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Barbara Dotto Fontana

EFEITOS NEUROCOMPORTAMENTAIS DA TAURINA EM MODELOS DE EXPOSIÇÃO AO ETANOL E DE CRISE CONVULSIVA EM PEIXE-ZEBRA

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, RS), como requisito parcial para obtenção do título de **Mestre em Ciências Biológicas: Bioquímica Toxicológica**

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DEDICATÓRIA

Dedico este trabalho a aqueles que sempre me apoiaram, me incentivaram a nunca desistir e também sempre me estimularam a dar o melhor de mim em tudo, meu pai Paulo Rogério Fontana e minha mãe Adriana Ribeiro Dotto. A vitória deste trabalho eu dedico a vocês com todo meu amor.

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“We keep moving forward, opening new doors, and doing new things, because we're curious and curiosity keeps leading us down new paths”.

Walt Disney

RESUMO

EFEITOS NEUROCOMPORTAMENTAIS DA TAURINA EM MODELOS DE EXPOSIÇÃO AO ETANOL E DE CRISE CONVULSIVA EM PEIXE-ZEBRA

AUTORA: Barbara Dotto Fontana
ORIENTADOR: Prof. Dr. Denis Broock Rosemberg

O uso de modelos translacionais alternativos vem sendo amplamente utilizado na neurociência para o estudo de diversas doenças neurológicas e neuropsiquiátricas. Nesse contexto, o peixe-zebra (*Danio rerio*) ganha grande destaque uma vez que possui alta similaridade genética com seres humanos e permite a triagem de novos agentes farmacológicos de forma bastante eficiente. Assim, o objetivo do nosso primeiro trabalho foi avaliar as perspectivas do uso do peixe-zebra como organismo modelo a fim de posteriormente utilizá-lo para a avaliação do potencial protetor da taurina (TAU) em diferentes situações. A TAU é uma molécula que desempenha funções fisiológicas essenciais nos seres vivos, tais como neuromodulação, atividade antioxidante, osmoregulação e estabilização de membrana. Sua ação neuromoduladora ocorre principalmente através de sua ação agonista em receptor $GABA_A$ e de glicina. Diversas drogas são capazes de alterar a funcionalidade de receptores $GABA_A$ e modificar o balanço das sinapses inibitórias/excitatórias, tais como o etanol (EtOH) e o pentilenotetrazol (PTZ). Portanto, no segundo e no terceiro trabalho tivemos por objetivo analisar o papel da TAU sobre as alterações na agressividade, no comportamento social e na avaliação de risco induzidas pelo EtOH. Os animais foram expostos por 1 hora e divididos em 8 grupos: Controle; TAU 42 mg/L; TAU 150 mg/L; TAU 400 mg/L; EtOH 0,25% (v/v); TAU 42/EtOH; TAU 150/EtOH e TAU 400/EtOH. Posteriormente, os animais foram submetidos ao teste de agressividade, comportamento em cardume, preferência social e ao teste do predador. De modo geral, a TAU apresentou um efeito bifásico no comportamento agressivo, onde a menor e a maior concentração testada (42 e 400 mg/L) modularam positivamente a agressão, enquanto que 150 mg/L exerceu uma ação anti-agressiva. Além disso, análise temporal do comportamento em cardume mostrou que EtOH sozinho e associado à TAU apresentam uma redução do comportamento social. No entanto, no teste de preferência social somente TAU 400/EtOH reduziram a preferência por coespecíficos. Em relação ao comportamento aversivo do peixe-zebra, TAU *per se* e em associação diminuiu as avaliações de risco na área do predador. Por fim, avaliamos o papel protetor da TAU frente às crises convulsivas induzidas pelo PTZ através da análise dos estágios de crise convulsiva, bem como dos parâmetros relacionados ao estresse oxidativo. Os peixes foram pré-tratados com TAU (42, 150 ou 400 mg/L) por 40 minutos e então expostos ao PTZ por 20 minutos, onde os escores de crise convulsiva foram analisados a cada 30 segundos durante a exposição e a intensidade da crise convulsiva mensurada. Para a análise de estresse oxidativo, os seguintes parâmetros foram quantificados: atividade das enzimas CAT, SOD e GST cerebral,níveis de peroxidação lipídica, carbonilação de proteínas, viabilidade e morte celular. Os resultados mostraram que a TAU 150 mg/L atenuou as crises convulsivas induzidas pelo PTZ, diminuindo a intensidade das crises convulsivas e prevenindo o dano oxidativo. Assim, a TAU é capaz de modular diferentes respostas neuroquímicas e comportamentais dependendo da concentração, sugerindo um complexo efeito da TAU em diferentes receptores e/ou em vias de transdução de sinal no SNC.

Palavras-chave: agressividade; comportamento aversivo; comportamento social; epilepsias.

ABSTRACT

NEUROBEHAVIORAL EFFECTS OF TAURINE IN ETHANOL EXPOSURE MODELS AND SEIZURES IN ZEBRAFISH

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ADVISOR: DENIS BROOCK ROSEMBERG

Alternative translational models have been widely used in neuroscience for assessing several neurological and neuropsychiatric diseases. In this context, the emergent use of zebrafish (*Danio rerio*) has been relevant due to the high genetic conservation and the ability to perform high-throughput screens. Thus, in our first article, we aimed to evaluate the developing utility of zebrafish as an experimental model organism to later use evaluate the potential protector effects of taurine (TAU) in different situations. TAU is an endogenous molecule that has pleiotropic actions, such as neuromodulation, antioxidant activity, osmoregulation and membrane stabilization. The neuromodulatory action of TAU action occurs mainly through $GABA_A$ and glycine agonism. Several drugs can alter the functionality of $GABA_A$ receptors and modify the balance of inhibitory/excitatory synapses, such as ethanol (EtOH) and pentylenetetrazol (PTZ). Therefore, in our second and third works we analyzed the effects of TAU in preventing different EtOH-induced behavioral modifications, such as aggression, social interaction deficits,and changes in risk evaluation. Animals were exposed for 1 hour and divided into 8 groups: Control; TAU 42 mg/L; TAU 150 mg/L; TAU 400 mg/L; 0.25% EtOH (v/v); TAU 42/EtOH; TAU 150/EtOH and TAU 400/EtOH. Afterwards, fish were submitted to the aggression test, shoal behavior test, social preference test, and predator test. TAU exerted a biphasic effect on the aggressive behavior, where the lowest and highest concentration tested (42 and 400 mg/L) positively modulated aggression, while 150 mg/L exerted an anti-aggressive action. Additionally, temporal analysis of shoal behavior showed that EtOH alone and associated to TAU reduced social behaviors. However, in the social preference test only TAU 400/EtOH reduced conspecific preference. Regarding the aversive behavior, TAU alone and in association decreased the number of risk assessment in the predator area. Finally, we evaluated the protective role of TAU in PTZ-challenged animals and analyzed the occurence of seizures as well as changes in oxidative stress-related parameters. In this experiment, fish were pretreated with TAU (42, 150 or 400 mg / L) for 40 minutes and then exposed to PTZ for 20 minutes. Seizure scores were analyzed every 30 seconds during PTZ exposure and seizure intensity was quantified. Regarding theoxidative stress, the following parameters were analyzed: CAT, SOD, and GST activities, lipid and protein damage, as well as cellular viability and cellular death. TAU 150 mg/L attenuated PTZ-induced seizures by reducing the seizure intensity and protecting against lipid and protein damage. Collectively, TAU modulates different neurochemical and behavioral responses depending on the concentration, suggesting a complex effect on different receptors and/or neural transduction pathways.

Keywords: aversive behaviors; aggressiveness; epilepsy; social behavior.

LISTA DE ABREVIATURAS

ACTH – Hormônio adrenocorticotrópico

CRF – Fator liberador de corticotropina (do inglês, ‘corticotropin release factor’)

CAT – Catalase

DZP – Diazepam

EtOH – Etanol

HPA – Eixo-hipotálamo-pituitária-adrenal

MEOS – Sistema de oxidação de EtOH microssomático

NPSH – Tióis não proteicos (do ingês, ‘nonprotein thiol content’)

PTZ – Pentilenotetrazol

Receptor $GABA_A$ – Receptor ionotrópico de GABA, subtipo A

SNC – Sistema nervoso central

TAU – Taurina

TauT – Transportador de taurina

SUMÁRIO

1. APRESENTAÇÃO

A presente dissertação está estruturada na seguinte forma: primeiramente é apresentada a **introdução** que inclui uma revisão da literatura sobre os temas abordados na dissertação e os objetivos do estudo. A seguir, as **metodologias** e **resultados** estão apresentadas no item **artigos e manuscrito científico**. O item **discussão** contém a análise integrada dos resultados obtidos nos manuscritos desta dissertação. O item **conclusão** apresenta interpretações gerais sobre os artigos científicos apresentados nesta dissertação. Por fim, o item **perspectivas** apresenta possibilidades de novos estudos a partir dos resultados obtidos. As **referências bibliográficas** apresentadas no final da dissertação referem-se às citações que aparecem nas sessões introdução e discussão.

2. INTRODUÇÃO

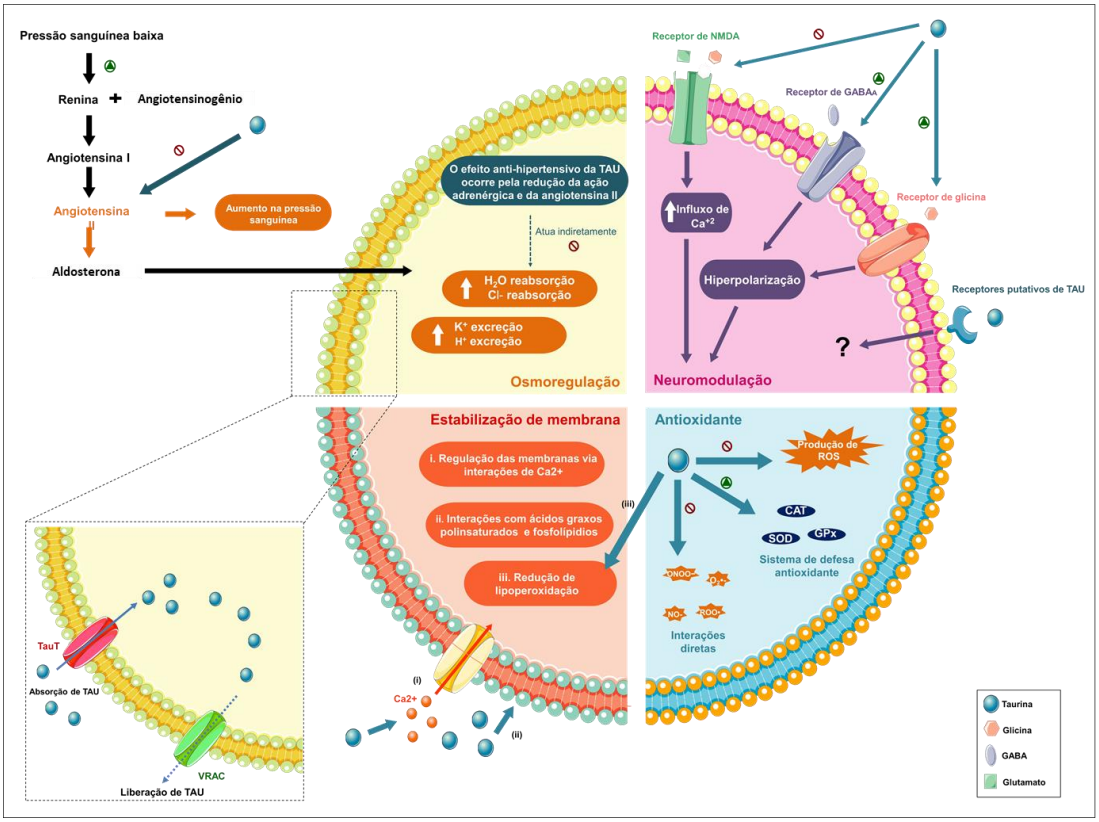
2.1. TAURINA E SEUS EFEITOS BIOLÓGICOS

A taurina (TAU) é um aminoácido sulfurado que, além de ser obtido através da dieta, pode ser sintetizado no sistema nervoso central (SNC) a partir da oxidação da cisteína (VITVITSKY, 2011). A biossíntese de TAU é altamente variável entre indivíduos, dependendo de estado nutricional, ingestão de proteínas e acessibilidade do aminoácido cisteína (HUXTABLE, 1992; DE LUCA et al., 2015). A disponibilidade de cisteína é dependente do equilíbrio metabólico entre a homocisteína e metionina, via ácido fólico, vitamina B12 e a atividade enzimática da metiltetrahidrofolato redutase (DE LUCA et al., 2015). *In vivo*, a TAU é absorvida pelo intestino e é liberada na corrente sanguínea por uma via não saturável (ROIG-PÉREZ et al., 2005; LAMBERT et al., 2015). Uma vez que atinge a circulação, a TAU é distribuída entre as células, carreada por transportadores específicos de membrana plasmática chamados TauT (codificados pelo gene *SLC6A6*) e/ou pelo transportador de aminoácidos acoplado a transporte de prótons (PAT1, codificado pelo gene *SLC36A1*) (RIPPS E SHEN, 2012; LAMBERT et al., 2015). As concentrações de TAU em fluidos extracelulares são menores do que aquelas relatadas intracelularmente, variando de 10 a 100 μM (HUXTABLE, 1992; SCHULLER-LEVIS E PARK, 2003; MARCINKIEWICZ E KONTNY, 2014; DE LUCA et al., 2015). Os efeitos extracelulares da taurina são atribuídos à ativação de alvos celulares específicos mesmo em concentrações muito baixas (HUXTABLE, 1992). Os níveis de TAU intracelular são mais elevados nos tecidos com atividade oxidante considerável, como coração (25-30 mM), pulmão (11-17 mM) e cérebro (30-40 mM), devido a sua importante ação antioxidante nestes tecidos (GREEN et al., 1991; STURMAN, 1993; MASSIEU et al., 2004; HANSEN et al., 2006; OLIVEIRA et al., 2010). Suas ações antioxidantes intracelulares ocorrem pela diminuição das espécies oxidativas e pelo estimulo das defesas antioxidantes (ROSEMBERG et al., 2010; SHIMADA et al., 2015). Assim, *in vitro*, a TAU pode agir diretamente em alguns radicais livres (peroxil, ânion superóxido, óxido nítrico e peroxinitrito) exercendo um efeito de captura destes radicais e diminuindo suas concentrações intracelulares (OLIVEIRA et al., 2010).

A TAU também desempenha uma variedade de funções no SNC, tais como regulação do metabolismo do Ca^{2+}, osmorregulação, manutenção do potencial de

membrana, além da sua neuromoduladora inibitória, como agonista de receptor $GABA_A$ (**Figura 1**) (WU et al., 2000; ROSEMBERG et al, 2012). Acredita-se que a TAU possa exercer seu papel através de outras moléculas sinalizadoras, tais como receptores de glicina ou, inclusive, receptores específicos para TAU ainda não totalmente elucidados (WU et al., 1992). Além disso, foi demonstrado que a TAU pode antagonizar os receptores de NMDA através de múltiplos mecanismos para reduzir a neurotoxicidade induzida pelo glutamato (CHAN et al., 2014). No cerebelo, por exemplo, a TAU aumenta a condutância de Cl- em membranas excitáveis, causando hiperpolarização em neurônios e reduzindo sua excitabilidade (CONTE-CAMERINO et al., 1987). Esses dados apoiam fortemente a ideia de que a TAU pode modular vários sistemas de segundos mensageiros antagonizando as ações do glutamato e, assim, capaz de atenuar os efeitos causados pela excitotoxicidade. Dessa maneira, o papel da TAU como molécula neuromoduladora, e neuroprotetora (WADE et al, 1988; CHEN, 2001; 2004; MENZIE et al., 2014) faz com que o seu uso terapêutico seja promissor para diversos distúrbios neurológicos e neuropsiquiátricos, tais como alterações comportamentais de sociabilidade e nas epilepsias.

Figura 1. Mecanismos de ação da taurina *in vivo*

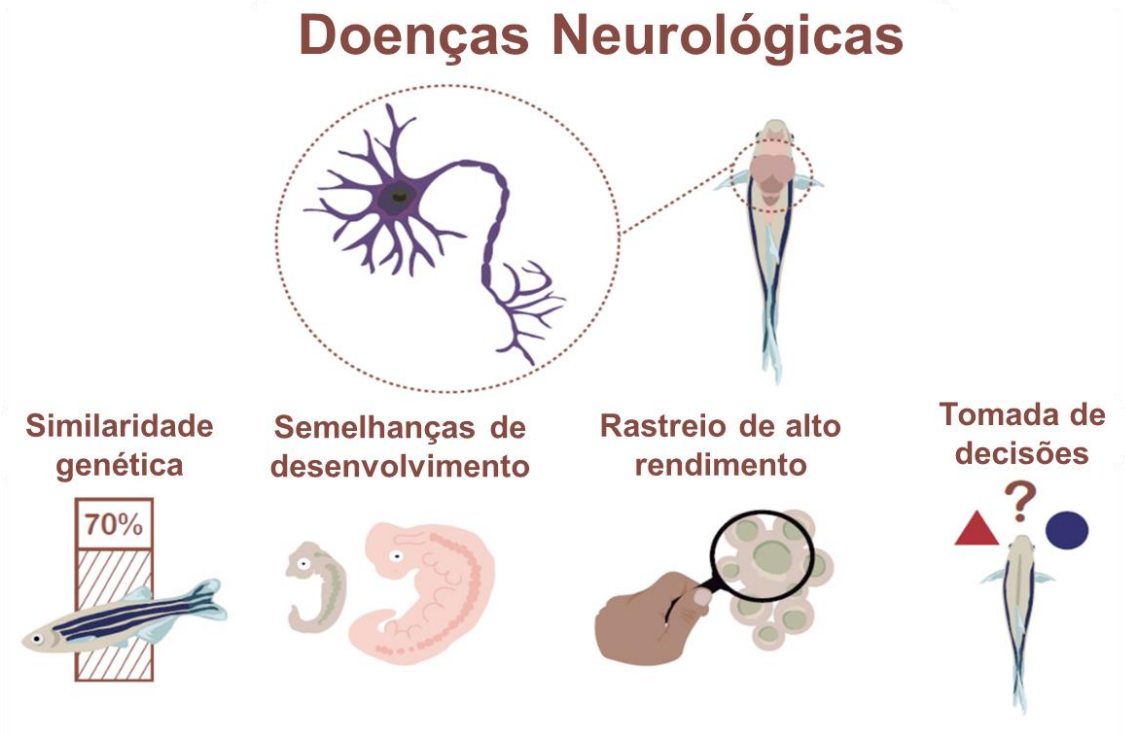


Fonte: Adaptada de MEZZOMO et al., (2017)

2.2. PEIXE-ZEBRA COMO ORGANISMO MODELO

No contexto da pesquisa biomédica translacional, o uso de organismos experimentais alternativos, como o peixe-zebra (*Danio rerio*) tem crescido exponencialmente na área de neurociências, oferecendo uma possibilidade real para a triagem de novas moléculas em média/larga escala (DINDAY e BARABARAN, 2015; GUPTA et al, 2014). Este organismo possui diversas vantagens em estudos de neuropsicofarmacologia e biologia do comportamento, tais como alta similaridade genética quando comparado aos genes de seres humanos (aproximadamente 70%) e os sistemas de neurotransmissão já caracterizados. Além disso, o peixe-zebra possui diversas semelhanças de desenvolvimento, é capaz da tomada de decisões em testes comportamentais e possibilita um rastreio de alto rendimento, permitindo a realização de grandes baterias de experimentos em um curto período (**Figura 2**) (MEZZOMO et al., 2017; KALUEFF et al, 2013). Neste trabalho, iremos focar em dois distúrbios que possuem grande impacto na sociedade, as alterações comportamentais induzidas pelo etanol e nas epilepsias em modelos experimentais translacionais.

