UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:
FARMACOLOGIA E TERAPÊUTICA

Patrícia Santos

EFEITOS ANSIOLÍTICOS DA N-ACETILCISTEÍNA E DE COMPOSTOS MODULADORES DE ESTRESSE OXIDATIVO, NEUROINFLAMAÇÃO E TRANSMISSÃO GLUTAMATÉRGICA

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Orientadora: Profa. Dra. Elaine Elisabetsky
Co-orientador: Prof. Dr. Angelo Piato

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“O segredo está no equilíbrio... Equilíbrio entre a ciência e a fé, entre o trabalho e o lazer, entre se fazer respeitar e o respeito ao próximo, entre a consciência da sabedoria que já possuímos e do quanto ainda temos a aprender na vida, com todas as pessoas e situações que encontramos em nossa caminhada evolutiva”.

(Patrícia Santos)

RESUMO

Evidências crescentes na literatura demonstram que a fisiopatologia da ansiedade e transtornos relacionados é multifatorial, envolvendo a hiperatividade glutamatérgica, o estresse oxidativo e a neuroinflamação. Na primeira parte deste trabalho, através de uma revisão da literatura, mostramos que a N-acetilcisteína (NAC), agomelatina e os ácidos graxos ômega-3 são os principais agentes com mecanismo de ação multialvo envolvendo a modulação da hiperatividade glutamatérgica, do estresse oxidativo e da neuroinflamação que demonstram efeitos ansiolíticos tanto em estudos pré-clínicos quanto em ensaios clínicos. Os resultados dos ensaios clínicos são geralmente preliminares, mas revelam efeitos ansiolíticos promissores e um bom perfil de segurança e tolerância aos efeitos adversos desses agentes. A NAC, a agomelatina e os ácidos graxos ômega-3 mostram efeitos benéficos em condições clínicas relacionadas a ansiedade nas quais os ansiolíticos disponíveis atualmente apresentam eficácia modesta, sendo considerados candidatos promissores a ansiolíticos inovadores, efetivos e bem tolerados pelos pacientes. Mostramos também que a NAC possui efeitos ansiolíticos em camundongos nos testes de campo aberto, claro/escuro, placa-perfurada, interação social e hipertermia induzida por estresse, tanto após o tratamento agudo quanto subagudo (4 dias). Esses resultados fornecem evidências adicionais para sustentar a validade dos ensaios clínicos com NAC no contexto dos transtornos de ansiedade, destacando-se o perfil de segurança de uso da NAC em longo prazo em comparação com ansiolíticos como o diazepam. A NAC é um fármaco seguro, de baixo custo e que demonstra benefícios em outras condições psiquiátricas que frequentemente se apresentam em comorbidade com os transtornos de ansiedade.

Palavras-chave: ansiedade, modelos animais, N-acetilcisteína, agomelatina, ômega-3

ABSTRACT

Increasing evidence in the literature demonstrates that the pathophysiology of anxiety and related disorders is multifactorial, involving glutamatergic hyperactivity, oxidative stress, and neuroinflammation. In the first part of this work, through a review of the literature, we showed that N-acetylcysteine (NAC), agomelatine and omega-3 fatty acids are the main agents with multi-target mechanism of action involving the modulation of glutamatergic hyperactivity, oxidative stress, and neuroinflammation that demonstrate anxiolytic effects in both preclinical studies and clinical trials. Clinical trials data are generally preliminary but show promising anxiolytic effects and an adequate safety profile and tolerance to adverse effects. NAC, agomelatine and omega-3 fatty acids show beneficial effects under clinical conditions related to anxiety in which available anxiolytics show moderate efficacy, and are considered promising candidates for innovative, effective and well tolerated anxiolytic agents by patients. We also showed that NAC has anxiolytic effects in mice in the open field, light/dark, hole-board, social interaction and stress-induced hyperthermia tests, either after acute or subacute treatment (4 days). These results provide additional evidence to support the validity of NAC clinical trials in the context of anxiety disorders, highlighting the long-term safety profile of NAC use compared to anxiolytics as diazepam. NAC is a safe, low-cost agent that demonstrates benefits in other psychiatric conditions that often present in comorbidity with anxiety disorders.

Keywords: anxiety, animal models, N-acetylcysteine, agomelatine, omega-3

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LISTA DE ABREVIATURAS E SIGLAS

AMPA	subtipo de receptor ionotrópico de glutamato
BZD	benzodiazepínicos
CAT	catalase
DHA	ácido docosahexaenóico
DSM	Manual Diagnóstico e Estatístico de Transtornos Mentais
EPA	ácido eicosapentaenóico
FDA	*Food and Drug Administration*
GSH	glutationa
HPA	eixo hipotálamo – hipófise - adrenal
IL	interleucina
ISRS	inibidores seletivos da recaptação de serotonina
IRSN	inibidores da recaptação de serotonina e noradrenalina
MT_1	receptores melatoninérgicos MT_1
MT_2	receptores melatoninérgicos MT_2
mGlu	receptor metabotrópico glutamatérgico
$mGlu_{2/3}$	receptor metabotrópico glutamatérgico do subtipo 2/3
NAC	N-acetilcisteína
NF-κB	fator de transcrição nuclear NF-κB
NMDA	subtipo de receptor ionotrópico de glutamato
Ômega-3	ácidos graxos poliinsaturados ômega-3
SNC	sistema nervoso central
SOD	superóxido dismutase
TAG	Transtorno de ansiedade generalizada
TAS	Transtorno de ansiedade social
TEPT	Transtorno de estresse pós traumático
Th	linfócitos *T-helper*
TNF-α	fator de necrose tumoral alfa
TOC	Transtorno obsessivo-compulsivo
TP	Transtorno de pânico
$5\text{-}HT_{1A}$	receptor de serotonina do subtipo $5\text{-}HT_{1A}$
$5\text{-}HT_{2C}$	receptor de serotonina do subtipo $5\text{-}HT_{2C}$

SUMÁRIO

1. REVISÃO DA LITERATURA

1.1. Ansiedade e Transtornos de Ansiedade

A ansiedade pode ser interpretada como uma antecipação emocional a uma situação aversiva, manifestando-se por respostas comportamentais espécie-específicas de medo a estímulos estressantes e ameaçadores (PITSIKAS, 2014). Caracterizada por alterações fisiológicas como sudorese, tontura, aumento da frequência cardíaca e pressão arterial, além de experiências subjetivas como preocupação persistente e tensão, a ansiedade pode ocorrer na forma de crises esporádicas em indivíduos sadios. Porém quando a ansiedade é persistente, perturbadora e desproporcional à demanda, se torna deletéria e pode comprometer a saúde do indivíduo (CALHOON; TYE, 2015).

Os transtornos de ansiedade tais como descritos até a IV edição do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-IV) incluíam o Transtorno de Ansiedade Generalizada (TAG), Transtorno de Ansiedade Social (TAS), Fobias Específicas e Transtorno de Pânico (TP), bem como o Transtorno de Estresse Pós Traumático (TEPT) e o Transtorno Obsessivo Compulsivo (TOC). Ainda que o DSM-V (2013) tenha alterado algumas dessas categorizações colocando-as em subgrupos próprios, o TOC e o TEPT são transtornos reconhecidos na clínica com marcante presença de sintomas de ansiedade. De acordo com o DSM-V, a classificação dos transtornos de ansiedade inclui o Transtorno de Ansiedade de Separação, Mutismo Seletivo, Fobia Específica, TAS (Fobia Social), TP, Agorafobia, TAG, Transtorno de Ansiedade Induzido pelo uso de medicamentos/ substâncias e o Transtorno de Ansiedade devido à outra condição médica (AMERICAN PSYCHIATRIC ASSOCIATION, 2013)..

Os transtornos de ansiedade geralmente se originam precocemente na vida (KESSLER et al., 2007), com taxas de prevalência global estimadas de aproximadamente 28,3% (BAXTER et al., 2013) e resultam em alto custo econômico, associado a problemas com o diagnóstico e tratamento

inapropriados e a utilização extensiva de serviços de saúde básicos (KESSLER, GREENBERG, 2002).

Postula-se que os transtornos de ansiedade resultam de perturbações em circuitos interconectados que servem normalmente para o processamento de estímulos ambientais. O processamento dessas informações é distribuído em circuitos córtico-límbicos, e quando resulta na interpretação de estímulos como ameaçadores são observadas respostas relacionadas à ansiedade. Uma perturbação em qualquer componente desses circuitos prejudica o equilíbrio no sistema, resultando em uma má interpretação de informações sensoriais neutras como potencialmente ameaçadoras e levando a respostas emocionais e comportamentais inapropriadas. Dentre as estruturas cerebrais pertencentes às vias neurais implicadas em comportamentos relacionados a ansiedade destacam-se a amígdala, o hipocampo ventral, o córtex pré-frontal, o núcleo leito da estria terminal, o hipotálamo e estruturas do tronco cerebral como a substância cinzenta periaquedutal e o núcleo dorsal da rafe (CALHOON; TYE, 2015).

1.2. Tratamento dos transtornos de ansiedade

As principais classes de fármacos utilizadas no tratamento dos transtornos de ansiedade atuam no sistema gabaérgico e na transmissão serotonérgica, tais como benzodiazepínicos (BZD), agonistas parciais do receptor serotonérgico 5-HT_{1A} (buspirona) e os inibidores seletivos da recaptação de serotonina (ISRS). Entretanto, algumas formas de ansiedade são relativamente resistentes ao tratamento com estes fármacos (HAMNER; ROBERT; FRUEH, 2004; VAN AMERINGEN et al., 2004).

Tanto os BZDs quanto os ISRS estão associados com efeitos adversos severos como sedação, déficits de memória, disfunção sexual e ganho de peso, o que prejudica a adesão dos pacientes ao tratamento. Além disto, o uso dos BZD a longo prazo está relacionado a ocorrência de dependência e síndrome de abstinência. Em relação à buspirona, este fármaco tem um uso limitado, pois apesar de ser bem tolerado, seu início de ação é mais lento em comparação a fármacos mais antigos como os BZD e possui eficácia

comprovada apenas no TAG (CRYAN; SWEENEY, 2011; LOANE; POLITIS, 2012).

Considerando os efeitos adversos dos fármacos ansiolíticos disponíveis no mercado, principalmente quando utilizados cronicamente, bem como a resposta insatisfatória observada em alguns transtornos de ansiedade, é relevante a busca por novas estratégias de tratamento para os transtornos de ansiedade.

O processo de redirecionamento ou reposicionamento ("*repurposing*") de fármacos é uma estratégia interessante para a descoberta de novos usos terapêuticos para fármacos já existentes no mercado, apresentando diversas vantagens, evitando o longo tempo necessário para os estágios de testes clínicos de um novo fármaco e estabelecimento do perfil farmacocinético e segurança de uso (ASHBURN; THOR, 2004; CORSELLO et al., 2017)

Destacam-se alguns exemplos importantes de sucesso em casos de redirecionamento de fármacos como a clorpromazina (originalmente desenvolvida como antiemético/anti-histamínico e posteriormente descoberta como um importante antipsicótico sedativo), a bupropiona (desenvolvida como antidepressivo e posteriormente utilizada como coadjuvante para cessação do tabagismo) e a sibutramina (desenvolvida como antidepressivo e posteriormente aprovada para o tratamento da obesidade) (ASHBURN; THOR, 2004). O sildenafil é outro exemplo interessante de redirecionamento, pois foi desenvolvido originalmente como um fármaco antianginoso e mostrou-se ineficaz para este propósito em testes clínicos. No entanto, vários voluntários relataram ereções persistentes após o uso do fármaco, que na sequência foi submetido a ensaios clínicos de disfunção erétil e tornou-se efetivo no seu tratamento (ASHBURN; THOR, 2004).

Neste contexto, a N-acetilcisteína (NAC) é um bom exemplo de redirecionamento de sucesso, pois desde sua aprovação inicial para o tratamento da superdosagem de paracetamol em 1985 nos Estados Unidos (YAREMA et al., 2009), sua utilização clínica vem sendo ampliada consideravelmente, incluindo as áreas da psiquiatria e neurologia (DEEPMALA et al., 2015).

1.3. Fisiopatologia da Ansiedade

1.3.1. Ansiedade e Glutamato

A maior parte dos neurônios e sinapses nas áreas e circuitos cerebrais que medeiam comportamentos cognitivos/emocionais complexos usam glutamato como neurotransmissor. Existem evidências na literatura de que mudanças em longo prazo nessas áreas e circuitos cerebrais representem a base biológica dos transtornos de humor e ansiedade, e por isso considera-se que o sistema glutamatérgico seja o mediador primário de várias psicopatologias (SANACORA; TRECCANI; POPOLI, 2012).

Múltiplas linhas de evidências indicam que anormalidades na neurotransmissão glutamatérgica estão envolvidas nos mecanismos biológicos subjacentes aos transtornos de ansiedade e à resposta ao estresse (RIAZA BERMUDO-SORIANO et al., 2012). Foram encontrados níveis anormais de glutamato e de receptores glutamatérgicos no sistema nervoso central (SNC) de pacientes com transtornos de ansiedade, além de níveis de glutamato aumentados em roedores após exposição a estressores (GRIEBEL; HOLMES, 2013; KRYSTAL et al., 2010; PHAN et al., 2005; POLLACK et al., 2008). Particularmente, tem sido sugerido que os transtornos de ansiedade são o resultado de um desequilíbrio no balanço inibitório/excitatório no SNC, devido a um aumento da atividade do sistema glutamatérgico (PITSIKAS, 2014).

Agentes com ação na neurotransmissão glutamatérgica são uma importante alternativa aos agentes gabaérgicos no desenvolvimento de novos fármacos ansiolíticos (GRIEBEL; HOLMES, 2013; WIEROŃSKA; PILC, 2013). Antagonistas dos receptores glutamatérgicos ionotrópicos NMDA e AMPA têm demonstrado efeitos ansiolíticos consistentes em estudos pré-clínicos (GRIEBEL; HOLMES, 2013; WIEROŃSKA; PILC, 2013), entretanto, o bloqueio indiscriminado de receptores NMDA não é uma opção bem tolerada para um ansiolítico em termos de riscos de efeitos adversos (GRIEBEL; HOLMES, 2013). Por outro lado, a D-cicloserina (que potencializa a sinalização no receptor NMDA agindo como um co-agonista do sítio da glicina neste receptor) tem demonstrado eficácia como um fármaco adjunto em transtornos de ansiedade facilitando a extinção do medo e o tratamento de exposição em

indivíduos com fobias (NORBERG; KRYSTAL; TOLIN, 2008; RESSLER et al., 2004). Adicionalmente, vários ensaios clínicos com pacientes com transtornos de ansiedade e transtornos relacionados (transtorno de ansiedade generalizada, transtorno de ansiedade social e transtorno de estresse pós-traumático) têm sido realizados com pregabalina, topiramato e riluzol (os quais exercem efeitos glutamatérgicos como parte de seu perfil farmacológico complexo), porém, a contribuição de seu efeito glutamatérgico para o efeito ansiolítico não está esclarecida (WIEROŃSKA; PILC, 2013), sendo necessário o desenvolvimento de fármacos glutamatérgicos com mecanismo de ação mais preciso (GRIEBEL; HOLMES, 2013).

Ensaios clínicos recentes estão focando predominantemente na busca de novos ansiolíticos capazes de inibir a hiperativação glutamatérgica e a pesquisa com moduladores de receptores glutamatérgicos metabotrópicos (mGlu) tem demonstrado resultados promissores (GRIEBEL; HOLMES, 2013; PITSIKAS, 2014; WIEROŃSKA; PILC, 2013). A família de receptores mGlu divide-se em 3 grupos baseados na homologia, perfil farmacológico e vias de transdução de sinal. Dentre as 3 famílias de receptores mGlu, os receptores $mGlu_{2/3}$ se destacam como alvos potencias para a ação de agentes moduladores da ansiedade (PITSIKAS, 2014). Localizados pré-sinapticamente e extra-sinapticamente em terminais nervosos glutamatérgicos, os receptores $mGlu_{2/3}$ estão presentes em diversas áreas cerebrais (incluindo córtex, tálamo, estriado, amígdalas e hipocampo) que parecem desempenhar um papel crítico na ansiedade. Acredita-se que a hiperatividade glutamatérgica nessas regiões está associada à fisiopatologia da ansiedade (LINDEN et al., 2004; SWANSON et al., 2005). A ativação dos receptores $mGlu_{2/3}$ aciona um mecanismo de feedback negativo que limita a liberação neuronal de glutamato (SCHOEPP, 2001) e agonistas desse receptor tem mostrado atividade ansiolítica em vários modelos animais de ansiedade (PITSIKAS, 2014).

1.3.2. Ansiedade e Estresse Oxidativo

O papel de alterações no equilíbrio redox na fisiopatologia dos transtornos de ansiedade é fortemente baseado em resultados obtidos com modelos animais e é compatível com dados bioquímicos obtidos de pacientes.

