was a negative correlation between the severity of depression and folic acid status in depressive patients.
Lewis et al., 2011	Folic acid supplements during pregnancy protected against depression 21 months postpartum

Considering that several clinical findings indicate a strong correlation between folic acid and depression, some preclinical studies have emerged to elucidate the possible antidepressant effect of this vitamin in animal model of depression, as reviewed in Table 2. The antidepressant potential of folic acid (administered either through the oral, intracerebroventricular, or intraperitoneal route) was investigated in mice that were submitted to the forced swimming and the tail suspension tests, two behavioral tests that are predictive of antidepressant activity (Brocardo et al., 2008a). In these tests, a reduction in immobility time is the parameter used to infer the antidepressant-like action of drugs (Porsolt et al., 1977, Steru et al., 1985). Moreover, in this study, Brocardo et al. (2008a) also used pharmacological tools to investigate the involvement of the serotonergic and noradrenergic systems in mediating the antidepressant-like effect of folic acid. The results of this study support the notion that the anti-

depressant effects of folic acid might result from the activation of both serotonergic (5-HT_{1A} and 5-$HT_{2A/2C}$ receptors) and noradrenergic (α_1- and α_2-adrenoceptors) systems (Brocardo et al., 2008a). Furthermore, the antidepressant-like effects of this vitamin were shown to be dependent on the inhibition of either *N*-methyl-D-aspartate (NMDA) receptors or nitric oxide (NO) and cyclic guonosine monophosphate (cGMP) synthesis (Brocardo et al., 2008b), as well as through an interaction with the opioid system (μ_1 and δ receptors) (Brocardo et al., 2009). It is also important to emphasize that co-administration of sub-effective doses of folic acid and fluoxetine in mice produced antidepressant-like effects as assessed with the forced swimming test (Brocardo et al., 2008a). Moreover, using the same behavioral assessment tool, it was possible to verify that folic acid alone or combined with estradiol or fluoxetine also has antidepressant-like effects in ovariectomized female rats (Molina-Hernández et al., 2011). Furthermore, it was recently reported that the antidepressant effect of folic acid might also be dependent, at least in part, on the inhibition of glycogen synthase kinase-3 (GSK-3β) and activation of peroxisome proliferator-activated receptor-γ (PPARγ) (Budni et al., 2011).

Although these studies clearly indicate that folic acid plays an important role in depression, the exact mechanisms of this relationship remain to be fully elucidated. Furthermore, future well-designed and adequately powered clinical trials are warranted in order to ascertain the prophylactic and therapeutic properties of folic acid, including its appropriate formulation, optimal dose, and ideal duration of treatment.

Table 2. Summary of findings from preclinical studies that document the antidepressant-like effect of folic acid

Study	Major Findings
Brocardo et al., 2008a	Folic acid had antidepressant-like effects in mice as assessed in the FST and TST. This effect involves serotonergic (5-HT_{1A} and 5-$HT_{2A/2C}$ receptors) and noradrenergic (α_1- and α_2-adrenoceptors) systems.
Brocardo et al., 2008b	The antidepressant-like effects of folic acid involve NMDA receptors and modulation of the L-arginine-NO-cGMP pathway.
Brocardo et al., 2009	The antidepressant-like effects of folic acid involve δ- and μ-opioid receptors.

Molina-Hernández et al., 2011	Folic acid alone or combined with estradiol or fluoxetine has antidepressant-like effects in ovariectomized female rats as assessed in the FST.
Budni et al., 2011	The antidepressant-like effects of folic acid involve inhibition of GSK-3β and activation of PPARγ.

Folic Acid and Bipolar Disorder

Bipolar disorder is a psychiatric disease characterized by mood alterations that are associated with recurrent depression and mania throughout lifetime (Swann, 2005). Mania is the most specific manifestation of bipolar disorder and is defined by an abnormally elevated mood state characterized by such symptoms as inappropriate elation, increased irritability, severe insomnia, grandiose notions, increased speed and/or volume of speech, disconnected and racing thoughts, increased sexual desire, markedly increased energy and activity level, poor judgment, and inappropriate social behavior (Berrios, 2004). Early studies have shown that folic acid deficiency is associated with a number of neurological and psychiatric disorders, including bipolar disorder (Hasanah et al., 1997; Ozbek et al., 2008).

Since monoamines are involved in mood regulation (Elhwuegi, 2004) and serotonin has been shown to contribute to mania (Shiah and Yatham, 2000), it is possible that bipolar patients have an increased need for folic acid (Coppen et al., 1986). In addition, several studies have also observed disturbances of homocysteine metabolism in bipolar disorder (Osher et al., 2004; Dittmann et al., 2007; 2008). Moreover, both genetic polymorphisms that alter enzymes involved in the metabolism of homocysteine such as MTHFR and folic acid deficiency, which can result in variations in the levels of homocysteine (Jacques et al., 1996), have been correlated with bipolar disorder. Indeed, Ozbek et al. (2008) investigated the effect of polymorphic variants of MTHFR (c.1298A>C and c.677C>T) on homocysteine, folic acid, and vitamin B12 levels in 197 bipolar patients, 278 unaffected first-degree relatives, and 238 normal controls and found that homocysteine levels are higher and folic acid levels are lower in patients with bipolar disorder as compared with the healthy controls.

Hyperhomocysteinaemia may also play a role in the pathophysiology of neurocognitive deficits in bipolar disorder, with a higher impact in older patients or in patients who had delayed onset of

illness (Osher et al., 2004; Dittmann et al., 2007; 2008). Osher et al. (2004) measured homocysteine levels in 41 euthymic outpatients with bipolar disorder and compared them with 305 subjects from a large employee health-screening program. They found that bipolar patients who show functional deterioration have elevated plasma levels of homocysteine as compared with control individuals. Dittmann et al. (2007) used neuropsychological tests in 55 euthymic bipolar patients and 17 healthy controls and observed an association between neuropsychological measures and elevated homocysteine levels in euthymic bipolar patients. In addition, a study was conducted between 2002 and 2006 in 75 euthymic bipolar patients and 42 healthy controls in order to investigate the potential relationship of elevated homocysteine levels and cognitive impairment in bipolar patients. In agreement with the previous studies, this trial also found an association between homocysteine levels and verbal learning, delayed memory, as well as executive function in the patient group (Dittmann et al., 2008).

In agreement with these clinical studies, we have recently shown that folic acid can prevent biochemical and behavioral alterations in an ouabain-induced animal model of mania in rats (Brocardo et al., 2010). In addition, other clinical studies have shown reduced folic acid levels in erythrocytes (Hasanah et al., 1997) and serum (Ozbek et al., 2008) of manic patients, demonstrating that folic acid may play an important role during the manic phase of bipolar disorder. Furthermore, it has been reported that folic acid can enhance the prophylactic effects of lithium on affective morbidity (Coppen and Abou-Saleh, 1982; Coppen et al., 1986). A recent clinical study has also shown that folic acid can be an effective adjuvant to sodium valproate in the treatment of the acute phase of mania in patients with bipolar disorder (Behzadi et al., 2009). A summary of the main findings are provided below in Table 3.

Taken together, these studies strongly indicate that folic acid can be a putative candidate for the treatment of bipolar disorder. Further preclinical and clinical studies are thus warranted in order to confirm this hypothesis and to investigate whether folic acid treatment has beneficial effects in patients with normal folic acid levels as well as in individuals with folic acid deficiency.

Table 3. Summary of findings from preclinical and clinical studies that investigated the role of folic acid in bipolar disorders

Study	Major Findings
Brocardo et al., 2010	Folic acid demonstrated antimanic-like effects in the animal model of mania induced by ouabain in rats.
Coppen and Abou-Saleh, 1982	Folic acid combined with lithium decreased affective morbidity.
Coppen et al., 1986	Folic acid enhanced the prophylactic effects of lithium on affective morbidity.
Hasanah et al., 1997	Reduced folate levels in erythrocytes of bipolar patients.
Ozbek et al., 2008	Folate levels were lower and homocysteine levels were higher in the serum of bipolar patients.
Behzadi et al., 2009	Folic acid was an effective adjuvant to sodium valproate in the treatment of the acute phase of mania.

Folic Acid and Schizophrenia

Schizophrenia is a complex psychiatric disease that is believed to result from multiple genetic and environmental factors (Levi and Waxman, 1975; Miyamoto et al., 2003). This disease is often described in terms of positive and negative symptoms. Positive symptoms can include delusions, disordered thoughts and speech, as well as tactile, auditory, visual, olfactory and gustatory hallucinations, typically regarded as manifestations of psychosis (Frith and Done, 1989). Negative symptoms are deficits of normal emotional responses or of other thought processes such as poverty of speech, impoverished ability to express emotion, lack of motivation, and apathy (Rummel et al., 2005). While antipsychotic drugs are very effective for the positive symptoms of schizophrenia, the treatment of negative symptoms is still a major problem (Crow, 1980; Andreasen, 1985).

Studies on the association of folic acid deficiency with schizophrenia (summarized in Table 4) extend back to the mid 1960's (Krebs et al., 2009). Carney (1967) found an incidence of folic acid deficiency in a group of 423 psychiatric patients. This study established that a high incidence (20%) of folic acid deficiency occurs in patients

with schizophrenia. In agreement, a study conducted on 243 psychiatric patients indicated that schizophrenic patients had red cell folic acid deficiency (below 200 μg/l) (Carney, 1990). However, a recent study by García-Miss et al. (2010) observed low levels of folic acid in RBC and increased levels of TNF-α, interleucyne-6 and homocysteine. It is believed that folic acid treatment in patients with hyperhomocysteinemia reduces the release of both homocysteine and proinflammatory cytokines from monocytes (Wang et al., 2005). However, folic acid treatment decreased not only the levels of homocysteine, but also the inflammatory response in schizophrenic patients (García-Miss et al., 2010). Furthermore, another recent study showed that deficiency of folic acid both in the serum and RBC is common in schizophrenic patients, suggesting that decreased levels of folic acid in the plasma may act as a risk factor for schizophrenia (Saedisomeolia et al., 2011). These findings corroborate the study by Kale et al. (2010) that showed significantly lower levels of folic acid and vitamin B12 in the plasma and of folic acid in RBC as well as a significant increase in plasma homocysteine and cortisol levels in schizophrenic patients. Moreover, a case reported by Ho et al. (2010) showed that a 13-year-old previously healthy subject with schizophrenic symptoms and progressively worsening catatonia had cerebral folic acid deficiency and elevated titers of folic acid receptor-blocking antibodies, indicating that folic acid deficiency may be related with catatonic schizophrenia.

Individuals with schizophrenia and low serum levels of folic acid are especially vulnerable to the negative symptoms of this disease, since serum folic acid concentration appears to be inversely correlated with the severity of negative symptoms on the Scale for Assessment of Negative Symptoms (Goff et al., 2004). These findings corroborate a similar study that examined the incidence of folic acid deficiency, based on serum determinations, in relation to the severity of negative symptoms in schizophrenic patients (Herran et al., 1999).

Several studies indicate that prenatal deficiencies of certain micronutrients, including folic acid, may play an important role in schizophrenia (Brown et al., 1996; Susser et al., 1996; Hoek et al., 1998; McGrath et al., 2011), and folic acid deficiency during early pregnancy has been hypothesized as a cause of schizophrenia in the offspring (Smits and Essed, 2001; Smits et al., 2004; McGrath et al., 2011). Postpartum restoration of folic acid levels to normal status may take up to 1 year (Smith et al., 1983; Açkurt et al., 1995; Bruinse and Van den

Berg, 1995). Accordingly, the risk of developing schizophrenia may be elevated for children conceived within this restorative period (Smits et al., 2004).

