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EFEITO DA N-ACETILCISTEÍNA-AMIDA SOBRE PARÂMETROS COMPORTAMENTAIS E BIOQUÍMICOS EM PEIXES-ZEBRA

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Dissertação apresentada ao Programa de Pós-graduação em Neurociências do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de Mestre em Neurociências.

Orientador: Prof. Dr. Angelo Piato

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Dedico este trabalho a todos os professores, especialmente aos brasileiros, que seguem trabalhando incansavelmente mesmo quando desvalorizados e em condições desfavoráveis.

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ABSTRACT

Anxiety disorders are a major cause of disability and have a high prevalence worldwide. These disorders are characterized by excessive and enduring fear and/or anxiety, being related to stress. Stress triggers normal adaptive responses in the organism but if persistent it can lead to a failure on this adaptation. Factors such as oxidative stress, excitotoxicity, and neuroinflammation are also related to the etiology of anxiety disorders. N-acetylcysteine (NAC) has antioxidant and anti-inflammatory activity, also being able to modulate the glutamatergic transmission. Recent studies shown that this drug has potential use in mental disorders. However, the lack of better results is related to the low bioavailability of this compound. N-acetylcysteine-amide (called AD4 or NACA) is a NAC-derived molecule that exhibits a change in its chemical structure in order to improve this property. In this study, we evaluated the effect of AD4 and NAC on behavioral and biochemical parameters in the novel tank (NTT) and light/dark (LDT) tests in zebrafish. In addition, the effects of AD4 were evaluated in an acute restraint stress (ARS) protocol. Both drugs did not show locomotor and exploratory effects in the NTT. AD4 and NAC demonstrated an anxiolytic effect in the LDT and also in the ARS. AD4 demonstrated greater potency in the light/dark test when compared to NAC. Both compounds did not alter oxidative status in the zebrafish brain. The results of this study contribute to the characterization of the effects of AD4 in zebrafish and its potential use in mental disorders.

RESUMO

Os transtornos de ansiedade são uma das principais causas de incapacitação e possuem grande prevalência global. São caracterizados pela presença de medo e/ou ansiedade excessivos e persistentes, estando relacionados ao estresse. O estresse desencadeia respostas adaptativas normais no organismo, porém se persistente pode levar a uma falha nessa adaptação. Fatores como estresse oxidativo, excitotoxicidade e neuroinflamação também estão relacionados à etiologia dos transtornos de ansiedade. A N-acetilcisteína (NAC) possui atividade antioxidante e anti-inflamatória além de ser capaz de modular a transmissão glutamatérgica. Estudos recentes têm demonstrado que esse fármaco possui um potencial uso em diversos transtornos mentais. Contudo, a ausência de melhores resultados é atribuída à baixa biodisponibilidade desse composto. A N-acetilcisteína-amida (AD4 ou NACA) é uma molécula derivada da NAC que apresenta uma modificação na estrutura química visando melhorar essa propriedade. Neste estudo, foi avaliado o efeito da AD4 e NAC sobre parâmetros comportamentais de ansiedade e bioquímicos nos testes de tanque-novo e claro/escuro em peixes-zebra. Além disso, foram avaliados os efeitos desses compostos em um protocolo de estresse agudo de contenção. No teste de tanque novo, nenhum dos compostos mostrou efeitos sobre aspectos locomotores e exploratórios. AD4 e NAC demonstraram efeitos ansiolíticos nos testes de claro/escuro e estresse agudo de contenção, sendo que a AD4 demonstrou maior potência do que a NAC no teste de claro/escuro. Nenhum dos compostos foi capaz de alterar o status oxidativo no encéfalo de peixes-zebra. Os resultados desse estudo contribuem para a caracterização dos efeitos de AD4 em peixes-zebra e de seu potencial uso em transtornos mentais.

LISTA DE ABREVIATURAS

5-HT - Serotonina

AD4 - N-acetilcisteína-amida

BDNF - Fator neurotófico derivado do encéfalo

BHE - barreira hematoencefálica

CPF - Córtex pré-frontal

DA - Dopamina

DSM - Manual Diagnóstico e Estatístico de Transtornos Mentais

EAC - Estresse agudo de contenção

GABA - Ácido gama-aminobutírico

HHA - Eixo hipotálamo-hipófise-adrenal

HHI – Eixo hipotálamo-hipófise-interrenal

IRND - Inibidor da recaptação de noradrenalina e dopamina

ISRS - Inibidor seletivo da recaptação de serotonina

NAC - N-acetilcisteína

NACA - N-acetilcisteína-amida

NE - Noradrenalina

pH - Potencial hidrogeniônico

TAG - Transtorno de ansiedade generalizada

TNF - Fator de necrose tumoral

SUMÁRIO

1. INTRODUÇÃO

1.1. Transtornos de Ansiedade

Os transtornos de ansiedade são uma das principais causas de incapacidade, tendo prevalência global em 12 meses estimada em aproximadamente 14% (Baxter et al., 2013; Bystritsky et al., 2013; Craske et al., 2017). São caracterizados pela presença de medo e/ou ansiedade intensos e persistentes em relação a ameaças percebidas, levando à antecipação de potenciais situações aversivas (Craske et al., 2017; Pitsikas, 2014). Alguns fatores de risco associados incluem o gênero (2 vezes mais comuns em homens do que em mulheres) e histórico de depressão e ansiedade na família (Craske et al., 2017; Grenier et al., 2018). No Manual Diagnóstico e Estatístico de Transtornos Mentais da Associação Americana de Psiquiatria (DSM, 5ª edição), os transtornos de ansiedade incluem: transtorno de ansiedade generalizada (TAG), transtorno do pânico, fobias específica, fobia social, agorafobia entre outros (Bandelow and Michaelis, 2015; Shah and Han, 2015).

O desenvolvimento de tais condições está relacionado ao estresse, com respostas comportamentais e neuroendócrinas adaptativas (chamada de alostase) para que o organismo seja capaz de responder adequadamente a determinada demanda (Pitsikas, 2014). Dentre as estratégias adaptativas adotadas para reestabelecer a homeostase estão a ativação do eixo hipotálamo-hipófise-adrenal (HHA), do sistema nervoso autônomo e componentes anti- e pró-inflamatórios (Chrousos, 2009; Duman et al., 2016; McEwen et al., 2012, 2015). Também ocorrem mudanças funcionais em estruturas anatômicas como a amígdala, córtex pré-frontal (CPF) e hipocampo (Arborelius et al., 1999; Etkin and Wager, 2007; Faravelli et al., 2012; Martin et al., 2009). Quando há superação dos processos adaptativos associados estabelece-se uma condição chamada de carga alostática, onde há um aumento na chance do indivíduo de desenvolver uma série de condições como hipertensão, diabetes, prejuízos cognitivos e transtornos mentais como ansiedade e depressão (Binder, 2019; Peters et al., 2017; Sandi and Haller, 2015; Sapolsky, 2000; Steimer, 2011).

As bases neurobiológicas relacionadas aos transtornos de ansiedade são complexas e ainda existem muitas questões a serem adequadamente

respondidas. Quanto à neurotransmissão, alguns sistemas de neurotransmissores se mostram alterados. Há liberação excessiva de glutamato, levando à excitotoxicidade, além de diminuição da expressão do fator neurotrófico derivado do encéfalo (BDNF), importante na neurogênese (Miller and Raison, 2016; Pitsikas, 2014; Riaza Bermudo-Soriano et al., 2012). Serotonina (5-HT), noradrenalina (NE) e dopamina (DA) também podem estar disfuncionais e interagem entre si na manifestação dos sintomas (figura 1) (Liu et al., 2018). O ácido gama-aminobutírico (GABA) é utilizado por diversas estruturas responsáveis pela modulação comportamental, e no caso da ansiedade, há hipofunção gabaérgica, contribuindo para a excitotoxicidade (Nuss, 2015).

