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EFEITOS DA N-ACETILCISTEÍNA EM MODELOS DE ESTRESSE CRÔNICO IMPREVISÍVEL E EXPOSIÇÃO AO ETANOL EM PEIXES-ZEBRA

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Tese 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 Doutor em Ciências Biológicas: Neurociências.

Orientador: Prof. Dr. Angelo Piato

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O êxito da vida não se mede pelo caminho que você conquistou,

mas sim pelas dificuldades que superou no caminho.

Abraham Lincoln

AGRADECIMENTOS

RESUMO

O estresse crônico e o abuso de álcool são fatores que predispõem os indivíduos a desenvolver transtornos mentais, condições que impactam na qualidade de vida do indivíduo e contribuem para a morbidade e a mortalidade global. A farmacoterapêutica disponível para tratar esses pacientes apresenta eficácia limitada e alta incidência de efeitos adversos. O estresse oxidativo, a hiperativação glutamatérgica, a depleção de glutationa e a neuroinflamação fazem parte dos achados observados em estudos pré-clínicos e clínicos nessas condições. A N-acetilcisteína (NAC) é uma molécula promissora para o tratamento de uma variedade de condições psiquiátricas. Esse fármaco atua em diversos alvos relevantes para o tratamento de transtornos mentais, incluindo ansiedade, depressão e abuso de álcool. Na presente tese, investigamos os efeitos da NAC nesses contextos utilizando como organismo modelo o peixe-zebra. No modelo de estresse crônico imprevisível, os animais apresentaram aumento na ansiedade (aumento no tempo na área inferior e diminuição nas entradas e tempo de permanência na área superior do aquário) no teste de tanque novo, lipoperoxidação, aumento nas espécies reativas de oxigênio e redução nos níveis de glutationa e na atividade das enzimas superóxido dismutase e catalase. A NAC reverteu o comportamento ansiogênico e o dano oxidativo. No modelo de exposição aguda ao etanol os animais apresentaram dano motor (diminuição da distância e número total de cruzamentos entre as diferentes áreas do aquário), comportamento ansiogênico (diminuição nas entradas e tempo de permanência na área superior do aquário), dano lipídico, aumento nas espécies reativas de oxigênio e depleção de glutationa. A NAC preveniu os efeitos comportamentais e bioquímicos induzidos pela exposição aguda ao etanol. Finalmente, avaliamos os efeitos da NAC em animais abstinentes após exposição crônica ao etanol por oito dias. 24 horas após oito dias de exposição intermitente, o comportamento dos peixes foi testado no teste de tanque novo. A abstinência induziu comportamento ansiogênico (aumento no tempo na área inferior e diminuição nas entradas e tempo de permanência na área superior do aquário) e desequilíbrio do status oxidativo, com peroxidação lipídica, diminuição da glutationa e das atividades da superóxido dismutase e catalase. NAC preveniu os danos. Nosso estudo agrega importantes achados que contribuem para o corpo de evidências existentes que apoiam a avaliação e utilização clínica da NAC em transtornos mentais e condições associadas ao abuso de substâncias.

ABSTRACT

Chronic stress and alcohol abuse are factors that predispose individuals to develop mental disorders, conditions that impact on the individual quality of life and contribute to overall morbidity and mortality. Pharmacotherapeutics available to treat these patients have limited efficacy and a high incidence of adverse effects. Oxidative stress, glutamatergic hyperactivation, glutathione depletion, and neuroinflammation are part of the findings observed in preclinical and clinical studies in these conditions. N-acetylcysteine (NAC) is a promising molecule for the treatment of a variety of psychiatric conditions. This drug acts on several relevant targets for the treatment of mental disorders, including anxiety, depression and alcohol abuse. In the present thesis, we investigated the effects of NAC in these contexts using zebrafish as an organism model. In the unpredictable chronic stress, the animals showed an increase in anxiety (increase in the time in the bottom area and decrease in the entries and time in the top area of the tank) in the novel tank test, lipoperoxidation, reactive oxygen species increase and reduction in glutathione levels and activity of the superoxide dismutase and catalase enzymes. NAC reversed anxiogenic behavior and oxidative damage. In the acute exposure to ethanol, the animals presented motor damage (decrease of distance and a total number of crossings between different areas of the tank), anxiogenic behavior (decrease in entries and time in the top area of the tank), lipid damage, reactive oxygen species increase and glutathione depletion. NAC prevented the behavioral and biochemical effects induced by acute exposure to ethanol. Finally, we evaluated the effects of NAC on abstinent animals after chronic exposure to ethanol for eight days. 24 hours after eight days of intermittent exposure, fish behavior was tested in the novel tank test. Abstinence induced anxiogenic behavior (increase in the time in the bottom and decrease in the entries and time in the top area of the tank) and oxidative status imbalance, lipid peroxidation, reduction of glutathione and superoxide dismutase and catalase activities. NAC prevented the damage. Our study aggregates important findings that contribute to the body of evidence supporting the assessment and clinical use of NAC in mental disorders and conditions associated with substance abuse.

LISTA DE ABREVIATURAS

AMPA	Alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico
BDNF	Fator neurotrófico derivado do cérebro
CAINATO	Ácido caínico
CYS	Cisteína
DSM-5	Manual Diagnóstico e Estatístico de Transtornos Mentais (5º edição)
ECI	Estresse crônico imprevisível
ERNs	Espécies reativas de nitrogênio
EROs	Espécies reativas de oxigênio
FDA	*Food and drug administration*
GLY	Glicina
GLU	Glutamato
GSH	Glutationa
H_2O_2	Peróxido de hidrogênio
IL-1β	Interleucina 1-beta
IL-6	Interleucina 6
ISRS	Inibidor seletivo da recaptação de serotonina
LPO	Lipoperoxidação
mGluR	Receptor metabotrópico de glutamato
NMDA	N-metil-D-aspartato
$O_2^{\bullet-}$	Radical superóxido
$OH^{\bullet}$	Radical hidroxila
SNC	Sistema nervoso central
TAG	Transtorno de ansiedade generalizada
TNF-α	Fator de necrose tumoral alfa
X_C^-	Trocador cistina-glutamato antiporter

SUMÁRIO

1. INTRODUÇÃO

1.1. ESTRESSE

O estresse induz alterações no organismo como reposta adaptativa frente a uma determinada demanda. A esse processo adaptativo dá-se o nome de alostase (GOLDSTEIN; MCEWEN, 2002; MCEWEN et al., 2015a). Quando a capacidade adaptativa é excedida, por exemplo, em situações de estresse mais intenso, outra fase é estabelecida, chamada de sobrecarga alostática. Essa fase pode predispor o indivíduo a diversas condições como hipertensão, diabetes, infecções, imunossupressão, distúrbios sexuais, obesidade e transtornos mentais (BAINS; CUSULIN; INOUE, 2015; DIAS-FERREIRA et al., 2009; GOLDSTEIN; MCEWEN, 2002; MCEWEN et al., 2015b; MCEWEN; WINGFIELD, 2003).

Eventos estressores, sejam agudos ou crônicos, podem contribuir para o estabelecimento de transtornos de ansiedade e humor (DAVIS et al., 2017; KRISHNAN; NESTLER, 2008; MCEWEN, 2007; MCEWEN et al., 2012, 2015a; MÉNARD; HODES; RUSSO, 2015; MUSAZZI; MARROCCO, 2016; RUSSO; NESTLER, 2013; SCHIAVONE; COLAIANNA; CURTIS, 2015). Os modelos pré-clínicos de estresse crônico imprevisível (ECI) podem mimetizar os achados neuroquímicos e comportamentais observados em pacientes submetidos a condições de estresse (BRUNELLO et al., 2006; FERNANDES; GUPTA, 2019; WILLNER, 2005, 2017; WILLNER; MUSCAT; PAPP, 1992). A base neurobiológica adjacente a essas condições é complexa, mas há evidências de alterações nos níveis de neurotransmissores, ativação de eixos neuroendócrinos, aumento do estresse oxidativo e neuroinflamação (MCEWEN, 2007; PATEL, 2016).

O glutamato é um neurotransmissor excitatório e apresenta importante papel sobre o comportamento, a aprendizagem, a função cognitiva e a memória (BURNETT; CHANDLER; TRANTHAM-DAVIDSON, 2016; HASHIMOTO, 2011; PITSIKAS, 2014). O ECI induz desequilíbrio glutamatérgico no sistema nervoso central (SNC). Esse desequilíbrio é

caracterizado por um aumento na liberação e redução na captação, desencadeando excitotoxicidade, responsável pela ativação de cascatas neuroinflamatórias com aumento de citocinas pró-inflamatórias IL-1β, IL-6 e TNF-α (FERNANDES; GUPTA, 2019; HASHIMOTO, 2011; KOO et al., 2010; MILLER; MALETIC; RAISON, 2009; MILLER; RAISON, 2016; MOGHADDAM, 2002; PITSIKAS, 2014; POPOLI et al., 2011). Além do desequilíbrio glutamatérgico, o ECI aumenta os níveis das espécies reativas de oxigênio (EROs) e de nitrogênio (ERNs). Isso causa lipoperoxidação (LPO) e diminuição da atividade das enzimas antioxidantes superóxido dismutase (SOD) e catalase (CAT), além de redução dos níveis de glutationa (GSH). O conjunto desses fatores são responsáveis por dano e morte de tecidos neurais (Figura 1) (MINEUR; BELZUNG; CRUSIO, 2006; NOLLET et al., 2019; PATEL, 2016; WOHLEB et al., 2016).

Além da relação do estresse crônico como fator etiológico dos transtornos mentais, há uma associação entre o estresse e o abuso de substâncias, como o etanol (KEYES et al., 2012; KEYES; HATZENBUEHLER; HASIN, 2011). Culturalmente, indivíduos utilizam o etanol para alívio de sintomas relacionados a ansiedade. Entretanto, o consumo acentuado pode causar, além de síndrome de abstinência e dependência, estresse oxidativo, neuroinflamação e redução de neurotrofinas (HARPER, 2007; IRWIN; MILLER, 2007; WARD; LALLEMAND; DE WITTE, 2009).

1.2. TRANSTORNOS MENTAIS

Os transtornos mentais como ansiedade e depressão são altamente deletérios, associados à perda de produtividade, incapacidade e morte prematura (INSEL et al., 2013; PATEL et al., 2018; WALKER; MCGEE; DRUSS, 2015). Além disso, esses transtornos geram elevados custos globais que podem atingir 6,5 trilhões de dólares por ano até 2030 (PATEL et al., 2018; WALKER; MCGEE; DRUSS, 2015).

A ansiedade constituí o maior grupo de transtorno mental crônico e uma das principais causas de incapacidade, afetando cerca de 30% da população mundial (CRASKE et al., 2017; MACHADO et al., 2016). De acordo com o Manual Diagnóstico e Estatístico de Transtornos Mentais 5ª edição (DSM-5, 2014), os transtornos de ansiedade são classificados em (1) de separação; (2) mutismo seletivo; (3) fobia específica; (4) social; (5) transtorno de pânico; (6) agorafobia; (7) generalizada; (8) devido a outra condição médica; (9) outro transtorno de ansiedade especificado e (10) não especificado. O transtorno de ansiedade generalizada (TAG) é um dos mais prevalentes (CRASKE et al., 2017). Pacientes com TAG apresentam preocupação persistente e excessiva, desproporcional à realidade e de difícil controle. Essas preocupações são acompanhadas de sintomas como taquicardia, sudorese, insônia, fadiga e dores musculares (CORDIOLI, 2015, DSM-V, 2014). Se não tratados, os pacientes podem apresentar comorbidade com outras condições como depressão ou doenças cardiovasculares (BATELAAN et al., 2014; KESSLER et al., 2005; SMOLLER et al., 2007). Sintomas depressivos aparecem em até 90% dos pacientes com ansiedade e 85% dos pacientes com depressão podem apresentar sintomas significativos de ansiedade, o que demonstra a complexidade e a similaridade neurobiológica entre esses transtornos (MÖLLER et al., 2016).

Estima-se que 40% dos pacientes diagnosticados com transtornos de ansiedade não apresentam redução dos sintomas durante o tratamento. As opções farmacológicas disponíveis são os inibidores seletivos da recaptação de serotonina (ISRS), os benzodiazepínicos ou agonistas parciais de serotonina (5-HT$_{1A}$). Esses fármacos interagem com diferentes sistemas neurotransmissores e neuromoduladores (CRASKE; STEIN, 2016; WU et al., 2012). Apesar das diferentes opções farmacológicas, nem todos pacientes respondem adequadamente ao tratamento (FLUYAU; REVADIGAR; MANOBIANCO, 2018). Os efeitos adversos induzidos por esses fármacos também são relevantes. Os ISRS, como por exemplo a fluoxetina e o citalopram, podem causar cefaleia, náusea, insônia, anorexia, ansiedade, diarreia, sonolência,

tremor, fraqueza, além de outros. Já os benzodiazepínicos, como por exemplo o diazepam e o bromazepam, podem causar hipotensão, fadiga, fraqueza muscular, depressão respiratória, retenção urinária, depressão, visão borrada, cefaleia, dentre outros. O uso crônico de benzodiazepínicos pode causar dependência, tolerância, síndrome de abstinência e ideação suicida. Além disso, a associação com outros depressores do SNC, como o etanol, pode potencializar o efeito, induzir coma e levar a morte (FLUYAU; REVADIGAR; MANOBIANCO, 2018; TIIHONEN et al., 2015).

Considerando a alta incidência de transtornos mentais, aliado às limitações dos fármacos disponíveis para tratamento, há uma clara necessidade por novas opções terapêuticas. Com exceção da esketamina, aprovada recentemente pelo FDA para pacientes deprimidos não-responsivos aos tratamentos disponíveis, nenhum novo fármaco com mecanismo de ação inovador chegou ao mercado nas últimas décadas (CRASKE; STEIN, 2016). Nesse contexto, compostos que atuem sobre diferentes alvos terapêuticos podem oferecer maior eficácia clínica e menores efeitos adversos do que as terapias atuais.

1.3. ETANOL

O álcool é consumido comumente pelas pessoas com o propósito de socializar, celebrar ou aliviar o estresse (GRANT et al., 2015; HASHIMOTO; WIREN, 2007). O consumo em demasia induz neurotoxicidade, distúrbios cognitivos, diminuição de produtividade e problemas na relação interpessoal, além de aumentar a incidência de acidentes automobilísticos e violência, contribuindo substancialmente para a morbidade e mortalidade global (ABRAHAO; SALINAS; LOVINGER, 2017; GRANT et al., 2015; NIAAA, 2017; WHO, 2018). O abuso de álcool é um problema grave de saúde, considerado um fator importante para o desenvolvimento de transtornos de ansiedade e humor (GRANT et al., 2015). Induz incapacidade, morte prematura e contribui para 5,3% (aproximadamente 3 milhões) de todas as

causas de morte. Responsável por ocasionar prejuízos sociais e econômicos significativos para os indivíduos e a sociedade em geral, o etanol é fator casual para mais de 200 doenças e condições clínicas, dentre eles os transtornos de ansiedade e de humor (DRIESSEN et al., 2001; GRANT et al., 2015; SCHWARZINGER et al., 2018; SMITH; RANDALL, 2012; WHO, 2018).

O etanol induz alterações comportamentais e cognitivas, além de neurotoxicidade e neuroinflamação. Além disso, pode promover alterações em diversos sistemas neurais e afetar o bem-estar do indivíduo (HARPER, 2007; IRWIN; MILLER, 2007; WARD; LALLEMAND; DE WITTE, 2009). É uma droga psicotrópica, hipnótica-sedativa que causa alterações comportamentais de maneira dose-dependente (WHITE; MATTHEWS; BEST, 2000). Em baixas doses, o etanol causa euforia, relaxamento e alívio do estresse. Moderadas doses ocasionam prejuízos motores. Doses altas induzem danos cognitivos e aumento no tempo de reação, fala arrastada, ataxia, além disso pode levar a perda de consciência e morte (ANKER et al., 2016; NIAAA, 2017). Apesar de um grande número de pesquisas pré-clínicas e clínicas, não é completamente consolidada a relação do abuso de etanol como um fator predisponente ao desenvolvimento de transtornos de ansiedade e humor ou vice-versa (ABRAHAO; SALINAS; LOVINGER, 2017; SPANAGEL, 2009).

O etanol induz neurotoxicidade pela geração de EROs como radical superóxido ($O_2^{\bullet-}$), peróxido de hidrogênio (H_2O_2) e radical hidroxila ($OH^{\bullet}$), além de alterações na homeostase de defesas antioxidantes (BARNHAM; MASTERS; BUSH, 2004; NG et al., 2008). A produção de EROs no SNC pode ser decorrente da metabolização do etanol a acetaldeído. Consequentemente, ocorre redução das defesas antioxidantes, tanto não-enzimáticas (como os níveis de GSH) como enzimáticas (SOD e CAT), além de aumento na peroxidação lipídica, causando dano lipídico, proteico e ao DNA (HARRISON et al., 2017; SEITZ et al., 2018; VARELA-REY et al., 2013). Estudos têm mostrado que a administração de antioxidantes,

especialmente agentes que aumentam o aporte de GSH, pode prevenir tais efeitos deletérios (GONZÁLEZ-REIMERS et al., 2014; IIMURO et al., 2000; KARADAYIAN et al., 2017; SCHNEIDER et al., 2015). Além disso, o excesso de etanol no organismo afeta a homeostase do sistema imune. Indivíduos que consomem quantidades elevadas de etanol apresentam aumento de citocinas pró-inflamatórias como IL-1β, IL-6 e TNF-α (ACHUR; FREEMAN; VRANA, 2010; KELLEY; DANTZER, 2011), contribuindo com a neurotoxicidade (Figura 1) (IRWIN et al., 2009; IRWIN; MILLER, 2007).