Figura 2. Diagrama esquemático mostrando vantagens do uso do peixe-zebra para investigar os mecanismos envolvidos nos distúrbios do SNC.



Fonte: Adaptada de MEZZOMO et al. (2017)

Os modelos mais utilizados em peixes-zebra para o estudo de alterações comportamentais de epilepsias são através da indução de crises convulsivas por fármacos, como o pentilenotetrazol (PTZ) (LÖSCHER, 2011; BARABAN et al, 2005; MUSSULINI et al, 2013). Por outro lado, a exposição aguda ao etanol (EtOH) em peixe-zebra possibilita o estudo de fenótipos relacionados à agressividade, alterações sociais e de avaliação de risco (VAN ERP e MICZEK, 1997; LEVINSON et al, 2011; GERLAI et al, 2000). Sabe-se, que a exposição ao EtOH em uma concentração de 0,25% (v/v) por uma hora aumenta da agressividade sem induzir sedação, o que se torna relevante para a análise de comportamentos agonísticos em peixe-zebra (DLUGOS e RABIN, 2003; GERLAI et al, 2000). Esta concentração também é capaz de aumentar o comportamento antipredatório frente ao predador e reduzir o comportamento social em cardume de peixes-zebra (GERLAI et al., 2000; KURTA e PALESTIS, 2010). EtOH 0,25% (v/v) também modula a resposta de camuflagem (PENG et al., 2009), onde alterações na pigmentação de vertebrados é uma resposta inata que desempenha funções importantes para sobrevivência destes animais relacionadas à defesa contra predadores e comunicação social (FUJII, 2000; NASCIMENTO et al., 2003). Do mesmo modo, o modelo de crises epilépticas induzidas pelo PTZ em peixe-zebra também já está bem estabelecido, onde 20 minutos de exposição ao PTZ induz quadros convulsivos e concentrações significativas do composto podem ser detectadas no SNC sem causar mortalidade nos peixes. Os escores de crise convulsiva são avaliados manualmente e consistem em respostas comportamentais robustas similares a de outros modelos animais (**Tabela 1**). Além disso, pelo fato do monitoramento ser realizado com o peixe imerso na solução contendo PTZ, a simples retirada do animal do tanque de exposição para recuperação em água declorada faz com que os fenótipos comportamentais retornem aos padrões do controle em um curto período de tempo (MUSSULINI et al, 2013).

Assim, sabe-se que a exposição ao EtOH e o PTZ são protocolos que induzem alterações neurocomportamentais e crises convulsivas, respectivamente, e que as metodologias mais comuns para o estudo relacionado a estes distúrbios são por meio de análises dos padrões comportamentais, moleculares e bioquímicos (LUKASIUK et al, 2003).

Tabela 1. Escores de crise convulsiva em peixe-zebra expostos ao PTZ

Escores	**Fenótipos comportamentais**
0	Nado principalmente no fundo do tanque
1	Aumento da atividade natatória e aumento da frequência de movimentos operculares
2	Aumento de movimentos abruptos para esquerda e direita, e movimentos erráticos
3	Movimentos circulares no topo do aquário
4	Comportamento do tipo clônico convulsivo (contração muscular rítmica anormal de corpo inteiro)
5	Comportamento convulsivo do tipo tônico (afundam no tanque e extensão rígida do corpo)

Tabela adaptada de MUSSULINI et al. (2013)

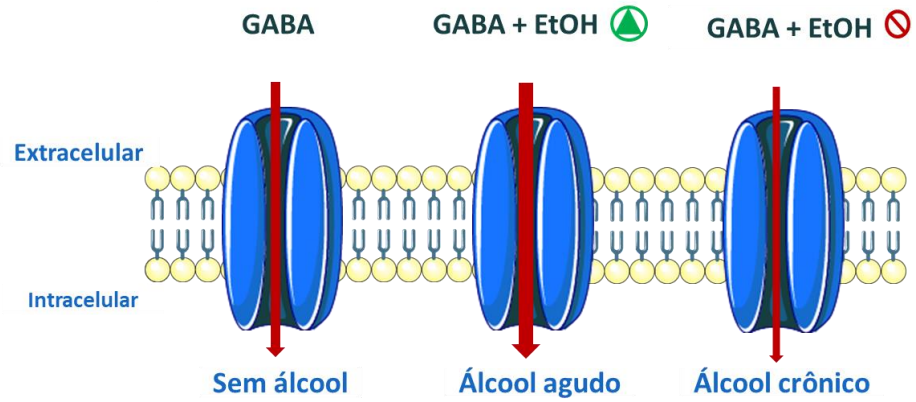
2.3. ETANOL E TAURINA

O uso abusivo do álcool e o alcoolismo representam sérios problemas para os pacientes e para a sociedade. Somente nos Estados Unidos, 86,4% dos adultos já beberam álcool em algum momento na vida e 70,1% destes beberam no último ano segundo dados da Pesquisa Nacional de Uso e Saúde de Drogas em 2015. No mundo, o uso abusivo EtOH é o quinto principal fator de risco para morte prematura e entre pessoas de 15 a 49 anos é considerada o primeiro fator de risco para a mesma, com mortalidade estimada em 25% do total em pessoas de 20 a 39 anos (WHO, 2014). Esses efeitos podem ser fortemente relacionados às alterações comportamentais induzidas pelo EtOH, onde a desinibição do comportamento punido, bem como o déficit cognitivo causado pelo mesmo pode alterar a percepção de situações de risco (MITCHELL e POTENZA, 2014). O consumo excessivo de EtOH também aumenta o risco de problemas sociais e de saúde, causando alterações comportamentais (ex. problemas de sociabilidade e comportamentos tipo depressivo) que muitas vezes podem isolar estas pessoas da sociedade (NAIMI et al.,

2003; ROSENQUIST et al., 2010). As alterações comportamentais induzidas pelo EtOH também são comumente encontradas em diversas doenças neurológicas e a investigação de como esta molécula induz estes comportamentos é importante para compreensão dos fenótipos associados. Assim, os estudos relacionados ao consumo do álcool e comportamento são de fundamental importância, uma vez que o EtOH é capaz de modular componentes relacionados à sociabilidade, à impulsividade e até mesmo à avaliação de risco (PARKER et al., 2014).

Os mecanismos pelos quais o EtOH afeta o sistema nervoso central (SNC) estão associados com a modulação de diversos sistemas de neurotransmissores, comprometimento da função mitocondrial, mudanças na expressão genética e alterações de vias de sinalização (HARPER E CORBETT, 1990; HARPER E MATSUMOTO, 2005; TONG et al, 2011). O mecanismo pelo qual o EtOH induz alterações comportamentais está fortemente ligado à modulação nos níveis de serotonina e dopamina que estimula os centros de recompensa do cérebro, o que explica por que as pessoas sentem uma sensação de bem-estar durante o consumo. Além disso, o álcool também modula a via gabaérgica, onde a estimulação de receptores $GABA_A$ leva à hiperpolarização e causa relaxamento e sedação do organismo (**Figura 3**) (DAVIES, 2003; MCBRIDE et al., 1990). Todavia, alguns estudos indicam que a ingestão crônica de EtOH pode causar uma inibição dos receptores GABAérgicos e consequentemente a despolarização das células neuronais, ocasionando um efeito reverso (BANERJEE, 2014). Sendo assim, o EtOH modula diversos sistemas de neurotransmissão, o que se caracteriza como um dos principais mecanismos relacionados a suas alterações sobre o comportamento.

Figura 3. Efeito do etanol agudo e crônico nos canais de GABA.



Fonte: Do autor.

Recentemente, a ingestão combinada de álcool e bebidas energéticas tem se tornado rapidamente popular e os usuários relatam frequentemente a diminuição da sonolência e aumento da sensação de prazer quando estas bebidas são combinadas (FERREIRA et al., 2004A; 2004B). A TAU é uma das principais moléculas encontradas nestas bebidas (HECKMAN et al., 2010) e sua ação neuromodulatória pode estar relacionada aos efeitos benéficos causados por essa associação. Além disso, como já mencionado, a TAU possui importantes propriedades antioxidantes (ARUOMA et al., 1988; GREEN et al., 1991; OLIVEIRA et al., 2010; SHIMADA et al., 2015; PATEL et al., 2016). O EtOH induz um aumento no nível das espécies reativas de oxigênio que estão fortemente associadas a muitas doenças relacionadas ao álcool (ALBANO, 2006). Com relação ao metabolismo do álcool, o EtOH é oxidado principalmente pela enzima álcool desidrogenase, pelo sistema de oxidação de EtOH microsomático (MEOS) e pela catalase. Essas enzimas produzem seu metabolito reativo, o acetaldeído, que afeta as respostas mediadas pelo EtOH e prejudica o sistema de defesa antioxidante (LIEBER, 1997; ZIMA et al., 2001; QUERTEMONT E DIDONE, 2006; DAS et al., 2007). Estudos recentes sobre o peixe-zebra mostram o papel protetor da TAU na exposição aguda ao EtOH (ROSEMBERG et al., 2010), uma vez que a TAU diminui a atividade da acetilcolinesterase e a peroxidação lipídica no cérebro dos peixes. Isso sugere que a exposição à TAU pode auxiliar na manutenção da homeostase redox, bem como na modulação da enzima responsável pelo término da transmissão colinérgica *in vivo*. Além disso, outras análises revelaram que TAU antagoniza os efeitos do álcool no peixe-zebra (ROSEMBERG et al., 2012), uma vez que previne a ação ansiolítica após um período de 20 minutos e impede a ocorrência de alterações locomotoras induzidas pelo EtOH após uma exposição de 60 minutos. Assim, alterações nas concentrações intracelular/extracelular de TAU desempenham papéis adaptativos no SNC e podem estar associadas aos efeitos fisiológicos do EtOH no cérebro (OLIVE, 2002). Considerando os efeitos pleiotrópicos da TAU nos modelos de administração ao EtOH, podemos sugerir que ela poderia apresentar um papel neuromodulator frente as alterações de sociabilidade, agressividade e avaliação de risco causadas pelo álcool, o que torna relevante a investigação dos efeitos gerados por esta associação (MCGLONE, 1986; CAMERLINK e TURNER, 2016).

2.4. CRISES EPILÉPTICAS E TAURINA

Estudos prévios com modelos animais demonstram que a TAU pode desempenhar seu papel neuroprotetor em doenças neurológicas como as epilepsias, bloqueando sinapses excitatórias no giro denteado do hipocampo (VAN GELDER et al, 1977; CARRUTHERS-JONES e VAN GELDER, 1978; RIPPS e SHEN, 2012). As epilepsias são um conjunto de distúrbios caracterizados pela recorrência dos episódios de crises epilépticas associadas a alterações comportamentais e distúrbios neurobiológicos (MUSSULINI et al, 2013; FISHER et al, 2005). Ela atinge cerca de 65 milhões de pessoas em todo o mundo (AFRIKANOVA et al, 2013) e a recorrência das crises convulsivas leva a um alto índice de mortalidade, problemas cognitivos e disfunções psicossociais (PINEDA et al, 2011). O tratamento para pacientes com epilepsias é complexo, uma vez que os fármacos antiepiléticos não controlam as crises convulsivas em até 30% dos pacientes (TORRES-HERNÁNDEZ et al, 2015). Outro ponto importante é que as crises convulsivas podem levar à neurodegeneração e à peroxidação de lipídios de membrana devido ao aumento da produção de radicais livres e/ou à diminuição nos mecanismos de defesa (GOMES et al, 2011).

Grande parte dos modelos animais utilizados para investigar fenótipos relacionados às crises convulsivas consistem na indução das mesmas por fármacos que alteram as transmissões sinápticas, como o pentilenotetrazol (PTZ) (LÖSCHER, 2011; BARABAN et al., 2005; MUSSULINI et al., 2013). O PTZ é uma molécula que atua no SNC como antagonista de receptores $GABA_A$, o qual consiste um dos mecanismos responsáveis por causar crises convulsivas em modelos animais (WHITE, 1997). O tratamento das crises convulsivas é muitas vezes realizado por meio de fármacos inibidores da enzima GABA transaminase (vigabatrina) ou pelo uso de fármacos que atuam como agonistas do receptor $GABA_A$ (fenobarbital e benzodiazepínicos) (ZACCARA E SCHIMDT, 2015). Porém, estes fármacos nem sempre controlam os episódios de crise em todos os pacientes, podendo causar efeitos indesejáveis como aumento da agressão, impulsividade, violência e até o aumento das crises epilépticas (GREENWOOD, 2000). Dessa maneira, torna-se importante a realização da triagem do papel da TAU em vista de sua potencial ação neuroprotetora em situações nas quais ocorrem alterações neuroquímicas que influenciem a sinalização gabaérgica e/ou glutamatérgica. Considerando o consumo de álcool e a ocorrência das crises epilépticas como problemas de saúde pública que levam a alterações neurocomportamentais, é relevante analisar se TAU modula as alterações

neurocomportamentais induzidas pelo EtOH e as crises convulsivas induzidas pela exposição aguda PTZ em peixe-zebra.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar os efeitos da TAU sobre fenótipos comportamentais induzidos pela exposição ao EtOH e sobre alterações comportamentais e neuroquímicas promovidas pelas crises convulsivas induzidas pelo PTZ em peixe-zebra.

3.2 OBJETIVOS ESPECÍFICOS

3.2.1 Artigo 1

- Elaborar uma revisão sistemática sobre as perspectivas do uso do peixe-zebra como organismo modelo para estudos de doenças relacionadas ao CNS.

3.2.2 Artigo 2

- Avaliar os efeitos da TAU *per se* e em associação ao EtOH sobre as alterações comportamentais relacionadas à agressividade;
- Verificar alterações pigmentares geradas pela exposição de TAU associada ao EtOH;

3.2.3 Manuscrito 1

- Avaliar os efeitos promovidos pela TAU e EtOH, *per* se e associados no comportamento social do cardume ao longo da exposição;
- Verificar os efeitos promovidos pela TAU, EtOH e sua interação na preferência social;

- Comparar os efeitos do EtOH *per se* e da sua interação com a TAU em respostas aversivas de avaliação de risco.

3.2.4 Artigo 3

- Avaliar os efeitos da TAU sobre as alterações comportamentais induzidas pela exposição aguda ao PTZ;

- Verificar os efeitos promovidos pela TAU e pelo PTZ sobre parâmetros relacionados ao estresse oxidativo em encéfalo de peixe-zebra;

- Comparar os efeitos da TAU com relação à ação promovida pelo diazepam (DZP) como controle positivo em animais expostos ao PTZ.

4. ARTIGOS E MANUSCRITOS CIENTÍFICOS

ARTIGO 1

The developing utility of zebrafish models of neurological and neuropsychiatric disorders: A critical review

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Review Article

The developing utility of zebrafish models of neurological and neuropsychiatric disorders: A critical review

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ABSTRACT

Zebrafish (*Danio rerio*) have become a powerful tool in neuroscience research due to their genetic tractability, molecular/physiological conservation, small body size, ease of experimental manipulations in vivo, and rich behavioral repertoire. Zebrafish models and tests are particularly useful in genetics research, neurophenotyping, CNS drug screening, as well as in modeling complex neurological and psychiatric disorders. Here, we discuss selected examples of successful application of zebrafish models to mimicking various aspects of brain pathology, and emphasize their developing utility for studying the underlying molecular and genetic mechanisms. We also summarize recent advances in zebrafish-based CNS disease modeling, and outline new research strategies that may significantly improve translational neuroscience and experimental neurology research, and drug discovery.

1. Introduction

As neurological and neuropsychiatric brain disorders are widespread globally and contribute substantially to public health costs (Kleinman et al., 2016; Vigo et al., 2016), animal models have become an important tool in translational research of these illnesses (McGonigle and Ruggeri, 2014; Nestler and Hyman, 2010). For decades, mammalian species (especially laboratory rodents) have been primarily used to model human brain disorders (Ellenbroek and Youn, 2016). Recent translational research in this field, however, is witnessing a rapidly growing interest to various novel model organisms, such as fruit flies, roundworms and zebrafish (*Danio rerio*) (Freires et al., 2017; Peterson et al., 2008). Sometimes termed 'alternative' or 'complementary' models (Orger and de Polavieja, 2017; Vasaikar et al., 2016), such organisms are gradually but steadily entering the mainstream neuroscience and experimental neurology research (Meshalkina et al., 2017; Orger and de Polavieja, 2017) (Fig. 1).

To address these challenges, here we discuss selected examples of successful application of zebrafish models to mimicking various aspects of neurological and psychiatric disorders, and summarize how such models can help explore molecular and genetic mechanisms underlying these maladies. The disorders specifically discussed here - epilepsy, Alzheimer's disease (AD), Parkinson's disease (PD), affective disorders and schizophrenia - were chosen among most common and debilitating neurological and psychiatric illnesses (Fig. 2). We also critically assess recent advances and limitations using zebrafish models, and discuss new strategies that may significantly improve translational CNS research and drug discovery.