However, although all evidence indicating that folic acid deficiency is a risk factor for schizophrenia, there are recent reports that contradict this view (Haidemenos et al., 2007; Petronijevic et al., 2008). For example, an increase in folic acid content in RBC of schizophrenic patients has been documented (Muntjewerff et al., 2003).

Surprisingly, despite numerous reports on the association between folic acid deficiency and schizophrenia, there are relatively few controlled clinical trials that have tested the potential beneficial effect of folic acid supplementation on this disorder (Godfrey et al., 1990; Levine et al., 2006). Godfrey et al. (1990) showed that methylfolate supplementation significantly improved clinical and social recovery among both depressed and schizophrenic patients in a double-blind, placebo-controlled trial. Additionally, another double-blind placebo-controlled trial of methylfolate was performed. The administration of this compound (15 mg/day) for 6 months resulted in the attenuation of the psychiatric symptoms of schizophrenia (Godfrey et al., 1990). In a placebo-controlled cross-over trial of 42 schizophrenic patients with elevated serum homocysteine levels, Levine et al. (2006) reported a significantly reduction in the total scores of the Positive and Negative Syndrome Scale in the group who received folic acid and vitamin B12 for 3 months.

Both hyperhomocysteinemia and polymorphisms in MTHFR, encoding a critical enzyme for the metabolism of both folic acid and homocysteine have been linked to schizophrenia (Muntjewerff et al., 2005; Muntjewerff et al., 2006; Yoshimi et al., 2010) and strong epidemiological evidence supports this relationship (Allen et al., 2008). Additionally, 677T allele load is associated with the severity of the negative symptoms in schizophrenic patients (Roffman et al., 2008). From a clinical point of view, it is interesting to note that homocysteine-reducing strategies improve psychopathology in chronic schizophrenic patients with hyperhomocysteinemia (Levine et al., 2006). Moreover, Brown et al. (2007) found that elevated third trimester homocysteine levels (caused by low folic acid levels) were associated with a 2-fold increase in the risk of developing adult schizophrenia. However, despite the evidence pointing towards an association between MTHFR C667T mutation and schizophrenia, there is a recent report that contradicts this idea (García-Miss et al., 2010), indicating that further studies are required in order to clarify this hypothesis.

Table 4. Summary of findings from clinical studies evaluating the involvement of folic acid in schizophrenia

Study	Major Findings
Carney, 1967	Patients suffering from various psychiatric syndromes had low serum folic acid concentrations.
Carney, 1990	Schizophrenic patients had RBC folic acid deficiency (below 200 μg/l).
Godfrey, 1990	33% of 123 patients with psychiatric disorders including schizophrenia had low RBC folic acid content and supplementation with methylfolate (15 mg/day, for 6 months) improved clinical and social recovery.
Herran et al., 1999	Schizophrenic patients had serum folic acid deficiency but normal RBC folic acid.
Smits and Essed, 2001	Patients conceived shortly after another birth (during maternal folic acid deficiency) had an increased risk for schizophrenia.
Smits et al., 2004	
Muntjewerff et al., 2003	Schizophrenic patients had low plasma folic acid status and high RBC folic acid concentration. MTFR C677T polymorphism was not associated with an increased risk for schizophrenia.
Goff et al., 2004	Schizophrenic patients had low serum folic acid concentration, which was inversely correlated with the Scale for Assessment of Negative Symptoms total score.
Smits et al., 2004	Patients conceived shortly after another birth (during maternal folic acid deficiency) had an increased risk for schizophrenia.
Muntjewerff et al., 2005	A meta-analysis showed that folic acid deficiency is associated to risk of schizophrenia.
Muntjewerff et al., 2006	A meta-analysis showed that MTHFR 677C>T polymorphism is associated to risk of schizophrenia.
Haidemenos et al., 2007	Schizophrenia was not associated with folic acid deficiency.

Petronijevic et al., 2008	
Roffman et al., 2008	MTHFR 677C>T polymorphism was associated with the severity of negative symptoms in schizophrenic patients.
García-Miss et al., 2010	Schizophrenic patients had low RBC folic acid levels and elevated homocysteine, interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) levels. MTHFR 677C>T polymorphism was not associated with the risk for schizophrenia.
Ho et al., 2010	A case study showed that a schizophrenic teenager with cerebral folic acid deficiency had catatonic symptoms.
Kale et al., 2010	Significantly low plasma and RBC folic acid and vitamin B12 concentrations were observed in schizophrenic patients.
Yoshimi et al., 2010	MTHFR 677C>T polymorphism was associated with the severity of negative symptoms in schizophrenic patients.
Saedisomeolia et al., 2011	Significantly low serum and RBC folic acid content was observed in schizophrenic patients.

Conclusion

Basic research and clinical studies have shown that folic acid plays a role in the pathophysiology and/or treatment of psychiatric disorders such as depression, bipolar disorder and schizophrenia. Regarding this issue, the majority of the studies published to date deal with the effects of folic acid deficiency/supplementation on depressive symptoms, indicating that the potential of this vitamin as an antidepressant drug should be further evaluated. The studies involving the relationship between the levels of folic acid and bipolar disorder are scarce and the possible role of this vitamin in the schizophrenia is not well established. Thus, we conclude that further experimental evidence is warranted in order to ascertain the involvement of folic acid in the pathophysiology and treatment of these psychiatric diseases.

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6. DISCUSSÃO

Os resultados do presente trabalho estão divididos em estudos *in vivo* e estudos *in vitro* e a discussão foi dividida da mesma forma.

Os estudos *in vivo* do presente trabalho demonstram que o efeito tipo-antidepressivo do ácido fólico no modelo animal preditivo para a ação antidepressiva, TNF, em camundongos, pode envolver, pelo menos em parte, a inibição da GSK-3β, canais de K^+ e ativação de PPAR-γ. Além disso, foi demonstrado que o ácido fólico reverte o comportamento tipo-depressivo, mas não o déficit cognitivo, induzido por estresse de contenção, acompanhado pela proteção contra o estresse oxidativo induzido por este estímulo estressante.

Vários estudos clínicos reportam que pacientes com depressão apresentam, no soro, eritrócitos e líquor, níveis reduzidos de ácido fólico (Carney, 1967; Reynolds et al., 1970; Carney e Sheffield, 1978; Ghadirian et al., 1980; Abou-Saleh e Coppen, 1989; Bottiglieri et al., 2000; Lindeman et al., 2000). A administração de ácido fólico associada com antidepressivos, nestes pacientes, mostrou melhora no quadro depressivo (Botez et al., 1982;1984; Godfrey et al., 1990; Alpert et al., 2002). Além disso, esta vitamina pode ser uma boa alternativa terapêutica em paciente resistente ao tratamento com antidepressivos (Papakostas et al., 2004a; Papakostas et al., 2005). Estes achados suportam a hipótese que a deficiência do ácido fólico está envolvida na fisiopatologia da depressão (Coppen e Bailey, 2000; Coppen e Bolander-Gouaille, 2005). Adicionalmente, é impotante mencionar que estudos pré-clínicos, todavia não muito abundantes, realizados em camundongos (Brocardo et al., 2008a; 2008b; 2009) e ratos (Molina-Hernández et al., 2011; 2012; Molina-Hernández e Tellez-Alcantara, 2011), mostram que o ácido fólico apresenta efeito tipo-antidepressivo no TNF ou TSC. Porém, os mecanismos responsáveis pelo papel antidepressivo do ácido fólico, ainda, necessitam de investigações adicionais.

Os estudos *in vivo* deste trabalho utilizaram o TNF e/ou o modelo de depressão induzida por estresse de contenção. O TNF é um teste muito utilizado devido sua validade preditiva, considerando o fato, de que este teste é sensível para antidepressivos clássicos (incluindo tricíclicos, ISRS, inibidores da MAO e antidepressivos atípicos), os quais diminuem o tempo de imobilidade dos camundongos (Porsolt et al., 1977; Petit-Demouliere et al., 2005). Quanto ao modelo de depressão induzida por estresse de contenção, muito estudos mostram que tanto ratos quanto camundongos expostos a estresse de contenção,

em diferentes tempos, exibem comportamento tipo-depressivo no TNF (Poleszak et al., 2006; Zafir et al., 2009; Capra et al., 2010; Park et al., 2010; Naert et al., 2011), bem como prejuízo cognitivo (Baker e Kim, 2002; Walesiuk et al., 2005; Nagata et al., 2009; Li et al., 2012). Esta alteração comportamental em roedores é comparável aos sintomas depressivos encontrados em humanos (Wong e Licínio, 2004; Marim et al., 2011). O presente trabalho utilizou o modelo de indução de comportamento tipo-depressivo por estresse de contenção durante 7 horas, o qual causou um aumento de tempo de imobilidade no TNF em camundongos e prejuízo cognitivo no TRO, sem alterar a atividade locomotora dos animais.

A atividade locomotora empregada em todos os estudos *in vivo* deste trabalho foi avaliada pelo teste do campo aberto. Sabe-se que drogas que induzem hiperlocomoção podem mostrar um efeito "falso" positivo no TNF, enquanto drogas que diminuem a atividade locomotora podem dar um resultado "falso" negativo. Portanto, o teste do campo aberto é utilizado para descartar a possibilidade de que o efeito tipo-antidepressivo ou tipo-depressivo não seja devido a uma alteração locomotora (hiper- ou hipolocomoção, respectivamente) (Borsini e Meli, 1988; Rodrigues et al., 2005). Realmente, o efeito tipo-antidepressivo ou tipo-depressivo no TNF, encontrados nos nossos estudos *in vivo*, não foram devido a alterações na atividade locomotora dos camundongos.

Os resultados apresentados no capítulo 1 indicam que o efeito tipo-antidepressivo do ácido fólico pode envolver via da PI3K/Akt/GSK-3β bem como receptores PPARγ.

A GSK-3β é regulada pela Akt, estas duas proteínas fazem parte da via de sinalização regulada pela fosfatidil inositol 3 cinase (PI3K) (Cross et al., 1995; Grimes e Jope, 2001). A ativação da PI3K induz ativação da Akt, a qual é alvo primário da PI3K (Katso et al., 2001; Beaulieu et al., 2009). Portanto, anormalidades na ativação e expressão da PI3K podem envolver o mecanismo fisiopatológico da depressão. Dwivedi et al. (2008) realizaram um estudo em pacientes com depressão, e encontraram no córtex pré-frontal e hipocampo destes pacientes, uma ativação reduzida da PI3K, quando comparado aos controles saudáveis, um grande indicativo da implicação da PI3K na fisiopatologia da depressão. Corroborando com este estudo, nossos resultados mostraram que o pré-tratamento dos animais com um inibidor da PI3K, LY294002, preveniu o efeito tipo-antidepressivo elicitado pelo ácido fólico no TNF em camundongos. Além disso, para reforçar nossa hipótese, um estudo de Seto et al. (2010) mostrou que o ácido fólico restaurou a inibição do relaxamento aórtico induzido por acetilcolina em

um modelo de hipertensão associada à diabetes mellitus em camundongos, via aumento da atividade da cascata PI3K/Akt.