A combinação de tratamento farmacológico, psicoterapia e grupos de apoio (como os Alcoólicos Anônimos) são abordagens para o tratamento dos transtornos de uso de álcool, entretanto, apenas 10% desses pacientes recebem alguma intervenção terapêutica. Apenas três fármacos são aprovados pelo FDA para o tratamento desses pacientes: a naltrexona, o acamprosato e o dissulfiram (NIAAA, 2017). Essas opções apresentam limitada efetividade e alta incidência de efeitos adversos, o que dificulta a adesão ao tratamento e contribui para as recaídas (ANTON et al., 2006; KOSTEN; O'CONNOR, 2003; LITTEN et al., 2015). Nesse sentido, a busca por novos tratamentos é indispensável.
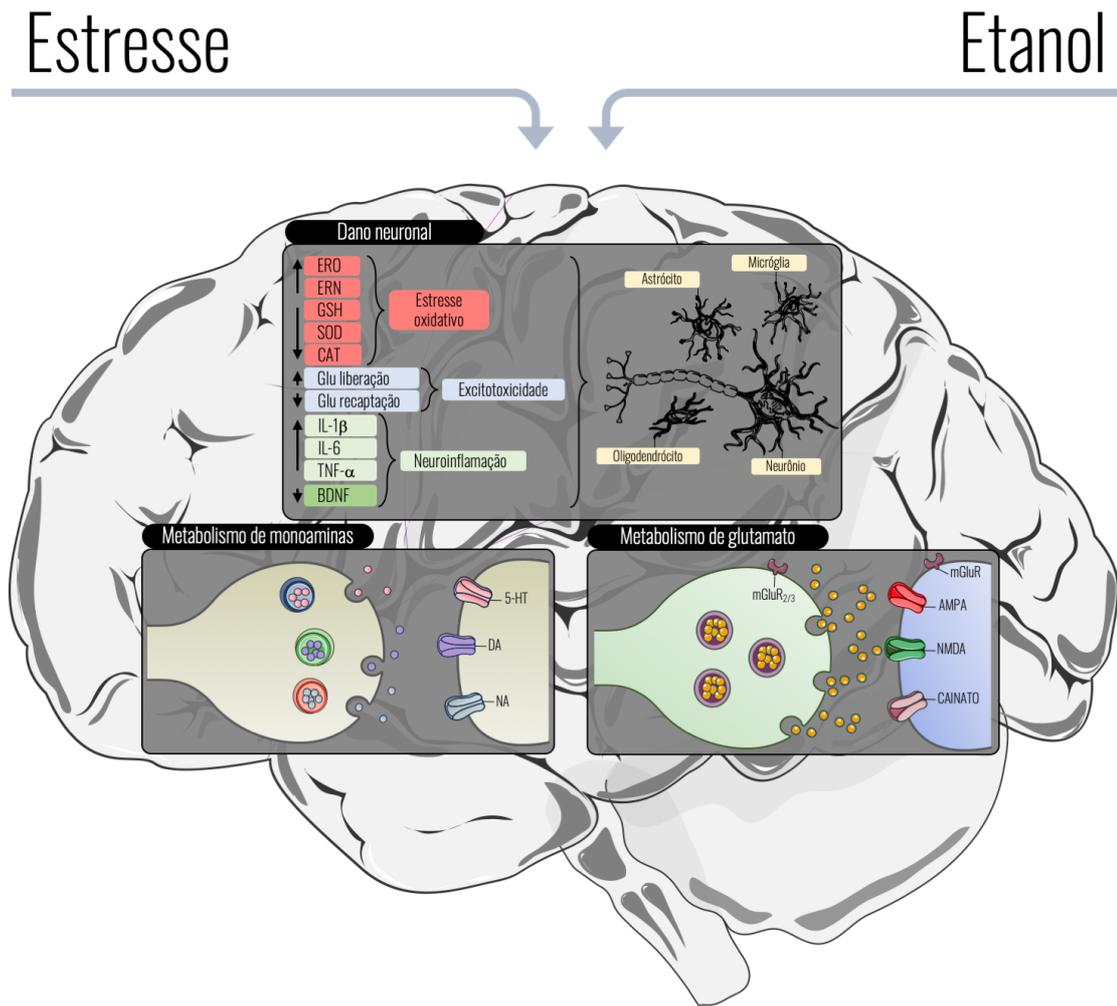
Figura 1. Resumo da neurobiologia dos transtornos relacionados ao estresse e abuso de etanol (Fonte: autor/adaptado de (MILLER; MALETIC; RAISON, 2009; MILLER; RAISON, 2016; RON; BARAK, 2016).

1.4. N-ACETILCISTEÍNA

A N-acetilcisteína (NAC) é um composto tiólico derivado do N-acetil do aminoácido L-cisteína, amplamente utilizado na clínica como mucolítico, antídoto em casos de overdose por paracetamol e na prevenção de doença pulmonar obstrutiva crônica. Apresenta boa tolerabilidade e baixa incidência de efeitos adversos (DEAN; GIORLANDO; BERK, 2011a; WHYTE; FRANCIS; DAWSON, 2007)

Quando administrada via oral, a NAC promove aumento de cisteína no SNC. Essa cisteína sofre oxidação formando duas moléculas de cistina, que age no trocador cistina-glutamato (X_C-) presente em astrócitos. Ocorre aumento de glutamato extracelular devido a

troca de cistina por glutamato no trocador X_C- (BAKER et al., 2008). O glutamato pode ativar os receptores $mGluR_{2/3}$ localizados no espaço extra-sináptico dos neurônios pré-sinápticos. Como resultado observa-se a diminuição da liberação de glutamato na fenda sináptica. Essa redução previne a excitotoxicidade e a ativação de receptores NMDA (DEAN; GIORLANDO; BERK, 2011a). Além da atividade indireta sobre os receptores NMDA, a NAC parece atuar sobre receptores AMPA (Figura 2) (LINCK et al., 2012). A modulação da transmissão glutamatérgica através dos receptores do tipo NMDA e AMPA parece ser um potencial alvo terapêutico no tratamento de transtornos mentais (AVERILL et al., 2017; HASHIMOTO, 2011; KADRIU et al., 2019). Estudos clínicos mostram que a NAC é capaz de reduzir os níveis de glutamato em diferentes regiões do SNC (AVERILL et al., 2017; GIRGIS et al., 2019; MCQUEEN et al., 2018; O'GORMAN TUURA et al., 2019; SWANSON et al., 2005).

Além desses efeitos sobre a transmissão glutamatérgica, a NAC possui efeito neuroprotetor regulando os sistemas antioxidantes como um *scavenger* de radicais livres e atuando como substrato para a formação de GSH. Nos astrócitos, a NAC fornece a cisteína que associada a glicina e o glutamato formam a GSH (Figura 2), principal antioxidante do SNC (BERK et al., 2013; DEAN; GIORLANDO; BERK, 2011a; DODD et al., 2008). Recentemente, foi demonstrado que a NAC é capaz de proteger os astrócitos contra dano oxidativo independente da produção de GSH, ou seja, a NAC é capaz de atuar como um antioxidante diretamente pois possui um grupamento tiol sulfidrila (SH) na sua estrutura (GLEIXNER et al., 2017). Esse grupamento sofre oxidação pelas EROs e aumento na capacidade de formar pontes dissulfeto, prevenindo que essas reações causem dano aos tecidos biológicos (POOLE, 2015; REQUEJO et al., 2010).

Além dos mecanismos discutidos até aqui, sabe-se que a NAC apresenta atividade anti-inflamatória e por isso poderia atuar contra a neuroinflamação presente em várias condições que afetam o SNC (BERK et al., 2013; DEAN; GIORLANDO; BERK, 2011a; ROBERTSON

et al., 2019). Em modelos pré-clínicos, a NAC diminuiu os níveis de citocinas pró-inflamatórias como IL-1β, IL-6 e TNF-α em animais submetidos ao ECI (FERNANDES; GUPTA, 2019). A redução dessas citocinas e o aumento da disponibilidade de BDNF podem estar relacionados ao efeito neuroprotetor da NAC (ADZIC et al., 2018; CAVIEDES et al., 2017; FERNANDES; GUPTA, 2019), entretanto, nenhum estudo clínico confirmou tais achados.
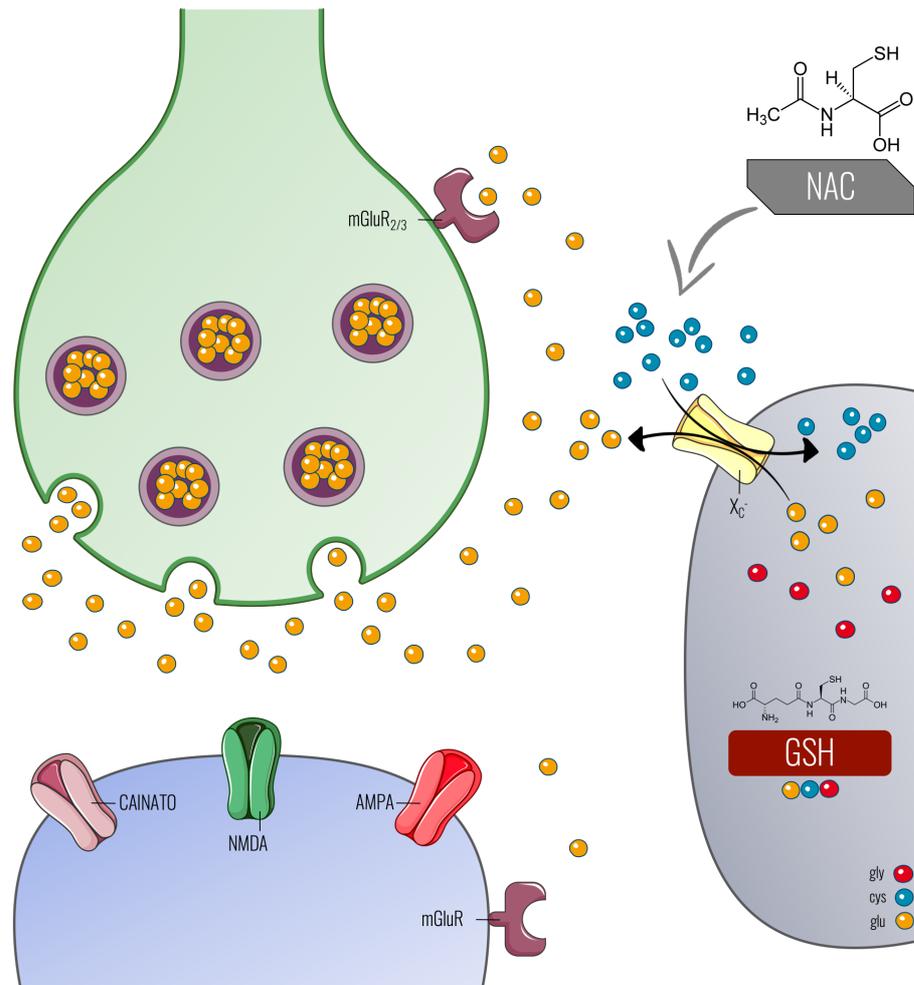
Figura 2. Efeitos multialvos da N-acetilcisteína (NAC). (Fonte: autor/adaptado de (BERK et al., 2013).

Essa ação multialvo da NAC desperta interesse de diversos grupo de pesquisa tanto pré-clínicas como clínicas que buscam avaliar seus efeitos em condições psiquiátricas relacionadas ao estresse e abuso de substâncias. Estudos demonstram potencial aplicabilidade da NAC como adjuvante terapêutico no tratamento de transtornos de ansiedade, humor, transtorno bipolar, esquizofrenia, autismo, transtorno obsessivo-compulsivo, tricotilomania e redução no consumo

e/ou recaídas a drogas de abuso como como o etanol, cocaína, maconha, dentre outras (BAUER et al., 2018; BAVARSAD SHAHRIPOUR; HARRIGAN; ALEXANDROV, 2014; BERK et al., 2019; DEEPMALA et al., 2015; GIPSON, 2016; GOENAGA et al., 2019; MCCLURE et al., 2014; PORCU et al., 2018; SEPEHRMANESH et al., 2018; YANG et al., 2018). Na base de dados do *Clinical Trials* (clinicaltrials.gov) é possível observar diferentes estudos em andamento com a NAC, investigando sua eficácia sobre diferentes transtornos mentais como transtorno bipolar (identificador: NCT02719392), depressão (identificador: NCT02972398), esquizofrenia (identificador: NCT02505477) e transtorno de uso de álcool (identificador: NCT02791945).

Estudos do nosso grupo mostraram que a NAC possui efeitos em modelos preditivos para drogas ansiolíticas tanto em peixes-zebra como em roedores (MOCELIN et al., 2015; SANTOS et al., 2017). Em modelos preditivos para drogas antidepressivas como é o caso do ECI, a NAC reverteu o comportamento tipo-depressivo e a neuroinflamação em ratos (FERNANDES; GUPTA, 2019). Outros trabalhos mostraram que a NAC foi capaz de prevenir os efeitos comportamentais e bioquímicos relacionados a exposição crônica de etanol em roedores (SCHNEIDER et al., 2015, 2017; YAWALKAR; CHANGOTRA; GUPTA, 2018). Entretanto, não há estudos investigando os efeitos de NAC sobre parâmetros comportamentais e bioquímicos em modelos de estresse crônico imprevisível e exposição ao etanol em peixes-zebra.

2. OBJETIVOS

2.1. OBJETIVO GERAL

O objetivo foi investigar os efeitos da N-acetilcisteína (NAC) em modelos de estresse crônico imprevisível e exposição ao etanol em peixes-zebra.

2.2. OBJETIVOS ESPECÍFICOS

a) Investigar os efeitos da NAC sobre parâmetros comportamentais e de estresse oxidativo em peixes-zebra submetidos ao modelo de estresse crônico imprevisível.

b) Investigar os efeitos da NAC sobre parâmetros comportamentais e de estresse oxidativo em peixes-zebra submetidos ao modelo de exposição aguda ao etanol.

c) Investigar os efeitos da NAC sobre parâmetros comportamentais e de estresse oxidativo em peixes-zebra submetidos ao modelo de abstinência ao etanol.

d) Investigar o efeito rápido, sustentado e profilático da NAC sobre parâmetros comportamentais, oxidativos e moleculares em peixes-zebra submetidos ao modelo de ECI.

3. COLETÂNEA DE ARTIGOS

A presente tese é apresentada no formato de coletânea de artigos científicos publicados em periódicos de acordo com cada objetivo específico.

3.1. CAPÍTULO I: Esse capítulo mostra os dados referentes ao primeiro objetivo específico (efeitos da NAC sobre parâmetros comportamentais e de estresse oxidativo em peixes-zebra submetidos ao modelo de estresse crônico imprevisível).

Artigo:

MOCELIN R, MARCON M, D'AMBROS S, MATTOS J, SACHETT A, SIEBEL AM, HERRMANN AP, PIATO A. N-acetylcysteine reverses anxiety and oxidative damage induced by unpredictable chronic stress in zebrafish. Mol Neurobiol. 2019 Feb;56(2):1188-1195. doi:10.1007/s12035-018-1165-y.

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N-Acetylcysteine Reverses Anxiety and Oxidative Damage Induced by Unpredictable Chronic Stress in Zebrafish

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Abstract

There is accumulating evidence on the use of N-acetylcysteine (NAC) in the treatment of patients with neuropsychiatric disorders. As a multi-target drug and a glutathione precursor, NAC is a promising molecule in the management of stress-related disorders, for which there is an expanding field of research investigating novel therapies targeting oxidative pathways. The deleterious effects of chronic stress in the central nervous system are a result of glutamatergic hyperactivation, glutathione (GSH) depletion, oxidative stress, and increased inflammatory response, among others. The aim of this study was to investigate the effects of NAC in zebrafish submitted to unpredictable chronic stress (UCS). Animals were initially stressed or not for 7 days, followed by treatment with NAC (1 mg/L, 10 min) or vehicle for 7 days. UCS decreased the number of entries and time spent in the top area in the novel tank test, which indicate increased anxiety levels. It also increased reactive oxygen species (ROS) levels and lipid peroxidation (TBARS) while decreased non-protein thiols (NPSH) and superoxide dismutase (SOD) activity. NAC reversed the anxiety-like behavior and oxidative damage observed in stressed animals. Additional studies are needed to investigate the effects of this agent on glutamatergic modulation and inflammatory markers related to stress. Nevertheless, our study adds to the existing body of evidence supporting the clinical evaluation of NAC in mood disorders, anxiety, post-traumatic stress disorder, and other conditions associated with stress.

Keywords N-acetylcysteine · Unpredictable chronic stress · Behavior · Oxidative status · Zebrafish

Introduction

Clinical studies have suggested that oral administration of N-acetylcysteine (NAC) may improve several outcomes in patients with neuropsychiatric disorders such as anxiety and depression [1–5]. The positive effects observed in these conditions are probably related to actions on oxidative status and the glutamatergic system [6], since NAC is a glutathione (GSH) precursor and modulates glutamatergic transmission [7]. In addition to clinical studies, preclinical findings have also demonstrated beneficial effects of NAC in different animal models, as evidenced by modulation of behavioral, biochemical, and molecular parameters involved in psychiatric and neurological conditions (e.g., anxiety, depression, Parkinson's and Alzheimer's disease, schizophrenia, alcoholism, drug addiction, gambling) [3, 6, 8–14].

Our group has previously reported that NAC induces anxiolytic-like effects in both mice and zebrafish in different protocols; specifically regarding stress-induced outcomes, NAC was able to prevent the deleterious effects observed in zebrafish submitted to an acute stress protocol [9, 10, 13]. Other studies have shown beneficial and

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protective effects of NAC against the imbalance on inflammatory (interleukin-1beta (IL-1b), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-a)) and oxidative stress markers (lipid peroxidation (TBARS), superoxide dismutase (SOD), catalase (CAT), and gluthatione peroxidase (GPx) activities), besides its neurotrophic actions (increase of brain-derived neurotrophic factor (BDNF)) [5, 11, 15, 16].

Chronic stress is a powerful trigger to the development of neuropsychiatric disorders and is particularly relevant in the context of modern life [17]. Its neurobiology is heterogeneous and includes dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and neurotransmitter systems (glutamatergic, noradrenergic, dopaminergic, serotoninergic, and GABAergic), decrease in GSH levels, imbalance in oxidative status parameters, activation of neuroinflammatory and apoptosis pathways, and behavioral changes in response to adverse situations [17–25].

As demonstrated by our laboratory and others, unpredictable chronic stress (UCS) protocols increased anxiety and reactive oxygen species (ROS) levels in zebrafish, besides increasing inflammatory cytokines (IL-1b, IL-6, and IL-10) and neurotrophic factor (BDNF) [26, 27]. Considering the multi-target effects of NAC in many systems related to stress and the deleterious effects of UCS, the aim of this study was to evaluate the potential of this drug in reversing the effects of UCS on anxiety-like behavior and oxidative markers in zebrafish.

Materials and Methods

Animals

A total of 44 adult (4–6-month-old) zebrafish (*Danio rerio*) of short-fin wild-type (WT) phenotype of both sexes (50:50 male:female ratio) was 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 2 weeks prior to testing in 16-L tanks (40 × 20 × 24 cm) filled with non-chlorinated water kept under constant mechanical, biological, and chemical filtration at 26 ± 2 °C. Fish were kept on a 14–10-h day/night cycle (lights on at 07:00 a.m.) and fed three times a day with brine shrimp (*Artemia salina*) and commercial flake fish food (Alcon BASIC®, Brazil). More detailed information on housing conditions is described in our previous study [8]. All protocols were approved by the Ethics Committee of Federal University of Rio Grande do Sul (process number #30914).

Drug

N-acetylcysteine (NAC, CAS number 616-91-1) was acquired from Sigma-Aldrich (St Louis, Missouri, USA). NAC concentration (1.0 mg/L) was based on a previous study published by our group [9].

Experimental Design and Procedures

Initially, fish were divided into control (non-stressed group, S−) and UCS (stressed group, S+). After 7 days, the experimental groups were subdivided again into control and NAC (1.0 mg/L). The animals were daily transferred at 08:00 a.m. to 5-L tanks containing fresh water (control groups) or NAC (treated groups) for 10 min and were gently returned to the housing tanks, resulting in 14 days of UCS and 7 days of treatment. The experimental design is summarized in Fig. 1.

The UCS protocol followed our previous studies [28–30]. Stressors were presented randomly twice a day for a total of 14 days to avoid habituation. The stressors included chasing with a net (8 min); low water level on housing tanks until dorsal body wall was exposed (2 min); crowding in a 250-mL beaker (50 min); cooling tank water to 23 °C (30 min); heating tank water up to 33 °C (30 min); tank change, three consecutive times with 30 min interval. All stressors were applied between 08:30 a.m. and 17:00 p.m. The non-stressed group was left undisturbed throughout the experiments. Aeration and temperature were controlled during each stressor presentation (except during heating and cooling stress). A white frosted cardboard (30 × 60 cm) was placed in between tanks to prevent visual contact of fish from different tanks in the same horizontal plane. Two identical tanks were ran in parallel for each experimental group; no tank effects were observed in the analysis of results, so data from tanks of the same experimental group were pooled together.

Behavioral Analyses

The behavioral test was performed on the 15th day. The animals were individually transferred to the novel tank test (NTT) and video recorded for 6 min. The videos were later analyzed by the ANY-Maze™ software (Stoelting Co., USA). Tests were performed between 08:00 a.m. and 01:00 p.m. The NTT apparatus and the parameters studied are described in detail in our previous studies [8, 9, 28].