2. General features of zebrafish

The zebrafish is a freshwater teleost fish from South-East Asia, where it is often the most abundant fish species usually found in shallow ponds and rice paddies (Spence et al., 2008). As defensive strategies in natural environment, zebrafish spend more time near the bottom, to mimimize predation risk (Maximino et al., 2010a; Rosemberg et al., 2011). The genetic tractability, small size, easy maintenance and breeding, low cost, and the presence of translucent

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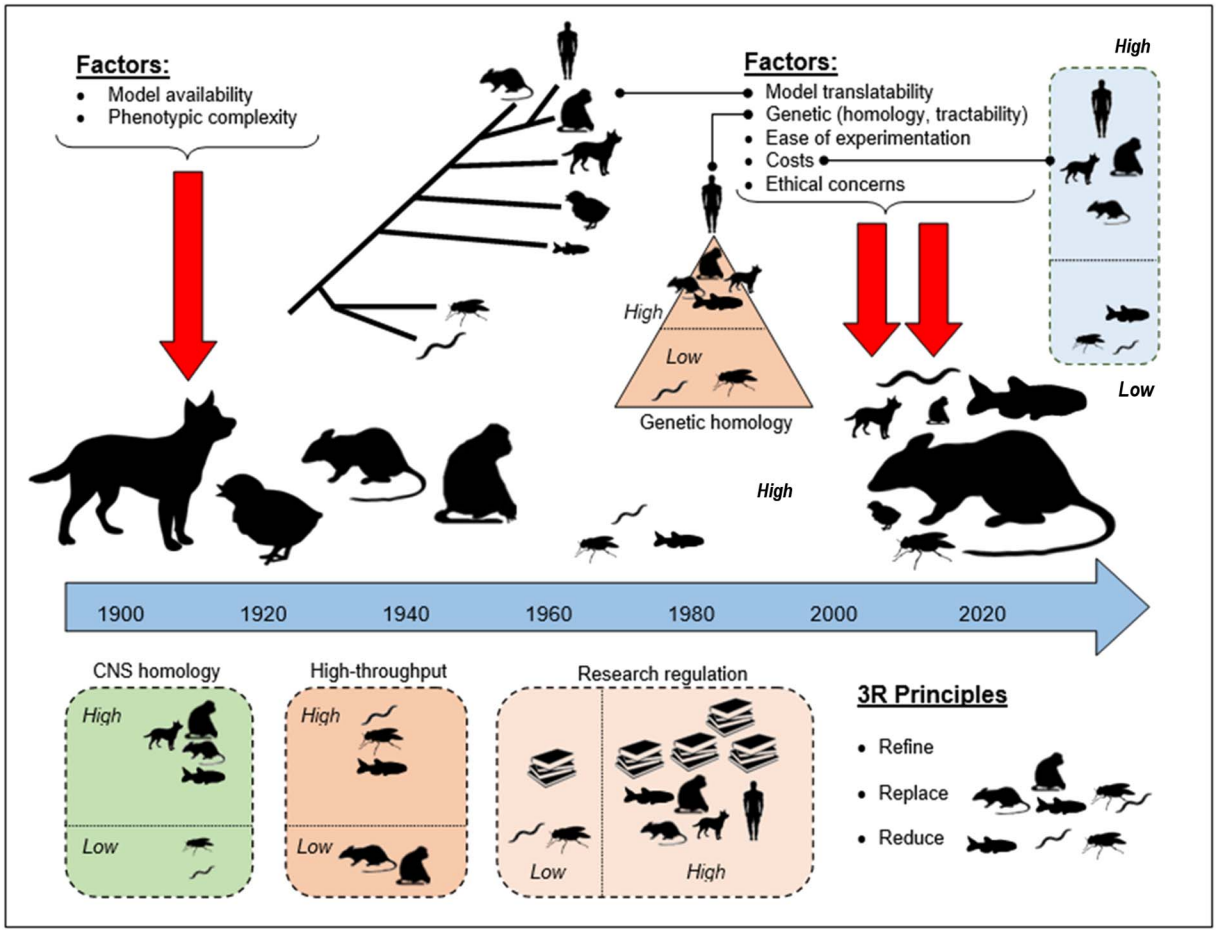

Fig. 1. The general timeline of model organism applications in neuroscience and various factors driving them (relative animal size reflects a general use of the model species in neuroscience research). Top insets – summary of evolutionary tree of model organisms (right), also paralleling their genetic homology to humans (middle), and reflecting relative costs (right). Bottom insets outline the models' relative CNS homology (left), potential for high-throughput, research regulation burden (middle), and following the 3R principles of ethical experimentations (right).

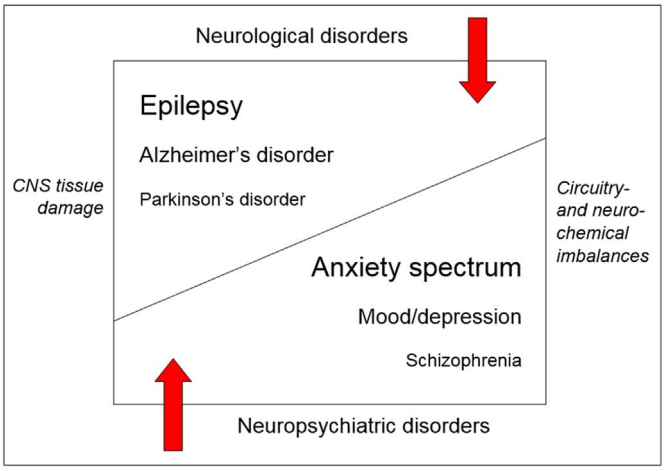

Fig. 2. Selected neurological and neuropsychiatric disorders (font size reflects their relative clinical occurrence). Red arrows indicate strong genetic and environmental determinants of both groups of CNS diseases.

embryos make zebrafish a useful model organism in biomedical research (Parker et al., 2012; Rico et al., 2011; Stewart et al., 2014; Stewart et al., 2015). The first reports using zebrafish as model organisms date back to the 1960s, when Streisnger suggested this species as an animal model for basic research, based on its embryonic and genetic features (Grunwald and Eisen, 2002). Zebrafish have a fast growth rate (e.g., adults can reproduce after 3 months of age) and display evident sexual dimorphism (males are torpedo-shaped with a pink or yellow tinge, and females are less pink but fatter, due to the presence of eggs) (Singleman and Holtzman, 2014). Embryonic transparency of zebrafish markedly improves monitoring the development of various organs, and helps trace ontogenetic profiles of gene expression using various fluorescent probes in vivo (MacRae and Peterson, 2003). Since zebrafish is a vertebrate species with several practical characteristics typical for invertebrate model organisms, this species fills the gap between cell culture and rodents, allowing an early in vivo validation and optimization of different experimental protocols (Langheinrich, 2003). For example, zebrafish embryos can easily grow in 96-microplate wells, which facilitates their maintenance and research use (Parng et al., 2002). Importantly, compared to rodents, both larvae and adult zebrafish are easier in husbandry, and need smaller space to work, constituting important characteristics for performing medium-to-high throughput pharmacological and genetic screening (Bilotta et al., 1999; Kalueff et al., 2013; Rico et al., 2011).

However, there are several important and indisputable (albeit addressable) limitations of using zebrafish models in translational neuroscience research. For instance, although pharmacological manipulations may be easily performed by adding chemical compounds in water (Rosemberg et al., 2012; Tran et al., 2015), such experiments cannot fully control the drug dose absorbed because substances can be rapidly absorbed through skin and gills, depending on the surface area of an individual fish and gill activity (Rubinstein, 2006). Moreover, pharmacokinetic studies in zebrafish are still limited, and the amount of the

drug that reaches different target tissues is poorly explored, although its presence and the amount can be confirmed in CNS by various chemo-analytical methods, such as mass-spectroscopy or high-performance liquid chromatography (Chatterjee and Gerlai, 2009; Kolesnikova et al., 2017; Rosemberg et al., 2012). The administration of drugs which are not soluble in water is another technical problem related to drug delivery in zebrafish studies, although the use of special solvents (e.g., dimethyl sulfoxide, DMSO) with little CNS activity at doses used per se, may circumvent this issue (Maes et al., 2012). Nonetheless, appropriate control groups must be run in order to avoid any potential non-specific toxicity of these solvents, which can also affect behavioral performance of aquatic animals (Chen et al., 2011). The injection of drugs in a particular nucleus of the brain, as well as microdialysis in freely moving animals, also present a considerable methodological challenge while using this organism due to its smaller size.

Furthermore, zebrafish also have external fertilization and show no parental care of their eggs and larvae, which may limit modeling certain CNS conditions, such as separation anxiety (Engeszer et al., 2007). Although researchers have been working extensively to characterize behavioral and genetic differences of various zebrafish strains and populations (Bos et al., 2017; Quadros et al., 2016; Seguret et al., 2016), future work is needed to select animal cohorts most appropriate for specific experimental conditions in question. Nevertheless, despite all these limitations, zebrafish are a powerful animal model for genetic, developmental and pharmacological screening, and exhibit a wide range of complex behaviors, including social, motor, affective and defensive, that may be useful for modeling various CNS disorders (Blaser and Vira, 2014; Egan et al., 2009; Gerlai, 2003; Kalueff et al., 2013; Norton, 2013).

3. Genetics and general neurobiology of zebrafish

The promise of zebrafish as an alternative organism for modeling human diseases is based on their well-characterized and conserved genome (Barbazuk et al., 2000; Howe et al., 2013), with over 70% of zebrafish genes sharing a high degree of similarity with their mammalian orthologs (MacRae and Peterson, 2015). Nearly 70% of zebrafish genes have at least one human orthologue, and 47% of human genes have a one-to-one relationship with a zebrafish counterpart (Howe et al., 2013). Clearly, this fosters markedly the use of zebrafish for studying the genetic bases of human CNS diseases (Lieschke and Currie, 2007). As a likely consequence of teleost-specific genome duplication, zebrafish express 26,206 protein-coding genes - more than any previously sequenced vertebrate, thereby presenting some duplicated genes, which, depending on research goals, can be a positive or a negative feature (Postlethwait et al., 1998). For example, gene duplication in zebrafish creates redundancy, an important source of novelty and variability during the evolution (Levasseur and Pontarotti, 2011; Wang et al., 2011). Thus, analyses of duplicated genes, especially those associated with neurobehavioral phenotypes, may be an important strategy to evaluate whether the functions of these genes are similar, divergent, or even lost. On the other hand, animals with duplicated genes may have a different regulation of their expression, which may, in turn, differently impact regulatory networks and output pathways, and can therefore be either advantageous or disadvantageous, depending on the experimental design (Comai, 2005). Although deletions of duplicated genes may provide novel information on how their encoded protein products contribute to a particular neurochemical mechanism in the brain, such studies often remain time- and cost-consuming, also requiring in-depth molecular, structural and pharmacological validation.

Despite considerable neuroanatomical differences between the mammalian and the teleost CNS, mounting evidence shows homologous functions in several key zebrafish brain areas (Randlett et al., 2015; Ullmann et al., 2010). For example, the lateral pallium of the telencephalic area of zebrafish is responsible for memory processes, whereas the habenula is associated with fear responses, similar to the hippocampus and the amygdala, respectively (Agetsuma et al., 2010; Perathoner et al., 2016). Zebrafish display excellent cognitive abilities and complex decision-making, and, like rodents, show robust behavioral responses to stimuli, with high sensitivity to pharmacological manipulations (Oliveira, 2013; Parker et al., 2012; Sison et al., 2006). In contrast to mammals, zebrafish brain has an extraordinary ability to regenerate, which is another advantageous characteristic for exploring the mechanisms underlying neuroprotection, neurogenesis and functional integration of newborn neural cells (Dorsemans et al., 2017; Marz et al., 2011).

A wide range of complex behaviors, ranging from aggression and anxiety (Fontana et al., 2016; Gerlai et al., 2000) to long- and short-term memory (Blank et al., 2009; Cognato Gde et al., 2012; Jia et al., 2014), object discrimination (May et al., 2016) and color preference (Bault et al., 2015), were recently comprehensively classified and characterized in zebrafish (Kalueff et al., 2013). Thus, tracing multiple behavioral endpoints contributes to drug screening and behavioral phenomics, especially when empowered by sophisticated automated analysis and high-resolution video-tracking systems (Cachat et al., 2010; Canzian et al., 2017). Collectively, this offers the opportunity to investigate the brain mechanisms and potential treatments associated with psychiatric and neurological disorders. As will be discussed here in-depth, relevant examples of application of zebrafish include modeling AD (Lee and Freeman, 2016), PD (Sarath Babu et al., 2016), schizophrenia (Giacomotto et al., 2016), epilepsy (Grone and Baraban, 2015; Grone et al., 2016), and fetal alcohol syndrome (FAS) (Fernandes and Gerlai, 2009; Fernandes et al., 2015b; Gerlai, 2015).

The characterization and validation of experimental models of neurological disorders also present a challenge for the search for novel effective pharmacological therapies. Considering complex neural mechanisms of behaviors (Price, 1999), the latter remains slow, expensive, and ineffective (Newman et al., 2011). Furthermore, it is difficult to create and validate reliable models for many CNS disorders. Indeed, behavioral endpoints that measure “anxiety-” and “depression-like” states are difficult to assess in animals, including zebrafish (Blaser and Rosemberg, 2012; Piato et al., 2011), whereas molecular biomarkers of neurodegeneration are often challenging to quantify in such alternative model systems (MacRae and Peterson, 2015; Menzie et al., 2014; Nunes et al., 2016).

4. Zebrafish models of selected neurological disorders

4.1. Epilepsy

Epilepsy is caused by recurrent unprovoked seizures (Banerjee et al., 2009; Fisher et al., 2005). One of the oldest pathological conditions described, and yet the most common neurological disorder (Afrikanova et al., 2013), it is caused by genetic factors and brain insults (Fisher et al., 2014). There are more than 40 epileptic syndromes described today, typically classified as partial or generalized. Partial seizures occur in a specific brain region (e.g., cerebral cortex or hippocampus), whereas generalized seizures appear through the forebrain to outset (Fisher et al., 2014; McCormick and Contreras, 2001; Nagae et al., 2016). Epilepsy results from sudden abnormal discharges of neurons (Dayapoglu and Tan, 2016; Fisher et al., 2005), which may cause irreversible damage to the brain and even death (Surges et al., 2009). Conventional anti-epileptic drugs (AEDs) reduce or prevent seizures by inhibiting the sodium currents (Dumoulin et al., 1992) or positively modulating gamma-aminobutyric acid (GABA)-ergic receptors (Czapinski et al., 2005). As nearly one third of epileptic patients remain treatment-resistant, the search for novel AEDs becomes an urgent unmet biomedical problem (Torres-Hernandez et al., 2015).

During the last decade, novel experimental (animal) models have been developed to study molecular bases of epilepsy (Baraban et al., 2013; Mussulini et al., 2013). The forward genetics in zebrafish has

been used to identify genes related to specific disorders and behaviors, such as epilepsy. The most common and well-characterized epilepsy model in this species is an N-Ethyl-N-nitrosourea (ENU)-induced model of Dravet syndrome, a disorder which involves 3 gene mutations (*scn1*, *scn8*, and *hcn1)* and presents as a severe early-onset childhood epilepsy with intellectual incapacity, drug-resistant seizures and unexpected deaths (Marini et al., 2011). Zebrafish *scn1a* mutants present haploinsufficiency for the voltage-gated sodium channel (which is one of the key mechanisms involved in Dravet syndrome (Escayg and Goldin, 2010; Griffin et al., 2016; Saitoh et al., 2012)), and are a good example of fish models of monogenic epilepsy disorders with high sensitivity to several clinical AEDs (Baraban et al., 2013). The *mind-bomb* mutant is another epilepsy model with disturbed E3 ubiquitin ligase activity and a downregulation of GABA-related gene that triggers defects of brain development and spontaneous seizures (Hortopan and Baraban, 2011; Hortopan et al., 2010). Additionally, mutations of the zebrafish *ocrl1* homolog gene facilitate hyperthermia-induced seizures (Oltrabella et al., 2015; Ramirez et al., 2012). Thus, the ongoing high-throughput mutagenesis efforts are expected to provide a valuable tool for characterizing additional epilepsy models (Grone and Baraban, 2015; Kettleborough et al., 2013; Moens et al., 2008) and to investigate spontaneous seizures in a simpler vertebrate system.

Epileptic states in zebrafish can be evoked pharmacologically, using classical convulsant drugs, such as pentylenetetrazole (PTZ) and kainic acid (KA). PTZ is a $GABA_A$ receptor Cl^- ionophore blocker that induces abnormal brain excitation and seizure-like behaviors (Huang et al., 2001). Epileptiform electrical discharges can be monitored by local field potential recordings using electrodes inserted into the tectum (Baraban et al., 2005; Cunliffe et al., 2015). In larval fish, PTZ exposure predictably up-regulates brain *c-fos* expression, a characteristic also observed in rodent seizure models (Baraban et al., 2005) as well as in adult zebrafish exposed to PTZ (Wong et al., 2010b). In the presence of distinct AEDs, both brain electroencephalogram (EEG) and behavioral analyses exhibit overlapping profiles with those found in rodents (Afrikanova et al., 2013). Diazepam, a classical AED that acts on the benzodiazepine site of $GABA_A$ receptors, increases the latency of corkscrew swimming and subsequent loss of posture with abnormal contractions of the body – the behavioral phenotypes that closely resemble tonic-clonic seizures (Mussulini et al., 2013). In addition, EEG analyses in adult PTZ-exposed zebrafish show higher-amplitude sharp transients analogous to the interictal epileptiform discharges of human epileptic EEG (Pineda et al., 2011).

Similar to larvae, adult fish seizure-like behaviors occur simultaneously to EEG responses (Pineda et al., 2011), enabling assessing different neurochemical mechanisms of epilepsy. For example, central purinergic system modulates zebrafish seizure-like behavior (Siebel et al., 2015), and exposure to some AEDs modulates adenosine deamination and delays tonic-clonic seizures (Siebel et al., 2013). Another pharmacogenic seizure model in zebrafish utilizes KA, activating the glutamatergic system and promoting excitotoxicity (Stafstrom et al., 1992). After intraperitoneal KA injection, seven characteristic stages of seizure-like behaviors in adult zebrafish include 1) immobility and hyperventilation); 2) whirlpool-like swimming; 3) erratic movements; 4) irregular and spasmodic muscular contractions; 5) fast whole-body clonic-like behavior; 6) dropping to the bottom of the tank and spasms for several minutes; 7) death (Alfaro et al., 2011). Although PTZ and KA do not share the same mechanism of epileptogenic action, both chemically-induced seizure models represent sensitive and suitable protocols for screening novel AEDs using zebrafish.

4.2. Alzheimer's disease

AD is a neurological disorder with progressive dementia, robust cognitive decline and neuromorphological changes, first described in 1907 (Alzheimer, 1907) but remaining one of the most common neurological diseases (Zhao et al., 2016). Behavioral symptoms of AD accompany synaptic degeneration and neuronal apoptosis in the limbic structures, and include escalating cognitive decline, social dependence and, eventually, death (Caltagirone et al., 2012; Carrettiero et al., 2015; Menzie et al., 2014). AD involves the deposition of amyloid beta (Aβ) (Louzada et al., 2004; Oz et al., 2009) as well as intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein, and neuronal loss, particularly in the cholinergic system (Braak and Braak, 1998; Newman et al., 2011). Although early evidence showed a neurotoxic effect of Aβ in vitro (Pike et al., 1991) and in vivo (Kowall et al., 1992), both Aβ- and tau protein-associated mechanisms of cell death remain poorly understood (Carrettiero et al., 2015; Louzada et al., 2004).

Despite the recent advances in studying NFTs and amyloid plaques in AD, biological processes associated with their formation and environmental influences on them, remain unclear (Mudher and Lovestone, 2002). One of three major AD hypotheses implicates activated amyloid cascades, culminating in the formation of Aβ1–42 peptide that increases tau aggregation, phosphorylation, neuronal attrition and clinical dementia (Chapman et al., 2001). However, the exact mechanisms responsible for these phenotypes are unclear (Mudher and Lovestone, 2002). Another popular hypothesis notes tau and tangle deficts in AD, where the normal role of tau in stabilizing microtubules is impaired, and microtubules of diseased neurons are gradually replaced by tangles (Gray et al., 1987). Tangles, usually accumulating in neurons, result in neuronal death, and their numbers correlate with clinical cognitive deficits (Nagy et al., 1995). Tangle formation occurs with increased tau phosphorylation and a posterior aggregation in critical regions responsible for memory processing (Braak and Braak, 1998). The third hypothesis links abnormal *wnt* signaling pathway with increased tau phosphorylation, which can trigger the progression of AD (Anderton et al., 2000; De Ferrari and Inestrosa, 2000; Mudher and Lovestone, 2002).

