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Talise Ellwanger Müller

ALTERAÇÕES COMPORTAMENTAIS MEDIADAS PELO ETANOL EM PEIXE-ZEBRA: INFLUÊNCIA DO ESTRESSE OXIDATIVO, DISFUNÇÃO MITOCONDRIAL E MODULAÇÃO SEROTONINÉRGICA

Santa Maria, RS
2020

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Tese apresentada ao programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM), como requisito parcial para a obtenção do título de **Doutora em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador: Prof. Dr. Denis Broock Rosemberg

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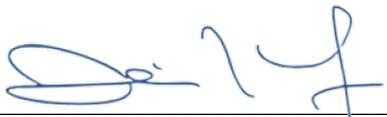

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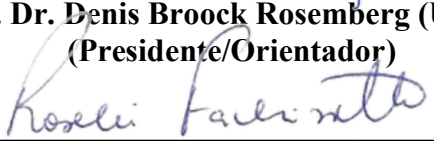

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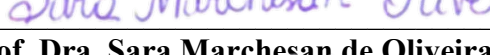

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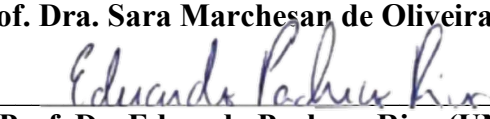

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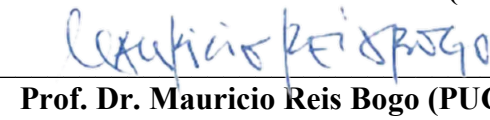

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(Viagem ao centro da Terra - Julio Verne)

RESUMO

ALTERAÇÕES COMPORTAMENTAIS MEDIADAS PELO ETANOL EM PEIXE-ZEBRA: INFLUÊNCIA DO ESTRESSE OXIDATIVO, DISFUNÇÃO MITOCONDRIAL E MODULAÇÃO SEROTONINÉRGICA

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ORIENTADOR: Denis Broock Rosemberg

O consumo de etanol é um grave problema de saúde pública devido aos impactos negativos que causa nos indivíduos e na sociedade. Pelo fato do mecanismo de ação do etanol no sistema nervoso central ser complexo e não totalmente compreendido, os medicamentos disponíveis para tratar as desordens relacionadas ao uso de álcool não apresentam boa eficácia. Assim, a validação de novos modelos experimentais para o estudo translacional das desordens induzidas pelo álcool é importante. O peixe-zebra (*Danio rerio*) tem sido utilizado com sucesso para investigar os mecanismos relacionados ao abuso e vício ao etanol. Porém, muitos aspectos bioquímicos e comportamentais ainda necessitam ser explorados e avaliados dentro dos modelos de exposição ao etanol em peixe-zebra. No presente estudo, utilizamos protocolos agudos e crônicos de exposição ao etanol em peixe-zebra com o objetivo de investigar aspectos relacionados ao estresse oxidativo, bioenergética e o potencial envolvimento do sistema serotoninérgico nas respostas neurocomportamentais induzidas pelo álcool. Em um primeiro estudo, verificamos que a exposição crônica ao etanol (1.0% v/v) por 20 minutos durante 8 dias gerou um efeito do tipo ansiogênico, aumentando o comportamento social e promovendo estresse oxidativo. Ao avaliar efeitos do etanol sobre a respiração mitocondrial no segundo estudo, observamos que a exposição aguda ao etanol (1.0% v/v por 1 hora) estimulou o processo de fosforilação oxidativa e aumentou a funcionalidade da mitocôndria em encéfalo de peixe-zebra, enquanto que a exposição crônica, similar ao protocolo do primeiro estudo, prejudicou a transferência de elétrons entre os complexos I e II da cadeia respiratória mitocondrial. No terceiro estudo, no qual avaliamos o envolvimento do sistema serotoninérgico nas respostas agudas ao etanol, verificamos que o comportamento de agressividade induzido pelo etanol (0.25% v/v por 1 hora) é modulado pela via serotoninérgica com ação principal do receptor 5-HT_{2A}, enquanto que as respostas do tipo ansiolíticas observadas após a exposição a uma concentração moderada de etanol (0.5% v/v por 1 hora) são moduladas principalmente pelo receptor 5-$HT_{1B.}$ As respostas depressoras induzidas pelo etanol (1.0% v/v por 1 hora) não foram moduladas por fármacos com ação serotoninérgica. Em suma, de modo similar ao que ocorre em humanos, verificamos que as repostas mediadas pelo etanol em diferentes protocolos experimentais envolvem alterações no comportamento social, estresse oxidativo, disfunção mitocondrial e modulação serotoninérgica em peixe-zebra. Nossos achados auxiliam na elucidação dos mecanismos centrais de ação do etanol e comportamentos associados, reforçando o valor preditivo, de face e de construto dos modelos de exposição ao etanol em peixe-zebra. Esses novos resultados permitirão a expansão dos estudos translacionais utilizando este organismo modelo em pesquisas científicas visando elucidar os mecanismos subjacentes ao abuso e vício ao etanol.

Palavras-chave: Álcool, Peixe-zebra, Comportamento, Bioquímica, Serotonina.

ABSTRACT

ETHANOL-INDUCED BEHAVIORAL CHANGES IN ZEBRAFISH: INFLUENCE OF OXIDATIVE STRESS, MITOCHONDRIAL DYSFUNCTION, AND SEROTONERGIC MODULATION

AUTHOR: Talise Ellwanger Müller
ADVISOR: Denis Broock Rosemberg

Ethanol consumption is a serious public health problem due to the negative impacts that affect individuals and society. Because the mechanism of action of ethanol in the central nervous system is complex and not fully understood, the drugs available to treat alcohol use-related disorders are not effective. Thus, the validation of new experimental models for translational studies of alcohol-induced disorders is important. The zebrafish (*Danio rerio*) has been used successfully to investigate the mechanisms related to ethanol abuse and addiction. However, several biochemical and behavioral features of ethanol exposure protocols in zebrafish still need to be explored. In the present study, we used acute and chronic ethanol exposure protocols in zebrafish to investigate biochemical aspects related to oxidative stress, bioenergetics, and the potential involvement of the serotonergic system in the neurobehavioral responses induced by alcohol. In a first study, we found that chronic exposure to ethanol (1.0% v/v) for 20 minutes for 8 days induced an anxiogenic effect, increasing social behavior and promoting oxidative stress. When assessing the effects of ethanol on mitochondrial respiration in the second study, we observed that acute exposure to ethanol (1.0% v/v for 1 hour) stimulated the oxidative phosphorylation process and increased the functionality of mitochondria in zebrafish brain, while the chronic exposure, similar to the protocol of the first study, impaired the transfer of electrons between I and II complexes of the mitochondrial respiratory chain. In the third study, we evaluated the involvement of the serotonergic system in acute responses to ethanol. Ethanol-induced aggressive behavior (0.25% v/v for 1 hour) is modulated by the serotonergic pathway with the main action of the 5-HT_{2A} receptor, while the anxiolytic-like responses observed after exposure to a moderate concentration of ethanol (0.5% v/v for 1 hour) are modulated mainly by the 5-HT_{1B} receptor. Depressive responses induced by ethanol (1.0% v/v for 1 hour) were not modulated by serotonergic drugs. In summary, similarly to what occurs in humans, we found that the responses mediated by ethanol in different experimental protocols involve changes in social behavior, oxidative stress, mitochondrial dysfunction, and serotonergic modulation in zebrafish. Our findings help elucidate the central mechanisms of action of ethanol and associated behaviors, reinforcing the predictive, face, and construct value of ethanol exposure models in zebrafish. These new results will allow the expansion of translational studies using this model organism in scientific research aimed at elucidating the mechanisms underlying the alcohol abuse and addiction.

Keywords: Alcohol, Zebrafish, Behavior, Biochemistry, Serotonin.

ABREVIAÇÕES

5-HT	Serotonina
ADH	Álcool desidrogenase
ADHL	Acetaldeído desidrogenase
Ca^{2+}	Íon cálcio
CAT	Catalase
DA	Dopamina
DUA	Desordens relacionadas ao uso de álcool
EO	Estresse oxidativo
ERO	Espécies reativas de oxigênio
FDA	Do inglês, “Food and Drug Administration”
GPx	Glutationa peroxidase
GR	Glutationa redutase
H_2O_2	Peróxido de hidrogênio
MEOS	Sistema microssomal de oxidação do etanol
NAD^+	Nicotinamida adenina dinucleotídeo
NADPH	Fosfato de dinucleotídeo de nicotinamida adenina
NT	Neurotransmissores
$O_2^{\cdot -}$	Radical superóxido
$OH^{\cdot -}$	Radical hidroxila
pCPA	*p*-clorofenilalanina
REDOX	Oxirredução
SNC	Sistema nervoso central
SOD	Superóxido dismutase

SUMÁRIO

1. APRESENTAÇÃO

Esta tese está apresentada na seguinte forma: primeiramente são descritas as partes referentes à introdução, justificativa, hipóteses e objetivos. As metodologias utilizadas, os resultados e a discussão dos dados obtidos são descritos na forma de três artigos publicados. A interpretação geral e integrada dos dados é descrita na sessão discussão e finalizada na conclusão do estudo. As referências bibliográficas utilizadas são apresentadas no final da tese.

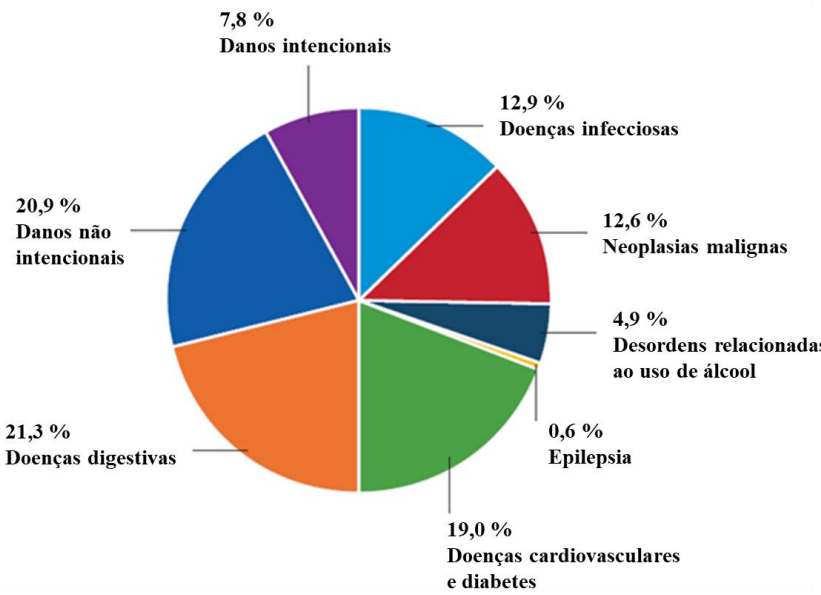
2. INTRODUÇÃO

2.1 Abuso de álcool e alcoolismo: um problema de saúde pública

Atualmente, o consumo excessivo de bebidas alcoólicas é um problema grave de saúde pública devido à alta prevalência e consequências multidimensionais à saúde da população (PREUSS et al., 2018; WHO, 2018). Estudos têm demonstrado que o álcool (etanol, álcool etílico) é a droga de abuso que mais causa prejuízos para o indivíduo e para a sociedade. Quando avaliados fatores como mortes relacionadas ao uso da droga, dependência química, crimes e danos (intencionais e não intencionais), o álcool é classificado como mais nocivo que drogas como heroína, crack e metanfetamina (NUTT, KING e PHILLIPS, 2010; VAN AMSTERDAM et al., 2015). Tradições socioculturais, baixo custo e fácil acesso facilitam o consumo de etanol, contribuindo para o desenvolvimento dos distúrbios relacionadas ao uso de álcool (DUA) (SUDHINARASET et al., 2016). Os DUA consistem em condições mentais caracterizadas pelo uso compulsivo de álcool, perda de controle sobre a ingestão de álcool e desenvolvimento de um estado emocional negativo quando a droga não está em uso (NIH, 2020). A adiccção o ao álcool foi recentemente recontextualizada como uma doença crônica cerebral passível de tratamento (NIH, 2020).

Anualmente, mais de 3 milhões de pessoas no mundo são vítimas dos efeitos do etanol (Figura 1), uma vez que seu consumo está relacionado com o desenvolvimento de mais de 200 doenças (WHO, 2018). A mortalidade resultante do consumo de álcool é maior do que a causada por doenças como tuberculose, HIV/AIDS e diabetes (WHO, 2018). Dentre as principais doenças associadas ao consumo de álcool estão os DUA, doenças gastrointestinais e cardiovasculares (OBAD et al., 2018; SHIELD, PARRY e REHM, 2013). Além disso, evidências sugerem que o consumo de álcool também está relacionado à incidência de doenças infecciosas como tuberculose e HIV/AIDS, uma vez que os indivíduos exibem mais comportamentos de risco (REHM et al., 2017). Danos intencionais (suicídio e violência) ou não-intencionais (devido ao comprometimento das habilidades psicomotoras) também contribuem com um número significativo na taxa de mortalidade, a qual representa 5,3 % das mortes do mundo. Ainda, estima-se que 50 a 75% dos alcoólatras exibem comprometimentos cognitivos relacionados à atenção e memória e dano cerebral estrutural (HARPER, 2009).

Figura 1 – Porcentagem de mortes relacionadas ao uso de álcool em 2016
(total líquido = 3 milhões de mortes)



Fonte: Adaptado de WHO, 2018.

A etiologia da dependência ao álcool é complexa e envolve fatores individuais como genótipo do indivíduo, gênero e idade, e fatores de interação indivíduo-ambiente como aspectos sociais e culturais (SAMOCHOWIEC et al., 2013; WALL, LUCZAK e HILLER-STURMHÖFEL, 2016). Apesar dos notáveis impactos dos DUA na saúde pública, os tratamentos farmacológicos para a dependência ao etanol são subutilizados. Atualmente, existem apenas três medicamentos aprovados pelo *Food and Drug Administration* (FDA, 2020) para tratar a dependência ao álcool. O dissulfiram que atua inibindo a enzima aldeído desidrogenase gerando acúmulo de aldeído no organismo, a naltrexona que age bloqueando receptores opioides envolvidos nos efeitos de recompensa da droga e o acamprosato que tem ação sobre os sistemas neurotransmissores (NT) glutamatérgicos e GABAérgicos e age reduzindo sintomas da retirada da droga (CASTRO e BALTIERI, 2004). Entretanto, estes medicamentos estão envolvidos no surgimento de inúmeros efeitos adversos (cefaleia, náusea, vômito) e não apresentam boa eficácia no tratamento da dependência ao álcool e na reabilitação do indivíduo (ANTONELLI et al., 2018; GOH e MORGAN, 2017). Esses fatos podem estar associados ao mecanismo de ação do etanol ainda não ser completamente elucidado. Portanto, entender a relação entre genes, função neuronal e comportamento é um

aspecto chave para identificar de forma rápida e eficaz novos alvos terapêuticos objetivando o tratamento da adicção ao etanol.

2.2 Efeitos neuroquímicos e comportamentais associados ao consumo de etanol

Devido a sua característica lipofílica, o álcool se difunde facilmente pelas membranas das células, distribuindo-se de forma rápida pela corrente sanguínea aos órgãos e tecidos (EŞEL e DINÇ, 2017). A maior parte da absorção do álcool acontece no trato gastrointestinal, mas tecidos como encéfalo e os pulmões, por serem altamente vascularizados, recebem doses iniciais de etanol mais rapidamente (MULLEN, 1977). Portanto, o etanol atravessa facilmente a barreira hematoencefálica e atua sob o sistema nervoso central (SNC) de modo rápido, podendo desencadear alterações moleculares, neuroquímicas e comportamentais dependendo da dose ingerida (EŞEL e DINÇ, 2017; SPANAGEL, 2009).

O consumo agudo de etanol exerce um efeito bifásico dependente de dose nos indivíduos: inicialmente promove euforia, desinibição e efeitos ansiolíticos, com posterior sedação, falta de coordenação e sonolência (HANDLER et al., 2013). Em baixas doses, o consumo de etanol promove estados de humor positivos, desinibição do comportamento punido e efeitos de alívio de ansiedade/estresse (HARRISON et al., 2017). No entanto, as alterações neuroquímicas desencadeadas pela droga não são apenas responsáveis pelos efeitos fisiológicos agudos, mas também pelo desenvolvimento da dependência ao etanol (GILPIN e KOOB, 2008). Cronicamente, o consumo de etanol induz a um processo neuroadaptativo que pode levar ao desenvolvimento das DUA, que são classificadas pela Organização Mundial da Saúde como distúrbios neuropsiquiátricos (WHO, 2018). Consumidores compulsivos de etanol exibem perda de controle comportamental em relação à busca pela droga, tolerância, síndrome de retirada e sintomas de abstinência, os quais podem gerar ansiedade, episódios depressivos, déficits cognitivos, insônia e náuseas (ABRAHAO, SALINAS e LOVINGER, 2017; BANERJEE, 2014; HAMMOUD e JIMENEZ-SHAHED, 2019). Além disso, alterações no comportamento social dos indivíduos também são observadas após um consumo crônico de etanol (LE BERRE, 2019).

Diferente de outras drogas psicotrópicas, o etanol apresenta uma ação pleiotrópica sobre o SNC, influenciando diferentes alvos moleculares e vias bioquímicas. Seu mecanismo de ação ainda não é completamente elucidado, mas sabe-se que o etanol modula as vias de transdução de sinais inibitórias e excitatórias do SNC, assim como indiretamente o sistema de recompensa mediado por dopamina (DA) e serotonina (5-HT) (CHASTAIN, 2006;

QUERTEMONT, TAMBOUR e TIRELLI, 2005). De uma forma geral, o etanol atua diminuindo a atividade metabólica do encéfalo (WANG et al., 2000) e a neurotransmissão glutamatérgica (HWA et al., 2017; ROBERTO e VARODAYAN, 2017), enquanto age potencializando as sinapses mediadas pelos NT ácido gama aminobutírico (GABA) e glicina (BREESE et al., 2006; SODERPALM et al., 2017; ZHU e LOVINGER, 2006). Ainda, o etanol reduz o fluxo transmembrana do íon cálcio (Ca^{2+}) pela inibição dos canais de Ca^{2+} tipo L, os quais desempenham um importante papel nos comportamentos depressivos e na neuroadaptação induzida pelo etanol (HENDRICSON et al., 2003; MULHOLLAND et al., 2011; NIMITVILAI et al., 2016).

O sistema de recompensa modulado por DA e 5-HT apresenta homeostase alterada de forma dependente de dose e de tempo após o consumo de etanol (ERDOZAIN e CALLADO, 2014; GILPIN e KOOB, 2008). Os sistemas serotoninérgico e dopaminérgico atuam na regulação de parâmetros como locomoção, cognição, emoção, agressividade e ansiedade (BISSONETE e ROESCH, 2016; PARSEY, 2010), contribuindo nos efeitos fisiológicos e comportamentais em indivíduos consumidores de álcool. Baixas doses de etanol aumentam bruscamente a liberação de 5-HT e DA no núcleo accumbens facilitando o reforço positivo pela droga. Esse reforço positivo é associado a sensações de prazer, fazendo com que a busca pela droga se torne cada vez mais constante (MARCINKIEWCZ, 2015; MOREL, MONTGOMERY e HAN, 2018; NUTT et al., 2015). De maneira oposta, o consumo crônico de etanol reduz os níveis desses NT no SNC, desencadeando estados emocionais negativos quando ocorre a retirada do etanol (ERDOZAIN e CALLADO, 2014). Assim, sugere-se que os sistemas modulados por 5-HT e DA estão envolvido nos efeitos de reforço tanto positivos (recompensa) quanto negativos (síndrome de abstinência) do etanol.

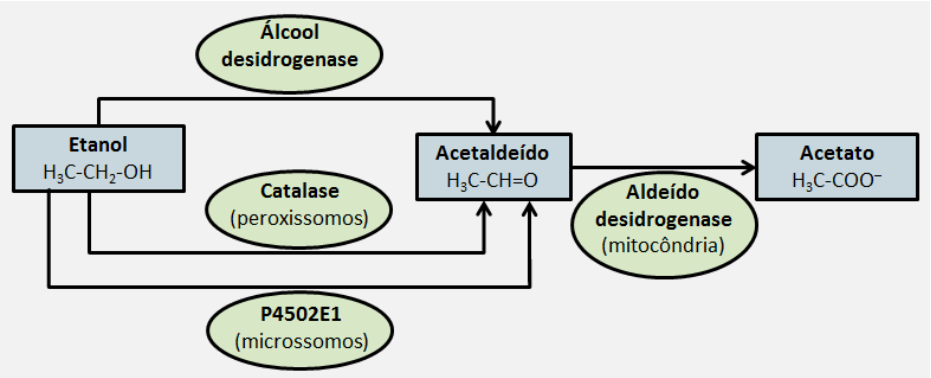
A via serotoninérgica é diretamente relacionada com o desenvolvimento da dependência ao álcool (CHASTAIN, 2006; EŞEL e DINÇ, 2017; MARCINKIEWCZ, 2015). O NT 5-HT tem grande papel no aumento do consumo do álcool, ciclo vicioso e reincidências, pois regula comportamentos de impulsividade, motivação, medo e agressividade (KIRBY, ZEEB e WINSTANLEY, 2011; SARI, JOHNSON e WEEDMAN, 2011). Estudos têm demonstrado que o consumo de álcool afeta a funcionalidade e expressão dos receptores 5-HT, alterando a homeostase e atividade do sistema serotoninérgico (SARI, JOHNSON e WEEDMAN, 2011; STORVIK et al., 2006). O etanol agudamente é capaz de aumentar a liberação de 5-HT no SNC (MCBRIDE, 2002; SARI, JOHNSON e WEEDMAN, 2011), enquanto que durante o uso crônico ou na retirada, as ações do sistema serotoninérgico são suprimidas, ocorrendo à diminuição da atividade de receptores, transportadores e de todas

as funções reguladas por 5-HT no encéfalo (SARI et al., 2011; STORVIK et al., 2006). Níveis anormais de 5-HT nas sinapses podem contribuir para o desenvolvimento do abuso de álcool, uma vez que estudos encontraram níveis reduzidos de 5-HT no encéfalo de indivíduos alcoólatras e de animais expostos ao etanol cronicamente. Tal redução é relacionada ao desenvolvimento de depressões clínicas e também a atos de violência ocasionalmente verificados sob seu efeito (GLICK, 2015; HEINZ et al., 2011; LOVINGER, 1997; MICZEK et al., 2015). Além disso, a liberação de 5-HT pode afetar o sistema GABAérgico e aumentar a produção de DA, NT que desempenham um papel importante no processo de tomada de decisões (SAMSOM e HARRIS, 1992) e comportamentos emocionais (CHASTAIN, 2006). Portanto, a análise do envolvimento do sistema serotoninérgico nos comportamentos mediados por etanol, bem como a exploração de mecanismos de ação sobre essa via possibilitará a descoberta de novos alvos terapêuticos para tratar o abuso de álcool e alcoolismo.