Os dados da literatura sugerem que os mecanismos oxidativos parecem ser um fator patogênico comum nos transtornos psiquiátricos, e, se essa hipótese for correta, pode-se considerar novos alvos para o desenvolvimento de intervenções terapêuticas (NG et al., 2008).

O estresse oxidativo ocorre quando a homeostase redox é alterada para um excesso de radicais livres e outras espécies reativas, devido ao aumento da sua produção ou deficiência nas defesas antioxidantes (SIES, 1997). O dano celular resultante pode variar de lesão celular estrutural e prejuízo na mitose a apoptose e necrose celular, dependendo da severidade do estresse oxidativo (DAVIES, 2000; FINKEL; HOLBROOK, 2000). O cérebro é considerado particularmente vulnerável ao estresse oxidativo por várias razões, incluindo a alta taxa de metabolismo e utilização de oxigênio com consequente geração de radicais livres, suas modestas defesas antioxidantes, sua constituição rica em lipídeos facilmente oxidável, o potencial redutor de certos neurotransmissores e a presença de metais redox – catalíticos como o ferro e o cobre (HALLIWELL, 2006; VALKO et al., 2007). Adicionalmente, o cérebro é também susceptível a lesões secundárias a injúria oxidativa, via efeitos neurotóxicos de aminas excitatórias (principalmente glutamato), ferro e resposta inflamatória ativada (HALLIWELL, 2006).

Os estudos relacionando o estresse oxidativo com ansiedade são relativamente recentes na literatura e os resultados tem demonstrado correlações importantes entre ansiedade e estresse oxidativo, tanto em estudos com animais quanto em humanos (ATMACA et al., 2004, 2008; BEHL et al., 2010; BULUT et al., 2013; CHAKRABORTY et al., 2009; EMHAN et al., 2015; ERSAN et al., 2006; HASSAN et al., 2014; KANDEMIR et al., 2013; KAYA et al., 2013; KROLOW et al., 2014; KULOGLU et al., 2002a, 2002b; NG et al., 2008). Um conjunto interessante de estudos tem ligado os genes da glioxalase I e glutationa redutase I (que conferem proteção contra o estresse oxidativo) com a ansiedade em camundongos (HOVATTA et al., 2005). Utilizando análise comportamental de linhagens isogênicas de camundongos visando determinar fenótipos de ansiedade e perfil de expressão gênica quantitativa em áreas cerebrais relevantes, foram identificados 17 genes candidatos, sendo que os genes da glioxalase I e glutationa redutase I demonstraram correlação positiva entre expressão e estados de ansiedade. A

super-expressão do gene da glioxalase I também tem sido relatada em camundongos naturalmente ansiosos (LANDGRAF et al., 2007). Bouayed e colaboradores (2007) encontraram uma correlação positiva entre marcadores de estresse oxidativo periférico e comportamento ansioso. Doses ansiogênicas de vitamina A induziram estresse oxidativo no hipocampo de ratos, com aumento de peroxidação lipídica, carbonilação proteica, oxidação de proteínas tiólicas e níveis alterados de superóxido dismutase (SOD) e catalase (CAT) (DE OLIVEIRA et al., 2007). Estudos na literatura relatam também aumento do comportamento ansioso acompanhado de estresse oxidativo aumentado em roedores expostos a estresse crônico por contenção (NOSCHANG et al., 2009), indutores de estresse oxidativo (SALIM et al., 2010a, 2010b) e estresse psicológico (LI et al., 2011).

Em humanos, estudos relacionando o estresse oxidativo à ansiedade também tem sido publicados. Foram relatadas elevação de produtos da peroxidação lipídica e alterações nas defesas antioxidantes no TAG (BULUT et al., 2013; EMHAN et al., 2015; KAYA et al., 2013), TOC (BEHL et al., 2010; CHAKRABORTY et al., 2009; ERSAN et al., 2006; KANDEMIR et al., 2013; KULOGLU et al., 2002a), TP (KULOGLU et al., 2002b) e TAS (fobia social) (ATMACA et al., 2004, 2008). Verificou-se que mulheres ansiosas mostraram reduzida capacidade antioxidante total quando comparadas aos controles, o que foi acompanhado por prejuízo de vários parâmetros de função imunológica (ARRANZ; GUAYERBAS; DE LA FUENTE, 2007).

1.3.3. Ansiedade e Inflamação

Citocinas tais como fator de necrose tumoral alfa (TNF-α) e as interleucinas (IL) 2 e 6 medeiam informação entre o sistema nervoso central (SNC) e o sistema imune periférico. As citocinas no SNC podem desempenhar vários papéis incluindo a iniciação de processos imunes como a resposta alérgica, envolvimento nos mecanismos de reparo após lesões e regulação do eixo hipotálamo – hipófise - adrenal (HPA) (KASPER et al., 2003). Associações entre a desregulação do eixo HPA e transtornos de ansiedade são reconhecidos na literatura científica, especialmente em relação aos níveis de

cortisol e modificações nos níveis de citocinas pró e anti-inflamatórias (FURTADO; KATZMAN, 2015).

Segundo Chesnokova e colaboradores (2016) a neurogênese hipocampal está implicada no prejuízo cognitivo que acompanha transtornos de humor, incluindo ansiedade e depressão. As citocinas pró-inflamatórias liberadas na periferia estão envolvidas na comunicação do sistema imune periférico com o cérebro através da ativação da micróglia, que ativada reduz a neurogênese diminuindo a sobrevivência de neurônios recentemente formados, bem como sua integração a circuitos neuronais existentes.

Os linfócitos T-helper (Th), subdivididos de acordo com as citocinas que produzem em células Th1 (que secretam as citocinas IL-2, TNF-α e interferon γ) e Th2 (que secretam as citocinas IL-4, 5, 6, 10 e 13), atuam primariamente ativando respectivamente a imunidade mediada por células e a humoral (GLIK; DOUVDEVANI, 2006). O equilíbrio entre Th1 e Th2 encontra-se frequentemente alterado em transtornos de ansiedade, tais como TOC e TEPT: especificamente há uma dominância de Th2, de modo que a IL-6 e o TNF-α estão tipicamente aumentados (MARTINO et al., 2012).

Em animais observou-se que um aumento da expressão de citocinas na periferia está associado com aumento da ansiedade (SAKIĆ et al., 1994; SCHROTT; CRNIC, 1996) e que camundongos com superexpressão de IL-6 e TNF-α exibem um fenótipo ansiogênico (CONNOR; LEONARD, 1998; FIORE et al., 1998). Camundongos com deficiência do fator nuclear kappa-B (NF-κB), um fator de transcrição chave no controle da resposta inflamatória, exibem diminuição de comportamentos do tipo ansioso (KASSED; HERKENHAM, 2004). Recentemente, foi demonstrado que a inflamação sistêmica induz comportamento do tipo ansioso em ratos (DO NASCIMENTO; LEITE-PANISSI, 2014) e camundongos (KRISHNA; DODD; FILIPOV, 2016; YANG et al., 2016).

O cortisol é um importante hormônio liberado em resposta ao estresse, com função moduladora da inflamação e do sistema imunológico (SORRELLS et al., 2009). O eixo HPA controla a liberação de cortisol, um hormônio essencial para que o organismo produza uma resposta eficiente ao estresse. Uma resposta dinâmica ao estresse, envolvendo um rápido aumento e subsequente declínio nos níveis de cortisol, facilita o organismo a lidar adequadamente com estímulos ambientais percebidos como ameaçadores. Por outro lado,

mudanças no padrão de liberação de cortisol em resposta ao estresse podem aumentar a susceptibilidade do organismo aos seus efeitos negativos, sendo que a ativação excessiva, prolongada ou insuficiente do eixo hipotálamo-hipófise-adrenal podem induzir modificações no cérebro resultando no desenvolvimento de doenças psiquiátricas (MCEWEN, 2004). A exposição ao estresse crônico é um importante fator de risco para transtornos de ansiedade, além de outras doenças psiquiátricas (MORENO-PERAL et al., 2014). O cortisol encontra-se aumentado em pacientes diagnosticados com diversos transtornos de ansiedade e transtornos relacionados, tais como TAG, TAS, TEPT e TOC (FURTADO; KATZMAN, 2015).

1.4. N-Acetilcisteína (NAC)

A NAC é um derivado acetilado do aminoácido cisteína, amplamente conhecida como um suplemento nutricional com propriedades antioxidantes (BERK et al., 2013). É um fármaco utilizado mundialmente no tratamento de diversas condições médicas nas últimas décadas, sendo considerada segura e bem tolerada (DEEPMALA et al., 2015; LAROWE et al., 2006). O uso terapêutico da NAC é amplamente reconhecido como um antídoto no tratamento da superdosagem de paracetamol, tendo sido aprovada pela *Food and Drug Administration* (FDA) para essa finalidade nos Estados Unidos desde 1985 (YAREMA et al., 2009). Adicionalmente, a NAC também é utilizada como agente mucolítico em doença pulmonar obstrutiva crônica (DEKHUIJZEN; VAN BEURDEN, 2006), como protetor renal em nefropatia induzida por contraste (QUINTAVALLE et al., 2013), como fármaco preventivo de fibrilação atrial (LIU; XU; FAN, 2014) e como terapia adjunta em pacientes com infecção pelo vírus HIV (DE ROSA et al., 2000).

O interesse em utilizar a NAC no tratamento de doenças psiquiátricas e neurológicas tem sido crescente desde a década passada, pois estudos pré-clínicos sugerem que a NAC é capaz de modular importantes processos envolvidos na fisiopatologia de diversas doenças psiquiátricas e neurológicas, tais como estresse oxidativo, disfunção mitocondrial, desregulação da neurotransmissão glutamatérgica e dopaminérgica, neuroinflamação, neurogênese e apoptose (DEAN; GIORLANDO; BERK, 2011; SAMUNI et al.,

2013). Têm sido relatadas evidências favoráveis de benefícios da NAC no TOC, tricotilomania, hábito de roer unhas e escoriações de pele, transtorno bipolar, depressão, esquizofrenia, dependência de cocaína e maconha, autismo, doença de Alzheimer, neuropatia induzida por fármacos e epilepsia progressiva mioclônica (AFSHAR et al., 2012; BERK et al., 2008, 2009, 2011; BERNARDO et al., 2009; DEEPMALA et al., 2015; FERNANDES et al., 2016; GHANIZADEH; DERAKHSHAN; BERK, 2013; GRANT et al., 2016; GRANT; ODLAUG; KIM, 2009; MINARINI et al., 2017; PAYDARY et al., 2016)

A NAC possui um mecanismo de ação inovador, que inclui a modulação do estresse oxidativo, neurotransmissão glutamatérgica e neuroinflamação. A glutationa (GSH) é um importante antioxidante endógeno, com maiores concentrações em células gliais que neuronais. A produção de GSH pelos astrócitos é proporcionalmente limitada pela cisteína disponível e pela enzima glutamato-cisteína ligase. Além de prover cisteína para a produção de GSH, a NAC também é capaz de sequestrar diretamente substâncias oxidantes (DEAN; GIORLANDO; BERK, 2011).

Somam-se aos efeitos da NAC no balanço oxidativo, a modulação da neurotransmissão, especialmente glutamatérgica e dopaminérgica (DEAN; GIORLANDO; BERK, 2011). A cisteína participa na regulação da liberação de glutamato através do antiporter cistina/ glutamato, localizado preferencialmente em astrócitos. O dímero cistina é captado pelos astrócitos e trocado por glutamato, o qual quando liberado no espaço extra-sináptico estimula receptores glutamatérgicos metabotrópicos (mGlu) inibitórios, reduzindo a liberação sináptica de glutamato. Por outro lado a GSH potencializa a resposta de receptores NMDA (DEAN; GIORLANDO; BERK, 2011).

Adicionalmente, a NAC possui propriedades anti-inflamatórias relacionadas a vias oxidativas, sendo este outro potencial mecanismo de ação da NAC em psiquiatria (BERK et al., 2013; DEAN; GIORLANDO; BERK, 2011). A NAC foi capaz de reverter o aumento de TNF-α e IL-1β em ratos submetidos a modelos animais de injúria cerebral por trauma e isquemia cerebral focal (CHEN et al., 2008; KHAN et al., 2004). Adicionalmente, a NAC foi capaz de reverter a inibição do desenvolvimento e mielinização de oligodendrócitos em cultura de células gliais de ratos tratada com lipopolissacarídeos (PAINTLIA et al., 2008) e preveniu o estresse oxidativo e perda da potenciação a longo prazo

que ocorrem em consequência da exposição à inflamação pré-natal (LANTÉ et al., 2008).

Apesar da potencial ação terapêutica da NAC em transtornos de ansiedade e algumas condições psiquiátricas relacionadas (como transtornos do espectro obsessivo-compulsivo) estar sendo sugerida em estudos de caso e ensaios clínicos (DEEPMALA et al., 2015; MINARINI et al., 2017), ainda são escassos na literatura estudos em modelos animais que possam comprovar seu efeito ansiolítico e subsidiar desenhos de provas clínicas.

1.5. Modelos animais de ansiedade

Os modelos animais de ansiedade são ferramentas importantes para o descobrimento de agentes com propriedades ansiolíticas e idealmente devem atender a três principais critérios de validade: validade de face (evocação de comportamentos relacionados a sintomas de ansiedade em humanos); validade preditiva (sensibilidade a ansiolíticos clinicamente eficazes) e validade de constructo (envolvimento de alguns dos mecanismos fisiopatológicos relacionados aos transtornos de ansiedade). Na realidade, todos os modelos animais de ansiedade disponíveis até o momento são capazes de atender apenas parcialmente a estes três critérios (BOURIN, 2015; GRIEBEL; HOLMES, 2013).

Os modelos de ansiedade são classificados de forma geral em testes de conflito entre aproximação-esquiva, também chamados de testes etológicos que envolvem respostas não-condicionadas e testes que envolvem respostas condicionadas (BOURIN, 2015; STEIMER, 2011). Os testes de conflito entre aproximação-esquiva tem sido a base principal da pesquisa pré-clínica de agentes ansiolíticos e incluem testes simples como o campo aberto, o teste claro/escuro e o labirinto em cruz elevado. São testes que exploram a tendência natural de roedores de preferir áreas fechadas a locais abertos, mais expostos e elevados. De forma geral considera-se que estes testes modelam aspectos com maior analogia ao transtorno de ansiedade generalizada e fobias específicas, baseando-se principalmente na sensibilidade dos mesmos a ansiolíticos BZDs e validade de face (GRIEBEL; HOLMES, 2013). Por outro lado, os modelos de ansiedade baseados em conflito, como por exemplo o

teste de Vogel (no qual agentes com propriedades ansiolíticas são capazes de manter uma resposta comportamental normalmente suprimida por choque), e os testes baseados em respostas de medo condicionado, como o condicionamento de medo Pavloviano clássico (no qual o animal aprende a associar um contexto ou estímulo ambiental específico com um choque elétrico, expressando respostas de medo condicionado) são menos comumente utilizados entre os testes clássicos na descoberta de ansiolíticos. Isto deve-se principalmente ao fato de os animais precisarem ser submetidos a treinos em vários dias, consumindo maior tempo e trabalho para a realização dos experimentos em comparação com os testes de aproximação-esquiva (GRIEBEL; HOLMES, 2013).

Entre os quinze modelos animais de ansiedade mais comumente utilizados na pesquisa de agentes ansiolíticos estão o campo aberto, claro/escuro, placa perfurada, teste de interação social, labirinto em T-elevado e hipertermia induzida por estresse (GRIEBEL; HOLMES, 2013), sendo que estes foram os modelos animais de ansiedade utilizados no presente trabalho para a avaliação das propriedades ansiolíticas da NAC.

O campo aberto é um teste amplamente utilizado para avaliar os efeitos de agentes sobre a atividade locomotora e seus efeitos sobre o comportamento tipo-ansioso dos animais. O aparato consiste geralmente em uma arena circular ou quadrada, iluminada e circundada por paredes altas. Neste teste, além da atividade locomotora, que pode ser avaliada através do número total de quadrantes cruzados no aparato experimental ou pela distância total percorrida, agentes com atividade ansiolítica aumentam o tempo gasto na zona central do aparato (CALABRESE, 2008).

O teste claro/escuro é um modelo baseado na aversão inata de roedores a áreas claras. O aparato consiste em uma caixa contendo um compartimento escuro interligado por uma abertura a um compartimento claro, no qual é gerado um conflito entre a tendência natural dos animais de explorar ambientes novos e a esquiva do compartimento claro e iluminado. O tratamento com fármacos ansiolíticos, como os BZDs, aumenta o tempo gasto no compartimento claro e o número de transições entre as duas áreas, sendo que este último parâmetro também está relacionado à atividade locomotora (BOURIN; HASCOËT, 2003; CALABRESE, 2008).