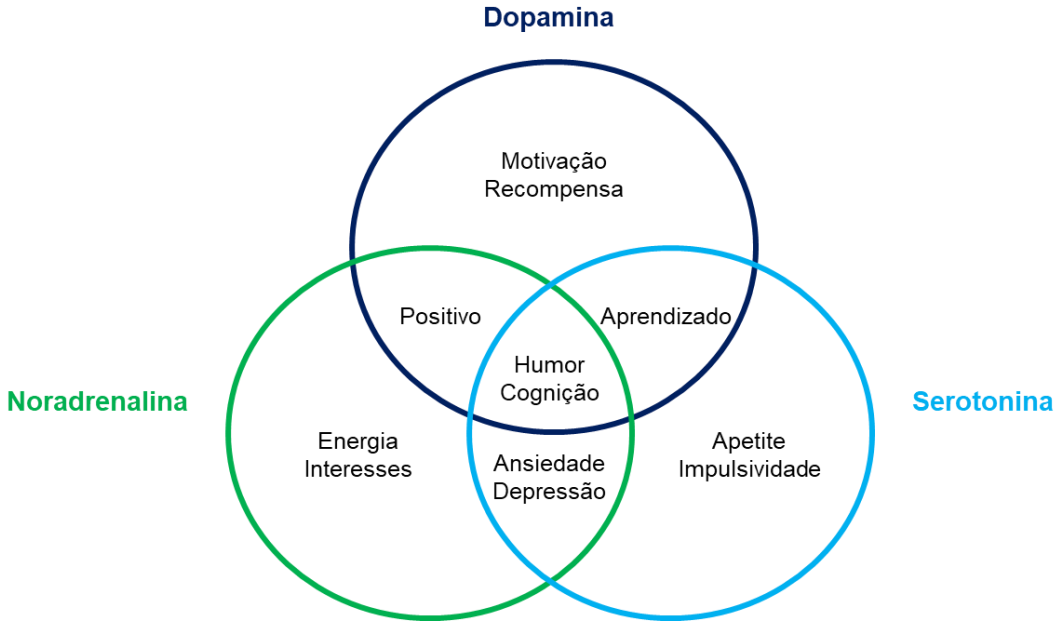

Figura 1. A complexa integração entre diferentes sistemas de neurotransmissores na regulação de transtornos de humor e ansiedade (adaptado de Liu et al., 2018).

O estresse oxidativo também está envolvido com os transtornos de ansiedade. Estudos demonstram uma diminuição nas defesas antioxidantes e uma produção aumentada de espécies reativas de oxigênio e nitrogênio em pacientes e em modelos animais de ansiedade (Fedoce et al., 2018). Isso gera dano oxidativo às proteínas, lipídeos e ácidos nucleicos, contribuindo para o estabelecimento e progressão da ansiedade e depressão (Aschbacher et al., 2013; Fedoce et al., 2018). A neuroinflamação também tem sido reportada, com

aumento na expressão de interleucinas inflamatórias, interferons e fator de necrose tumoral (TNF), que interagem com as sinapses podendo diminuir a disponibilidade de monoaminas em indivíduos com transtornos de humor e ansiedade (Gałecki and Talarowska, 2018; Miller and Raison, 2016).

Contudo, sendo a etiologia dos transtornos de ansiedade multifatorial, ainda há a necessidade de mais estudos que elucidem suas causas e desvendem os mecanismos relacionados de maneira mais completa (Coleman and Pierre, 2014; Fedoce et al., 2018).

1.1.2 Tratamentos disponíveis e novas abordagens

O tratamento para os transtornos de ansiedade associa psicoterapia com farmacoterapia. Entre os fármacos, os inibidores seletivos da recaptação de serotonina (ISRS) e os benzodiazepínicos são amplamente utilizados (Baldwin et al., 2017; Bandelow et al., 2017; Thibaut, 2017). Os ISRS como a fluoxetina e a sertralina aumentam a concentração extracelular de serotonina, um neurotransmissor importante no transtorno (Liu et al., 2018). Os benzodiazepínicos como bromazepam e diazepam promovem a ativação de subtipos dos receptores $GABA_A$, desencadeando um efeito inibitório na neurotransmissão (Griebel and Holmes, 2013). Contudo, o uso de fármacos de ambas as classes está associado a efeitos adversos agudos e crônicos como ansiedade, sedação, déficits de memória, dependência, abstinência, disfunção sexual e ganho/perda de peso (Cryan and Sweeney, 2011). Considerando a complexidade da neurobiologia dos transtornos mentais, é evidente a necessidade de fármacos que modulem novos alvos, a fim de melhorar a resposta terapêutica e o perfil de efeitos adversos.

Nesse contexto, observa-se na indústria farmacêutica um esgotamento na capacidade de desenvolver psicofármacos. O alto custo, o longo tempo de estudo e a alta taxa de insucesso faz com que a indústria tenha diminuído significativamente os investimentos na pesquisa e desenvolvimento de novos psicofármacos (Insel et al., 2013). Uma abordagem para contornar essa problemática chama-se “drug repurposing” que consiste no redirecionamento de fármacos já existentes no mercado para outras aplicações clínicas diferentes da inicialmente aprovada (Li and Jones, 2012; Pushpakom et al., 2018; Strittmatter,

2014). É uma abordagem vantajosa já que é possível abreviar as etapas farmacodinâmica, farmacocinética e estudos de toxicidade, uma vez que esses dados já estão disponíveis. Um exemplo de psicofármaco reposicionado é a bupropiona, utilizado como coadjuvante na cessação do tabagismo, um inibidor da recaptação de noradrenalina e dopamina (IRND), inicialmente indicado no tratamento da depressão. Também pode ser citado o antidepressivo fluoxetina, um inibidor seletivo da recaptação de serotonina (ISRS), que posteriormente foi aprovado para o tratamento de transtornos de ansiedade (Ashburn and Thor, 2004).

1.2 N-acetilcisteína e N-acetilcisteína-amida

A N-acetilcisteína (NAC) é derivada da L-cisteína, um aminoácido que compõe a glutationa, principal antioxidante do sistema nervoso central (Dean et al., 2011a; Racz et al., 2015). De uso consagrado no tratamento de intoxicações por paracetamol e como agente mucolítico, a NAC vem recebendo grande atenção por apresentar potencial aplicabilidade em transtornos neuropsiquiátricos como depressão, ansiedade, transtorno bipolar, esquizofrenia, autismo, transtorno obsessivo-compulsivo e em abuso de substâncias (Berk et al., 2013; Deepmala et al., 2015; Jastrzębska et al., 2016; Ooi et al., 2018). Sua eficácia no tratamento dessas condições é explicada por sua capacidade antioxidante, anti-apoptótica, anti-inflamatória, moduladora glutamatérgica e neurotrófica (Dean et al., 2011b; Minarini et al., 2017; Tardiolo et al., 2018). Estudos do nosso grupo demonstraram que a NAC possui atividade antiestresse e ansiolítica, tanto em camundongos como em peixes-zebra (Mocelin et al., 2015, 2018; Santos et al., 2017).

Apesar de ser uma molécula com grande potencialidade, a NAC possui baixa biodisponibilidade e isso pode comprometer a sua atividade biológica. Isso ocorre porque em pH fisiológico (7,4) o grupamento carboxila perde o próton, tornando a molécula carregada negativamente e dificultando sua passagem pelas membranas biológicas e barreira hematoencefálica (BHE) (Samuni et al., 2013; Wu et al., 2008).