2.3 Estresse oxidativo e disfunções mitocondriais como consequências do consumo de etanol

Além dos efeitos neuroquímicos citados, o etanol também age causando desequilíbrios no sistema de oxirredução (REDOX) do SNC, o que pode culminar em estresse oxidativo (EO) (AUGUSTYNIAK; MICHALAK e SKRZYDLEWSKA, 2005; PEREIRA, ANDRADE e VALENTÃO, 2015). O etanol é oxidado a acetaldeído no encéfalo por três vias: pela via da enzima álcool desidrogenase (ADH) no citosol, utilizando o cofator nicotinamida adenina dinucleotídeo (NAD^+) que é reduzido a NADH; pela via da enzima catalase nos peroxissomos, onde o etanol doa elétrons reduzindo o peróxido de hidrogênio (H_2O_2) em água; e pela via do sistema microssomal de oxidação do etanol (MEOS) no retículo endoplasmático liso, através do citocromo P450 (isoforma CYP2E1) e seu cofator fosfato de dinucleotídeo de nicotinamida adenina (NADPH) (ZIMATKIN, 2006). Uma vez formado, o acetaldeído é rapidamente metabolizado em acetato pela família de isoenzima acetaldeído desidrogenase (ADHL) (Figura 2). Este processo ocorre dentro das mitocôndrias, onde o acetaldeído é oxidado em acetato com o auxílio do cofator NAD^+, gerando também como outro produto final o NADH. Grande parte do acetato formado segue para corrente sanguínea e é oxidado em dióxido de carbono ou metabolizado em acetil-CoA (ISRAEL, ORREGO e CARMICHAEL, 1994).

Figura 2 – Metabolismo oxidativo do etanol



Fonte: Adaptado de Alcohol Alert (2001).

O acetaldeído apresenta um importante papel da neurotoxicidade induzida pelo etanol. Além de causar danos diretos como a formação de adutos com estruturas celulares, sua produção é associada à formação de espécies reativas de oxigênio (ERO) como o ânion superóxido ($O_2^{\cdot-}$), peróxido de hidrogênio (H_2O_2) e radical hidroxila ($OH^{\cdot-}$) (BORJA-OLIVEIRA, 2014; LAMBETH e NEISH, 2014; PEREIRA et al., 2015). Essas espécies reativas formadas podem afetar diretamente biomoléculas como DNA, lipídeos e proteínas, causando danos estruturais que modificam os sistemas de NT, a fluidez das membranas e a função mitocondrial, levando à ativação de vias pró-apoptóticas (COMPORTI et al., 2010; GARCÍA-SUÁSTEGUI et al., 2017; GONZÁLEZ-REIMERS et al., 2004; WU e CEDERBAUM, 2003).

As enzimas antioxidantes presentes no SNC como a superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa redutase (GR) tem atividade alterada conforme o tempo e quantidade de ingestão de álcool (AUGUSTYNIAK et al., 2005; HERNÁNDEZ et al., 2016; ZIMATKIN e BUBEN, 2007). Os níveis de glutationa, uma das principais defesas não enzimáticas que atua neutralizando xenobióticos, também se mostram diminuídos no SNC após exposição crônica ao etanol (AUGUSTYNIAK et al., 2005). É de extrema importância que as enzimas do sistema de defesa antioxidante atuem corretamente para neutralizar as ERO produzidas pela oxidação do etanol, a fim de manter a homeostasia do SNC (AUGUSTYNIAK et al., 2005).

O consumo de etanol também pode afetar a atividade neuronal pela alteração da função mitocondrial (ALMANSA et al., 2009; MANZO-AVALOS e SAAVEDRA-MOLINA, 2010). As mitocôndrias são as maiores fontes geradoras de ERO como produto

secundário da respiração aeróbica, porém, também são alvos das ERO (JUNG, 2015). Durante o metabolismo do etanol, o NADH é convertido em sua forma oxidada NAD^+ e a geração de radicais livres aumenta (SLATER, 1984). Os radicais livres reagem causando a peroxidação dos fosfolipídios da membrana mitocondrial interna e externa, alterando a permeabilidade e homeostase dessas membranas (REDDY et al., 2013). As membranas da mitocôndria regulam o transporte de substratos energéticos via β-oxidação, e um desequilíbrio neste processo pode prejudicar a fosforilação oxidativa e causar a depleção da produção de energia no encéfalo (REDDY et al., 2013). A oxidação das proteínas mitocondriais (HOEK, CAHILL e PASTORINO, 2002) e alterações funcionais entre os complexos I e III da cadeia transportadora de elétrons também são relatados após a exposição ao etanol (BAILEY; PIETSCH e CUNNINGHAM, 1999). É sabido que essas condições comprometem o funcionamento da cadeia respiratória, resultando em diminuição da produção energia e morte celular (ESTAQUIER et al., 2011; JUNG, 2015; PEREIRA, ANDRADE e VALENTÃO, 2015). O desequilíbrio redox e as alterações no metabolismo energético parecem estar diretamente envolvidos na neurotoxicidade mediada pelo etanol.

2.4 O peixe-zebra como modelo para estudar os efeitos neurocomportamentais do etanol

O uso de modelos animais não tradicionais é crescente nas pesquisas relacionadas à neurociência comportamental, e o peixe-zebra (*Danio rerio*) tem se mostrado um organismo de ampla utilidade para estudar os efeitos do etanol em diferentes protocolos (KLEE et al., 2012; NINKOVIC e BALLY-CUIF, 2006; STEWART et al., 2011; TRAN, FACCIOL e GERLAI, 2016). O estudo dessa espécie começou no final da década de 60 por George Streisinger através de técnicas de mutagênese e foi de grande valia para o avanço no conhecimento da embriogênese e ciclo de vida dos vertebrados (GRUNWALD e EISEN, 2002). O peixe-zebra é um pequeno teleósteo (família Cyprinidae) que possui cerca de 3 a 4 cm de comprimento, ovos translúcidos, grande prole e rápido desenvolvimento (DAHM e GEISLER, 2006; GRUNWALD e EISEN, 2002). A espécie é atraente por necessitar um pequeno espaço para manutenção, apresentar baixo custo e por ser de fácil manipulação genética (GOLDSMITH, 2004; LIESCHKE e CURRIE 2007; KALUEFF et al., 2013). O peixe-zebra também tem sido bastante utilizado em triagens em larga escala para identificar compostos terapêuticos e possíveis alvos moleculares de forma rápida e eficaz. Reagentes e drogas podem ser testados diretamente na água, ou aplicados via intraperitoneal, o que reduz significativamente a quantidade das substâncias a serem testadas (GOLDSMITH, 2004). Por

exemplo, a administração de etanol nesta espécie é feita pela imersão do animal em uma solução contendo etanol, o qual é absorvido pelos vasos sanguíneos das brânquias e da pele, fazendo com que os níveis sanguíneos de etanol atinjam rapidamente um equilíbrio com os niveis externos do tanque (GERLAI et al., 2000; PAN et al., 2011).

O peixe-zebra possui o genoma evolutivamente conservado e completamente sequenciado, compartilhando um alto grau de similaridade com seus ortólogos em mamíferos (aproximadamente 70%) (BARBAZUK et al., 2000; HOWE et al., 2013; LIESCHKE e CURRIE, 2007; MACRAE e PERTERSON, 2015). Vários desses genes ortólogos estão envolvidos nos processos de abuso e adicção ao etanol (CHATTERJEE, SHAMS e GERLAI, 2014; GERLAI et al., 2009; KLEE et al., 2012; MILLER et al., 2013), destacando o valor translacional das pesquisas com peixe-zebra nesta área de estudo. Diversos sistemas de NT, como glutamatérgico (EDWARDS e MICHEL, 2002; RICO et al., 2010), colinérgico (BEHRA et al., 2002), dopaminérgico (BOEHMLER et al., 2004), serotoninérgico (HERCULANO et al., 2013), GABAérgico (KIM et al., 2004) também já foram caracterizados nesta espécie. Apesar do evento da duplicação do genoma dos teleósteos ter modificado o número de genes que codificam proteínas relacionadas à síntese, transporte e sinalização dos sistemas de NT, o padrão de expressão dessas proteínas em peixe-zebra é similar ao de outros vertebrados, e suas funcionalidades são evolutivamente conservadas (HORZMANN e FREEMAN, 2016). Além disso, mesmo sendo observadas diferenças anatômicas entre o encéfalo do peixe-zebra e dos mamíferos, diversas áreas são identificadas como homólogas e desempenham funções semelhantes (RANDLETT et al., 2015; ULLMAN et al., 2010). Por exemplo, o pálio lateral da área telencefálica do peixe-zebra é responsável pelo processamento da memória e é homólogo ao hipocampo em mamíferos. Da mesma forma, a habenula do peixe zebra, associada às respostas ao medo, corresponde anatomicamente à amígdala em mamíferos (AGETSUMA et al., 2010; PERATHONER et al., 2016). O sistema de recompensa do peixe-zebra também apresenta regiões homólogas aos mamíferos, por exemplo, o núcleo tubular posterior é homólogo a área tegumental ventral em mamíferos e o núcleo telencefálico ventral e dorsal é homólogo ao núcleo de accumbens em mamíferos, homologia que permite o desenvolvimento de comportamento motivacional e busca por drogas em peixe-zebra (PARKER et al., 2013).

Estudos relatam uma correlação entre a liberação de 5-HT no encéfalo de peixe-zebra com comportamentos específicos (por exemplo, medo, ansiedade e agressão) (HERCULANO e MAXIMINO, 2014; MAXIMINO et al., 2013a). Embora o sistema serotoninérgico do peixe-zebra apresente algumas diferenças genéticas e neuroanatômicas em relação ao dos

mamíferos, os genes que codificam proteínas relacionadas à síntese, transporte, sinalização e degradação de 5-HT são similares aos de outros vertebrados (MAXIMINO et al., 2013b). Dessa forma, os efeitos das drogas que modulam o metabolismo da 5-HT são conservados entre as espécies (MAXIMINO e HERCULANO, 2010). Tanto as drogas agonistas/antagonistas serotoninérgicas, quanto a *p*-clorofenilalanina (pCPA), um inibidor da triptofano hidroxilase, modulam parâmetros do tipo ansiedade e locomoção em larvas e peixes-zebra adultos (AIRHART et al., 2012; SALLINEN et al., 2009). Considerando que manipulações farmacológicas no sistema serotoninérgico produzem efeitos comportamentais e neuroendócrinos robustos em peixe-zebra, o uso desta espécie em estudos translacionais relacionados a mecanismos serotoninérgicos no SNC é bastante promissor.

O peixe-zebra adulto apresenta um repertório comportamental bastante complexo e já caracterizado (KALUEFF et al., 2013). A exposição a agentes estressores pode evocar medo ou comportamentos do tipo ansiedade facilmente quantificáveis através de exploração reduzida, aumento da escototaxia (preferência pela escuridão), geotaxia (resposta de mergulho), tigmotaxia (preferência pela periferia do tanque), avaliação de risco (entrada parcial no compartimento claro e rápido retorno para o compartimento escuro), preferência por coespecíficos e/ou agressividade (BLASER e GERLAI, 2006; EGAN et al., 2009; MAXIMINO et al., 2015). Esses parâmetros comportamentais, em conjunto, podem ser utilizados para predizer efeitos neurotóxicos de drogas como o etanol (KALUEFF et al., 2013). Portanto, considerando as características genéticas e comportamentais, é possível afirmar que a investigação de fenótipos comportamentais evolutivamente conservados subjacentes ao abuso, recompensa e dependência ao álcool podem ser estudados com sucesso em peixe-zebra, de forma complementar aos estudos com roedores.

Em peixe-zebra, sabe-se que a exposição aguda ao etanol altera parâmetros comportamentais, onde o efeito bifásico do etanol pode ser observado (GERLAI et al., 2000). Concentrações baixas a moderadas (0,25 e 0,5% v/v) aumentam a atividade exploratória e exercem efeito ansiolítico, enquanto altas concentrações (geralmente maiores do que 1% v/v) inibem a atividade locomotora causando efeito depressor, similar aos efeitos estimulantes e sedativos que ocorrem em humanos. Em peixe-zebra, após a exposição aguda a concentrações baixas a moderadas de etanol observa-se uma diminuição de comportamentos do tipo ansiedade, como movimentos erráticos (EGAN et al., 2009), congelamento (BLASER e PENALOSA, 2011), fuga do predador (PANNIA et al., 2014) e tempo no fundo do tanque (WONG et al., 2010). Nessas concentrações, o etanol também pode desencadear comportamentos agressivos, afetando a formação de cardumes e modulando respostas

semelhantes ao medo (FONTANA et al., 2016; FONTANA et al., 2018; GERLAI et al., 2000). A exposição aguda ao etanol eleva os níveis cerebrais de etanol em peixe-zebra (ROSEMBERG et al., 2012), bem como aumenta os níveis de DA, 5-HT e seus metabólitos no encéfalo, sugerindo o envolvimento de mecanismos dopaminérgicos e serotoninérgicos nos fenótipos neurocomportamentais (CHATTERJEE e GERLAI, 2009; CHATTERJEE, SHAMS e GERLAI, 2014). Além disso, o etanol agudamente aumenta a atividade da enzima acetilcolinesterase e altera as defesas antioxidantes pela diminuição da atividade da enzima SOD e aumento da atividade da enzima CAT, fato que culmina em peroxidação lipídica no SNC do peixe-zebra (ROSEMBERG et al., 2010).

Quanto à exposição crônica de etanol em peixe-zebra, a administração pode ser realizada utilizando um protocolo de exposição intermitente (MATHUR e GUO, 2011) ou contínua (DAMODARAN et al., 2006; DLUGOS e RABIN, 2003; EGAN et al., 2009). O modelo de exposição intermitente possui maior valor translacional porque imita o consumo de etanol observado em seres humanos, mas ambos os protocolos induzem tolerância, efeitos motores, ansiolíticos, ansiogênicos e alterações nos sistemas modulados por 5-HT e DA e seus metabólitos (DLUGOS e RABIN, 2003; MATHUR e GUO, 2011). Essas respostas comportamentais podem estar associadas a alterações nos processos oxidantes no encéfalo, uma vez que o etanol exerce efeitos pró-oxidativos em peixe-zebra (ROSEMBERG et al., 2010). Em peixe-zebra, protocolos para o estudo de tolerância e retirada do etanol (DLUGOS e RABIN, 2003; GERLAI et al., 2009; MATHUR e GUO, 2011; TRAN, CHATTERJEE e GERLAI, 2015; BERNARDO et al., 2019) também são descritos. Entretanto, esses protocolos têm produzido alguns resultados conflitantes. Alguns estudos relatam efeitos robustos da abstinência no comportamento tipo ansiedade (da SILVA CHAVES et al., 2018; TRAN, CHATTERJEE e GERLAI, 2015), enquanto outros trabalhos (CACHAT et al., 2010) não observam respostas comportamentais significativas. Uma possível explicação para a heterogeneidade dos dados pode estar relacionada a diferenças na concentração de etanol e no período de exposição empregado que diferem entre os protocolos (da SILVA CHAVES et al., 2018).

De modo geral, o peixe-zebra tem se mostrado uma espécie atraente para o estudo neurocomportamental de protocolos relacionados ao abuso de álcool e dependência. Assim, estudos utilizando este organismo modelo podem servir como um ponto inicial para a busca de novos compostos com potencial ação terapêutica. Portanto, aprofundar a investigação dos mecanismos neuroquímicos envolvidos nas respostas promovidas pelo etanol em diferentes modelos experimentais são de extrema importância.

3. JUSTIFICATIVA

O álcool é uma substância psicoativa amplamente consumida no mundo com propriedades capazes de causar dependência química. Seu uso nocivo pode acarretar em diversas consequências para o indivíduo e a sociedade e seu consumo pode ser relacionado com o desenvolvimento de mais de 200 doenças (SHIELD, PARRY e REHM, 2013; WHO, 2018). Dentre essas doenças, as doenças neuropsiquiátricas são as principais condições patológicas que se associam ao uso do etanol, uma vez que o álcool penetra facilmente no SNC desencadeando desequilíbrios fisiológicos, bioquímicos e neuroquímicos (DEITRICH, ZIMATKIN e PRONKO, 2004; EŞEL e DINÇ, 2017; QUERTEMONT, TAMBOUR e TIRELLI, 2005).

Dados sobre o consumo de álcool levantados pelo Instituto Nacional de Políticas Públicas do Álcool e Outras Drogas da Universidade Federal de São Paulo (2012) revelaram que aproximadamente 64% dos homens e 39% das mulheres adultas tendem a consumir álcool regularmente, onde 32% dos entrevistados bebem moderadamente e 16% consomem quantidades nocivas de álcool. Em virtude da elevada prevalência do consumo de bebidas alcoólicas entre os brasileiros, a busca de estratégias preventivas e de tratamentos para os sintomas nocivos do álcool acarretam em enormes custos financeiros (GARCIA e FREITAS, 2015).

Nessas condições, a utilização de modelos animais alternativos como o peixe-zebra apresenta muitas vantagens. Além de ser um modelo financeiramente acessível, o peixe-zebra possui alta similaridade genética com os mamíferos, e a maioria dos seus sistemas de NT já foram caracterizados. O peixe-zebra tem sido utilizado com sucesso nas pesquisas relacionadas ao alcoolismo, pois responde de forma rápida a nível neuroquímico, bioquímico e comportamental a diferentes protocolos de exposição. Entretanto, ainda existem muitos aspectos a serem aprofundados nestes modelos que contribuirão para e elucidação dos mecanismos envolvidos nos processos fisiopatológicos desencadeadas pelo etanol no SNC. Dessa forma, a utilização do peixe-zebra também poderá favorecer a descoberta de novos tratamentos para os processos relacionados ao abuso de álcool de forma rápida e eficaz.

4. HIPÓTESES

• A exposição crônica ao etanol altera o comportamento social e induz estresse oxidativo em peixe-zebra.

• A exposição aguda e crônica ao etanol alteram parâmetros bioenergéticos e podem levar à disfunção mitocondrial em peixe-zebra.

• A ativação da via serotoninérgica está envolvida em respostas comportamentais de agressão e ansiedade mediadas pela exposição aguda ao etanol em peixe-zebra.

5. OBJETIVOS

5.1. Objetivo geral

Investigar a influência do estresse oxidativo e do sistema serotoninérgico nas respostas neurocomportamentais causadas pelo etanol em peixe-zebra.

5.2. Objetivos específicos

• Verificar se a exposição crônica ao etanol influencia o comportamento social e modula parâmetros relacionados ao estresse oxidativo em peixe-zebra;

• Investigar se o modelo de exposição aguda e crônica ao etanol alteram parâmetros bioenergéticos e promovem disfunção mitocondrial em peixe-zebra;

• Verificar o envolvimento da via serotoninérgica em respostas comportamentais mediadas pela exposição aguda ao etanol em peixe-zebra.

6. DESENVOLVIMENTO

As metodologias utilizadas e os resultados desta tese estão demonstrados na forma de três artigos científicos publicados em periódicos internacionais de relevante fator de impacto na área.

• O **Artigo 1** foi publicado na revista *Progress in Neuro-Psychopharmacology and Biological Psychiatry* (Qualis Referência CAPES A1, FI: 4.315) em 2017 e se intitula: "Repeated ethanol exposure alters social behavior and oxidative stress parameters of zebrafish".

• O **Artigo 2** foi publicado na revista *Neurochemistry International* (Qualis Referência CAPES A2, FI: 3.994) em 2019 e se intitula: "Neurochemical mechanisms underlying acute and chronic ethanol-mediated responses in zebrafish: the role of mitochondrial bioenergetics".

• O **Artigo 3** foi publicado na revista *European Neuropsychopharmacology* (Qualis Referência CAPES A1, FI: 4.468) em 2019 e se intitula: "Role of the serotonergic system in ethanol-induced aggression and anxiety: a pharmacological approach using the zebrafish model".

ARTIGO 1

Repeated ethanol exposure alters social behavior and oxidative stress parameters of zebrafish

Talise Ellwanger Müller, Stenio Zimermann Nunes, Ariane Silveira, Vania Lucia Loro, Denis Broock Rosemberg

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Repeated ethanol exposure alters social behavior and oxidative stress parameters of zebrafish

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ABSTRACT

Repeated ethanol (EtOH) consumption induces neurological disorders in humans and is considered an important public health problem. The physiological effects of EtOH are dose- and time-dependent, causing relevant changes in the social behavior. In addition, alcohol-induced oxidative stress has been proposed as a key mechanism involved in EtOH neurotoxicity. Here we investigate for the first time whether repeated EtOH exposure (REE) alters the social behavior of zebrafish and influences brain oxidation processes. Animals were exposed to water (control group) or 1% (v/v) EtOH (EtOH group) for 8 consecutive days (20 min per day). EtOH was added directly to the tank water. At day 9, the social behavior and biochemical parameters were assessed. REE increased shoal cohesion by reducing inter-fish and farthest neighbor distances. SOD and CAT activities, as well as NPSH levels decreased in brain tissue. Moreover, REE increased lipid peroxidation suggesting oxidative damage. In summary, changes in oxidation processes may play a role in the CNS effects of EtOH, influencing the social behavior of zebrafish. Furthermore, in a translational neuroscience perspective, our data reinforces the utility of zebrafish to clarify the biochemical and behavioral effects of intermittent EtOH administration.

1. Introduction

Alcohol consumption is associated with a wide spectrum of negative health outcomes including morbidity, disability, and mortality (Global Status Report on Alcohol and Health - World Health Organization, 2014). Alcohol abuse and dependence lead to economic problems due to the costs of healthcare (Sacks et al., 2015) since alcoholic individuals are more susceptible to develop severe neurological disorders (Costardi et al., 2015). Importantly, different neurotransmitters and intricate transduction signaling pathways mediate the psychotropic effects of ethanol (EtOH) (Esel, 2006; Rico et al., 2011a, 2011b).

At a behavioral level, low to moderate EtOH doses induce stimulant effects on behavior, decreasing anxiety and contributing to drug abuse. Conversely, chronic alcohol consumption increases anxiety, leading to deleterious effects on brain functions (Camarini et al., 2010; Gerlai et al., 2000; Rosemberg et al., 2012). Several mechanisms involved in EtOH-mediated neurotoxicity have been proposed and oxidative stress is usually associated with deleterious effects (Augustyniak et al., 2005; Pereira and Andrade, 2015; Sun and Sun, 2001; Sun et al., 2001). Brain EtOH catabolism involves enzyme activities (e.g. catalase, alcohol dehydrogenase, and cytochrome P450) and naturally produces reactive oxygen species (ROS) (e.g. superoxide free radicals, hydrogen peroxide, and hydroxyl radicals) (Haorah et al., 2008; Hipólito et al., 2007; Zakhari, 2006). However, an excessive ROS production may alter the central nervous system (CNS) redox state, impairing DNA, lipid, and protein metabolism (Bondy, 1992; Comporti et al., 2010). Animals chronically intoxicated with EtOH showed significant changes in superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) activities (Augustyniak et al., 2005; Hernández et al., 2016; Zimatkin and Buben, 2007). These data clearly suggest an involvement of oxidant processes in the neurobehavioral actions of alcohol.

