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Flavia Vestena Stefanello

EFEITOS DA TAURINA NA EXPOSIÇÃO REPETIDA AO ETANOL EM PEIXE-ZEBRA: PARÂMETROS COMPORTAMENTAIS E ATIVIDADE DA MONOAMINA OXIDASE CEREBRAL

Santa Maria, RS
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Dissertação apresentada ao curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM), como requisito parcial para obtenção do título de **Mestra em Ciências Biológicas: Bioquímica Toxicológica**.

Orientador: Prof. Dr. Denis Broock Rosemberg

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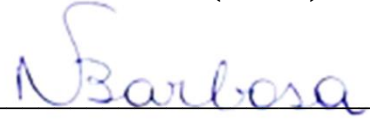

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“Não existe outro meio, é preciso trabalhar duro para realizar sonhos. A sorte sempre vai sorrir para os dedicados, exaustos e, principalmente para aqueles que não perdem a fé e vão à luta todos os dias”.

DIEGO VINICIUS

RESUMO

EFEITOS DA TAURINA NA EXPOSIÇÃO REPETIDA AO ETANOL EM PEIXE-ZEBRA: PARÂMETROS COMPORTAMENTAIS E ATIVIDADE DA MONOAMINA OXIDASE CEREBRAL

AUTORA: Flavia Vestena Stefanello
ORIENTADOR: Denis Broock Rosemberg

O abuso de álcool está associado a impactos negativos na saúde incluindo a alta taxa de morbidade e mortalidade, além de induzir diversas mudanças neurocomportamentais. Contudo, os medicamentos disponíveis para tratar os transtornos relacionados ao uso do álcool apresentam pouca eficácia terapêutica e a busca por novas alternativas de tratamento é fundamental. Assim, a taurina (TAU) surge como um potencial alvo de estudo devido a sua ação pleiotrópica no cérebro, a qual está envolvida na manutenção da integridade da membrana, osmorregulação, neuromodulação, além de possuir atividade antioxidante. Aqui, investigamos se a TAU desempenha um papel benéfico contra os efeitos da exposição repetida ao etanol (EtOH) em diferentes domínios comportamentais do peixe-zebra especialmente com foco no comportamento social, respostas do tipo ansiedade e memória. Além disso, analisamos os efeitos dos tratamentos sobre a atividade da monoamina oxidase (Z-MAO) cerebral, uma vez que as monoaminas podem desempenhar um papel importante nas respostas mediadas pelo EtOH. No presente estudo, os peixes foram expostos a água não-clorada ou ao EtOH 1% por 8 dias consecutivos (20 minutos por dia). A partir do quinto dia, imediatamente após a exposição ao EtOH, os animais foram expostos na ausência ou presença de TAU (42, 150 ou 400 mg/L) durante 1 hora por dia (totalizando 4 exposições) até o final do período experimental (oitavo dia). Vinte e quatros horas após a última exposição ao EtOH (nono dia), os peixes foram submetidos aos testes comportamentais (comportamento social, seguido pelo teste do tanque novo ou esquiva inibitória) e ao teste bioquímico para a avaliação da atividade da Z-MAO cerebral. Observamos que TAU 150 aboliu as respostas induzidas pelo protocolo de exposição repetida ao EtOH, enquanto que as demais concentrações testadas mostraram uma modesta atenuação deste efeito ansiogênico mensurado a nível social em peixes-zebra. Além disso, os animais expostos repetidamente ao EtOH apresentaram um aumento do comportamento semelhante ao de ansiedade no teste do tanque novo, enquanto TAU 42 e TAU 400 atenuaram algumas respostas comportamentais. No teste da esquiva inibitória, TAU 42 e TAU 150 apresentaram um papel protetor na reversão do déficit na aquisição da memória causado pelo EtOH. A análise bioquímica revelou que a TAU não modulou o aumento da atividade da Z-MAO cerebral induzido pela exposição repetida ao EtOH. De modo geral, nossos resultados sugerem um potencial efeito benéfico da TAU contra a exposição repetida ao EtOH em peixes-zebra, reforçando a crescente utilidade deste organismo modelo em pesquisas científicas para investigar os mecanismos subjacentes as respostas neurocomportamentais do EtOH e da TAU em vertebrados.

Palavras-chave: Comportamento. Exposição repetida ao etanol. Atividade da monoamina oxidase cerebral. Peixe-zebra. Taurina.

ABSTRACT

EFFECTS OF TAURINE ON REPEATED ETHANOL EXPOSURE IN ZEBRAFISH: BEHAVIORAL PARAMETERS AND BRAIN MONOAMINE OXIDASE ACTIVITY

AUTHOR: Flavia Vestena Stefanello
ADVISOR: Denis Broock Rosemberg

Alcohol abuse is associated with negative health impacts including high mortality and morbidity rates, as well as induces several behavioral changes. However, drugs available to treat disorders related to alcohol use have low therapeutic efficacy and the search for new treatment alternatives is essential. Thus, taurine (TAU) appears as a potential target for study due to the pleiotropic action in the brain, which is involved in maintaining the integrity of the membrane, osmoregulation, neuromodulation, in addition to having antioxidant activity. Here, we investigate whether TAU plays a beneficial role against the effects of repeated ethanol (EtOH) exposure on different behavioral domains of zebrafish, especially focusing on social behavior, anxiety-like responses, memory and brain monoamine oxidase (Z-MAO) activity, since monoamines can play an important role in EtOH-mediated responses. In the present study, fish were exposed to non-chlorinated water or 1% EtOH for 8 consecutive days (20 minutes per day). From the fifth day, immediately after EtOH exposure, animals were exposed in the absence or presence of TAU (42, 150 or 400 mg/L) 1 hour per day (totalizing 4 exhibitions) until the end of the experiment period (eight day). Twenty four hours after the last exposure to EtOH (ninth day), fish were introduced to behavioral tests (shoaling behavior followed by the novel tank diving test or the inhibitory avoidance test) and biochemical assays were performed to evaluate the brain Z-MAO activity. We observed that TAU 150 abolished the responses induced by the repeated exposure protocol to EtOH, while the other concentrations tested showed a modest attenuation of this socially measured anxiety-like effect in zebrafish. Moreover, animals repeatedly exposed to EtOH showed increase in anxiety-like behaviors in the novel tank test, while TAU 42 and TAU 400 attenuated some behaviors responses. In the inhibitory avoidance test, TAU 42 and TAU 150 exerted a protective role by reversing the memory acquisition deficit caused by EtOH. Biochemical analysis revealed that TAU did not modulate the increase in brain Z-MAO activity induced by repeated EtOH exposure. Overall, our results show a potential beneficial effect of TAU against repeated EtOH exposure in zebrafish, reinforcing the growing utility of this model organism in scientific research to investigate the mechanism underlying the neurobehavioral responses of EtOH and TAU in vertebrates.

Keywords: Behavior. Repeated ethanol exposure. Brain monoamine oxidase activity. Zebrafish. Taurine.

LISTA DE ABREVIATURAS

EtOH – Etanol
FDA – do inglês, “Food and Drug Administration”
GABA – Ácido gama-aminobutírico
GABAa – Receptor do ácido gama-aminobutírico do subtipo A
MAO – Monoamina oxidase
MAO-A – Monoamina oxidase do tipo A
MAO-B – Monoamina oxidase do tipo B
NMDAR – Receptor glutamatérgico ionotrópico do tipo N-metil-D-aspartato
PeNSe – Pesquisa Nacional de Saúde do Escolar
SNC – Sistema nervoso central
TAU – Taurina
TauT – Transportador de taurina
Z-MAO – Monoamina oxidase do tipo Z

SUMÁRIO

1 APRESENTAÇÃO

A presente Dissertação está estruturada da seguinte forma: primeiramente são descritas as partes referentes à **introdução** que consta com uma revisão bibliográfica sobre os itens abordados, bem como a **justificativa** e **objetivos** do presente estudo. A seguir, os **materiais e métodos**, os **resultados** e a **discussão** estão apresentados na forma de um **manuscrito científico**. Na **conclusão**, são aceitas/refutadas as hipóteses iniciais a partir dos principais achados do trabalho apresentado nesta Dissertação. Por fim, as **perspectivas** apresentam as possibilidades de novos estudos a partir de resultados obtidos. As **referências bibliográficas** referem-se às citações que aparecem na sessão introdução.

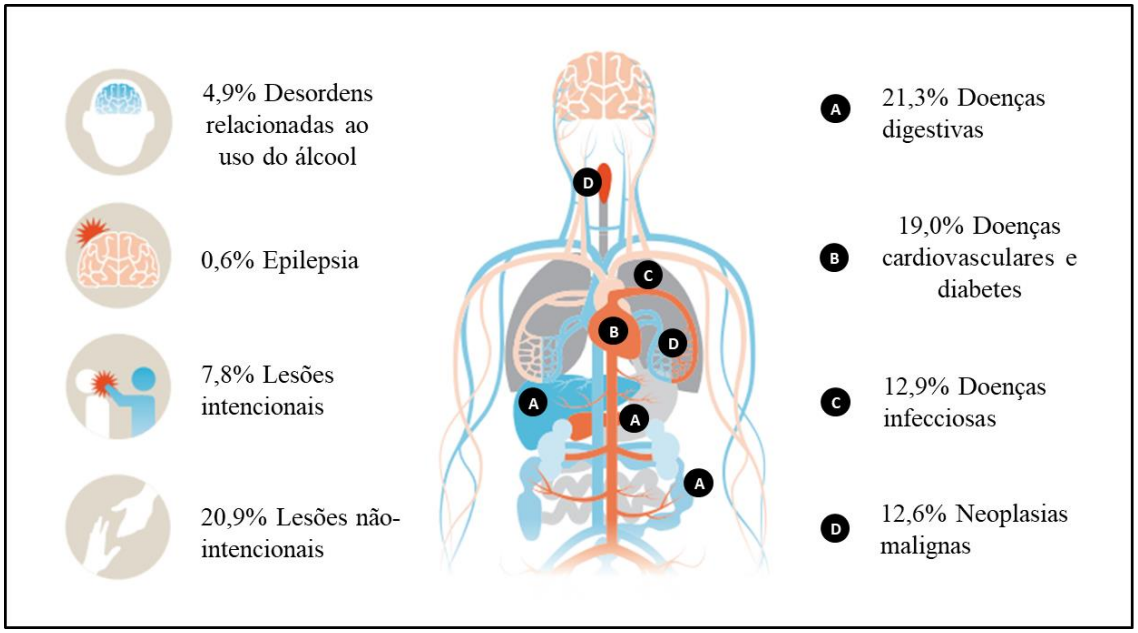
2 INTRODUÇÃO

2.1 ABUSO DE ÁLCOOL

O álcool é uma das drogas aditivas mais aceitas socialmente pelo mundo (GNEITING; SCHMITZ, 2016). Seu consumo está relacionado ao fato de apresentar um custo relativamente baixo, ser de fácil acesso, estar associado com atividades de lazer e também devido às tradições socioculturais que facilitam a experiência precoce do seu uso (SUDHINARASET; WIGGLESWORTH; TAKEUCHI, 2016). Dados da Pesquisa Nacional de Saúde do Escolar (PeNSE) de 2015, mostram que o uso precoce de bebidas alcoólicas entre estudantes brasileiros do 9º ano do ensino fundamental, com idades entre 13 e 15 anos, aumentou de 50,3% para 55,5% em 3 anos, sendo a idade média de experimentação de 12,5 anos. Além do mais, 17,9% da população brasileira adulta fazem uso abusivo de bebida alcoólica, onde as mulheres (11%) apresentaram maior crescimento em relação aos homens (26%), no período de 2006 a 2018, os quais eram respectivamente 7,7% e 24,8% no ano de 2006 (MINISTÉRIO DA SAÚDE, 2019).