Visando contornar as limitações farmacocinéticas da NAC, pesquisadores israelenses associados ao nosso grupo de pesquisa sintetizaram um composto

derivado da NAC, denominado N-acetilcisteína-amida (AD4 ou NACA), no qual o grupamento carboxila é substituído por um grupamento amida no intuito de melhorar a biodisponibilidade. Estudos já demonstraram vantagens da AD4 sobre a NAC como antioxidante, anti-apoptótica, anti-inflamatório, quelante e agente sequestrante de radicais livres (Ates et al., 2008; Sunitha et al., 2013; Wu et al., 2008; Zhou et al., 2018). Também foi demonstrado que a AD4 é neutra em pH 7,4 e a administração oral ou intraperitoneal em camundongos de NAC ou AD4 resulta no aparecimento apenas de AD4 no encéfalo dos animais (Offen et al., 2004). Isto atesta uma maior permeabilidade na BHE. Na figura 2 abaixo é possível visualizar a diferença estrutural entre as moléculas:

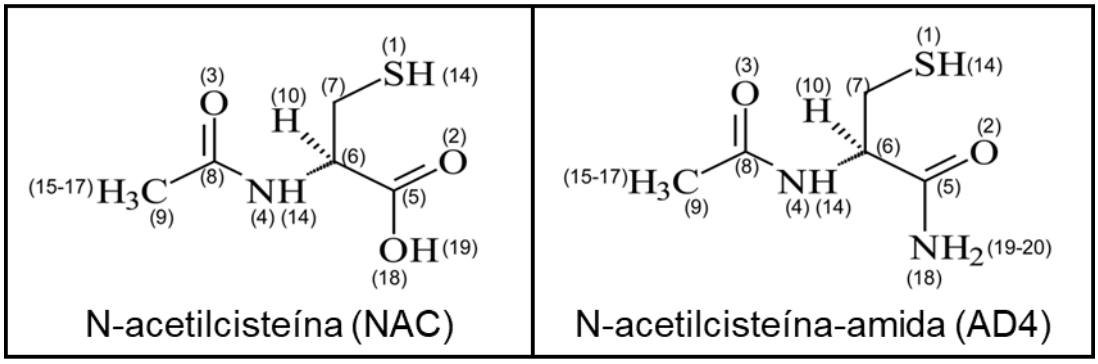

Figura 2. Estrutura química da N-acetilcisteína (NAC) e da N-acetilcisteína-amida (AD4) (adaptado de Chen et al., 2012).

1.3 Peixes-zebra como organismo modelo para estudo da ansiedade

O peixe-zebra ou paulistinha (*Danio rerio,* Hamilton 1822*)* pertence à família dos ciprinídeos. É um pequeno teleósteo de água doce, nativo de regiões tropicais quentes localizadas no sudeste asiático. Internacionalmente é conhecido como “zebrafish”. Sua crescente popularização como modelo animal tem início na década de 1960, quando foram realizados os primeiros estudos comportamentais da espécie. Nos anos 1980, George Streisinger introduziu a utilização do peixe-zebra em estudos de genética, dando visibilidade ao potencial desse vertebrado na pesquisa experimental (Walker and Streisinger, 1983).

O interesse cada vez maior nesse organismo modelo se deve a diversas vantagens em relação a outros organismos (d’Amora and Giordani, 2018;

Lieschke and Currie, 2007). Em comparação a roedores, espécies muito empregadas por serem mamíferos, o peixe-zebra exige menor custo e espaço para manutenção. Apresenta fecundação e reprodução externas com formação de embrião transparente, tendo o ciclo de vida curto atingindo rapidamente a maturidade, características que favorecem o acompanhamento do desenvolvimento embrionário. Seu amplo e bem conhecido repertório comportamental aliado a semelhanças de diversos sistemas permitem extrapolar os dados obtidos traçando um paralelo confiável com os humanos (Kalueff et al., 2014; MacRae and Peterson, 2015).

Em neurociências muitos estudos demonstram semelhanças entre o sistema nervoso central de mamíferos e as principais regiões encefálicas do peixes-zebra (Cheng et al., 2014; Pickart and Klee, 2014). Uma comparação estrutural com um roedor pode ser vista abaixo na figura 3.

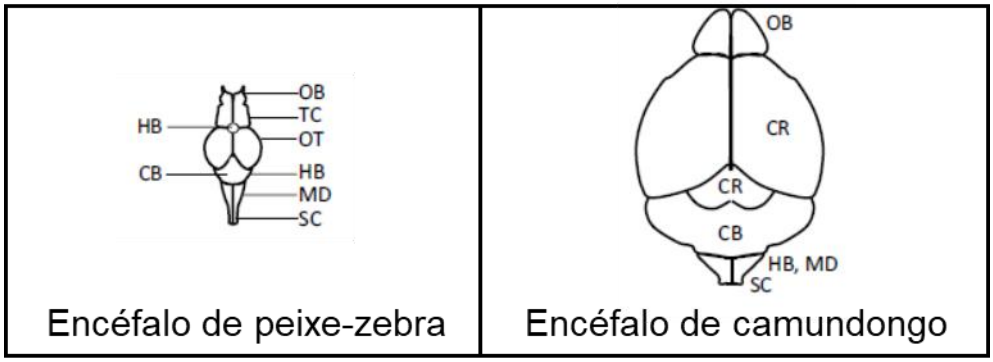

Figura 3. Encéfalos de peixe-zebra (esquerda) e de camundongo (direita). OB = bulbo olfatório. TC = telencéfalo. OT = teto ótico. HB = habênula. CB = cerebelo. HB = rombencéfalo. MD = medula. SC = medula espinhal. CR = córtex (adaptado de Kalueff et al., 2014).

A organização macroscópica e a morfologia celular são extremamente parecidas entre os encéfalos e se apresentam conservadas em comparação com seus homólogos humanos. Funcionalmente, estruturas como, por exemplo, a amígdala e habênula regulam o comportamento emocional de maneira análoga nas duas espécies, sendo os principais neurotransmissores presentes nos humanos comuns ao peixe-zebra. Como resposta ao estresse também produzem cortisol por meio da ativação de um eixo análogo ao HHA em humanos, denominado eixo hipotálamo-hipófise-interrenal (HHI). Aliada a essas

vantagens, está a existência de paradigmas já consolidados para o estudo do comportamento e ansiedade de maneira translacional nessa espécie em relação à validação preditiva (Kalueff et al., 2014; Kanungo et al., 2014). Outro aspecto que deve ser citado é a alta homologia genética (70%) entre o peixe-zebra e humanos (Howe et al., 2013).

Tudo isso faz do peixe-zebra um organismo modelo adequado para testes farmacológicos e de toxicidade, descoberta e redirecionamento de fármacos (Kanungo et al., 2014; Kari et al., 2007; Lieschke and Currie, 2007). Pesquisas também apontam seu uso com sucesso no estudo de diversas doenças (Bencan et al., 2009; Egan et al., 2009; Gebauer et al., 2011; Guo, 2004; Rosemberg et al., 2011).

Considerando a problemática abordada, a hipótese desse estudo é que, comparativamente à NAC, a AD4 possui propriedades ansiolíticas e antioxidantes mais potentes em peixes-zebra.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar os efeitos da N-acetilcisteína-amida (AD4) sobre parâmetros comportamentais e bioquímicos em peixes-zebra adultos.

2.2 Objetivos específicos

a. Avaliar o efeito da AD4 e NAC nos testes de tanque novo e claro/escuro em peixes-zebra;

b. Avaliar o efeito da AD4 e NAC em um protocolo de estresse agudo de contenção (EAC);

c. Avaliar o efeito da AD4 e NAC sobre parâmetros de estresse oxidativo em encéfalos de peixes-zebra submetidos a um protocolo de estresse agudo de contenção (EAC).