During the last decades, new experimental models have been validated to assess the effects of EtOH in vertebrates (Kaun et al., 2011; Spanagel, 2010; Tran and Gerlai, 2014). In this context, the investigation of evolutionarily conserved mechanisms is a valuable strategy to understand the basis of alcohol abuse and addiction in translational neuroscience. Zebrafish (*Danio rerio*) is a prominent model organism to

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assess the neurochemical and behavioral effects of EtOH (Tran et al., 2016). In addition to the high genome conservation (Howe et al., 2014), this species displays the major neurotransmitter systems that mediate EtOH responses (Chatterjee et al., 2014; Gerlai et al., 2009; Miller et al., 2013). Importantly, EtOH also exerts a biphasic effect on zebrafish behavior by exacerbating vertical activity and aggression at low concentrations, whereas locomotion and social preference decrease at higher concentrations (Fontana et al., 2016; Ladu et al., 2014; Rosemberg et al., 2012).

Regarding the social behavior, adult zebrafish have a natural tendency to form shoals. Shoaling is a highly complex behavior involved in foraging strategies, anti-predatory behaviors, and mating (Buske and Gerlai, 2011; Fernandes et al., 2015; Miller et al., 2013; Miller and Gerlai, 2007; Miller and Gerlai, 2011). Various human brain diseases are linked with disrupted group behavior and chronic alcohol intoxication may influence social behavior components (Gerlai, 2014; Kalueff et al., 2015). In this report, we investigate whether REE alters the social behavior of zebrafish and modulates oxidative stress parameters in the brain.

2. Material and methods

2.1. Animals

All experiments were performed using 64 adult (4–6 months-old) zebrafish (*Danio rerio*). Considering the conflicting data about the sex and EtOH influence on social behavior (Etinger et al., 2009; Kurta and Palestis, 2010; Fernandes et al., 2015), and the random use of male and female fish in different behavioral paradigms with reproducible data (Canzian et al., 2017; Egan et al., 2009; Green et al., 2012; Maximino et al., 2010), zebrafish of mixed genders (50:50 male:female ratio) were used. Short fin wild-type (*WT*) zebrafish were obtained from a local supplier (Hobby Aquários, RS, Brazil) and acclimated in 40-L tanks for two weeks in a maximum density of four fish per liter. Tanks were filled with non-chlorinated water kept under constant mechanical, biological, and chemical filtration at 26 ± 2 °C. The pH and conductivity were monitored and set at 7.0–8.0 and 1500–1600 μS/cm, respectively. Illumination was provided by ceiling-mounted fluorescent light tubes kept on a 14/10 light/dark photoperiod cycle (lights on at 7:00 am). Animals were fed with a commercial flake fish food (Alcon BASIC®, Alcon, Brazil) twice daily. All experiments were performed in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals and the protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (process number 026/2014).

2.2. Exposure protocol

REE was performed as described previously (Mathur and Guo, 2011). Initially, zebrafish were kept in housing tanks (25 cm length × 15 cm height × 6 cm width) separated in groups of 4 animals per shoal. Although zebrafish form larger shoals in their natural environment, previous studies showed reproducible data of social behavior using 4-fish shoals (Canzian et al., 2017; Schmidel et al., 2014; Green et al., 2012). Zebrafish shoals (n = 8) were exposed to 1% v/v EtOH for 8 consecutive days (20 min per day). Control fish (n = 8 shoals) were handled in a similar manner, except that no EtOH was added. Later, fish were returned into their housing tanks. We used the intermittent ethanol exposure protocol due to its translational relevance since it closely resembles what human drinkers would experience (Alcohol-Alert, 2001). No physical abnormalities were observed during the exposure period and at day 9, the behavioral and biochemical parameters were evaluated. Fig. 1 shows a schematic representation of the experimental protocol.

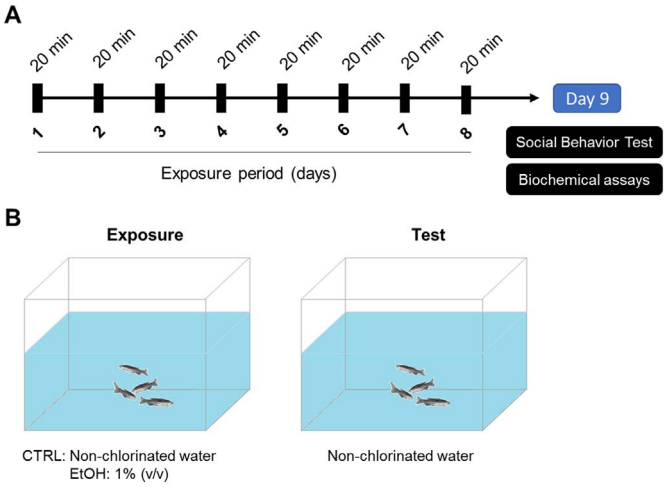

Fig. 1. Schematic representations of the methodological approach used for the evaluation of the social behavior and biochemical parameters in zebrafish using a REE protocol. (A) Experimental design and endpoints measured. (B) Illustration of the zebrafish group and experimental conditions during exposure period and behavioral tests.

2.3. Social behavior task

After the exposure period, each zebrafish group was placed in the test tank (25 cm length × 15 cm height × 6 cm width). Tank dimensions were similar to those used in previous reports that assessed the group behavior of zebrafish (Canzian et al., 2017; Green et al., 2012). Water column was 10 cm depth and the social behavior was recorded during a 6-min period. The videos were further exported to Image J 1.49 software for Windows™ and shoaling was determined using screenshots made every 15 s over the test period (with a total of 25 screenshots per group). Screenshots were further calibrated to the size of the tank and each fish was marked to allow automated quantification of the proximity between the fish (inter-fish distance, nearest neighbor distance, and farthest neighbor distance), and the mean dispersion (shoal area). The vertical distribution of zebrafish (number of animals in the top area of the tank) was evaluated by manually scoring the number of animals in the upper half of the apparatus every 15 s over the test period. Two trained observers (inter-rater reliability > 0.85) blind to the experimental conditions analyzed the videos and all measures were performed as described previously (Canzian et al., 2017; Schmidel et al., 2014). All behavioral analyses were performed using eight independent treatments per shoal of 4 fish (n = 8) for both control and EtOH groups.

2.4. Biochemical assays

2.4.1. Tissue preparation

At day 9, zebrafish were gently netted from their home tanks and rapidly euthanized by decapitation. For each independent preparation, four brains were pooled and homogenized on ice in 1 mL Tris HCl buffer (50 mM pH 7.4). Samples were further centrifuged at 3000 × g for 10 min at 4 °C and the supernatants were kept in microtubes at − 80 °C for posteriors assays.

2.4.2. Quantification of SOD activity

SOD activity was assessed by testing the inhibition of the radical superoxide reaction in the presence of adrenalin (Misra and Fridovich, 1972). The reaction was quantified by monitoring adrenochrome formation at 480 nm in a medium containing glycine-NaOH buffer (50 mM, pH 10), adrenaline (1 mM) and homogenate (20–30 μg protein) (Müller et al., 2017; Nunes et al., 2016). A unit of SOD was defined as the amount of enzyme that inhibits 50% of adrenaline oxidation rate. SOD activity was determined in a microplate reader and expressed as U

SOD/mg protein.

2.4.3. Determination of CAT activity

CAT activity was measured by monitoring the decrease in H_2O_2 absorbance at 240 nm (Aebi, 1984). The assay mixture consisted of 1 mL potassium phosphate buffer (50 mM, pH 7.0), 0.05 mL H_2O_2 (0.3 M), and 0.01 mL homogenate (20–30 μg protein). CAT activity was expressed as μmol/min/mg protein.

2.4.4. GST activity

GST activity was analyzed as described previously (Habig et al., 1974; Müller et al., 2017). The assay mixture contained 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol, 10 mM reduced glutathione, 20 mM potassium phosphate buffer (pH 6.5), and 20 μL of tissue homogenate (40–60 μg protein). Absorbance was monitored at 340 nm and the enzyme activity was further calculated by using the molar extinction coefficient (9.6 mM/cm). A unit of GST was defined as the amount of enzyme required to catalyze the conjugate 1 mol of CDNB with GSH/min at 25 °C. Data were expressed as μmol GS-DNB/min/mg protein.

2.4.5. Quantification of NPSH levels

To determine non-protein thiols levels (NPSH), equal volumes of brain preparation and 10% trichloroacetic acid (100 μL) were mixed and centrifuged (3.000 × g, 10 min at 4 °C). Supernatants (60–80 μg protein) were added to 10 mM DTNB (5,5-dithio-bis-2-nitrobenzoic acid) dissolved in ethanol and the intense yellow color developed was measured at 412 nm after 1 h (Ellman, 1959) in a microplate reader. Results were expressed as nmol SH/mg protein.

2.4.6. Lipid peroxidation

Lipid peroxidation was estimated by quantifying thiobarbituric acid-reactive substance (TBARS) production (Draper and Hadley, 1990). Briefly, 80 μL of sample (80–100 μg protein) and 160 μL of 10% trichloroacetic acid were mixed and then centrifuged (10,000 × g for 10 min) at 4 °C. Supernatants (100 μL) were heated at 100 °C for 30 min in the presence of 0.67% thiobarbituric acid (TBA) (100 μL). The absorbance was measured at 532 nm in a microplate reader. Malondialdehyde (MDA) was used as standard and results were expressed as nmol MDA/mg protein.

2.4.7. Protein determination

Protein was determined by the Coomassie blue method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm (Bradford, 1976).

2.4.8. Statistics

Data normality and homogeneity of the variances were analyzed by Shapiro-Wilk and Kolmogorov-Smirnov tests, respectively. Results were expressed as mean ± standard error of the mean (S.E.M.) and analyzed by unpaired Student's *t*-test. The significance level was set at $p \leq 0.05$. All biochemical experiments were performed in duplicate.

3. Results

3.1. REE increases shoaling behavior

Behavioral endpoints of shoaling are depicted in Fig. 2. EtOH-exposed fish showed a decrease in inter-fish distance ($t_{(0.05;14)} = 2.680$, $p = 0.0179$) when compared to control group. Moreover, REE decreased the farthest neighbor distance ($t_{(0.05;14)} = 2.390$, $p = 0.0314$) while the nearest neighbor distance and the shoal area did not alter. No significant differences in the vertical distribution of fish were observed (data not shown).

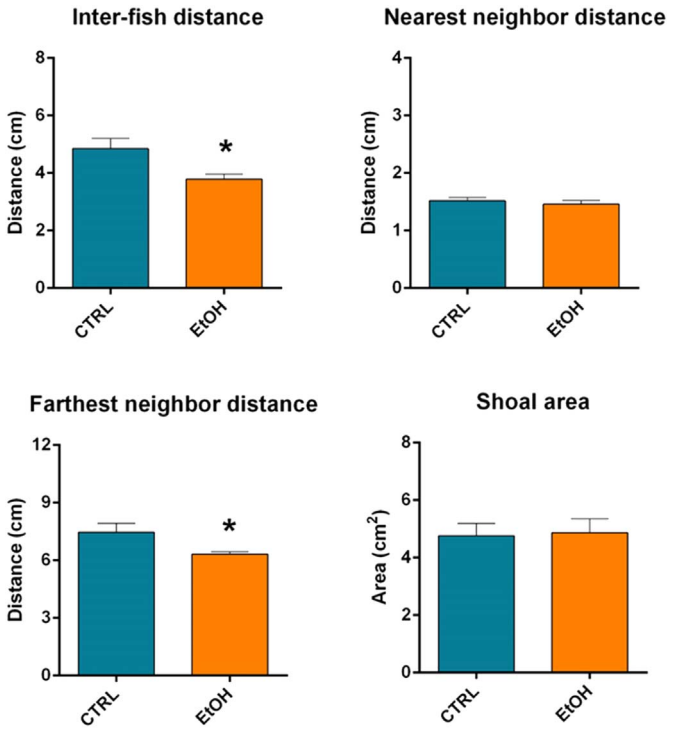

Fig. 2. Effects of REE on social behavior of zebrafish. The figure shows the results of control (CTRL) and ethanol (EtOH) groups related to shoaling (inter-fish distance, nearest neighbor distance, farthest neighbor distances, and shoal area). Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test (*$p < 0.05$, $n = 8$ shoals per group).

3.2. REE changes antioxidant parameters and induces oxidative stress in the zebrafish brain

Biochemical parameters associated with antioxidant mechanisms and oxidative stress were tested. Concerning the antioxidant mechanisms (Fig. 3), EtOH decreased SOD ($t_{(0.05;\ 8)} = 3.158$, $p = 0.0134$) and CAT activities ($t_{(0.05;\ 8)} = 5.546$, $p = 0.0005$). NPSH levels significantly decreased ($t_{(0.05;\ 8)} = 3.106$, $p = 0.0145$) after EtOH exposure, while GST activity did not change. Increased TBARS levels ($t_{(0.05;\ 8)} = 2.946$, $p = 0.0185$) were observed in EtOH-exposed group (Fig. 4).

3.3. Overview of REE actions on behavioral and biochemical parameters of zebrafish

Fig. 5 illustrates the main alterations observed in zebrafish after REE. Basically, EtOH does not change vertical distribution, but increases social cohesion (Fig. 5A) and modulates oxidative stress parameters in brain samples causing lipid peroxidation (Fig. 5B).

4. Discussion

In this study, we evaluated whether REE alters social behavior of zebrafish and changes oxidation mechanisms in the CNS. For the first time, we demonstrate that REE increases shoal cohesion, modulates antioxidant enzyme mechanisms, and induces oxidative stress in brain samples. Therefore, we suggest a role of oxidative damage in the CNS effects of EtOH, which may be associated with changes in social behavior domain after REE.

Repeated and chronic alcohol consumption is associated with addiction and tolerance in humans (Michalak and Biała, 2016; Novier et al., 2015). In this case, higher EtOH doses are necessary to achieve the same rewarding effects. Chronic alcohol exposure affects the CNS, influences behavior, and induces neuroadaptive changes in vertebrate species (Gerlai et al., 2006; Novier et al., 2016; Rico et al., 2011b; Tran

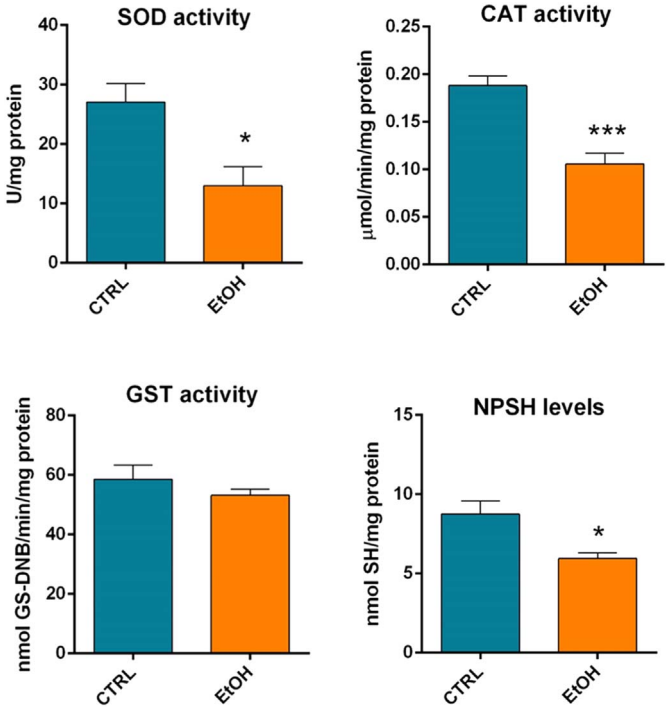

Fig. 3. Effects of REE on antioxidant mechanisms of zebrafish brain. Enzyme activities of SOD, CAT and GST, as well as NPSH levels were measured in control (CTRL) and ethanol (EtOH) groups. Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test (*$p < 0.05$, ***$p < 0.005$, $n = 5$ biological preparations per group).

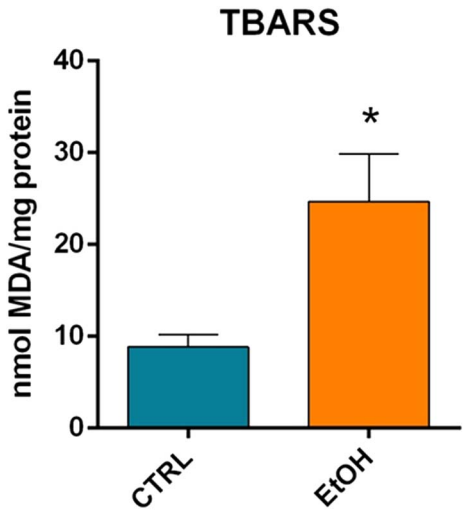

Fig. 4. Effects of REE on lipid peroxidation in zebrafish brain tissue. TBARS levels of control (CTRL) and ethanol (EtOH) groups are shown. Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test (*$p < 0.05$, $n = 5$ biological preparations per group).

and Gerlai, 2014). Although there are few studies assessing the behavioral effects of intermittent EtOH exposure in zebrafish, both REE and continuous EtOH administration modify anxiety-like behaviors (Chacon and Luchiari, 2014; Dlugos and Rabin, 2003; Egan et al., 2009; Mathur and Guo, 2011). We used a REE protocol because it models human drinking more closely than a continuous exposure to alcohol. Indeed, instead of an extensive period of continuous EtOH intake, alcohol abuse is driven by a cycle of drinking EtOH, and then craving more alcohol as blood alcohol levels falls (Alcohol-Alert, 2001).

Behavioral neurophenotyping of adult zebrafish after chronic ethanol exposure paradigms have been described (Cachat et al., 2010; Holcombe et al., 2014; Tran et al., 2015). Disturbances in social

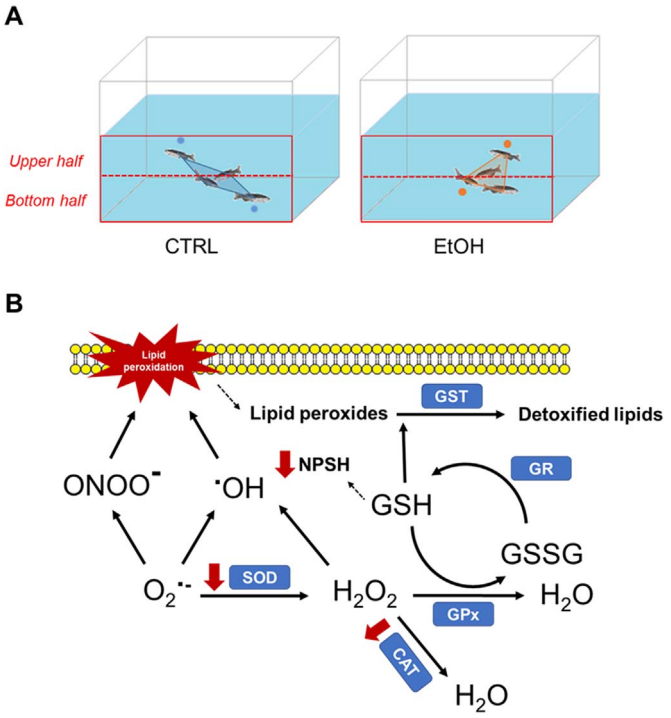

Fig. 5. Representative diagrams of alcohol effects on social behavior and oxidative-stress related parameters in zebrafish brain after REE. (A) Ethanol (EtOH) increases shoal cohesion when compared to control group (CTRL) without altering vertical distribution. (B) REE causes oxidative damage by increasing lipid peroxidation promoting markedly changes in antioxidant mechanisms. The panel depicts a putative involvement of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GR) in mediating EtOH responses.

parameters are easily to observe in experimental models of alcoholism, autism spectrum disorders, schizophrenia, and personality disorders (Kalueff et al., 2014; Stewart et al., 2015). Using full genome DNA microarrays and qRT-PCR experiments, Pan et al. (2011) characterized several alcohol responsive genes in zebrafish chronically exposed to EtOH. These authors observed significant changes in transcript levels that encode proteins associated with different neurotransmitter systems (e.g. dopaminergic, serotonergic, glutamatergic, and GABAergic) and mitochondrial energy metabolism (e.g CYP450 family). Since neurochemicals play a key role in social cognitive functioning (Henry et al., 2016) and mitochondrial physiology is linked with ROS formation, we hypothesize that REE could affect the social behavior domain and induce oxidative stress in the brain.

Here we use the shoaling response as an effective protocol for assessing the effects of drugs on group behavior (Fernandes et al., 2015; Gerlai, 2014; Miller et al., 2013). In general, zebrafish live in shoals (Engeszer et al., 2007; Miller and Gerlai, 2011) which are susceptible to external factors (Miller and Gerlai, 2007). For example, a stronger cohesion characterizes increased defensive responses, while decreased social behavior is indicative of impaired shoaling (Fernandes et al., 2015; Kurta and Palestis, 2010). EtOH engages several molecular mechanisms in a complex dose- and administration regimen-dependent manner (Vengeliene et al., 2008). After REE, fish showed increased shoal cohesion (decreased in inter-fish distance and farthest neighbor distance). Studies have shown a shoal disruption after EtOH exposure possibly due to its depressant effects at high concentrations (Dlugos and Rabin, 2003; Echevarria et al., 2011; Gerlai et al., 2006; Miller and Gerlai, 2012). One possible reason for why EtOH-exposed zebrafish differentially present distinct social behavior could be due to altered locomotion. However, using the same REE protocol, Mathur and Guo (2011) did not observe alterations in swim velocity when fish were placed in a novel tank. Thus, the increased shoaling observed herein are

not due to general effects in locomotion suggesting that REE affects social behavior domain.

In order to avoid a possible influence of acute exposure in the behavioral responses, all tests were performed 24 h after the last EtOH administration, which could also reflect the influence of withdrawal. Our novel findings showed that REE increases social behavior, suggesting an anxiogenic-like effect. Accordingly, Cachat et al. (2010) showed increased anxiety-like behaviors in zebrafish following repeated withdrawal from EtOH, which is consistent with our findings. Regarding the vertical distribution, Mathur and Guo (2011) showed a decrease in vertical exploration 6 days after REE, which was not observed 2 days after intermittent EtOH exposure. Thus, in addition to the influence of EtOH concentration in tank water, we postulate that EtOH could exert a time-dependent effect on distinct behavioral domains of zebrafish.