A população que faz a ingestão abusiva de bebidas alcoólicas pode se envolver em situações graves, cujos danos individuais, sociais e econômicos são expressivos (CISA, 2019). O uso nocivo está associado com os acidentes de trânsito, sexo desprotegido, diminuição da produtividade no ambiente de trabalho e aos próprios transtornos relacionados ao uso do álcool (abuso e dependência) (CISA, 2019). No Brasil, a análise de custos das internações totalmente atribuíveis ao álcool mostrou um aumento de 4,3% no período de 2010 a 2016, correspondendo a cerca de R$ 4,6 milhões (CISA, 2019). Além disso, anualmente, mais de 3 milhões de pessoas morrem pelo uso abusivo do álcool, uma vez que seu consumo está relacionado com o desenvolvimento de mais de 200 doenças. Dentre elas, podemos destacar as doenças gastrointestinais e cardiovasculares, doenças infecciosas, cânceres e outras condições de saúde. Lesões intencionais (violência interpessoal e suicídio) e lesões não-intencionais (comprometimento das habilidades psicomotoras) também contribuem para a taxa de mortalidade (WHO, 2018) **(Figura 1)**.

Figura 1 - Porcentagem de mortes atribuídas ao uso abusivo do álcool em 2016.



Fonte: Adaptado de WHO, 2018.

Apesar dos impactos dos transtornos do uso do álcool na saúde pública, os tratamentos disponíveis para as pessoas com dependência a droga incluem uma combinação de aconselhamento, medicação e apoio social (NIAAA, 2020). Atualmente, existem três medicamentos aprovados pelo *Food and Drug Administration (FDA)* para tratar a dependência ao álcool: o dissulfiram, que atua inibindo a enzima acetaldeído desidrogenase gerando um aumento de acetaldeído no organismo; a naltrexona, que age bloqueando os receptores opioides envolvidos nos efeitos de recompensa da droga; e o acamprosato, que tem ação sobre os receptores glutamatérgicos e GABAérgicos e age reduzindo alguns sintomas negativos da retirada da droga (ANTONELLI et al., 2018; FDA, 2020). Entretanto, estes medicamentos não apresentam boa eficácia no tratamento da dependência ao álcool, pois as taxas de recidivas são altas, em parte devido ao surgimento de vários efeitos adversos como náusea, vômito e cefaleia (ANTONELLI et al., 2018; GOH; MORGAN, 2017). Nesse contexto, torna-se necessário investigar novas alternativas terapêuticas objetivando o tratamento dos efeitos do abuso de álcool e alcoolismo.

2.2 ETANOL E SEUS EFEITOS BIOLÓGICOS

Em humanos, o álcool (álcool etílico, etanol) é absorvido pelo trato gastrointestinal, distribuindo-se rapidamente pela circulação sanguínea até os órgãos e tecidos (NORBERG et al., 2003). Por ser uma molécula lipofílica, o etanol (EtOH) atravessa facilmente a barreira hematoencefálica e afeta o sistema nervoso central (SNC), podendo desencadear alterações neurocomportamentais de maneira dependente de dose e de tempo (EŞEL; DINÇ, 2017; SPANAGEL, 2009). O consumo agudo de EtOH apresenta um efeito bifásico nos indivíduos dependendo da dose ingerida: em doses baixas a moderadas promove euforia, desinibição e alivia a ansiedade/estresse, enquanto em doses mais altas promove efeitos depressores, causando perda da coordenação motora, desorientação e sedação (HENDLER et al., 2013). No entanto, o consumo crônico induz processos neuroadaptativos e desencadeia tolerância, dependência e síndrome de abstinência após a interrupção do consumo de EtOH, o qual pode gerar insônia, náuseas, tremores, ansiedade e episódios depressivos (BANERJEE, 2014; BECKER; MULHOLLAND, 2014). Consumidores compulsivos apresentam déficits cognitivos em tarefas de memória de trabalho (AMBROSE; BOWDEN; WHELAN, 2001) e resolução de problemas (BEATTY et al., 1993), bem como deficiências nas habilidades motoras como coordenação e equilíbrio (SULLIVAN; ROSENBLOOM; PFEFFERBAUM, 2000). Além disso, alterações emocionais e interpessoais também são observadas em alcoólatras humanos (LE BERRE, 2019).

Os mecanismos pelos quais o EtOH atua no SNC estão associados com as alterações nas vias de transdução de sinal, comprometimento da função mitocondrial, mudanças na expressão gênica e a modulação de diversos sistemas de neurotransmissores (HARPER; MATSUMOTO, 2005; TONG et al., 2011). O consumo de EtOH em doses mais elevadas de modo agudo induz efeitos depressores devido à estimulação do receptor do ácido gama-aminobutírico (GABA) (TRAN; GERLAI, 2013; WHITE; MATTHEWS; BEST, 2000). Além disso, também ocorre a inibição do receptor glutamatérgico ionotrópico do tipo N-metil-D-aspartato (NMDAR), sugerindo que esse bloqueio nos receptores glutamatérgicos possa contribuir com os danos cognitivos induzidos pelo EtOH (LOVINGER; ROBERTO, 2013). Por outro lado, o consumo crônico de EtOH leva a um aumento na estimulação glutamatérgica para compensar a inibição contínua da função do NMDAR (GONZALES; JAWORSKI, 1997). Acredita-se que essas alterações adaptativas nos NMDAR contribuam para o desenvolvimento da tolerância, dependência e recaída no comportamento de busca pela droga (MULHOLLAND et al., 2011).

2.3 SISTEMA MONOAMINÉRGICO E RESPOSTAS MEDIADAS PELO ETANOL

Além dos efeitos conhecidos sobre os sistemas de neurotransmissão GABAérgico e glutamatérgico, o EtOH também modula os níveis de serotonina e dopamina que estimulam o sistema de recompensa do cérebro (ERDOZAIN; CALLADO, 2014), o qual é responsável por influenciar o consumo de EtOH por causar uma sensação de bem-estar (ALEXANDRE et al., 2019). Os sistemas serotoninérgico e dopaminérgico contribuem para os efeitos comportamentais promovidos pelo EtOH, pois são responsáveis pela regulação de parâmetros como ansiedade, agressividade, cognição e emoção (BISSONETTE; ROESCH, 2016; PARSEY, 2010). Estudos mostram que em doses baixas, o EtOH aumenta a liberação de serotonina e dopamina, alterando comportamentos relacionados a emoções (MARCINKIEWCZ, 2015) e facilita o reforço (MOREL; MONTGOMERY; HAN, 2019; NUTT et al., 2015), respectivamente. Entretanto, a ingestão crônica de EtOH reduz os níveis desses neurotransmissores causando alterações emocionais negativas quando ocorre a retirada de EtOH (ERDOZAIN; CALLADO, 2014; SARI; JOHNSON; WEEDMAN, 2011).

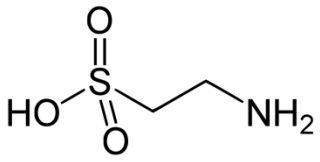
A atividade da monoamina oxidase (MAO) é um mecanismo importante para controlar os níveis de monoaminas no SNC (NIKOLAC PERKOVIC et al., 2016), uma vez que a MAO é uma enzima responsável pela desaminação oxidativa das aminas biogênicas como a serotonina e a dopamina (BORTOLATO; SHIH, 2011; SALLINEN et al., 2009). Os mamíferos apresentam duas isoformas da MAO, a MAO-A e a MAO-B, que embora apresentem origem de um mesmo gene ancestral, são diferenciadas pela afinidade por substratos, sensibilidade a inibidores e localização anatômica (BORTOLATO; SHIH, 2011). A MAO-A desamina preferencialmente serotonina e norepinefrina, sendo inibida pela clorgilina e é caracteristicamente abundante nos fibroblastos e na placenta; em contraste, a MAO-B desamina preferencialmente a 2-feniletilamina e a benzilamina, sendo inibida pelo deprenil e é expressa em plaquetas e linfócitos (BORTOLATO; SHIH, 2011; NIKOLAC PERKOVIC et al., 2016). Inibidores da MAO (tanto inibidores da MAO-A, befloxatana e clorgilina, quanto da MAO-B, pargilina e l-deprenil) diminuem a ingestão de EtOH em ratos (COHEN et al., 1999), o que faz com que esta enzima seja uma forte candidata para estudos envolvendo abuso/dependência de EtOH. Além disso, evidências clínicas e pré-clínicas indicam que variações na atividade da MAO podem desencadear alteração comportamental bem como distúrbios neurodegenerativos (BORTOLATO; SHIH, 2011). Em humanos, mutações do gene da MAO-A, que levam à perda de função desta enzima, estão relacionadas a comportamentos antissociais e de agressão (GODAR et al., 2016; TAKAHASHI et al.,

2011), enquanto que alterações na atividade e expressão da MAO-B estão associados com os transtornos de déficit de atenção, depressão, impulsividade, doenças neurodegenerativas e alcoolismo (BORTOLATO; SHIH, 2011). Portanto, o EtOH modula diversos sistemas de neurotransmissão, que podem estar diretamente associados com as modificações observadas no comportamento, tornando-se relevante investigar novos alvos terapêuticos para tratar os efeitos do abuso do EtOH.

2.4 TAURINA E SEUS EFEITOS BIOLÓGICOS

A taurina (TAU), ácido 2-aminoetanossulfônico, **(Figura 2)** é o segundo aminoácido mais encontrado no SNC dos vertebrados (JACOBSEN; SMITH, 1968). Essa molécula pode ser sintetizada endogenamente a partir da oxidação sequencial da cisteína em ácido cisteína sulfínico catalisada pela cisteína dioxigenase, seguida de uma descarboxilação pela cisteína sulfinato descarboxilase e oxidação da hipotaurina em TAU pela hipotaurina desidrogenase (DE LUCA; PIERNO; CAMERINO, 2015). No entanto, essa taxa de biossíntese é variável entre os indivíduos, pois além da acessibilidade ao aminoácido cisteína, também depende do estado nutricional e da ingestão de proteínas (DE LUCA; PIERNO; CAMERINO, 2015; HUXTABLE, 1992). A TAU é absorvida pelo intestino, liberada na circulação sanguínea e é distribuída entre as células, onde é carreada por transportadores específicos de membrana plasmática denominados TauT (SLC6A6) e/ou pelo transportador de aminoácidos acoplado a transporte de prótons (SLC36A1) (LAMBERT et al., 2015; RIPPS; SHEN, 2012). As concentrações de TAU em fluidos extracelulares são menores do que aquelas relatadas intracelularmente, variando de 10 a 100 μM (DE LUCA; PIERNO; CAMERINO, 2015; HUXTABLE, 1992), sugerindo que ela é realmente necessária para modular funções celulares importantes. Os efeitos extracelulares da TAU são atribuíveis à ativação de alvos celulares específicos mesmo em concentrações muito baixas (HUXTABLE, 1992). As concentrações de TAU intracelulares são mais elevadas nos tecidos com considerável atividade oxidativa, como encéfalo, músculo esquelético e cardíaco, devido a sua importante ação antioxidante nestes tecidos (DE LUCA; PIERNO; CAMERINO, 2015; HANSEN et al., 2006; OLIVEIRA et al., 2010). *In vitro*, a TAU pode interagir diretamente com alguns radicais livres como "scavenger", diminuindo suas concentrações intracelulares (OLIVEIRA et al., 2010) ou aumentando a produção das defesas antioxidantes *in vivo* (ROSEMBERG et al., 2010).