Os resultados desse trabalho são apresentados na forma de artigo científico completo que será submetido à publicação no periódico Pharmacology Biochemistry and Behavior (ISSN: 0091-3057)**.**

3. ARTIGO CIENTÍFICO

COMPARISON OF N-ACETYLCYSTEINE AND N-ACETYLCYSTEINE AMIDE ON ANXIETY AND STRESS BEHAVIOR IN ZEBRAFISH

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ABSTRACT

Anxiety disorders are highly prevalent and a leading cause of disability worldwide. The etiology is related to stress, an adaptive response of the organism to restore homeostasis, in which oxidative stress and glutamatergic hyperactivity are involved. N-acetylcysteine (NAC) is a multitarget approved drug that possesses several psychopharmacology properties and proved beneficial in the treatment of various mental disorders. Nevertheless, NAC low membrane permeability and poor bioavailability limits its delivery to the brain and may explain inconsistencies in the literature. N-acetylcysteine amide (AD4 or NACA) is a synthetic derivative of NAC in which the carboxyl group was modified to an amide. The amidation of AD4 improved lipophilicity, permeability, allowed crossing the blood brain barrier, and enhanced its antioxidant activity. Although AD4 was shown to be very effective in animals models of multiple scleroses, Parkinson's disease, β thalassemia and allergic asthma, as well as in reducing cocaine-induced reinstatement, the effects of AD4 on behavior parameters in animal models are understudied. The purpose of the present study was to investigate the effects of AD4 on behavioral and biochemical parameters in zebrafish anxiety models. Neither AD4 nor NAC induced any effects on locomotion and anxiety-related parameters in the novel tank test. However, in the light/dark test, AD4 (0.001 mg/L) increased the time spent in the lit side in a concentration 100 times lower than NAC (0.1 mg/L), indicating a significant improvement in potency. In the acute restraint stress protocol, NAC and AD4 (0.001 mg/L) showed anxiolytic properties without meaningful effects on the accompanying oxidative status. We provide data that AD4 has anxiolytic effects in zebrafish, as expected with higher potency than the parent compound. Additional studies are warranted to better characterize the anxiolytic profile of AD4 and its potential in the management of anxiety disorders.

KEYWORDS

N-acetylcysteine amide - Anxiety - Acute restraint stress - AD4 - NACA

INTRODUCTION

Anxiety disorders are a leading cause of disability, with a global 12-month prevalence estimated to be circa 14% (Baxter et al., 2013; Bystritsky et al., 2013; Craske et al., 2017). Affected individuals show excessive and enduring fear, anxiety and/or avoidance of perceived threats, and anticipation of potential aversive situations (Craske et al., 2017; Pitsikas, 2014). The development of anxiety disorders is related to stress, a physiological adaptative response that prepares the organism to cope with adversities and restore homeostasis. The intended adaptation occurs via allostasis, a process that involves the hypothalamic-pituitary-adrenal (HPA) axis, the autonomic nervous system and pro- and anti-inflammatory mediators, among others (Chrousos, 2009; McEwen et al., 2015). Despite its adaptative nature, if anxiety is persistent and intense, an allostatic overload can trigger mental disorders (Sandi and Haller, 2015; Sapolsky, 2000). Oxidative stress also plays an important role in stress and anxiety, primarily by the excessive production of reactive oxygen species (ROS) (Aschbacher et al., 2013). Additionally, the neurotransmitter glutamate has been implicated in the pathophysiology of anxiety disorders, specifically its excessive liberation and resulting excitotoxicity (Pitsikas, 2014; Riaza Bermudo-Soriano et al., 2012).

The mainstream pharmacotherapy of anxiety disorders includes selective serotonin reuptake inhibitors (SSRI) and benzodiazepines (Baldwin et al., 2017; Bandelow et al., 2017; Thibaut, 2017). However, these drugs are not adequately effective and are associated with undesirable side effects, including anxiety, abstinence, weight gain, and sexual dysfunction (Cryan and Sweeney, 2011), drawing attention to the need for better drugs.

The potential use of N-acetylcysteine (NAC) in mental disorders have been documented in experimental models and clinical trials (Dean et al., 2011; Minarini et al., 2017; Ooi et al., 2018; Tardiolo et al., 2018). NAC possesses a multitarget mechanism including glutamatergic modulation, cysteine precursor, anitinflammatory and neurotrophic properties (Kowalczyk-Pachel et al., 2016). The use of NAC is somewhat limited due to its poor bioavailability and blood brain barrier impermeability (Ates et al., 2008; Bahat-Stroomza et al., 2005; Bartov et al., 2006; Grinberg et al., 2005; Offen et al., 2004; Sadan et al., 2005;

Samuni et al., 2013; Sunitha et al., 2013). N-acetylcysteine amide (AD4 or NACA) is the amide form of NAC, in which the carboxyl group was modified to amide, for neutralizing the charge and thereby improving membrane permeability, and overcoming pharmacokinetic limitations. AD4 proved to be superior to NAC as antioxidant, free radical scavenger, anti-inflammatory and antiapoptotic (Chen et al., 2012; Wu et al., 2008; Zhang et al., 2009; Zhou et al., 2018). However, the behavioral effects of AD4 in animal models is poorly studied. The aim of our study was to evaluate AD4's in vivo properties by using behavioral paradigms and biochemical assays in adult zebrafish.

MATERIALS AND METHODS

Animals

A total of 512 adult (4-6-month-old, 3-4 cm long) zebrafish (*Danio rerio*, F. Hamilton 1822) of short-fin wild-type phenotype (which better represent the population heterogeneity due to their increased genetic variability) of both sexes (50:50 male: female ratio) were obtained from a local specialized commercial supplier (Delphis, RS, Brazil). Fish were housed in a maximum density of two fish per liter and acclimatized for four weeks prior to testing in 40-L tanks (45 x 35 x 30 cm) with heater, filter, aeration system, and non-chlorinated water kept under constant mechanical, biological and chemical filtration at 26 ± 2 ºC. The fish were randomly divided into the experimental groups by a computerized random number generator (www.random.org). Fish were kept on a 14-10 h day/night cycle (lights on at 07:00 am) and fed three times a day with brine shrimp (*Artemia salina*) and commercial flake fish food (Poytara®, Brazil). More detailed information on housing conditions is described in our previous study (Mocelin et al., 2018a). Protocols were approved by the University Ethics Committee (#33406/2017).

Drugs

N-acetylcysteine amide (AD4) was provided by Professor Daphne Atlas from the Hebrew University of Jerusalem (Jerusalem, Israel) and N-acetylcysteine (NAC, CAS number 616-91-1) was acquired from Sigma-Aldrich (St Louis, Missouri, USA). Fluoxetine (FLU, Daforin™) and bromazepam (BMZ,

Somalium™) were acquired from Sigma Pharma and Ache (São Paulo, Brazil), respectively. The concentrations of NAC (0.0001, 0.001 and 0.01 mg/L) were based on previous studies (Mocelin et al., 2015, 2018a) and pilot experiments. AD4 concentrations were based on those of NAC in order to compare potencies. FLU (10.0 mg/L) and BMZ (1.5 mg/L) were used as positive controls (CTRL) in concentrations based on previous studies (Mocelin et al., 2015).

Experimental design and procedure

The experimental design is shown in Figure 1. Animals were exposed to AD4, NAC, FLU, BMZ in a beaker for 10 min in groups of 4 fish per treatment. In the novel tank test (NTT) and light/dark test (LDT) fish were video recorded immediately after treatment for 6 and 5 min, respectively. In the acute restraint stress (ARS), the animals were simultaneously treated and submitted to ARS for 15 min and then placed in the NTT. Different sets of animals were used for each experiment. Control groups were submitted to the same experimental conditions but exposed to a beaker filled just with tank water. Tank temperature (26 ± 2 °C) was kept constant during all tests. Videos were analyzed by experimenters blinded to treatment (experimental groups were coded by a researcher not participating in the analyses and revealed only after statistical analyses).