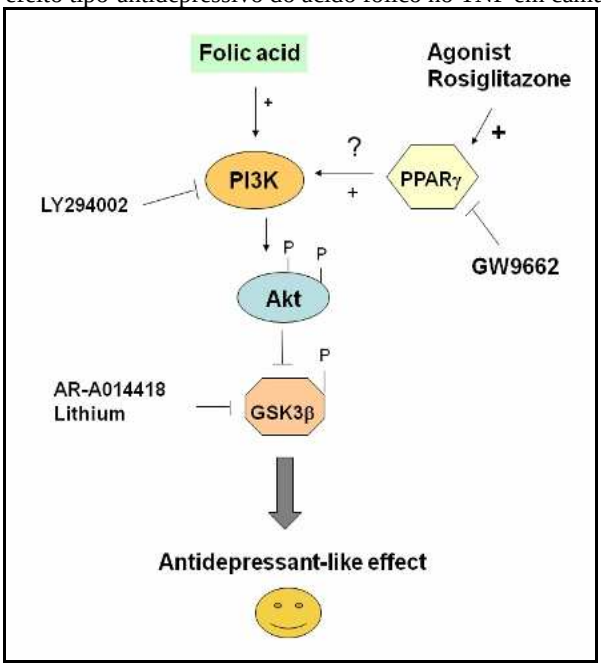
A via PI3K/Akt age como um mecanismo *upstream* que regula a atividade da GSK-3β, uma vez que este sistema pode fosforilar diretamente o resíduo de serina 9 da GSK-3β, inativando-a (Grimes e Jope, 2001; Bhat et al., 2004; Beaulieu et al., 2009). Muitos estudos mostram que a GSK-3β está associada com depressão, já que inibidores desta enzima, como o lítio, AR-A014418 e uma tiazolidona NP031115 reduzem o tempo de imobilidade dos camundongos no TNF, mimetizando uma ação tipo-antidepressiva (Gould et al., 2004; Rosa et al., 2008). Os resultados do presente trabalho mostram que o efeito tipo-antidepressivo do ácido fólico pode envolver a inibição da GSK-3β, levando em conta, que o tratamento dos animais com inibidores da GSK-3β, AR-A014418 ou lítio, combinado com uma dose sub-ativa de ácido fólico é capaz de produzir um efeito tipo-antidepressivo no TNF. É importante mencionar que a eficácia terapêutica do lítio pode resultar da inibição direta da GSK-3β, porém, sabe-se que este fármaco age em muitos outros alvos moleculares, não sendo um inibidor seletivo da enzima GSK-3β (Li et al., 2002; Gould e Manji, 2005) como o AR-A014418 e o NP031115 (Rosa et al., 2008). Apesar do lítio não ser seletivo para a inibição da enzima GSK-3β, pode ser considerado, neste trabalho, uma importante ferramenta confirmatória do envolvimento da GSK-3β no efeito tipo-antidepressivo do ácido fólico no TNF.

Além da implicação da GSK-3β na fisiopatologia da depressão, em um prévio trabalho, nosso grupo foi o primeiro a mostrar que a administração intracerebroventricular do agonista PPARγ, rosiglitazona, produziu um efeito tipo-antidepressivo no TNF em camundongos (Rosa et al., 2008). Esta idéia foi reforçada recentemente através de um estudo realizado por Eissa Ahmed et al. (2009), o qual mostrou que a administração oral de rosiglitazona produziu um efeito tipo-antidepressivo no TNF em ratos e no TSC em camundongos. Além disso, o nosso grupo mostrou que o pré-tratamento dos animais com GW-9662, um antagonista PPARγ, foi efetivo em prevenir o efeito tipo-antidepressivo elicitado por NP031115, AR-A014418 (inibidores da GSK-3β) e rosiglitazona (agonista PPARγ) (Rosa et al., 2008). Adicionalmente, foi mostrado que NP031115 e AR-A014418 ativam PPARγ em células de ovário de hamsters chineses (CHO) transfectadas com o plasmídio pPPRE-tk-luc contendo três consensos de ligação para PPARγ (Rosa et al., 2008). Desta forma, foi visto que a ativação do PPARγ pode inibir a GSK-3β. A inibição desta enzima pode

desempenhar um papel importante no efeito tipo-antidepressivo de alguns compostos no TNF. Portanto, o presente estudo também se propôs a verificar se a ativação de PPARγ poderia estar envolvida no efeito tipo-antidepressivo do ácido fólico. Os resultados obtidos do presente estudo indicam que a administração de uma dose sub-ativa de ácido fólico combinada com uma dose sub-ativa de rosiglitazona reduziu o tempo de imobilidade no TNF em camundongos, sugerindo que o efeito tipo-antidepressivo do ácido fólico pode ser dependente da ativação de PPARγ. Adicionalmente, para reforçar esta idéia, nós mostramos que a administração de GW-9662 foi capaz de reverter o efeito anti-imobilidade do ácido fólico.

Portanto, este estudo indica que o efeito tipo-antidepressivo do ácido fólico no TNF em camundongos pode ser dependente da ativação da via PI3K/Akt, inibição da GSK-3β e ativação do PPARγ **(Figure 9)**.

Figura 9. Esquema ilustrativo do envolvimento da via PI3K/Akt/GSK-3β e PPARγ no efeito tipo-antidepressivo do ácido fólico no TNF em camundongos.



Os resultados do presente estudo mostram que o ácido fólico pode agir por ativar a via PI3K/Akt, inibir a GSK-3β e ativar PPARγ. Neste estudo LY294002, um inibidor da PI3K e GW9662, um antagonista de PPARγ preveniram o efeito tipo antidepressivo do ácido fólico. Além disso, inibidores

da GSK-3β (AR-A014418 e lítio) e o agonista PPARγ rosiglitazona, todos administrados em doses sub-ativas, apresentaram um efeito sinérgico com uma dose sub-ativa de ácido fólico. Portanto, ácido fólico pode causar ativação da PI3K, que por sua vez, induz inibição da GSK-3β, por fosforilação dependente de Akt. Além disso, o ácido fólico pode ativar receptores PPARγ causando inibição da GSK-3β, por um mecanismo também dependente da ativação da PI3K. GSK3β, glicogênio sintase cinase 3-β; PI3K, fosfoinositol 3 cinase; PPARγ, receptor ativado por proliferador peroxissomal-γ.

Como mostrado no capítulo 2, o efeito antidepressivo do ácido fólico também pode ser modulado por um bloqueio de diferentes tipos de canais de K^+, uma vez que o tratamento dos animais com dose sub-ativa de ácido fólico associado com uma dose sub-ativa de diferentes bloqueadores de canais de K^+ (glibenclamida, caribdotoxina ou apamina) produziu um efeito tipo-antidepressivo no TNF.

Bloqueadores de canais de K^+ como a glibenclamida, caribdotoxina e apamina, agem por diferentes mecanismos, uma vez que bloqueam seletivamente canais de K^+ sensíveis ao ATP, canais de K^+ ativados por Ca^{2+} de alta e intermediária condutância e canais de K^+ ativados por Ca^{2+} de baixa condutância, respectivamente (Hugues et al., 1982; Gimenez-Gallego et al., 1988; Gehlert e Gackenheimer, 1993; Clapp, 1995). Glibenclamida é uma sulfoniluréia, utilizada como agente hipoglicemiante que bloqueia canais de K^+ sensíveis ao ATP de células β-pancreáticas, cardíacas, musculares lisas, musculares esqueléticas e de alguns neurônios (Clapp, 1995; Proks et al., 2002). Caribdotoxina é um peptídio, componente do veneno de escorpião da espécie *Leiurus quinquestriatus* (Nelson e Quayle, 1995) causa um potente bloqueio seletivo de canais de K^+ ativados por Ca^{2+} presentes em células da pituitária anterior (GH3) e em células musculares lisas primárias da aorta bovina (Gimenez-Gallego et al., 1988). Apamina é um peptídio presente no veneno de abelhas da espécie *Apis mellifera* (Stocker, 2004) que bloqueia seletivamente a condutância de K^+ dependente de Ca^{2+} (Hugues et al., 1982).

Estes e outros bloqueadores de canais de K^+ (3,4-diaminopiridina e gliquidona) exercem efeito tipo-antidepressivo no TNF, em doses maiores (doses ativas) daquelas encontradas no presente estudo (doses sub-ativas) (Galeotti et al., 1999; Inan et al., 2004; Kaster et al., 2005). Além disso, antidepressivos como a fluoxetina, desipramina, amitriptilina, nortriptilina, clomipramina, maprotilina, citalopram e paroxetina também produzem bloqueio das correntes de K^+, o que pode também justificar seus efeitos terapêuticos (Tygat et al., 1997; Yeung et

al., 1999; Nicholson et al. 2002; Choi et al., 2004; Kobayashi et al., 2004, 2006).

Suportando a idéia de que o bloqueio de canais de K^+ está relacionado com a fisiopatologia e o tratamento da depressão, Takahashi et al. (2006) demonstraram que a inibição contínua dos canais GIRK2 (canais de K^+ retificadores interno ativados pela protein G) induzida por antidepressivos como a fluoxetina e a desipramina causou uma supressão substancial da morte neuronal resultando em melhora do distúrbio motor, em camundongos *weave* mutantes (camundongos com mutação nos canais GIRK2, que induz morte neuronal e déficit motor). Além disso, muitos estudos mostraram que a administração combinada de antidepressivos com bloqueadores de canais de K^+ produziram efeito tipo-antidepressivo no TNF em camundongos (Guo et al., 1995, 1996; Inan et al., 2004; Kaster et al., 2007; Bortolatto et al., 2010). Outros compostos com propriedades antidepressivas como a agmatina, adenosina e tramadol associados com bloqueadores de canais de K^+ também produziram efeito tipo-antidepressivo no TNF (Budni et al., 2007; Kaster et al., 2007; Jesse et al., 2009).

É importante mencionar ainda, que um tipo particular de canais de K^+ de dois poros (TREK-1) também está envolvido na fisiopatologia da depressão (Kennard et al., 2005). A inibição destes canais está envolvida na liberação de serotonina em neurônios do núcleo da rafe, confirmando a íntima relação destes canais de K^+ com o mecanismo de resposta aos antidepressivos (Heurteaux et al., 2006). Portanto, levando em consideração que: a) canais de K^+ estão envolvidos no efeito tipo-antidepressivo de diferentes agentes dotados de propriedades antidepressivas; b) canais de K^+ podem modular o sistema serotoninérgico; c) o ácido fólico apresenta efeito tipo-antidepressivo no TNF em camundongos e este efeito parece envolver o sistema serotoninérgico (Brocardo et al., 2008a), é de se esperar que o ácido fólico possa modular, de alguma forma, estes canais. De fato, os resultados do presente estudo mostram que o tratamento combinado com ácido fólico e bloqueadores de canais de K^+ (glibenclamida, caribdotoxina e apamina) produziu um efeito tipo-antidepressivo no TNF em camundongos.

Diferentes bloqueadores de canais de K^+ como o TEA, glibenclamida, apamina e caribdotoxina produzem um efeito tipo-antidepressivo no TNF e este efeito foi revertido pelo tratamento dos animais com L-arginina (precursora de NO) ou sildenafil (inibidor da enzima fosfodiesterase-4 que degrada o GMPc). Portanto, os resultados sugerem que NO e GMPc são importantes moduladores de alguns canais

de K^+ (Kaster et al., 2005). Realmente, estudos mostram que canais de K^+ ativados por Ca^{2+} e dependente de voltagem desempenham um importante papel na modulação do tempo de imobilidade no TNF em camundongos (Inan et al., 2004), e os canais de K^+ ativados por Ca^{2+} de alta condutância parece ser um alvo fisiológico do NO no cérebro (Jeong et al., 2001). Considerando que os canais de K^+ representam um dos maiores alvos *downstream* regulados pela ativação de receptors NMDA e da via L-arginina-NO, acredita-se que a inibição de canais de K^+ pode ser uma consequência da inibição de receptors NMDA e da produção de NO induzido por ácido fólico, uma vez que estudos mostram que o efeito desta vitamina pode ser mediado por inibição de receptores NMDA e síntese de NO (Brocardo et al., 2008b). Por isso, a modulação indireta dos canais de K^+ por ácido fólico mediado pela via NMDA-L-arginina-NO pode ser a justificativa do efeito comportamental observado neste estudo. Contudo, não se pode descartar a possibilidade de que este efeito seja em conseqüência de uma inibição direta dos canais de K^+.