Chronic EtOH abuse leads to several biochemical changes in the CNS (Haorah et al., 2008; Sun and Sun, 2001). EtOH oxidation increases the formation of its metabolite, acetaldehyde (ACH), through pathways involving CAT, cytochrome CYP2E1, and alcohol dehydrogenase (ADH) (Comporti et al., 2010; Hipólito et al., 2007). ACH can mediate the neurotoxic effects of EtOH (Borja-Oliveira, 2014; Pereira et al., 2015) and its formation increases ROS production (e.g. superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot -}$)) (Halliwell and Gutteridge, 2007; Lambeth and Neish, 2014). The processes involved in EtOH–induced neurotoxicity also include changes in the NAD^+/NADH ratio, mitochondrial impairments, altered signal transduction pathways, and structural damages (Pereira and Andrade, 2015; Wu and Cederbaum, 2001; Yang and Luo, 2015; Zima et al., 2001). Importantly, the brain is an organ highly susceptible to oxidative stress due to its high O_2 consumption and low antioxidant capacity (Cohen-Kerem and Koren, 2003). Thus, to maintain the brain redox homeostasis, specific enzymatic/non-enzymatic antioxidant mechanisms are necessary.

Although SOD can play a prooxidant role due to its peroxidase activity, its primordial role is to neutralize $O_2^{\cdot -}$ to H_2O_2 acting in synergism with CAT. In this context, SOD and CAT play a key role in regulating $O_2^{\cdot -}$ and H_2O_2 levels (Hernández et al., 2016; Hipólito et al., 2007; Reddy et al., 1999; Zimatkin et al., 2006). In our study, both SOD and CAT enzymes activities were decreased after REE. A previous report evaluated the effects of a single EtOH exposure for 1 h in brain tissue of zebrafish (Rosemberg et al., 2010). In the respective study, EtOH acutely decreased SOD and increased CAT activities leading to oxidative stress. Contrastingly, studies have shown that chronic EtOH administration decreases antioxidant responses (Augustyniak et al., 2005; Bosch-Morell et al., 1998; Calabrese et al., 1998; Sun et al., 2001; Vidhya et al., 2013), indicating adaptive changes on oxidant processes after prolonged exposure. Therefore, the effects of EtOH in SOD/CAT activities are dependent on the model, amount, and time of alcohol intake. Since CAT is an enzyme responsible for oxidizing brain EtOH to ACH (Hipólito et al., 2007), changes on CAT activity may reflect impaired EtOH metabolism. Considering the differences in CAT activity after acute and chronic EtOH exposures, the brain EtOH metabolism could differ according with the protocol. Although the involvement of ACH in chronic EtOH responses should not be completely ruled out, impaired SOD and CAT activities could increase reactive species (e.g. $O_2^{\cdot -}$ and H_2O_2) triggering oxidative damage.

Glutathione (GSH) is an essential component of the antioxidant system and serves as a cofactor for GST, detoxifying certain ROS and eliminating lipid peroxides from the cell (Dringen, 2000; Pisoschi and Pop, 2015). Although GST activity remained unchanged after REE, decreased NPSH levels (which reflect GSH amounts) were observed. Additionally, TBARS levels significantly increased, suggesting lipid peroxidation. Chronic EtOH exposure influences lipid metabolism (Hernández et al., 2016) and promotes numerous changes in oxidative metabolism, such as GSH depletion (Fernandez-Checa et al., 1997; Koch et al., 2004; Wu and Cederbaum, 2001). Since these effects are also observed in a REE protocol, it may serve as an interesting approach to investigate the neural basis underlying alcohol abuse and alcoholism in alternative zebrafish models.

Antioxidant mechanisms play a crucial role in maintaining brain redox homeostasis and EtOH administration can disrupt CNS oxidation processes (Bosch-Morell et al., 1998; Calabrese et al., 1998; Comporti et al., 2010; Hernández et al., 2016). Oxidative/nitrosative stress can activate intracellular signaling pathways that have been implicated in social deficits (Maes et al., 2011). Additionally, recent data correlate brain oxidation processes with changes in different social behavior domains. For example, quercetin and resveratrol exert antioxidant action, prevent cell damage, and reverse social defeat-induced behavioral and cognitive deficits in rats (Solanki et al., 2017). Another study demonstrated a disruption of mitochondrial function and an overproduction of nitric oxide in the cortical areas of mice following a 4-week social isolation period (Haj-Mirzaian et al., 2016). Importantly, the respective stress protocol provoked depressive-like behaviors, suggesting a CNS redox imbalance in the respective phenotype. Although the central mechanisms underlying EtOH-mediated responses have not been fully described in alternative fish models, we suggest an involvement of oxidative stress in the behavioral changes of zebrafish after REE.

5. Conclusion

To our knowledge, we report the first evidence regarding the effects of REE in social behavior and oxidative stress parameters of zebrafish. Although more studies are needed, our results are relevant to clarify the central mechanisms of EtOH and their potential implication with behavioral functions. In a translational perspective, our data could have therapeutic relevance to prevent or ameliorate the deleterious effects of intermittent EtOH administration at neurochemical and behavioral levels.

Conflict of interest

The authors declare that no competing interests exist.

Ethical statement

1) This material has not been published in whole or in part elsewhere;
2) The manuscript is not currently being considered for publication in another Journal;
3) All authors have been personally and actively involved in substantive work leading to the manuscript and are responsible for its content.

Author contributions

1. Conceived and designed the experiments: DBR, TEM.
2. Performed the experiments: TEM, SZN, AS.
3. Analyzed the data: DBR, TEM, SZN, AS, VL.
4. Contributed reagents/materials/analysis tools DBR, VL.
5. Wrote the paper: DBR, TEM.

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ARTIGO 2

Neurochemical mechanisms underlying acute and chronic ethanol-mediated responses in zebrafish: the role of mitochondrial bioenergetics

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Neurochemical mechanisms underlying acute and chronic ethanol-mediated responses in zebrafish: The role of mitochondrial bioenergetics

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ABSTRACT

Ethanol (EtOH) is a socially-accepted drug, whose consumption is a risk factor for non-intentional injuries, development of pathologies, and addiction. In the brain, EtOH affects redox signaling and increases reactive oxygen species (ROS) production after acute and chronic exposures. Here, using a high-resolution respirometry assay, we investigated whether changes in mitochondrial bioenergetics play a role in both acute and chronic EtOH-mediated neurochemical responses in zebrafish. For the first time, we showed that acute and chronic EtOH exposures differently affect brain mitochondrial function. Acutely, EtOH stimulated mitochondrial respiration through increased baseline state, CI-mediated OXPHOS, OXPHOS capacity, OXPHOS coupling efficiency, bioenergetic efficiency, and ROX/ETS ratio. Conversely, EtOH chronically decreased baseline respiration, complex I- and II-mediated ETS, as well as increased ROX state and ROX/ETS ratio, which are associated with ROS formation. Overall, we observed that changes in mitochondrial bioenergetics play a role, at least partially, in both acute and chronic effects of EtOH in the zebrafish brain. Moreover, our findings reinforce the face, predictive, and construct validities of zebrafish models to explore the neurochemical bases involved in alcohol abuse and alcoholism.

1. Introduction

Ethanol (EtOH) is one of the most socially-accepted addictive drug worldwide (Gneiting and Schmitz, 2016). Alcohol consumption is a risk factor for accidents, development of pathologies, as well as addiction and alcoholism (Rehm, 2011). Alcohol-related chronic disorders constitute a substantial health and economic burden due to the occurrence of different types of diseases, including neuropsychiatric conditions (Ridley et al., 2013). These disorders contribute to the alcoholism-related high morbidity and mortality (Shield et al., 2013).

Evidence shows that acute and chronic ethanol exposures affect redox signaling and increase free radicals production in the central nervous system (CNS), which impair proteins, carbohydrate, and fatty acid metabolism (Manzo-Avalos and Saavedra-Molina, 2010). Mitochondria play a key role in energy production via aerobic metabolism, and mitochondrial electron transport chain has been widely recognized as an endogenous source of reactive oxygen species (ROS) (Bolisetty and Jaimes, 2013). EtOH oxidation can affect mitochondria physiology, which culminates in the overproduction of ROS (Almansa et al., 2009). EtOH also impairs the membrane potential, decreases Ca^{2+} intracellular levels (Goodlett and Horn, 2001), and affects the mitochondrial electron transport system, thereby reducing ATP production and triggering neuronal death (Bailey et al., 1999; Cunningham and Van Horn, 2003; Guo et al., 2013).

In translational neuroscience research, the zebrafish (*Danio rerio*) has been considered a suitable vertebrate for modeling human-related

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disorders (Fontana et al., 2018; Stewart et al., 2015). This species shares a high genomic conservation when compared to humans (Howe et al., 2013), and presents an evolutionarily conserved physiology (Holzschuh et al., 2001; Horzmann and Freeman, 2016; MacRae and Peterson, 2015). In zebrafish, EtOH-mediated effects on behavior are concentration- and time-dependent, and redox imbalances occur following acute and chronic exposures (Gerlai et al., 2000; Muller et al., 2017; Rosemberg et al., 2010, 2012). Because EtOH affects energy metabolism, as well as modulates redox signaling, which culminates in oxidative stress, we hypothesized that such responses could be related to changes in mitochondrial functionality in zebrafish. Thus, the goal of this study was to verify whether changes in mitochondrial bioenergetics play a role in EtOH-mediated effects on the CNS of zebrafish using a high-resolution respirometry assay.

2. Materials and methods

2.1. Animals

Subjects were 88 adult (4–6 months-old) short fin wild-type zebrafish (*Danio rerio*) of mixed genders (50:50 male:female ratio). Fish were obtained from a local supplier (Hobby Aquários, RS, Brazil) and acclimated in 40-L tanks for two weeks in a maximum density of four fish per liter. Tanks were filled with non-chlorinated water under constant mechanical, biological, and chemical filtration. Water temperature, pH, and conductivity were set at 28 ± 1 °C, 7.2 ± 0.5, and 400 ± 50 μS, respectively. Ammonia, nitrite, and nitrate values were kept lower than 0.2 ppm, 0.05 ppm, and 0.05 ppm, respectively. Animals were kept on a 14/10 light/dark photoperiod cycle (lights on at 7:00 a.m.), water dissolved oxygen equal or above 95% saturation and fed with a commercial flake fish food (Alcon BASIC®, Alcon, Brazil) twice daily. All protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (process number 026/2014).

2.2. Alcohol exposure protocols

We used two protocols to investigate whether EtOH modulates mitochondrial bioenergetics. To evaluate the acute effects of EtOH, 40 fish were individually exposed to non-chlorinated water (control) or 1.0% (v/v) EtOH (Merck, Darmstadt, Germany) for 1 h (20 animals per group). EtOH concentration used here is known to induce sedative/depressant-like behavior, as well as impairs oxidant processes in the zebrafish brain (Chatterjee and Gerlai, 2009; Rosemberg et al., 2010, 2012). Chronically, EtOH was administered as described previously, using the intermittent exposure protocol (Mathur and Guo, 2011; Muller et al., 2017). Briefly, 48 zebrafish were kept in housing tanks and exposed to non-chlorinated water (control) or 1.0% (v/v) EtOH for 8 consecutive days (20 min per day) and euthanized at 9th day (24 animals per group). Importantly, no physical abnormalities were observed during the exposure period. After euthanasia, the brains were dissected and samples were prepared to further biochemical analyses.

2.3. Mitochondrial respiration assays

Mitochondrial activity was measured by high-resolution respirometry using an Oxygraph-2k (O2k, Oroboros Instruments, Innsbruck, Austria). For each independent preparation, four brains were pooled (~24 mg of tissue) and homogenized in 240 μL of medium containing 5 mM Tris-HCl (pH 7.4), 250 mM sucrose, and 2 mM EGTA. Samples were homogenized gently with a pestle and 100 μL of homogenate was further transferred to 2 mL respiration buffer (115 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$), 3 mM HEPES, 1 mM EGTA, essentially fatty acid-free BSA (0.2%, pH 7.2). All experiments were performed in duplicate at 28 °C using DatLab 4.0 software (Oroboros Inc., Austria), with continuous stirring at 750 rpm (de Carvalho et al., 2017).

Using titration protocols based on previous reports (Carvalho et al., 2013; Gnaiger, 2009; Pesta and Gnaiger, 2012), we assessed the influence of various substrates and inhibitors in mitochondrial function as reflected in different respiration states. Glutamate + pyruvate + malate and succinate were used as oxidizable substrates. We determined the changes in mitochondrial respiratory chain complexes, respiratory rates, and the production of oxidative oxygen species.

After signal stabilization, the baseline respiration supported by endogenous substrates was measured. The complex I (CI)-mediated leak (LEAK; L(n)) respiration was determined using 5 mM pyruvate, 5 mM glutamate and 1 mM malate. CI-mediated oxidative phosphorylation (OXPHOS) was tested using 2.5 mM ADP. The convergent electron flow during the maximal OXPHOS respiration (CI + CII_{OXPHOS}) was determined with substrates of CI and CII (10 mM succinate). To induce LEAK state, we added 2 μg/mL oligomycin, an inhibitor of ATP synthase by blocking its proton channel. The electron transport system (ETS) respiration represents the uncoupled respiration, which was measured using carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) as uncoupler (optimum concentration reached between 0.5 and 1.5 μM); CI + CII-mediated ETS respiration (CI + CII_{ETS}) was determined in the presence of FCCP, while CII-mediated ETS respiration (CII_{ETS}) was measured in the presence of 0.5 μM rotenone. The addition of 2.5 μM antimycin A was performed to inhibit complex III activity, which abolished mitochondrial respiration. Then, the residual oxygen consumption (ROX) with small contributions from electron leak in the uncoupled state was measured. We also determined the magnitude of residual oxygen consumption relative to the maximum oxygen consumption (expressed as fold change of ROX/ETS ratio), ETS/OXPHOS ratio, OXPHOS capacity, and OXPHOS coupling efficiency, which is based on the ratio of free to total OXPHOS capacity (1-L/P). Mitochondrial bioenergetics capacity was quantified by subtracting the ADP-induced CI_{OXPHOS} values from the CI_{LEAK}. Moreover, the respiratory control rates (RCR) were measured as indicators of the mitochondrial coupling state (RCR = CI_{OXPHOS}/CI_{LEAK} ratio), as well as the succinate control factor (CI_P/CI + CII_O, fold change). Substrate control ratio (SCR) (CI_{OXPHOS}/CII_{ETS} ratio) was quantified to evaluate the effects of EtOH on mitochondrial respiratory control. Using the high-resolution respirometry protocol measured by Oxygraph-2k, the limit of detection of respiratory flux was 1 $\rho mol \cdot s^{-1} \cdot cm^{-3}$ (0.001 $\mu M\, s^{-1}$) and the limit of detection of oxygen concentration extends to 0.005 μM O_2. Low intra- and inter-assay CV values (ranging from 4.4–9.2% and 7.7–11.8%, respectively) were observed for each endpoint measured (Table 1).

2.4. Statistics

Normality of data and homogeneity of variances were analyzed by Kolmogorov-Smirnov and Bartlett's tests, respectively. Because results were normally distributed and homoscedastic, data were expressed as means ± standard error of the mean (S.E.M.) and the effects on mitochondrial activity were analyzed by unpaired Student's *t*-test, considering $p \leq 0.05$ as significant. Statistical analyses were performed using the GraphPad Prism software (version 7.0 for Macintosh OS X,

Table 1
Coefficient of variation (CV) obtained from each endpoint measured.

Endpoints	Coefficient of variation (%)	
	Intra-assay	Inter-assay
Basal	5.32	10.04
Cl_{LEAK}	4.46	7.79
Cl_{OXPHOS}	6.75	10.84
Cl + CII_{OXPHOS}	5.20	11.16
Cl + CII_{ETS}	4.46	9.30
CII_{ETS}	9.22	7.92
ROX	4.35	11.86

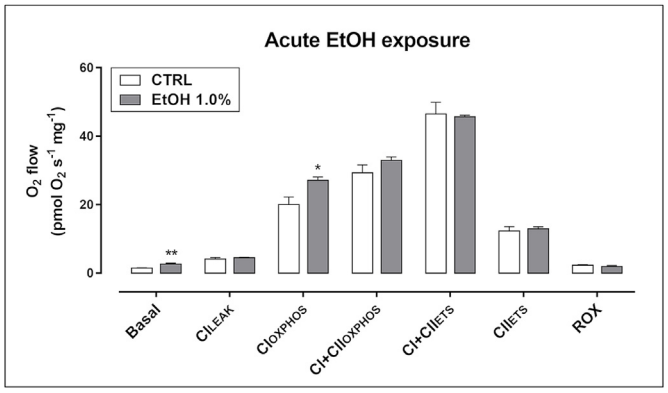

Fig. 1. Mitochondrial O_2 consumption in the zebrafish brain following acute 1.0% (v/v) EtOH exposure. Mitochondrial functions are presented with the abbreviation(s) of the complex(es) involved followed by the state of respiration measured in the presence of endogenous substrates (baseline), pyruvate + malate + glutamate (CI_{LEAK}), + ADP (CI_{OXPHOS}), + succinate (CI + CII_{OXPHOS}), + FCCP (CI + CII_{ETS}), + rotenone (CII_{ETS}), + antimycin A (Ama) used to correct for residual O_2 consumption (ROX). Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test (*$p < 0.05$, $n = 5$ independent preparations per group).

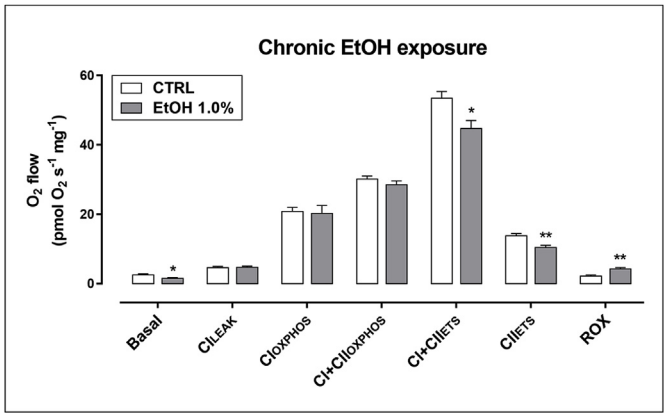

Fig. 3. EtOH (1.0%, v/v) chronically alters mitochondrial O_2 flow in the zebrafish brain. Mitochondrial functions are presented with the abbreviation(s) of the complex(es) involved followed by the state of respiration measured in the presence of endogenous substrates (baseline), pyruvate + malate + glutamate (CI_{LEAK}), + ADP (CI_{OXPHOS}), + succinate (CI + CII_{OXPHOS}), + FCCP (CI + CII_{ETS}), + rotenone (CII_{ETS}), + antimycin A (Ama) used to correct for residual O_2 consumption (ROX). Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test. Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test (*$p < 0.05$, **$p < 0.01$, $n = 6$ independent preparations per group).

San Diego, CA).

3. Results

3.1. EtOH acutely stimulates mitochondrial O_2 consumption

Fig. 1 depicts the acute effects of 1.0% EtOH exposure on the mitochondrial bioenergetics. EtOH-exposed group showed higher baseline respiration ($t_{(0.05;\ 8)} = 3.991$, $p = 0.004$) and complex I-induced oxidative phosphorylation (CI_{OPHOX}) ($t_{(0.05;\ 8)} = 3.265$, $p = 0.0114$) than control. However, CI_{LEAK} respiration, and complex I- and II-induced oxidative phosphorylation (CI + CII_{OPHOX}) did not change between groups. When the respiration was uncoupled by FCCP (CI + CII_{ETS}), no differences were observed. Moreover, the C_{IIETS} respiration and ROX values did not show significant differences between groups. EtOH exposure also increased ROX/ETS ratio ($t_{(0.05;\ 8)} = 3.639$, $p = 0.0066$) (Fig. 2A) and decreased ETS/OXPHOS ($t_{(0.05;\ 8)} = 6.088$, $p = 0.0006$) (Fig. 2B). The OXPHOS capacity ($t_{(0.05;\ 8)} = 2.391$, $p = 0.0438$) (Fig. 2C), OXPHOS coupling efficiency ($t_{(0.05;\ 8)} = 3.017$, $p = 0.0116$) (Fig. 2D), bioenergetic efficiency ($t_{(0.05;\ 8)} = 2.695$, $p = 0.0273$) (Fig. 2E), RCR ($t_{(0.05;\ 8)} = 2.791$, $p = 0.0235$) (Fig. 2F) increased after EtOH exposure. Succinate control factor was lower ($t_{(0.05;\ 8)} = 2.528$, $p = 0.0353$) (Fig. 2G) in EtOH group, while SCR did not change following acute EtOH regimen (Fig. 2H).

3.2. EtOH chronically impairs mitochondrial respiration

Fig. 3 shows the effects of 1.0% chronic EtOH exposure on mitochondrial respiration. EtOH decreased the baseline state ($t_{(0.05;\ 10)} = 2.783$, $p = 0.0193$), while no changes in CI_{LEAK} respiration, as

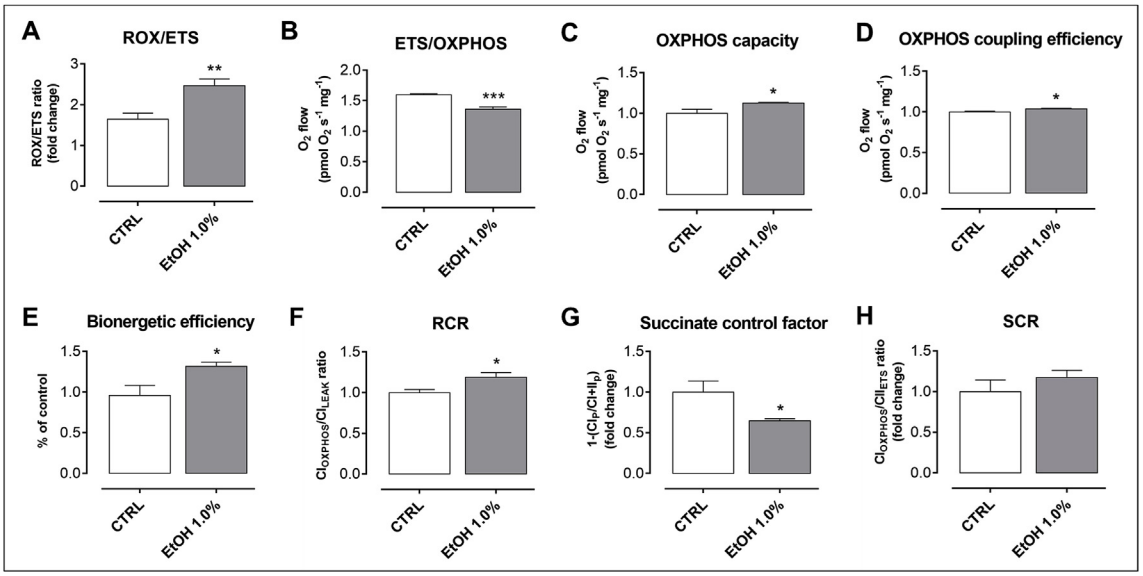

Fig. 2. Acute effects of 1.0% (v/v) EtOH on residual oxygen consumption (ROX/ETS ratio, fold change) **(A)**, ETS/OXPHOS **(B)**, OXPHOX capacity **(C)**, OXPHOS coupling efficiency **(D)**, bioenergetic efficiency (by subtracting the ADP-induced CI_{OXPHOS} values from the CI_{LEAK}) **(E)**, respiratory control rate (RCR = CI_{OXPHOS}/CI_{LEAK} ratio) **(F)**, succinate control ratio (CI_P/CI + CII_O, fold change) **(G)**, and substrate control ratio (SCR) (CI_{OXPHOS}/CII_{ETS} ratio, fold change) **(H)**. Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 5$ independent preparations per group).