Analyses of genetic bases of AD identified autosomal dominant mutations in *app* (Chartier-Harlin et al., 1991; Murrell et al., 1991), *presenilin 1 (ps1)* (Campion et al., 1995; Cruts et al., 1995) and *presenilin 2 (ps2)* (Sherrington et al., 1996) genes. Familial AD (FAD) accounts for approximately 4–8% of all AD cases, in which mutations of *ps1* gene is the most frequent (Chapman et al., 2001). Despite rigorous research, however, various therapeutic agents aiming to reduce Aβ production and/or accumulation, have failed in clinical trials (Caltagirone et al., 2012; Newman et al., 2011). Among these agents, there are peptides that inhibit Aβ aggregation and molecules that can either inhibit γ-secretase activity or target the production of the most neurotoxic Aβ1–42 (Newman et al., 2011). Neurological disorders and brain aging have been linked to glutamate-mediated excitotoxicity, strongly supporting the involvement of glutamate receptors in Aβ-mediated neurotoxicity in vitro (Louzada et al., 2004). Indeed, the Aβ peptide is associated with AD pathogenesis, and the blockade of glutamate receptors prevents Aβ–induced neuronal death (Lipton and Rosenberg, 1994; Mattson, 2003).

Dominant mutations in the *ps1* and *ps2* genes are the major causes of early-onset AD (Tang and Gershon, 2003). Analyses of PSEN1 and PSEN2 expression during zebrafish embryogenesis became particularly useful since presenilin proteins express early and are highly evolutionarily conserved in vertebrates (Nornes et al., 2003). PSEN1 and PSEN2 holoproteins proteolytically processed are relatively abundant in zebrafish embryos. The PSEN1 C-terminal proteolytic fragment (CTF) is present at embryogenesis, indicating the existence of regulatory mechanisms during development. Thus, reduction of PSEN1 activity in zebrafish embryos produces similar developmental defects to those seen in knockout PS1 mice (Nornes et al., 2003). Additionally, zebrafish presenilin enhancer (*pen-2)* knockdown shows the activation of the p53-dependent apoptotic pathway that contributes to neuronal death (Campbell et al., 2006). Collectively, this implicates *pen-2* in promoting neuronal cell survival by preventing apoptosis in vivo in zebrafish models.

Presenilin fragments, along with mature nicastrin, anterior pharynx defective (*aph-1*) and *pen-2* are functional components of the γ-secretase complex. Aberrant proteolytic processing of amyloid-β protein precursor by γ-secretase complexes may impair the balance between the production and the clearance of the Aβ proteolytic product, thereby triggering neuronal dysfunction and death (Wilson and Lardelli, 2013). A novel, conserved and specific genetic interaction between APP and PRP has recently been reported in zebrafish, whose APP and/or PRNP knockdown (*appa, appb, prp1,* and *prp2*) reduced cell adhesion and CNS cell death (Kaiser et al., 2012). This genetic interaction is surprising because *prp1* genetically interacts with zebrafish *appa,* but not with *appb*, while the zebrafish paralog *prp2* fails to interact with *appa*. Intriguingly, *appa* and *appb* are essentially redundant in early zebrafish development even though their abilities to rescue CNS cell death are differentially contingent upon *prp1* abundance (Kaiser et al., 2012). These findings suggest the use of zebrafish as a promising model organism for assessing the phenotypes and mechanisms of CNS disorders in early stages of brain development. Because AD is the most frequent disorder in aged humans with Aβ deposition, the use of morpholino antisense oligonucleotides in zebrafish remains problematic being restricted to embryo/larvae phases. Interestingly, early transient alterations in dopaminergic system produce persistent alterations in adult brain function (Formella et al., 2012). Thus, early alterations in gene and protein expression may precede the onset of certain psychiatric or neurological conditions and zebrafish appear to be well-suited to model this aspect of pathogenesis.

Likewise, a mutant human tau-GFP hyperphosphorylated in stable transgenic zebrafish expressing human tau with a mutation (TAU-P301L) (Tomasiewicz et al., 2002) may be useful to understand how the microtubule-associated tau protein contributes to AD in vivo. These mutants can also help to clarify how tau redistributes from neuronal axons to neuronal soma forming pathogenic NFTs. TAU-P301L mutation may be easily assessed in fluorescent embryos displaying neurodegeneration in the spinal cord (Paquet et al., 2009). Moreover, tau-expressing embryos have behavioral deficits in escape response after a mechanical stimulus (Xi et al., 2011).

Notably, in transgenic mice, amyloid production does not trigger the predicted cascade of humans since plaques and tangles are separated temporally and spatially (Mudher and Lovestone, 2002). Different transgenic mouse models bearing FAD mutations show increased Aβ1–42 levels and plaque pathology (Citron et al., 1997; Duff et al., 1996). Interestingly, while most of these models do not show significant neuronal degeneration (Holcomb et al., 1998; Irizarry et al., 1997), the same occurs in transgenic zebrafish, which fail to stimulate tau pathology, thereby necessitating further studies of AD pathogenesis using this alternative vertebrate model.

AD-related phenotypes can also be modeled in animals using cholinergic (e.g., scopolamine, pilocarpine and physostigmine) and glutamatergic (e.g., MK-801, ketamine, APV, memantine, kainate, domoate and CNQX) neurotoxins. Cholinergic neurotoxins act as agonists or antagonists of muscarinic receptor, or as acetylcholinesterase inhibitors. They usually impair acquisition of passive avoidance, retention of the learned response, and suppress the electrically evoked field potentials in the telencephalon of adult zebrafish (Kim et al., 2010; Park et al., 2008). At the same time, glutamatergic neurotoxins inhibit N-Methyl-D-aspartate receptor (NMDA) and AMPA receptors, and also can stimulate KA receptors (Best et al., 2008; Cognato Gde et al., 2012; Richetti et al., 2011; Sison and Gerlai, 2011; Swain et al., 2004; Tiedeken et al., 2005). As an antagonist of NMDA receptors, MK-801 impairs the inhibitory avoidance behavior and alters extracellular signal-regulated kinase phosphorylation levels in zebrafish telencephalon after training sessions (Ng et al., 2012). Therefore, administration of neurotoxins represents another promising strategy to model AD-like phenotypes in zebrafish.

4.3. Parkinson's disease

PD is the second most common progressive neurodegenerative disorder after AD (Driver et al., 2009; Ricciardi et al., 2015; Shulman et al., 2011), increasing exponentially in humans aged 55–79 (Driver et al., 2009). First described in individuals with “paralysis agitans” (Parkinson, 1817), PD was later conceptualized as a syndrome presenting as tremor, slower movements and motor impairments (Lanska, 2010). Cardinal neuropathological lesions (the Lewy bodies, LBs) (Lewy, 1912; Shulman et al., 2011), degeneration of dopaminergic nigrostriatal neurons, intracytoplasmic LBs and intra-axonal Lewy neurites (LNs) have also been reported for clinical PD (Calabresi et al., 2013; Forno, 1996). Both LBs and LNs contain fibrillary aggregated α-synuclein (Spillantini et al., 1998), a presynaptic protein that modulates lipid binding to membranes and vesicular trafficking (George et al., 1995; Jensen et al., 2000). Mutation of the α-synuclein gene triggers aggregates of the β-pleated sheath observed in LBs and LNs (Eliezer et al., 2001). Although conformational changes of α-synuclein are responsible for its neurotoxic effects (Dauer et al., 2002; Menzie et al., 2014), PD may also result from genetic mutations in *parkin*, (Kitada et al., 1998), *dj-1* (Bonifati et al., 2003), *pink1* (Hofer and Gasser, 2004) and *lrrk2* genes (Zimprich et al., 2004).

Clinical PD symptoms include bradykinesia, rigidity, resting tremor, postural instability (Calabresi et al., 2013) and cognitive decline (Kehagia et al., 2010; Ricciardi et al., 2015). Albeit motor symptoms of PD are treated with dopamine- replacement, most disabilities in progressive PD come from motor symptoms that do not respond to such treatment, thereby implicating other neurotransmitters (Calabresi et al., 2013; Kehagia et al., 2010). Moreover, non-motor symptoms (e.g., hyposmia, disturbed rapid eye movement sleep, depression/anxiety, and apathy) precede the onset of motor deficits in PD (Chaudhuri, 2009; Chaudhuri et al., 2006). Although the mechanisms underlying these symptoms are multifaceted, neuropathological and clinical findings implicate cholinergic, noradrenergic and serotonergic systems in PD (Calabresi et al., 2013; Kehagia et al., 2010; Voon et al., 2009).

Similar to other neurodegenerative disorders, degenerating dopaminergic neurons in PD express glutamate receptors and are, therefore, vulnerable to excitotoxicity (Miranda et al., 1997). Thus, while mounting evidence supports the role of excitotoxicity in PD (Louzada et al., 2004; Mattson, 2003), other compounds (e.g., modulating GABAergic activity) that counteract excitation may be further explored as possible neuroprotective agents against PD. As various animal models have been validated for testing potential neuroprotective agents, zebrafish have become a valuable organism for modeling PD and other neurodegenerative disorders. Zebrafish brain contains three main regions (forebrain, midbrain, and hindbrain), allowing for region-specific analyses of neurochemicals and structures involved in motor and non-motor behaviors related to PD (Zenki et al., 2014). The neuronal structure of zebrafish possesses typical features observed in mammals, including the soma, dendrites, myelinated or demyelinated axons (Kimmel, 1993), and the blood-brain barrier (Cuoghi and Mola, 2007). Permeability tests on the zebrafish blood-brain barrier further indicate conserved physiological properties, which straightforward the assessment of neuroprotective strategies using this model organism (Cuoghi and Mola, 2007; Wager and Russell, 2013).

Another relevant advantage of zebrafish is their ability to provide in vivo tests for toxicity and to screen potential therapeutic molecules in a medium-to-high throughput manner (Parng et al., 2002; Stewart et al., 2014; Stewart et al., 2015). Extensive information is available regarding the neurotransmitter systems in zebrafish, showing significant similarities to humans (Bretaud et al., 2011; Wager and Russell, 2013). Additionally, the dopaminergic system has already been characterized in both embryonic and adult zebrafish (Panula et al., 2010). After fertilization, dopaminergic neurons are detected at 18–19 h post-fertilization (hpf) in the ventral diencephalon (Holzschuh et al., 2001) which ascends to the striatum, and resembles human nigrostriatal system

(Bretaud et al., 2011). Tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis and the TH-containing neurons located in the ventral diencephalon, may be detected using fluorescent probes and immunohistochemistry (Son et al., 2003). Other brain structures from prosencephalic regions display physiological similarities to mammalian striatum (Rink and Wullimann, 2001, 2002). Furthermore, there is extensive genomic information available of zebrafish PD-related genes. Some molecular markers, such as *dj-1* (Bai et al., 2007; Bai et al., 2006; Bretaud et al., 2007), *uch-l1* (Son et al., 2003), *snca*, *pink1*, *park2*, and *lrrk2* (Flinn et al., 2008) are evolutionarily conserved and their protein products are expressed in zebrafish ventral diencephalic dopaminergic neurons (Pienaar et al., 2010). Molecular analyses of *pink1* and *park2* highlight the role of mitophagy in the pathogenesis of different neurological disorders (Wager and Russell, 2013). Decreased PINK1 expression is the second most common cause of autosomal recessive PD, and the loss of *pink1* function in zebrafish reduces the number of dopaminergic neurons and impairs their mitochondrial function (Anichtchik et al., 2008). Locomotor deficits are classical symptoms of PD, and *pink1* knockdown zebrafish show impaired response to tactile stimuli and reduced swimming activity (Xi et al., 2010), thereby emerging as a promising model for studying PD-like phenotypes in zebrafish.

In addition to their rapid development, genetic manipulation in zebrafish is relatively easy (compared to mammals), which provides a good opportunity to assess CNS gene and protein expression during ontogeny. Directly relevant to PD, various aspects of neurodegeneration have been modeled in zebrafish using mutant forms of *mapt* (Bai et al., 2006; Tomasiewicz et al., 2002), *sod1* (Ramesh et al., 2010), and *htt* (Williams et al., 2008). PD-like phenotypes are also observed in pharmacogenic models, for example, in zebrafish treated with dopaminergic neuron-selective toxins, indicating the existence of equivalent 'PD' circuitry (Wager and Russell, 2013). PD-like states can be modeled in zebrafish using dopaminergic neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat – the agents which affect energy metabolism and cause oxidative stress, proteasomal dysfunction, and loss of dopaminergic neurons (Sarath Babu et al., 2016). MPTP has been used for inducing PD-like symptoms in zebrafish, inducing more erratic swimming and freezing, and also downregulating key brain proteins, such as NEFL, MUNC13–1, NAV2 and GAPVD1 (Sarath Babu et al., 2016). Rotenone and paraquat alter mitochondrial physiology causing PD-like behaviors, decreasing dopamine levels in the brain and modulating some PD-related genes (Bortolotto et al., 2014; Muller et al., 2017; Nunes et al., 2017; Wang et al., 2017). Thus, zebrafish PD models target a wide range of PD-related phenotypes, including spontaneous motor behavior, gene expression, the number of dopaminergic neurons and susceptibility to neurotoxins, also allowing for studying the effect of gene knockdown (Bretaud et al., 2011). Collectively, this renders zebrafish a useful tool to improve our understanding of PD pathogenesis and its pharmacological interventions.

5. Zebrafish models of selected neuropsychiatric disorders

5.1. Stress-related (affective) disorders: anxiety, depression and related behavioral deficits

Stress is a general term used to describe emotionally and physiologically stimulated experiences (Selye, 1956, 1985). While the "good stress" (eustress) involves experiences of limited duration with a sense of excitement and accomplishment, the "bad stress" (distress) refers to experiences often prolonged/recurrent, irritating, emotionally and physically exhausting or dangerous (McEwen, 2007). When the hypothalamus–pituitary–adrenal (HPA) axis is activated, the paraventricular nucleus of the hypothalamus releases corticotropin-releasing factor (CRF) to the pituitary, stimulating the secretion of adrenocorticotropic hormone (ACTH). ACTH promotes the secretion of cortisol from the adrenal cortex in humans and some mammals, whereas in rats, mice, birds and most reptiles, the 'main' stress hormone is corticosterone (Li and Hu, 2016; Lv et al., 2015; Spencer and Deak, 2016). Like in humans, cortisol is the main stress hormone in zebrafish, released from interrenal cells (adrenal gland homolog) following activation of the hypothalamus–pituitary–interrenal (HPI) axis (Alderman and Vijayan, 2012; Alsop and Vijayan, 2009; Baiamonte et al., 2015).

HPA dysfunctions are associated with various psychiatric disorders, such as depression, post-traumatic stress disorder, and anxiety (Holsboer, 2000; Moreno-Peral et al., 2014; Newport and Nemeroff, 2000; Walker et al., 2013). Exposure to repeated stress contributes, as the major risk factor, to various CNS disorders, accompanied by robust changes in both basal and stress-evoked glucocorticoids (Moreno-Peral et al., 2014; Spencer and Deak, 2016). Clinical anxiety is associated with excessive worries, and includes several 'anxiety spectrum' psychiatric disorders, such as panic disorders, specific phobias, separation anxiety disorder and social anxiety disorder (Andlin-Sobocki and Wittchen, 2005; Kessler et al., 2012; Landgraf and Wigger, 2002; McEwen et al., 2007; Suveg et al., 2010). Although comorbidity between anxiety and depressive disorders are common, depression is a more complex disorder that may occur following chronic stress, intermittent fear and/or anxiety (Dean and Keshavan, 2017). Patients with major depression show increased CRF levels in various brain regions, and, thus, variations in glucocorticoid hormones may serve as potential biomarkers of depression in animal models (Holsboer, 2000).

Numerous studies have applied psychosocial stressors to examine whether psychiatric disorders are associated with fluctuations in cortisol levels and HPA reactivity (Holsboer, 2000; Kiefer et al., 2006; McEwen, 2007; Walker et al., 2013). While studying the HPA axis functionality is informative, the interpretation of such data is problematic (Zorn et al., 2017). For example, phenotypic data cannot be interpreted correctly without considering parameters that influence stress-induced cortisol levels and modulate HPA axis reactivity, such as sex and age (Kirschbaum et al., 1999; Kudielka et al., 2004). Epigenetic factors also exert an important influence on adult stress responsiveness. For example, the early-life maternal care is a determinant of life-long emotional reactivity and stress hormone reactivity, associated with cognitive functions and lifespan in rodents (Cavigelli and McClintock, 2003; Cerqueira et al., 2005). A prolonged separation of pups from mother increases emotionality and stress reactivity, in part by decreasing maternal care when pups are returned to their mothers (Plotsky et al., 2005). As a therapeutic strategy, animals kept in enriched environments during the peripubertal period present amelioration of these deficits (Francis et al., 2002) – effects which can be seen cross-generationally, and are accompanied by DNA methylation of key genes (Francis et al., 1999; McEwen, 2007).

Over the last decades, animal models have been increasingly used to integrate novel protocols and biomarkers. Numerous reports show the utility of zebrafish for assessing behavioral, neurochemical, physiological and epigenetic effects of stress (Barcellos et al., 2016; Barcellos et al., 2014; Nesan and Vijayan, 2016). In zebrafish, during embryogenesis, *crf*, proopiomelanocortin (*pomc*), melanocortin 2 receptor (*mc2r*), steroidogenic acute regulatory protein (Oltrabella et al., 2015), and *P450scc* are expressed before hatching. Upregulated *mc2r*, *StAR* and *P450scc* levels are detected prior to the rise in basal cortisol levels after hatching. Although all components of the HPI axis are expressed and cortisol is synthesized at hatch, stressor-induced cortisol responses are not evident until 97 hpf (Alsop and Vijayan, 2009). Since zebrafish mutants that lack pituitary corticotropic cells do not have the ability to synthesize ACTH (Herzog et al., 2004), these fish offer an excellent opportunity to study the role of ACTH in biochemical and behavioral stress responses. Importantly, zebrafish possess a single copy of genes that encode CRF (Chandrasekar et al., 2007) and GR (Alsop and Vijayan, 2009). Although *pomc* genes are duplicated, only one is involved in ACTH synthesis (de Souza et al., 2005). Because zebrafish embryos are transparent and all HPI-related genes have already been

described for this species, the use of genetic tools including transgenesis, gene silencing (morpholino), and chemically-induced mutagenesis (ENU) could foster future molecular and pharmacological analyses of the HPI axis reactivity.

Paralleling physiological responses to stress with behavioral analyses is an important direction of translational stress neuroscience research (Kalueff et al., 2014a; Kalueff et al., 2013; Kalueff et al., 2014b). The novel tank diving test is a commonly used behavioral task to assess behavioral phenotypes in zebrafish (Cachat et al., 2010; Egan et al., 2009; Levin et al., 2007; Mezzomo et al., 2016; Quadros et al., 2016; Rosemberg et al., 2011; Wong et al., 2010a). This test assesses vertical and horizontal swimming activity of fish, their habituation to novelty, overall locomotor activity, exploration, as well as other anxiety-like responses, such as freezing and hyperarousal-like erratic swimming (Kalueff et al., 2013). The behavioral measures in this test are highly sensitive to anxiolytic and anxiogenic compounds, and can be easily analyzed using ethograms, occupancy plots, and three-dimensional reconstructions of the swimming traces (Cachat et al., 2010; Maximino et al., 2010b; Rosemberg et al., 2012). Another popular anxiety model is the light-dark task, which reflects scototaxis, a natural tendency of zebrafish to avoid brightly lit environments and to seek protection in the dark compartment of the apparatus, sensitive to a wide spectrum of anxiolytic drugs (Blaser and Rosemberg, 2012; Maximino et al., 2010a; Maximino et al., 2010b; Mezzomo et al., 2016; Rosemberg et al., 2011).