Figura 2 - Estrutura química da molécula da taurina.



Fonte: Adaptado de DE LUCA; PIERNO; CAMERINO, 2015.

A TAU também desempenha uma variedade de funções no SNC, sendo responsável pela regulação no metabolismo do cálcio (FOOS; WU, 2002), osmorregulação (SCHAFFER et al., 2010), manutenção da integridade da membrana (LAMBERT et al., 2015), além de sua ação neuromodulatória agonística sobre os receptores $GABA_A$ e de glicina (HUXTABLE, 1992). Ademais, estudos demonstram a capacidade da TAU em proteger os neurônios da excitotoxicidade diminuindo o nível de cálcio intracelular, sugerindo uma interação entre a TAU e o NMDAR (CHAN et al., 2014; CHEN et al., 2011; WU et al., 2005). Dessa forma, a TAU pode antagonizar a excitotoxicidade glutamatérgica através de diferentes mecanismos de ação (YE; SHI; YIN, 2013).

Estudos mostram os efeitos benéficos da TAU no tratamento de uma variedade de doenças neurológicas (doença de Parkinson, Alzheimer e epilepsia), dos transtornos relacionados ao estresse e também para prevenir efeitos agudos mediados pelo EtOH (JAKARIA et al., 2019; MENZIE et al., 2014; MEZZOMO et al., 2018). Além disso, a atividade moduladora da TAU regula comportamentos distintos como a agressividade (FONTANA et al., 2016; OJA; SARANSAARI, 2007), medo e ansiedade (KONG et al., 2006; MEZZOMO et al., 2016; ROSEMBERG et al., 2012) e melhora o déficit de memória em modelos experimentais (BERTONCELLO et al., 2019; FRANSCESCON et al., 2020; LU et al., 2014). Na clínica, o acamprosato (N-acetil- homotaurina), um análago da TAU, é usado no tratamento do alcoolismo reduzindo alguns efeitos da retirada do EtOH (ANTONELLI et al., 2018). É importante ressaltar que a TAU também pode compensar os efeitos do EtOH devido as suas características pleiotrópicas no cérebro, além de ser um aminoácido importante na regulação de vários efeitos fisiológicos no organismo. Assim, a caracterização de modelos alternativos para investigar os efeitos neurocomportamentais modulados pela TAU é de extrema importância.

2.5 PEIXE-ZEBRA COMO ORGANISMO MODELO EXPERIMENTAL

O peixe-zebra (*Danio rerio*), conhecido popularmente como “paulistinha”, é uma espécie de peixe de água doce, nativa da Ásia, a qual pertence à família Cyprinidae (DAHM; GEISLER, 2006). Este teleósteo apresenta diversas vantagens como tamanho pequeno (adultos podem medir de 3 a 4 centímetros de comprimento), rápido desenvolvimento até a fase adulta (aproximadamente 2-3 meses), uma grande prole (50 a 200 ovos por dia para cada fêmea em condições otimizadas de reprodução), ovos translúcidos, além de ser um organismo modelo atraente por apresentar um baixo custo e necessitar de um pequeno espaço para manutenção (DAHM; GEISLER, 2006; LELE; KRONE, 1996).

O peixe-zebra possui um genoma altamente conservado e com alto grau de similaridade em comparação com os genes de mamíferos (aproximadamente 70%) (HOWE et al., 2013). Os principais sistemas de neurotransmissores já estão caracterizados na espécie (DEMIN et al., 2018; RICO et al., 2011), a qual possui um repertório comportamental bastante complexo que já foi descrito previamente (KALUEFF et al., 2013), permitindo o desenvolvimento de modelos em pesquisas da neurociência translacional. Esta espécie também tem sido bastante utilizada em triagens em larga escala para identificar compostos terapêuticos e possíveis alvos moleculares de forma rápida e eficaz (FONTANA et al., 2018a; MEZZOMO et al., 2018). Ademais, outra característica importante está relacionada ao método como a droga é administrada, onde os animais são imersos em compostos hidrossolúveis que podem ser facilmente dissolvidos na água do tanque e absorvidos pelo peixe através da pele e brânquias (FONTANA et al., 2018a).

Por mais que a organização do SNC do peixe-zebra seja mais simples do que a de mamíferos, o pálio lateral da área telencefálica deste peixe é estruturalmente semelhante ao hipocampo dos mamíferos, permitindo avaliar aspectos relacionados ao aprendizado e a memória (PERATHONER; CORDERO-MALDONADO; CRAWFORD, 2016). Além disso, o peixe-zebra expressa apenas uma isoforma da MAO (Z-MAO), cuja estrutura se assemelha à da MAO humana, pois compartilha aproximadamente 70% de identidade com a MAO-A e MAO-B de mamíferos (ALDECO; ARSLAN; EDMONDSON, 2011; SETINI et al., 2005). Essas características tornam o peixe-zebra uma ferramenta adequada para avaliar como as manipulações farmacológicas afetam uma ampla gama de domínios neurocomportamentais.

Alguns dos efeitos após a exposição repetida (ou intermitente) ao EtOH no comportamento em peixe-zebra já estão bem caracterizados, onde ocorre alteração no comportamento social (aumenta a coesão do cardume) (MÜLLER et al., 2017) e observa-se

um aumento de comportamentos do tipo ansiedade (diminui o número de entradas e o tempo gasto na área superior do tanque) (MATHUR; GUO, 2011; MOCELIN et al., 2019). Agudamente, em um modelo de exposição concomitante com EtOH, a TAU neutraliza os efeitos promovidos pelo EtOH no comportamento do tipo ansiogênico (FONTANA et al., 2020), na agressividade (FONTANA et al., 2016) e no déficit no comportamento social (FONTANA et al., 2018b) dependendo da concentração testada. O pré-tratamento com TAU diminui os níveis de EtOH no cérebro e previne a hipolocomoção e o comportamento do tipo ansiedade causadas pelo EtOH na exposição de 60 minutos (ROSEMBERG et al., 2012). Da mesma forma, a TAU também previne o comprometimento da consolidação da memória induzida pela exposição aguda de 60 minutos ao EtOH 1% em peixe-zebra (BERTONCELLO et al., 2019). Além disso, após a exposição semanal intermitente ao EtOH observa-se alterações no nível de dopamina e na atividade do seu transportador, bem como na atividade da Z-MAO no encéfalo desses animais (ALEXANDRE et al., 2019), sugerindo o envolvimento do sistema monoaninérgico nos fenótipos neurocomportamentais. Em peixes, o EtOH diminui a atividade da Z-MAO cerebral (ALEXANDRE et al., 2019), porém em estudos com roedores relatam um aumento da atividade da MAO (MATTHEWS et al., 2018; ZIMATKIN; TSYDIK; LELEVICH, 1997). Por mais que sejam trabalhos realizados com diferentes modelos experimentais, os resultados ainda carecem de mais estudos para fornecer uma possível explicação para a heterogeneidade de dados (a qual pode estar relacionada com diferentes concentrações de EtOH, modelo de organismo testado e período de exposição) (DA SILVA CHAVES et al., 2018). Dessa maneira, utilizamos um protocolo de exposição repetida ao EtOH por apresentar maior valor translacional, pois mimetiza o consumo de álcool observado em seres humanos (MATHUR; GUO, 2011; MÜLLER et al., 2017).

Assim, considerando que o EtOH tem potencial de causar alterações neurocomportamentais e pelo fato da TAU apresentar ações neuromoduladora e neuroprotetora, a avaliação desses efeitos utilizando o peixe-zebra como organsimo modelo pode contribuir para a busca de um novo composto com ação terapêutica para o abuso do álcool e alcoolismo.

3 JUSTIFICATIVA

O álcool é uma droga amplamente consumida pela população e que representa uma preocupação a saúde pública devido aos impactos negativos individuais, sociais e econômicos (CISA, 2019). Seu consumo excessivo pode causar efeitos complexos no organismo, pois além de estar associado ao desenvolvimento de mais de 200 doenças (WHO, 2018) também pode causar alterações neurocomportamentais (EŞEL; DINÇ, 2017; SPANAGEL, 2009). Dados sobre o consumo de álcool durante a pandemia levantada pelo Ministério da Cidadania e Organização Pan-Americana da Saúde (2020) revelam que entre os 33 países avaliados nessa pesquisa, o Brasil teve a maior porcentagem de pessoas entrevistadas que relataram exagero no consumo de álcool, onde os resultados mostram que 74% dos brasileiros beberam durante a pandemia do novo coronavírus. Devido à alta prevalência do consumo de bebidas alcoólicas entre os brasileiros e os medicamentos disponíveis para tratar os transtornos do uso do álcool apresentam pouca eficácia devido ao surgimento de inúmeros efeitos adversos (ANTONELLI et al., 2018; GOH; MORGAN, 2017), a busca por estratégias terapêuticas torna-se necessária. Estudos já demostram que a TAU previne alguns efeitos neurocomportamentais causados pela exposição aguda ao EtOH (BERTONCELLO et al., 2019; FONTANA et al., 2016, 2018b, 2020; ROSEMBERG et al., 2012). Assim, torna-se importante investigar se a TAU também exerce essa ação moduladora sobre respostas comportamentais (especialmente no comportamento social, ansiedade e memória) e bioquímica (atividade da Z-MAO) em um protocolo de exposição repetida ao EtOH utilizando o peixe-zebra. Um organismo modelo amplamente utilizado na pesquisa neuroquímica comportamental, por apresentar diversas vantagens como alta similaridade genética com os mamíferos, principais sistemas de neurotransmissores e um repertório comportamental bastante complexo, o que permite a descoberta de novas alternativas de tratamento para o abuso do álcool e alcoolismo.

4 OBJETIVOS

4.1 OBJETIVO GERAL

Avaliar um possível efeito protetor da TAU em diferentes domínios comportamentais e na atividade da Z-MAO cerebral modulados por um protocolo de exposição repetida ao EtOH em peixe-zebra.