Behavioral analyses

The novel tank test (NTT), an exploratory test analogous to the open field test in rodents, was described in previous studies (Marcon et al., 2018; Mocelin et al., 2018b). The NTT apparatus is virtually divided into three equal horizontal areas and reduction in the number of entries and time in the top area reflects anxiety-like behavior (Egan et al., 2009; Levin et al., 2007). Animals were individually placed in the NTT apparatus (2.7-L tanks, 24 x 8 x 20 cm) 10 min after the treatment (or the ARS protocol described below) and recorded for 6 min. The software ANY-maze™ (Stoelting Co., USA) was used to track the movement of the animals. Exploratory behavior is indicated by the total distance traveled (meters), total number of crossings (transitions among areas), and number of entries and time spent (seconds) in the top area of the tank.

The light/dark test (LDT) procedure was performed as described in previous studies (Gebauer et al., 2011). Immediately after treatment, animals were individually placed in the white compartment of the apparatus (18 x 9 x 7 cm, divided into a dark and white compartment of equal sizes) and recorded for 5 min. The time spent in the lit side compartment (seconds) was analyzed, and a reduction in the time spent in the lit side reflects anxiety-like behavior (Gebauer et al., 2011; Maximino et al., 2010).

The acute restraint stress (ARS) method was detailed in previous studies (Dal Santo et al., 2014; Ghisleni et al., 2012; Piato et al., 2011). Briefly, each animal was gently moved into 2-mL plastic microcentrifuge tubes with openings at both ends (to allow adequate water circulation) and placed in a 1.7-L (13 x 13 x 13 cm) tank, containing AD4 (0.001 mg/L) or NAC (0.001 mg/L) or no drug (controls) for 15 min. Immediately after, the animals were individually transferred to the NTT and behavioral parameters quantified as described above.

Oxidative stress analyses

A proportion of 4 brains to 600 μL of phosphate buffered saline (PBS, pH 7.4, Sigma-Aldrich®) were used for the biochemical analyses (Mocelin et al., 2018a). Immediately after the ARS protocol and behavioral tests, each fish was cryoeuthanized, the brain dissected and gently homogenized. The homogenate was centrifuged at 10,000 g for 10 min at 4 ºC in a cooling centrifuge, and the supernatant packed in microtubes was used for assays. Protein was quantified according to the Coomassie blue method (Bradford, 1976) using bovine serum albumin (Sigma-Aldrich®) as a standard.

The following parameters were quantified for the oxidative stress assessment: lipid peroxidation (thiobarbituric acid reactive species - TBARS), total content of reduced thiol (SH), non-protein thiols (NPSH), and antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT), as detailed in previous studies (Mocelin et al., 2018a, 2018b).

Statistical analysis

Normality and homogeneity of variances were confirmed for all data sets using D'Agostino-Person and Levene tests, respectively. Results were analyzed

by Student's T-test, one- or two-way ANOVA/Tukey as applicable. The sex effect was tested in all comparisons, but since no effects were observed data were pooled. Data are expressed as mean ± standard error of the mean (S.E.M). The level of significance was set at $p<0.05$.

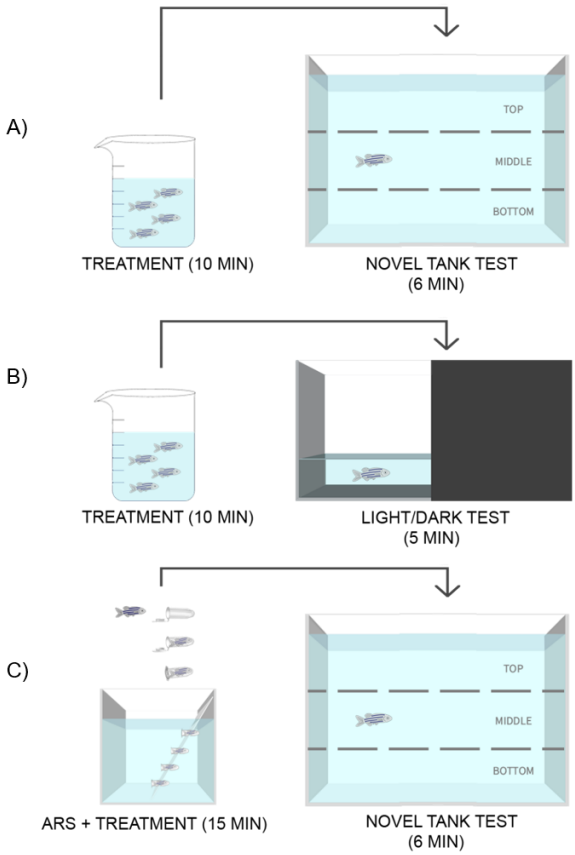

Figure 1. Schematic representation of the experimental protocols for the novel tank (A), light/dark (B), and acute restraint stress (C) tests.

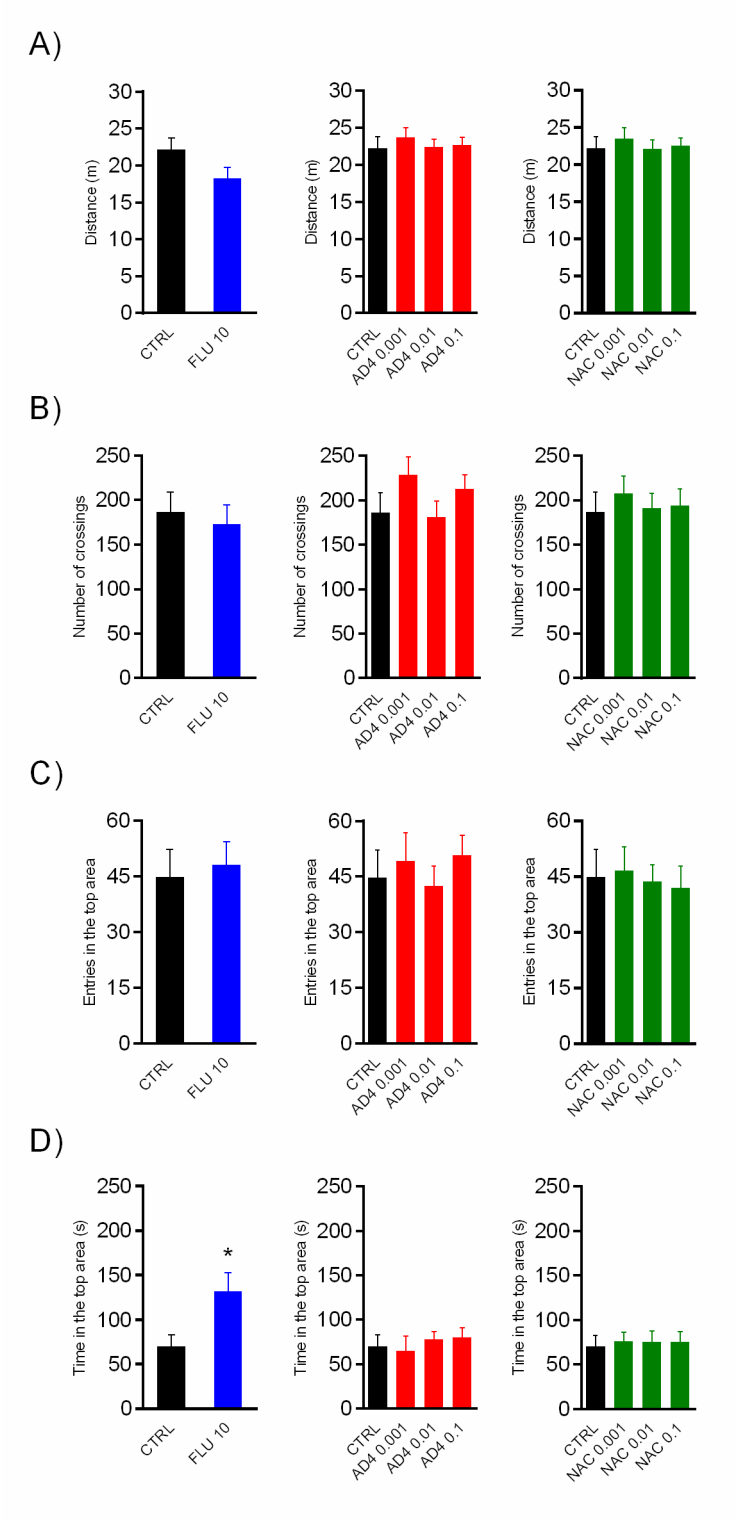

Figure 2. Effects of fluoxetine, AD4 and NAC in the novel tank test (NTT). Distance traveled (A), number of crossings (B), entries (C) and time spent in the top area (D). Concentrations are indicated as mg/L. Data are expressed as mean ± S.E.M. *p<0.05 x CTRL. Student t-test or one-way ANOVA/Tukey. CTRL = control. FLU = fluoxetine. NAC = N-acetylcysteine. AD4 = N-acetylcysteine amide. N=11-12.