In rodents, standard methods to investigate depressive-like behaviors are the forced swim and tail suspension tests which measure 'behavior despair'-like immobility and are highly sensitive to conventional antidepressants (Castagne et al., 2011). Another, conceptually different, approach is to evaluate rodent anhedonic responses, which parallel anhedonic behaviors often seen in clinical depression (Moreau, 2002; Strekalova et al., 2004). In zebrafish, depressive-like behaviors can be assessed by motor phenotypes (e.g., the novel tank test motor retardation-like behaviors corrected by chronic antidepressants) and anhedonia-like states (e.g., reduced preference for food, conspecifics and sexual interactions) (Nguyen et al., 2014). HPA deficits and neuroinflammation (associated with major depression in humans) can be assessed by measuring stress-associated hormones (e.g., cortisol) as well as pro- and anti-inflammatory cytokines. Like rodents, zebrafish have highly robust, quantifiable behaviors and the HPI axis homologous to the human HPA axis (Kalueff et al., 2013; Nguyen et al., 2014). Thus, complex analyses of behavioral endpoints, endocrine responses, and cytokines may help assess zebrafish stress-related depressive-like phenotypes and their prospective correction by antidepressants (Song et al., 2017).

Social withdrawal is another hallmark symptom of clinical depression. Several existing tests are not ideal for testing zebrafish sociality because they typically assay individual animals (Egan et al., 2009; Pagnussat et al., 2013; Sackerman et al., 2010). Isolation-induced stress has been described in several species, including zebrafish (Stewart et al., 2012), where, like in rodents, unpredictable social isolation is stressful (Piato et al., 2011). Interestingly, behavioral testing of adult zebrafish triplets previously kept in groups produces faster habituation to the novel tank test and reduced anxiety-like responses (Pagnussat et al., 2013). Moreover, because in their natural habitat zebrafish live in relatively large shoals, environment affects fish stress reactivity and potently modifies their physiological, neuroendocrine and behavioral responses (Giacomini et al., 2015). Endocrine analyses show lower basal cortisol levels in individually housed zebrafish, suggesting some adaptive changes of stress reactivity and habituation to isolation (Parker et al., 2012). Overall, the environment has a fundamental role in stress models since enriched environments reduce stress and promote zebrafish welfare. Environmental enrichment blunts the stress response in both isolated and grouped fish, strikingly paralleling a similar effect observed in zebrafish exposed to anti-stress drugs diazepam (an anxiolytic) or fluoxetine (an antidepressant with anxiolytic chronic effects) (Giacomini et al., 2016). Thus, housing conditions have a crucial role in behavioral and neuroendocrine responses of zebrafish, and should be considered carefully in translational models of anxiety and depression, in order to improve their validity and data reproducibility.

Acute stress has been successfully modeled in zebrafish exposed to conspecific 'alarm substance' (CAS), which increases bottom dwelling, scototaxis and shoaling behavior (Canzian et al., 2017; Quadros et al., 2016). CAS-exposed zebrafish also present freezing, erratic movements and increased *c-fos* expression in the habenula, a brain region involved in fear responses (Canzian et al., 2017; Nathan et al., 2015; Ogawa et al., 2014). A time-dependent sensitization 24 h following acute CAS exposure provides a simple protocol for studying stress/trauma related responses in zebrafish (Lima et al., 2016). Other studies using mechanical stressors (e.g., restraint stress and net chasing) reported elevated whole-body cortisol which can be modulated pharmacologically (Barcellos et al., 2016; Giacomini et al., 2016; Idalencio et al., 2015). In addition to changes in cortisol levels and *gr* expression, the restraint stress also impairs zebrafish CNS antioxidant responses (Dal Santo et al., 2014). Hence, similarly to other vertebrate species, various acute stress protocols offer powerful tools for modeling a wide range of clinically relevant affective phenotypes in zebrafish.

Importantly, life stressors often have a recurrent nature – an important aspect commonly factoring into affective pathogenesis in humans. Therefore, experimental models of chronic stress become particularly important. A useful model for studying the effects of chronic stress in zebrafish is the unpredictable chronic mild stress (UCMS) (Piato et al., 2011) which exposes fish to various natural stressors (e.g., social isolation, exposure to predator, crowding) for 1–2 weeks. Together with cognitive deficits, animals display increased anxiety-like behavior, *crf* gene expression, and higher cortisol levels (Piato et al., 2011). UCMS promotes cognitive impairment, neuroendocrine disturbances and anxiety-like behavior, thereby representing an important new strategy for modeling anxiety- and depression-like phenotypes in zebrafish. For example, animals stressed for 7 days show lower vertical exploration and increased social cohesion, which could reflect an adaptive 'anxiety-like' behavior. In contrast, fish stressed for 14 days present disrupted social behavior and impaired locomotion – the likely maladaptive loss of resilience, similar to depressive-like social withdrawal and motor retardation (Piato et al., 2011). Recent data demonstrate a preventive effect of anxiolytic and antidepressant drugs in stress models, and also emphasize a crucial modulatory role on neuroinflammatory responses in zebrafish (Marcon et al., 2016).

Extending chronic stress models even further, a recent study has developed a new zebrafish model of clinically relevant, prolonged unpredictable chronic stress (PUCS). The 5-week PUCS procedure induced overt anxiety-like and motor retardation-like behaviors in adult zebrafish, also elevating whole-body cortisol and proinflammatory cytokines interleukins IL-1β and IL-6 (Song et al., 2017). PUCS also elevated whole-body levels of the anti-inflammatory cytokine IL-10 and increased the density of dendritic spines in telencephalic neurons, whereas chronic treatment with an antidepressant fluoxetine normalized the behavioral and endocrine phenotypes, as well as stress-elevated IL-1β and IL-6 levels, similar to clinical and rodent data (Song et al., 2017). Although the *bdnf*, *trkB*, *p75*, and *gfap* mRNA levels were unaltered in brain tissue, PUCS elevated whole-body BDNF levels and telencephalic dendritic spine density (which were also corrected by fluoxetine) (Song et al., 2017). Taken together, these findings support zebrafish as a useful in-vivo model of chronic stress, also calling for further cross-species studies of both shared and distinct neurobiological responses to chronic stress. Finally, increasing both the construct and face validity of zebrafish models, such studies also show good predictive validity and can be used for testing new anxiolytic and antidepressant drugs.

As already mentioned, social factors play a key role in modulating stress responses in both clinical and animal studies. Since zebrafish have no parental care and increase shoaling with age (Buske and Gerlai, 2011), they may serve as an interesting model organism to investigate

the intrinsic and extrinsic factors of social behaviors (Engeszer et al., 2007). Developmentally isolated and socially-reared zebrafish display distinct phenotypes following UCMS, which increases anxiety-like behaviors whereas developmental isolation alters motor responses to conspecific images (Fulcher et al., 2017). Moreover, this protocol also reveals reduced weight gain and lower whole-brain levels of dopamine and 5-hydroxyindoleacetic acid in developmentally isolated, but not socially reared, zebrafish (Fulcher et al., 2017). Thus, combining developmental isolation with chronic stress, as well as the wider use of both chronic (e.g., UCMS, PUCS) and acute (e.g., CAS) stress protocols, may be a useful strategy for modeling affective phenotypes in zebrafish.

Finally, aberrant sociality is a core symptom of many neuropsychiatric disorders (Henry et al., 2016). Given the complexity of brain pathogenesis, validation of novel experimental models to understand the biology of social behavior is essential (Shams et al., 2017). Shoaling behavior in zebrafish is a powerful tool for modeling social abnormalities (Jones and Norton, 2015). For example, since zebrafish embryos are transparent and develop externally, it is easy to assess the influence of ethanol administration at different developmental stages, which can elicit distinct molecular, biochemical and behavioral phenotypes (Fernandes et al., 2017; Jones and Norton, 2015; Lovely and Eberhart, 2014). Maternal alcohol consumption during pregnancy can trigger several morphological abnormalities to the fetus, resulting in fetal alcohol spectrum disorder (FASD), which includes fetal alcohol syndrome (FAS), partial FAS, and alcohol-related neurodevelopmental disorders (ARND) (Abel, 2009; Fernandes et al., 2015a; Jones and Bass, 2003; Joya et al., 2014). Clinical ARND symptoms include behavioral abnormalities such as cognitive deficits, impaired academic performance, abnormal emotional functioning and maladaptive social behavior (Furuya et al., 2006; Greenbaum et al., 2002). Early ethanol exposure affects neuronal differentiation without impairing neurogenesis, which makes zebrafish a promising animal model to elucidate neuromolecular mechanisms of ethanol-induced developmental toxicity (Joya et al., 2014). Embryonic alcohol exposure alters social behavior without changing locomotion or visual perception (Fernandes et al., 2015a). Embryos exposed to alcohol also display FAS-related phenotypes, such as microphthalmia and reduced hindbrain *gad1* gene expression (Zhang et al., 2014). Interestingly, although adult zebrafish exposed to alcohol during developmental stages do not present delayed alterations in fear- and anxiety-like behaviors (Seguin et al., 2016), impairments of social behavior remain (Fernandes et al., 2015a; Fernandes et al., 2015b), suggesting persistent effects of ethanol on social behavior domain even at low concentrations. Additionally, early ethanol exposure modulates both dopaminergic and glutamatergic systems in zebrafish, affecting both reward responses and brain glutamate uptake (Baggio et al., 2017; Fernandes et al., 2015b). Thus, zebrafish offer an excellent opportunity to explore neurochemical mechanisms underlying affective disorders and their related social behavior deficits.

5.2. Schizophrenia

Schizophrenia was first described as the separation of function between personality, thinking, memory and perception (Bleuler, 1908). Positive (e.g., hallucinations, delusions) and negative symptoms (e.g., social withdrawal, anhedonia) are both present in schizophrenia, as patients usually display a confused mental state, social and emotional disengagement, the loss of motivation, pleasure and interest, as well as hallucinations (Heinrichs, 2003; Jenkins, 2013; Nasyrova et al., 2015). Schizophrenic patients also often have additional comorbidities (e.g., with anxiety spectrum disorders, major depressive illness, or drug abuse) (Buckley et al., 2009; Nasyrova et al., 2015).

While schizophrenia is a multifactorial disorder influenced by genes, environmental factors, and their interactions (Stefansson et al., 2009), its underlying mechanisms remain unclear (Keshavan et al., 2013; Nasyrova et al., 2015). Although some schizophrenia cases have specific genetic causes (Kakela et al., 2014; Sullivan et al., 2003), the exact role of some other candidate genes (for example *nrgn*, *znf804a*, *tcf4* and *tsnare1*) is poorly understood (Sleiman et al., 2013; Stefansson et al., 2009). The early hypothesis postulated schizophrenia as a consequence of disturbed dopamine metabolism (Creese et al., 1976; Howes and Kapur, 2009), and was based on two observations: (i) the abuse of stimulants increases dopaminergic neurotransmission, causing psychosis and (ii) all drugs used to treat schizophrenic psychosis act by blocking dopamine D2 receptors (Coyle, 2006). This idea was later reconceptualized, noting that the dopaminergic system is disturbed in two ways: (i) the levels of dopamine increase in subcortical (striatum) and (ii) decrease in prefrontal structures of the brain, culminating in prefrontal hypodopaminergia (Howes and Kapur, 2009; Scatton et al., 1982). Lastly, the “final common pathway” hypothesis noted the interplay of endogenous and exogenous factors acting on presynaptic dopamine neurotransmission in striatum (Howes and Kapur, 2009). In addition to the dopaminergic hypothesis, the role of central glutamatergic and GABAergic systems is now also recognized (Hu et al., 2015; Timms et al., 2013).

The pathogenesis of schizophrenia has long been linked to environmental factors affecting the organism during the development. For example, modifications in gray matter occur during childhood to adolescence (Clarke et al., 2011). Mounting evidence implicates inflammation and oxidative stress in the pathogenesis of mental diseases, especially schizophrenia (Bergink et al., 2014; Haller et al., 2014; Song et al., 2013). Besides, inflammatory processes are associated with oxidative stress, leading to structural and biochemical changes in various tissues, and are particularly critical for brain physiology (Bitanihirwe and Woo, 2011). Multiple epigenetic and environmental factors may influence gene expression, increasing the risk of schizophrenia (Haller et al., 2014), including the interaction between cannabis and the *akt1* gene (Alemany et al., 2013; Di Forti et al., 2012) and the link between fetal hypoxia, hypoxia-related genes and the volume of the hippocampus (Haukvik et al., 2010).

Since the introduction of chlorpromazine, antipsychotic drugs have been used to mitigate psychotic episodes as well as to improve the functional recovery of patients (Nur and Adams, 2016). However, the side effects associated with the first-generation of antipsychotics have fostered the development of new serotonin 2A receptor ($5HT_{2A}$) antagonists, which decrease extrapyramidal effects (Meltzer et al., 1999; Miyamoto et al., 2012). The failure of antipsychotic medications raises questions about alternative approaches to modeling schizophrenia (Coyle, 2006; Coyle and Tsai, 2004; Coyle et al., 2002).

Given their role as an excellent model organism to assess cognitive and behavioral dysfunctions (Blaser and Vira, 2014), the use of genetic manipulations (e.g., gene knockdown, knockout or transgenics) in zebrafish to probe altered dopamine signaling in neuropsychiatric disorders is warranted (Souza and Tropepe, 2011). Recent evidence suggests conserved mechanisms of neurogenesis of dopaminergic neurons in zebrafish and mammals (Filippi et al., 2007; Ryu et al., 2007). For instance, the *disc1* gene mutations in both mice and zebrafish are associated with schizophrenia, autism, bipolar disorder and major depression (Chubb et al., 2008; Niwa et al., 2010; Souza and Tropepe, 2011). Four classes of dopaminergic receptors, identified in zebrafish, correspond to the mammalian D1, D2, D3 and D4 orthologs (Souza and Tropepe, 2011). Gene expression data support a crucial role of dopaminergic signaling during development of several distinct brain regions. The expression of *drd1* receptor is first detected at 30 hpf mainly in the diencephalon and hindbrain (Li et al., 2007). Three *drd2* genes (*drd2a, drd2b*, *and drd2c*) are detected at early mid-somitogenesis, and together with the *drd3* gene, are mainly expressed in larval diencephalon, optic tectum, hindbrain and telencephalon at 5 dpf (Boehmler et al., 2004). The three D4 receptors (*drd4a, drd4b,* and *drd4c*) have been later identified in zebrafish, with both *drd4a* and *drd4b* genes expressed predominantly in the forebrain, and *drd4b* - in telencephalon, diencephalon, and midbrain (Boehmler et al., 2007). Dopaminergic receptors

are involved in different behaviors, such as motor control, learning, and reward (Bromberg-Martin et al., 2010). However, the association of behavioral phenotypes with dopamine signaling is complex, and may often result in similar behavioral outcomes. Since brain development occurs during early larval stages in zebrafish, it may have long-term consequences for normal adult behavior, and may help link dopamine to brain morphogenesis and specific neural circuits (Souza and Tropepe, 2011).

The function of *disc1* gene has already been evaluated in zebrafish (Wood et al., 2009), where the *disc1* and *neuregulin 1* knockdown embryos display defective oligodendrocyte development and lose olig2-positive cerebellar neurons. The *disc1* gene is expressed in cranial neural crest cells, critical for the development of the dorsal neural tube that produces multiple cells types, including neurons and glia (Knight and Schilling, 2006). Additionally, *disc1* regulates transcription factors (*sox10* and *foxd3*) and the continued expression of *sox10* factor culminates in craniofacial abnormalities and expansion of peripheral cranial glia population (Knight and Schilling, 2006). The overlap of schizophrenia with oligodendrocyte dysfunction (Hoistad et al., 2009) may involve *sox10*, a gene that regulates oligodendrocyte differentiation (Wegner and Stolt, 2005). Thus, the use of zebrafish may help clarify the role of *disc1* in modulating cell migration, fate determination and differentiation in schizophrenia models (Morris, 2009).

Likewise, molecules that modulate glutamatergic neurotransmission are also used to mimic psychosis in zebrafish. For instance, dizocilpine (MK-801), an antagonist of NMDA receptor, increases oxidative damage and impairs Na^+-K^+-ATPase activity in zebrafish CNS (Seibt et al., 2012). These alterations accompany schizophrenia-like phenotypes, in which animals display positive and negative symptoms, including hyperlocomotion, memory deficits and disrupted social behavior (Seibt et al., 2010; 2011; Seibt et al., 2012). Therefore, further probing dopaminergic and glutamatergic signaling using specific molecular biomarkers is needed for clarifying the mechanisms involved in neurodevelopmental aspects of schizophrenia, and for screening potential therapeutic targets using zebrafish models.

6. Conclusions

In summary, we emphasize the developing utility of zebrafish models of neurological and neuropsychiatric disorders. Since brain disorders continue to affect the growing population worldwide, further development, validation and refinement of mammalian and non-mammalian models in translational neuroscience is critical for our improved understanding of the etiology of human diseases and their evolution (Garakani et al., 2006; Griebel and Holmes, 2013; Khan et al., 2017).

Zebrafish have been successfully used to investigate neurobehavioral bases of various neurological and neuropsychiatric conditions, such as epilepsy, AD, PD, schizophrenia, affective disorders and drug-related disorders (Baraban et al., 2005; Fonseka et al., 2016; Giacomotto et al., 2016; Grone et al., 2016; Lee and Freeman, 2016; Sarath Babu et al., 2016; Wood et al., 2009). However, as an intrinsic characteristic of animal experimentation in general, these models present some limitations. For example, most of the neurodegenerative disease models do not appropriately describe a permanent neuronal death, the main relevant human condition observed in age-related neurodegeneration. So far, translational studies related to schizophrenia and depression have been limited in assessing the 'true' complexity of evoked emotions, cognition and behavioral deficits in animals, especially zebrafish. Furthermore, although the use of morpholino antisense oligonucleotides in zebrafish is restricted to embryo/larvae phases, they continue to serve as genetic tools to investigate the molecular mechanisms underlying neurological diseases. For in-vivo epilepsy models, a key limitation is modeling recurrent and/or focal seizures in adult animals, since chemical agents induce generalized seizure episodes. Thus, moving forward, substantial innovation will be required to design new experimental protocols improving predictive, face, and construct validity of zebrafish disease models. The growing availability of molecular tools and the rapidly increasing number of behavioral tests in larvae and adult zebrafish offer interesting approaches to characterize novel physiological biomarkers involved in CNS disorders. Finally, the biologically conserved nature of zebrafish and human CNS traits offers exciting opportunities for advancing translational neuroscience and phenotype-based drug discovery.