4.2 OBJETIVOS ESPECÍFICOS

- Investigar os efeitos da TAU sobre alterações no comportamento social induzidas pelo EtOH;

- Avaliar os efeitos da TAU e do EtOH sobre parâmetros locomotores e de ansiedade;

- Avaliar os efeitos promovidos pela TAU no déficit na aquisição da memória induzido pelo EtOH;

- Investigar os efeitos da TAU e da exposição repetida ao EtOH sobre a atividade da Z-MAO cerebral.

5 MANUSCRITO CIENTÍFICO

Taurine modulates behavioral effects of intermittent ethanol exposure without changing brain monoamine oxidase activity in zebrafish: attenuation of shoal- and anxiety-like responses, and abolishment of memory acquisition deficit

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Taurine modulates behavioral effects of intermittent ethanol exposure without changing brain monoamine oxidase activity in zebrafish: attenuation of shoal- and anxiety-like responses, and abolishment of memory acquisition deficit

Flavia V. Stefanello[a,b,*], Talise E. Müller[a,b], Francini Franscescon[a,b], Vanessa A. Quadros[a,b], Thiele P. Souza[a,b], Julia Canzian[a,b], Jossiele Leitemperger[b,c], Vania L. Loro[b,c,d], Denis B. Rosemberg[a,b,e,*]

[a] *Laboratory of Experimental Neuropsychobiology, Department of Biochemistry and Molecular Biology, Natural and Exact Sciences Center, Federal University of Santa Maria, 1000 Roraima Avenue, Santa Maria, RS, 97105-900, Brazil.*

[b] *Graduate Program in Biological Sciences: Toxicological Biochemistry, Federal University of Santa Maria. 1000 Roraima Avenue, Santa Maria, RS, 97105-900, Brazil.*

[c] *Laboratory of Aquatic Toxicology, Federal University of Santa Maria, 1000 Roraima Avenue, Santa Maria, RS, 97105-900, Brazil.*

[d] *Graduate Program in Animal Biodiversity, Federal University of Santa Maria, 1000 Roraima Anenue, Santa Maria, RS, 97105-900, Brazil.*

[e] *The International Zebrafish Neuroscience Research Consortium (ZNRC), 309 Palmer Court, Slidell, LA 70458, USA.*

[*]Correspondence to:

Flavia V. Stefanello

Graduate Program in Biological Sciences: Toxicological Biochemistry, Natural and Exact Sciences Center, Federal University of Santa Maria. 1000 Roraima Avenue, Santa Maria, RS, 97105-900, Brazil.

E-mail: flaviavestena@gmail.com

Denis B. Rosemberg

Department of Biochemistry and Molecular Biology, Natural and Exact Sciences Center, Federal University of Santa Maria. 1000 Roraima Avenue, Santa Maria, RS, 97105-900, Brazil.

E-mail: dbrosemberg@gmail.com

Highlights

- We tested the chronic effects of taurine and ethanol on zebrafish behaviors.
- Taurine attenuated the effects of ethanol on shoaling and anxiety-like behaviors.
- Taurine reversed the memory acquisition deficit caused by repeated ethanol exposure.
- Taurine did not modulate the ethanol-induced increase on monoamine oxidase activity.

Abstract

Prolonged alcohol consumption has been considered as an important risk factor for various diseases. Chronic ethanol (EtOH) intake is associated with deleterious effects on brain functions culminating in robust behavioral changes. Notably, drugs available to treat the effects of EtOH have low therapeutic efficacy so far. Taurine (TAU) appears as a promising neuroprotective molecule due to its pleiotropic action in the brain. Here, we investigated whether TAU plays a beneficial role on different behavioral domains of zebrafish submitted to an intermittent EtOH exposure model, specially focusing on social behavior, anxiety-like responses, and memory. Moreover, since monoamines can play a role in EtOH-mediated responses, we also evaluated the influence of both TAU and EtOH exposures in brain monoamine oxidase (Z-MAO) activity. Fish were exposed to non-chlorinated water or 1% EtOH for 8 consecutive days (20 min per day). From the 5th day until the end of the experimental period, animals were kept in the absence or presence of TAU (42, 150, or 400 mg/L) 1 h per day immediately after EtOH exposure. Behaviors measurements started 24 h after the last EtOH exposure. We observed that TAU showed modest attenuating effects on shoaling behavior and anxiety-like responses, while 42 and 150 mg/L TAU abolished the memory acquisition deficit in the inhibitory avoidance task. Biochemical analysis revealed that TAU did not modulate EtOH-induced increase on brain Z-MAO activity. Collectively, our novel data show a potential beneficial effect of TAU in an intermittent EtOH exposure model in zebrafish. Moreover, these findings foster the growing utility of this aquatic species to investigate the neurobehavioral basis of EtOH- and TAU-mediated responses in vertebrates.

Keywords: behavioral domains; chronic exposure; ethanol; taurine; zebrafish; Z-MAO.

1. Introduction

Ethanol (EtOH) is a widely consumed substance worldwide that causes negative impacts on human health (*e.g.*, high mortality rate, development of various pathologies, and dependence) (MICHALAK; BIAŁA, 2016). More than 3 million people die annually from alcohol abuse and approximately 65% are related to various alcohol-related diseases (WHO, 2018). EtOH acts in the central nervous system (CNS) by altering transduction signaling pathways, disrupting mitochondrial function, changing gene expression, and modulating different neurotransmitters systems (TONG et al., 2011). At higher doses, EtOH acute consumption induces depressant effects due to the stimulation of gamma-aminobutyric acid (GABA) receptors, causing sedation and disorientation (TRAN; GERLAI, 2013; WHITE; MATTHEWS; BEST, 2000). EtOH also inhibits N-methyl-D-aspartate (NMDA) receptors, which contributes to EtOH-induced cognitive impairments (LOVINGER; ROBERTO, 2013; LOVINGER; WHITE; WEIGHT, 1990). Conversely, chronic EtOH consumption increases glutamatergic activity to compensate the inhibition of NMDA receptors, facilitating tolerance and dependence (CHEFER et al., 2011; GONZALES; JAWORSKI, 1997; MULHOLLAND et al., 2011). Furthermore, EtOH modulates the reward system mediated by biogenic amines (*e.g.*, serotonin and dopamine) (ERDOZAIN; CALLADO, 2014), which play a role in modulating various behavioral domains, such as anxiety, aggression, cognition, and emotion (BISSONETTE; ROESCH, 2016; PARSEY, 2010). Moreover, because biogenic amines play a role in EtOH-mediated responses, the monoamine oxidase (MAO) activity represents an important enzymatic mechanism responsible for inactivating these monoamines (BORTOLATO; SHIH, 2011). In humans, preclinical and clinical evidence indicates that changes in MAO activity are associated with antisocial behavior and aggression, as well as play a role in attention-deficit hyperactivity disorder, neurodegenerative diseases, and alcoholism (BORTOLATO; SHIH, 2011).

Pharmacological treatments for patients with alcohol-related disorders have shown little therapeutic efficacy, mainly due to the appearance of several adverse effects (ANTON et al., 2006; LITTEN et al., 2016). Taurine (TAU), also known as 2-aminoethanesulfonic acid, is a promising molecule for treating the effects of alcohol in experimental models (PUSHPAKIRAN; MAHALAKSHMI; ANURADHA, 2004; ROSEMBERG et al., 2012). TAU is synthesized endogenously from sequential cysteine oxidation reactions in tissues with high oxidative metabolism such as brain, skeletal and cardiac muscle (DE LUCA; PIERNO; CAMERINO, 2015). TAU plays a physiological role in osmoregulation (SCHAFFER et al., 2010), as well as regulates intracellular Ca^{2+} metabolism (FOOS; WU, 2002), membrane stability (LAMBERT et al., 2015), and displays antioxidant properties (ROSEMBERG et al., 2010). In the CNS, TAU acts as agonist of $GABA_A$ and glycine receptors (HUXTABLE, 1992) and decreases Ca^{2+} influx, exerting a beneficial role against the glutamatergic excitotoxicity (YE; SHI; YIN, 2013).

In humans, acamprosate (N-acetyl-homotaurine), an analogue of TAU, has been used to treat of alcoholism-related symptoms, since this molecule can reduce some undesirable effects of withdrawal (ANTONELLI et al., 2018). Importantly, TAU can also counteract the effects of EtOH due to its pleiotropic role in the brain. Mounting evidence shows that TAU prevents some neurobehavioral changes induced by acute EtOH exposure in zebrafish (*e.g.*, anxiety-like responses, hypolocomotion, decreased social preference, memory impairment, and oxidative stress in the brain) (BERTONCELLO et al., 2019; FONTANA et al., 2018b, 2020; ROSEMBERG et al., 2010, 2012). This aquatic species shows a high degree of genomic conservation (HOWE et al., 2013), expressing the main neurotransmitter systems found in mammals (DEMIN et al., 2018; RICO et al., 2011). Zebrafish also express a single MAO isoform (Z-MAO), whose structure closely resembles human MAO (ALDECO; ARSLAN; EDMONDSON, 2011; SETINI et al., 2005). These features make zebrafish a

suitable tool to assess how distinct pharmacological manipulations affect a wide range of well-characterized behavioral domains (KALUEFF et al., 2013). Here, aiming to verify whether TAU can exert a beneficial role in an intermittent EtOH exposure model in zebrafish, we tested the role of TAU on shoaling behavior, anxiety-like responses, and memory, as well as on brain Z-MAO activity. Importantly, behavioral analysis started 24 h after the last EtOH exposure and we chose to use the intermittent EtOH exposure due to its higher translational value as it mimics the consumption of alcohol observed in humans (MATHUR; GUO, 2011; MÜLLER et al., 2017).

2. Materials and Methods

2.1. Animals

Wild-type adult zebrafish (*Danio rerio*) (4-6 months-old, ~50:50, male:female ratio from the short-fin phenotype) were obtained from a commercial supplier (Hobby Aquários, RS, Brazil). Animals were acclimated for 15 days before the experiments in 40 L tanks (density of four animals per liter) filled with non-chlorinated water under constant filtration and aeration (25 ± 2°C at pH 7.0-7.2). The water was changed twice a week and chemical parameters were monitored with commercial kits for determining pH, ammonia, nitrite, and chloride. Illumination was provided by fluorescent lamps under a controlled 14h/10 h light/dark photoperiod cycle (lights on at 7:00 am). Animals were fed with commercial flake fish food (Alcon Basic^{TM}, Alcon, Brazil) two times per day. All procedures were approved by the Institutional Animal Care and Use Committee (process number 026/2014).

2.2. Drugs

Ethanol (EtOH, CAS number 64-17-5) and taurine (TAU, CAS number 107-35-7) were acquired from Dinâmica Química Contemporânea LTDA (São Paulo, SP, Brazil) and

Sigma-Aldrich (St. Louis, MO, USA), respectively. All solutions were prepared daily before the experiments.