Tissue Preparation

For the biochemical analyses, a proportion of two brains to 300 μL of phosphate-buffered saline (PBS, pH = 7.4, Sigma-Aldrich®) was used. Immediately after the behavioral test, fish were anesthetized by rapid cooling (immersion in water at 2–4 °C until unable to swim and cessation of opercular

Fig. 1 Outline of the experimental design. Unpredictable chronic stress (UCS); *N*-acetylcysteine (NAC)
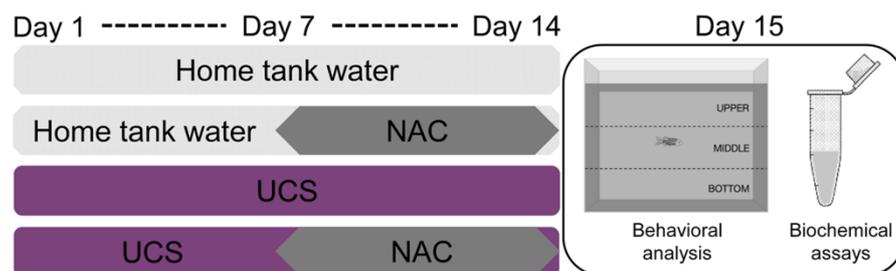
movement). Each animal was then euthanized by decapitation for brain tissue sampling. The brains were dissected and gently homogenized. The homogenate was centrifuged at $10,000 \times g$ for 10 min at 4 °C in a cooling centrifuge, and the supernatant packed in microtubes was used for assays. We quantified the following parameters: reactive oxygen species (ROS) levels (dichlorofluorescein—DCF), lipid peroxidation (thiobarbituric acid reactive species—TBARS), non-protein thiols (NPSH), and antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)). All biochemical measures were performed in duplicate. Details of each procedure are described in previous studies [8, 31].

Statistics

Data were analyzed after confirmation of normality and homogeneity of variances using D'Agostino-Person and Levene tests, respectively. Two-way ANOVAs were carried out to identify the main effects of stress and treatment, as well as their interactions. Bonferroni post hoc test was performed when significant interactions were obtained. Data are expressed as a mean + standard error of the mean (S.E.M). The level of significance was set at $p < 0.05$.

Results

Figure 2 shows the influence of NAC on behavioral parameters in zebrafish submitted to UCS. As expected, UCS increased the time spent in the bottom area (Fig. 2d) and decreased the entries and time in the top area (Fig. 2e, f, respectively). Two-way ANOVA revealed a main effect of treatment ($F_{1, 41} = 9.85$, $p = 0.0031$) and an interaction effect ($F_{1, 41} = 15.65$, $p = 0.0003$) for time in bottom area (Fig. 2d), as well as interaction effects for entries in top area (Fig. 2e, $F_{1, 41} = 6.01$, $p = 0.0185$) and time in the top area (Fig. 2f, $F_{1, 41} = 10.66$, $p = 0.0022$). NAC reversed the UCS-induced decrease in top swimming and the complementary increase in bottom dwelling. The distance traveled (Fig. 2a), the number of crossings (Fig. 2b) and entries to the bottom area (Fig. 2c) was not affected by any intervention.

The effects of the UCS on oxidative status are presented in Figs. 3 and 4. As shown in Fig. 3a, b, respectively, UCS promoted an increase in ROS levels and lipid peroxidation.

Two-way ANOVA revealed an interaction between the factors ($F_{1, 18} = 21.49$, $p = 0.0002$) for ROS levels (DCF assay), and main effects of stress ($F_{1, 18} = 15.32$, $p = 0.0010$), treatment ($F_{1, 18} = 5.33$, $p = 0.0330$), and interaction ($F_{1, 18} = 12.71$, $p = 0.0022$) for lipid peroxidation (TBARS assay). NAC was able to reverse the increase on ROS levels and lipid peroxidation in zebrafish submitted to UCS, while it did not alter such parameters in non-stressed animals.

Regarding SOD activity (Fig. 4a), two-way ANOVA revealed an interaction between treatment and stress ($F_{1, 18} = 7.95$, $p = 0.0113$); UCS decreased SOD activity, while NAC blocked this effect in stressed fish, without affecting non-stressed animals. For NPSH (Fig. 4c), the two-way ANOVA revealed main effects of stress ($F_{1, 18} = 5.19$, $p = 0.0351$), treatment ($F_{1, 18} = 8.94$, $p = 0.0078$), and interaction ($F_{1, 18} = 7.45$, $p = 0.0137$); UCS decreased NPSH levels, and NAC again protected against this UCS-induced reduction in antioxidant defenses, without effects in non-stressed animals. No effects of stress or treatment were observed for CAT activity (Fig. 4b).

Discussion

Here, we provide the first evidence that *N*-acetylcysteine (NAC) reverses the behavioral alterations and oxidative stress induced by an unpredictable chronic stress (UCS) protocol in zebrafish. Fish submitted to 14 days of stress presented behavioral changes in the number of entries and time spent in the top area in the novel tank test (NTT); moreover, the protocol increased ROS levels (DCF) and lipid peroxidation (TBARS), while decreased non-protein thiols (NPSH) and superoxide dismutase (SOD) activity, without affecting catalase (CAT). All these effects were reversed by treatment with NAC in the last 7 days of UCS. This suggests that the behavioral changes induced by the UCS protocol are accompanied by oxidative damage in the zebrafish brain. Although a causal link cannot be established, it is possible that oxidative stress is somehow involved in the behavioral alterations induced by stress, since treatment with the antioxidant NAC reversed such changes.

We opted to submit zebrafish to UCS for 7 days before starting the treatment with NAC to recapitulate a more realistic scenario considering the potential use of NAC in patients with

Fig. 2 Effects of NAC treatment against UCS-induced changes on behavioral parameters in zebrafish. **a** Distance traveled. **b** Crossings. **c** Entries in the bottom area. **d** Time in the bottom area. **e** Entries in the top area. **f** Time in the top area. Non-stressed group (S−); stressed group (S+). Data are expressed as mean + S.E.M. Two-way ANOVA followed by Bonferroni's test. $n = 10$–12. *$p <$ 0.05 vs. control (S−)
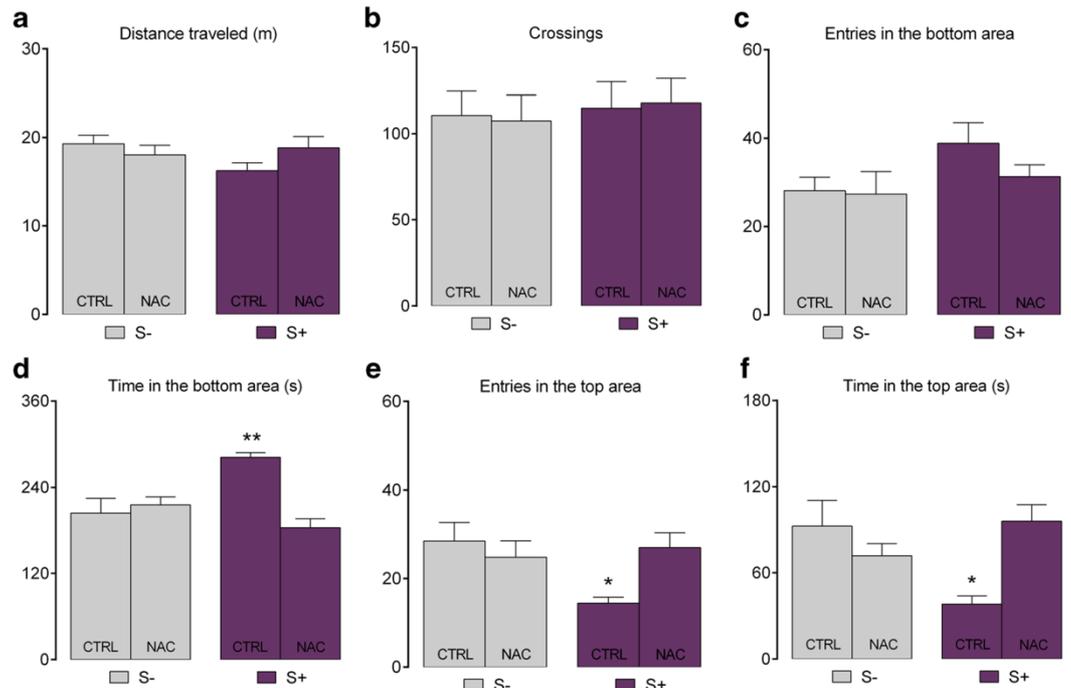
mental disorders related to stress. In the NTT, total distance traveled and crossings were used as an indicator of locomotor activity. The increase in time spent in the bottom area and decrease in time spent in the top area is used as a proxy for anxiety behavior [32], which corresponds in rodents to thigmotaxis in the open-field test [33, 34]. As expected, our protocol increased anxiety-like behavior, as evidenced by the increased time spent in the bottom area and decreased entries and time in the upper zone of the tank, in agreement with previous works from our group [27, 28]. NAC did not affect locomotor activity and was devoid of effect in non-stressed animals. Similar effects in the novel tank test were observed for different anxiolytics such as bromazepam, diazepam, buspirone, and fluoxetine [9, 28, 35].

As all animals were daily transferred to control or NAC-containing tanks during the treatment exposure period, all groups were habituated to handling and to the presence of a net. On the testing day, the handling was performed carefully in the same manner as previous days. This circumvented the possibility of the UCS group being less stressed on the testing day if compared to a never handled control group. Furthermore, stressors were applied twice a day in a randomized unpredictable fashion, precisely to avoid habituation in stressed groups.

Our group has recently shown that UCS for 7 days is able to increase the production of ROS in zebrafish brain [27]. Here, we show for the first time the effects of UCS for 14 days on oxidative status parameters in zebrafish. We observed that ROS levels and TBARS significantly increased after UCS, an indication of lipid peroxidation that corroborates studies in other animal models [36, 37]. We also detected a decrease in NPSH levels, probably as a result of reduction in the amount of GSH [38]. NAC, as a GSH precursor, blocked the decrease in NPSH levels induced by UCS. Our results are in agreement

Fig. 3 Effects of NAC treatment against UCS-induced changes in levels of reactive oxygen species (**a**) and lipid peroxidation (**b**) in whole-brain zebrafish. Non-stressed group (S−); stressed group (S+). Data are expressed as mean + S.E.M. Two-way ANOVA followed by Bonferroni's test. $n = 5$–6. **$p <$ 0.01; ***$p <$ 0.001 vs. control (S −)
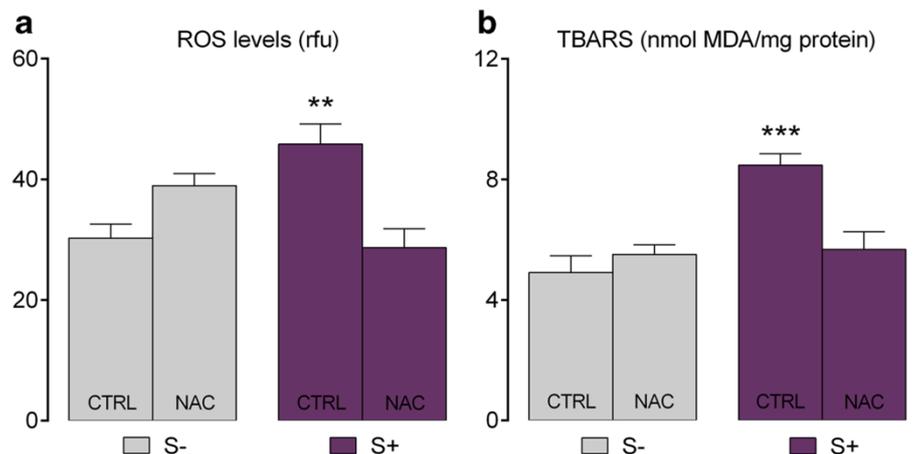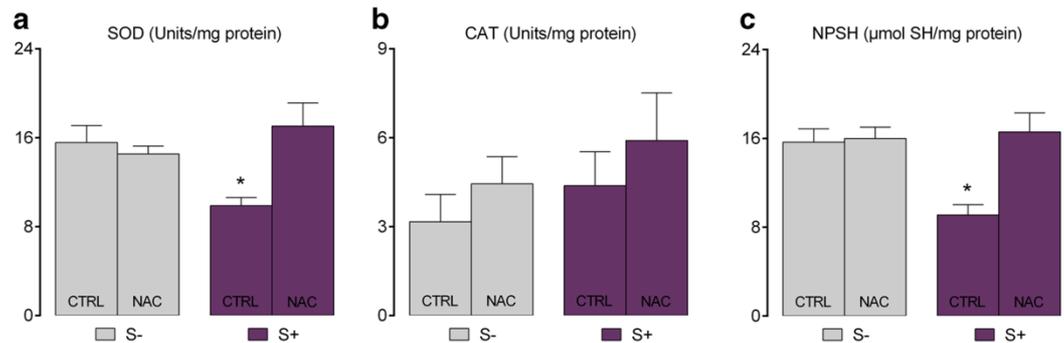
Fig. 4 Effects of NAC treatment against UCS-induced changes on oxidative status in zebrafish whole-brain. **a** SOD activity. **b** CAT activity. **c** Non-protein thiols. Non-stressed group (S−); stressed group (S+). Data are expressed as mean + S.E.M. Two-way ANOVA followed by Bonferroni's test. $n = 5$–6. *$p < 0.05$ vs. control (S−)

with rodent studies, in which chronic stress impaired antioxidant defenses and resulted in lipid peroxidation and damage to the brain [39–41].

One of the defense lines against the excessive production of ROS and consequent brain damage is the increased activity of antioxidant enzymes, for instance, SOD and CAT [42]. SOD catalyzes the reaction of superoxide anion radical ($O_2^{\bullet-}$) dismutation into hydrogen (H_2) and hydrogen peroxide (H_2O_2), while the reduction of H_2O_2 to O_2 and H_2O is catalyzed by CAT or glutathione peroxidase (GPx) [21, 43]. UCS significantly decreased SOD, but not CAT activity in zebrafish. This decrease in enzymatic antioxidant defense induced by UCS indicates a possible maladaptive response to the long-term effects of stressful conditions, that is, the organism was not able to properly cope with the increased ROS levels and prevent oxidative damage [44]. Nevertheless, the oxidative damage induced by UCS was reversed by NAC, which blocked SOD activity decrease in stressed fish (Fig. 5).

Manganese superoxide dismutase (MnSOD) is the most important specific SOD enzyme present in the inner membrane and matrix of mitochondria [45, 46]. Chronic stress increases peroxynitrite levels, thus causing inhibition of MnSOD enzyme through nitration of its tyrosine residues [47–50]. Overexpression of MnSOD, however, is able to avoid stress-induced lipid peroxidation and neuronal death in rats [51]. NAC is an indirect antioxidant and increases SOD activity by increasing MnSOD expression [52]. The

increased levels of MnSOD associated with NAC may reflect augmented de novo synthesis or decreased elimination rates due to lower oxidation levels [53]. Studies report that MnSOD mRNA is regulated at different transcriptional levels, and while NAC may potentially modulate its expression, the exact molecular mechanisms involved are still unknown [54–57]. We should also consider the possibility that peroxynitrite scavenging by NAC may underlie its ability to preserve SOD activity in stressed animals.

A limitation of our study is that we cannot assign the benefits of NAC exclusively to its antioxidant properties since it also modulates glutamatergic transmission and other relevant pathways [3, 5]. Chronic stress causes glutamate release and leads to maladaptive synaptic changes, including reduced extracellular glutamate clearance by glial cells, increased activation of extrasynaptic N-methyl-D-aspartate receptors and excitotoxicity, which may result in neuronal death [23]. Recent studies evidence the potential therapeutic usefulness of mGlu2/3 ligands and mGlu5 receptor antagonists in stress-related disorders [58]. NAC regulates intra and extracellular glutamate [59] by increasing the activity of the cystine-glutamate antiporter expressed by astrocytes, which inhibits glutamate release and excitotoxicity through activation of mGluR2/3 receptors [1, 3, 5].

Considering our observations of the effects of NAC in reversing the deleterious effects caused by UCS, we reinforce the idea that NAC would be a beneficial and promising agent
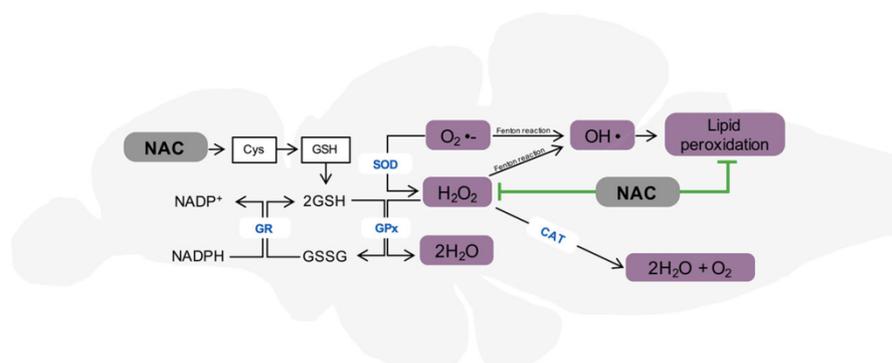
Fig. 5 Schematic representation of the mechanism of NAC on UCS-induced oxidative damage in the zebrafish brain. The illustration depicts an involvement of NPSH and SOD in mediating UCS responses and the effects of NAC on TBARS and SOD. NAC is a precursor of GSH, which is the main non-protein thiol. N-acetylcysteine (NAC); lipid peroxidation (TBARS); non-protein thiols (NPSH); superoxide dismutase (SOD); catalase (CAT); cysteine (Cys); glutathione (GSH); glutathione peroxidase (GPx); glutathione reductase (GR)

for the treatment of stress and associated psychiatric disorders. Additional studies are therefore needed, but the data available thus far are encouraging for the clinical trials needed to further assess the efficacy of NAC in neuropsychiatric conditions.

Compliance with Ethical Standards

All protocols were approved by the Ethics Committee of Federal University of Rio Grande do Sul (process number #30914).

Conflict of Interest The authors declare that they have no conflict of interest.

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3.2. CAPÍTULO II: Esse capítulo mostra os dados referentes ao segundo objetivo específico (efeitos da NAC sobre parâmetros comportamentais e de estresse oxidativo em peixes-zebra submetidos ao modelo de exposição aguda ao etanol).

Artigo:

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ORIGINAL PAPER

Behavioral and Biochemical Effects of *N*-Acetylcysteine in Zebrafish Acutely Exposed to Ethanol

Ricieri Mocelin[1] · Matheus Marcon[1] · Simone D'ambros[1] · Ana P. Herrmann[2] · Alex Sander da Rosa Araujo[3] · Angelo Piato[1,4]

Abstract

Alcohol hangover refers to unpleasant symptoms experienced as a direct consequence of a binge drinking episode. The effects observed in this condition are related to the increase in alcohol metabolites and imbalance in oxidative status. *N*-acetylcysteine (NAC) is a mucolytic agent and an antidote for paracetamol overdose. Preclinical and clinical studies have shown that NAC is a multi-target drug acting through neuroprotective, antioxidant and neurotrophic mechanisms as well as a glutamate modulator. The aim of this study was to investigate the effects of NAC in zebrafish acutely exposed to ethanol (EtOH). Animals pretreated or not with NAC (1 mg/L, 10 min) were exposed for 60 min to standard tank water (EtOH−) or to 1% EtOH (EtOH+) to evaluate anxiety-like behavior and locomotion in the novel tank test and oxidative damage in the brain. Zebrafish (*Danio rerio*) exposed to EtOH displayed a decrease in the distance traveled, crossings, entries and time spent in the top area in the novel tank test. Exposure to EtOH also caused oxidative damage, shown by increased lipid peroxidation, decreased non-protein thiols and increased production of reactive oxygen species (DCF assay). NAC prevented both the behavioral alterations and the oxidative stress observed in EtOH+ animals. Given the effects of NAC in preventing the acute behavioral and biochemical effects of EtOH, additional studies are warranted to further investigate the basis of its anecdotal use to prevent hangover.