O teste da placa perfurada baseia-se na atividade exploratória natural dos animais em um ambiente novo, o qual consiste em uma placa quadrada contendo perfurações equidistantes. Neste teste os animais exploram as perfurações fazendo o comportamento de espreitamento (onde o animal submerge a cabeça nas perfurações da placa), o qual foi validado como uma medida de ansiedade. Agentes ansiolíticos aumentam o número de espreitamentos neste teste (CALABRESE, 2008; TAKEDA; TSUJI; MATSUMIYA, 1998).

No teste de interação social pares de animais não-familiares são colocados em uma arena iluminada e o tempo de interação social entre eles é avaliado, sendo que agentes ansiolíticos causam um aumento no tempo de interação social (CALABRESE, 2008).

O labirinto em T-elevado foi desenvolvido a partir do labirinto em cruz elevado, consistindo em um braço fechado perpendicular a dois braços abertos e baseia-se na aversão dos animais pelos braços abertos do aparato. Neste teste a exposição sucessiva dos animais ao aparato aumenta a latência de saída para os braços abertos (aquisição de esquiva inibitória), sendo que agentes ansiolíticos diminuem a latência de saída para os braços abertos, prejudicando a aquisição de esquiva inibitória (CARVALHO-NETTO; NUNES-DE-SOUZA, 2004; GRAEFF; NETTO; ZANGROSSI, 1998; GRAEFF; VIANA; TOMAZ, 1993).

O teste de hipertermia induzida por estresse baseia-se no aumento de temperatura que ocorre nos indivíduos em resposta a situações percebidas como ameaçadoras ou estressantes. É um evento mediado pelo sistema nervoso autonômico e pode ser desencadeado por diversos estímulos estressores (OLIVIER et al., 2003; VAN DER HEYDEN; ZETHOF; OLIVIER, 1997). Neste teste é medida a temperatura basal retal dos animais, e esta medida é um estímulo estressor que leva a um aumento de temperatura, que ocorre após aproximadamente 10 a 15 minutos, sendo que após este intervalo de tempo a temperatura é medida novamente. Agentes ansiolíticos diminuem o aumento de temperatura induzida pelo estresse neste modelo (OLIVIER et al., 2003).

2. OBJETIVOS

2.1. Objetivo geral

Avaliar as propriedades ansiolíticas da N-acetilcisteína e de compostos com mecanismo de ação multialvo envolvendo a modulação do estresse oxidativo, neuroinflamação e neurotransmissão glutamatérgica.

2.2. Objetivos específicos

- Realizar uma revisão da literatura buscando compostos que tenham mecanismo de ação multialvo envolvendo a modulação do estresse oxidativo, neuroinflamação e neurotransmissão glutamatérgica e que tenham sido estudados tanto em modelos animais de ansiedade, quanto em ensaios clínicos randomizados duplo-cegos controlados por placebo em pacientes com transtornos de ansiedade e transtornos relacionados (Artigo 1).

- Estudar as propriedades ansiolíticas da NAC em camundongos avaliados em diversos modelos animais de ansiedade (Artigo 2).

3. ABORDAGEM METODOLÓGICA

Para realizar a revisão da literatura (Artigo 1) foi feita uma busca na base de dados Pubmed por referências disponíveis até março de 2017. A estratégia de busca foi realizada com combinações sucessivas das seguintes palavras-chave (selecionadas com base em agentes que tem mecanismo de ação multi-alvo bem estabelecido na literatura, incluindo a modulação do estresse oxidativo e/ou neuroinflamação e/ou hiperatividade glutamatérgica): ascorbic acid, vitamin C, vitamin A, vitamin E, tocopherol, vitamin D, polyphenols,

flavonoids, metabotropic glutamate receptor 2/3 modulator, melatonin, agomelatine, N-acetylcysteine, omega-3 fatty acids, omega-3 PUFA AND Anxiety. Inicialmente os resultados selecionados foram limitados a ensaios clínicos. Evidências de efeitos ansiolíticos dos agentes tanto em ensaios clínicos randomizados duplo-cegos controlados por placebo quanto em modelos animais foi uma condição para incluir os agentes neste trabalho. Para os agentes que foram testados em ensaios clínicos, nós realizamos uma busca adicional para o agente e cada uma das seguintes condições (as quais são classificadas como transtornos de ansiedade ou possuem uma forte relação com sintomas relacionados a ansiedade: generalized anxiety disorder, social phobia, specific phobia, panic disorder, obsessive-compulsive disorder, post-traumatic stress disorder, trichotillomania, nail biting, skin-picking and excoriation disorder. Os artigos foram acessados de acordo com sua relevância nos tópicos selecionados e a busca foi limitada a artigos em inglês.

Para a avaliação das propriedades ansiolíticas da NAC (Artigo 2), camundongos foram testados em seis modelos animais de ansiedade (campo aberto, claro/escuro, placa perfurada, interação social, labirinto em T-elevado e hipertermia induzida por estresse) após tratamento agudo ou subagudo com NAC, administrada intraperitonealmente.

4. ARTIGOS

Artigo 1 - Submetido a Revista Brasileira de Psiquiatria

ANXIOLYTIC PROPERTIES OF COMPOUNDS THAT COUNTERACT OXIDATIVE STRESS, NEUROINFLAMMATION, AND GLUTAMATERGIC DYSFUNCTION: A REVIEW

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Running title: Anxiolytic effects of multitarget agents

Abstract

Objective: Anxiety disorders are highly prevalent and the efficacy of the available anxiolytic drugs is less than desired. Additionally, adverse effects compromise the quality of life and the adherence to treatment. Accumulating evidence shows that the pathophysiology of anxiety and related disorders is multifactorial, with the involvement of oxidative stress, neuroinflammation, and glutamatergic dysfunction. The aim of this review is to evaluate data from animal studies and clinical trials showing anxiolytic effects of agents with mechanisms of action targeting these multiple domains.

Methods: A search at Pubmed database was conducted looking for multi-target agents that had been evaluated in animal models of anxiety and randomized double-blind placebo-controlled clinical trials of anxiety and/or anxiety related disorders.

Results: The main multi-target agents that have shown consistent anxiolytic effects in various animal models of anxiety as well in clinical trials are agomelatine, N-acetylcysteine, and omega-3 fatty acids. Data from clinical trials are preliminary at best, but reveals good safety profiles and tolerance to adverse effects.

Conclusion: Agomelatine, N-acetylcysteine and omega-3 fatty acids show beneficial effects in clinical conditions where mainstream treatments are ineffective. These three multi-target agents are considered as promising candidates for innovative, effective and better-tolerated anxiolytics.

Keywords: Anxiety, agomelatine, N-acetylcysteine, omega-3 fatty acids.

Introduction

Anxiety has been defined as a state of high arousal and enhanced vigilance in the absence of immediate threat [1]. It is characterized by subjective experiences (such as persistent worry and tension) in addition to physiological changes (such as sweating and increased heart rate). Though healthy individuals may present sporadic anxiety, it becomes pathological if persistent, disruptive and disproportionate [2]. Anxiety disorders have global lifetime prevalence rates as high as 28% [3], and include social phobia, panic disorder, agoraphobia and generalized anxiety disorder [4]. Though obsessive-compulsive disorders (OCD) and posttraumatic stress disorder (PTSD) present marked anxiety symptoms, the DSM-5 categorizes these conditions within the obsessive-compulsive and related disorders and the trauma and stressor-related disorders, respectively.

In addition to drug therapy, the current treatment of anxiety disordes involves lifestyle interventions, such as physical exercise and mindfulness-based stress reduction, and psychological interventions, such as cognitive behavioral therapy, which are knowingly difficult to implement. The main drug classes of used to treat anxiety disorders are GABAergic or serotonergic agents, including benzodiazepines (BZD), 5-HT_{1A} serotonin receptor agonists, and selective serotonin reuptake inhibitors (SSRIs) [5]. Unfortunately, however, not all patients respond to the available medications [6]. Moreover, BZDs and SSRIs are associated with unwanted adverse effects, including sedation, memory deficits, dependence, withdrawal syndrome, sexual dysfunction and weight gain [5]. While these adverse effects decrease adherence to treatment,

the better-tolerated 5-HT_{1A} agonist buspirone has the slowest onset of action and its efficacy is limited to generalized anxiety disorder [7,8].

Despite its high prevalence, few therapeutic targets have been identified for the treatment of anxiety disorders. The expectation that highly selective agents acting on specific molecular targets would yield better and safer psychiatric drugs has not been fulfilled [9]. The newer multi-target agents approach [10,11] is in resonance with the recognition of the complex pathophysiology underlying psychiatric disorders. In the case of anxiety disorders, oxidative stress [12–14], neuroinflammation [15] and glutamatergic hyperactivity [16–18] are now recognized as key contributing factors.

Anxiety and neurochemical damage

It is now recognized that glutamatergic hyperactivity, a key feature in brain insults, triggers a complex chain of events including oxidative stress, mitochondrial dysfunction and cellular signaling that result in inflammatory responses and/or cell death [19,20]. As glutamatergic hyperactivity is characteristic of anxiety [17,18], oxidative stress and neuroinflammation are of relevance.

Abnormalities in glutamate neurotransmission are among the biological mechanisms underlying the stress response and anxiety disorders [17]. Anxiety disorders seem to result from a hyperactive glutamatergic system deregulating the inhibitory/excitatory balance in the brain [16,18]. The metabotropic glutamatergic receptors 2/3 ($mGlu_{2/3}$ receptors) stand out as a potential target for anxiety-modulating drugs (Pitsikas, 2014) [16]: presynaptically located, $mGlu_{2/3}$ receptors are present in several brain areas where glutamate hyperactivity is associated with anxiety, including cortex, thalamus, striatum, amygdala, and

hippocampus [21,22]. The activation of $mGlu_{2/3}$ receptors limits neuronal glutamate release [23], and agonists of such receptors show anxiolytic activity in diverse animal models of anxiety [16].

The association of anxiety and oxidative stress has been documented in rodents and humans. Hovatta *et al.* (2005) revealed a positive correlation between the glyoxalase I and glutathione reductase I genes expression and anxiety phenotypes on strain-related behaviors in isogenic mice [24]. Overexpression of the glyoxalase I gene has also been reported for naturally anxious mice [25]. Bouayed et al. (2007) reported a positive correlation between markers of peripheral oxidative stress and anxious behavior in mice [26]. Increased anxiety-like behavior accompanied by oxidative stress has been documented in rodents exposed to psychological stress [27], chronic restraint stress [28] and oxidative stress inducers [29–31]. Changes in antioxidant defenses and elevation of lipid peroxidation products have been reported in generalized anxiety disorder [32–34], obsessive-compulsive disorder (OCD) [35–39], panic disorder (Kuloglu et al., 2002) [40] and social phobia [41,42]. Anxious women showed reduced total antioxidant capacity in the blood when compared to controls [43].

Associations between deregulation of the hypothalamic pituitary adrenal axis (HPA) and anxiety disorders are widely recognized, resulting in changes in the levels of pro- and anti-inflammatory cytokines and cortisol [15,44]. Inflammatory cytokines and immune cells can access the brain and alter behavior, as the synthesis, release, and reuptake of neurotransmitters such as glutamate, serotonin, and dopamine are affected by cytokines and their signaling pathways [45]. The kynurenine pathway is also activated by cytokines, generating

neuroactive metabolites that influence dopamine and glutamate transmission and, by depleting tryptophan, regulate the synthesis of serotonin [45].

Increased cytokine expression in the periphery is associated with increased anxiety in mice [46,47]. Mice overexpressing IL-6 or TNF exhibit an anxiogenic phenotype [48,49]. Several human studies showing a correlation between anxiety, neuroinflammation, and immune system have also been documented in humans [15,44]. Injection of the immune activator lipopolysaccharide (LPS) induced anxiety symptoms in normal volunteers [50], and a positive correlation between anxiety and increased levels of inflammatory markers (such as TNF-α and IL-6) was repeatedly documented in anxiety disorders [15,43,51,52].

Strategies to minimize and/or counteract the damage resulting from these accompanying neurochemical processes may lead to innovation in the field of anxiolytic drugs research. As a key step in translational research is target validation, the aim of this study is to review drug candidates known to counteract oxidative stress, neuroinflammation, and glutamatergic hyperfunction, which were subjected to preclinical and clinical analyses relevant to anxiety disorders.

Methods

A search at PubMed database was conducted up to March 2017. The search strategy used the successive combinations of the following terms (chosen based on compounds that have well stablished multitarget mechanisms of action in the literature, including modulation of oxidative stress and/or neuroinflammation and/or glutamate hyperactivity): ascorbic acid, vitamin C,

vitamin A, vitamin E, tocopherol, vitamin D, polyphenols, flavonoids, metabotropic glutamate receptor 2/3 modulator, melatonin, agomelatine, N-acetylcysteine, omega-3 fatty acids, omega-3 PUFA AND anxiety. Firstly, results were limited to clinical trials. Evidence of anxiolytic effects in both randomized double-blind placebo-controlled clinical trials and animal models was a condition to include a compound in this review. When no such studies were found for a given compound, it was excluded from further analysis. For the compounds that had been tested in clinical trials, we also carried out searches for the compound AND each of these conditions (which are classified as anxiety disorders or have strong relation with anxiety-related symptoms): generalized anxiety disorder, social phobia, specific phobia, panic disorder, obsessive-compulsive disorder, post-traumatic stress disorder, trichotillomania, nail biting, skin-picking and excoriation disorder. The manuscripts were assessed for relevance to the topics selected. The search was limited to texts in English and at all the abstracts found according to the search criteria were read to select the articles for inclusion.

Results and Discussion

We found that agomelatine, N-acetylcysteine and omega-3 polyunsaturated fatty acids (PUFAs) are the main agents that fit the inclusion criteria and possess antioxidant, anti-inflammatory and glutamatergic effects.

Agomelatine

Agomelatine, a synthetic analog of melatonin, is a high-affinity agonist of the MT_1 and MT_2 melatonin receptors [53,54]. Agomelatine antagonizes 5-HT_{2C} serotonin receptors, an effect thought to be involved in its anxiolytic effects [55]. Agomelatine also modulates glutamate neurotransmission in regions associated with mood and cognition, such as prefrontal and frontal cortex [55], hippocampus and amygdala [56]. In rats submitted to prenatal restraint stress, agomelatine blocked the stress-induced glutamate release in the prefrontal cortex [57] and regularized glutamate release and the expression of $mGlu_{2/3}$ receptor mRNA in the hippocampus [58].

Agomelatine decreased lipid peroxidation levels and nitrite contents in the brain of mice submitted to chemically induced seizures [59] and also protected cultured PC-12 neuronal cells from cytosolic reactive oxygen species production, decrease in glutathione and lipid peroxidation [60].

Agomelatine was able to reduce the LPS-induced upregulation of proinflammatory cytokines IL-6 and IL1-β in and out rat brain; centrally, these effects were accompanied by inhibition of NF-κB translocation and microglia activation [61]. Microglia are resident macrophages normally present in the healthy brain, which perform active tissue scanning and are ready to respond quickly to any microenvironment change [62]. Agomelatine also modified the expression of enzymes associated with the kynurenine pathway, possibly protecting the brain from the neurotoxic consequences of the conversion of kynurenine to quinolinic acid, an N-methyl-D-aspartate (NMDA) receptor agonist [61].

Though the antidepressant properties of agomelatine have been better characterized [63], its anxiolytic effects were reported in different animal models

[58,64–66] (Table 1). In most animal studies, agomelatine anxiolytic effects were documented after acute administration. However, Morley-Fletcher et al. (2011) reported that agomelatine administered for 3 or 6 weeks prevented prenatal restraint stress-induced anxiety (in the elevated plus-maze) as well as reversed the reduced hippocampal levels of $mGlu_{2/3}$ and $mGlu_5$ receptors in rats [58]. These effects were restricted to the rats submitted to restraint stress, suggesting that agomelatine modulation of $mGlu_{2/3}$ receptors may be especially relevant in stressed subjects.

Most clinical data available for agomelatine as an anxiolytic refer to generalized anxiety disorder (GAD) patients and were published by the same group. The first clinical trial was published in 2008 (see Table 2), where GAD patients (comorbidity free) were randomized to agomelatine or placebo for 12 weeks [65]. This randomized double-blind placebo controlled trial (RDBCT) revealed that agomelatine (25-50 mg/day) was superior to placebo in the primary outcome (Hamilton Anxiety Rating Scale), as well as secondary outcome measures (clinical response, insomnia and associated disability). In this study, agomelatine was well tolerated and discontinuation symptoms were lower on agomelatine than on placebo patients [67]. An open-label study with agomelatine 25-50 mg/day for 16 weeks, followed by a multicenter RDBCT (with the same doses of agomelatine) for 26 weeks was conducted in order to evaluate the agomelatine long-term tolerability and its efficacy in preventing relapse. The results showed that agomelatine was well tolerated and superior to placebo in preventing relapse [68]. A third trial compared agomelatine with escitalopram and placebo. The multicenter RDBCT showed that agomelatine and escitalopram were comparable regarding improved symptomatology but

escitalopram had a higher incidence of adverse events in comparison to placebo [69]. A recent trial evaluated the minimal effective optimal dose of agomelatine in GAD patients: a 12-week multicenter RDBCT showed that 10 and 25 mg/kg are better than placebo, with the best response obtained with 25 mg [70].