2.3. Experimental design

Before the exposure period, groups of eight fish were randomly allocated into tanks (22 cm length x 12 cm height x 14 cm width) to perform the treatments. Eight experimental groups were separated in two major classifications as follows i) experimental controls (CTRL): water CTRL; TAU at 42 mg/L (TAU 42), 150 mg/L (TAU 150), or 400 mg/L (TAU 400) alone; ii) EtOH-exposed groups: 1% EtOH; and EtOH plus TAU at 42, 150, or 400 mg/L. The water CTRL group was maintained only in non-chlorinated water, in the absence of drugs. The respective TAU concentrations were chosen based on previous reports, in which 42, 150, and 400 mg/L exert significant neuromodulatory effects on multiple zebrafish behavioral domains (FONTANA et al., 2018b; MEZZOMO et al., 2016; ROSEMBERG et al., 2012). Moreover, the 1% EtOH concentration used has been associated with deleterious effects on biochemical and behavioral responses 24 h after the intermittent EtOH exposure protocol in zebrafish (MATHUR; GUO, 2011; MOCELIN et al., 2019; MÜLLER et al., 2017). Three replicate experiments have been performed, and subjects were 192 fish obtained from two independent batches.

2.3.1. Repeated EtOH and TAU exposures

The intermittent EtOH exposure protocol was performed as described elsewhere (MATHUR; GUO, 2011; MOCELIN et al., 2019; MÜLLER et al., 2017). Initially, four zebrafish were exposed simultaneously in 1 L tanks (15 cm length x 13 cm height x 10 cm width) containing non-chlorinated water or 1% EtOH (95% stock solution), for 20 min for 8 consecutive days. From the 5^{th} day until the end of the experiment (8^{th} day), immediately after

exposure to EtOH, fish were placed in tanks with similar dimensions containing non-chlorinated water or TAU (42, 150 or 400 mg/L) for 1 h (MEZZOMO et al., 2016; ROSEMBERG et al., 2012). Notably, TAU treatment started from the 5th day of EtOH exposure aiming to improve data translatability, since pharmacological treatments only starts after alcohol consumption in clinic.

Immediately after the exposure to non-chlorinated water or TAU, fish were returned to their home tanks. On the 9th day (24 hours after finishing the treatments), four animals per group were tested simultaneously in the shoaling behavior test. Subsequently, fish were immediately tested in the novel tank diving test or separated into perforated housing tanks to assess their behavior in the inhibitory avoidance task. We did not use the same animals for all experiments in order to reduce a potential interference between different tests (test battery effect). However, we also aimed to minimize the number of subjects tested, fully adhering to the reduction criterion of the 3Rs principle of animal experimentation. As reported in both zebrafish (MATHUR; GUO, 2011; MOCELIN et al., 2019; MÜLLER et al., 2017) and rodents (LEBOURGEOIS et al., 2019; MATTHEWS et al., 2019), animals testing started 24 h after the last EtOH exposure to exclude a possible acute influence on the neurobehavioral responses measured here. Importantly, although the effects of withdrawal should not be ruled out, in our protocol zebrafish were not continuously kept into EtOH solution during the exposure period, thereby reducing the responses caused by abrupt interruption of administration. Fish used in the novel tank diving test were euthanized after the trial, and brain samples removed to further biochemical assays. All behavioral tests were recorded in the same period (between 13:30 and 17:00). **Fig. 1** shows a schematic representation of the experimental design.

2.4. Behavioral tests

2.4.1. Shoaling behavior test

On the 9th day, four fish (representing each zebrafish shoal tested) were placed simultaneously in the test tank (25 cm length x 15 cm height x 10 cm width). Tank dimensions were similar to those used in a previous study that evaluated the group behavior of zebrafish (FONTANA et al., 2018b). The tank was filled with 2.5 L of non-chlorinated water and the shoaling behavior was recorded for 6 min. Videos were exported to the Image J 1.49 software and shoaling was assessed by screenshots taken every 15 s over the test period, totaling 24 screenshots per group (GREEN et al., 2012; MÜLLER et al., 2017). Screenshots were further calibrated for the size of the tank and each fish was marked to allow the measurement of the inter-fish distance, shoal area, farthest neighbor distance, and nearest neighbor distance. Two trained observers blinded to the experimental condition (inter-rater reliability ≥ 0.90) analyzed the results.

2.4.2. Novel tank diving test

After the shoaling behavior test, two fish were kept randomly and tested individually in a tank (25 cm length x 15 cm height x 6 cm width) containing 1.3 L non-chlorinated water. Behaviors were recorded for 6 min and videos were further analyzed offline using appropriate automated video-tracking system (Any-Maze™, Stoelting, CO, USA). The apparatus was virtually divided into two horizontal areas (top and bottom) and all the experimental conditions were similar as described previously (DUARTE et al., 2019). Distance traveled and absolute turn angle were quantified as locomotion-related endpoints. The latency to enter the top area, time spent in top area, and the number of transitions to the top area were used to assess the vertical exploration of zebrafish, which reflects anxiety-like responses during habituation to novelty stress (KALUEFF et al., 2013).

2.4.3. Inhibitory avoidance task

After the shoaling behavior test, another cohort of fish (2 animals) was randomly separated in tanks (50 cm length x 35 cm width x 6 cm height), which had equal divisions for each fish with small perforations (0.5 cm diameter) that allow the free water circulation inside the tank. These perforated Plexiglas tanks have transparent divisions that allow visual contact with the conspecifics, minimizing the isolation stress as well as ensuring the identification of each subject throughout the experimentation (MAXIMINO et al., 2018). Importantly, water conditions were similar to those of the housing tanks.

The inhibitory avoidance apparatus (30 cm length x 10 cm height x 10 cm width) was equally divided into two compartments (white and black) separated by a guillotine-type door and was filled with 1.3 L of non-chlorinated water. The black compartment contained three pairs of steel metal bars connected to an electric 12 V stimulator and shock frequency was set at 100 Hz (50 pulses of 5 ms every 500 ms) (BERTONCELLO et al., 2019; FRANSCESCON et al., 2020).

On the 10^{th} day, animals were trained to assess the effects EtOH and TAU on the memory acquisition of zebrafish. In the training session, zebrafish were individually placed on the white compartment of the tank and, after 1 min of acclimatization, the guillotine door was partially open, allowing free movement of the animal in the apparatus. When fish crossed to the black compartment, the door was closed and a mild electric shock (3 ± 0.2 V, 100 Hz) was administered for 5 s. Then, fish was removed from the apparatus and placed in their respective perforated housing tanks. As exclusion criterion, animals that did not cross to the black compartment within 300 s were removed from the trial.

The test session (11^{th} day) was performed 24 h after the training with a similar protocol, except that no shock was administrated when fish crossed to the black compartment. Two trained observers blinded to the experimental conditions (reliability between evaluators ≥

0.90) manually quantified the latency to enter the black compartment of the apparatus using a stopwatch. Fish were euthanized by immersion in water at 4°C immediately after the test session.

2.5. Determination of Z-MAO activity

After the novel tank diving test, fish were anesthetized by immersion in water at 4°C in a 500 mL beaker and euthanized by decapitation for brain tissue sampling. Z-MAO activity was determined based on the quantification of the 4-hidroxyquinoline produced by the reaction (KRAJL, 1965) previously characterized for zebrafish (QUADROS et al., 2018). According to pilot studies, two brains were pooled for each sample and, thus, a total of 6 to 8 fish for each experimental group (n = 3–4 independent preparations per group). Samples were homogenized in 500 µL of buffer solution (0.0168 M Na_2HPO and 0.0106 M KH_2PO_4, in pH 7.4, and 0.32 M sucrose) and centrifuged at 1.000 x g for 5 min. Experiments were performed using 90 µg protein from supernatants, mixed with 100 µL of distilled water, 460 µL of assay buffer (0.022 M Na_2HPO_4 and 0.016 M KH_2PO_4, pH 7.4, with 0.047 M KCl). The reaction medium described above was preincubated for 5 min at 37°C. Subsequently, 50 µL of kynuramine hydrobromide was added (totalizing a final volume of 700 µL) and after 30 min the reaction was stopped with 300 µL of 10% trichloroacetic acid. Samples were centrifuged for 5 min at 16.000 x g at 4°C and 800 µL of supernatant was mixed with 1 mL of 1 M NaOH. The fluorescent intensity was monitored at excitation and emission wavelengths of 315 nm and 380 nm, respectively. The amount of 4-hidroxyquinoline produced was estimated from a corresponding standard fluorescence curve. Specific activity was expressed as nmol 4-OH quinolone/min/mg protein. Protein in brain samples were measured in duplicate at 595 nm using the Coomassie Blue method (BRADFORD, 1976) and bovine serum albumin as standard.

2.6. Statistical analyses

Data normality and homogeneity of variances were analyzed by Kolmogorov-Smirnov and Bartlett's tests, respectively. The effects of EtOH and TAU on the shoaling behavior, anxiety-like responses, and Z-MAO activity were analyzed by two-way ANOVA (using EtOH and TAU as factors) followed by Student-Newman-Keuls multiple comparisons test when necessary. Results were expressed as means ± standard error of the mean (S.E.M). In the inhibitory avoidance test, non-parametric data of latencies were expressed as median ± interquartile range and analyzed by Wilcoxon matched-pairs signed rank test. To evaluate the effects of different experimental conditions on memory performance in the test session, non-parametric data were log-transformed and reported as fold change in relation to water CTRL group. These results were expressed as means ± S.E.M and analyzed by two-way ANOVA followed by Student-Newman-Keuls multiple comparisons test. The level of significance was set at $p \leq 0.05$.

3. Results

In the shoaling behavior test **(Fig. 2)**, two-way ANOVA yielded significant effects of EtOH x TAU interaction for inter-fish distance ($F_{(3,34)} = 8.247$, $p = 0.003$), shoal area ($F_{(3,34)} = 3.857$, $p = 0.0177$), farthest neighbor distance ($F_{(3,34)} = 6.869$, $p = 0.0010$), and nearest neighbor distance ($F_{(3,34)} = 3.543$, $p = 0.0246$). Moreover, significant effects of EtOH were observed for shoal area ($F_{(1,34)} = 4.737$, $p = 0.0366$). Post-hoc analyses showed that EtOH decreased the shoal area, as well as TAU 42, TAU 150, and EtOH alone decreased the inter-fish distance and farthest neighbor distance when compared to water CTRL group. TAU 150 abolished the effects of intermittent EtOH exposure on inter-fish distance and farthest neighbor distance. Although behavioral responses observed in fish treated with EtOH and

other TAU concentrations tested did not differ from EtOH alone, they were also similar to those found in water CTRL group.

Using the novel tank diving test, we further evaluated the effects of EtOH and TAU on locomotor and anxiety-like responses **(Fig. 3)**. For distance traveled and absolute turn angle no significant differences between groups were observed (**Fig. 3A**). **Fig. 3B** shows the effects of EtOH and TAU on vertical exploration, which may reflect anxiety-like responses. For the time spent in top area, two-way ANOVA revealed a significant effects of EtOH x TAU interaction ($F_{(3,72)}$ = 6.067, p = 0.0010) and EtOH ($F_{(1,72)}$ = 10.27, p = 0.0020). Post-hoc analyses showed that TAU 42, EtOH, and EtOH/TAU 150 groups spent less time in top area when compared to water CTRL group. EtOH/TAU 150 group also remains less time in top area when compared to TAU 150 group. Notably, EtOH-induced changes in vertical activity were attenuated in both EtOH/TAU 42 and EtOH/TAU 400 groups since no differences from water CTRL were found.