Keywords Alcohol abuse · Novel tank test · Oxidative stress · Hangover

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Introduction

Acute ingestion of alcohol is associated with undesired consequences, including the unpleasant experience of hangover [1]. The mechanisms underlying hangover symptoms, which include apathy and cognitive deficits, seem to involve the metabolism of alcohol [2–5]. The consumption of alcohol generates a major and highly toxic metabolite, acetaldehyde, which reacts with proteins, phospholipids, and nucleic acids, actively participating in the production of reactive oxygen species (ROS) [6]. The excessive generation of ROS such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) may exceed the cellular antioxidant capacity, causing oxidative damage [7, 8]. The increase in acetaldehyde concentrations and imbalance to the cellular redox homeostasis has been suggested to cause the unpleasant effects of acute alcohol consumption, such as fatigue, headache, increased sensitivity to light and sound, dizziness, vertigo, and mood changes [9, 10].

N-acetylcysteine (NAC) is a glutathione (GSH) precursor with potent antioxidant properties, which also presents anti-inflammatory (decreasing proinflammatory cytokines interleukin-1beta (IL-1b), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-a)) and neurotrophic (increasing brain-derived neurotrophic factor) activities, and modulates different neurotransmitter systems, especially glutamate [11, 12]. Preclinical and clinical evidence show that NAC modulates pathophysiological processes related to different psychiatric disorders, including drug addiction, gambling and hoarding [11, 13–18]. Specifically regarding alcohol abuse, studies have shown beneficial effects of NAC on behavior (preventing the decrease in exploratory behavior), pro-inflammatory cytokines (preventing the increase in interleukin-18, IL-1b, IL-6 and TNF-a), and oxidative stress (reducing total oxidant status) [17–20].

Despite the anecdotal use of NAC [21] for hangover, there are no studies investigating the effects of NAC on acute models of alcohol exposure. There are preclinical and clinical indications for a variety of novel roles for NAC, however further studies are required to investigate whether it has any effect in this context. Considering its multi-target properties, the aim of this study was to evaluate the preventive effects of NAC in behavioral parameters and oxidative stress in a model of acute alcohol exposure in zebrafish.

Materials and Methods

Animals

36 Adult (4–6 month-old) zebrafish (*Danio rerio*) of short fin wild-type (WT) phenotype of both sexes (50:50 male: female ratio) were obtained from a local specialized commercial supplier (Delphis, RS, Brazil). Fish were housed and acclimatized in 40-L tanks prior to testing for 14 days (80–100 fish per tank), filled with non-chlorinated water kept under constant mechanical, biological and chemical filtration at 26 ± 2 °C. Fish were fed three times a day with brine shrimp (*Artemia salina*) and commercial flake fish food (Alcon BASIC®, Alcon, Brazil). More detailed information on housing conditions is described in our previous study [15]. All protocols were approved by the Ethics Committee of Federal University of Rio Grande do Sul (process number #30914).

Drugs and Experimental Design

Ethanol (EtOH, C_2H_6O, CAS number 64-17-5) and *N*-acetylcysteine (NAC, CAS number 616-91-1) were acquired from Merck (Darmstadt, Germany) and Sigma-Aldrich (St Louis, Missouri, USA), respectively. EtOH protocol and NAC concentration were based and adapted from previous studies [15, 22]. Initially, fish were individually pretreated in a beaker with home tank water or NAC 1 mg/L for 10 min. Then the animals were gently transferred to another beaker and exposed to the home tank water or 1% EtOH (v/v) for 60 min (Fig. 1). The experimental groups used were Control (water and water), EtOH (water and EtOH 1%), NAC (NAC 1 mg/L and water), and NAC-pretreated group (NAC 1 mg/L and EtOH 1%).

Novel Tank Test

The behavioral test was performed immediately after treatments. The animals were individually transferred to the apparatus and video recorded for 6 min. The videos were later analyzed by the ANY-maze™ software (Stoelting Co., USA). Tests were performed between 09:00 am and 14:00 pm. The novel tank apparatus consists of a 2.7-L tank ($24 \times 8 \times 20$ cm; width × depth × height), virtually divided into three equal horizontal zones and filled with standard tank water up to 15 cm as previously reported [15]. The following parameters were analyzed to evaluate exploratory behavior and locomotion: total distance traveled (m), number of crossings (transitions between zones of the tank), and number of entries and time spent in the upper (s) zone (increased by anxiolytic and decreased by anxiogenic interventions).

Tissue Preparation

Immediately after the behavioral test, fish were cryoanesthetized and euthanized by decapitation. The brains were

Fig. 1 Schematic representation of the methodological approach used for evaluation of behavioral and biochemical effects of NAC in acute EtOH model in zebrafish
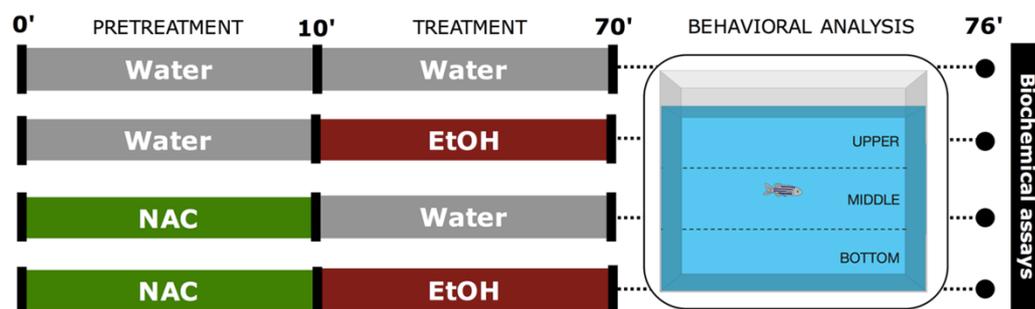
dissected and gently homogenized in a proportion of 1 brain to 150 μL of phosphate buffered saline (PBS, pH 7.4, Sigma-Aldrich®). The homogenate was centrifuged at 10,000×g for 10 min at 4 °C in a cooling centrifuge, and the supernatant packed in microtubes was used for assays.

Thiobarbituric Acid Reactive Species (TBARS)

Lipid peroxidation was determined by quantifying TBARS production [23]. Briefly, 50 μL of the sample (80–100 μg protein) were mixed with 75 μL of 15% trichloroacetic acid (TCA, Sigma-Aldrich®) and centrifuged (6000 rpm, 5 min). Supernatants were added to 75 μL of 0.67% thiobarbituric acid (TBA, Sigma-Aldrich®), then homogenized in a vortex for 5 s and heated at 100 °C for 30 min. TBARS levels were measured through absorbance (532 nm) in a microplate reader, using malondialdehyde (MDA, Sigma-Aldrich®) as standard. Results were expressed as nmol MDA/mg protein.

Quantification of Non-protein Thiols

Levels of non-protein thiols were determined and measured at 412 nm in a microplate reader. Briefly, 40 μL of the sample (60–80 μg protein) was mixed with equal volume of 10% trichloroacetic acid and centrifuged (6.000 rpm, 5 min). Supernatants were added to 10 mM DTNB (5,5-dithio-bis-2-nitrobenzoic acid, Sigma-Aldrich®) dissolved in ethanol, developing yellow color after 60 min [24]. Results were expressed as μmol SH/mg protein.

Reactive Oxygen Species (ROS) Assays

ROS levels were measured using the fluorescent dye 2′7′-dichlorofluorescein-diacetate (DCFH-DA) [25, 26]. One aliquot of homogenate (25 μL) was mixed with 0.1 mM 2′7′-dichlorofluorescein-diacetate (DCFH-DA, Sigma-Aldrich®) and PBS buffer. The product of this reaction is oxidized by ROS present in samples yielding fluorescence. ROS levels were determined by fluorescence at emission (520 nm) and excitation (480 nm). Results were represented by relative fluorescence units.

Protein Determination

Protein was determined by Coomassie blue method using bovine serum albumin (Sigma-Aldrich®) as standard. The absorbance of samples was measured at 595 nm [27].

Statistics

Data were analyzed after normality and homogeneity of variance (D'Agostino-Person and Levene tests, respectively) confirmation using two-way ANOVA to identify the main behavioral and biochemical effects of pretreatment (NAC exposure or not) and treatment (EtOH exposure or not) and their interactions followed by Bonferroni post hoc test. Data are expressed as mean $\pm$ standard error of the mean (S.E.M.). The level of significance was set at $p < 0.05$. Correlation analysis between TBARS and ROS levels (DCF) were assessed using Pearson's correlation analysis.

Results

Figure 2 shows the influence of NAC and EtOH on distance traveled, number of crossings, entries and time in the top area in the novel tank test. Two-way ANOVA revealed a main effect of treatment ($F_{1,32} = 9.55$, $p = 0.0041$) and an interaction effect ($F_{1,32} = 4.33$, $p = 0.0455$) for distance traveled; post hoc analysis indicated that pretreatment with NAC prevented the effect of EtOH exposure in distance traveled ($p < 0.05$, Fig. 2a). Two-way ANOVA revealed a main effect of treatment ($F_{1,32} = 16.86$, $p = 0.0003$) as well as an interaction effect ($F_{1,32} = 6.05$, $p = 0.0195$) for a number of crossings; the post hoc test showed that EtOH decreased the number of crossings ($p < 0.001$) and NAC prevented this effect ($p < 0.05$, Fig. 2b). Two-way ANOVA revealed a main effect of treatment ($F_{1,32} = 47.34$, $p < 0.0001$) and an interaction effect ($F_{1,32} = 11.6$, $p = 0.0018$) for entries in top area; since post hoc comparisons showed that the NAC-EtOH + group significantly ($p < 0.01$) differed from CTRL-EtOH-group, pretreatment with NAC attenuated the effects of EtOH in this parameter (Fig. 2c). Two-way ANOVA revealed pretreatment ($F_{1,32} = 4.99$, $p = 0.0326$), treatment ($F_{1,32} = 7.15$, $p = 0.0117$), and interaction effects ($F_{1,32} = 4.68$, $p = 0.0382$) for the time in the top area; post hoc analysis indicated that pretreatment with NAC prevented the effects of EtOH in this parameter ($p < 0.05$, Fig. 2d).

Biochemical parameters associated with oxidative stress and levels of ROS are shown in Fig. 3. Two-way ANOVA revealed pretreatment ($F_{1,31} = 4.76$, $p = 0.0368$) and an interaction effect ($F_{1,31} = 5.59$, $P = 0.0245$) for lipid peroxidation; post hoc analysis indicated that pretreatment with NAC prevented the effects of EtOH ($p < 0.05$, Fig. 3a). Two-way ANOVA revealed treatment ($F_{1,31} = 9.59$, $p = 0.0041$) and an interaction effect ($F_{1,31} = 7.91$, $p = 0.0084$) for non-protein thiols; post hoc analysis indicated that pretreatment with NAC prevented the effects of EtOH ($p < 0.05$, Fig. 3b). Two-way ANOVA revealed a significant main effect of the pretreatment ($F_{1,31} = 13.24$, $p = 0.0010$) on ROS levels; NAC prevented the effect of EtOH on this parameter (Fig. 3c).

Figure 4 shows the Pearson's correlations coefficient (r) between ROS levels (DCF) and TBARS. Analysis showed a positive ($r = 0.3376$) and significant ($p = 0.0473$) correlation between variables; the higher the ROS levels, the higher the lipid peroxidation levels.
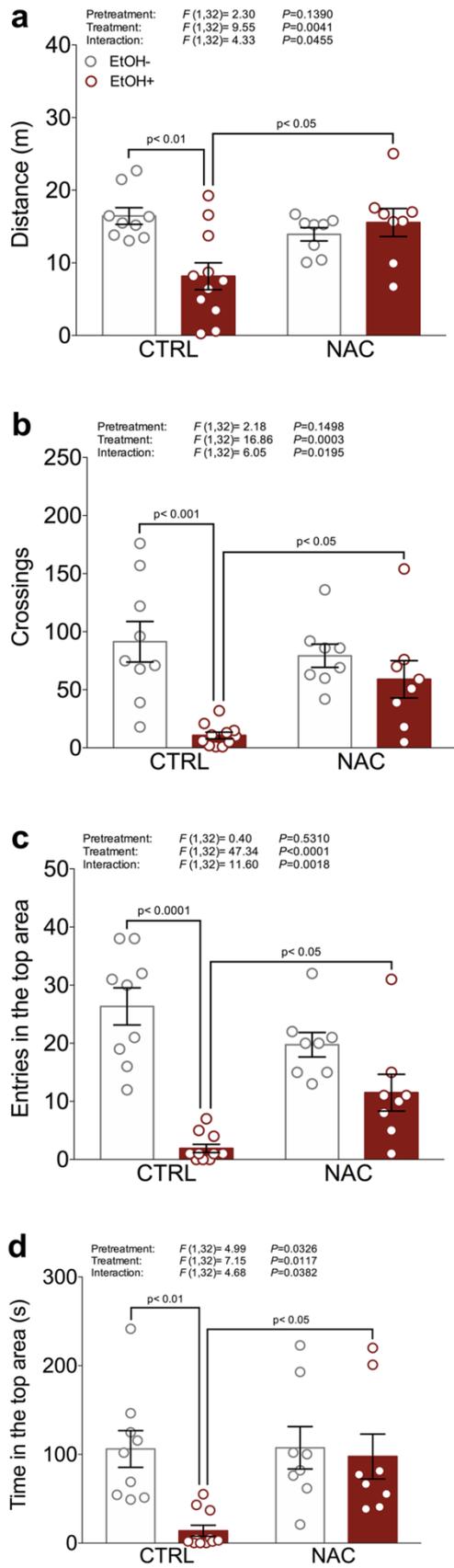
Fig. 2 Effects of NAC pretreatment against EtOH-induced changes on behavioral parameters in zebrafish. **a** Distance traveled, **b** crossings, **c** entries in the top area and **d** time in the top area. Data were expressed as mean ± S.E.M. Two-way ANOVA followed by Bonferroni's test. n = 8–11
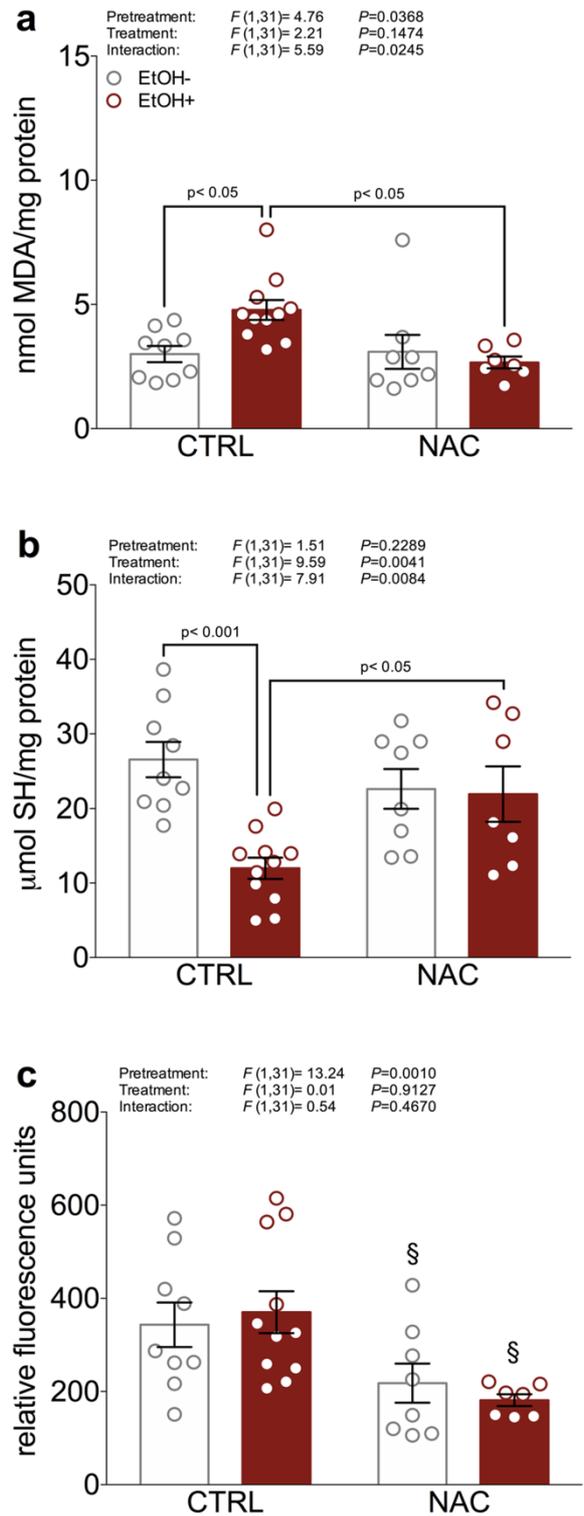
Fig. 3 Effects of NAC pretreatment against EtOH-induced changes on oxidative stress in whole-brain zebrafish. **a** Thiobarbituric acid reactive species, **b** non-protein thiols, and **c** levels of reactive oxygen species. Data were expressed as mean ± S.E.M. Two-way ANOVA followed by Bonferroni's test. n = 7–11. [§] represents the main effect of NAC
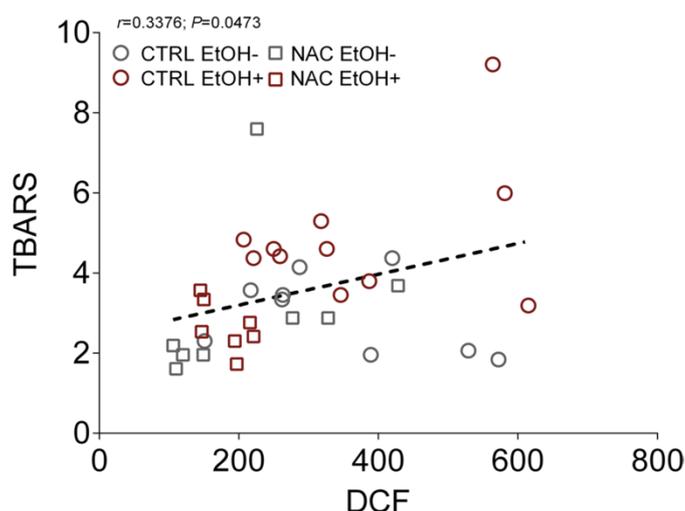
Fig. 4 Pearson's correlation analysis between TBARS and DCF

Discussion

In this study, we demonstrated that NAC prevented the effects of acute EtOH exposure on behavioral and oxidative stress parameters in zebrafish. EtOH decreased distance traveled, number of crossings, entries and time in the top area in the novel tank test; furthermore, it increased lipid peroxidation (TBARS) and decreased non-protein thiols, without affecting ROS levels (DCF assay). All these changes were prevented by pretreatment with NAC. This suggests that the behavioral changes induced by EtOH are accompanied by oxidative damage in the CNS. Since pretreatment with an antioxidant prevented such changes, oxidative stress may underlie the behavioral observations. Although speculative, the results observed indicate that NAC may indeed have a potential in preventing the symptoms of acute ethanol ingestion and possibly decrease hangover effects.

Regarding behavioral results, a previous study has shown that exposure to 1% EtOH for 60 min induces a depressor effect with reduced vertical exploration, decreased distance traveled, crossings and time in the top area in the novel tank [22]. Others studies corroborate with the data presented here [28, 29]. The effects of ROS in some areas of the brain related to behavioral control (e.g. amygdala, cortex, hippocampus) could contribute to the effects observed in the novel tank test after acute EtOH exposure.