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ARTIGO 2

Modulatory action of taurine on ethanol-induced aggressive behavior in zebrafish

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ABSTRACT

Alcohol is a potent agent for eliciting aggression in vertebrates. Taurine (TAU) is an amino sulfonic acid with pleiotropic actions on brain function. It is one of the most abundant molecules present in energy drinks frequently used as mixers for alcoholic beverages. However, the combined effects of TAU and ethanol (EtOH) on behavioral parameters such as aggression are poorly understood. Considering that zebrafish is a suitable vertebrate to assess agonistic behaviors using noninvasive protocols, we investigate whether TAU modulates EtOH-induced aggression in zebrafish using the mirror-induced aggression (MIA) test. Since body color can be altered by pharmacological agents and may be indicative of emotional state, we also evaluated the actions of EtOH and TAU on pigment response. Fish were acutely exposed to TAU (42, 150, and 400 mg/L), EtOH (0.25%), or cotreated with both molecules for 1 h and then placed in the test apparatus for 6 min. EtOH, TAU 42, TAU 400, TAU 42/EtOH and TAU 400/EtOH showed increased aggression, while 150 mg/L TAU only increased the latency to attack the mirror. This same concentration also prevented EtOH-induced aggression, suggesting that it antagonizes the effects of acute alcohol exposure. Representative ethograms revealed the existence of different aggressive patterns and our results were confirmed by an index used to estimate aggression in the MIA test. TAU did not alter pigment intensity, while EtOH and all cotreated groups presented a substantial increase in body color. Overall, these data show a biphasic effect of TAU on EtOH-induced aggression of zebrafish, which is not necessarily associated with changes in body color.

1. Introduction

Taurine (TAU) is a β-amino sulfonic acid that acts as an osmoregulator, antioxidant, Ca^{2+} modulator and as an inhibitory neuromodulator in the central nervous system (CNS) (Huxtable, 1992; Oja and Saransaari, 1996; Saransaari and Oja, 2000; Menzie et al., 2013). This molecule appears to exert its inhibitory effects on neurons by enhancing the function of $GABA_A$ and glycine receptors and it also counteracts the action of glutamate and inhibits Ca^{2+} channel influx (Banerjee et al., 2013). In addition to its biosynthetic pathway derived from cysteine oxidation, TAU may be obtained from the diet (Lambert et al., 2015; Vitvitsky et al., 2011). TAU is one of the most abundant molecules present in energy drinks frequently used as mixers for alcoholic beverages, so the interaction of TAU and EtOH may constitute a public health concern (Marczinski and Fillmore, 2014). Evidence has shown that consuming energy drinks in combination with alcohol may decrease perceived intoxication, enhance stimulation, and increase drinking compared to consuming ethanol (EtOH) alone (Franklin et al., 2013; Marczinski et al., 2012).

Acute EtOH intake can cause adverse effects on brain function (Collier et al., 2014). The mechanisms by which EtOH affects the CNS are associated with modulation of different neurotransmitter systems, impairment of mitochondrial function, changes in gene expression and alterations of intricate transduction signaling pathways (Davies, 2003; Harper and Corbett, 1990; Harper and Matsumoto, 2005; Tong et al., 2011). In rodents, studies have shown that acute EtOH administration elicits a significant efflux of TAU from neurons and astrocytes, increasing its extracellular levels in different brain regions (Dahchour et al., 1996; Quertemont et al., 1999). It has been postulated that

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variations in intracellular/extracellular TAU contents could play an adaptive role in the CNS, mediating several of the physiological effects of EtOH in brain (Olive, 2002).

In humans, alcohol consumption is usually associated with cognitive deficits, depression, vulnerability to stress, impulsivity, inattention and boldness (Parker et al., 2014). Although a link between boldness, aggression and acute EtOH consumption is well established, the nature of these phenomena is not completely understood. Considering the pleiotropic actions of EtOH on CNS, the specific pharmacological mechanisms underlying the EtOH-induced aggression are multifaceted (Heinz et al., 2011). Hypotheses concerning the route by which EtOH affects aggression include: I) via cognitive mechanisms, implying a role for learned associations; II) via disinhibition of behavior, by disrupting the frontal regulatory systems after alcohol consumption; III) indirectly, via anxiolytic properties, in which alcohol reduces anxiety, increasing approach behaviors or/and reducing propensities to relent when threatened (Attwood and Mufanò, 2014). Thus, the validation of alternative/complementary models to evaluate agonistic behaviors following acute exposure to different drugs may serve as valuable tools to understand the neural basis of aggression in vertebrates.

The zebrafish (*Danio rerio*) is a model organism that has been widely used in neurobehavioral studies to investigate phenotypes related to drug abuse on a medium/large scale (Stewart et al., 2011; Wyatt et al., 2015; Zon, 1999). As a vertebrate species, it exhibits a considerable genetic and physiological homology to humans and also presents the major neurotransmitter systems described for mammalian models (Kyzar et al., 2012). The drug delivery method is one of its great advantages because soluble drugs can be mixed directly in tank water and promptly absorbed by the immersed zebrafish (Rosemberg et al., 2012; Tran et al., 2015). EtOH also modulates the color of zebrafish, a phenomenon known as the camouflage response (Peng et al., 2009). As an innate response exhibited by many vertebrates, pigment patterning plays a role in facilitating foraging, anti-predator responses and social communication (Fujii, 2000; Nascimento et al., 2003). Additionally, an extensive repertoire of behaviors has been described for zebrafish, providing a sound basis for the use of this model to study complex behaviors such as aggression (Filby et al., 2010).

Aggression in zebrafish can be measured by different protocols (Way et al., 2015). One protocol involves putting two fish in a same tank to observe their behavior (Oliveira et al., 2011). This method allows the observation of all natural interactions between animals in order to quantify agonistic behaviors. However, dyadic measures of aggression may be ethically questionable because fish can harm each other and experience increasing levels of stress during the interaction (Jones and Norton, 2015). Additionally, this procedure offers reduced experimental control since the behavior of the experimental subject will be affected by the activities of its test partner. Another protocol evaluates the reaction of a single fish facing its image in a mirror, known as the mirror-induced aggression test (MIA) (Gerlai et al., 2000). Although it may not represent all natural interactions between fish, this method is relatively simple and does not present ethical concerns. Moreover, the results may be more reliable because there is a greater degree of experimental control over the stimuli. Due to the practical advantages of using zebrafish for modeling behavioral phenotypes, the MIA test represents a time-efficient strategy to screen for potential drugs that modulate aggression. Since the potential actions of TAU and EtOH on aggressive behavior are poorly understood, we evaluate whether TAU modulates agonistic behavior and pigment response in zebrafish acutely exposed to EtOH. For this purpose, we described the behavioral patterns of aggression, exploring how different variables may influence the results of behavioral endpoints using ethograms. Moreover, we have proposed an index for measuring aggression in the MIA test that includes the behavioral endpoints most relevant to the expression of aggression in this task (e.g. approach to the mirror, number, and duration of attacks).

2. Materials and methods

2.1. Animals

Wild type adult zebrafish (*D. rerio*) (4–6 months-old, ~50:50 male:female ratio, short fin strain) were obtained from a commercial supplier (Hobby Aquarios, RS, Brazil) and kept for two weeks in a 50-L thermostatic aquarium under constant mechanical and chemical filtration before the experiments to acclimate to the laboratory facility. The water was previously treated with AquaSafe™ (Tetra, USA) and the temperature was set at 27 ± 1 °C, pH 7.2. Room illumination was provided by ceiling-mounted fluorescent light tubes on a 14/10 light/dark photoperiod cycle (lights on at 7:00 am and off at 9:00 pm). Fish were fed thrice daily with commercial flake fish food (alcon BASIC™, Alcon, Brazil). Animals were maintained in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. The protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (process number 026/2014).

2.2. Pharmacological manipulations

EtOH and TAU were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich (St. Louis, MO, USA), respectively. All exposure periods were set for 1 h as previously described and the animals were placed individually in a 500-mL beaker in the presence or absence of drugs (Rosemberg et al., 2010). The following groups were tested: i) Control group; ii) Taurine groups at 42 mg/L (TAU 42), 150 mg/L (TAU 150) and 400 mg/L (TAU 400); iii) EtOH group (EtOH, 0.25% *v*/*v*); Cotreatment groups (TAU 42/EtOH, TAU 150/EtOH and TAU 400/EtOH). The control group was kept only in non-chlorinated water, in the absence of drugs. The 0.25% EtOH concentration was chosen because it induces anxiolytic-like effects in zebrafish, characterized by increased vertical exploration in novel apparatuses, decreased scototaxis in the light–dark test, and increased aggression without inducing sedation (Dlugos and Rabin, 2003; Gerlai et al., 2000). TAU treatments were performed as described elsewhere (Rosemberg et al., 2010), varying from 0.33 to 3.2 mM. Previous studies demonstrated that these TAU concentrations modulate EtOH-induced changes in locomotion and vertical activity of zebrafish, antagonizing the oxidative effects caused by acute EtOH exposure in brain (Rosemberg et al., 2010, 2012). TAU solutions were prepared just before the experiments and buffered to pH 7.0 using 0.1 M NaOH.

2.3. Mirror-induced aggression test

The MIA test was based on the protocol described previously (Gerlai et al., 2000). After each treatment, fish were individually placed in the test apparatus (25 cm length × 15 cm height × 6 cm width). In one back wall of the tank, an inclined mirror was placed with an angle of 22.5° so that the left vertical edge of the mirror was touching the side of the tank and the right edge was further away. All other tank sides were covered with opaque partitions in order to keep a minimal distraction and to allow the simultaneous recording of behavior from two animals. The tanks were virtually divided into four areas related to their proximity to mirror: a1 and a2 (proximal), a3 and a4 (distal), in which a1 and a3 represent the close area, while a2 and a4 are the far area in relation to the inclined mirror (Fig. 1).

The behavioral tests were recorded at the same time period (between 09:00 am and 4:00 pm). All apparatuses were filled with non-chlorinated water and the experimental procedures were performed on a stable surface in an isolated environment with minimal external interference. Behavior was recorded in a single 6-min trial immediately after the exposure period using a webcam connected to a laptop with appropriate video-tracking software (ANY-maze™, Stoelting CO, USA) at a rate of 30 frames/s.

Aggressive behavior was analyzed by the following parameters: number of aggressive episodes, duration of aggressive episodes, average

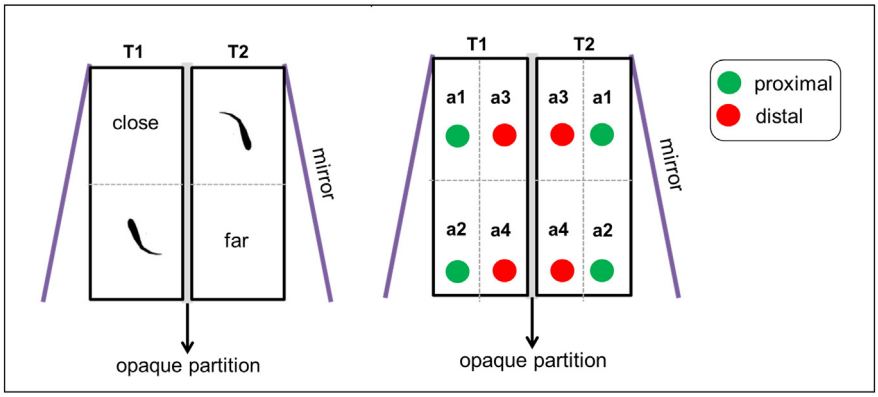


Fig. 1. Schematic model illustrating the behavioral apparatuses used for the MIA test. The Figure shows the inclined mirrors (22.5°) and the two tanks divided by an opaque partition. Virtual divisions in the tanks were made to analyze the behavioral pattern of zebrafish, in which two areas (close and far) represent the apparent distance to the opponent image and four sections (a1, a2, a3, and a4) indicate the proximity to mirror. Proximal sections (a1 and a2) are depicted as green and distal sections (a3 and a4) are shown as red color, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

duration of aggressive episodes and latency to attack the mirror. Locomotor and motor activities were assessed by distance traveled and angular velocity using automated analysis to evaluate the overall swimming pattern of zebrafish in the test apparatuses. In order to investigate the exploration of zebrafish, the transitions to tank areas, time spent in tank areas and the average duration of entry in tank areas were determined. To analyze the aggressive profile of fish, the following parameters were measured: number of aggressive episodes per tank area, duration of aggressive episodes per tank area and proportion of time attacking the mirror per tank area. Aggression was coded manually by two trained experimenters blind to the experiment (inter-rater reliability > 0.85). Aggressive episodes were counted when fish presented erection of fins, associated with undulating body movements, fast swimming and biting towards the mirror (Gerlai et al., 2000; Kalueff et al., 2013). The end of aggression was determined when animals stopped biting the opponent image.

2.4. Aggression index

To determine a general aggression index, we considered several variables that are usually involved in the aggressive behavior of zebrafish subjected to the MIA test. The selected endpoints were: number and duration of aggressive episodes (aggressive behavior); number of entries in the proximal sections of the tank that allow the fish to attack the opponent image (a1 and a2) (approach). These parameters were used for calculating an aggression index as follows: $f(\rho) = k\,(\frac{\log(DAE)X\ AE}{\sum NE(a1+a2)})$, where k is a constant with value 4; DAE is the duration of aggressive episodes; AE is the number of aggressive episodes; and NE represents the number of entries in the proximal sections of the test apparatuses. This equation was chosen to weigh aggressive behavior of control animals with an index value of approximately 1. A higher aggression score is produced by an increase in the number and duration of attacks to the mirror, while a lower score is produced by more transitions to a1 and a2 in the absence of aggressive displays, indicating exploration of the opponent image with decreased aggression.

2.5. Construction of ethograms

In order to represent the key behaviors of zebrafish in the MIA test, we have constructed representative ethograms including the time spent and the frequencies of transitions between the tank areas (a1, a2, a3, and a4) as well as the duration and the number of aggressive episodes. The diameter of each circle represents the duration of the behavioral endpoints analyzed, whereas the arrow width and direction reflect the frequency and direction of these behaviors. All measures were previously normalized to proportionally express the behavioral activity of each experimental group (Grossman et al., 2010; Rosemberg et al., 2011).

2.6. Pigment response

Zebrafish were euthanized by decapitation immediately after the behavioral analysis and quickly placed on a white paper that served as a clear background. For measuring color intensity, we did not use any drug or ice-cold anesthesia because such procedures could affect the pigment response. Pictures of each side of the body were taken with an 8.0-megapixel camera from a Samsung Galaxy S3™ smartphone mounted on a ring stand 8-cm distance above the fish. The pictures were analyzed on a computer using the Image J 1.48 for Windows software to quantify the pigmentation. A standard lateral area of the fish was selected, which represented a substantial melanosome-filled region of zebrafish body (Cachat et al., 2013). Results were normalized based on the pigment intensity of fish subtracted from the respective background and expressed as a saturation score index (SSI), calculated by the following formula: $SSI = \frac{1}{MGV} * 100$, where the MGV represents the mean gray value of the selected region of zebrafish body, ranging from 0 (black) to 255 (white). Data were expressed as percentage of control group.

2.7. Statistics

Normality of data and homogeneity of variance were analyzed by Kolmogorov–Smirnov and Bartlett's tests, respectively. Results were expressed as means ± standard error of the mean (S.E.M.) and analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA using treatment and tank area as factors when different areas were considered. Differences between groups were further assessed by the Newman Keuls multiple comparisons test. The latency to attack the mirror was expressed as median ± interquartile range and analyzed by Kruskal–Wallis test followed by Dunn's multiple comparison test whenever appropriate. The level of significance was set at $p \le 0.05$ ($n = 10$–15 per group).

3. Results

3.1. Effects of TAU in zebrafish subjected to MIA task

The actions of acute TAU exposure in the behavior of fish subjected to the MIA test are shown in Fig. 2. TAU per se did not significantly change total distance traveled and angular velocity in comparison to

control (Fig. 2A). Considering the aggression-related parameters (Fig. 2B), one-way ANOVA yielded a significant increase in the number ($F_{3,33} = 12.38$, $p < 0.0001$) and duration ($F_{3,33} = 6.734$, $p < 0.005$) of aggressive episodes for TAU 42 and TAU 400 groups. Moreover, TAU 400 revealed a substantial increase in the average duration of aggressive episodes when compared to control ($F_{3,33} = 5.319$, $p < 0.005$). Nonparametric Kruskal–Wallis test showed that TAU 150 presented a significant increase in the latency to attack the mirror ($p < 0.05$). Two-way ANOVA using area and treatment as factors revealed that all groups showed increased transitions ($F_{1,66} = 151.5$, $p < 0.0001$), time spent ($F_{1,66} =$

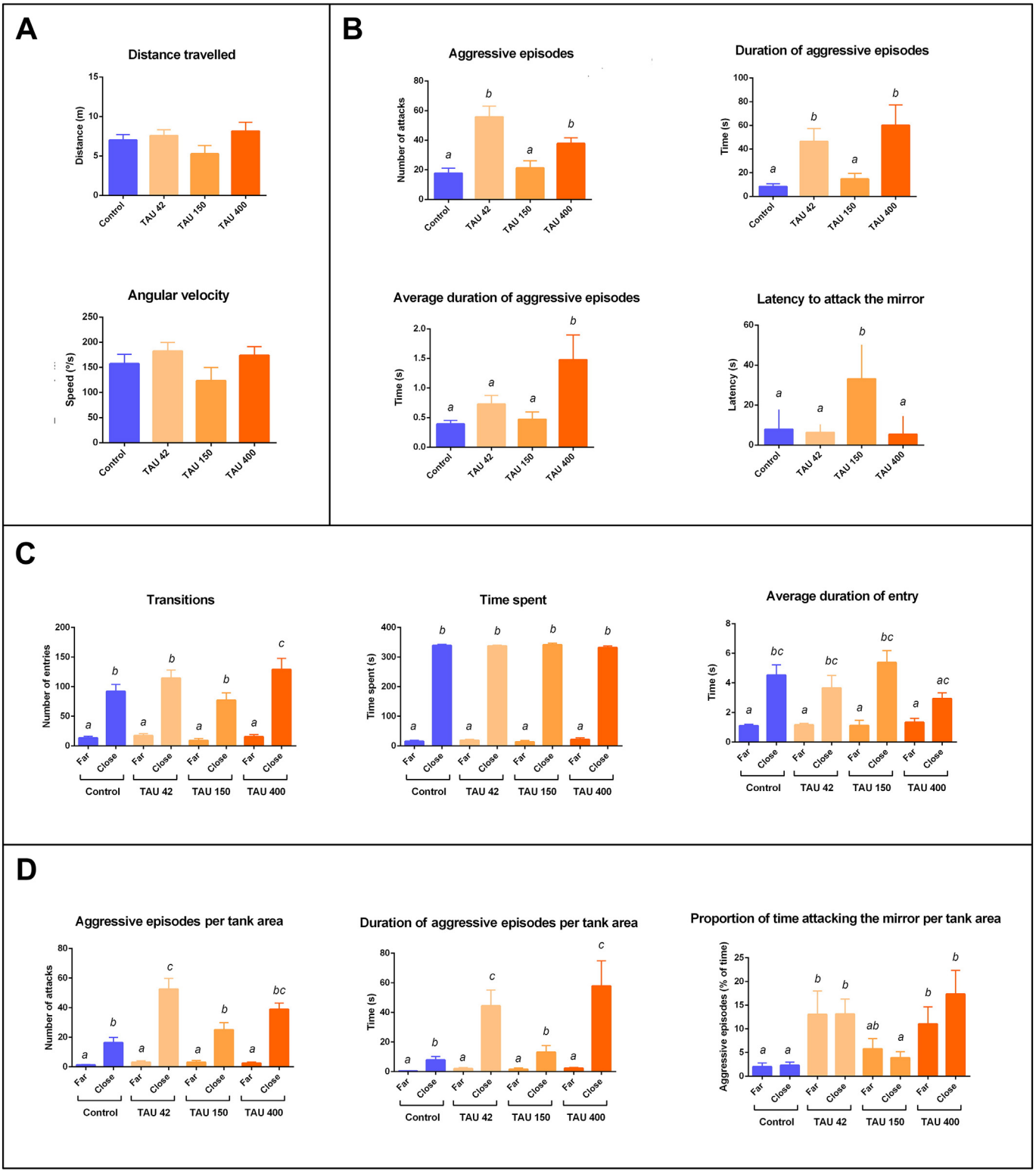


Fig. 2. Effects of TAU in zebrafish subjected to the MIA test. (A) Locomotor parameters. (B) Aggressive behavior parameters of zebrafish. (C) Exploration in close and far areas. (D) Aggressive profile per tank area. Data were represented as mean ± S.E.M. and analyzed by one or two-way ANOVA, following by Newman–Keuls multiple comparison test when appropriate. The latency to attack the mirror was expressed as median ± interquartile range and analyzed by Kruskal–Wallis test followed by Dunn's post hoc test. Distinct letters indicate statistical differences between groups ($p < 0.05$, $n = 10$–12 per group).