Two-way ANOVA also revealed a significant effect of EtOH on the latency to enter the top area ($F_{(1,72)}$ = 5.831, p = 0.0183), in which animals exposed to EtOH spent more time to enter the top when compared to water CTRL. No differences were observed in transitions in top area.

The effects of repeated EtOH and TAU exposures on memory acquisition were assessed in the inhibitory avoidance task **(Fig. 4)**. Wilcoxon matched-pairs rank test revealed that water CTRL (W = 50.00, p = 0.0244), TAU 42, 150, and 400 alone (W = 51.00, p = 0.0205; W = 60.00, p = 0.0161, and W = 77.00, p = 0.0046, respectively), as well as EtOH/TAU 42 and EtOH/TAU 150 groups (W = 37.00, p = 0.0273; and W = 53.00, p = 0.0039, respectively) showed a significant increase in the latency to enter the black compartment 24 h after the training session (**Fig. 4A**). Moreover, two-way ANOVA showed a significant effect of EtOH x TAU interaction ($F_{(3,77)}$ = 4.401, p = 0.0065) and EtOH ($F_{(1,77)}$ =

4.397, p = 0.0393) for the memory performance in the test session (**Fig. 4B**). Post-hoc analyses showed that EtOH group had lower memory performance when compared to water CTRL group. While EtOH/TAU 42 and EtOH/TAU 150 showed significant protective effects, EtOH/TAU 400 treatment did not reverse the EtOH-induced memory acquisition impairment.

For brain Z-MAO activity **(Fig. 5)**, two-way ANOVA showed significant effects of EtOH ($F_{(1,21)}$ = 44.31, $p < 0.0001$) and TAU ($F_{(3,21)}$ = 8.232, p = 0.0008). Post-hoc analyses showed TAU 150, TAU 400, EtOH alone and all groups treated with EtOH and TAU (EtOH/TAU 42, EtOH/TAU 150, and EtOH/TAU 400) have higher the Z-MAO activity when compared to water CTRL. Moreover, EtOH/TAU 42 and EtOH/TAU 150 show higher enzyme activity when compared to TAU 42 and TAU 150 groups, respectively.

4. Discussion

Here, we demonstrate for the first time the effects of TAU on behavioral and biochemical parameters following intermittent EtOH exposure in zebrafish. Fish exposed to EtOH had increased shoaling behavior, while TAU 150 abolished this response. The other TAU concentrations tested also showed a potential mild attenuation of this effect. Moreover, animals showed increased anxiety-like behavior in the novel tank diving test, while TAU 42 and TAU 400 attenuated some behavioral responses. In the inhibitory avoidance test, TAU 42 and TAU 150 reverted the memory acquisition deficit caused by intermittent EtOH exposure, while TAU did not modulate EtOH-induced enhancement on brain Z-MAO activity. Thus, although some beneficial properties have been observed in shoaling behavior and habituation to novelty stress, TAU seems to exert a prominent effect in reversing the memory acquisition impairment in zebrafish.

Chronic alcohol consumption affects physiological and behavioral parameters in mammals (KLIETHERMES, 2005; NOVIER; DIAZ-GRANADOS; MATTHEWS, 2015). Zebrafish are suitable to explore the effects of drugs on social behavior due to their ability to form shoals in nature and also in laboratory conditions, requiring a relatively small space to test a large number of subjects (GERLAI, 2014). Usually, increased shoal cohesion reflects pronounced defensive responses, while decreased shoaling is indicative of impaired social behavior (FERNANDES; RAMPERSAD; GERLAI, 2015; KURTA; PALESTIS, 2010). In our experiments, fish showed increased shoaling behavior, supported by a decreased in inter-fish distance, shoal area, and farthest neighbor distance, suggesting an anxiogenic effect as reported elsewhere (MÜLLER et al., 2017). These results are supported by the behavioral responses observed in the novel tank diving test, which measures habituation to novelty stress (EGAN et al., 2009; LEVIN; BENCAN; CERUTTI, 2007). Corroborating previous findings (MATHUR; GUO, 2011; MOCELIN et al., 2019), intermittent EtOH exposure induced anxiety-like behavior, since fish spent less time in the top and showed increased latency to enter the top area of the tank. These set of data reinforce that the protocol used here induces robust behavioral changes in zebrafish.

Acutely, TAU elicits anxiolytic-like behavior in zebrafish (MEZZOMO et al., 2016), preventing some behavioral alterations following acute EtOH exposure (FONTANA et al., 2018b; ROSEMBERG et al., 2012). Although the acute effects of TAU and EtOH have been widely studied, the influence of intermittent exposures of TAU and EtOH in zebrafish behaviors have not been explored yet. In mice, chronic TAU supplementation promotes anxiety (EL IDRISSI et al., 2009), which may help explain the increased shoaling responses in both TAU 42 and TAU 150 groups, well as the increased anxiety-like responses in TAU 42 group. Importantly, TAU appears to have context- and concentration-dependent effects, since fish exposed to 42 and 150 mg/L TAU showed prominent responses depending on the

behavioral parameters assessed. We also verified that EtOH/TAU 150 group spent less time in top area of the tank, indicating pronounced anxiety-like behavior. However, 150 mg/L TAU abolished EtOH-increased shoal responses, supporting that both tests may also not reflect the same underlying construct (*i.e.*, sociability and habituation to novelty). As observed in the shoaling behavior task, both TAU 42 and TAU 400 showed a modest attenuation of the effects of EtOH in the novel tank diving test. Since both EtOH and TAU interact with the GABAergic (HUXTABLE, 1992; WHITE; MATTHEWS; BEST, 2000) and glutamatergic system (CHAN et al., 2014; GONZALES; JAWORSKI, 1997), these molecules could act in the same receptor, even attenuating or potentiating their responses alone. Furthermore, anxiety-like responses observed may also be associated with oxidative processes in the brain, since intermittent EtOH exposure modulates antioxidant enzymatic defenses and induces oxidative stress in zebrafish (MÜLLER et al., 2017). Thus, the antioxidant profile of TAU may partially help explain the potential beneficial role in the CNS, which requires further scrutiny. Importantly, both EtOH and TAU concentrations tested did not affect locomotion, reinforcing that the behavioral changes measured here are not associated with altered motor coordination.

It is conceivable that EtOH chronically increases glutamatergic activity (CHEFER et al., 2011; GONZALES; JAWORSKI, 1997; MULHOLLAND et al., 2011). Although glutamate plays an essential role in synaptic plasticity, learning, and memory (MCENTEE; CROOK, 1993; RIEDEL; PLATT; MICHEAU, 2003), increased glutamatergic stimulation in the brain can promote excitotoxicity (GONZALES; JAWORSKI, 1997), which is one of the mechanisms associated with cognitive deficits observed in the alcoholism (RON; WANG, 2009; TSAI; GASTFRIEND; COYLE, 1995). The exposure protocol used here impaired memory acquisition, since zebrafish showed similar latencies to enter the dark compartment during training and test sessions as well as reduced memory performance in the test session.

Similarly, evidence shows that chronic 0.25% and 1.0% EtOH exposures for at least 18 days impair associative learning in zebrafish subjected to the cognition test (CHACON; LUCHIARI, 2014). Due to the pleiotropic action on the brain, TAU can act as a inhibitory neuromodulator protecting neurons from glutamate-induced excitotoxicity (YE; SHI; YIN, 2013). When administered for 10 or 30 days, TAU alone does not modulate memory retention in mice, but shows beneficial effects when associated with EtOH (VOHRA; HUI, 2000). We observed that TAU alone did not alter the memory acquisition, while treatments with EtOH associated to TAU 42 or TAU 150 reversed the EtOH-induced memory impairment. We hypothesize that the effect of TAU may be due to the inhibition of NMDA receptor responses (CHAN et al., 2014), supporting the neuromodulatory role in the CNS (HUXTABLE, 1992). Moreover, TAU 400 did not reverse the memory acquisition impairment caused by EtOH, reinforcing a concentration-dependent effect, as occur in zebrafish after acute exposure (FONTANA et al., 2018b, 2020).

In the brain, MAO activity represents an important mechanism to control the monoamine levels (NIKOLAC PERKOVIC et al., 2016). Because zebrafish expresses a single MAO isoform (ANICHTCHIK et al., 2006; SETINI et al., 2005), this species may be a suitable model organism to investigate the relationship of brain monoamine catabolism and alcoholism. Here, brain Z-MAO activity increased 24h after the last EtOH exposure. Similarly, chronic EtOH administration in rodents increases MAO-A activity 24 h after the exposure period (MATTHEWS et al., 2018). Evidence also demonstrates increased dopamine levels, associated to decreased norepinephrine in the rat striatum 48 h after one-week EtOH exposure (VASCONCELOS et al., 2003). Similar effects were also observed in zebrafish, where dopamine levels were raised 2 days and 9 days after intermittent EtOH exposure, but no changes were found in noradrenaline levels (ALEXANDRE et al., 2019). Notably, EtOH increases the levels of TAU and dopamine in the nucleus accumbens of rodents, in which

extracellular TAU facilitates dopamine release in the brain (ERICSON et al., 2006, 2011). Here, we observed a significant increase in Z-MAO activity in TAU 150 and TAU 400 alone, as well as in all EtOH/TAU groups. Although we do not have a clear explanation of the underlying mechanisms of EtOH and TAU in zebrafish brain, this response could be associated with a compensatory mechanism in the CNS triggered by repeated EtOH exposure and/or even related to a stimulatory effect of both molecules on the reward system.

Mounting evidence shows the influence of monoamines on different behavioral responses. For example, MAO-A deficiency increases serotonin levels producing antisocial behavior in mice (BORTOLATO et al., 2013) and anxiolytic-like effects in rats (CAILLE et al., 1996). Likewise, the selective MAO-B inhibitor, L-deprenil, improves learning and memory deficits in aged rats (BRANDEIS et al., 1991). Because zebrafish treated with TAU 42 and TAU 150 showed a markedly reversion of memory acquisition deficit caused by EtOH with a similar effect on brain Z-MAO, changes in this enzyme activity are probably unrelated with the protective effect observed. However, an involvement of monoamines in regulating shoal behaviors and anxiety-like responses after EtOH and TAU exposures should not be ruled out. Notably, since our analyses were performed after the exposure period, behavioral and biochemical data reported here result from indirect effects of treatments rather than acute/direct effects per se.

5. Conclusion

To our knowledge, we report the first evidence regarding the beneficial role of TAU in counteracting the effects of intermittent EtOH exposure on multiple behavioral domains in zebrafish. Because the available treatments for alcohol abuse disorders and alcoholism are limited so far, our results demonstrate a potential utility of TAU to attenuate/reverse some behavioral responses induced by EtOH. As a limitation, since both EtOH- and TAU-mediated

effects in the brain can be multifaceted, the precise analysis of the neurochemical mechanisms underlying the behaviors measured merits future scrutiny. Collectively, our data foster the growing utility of zebrafish models to assess potential neuroprotective strategies against chronic alcohol administration.

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Conflict of interest

The authors declare that there is no conflict of interest.