Data on other anxiety disorders are very limited, and present too many confounding factors to allow meaningful conclusions [71]. Stein et al. (2013) reviewed data from three placebo-controlled short-term trials [72–74] and three comparative studies of agomelatine versus the SSRI antidepressants venlafaxine [75], fluoxetine [76] and sertraline [77] in major depression patients with anxiety symptoms, and agomelatine showed a greater effect on anxiety symptoms than placebo or comparator antidepressants [78].

Adverse events reported with agomelatine are mostly perceived as mild to moderate and include headache, dizziness, somnolence, fatigue and gastrointestinal symptoms [79]. Elevation of liver transaminases levels and rare cases of hepatic failure were seen only with 50 mg/day [79]. The use of agomelatine was not associated with discontinuation symptoms [80,81], a relevant aspect considering its beneficial effects in the sleep disturbances observed in patients with depression and/or anxiety [67,69].

Insert Table 1 here

N-Acetylcysteine

N-Acetylcysteine (NAC) is a precursor of cysteine (required for the production of the primary endogenous antioxidant glutathione), also able to

directly sequester oxidants [82]. NAC supplementation results in additional cysteine that activate the cystine/glutamate antiporter (also called x(c)-system), predominantly expressed by astrocytes in the brain. The dimer cystine is taken up by astrocytes and exchanged for glutamate, which activates $mGluR_{2/3}$ receptors on presynaptic neurons and reduces the synaptic release of glutamate [82].

NAC possesses anti-inflammatory properties as result of multiple mechanisms. Through its direct antioxidant effect and as glutathione (GSH) precursor, NAC inhibits the activation of the proinflammatory transcription factor NF-κB, which downregulates the expression of several proinflammatory genes [83–85]. Microglia inhibition seems to be also important for the ability of NAC to reduce neuroinflammation [86,87]. Therefore, through the stimulation of GSH synthesis and regulation of the cystine/glutamate antiporter, glutamate excitotoxicity, and oxidative stress, NAC inhibits microglia, macrophage activation and production of cytokines and oxidative species [86,88].

NAC anti-inflammatory properties were documented in animal models of ischmic and traumatic brain injury [89-91], LPS-induced pulmonary edema [92] and lethal endotoxemia [93]. In humans, NAC was able to reduce lung inflammation (Blackwell et al., 1996) [94], decrease proinflammatory cytokines in burn [95] and dialysis patients [96] and also caused a reduction of proinflammatory cytokines along with antioxidant effects in cardiac injury after aortic aneurysm repair [97].

Egashira et al. (2012) showed that acute NAC (but not α-tocopherol) inhibited marble-burying behavior in mice (Table 1), suggesting that this anxiolytic-like effect is related to glutamate modulation rather than antioxidant effects [98]. Chen et al. (2014) showed that NAC reversed valproate-induced

social interaction deficit and anxiety-like behavior in rats (considered an experimental model of autism). The effects were $mGLU_{2/3}$ receptors dependent since blocked by the $mGlu_{2/3}$ receptor antagonist LY341495. Accordingly, NAC reduced to normal levels the enhanced presynaptic excitatory neurotransmission in the amygdala of valproate-exposed rats [99]. NAC prevented the rhythm disruption-induced anxiety in mice [100]. Anxiolytic effects of NAC were also documented in the light/dark model and stress-induced anxiety behavior in zebrafish [101], as well as in the hole-board, light/dark, open field, social interaction and stress-induced hyperthermia in mice [102]. One report suggested that NAC may have anxiogenic properties, but the conclusion is questionable since it was based only on the decrease in time spent in the center of an open field, which was accompanied by decreased locomotion [103].

Studies designed to evaluate the effects of NAC specifically in patients diagnosed with an anxiety disorder are very limited. Back et al. (2016) conducted a study with NAC in veterans diagnosed with comorbid posttraumatic stress disorder (PTSD) and substance user disorder (SUD), where patients were treated with NAC or placebo, along with group cognitive-behavioral therapy. NAC decreased self-reported and clinician-rated PTSD symptoms, and the symptoms remained significantly reduced after drug discontinuation at the one-month follow-up. Patients receiving NAC also reported decreased craving [104].

NAC has been evaluated in obsessive-compulsive and related disorders where anxiety is a key component. A RDBCT conducted by Grant et al. (2009) revealed significant improvement in trichotillomania patients after 12 weeks of treatment [105]; this result was substantiated by a number of case reports [106–108].

In pediatric trichotillomania patients, a RDBCT did not find a significant reduction of hair pulling in comparison with placebo [109]; in this trial, the authors suggest that the improvement was associated with psychoeducation about trichotillomania rather than drug treatment, since significant improvement in several measures of hair pulling was observed regardless of treatment time. A series of case reports showed NAC beneficial effects in reducing skin-picking (excoriation disorder) [106,110–112]. A RDBCT concluded that NAC resulted in significant reductions in skin-picking symptoms [113]. Berk et al. (2009) found a reduced frequency of nail biting in three patients enrolled in a bipolar disorder treatment protocol with NAC [114]. The short, but not long term, decreased nail biting reported by Ghanizadeh et al. (2013) is somewhat questionable considering the lower dose and shorter NAC treatment time used in the RDBCT as compared to the case reports [115].

Beneficial effects of NAC in children, adolescents, and adults with obsessive-compulsive disorder (OCD) have been reported [116,117]. Afshar et al. (2012) performed a RDBCT with NAC as add-on treatment in OCD patients refractory to SSRIs. NAC improved the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) and Clinical Global Impression-Severity of Illness (CGI-S) scores, but not the Clinical Global Impression–Improvement Scale; full responders at the end of the study were significantly higher than placebo [118]. Sarris et al. (2015) conducted a RDBCT using NAC as add-on treatment (mainly to SSRIs) in OCD patients; the primary outcome measure was the Y-BOCS, conducted every 4 weeks. At week 12 a significant reduction in Y-BOCS score was observed, the difference dissipated at week 16 [119]. A third RDBCT was performed with moderate-to-severe OCD patients, randomized to receive

fluvoxamine plus placebo or fluvoxamine plus NAC. NAC showed a significant effect on the Y-BOCS [120].

Insert Table 2 here

Omega-3

Adequate dietary levels of polyunsaturated fatty acids (PUFAs), including omega-3 fatty acids, are important for health maintenance since PUFAs are important components of cholesterol esters and phospholipids of the neuronal cell membrane. Changes in the composition of these membrane phospholipids can affect the regulation of neurotransmitter release, receptors, ion channels, and enzyme activity [121,122]. Omega-3 and omega-6 PUFAs are cleaved from membrane phospholipids and converted via different pathways to mediators that have opposing effects: arachidonic acid mediators are derived from omega-6 fatty acids and are proinflammatory, while mediators derived from omega-3 fatty acids have anti-inflammatory effects. Ecosapentanoic acid (EPA) and docosahexanoic acid (DHA) are the two main representatives of omega-3 PUFAs, and fish oil is their main dietary source. It has been suggested that EPA may play a role in brain function by counteracting the arachidonic acid-mediated signaling, decreasing the immune-inflammatory responses mediated by omega-6 derived eicosanoids, which have been linked to the pathophysiology of anxiety and other mental disorders [123,124]. Moreover, through the inhibition of proinflammatory cytokines secretion, omega-3 may also decrease corticosteroid release from the adrenal gland, reducing the mood-altering effects associated with increased cortisol [125], and hence reducing the impact of cortisol in anxiety.

Several studies have been performed to investigate the effects of omega-3 fatty acids in animal models of anxiety (Table 1). Most of the rodent studies were designed with long-term administration of diet supplemented with DHA or a combination of EPA and DHA. Carrié et al. (2002) used DHA-supplemented diet to mice previously fed with a semi-synthetic balanced diet or a diet deficient in alpha-linolenic acid (ALA, antother type of omega-3 fatty acid) until the age of 8 months. The supplemented diet showed anxiolytic effects regardless of the previous diet condition and restored water maze performance impaired in the ALA deficient diet group [126]. Jašarević et al. (2014) treated female mice for three generations with omega-6/omega-3 supplemented diet and found that the male offspring of the third generation showed decreased anxiety-like behavior [127]. Diets supplemented with different combinations of PUFAs counteracted the anxiogenic effects of intracerebroventricular administered IL-1 beta [128,129] and restraint stress in rats [130,131]. Anxiolytic effect of omega-3 supplementation was also demonstrated in adult male grey mouse lemur (*Microcebus murinus*), a nocturnal Malagasy prosimian primate [132,133].

Low omega-3 levels in erythrocyte membrane have been observed in patients with anxiety disorders [134–136]. Nevertheless, most trials investigating omega-3 in anxiety focused on anxiety symptoms in different conditions rather than anxiety disorders properly. In healthy young adults, Kiecolt-Glaser et al. (2011) showed in a RDBCT that EPA and DHA supplementation decreased anxiety symptoms and the LPS-stimulated production of IL-6 [137]. Yehuda et al. (2005) showed that a mixture of alpha-linolenic acid and linolenic acid, given to university students experiencing significant anxiety associated with upcoming exams (test anxiety), improved variables associated with test anxiety (e.g.

appetite, mood, concentration, fatigue, academic organization, poor sleep) and lowered cortisol levels [138]. Anxiolytic effects of omega-3 supplementation were found in patients with acute myocardial infarction [139] and women diagnosed with premenstrual syndrome (PMS) [140]. Buydens-Branchey and Branchey (2006) investigated the effects of a mixture of EPA + DHA supplementation in patients with a history of substance abuse: the RDBCT showed that the supplementation decreased anxiety scores progressively, which remained decreased 3 months after treatment discontinuation [122]. In a subsequent similarly designed RDBCT the same group showed that increases in circulating omega-3 levels paralleled the decreases in anxiety scores [141]. Similar results were found with male alcoholic patients undergoing residential rehabilitation program: the small sample RDBCT showed that fish-oil (a source of omega-3 fatty acids) decreased the stress/anxiety ratings accompanied by a reduction of cortisol basal levels [142]. In a placebo-controlled crossover trial, Fux et al. (2004) showed that EPA is ineffective as add-on treatment to SSRI in OCD patients, though results are tainted by the small number of patients and the high placebo response [143]. MATSUOKA et al. (2015) reported that omega-3 supplementation was not superior to placebo for PTSD symptoms prevention at 3 months after accidental injury [144]. In a cohort of Japanese accident survivors, at risk for developing PTSD, the same group reported that a short-term supplementation of DHA and EPA lowered heart rate during script-driven imagery and/or resting, whereas baseline heart rate did not differ from the placebo group [145].

In addition to the compounds discussed above (agomelatine, NAC and omega-3 fatty acids) we also found some evidence of anxiolytic effects in clinical trials and animal studies for ascorbic acid (vitamin C) and the $mGlu_{2/3}$

receptor agonist LY354740. Although ascorbic acid has presented anxiolytic effects in different animal models in rats [146], mice [147] and zebrafish [148], the evidences of anxiolytic effects in humans are limited. Only one small size randomized double-blind placebo controlled clinical trial (n=42) with ascorbic acid was conducted with normal volunteers, and results showed that ascorbic acid decreased anxiety levels in students [149]. Studies with LY354740 showed a robust anxiolytic activity in diverse animal models and also in a few clinical trials, however the larger clinical trials were interrupted due to reports of seizures in animal studies [16].

A limitation of our study is the likely existence of publication bias in this field. Altough many negative results concerning the topic of our review may have been deterred from publication, our main goal was to present the available data for compounds with a robust body of evidence.

Conclusion

We reviewed three compounds able to counteract key biochemical correlates of anxiety states. Despite a reasonable body of evidence showing anxiolytic properties, the review shows that clinical data is deficient. Data from clinical trials are more indicative than conclusive and larger trials specifically designed for anxiety disorders are needed. Yet, the beneficial effect observed in clinical conditions where mainstream treatments are ineffective should not be overlooked.

Regarding safe and tolerability, clinical trials and toxicity studies show that agomelatine [78,150], NAC [83] and omega-3 [151] were generally well tolerated and free of serious adverse effects. The most common side effects reported

were headache, dizziness, somnolence, fatigue and gastrointestinal symptoms for agomelatine [150], gastrointestinal symptoms and headache for NAC [83] and a fish aftertaste and nausea with omega-3 [125,151].

In conclusion, the prevalence and morbidity of anxiety disorders, the potential translational value of the biochemical basis of anxiety, and the safety profile of these compounds seem to justify the investment in larger clinical trials.

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Disclosure

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Table 1 – Anxiolytic-like effects of multi-target compounds: preclinical studies

Compound/ dose	Dose	Treatment duration	Species	Behavioral tests	Effects	Reference
Agomelatine	2.5–80 mg/kg, i.p.	Acute	Rats	EPM , SI, UV, VCT	Anxiolytic	Millan et al. [65]
Agomelatine	10–75 mg/kg, i.p.	Acute	Rats	Conditioned footshock-induced UV, EPM, VCT,	Anxiolytic	Papp et al. [66]
Agomelatine	20–40 mg/kg i.p., acute	Acute	Rats	EPM, NIH, PD, SSWS	Anxiolytic in the EPM.	Loiseau et al. [64]
Agomelatine	40-50 mg/kg i.p.	Chronic	Rats	EPM, FST	Prevented prenatal restraint-induced anxiety-like behavior in the EPM.	Morley-Fletcher et al. [58]
NAC	50 mg/kg i.p.,	Acute	Mice	MBB	Inhibited marble-burying behavior.	Egashira et al. [98]
NAC	150 mg/kg, i.p.	10 days	Rats	EPM, OF, SI	Reversed valproate-induced anxiety-like behavior and social interaction deficit.	Chen et al. [99]

NAC	30 or 60 mg/kg i.p.	11 days	Mice	HB, SP	Prevented rhythm disruption-induced anxiety in the HB.	Pilz et al. [100]
NAC	0.1, 1.0 and 10 mg/L of tank water	Acute	Zebrafish	L/D, NT	Anxiolytic in the L/D, prevented acute stressor-induced anxiety-like behavior in NT.	Mocelin et al. [101]
NAC	60–150 mg/kg i.p.	Acute and subacute (4 days)	Mice	ETM, HB, L/D, OF, SI, SIH	Anxiolytic (except at the elevated T-maze).	Santos et al. [102]
Omega-3	Diet supplemented with DHA	Chronic	Mice	OF, L/D, MWM	Anxiolytic in the L/D.	Carrié et al. [126]
Omega-3	Diet supplemented, with different combinations of omega-3 PUFAs	Chronic	Rats	EPM, OF	Attenuated i.c.v. IL-1 beta- induced anxiety.	Song et al. [128]

Omega-3	Diet supplemented with different proportions of ethyl-EPA	Chronic	Rats	EPM, OF	Attenuated the i.c.v. IL-1 beta- induced anxiety.	Song et al. [129]
Omega-3	Diet supplemented with EPA + DHA	Chronic	Rats	EPM, modified FST, MWM	Counteracted the restraint-induced anxiety.	Ferraz et al. [130]
Omega-3	Diet supplemented with long-chain omega-3 PUFAs	Chronic	Grey mouse lemur (*Microcebus murinus*)	OF	Anxiolytic	Vinot et al. [133]
Omega-3	Diet supplemented with EPA + DHA	Chronic	Rats	Avoidance conditioning, EPM	Prevented restraint stress-induced anxiety.	Pérez et al. [131]
Omega-3	10:1 omega-6/omega-3 diet	Chronic	Mice	EPM, OF	Anxiolytic in male offspring of the third	Jašarević et al. [127]

	supplemented with DHA, for 3 generations				generation.	
Omega-3	Diet supplemented with long-chain omega-3 PUFA	Chronic	Grey mouse lemur (*Microcebus murinus*)	OF, Barnes maze	Anxiolytic	Pifferi et al. [132]

DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, EPM: elevated plus maze, ETM: elevated T-maze, FST: forced swim test, HB: hole-board, i.c.v: intracerebroventricular, i.p.: intraperitoneal, L/D: light/dark, MBB: marble-burying behavior, MWM: Morris water maze, NAC: N-acetylcysteine, NIH: novelty-induced hypophagia, NOR: novel object recognition test, NT: novel tank, OF: open field, PD: punished drinking test, PUFA: polyunsaturated fatty acid, SI: social interaction, SIH: stress-induced hyperthermia, SP: social preference, SSWS: safety signal withdrawal schedule (operant conflict procedure), UV: ultrasonic vocalization test, VCT: vogel conflict test.