The oxidation of EtOH to acetaldehyde is associated with generation of ROS. In the brain, EtOH is mainly metabolized by pathways that involve catalase, cytochrome P450 enzymes and alcohol dehydrogenase, which may induce oxidative damage in neurons and glial cells [6, 30, 31]. Nevertheless, in the DCF assay, we did not observe a significant difference between control and EtOH exposure animals. On the other hand, NAC was able to decrease ROS levels in zebrafish exposed or not to alcohol. One possible explanation for these

data is that the novel environment and the EtOH exposure procedure can generate ROS, since the manipulation of the animal, even if careful, and the removal of group-housed fish for individual treatment produces stress [32, 33]. In addition, it is also thought that the reactive oxygen species are very unstable and may be used up rapidly; therefore, the detection of reactive species concentration and its alterations may become sometimes difficult. However, it is important to also consider other markers, such as lipid peroxidation, in order to understand the key role of oxidative stress on cellular function.

We show that TBARS levels significantly increased after EtOH exposure, indicating lipid peroxidation, one important cause of neuronal damage [34]. Our study also shows that pretreatment with NAC prevented the increase in TBARS levels. The positive and significant correlation between ROS levels and TBARS suggest that lipid peroxidation accompanies the increase in ROS production, but a causal relationship cannot be established. The protection against ROS generation provided by NAC may be related to neutralization of $O_2{}^{\cdot-}$ and $\cdot OH$ by non-protein thiols, since NAC is a precursor of glutathione, an important non-enzymatic antioxidant that maintains redox homeostasis [35].

In our study, we observed a decrease in non-protein thiols levels induced by acute exposure to EtOH. However, NAC increased the amount of non-protein thiols, probably by elevating the levels of reduced form GSH, since NAC acts as its precursor and consequentially increases its production, preventing the deleterious effects of EtOH [36]. The imbalance between oxidants and antioxidant defenses results in decreased intracellular of GSH/GSSG (oxidized form) ration, leading to an increase in mitochondrial permeability and activating caspases and subsequent cell death [37, 38]. Moreover, some studies have reported that acute EtOH administration leads to glutathione depletion in the liver and other tissues [39, 40]. In an alcohol hangover model in mice, glutathione was decreased by 43 and 17% in synaptosomes and cytosol, respectively [9]. However, administration of glutathione or its precursors prevented its depletion by EtOH, decreasing oxidative toxicity [41, 42], as observed in our study.

The increase in glutathione levels by NAC improves the antioxidant defenses and is probably involved in the protection against lipid peroxidation and ROS formation. The association of these factors could be related to the preventive effects of NAC. These data are consistent with previous studies showing that acute exposure to EtOH altered behavior and antioxidant defenses, and compounds with antioxidant properties and glutamatergic signaling modulators, such as taurine, for example, were able to prevent these effects [22, 43].

EtOH blocks NMDA receptors and potentiates GABAergic neurotransmission by increasing the presynaptic release of GABA. During EtOH withdrawal, however, GABA decreases [44, 45] and glutamate activity increases [44, 45].

Such neurochemical alterations have also been linked to neuroinflammation, with increases in proinflammatory cytokines reported in humans, rodents and zebrafish [7, 18, 46]. NAC is able to reduce neuroinflammation through inhibition of cytokine production by macrophages and activated microglia [14, 47]. Through its action on the cystine-glutamate antiporter, NAC can also modulate several neurotransmitter systems such as glutamate and dopamine; however, its role on GABAergic transmission is less well studied. Even though we did not access inflammatory or neurotransmitter markers, this is an interesting line of investigation worth pursuing in future studies.

A limitation of our study is that we cannot attribute the benefits of NAC exclusively to its antioxidant properties. NAC also modulates neurotransmitter release by indirectly activating mGluR2/3 receptors due to increased activity of the cystine-glutamate antiporter expressed by astrocytes [14]. It has been previously reported that acute EtOH exposure also results in neurotransmitter dysfunctions (i.e., increased inhibitory neurotransmitter GABA; decreased excitatory neurotransmitter glutamate) [48, 49], leading to an imbalance between inhibitory and excitatory neurotransmitters. Therefore, neurotransmitter modulation could also be a mechanism involved in the protective effects of NAC on the behavioral alterations induced by EtOH.

The present study highlights for the first time that NAC can play a protective role in the behavioral and biochemical functions impaired by EtOH in zebrafish. NAC can prevent changes in different behavioral paradigms and increase antioxidant defenses. Given the preventive effects of NAC on behavioral and oxidative stress responses to EtOH, additional studies could investigate the effects and mechanisms of NAC in EtOH-induced hangover and abstinence responses.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Ethical Approval All protocols performed in this study were in accordance with the Ethics Committee of Federal University of Rio Grande do Sul (process number #30914) and followed national and international guidelines for the care and use of laboratory animals.

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3.3. CAPÍTULO III: Esse capítulo mostra os dados referentes ao terceiro objetivo específico (efeitos da NAC sobre parâmetros comportamentais e de estresse oxidativo em peixes-zebra submetidos ao modelo de abstinência ao etanol).
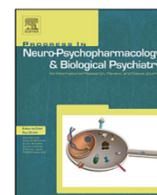
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Withdrawal effects following repeated ethanol exposure are prevented by *N*-acetylcysteine in zebrafish

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ABSTRACT

Alcohol abuse is a highly prevalent condition that substantially contributes to global morbidity and mortality. Most available pharmacological treatments offer little efficacy as relapse rates are high, due in part to the symptoms experienced during abstinence. The roles of oxidative stress and glutamatergic transmission in alcohol withdrawal have been demonstrated in several studies, suggesting that restoration of oxidative status and glutamatergic function may represent a new pharmacological target to prevent the behavioral and biochemical alterations observed during withdrawal. A well-known antioxidant and glutamatergic modulator, *N*-acetylcysteine (NAC), has shown promise in treating a variety of psychiatric conditions, including substance use disorders, and is a promising molecule in the management of alcohol withdrawal syndrome. Thus, the aim of this study was to investigate whether NAC is able to prevent the expression of behavioral and biochemical alterations induced by ethanol withdrawal in chronically exposed zebrafish. Animals were exposed to ethanol (1% *v*/v, 20 min) or control water, followed by treatment with NAC (1 mg/L, 10 min) or control water daily for 8 days; 24 h later, experimental animals were submitted to the novel tank test (NTT). Ethanol withdrawal decreased the distance traveled and increased the number of immobile episodes, indicating locomotor deficits; moreover, withdrawal decreased the number of entries and time spent in the top area, while increasing time spent in the bottom area, indicating anxiety-like behavior. Alcohol withdrawal also increased lipid peroxidation (TBARS) and decreased non-protein reduced sulfhydryl (NPSH) and superoxide dismutase (SOD) and catalase (CAT) activities. NAC attenuated these locomotor deficits and prevented the manifestation of anxiety-like behavior as well as the oxidative damage observed following ethanol withdrawal. Given its favorable safety profile, additional clinical and preclinical studies are warranted to unravel the long-term effects of NAC in the context of alcohol abuse and the exact mechanisms involved. Nevertheless, our study adds to the existing body of evidence supporting the clinical evaluation of NAC in substance abuse disorders.

1. Introduction

Alcohol abuse is associated with neurotoxicity, cognitive disruption, loss of productivity and interpersonal functioning, substantially contributing to global morbidity and mortality (Abrahao et al., 2017; Grant et al., 2015; NIAAA, 2017; WHO, 2017). Chronic alcohol abusers may experience symptoms of alcohol withdrawal, such as insomnia, tremors, anxiety, dysphoria, nausea or vomiting, restlessness and, in more severe cases, seizures and *delirium tremens* (a state of mental confusion) (Kosten and O'Connor, 2003; Schuckit, 2014). This symptomatology presents itself as a multifaceted disorder with neurobiological and behavioral complexity (Campbell et al., 2018; DeHaas et al., 2001; Gatch and Lal, 2001; Grant et al., 2015). Such experiences contribute to the difficulty in maintaining abstinence faced by alcohol dependent

individuals (Abrahao et al., 2017; Amato et al., 2011; Grant et al., 2015; Lovinger and Crabbe, 2005).

According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA, 2017), current approaches for the treatment of alcohol use disorder include a combination of counseling, medication, and social support. There are three drugs approved by the FDA for alcohol withdrawal: naltrexone, acamprosate, and disulfiram; their efficacy, however, is low, and relapse rates are high (Anton et al., 2006; Litten et al., 2016, 2015). In this context, continued efforts are needed to identify pharmacological alternatives, and our increased understanding of the underlying neurobiology of alcohol addiction is helpful for identifying novel drug targets. Preclinical studies have evidenced the role of oxidative stress and glutamate in alcohol withdrawal, suggesting that restoration of oxidative status and glutamatergic function may represent new pharmacological targets to help individuals cope during abstinence (Campbell et al., 2018; Lebourgeois et al., 2018; Schneider et al., 2015; Yawalkar et al., 2018).

Alcohol consumption is strongly associated with oxidative stress due to the production of reactive oxygen species (ROS) that result from ethanol metabolism (Prendergast et al., 2004). Excessive generation of ROS was observed in human neurons in response to chronic alcohol consumption, and it is believed that chronic oxidative stress conditions cause oxidative neuronal injury (Haorah et al., 2008). In rodents, excess ROS decreases antioxidant defenses, such as superoxide dismutase (SOD) and catalase (CAT) enzymes, disturbs glutathione (GSH) homeostasis and increases lipid peroxidation (LPO), promoting oxidative damage and contributing to the occurrence of neuropsychiatric symptoms (Haorah et al., 2008; Koop, 2006; Zhou et al., 2015). Excess glutamate release has also been implicated in the adverse behavioral effects of alcohol abuse (Burnett et al., 2016; Holmes et al., 2013), and glutamatergic modulation has been described to mediate the effects of alcohol on behavior and the pathogenesis of alcohol-induced psychiatric disorders (Yawalkar et al., 2018).

N-acetylcysteine (NAC) is a well-known antioxidant that increases the cellular pool of cysteine, an amino acid required for GSH biosynthesis, and has been tested for conditions associated with GSH depletion and altered redox status (Bosch-Morell et al., 1998; Dean et al., 2011b; Grinberg et al., 2005; Whyte et al., 2007). NAC has shown promise in treating a variety of psychiatric disorders, including substance abuse, not only due to its antioxidant capacity but also for its attenuation of glutamate release. The increased supply of cysteine induced by NAC leads to activation of the cystine-glutamate antiporter (system X$_C$-) expressed by astrocytes, resulting in higher extrasynaptic glutamate levels, which in turn reduce neuronal glutamate release through activation of inhibitory metabotropic glutamate receptors (Berk et al., 2013; Couto and Moreira, 2018; Dean et al., 2011a; Deepmala et al., 2015; McClure et al., 2014).

Several studies have reported the effects of different schedules of ethanol exposure on behavior and oxidative damage in zebrafish (da Silva Chaves et al., 2018; Mathur and Guo, 2011; Müller et al., 2017); however, none of them have addressed the potential protective effects of pharmacological therapies. Considering the need for further studies in this area, the aim of this study was to evaluate the preventive effects of NAC treatment on behavioral and oxidative status parameters altered by ethanol withdrawal in adult zebrafish.

2. Methods

2.1. Animals

Behavioral and biochemical experiments were performed using 64 4-month-old zebrafish of the short fin phenotype (50:50 male:female ratio). To better represent population heterogeneity, a wild-type strain zebrafish population was used due to their increased genetic variability.

2.2. Housing standards

Fish were housed at a maximum density of two fish per liter and acclimated for 30 days in 40-L tanks (45 × 35 × 30 cm). After this quarantine period, fish were transferred to 16-L home tanks (40 × 20 × 24 cm) with non-chlorinated water kept under constant mechanical, biological and chemical filtration, where they acclimated for another 14 days prior to testing. Fish were maintained and monitored under 14/10 h day/night cycle (lights on at 07:00 am), pH of 7.0–8.0 and conductivity of 1500–1600 μS/cm at 26 ± 2 °C. Levels of available food were based on zebrafish literature (4% of body weight in food per fish per day), and fish were fed three times a day with brine shrimp (*Artemia salina*) and commercial flake fish food (Alcon BASIC, Brazil). Housing standards followed our previous studies and are supported by the literature (Mocelin et al., 2018b, 2018a; Westerfield, 2007).

2.3. Drugs

N-acetylcysteine (NAC, CAS number 616-91-1) and ethanol (EtOH, C$_2$H$_6$O, CAS number 64-17-5, ≥ 99.9%) were acquired from Sigma-Aldrich (St Louis, Missouri, USA) and Merck (Darmstadt, Germany), respectively. Concentrations utilized were based on previous studies from our group (Mocelin et al., 2018b, 2018a, 2015). EtOH and NAC solutions were prepared daily.

2.4. Study design

Ethanol withdrawal was induced following a chronic ethanol exposure protocol adapted from previous studies (Mathur and Guo, 2011; Müller et al., 2017). Fish were randomly allocated to experimental groups with the aid of computerized random numbers (www.random.org). Investigators were blind to the experimental groups, and each exposure tank was given a code. Codification was performed by a researcher who did not participate in the experiments. The codes of experimental groups were revealed only during statistical analysis.

Experimental groups included CRTL (water and water), NAC (water and NAC), EtOH (ethanol and water) and EtOH-NAC (ethanol and NAC). Fish were gently transferred daily at 08:00 am in groups of 8 to exposure tanks containing freshwater or EtOH (1% *v/v*) for 20 min. Immediately afterward, animals were placed in treatment tanks containing freshwater or NAC (1 mg/L) for 10 min and then returned to their home tanks. Exposure and treatment tanks were of equal dimensions (2.7-L; 24 × 8 × 20 cm), and white frosted cardboard was placed between the tanks to avoid visual contact among fish from different groups (Marcon et al., 2018b). Each experimental group originated from two identical home tanks, and no tank effects were observed during analysis, so data were pooled together. The exposure plus treatment protocol occurred for 8 consecutive days, and the behavioral and biochemical analysis took place on the 9th day, as shown in Fig. 1.

2.5. Behavioral testing

Behavioral testing was performed 24 h after the last drug exposure (n = 16). Fish were gently handled to avoid any stressors interfering with the behavioral analysis. To minimize any potential confounders that could arise due to variable fasting duration and because testing started before the first feeding was scheduled, fish were not fed on the day of the test. Animals were individually transferred and recorded for 6 min in the novel tank test (NTT), and results were analyzed using ANY-Maze software (Stoelting Co., USA). The NTT apparatus consists of a 2.7-L tank (24 × 8 × 20 cm) filled with water up to 15 cm, virtually divided into three equal horizontal zones (bottom, middle and top) (Marcon et al., 2018a, 2016; Pancotto et al., 2018). The vertical location in the NTT is considered a parameter of anxiety-like behavior analogous to the thigmotaxic behavior that rodents display in an open
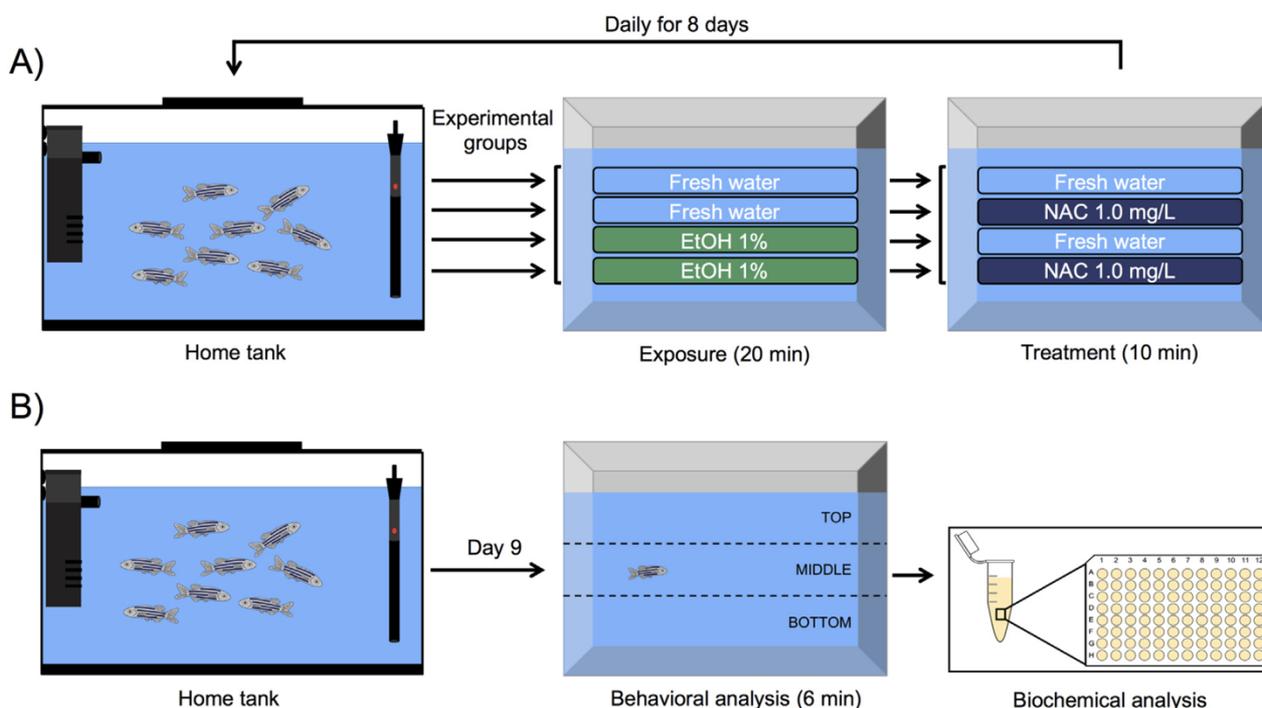
Fig. 1. Outline of the study design. Fish were exposed daily for 8 days to EtOH or fresh water for 20 min and then treated for 10 min with NAC or freshwater (A). On day 9 (B), fish behavior was analyzed, and brain samples were collected for biochemical assays. Ethanol (EtOH); *N*-acetylcysteine (NAC).

field (Levin et al., 2007).

The parameters studied included total distance traveled, number of crossings (transitions between zones of the tank), number of immobile episodes, and number of entries and time spent in the top and bottom zones of the tank. Distance, crossings and immobile episodes were used to evaluate locomotion, while number of entries and time in the top zone was used to evaluate anxiety-like behavior (Kalueff et al., 2013).

2.6. Biochemical assays

Immediately after the behavioral test, fish were anesthetized by immersion in water at 2–4 °C (rapid cooling) in a beaker (500 mL) and euthanized afterward by decapitation for brain tissue sampling. Tissue homogenates were prepared and stored at −80 °C in microtubes for further analysis. Each sample consisted of 4 pooled dissected brains ($n = 4$) that were gently homogenized in 600 μL phosphate buffered saline (PBS, pH 7.4, Sigma-Aldrich) using a disposable tissue grinder pestle (Axygen, PES-15-B-SI) in 1.5-mL microcentrifuge tubes (Axygen, MCT-150-C) 60 times. Homogenates were centrifuged at $10,000 \times g$ for 10 min at 4 °C in a cooling centrifuge (ALC, PK121R), and the supernatant packed in microtubes was used for experimental assays. Protein concentrations were determined by the Coomassie blue method using bovine serum albumin (Sigma-Aldrich) as a standard and measured at 595 nm (Bradford, 1976). All biochemical procedures were adapted from previous studies in zebrafish (Marcon et al., 2018b; Mocelin et al., 2018b, 2018a; Sachett et al., 2018).

2.6.1. Lipid peroxidation

Thiobarbituric acid reactive species (TBARS) were estimated by quantifying TBARS production as described by Draper and Hadley (1990). More specifically, a volume containing 80 μg protein sample was mixed with 75 μL trichloroacetic acid (TCA 10%, Sigma-Aldrich), adjusted with deionized water (totaling 175 μL) and centrifuged at $3400 \times g$ for 5 min at 4 °C in a cooling centrifuge. Supernatants were collected and added to 75 μL thiobarbituric acid (TBA 0.67%, Sigma-Aldrich), then homogenized in a vortex for 5 s followed by heating at

100 °C for 30 min. TBARS levels were quantified in a microplate reader at 532 nm using malondialdehyde (MDA, Sigma-Aldrich) as a standard, and results are expressed as nanomoles (nmol) MDA/mg protein.