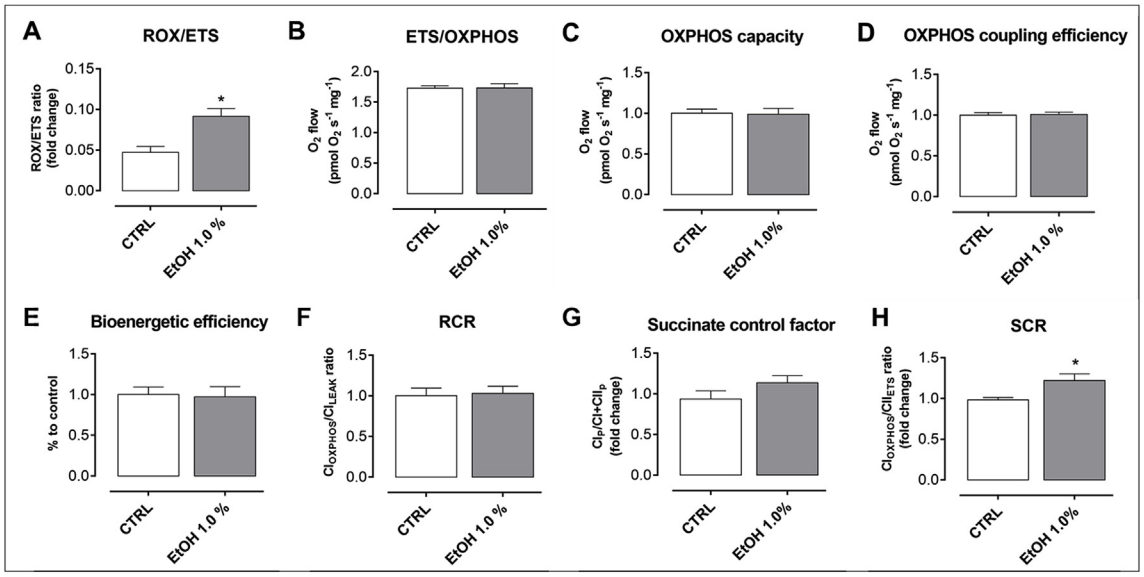

Fig. 4. Effects of 1.0% (v/v) chronic EtOH exposure on residual oxygen consumption (ROX/ETS ratio, fold change) **(A)**, ETS/OXPHOS **(B)**, OXPHOX capacity **(C)**, OXPHOS coupling efficiency **(D)**, bioenergetic efficiency (by subtracting the ADP-induced CI_{OXPHOS} values from the CI_{LEAK}) **(E)**, respiratory control rate (RCR = CI_{OXPHOS}/CI_{LEAK} ratio) **(F)**, succinate control ratio ($CI_P/CI + CII_O$, fold change) **(G)**, and substrate control ratio (SCR) (CI_{OXPHOS}/CII_{ETS} ratio, fold change) **(H)**. Data were expressed as mean ± SEM and analyzed by unpaired Student's *t*-test (*$p < 0.05$, $n = 6$ independent preparations per group).

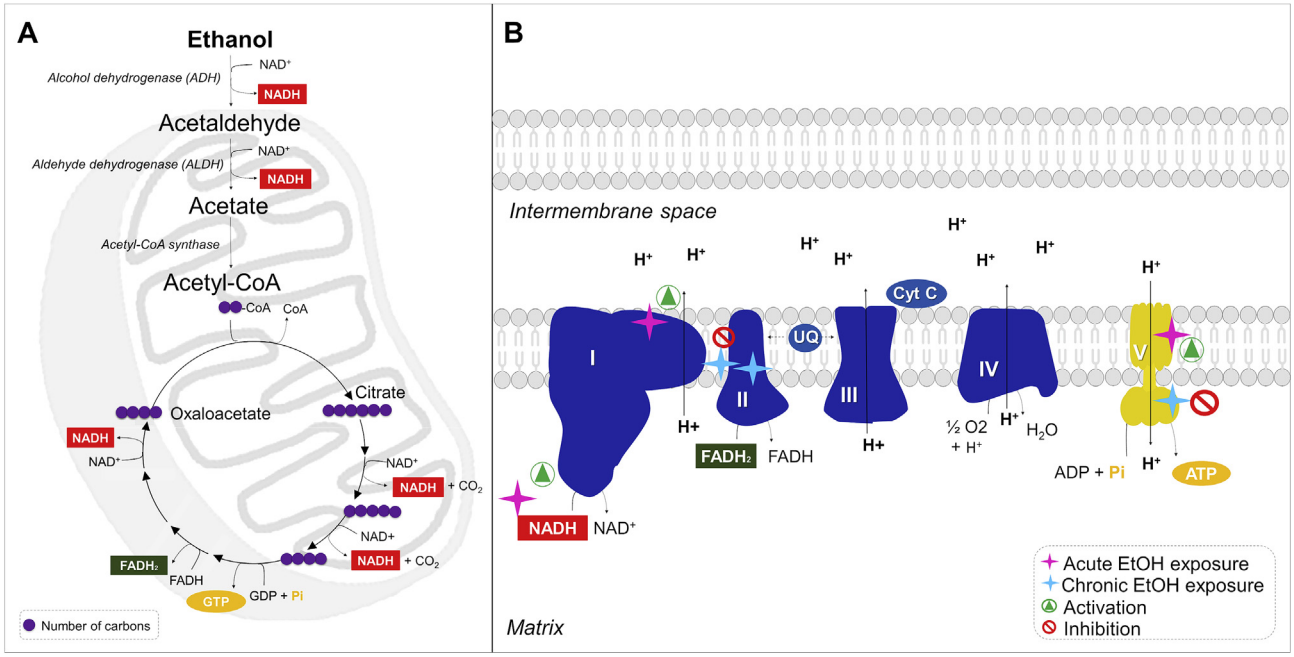

Fig. 5. Schematic representation of the production of reduced equivalents via EtOH catabolism **(A)** and mechanisms underlying the effects of acute and chronic EtOH exposure in the zebrafish brain mitochondria **(B)**. EtOH acutely stimulates CI-mediated OXPHOS, while the chronic exposure decreases complex I- and II-mediated ETS.

well as in complex I- and II-induced oxidative phosphorylation (CI_{OPHOX}, CI + CII_{OPHOX}) were verified. EtOH-exposed fish showed decreased CI + CII_{ETS} ($t_{(0.05;\ 10)} = 2.817$, $p = 0.0183$) and C_{IIETS} ($t_{(0.05;\ 10)} = 4.048$, $p = 0.0023$) and higher ROX values ($t_{(0.05;\ 10)} = 3.84$, $p = 0.0033$) and ROX/ETS ratio ($t_{(0.05;\ 10)} = 3.696$, $p = 0.0031$) than control (Fig. 4A). Although the ETS/OXPHOS (Fig. 4B), OXPHOS capacity (Fig. 4C), OXPHOS coupling efficiency (Fig. 4D), bioenergetic efficiency (Fig. 4E), RCR (Fig. 4F), and succinate control factor (Fig. 4G) did not change, SCR was increased ($t_{(0.05;\ 8)} = 2.593$, $p = 0.0268$) after EtOH exposure (Fig. 4H). Fig. 5 shows a schematic representation of the energy metabolism of acetate from EtOH catabolism (Fig. 5A) and the main effects of acute and chronic EtOH exposures on mitochondrial bioenergetics described (Fig. 5B).

4. Discussion

Evidence has shown that EtOH can modulate redox signaling and induce oxidative stress in the zebrafish brain (Muller et al., 2017; Rosemberg et al., 2010). Oxidative stress is one of the main mechanisms associated with the harmful effects of EtOH on the CNS (Augustyniak et al., 2005; Pereira et al., 2015; Sun et al., 2001; Sun and Sun, 2001), and mounting data support a crucial role of mitochondrial dysfunction

in alcohol-related neurotoxicity in various animal models (Pereira et al., 2015; Wu and Cederbaum, 2003; Yang and Luo, 2015; Zimatkin et al., 2006). To date, there are no data reporting whether redox alterations in the CNS occur due to changes in mitochondrial respiration in zebrafish. Here, we observed that acute EtOH exposure overstimulated mitochondrial O_2 consumption, while EtOH chronically decreased mitochondrial respiration by negatively modulating the ETS activity. Therefore, our novel findings demonstrate that both acute and chronic EtOH exposures affect, at least in part, the mitochondrial function by different mechanisms depending on the administration protocol.

EtOH acutely stimulated mitochondrial respiration through increased baseline respiration and CI_{OXPHOS}. OXPHOS capacity (directly related to CI electron flux), coupling efficiency. Furthermore, bioenergetics efficiency increased after acute EtOH exposure, reinforcing the EtOH stimulatory effect on mitochondrial O_2 consumption. EtOH acutely also increased RCR, which is related to the mitochondrial functionality and state of mitochondrial coupling, suggesting an enhancement of OXPHOS process. The enhanced baseline respiration and CI_{OXPHOS} may be related with EtOH metabolism pathway in the brain, which increases NADH levels during the oxidation process (Deitrich et al., 2006; Hipolito et al., 2007). The acetate from EtOH metabolism can be incorporated into acetyl-coenzyme A (acetyl-CoA), a substrate of the Krebs cycle, which increases the formation of reducing equivalents (Deng and Deitrich, 2008; Lieber, 2005). NADH plays a role in ATP generation during the OXPHOS, facilitating ATP production. However, excessive NADH formation may overstimulate CI complex, thereby generating the leak of electrons (Vinogradov and Grivennikova, 2016). This phenomenon may reflect higher mitochondrial O_2 consumption, which facilitates ROS formation (*e.g.*, $O_2^{\cdot}$, H_2O_2) (Bailey and Cunningham, 2002; Bailey et al., 1999; Hoek et al., 2002). Importantly, the reduction of NAD^+/NADH ratio as a consequence of EtOH metabolism can disrupt fatty acid oxidation, inducing ketogenesis, lactic acidosis, and hypoglycemia (Cunningham and Bailey, 2001; Haorah et al., 2013; Lieber, 2005; McGuire et al., 2006). Based on our findings showing a decreased succinate control factor, the increased OXPHOS following acute EtOH exposure does not result from changes in complex II activity. Although EtOH can acutely decrease ATP production (Budd and Nicholls, 1996; Liu et al., 2014), the mitochondrial overstimulation could facilitate ROS formation in the CNS (Hoek et al., 2002), corroborating the higher ROX/ETS ratio observed here. These results support a role of mitochondria in mediating oxidative stress in zebrafish, which showed impaired brain antioxidant enzyme activities and increased lipid peroxidation in our previous report (Rosemberg et al., 2010).

In addition to the acute exposure protocol, we explored the chronic effects of EtOH in zebrafish. Chronically, EtOH-exposed group showed a reduced baseline respiration as well as an impaired ETS, reflected by the lower CI + CII- and CII-mediated ETS. A dysfunction of CII-mediated respiration may overload other mitochondrial complexes, thereby affecting ETS and accentuating endogenous ROS formation. Importantly, the increased SCR suggest a main involvement of complex I in ETS. Moreover, the higher ROX state and ROX/ETS ratio corroborate with the increased ROS levels and pro-oxidant effects in the zebrafish brain described elsewhere (Muller et al., 2017). Thus, we suggest that part of the O_2 is not being consumed by mitochondria, but rather by other EtOH detoxification pathways (*e.g.*, catalase and CYP450 enzymes), which are directly involved in EtOH metabolism (Moghe et al., 2011). Oxidative damage after chronic EtOH exposure can alter the fluidity of the mitochondrial membrane (Kowaltowski et al., 2009; Tapia-Rojas, 2018), disrupt the mitochondrial membrane potential (Karadayian et al., 2015), and reduce the mitochondrial complexes I, III, and IV activities, which are necessary for ATP formation (Bustamante et al., 2012; Karadayian et al., 2015).

The use of the high-resolution respirometry assay can be a promising strategy to assess mitochondrial bioenergetics in zebrafish models. However, to perform such analysis, the zebrafish presents some limitations. For example, the small size of brain tissue requires more than two brains per independent sample to perform replicate experiments. Furthermore, the use of other oxidizable substrates and inhibitors, as well as the investigation of enzyme activities related to the Krebs cycle could provide a more detailed response involving the mechanistic bases of EtOH in brain energy metabolism of zebrafish. Although we show distinct effects of alcohol depending on the exposure period, we cannot affirm whether such responses are mediated by EtOH alone and/or by its metabolite, acetaldehyde. Because acetaldehyde can mediate deleterious effects of EtOH in the CNS (*e.g.*, lipid peroxidation, ROS formation, DNA damage) (Balino et al., 2019; Pereira et al., 2015; Quertemont et al., 2005), further studies are needed to investigate a putative involvement of this metabolite on the biochemical responses measured here.

In conclusion, our novel findings show that EtOH affects the mitochondrial respiration in the zebrafish brain. These effects on bioenergetic in the zebrafish CNS could be related to multifactorial mechanisms (*e.g.*, pro-oxidant properties of EtOH concomitant with its toxic metabolite acetaldehyde, ROS generation, and OXPHOS dysfunction), playing a central role in EtOH-mediated neurotoxicity. Due to the similarity of zebrafish CNS physiology with those of rodents and humans, this species can provide robust and translational data regarding the neurobiological bases of alcohol abuse and addiction, contributing to unravel novel therapeutic strategies.

Declaration of competing interest

The authors declare no conflict of interest.

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ARTIGO 3

Role of the serotonergic system in ethanol-induced aggression and anxiety: a pharmacological approach using the zebrafish model

Talise E. Müller, Paola R. Ziani, Barbara D. Fontana, Tâmie Duarte, Flavia V. Stefanello, Julia Canzian, Adair R. S. Santos, Denis B. Rosemberg

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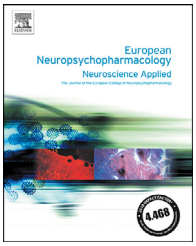

Role of the serotonergic system in ethanol-induced aggression and anxiety: A pharmacological approach using the zebrafish model

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Abstract
Acute ethanol (EtOH) consumption exerts a biphasic effect on behavior and increases serotonin levels in the brain. However, the molecular mechanisms underlying alcohol-mediated behavioral responses still remain to be fully elucidated. Here, we investigate pharmacologically the involvement of the serotonergic pathway on acute EtOH-induced behavioral changes in zebrafish. We exposed zebrafish to 0.25, 0.5, 1.0% (v/v) EtOH for 1 h and analyzed the effects on aggression, anxiety-like behaviors, and locomotion. EtOH concentrations that changed behavioral responses were selected to the subsequent experiments. As a pharmacological approach, we used pCPA (inhibitor of tryptophan hydroxylase), WAY100135 (5-HT_{1A} antagonist),

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buspirone (5-HT_{1A} agonist), CGS12066A and CGS12066B (5-HT_{1B} antagonist and agonist, respectively), ketanserin (5-HT_{2A} antagonist) and (±)-DOI hydrochloride (5-HT_{2A} agonist). All serotonergic receptors tested modulated aggression, with a key role of 5-HT_{2A} in aggressive behavior following 0.25% EtOH exposure. Because CGS12066B mimicked 0.5% EtOH anxiolysis, which was antagonized by CGS12066A, we hypothesized that anxiolytic-like responses are possibly mediated by 5-HT_{1B} receptors. Conversely, the depressant effects of EtOH are probably not related with direct changes on serotonergic pathway. Overall, our novel findings demonstrate a role of the serotonergic system in modulating the behavioral effects of EtOH in zebrafish. These data also reinforce the growing utility of zebrafish models in alcohol research and help elucidate the neurobiological mechanisms underlying alcohol abuse and associated complex behavioral phenotypes.

1. Introduction

Alcohol abuse is a serious problem worldwide due to the potential alcohol-mediated toxicity and dependence-producing properties (Spanagel, 2009). Alcohol consumption triggers more individual and social damages than other drugs (*e.g.*, heroin, crack, and methamphetamine) (Nutt et al., 2010). Annually, 3.3 million people die from one of more than 200 conditions and diseases caused by alcohol consumption (*e.g.*, neuropsychiatric disorders, fetal alcohol syndrome, cancers, and gastrointestinal diseases) (WHO, 2014). The etiology of alcohol abuse and alcoholism is complex and involves both intrinsic and extrinsic factors (Wall et al., 2016).

Acute ethanol (EtOH) consumption exerts a biphasic effect on behavior. At lower doses, EtOH promotes euphoria by disinhibiting punished operant behaviors, while high doses promote depressant symptoms, causing lack of coordination, and dormancy (Hendler et al., 2013). Various mechanisms contribute to the neurobiological effects of EtOH, since it acts directly on cellular membranes and/or indirectly by modulating signaling transduction pathways (Chastain, 2006). Moreover, alcohol abuse impairs the physiology of various neurotransmitters in the brain (Esel and Dinc, 2017), which contributes for euphoric/reinforcing effects, neuroadaptation, dependence, tolerance, and withdrawal symptoms (Trudell et al., 2014).

The serotonergic pathway is associated with alcohol dependence, playing a key role in EtOH consumption, vicious cycle, and recidivism (Kirby et al., 2011). Acutely, EtOH increases serotonin (5-HT) release in the central nervous system (CNS) (Chatterjee and Gerlai, 2009; McBride, 2002), affecting thinking behaviors, mood, fear, and aggressiveness (Chastain, 2006; Sari et al., 2011). Changes in 5-HT levels also play a role in dependence by modulating reward and stress systems (Marcinkiewcz, 2015). Furthermore, 5-HT release may affect the GABAergic system and increase dopamine production, which play a role in decision-making (Samson and Harris, 1992), and emotional behaviors (Chastain, 2006), respectively.

To understand the evolutionarily conserved mechanisms involved in alcohol abuse and alcoholism, alternative animal models have been successfully used in neuropharmacology (Grotewiel and Bettinger, 2015; Tran et al., 2016b). The zebrafish (*Danio rerio*) is gaining popularity in alcohol research, biological psychiatry, and behavioral pharmacology (Fontana et al., 2018; Ninkovic and Bally-Cuif, 2006). This species has a high degree of genomic conservation (Barbazuk et al., 2000), well-characterized neurotransmitter systems (Demin et al., 2018; Rico et al., 2011), and displays an extensive behavioral repertoire (Kalueff et al., 2013). EtOH exposure in zebrafish affects behavioral functions in a concentration- and time-dependent manner (Gerlai et al., 2000; Mathur and Guo, 2011; Muller et al., 2017), elevates blood alcohol concentrations (Echevarria et al., 2011) and increases 5-HT, dopamine, and their metabolites in the brain (Chatterjee and Gerlai, 2009).

Although the serotonergic system presents some genetic differences between zebrafish and mammals, the effects of drugs that modulate 5-HT metabolism are conserved (Maximino and Herculano, 2010; Maximino et al., 2013a). In zebrafish, a correlation between 5-HT release in the brain and specific behaviors (*e.g.*, fear, anxiety, and aggression) has been reported (Herculano and Maximino, 2014; Maximino et al., 2013b). Both serotonergic agonists/antagonists, as well as *p*-chlorophenylalanine (pCPA), an inhibitor of tryptophan hydroxylase, modulate anxiety and locomotion in larvae and adult zebrafish (Airhart et al., 2012; Sallinen et al., 2009). Because EtOH affects specific behavioral functions (*e.g.*, anxiety, locomotion, and aggression), which can be modulated by 5-HT, we sought to investigate pharmacologically a putative involvement of the serotonergic pathway on acute EtOH-induced behavioral responses in zebrafish.

2. Experimental procedures

2.1. Animals

Subjects were adult (4–6 months-old) short fin wild-type zebrafish (*Danio rerio*) of mixed genders (50:50 male:female ratio). Fish were obtained from a local supplier (Hobby Aquários, RS, Brazil) and acclimated in 40-L tanks for two weeks in a maximum density of four fish per liter. Tanks were filled with non-chlorinated water kept under constant mechanical, biological, and chemical filtration. Water temperature, pH, and conductivity were set at 26 ± 2 °C, 7.0–8.0, and 1500–1600 μS/cm, respectively. Animals were kept on a 14/10 light/dark photoperiod cycle (lights on at 7:00 am) and fed with a commercial flake fish food (Alcon BASIC®, Alcon, Brazil) twice daily. All protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (process number 026/2014).

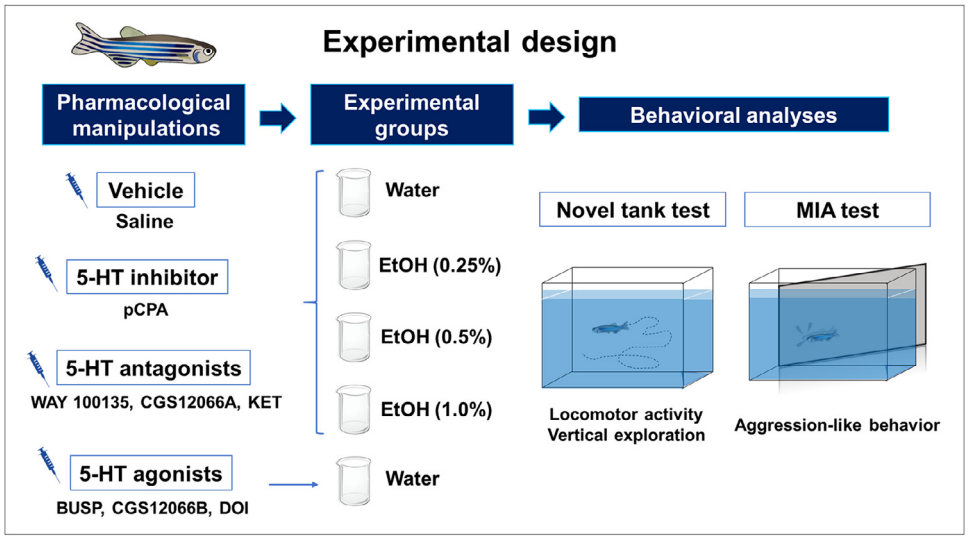

Fig. 1 Schematic representation of the experimental design. The figure shows the pharmacological manipulations of the serotonergic system using pCPA (inhibitor of tryptophan hydroxylase), as well as 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{2A} antagonists (WAY100135, CGS12066A, and KET, respectively) to analyze the influence on EtOH-mediated responses (0.25–1.0% v/v). The effects of 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{2A} agonists (buspirone, CGS12066B, and DOI, respectively) alone were tested to verify whether their response profile would be similar to those observed in EtOH-treated group. Locomotor and anxiety-like behaviors were measured in the novel tank test, while aggression-like behaviors were measured using the mirror-induced aggression (MIA) task (pCPA = *p*-chlorophenylalanine; EtOH = ethanol; KET = ketanserin; BUSP = buspirone; DOI = (±)-DOI hydrochloride).