Para reforçar a hipótese de que ácido fólico pode inibir canais de K^+, nós também pré-tratamos os animais com cromacalim, um ativador de canais de K^+, o qual reverteu o efeito tipo-antidepressivo elicitado por uma dose ativa de ácido fólico (50 mg/Kg), administrada por via oral, no TNF, sem alterar a atividade locomotora dos camundongos no teste do campo aberto. Cromacalim é um ativador de canais de K^+ com alta sensibilidade para canais de K^+ sensíveis ao ATP (Clapp et al., 1995). Dados encontrados na literatura indicam que a administração de doses ativas de cromacalim, maiores que a dose utilizada no presente estudo, aumentaram o tempo de imobilidade no TNF, indicando um efeito tipo-depressivo induzido pelos ativadores destes canais (Galeotti et al., 1999). Além disso, Redrobe et al. (1996) demonstraram que o pré-tratamento dos animais com cromacalim preveniu o efeito tipo-antidepressivo de fármacos antidepressivos como a imipramina, amitriptilina, desipramina e paroxetina. Ainda, outros estudos mostram que este ativador de canais de K^+, também reverteu o efeito anti-imobilidade elicitado por adenosina e agmatina, compostos dotados de propriedades antidepressivas no TNF em camundongos (Kaster et al., 2007; Budni et al., 2007).

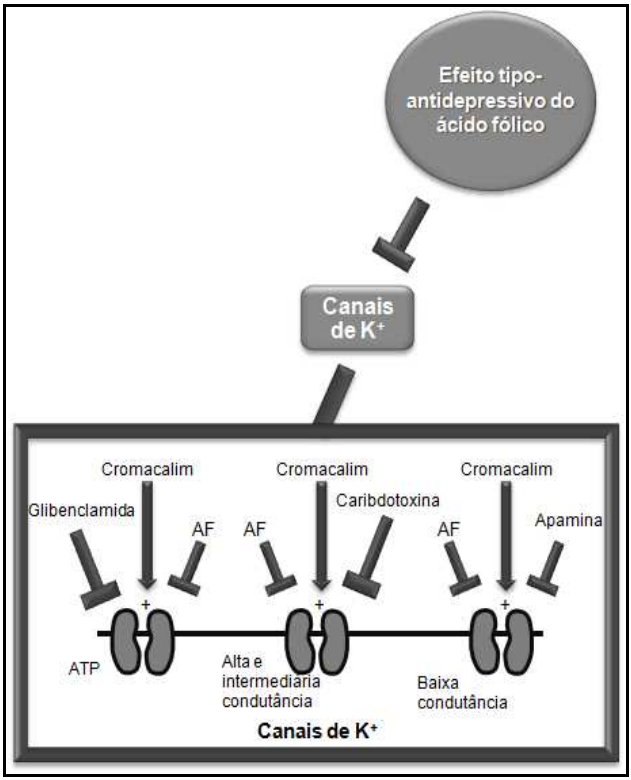
É importante ressaltar ainda, que Kaster et al. (2007) mostraram que uma dose sub-ativa de fluoxetina combinada com uma dose sub-ativa de bloqueadores de canais de K^+ produziu um efeito tipo-antidepressivo no TNF e este efeito foi prevenido pela administração, nos animais, de ativadores de canais de K^+. Estes resultados são

comparáveis com os resultados encontrados no presente estudo com ácido fólico, o qual pode igualmente exercer um efeito tipo-antidepressivo no TNF por um mecanismo similar à fluoxetina ou bloqueadores de canais de K^+.

Finalmente, é igualmente importante enfatizar que os antidepressivos, principalmente os tricíclicos, apresentam efeitos colaterais relacionados a problemas cardíacos, ou seja, causam anormalidades cardíacas relacionada à síndrome do QT longo em função de um bloqueio direto da corrente de K^+ cardíaca (I(Kr)/hERG), a qual é importante para repolarização terminal no coração humano (Dennis et al., 2011). Em contraste com os antidepressivos, o ácido fólico apresenta efeito benéfico, ou pelo menos, nenhum risco para o desenvolvimento de doenças cardíacas (Czeizel, 1996; Bazzano et al., 2006). Além disso, um recente estudo mostrou que o ácido fólico apresenta propriedades protetoras contra o estresse nitrosativo induzido por homocisteína no coração de ratos (Kolling et al., 2011). Portanto, é viável supor que o ácido fólico não bloqueia significativamente canais de K^+ implicados com riscos cardiovasculares e que a associação desta vitamina com bloqueadores de canais de K^+, como a glibenclamida e/ou antidepressivos para o tratamento da depressão, pode ser uma estratégia para a prevenção ou redução dos riscos cardiovasculares associados com o bloqueio dos canais de K^+. Porém, adicionais estudos são necessários para elucidar melhor esta proposta.

Os resultados deste estudo mostram, portanto, que o efeito tipo-antidepressivo do ácido fólico pode ser mediado por uma inibição de diferentes canais de K^+, um mecanismo que envolve a modulação da excitabilidade neuronal **(Figura 10)**.

Figura 10. Esquema ilustrativo do envolvimento de canais de K+ no efeito tipo-antidepressivo do ácido fólico no TNF em camundongos. Os resultados do presente estudo mostram que o ácido fólico pode agir por inibir canais de K^+, envolvendo a modulação da excitabilidade neuronal no efeito tipo-antidepressivo desta vitamina. Neste trabalho, doses sub-ativas de glibenclamida (bloqueador de canais de K^+ sensível ao ATP), caribdotoxina (bloqueador de canais de K^+ ativados por Ca^{2+} de alta e intermediária condutância) e apamina (bloqueador de canais de K^+ ativados por Ca^{2+} de baixa condutância) combinadas com uma dose sub-ativa de ácido fólico produziram um efeito tipo-antidepressivo no TNF. Adicionalmente, o pré-tratamento dos animais com um ativador de canais de K^+ (cromacalim), reverteu o efeito tipo-antiimobilidade do ácido fólico. K^+, canais de potássio; TNF, teste do nado forçado.



Os resultados do estudo *in vivo* apresentado no capítulo 3 mostram que o ácido fólico foi capaz de reverter o efeito tipo-depressivo no TNF induzido por estresse de contenção durante 7 h, porém, não foi

capaz de reverter o déficit cognitivo induzido por este modelo de depressão elicitado por estímulo estressante. O ácido fólico também foi importante na prevenção da peroxidação lipídica e do aumento da atividade das enzimas antioxidantes induzida pelo estresse de contenção.

O estresse de contenção é um modelo de estresse que induz comportamento tipo-depressivo (Poleszak et al., 2006; Sevgi et al., 2006; Capra et al., 2010; O'Mahony et al., 2010; Christiansen et al., 2011; Huynh et al 2011) e prejuízo cognitivo (Baker e Kim, 2002; Nagata et al., 2009; Li et al., 2012). Nós observamos que o ácido fólico *per se* foi capaz de induzir comportamento tipo-antidepressivo no protocolo utilizado (depois de 8 horas e 40 minutos do tratamento) e, além disso, foi capaz de reverter o aumento do tempo de imobilidade induzido pelo estresse de contenção. Estes dados corroboram com dados da literatura que monstram que o ácido fólico apresenta efeito-antidepressivo no TNF e TSC em camundongos e ratos (Brocardo et al., 2008a, 2008b, 2009; Molina-Hernández et al. 2011; 2012; Molina-Hernández e Tellez-Alcantara 2011). Além disso, nós observamos que o tratamento com ácido fólico *per se* pode aumentar a exploração do objeto novo em relação ao objeto original quando comparado ao grupo controle (vehicle/non-stressed) na sessão teste do teste de reconhecimento de objeto (TRO). Por outro lado, o ácido fólico não foi efetivo em reverter o déficit cognitivo induzido pelo estresse de contenção. Estudos da literatura indicam que ácido fólico é efetivo em melhorar a performance cognitiva de ratos idosos (Singh et al., 2011) e proteger contra o déficit cognitivo induzido por hiperhomocisteína (Matté et al., 2007; Matté et al., 2009b) em roedores. Consequentemente há indícios de que a deficiência desta vitamina está associada ao prejuízo cognitivo em roedores (Troen et al., 2008). Além disso, muitos estudos clínicos indicam que o ácido fólico é importante na cognição, e a sua deficiência pode ser um fator de risco para o prejuízo cognitivo (Kado et al., 2005; Ramos et al., 2005; Durga et al 2006; De Lau et al., 2007). Portanto, estes estudos podem justificar a melhor performance cognitiva do ácido fólico *per se* em relação ao grupo controle no TRO. Já a ineficácia na proteção contra o déficit cognitivo induzido por estresse de contenção, pode ser explicada pela baixa dose de ácido fólico e/ou pelo tratamento agudo. Por isso, podemos concluir que o efeito do ácido fólico foi específico para a depressão, ou seja, apresentou um efeito tipo-antidepressivo específico no modelo de depressão induzida por estresse de contenção, já que não reverteu o déficit congnitivo induzido por este modelo.

Os resulatdos do presente trabalho também indicam um desbalanço oxidativo, alteração dos marcadores de dano oxidativo para lipídios e alterações nas defesas antioxidantes em camundongos submetidos ao estresse de contenção durante 7 horas. Os resultados mostram que o comportamento tipo-antidepressivo e o prejuízo cognitivo induzido pelo estresse de contenção foram acompanhados por um significativo aumento nos níveis de TBARS no córtex cerebral e hipocampo de camundongos. Nossos resultados corroboram com dados da literatura, os quais mostram que o estresse de contenção realizado em diferentes tempos, induziu aumentos significativos de MDA no cérebro de roedores (García-Bueno et al., 2005; Kumari et al., 2007; Zafir e Banu, 2007; Kumar e Goyal, 2008; Zafir et al., 2009; Kumar et al., 2010). Suportando nossos dados, estudos clínicos indicam níveis aumentados de MDA em pacientes deprimidos, indicando uma forte associação entre estresse oxidativo e depressão (Bilici et al., 2001; Khanzode et al., 2003). Portanto, consistente com dados da literatura, nossos dados sugerem que o estresse de contenção durante 7 horas causa peroxidação lipídica no córtex cerebral e hipocampo de camundongos, duas estruturas extremamente relacionadas com a fisiopatologia da depressão (Bennett, 2011; Duman e Voleti, 2011; Yu e Chen, 2011). Considerando que a peroxidação lipídica é uma das maiores consequências de dano oxidativo mediado por radicais livres no cérebro (Dotan et al., 2004), o presente estudo sugere que as alterações comportamentais observadas com o estresse de contenção pode estar associada com esta alteração neuroquímica (aumento de TBARS).

Foi possível observar no presente estudo que a peroxidação lipídica induzida por estresse agudo de contenção foi revertida pelo tratamento com ácido fólico no hipocampo, mas não no córtex cerebral. Este achado no hipocampo é similar aos resultados encontrados por Brocardo et al. (2010), os quais mostraram que o tratamento com ácido fólico preveniu o aumento da peroxidação lipídica induzida por ouabaína no hipocampo. Porém, neste estudo, também foi observado este efeito neuroprotetor no córtex, efeito que não foi observado no presente estudo (Brocardo et al., 2010). Isso indica que o efeito do ácido fólico, no modelo de estresse de contenção, é hipocampo específico. Além disso, outros estudos mostram que o ácido fólico protege contra a peroxidação lipídica em diferentes regiões cerebrais de ratos (Singh et al., 2011) e a deficiência de ácido fólico induz aumento nos níveis de TBARS no hipocampo (Chen et al., 2011), fígado (Huang et al., 2001; Chen et al., 2011) e em cultivo de células humanas Hep G2 (Chern et al., 2001). É importante mencionar que a suplementação com ácido

fólico em pacientes com hiperhomocisteinemia promoveu prevenção parcial da peroxidação lipídica plasmática (Racek et al., 2005). Portanto, o ácido fólico pode ser importante na detoxificação de peróxidos lipídicos, possivelmente devido a sua capacidade de atuar como *scavanger* de EROs (Joshi et al., 2001).