2.6.2. Non-protein thiols levels

Non-protein thiol (NPSH) levels were estimated in a microplate reader at 412 nm as described by Ellman (1959). More specifically, a volume containing 60 μg of protein sample was mixed with an equal volume of TCA (10%) and centrifuged at $3400 \times g$ for 5 min at 4 °C in a cooling centrifuge. To the supernatant, 255 μL potassium phosphate buffer (TFK, 1 M, pH 7) and 15 μL 5,5-dithio-bis-2-nitrobenzoic acid (DTNB 10 mM, Sigma-Aldrich) dissolved in EtOH were added, totaling approximately 300 μL. A yellow color developed after 60 min. The results are expressed as micromole (μmol) SH/mg protein.

2.6.3. Superoxide dismutase

Superoxide dismutase (SOD) activity was estimated by testing the amount of enzyme capable of inhibiting 50% of the adrenaline oxidation rate as described by Misra and Fridovich (1972). More specifically, in brain samples (volumes containing 15, 30 and 60 μg of protein), adrenochrome formation rate was monitored in a medium containing glycine-NaOH buffer (50 mM, pH 10) and 5 μL epinephrine (60 mM, pH 1.7, Sigma-Aldrich), totaling approximately 200 μL, in a microplate reader at 480 nm. The results are expressed as SOD units/mg protein.

2.6.4. Catalase

Catalase (CAT) activity was estimated by monitoring the rate of decrease in hydrogen peroxide (H_2O_2) absorbance as described by Aebi (1984). More specifically, a volume containing 30 μg protein sample was mixed with TFK (50 mM, pH 7) and 5 μL H_2O_2 (1 M), totaling approximately 250 μL. The amount of enzyme required to degrade 1 μmol (μmol) H_2O_2 is defined as one unit of CAT, measured in the microplate reader at 240 nm. The results are expressed as CAT units/mg protein.

2.7. Statistical analysis

Power analysis revealed a minimum sample size of 15 to detect a difference in time spent in the upper zone of the tank (we used $n = 16$ to ensure equal numbers of males and females). This was calculated using Minitab 18 for Windows considering an alpha = 0.05, power = 0.9, effect size = 0.5 and standard deviation = 0.4 (based on pilot experiments and historical data from our group). Data normality and homogeneity of variances were analyzed using D'Agostino-Person and Levene tests, respectively. Two-way ANOVA was performed to identify the main effects of ethanol exposure and NAC treatment, as well as their interaction. When ANOVA revealed significant interactions, we conducted post hoc comparisons using the Bonferroni test. Data are expressed as the mean ± standard error of the mean (S.E.M.). In all cases, statistical comparisons were two-tailed. The level of significance was set at $p < .05$.

3. Results

3.1. Locomotor parameters

Ethanol withdrawal after chronic exposure altered locomotor parameters in zebrafish. Fig. 2 shows the effects on total distance, crossings, immobile episodes and occupancy plots in the NTT. For total distance (Fig. 2A), two-way ANOVA revealed a main effect of EtOH (F $(1,60) = 5.42$, $p = .0233$), indicating a decrease in total distance traveled following withdrawal. Concerning immobile episodes (Fig. 2C), two-way ANOVA revealed a main effect of EtOH (F$(1,60) = 7.29$, $p = .0090$), which increased this parameter. Locomotor parameters and anxiogenic behavior observed following ethanol withdrawal is visualized in occupancy plots (Fig. 2D) obtained by the automated video-tracking system (ANY-maze). The colors represent the time fish spent in each region of the apparatus, with low wavelengths (e.g., red) indicating high occupancy and high wavelengths (e.g., blue) indicating

low occupancy (frontal view recordings). No significant differences were observed in the number of crossings (Fig. 2B). Although the interaction did not reach the threshold for significance, NAC appears to attenuate the effects induced by EtOH, as measured by distance traveled and immobile episodes (see supplementary video).

3.2. Anxiety-like behavior

Fig. 3 shows the effects of ethanol withdrawal and NAC treatment on anxiety-like behavior. For entries and time in the top area (Fig. 3A and B, respectively), two-way ANOVA revealed main effects of EtOH (F $(1,60) = 8.30$, $p = .0055$ and F$(1,60) = 24.36$, $p < .0001$, respectively) and NAC (F$(1,60) = 10.60$, $p = .0019$ and F$(1,60) = 10.99$, $p = .0016$, respectively), as well as an interaction between both factors (F$(1,60) = 6.19$, $p = .0157$ and F$(1,60) = 17.15$, $p = .0001$, respectively). Post hoc analysis revealed that ethanol withdrawal decreased the number of entries ($p < .01$) and time spent in the top area ($p < .0001$), while NAC was able to prevent these effects. For time spent in the bottom area (Fig. 3D), two-way ANOVA revealed main effects of EtOH (F$(1,60) = 7.22$, $p = .0093$) and NAC (F$(1,60) = 12.28$, $p = .0009$), as well as an interaction effect (F$(1,60) = 11.27$, $p = .0014$). Post hoc analysis revealed that ethanol withdrawal increased the time spent in the bottom area ($p < .001$), while NAC prevented this alteration. No significant effects were observed for entries in the bottom area (Fig. 3C).

3.3. Oxidative status

The influence of ethanol withdrawal and NAC treatment on biochemical parameters associated with oxidative status is presented in Fig. 4. For TBARS (Fig. 4A), two-way ANOVA revealed the main effects of ethanol withdrawal (F$(1,12) = 15.67$, $p = .0019$) and NAC treatment (F$(1,12) = 6.62$, $p = .0354$), as well as an interaction effect (F $(1,12) = 4.91$, $p = .0468$). Post hoc analysis indicated that ethanol

A)

EtOH:	$F_{1,60} = 5.42$	p=0.0233
NAC:	$F_{1,60} = 0.40$	p=0.5311
Interaction:	$F_{1,60} = 3.46$	p=0.0677

Distance (m) — CTRL / NAC (CTRL), CTRL / NAC (EtOH)

B)

EtOH:	$F_{1,60} = 1.32$	p=0.2552
NAC:	$F_{1,60} = 2.88$	p=0.0946
Interaction:	$F_{1,60} = 2.41$	p=0.1256

Crossings — CTRL / NAC (CTRL), CTRL / NAC (EtOH)

C)

EtOH:	$F_{1,60} = 7.29$	p=0.0090
NAC:	$F_{1,60} = 2.50$	p=0.1188
Interaction:	$F_{1,60} = 3.82$	p=0.0553

Immobile episodes — CTRL / NAC (CTRL), CTRL / NAC (EtOH)

D) CTRL / NAC; CTRL / EtOH
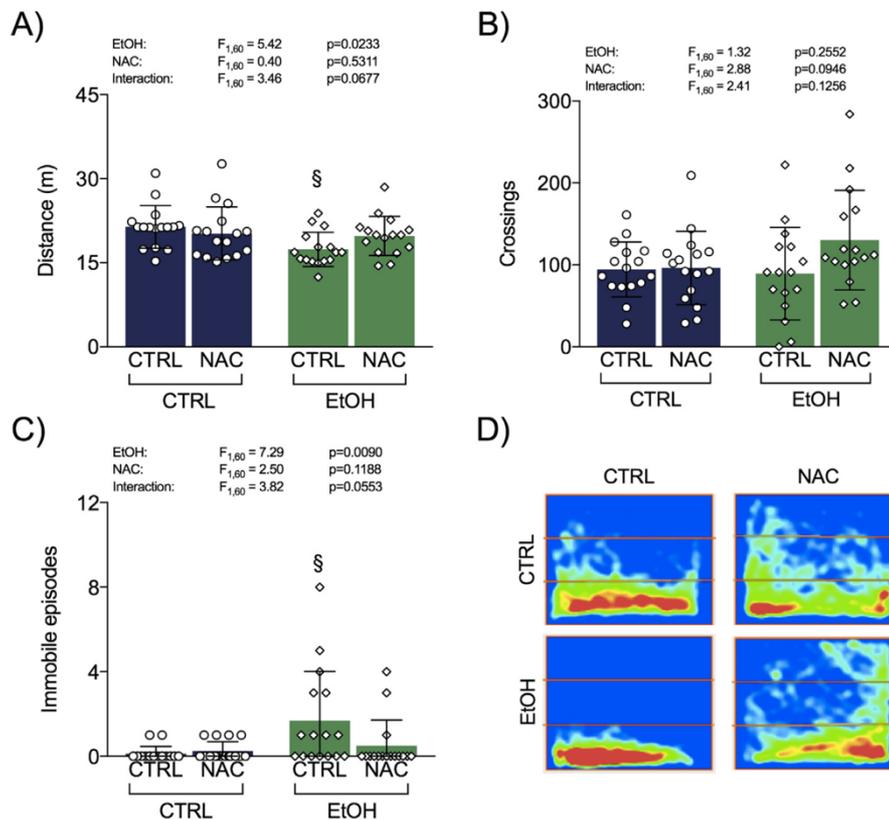
Fig. 2. Effects of NAC (1.0 mg/L) treatment against ethanol withdrawal-induced changes in locomotor parameters. Distance traveled (A), number of crossings (B), immobile episodes (C) and representative color heat map of the behavior of one fish from each treatment group during the 6-min trial (D). The data are presented as the mean ± S.E.M. Two-way ANOVA followed by Bonferroni's test. $n = 16$. § represents the main effect of EtOH.

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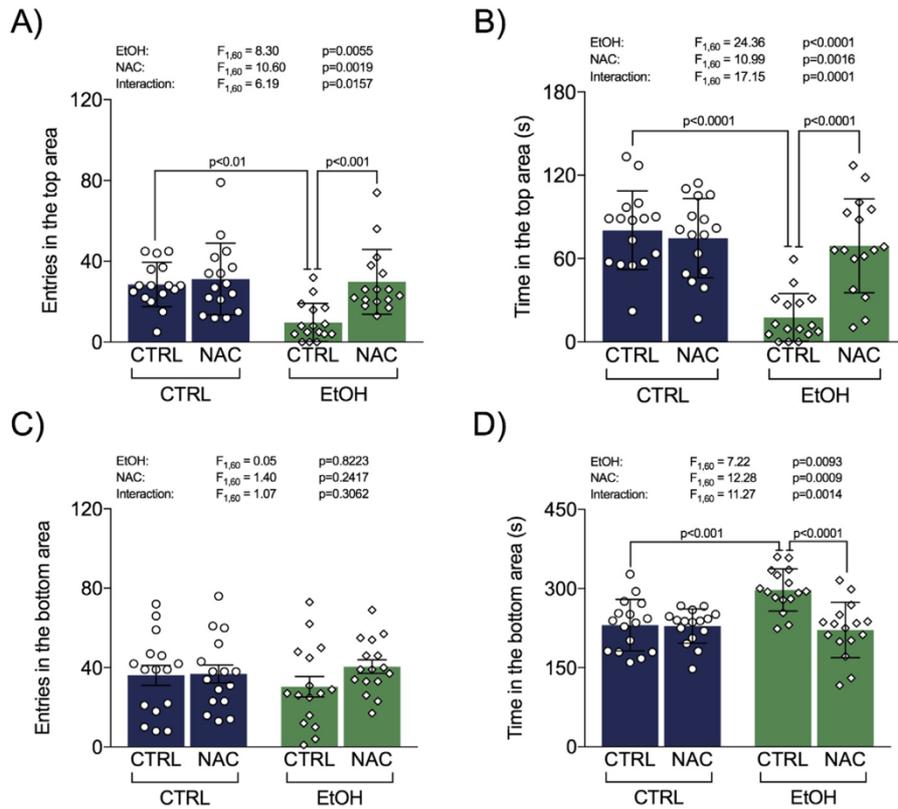
Fig. 3. Effects of NAC (1.0 mg/L) treatment against ethanol withdrawal-induced changes in behavioral parameters. Entries (A) and time (B) in the top area, entries (C) and time (D) in the bottom area. The data are presented as the mean ± S.E.M. Two-way ANOVA followed by Bonferroni's test. $n = 16$.

Fig. 4. Effects of NAC (1.0 mg/L) treatment against ethanol withdrawal-induced changes in biochemical parameters. Thiobarbituric acid reactive substances (A), non-protein sulfhydryl (B), superoxide dismutase (C), and catalase (D). The data are presented as the mean ± S.E.M. Two-way ANOVA followed by Bonferroni's test. $n = 4$.
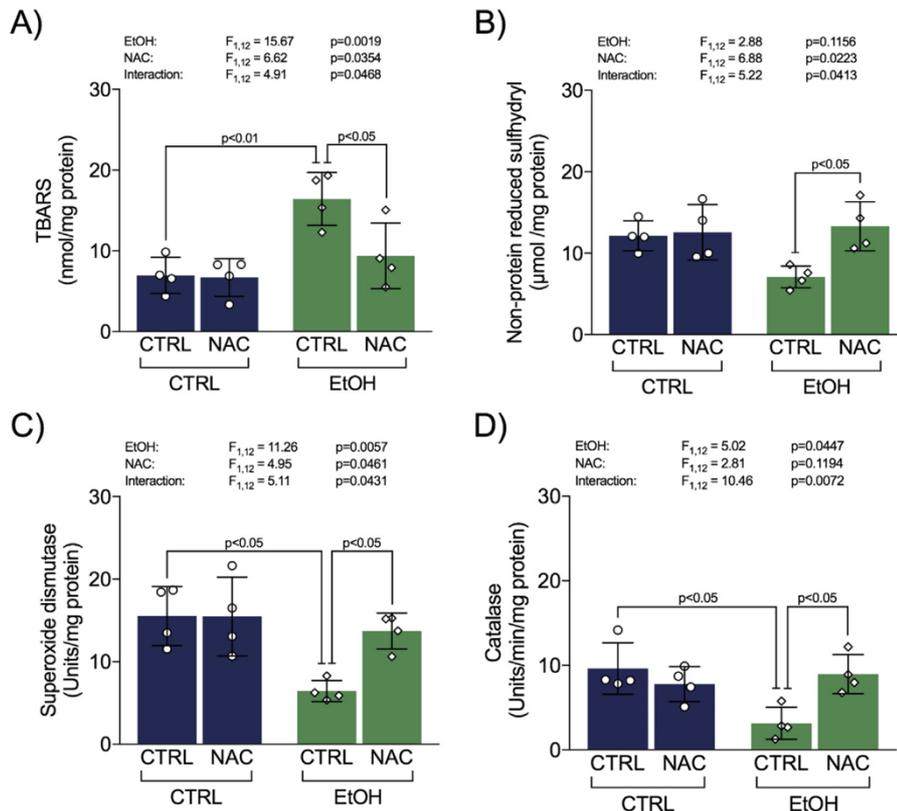
withdrawal increased lipid peroxidation ($p < .01$) but not in NAC treated fish.

A main effect of NAC ($F(1,12) = 6.88$, $p = .0223$) and an interaction effect were observed ($F(1,12) = 5.22$, $p = .0413$) for NPSH levels (Fig. 4B); ethanol withdrawal decreased NPSH levels only in the control group.

Two-way ANOVA revealed the main effects of EtOH ($F(1,12) = 11.26$, $p = .0057$) and NAC ($F(1,12) = 4.95$, $p = .0461$), as well as an interaction ($F(1,12) = 5.11$, $p = .0431$) between both factors for SOD activity (Fig. 4C). For CAT activity (Fig. 4D), a main effect of EtOH ($F(1,12) = 5.02$, $p = .0447$) and an interaction effect ($F(1,12) = 10.46$, $p = .0072$) were observed. Ethanol withdrawal decreased SOD ($p < .05$) and CAT ($p < .05$) activities in untreated fish, while NAC-treated animals were protected from these effects.

4. Discussion

There are limited clinical and preclinical studies exploring the neurobiology underlying the symptoms and signs associated with ethanol withdrawal or aimed at evaluating the potential of pharmacological interventions to reduce the severity of this condition and increase the chances of abstinence (Litten et al., 2016; Schneider et al., 2015; Yawalkar et al., 2018). Herein, we demonstrate the beneficial effects of NAC treatment in zebrafish submitted to ethanol withdrawal. We demonstrated that treatment with NAC protects zebrafish from the effects of ethanol withdrawal after repeated exposure for 8 days. NAC treatment prevented locomotor deficits and anxiety-like behavior, as well as the lipid peroxidation and ethanol withdrawal-induced depletion of antioxidant defenses observed during ethanol withdrawal.

Studies have shown that alcohol consumption affects physiological and behavioral parameters in humans and induces anxiety-like behavior in animal models (Abrahao et al., 2017; Kliethermes, 2005; Litten et al., 2015; Novier et al., 2015). As expected, zebrafish exposed to 8 days of ethanol displayed increased anxiety-like behavior in the NTT after withdrawal, as demonstrated by increased time spent in the bottom and decreased time and entries to the top area (Mathur and Guo, 2011). Furthermore, we also observed that withdrawal from repeated ethanol exposure altered locomotor parameters, including decreased total distance traveled and increased number of immobile episodes.

Chronic ethanol consumption increases acetaldehyde and exerts deleterious effects on the central nervous system (CNS) and in the peripheral system (Lieber, 2005). Indeed, chronic ethanol consumption impacts oxidative status through the generation of ROS and increases both mRNA and protein expression levels of CYP2E1, leading to increased peroxide radical formation and lipid peroxidation (see Fig. 5A) (Berg et al., 2002; Huang et al., 2009; Lieber, 2005; Tsai et al., 1998). Therefore, it is possible that ethanol oxidation in the brain results in ROS formation and oxidative stress, which may contribute to withdrawal symptoms, especially in frequent ethanol consumers (Burnett et al., 2016; Haorah et al., 2008; Müller et al., 2017). At the same time, defense mechanisms are unable to control the excess ROS, leading to oxidative damage (Koop, 2006; Slot et al., 1986; Weydert and Cullen, 2010). In support of such claims, we demonstrated that ethanol withdrawal increases lipid peroxidation and impairs different defense mechanisms against oxidative damage, manifested as reduced glutathione (GSH) (as measured by NPSH levels), SOD and CAT enzymes.

The imbalance between the generation of oxidizing compounds and the performance of the antioxidant defense systems leads to oxidative stress and can cause tissue injury (Haorah et al., 2008; Huang et al., 2009). Studies demonstrate that administering antioxidants, such as glutathione repletion agents, can prevent or ameliorate the toxic effects induced by alcohol (Aydin et al., 2002; Iimuro et al., 2000; Karadayian et al., 2017).

SOD is most sensitive to and is the primary defense against the superoxide anion radical (Fridovich, 1997; Slot et al., 1986).

Nevertheless, SOD activity is decreased in alcoholics, possibly because a greater amount of peroxide radical, which is a product of the superoxide dismutation reaction, reduces SOD activity (Halliwell, 2007). A reduction in SOD activity has been previously observed in a similar protocol of chronic ethanol exposure using zebrafish (Müller et al., 2017) and rodents (Aydin et al., 2002; Elbini Dhouib et al., 2016). Such reduced enzymatic activity may increase $O_2^{•-}$ levels, which are also able to reduce the antioxidant capacity of CAT (Halliwell, 2007; Haorah et al., 2011; Perera et al., 1995).