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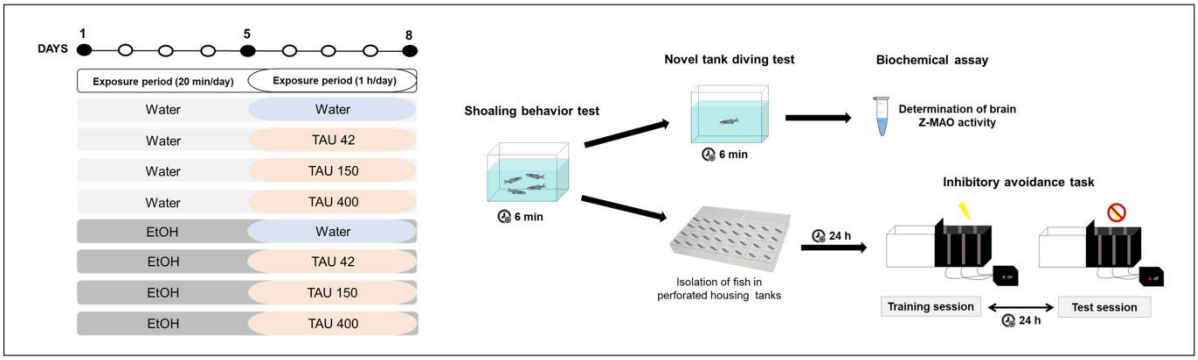

Fig. 1. Schematic representation of the experimental design. Zebrafish were exposed to EtOH (20 min per day for 8 days) and TAU treatment started at the 5th day of exposure until the end of the experimental period (1 h per day). Fish were later tested on shoaling behavior and novel tank diving test (9th day) or in the inhibitory avoidance task (10th and 11th days). Immediately after the novel tank diving test, fish were euthanized and brain samples were prepared for measuring Z-MAO activity (EtOH: 1% ethanol; TAU 42: 42 mg/L taurine; TAU 150: 150 mg/L taurine; TAU 400: 400 mg/L taurine).

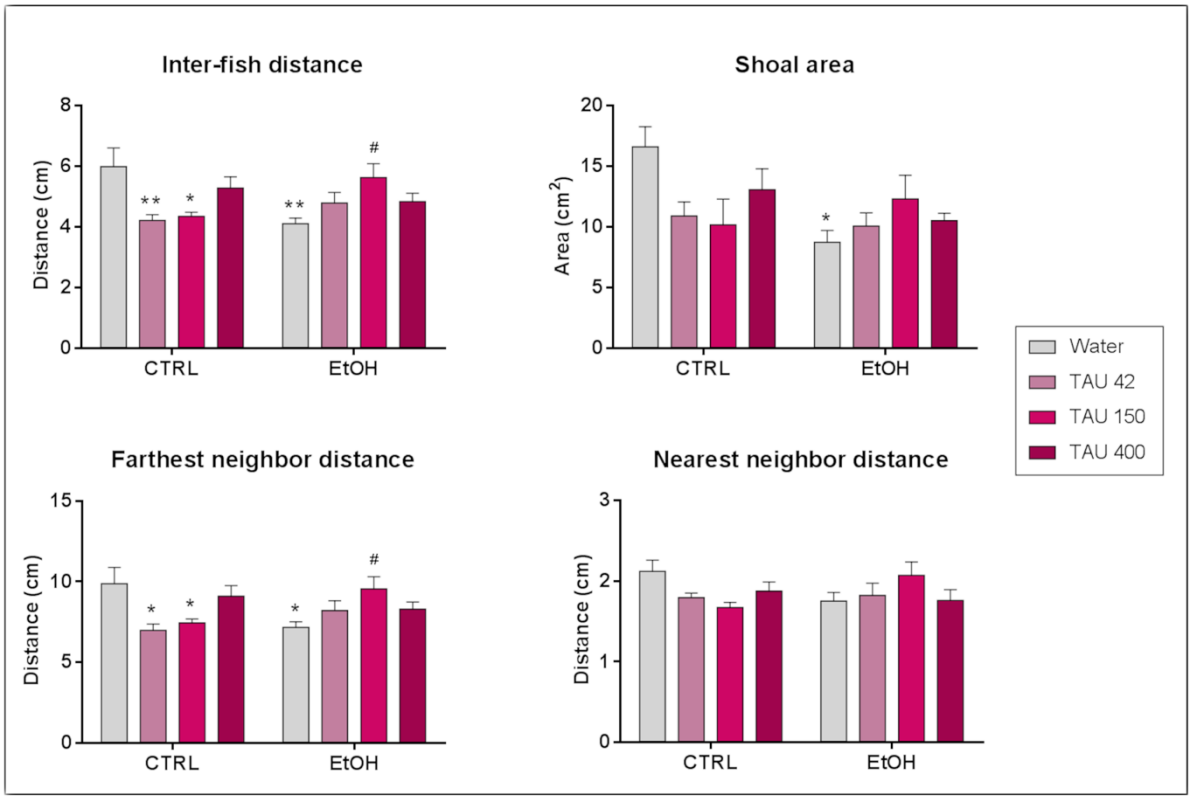

Fig. 2. Effects of TAU and EtOH on shoaling behavior of zebrafish. Data are expressed as mean ± S.E.M. and analyzed by two-way ANOVA followed by Student-Newman-Keuls multiple comparisons test when necessary. Asterisks above bars express significant differences compared to the water CTRL group and symbols (#) represent significant differences compared to EtOH group (n = 4–6 shoals per group; * $p < 0.05$, ** $p < 0.01$; CTRL: experimental controls of water or TAU alone, EtOH: ethanol; TAU: taurine).

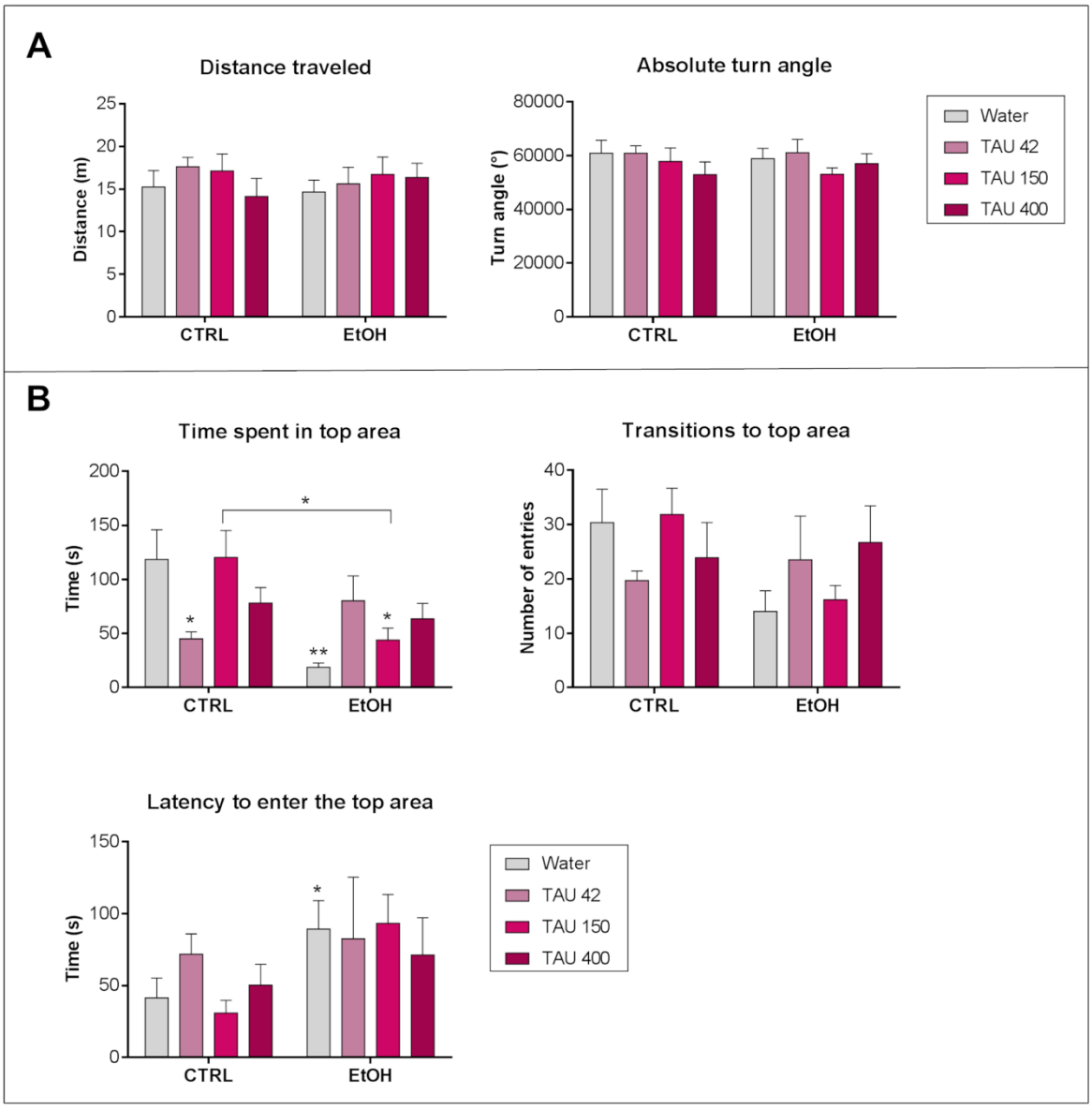

Fig. 3. Effects of TAU and EtOH on locomotion and anxiety-like responses in the novel tank diving test. **(A)** Locomotion-related endpoints, measured by the distance traveled and absolute turn angle. **(B)** Vertical exploration, assessed by the time spent in top area, transitions to top area, and latency to enter the top area. Data are expressed as mean ± S.E.M. and analyzed by two-way ANOVA followed by Student-Newman-Keuls multiple comparisons test when necessary. Asterisks above bars express significant differences compared to the water CTRL group, while asterisks above brackets indicate statistical differences compared to TAU alone

(n = 8–11 animals per group; * $p < 0.05$, ** $p < 0.01$; CTRL: experimental controls of water or TAU alone, EtOH: ethanol; TAU: taurine).