O presente estudo também encontrou um aumento das atividades da SOD (somente no hipocampo), CAT, GR e GPx (córtex cerebral e hipocampo) em camundongos estressados, indicando alterações nas defesas antioxidantes em camundongos expostos ao estresse agudo de contenção que apresentaram comportamento tipo-depressivo associado a déficit cognitivo.

As principais enzimas antioxidantes envolvidas diretamente na neutralização das EROs são SOD, CAT, GPx e GR. A SOD é a primeira linha de defesa contra as EROs e cataliza a dismutação do radical ânio superóxido ($O_2^{\cdot}$) ao peróxido de hidrogênio (H_2O_2) (McCord e Fridovich, 1988) que pode ser reduzido a H_2O e O_2 pela CAT (Chelikani et al., 2004) ou GPx (Flohe, 1971). Além de detoxificar H_2O_2, GPx pode reduzir hidroperóxidos lipídicos e não-lipídicos às custas da glutationa (GSH), a qual, torna-se oxidada, formando a glutationa dissulfeto (GSSG) (Flohe, 1971). No presente estudo, as atividades aumentadas destas enzimas antioxidantes em resposta ao estresse de contenção corroboram com vários estudos clínicos. Por exemplo, estudos mostram que pacientes deprimidos podem apresentar atividade aumentada da GR no plasma, e atividade aumantada da GPx (Bilici et al., 2001), SOD (Bilici et al., 2001; Galecki et al., 2009; Kotan et al., 2011) e da CAT (Galecki et al., 2009) nos eritrócitos. Um estudo, *post-mortem* de pacientes deprimidos, encontrou a atividade da SOD aumentada no cortex pré-frontal destes pacientes (Michel et al., 2007). Similarmente, estudos pré clínicos também mostram estas alterações, como o estudo de Fontella et al. (2005), o qual mostrou que o estresse crônico de contenção (1 h/dia durante 40 dias) induziu aumento da atividade da GPx no hipocampo de rato. Kim et al. (2005) encontraram atividades aumentadas da SOD e CAT no cérebro de camundongos submetidos ao estresse agudo de contenção (2 h/dia durante 3 dias). E mais recentemente, um estudo realizado por Balk et al. (2010) encontrou aumentada a atividade da CAT no estriado de ratos submetidos ao estresse crônico de contenção (1 h/dia durante 40 dias).

Estudos em que utilizam protocolos de estresse como modelos animais tem encontrado diferentes efeitos nas atividades das enzimas antioxidantes. Especialmente, estudos pré-clínicos, tem observado um aumento na atividade das enzimas antioxidantes em animais submetidos

a modelos repetidos ou crônicos de estresse de contenção (Fontella et al., 2005; Kim et al., 2005; Balk et al., 2010), enquanto que em protocolos de estresse de contenção agudo (somente uma exposição), foi encontrado redução nas atividades da CAT e GR (Kumari et al., 2007; Kumar e Goyal, 2008; Kumar et al., 2010), resultados que contrapõem os resultados do presente trabalho. Além disso, estudos realizados com estresse crônico de contenção (4 h/21 dias) encontraram atividades reduzidas da SOD, CAT e GR (Zafir e Banu, 2007; Zafir et al., 2009). Estas inconsistências podem ocorrer em função de uma série de variações quanto aos procedimentos ou animais utilizados, como idade e sexo dos animais, intensidade, duração, freqüência e tipo do estressor (Buynitsky e Mostofsky, 2009).

As atividades aumentadas da SOD, CAT, GPx e GR (principalmente no hipocampo) encontradas neste estudo corroboram com alguns estudos pré-clínicos (Fontella et al., 2005; Kim et al., 2005; Balk et al., 2010) e clínicos (Bilici et al., 2001; Michel et al., 2007; Galecki et al., 2009; Kotan et al., 2011). O aumento nas atividades das defesas antioxidantes pode ter ocorrido em função de uma resposta compensatória ao aumento da formação de radicais livres, induzido pelo estresse agudo de contenção em camundongos. Associados com os resultados do TBARS, estes dados suportam a idéia de que o estresse agudo de contenção é capaz de induzir a formação das EROs. Portanto, o aumento do nível de TBARS (cortex cerebral e hipocampo) e o aumento compensatório das atividades da SOD, CAT, GPx e GR (especialmente no hipocampo) em camundongos submetidos ao estresse agudo de contenção pode ser correlacionado como o efeito tipo-depressivo no TNF e o déficit cognitivo no TRO.

Nossos resultados mostram que ácido fólico foi capaz de restaurar as alterações nas atividades da SOD, CAT, GPx e GR induzidas pelo estresse agudo de contenção em camundongos, especialmente no hipocampo. Este efeito protetor do ácido fólico pode ser em consequência da redução, induzida por ácido fólico, na formação de radicais livres e estresse oxidativo, uma vez que a SOD, CAT, GPx e GR são as maiores enzimas antioxidantes envolvidas diretamente na neutralização das EROs (McCord e Fridovich, 1988). Nossos resultados corroboram com dados de Matté et al. (2009a) os quais mostraram que a suplementação com ácido fólico reverteu o aumento das atividades da SOD e GR no sangue de ratos com hiperhomocisteinemia. O ácido fólico apresenta propriedades antioxidantes intrínsecas e pode eliminar as EROs (Patro et al., 2006; Joshi et al., 2001). Além disso, esta vitamina apresenta importante papel na síntese e estabilidade do DNA,

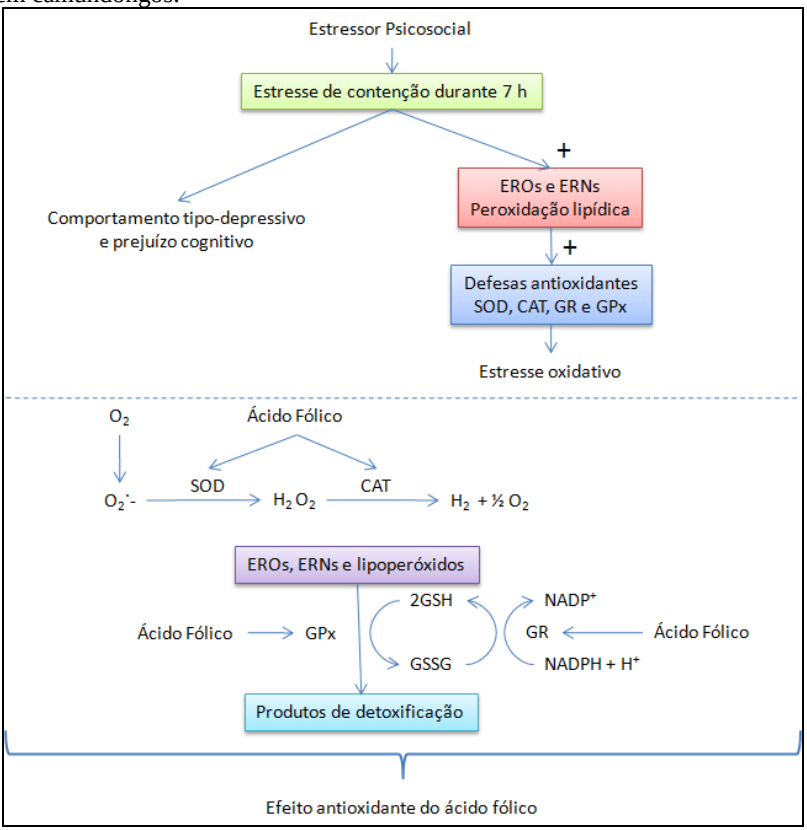
previne a incorporação errônea de uracila, a hipometilação e quebra da fita de DNA (Fenech, 2001; Fenech et al., 2005). Além disso, muitos outros estudos, não menos importantes, mostram que o ácido fólico é um potente agente neuroprotetor (Ho et al., 2003; Tjiattas et al., 2004; Lin et al 2004; Yu et al., 2009).

É importante mencionar que os resultados do presente estudo mostram que o tratamento com ácido fólico em camundongos submetidos ao estresse agudo de contenção afeta diferentemente o *status* oxidativo do hipocampo e do córtex cerebral, uma vez que o ácido fólico protege contra o estresse oxidativo induzido pelo estresse de contenção, especialmente no hipocampo. Isso sugere que estas áreas cerebrais apresentam diferenças específicas nos mecanismos de defesas quando submetidas a um estímulo estressante. Estas diferenças podem justificar as diferentes susceptibilidades de distintas áreas do cérebro ao tratamento com ácido fólico frente a um estímulo estressante.

Nossos resultados mostram que não houve nenhuma alteração nos níveis da glutationa (GSH) tanto no córtex cerebral quanto no hipocampo de camundongos, independente das condições de estresse ou do tratamento com ácido fólico. Nossos resultados corroboram com o estudo de Méndes-Cuesta et al. (2011), o qual observou ausência de alteração nos níveis de GSH no cérebro de ratos submetidos ao estresse de contenção durante 24 h. GSH é o mais importante antioxidante endógeno não-enzimático que pode ser regenerado pela GR com o consumo de nicotinamida adenina dinucleotídio fosfato na forma reduzida (NADPH) (Krohne-Ehrich et al., 1977). Este antioxidante não-enzimático desempenha um importante papel na detoxificação de uma grande variedade de xenobióticos eletrofílicos, reduzindo a produção de compostos tóxicos (Jakoby, 1978). A ausência de alterações nos níveis de GSH como resultado do estresse e do tratamento com ácido fólico sugere que este antioxidante não desempenha um papel importante neste modelo, sob as condições experimentais empregadas neste estudo.

Portanto, estresse psicosocial, como o estresse de contenção pode induzir comportamento tipo-depressivo no TNF e déficit cognitivo no TRO, acompanhados por níveis aumentados de TBARS (córtex cerebral e hipocampo) e atividades aumentadas das enzimas antioxidantes (SOD, CAT, GPx e GR), principalmente no hipocampo. Ácido fólico foi capaz de reverter o efeito tipo-depressivo, mas não o déficit cognitivo, bem como reverter os níveis aumentados de TBARS e as atividades aumentadas das enzimas antioxidantes (SOD, CAT, GPx e GR), principalmente no hipocampo **(Figura 11)**.

Figura 11. Esquema ilustrativo do provável efeito antioxidante do ácido fólico em um modelo animal de depressão induzida por estresse de contenção no TNF em camundongos.



Os resultados do presente estudo mostram que um estressor psicosocial, como o estresse de contenção durante 7 horas, induz comportamento tipo-depressivo no TNF e déficit cognitivo no TRO. Paralelamente, o estresse de contenção induziu peroxidação lipídica (evidenciado pelos níveis aumentados de TBARS no córtex cerebral e hipocampo) e provavelmente através de uma resposta compensatória, induziu aumento nas atividades das enzimas antioxidantes (CAT, GR, GPx, no córtex cerebral e hipocampo e SOD, somente no hipocampo), induzindo estresse oxidativo. Por outro lado, ácido fólico foi capaz de reverter o comportamento tipo-depressivo, mas não o déficit cognitivo. Também foi capaz de reverter o aumento de TBARS e reverter o aumento das atividades da SOD, CAT, GR e GPx no hipocampo, revertendo o estresse oxidativo induzido pelo estresse de contenção, indicando um importante papel antioxidante do ácido fólico. CAT, catalase; GPx, glutationa peroxidase; GR, glutationa redutase; ERNs, espécie reativa de nitrogênio; EROs, espécie reativa

de oxigênio; SOD, superóxido dismutase; TBARS, espécies reativas ao ácido tiobarbitúrico; TNF, teste do nado forçado; TRO, teste do reconhecimento de objeto.