Table 2 –Anxiolytic effects of multi-target compounds: clinical trials

Compound	Disorder	Study design	Study size	Daily dose and treatment duration	Main measures/ Instruments	Results	Reference
Agomelatine	GAD	RDBCT	121	25-50 mg, 12 weeks	CGI, HARS, LSEQ, SDS	Anxiolytic	Stein et al. [67]
Agomelatine	GAD	Open-label treatment followed by a multicenter RDBCT	477	25-50 mg, 16 weeks (open-label) followed by 26 weeks (RDBCT)	CGI, DESS, HAD, HARS, LSEQ, SDS	Anxioltytic and well-tolerated in long-term treatment. Superior to placebo in preventing relapse.	Stein et al. [68]
Agomelatine	GAD	Multicenter, RDBCT	412	25-50 mg, 12 weeks	CGI, HADS, LSEQ, SDS	Anxiolytic effect similar to escitalopram, with lower adverse events incidence.	Stein et al. [69]
Agomelatine	GAD	RDBCT	412	10-25 mg, 12 weeks	HARS	Anxiolytic, placebo-	Stein et al. [70]

						agomelatine difference greater with the higher dose.	
NAC	TTM	RDBCT	50	1200-2400 mg, 12 weeks	CGI, HARS MGH-HPS, PITS	Reduced hair-pulling.	Grant et al. [105]
NAC	OCD (refractory to SRI)	RDBCT	39	Initially 600 mg, doubling weekly to a maximum dose of 2400 mg (add-on treatment to SRI), 12 weeks	CGI-S, Y-BOCS	Improved mean CGI-S and Y-BOCS scale scores.	Afshar et al. [118]
NAC	Chronic nail biting	RDBCT	25	800 mg, 2 months	Nail length	Decreased nail biting over the short term.	Ghanizadeh et al. [115]
NAC	OCD	RDBCT	44	3000 mg (add-on treatment), 16 weeks	Y-BOCS	Decreased Y-BOCS score.	Sarris et al. [119]
NAC	PTSD and SUD	RDBCT	35	2400 mg, 8 weeks	CAPS, PCL-M, VAS	Improved PTSD and craving.	Back et al. [104]
NAC	Skin-picking	RDBCT	53	1200-3000 mg, 12	Measures of	Decreased skin-	Grant et al.

	disorder			weeks	skin-picking severity: CGI-S and modified Y-BOCS	picking.	[113]
NAC	OCD	RDBCT	44	2000 mg (add-on treatment to fluvoxamine), 10 weeks	Y-BOCS	Decreased scores in Y-BOCS.	Paydary et al. [120]
Omega-3	Test anxiety	Placebo controlled trial	126	90 mg of α-linolenic acid (omega-3) and 360 mg of linoleic acid (omega-6 fatty acid), 3 weeks	Standardized rating scale	Improved variables associated with test anxiety.	Yehuda et al. [138]
Omega-3	SUD	RDBCT	24	3 g, 3 months	Modified version of the POMS (baseline and monthly)	Decreased anxiety scores progressively.	Buydens-Branchey; Branchey. [123]
Omega-3	SUD	RDBCT	22	3 g, 3 months	Modified version of POMS	Decreased anxiety scores.	Buydens-Branchey et

							al. [141]
Omega-3	Healthy young adults	RDBCT	68	2.5 g, 12 weeks	BAI, CES-D	Decreased anxiety.	Kiecolt-Glaser et al. [137]
Omega-3 (fish oil)	Alcoholic patients	RDBCT	31	60 mg EPA + 252 mg DHA, 3 weeks	PSS	Decreased anxiety/stress.	Barbadoro et al. [142]
Omega-3	Early post-myocardial infarction	RDBCT	52	1 g + standard pharmacotherapy, 1 month	BDI, ESQ, STAI-S, STAI-T, used at the baseline (3rd day of AMI) and after one month	Decreased anxiety (STAI-S)	Haberka et al. [139]
Omega-3	PMS	RDBCT	124	2 g, 3 months	VAS	Decreased anxiety severity and duration.	Sohrabi et al. [140]
Omega-3	Japanese accident survivors (at risk for developing	RDBCT	83	1470 mg DHA + 147 mg EPA, 12 weeks	Monitoring of heart rate and skin conductance, Script-driven	Decreased heart rate.	Matsumura et al. [145]

	PTSD)	imagery of their traumatic event,

BAI: Beck Anxiety Inventory, BDI: Beck Depression Inventory, CAPS: Clinician Administered PTSD Scale, CES-D: Center for Epidemiological Studies Depression Scale, CGI: Clinical Global Impression Scale, CGI-S: Clinical Global Impression – Severity of Illness, DESS: Discontinuation Emergent Signs and Symptoms checklist, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, ESQ: Emotional State Questionnaire, GAD: generalized anxiety disorder, HAD: Hospital Anxiety and Depression Scale, HARS: Hamilton Anxiety Rating Scale, LSEQ: Leeds Sleep Evaluation Questionnaire, MGH-HPS: Massachusetts General Hospital Hair Pulling Scale, NAC: N-Acetylcysteine, OCD: obsessive-compulsive disorder, PCL-M: PTSD Checklist-Military, PITS: Psychiatric Institute Trichotillomania Scale, PMS: premenstrual syndrome, POMS: Profiles of Mood States, PSS: Perceived Stress Scale, PTSD: post-traumatic stress disorder, PUFA: polyunsaturated fatty acid, RDBCT: randomized double-blind placebo-controlled trial, SDS: Sheehan Disability Scale, SRI: serotonin reuptake inhibitor, STAI-S: State-Trait Anxiety Inventory in a Specific Situation, STAI-T: State-Trait Anxiety Inventory as a General Trait, SUD: substance use disorder, TTM: trichotillomania, VAS: Visual Analog Scale, Y-BOCS: Yale-Brown Obsessive-Compulsive Scale.

Artigo 2

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Research report

Anxiolytic properties of *N*-acetylcysteine in mice

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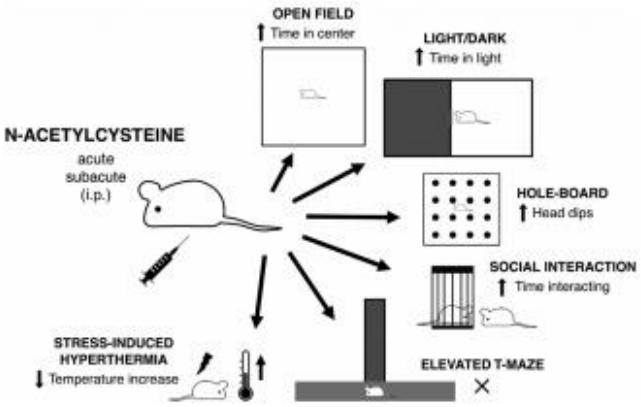
HIGHLIGHTS

- NAC shows anxiolytic effects on five mice models of anxiety.
- Subacute NAC results in lower effective anxiolytic doses than acute treatment.
- Anxiolytic doses of NAC do not affect locomotion.

GRAPHICAL ABSTRACT



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ABSTRACT

Anxiety disorders are highly prevalent and often result in poor quality of life. Available anxiolytics show significant adverse effects as well as partial efficacy in a sizable part of patients. Innovative treatments with more favorable risk-benefit ratio are sorely needed. A growing body of clinical data indicates the benefits of *N*-acetylcysteine (NAC) in psychiatric conditions. NAC modulates antioxidant, glutamatergic, inflammatory and neurotrophic pathways in the central nervous system, all of which are relevant to anxiety pathology. We evaluated the effects of NAC in mice models commonly used to characterize anxiolytic compounds. Male adult CF1 or BALB/c mice were treated (i.p.) acutely or subacutely (4 consecutive days) with NAC (60–150 mg/kg) 60 min before open field, light/dark, hole-board, social interaction, elevated T-maze or stress-induced hyperthermia tests. Diazepam (2 mg/kg) was used as positive control. We found that NAC presents anxiolytic effects in all models, except for the elevated T-maze. Subacute treatments

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resulted in lower effective doses in comparison to acute treatment. The anxiolytic effects of NAC were comparable to diazepam. NAC is a safe and low cost medicine with suggested benefits in psychiatric conditions often presenting co-morbidity with anxiety. This study contributes evidence to support the validity of clinical trials with NAC in the context of anxiety disorders, especially considering the safety profile in comparison to the limitations of diazepam for long term treatment.

1. Introduction

Anxiety can be defined as the emotional anticipation of aversive situations, and is associated with behavioral and endocrine adaptive responses to cope with threatening stimuli [1]. Anxiety can become pathological when unsuccessful in its adaptive function, especially if combined with predisposing factors [2]. Anxiety disorders have an estimated prevalence of 28% [3], resulting in significant impairments in quality of life and high economic costs related to diagnostic problems, inappropriate treatment, and extensive use of health services [4].

Current available anxiolytics act on GABAergic and serotonergic systems, including benzodiazepines (BZDs), partial 5-HT_{1A} receptor agonist (buspirone) and selective serotonin reuptake inhibitors (SSRIs). The adverse effects of BZDs and SSRIs [5], the slow onset of action and limited scope of buspirone [6], as well as the various forms of treatment resistant anxiety [7,8], reinforce the need for innovative treatments. While congruent with the less than desirable efficacy of typical treatments, the recognition that anxiety disorders may result from hyperactivity of the excitatory glutamatergic system [1,9] and is accompanied by neuroinflammation [10,11] and oxidative stress [12–14] has opened new scenarios in the field.

N-acetylcysteine (NAC), a precursor of glutathione, possesses an original mechanism of action composed by the modulation of antioxidant, glutamatergic, inflammatory and neurotrophic pathways [15,16]. Long marketed as treatment for paracetamol poisoning, chronic obstructive pulmonary disease and contrast-induced nephropathy [16], a growing body of clinical data bespeaks on the benefits of NAC in psychiatric conditions. Favorable evidence for NAC has been reported for schizophrenia, autism, Alzheimer's disease, drug-induced neuropathy, progressive myoclonus epilepsy, bipolar disorder, depression, addiction (cocaine, heroin, cigarettes and marijuana), obsessive compulsive disorder, trichotillomania, nail biting, and skin picking [15–21]. Relevant to this study, marked anxiety is frequently presented in several of the conditions where NAC seems to be clinically useful and a core symptom in obsessive-compulsive disorder, trichotillomania, nail biting, and skin picking.

Considering the paucity of data on properties of NAC on anxiety [22,23], the purpose of this study was to verify the effects of NAC in mice. The effects of various doses of acute and subacute (4 days) NAC were evaluated in the open field, light/dark, hole-board, social interaction, elevated T–maze and stress-induced hyperthermia models.

2. Materials and methods

2.1. Animals

Two month-old male mice were used: CF1 (from Universidade Federal do Rio Grande do Sul, UFRGS) or BALB/c mice (from Universidade Federal de Pelotas, UFPel). Mice were housed in groups of 4 animals per cage (30 × 19 × 13 cm), under controlled environmental conditions (22 ± 2 °C, 12-h light/dark cycle, lights on 07:00 h, food and water *ad libitum*). The animals were kept in our animal facility for at least 14 days before experiments. All procedures were carried out according to institutional policies on experimental animals handling and approved by the University Ethics Committee (approval #22308 and #27553). A total of 535 mice were used in this study (447 CF1 and 88 BALB/c).

2.2. Drugs

N-acetylcysteine (NAC) was purchased from Sigma-Aldrich (St Louis, Missouri, USA). Diazepam (DZP) was used from commercial source (injectable ampoules from Teuto Laboratories, GO, Brazil). All drugs were solubilized in saline (NaCl 0.9%), which was used as the negative control. Injection volume was 0.1 ml/10 g of body weight. All drugs were administered intraperitoneally.

2.3. Experimental design

Mice were habituated to the experimental room for at least 30 min before behavioral testing. Except for stress-induced hyperthermia, tests were performed in dimly lit (red light 26 W) experimental room. Animals were randomly assigned to the treatment groups. All parameters were quantified by researchers blind to the treatment groups. Behavioral tests were performed in separate groups of animals; each mouse was used only once.

Acute treatment: animals were treated with saline, DZP 1 or 2 mg/kg (as specified), or NAC 60, 100 or 150 mg/kg. NAC was administered 60 min and DZP 30 min before behavioral tests, except for stress-induced hyperthermia where all treatments were administered 60 min before tests (because temperature returns to basal levels 60 mins after injections [24]).

Subacute treatment: animals were treated for four consecutive days with saline, DZP 1 or 2 mg/kg (as specified), or NAC 10, 30, 60 or 100 mg/kg. The last administration of NAC was realized 60 min, and that of DZP 30 min, before behavioral tests, except for the stress-induced hyperthermia where all treatments were administered 60 min before tests. The time course for drug administration was chosen according to specificities in pharmacokinetics, the literature and upon pilot experiments showing that NAC was effective 1 h after i.p. administration whereas diazepam within 30 min [25–31].

2.4. Behavioral tests

2.4.1. Open field

To discard non-specific reactions to the pharmacological treatments or changes in locomotion, CF1 mice (n = 7–10) were tested in a gray wooden apparatus (40 × 40 × 40 cm). In addition to locomotor activity, the time spent in the center of the open field was assessed to index anxiety levels [32]. Animals were allowed to explore the arena for 15 min; the first 5 min were considered as exploratory behavior and the last 10 min as locomotion. The experiment was recorded by a digital camera installed above the arena and videos were analyzed using ANY-Maze tracking software (Stoelting Co., Wood Dale, IL, USA).

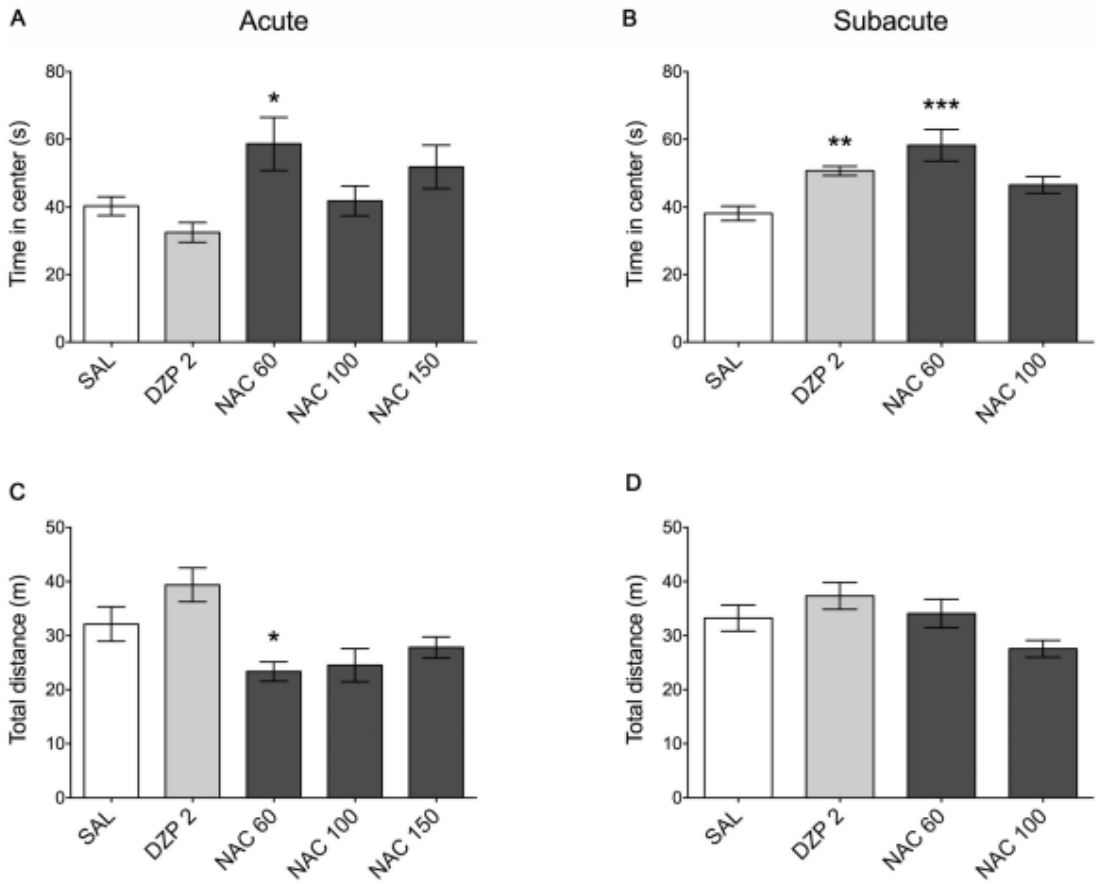

Fig. 1. Effects of acute (A and C) and subacute (B and D) *N*-acetylcysteine (NAC) on the open field test in CF1 mice. Saline (SAL) was used as the vehicle and diazepam (DZP) was used as the positive control. Doses are indicated as mg/kg. Bars represent mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001 vs. SAL. One-way ANOVA followed by LSD. n = 7–10.