Modulation of CAT activity may alter behavioral responses to ethanol, seemingly by controlling levels of acetaldehyde or by influencing the rate of ethanol elimination from the brain (Correa et al., 2001; Manrique et al., 2006; Sanchis-Segura et al., 1999). In the present study, CAT activity was reduced in zebrafish after withdrawal from repeated ethanol exposure, replicating observations in previous zebrafish studies (da Silva Chaves et al., 2018; Müller et al., 2017). Our results corroborate a previous study that observed changes in redox status following 24-h withdrawal of exposure to alcohol (1%) for 20 min daily over 8 days in zebrafish (Müller et al., 2017). In addition, CAT activity is decreased in alcohol withdrawal patients and is a key enzyme for ethanol oxidation, accounting for approximately 60% of total acetaldehyde formed in the brain of rodents (Huang et al., 2009; Zimatkin et al., 2006).

In contrast, there is a lack of studies investigating a more complete profile of NAC involvement in protection against behavioral and oxidative damage in the brain, which strengthens the interest for using NAC in the context of substance use disorders. Our group previously reported that NAC decreased anxiety-like behavior in zebrafish submitted to an acute ethanol exposure (Mocelin et al., 2018a), and now we show the beneficial effects of NAC in ethanol withdrawal, which prevented anxiety-like behavior and oxidative damage in zebrafish. The positive and promising effects of NAC are likely due to its antioxidant potential, as well as its anti-inflammatory properties and ability to modulate different neurotransmitter systems, especially glutamate, which is implicated in the pathophysiology of drug abuse (Deepmala et al., 2015; Minarini et al., 2017). Previous rodent studies have shown that NAC prevented ethanol withdrawal-induced anxiety-like behavior in the open field test, depression in the forced swim test and tail suspension test, as well as reduced ethanol-seeking behavior and reacquisition in abstinent rats (Lebourgeois et al., 2018; Schneider et al., 2015; Yawalkar et al., 2018). A clinical study has also reported the efficacy of NAC in reducing alcohol consumption during cannabis cessation (Squeglia et al., 2018), and other investigations are ongoing to more directly investigate the efficacy of NAC to treat alcohol use disorder (ClinicalTrials.gov identifier: NCT02791945 and NCT02966873). This positive effect of NAC may involve an improvement in redox homeostasis because it plays a key role in maintaining cysteine levels and boosting GSH biosynthesis.

In this sense, NAC may have prevented lipid peroxidation by reacting with hydroxyl radicals, enhancing the formation of the main intracellular non-protein thiol (GSH) or increasing the pool of free thiols, and preserving antioxidant enzyme activities (Ahmad et al., 2013; Gleixner et al., 2017; Halliwell, 2007; Nouri et al., 2017). Moreover, NAC is a sulfur compound and a precursor of GSH, as it supplies cysteine for its synthesis (Grinberg et al., 2005). In rats chronically exposed to ethanol, a decrease in NPSH (GSH levels) was observed, while NAC restored GSH content (Bosch-Morell et al., 1998; Calabrese et al., 1998). We can consider that NAC prevented the reduction of NPSH levels in our study by increasing GSH content since it provides its rate-limiting substrate (Grinberg et al., 2005). Acting as an indirect antioxidant, NAC increases SOD activity, being able to potentially modulate MnSOD expression by augmenting de novo synthesis or decreasing elimination rates due to lower oxidation levels, but the exact mechanism for this remains unknown (Barreiro et al., 2005; Mao et al., 2016; Nagata et al., 2007). In parallel, we showed that NAC was also able to prevent the decrease in CAT activity in zebrafish brain, though
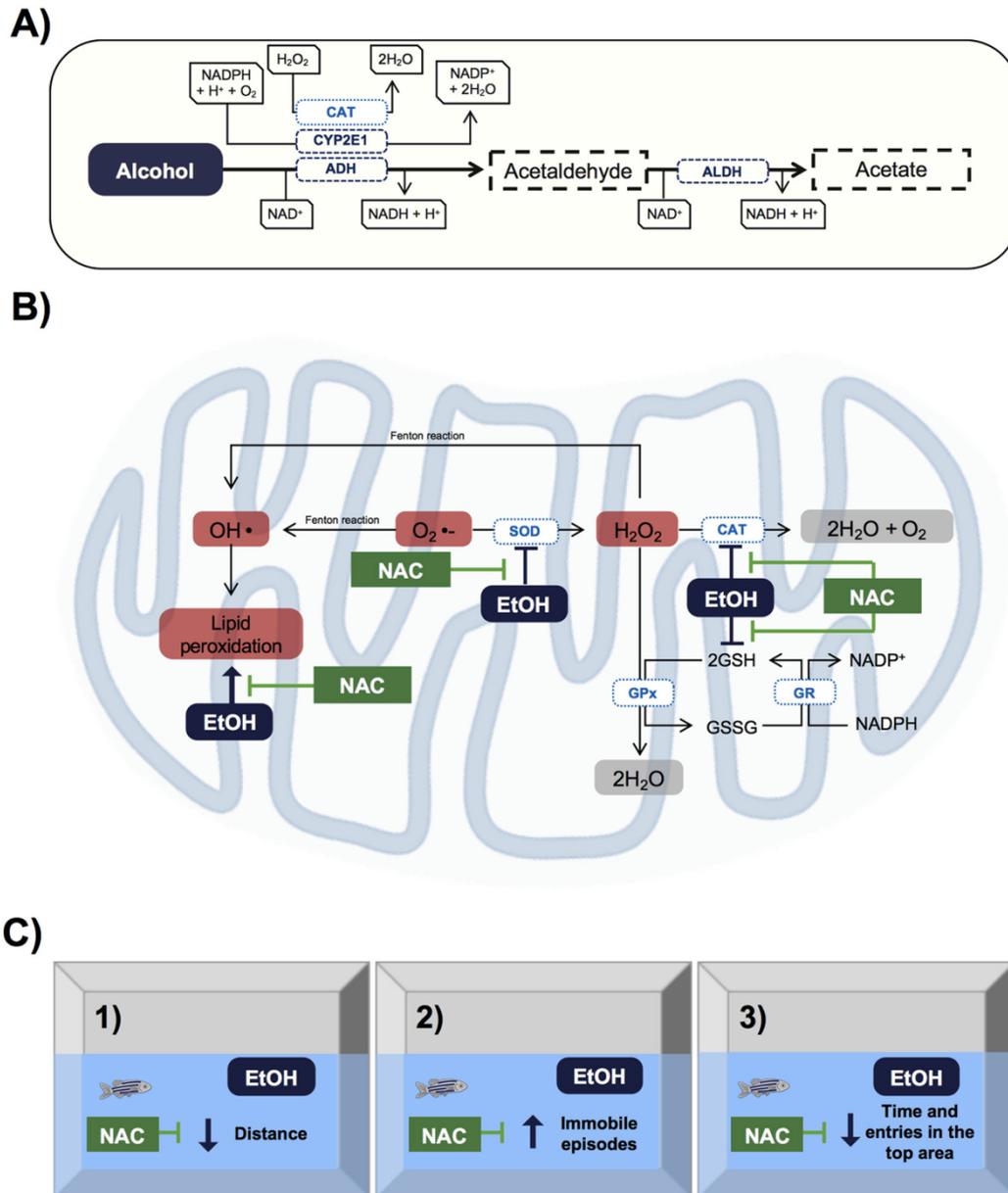
Fig. 5. The panel depicts a putative mechanism of behavioral alterations and oxidative damage induced by chronic ethanol withdrawal and the protective effects of *N*-acetylcysteine (NAC). (A) Alcohol is converted into acetaldehyde by alcohol dehydrogenase (ADH) and/or cytochrome P450 2E1 (CYP2E1). Acetaldehyde is oxidized to acetate by acetaldehyde dehydrogenase (ALDH) and catalase (CAT). Oxidation by enzymes (ADH, CYP2E1, and ALDH) leads to increased reactive oxygen species (ROS) formation. (B) Chronic EtOH increases lipid peroxidation, decreases superoxide dismutase (SOD) and CAT activities, and the levels of reduced glutathione (GSH) (blue arrows). NAC abolished all such effects elicited by ethanol (green arrows) in the zebrafish brain. (C) Chronic EtOH reduces the entries (1) and time in the top area (2), as well as increases the time in the bottom area (3); NAC abolished these alterations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

this decrease was not observed in rats (Ozkol et al., 2017). The molecular mechanisms by which NAC acts to maintain CAT activity remain unclear, but one study revealed that NAC may upregulate gene expression levels of CAT (Doi et al., 2011). Taken together, our findings suggest the presence of oxidative stress and weakened antioxidant capacity in zebrafish following chronic ethanol withdrawal (Fig. 5B), which is possibly involved in observed behavioral alterations (Fig. 5C), most of which were abolished by co-administration of NAC.

We must consider that repeated alcohol consumption leads to a hyperglutamatergic state, with behavioral consequences in rodents and human (Holmes et al., 2013; Tsai et al., 1998). An alternative approach for the treatment of ethanol withdrawal would be modulation of AMPA and NMDA receptors, as blocking these receptors positively affects various ethanol-related behaviors in rodents (Rao et al., 2015). In this context, one study showed that NAC was able to reverse the increased expression of subunits 2A and 2B of the NMDA glutamate receptor in abstinent rats (Yawalkar et al., 2018). Moreover, glutamate transporter upregulation may attenuate the behavioral and neurotoxic damage of excess glutamate induced by ethanol (Goodwani et al., 2017). Importantly, NAC also modulates glutamatergic transmission, including AMPA and NMDA receptors (Gipson, 2016; Linck et al., 2012), providing another mechanism of action that may explain the protective effects reported for this molecule.

5. Conclusions

NAC treatment 10 min daily for 8 days was effective in preventing the behavioral and oxidative changes observed after 24 h of alcohol cessation. Considering that treatment for people with alcohol abuse disorder is limited and current drugs are of little efficacy, our results demonstrate a potential use for NAC in this context. Moreover, NAC has a favorable safety profile and has been around for decades. Nevertheless, further preclinical and clinical studies are needed to unravel its mechanisms of action, efficacy and long-term effects.

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Author contribution

Conceived and designed the experiments: RM, APH, AP; Performed the experiments: RM, MM; Analyzed the data: RM, APH, AP; Contributed reagents/materials/analysis tools: AP, ASA; Wrote the paper and prepared figures: RM; Authored or reviewed drafts of the paper: AP, APH, ASA, MM; Approved the final draft: RM, MM, ASA, APH, AP.

Acknowledgments

Conflict of interest

All authors declare that they have no conflicts of interest.

Ethical statement

All protocols were approved by the Ethics Committee of the Federal University of Rio Grande do Sul (process number #30914). The manipulation and animal care were conducted according to the National Institute of Health Guide for Care and Use of Laboratory Animals and aimed to minimize the discomfort, suffering, as well as the number of animals used.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2019.03.014.

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4. RESULTADOS ADICIONAIS

Aqui mostramos dados parciais referente ao quarto objetivo específico (efeito rápido, sustentado e profilático da NAC sobre parâmetros comportamentais, oxidativos e moleculares em peixes-zebra submetidos ao modelo de estresse crônico imprevisível).

QUALIFICAÇÃO DO PROBLEMA

A NAC é capaz de modular o sistema glutamatérgico através do trocador X_C^-, aumentando quantidade de glutamato. Como resposta, indiretamente a NAC atua sobre receptores NMDA e AMPA (BERK et al., 2013; DEAN; GIORLANDO; BERK, 2011a). Essa modulação sobre o sistema glutamatérgico demonstra uma certa similaridade com o mecanismo de ação da cetamina.

Moduladores glutamatérgicos, como a cetamina, são compostos promissores no tratamento de transtornos mentais (MCEWEN et al., 2015c; MURROUGH; ABDALLAH; MATHEW, 2017; POPOLI et al., 2011; SWANSON et al., 2005). Um estudo clínico realizado em 2006 mostrou que uma dose subanestésica desse fármaco foi capaz de reverter os sintomas depressivos. Surpreendentemente esse efeito foi sustentado por uma semana (ZARATE et al., 2006). Essa rápida resposta antidepressiva, em comparação com as semanas ou meses necessários para os fármacos atualmente disponíveis, fortalece a hipótese de que moduladores desse sistema podem ser agentes inovadores no tratamento de transtornos mentais (ANDRADE, 2017; GLUE et al., 2017; KRYSTAL; SANACORA; DUMAN, 2013; TAYLOR et al., 2018; VIDAL et al., 2018; ZANOS et al., 2016; ZARATE et al., 2012, 2006). Recentemente, o FDA aprovou a esketamina (SPRAVATO[TM], Jonhson & Jonhson) para o tratamento de pacientes com depressão maior não-responsivos a antidepressivos. SPRAVATO[TM] é um enantiômero S da cetamina sob formulação farmacêutica de spray nasal, disponível a um alto custo (AUST et

al., 2019; FREEMAN et al., 2019; JANSEN; DARRACOT-CANKOVIC, 2001; PAL et al., 2002).

A cetamina é um antagonista de receptores pós-sinápticos glutamatérgicos do tipo NMDA, mecanismo pelo qual promove o efeito antidepressivo rápido. Já o efeito sustentando é dependente da ativação dos receptores AMPA, não diretamente pela cetamina, mas pelo seu metabólito (2R,6R-hidroxinorcetamina, HNK) (KOIKE; CHAKI, 2014; ZANOS et al., 2016, 2018). Além disso, a cetamina foi capaz de prevenir o comportamento tipo-depressivo induzido pelo estresse crônico em roedores (ABDALLAH et al., 2018; BRACHMAN et al., 2016; MASTRODONATO et al., 2018; MCGOWAN et al., 2018). Tal efeito antidepressivo estaria também relacionado a ativação de mTOR e produção de BDNF, importantes aspectos relacionados à regulação da plasticidade sináptica (ABDALLAH et al., 2016; LI et al., 2010; MAENG et al., 2008). Entretanto, esses mecanismos ainda não estão totalmente elucidados.

Apesar desses efeitos clínicos evidentes, a cetamina pode ser utilizada como droga de abuso devido aos seus efeitos alucinógenos e estimulantes (DILLON; COPELAND; JANSEN, 2003, 2003; KOHRS; DURIEUX, 1998; SMITH; LARIVE; ROMANELLI, 2002). Além disso, pode causar hipertensão, taquicardia, movimentos tônico-clônicos, sonhos vívidos e diplopia (AZARI et al., 2012; PELTONIEMI et al., 2016). Em roedores, já foi demonstrado que a cetamina pode induzir déficit cognitivo, abstinência, tolerância e ansiedade (CADDY et al., 2014; JIANG et al., 2017; TROFIMIUK et al., 2019).

A NAC aumenta os níveis de glutamato extra-sináptico que atua em receptores metabotrópicos, diminuindo a liberação de glutamato na fenda sináptica. Essa redução é benéfica por prevenir a excitotoxicidade e a ativação de receptores do tipo NMDA (DEAN; GIORLANDO; BERK, 2011b). Além do efeito indireto sobre receptores NMDA, um estudo pré-clínico demonstrou que a atividade tipo-antidepressiva pela NAC parece ser dependente da ativação de receptores AMPA. Esse efeito foi abolido por um antagonista de AMPA, chamado

NBQX (LINCK et al., 2012). Entretanto, a similaridade da NAC com o mecanismo de ação da cetamina, bem como sua atividade antidepressiva e ansiolítica, não está totalmente esclarecido.

Dessa forma, considerando o mecanismo de ação da NAC, nossa hipótese é que esse composto possui efeito tipo-antidepressivo/ansiolítico rápido, sustentado e profilático em peixes-zebra.

OBJETIVO

Investigar o efeito rápido, sustentado e profilático da N-acetilcisteína (NAC) sobre parâmetros comportamentais, oxidativos e moleculares em peixes-zebra submetidos a um modelo de estresse crônico imprevisível (ECI).

OBJETIVOS ESPECÍFICOS

i. Investigar o efeito rápido, sustentado e profilático da NAC sobre parâmetros comportamentais em peixes-zebra submetidos ao ECI.

ii. Investigar a participação do sistema glutamatérgico no mecanismo de ação da NAC em peixes-zebra submetidos ao ECI.

iii. Investigar o efeito da NAC sobre parâmetros de estresse oxidativo e moleculares em peixes-zebra submetidos ao ECI.

MATERIAIS E MÉTODOS

Animais

Foram usados 96 peixes-zebra (*Danio rerio*) adultos com aproximadamente 4 meses de idade (proporção 50:50 machos: fêmeas) do tipo selvagem (*wild-type,* fenótipo *short fin*) para melhor representar a heterogeneidade e variabilidade genética da população.

Habituação

Os peixes foram adquiridos em loja especializada (Delphis, Porto Alegre/RS), aclimatados em quarentena por 30 dias em tanques de 40 litros (45 x 35 x 30 cm) e alojados com densidade máxima de 2 peixes por litro. Após período de habituação, os animais foram transferidos para tanques de aclimatação de 16 litros (40 x 20 x 24 cm) de forma randomizada (com o auxílio de números aleatórios computadorizados, www.random.org), totalizando 12 animais (6 machos e 6 fêmeas) e mantidos durante 5 dias. Os peixes foram mantidos sobre condições ideais para a espécie, alimentados três vezes ao dia com ração comercial (Poytara, tropicais dia a dia, Araraquara/SP) e suplementados com *Artemia salina,* seguindo padrões do nosso laboratório (MOCELIN et al., 2018a, 2018b). A codificação dos tratamentos para os tanques de aclimatação foi realizada de forma aleatória e randomizada. Para a análise comportamental, os experimentadores foram cegados e cada aquário recebeu um código, realizado por um pesquisador que não participou dos experimentos, sendo os códigos revelados apenas durante a análise estatística.

Drogas

A N-acetilcisteína (NAC) foi adquirida da Sigma-Aldrich (St Louis, Missouri, EUA), a fluoxetina (FLU, Daforin®, EMS) e a cetamina (CET, Cetamin 10%, Syntec) foram adquiridos de lojas especializadas. As concentrações foram baseadas em estudos anteriores (MOCELIN et al., 2015; RIEHL et al., 2011). As soluções de NAC, fluoxetina e cetamina foram preparadas no dia da exposição.

Desenho experimental

O protocolo experimental foi baseado em estudos em roedores que mostraram que a cetamina apresenta efeito rápido, sustentado e profilático em modelos de estresse crônico (LI

et al., 2011; NEIS et al., 2016; ZANOS et al., 2016). Os grupos experimentais foram divididos em: controle (água do sistema), cetamina (20 mg/L), NAC (10 mg/L) e fluoxetina (10 mg/L). Após 5 dias de aclimatação, os peixes foram gentilmente transferidos em grupos de 12 animais para os respectivos tanques de exposição (4 litros; 18 x 18 x 18 cm). Cada grupo experimental originou-se de 2 tanques de aclimatação idênticos, totalizando 24 animais por grupo experimental. Os animais foram expostos às 8:00 durante 20 minutos, imediatamente após os animais retornaram aos seus tanques moradia. A análise comportamental foi realizada 24 horas após exposição, conforme mostra a figura 1:
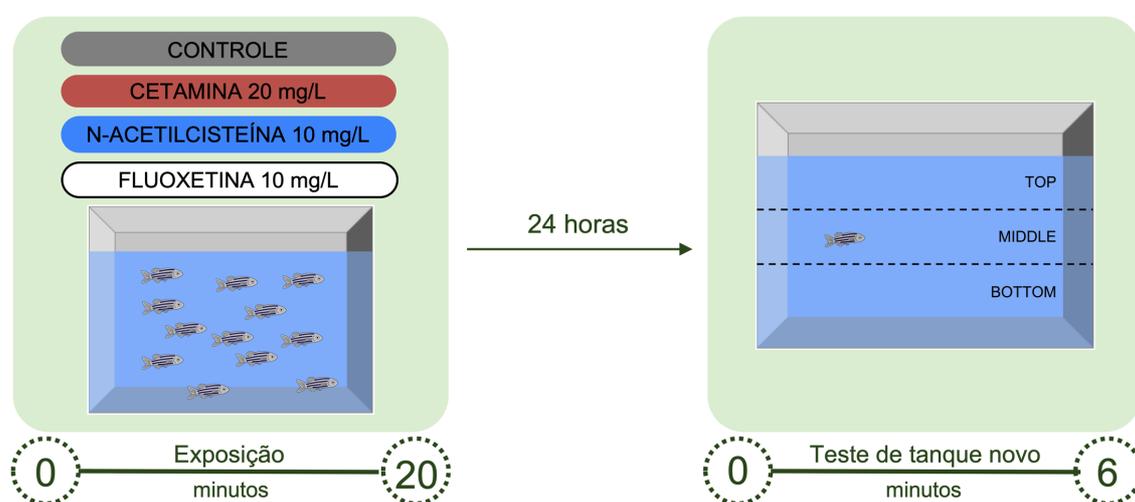
Figura 1. Desenho experimental. Os animais foram durante 20 minutos. Após 24 horas, os animais foram submetidos ao teste de tanque novo, filmados durante 6 minutos.