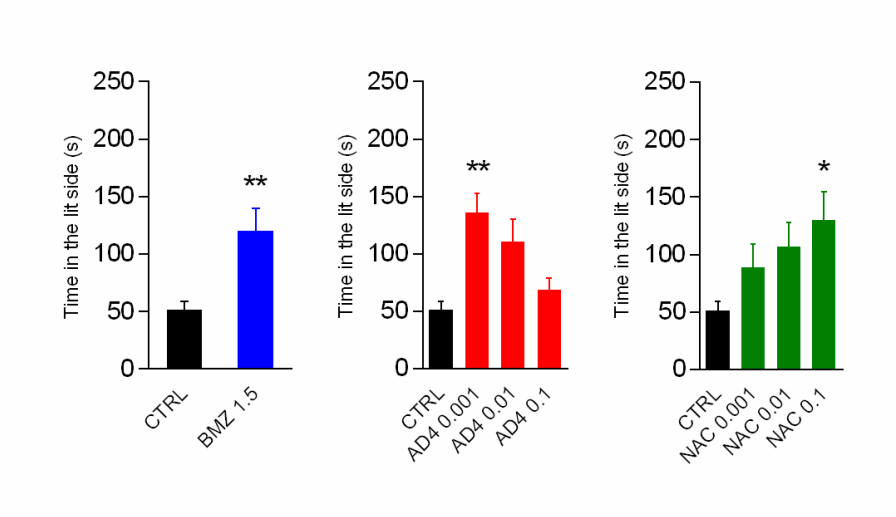

Figure 3. Effects of bromazepam, AD4 and NAC on the time in the lit side in the light dark test. Concentrations are indicated as mg/L. Data are expressed as mean ± S.E.M. Student t-test or one-way ANOVA/Tukey. *p<0.05 x CTRL. **p<0.01 x CTRL. BMZ = bromazepam. CTRL = control. NAC = N-acetylcysteine. AD4 = N-acetylcysteine amide. N=10-16.

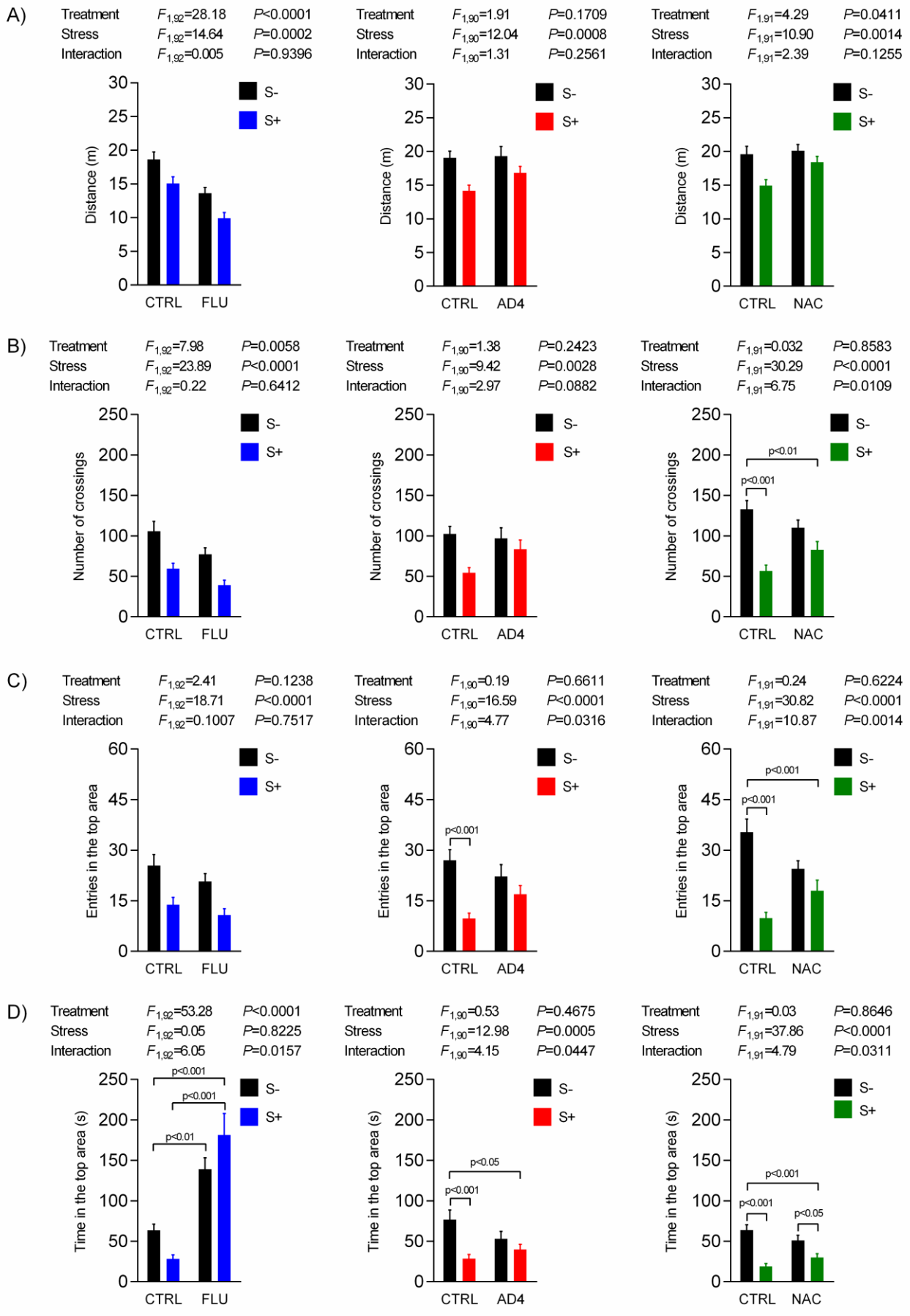
A)
Treatment $F_{1,92}$=28.18 P<0.0001
Stress $F_{1,92}$=14.64 P=0.0002
Interaction $F_{1,92}$=0.005 P=0.9396
Treatment $F_{1,90}$=1.91 P=0.1709
Stress $F_{1,90}$=12.04 P=0.0008
Interaction $F_{1,90}$=1.31 P=0.2561
Treatment $F_{1,91}$=4.29 P=0.0411
Stress $F_{1,91}$=10.90 P=0.0014
Interaction $F_{1,91}$=2.39 P=0.1255
Distance (m)
S-
S+
CTRL FLU
CTRL AD4
CTRL NAC
B)
Treatment $F_{1,92}$=7.98 P=0.0058
Stress $F_{1,92}$=23.89 P<0.0001
Interaction $F_{1,92}$=0.22 P=0.6412
Treatment $F_{1,90}$=1.38 P=0.2423
Stress $F_{1,90}$=9.42 P=0.0028
Interaction $F_{1,90}$=2.97 P=0.0882
Treatment $F_{1,91}$=0.032 P=0.8583
Stress $F_{1,91}$=30.29 P<0.0001
Interaction $F_{1,91}$=6.75 P=0.0109
Number of crossings
p<0.01
p<0.001
C)
Treatment $F_{1,92}$=2.41 P=0.1238
Stress $F_{1,92}$=18.71 P<0.0001
Interaction $F_{1,92}$=0.1007 P=0.7517
Treatment $F_{1,90}$=0.19 P=0.6611
Stress $F_{1,90}$=16.59 P<0.0001
Interaction $F_{1,90}$=4.77 P=0.0316
Treatment $F_{1,91}$=0.24 P=0.6224
Stress $F_{1,91}$=30.82 P<0.0001
Interaction $F_{1,91}$=10.87 P=0.0014
Entries in the top area
p<0.001
D)
Treatment $F_{1,92}$=53.28 P<0.0001
Stress $F_{1,92}$=0.05 P=0.8225
Interaction $F_{1,92}$=6.05 P=0.0157
Treatment $F_{1,90}$=0.53 P=0.4675
Stress $F_{1,90}$=12.98 P=0.0005
Interaction $F_{1,90}$=4.15 P=0.0447
Treatment $F_{1,91}$=0.03 P=0.8646
Stress $F_{1,91}$=37.86 P<0.0001
Interaction $F_{1,91}$=4.79 P=0.0311
Time in the top area (s)
p<0.001
p<0.01
p<0.05