2.4.2. Light/dark

The light/dark apparatus consists of a rectangular wooden box (46 × 27 × 30 cm) divided into a smaller (18 × 27 cm) and a larger area (27 × 27 cm) by a square opening (7 × 7 cm). The smaller compartment is painted black and not lighted, while the larger is painted white and lightened by a 60 W lamp. CF1 mice (n = 9–14) were individually placed in the light compartment facing the opening. The experiment was video recorded from above for 5 min and the time spent in the light compartment was analyzed [27].

2.4.3. Hole-board

The hole-board apparatus (Ugo Basile, Italy) consists of a gray Perspex panel (40 × 40 cm, 2.2 cm thick) with 16 equidistant holes (3 cm diameter) in the floor. Photocells below the surface of the hole provided the measures of the number of head dips. The board was positioned 15 cm above the table. The floor was divided (with gray water resistant marker) into 9 squares of 10 × 10 cm. A transparent acrylic rectangle was positioned over the board during the experiments. CF1 mice (n = 9–15) were placed individually in the center of the board and the following parameters assessed for 5 min: number of head dips, latency to the first head dip, and number of squares crossed with all four paws [33].

2.4.4. Social interaction

The social interaction protocol was modified from Silverman et al. [34]. CF1 mice (n = 9–10) were tested in a wooden gray apparatus (40 × 40 × 40 cm), containing two small metal barred cups placed in opposite corners of the apparatus. Pairs of mice unknown to each other were placed in the apparatus, one inside one of the barred cups. The subject mouse under observation was placed in the apparatus facing the cuped animal; interaction was defined as the time spent by the subject mouse exploring the containment cup housing the stranger mouse. The experiment was videotaped for 10 min by a digital camera installed above the arenas; the videos were analyzed with The Observer ® XT 5.0 (Noldus Information Technology, Wageningen, The Netherlands).

2.4.5. Elevated T-maze

Described by Graeff et al. [35,36] and adapted for mice by Carvalho-Netto and Nunes-de-Souza [37], the elevated T-maze (ETM) consists of a T-shaped black acrylic apparatus with three arms of equal dimensions (30 × 5 cm) elevated 50 cm above the floor. One arm enclosed by 20 cm high dark walls is perpendicular to two opposed open arms, both surrounded by a 0.25 cm high border. BALB/c mice (n = 9–17) were placed at the end of the enclosed arm and the latency to leave the enclosed arm (inhibitory avoid-

ance) was measured in 3 consecutive trials, with a 30-s intertrial interval and a cut-off time of 300 s.

2.4.6. Stress-induced hyperthermia

Each experimental group (n = 9–10) was subjected to a modified stress-induced hyperthermia (SIH) test adapted from Van der Heyden et al. [24]. CF1 mice were housed individually (30 × 19 × 13 cm cages) 24 h before testing. Body temperature was measured to the nearest 0.1 °C by using a digital thermometer (Geratherm Medical AG, Geschwenda, Germany) lubricated with liquid paraffin. The thermometer was inserted 2 cm into the rectum while the mouse was gently hand held near the base of the tail; the probe was left in place until steady readings were obtained (approximately 30 s). Body temperature was measured at baseline (T1) and 15 min after the first recording (T2). The first measurement induces a mild stress that elevates body temperature and the stress-induced hyperthermia was determined as T2 – T1.

2.5. Statistical analysis

Statistical analyses for acute and subacute conditions were carried out separately. Data were analyzed by analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc comparisons. A single factor (treatment) was analyzed by one-way ANOVA in all tests except the elevated T-maze, which was analyzed by two-way ANOVA with drug as the independent variable and trial as the repeated measure; restricted one-way ANOVAs were carried out when appropriate. ANOVA results are fully disclosed in the results section, and the p value of each significant post hoc comparison between treatment and control groups is depicted in the figures. Statistical significance was set at $p < 0.05$. Values are expressed as mean ± standard error of mean (S.E.M.).

3. Results

3.1. Open field

Acute and subacute NAC 60 mg/kg increased the time spent in the arena center ($F_{4,37} = 4.08$, $p = 0.007$ and $F_{3,34} = 8.26$, $p = 0.0003$, Fig. 1A and B, respectively) suggesting anxiolytic activity. Diazepam induced the same effect only after subacute treatment. Acute NAC 60 mg/kg decreased the total distance moved ($F_{4,37} = 6.09$, $p = 0.0007$) (Fig. 1C), while all other acute and subacute treatments did not affect locomotion ($F_{3,34} = 2.66$, $p = 0.064$) (Fig. 1D).

3.2. Light/dark

Acute diazepam and NAC 150 mg/kg increased the time spent in the light compartment ($F_{4,47} = 6.05$, $p = 0.0005$) (Fig. 2A), as observed with anxiolytics. Subacute diazepam and NAC 100 mg/kg treatment also presented anxiolytic effect ($F_{3,35} = 7.21$, $p = 0.0007$) (Fig. 2B).

3.3. Hole-board

Diazepam and NAC at all tested doses increased the number of head dips after acute ($F_{4,49} = 4.45$, $p = 0.004$) (Fig. 3A) and subacute treatments ($F_{3,36} = 6.99$, $p = 0.0008$) (Fig. 3B). Subacute diazepam and NAC 100 mg/kg decreased the latency to the first head dip ($F_{3,36} = 3.25$, $p = 0.033$) (Fig. 3D), while acute treatments did not alter this parameter ($F_{4,49} = 1.04$, $p = 0.39$) (Fig. 3C). Number of crossings was not altered by acute ($F_{4,49} = 1.25$, $p = 0.356$) or subacute treatments ($F_{3,36} = 0.38$, $p = 0.769$) (data not shown).

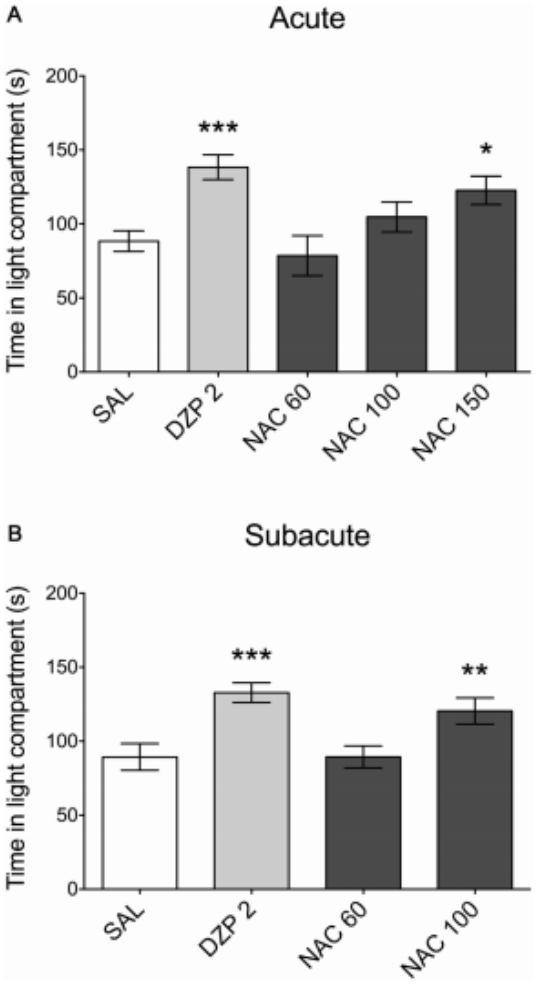

Fig. 2. Effects of acute (A) and subacute (B) *N*-acetylcysteine (NAC) on the light/dark test in CF1 mice. Saline (SAL) was used as the vehicle and diazepam (DZP) was used as the positive control. Doses are indicated as mg/kg. Bars represent mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001 vs. SAL. One-way ANOVA followed by LSD. n = 9–14.

3.4. Social interaction

Acute diazepam and NAC 150 mg/kg ($F_{4,42} = 7.38$, $p = 0.0001$) (Fig. 4A), as well as subacute diazepam ($F_{3,34} = 3.74$, $p = 0.02$) (Fig. 4B), increased social interaction.

3.5. Elevated T-maze

For acute experiments (Fig. 5A), two-way ANOVA revealed a significant main effect of trial ($F_{2,48} = 11.35$, $p < 0.0001$). There was no main effect of treatment ($F_{2,24} = 0.86$, $p = 0.437$) or interaction effect ($F_{4,48} = 0.73$, $p = 0.579$).

For subacute experiments (Fig. 5B), two-way ANOVA revealed significant main effects of trial ($F_{2,114} = 9.09$, $p = 0.0002$) and treatment ($F_{3,57} = 3.43$, $p = 0.023$). There was no interaction effect ($F_{6,114} = 1,46$, $p = 0.198$). Restricted one-way ANOVAs to compare different treatments within each trial revealed that diazepam sig-

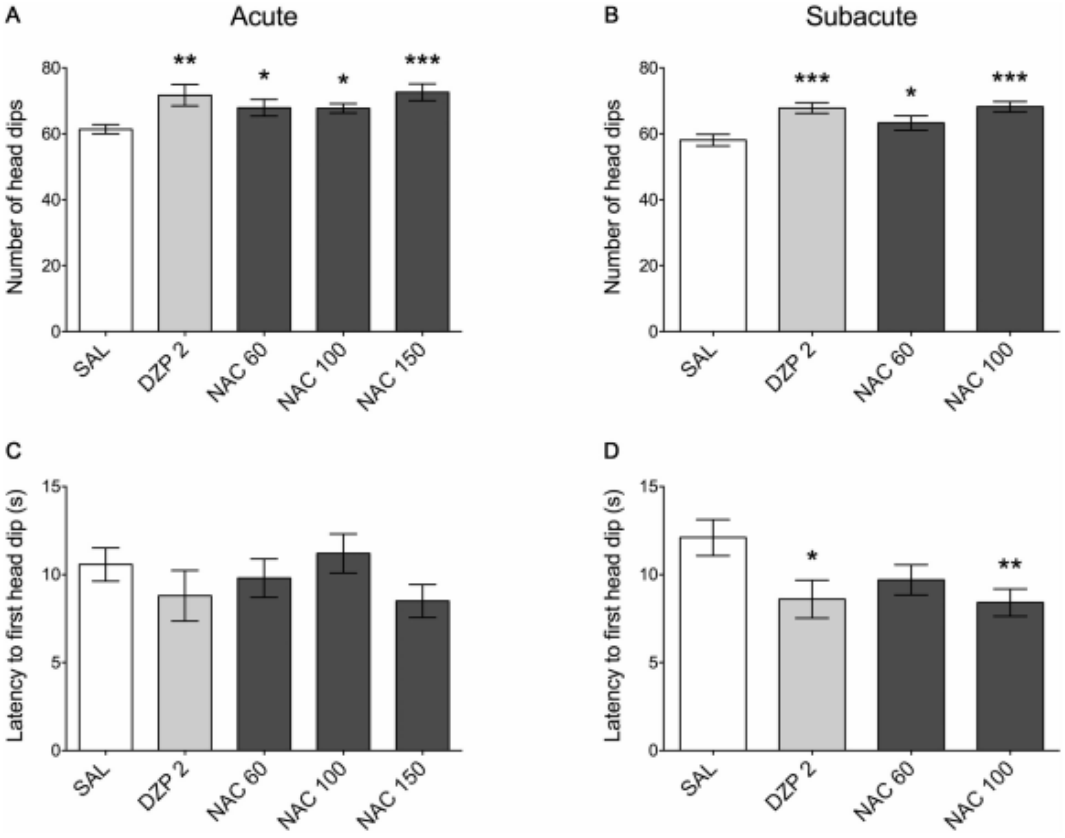

Fig. 3. Effects of acute (A and C) and subacute (B and D) *N*-acetylcysteine (NAC) on the hole-board test in CF1 mice. Saline (SAL) was used as the vehicle and diazepam (DZP) was used as the positive control. Doses are indicated as mg/kg. Bars represent mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001 vs. SAL. One-way ANOVA followed by LSD. n = 9–15.

nificantly reduced latency to exit the closed arm compared to control group in trial 3 ($F_{3,57} = 3.36$, $p = 0.025$), demonstrating its anxiolytic effect.

3.6. Stress-induced hyperthermia

Basal body temperatures (T1) fell within the range (circa 37 °C) reported in the literature for mice [24]. Acute NAC 100 and 150 mg/kg, but not diazepam, decreased T1 ($F_{4,44} = 13.45$, $p < 0.0001$) (Fig. 6A). Subacute treatments did not affect T1 ($F_{4,45} = 0.56$, $p = 0.565$) (Fig. 6B).

Only acute diazepam reduced the stress-induced hyperthermia ($F_{4,44} = 7.8$, $p < 0.0001$) (Fig. 6C). Subacute diazepam and NAC (all doses) reduced the stress induced hyperthermia ($F_{4,45} = 6.27$, $p = 0.0004$) (Fig. 6D).

4. Discussion

We found that NAC presents anxiolytic effects in the open field, light/dark, hole-board, social interaction and stress-induced hyperthermia models. No effects were seen with the elevated T-maze model. In most models the anxiolytic effect of NAC was comparable to that of diazepam. In the models in which anxiolytic effects were seen with acute and subacute NAC, subacute treatments resulted in lower effective doses. This study corroborates and complements our recent report suggesting an anti-stress effect of NAC in zebrafish [22]. Notably, the open field, light/dark, hole-board, social interaction, and stress-induced hyperthermia are among the rodent tests most frequently used to detect and evaluate anxiolytic/anxiogenic properties in drug discovery [38,39]. Though Durieux et al. [23] reported that NAC 150 mg/kg resulted in increased anxiety after 130 min in the open field with C57BL/6 mice, our more comprehensive analyses point to anxiolytic, rather than anxiogenic, overall effect.

Our choice for the acute doses was based on the reported inhibitory effects of NAC on the mice marble burying, a model of obsessive compulsive disorder [40]. The choice of 4 days of administration for subacute treatment was based on the anxiolytic-like effects of NAC after alcohol cessation in rats [41] and the ability of NAC to quickly reset hyperactive glutamate transmission [15]. Accumulating evidence suggests that increased glutamate excitatory activity is the foundation of anxiety disorders [1,42]. Mice with high susceptibility to stress-induced anxiety exhibited lower $mGlu_2$ receptors mRNA transcript levels in the hippocampus than mice with low susceptibility or non-stressed controls [43]. Agonists of $mGlu_{2/3}$ receptors present anxiolytic activity in various animal models of anxiety [1,44], though clinical trials are still lacking. Located presynaptically, $mGlu_{2/3}$ receptors are expressed in brain areas (cortex, thalamus, striatum, amygdala and hippocampus) associated with pathologic anxiety [45,46]. Activation of $mGlu_{2/3}$

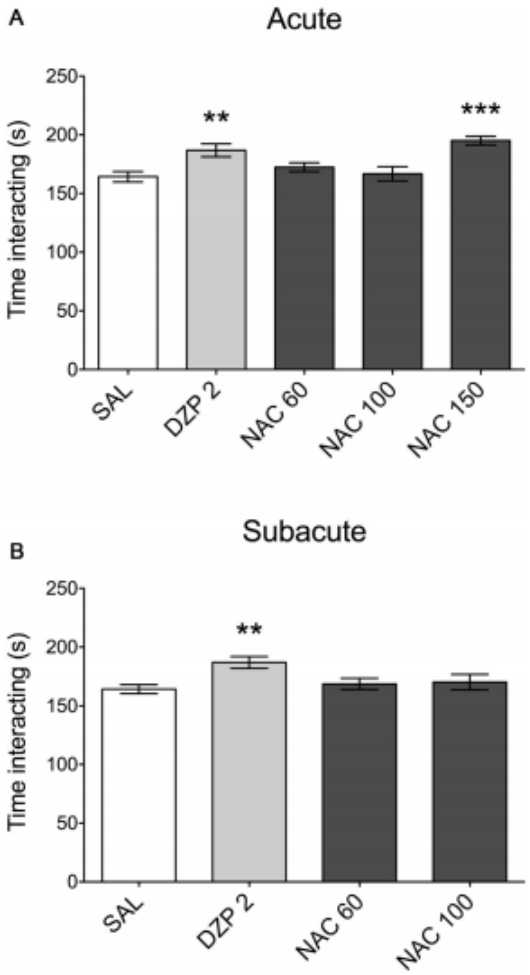

Fig. 4. Effects of acute (A) and subacute (B) *N*-acetylcysteine (NAC) on the social interaction test in CF1 mice. Saline (SAL) was used as the vehicle and diazepam (DZP) was used as the positive control. Doses are indicated as mg/kg. Bars represent mean ± S.E.M. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. SAL. One-way ANOVA followed by LSD. n = 9–10.