Análise comportamental

Os animais foram transferidos individualmente e cuidadosamente para o teste de tanque novo, filmados por 6 minutos e posteriormente analisados usando o software ANY-Maze (Stoelting Co., EUA). Os parâmetros avaliados foram: distancia total percorrida, número total de cruzamentos entre áreas, bem como número de entradas e tempo de permanência na área superior e inferior do aparato (MOCELIN et al., 2015, 2018a, 2018b).

Análise estatística

A normalidade dos dados foi analisada pelo teste de D'Agostino-Person e a homogeneidade das variâncias pelo teste de Levene. ANOVA de uma via seguida do teste *post hoc* de Tukey, foi utilizada para identificar os efeitos da exposição aos compostos. Os dados são expressos como média ± erro padrão da média (S.E.M). O nível de significância adotado foi p<0,05.

RESULTADOS E PERSPECTIVAS

A figura 2 mostra os efeitos da cetamina, NAC e fluoxetina no teste de tanque novo. Fluoxetina diminuiu significativamente a distância percorrida ($F_{3,92}$=16,24, p<0,0001, Fig. 2A), o número de entradas ($F_{3,92}$=8,54, p=0,0002, Fig. 2C) e tempo de permanência na área inferior do aparato ($F_{3,92}$=10,69, p=0,0002, Fig. 2E). Ao mesmo tempo, a fluoxetina aumentou significativamente o tempo de permanência na área superior do aparato ($F_{3,92}$=14,19, p<0,0001, Fig. 2F). Não foram observados efeitos dos tratamentos sobre o número de cruzamentos entre as diferentes áreas (Fig. 2B) e número de entradas na área superior do aparato (Fig. 2D).
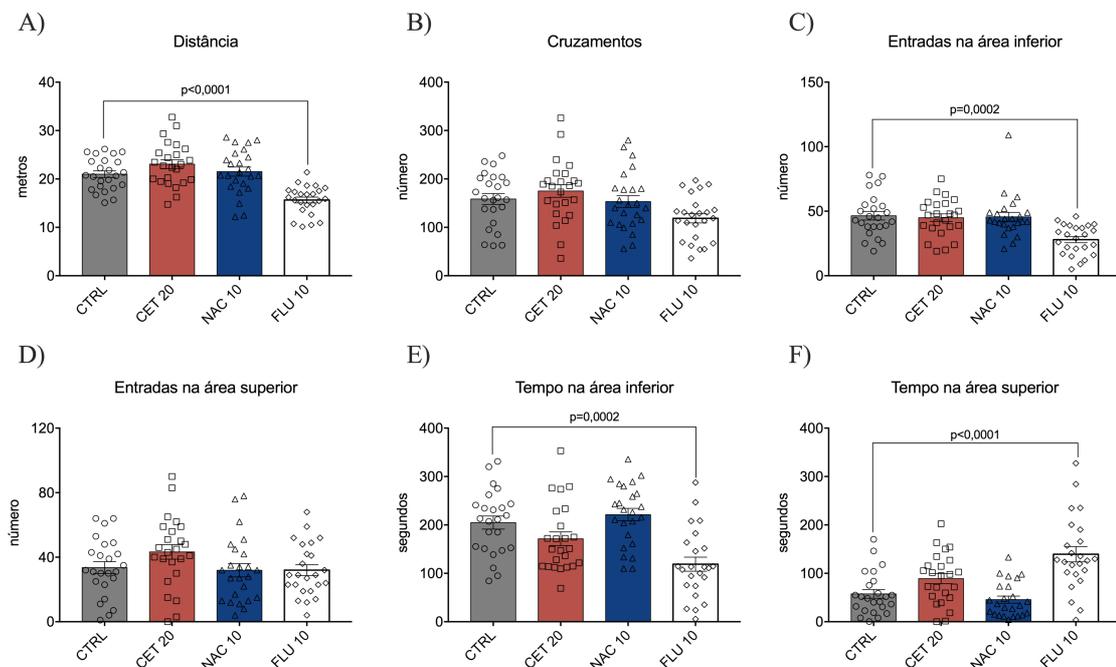
Figura 2. Efeitos da cetamina (CET, 20 mg/L), NAC (10 mg/L) e fluoxetina (FLU, 10 mg/L) sobre parâmetros comportamentais no teste de tanque novo em peixes-zebra. Distância percorrida (A), número de cruzamentos (B), entradas na área inferior (C), entradas na área superior (D), tempo na área inferior (E) e tempo na área superior (F). Os dados são apresentados como a média ± S.E.M. n=24

Os resultados aqui apresentados não corroboram com dados da literatura em roedores (ABDALLAH et al., 2018; BRACHMAN et al., 2016; KOIKE; CHAKI, 2014; KOIKE; IIJIMA; CHAKI, 2013; LI et al., 2011; MASTRODONATO et al., 2018; MCGOWAN et al., 2018; NEIS et al., 2016; ZANOS et al., 2016). Dessa forma, consideramos necessário mais experimentos a fim de demonstrar ou refutar nossa hipótese. Além disso, é imprescindível a ampliação de testes comportamentais que avaliem outros contextos neurobiológicos, como anedonia e cognição.

5. DISCUSSÃO

Esse trabalho pode ser sumarizado em três achados principais: (1) a N-acetilcisteína (NAC) reverte o comportamento tipo-ansiogênico e o dano oxidativo induzidos por um modelo de estresse crônico imprevisível (ECI), (2) a NAC previne as alterações comportamentais e o estresse oxidativo induzidos pela exposição aguda ao etanol, bem como (3) na abstinência após exposição crônica intermitente ao etanol.

Em estudo anterior mostramos que a NAC previne o efeito ansiogênico do estresse agudo por perseguição em peixes-zebra (MOCELIN et al., 2015). Nesse mesmo estudo, a NAC aumentou o tempo de permanência dos animais no lado claro do aparato claro/escuro, ou seja, possui atividade tipo-ansiolítica, comparável ao benzodiazepínico bromazepam (MOCELIN et al., 2015). Para ampliar a caracterização dos efeitos da NAC em modelo de estresse, no capítulo I mostramos os efeitos da NAC sobre parâmetros comportamentais e de estresse oxidativo em animais submetidos ao ECI. Em peixes-zebra, esse modelo é amplamente utilizado para a investigação dos mecanismos neurobiológicos e avaliação de novos compostos ansiolíticos/antidepressivos. É um modelo que apresenta validade de face, pois há correlatos comportamentais semelhantes aos observados em humanos submetidos a situações estressantes (aumento da ansiedade e redução do comportamento social), validade de constructo, pois as bases neurobiológicas são semelhantes às observadas em pacientes deprimidos (por exemplo, aumento de cortisol, aumento estresse oxidativo, neuroinflamação), e validade de predição, já que fármacos que são utilizados na clínica são efetivos nesse modelo (CHAKRAVARTY et al., 2013; FULCHER et al., 2017; MANUEL et al., 2014; MARCON et al., 2016, 2018; PAVLIDIS; THEODORIDI; TSALAFOUTA, 2015; PIATO et al., 2011; RAMBO et al., 2017; SONG et al., 2017; ZIMMERMANN et al., 2015). Nosso grupo mostrou que fármacos como fluoxetina, nortriptilina e bromazepam previnem o comportamento ansiogênico induzido pelo ECI (MARCON et al., 2016). O ECI induziu comportamento tipo-ansiogênico, ou seja,

diminuiu o tempo de permanência e o número de entradas na área superior, bem como aumentou o tempo de permanência na área inferior do aparato no teste de tanque novo. A NAC reverteu os efeitos comportamentais induzidos pelo ECI.

Para contribuir com a elucidação do mecanismo de ação de NAC no ECI, foram avaliados parâmetros de estresse oxidativo em encéfalo de peixes-zebra. Já foi demonstrado uma estreita relação entre transtornos mentais e o estresse oxidativo, ainda que não seja possível determinar causalidade (ANDERSON et al., 2014; MAES et al., 2011; MILLER; RAISON, 2016). Antidepressivos como a fluoxetina, imipramina, duloxetina e desipramina previnem o estresse oxidativo em modelos animais de estresse crônico (DEMIRDAŞ; NAZIROĞLU; ÖVEY, 2017; GŁOMBIK et al., 2017; NOVÍO et al., 2011; VILLA et al., 2017). Nosso modelo induziu lipoperoxidação (LPO) e aumento das espécies reativas de oxigênio (EROs), além de reduzir os níveis de glutationa (GSH) e a atividade das enzimas superóxido dismutase (SOD) e catalase (CAT). A NAC reverteu o dano oxidativo, confirmando seu efeito neuroprotetor já demonstrado em roedores (AYDIN et al., 2002; QUINTANILLA et al., 2018; SCHNEIDER et al., 2015; SEIVA et al., 2009).

A ingestão aguda de álcool está associada a sintomas periféricos e centrais desagradáveis (NIAAA, 2017). Além disso, em doses elevadas pode causar ressaca. A ressaca é resultado dos efeitos neurotóxicos do etanol e do seu metabólito acetaldeído por vias que envolvem as enzimas do citocromo P450 (CYP2E1), CAT e a álcool desidrogenase (ADH), responsáveis pelo aumento de radicais livres e dano oxidativo (HERNÁNDEZ; LÓPEZ-SÁNCHEZ; RENDÓN-RAMÍREZ, 2016; LOUVET; MATHURIN, 2015; REIMERS; FLOCKTON; TANGUAY, 2004; ZIMATKIN; DEITRICH, 1997). Em peixes-zebra, a exposição aguda ao etanol induz comportamento tipo-ansiogênico e dano oxidativo (ROSEMBERG et al., 2012). No capítulo II, mostramos os efeitos da NAC sobre parâmetros comportamentais e de estresse oxidativo em animais submetidos à exposição aguda ao etanol.

Animais expostos ao etanol apresentaram diminuição na distância percorrida, no número de cruzamentos, no número de entradas e no tempo de permanência na área superior do aparato no teste de tanque novo. Além disso, esses animais apresentaram aumento de EROs e LPO, com diminuição de GSH. Em roedores, a exposição aguda ao etanol induz dano oxidativo em encéfalos de ratos e a NAC previne tais alterações (OZKOL et al., 2017). Em nosso estudo, o pré-tratamento com NAC também preveniu o aumento da produção de EROs e LPO, além de bloquear a redução dos níveis de GSH, ampliando a caracterização neuroprotetora e colaborando com estudos em roedores. O uso anedótico da NAC para aliviar sintomas de ressaca tem sido previamente evidenciado em estudos clínicos (*Clinical Trials* identificador: NCT03104959 e NCT02541422). Em nosso estudo, expandimos a caracterização comportamental e antioxidante, defendendo a hipótese de que a NAC poderia ser indicada para prevenir os sintomas de ressaca, especialmente aqueles associados ao dano oxidativo.

Já a ingestão crônica de álcool está relaciona a efeitos deletérios graves para o indivíduo, sua família e a sociedade. Esse regime de consumo pode causar dependência e síndrome de abstinência, o que favorece a recaída de pacientes em tratamento (GRANT et al., 2015; KOSTEN; O'CONNOR, 2003; LITTEN et al., 2015; NIAAA, 2017; WHO, 2018). Para o tratamento do transtorno de uso de álcool são utilizados fármacos como o acamprosato, a naltrexona e o dissulfiram. Entretanto, o uso desses compostos está relacionado a eficácia limitada e a vários efeitos adversos como depressão, ansiedade, insônia, diarreia, anorexia, tontura, cefaleia, sonolência, dentre outros (ANTON et al., 2006; LITTEN et al., 2015; NIAAA, 2017; WHO, 2018). Portanto, há uma evidente necessidade de novos compostos para auxiliar os pacientes no tratamento do alcoolismo e manutenção da abstinência. Em roedores cronicamente expostos ao etanol, a retirada abrupta induz comportamento semelhante ao observado em humanos, ou seja, ansiedade, depressão, distúrbios autonômicos, cognitivos e motores (FERNANDES; GUPTA, 2019; HEILIG et al., 2010; KUMAR et al., 2016;

LEBOURGEOIS et al., 2018; SCHNEIDER et al., 2015; SLAWECKI; ROTH, 2004). Além dos efeitos comportamentais, o metabolismo do etanol no SNC induz a geração excessiva de EROs, aumento da expressão de RNAm e proteínas do CYP2E1, promovendo dano lipídico e redução de sistemas de defesa antioxidante não enzimáticos e enzimáticos (DEITRICH; ZIMATKIN; PRONKO, 2006; HAORAH et al., 2008; HUANG et al., 2009; KOOP, 2006; PARTHASARATHY; KATTIMANI; SRIDHAR, 2015; ZIMATKIN; DEITRICH, 1997). Em peixes-zebra foi demonstrado que a retirada abrupta do etanol é ansiogênica (MATHUR; GUO, 2011), mas não havia até o momento dados sobre o mecanismo relacionado a essas alterações. No capítulo III mostramos os efeitos da NAC sobre os parâmetros comportamentais e de estresse oxidativo em animais submetidos a um modelo de abstinência após exposição crônica intermitente ao etanol. O modelo diminuiu a distância percorrida, o número de entradas e tempo na área superior, bem como aumentou os episódios de imobilidade e o tempo de permanência na área inferior do aparato no teste de tanque novo. NAC preveniu esses efeitos comportamentais. Em estudos anteriores, NAC reduziu o comportamento de autoadministração, motivação e busca pelo etanol em modelo de abstinência em ratos (LEBOURGEOIS et al., 2019). Além disso, preveniu o comportamento tipo-depressivo e o dano locomotor em modelos semelhantes (LEBOURGEOIS et al., 2018; QUINTANILLA et al., 2018; SCHNEIDER et al., 2015; YAWALKAR; CHANGOTRA; GUPTA, 2018).

Em relação aos parâmetros de estresse oxidativo, peixes-zebra expostos cronicamente ao etanol apresentaram um aumento na LPO, diminuição dos níveis de GSH e da atividade das enzimas SOD e CAT (MÜLLER et al., 2017). Nossos dados corroboram com os observados, mas mostram que NAC foi capaz de prevenir tal desiquilíbrio no status oxidativo. Resultados semelhantes foram observados em estudos com roedores tratados com NAC (AYDIN et al., 2002; QUINTANILLA et al., 2018; SCHNEIDER et al., 2015; SEIVA et al., 2009). O papel do estresse oxidativo na abstinência ao etanol é demonstrada em diversos estudos (HAORAH

et al., 2008; HUANG et al., 2009; MARINO; AKSENOV; KELLY, 2004; TSAI et al., 1998), sugerindo que alterações em vias oxidativas podem estar ligadas às alterações comportamentais de pacientes alcoolistas. Nesse contexto, a restauração do status oxidativo pode representar uma nova proposta terapêutica na prevenção da neurotoxicidade desses pacientes. Nosso estudo corrobora e dá suporte científico ao corpo de evidências que mostram que a NAC é um composto promissor na terapia para abstinência de drogas de abuso, especialmente ao etanol, entretanto mais estudos clínicos são necessários para comprovar tais benefícios.

No capítulo IV avaliamos o possível efeito rápido da NAC sobre parâmetros comportamentais 24 horas após exposição aguda. Supreendentemente, a fluoxetina diminuiu a distância percorrida, o número de entradas e tempo na área inferior, além de aumentar o tempo na área superior no teste de tanque novo. Esses efeitos não foram observados em animais expostos à NAC ou cetamina, diferentemente do que já foi observado quando testados imediatamente após o tratamento (MOCELIN et al., 2015; RIEHL et al., 2011). Considerando o papel da NAC como neuromodulador de diversas vias relevantes aos transtornos mentais e sua similaridade com o mecanismo de ação da cetamina, justifica-se uma maior investigação da NAC para efeito rápido, sustentando e profilático em transtornos mentais. Mais estudos são necessários para ampliar a caracterização desses efeitos em peixes-zebra e para subsequente aplicação em estudos clínicos.

6. CONCLUSÃO

Os dados obtidos demonstram pela primeira vez que a NAC possui propriedades ansiolíticas e antioxidantes em modelos de estresse crônico imprevisível e na exposição aguda ou crônica ao etanol em peixes-zebra. Nossos resultados fornecem evidências e justificam a continuidade dos estudos com a NAC em transtornos mentais associados ao estresse e ao abuso de substâncias. Mostramos também que o peixe-zebra é um excelente organismo modelo para a investigação de novos compostos bem como para a elucidação dos mecanismos neurobiológicos associados.

7. PERSPECTIVAS

Com o objetivo de responder a hipótese do quarto objetivo, experimentos futuros serão realizados para a continuidade na investigação do mecanismo de ação neuroprotetor da NAC. Será investigado:

i. O efeito rápido, sustentando e profilático da NAC sobre parâmetros comportamentais, oxidativos e neuroinflamatórios em peixes-zebra submetidos ao ECI.

ii. O efeito da NAC sobre parâmetros cognitivos e anedonia no teste de labirinto em T (T-maze) em peixes-zebra submetidos ao ECI.

iii. A participação do sistema glutamatérgico no mecanismo de ação da NAC sobre parâmetros comportamentais, oxidativos e neuroinflamatórios em peixes-zebra submetidos ao ECI.

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ANEXO

7.1. CARTA DE APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA-UFRGS).

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CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 30914
Título: Investigação dos efeitos neuropsicofarmacológicos de N-acetilcisteína em peixes-zebra

Vigência: 04/04/2016 à 04/04/2020

Pesquisadores:

Equipe UFRGS:

ÂNGELO LUIS STAPASSOLI PIATO - coordenador desde 04/04/2016
DÉBORA MOCELLIN VILLANOVA - Outra Função desde 04/04/2016
Ricieri Naue Mocelin - Aluno de Doutorado desde 04/04/2016
Matheus Felipe Marcon - Aluno de Doutorado desde 04/04/2016

Comissão De Ética No Uso De Animais aprovou o mesmo em seus aspectos éticos e metodológicos, para a utilização de 1020 peixes-zebra, adultos, jovens, provenientes 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, 4 de Maio de 2016

MARCELO MELLER ALIEVI
Coordenador da comissão de ética

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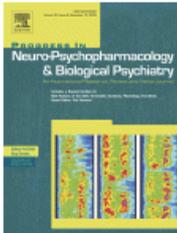Autorização para reprodução e utilização do artigo científico do Capítulo III.

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Author: Ricieri Mocelin,Matheus Marcon,Alex Sander da Rosa Araujo,Ana Paula Herrmann,Angelo Piato

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7.3. PARTICIPAÇÃO EM PUBLICAÇÕES DO GRUPO DE PESQUISA AO LONGO DA TESE

MARCON M, MOCELIN R, DE OLIVEIRA DL, DA ROSA ARAUJO AS, HERRMANN AP, PIATO A. Acetyl-L-carnitine as a putative candidate for the treatment of stress-related psychiatric disorders: Novel evidence from a zebrafish model. Neuropharmacology. 2019 Mar;150:145-152. doi: 10.1016/j.neuropharm.2019.03.024.

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