Figure 4. Effects of fluoxetine, AD4 and NAC on distance traveled (A), number of crossings (B), entries (C) and time in the top area (D) in the acute restraint stress. Data are expressed as mean ± S.E.M. Two-way ANOVA/ Tukey. CTRL = control. FLU = fluoxetine. NAC = N-acetylcysteine. AD4 = N-acetylcysteine amide. N= 22-24.

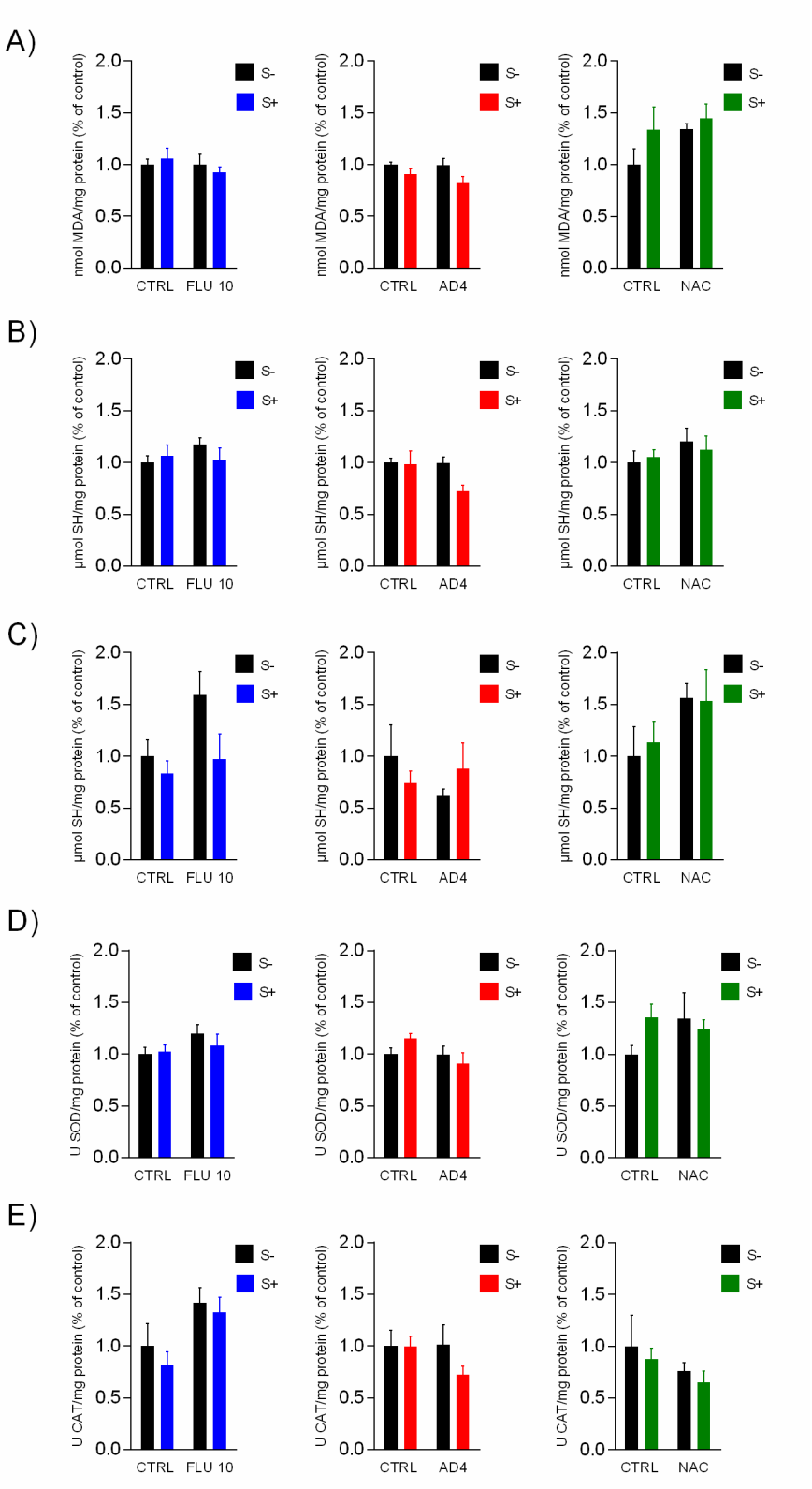

Figure 5. Effects of fluoxetine, AD4 and NAC on oxidative stress parameters. Thiobarbituric acid reactive substances (TBARS) (A), total thiols levels (SH) (B), non-protein thiols levels (NPSH) (C), superoxide dismutase (SOD) (D) and catalase activities (CAT) (E) in the acute restraint stress. Data are expressed as

% of control. Two-way ANOVA/ Tukey. CTRL = control. FLU = fluoxetine. NAC = N-acetylcysteine. AD4 = N-acetylcysteine amide. N= 5-6.

RESULTS

Figure 2 shows the results of the novel tank test. AD4 and NAC in all concentrations did not alter any of the observed parameters. As expected, and replicating previous studies, fluoxetine increased the time spent in the top area in comparison to the control group (Fig. 2D).

Figure 3 shows the results of the light/dark test. BMZ (1.5 mg/L), AD4 (0.001 mg/L) and NAC (0.1 mg/L) increased the time spent in the lit side when compared to the control group. The treatments did not alter the number of crossings (data not shown).

Figure 4 shows the results from the acute restraint stress on behavioral parameters in zebrafish. Stress and fluoxetine decreased distance and number of crossings (main effects of stress and treatment). ARS decreased the entries and time in the top area, while fluoxetine increased the latter parameter in stressed (S+) and non-stressed (S-) groups. AD4 attenuated the effects of ARS on the number of entries and time spent in the top area. NAC attenuated the effects of ARS on crossings and number of entries in the top area.

Figure 5 shows the data on oxidative status in zebrafish brain. Neither the ARS nor the treatments induced alterations in any of the parameters analyzed.

DISCUSSION

The main findings of this study are the anxiolytic effect of AD4 in zebrafish, its comparable profile to NAC as anxiolytic in this species and the much higher potency of AD4 in comparison to NAC. This study replicates the lack of effect of NAC in the novel tank test (Mocelin et al., 2015) and AD4 also proved to be inactive. AD4 and NAC presented anxiolytic effects in the light and dark test and prevented the effects of stress in zebrafish. The potency of AD4 in the light dark test is 100 times higher than NAC.