Em conclusão, os resultados *in vivo* descritos nos capítulos 1, 2 e 3 mostram que o efeito antidepressivo do ácido fólico no TNF em camundongos pode ser mediado, pelo menos em parte, por uma modulação da via PI3K/Akt/GSK-3β, ativação do PPARγ e inibição de canais de K^+, reforçando a noção de que estes são importantes alvos para a atividade antidepressiva. Além disso, o tratamento com ácido fólico foi capaz de reverter o comportamento tipo-depressivo induzido pelo estresse agudo de contenção (7 h), mas não o déficit cognitivo induzido por este modelo. A prevenção, pelo tratamento com ácido fólico, da alteração comportamental induzida pelo estresse de contenção foi acompanhada pela reversão do dano oxidativo (níveis aumentados de TBARS e atividades aumentadas das enzimas antioxidantes SOD, CAT, GPx e GR) induzido por este estínulo estressante, reforçando a idéia que o ácido fólico pode ser uma importante vitamina contra o estresse psicossocial e oxidativo.

Os resultados *in vitro* do presente trabalho encontram-se descritos em dois capítulos. Os resultados do capítulo 4 demonstram que o ácido fólico pode proteger células SH-SY5Y da toxicidade induzida por dexametasona por envolvimento da via PI3K/Akt, CaMKII e PKA. Os resultados do capítulo 5 evidenciaram que esta vitamina foi capaz de proteger fatias hipocampais de ratos contra a morte celular induzida por glutamato via modulação da via PI3K/Akt/GSK-3β/β-catenina e inibição da iNOS.

Os resultados descritos no capítulo 4 mostram que dexametasona (1 mM) causou dano em células neuroblastoma humano SH-SY5Y e ácido fólico (300 uM) foi capaz de proteger contra esta injúria. Além disso, nós investigamos a via de transdução de sinal que regula a resposta neuroprotetora do ácido fólico neste protocolo de morte celular. A morte celular induzida por dexametasona foi bloqueada por inibidores de cinases como o LY294002, KN-93 e H-89, mas não por PD98059 ou queleritrina. Portanto, nós mostramos que a proteção induzida por ácido fólico neste protocolo experimental foi provavelmente mediada por ativação da via de sinalização PI3K/Akt, CaMKII e PKA mas não pela via MEK1/2 e PKC.

Estímulos estressores (físicos e psicológicos) ativam o eixo HPA, culminando na liberação pela glândula adrenal de glicocorticóides (cortisol em humanos e corticosterona em roedores), os quais são

responsáveis pelo *feedback* inibitório, principalmente no hipocampo e glândula pituitária, que regula a secreção destes hormônios (Kunugi et al., 2010). Dependendo da intensidade ou duração do estímulo estressante, a resposta ao estresse pode tornar-se patológica, induzindo hiperativação do eixo HPA, fenômeno envolvido em diferentes transtornos como doença de Cushing, transtorno do estresse pós-traumático, transtorno bipolar e depressão (Conrad, 2008; Yu et al., 2008; Kunugi et al., 2010). Corroborando com esta hipótese, Haynes et al. (2004) mostraram que o tratamento crônico com antidepresivos resultou na proteção contra morte neuronal induzida por dexametasona no estrido e hipocampo de ratos.

Considerando que a hipersecreção de glicocorticóides está presente em muitos pacientes deprimidos (Wolkowitz et al., 2009), neste estudo nós utilizamos um modelo de morte celular que mimetiza a hipersecreção de glicocorticóides por incubação das células SH-SY5Y com dexametasona, um glicocorticóide sintético (Mitchell et al., 1998; Haynes et al., 2001; Jacobs et al., 2006; Zhu et al., 2006; Tazik et al., 2009). Nossos dados mostram que a dexametasona (1 mM) induziu morte neuronal, corroboram com a dados da literatura, uma vez que relatam dano neuronal *in vivo* e *in vitro*, causado por glicocorticóides (Woolley et al., 1990; Virgin et al., 1991; Hassan et al., 1996; Mitchell et al., 1998; Ahlbom et al., 2000; Joels, 2001; Lu et al., 2003; Crochemore et al., 2005; Zhu et al., 2006). Muitos mecanismos podem estar implicados no dano induzido por glicocorticóides, como a inibição da via PI3K/Akt (Nuutinen et al., 2006), excitotoxicidade glutamatérgica (Sapolsky, 2000) e prejuízo na função e expressão do fator neurotrófico BDNF (Kumamaru et al., 2008; Kunugi et al., 2010).

Neste estudo, 24 horas de pré-incubação com ácido fólico (10-300 µM) e co-incubação de ácido fólico mais dexametasona por mais 48 horas, reduziu a neurotoxicidade induzida por dexametasona, de forma dependente de concentração. A maxima proteção ocorreu com 300 µM de ácido fólico. Dados da literatura indicam que a deficiência de ácido fólico pode induzir morte celular por aumeto do Ca^{2+} e EROs (Ho et al., 2003; Tjiattas et al., 2004). Além disso, ácido fólico pode proteger contra o dano causado por NMDA e glutamato em cultura de neurônios granulares cerebelares de camundongos (Lin et al 2004) e contra o prejuízo causado por peptídio $A\beta_{25-35}$ em neurônios (Yu et al., 2009).

Adicionalmente, nossos resultados indicam que a presença de LY294002, um inibidor da PI3K, parcialmente reverteu o efeito neuroprotetor do ácido fólico em células expostas à dexametasona. A via de sinalização PI3K é muito importante para o crescimento e

sobrevivência neuronal (Brunet et al., 2001; Hennessy et al. 2005; Shao et al., 2010) e em alguns casos, é necessária para neuroproteção mediada por fatores tróficos, como o fator de crescimento tipo-insulina (IGF-1) em protocolos de morte celular induzida por corticosterona em neurônios hipocampais (Nitta et al., 2004). Além disso, a dexametasona pode reduzir a proliferação e induzir apoptose em cultivo de condrócitos através da inibição desta via (Chrysis et al., 2005). Para reforçar nossos dados, Seto et al. (2010) mostraram que o ácido fólico pode ser benéfico em um modelo de hipertensão associado a diabetes mellitus, por ativação da via PI3K/Akt. Corroborando com estes dados, nossos resultados indicam que o LY294002 parcialmente bloqueou o efeito neuroprotetor do ácido fólico contra a morte celular induzida por dexametasona nas células SH-SY5Y. Estes resultados reforçam que a via de sinalização PI3K/Akt pode estar implicada no efeito neuroprotetor do ácido fólico nestas células.

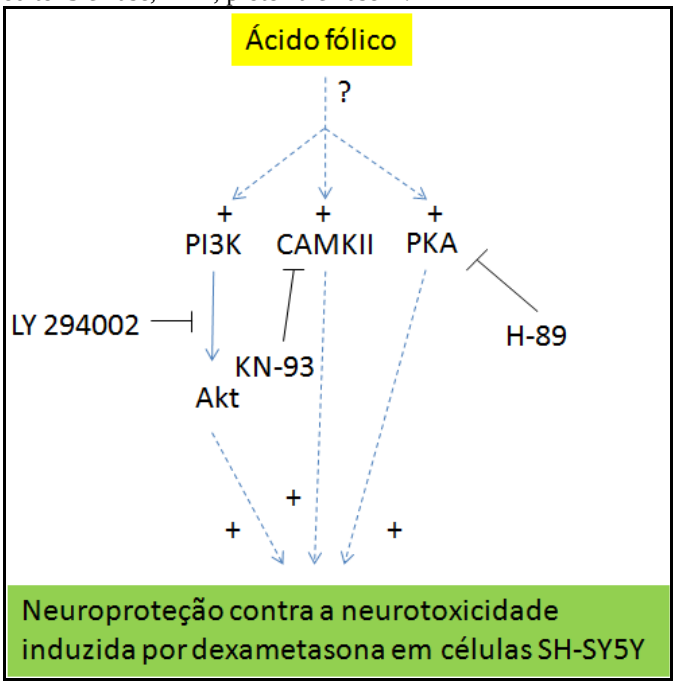
Nossos resultados também mostram que PD98059, inibidor da MEK1/2, e cheleritrina, inibidora da PKC, não bloquearam o efeito neuroprotetor do ácido fólico contra a morte celular induzida por dexametasona. Estes resultados sugerem que esta via de sinalização não participa no efeito neuroprotetor do ácido fólico neste protocolo de insulto celular.

CaMKII (Cálcio calmodulina cinase II) é a uma proteína cinase multifuncional que responde a aumentos intracelular de Ca^{2+} e fosforila uma grande variedade de substratos e portanto está implicada na transmissão sináptica (Houston et al., 2009). É portanto, considerada a mais abundante proteina cinase envolvida na plasticidade neuronal (Popoli et al., 2000; Cammarota et al., 2002;). Nossos resultados mostram que KN-93, inibidor da CaMKII, suprimiu o efeito neuroprotetor do ácido fólico, indicando que a ativação desta via de sinalização pode estar envolvida no efeito neuroprotetor do ácido fólico neste protocolo de morte celular.

Finalmente nós também mostramos que H-89 reverteu o efeito protetor do ácido fólico contra a morte celular induzida por dexametasona. O H-89 é um inibidor competitivo da PKA, que por sua vez, está envolvida em várias funções fisiológicas no cérebro, incluindo síntese e liberação de neurotrasmissores, expressão gênica, plasticidade sináptica, memória, crescimento, diferenciação e sobrevivência celular (D'Sa e Duman, 2002; Gould e Manji, 2002; Blendy 2006). Em nosso estudo, o H-89 foi capaz de reverter a neurotoxicidade induzida por dexametasona nas células SH-SY5Y, sugerindo que esta via de sinalização está implicada no efeito neuroprotetor do ácido fólico.

Portanto, os resultados deste estudo indicam que a dexametasona pode induzir morte em células SH-SY5Y e o ácido fólico pode proteger contra esta morte celular, pelo menos em parte por uma ativação de alvos moleculares como PI3K/Akt, CaMKII, e PKA, mas não MEK1/2 e PKC **(Figura 12)**.

Figura 12. Esquema ilustrativo da provável via de sinalização ativada por ácido fólico na proteção contra a morte celular induzida por dexametasona em células neuroblastoma humano SH-SY5Y. Os resultados do presente estudo mostram que o ácido fólico pode proteger contra a morte celular induzida por dexametasona em células SH-SY5Y por ativação de alvos moleculares como a PI3K/Akt, CaMKII e PKA. A pré-incubação destas células com LY294002 (inibidor da PI3K/Akt), KN-93 (inibidor da CaMKII) ou H-89 (inibidor da PKA) reverteu o efeito neuroprotetor do ácido fólico contra a morte celular induzida por dexametasona. CaMKII, cálcio calmodulina cinase II; PI3K, fosfoinoditol 3 cinase; PKA, proteína cinase A.



No capítulo 5 apresentamos dados que mostram que o ácido fólico foi capaz de proteger fatias hipocampais de ratos jovens contra a excitotoxicidade glutamatérgica (redução da viabilidade celular e

liberação de glutamato). Este efeito neuroprotetor pode envolver a modulação da via PI3K/Akt/GSK-3β/β-catenina e inibição da iNOS.