104.34, $p < 0.0001$) and average duration of entry ($F_{1,66} = 60.01$, $p < 0.0001$) in the close area. However, animals treated with 400 mg/L TAU performed more entries to the close area in comparison to the other experimental groups, and did not show a significant effect of entry duration in both close (a1 and a3) and far (a2 and a4) areas (Fig. 2C). The analysis of aggressive behavior in the areas of the apparatus (Fig. 2D) revealed that all groups displayed more attacks ($F_{1,66} = 58.43$, $p < 0.0001$) and a higher duration of aggressive episodes ($F_{1,66} = 73.69$, $p < 0.0001$) in the region where the mirror was closer to the tank wall. TAU 42 showed a significant increase in aggressive episodes in the close area, and both TAU 42 and 400 groups had increased duration of attack in the respective area when compared to control and TAU 150 groups. The proportion of time attacking the mirror in both tank areas was significantly increased for TAU 42 and TAU 400 ($F_{1,66} = 4.012$, $p < 0.05$).

3.2. Modulatory role of TAU on EtOH-induced aggressive behavior

The effects of EtOH and TAU cotreatment on zebrafish behavior during the MIA test are depicted in Fig. 3. A 2 × 4 two-way ANOVA revealed a significant interaction of TAU and EtOH for distance traveled ($F_{3,80} = 4.201$, $p < 0.01$) and angular velocity ($F_{3,80} = 4.087$, $p < 0.01$), with a significant increase for both measures in TAU 42/EtOH and TAU 400/EtOH groups (Fig. 3A). Regarding the aggression-related parameters (Fig. 3B), EtOH, TAU 42/EtOH and TAU 400/EtOH presented a significant increase in the frequency and time attacking the opponent image ($F_{4,57} = 12.24$, $p < 0.0001$ and $F_{4,57} = 6.998$, $p < 0.001$, respectively) as well as in the average duration of aggressive episodes ($F_{4,57} = 3.888$, $p < 0.01$) when compared to control. On the other hand, the latency to attack the mirror significantly increased in TAU 150/EtOH group ($p < 0.05$). Two-way ANOVA showed a significant interaction of TAU and EtOH for the number ($F_{3,80} = 5.213$, $p < 0.005$) and duration ($F_{3,80} = 4.382$, $p < 0.01$) of aggressive episodes.

Fig. 3C shows the exploratory parameters of zebrafish during the trial. A 2 × 5 two-way ANOVA using area and treatment as factors revealed that all groups performed more entries and spent more time in the area in which the mirror appeared closer to fish ($F_{1,114} = 23.83$, $p < 0.0001$ and $F_{1,114} = 141.9$, $p < 0.0001$, respectively). Although the average duration of entry did not significantly differ between close and far in all TAU-cotreated groups, both control and EtOH groups showed a significant increase of this parameter in the close area ($F_{1,114} = 4.283$, $p < 0.0001$). Considering aggressive behavior in each area of the apparatus, the number ($F_{1,114} = 19.67$, $p < 0.0001$) and duration ($F_{1,114} = 13.63$, $p < 0.0001$) of aggressive episodes per area was significantly higher in the close area for all groups. Post-hoc analyses indicated that the number of aggressive episodes was significantly increased in TAU 400/EtOH, while the duration of aggressive episodes was prolonged in both TAU 42/EtOH and TAU 400/EtOH groups. The proportion of time in which animals attacked the mirror in the close and far areas did not differ between groups, but TAU 400/EtOH showed higher values as compared to the other groups (Fig. 3D).

3.3. Pigment response

The body color of zebrafish after the behavioral experiments is shown in Fig. 4. No significant difference was observed between all TAU groups in relation to the control (Fig. 4A). However, EtOH presented a significant increase in pigmentation and this response was pronounced in all TAU-cotreated groups ($F_{4,57} = 10.37$, $p < 0.0001$) (Fig. 4B). A significant interaction of TAU and EtOH in pigment response was also revealed by two-way ANOVA ($F_{3,80} = 2.811$, $p < 0.05$).

3.4. Aggression index and ethograms

The aggression indexes calculated for each experimental group are represented in Fig. 5. The results showed that TAU 42 and TAU 400 were more aggressive than control and TAU 150 groups ($F_{3,33} = 8.311$, $p < 0.0005$) (Fig. 5A). Moreover, EtOH, TAU 42/EtOH and TAU 400/EtOH groups displayed higher indexes in comparison to control and TAU 150/EtOH ($F_{4,57} = 6.080$, $p < 0.001$). (Fig. 5B).

Representative ethograms were constructed to demonstrate the main behaviors, such as the frequency of transitions, time spent in tank areas, number of attacks and proportion of time with aggressive displays (Fig. 6). These diagrams illustrated the increase of aggressive behavior in TAU 42, TAU 400, EtOH, TAU 42/EtOH, and TAU 400/EtOH groups. Although TAU 42/EtOH and TAU 400/EtOH had similar indexes, they presented a different pattern of aggression since the number and duration of aggressive episodes were distinct.

4. Discussion

The novel findings of the current study demonstrate that acute TAU exposure modulates EtOH-induced aggression in zebrafish. These results were confirmed by representative ethograms, which revealed distinct behavioral patterns of agonistic behavior. We also proposed, for the first time, an integration of different behavioral endpoints to express aggression of zebrafish and the calculated indexes were consistent with the main effects observed for each experimental condition.

In the literature, studies regarding the actions of TAU on aggressive behavior of animal models are scarce. It is known that positive allosteric modulators of $GABA_A$ receptors, like TAU, may exacerbate aggressive behavior in animal models (McDonald et al., 2012; Miczek et al., 2003). We demonstrated that TAU per se at 42 and 400 mg/L enhanced aggressive behavior without altering locomotor and motor patterns, while no significant effects were observed after 150 mg/L TAU exposure. Although we do not have a precise explanation regarding the underlying mechanisms involved in the U-shaped response observed, there is evidence demonstrating that TAU may negatively modulate aggression, exerting an anti-aggressive action on the CNS (Gupta et al., 2005, 2006; Mandel et al., 1985). Our results reinforce the hypothesis that the different responses observed may be largely dependent on the concentration used, suggesting a biphasic effect of TAU on aggression-related behaviors.

Exposure to 0.25% EtOH increased the number and duration of aggressive episodes, corroborating with previous reports (Echevarria et al., 2011; Gerlai et al., 2000). The alterations of neurotransmitter systems implicated in alcohol-associated aggression are influenced by genetic background and environmental situations. In the mammalian brain, the neurobiological basis of EtOH-associated aggression after acute administration involves impairment of prefrontal cortex function along with increased dopamine release in ventral striatum (Heinz et al., 2011). Previous studies have shown that, similar to rodents, zebrafish acutely exposed to EtOH present a robust increase in dopamine and serotonin in whole brain extracts (Chatterjee and Gerlai, 2009). Additionally, we reported that EtOH and acetaldehyde in vitro differently affect glutamate uptake and cell viability in the forebrain, midbrain, and hindbrain, suggesting that the effects of alcohol in the zebrafish CNS are molecule- concentration- and structure-dependent (Zenki et al., 2014). When TAU was concomitantly administered with EtOH, TAU 42/EtOH and TAU 400/EtOH were more aggressive than control and TAU 150/EtOH, and also presented a significant increase in total distance traveled and angular velocity. This pattern of results suggests a potential interaction in which both molecules trigger a similar mechanism, at least for these behavioral endpoints. In contrast to the effects on locomotor activity, the actions of TAU and EtOH on aggression appear to be additive, suggesting that both molecules may act through independent mechanisms. At the present, even though locomotor alterations may be involved in aggressive behavior, it is difficult to determine whether the changes in aggression observed in groups TAU 42/EtOH and TAU 400/EtOH can be attributed entirely to locomotor effects. It seems unlikely, however, because TAU alone exerts different effects on locomotor activity and aggression. Additionally, because TAU

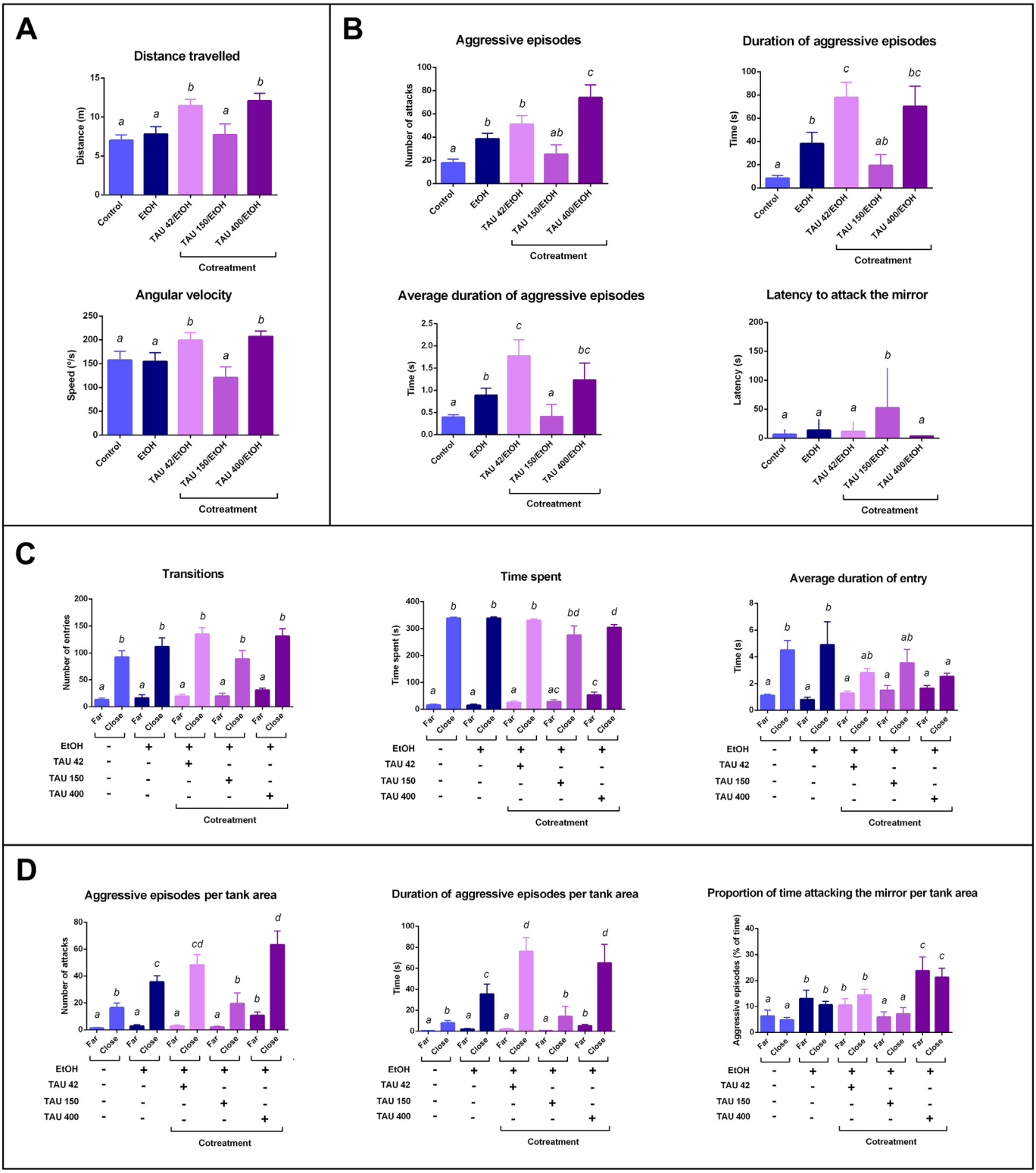


Fig. 3. Effects of EtOH and TAU/EtOH cotreatment in zebrafish subjected to the MIA test. (A) Locomotor parameters. (B) Aggressive behavior parameters of zebrafish. (C) Exploration in close and far areas. (D) Aggressive profile per tank area. Data were represented as mean ± S.E.M. and analyzed by one or two-way ANOVA, following by Newman–Keuls multiple comparison test when appropriate. The latency to attack the mirror was expressed as median ± interquartile range and analyzed by Kruskal–Wallis test followed by Dunn's post hoc test. Distinct letters indicate statistical differences between groups ($p < 0.05$, $n = 10$–15 per group).

and EtOH modulate different neurotransmitter systems (e.g. glutamatergic, GABAergic, dopaminergic and serotonergic) (Huxtable, 1992; McCool, 2011; Rosemberg et al., 2012; Wu et al., 2005), the dissociable actions on the behaviors measured may involve the activation of different brain regions and/or distinct neurochemical signaling pathways in zebrafish brain.

It is conceivable that multiple behaviors traits are correlated at the population or species level, termed behavioral syndromes (Sih et al.,

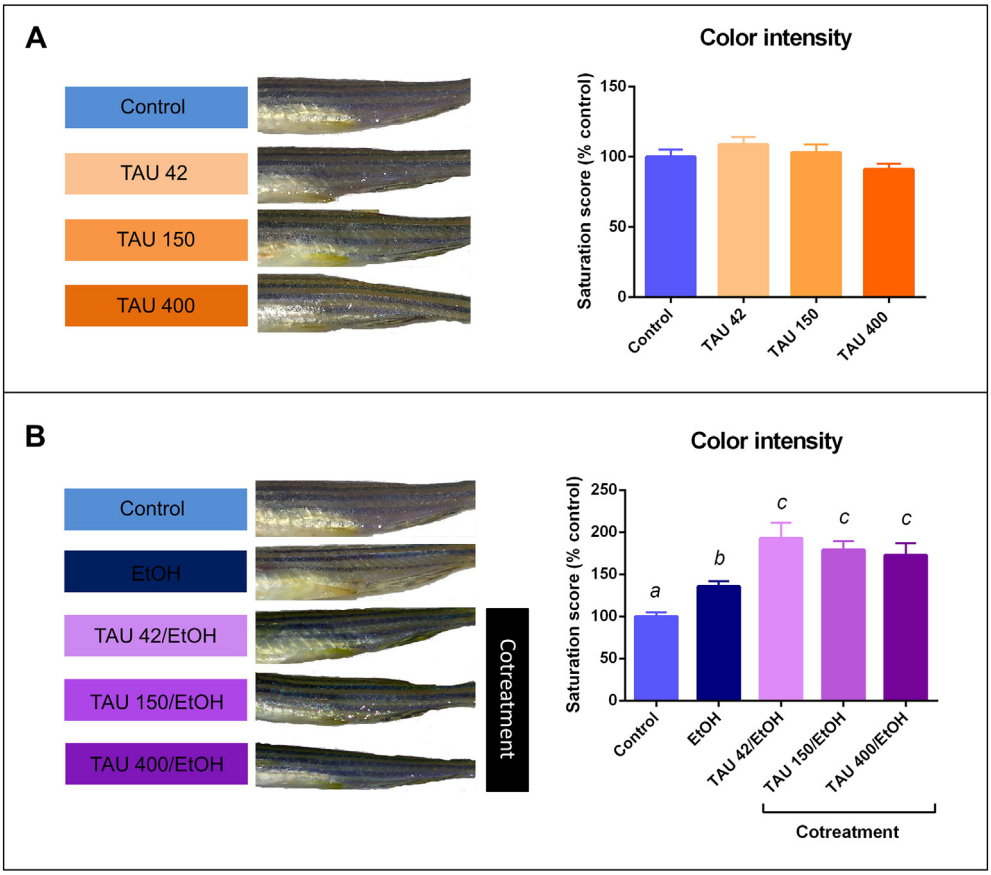


Fig. 4. Pigment response of zebrafish shown as representative images of body color intensities measured as saturation score for each group. (A) Effects of TAU in zebrafish pigmentation. (B) Effects of EtOH and TAU/EtOH cotreatment in body color. Data were represented as mean ± S.E.M. and analyzed by one-way ANOVA, following by Newman–Keuls multiple comparison test when appropriate. Distinct letters indicate statistical differences between groups ($p < 0.05$, $n = 10–15$ per group).