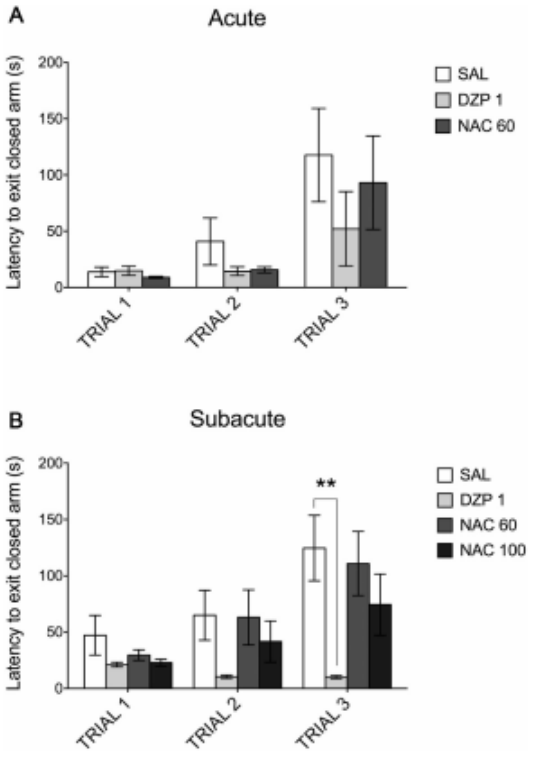

Fig. 5. Effects of acute (A) and subacute (B) *N*-acetylcysteine (NAC) on the elevated T-maze test in BALB/*c* mice. Saline (SAL) was used as the vehicle and diazepam (DZP) was used as the positive control. Doses are indicated as mg/kg. Bars represent mean ± S.E.M. $^{**}p < 0.05$ for the indicated comparison. Two-way ANOVA followed by restricted ANOVA/LSD. n = 9–17.

receptors limits the neuronal release of glutamate by negative feedback [47]. By promoting glutamate release from astrocytes through the cystine/glutamate antiporter, NAC stimulates extra-synaptic $mGlu_{2/3}$ receptors [48], ultimately decreasing glutamate neurotransmission.

Glutamate hyperactivation can lead to oxidative stress and inflammation [49,50], both of which have been linked to anxiety [10,51,52]. Increased anxiety in rodents has been correlated with increased levels of reactive oxygen species [53] and overexpression of enzymes that protect against oxidative stress (e.g., brain glyoxalase-1 and glutathione reductase-1) [54]. Increased anxiety-like behavior was observed in mice after the induction of oxidative stress in amygdala and hypothalamus [55] and in rats treated with pro-oxidant levels of vitamin A [13] and oxidative stress inducers [52]. While oxidative stress contributes to inflammation and cytotoxicity, pro-inflammatory cytokines induce oxidative stress and neurotoxicity [56]. Systemic inflammation induces anxiety-like behavior in mice [57,58] and rats [59]. Mice deficient in NF-κB (nuclear factor kappa-B), a transcription factor key in controlling inflammatory response, exhibit decreased anxiety-like behaviors [60]. Higher levels of cortisol and the pro-inflammatory cytokines IL-6 (interleukin-6) and TNF-α (tumor necrosis factor-α) have been repeatedly documented in patients with anxiety disorders [10,51]. The intertwined antioxidant and anti-inflammatory properties of NAC are therefore relevant to its anxiolytic effects.

Our finding that the anxiolytic effect of NAC was comparable to that of diazepam requires further exploration (e.g., dose effect curves). The finding is clinically relevant considering the side-effect profile of NAC compared to diazepam. BZDs in general and specifically diazepam, the most widely used BZD, are drugs to treat a range of conditions, including anxiety and panic attacks. Diazepam possess a wide safety window, low toxicity potential at high doses and rare serious acute side effects. Nevertheless, BZDs long-term use can result in tolerance, dependence, and withdrawal symptoms on dose reduction [61], limiting its chronic usage. It is therefore highly desirable to explore the role of NAC as an agent for the long term treatment of anxiety related disorders. Because anxiety, as well as BZD dependence, is more frequent in women [62], replicating this study in female mice is desirable, though the issue of the confounding interference of phases of estrous cycle needs to be properly addressed [63].

A limitation of our study is that we did not investigate the exact mechanism(s) underlying the anxiolytic effects of NAC in

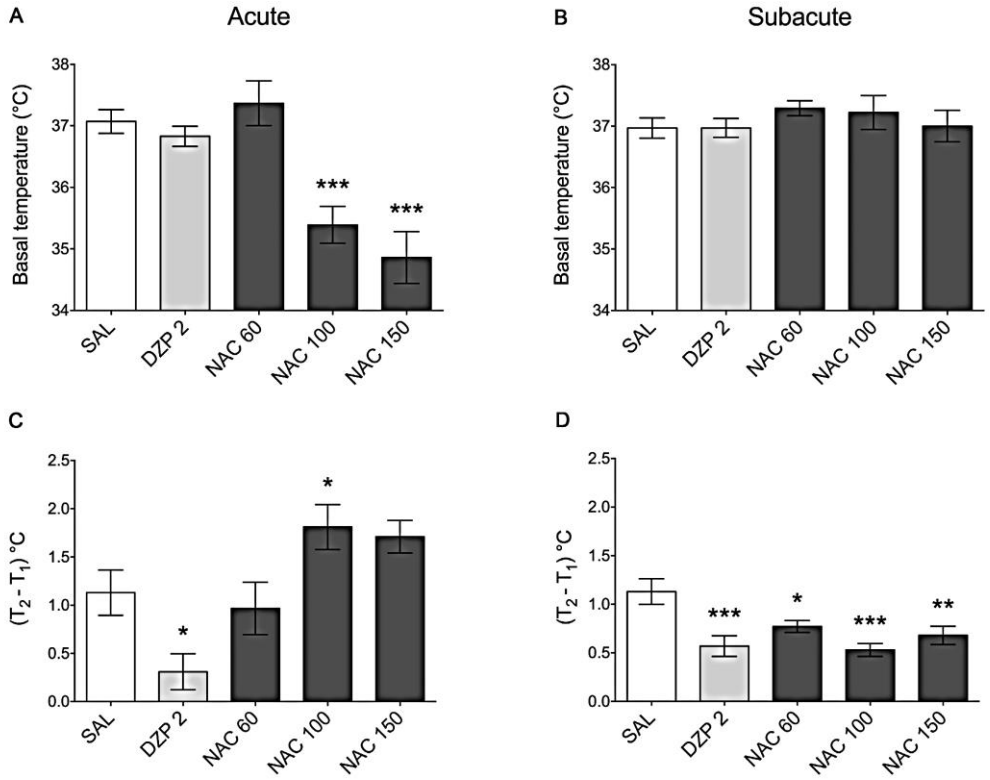

Fig. 6. Effects of acute (A and C) and subacute (B and D) *N*-acetylcysteine (NAC) on stress-induced hyperthermia in CF1 mice. Saline (SAL) was used as the vehicle and diazepam (DZP) was used as the positive control. Doses are indicated as mg/kg. Bars represent mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. SAL. One-way ANOVA followed by LSD. n = 9–10.

the mouse models. It is nevertheless arguable that the modulation of antioxidant, inflammatory and glutamatergic pathways are all in place. NAC inhibits NF-κB, downregulating the expression of pro-inflammatory genes [64]. Doses of NAC compatible with the ones used in this study (150–300 mg/kg, 1–4 injections) had anti-inflammatory effects on various rat models of neuroinflammation [65–68]. Antioxidant effects were likewise documented with NAC regimens compatible with this study. Subacute NAC (50–100 mg/kg, 10 days) reversed the olfactory bulbectomy-induced decrease in superoxide dismutase activity in various brain areas [69]. Subacute NAC (60 mg/kg, 7 days) decreased the oxidative damage in prefrontal cortex, amygdala and hippocampus of rats submitted to chronic mild stress [70]. Though it is arguable that the anti-inflammatory and antioxidant effects of NAC are likely to participate in the anxiolytic effects here presented, direct evidence of neuroinflammation and oxidative stress in the experimental models, and the combined effects of NAC on biochemical markers and behavior are needed to elucidate the molecular basis of NAC anxiolytic-like effects.

5. Conclusion

We identified the anxiolytic effect of NAC using unconditioned responses in ethologically based anxiety models. Though the models used in this study are better related to human anxiety than the conditioned responses elicited by conflict based models [32], investigating the effects of NAC in anxiety models associated with early life manipulation, oxidative stress, inflammation and glutamate deregulation would further increase the translational value of the present data.

Conflict of interest

The authors declare no conflict of interest.

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5. DISCUSSÃO

A ansiedade pode ser definida como um sentimento de inquietação, angústia, nervosismo ou preocupação sobre eventos futuros, cujos resultados são incertos (MAH; SZABUNIEWICZ; FIOCCO, 2016), ou ainda como uma emoção relacionada ao comportamento de avaliação de risco que é evocado em situações em que as demandas são interpretadas como ameaça potencial (GRAEFF, 2007). Esses sentimentos subjetivos são acompanhados geralmente de sintomas como agitação, sudorese, tontura e taquicardia. A ansiedade é uma condição que faz parte da vida e é benéfica e quando ocasional e temporária e tem valor adaptativo, levando o indivíduo a evitar danos físico ou prejuízos psicológicos ao organismo, sendo que um certo nível de ansiedade é importante para a eficiência no desempenho de tarefas intelectuais (GRAEFF, 2001). No entanto, a ansiedade pode se tornar patológica quando é frequente, consistindo em sintomas que interferem significativamente na vida cotidiana, prejudicando o desempenho intelectual, o trabalho e as relações pessoais (MAH; SZABUNIEWICZ; FIOCCO, 2016).

Apesar da alta prevalência dos transtornos de ansiedade, seu tratamento com fármacos ansiolíticos atualmente conta com fármacos que tem uma resposta abaixo do esperado e ocorrência de efeitos adversos pouco tolerados, o que compromete a adesão do paciente ao tratamento. Neste contexto, é importante ressaltar que a ansiedade patológica quando não tratada adequadamente pode causar dano ao cérebro, via degeneração estrutural e prejuízo no funcionamento do hipocampo e córtex pré-frontal, o que pode ser relacionado ao risco aumentado de desenvolvimento de transtornos neuropsiquiátricos como a depressão e demência (MAH; SZABUNIEWICZ; FIOCCO, 2016).

O princípio dominante para o desenvolvimento de fármacos ansiolíticos nos últimos 50 anos foi identificar compostos que agissem seletivamente em alvos moleculares específicos, o que levaria a tratamentos mais efetivos e com menos efeitos adversos (GRIEBEL; HOLMES, 2013). Esta abordagem reducionista não teve sucesso significativo e satisfatório, pois apesar de relativamente seletivas em relação ao alvo de ação os fármacos desenvolvidos até então não são isentos de efeitos adversos significativos. Atualmente existe

uma tendência de desenvolvimento de novos agentes com mecanismos de ação multi-alvo em várias áreas, incluindo o sistema nervoso central (HOPKINS, 2008; YOUDIM; BUCCAFUSCO, 2005).

Existem fortes evidências de que as bases fisiopatológicas da ansiedade são multifatoriais, com importante envolvimento da hiperatividade glutamatérgica (KRYSTAL et al., 2010; PITSIKAS, 2014; RIAZA BERMUDO-SORIANO et al., 2012; WIEROŃSKA et al., 2011; WIEROŃSKA; PILC, 2013), estresse oxidativo (BOUAYED et al., 2007; BOUAYED; RAMMAL; SOULIMANI, 2009; HASSAN et al., 2014; HOVATTA et al., 2005; KROLOW et al., 2014; LANDGRAF et al., 2007; NG et al., 2008; SALIM et al., 2010b; SALIM, 2017) e neuroinflamação (FIORE et al., 1998; FURTADO; KATZMAN, 2015; REICHENBERG et al., 2001; SAKIĆ et al., 1994; SALIM; CHUGH; ASGHAR, 2012; SCHROTT; CRNIC, 1996).

Considerando a natureza multifatorial da ansiedade, a disponibilidade atual de fármacos ansiolíticos com respostas terapêuticas abaixo do esperado e a ocorrência de efeitos adversos significativos relacionados ao uso desses compostos, o primeiro objetivo deste estudo foi investigar estudos na literatura sobre agentes com mecanismo de ação multialvo agindo como moduladores da neurotransmissão glutamatérgica, do estresse oxidativo e da neuroinflamação.

Os resultados demonstram que os três agentes com o referido mecanismo de ação multialvo que demonstram propriedades ansiolíticas consistentes na literatura são a agomelatina, a NAC e os ácidos graxos poliinsaturados ômega-3 (ômega-3) (artigo 1).

A agomelatina possui um mecanismo de ação multialvo que envolve efeitos como: agonista de receptores melatoninérgicos MT_1/MT_2 (MILLAN et al., 2003; SAN; ARRANZ, 2008), antagonista de receptores serotonérgicos 5-HT_{2C} (RACAGNI et al., 2011), modulador da neurotransmissão glutamatérgica (MORLEY-FLETCHER et al., 2011; RACAGNI et al., 2011; REAGAN et al., 2012; TARDITO et al., 2010), da resposta inflamatória (MOLTENI et al., 2013), além de efeitos antioxidantes (AKPINAR; UĞUZ; NAZIROĞLU, 2014). Provavelmente, todos esses mecanismos são relevantes para os efeitos ansiolíticos, considerando que os efeitos antioxidantes, moduladores da inflamação e da neurotransmissão glutamatérgica são capazes de interferir com vias importantes envolvidas nas bases biológicas da ansiedade. Além do

efeito ansiolítico demonstrado em estudos pré-clínicos utilizando diferentes paradigmas (LOISEAU et al., 2006; MILLAN et al., 2005; MORLEY-FLETCHER et al., 2011; PAPP et al., 2006), os estudos clínicos atualmente indicam que a agomelatina é um fármaco promissor no tratamento do TAG (STEIN et al., 2012, 2014, 2017; STEIN; AHOKAS; DE BODINAT, 2008).

Já a NAC é um fármaco com potencial para agir simultaneamente em múltiplos alvos atualmente relacionados com os transtornos de ansiedade (DEAN; GIORLANDO; BERK, 2011). Como esse fármaco é amplamente utilizado para o tratamento de diversas condições há anos, não são necessários novos estudos para avaliação da sua segurança e toxicidade (ASHBURN; THOR, 2004).

Os resultados de ensaios clínicos indicam efeitos benéficos da NAC em tricotilomania (GRANT; ODLAUG; KIM, 2009), no hábito de roer unhas (GHANIZADEH; DERAKHSHAN; BERK, 2013), no transtorno de escoriação (GRANT et al., 2016), no TOC (AFSHAR et al., 2012; PAYDARY et al., 2016; SARRIS et al., 2015) e no TEPT associado a transtorno por abuso de substâncias (BACK et al., 2016). Considerando que existe um importante papel da desregulação da neurotransmissão glutamatérgica na fisiopatologia do TOC (CHAKRABARTY et al., 2005; PITTENGER; KRYSTAL; CORIC, 2006) e de outros transtornos relacionados (GRADOS et al., 2015), os efeitos modulatórios da NAC sobre a neurotransmissão glutamatérgica parecem ser um importante aspecto de seu mecanismo de ação responsável pelo controle de comportamentos compulsivos, tais como tricotilomania, escoriações, hábito de roer as unhas (onicofagia) e sintomas de TOC. Por outro lado, estudos demonstram que o estresse oxidativo também está aumentado em pacientes com TOC e outros transtornos de ansiedade (KROLOW et al., 2014; NG et al., 2008), o que indica que os efeitos antioxidantes da NAC também devem contribuir para seus efeitos ansiolíticos e efeitos anti-obsessivo-compulsivos. Adicionalmente, considerando-se que o estresse oxidativo induz resposta inflamatória e vice-versa (NAJJAR et al., 2013; SATTLER; TYMIANSKI, 2001), pode ser que o efeito da NAC na modulação da resposta inflamatória também contribua para seus efeitos ansiolíticos. Recentemente, foram demonstrados efeitos ansiolíticos da NAC em estudos pré-clínicos com diferentes modelos animais em roedores (CHEN et al., 2014; EGASHIRA et al., 2012; PILZ et al.,

2015; SANTOS et al., 2017) e também em peixes-zebra (MOCELIN et al., 2015). Esses resultados corroboram a necessidade de ensaios clínicos de larga escala com a NAC em transtornos de ansiedade como TAG, TAS, TP e TEPT. É também importante ressaltar também que a NAC tem demonstrado benefícios em estudos clínicos em comorbidades psiquiátricas comuns de transtornos de ansiedade (DEEPMALA et al., 2015).