The NTT, in which locomotor and anxiety-related parameters are quantified, has been used to study the effects of pharmacological and non-pharmacological interventions in zebrafish. Fluoxetine, escitalopram, and buspirone have been demonstrated to increase the time spent by zebrafish in the upper area of the tank (Kalueff et al., 2014; Marcon et al., 2016; Mocelin et al., 2015; Sackerman et al., 2010; Schaefer et al., 2015; Stewart et al., 2011, 2012). Neither AD4 nor NAC were effective in the NTT.

The LDT is a classical protocol used in rodents and it was adapted to zebrafish (Maximino et al., 2010). Bromazepam, buspirone, and ethanol increase the time spent in the lit side of the tank (Gebauer et al., 2011; Maximino et al., 2010; Mocelin et al., 2015). Anxiogenic compounds such as caffeine and alarm substance increase the time spent in the dark side (Kysil et al., 2017; Maximino et al., 2014, 2011; Quadros et al., 2016). In the LDT, 0.001 mg/L AD4 increased the time spent in the lit side of the tank, whereas NAC had no effect at this concentration. Though more comprehensive dose-response relationships curves will allow for conclusive comparison, the data suggest, as hypothesized by its higher bioavailability, that AD4 is more potent than NAC. In another study, AD4 (but not NAC) was able to reduce cocaine seeking and relapse in bulbectomized rats (Jastrzębska et al., 2016).

The lack of effects in the NTT can be explained by different subjacent neurobiological substrates and the lower sensitivity of this test as compared to the LDT to detect anxiolytic interventions (Kalueff et al., 2014; Kysil et al., 2017). Accordingly, different drugs with distinct mechanisms of action were employed as positive controls: a selective serotonin reuptake inhibitor (SSRI) for the NTT and a benzodiazepine for the LDT (Mocelin et al., 2015).

We have previously showed that ARS applied for 90 min decreased the time in the top area of the tank, also increasing the color intensity of the animals, while ARS for 60 or 90 min increased distance traveled and number of crossings (Ghisleni et al., 2012; Piato et al., 2011). Biochemical parameters as whole-body cortisol levels were also increased for ARS during 15, 60, and 90 min (Ghisleni et al., 2012), and the later duration also decreased CAT activity and increased TBARS and non-protein thiol levels (Dal Santo et al., 2014). Decreased corticotropin-releasing hormone (CRH) expression in the zebrafish brain (Ghisleni et al., 2012) was observed following ARS for 15-90 min. Here,

ARS for 15 min decreased distance traveled, crossings among different tank areas and decreased entries and time in the top area, compatible with the expected anxiogenic effects. AD4 blocked the effect of stress on entries in the top area and attenuated the effect of stress on time in the top area. NAC was able to attenuate changes in the crossings and entries in the top area. Therefore, only AD4 blocked the effect of stress on entries in the top and attenuated the decrease in time in the top area, demonstrating a more powerful effect than NAC. The difference may again be attributed to AD4 superior bioavailability (Sunitha et al., 2013). At physiological pH (~ 7.4), NAC loses one proton and acquires a negative charge, which limits its crossing through the blood brain barrier (Samuni et al., 2013). The percentage of the molecule that remains in the neutral membrane permeating form is circa 0.001% of the total NAC dose (Samuni et al., 2013). AD4 is neutral at pH 7.4 and demonstrated to reach mice brain after oral and intraperitoneal administration (Offen et al., 2004). The shorter restraint period here employed and subtle variations in behavioral quantification may explain the lack of effects of ARS in oxidative stress.

Regarding oxidative status, although previous studies have shown that ARS for 90 min induces an imbalance in the antioxidant status in the zebrafish (Dal Santo et al., 2014), our 15-min protocol did not induce alterations in any of the oxidative parameters evaluated. Thus, we propose that the demonstrated behavioral effects are independent of oxidative status. Since AD4 and NAC possess a multitarget action, it is possible that the effects of AD4 in ARS in fish result from glutamatergic modulation. It is known that NAC modulates glutamatergic transmission through mechanisms related to cystine synthesis and activation of the astrocytic cystine-glutamate antiporter (Dean et al., 2011; Ooi et al., 2018). Cystine derived from NAC is taken up by astrocytes through the cystine-glutamate antiporter and exchanged for glutamate, which is released in the extrasynaptic millieu. This free glutamate acts on inhibitory metabotropic glutamate receptors (mGluRs2/3) in the pre-synaptic neuron, resulting in reduced glutamate release to nerve terminals (Berk et al., 2013). AD4 probably exerts the same glutamatergic effects as its parent compound, and although confirmatory studies are needed, this mechanism may underlie what we observed here.

A limitation of this study was the use of a shorter ARS exposition, which precluded the comparison of in vivo antioxidant capacity of AD4 and NAC. Another limitation is the employment of a single brief exposure to the drugs. Comparing the effects of AD4 and NAC in continuous or chronic intermitent schedules is necessary due to the chronic course of anxiety disordes.

Considering the substantial amount of data on the benefits of NAC on various mental disorders, and the apparent advantage in effectiveness, a more comprehensive comparison of AD4 and NAC is warranted.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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4. CONCLUSÃO

Esse estudo caracterizou os efeitos da AD4 e NAC sobre parâmetros comportamentais e bioquímicos em peixes-zebra. No teste de tanque novo, nenhuma das moléculas demonstrou efeitos sobre parâmetros locomotores e de ansiedade. Entretanto, foi demonstrado que a AD4 e a NAC possuem efeito ansiolítico no teste de claro/escuro. Nesse teste, AD4 possui efeito em concentração 100 vezes menor em relação à NAC, atestando uma maior potência da molécula. No protocolo de estresse agudo por contenção, ambas foram capazes de atenuar os efeitos do estresse sobre parâmetros comportamentais. Porém, em relação às entradas na área superior do aquário, AD4 bloqueou o efeito enquanto NAC atenuou. Em relação aos parâmetros de estresse oxidativo, não houve efeito de nenhum dos compostos. Esse trabalho contribui para a caracterização dos efeitos da AD4 em peixes-zebra, corroborando para a sua potencial aplicação em transtornos de ansiedade.

5. PERSPECTIVAS

A partir dos dados obtidos nessa dissertação serão realizados experimentos para avaliar o potencial desse composto na reversão/prevenção dos efeitos comportamentais e bioquímicos induzidos por um protocolo de estresse crônico imprevisível em peixes-zebra. Além disso, pretende-se verificar se a capacidade antioxidante de AD4 é superior à de NAC em encéfalo de peixes-zebra submetidos a esse protocolo experimental.

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7. ANEXOS

7.1 Carta de aprovação da Comissão de Ética no Uso de Animais da 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: 33406
Título: ESTUDOS DOS EFEITOS DE N-ACETILCISTEÍNA-AMIDA (NACA) EM PEIXES-ZEBRA

Vigência: 08/06/2017 à 28/02/2019

Pesquisadores:

Equipe UFRGS:

ÂNGELO LUIS STAPASSOLI PIATO - coordenador desde 08/06/2017
RADHARANI BENVENUTTI - Aluno de Mestrado desde 08/06/2017
Ricieri Naue Mocelin - Aluno de Doutorado desde 08/06/2017
Matheus Felipe Marcon - Aluno de Doutorado desde 08/06/2017
Carlos Guilherme Rosa Reis - Aluno de Mestrado desde 08/06/2017

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 31/07/2017 - SALA 330 DO ANEXO I DO PRÉDIO DA REITORIA - CAMPUS CENTRO - UFRGS-PAULO DA GAMA,110 BAIRRO FARROUPILHA -, em seus aspectos éticos e metodológicos, para a utilização de de 439 peixes-zebra (Danio rerio) selvagens adultos jovens de ambos os sexos (mais de 3 meses de vida), pesando aproximadamente 200-300mg, obtidos da colônia a partir de cruzamentos entre animais adquiridos do Departamento de Bioquímica da UFRGS, 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, Quarta-Feira, 14 de Março de 2018

MARCELO MELLER ALIEVI
Coordenador da comissão de ética