2.2. Ethanol exposure

For acute EtOH exposure, zebrafish were individually exposed to 0.25, 0.5, or 1.0% (v/v) in beakers for 1 h (Chatterjee and Gerlai, 2009; Rosemberg et al., 2012) and the solution was changed for each fish tested. The respective protocol allows the detection of a biphasic effect of alcohol in zebrafish – anxiolysis at lower concentrations and depressant/sedative-like effects at higher EtOH concentrations (Gerlai et al., 2000). Because our intention was to test the potential involvement of the serotonergic system on EtOH-induced effects, we analyzed specific behavioral endpoints modulated by EtOH in subsequent experiments.

2.3. Pharmacological manipulations of serotonergic system

To assess the involvement of serotonergic system in EtOH-mediated responses, all drugs were administered via intraperitoneal injection (volume of 10 μL) as described previously (Kinkel et al., 2010). The tryptophan hydroxylase inhibitor (pCPA) was diluted in saline solution (0.9% NaCl) and administered for 2 days (1 injection of 300 mg/kg per day) prior to EtOH exposure, since the respective concentration reduces 5-HT content in zebrafish brain (Maximino et al., 2013b). Control fish were injected with 10 μL saline. To analyze whether serotonergic receptors mediate EtOH responses, we tested the behavioral effects in the presence or absence of agonists and antagonists. To assess the involvement of 5-HT_{1A} receptors, we used WAY100135 (10 mg/kg) and buspirone (50 mg/kg) as antagonist and agonist drugs, respectively. As a partial agonist of 5-HT_{1A} receptors, buspirone has been extensively used to study the anxiolytic-like effect of 5-HT_{1A} agonism in both zebrafish (Bencan et al., 2009; Connors et al., 2014; Maximino et al., 2013b) and rodents (Mohajjel Nayebi and Sheidaei, 2010; Pokorny et al., 2016; Sivarao et al., 2004). To verify the involvement of 5-HT_{1B} receptors, we used the antagonist CGS12066A (30 mg/kg), and the agonist CGS12066B (14 mg/kg). To assess the involvement of 5-HT_{2A} receptors, the antagonist ketanserin (5 mg/kg) and the agonist (±)-DOI hydrochloride (5 mg/kg) were used. While the 5-HT antagonists were administered 20 min prior EtOH exposure, the 5-HT agonists were administered 20 min before the behavioral tests (without EtOH exposure) to verify whether their response profiles would be similar to those observed in EtOH-treated group. All drug concentrations used here were based on previous protocols (Bjorvatn and Ursin, 1994; Brumley and Robinson, 2005; Cui et al., 2018; Kunisawa et al., 2017; Maximino et al., 2013b). Fig. 1 shows a schematic representation of the experimental procedures.

2.4. Behavioral measurements

The behavioral tests were performed immediately after EtOH exposure between 10:00 am and 4:00 pm using $n = 8$–12 per group. Experimental tanks were filled with non-chlorinated water (27 ± 1 °C) and kept on a stable surface. Behaviors were recorded using a webcam connected to a laptop at 30 frames/s using appropriate video-tracking software (ANY-maze™, Stoelting CO, USA).

2.4.1. Mirror-induced aggression (MIA) test

Aggressive behavior was assessed using the mirror-induced aggression (MIA) test (Gerlai et al., 2000; Fontana et al., 2016). After EtOH exposure, fish were immediately transferred individually to the experimental tank (25 cm length × 15 cm height × 6 cm width) and a mirror was placed inclined at 22.5° to the back wall of the tank so that the left vertical edge of the mirror was touching the side of the tank and the right edge was further away. Thus, when the experimental fish swam to the one side of the tank their reflected image appeared closer, evoking aggression-like responses. Tanks were virtually divided into three areas related to their proximity to mirror (Gerlai et al., 2000) and the following behaviors were determined in a single 5-min session: number of entries and time spent in the mirror area, as well as the number and duration of aggressive episodes. Aggressive display was counted when fish presented erection of dorsal, caudal, pectoral, and anal fins, usually associated

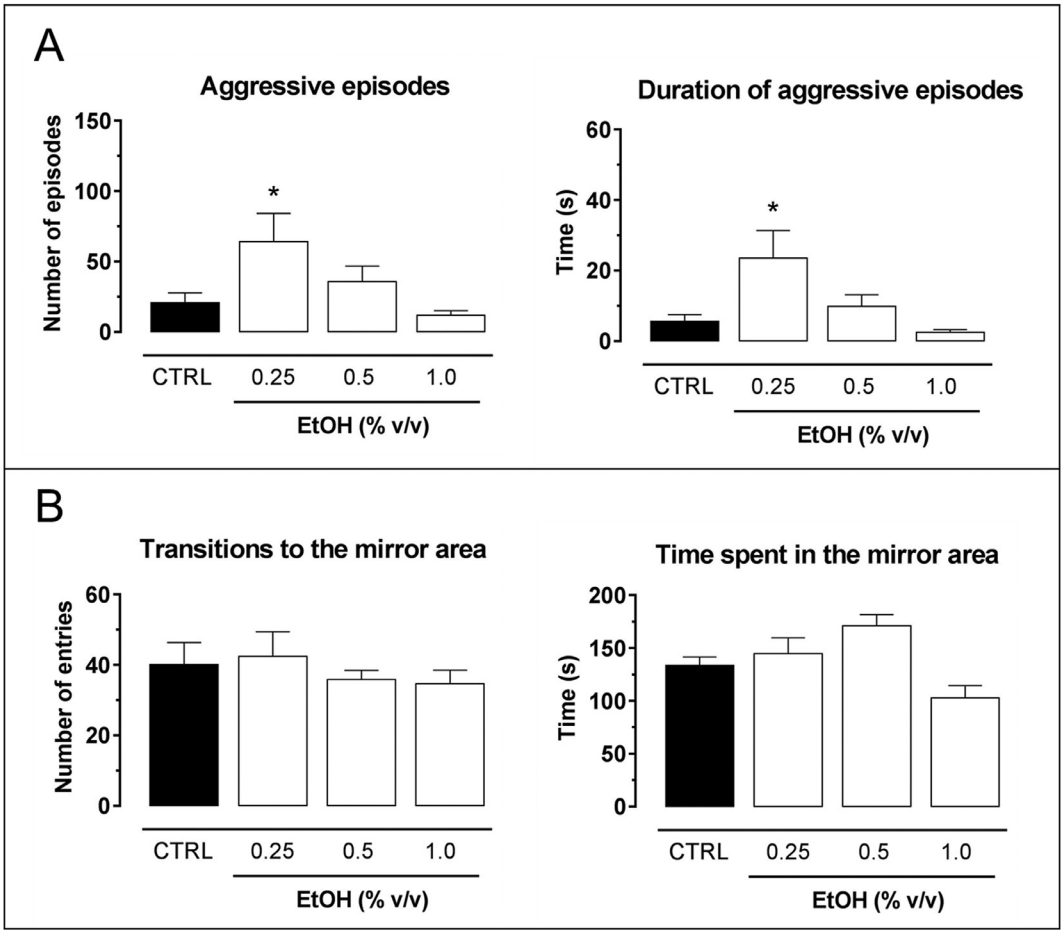

Fig. 2 Effects of acute EtOH exposure (0.25–1.0% v/v) on the aggression-like profile. Data were expressed as mean ± SEM and analyzed by one-way ANOVA followed Student-Newman-Keuls multiple comparisons test ($^{*}p < 0.05$, $n = 8$–12 per group; CTRL = control; EtOH = ethanol).

with undulating body movements and attacks. Attack episodes were manually counted by two trained observers blind to the experimental condition (inter-rater reliability > 0.85) when fish displayed short bout of fast swimming directed towards the opponent associated with bites towards the mirror image (Kalueff et al., 2013).

2.4.2. Novel tank test (NTT)

Locomotor and exploratory activities were analyzed using the novel tank test (NTT), which may reflect habituation to novelty stress (Cachat et al., 2010). Immediately after EtOH exposure, zebrafish were individually placed in a novel apparatus (25 cm length × 15 cm height × 6 cm width) filled with 2 L non-chlorinated water for 6 min and their swimming behavior was recorded. The tank was virtually divided into two horizontal sections (bottom and top) to assess the vertical exploration using the following endpoints: transitions and time spent in top and latency to enter the top area. Distance traveled, absolute turn angle, and time immobile were used as locomotor measures.

2.5. Statistics

Normality of data and homogeneity of variances were analyzed by Kolmogorov-Smirnov and Bartlett's tests, respectively. The effects of EtOH on behaviors were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. To investigate the effects of serotonergic antagonists, we used a two-way ANOVA (ethanol and antagonists as factors), followed Student-Newman-Keuls multiple comparison test. The effects of serotonergic agonists were analyzed by one-way ANOVA followed Student-Newman-Keuls multiple comparisons test. Data were expressed as mean ± standard error of the mean (S.E.M.) and the significance level was set at $p \leq 0.05$.

3. Results

3.1. Involvement of serotonergic system on aggressive behavior

Fig. 2 shows the effects of acute EtOH exposure on aggressive behavior. At 0.25%, EtOH increased the number ($F_{(3,30)} = 4.018$, $p = 0.0162$) and duration ($F_{(3,30)} = 5.172$, $p = 0.0053$) of aggressive episodes when compared to control. Both transitions and time spent in the mirror area did not differ.

The effects of pCPA, antagonists, and agonists on the number and duration of aggressive episodes following 0.25% EtOH exposure are shown in Fig. 3. Significant effects of pCPA x EtOH interaction ($F_{(1,28)} = 7.124$, $p = 0.0125$) and pCPA ($F_{(1,28)} = 12.91$, $p = 0.0012$) on the number of aggressive episodes were observed. Additionally, the duration

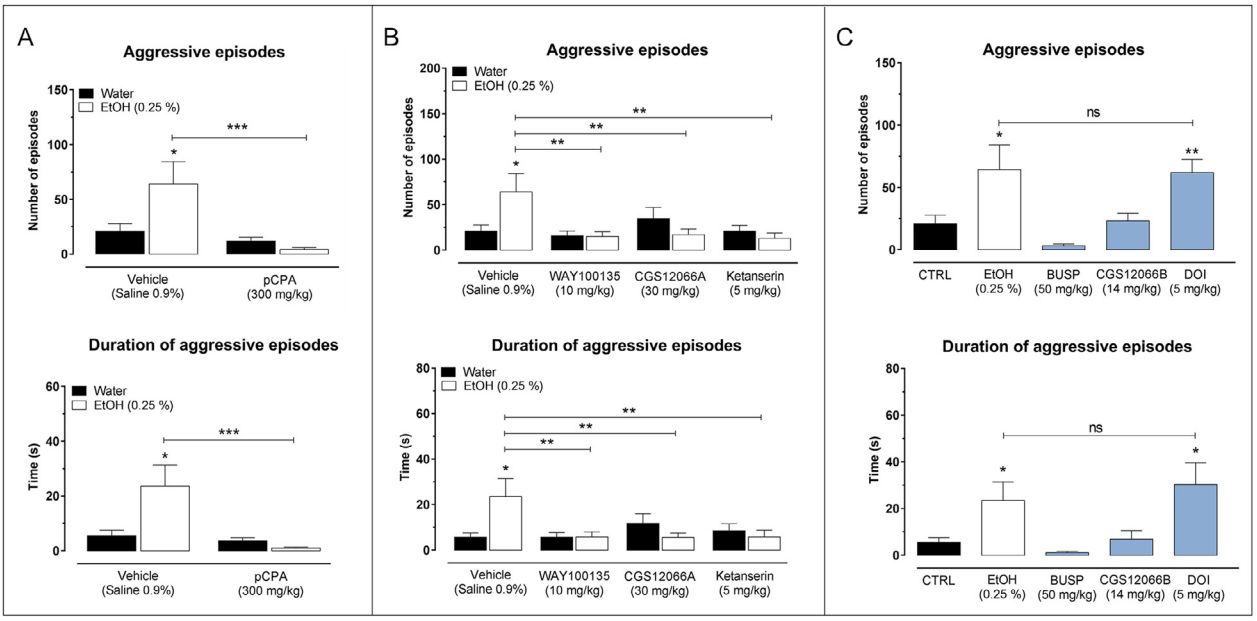

Fig. 3 Effects of pCPA, 5-HT antagonists, and 5-HT agonists on the number and duration of aggressive episodes. EtOH was tested at the concentration of 0.25% (v/v). **(A)** Effects of pCPA on aggression; **(B)** effects of 5-HT antagonists on EtOH-mediated aggressive behaviors; **(C)** effects of EtOH and 5-HT agonists on aggression. Data were expressed as mean ± SEM and analyzed by one or two-way ANOVA followed Student-Newman-Keuls multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, ns = non-significant; $n = 8$–12 per group; CTRL = control; pCPA = *p*-chlorophenylalanine; EtOH = ethanol; BUSP = buspirone; DOI = (±)-DOI hydrochloride).

of aggressive episodes also showed significant effects of pCPA x EtOH interaction ($F_{(1,28)} = 8.106$, $p = 0.0082$), pCPA ($F_{(1,28)} = 11.56$, $p = 0.0020$), and EtOH ($F_{(1,28)} = 4.518$, $p = 0.0425$) (Fig. 3A). Basically, pCPA abolished the effects of 0.25% EtOH on aggression and did not modulate the aggressive behavior alone. Fig. 3B shows the effects of serotonergic antagonists on 0.25% EtOH-induced aggressive responses. We observed significant effects of antagonists x EtOH interaction ($F_{(3,65)} = 3.325$, $p = 0.0250$) and antagonists ($F_{(3,65)} = 3.857$, $p = 0.0133$) on the number of aggressive episodes. The duration of aggressive episodes also showed a significant effect of antagonists x EtOH interaction ($F_{(3,65)} = 4.089$, $p = 0.0101$). In general, WAY100135, CGS12066A, and ketenserin abolished the effects of 0.25% EtOH on the aggression-related phenotypes. Moreover, DOI administration induced similar responses when compared to 0.25% EtOH group, with a significant increase in the number ($F_{(4,37)} = 7.842$, $p = 0.0001$) and duration ($F_{(4,39)} = 4.278$, $p = 0.0058$) of aggressive episodes when compared to control (Fig. 3C).

3.2. Influence of the serotonergic system on anxiety-like behavior and locomotion

Fig. 4A shows the acute effects of EtOH on vertical exploration in the NTT. Fish exposed to 0.5% EtOH spent more time in top area ($F_{(3,37)} = 28.23$, $p < 0.00010$) than control. Conversely, 1.0% EtOH reduced both transitions and time spent in top area ($F_{(3,36)} = 12.41$, $p < 0.0010$; $F_{(3,37)} = 28.23$, $p < 0.00010$), as well increased the latency to enter the top ($F_{(3,38)} = 23.48$, $p < 0.00010$). Fig. 4B shows the acute effects of EtOH on locomotor activity. We verified that 1.0% EtOH reduced the distance traveled ($F_{(3,36)} = 7.252$, $p = 0.0006$), absolute turn angle ($F_{(3,36)} = 9.196$, $p = 0.0001$), and increased time immobile ($F_{(3,36)} = 6.796$, $p = 0.0010$).

The influence of pCPA, antagonists, and agonists on the effects of 0.5% EtOH are shown in Fig. 5. Two-way ANOVA revealed significant effects of EtOH x pCPA interaction ($F_{(1,34)} = 4.968$, $p = 0.0325$), pCPA ($F_{(1,34)} = 89.24$, $p < 0.0001$), and EtOH ($F_{(1,34)} = 15.11$, $p = 0.0004$) on the time spent in top area. pCPA alone decreased the time spent in top area when compared to control and also abolished the effects of EtOH on the respective parameter (Fig. 5A). When the effects of serotonergic antagonists were tested, two-way ANOVA yielded significant effects of EtOH x antagonists interaction ($F_{(3,73)} = 4.819$, $p = 0.0041$) and antagonists ($F_{(3,73)} = 5.45$, $p = 0.0019$). Basically, CGS12066A antagonized the effects of EtOH on time spent in top area (Fig. 5B). Although BUSP and CGS12066B alone increased the time spent in top area ($F_{(4,41)} = 47.23$, $p < 0.0001$) when compared to control group, only CGS12066B group showed a similar response when compared to 0.5% EtOH (Fig. 5C).

Regarding the effects of pCPA on depressant-like responses following 1.0% EtOH (Fig. 6A), two-way ANOVA revealed significant effects of pCPA ($F_{(1,38)} = 31.33$, $p = 0.00172$), and EtOH ($F_{(1,38)} = 17.87$, $p = 0.0001$) on transitions to top area. The time spent in top area also showed significant effects of EtOH x pCPA interaction ($F_{(1,38)} = 14.05$, $p = 0.005$), pCPA ($F_{(1,38)} = 31.33$, $p < 0.0001$), and EtOH ($F_{(1,38)} = 28.01$, $p < 0.0001$). Both pCPA and pCPA/EtOH groups reduced both transitions and time spent in top area when compared to control. Two-way ANOVA revealed a significant effect of EtOH ($F_{(1,38)} = 11.71$, $p = 0.0015$) on the latency to enter the top area. pCPA alone

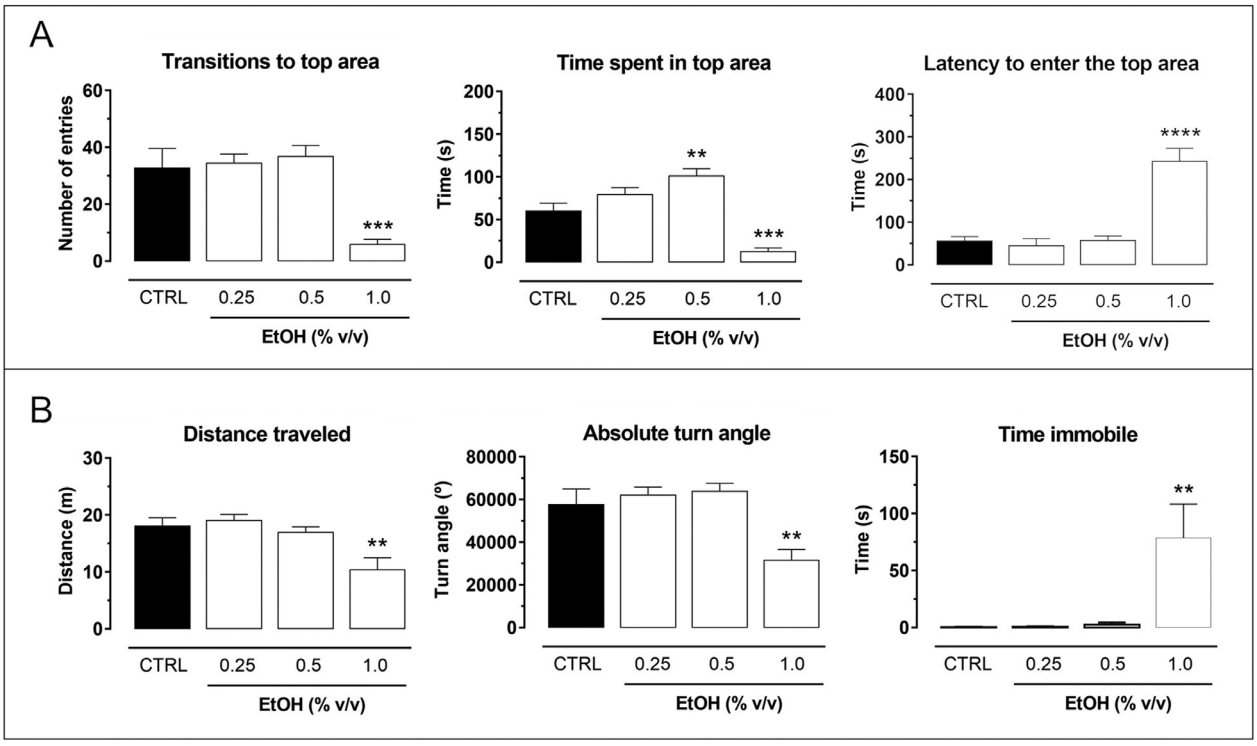

Fig. 4 Effects of acute EtOH exposure (0.25–1.0% v/v) on anxiolytic-like behaviors and locomotion. **(A)** Vertical exploration that reflects anxiety-like responses; **(B)** locomotor parameters. Data were expressed as mean ± SEM and analyzed by one-way ANOVA followed Student-Newman-Keuls multiple comparisons test (** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, $n = 8$–12 per group; CTRL = control; EtOH = ethanol).

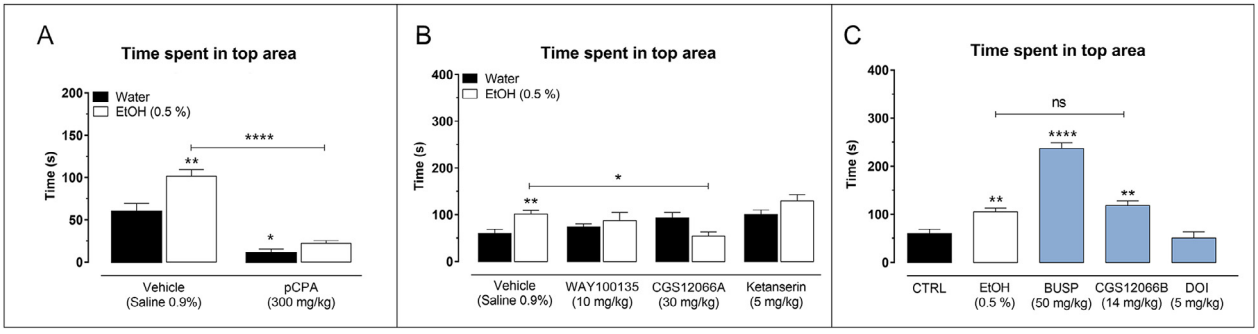

Fig. 5 Involvement of the serotonergic system on 0.5% EtOH-mediated anxiolysis. **(A)** Effects of pCPA on time spent in top area; **(B)** effects of 5-HT antagonists on time spent in top area; **(C)** effects of EtOH and 5-HT agonists on time spent in top area. Data were expressed as mean ± SEM and analyzed by one or two-way ANOVA followed Student-Newman-Keuls multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$, ns = non-significant; $n = 8$–12 per group; CTRL = control; pCPA = *p*-chlorophenylalanine; EtOH = ethanol; BUSP = buspirone; DOI = (±)-DOI hydrochloride).

and previously to EtOH showed higher latency to enter the top area than control.