Os ácidos graxos poli-insaturados ômega-3 possuem uma diversidade de ações relevantes para seus efeitos psicotrópicos. Alguns ácidos graxos poli-insaturados são componentes dos fosfolipídeos de membranas neuronais, principalmente o ácido araquidônico (ácido graxo ômega-6) e os ácidos graxos ômega-3 DHA (ácido docosahexaenóico) e EPA (ácido eicosapentaenóico) (BOURRE et al., 1991; YEHUDA; RABINOVITZ; MOSTOFSKY, 1999). Mudanças na composição desses fosfolipídeos de membrana podem afetar muitas funções importantes que os ácidos graxos ômega-3 desempenham nas células neuronais, tais como a regulação da função dos receptores de neurotransmissores, função dos canais iônicos, liberação de neurotransmissores e atividade enzimática (POLITI et al., 2013; PRIOR; GALDURÓZ, 2012). Em relação aos estudos pré-clínicos, os ácidos graxos ômega-3 (geralmente associações de DHA + EPA em suplementação dietética) demonstraram efeitos ansiolíticos em roedores em diferentes modelos animais e paradigmas em roedores (CARRIÉ et al., 2002; FERRAZ et al., 2011; JAŠAREVIĆ et al., 2014; PÉREZ; TERREROS; DAGNINO-SUBIABRE, 2013; SONG et al., 2003; SONG; LEONARD; HORROBIN, 2004) e também em primatas (lêmures de Madagascar, *Microcebus murinus*) (PIFFERI et al., 2015; VINOT et al., 2011). Em ensaios clínicos, os ácidos graxos ômega-3 demonstraram efeito ansiolítico em indivíduos normais (KIECOLT-GLASER et al., 2011) e com ansiedade pré-exames (YEHUDA; RABINOVITZ; MOSTOFSKY, 2005), indivíduos com transtorno por abuso de substâncias (BARBADORO et al., 2013; BUYDENS-BRANCHEY; BRANCHEY, 2006; BUYDENS-BRANCHEY; BRANCHEY; HIBBELN, 2008), pacientes após infarto do miocárdio (HABERKA et al., 2013), mulheres com transtorno disfórico pré-menstrual (SOHRABI et al., 2013) e indivíduos com risco de desenvolver TEPT (MATSUMURA et al., 2016).

Além destes três agentes, também foram observados na literatura estudos demonstrando efeitos ansiolíticos em estudos pré-clínicos e ensaios clínicos do agonista dos receptores $mGLU_{2/3}$ LY354740 (DUNAYEVICH et al., 2008) e da vitamina C (ácido ascórbico) (ANGRINI; LESLIE, 2012; DE OLIVEIRA et al., 2015; HUGHES; LOWTHER; VAN NOBELEN, 2011; MAZLOOM; EKRAMZADEH; HEJAZI, 2013; PUTY et al., 2014). Apesar de o composto LY354740 ter apresentado resultados preliminares encorajadores no tratamento do TAG, os ensaios clínicos com esta substância foram interrompidos devido a sua atividade pró-convulsivante observada em estudos pré-clínicos (DUNAYEVICH et al., 2008; PITSIKAS, 2014). Em relação aos efeitos ansiolíticos da vitamina C, apesar das evidências de efeitos ansiolíticos em estudos pré-clínicos, os ensaios clínicos realizados possuem limitações como pequeno número de pacientes, os quais não possuíam diagnóstico de transtorno de ansiedade, o que não permite chegar a uma conclusão sobre o efeito ansiolítico potencial dessas intervenções em pacientes com transtornos de ansiedade. Vale ainda ressaltar que a vitamina E (tocoferol), ao contrário do esperado, tem demonstrado efeitos principalmente ansiogênicos em estudos pré-clínicos com roedores (CARVALHO et al., 2013; KOLOSOVA; TROFIMOVA; FURSOVA, 2006; OKURA et al., 2009; TERADA et al., 2011).

Considerando que: (1) a NAC possui mecanismo de ação multialvo envolvendo vias importantes relacionadas à fisiopatologia da ansiedade (DEAN; GIORLANDO; BERK, 2011), (2) algumas evidências que apontam para um potencial efeito ansiolítico em estudos clínicos (DEEPMALA et al., 2015; MINARINI et al., 2017), o último objetivo desse estudo foi verificar as propriedades ansiolíticas da NAC em diversos modelos animais de ansiedade em camundongos. Para isso, camundongos foram tratados com NAC por via intraperitoneal (60-150 mg/Kg) agudamente (1h antes do teste) ou subagudamente (por 4 dias consecutivos) e testados em seis modelos animais de ansiedade (campo aberto, claro/escuro, placa perfurada, interação social, labirinto em T-elevado e hipertermia induzida por estresse). Neste trabalho optamos pela administração por via intraperitoneal, ao invés da via de administração oral, por vários motivos: devido a uma maior precisão da dose de fármaco administrado quando os animais recebem a injeção do fármaco em relação a sua administração por via oral, ao possível estresse de imobilização e

a possiblidade de ocorrência de lesões esofágicas quando administra-se soluções através de cânulas de gavagem, e também considerando que a ardência gástrica é um efeito adverso comum da NAC quando administrada por via oral. Estes problemas poderiam causar interferências potenciais na análise comportamental dos animais, nos modelos de ansiedade utilizados.

Os resultados obtidos demonstram que a NAC apresenta efeitos ansiolíticos em cinco dos modelos de ansiedade utilizados: campo aberto, claro/escuro, placa perfurada, interação social e hipertermia induzida por estresse (artigo 2). Dentre esses, quatro estão entre os dez modelos animais de ansiedade mais comumente utilizados para a triagem de substâncias com atividade ansiolítica (BOURIN, 2015; GRIEBEL; HOLMES, 2013). É importante a avaliação das propriedades ansiolíticas de substâncias em diferentes modelos animais de ansiedade, pois os resultados obtidos em diferentes modelos podem refletir o potencial ansiolítico da substância para o tratamento de transtornos de ansiedade mais específicos (BOURIN, 2015; GRIEBEL; HOLMES, 2013). Além disso, alguns modelos possuem maior sensibilidade a fármacos semelhantes aos BZD, muitas vezes produzindo resultados inconsistentes com fármacos que agem sobre a neurotransmissão serotonérgica como os ISRS (aprovados para o tratamento de vários transtornos de ansiedade e considerados atualmente os ansiolíticos de maior espectro) (GRIEBEL; HOLMES, 2013). Outra questão importante na avaliação de candidatos com potencial ansiolítico é a necessidade de não avaliar apenas o efeito agudo desses compostos, considerando-se que muitas vezes são utilizados cronicamente e muitos deles, como os ISRS, podem exacerbar inicialmente os sintomas de ansiedade, produzindo efeito ansiolítico consistente somente após uso crônico (GRIEBEL; HOLMES, 2013). Logo, a avaliação somente de efeitos agudos de agentes com potencial ansiolítico poderia levar a um resultado falso negativo quanto às suas propriedades ansiolíticas.

Nossos resultados demonstram que a NAC produz efeitos ansiolíticos agudos, bem como após administração subaguda (4 dias) em vários modelos de ansiedade, sendo que geralmente após administração subaguda a dose de NAC necessária para promover efeito ansiolítico é diminuída em relação as

doses efetivas agudas. Isto pode sugerir a ocorrência de uma adaptação cerebral mediante efeitos cumulativos e duradouros durante a administração subaguda de NAC, o que permite efeito ansiolítico em doses menores que a administração aguda. Neste sentido, em estudos anteriores do nosso grupo observou-se que a NAC previne a ansiedade induzida por perturbação no ritmo circadiano em camundongos (após 11 dias de administração) em uma faixa de dose menor (30-60 mg/kg) (PILZ et al., 2015) que as utilizadas no presente estudo (60-150 mg/kg), o que reforça a hipótese de que em tratamentos mais prolongados, a NAC requer doses menores para promover efeito ansiolítico. Nossos resultados também corroboram resultados de outros estudos que demonstram efeito ansiolítico da NAC em diferentes paradigmas em roedores (CHEN et al., 2014; EGASHIRA et al., 2012) e peixes-zebra (MOCELIN et al., 2015).

Existem crescentes evidências de que uma das bases biológicas dos transtornos de ansiedade é a hiperativação glutamatérgica (KRYSTAL et al., 2010; PITSIKAS, 2014; RIAZA BERMUDO-SORIANO et al., 2012; WIEROŃSKA et al., 2011; WIEROŃSKA; PILC, 2013). Os receptores $mGlu_{2/3}$ estão localizados pré-sinapticamente e são expressos em áreas cerebrais relacionadas à ansiedade (córtex cerebral, tálamo, estriado, amígdala e hipocampo) (NICOLETTI et al., 2011; SWANSON et al., 2005). A NAC é um precursor do aminoácido cisteína, formando o dímero cistina que age no antiporter cistina-glutamato astrocitário liberando glutamato, o qual estimula então os receptores $mGlu_{2/3}$ extra-sinápticos (DEAN; GIORLANDO; BERK, 2011), resultando na diminuição da neurotransmissão glutamatérgica através de um efeito de retroalimentação negativa (SCHOEPP, 2001). Agonistas do receptor $mGlu_{2/3}$ tem demonstrado efeito ansiolítico em diversos modelos animais de ansiedade (KOLTUNOWSKA; GIBULA-BRUZDA; KOTLINSKA, 2013; NICOLETTI et al., 2011; PITSIKAS, 2014; WIEROŃSKA; PILC, 2013). Entretanto, nenhum agonista deste alvo foi aprovado até o momento para uso clínico em transtornos de ansiedade, apesar de já terem demonstrado efeitos ansiolíticos em ensaios clínicos para TAG (DUNAYEVICH et al., 2008; PITSIKAS, 2014). No caso do agonista do receptor $mGlu_{2/3}$ LY354740, como já citado anteriormente, os ensaios clínicos com o composto foram interrompidos

pelo fato de este agente ter demonstrado efeito pró-convulsivante em testes pré-clínicos (PITSIKAS, 2014).

A inflamação pode ocorrer em decorrência do estresse oxidativo gerado pela hiperatividade da transmissão glutamatérgica (NAJJAR et al., 2013; SATTLER; TYMIANSKI, 2001). As citocinas pró-inflamatórias induzem estresse oxidativo e neurotoxicidade, enquanto o estresse oxidativo contribui para a reação inflamatória e citotoxicidade (MATTSON; CAMANDOLA, 2001). Uma limitação deste estudo foi o fato de não termos investigado os mecanismos de ação responsáveis pelos efeitos ansiolíticos da NAC nos modelos animais utilizados. Entretanto, é provável que a modulação de vias antioxidantes e inflamatórias, além da modulação da neurotransmissão glutamatérgica, esteja relacionada aos seus efeitos ansiolíticos observados nos modelos animais. Foram relatados efeitos antioxidantes da NAC em doses e regimes de tratamento compatíveis com as utilizadas neste estudo. A NAC demonstrou efeito antioxidante em ratos submetidos a um modelo de estresse crônico (ARENT et al., 2012) e bulbectomia olfatória (SMAGA et al., 2012). Com relação aos efeitos da NAC na modulação da inflamação, sabe-se que a NAC inibe o fator de transcrição NF-κB, causando a *downregulation* da expressão de genes pró-inflamatórios (YANG et al., 2002). A NAC também demonstrou efeito anti-inflamatório em vários modelos de neuroinflamação em roedores (BELOOSESKY et al., 2012; CHEN et al., 2008; KHAN et al., 2004; WANG et al., 2007), em regime de tratamento compatível com o utilizado em nosso trabalho e preveniu o aumento de corticosterona plasmática observado em ratos em abstinência de álcool (SCHNEIDER et al., 2015).

Portanto, as propriedades antioxidantes e anti-inflamatórias da NAC certamente são relevantes para os efeitos ansiolíticos observados. De acordo com Dean e colaboradores (2011), o efeito da NAC promovendo a redução de citocinas inflamatórias pode ser um potencial mecanismo de modulação de sintomas psiquiátricos por este fármaco, sendo que este efeito pode estar relacionado com processos oxidativos associados com inflamação, ou diretamente associado com vias inflamatórias.

Os efeitos adversos da NAC geralmente são leves e bem tolerados pelos pacientes, sendo isto uma grande vantagem em relação aos efeitos adversos causados por outros psicofármacos que possuem atualmente

indicação como ansiolíticos, como os ISRS e IRSN. De fato, na maior parte dos ensaios clínicos controlados com a NAC não foram relatados efeitos adversos significativos ou graves em comparação ao grupo placebo, que levassem a descontinuação do tratamento (DEEPMALA et al., 2015).

6. CONCLUSÃO

Os resultados obtidos nesse trabalho demonstraram que três agentes com mecanismo de ação multialvo envolvendo a modulação da neurotransmissão glutamatérgica, estresse oxidativo e neuroinflamação têm demonstrado efeitos ansiolíticos tanto em estudos pré-clínicos, envolvendo diferentes protocolos experimentais, quanto em ensaios clínicos randomizados duplo-cegos controlados por placebo em pacientes com transtornos de ansiedade e condições relacionados. Mais especificamente, a agomelatina apresenta evidências de efeitos ansiolíticos robustos no TAG; os ácidos graxos ômega-3 demonstraram efeitos ansiolíticos em indivíduos normais, na ansiedade pré-exames, em indivíduos com transtorno por abuso de substâncias, pacientes após infarto do miocárdio, mulheres com transtorno disfórico pré-menstrual e indivíduos com risco de desenvolver TEPT; e a NAC demonstrou efeitos ansiolíticos promissores no tratamento do TOC (principalmente como medicação adjunta aos fármacos convencionais, em pacientes com baixa resposta ao tratamento), em outros transtornos do espectro obsessivo-compulsivo como a tricotilomania, hábito de roer unhas e transtorno de escoriação e no TEPT associado a transtorno por abuso de substâncias.

A NAC possui efeitos ansiolíticos consistentes identificados em diferentes modelos animais etologicamente baseados, os quais se correlacionam bem com a ansiedade em humanos. Esses efeitos estão provavelmente relacionados com sua atividade reguladora da neurotransmissão glutamatérgica, do estresse oxidativo e da neuroinflamação.

Esse estudo fornece subsídios científicos para a realização de ensaios clínicos de larga escala com a NAC em outros transtornos de ansiedade como o TAG, TP e TAS. Esses transtornos muitas vezes possuem resposta terapêutica insatisfatória com os fármacos preconizados, ou ainda os pacientes possuem baixa adesão ao tratamento devido a efeitos adversos pouco tolerados. Nesse contexto a NAC se destaca como um fármaco com grande potencial de aplicação no campo do tratamento dos transtornos de ansiedade, considerando que é um fármaco seguro, com baixa incidência de efeitos

adversos, cujo mecanismo de ação multialvo envolve a modulação de algumas das principais vias envolvidas na fisiopatologia da ansiedade.

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ANEXOS

ANEXO A – Carta de aprovação do projeto pelo CEUA/UFRGS.

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 28528
Título: AVALIAÇÃO DOS EFEITOS DA N-ACETILCISTEÍNA AMIDA (NACA) EM MODELOS ANIMAIS DE DEPRESSÃO, ANSIEDADE E ESQUIZOFRENIA

Vigência: 01/03/2015 à 28/02/2020

Pesquisadores:

Equipe UFRGS:

ELAINE ELISABETSKY - coordenador desde 01/03/2015
ÂNGELO LUIS STAPASSOLI PIATO - pesquisador desde 01/03/2015
Ana Paula Herrmann - Aluno de Doutorado desde 01/03/2015
RADHARANI BENVENUTTI - Aluno de Especialização desde 01/03/2015
YASMINE TROJAN DOS SANTOS - Aluno de Especialização desde 01/03/2015
Patrícia Santos - Aluno de Doutorado desde 01/03/2015

Equipe Externa:

Roberta Andrejew - pesquisador desde 01/03/2015
Rafael Pires Lima - pesquisador desde 01/03/2015

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 15/06/2015 - Sala 330 - Prédio do Anexo I da Reitoria - Campus Centro - Porto Alegre - RS, em seus aspectos éticos e metodológicos, para a utilização de 108 camundongos BALB/c , 156 CF1 e 116 C57/BL6 todos machos, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Quinta-Feira, 25 de Junho de 2015

Cristiane Matte

CRISTIANE MATTE
Coordenador da comissão de ética

ANEXO B – Comprovante de submissão do artigo 1 para publicação na Revista Brasileira de Psiquiatria.

Assunto: Revista Brasileira de Psiquiatria - Manuscript ID RBP-2017-RA-2508
Data: 25/09/2017 09:55
Remetente: Revista Brasileira de Psiquiatria <onbehalfof+editorial+abpbrasil.org.br@manuscriptcentral.com>
Para: angelopiato@ufrgs.br
Responder para: editorial@abpbrasil.org.br

25-Sep-2017

Dear Dr. Piato:

Your manuscript entitled "ANXIOLYTIC PROPERTIES OF COMPOUNDS THAT COUNTERACT OXIDATIVE STRESS, NEUROINFLAMMATION, AND GLUTAMATERGIC DYSFUNCTION: A REVIEW" has been successfully submitted online and is presently being given full consideration for publication in Revista Brasileira de Psiquiatria.

Your manuscript ID is RBP-2017-RA-2508.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to ScholarOne Manuscripts at https://mc.manuscriptcentral.com/rbp and edit your user information as appropriate.

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Thank you for submitting your manuscript to Revista Brasileira de Psiquiatria.

Sincerely,
Revista Brasileira de Psiquiatria Editorial Office