Neste estudo, nós utilizamos um protocolo *in vitro* de morte cellular induzida pelo glutamato em fatias de hipocampo de ratos jovens. A excitotoxicidade mediada por receptores glutamatérgicos está associada com um grande número de condições patológicas ou transtornos neurológicos (Sanacora et al., 2008; Dong et al., 2009; Lau e Tymianski, 2010). Em certas condições patológicas, por exemplo, na injúria ou isquemia cerebral, o glutamato cerebral é rapidamente elevado, o qual resulta em ativação excessiva dos seus receptores e consequentemente, induz injúria neuronal (Lau e Tymianski, 2010). A excitotoxicidade pode ser modificada através da remoção do glutamato da fenda sináptica, função esta, desempenhada por transportadores de glutamato acoplados à atividade da Na^+/K^+-ATPase presente nos astrócitos e em menor extensão, nos neurônios (Anderson e Swanson, 2000; Danbolt, 2001; Beart e O'shea, 2007; Markowitz et al., 2007; Sheldon e Robinson, 2007). Portanto, estes transportadores removem o excesso de glutamato dos espaços extracelulares para prevenir a excitotoxicidade (Choi et al., 1987; Danbolt, 2001) e modular a transmissão sináptica (Huang e Bergles, 2004). Porém, quando ocorre dano nos transportadores de glutamato, seja por oxidação ou depleção de ATP, pode causar o transporte reverso de glutamato e seu acúmulo nos espaços extracelulares (Rossi et al., 2000; Camacho e Massieu, 2006; Struzyńska, 2009). Molz et al. (2008b) mostraram que a morte celular de fatias hipocampais induzida por glutamato pode ocorrer via transporte reverso deste neurotransmissor, uma vez que, um inibidor do transporte de glutamato, DL-TBOA, reverteu a redução da viabilidade celular e a liberação de L-[3H]glutamato induzidas por glutamato.

O presente trabalho mostrou que o tratamento de fatias hipocampais com ácido fólico (100 μM) protegeu contra a redução da viabilidade celular induzida por glutamato. Além disso, nossos resultados também indicaram que o glutamato induziu liberação de D-[3H]aspartato (glutamato) em fatias hipocampais e o ácido fólico reverteu este efeito. Estes achados suportam a idéia de que o ácido fólico reduz a hiperexcitabilidade glutamatérgica por reverter a redução da viabilidade celular e inibir a liberação excessiva de glutamato. Portanto, esta vitamina pode agir com um neuroprotetor endógeno, prevenindo a excitotoxicidade. De fato, um estudo de Lin et al. (2004), demonstrou que o ácido fólico foi capaz de proteger contra a toxicidade induzida por glutamato/NMDA em células granulares cerebelares de roedores. Além disso, resultados *in vivo* obtidos por nosso grupo

mostrou que o efeito tipo-antidepressivo do ácido fólico pode envolver a inibição de receptores NMDA, implicando a modulação do sistema glutamatérgico no efeito desta vitamina (Brocardo et al., 2008).

É importante mencionar que existem poucos estudos *in vitro* associando ácido fólico e glutamato. Portanto, os achados do presente trabalho tornam-se importantes para a comunidade científica, uma vez que, as evidências do presente trabalho mostram uma forte asociação entre ácido fólico e o sistema glutamatérgico *in vitro*.

O presente estudo mostrou que a pré-incubação com LY294002, um inibidor da PI3K, aboliu significativamente o efeito protetor do ácido fólico contra a redução da viabilidade celular induzida pelo glutamato. Além disso, a redução da liberação do D-[3H]aspartato (glutamato) induzida por ácido fólico foi abolida pela presença de LY294002. Estas evidências sugerem que o ácido fólico pode proteger contra a excitotoxicidade glutamatérgica (redução da viabilidade celular e liberação de D-[3H]aspartato) por ativação da PI3K.

Como mencionada anteriormente a PI3K é uma cinase lipídica que participa de muitas funções fisiológicas no cérebro e é utilizada, principalmente, por neurotrofinas para mediar a plasticidade neuronal, sobrevivência, proliferação, migração celular e inibição da apoptose (Katso et al., 2001; Marone et al., 2008; Beaulieu et al., 2009; Beaulieu, 2012; Mellor et al., 2012).

Para reforçar nossa hipótese, como mencionado anteriormente, dados da literatura mostram envolvimento da ativação da PI3K no efeito protetor *in vivo* do ácido fólico (Seto et al., 2010). Além disso, estes resultados corroboram com os nossos dados prévios *in vitro* encontrados no capítulo 4 e *in vivo* no capítulo 1 deste trabalho.

Com a finalidade de investigar o mecanismo *downstream* da PI3K envolvido no efeito neuroprotetor do ácido fólico contra a excitotoxicidade glutamatérgica (redução da viabilidade celular e liberação de D-[3H]aspartato), nós verificamos a participação da GSK-3β no efeito desta vitamina. Nós observamos que a pré-incubação de fatias hipocampais com ácido fólico (durante 30 minutos) foi suficiente para aumentar a forforilação da GSK-3β no resíduo de serina 9 (Ser9), porém este aumento não foi observado depois de 60 minutos de incubação com esta vitamina . Este rápido efeito do ácido fólico em aumentar a fosforilação da GSK-3β no sítio Ser9 (inibição da GSK-3β) pode estar relacionado com o efeito neuroprotetor desta vitamina contra o dano causado pelo glutamato avaliado 6 horas depois da retirada do glutamato do meio de incubação das fatias. Para reforçar esta hipótese,

nós avaliamos a fosforilação Ser9 da GSK-3β decorridos 6 horas de incubação das fatias em meio de cultura depois da retirado do glutamato. Foi observado que somente o grupo pré-incubado com ácido fólico e incubado com glutamato foi capaz de aumentar a forforilação Ser9 da GSK-3β, ou seja, inibição da GSK-3β. Isso indica que o ácido fólico *per se* em fatias incubadas durante 6 horas, não aumenta a fosforilação Ser9 da GSK-3β, mas frente a um estímulo tóxico, como o glutamato, protege a célula contra o dano celular, pelo menos em parte, por inibir a GSK-3β. Além disso, a incubação com ácido fólico *per se* durante 30 minutos foi capaz de aumentar a fosforilação Ser9 da GSK-3β. Este efeito do ácido fólico *per se* não foi observado depois de 6 horas de incubação, indicando que o efeito do ácido fólico em aumentar a forforilação Ser9 da GSK-3β, frente a um estímulo tóxico (glutamato), foi mantido para proteger os neurônios da morte celular induzida pelo glutamato. Estes dados corroboram com os dados *in vivo* decritos no capítulo 1 deste trabalho que mostra que o efeito tipo-antidepressivo do ácido fólico pode envolver a inibição da GSK-3β, indicando que GSK-3β pode ser um importante alvo de sinalização *in vivo* e *in vitro* envolvido no efeito do ácido fólico.

A GSK-3, como mencionado anteriormente, é uma serina/treonina cinase altamente expressa no cérebro (Woodgett, 1990; Yao et al., 2002; Perez-Costas et al., 2010), em neurônios e células da glia (Ferrer et al., 2002). A isoforma, GSK-3β, objeto de estudo do presente trabalho, desempenha importantes funções nos mecanismos de sobrevivência celular (Kaidanovich-Beilin e Woodgett, 2011; Sutherland, 2011).

Afim de confirmar a participação da inibição da GSK-3β no efeito neuroprotetor do ácido fólico contra o dano induzido por glutamato, nós analisamos a expressão da β-catenina decorridas 6 horas de incubação depois da retirada do glutamato. Os resultados mostram que o grupo pré-incubado com ácido fólico e incubado com glutamato foi capaz de aumentar a expressão da β-catenina, similar aos resultados da fosforilação da GSK-3β.

A β-catenina é uma proteína citoplasmática, um mediador *downstream* da via de sinalização Wnt, a qual regula um grande número de genes essenciais para a proliferação e diferenciação celular (Takahashi-Yanaga e Sasaguri, 2007; Bauer e Willert, 2012). No citoplasma, o nível de β-catenina é mantido baixo através de uma contínua ubiquitinação proteossômica mediada pela degradação regulada pelas proteínas adenomtous polyposis coli (APC), caseína

cinase 1α (CK1α) e GSK-3β (Takahashi-Yanaga e Sasaguri, 2007). Focando na GSK-3β, uma vez ativada, induz degradação da β-catenina, através da fosforilação da mesma. Em contraste, a inibição da GSK-3β culmina na defosforilação e estabilização da β-catenina, resultando em acúmulo da mesma, a qual transloca para o núcleo e induz transcrição, em cooperação com fatores de transcrição Tcf/Lef, de genes alvos da via Wnt, promovendo efeitos antiapoptóticos e estimulando o crescimento neuronal (Coyle e Duman, 2003). Portanto, os resultados do presente estudo, indicam que o ácido fólico pode promover a sobrevivência neuronal contra o dano induzido por glutamato através do acúmulo de β-catenina, provavelmente em resposta a inibição da GSK-3β.

Nossos resultados também demonstraram que a presença de LY294002 reverteu o efeito do ácido fólico em aumentar a fosforilação ser9 da GSK-3β e a expressão da β-catenina frente ao estímulo tóxico induzido pelo glutamato. Estes resultados podem confirmar que a ativação da PI3K está envolvida no efeito neuroprotetor do ácido fólico e que a ativação da PI3K pode culminar na inibição da GSK-β e acúmulo de β-catenina, indicando também que o efeito neuroprotetor do ácido fólico pode envolver a ativação da Akt. Akt pode ser uma ligação entre a ativação da PI3K por ácido fólico e modulação da via GSK-β/β-catenina, já que a ativação da PI3K induz ativação da Akt que por sua vez, inibe a GSK-β, induzindo acúmulo de β-catenina **(Figura 13)**. Realmente, está bem documentado que a Akt, também denominada protein cinase B (PKB), é regulada através da sinalização mediada pela PI3K, uma vez que a Akt é alvo *downstream* da PI3K. Akt, por sua vez, inibe GSK-3β, que consequentemente promove acúmulo de β-catenina, induzindo sobrevivência neuronal (Toledo et al., 2008; Beaulieu et al., 2009; Sutherland, 2011; Beaulieu, 2012).

Já é bem reconhecido que glutamato desempenha um importante papel na neuroinflamação, uma vez que este aminoácido excitatório pode induzir a síntese de citocinas pró-inflamatórias (interleucina 1β, IL-1β, e fator de necrose tumoral-α, TNF-α), as quais induzem a expressão da iNOS (Cardenas et al., 2000; Moro et al., 2004). Um estudo realizado por Molz et al. (2011) mostrou que glutamato promoveu aumento da expressão da iNOS em fatias hipocampais de ratos jovens. Similarmente, nossos resultados mostram que glutamato provocou indução da iNOS, no mesmo protocolo experimental utilizado por Molz et al. (2011). Já está bem documentado que a iNOS é induzida principalmente em células da glia (microglia e astrócitos) e esta isoforma da iNOS pode induzir morte neuronal em situações patológicas (Bal-Prince e Brown, 2001; Brown e Bal-Prince, 2003; Brown, 2010).