Fig. 6B shows the influence of 5-HT antagonists on the effects of 1.0% EtOH on transitions, time spent in top area, and latency to enter the top. Two-way ANOVA revealed significant effects of EtOH ($F_{(1,74)} = 95.17$, $p < 0.0001$) on transitions and time spent in top area ($F_{(1,77)} = 57.2$, $p < 0.0001$). WAY100135, CGS12066A, and ketanserin alone did not differ to control. We observed a significant decrease in both transitions and time in top area when WAY100135, CGS 12066A, and ketanserin were administered previously to EtOH when compared to their respective treatments alone. The latency to enter the top area also showed a significant effect of EtOH ($F_{(1,74)} = 93.14$, $p < 0.0001$). WAY100135, CGS12066A, and ketanserin alone did not change the behavioral responses when compared to control. However, we observed increased latency to enter the top area in WAY100135/EtOH, CGS12066A/EtOH, and ketanserin/EtOH when compared to the effects of antagonists alone. In sum, pCPA and antagonists did not abolish the depressant-like effects of EtOH.

Concerning the effects of agonists, BUSP, DOI, and CGS16066B decreased the transitions to top area ($F_{(4,41)} = 7.356$, $p = 0.0001$), while BUSP and DOI showed

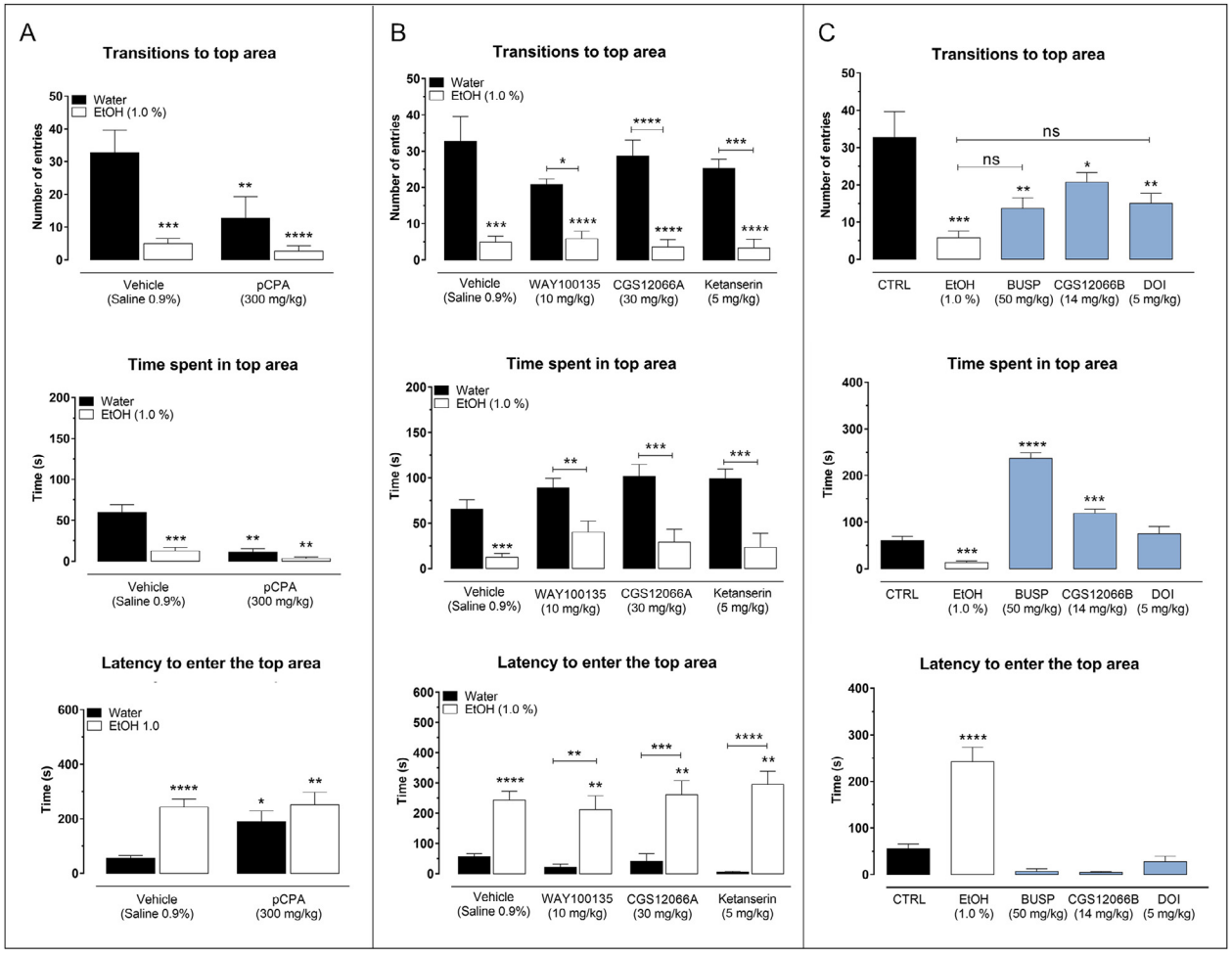

Fig. 6 Effects of pCPA **(A)** and 5-HT antagonists **(B)** on 1.0% EtOH-mediated sedative/depressant-like responses. The effects of 5-HT agonists **(C)** when compared to control and EtOH groups are also shown. The involvement of 5-HT system was tested on the transitions to top area, time spent in top area, and latency to enter the top area. Data were expressed as mean ± SEM and analyzed by one or two-way ANOVA followed Student-Newman-Keuls multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, ns = non-significant; $n = 8$–12 per group; CTRL = control; pCPA = *p*-chlorophenylalanine; EtOH = ethanol; BUSP = buspirone; DOI = (±)-DOI hydrochloride).

similar effects when compared to 1.0% EtOH group. However, BUSP and CGS12066B increased the time spent in top area when compared to control ($F_{(4,43)} = 73.45$, $p < 0.0001$). The 5-HT agonists tested here did not change the latency to enter the top (Fig. 6C).

Fig. 7 shows the effects of pCPA, 5-HT antagonists, and agonists on 1.0% EtOH-induced changes in locomotion. Two-way ANOVA revealed a significant effect of EtOH on distance traveled ($F_{(1,38)} = 24.05$, $p < 0.0001$), absolute turn angle ($F_{(1,38)} = 30.31$, $p < 0.0001$), and time immobile ($F_{(1,40)} = 23.24$, $p < 0.0001$). Although pCPA alone did not alter these behaviors, 1.0% EtOH also impaired locomotion-related phenotypes in pCPA-treated fish (Fig. 7A). Fig. 7B shows the effects of serotonergic antagonists on distance traveled, absolute turn angle, and time immobile following 1.0% EtOH exposure. Two-way ANOVA yielded significant effects of antagonists ($F_{(3,74)} = 7.143$, $p < 0.0001$), and EtOH ($F_{(1,74)} = 54.26$, $p = 0.0003$) on distance traveled. WAY100135, CGS12066A, and ketanserin alone decreased the distance traveled when compared to control group. Pretreatment with CGS12066A and ketanserin reduced the distance traveled in 1.0% EtOH-exposed group when compared to CGS12066A and ketanserin alone. Concerning the absolute turn angle, two-way ANOVA revealed significant effects of antagonists ($F_{(3,74)} = 3.493$, $p = 0.00197$) and EtOH ($F_{(1,74)} = 53.4$, $p < 0.0001$). Although WAY100135, CGS12066A, and ketanserin alone did change this behavior, CGS12066A/EtOH and ketanserin/EtOH groups showed reduced absolute turn angle when compared to CGS12066A and ketanserin alone. The administration of serotonergic antagonists did not change the time immobile. In sum, neither pCPA, nor the antagonists tested did change locomotion when compared to EtOH group.

Fig. 7C shows the effects of serotonergic agonists on locomotor activity. BUSP, CGS12066B, and DOI reduced both distance traveled ($F_{(4,41)} = 14.15$, $p < 0.0001$) and absolute turn angle ($F_{(4,41)} = 7.777$, $p < 0.0001$) when compared to control, showing a similar response to EtOH group. However, the agonists used here did not change the time immobile.

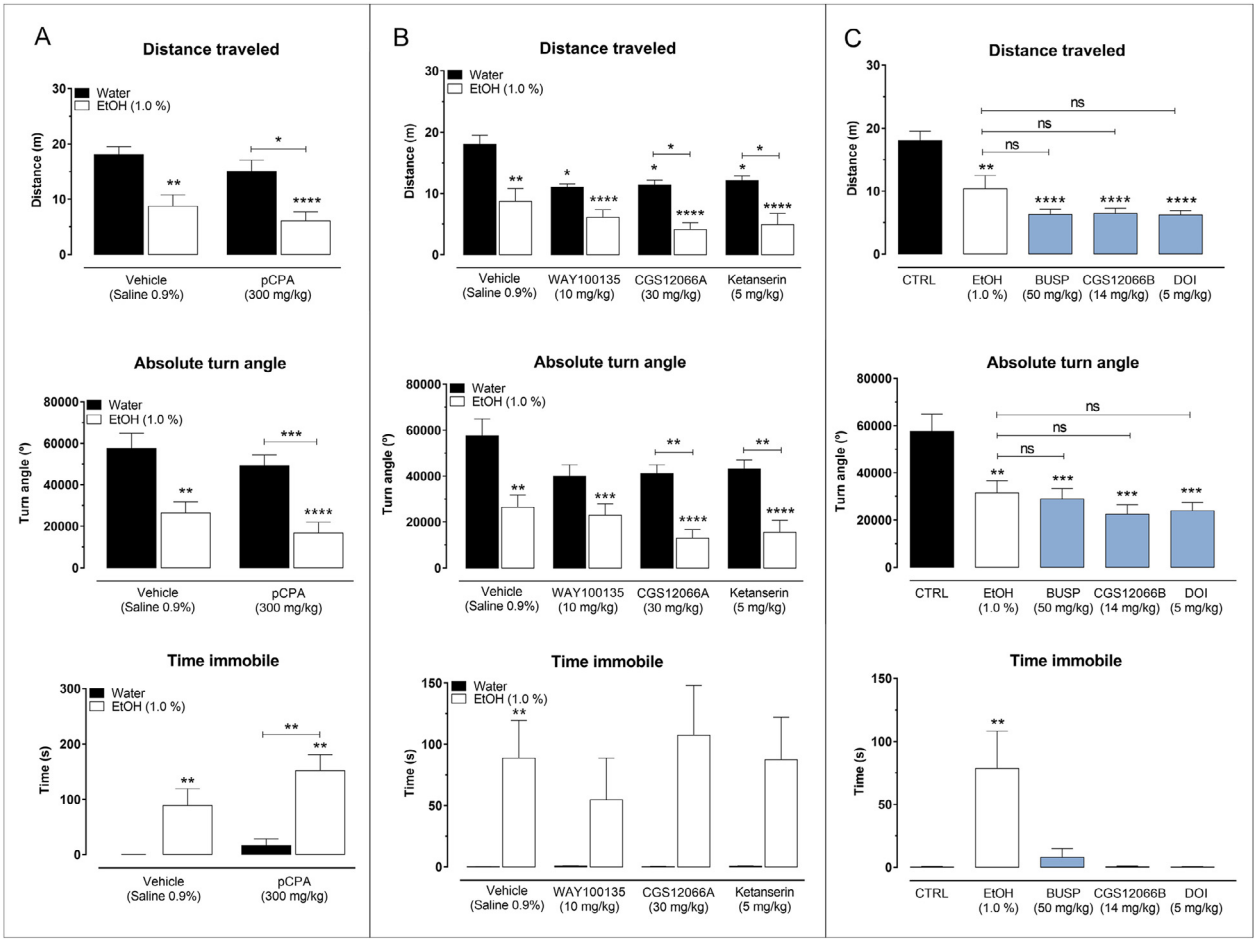

Fig. 7 Involvement of the serotonergic system on 1.0% EtOH-mediated sedative/depressant-like effects. The figure shows the effects of pCPA **(A)**, 5-HT antagonists **(B)** and 5-HT agonists **(C)** on distance traveled, absolute turn angle, and time immobile. Data were expressed as mean ± SEM and analyzed by one or two-way ANOVA followed Student-Newman-Keuls multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, ns = non-significant; n = 8-12 per group; CTRL = control; pCPA = *p*-chlorophenylalanine; EtOH = ethanol; BUSP = buspirone; DOI = (±)-DOI hydrochloride).

4. Discussion

Here, we verify the putative involvement of serotonergic pathway on EtOH-induced behavioral changes in zebrafish. To our knowledge, we demonstrate, for the first time, the role of the serotonergic system in the biphasic responses of acute EtOH exposure in this aquatic species. We observed a direct involvement of the serotonergic system in both aggressive behavior and anxiolytic-like responses following 0.25% and 0.5% EtOH exposure, respectively. However, the sedative/depressant effects are probably not related with changes on serotonergic pathway in zebrafish due to the lack of effects of the antagonists tested on 1.0% EtOH-mediated responses.

Acute alcohol exposure results in biphasic dose- and time-dependent effects, culminating in various behavioral and neurochemical changes (Hendler et al., 2013). Although previous data show anxiolytic-like effects following 1.0% EtOH exposure (Li et al., 2015; Mathur and Guo, 2011; Tran et al., 2016a) most of them used a different treatment regimen (exposure period) to evaluate the behavioral responses of ethanol in zebrafish, thereby eliciting distinct effects on behavior. Here, similar to what occurs in humans, we observed that EtOH acutely increases aggression, elicits anxiolytic-like behaviors, and induces depressant-like effects at lower and higher concentrations in zebrafish, corroborating previous findings (Gerlai et al., 2000; Mocelin et al., 2018; Rosemberg et al., 2012). Thus, we investigated the involvement of the serotonergic pathway in EtOH-mediated behavioral responses by testing specific EtOH concentrations that modulate aggression, locomotion, and anxiety-like parameters.

A link between aggression and acute alcohol consumption has been postulated (Heinz et al., 2011; Miczek et al., 2015). Although the exact nature of this relationship is not fully understood (Attwood and Munafo, 2014), evidence has shown that EtOH acutely enhances serotonergic activity in the mammalian brain (Lovinger, 1997; Sari et al., 2011). The activation of serotonergic pathway is one of the mechanisms involved in EtOH-induced violence and aggression (Glick, 2015). In rodents, low doses of alcohol facilitate aggression through activation of 5-HT neurons, as-

sociated with the stimulation of serotonin and dopamine release – for example, in the ventral and dorsal striatum (Heinz et al., 2011, van der Vegt, 2003). Similarly, acute EtOH exposure also increases 5-HT and its metabolite, 5-HIAA, in whole brain extracts of zebrafish (Chatterjee and Gerlai, 2009), suggesting a conserved role of serotonergic pathway in EtOH-mediated behavioral responses. Among all psychoactive substances, EtOH is arguably the most potent agent that elicits aggression by reducing punished operant behavior (Heinz et al., 2011). EtOH may increase aggression directly or indirectly, via disruption of cognitive-mediated systems or via anxiolysis (Bushman, 1997). As occur in mammals, lower alcohol concentrations predictably increases aggression in zebrafish (Gerlai et al., 2000). Here, although pCPA and the serotonergic antagonists tested abolished EtOH-induced aggression, the aggressive behavior was mimicked only when DOI, a 5-HT_{2A} receptor agonist, was tested. Although the role of $5HT_{2A}$ receptors in aggression is controversial (Nichols, 2004, 2016; Sakaue et al., 2002), genetic studies revealed an association between 5-HT_{2A} receptor polymorphisms, aggression, and impulse control disorders in humans (Giegling et al., 2006).

Our findings corroborate previous report, in which 5-HT_{2A} activation increases aggressive behavior in isolated mice (Sakaue et al., 2002). Importantly, the involvement of 5-HT in the aggressive profile is complex and depends on multiple receptor activations, 5-HT synthesis, and availability at the synaptic cleft (Seo et al., 2008). Moreover, other signaling molecules (*e.g.*, dopamine, GABA, neuropeptides, oxytocin, vasopressin, and norepinephrine) may contribute, at least partially, to the modulatory effects on the serotonergic system (Glick, 2015). Preclinical experiments have also shown a significant role of 5-HT_2 receptors in aggression (Jager et al., 2018). Indeed, the activation of postsynaptic 5-HT_{2A} receptors can be involved in the etiology, pathogenesis, and pathophysiology of impulsive aggression (Rosell and Siever, 2015). Importantly, this behavior is abolished in the presence of M100907, a 5-HT_{2A} receptor antagonist (Winstanley et al., 2004) reinforcing the anti-aggressive and mood stabilizing effects described in the zebrafish model.

The intermediate EtOH concentration tested here (0.5% EtOH) increased the time spent in top area, suggesting anxiolytic-like effects (Gerlai et al., 2000; Rosemberg et al., 2012). This response was mimicked by CGS12066B, a 5-HT_{1B} agonist, and abolished in the presence of pCPA and CGS12066A. Although fish treated with BUSP showed a less anxious behavior, this profile was different than that observed in EtOH group. Importantly, these effects can be associated with the anxiolytic proprieties of BUSP and CGS12066B (Sari, 2004). The serotonergic system has long been implicated in the control of fear, anxiety, and stress in zebrafish (Maximino et al., 2012). Evidence shows that 5-HT_{1B} receptors play a role in anxiety-like states, impulsivity, and aggression (Sari, 2004). Furthermore, these receptors control the release of several neurotransmitters, such as glutamate and GABA, showing a complex neurochemical regulation in anxiety states (Boeijinga and Boddeke, 1996). Pharmacological, molecular, and genetic studies have shown the involvement of 5-HT_{1B} receptors in alcohol dependence (Hoplight et al., 2006; Sari, 2013). Thus, our results are in line with previous findings, suggesting a key role of 5-HT_{1B} receptors in EtOH-induced anxiolysis in zebrafish. As pCPA blocked the effects of EtOH more markedly than CGS12066A, we suggest that other serotonergic receptors families (Zmudzka et al., 2018) may also be involved in anxiolytic-like responses of EtOH in zebrafish. Therefore, further studies should evaluate the implication of the other serotonergic receptors in the EtOH-induced anxiolysis in zebrafish.

Conversely, 1.0% EtOH reduced locomotor activity and vertical exploration. These data reflect a sedative/depressant-like state probably due to the general slowness, impaired coordination, and increased immobility in the bottom of the tank (Gerlai et al., 2000; Mocelin et al., 2018; Rosemberg et al., 2012). Decreased locomotion was also observed when 5-HT_{1A}, 5-HT_{1B}, and 5-HT_2 agonists were tested. Because serotonergic antagonists did not modulate EtOH-induced responses, we suggest that EtOH-induced depressant effects are not directly related to changes in serotonergic system. Accordingly, the agonists tested did not induce immobility, while BUSP and CGS12066B increased the time spent in top area, suggesting a distinct behavioral pattern. In vertebrates, 5-HT plays an important role in locomotion and modulates the motor output (Gabriel et al., 2009). However, since EtOH acts on different signaling pathways, we hypothesized that other neurotransmitters systems may be involved in the sedative effects observed here. For example, high alcohol concentrations potentiate GABAergic activity and antagonize glutamate excitatory actions (Lovinger and Roberto, 2013). Moreover, impaired motor functions and cognitive deficits following EtOH exposure may occur via the agonistic effects on $GABA_A$ receptors (Koob, 2004). Interestingly, 5-HT also interacts with GABAergic neurotransmission by exciting the neurons that produce and secrete GABA, intensifying depressant responses. Therefore, considering the lack of a precise molecular location of serotonergic receptors in zebrafish, more studies are needed to clarify the involvement of 5-HT on EtOH-induced hypolocomotion. Although pCPA alone decreased vertical exploration, the possible involvement of other serotonergic receptors in depressant-like behaviors still merits further scrutiny.

In conclusion, we observed a role of the serotonergic system in modulating EtOH-mediated aggression and anxiolysis in zebrafish. The model used here shows high predictive, face, and construct validities and highlights the use of zebrafish as a pharmacologically tractable tool to explore the neurobiological mechanisms underlying acute EtOH responses. Although the involvement of other serotonergic receptors (5-HT_{2B}, 5-HT_3) in aggression and anxiety-like behaviors of zebrafish still remains to be fully explored, our data suggest the serotonergic system as a possible pharmacological target to counteract some EtOH-mediated responses.

Contributors

TEM, ARSS, and DBR designed the experimental procedures; ARSS and DBR contributed with reagent/analyses tools; TEM, PRZ, BDF, TD, FVS, and JC performed the experiments; TEM, ARSS, and DBR analyzed the data; TEM and DBR wrote the manuscript.

Conflict of Interest

The authors declare that no competing interests exist.

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7. DISCUSSÃO

Sabe-se que o consumo de etanol está relacionado ao desenvolvimento de diversas doenças e que milhares de indivíduos morrem por ano vítimas dos efeitos multidimensionais desta droga (WHO, 2018). Os fármacos disponíveis atualmente não apresentam resultados satisfatórios no tratamento e na reabilitação do indivíduo com adicção em álcool (ANTONELLI et al., 2018). Esta dificuldade em desenvolver tratamentos eficazes é relacionada com a complexidade dos mecanismos de ação do etanol no SNC, os quais são de fundamental importância na busca por novos alvos terapêuticos e tratamentos para os DUA, porém ainda não totalmente compreendidos.