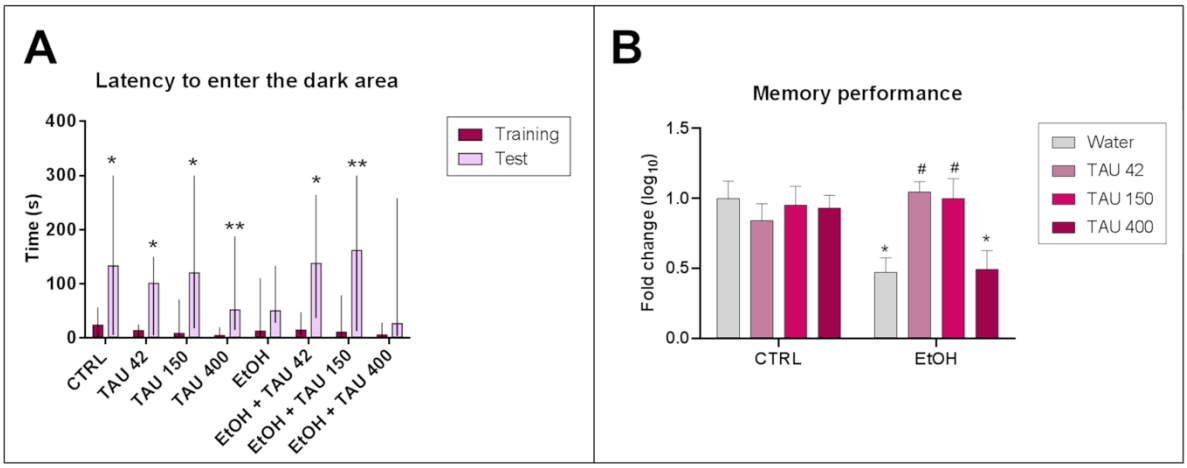

Fig. 4. TAU reverses EtOH-induced impairment on memory acquisition in the inhibitory avoidance task. **(A)** Latency to enter the black compartment in training and test sessions. Data are expressed as median ± interquartile range and analyzed by Wilcoxon matched-pairs signed rank test. Asterisks express significant differences compared to the training session. **(B)** Memory performance in the test session of the experimental groups. Data are expressed as mean ± S.E.M. and analyzed by two-way ANOVA followed by Student-Newman-Keuls multiple comparisons test. Asterisks above bars express significant differences compared to the water CTRL group and symbols (#) represent significant differences compared to EtOH group (n = 7–13 animals per group; * $p < 0.05$, ** $p < 0.01$; CTRL: experimental controls of water or TAU alone, EtOH: ethanol; TAU: taurine).

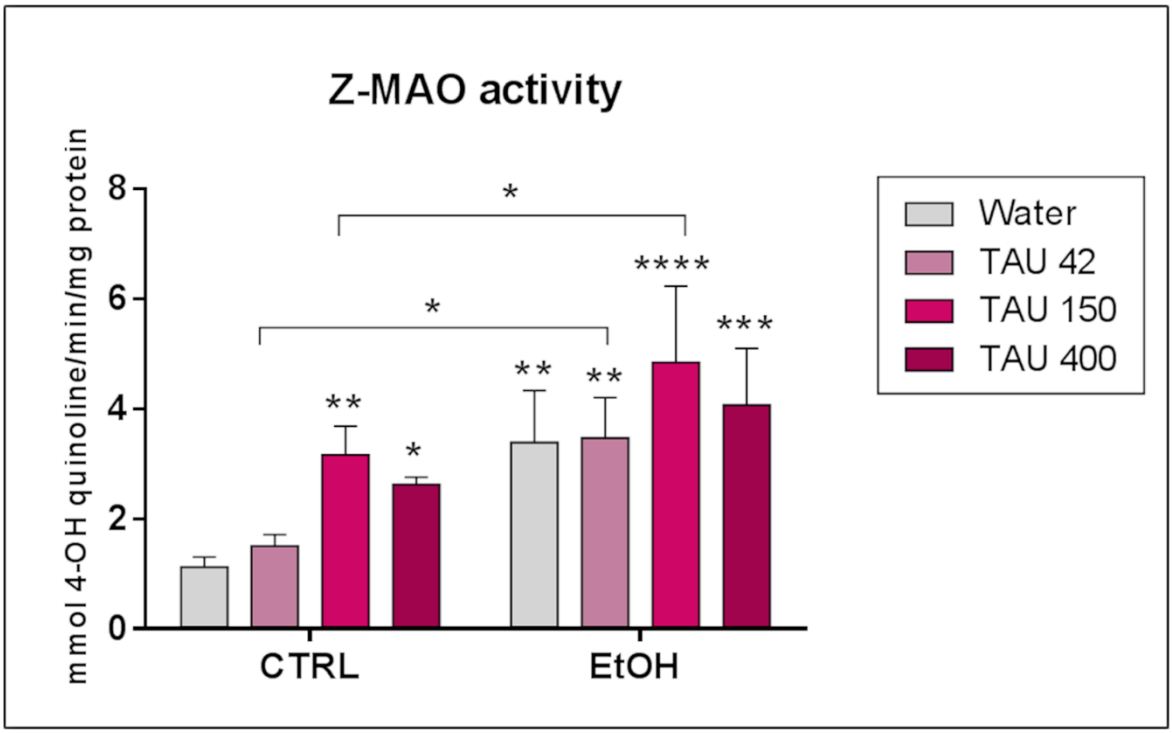

Fig. 5. Effects of repeated TAU and EtOH exposures on brain Z-MAO activity of zebrafish. Data are expressed as mean ± S.E.M. and analyzed by two-way ANOVA followed by Student-Newman-Keuls multiple comparisons test. Asterisks above bars express significant differences compared to the water CTRL group, while asterisks above brackets indicate statistical differences compared to TAU alone (n = 3–4 independent preparations per group; * $p < 0.05$, ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.001$; CTRL: experimental controls of water or TAU alone, EtOH: ethanol; TAU: taurine).

6 CONCLUSÃO

6.1 CONCLUSÕES PARCIAIS

A partir dos resultados apresentados nesta dissertação podemos concluir especificamente que:

- Vinte e quatros horas após a última exposição repetida ao EtOH, os animais apresentaram um aumento na coesão do cardume. O tratamento com TAU 150 mostrou reversão de respostas induzidas pelo EtOH, enquanto as demais concentrações testadas mostraram uma modesta atenuação deste efeito ansiogênico mensurado a nível social em peixes-zebra.

- Os peixes expostos repetidamente ao EtOH apresentaram um aumento do comportamento semelhante ao da ansiedade, enquanto TAU 42 e TAU 400 atenuaram algumas respostas comportamentais (o tempo gasto e o número de entradas na área superior do tanque);

- Os animais expostos repetidamente a TAU 42 e TAU 150 per se apresentaram um aumento no comportamento de cardume, enquanto TAU 42 per se também apresentou um aumento no comportamento semelhante ao de ansiedade no teste do tanque novo.

- Tanto o EtOH quanto a TAU não afetaram os parâmetros relacionados à locomoção, sugerindo que as mudanças comportamentais dos animais não estão associadas com alterações locomotoras;

- No teste da esquiva inibitória, TAU 42 e TAU 150 reverteram o déficit na aquisição da memória causado pelo EtOH;

- A TAU não modulou o aumento na atividade da Z-MAO cerebral em peixes-zebra induzidos após a última exposição repetida ao EtOH. Além disso, TAU 150 e TAU 400 per se apresentaram um aumento na atividade desta enzima.

6.2 CONCLUSÃO FINAL

Em suma, os resultados apresentados nessa dissertação demonstram que a TAU de um modo geral atenua as alterações causadas pela exposição repetida ao EtOH no comportamento social e nas respostas do tipo ansiedade, bem como exerce um papel protetor na reversão do déficit na aquisição da memória em peixes-zebra. Apesar dos tratamentos disponíveis para os transtornos do abuso de álcool e alcoolismo serem limitados até agora, nossos resultados demonstram um papel benéfico da TAU em algumas respostas induzidas vinte e quatro horas após a última exposição repetida ao EtOH. Além disso, em uma expansão de estudos translacionais, nossos dados reforçam a utilidade do peixe-zebra como organismo modelo para investigar os mecanismos subjacentes às respostas neurocomportamentais do EtOH e da TAU em humanos.

7 PERSPECTIVAS

Este trabalho mostrou o potencial efeito benéfico da TAU nas alterações no comportamento social, nas respostas do tipo ansiedade e na aquisição da memória causadas pela exposição repetida ao EtOH. Assim, as perspectivas do estudo são:

- Quantificar os níveis de monoaminas cerebral após a exposição repetida ao EtOH e TAU no encéfalo de peixe-zebra;

- Investigar a influência da atividade da Z-MAO cerebral na modulação de diferentes fenótipos comportamentais (comportamento social, ansiedade e memória) no peixe-zebra, utilizando inibidores como, por exemplo, clorgilina e deprenil;

- Avaliar se a TAU é capaz de reverter o estresse oxidativo causado pela exposição repetida ao EtOH em peixe-zebra.

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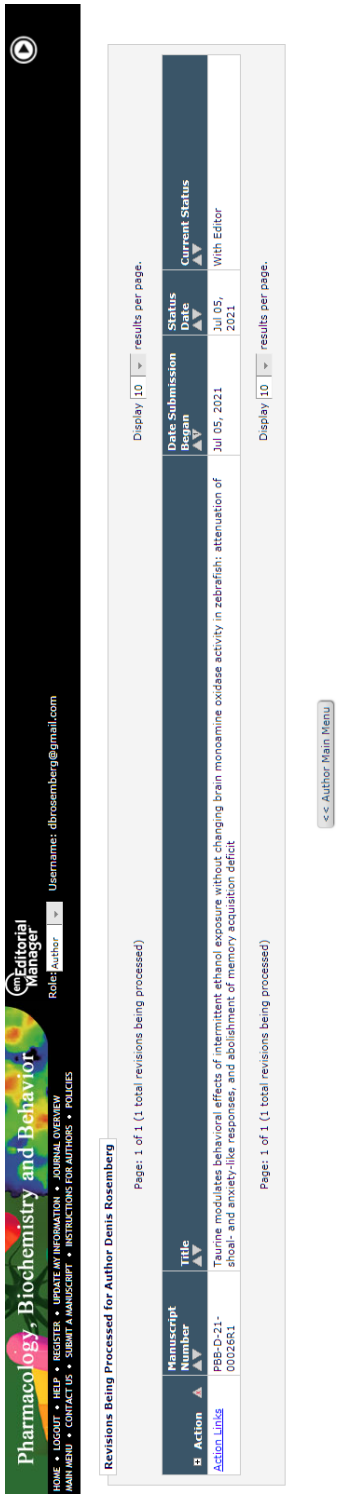
ANEXO A – COMPROVANTE DE SUBMISSÃO DO MANUSCRITO CIENTÍFICO



ANEXO B – LISTA DE TRABALHOS CIENTÍFICOS EM COLABORAÇÕES

Artigos colaborativos desenvolvidos durante o período do Mestrado:

1) Müller TE, Ziani PR, Fontana BD, Duarte T, **Stefanello FV**, Canzian J, Santos ARS, Rosemberg DB. Role of the serotonergic system in etanol-induced aggression and anxiety: A pharmacological approach using the zebrafish model. Eur Neuropsychopharmacol. 2020 Mar;32:66-76. doi: 10.1016/j.euroneuro.2019.12.120.

2) Müller TE, Fontana BD, Bertoncello KT, Franscescon F, Mezzomo NJ, Canzian J, **Stefanello FV**, Parker MO, Gerlai R, Rosemberg DB. Understanding the neurobiological effects of drug abuse: Lessons from zebrafish models. Prog Neuropsychopharmacol Biol Psychiatry. 2020 Jun 8;100:109873. doi: 10.1016/j.pnpbp.2020.109873.

3) Canzian J, Franscescon F, Müller TE, **Stefanello FV**, Souza TP, Rosa LV, Rosemberg DB. Stress increases susceptibility to pentylenetetrazole-induced seizures in adult zebrafish. Epilepsy Behav. 2021 Jan; 114(Pt A); 107557. doi: 10.1016/j.yebeh.2020.107557.