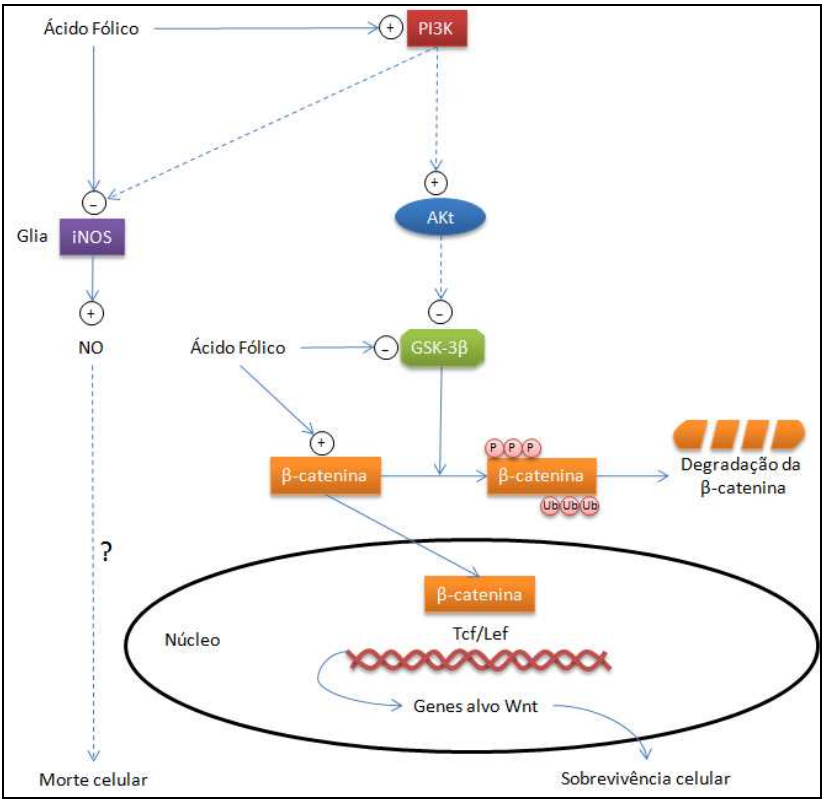
Existem muitos mecanismos pelos quais a glia ativada pode matar neurônios. O mecanismo primário envolve a expressão aumentada de iNOS, que produz NO. Esta molécula, por sua vez (i) causa mobilização de Ca^{2+} a partir do retículo endoplasmático e (ii) inibe a respiração motocondrial nos neurônios, culminando em liberação de glutamato. Estes eventos levam à morte neuronal. Além disso, a microglia ativada pode também liberar glutamato, que ativa receptores NMDA e induz formação de EROs, as quais reagem com o ânio superóxido, produzindo peróxido nitrito, culminando em estresse oxidativo e morte celular (Brown, 2010).

Neste trabalho nós observamos que o ácido fólico foi capaz de prevenir o aumento da expressão da iNOS induzida por glutamato. Os nossos dados concordam com dados da literatura que mostram que o ácido fólico induz neuroproteção frente a diferentes estímulos tóxicos através da inibição da iNOS e consequentemente redução nos níveis de NO (Majumdar et al 2010; Figueiredo et al., 2011). Além disso, um estudo *in vivo* mostrou que o tratamento com ácido fólico significativamente atenuou os níveis de homocisteína plasmática, suprimiu a ativação da microglia e astrócitos e inibiu a expressão da iNOS e TNF-α na medula espinhal de camundongos deficientes de SOD1 e com altos níveis de homocisteína, um modelo animal de esclerose amiotrófica lateral (Zhang et al., 2008). Adicionalmente, um estudo de Feng et al. (2011) demonstrou que ácido fólico inibiu a produção de NO, TNF-α e IL-1β induzida por LPS (lipopolissacarídeo), acompanhada com a diminuição do RNAm para iNOS, TNF-α e IL-1β em células RAW264.7. Estes estudos reforçam a noção de que o ácido fólico pode induzir neuroproteção por inibir a expressão da iNOS.

Finalmente, nós também encontramos no presente estudo que a redução da expressão da iNOS induzida por ácido fólico em um modelo de dano celular induzido por glutamato em fatias hipocampais de ratos jovens, foi completamente abolida na presença de LY294002. Similar aos resultados do presente estudo, um inibidor da PI3K induziu um aumento na expressão da iNOS em resposta a LPS ou citocinas em astrócitos ou em células glial C6 de ratos, indicando que a inibição da PI3K pode ser necessária para a expressão da iNOS e produção de NO (Pahan et al., 1999). Além disso, Molz et al. (2011) mostraram que o efeito neuroprotetor da guanosina, um nucleosídeo da guanina, nas mesmas condições experimentais do presente estudo, também foi abolido pela presença de LY294002, uma importante evidência adicional que associa a via PI3K e expressão da iNOS. Portanto, o ácido fólico possivelmente pode induzir sobrevivência celular frente a um

estímulo tóxico por inibir a expressão da iNOS, seja agindo diretamente na enzima ou indiretamente, ativando a enzima PI3K **(Figura 13)**.

Figura 13. Esquema ilustrativo da provável via de sinalização ativada por ácido fólico contra a morte celular induzida por glutamato em fatias de hipocampo de ratos.



Os resultados do presente estudo, descritos no texto, mostram que o ácido fólico pode proteger contra a morte celular induzida por glutamato por ativação da via PI3K/Akt/GSK-3β/β-catenina e inibição da iNOS. Portanto, ácido fólico, frente ao estímulo tóxico do glutamato, causa sobrevivência celular provavelmente por ativar a PI3K, que por sua vez, induz inibição da GSK-3β, por ativação da Akt. GSK-3β inibida, não fosforila a β-catenina (não ocorrendo sua degradação proteossomal) induzindo acúmulo da mesma. β-catenina, por sua vez, transloca para o núcleo e se associada a fatores de transcrição (Tcf/Lef) induzindo transcrição de genes alvos da via Wnt responsáveis pela sobrevivência neuronal. Além disso, ácido fólico pode inibir a expresssão da iNOS diretamente ou através da ativação da PI3K. A iNOS, é induzida nas

células gliais através de estímulos tóxicos (excitotoxicidade glutamatérgica) e produz NO, que por uma série de mecanismos descritos no texto, causa morte neuronal. Ácido fólico, provavelmente, por inibir esta via, induz sobrevivência neuronal. GSK3β, glicogênio sintase cinase 3-β; iNOS, óxido nítrico sintase induzida; NO, óxiod nítrico, PI3K, fosfoinositol 3 cinase.

Juntos estes resultados demonstram que o ácido fólico pode proteger fatias hipocampais contra a injúria induzida por glutamato (redução da viabilidade celular, liberação de D-[3H]aspartato e indução da iNOS). Este efeito neuroprotetor do ácido fólico pode ser possivelmente mediado através da modulação da PI3K/GSK-3β/β-catenina e inibição da iNOS.

Em conclusão, os resultados *in vitro* descritos nos capítulos 4 e 5 mostram que o ácido fólico pode proteger contra diferentes insultos, dexametasona e glumato, provavelmente por ativação de vias PI3K/Akt, CaMKII e PKC ou modulação da via PI3K/Akt/GSK-3β/β-catenina e inibir iNOS, respectivamente.

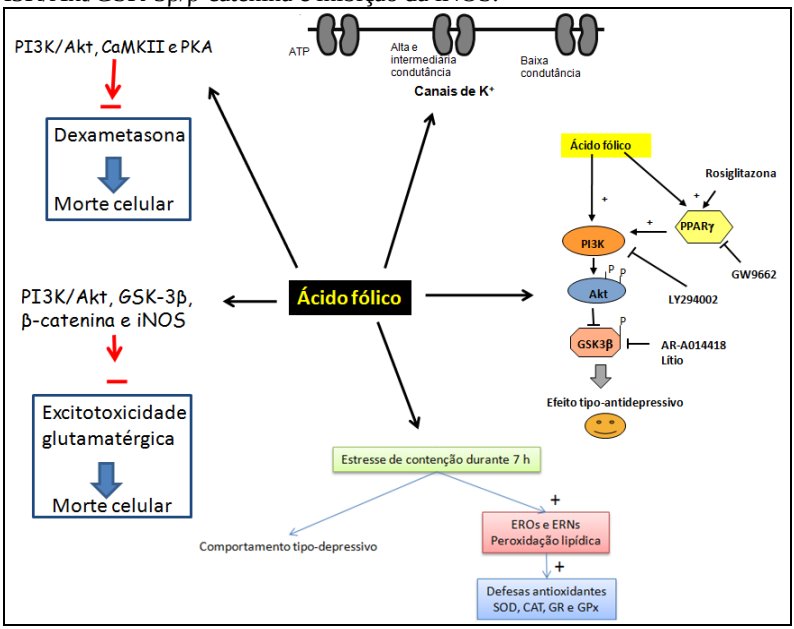
7. CONCLUSÃO

Podemos concluir com os resultados *in vivo* e *in vitro* descritos nos capítulos 1, 2, 3, 4 e 5 que:

- O efeito antidepressivo do ácido fólico no TNF em camundongos pode ser mediado, pelo menos em parte, por uma modulação da via PI3K/Akt/GSK-3β, ativação do PPARγ e inibição de canais de K^+, reforçando a noção de que estes são importantes alvos para sua atividade antidepressiva.
- O tratamento com ácido fólico pode ser um promissor agente contra o estresse, uma vez que o comportamento tipo-depressivo induzido pelo estresse agudo de contenção, mas não o déficit cognitivo, foi revertido pelo ácido fólico. Este efeito foi acompanhado pela reversão do dano oxidativo (níveis aumentados de TBARS e atividades aumentada das enzimas antioxidantes SOD, CAT, GPx e GR) induzido por este estínulo estressante no hipocampo.
- Esta vitamina pode proteger células neuronais contra a neurotoxicidade induzida pela dexametasona por ativação de vias PI3K/Akt, CaMKII e PKC.
- Ácido fólico apresenta efeito protetor contra a toxicidade glutamatérgica em fatias hipocampais de ratos possivelmente por modular a via PI3K/Akt/GSK-3β/β-catenina e inibir iNOS.

Juntos os resultados do presente estudo reforçam a idéia que o ácido fólico pode ser uma importante vitamina com propriedades antidepressivas e neuroprotetoras, mostrando um grande potencial antioxidante e modulador da via PI3K/Akt/ GSK-3β **(Figura 14)**.

Figura 14. Esquema ilustrativo da integração dos possíveis efeitos in vivo e in vitro do ácido fólico. Os resultados *in vivo* do presente trabalho indicam que o efeito tupo-antidepressivo desta vitamina pode ser mediado por uma modulação da via PI3K/Akt/GSK-3β, ativação do PPARγ e inibição dos canais de K^+. Além disso, o ácido fólico pode ser um importante agente anti-estresse e este efeito pode ser acompanhado pela atenuação do dano oxidativo (níveis aumentados de TBARS e atividades aumentada das enzimas antioxidantes SOD, CAT, GPx e GR) induzido pelo estresse de contenção no hipocampo de camundongos. Adicionamente, estudos *in vitro* indicam que o ácido fólico pode ser importante neuroprotetor contra a morte celular induzida por dexametasona por ativação de vias PI3K/Akt, CaMKII e PKC. O ácido fólico também pode ser neuroprotetor contra a excitotoxicidade glutamatérgica por modulação da via PI3K/Akt/GSK-3β/β-catenina e inibição da iNOS.



8. PERSPECTIVAS

- Realizar tratamento crônico com ácido fólico e fluoxetina por via oral durante 21 dias e avaliar o efeito antidepressivo desta vitamina no TSC, bem como avaliar o imunoconteúdo de proteínas implicadas na via de sinalização (ERK1/2, P38, Akt) e do fator neurotrófico BDNF no córtex cerebral e hipocampo.
- Avaliar o efeito tipo-antidepressivo do ácido fólico em um modelo de depressão induzida por TNF-α, uma citocina pró-inflamatória, no TSC e avaliar o possível efeito antidepressivo sinérgico de doses sub-ativas de ácido fólico combinada com doses sub-ativas de antidepressivos clássicos (fluoxetina, imipramina e bupropiona), um antagonista de receptores NMDA (MK-801) ou um inibidor da nNOS (7-nitroindazol) no modelo de depressão induzida por TNF-α, bem como o imunoconteúdo de proteínas envolvidas na via de sinalização das MPKs (proteínas cinases ativadas por mitógenos) (P38, Akt, ERK1/2 e JNK1/2) no córtex cerebral e hipocampo destes animais.
- Avaliar o efeito tipo-antidepressivo do ácido fólico em um modelo de depressão induzida pelo tratamento crônico com corticosterona durante 21 dias em testes comportamentais (TNF e TSC), verificar a atividade das enzimas antioxidantes (CAT, GR e GPx) e o conteúdo de GSH no córtex cerebral e hipocampo, bem como, analisar em cortes histológicos destas estruturas o nível de células apoptóticas por Kit de TUNEL.

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