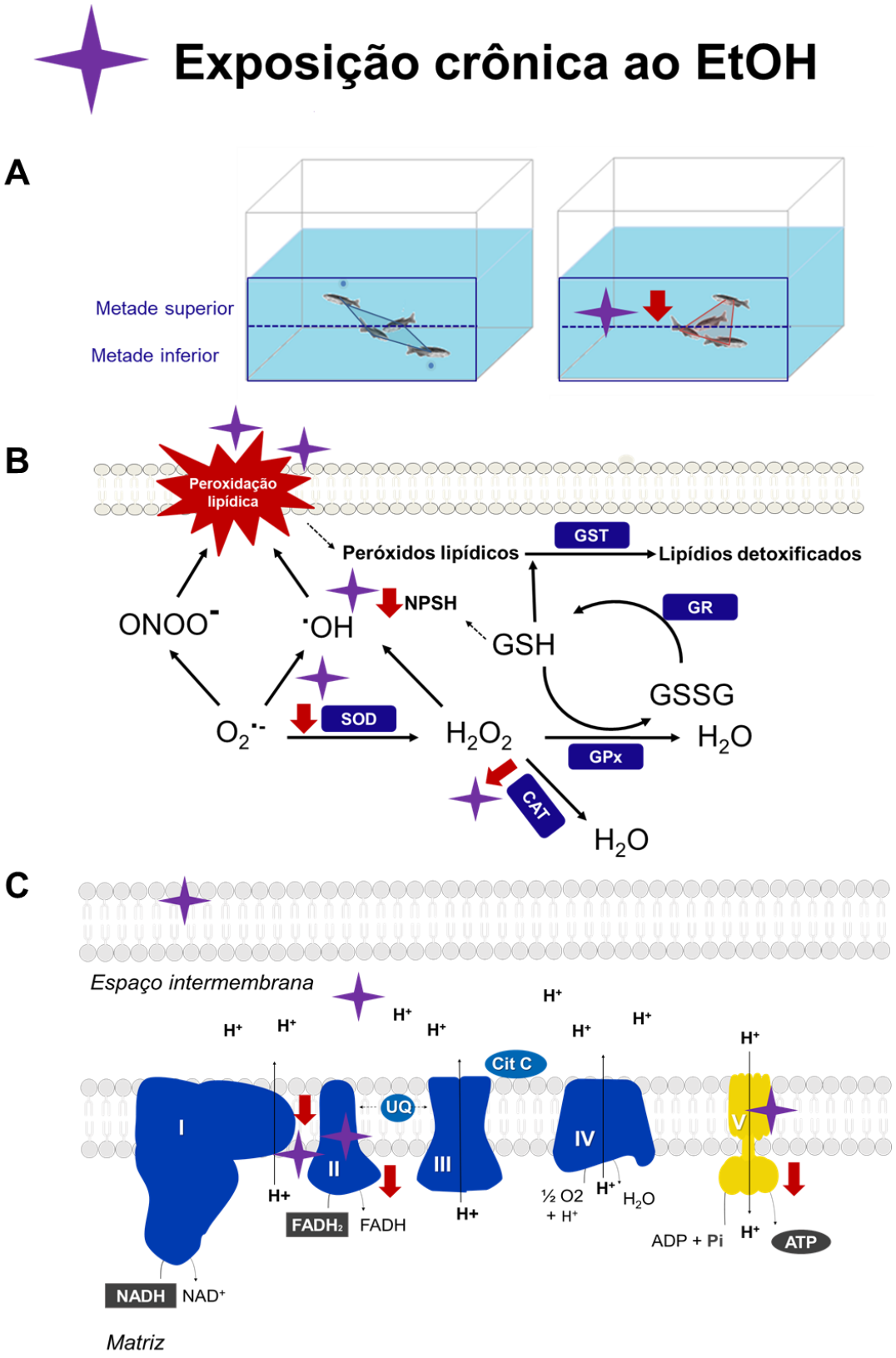
Dessa forma, para o avanço das pesquisas que visam elucidar os mecanismos de ação do etanol e a busca de compostos terapêuticos, torna-se indispensável à utilização de modelos experimentais. O peixe-zebra, por suas características genéticas, neuroquímicas e comportamentais conservadas (HOWE et al., 2013; KALUEFF et al., 2013), tem sido utilizado com sucesso em protocolos translacionais para estudar o abuso e a adicção ao etanol de forma rápida e eficiente (ECHEVARRIA et al., 2011; TRAN et al., 2016). Similar ao que ocorre em humanos, os efeitos neurocomportamentais do etanol em peixe-zebra são dependentes do tempo de exposição e da concentração testada (HANDLER et al., 2013; ROSEMBERG et al., 2012). Concentrações baixas a moderadas aumentam a atividade locomotora e exercem efeitos ansiolíticos, enquanto concentrações altas causam efeito depressor, inibindo a atividade locomotora (GERLAI et al., 2000). A exposição aguda ao etanol aumenta os níveis cerebrais de DA, 5-HT e de seus metabólitos (CHATTERJEE e GERLAI, 2009), além de causar alterações no sistema de defesa antioxidante e consequentemente estresse oxidativo em peixe-zebra (ROSEMBERG et al., 2010). A exposição crônica ao etanol promove tolerância, efeitos motores e ansiolíticos (MATHUR e GUO, 2011). Alterações nos sistemas neurotransmissores modulados por 5-HT e DA e pelos seus metabólitos (CHATTERJEE et al., 2014), também são observadas após protocolos crônicos de exposição ao etanol em peixe-zebra.

Com base nessas informações, identificamos que muitos aspectos bioquímicos e comportamentais poderiam ser explorados a fim de complementar os protocolos de abuso e a adicção ao etanol em peixe-zebra. Portanto, no presente estudo, utilizamos protocolos agudos e crônicos de exposição ao etanol em peixe-zebra e investigamos aspectos relacionados ao estresse oxidativo, bioenergética, bem como o envolvimento do sistema serotoninérgico nas respostas neurocomportamentais induzidas pelo álcool. Estas abordagens objetivam contribuir

para a construção e validação dos modelos de exposição ao etanol em peixe-zebra, para que sejam utilizados de forma robusta e tenham um maior valor preditivo para a triagem de novos compostos terapêuticos.

O estresse oxidativo é proposto como um dos principais mecanismos envolvidos na neurotoxicidade induzida pelo etanol (PEREIRA e ANDRADE, 2015) e sabe-se que os efeitos fisiológicos desta droga podem levar a alterações no comportamento social dos indivíduos (HANDLER et al., 2013). Portanto, o primeiro estudo objetivou verificar, pela primeira vez, se uma exposição crônica ao etanol afeta parâmetros bioquímicos relacionados ao estresse oxidativo e comportamento social em peixe-zebra. Utilizamos um protocolo de exposição intermitente ao etanol por ser o que mais se assemelha com o consumo de álcool pelos humanos (ALCOHOL-ALERT, 2001; MATHUR e GUO, 2011). Como resultado, verificamos que após a exposição intermitente ao etanol, ocorreu um aumento da coesão do cardume, caracterizando um efeito do tipo ansiogênico (CACHAT et al., 2010) (Figura 3A). A análise do comportamento de cardume é um protocolo efetivo para avaliar os efeitos de drogas em componentes sociais da espécie (FERNANDES et al., 2015; MILLER et al., 2013). Ainda, verificamos que a atividade das enzimas SOD e CAT, assim como os níveis de NPSH diminuíram após a exposição ao etanol, enquanto que os níveis de peroxidação lipídica aumentaram, sugerindo dano oxidativo (Figura 3B). Estudos têm demonstrado uma diminuição nas respostas antioxidantes e geração de estresse oxidativo após a exposição crônica ao etanol devido às mudanças adaptativas no SNC (AUGUSTYNIAK et al., 2005; BOSCH-MOREL et al., 1998; CALABRESE et al.,1998; HALLIWELL e GUTTERIDGE, 2007; SUN et al., 2001). Sabe-se que o estresse oxidativo/nitrosativo pode estar relacionado com a ativação de vias celulares envolvidas em déficits sociais (MAES et al., 2011), sugerindo que alterações nos processos oxidativos estão diretamente envolvidas nos efeitos neurocomportamentais do etanol em peixe-zebra.

Figura 3 – Resumo geral dos efeitos da exposição crônica ao etanol em peixe-zebra



Fonte: do autor.

Pelo fato do etanol modular a sinalização redox e gerar estresse oxidativo em protocolos de exposição aguda e crônica em peixe-zebra (MÜLLER et al., 2017; ROSEMBERG et al., 2010), hipotetizamos que essas respostas poderiam estar relacionadas com alterações na funcionalidade mitocondrial (por exemplo, alterações no potencial de membrana, transporte de elétrons e produção de ATP) (BAILEY et al., 1999; CUNNINGHAM e VAN HORN, 2003; GOODLETT e HORN, 2001). Portanto, o segundo estudo teve como objetivo central investigar se alterações na bioenergética mitocondrial, após exposições aguda e crônica, poderiam estar envolvidas nos efeitos neuroquímicos, bioquímicos e comportamentais do etanol em peixe-zebra. Para isso, realizamos uma exposição aguda (ROSEMBERG et al., 2000) e intermitente ao etanol (MÜLLER et al., 2017; MATHUR e GUO, 2011) em peixe-zebra e analisamos alterações na respiração mitocondrial pela técnica analítica de respirometria de alta resolução (GNAIGER, 2009). Agudamente, a exposição ao etanol aumentou a funcionalidade mitocondrial, estimulando o consumo de oxigênio em encéfalo de peixe-zebra. Este fato foi observado através do aumento da respiração basal, da fosforilação oxidativa mediada pelo complexo I, da capacidade de fosforilação oxidativa e da eficiência de acoplamento da mitocôndria (Figura 4A). O estímulo da fosforilação oxidativa pode estar relacionado à própria metabolização do etanol como um substrato energético (DEITRICH, ZIMATKIN e PRONKO, 2004; HIPÓLITO et al., 2007) e também ao aumento da formação de ERO pela mitocôndria (HOEK, CAHILL e PASTORINO, 2002; BAILEY e CUNNINGHAM, 2002).

Ao contrário, a exposição intermitente ao etanol promoveu uma diminuição da respiração basal e da transferência de elétrons entre o complexo I e II e complexo II (Figura 3C). Sabe-se que uma disfunção na respiração mitocondrial mediada pelo complexo II pode sobrecarregar os outros complexos da mitocôndria (KOWALTOWSKI et al., 2009; TAPIA-ROJAS, 2018), afetando o sistema de transporte de elétrons como um todo e acentuando a produção de ERO endógeno (BUSTAMANTE et al., 2012; KARADAYAN et al., 2015). Em conjunto, os dados demonstram que a exposição aguda ao etanol causou uma superestimulação na mitocôndria, enquanto que a exposição crônica modulou negativamente o processo de transporte de elétrons. Estes novos achados mostram que o etanol age por diferentes mecanismos, dependendo do protocolo de exposição. As alterações mitocondriais desencadeadas pelo etanol em peixe-zebra podem ser relacionadas a mecanismos multifatoriais, como por exemplo, ao próprio efeito pró-oxidante do etanol e do acetaldeído, a geração de ERO e disfunção oxidativa (AUGUSTYNIAK et al., 2005). Assim, podemos afirmar que as alterações bioenergéticas observadas neste estudo estão envolvidas, no mínimo

em parte, no estresse oxidativo, na neurotoxicidade e nas alterações comportamentais observadas previamente em protocolos agudo e crônico de exposição ao etanol em peixe-zebra (ROSEMBERG et al., 2010; MÜLLER et al., 2017).

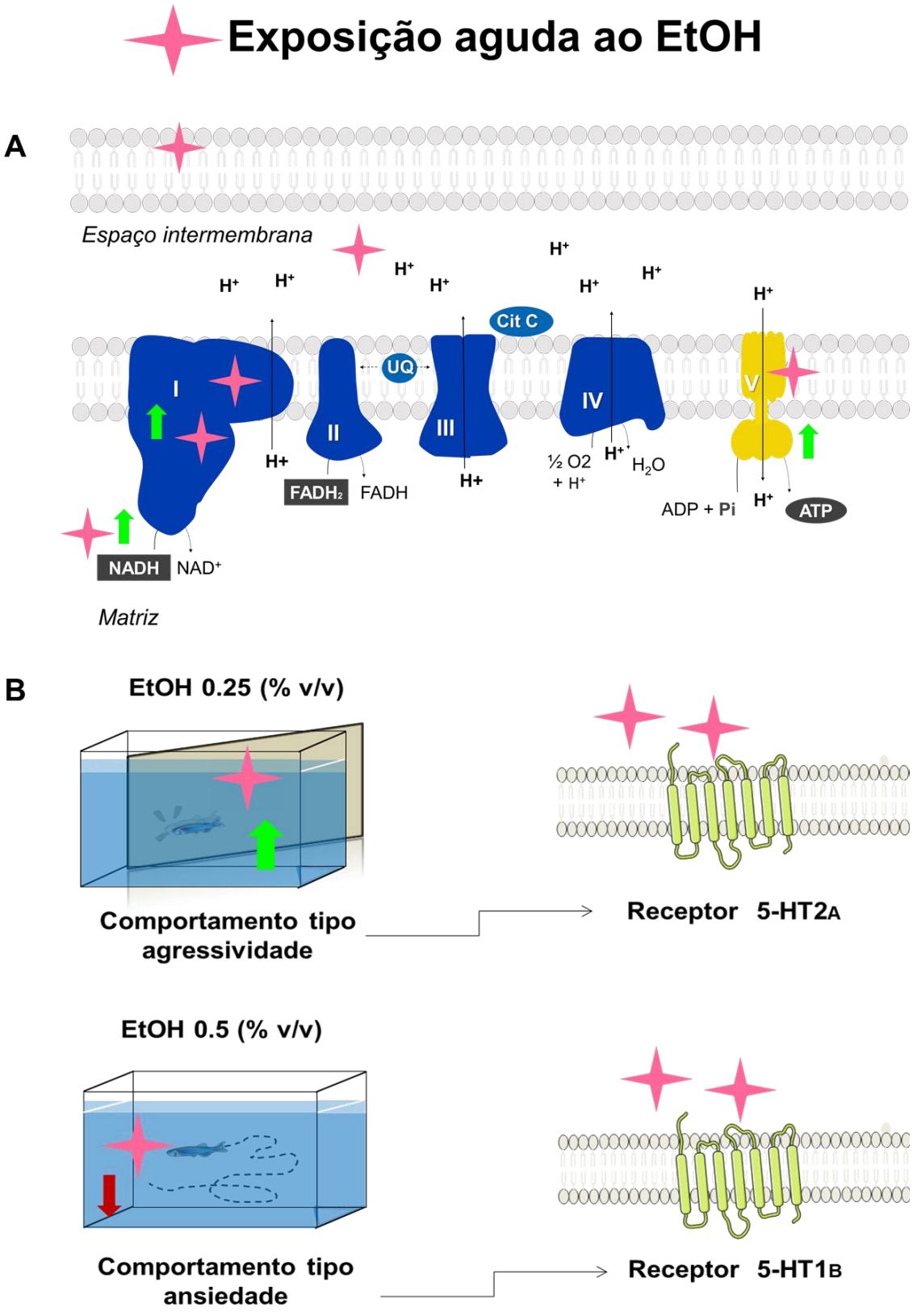
Com relação aos sistemas de neurotransmissão modulados pelo etanol, a via serotoninérgica é associada ao desenvolvimento da dependência ao álcool, principalmente nos processos de busca, ciclo vicioso e reincidências (KIRBY et al., 2011). Sabe-se que agudamente o consumo de etanol exerce um efeito comportamental bifásico e aumenta os níveis de 5-TH e seu metabólito em encéfalo de peixe-zebra (GERLAI et al., 2000; CHATTERJEE e GERLAI, 2009; CHATTERJEE, SHAMS e GERLAI, 2014). Entretanto, pouco se sabe sobre o modo que o etanol age agudamente no sistema serotoninérgico, em quais receptores atua e se os comportamentos bifásicos desencadeados por essa droga são influenciados pela ativação deste sistema de neurotransmissão. Portanto, no terceiro estudo, investigamos farmacologicamente o envolvimento da via serotoninérgica nas alterações comportamentais agudas mediadas pelo etanol em peixe-zebra. Realizamos uma exposição aguda dos animais utilizando as concentrações de 0.25, 0.5, 1.0% (v/v) de etanol por uma hora (CHATTERJEE e GERLAI, 2009; ROSEMBERG et al., 2012) e posteriormente avaliamos os efeitos nos comportamentos de agressividade, do tipo ansiedade e locomoção. Similar ao que ocorre em humanos, observamos que baixas concentrações de etanol aumentaram o comportamento agressivo e do tipo ansiedade nos animais, enquanto que altas concentrações induziram a efeitos do tipo depressores, corroborando com achados prévios (GERLAI et al., 2000; MOCELIN et al., 2018; ROSEMBERG et al., 2012). As concentrações de etanol que alteraram estas respostas comportamentais foram selecionadas para verificar o envolvimento da 5-HT e de receptores serotoninérgicos 5-HT_{1A}, 5-HT_{1B} e 5-HT_{2A}.

Verificamos um envolvimento de todos os receptores serotoninérgicos testados no comportamento de agressividade desencadeado pelo etanol 0.25%, com uma ação principal do receptor 5-HT_{2A} (Figura 4B). A ativação da via serotoninérgica é um dos principais mecanismos envolvidos na agressividade induzida pelo etanol (GLICK, 2015). O etanol pode aumentar a agressividade por causar uma disfunção nos sistemas que regulam processos cognitivos e desencadear um estado de ansiólise nos indivíduos (BUSHMAN, 1997). Estudos têm demonstrado que a ativação do receptor 5-HT_{2A} pode estar envolvida na etiologia, patogênese e patofisiologia da agressividade impulsiva (ROSELL e SIEVER, 2015; WINSTANLEY et al., 2004). Observamos que as respostas do tipo ansiedade observadas no peixe-zebra após exposição ao etanol 0.5% são principalmente mediadas pelo receptor 5-HT_{1B}, pois o agonista deste receptor mimetizou o efeito do etanol, enquanto que o antagonista

bloqueou as respostas do etanol sobre este comportamento (Figura 4B). O sistema serotoninérgico tem sido implicado no controle do medo, ansiedade e estresse no peixe-zebra (MAXIMINO et al., 2012) e os receptores 5-HT_{1B} estão envolvidos na dependência ao álcool e também em estados de ansiedade, impulsividade e agressão (HOPLIGHT et al., 2006 ; SARI, 2004; SARI, 2013).

Além destes aspectos, a serotonina tem um papel importante nas respostas locomotoras em vertebrados (GABRIEL et al., 2009). Contudo, verificamos que os efeitos depressores/sedativos observados após a exposição ao etanol 1.0% em peixe-zebra provavelmente não são mediados pela via serotoninérgica, pois agonistas e antagonistas deste sistema não modularam estes comportamentos. Como o etanol age modulando diferentes vias de sinalização, acreditamos que outros sistemas neurotransmissores podem estar envolvidos nestas respostas. Como exemplo, podemos destacar o sistema inibitório GABAérgico, que tem sua atividade potencializada pelo uso do etanol (KOOB, 2004) e o sistema glutamatérgico, o qual tem as ações inibidas pelo etanol (LOVINGER e ROBERTO, 2013). Em conjunto, os resultados do terceiro estudo demonstram, de forma inédita, que o sistema serotoninérgico está envolvido nos comportamentos de agressividade (principalmente via receptor 5-$HT_{2A)}$ e do tipo ansiedade (via receptor 5-HT_{1B}) mediados por etanol em peixe-zebra. Apesar da necessidade em explorar outros aspectos relacionados à ação do etanol no SNC, nossos dados sugerem que o sistema serotoninérgico é um possível alvo farmacológico para tratar os DUA.

Figura 4 – Resumo geral dos efeitos da exposição aguda ao etanol em peixe-zebra



Fonte: do autor.

8. CONCLUSÕES

A partir dos resultados apresentados, podemos observar uma clara evolução no conhecimento sobre parâmetros bioquímicos e comportamentais nos protocolos de exposição ao etanol em peixe-zebra. Em suma, de modo similar ao que ocorre em humanos, verificamos que as repostas mediadas pelo etanol em diferentes protocolos experimentais envolvem alterações no comportamento social, estresse oxidativo, disfunção mitocondrial e modulação serotoninérgica em peixe-zebra. Os resultados obtidos nesta tese reforçam a utilidade do peixe-zebra para estudar os efeitos bioquímicos, neuroquímicos e comportamentais do etanol.

Nossos dados são relevantes, pois auxiliam na elucidação dos mecanismos centrais de ação do etanol e comportamentos associados, reforçando o valor preditivo, de face e de construto dos modelos de exposição ao etanol em peixe-zebra. Em uma perspectiva translacional, destacamos este organismo modelo como uma ferramenta para explorar os mecanismos neurobiológicos do etanol e para auxiliar na busca de possíveis moléculas terapêuticas para prevenir ou atenuar os efeitos deletérios do etanol no SNC.

9. PERSPECTIVAS

Os resultados demonstrados ao longo desta tese contribuem para a consolidação do peixe-zebra como um organismo modelo eficaz nas pesquisas relacionadas ao abuso e adicção ao etanol. Entretanto, objetivando o avanço científico da área, muitos aspectos dos modelos de exposição ao etanol em peixe-zebra ainda necessitam ser abordados e explorados.

Por exemplo, a validação de novos protocolos e testes comportamentais para avaliar comportamentos de busca e retirada de etanol seria uma perspectiva interessante para expandir os estudos de dependência ao álcool e a busca de tratamentos que auxiliem nos sinais e sintomas da adicção e síndrome de abstinência. Além disso, a investigação do envolvimento dos sistemas dopaminérgico e serotoninérgico nestas respostas serviria para elucidar os mecanismos relacionados a estes comportamentos em modelos de exposição crônica, visando auxiliar na busca de alvos terapêuticos. Em relação às alterações bioquímicas desencadeadas pelo etanol em peixe-zebra, pouco se sabe se estes efeitos são mediados diretamente pelo etanol ou por seu metabólito tóxico acetaldeído. Portanto, investigar o papel do acetaldeído nestas respostas seria de grande contribuição para elucidar os mecanismos diretos e indiretos destas moléculas no SNC do peixe-zebra. Através de análises mais refinadas, a investigação de quais espécies reativas de oxigênio/nitrogênio são responsáveis pelos danos às biomoléculas gerados pelo acetaldeído e etanol também é uma importante perspectiva deste trabalho.

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ANEXO I - PRODUÇÕES CIENTÍFICAS EM COLABORAÇÃO

Artigos produzidos em parceria durante o período do doutorado:

1) Fontana BD, Duarte T, **Müller TE**, Canzian J, Ziani PR, Mezzomo NJ, Parker MO, Rosemberg DB. Concomitant taurine exposure counteracts ethanol-induced changes in locomotor and anxiety-like responses in zebrafish. Psychopharmacology (Berl). 2019 Nov 30. doi: 10.1007/s00213-019-05410-0.

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10) Canzian J, Fontana BD, Quadros VA, Müller TE, Duarte T, Rosemberg DB. Single pentylenetetrazole exposure increases aggression in adult zebrafish at different time intervals. Neurosci Lett. 2019 Jan 23;692:27-32. doi: 10.1016/j.neulet.2018.10.045.

11) Fontana BD, Stefanello FV, Mezzomo NJ, **Müller TE**, Quadros VA, Parker MO, Rico EP, Rosemberg DB. Taurine modulates acute ethanol-induced social behavioral deficits and fear responses in adult zebrafish. J Psychiatr Res. 2018 Sep;104:176-182. doi: 10.1016/j.jpsychires.2018.08.008.

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