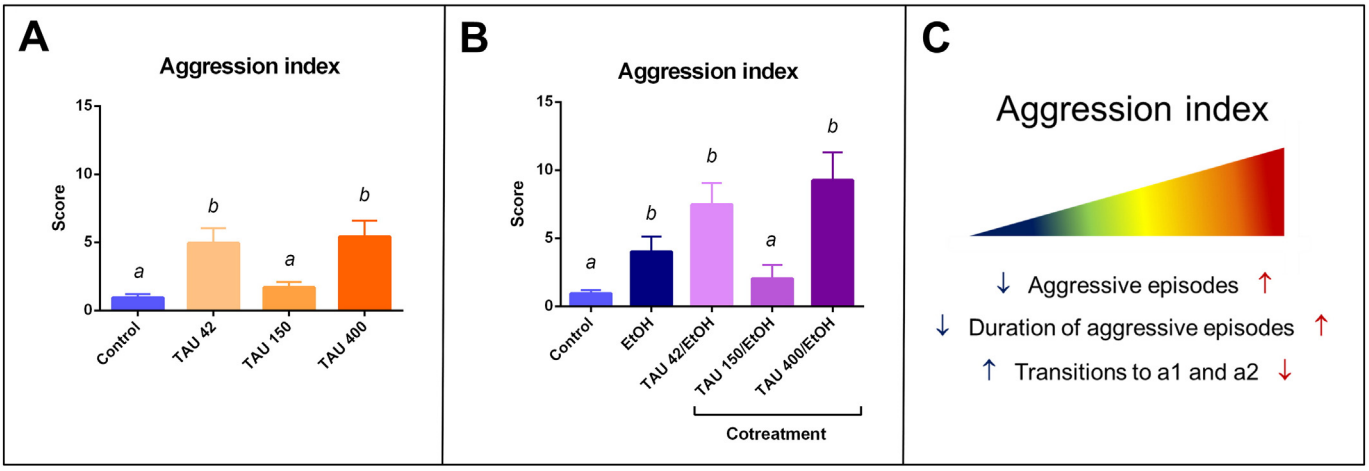
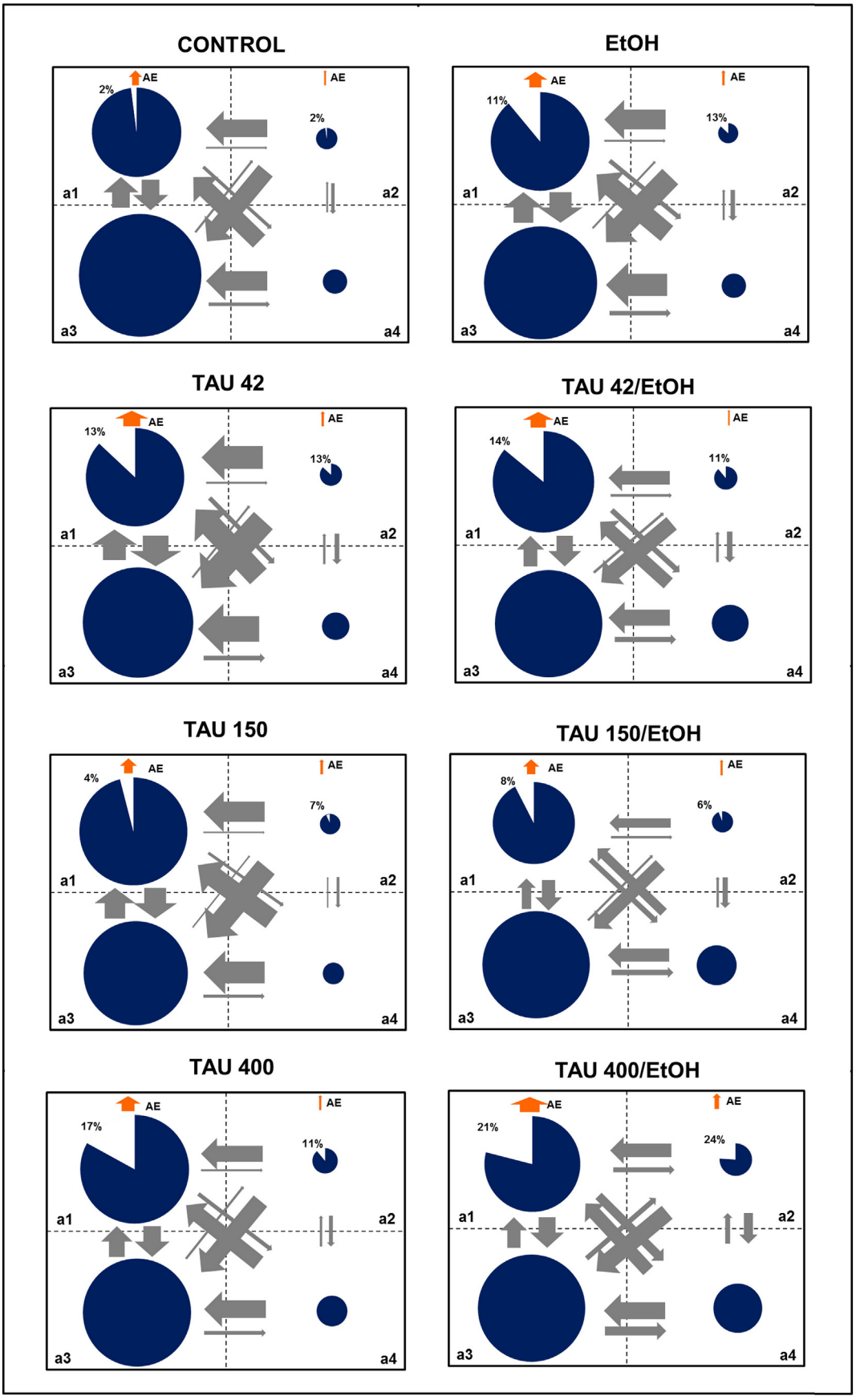


Fig. 5. Aggression index for MIA test estimated by the number and duration of aggressive episodes and transitions to a1 and a2 sections. (A) Effects of TAU on zebrafish aggression. (B) Effects of EtOH and TAU/EtOH cotreatment. (C) Heat map showing parameters that can increase (red) or decrease (blue) aggression in zebrafish. Data were represented as mean ± S.E.M. and analyzed by one-way ANOVA, following by Newman–Keuls multiple comparison test when appropriate. Distinct letters indicate statistical differences between groups ($p < 0.05$, $n = 10–15$ per group). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Behavioral profile of EtOH, TAU and TAU/EtOH groups in the MIA test during a 6-min observation period. Representative ethograms were generated based on frequencies and transitions between each individual behavior, showing the existence of a distinct aggression-related profile of zebrafish. The diameter of each circle corresponds to the time of each individual behavioral activity, whereas the arrow width and direction reflect the frequency of transitions between these behaviors. The figure shows the tank sections correspondent to close (a1 and a3) and far (a2 and a4) areas. AE represents the number of aggressive episodes. The percentage of time in which animals show aggressive profile (hachured white diagrams) in each proximal section is demonstrated.


CONTROL
AE
AE
2%
2%
a1
a2
a3
a4
EtOH
AE
AE
11%
13%
a1
a2
a3
a4
TAU 42
AE
AE
13%
13%
a1
a2
a3
a4
TAU 42/EtOH
AE
AE
14%
11%
a1
a2
a3
a4
TAU 150
AE
AE
4%
7%
a1
a2
a3
a4
TAU 150/EtOH
AE
AE
8%
6%
a1
a2
a3
a4
TAU 400
AE
AE
17%
11%
a1
a2
a3
a4
TAU 400/EtOH
AE
AE
21%
24%
a1
a2
a3
a4

2004). One example is the aggression-boldness syndrome in zebrafish, also known as proactive behavior, characterized by above-average boldness, exploratory behavior and aggressiveness in some individuals (Norton et al., 2011). These apparent correlations reinforce the idea that aggression is not a simple unitary phenomenon, but may include several related behaviors such as exploration of the environment along with investigation and approach to conspecifics (Wilson et al., 2011). In the MIA test, detection and approach to the opponent involves locomotion and exploration of the mirror image, which can reflect social or agonistic motivation.

Taking into consideration the effects of TAU and EtOH on zebrafish behavioral functions, we have previously shown that 42, 150, and 400 mg/L TAU prevented 1% EtOH-induced impairments on locomotor activity, while only 42 and 400 mg/L prevented EtOH-induced changes in vertical activity (Rosemberg et al., 2012). This U-shaped response indicates that the actions of TAU on exploration are not directly linked to changes in locomotor activity, suggesting that TAU also modulates motivational aspects of zebrafish behavior. The construction of ethograms, which demonstrate the key behaviors of animal models, facilitates the visualization and characterization of complex behavioral phenotypes (e.g. approach to the opponent image, social investigation, and agonistic interaction). Although the aggression indexes of TAU 42/EtOH and TAU 400/EtOH groups did not significantly differ, the ethograms revealed distinct patterns of approach and attack to the opponent image. Overall, this pattern of results suggests that exploration-related parameters may affect aggressive profiles, and that TAU and EtOH affect both, but not necessarily through identical pathways.

The modulation of pigment response is associated with changes in anxiety-related parameters, a natural response to lighting/environmental conditions (camouflage response), social behavior (e.g., display, fight or courtship) and also a result of stress or specific drug exposures (Kalueff et al., 2013; Price et al., 2008; Wagle et al., 2011). The pigment response involves a general change in body color resulting in a darker or lighter appearance of zebrafish (Echevarria et al., 2011; Gerlai et al., 2000; Logan et al., 2006). We verified that TAU per se altered aggression at 42 and 400 mg/L, but not the pigment intensity. All cotreated groups displayed a robust increase in body color when compared to fish exposed to EtOH, but 150 mg/L TAU prevented aggression induced by alcohol. Since our results point to a complex relationship between changes in body color and aggressive behavior triggered by EtOH and TAU, more studies combining gene expression, behavioral analyses, and pharmacological manipulations are required to investigate the mechanisms involved in pigment response and aggression.

5. Conclusion

In summary, acute TAU exposure exerts a biphasic effect on EtOH-induced aggressive behavior of zebrafish. At lower and higher concentrations, TAU positively modulates aggression, while 150 mg/L exerts an anti-aggressive action. The integration of ethograms with the aggression index described herein can provide reliable measurements for analyzing the agonistic behavioral patterns using the MIA test in future translational neuroscience and biological psychiatry studies. Moreover, our findings point to a complex relationship between locomotor, motor patterns, pigment response and aggression. In this regard, more studies are necessary to clarify the neurochemical mechanisms triggered by EtOH and TAU in vertebrate brain.

Competing interests

The authors declare no competing interests.

Author contributions

Conceived and designed the experiments: BDF, DLM, DBR. Performed the experiments: BDF, DLM, AS, GSG, VAQ. Analyzed the data: BDF, DLM, LVCR, NJM, AS, GSG, VAQ, GLBF, REB, DBR. Contributed reagents/materials/analysis tools: GLBF, DBR. Wrote the paper: BDF, DLM, LVCR, NJM, REB, DBR. All authors read and approved the final manuscript.

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MANUSCRITO 1

Taurine modulates acute ethanol-induced social behavioral deficits and fear responses in adult zebrafish

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Taurine modulates acute ethanol-induced social behavioral deficits and fear responses in adult zebrafish

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1. Introduction

Alcohol misuse represents a critical public health concern due to the high prevalence of alcohol-related morbidity and mortality in adults (WHO, 2014). Ethanol (EtOH) directly affects the central nervous system (CNS) resulting in behavioral changes, such as disinhibition of punished operant behavior and cognitive deficits, that may impair threat-perception (Mitchell and Potenza, 2014). Moreover, alcohol consumption increases the risk of social and health problems leading to alterations in social behavior (e.g., sociability deficits and depressive-like behavior) (Muller et al., 2017; Naimi et al., 2003; Rosenquist et al., 2010). Since EtOH modulates brain functions involved in sociability, impulsivity, and risk assessment (Parker et al., 2014), studies related to alcohol consumption and behavior are imperative. EtOH acts in the CNS by impairing various neurotransmitter systems, disrupting mitochondrial function, changing gene expression, and altering transduction signaling pathways (Davies et al., 2003; Harper and Matsumoto, 2005; Harper and Littleton, 1990; Tong et al., 2011). Because alcohol has pleiotropic actions in the brain, interrelated neural mechanisms are likely to be involved in the pharmacological mechanisms associated with changes in sociability and critical judgment (Heinz et al., 2011).

In young adults, alcohol beverages are often consumed mixed with energy drinks, with users reporting decreased drowsiness and improved pleasure sensation (Ferreira et al., 2004a; Ferreira et al., 2004b). Taurine (TAU) is one of the main molecules present in energy drinks (Heckman et al., 2010) and its neuromodulatory function plays a key role in behavior modification (Mezzomo et al., 2017). In vertebrates, TAU can be produced endogenously and its beneficial roles in the CNS physiology include inhibitory modulation (analogous to GABA and glycine), antioxidant potential, membrane stability, osmoregulation, as well as the regulation of intracellular Ca^{2+} metabolism (Huxtable,

1992; Wu et al., 2000; Wu et al., 1992). TAU also modulates complex behaviors, such as aggressiveness (Fontana et al., 2016; Oja and Saransaari, 2007), fear, and anxiety (Kong et al., 2006; Mezzomo et al., 2016; Rosemberg et al., 2012), and even has been shown to modify hippocampal functions permanently (Franconi et al., 2004). Acamprosate (*N*-acetyl-homotaurine; a TAU analog) is used for treating alcoholism, and is believed to work by modulating inhibitory (GABA) and excitatory (glutamate) neurotransmitter activity that stimulate EtOH-mediated withdrawal responses (Witkiewitz et al., 2012).

Although TAU analogs exert positive effects to treat alcoholism, the influence of simultaneous TAU and EtOH consumption on both social and aversive behaviors is still poorly understood (Ferreira et al., 2006). Thus, considering that EtOH and TAU alone may affect different behavioral domains, the aim of the present study was to evaluate a potential effect of co-exposure to TAU and EtOH in the modulation of social and fear responses. Zebrafish are a common model species in alcohol psychopharmacology, and adults show several behavioral responses to both acute and chronic EtOH exposure that are similar to those observed at comparable doses and exposure regimens in mammals. For example, adult zebrafish show concentration-dependent decreases in both social behavior (shoaling) and fear behavior (predator avoidance) following acute exposure (Gerlai et al., 2006; Parker et al., 2012). Therefore, in the present study we tested the hypothesis that TAU would concentration-dependently affect EtOH-induced decreases in shoaling and antipredator responses in adult zebrafish.

2. Materials and Methods

2.1. Animals

A total of 192 wild-type zebrafish (*Danio rerio*) (4-6 months-old, ~50:50, male: female ratio, short fin strain) were obtained from a commercial supplier (Hobby Aquários,

RS, Brazil). Animals were acclimatized for 15 days before the experiments under standard laboratory conditions. Water condition was set at 25 ± 2 ºC and pH 7.1, while the illumination was provided by fluorescent lamps on a 14/10 light/dark photoperiod cycle (lights on at 7:00 a.m. and off 9:00 p.m). Fish were fed twice daily with commercial flake fish food (Alcon BASIC®, Alcon, Brazil) and the water quality was monitored by commercial kits for pH, nitrite, and ammonia (Alcon BASIC®, Alcon, Brazil). Animals were maintained in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. All protocols were previously approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (process number 026/2014).

2.2. Experimental design

TAU and EtOH were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany), respectively. TAU (42, 150, and 400 mg/L) and EtOH (0.25 v/v) were added directly to the tank water concurrently or alone, and the control group was exposed to non-chlorinated water **(Fig. 1A)**. Shoaling behavior was assessed during the exposure period and fish were later tested in the social preference test or in the predator exposure task **(Fig. 1B)**. TAU concentrations were chosen based on previous reports, where 42, 150, and 400 mg/L exerted significant neuromodulatory effects on zebrafish behavior (Fontana et al., 2016; Mezzomo et al., 2016; Rosemberg et al., 2010). EtOH concentration was chosen as described elsewhere (Fontana et al., 2016; Gerlai et al., 2000), in which 0.25% (v/v) positively modulates aggression in zebrafish.

2.3. Shoaling behavior test

Four fish were simultaneously placed in the test tank (25×15×10 cm length x height x width) and group behavior was analyzed during the 1 h exposure period. Although zebrafish form larger shoals in their natural environment, previous data show reproducible social behavior data using four fish per shoal (Canzian et al., 2017; Green et al., 2012; Muller et al., 2017; Schmidel et al., 2014). To investigate the temporal effects of TAU and EtOH on fish behavior across the exposure period, we assessed group activity at different time intervals (T1: 0–5 min; T2: 30–35 min; T3: 55–60 min) (n = 6 shoals *per* treatment group). After recording, the apparatus was cleaned, and a new group was tested. Videos were exported to Image J 1.49 software and shoaling behavior was assessed using screenshots taken every 15 s during the 5-min trials (20 screenshots *per* trial) (Green et al., 2012; Schmidel et al., 2014). A social interaction index was measured as described previously, which included fish proximity to conspecifics with visual contact to other member of shoal at a maximum distance of 3 body lengths (6 cm) (Canzian et al., 2017). The indexes vary from "0" (low cohesion) to "6" (complete cohesion). Screenshots were also calibrated proportional to the size of the tank to allow the quantification of total inter-fish distance and shoal area using Image J 1.49 software. Zebrafish vertical position was measured manually by counting the number of animals in the upper half of apparatus every 15 s. Two trained observers (inter-rater reliability > 0.90) blind to the experimental condition analyzed the results.

2.4. Social preference test

Zebrafish is a social species that exhibits natural preference for conspecifics under neutral and slightly aversive conditions (Saverino and Gerlai, 2008). To assess the social preference, fish were placed individually in the social preference apparatus (25×15×10 cm length x height x width) (n = 12 *per* treatment group). This experimental tank was

virtually divided in 4 segments, where S4 represents the closest segment to conspecifics, S3 and S2 represent transition segments, and S1 represents the farthest segment in relation to conspecifics. In one side of the tank (S1 area), zebrafish had visual contact with empty aquarium, while in the other segment (S4 area) four conspecifics were placed in an identical tank as stimulus. After the exposure period, zebrafish were acclimated for 30 s in the test tank. Behavioral recordings were further performed for 60 s based on the protocol described previously (Gerlai et al., 2000). The number of transitions and time spent in each segment were quantified using the ANY-maze^{TM} software (Stoelting, CO, USA) at 30 frames/s.

2.5. Predator exposure test

Antipredatory behavior is an adaptive response exhibited spontaneously in nature with a key importance for survival that may reflect aversion to a dangerous situation (Csanyi and Gervai, 1986; Gerlai, 1993). Fish were individually placed in a tank (25×15×10 cm length x height x width) (n = 8 per treatment group) and the experiments were performed based on previous protocols (Gerlai et al., 2000; Ladu et al., 2015). Briefly, the experimental tank was virtually divided in 4 segments, in which one side of the tank allowed a visual contact to an empty aquarium (S1), while the other side had an identical tank containing a tiger Oscar fish (*Astronotus ocellatus*) (S4), a natural predator of zebrafish. The other areas (S2 and S3) represent transition segments.

Behavioral activities were recorded for 6 min and both transitions and time spent in the predator area were quantified (ANY-maze^{TM}, Stoelting, CO, USA). Freezing was defined as complete immobility of fish for > 2 s with increased opercular beating rate, while 'risk assessments' were defined as partial or fast (< 1 s) entries in the predator area with subsequent fast movements towards the safer area of the tank (Kalueff et al., 2013).

Freezing duration and risk assessment episodes were manually counted by two trained observers (inter-rater reliability >0.85) blind to the experimental condition.

2.6. Statistical analyses

Data normality and homogeneity of variances were performed by the Kolmogorov-Smirnov and Bartlett's test, respectively. Since all data were normally distributed and homoscedastic, data were expressed as means ± standard error of the mean (S.E.M) and further analyzed by one or repeated measures analysis of variance (ANOVA or RM-ANOVA) followed by Student Newman Keuls multiple comparisons test. RM-ANOVA was performed using treatment and time as factors when shoaling behavior was assessed in different time intervals. The significance was set at $p \leq 0.05$.

3. Results

3.1. Shoaling behavior task

We first evaluated the effect of TAU alone **(Fig. 2)** on shoaling behavior across the exposure period. Behavioral values obtained for each shoal were depicted as representative heat maps **(Fig. 2A)**. RM-ANOVAs showed a significant time effect for inter-fish distance ($F_{(2,40)} = 4.592$, $p < 0.05$), number of interactions ($F_{(2,40)} = 6.71$, $p < 0.01$), and animals in the upper segment ($F_{(2,40)} = 8.475$, $p < 0.001$). There was a significant treatment × time interaction observed for shoal area ($F_{(6,40)} = 3.595$, $p < 0.01$). The interaction was characterized by TAU 150 mg/L increasing the inter-fish distance at T3 when compared to T2, T1, and to its respective control. Shoal area increased in TAU 150 and TAU 400 groups, with higher T2 values in TAU 400 when compared to its respective control. Additionally, the control group showed more interaction at T2, while TAU 150 mg/L decreased the number of interactions at T3. Regarding the vertical

distribution, the number of fish in the upper half increased in TAU 150 and TAU 400 groups at T2 and T3 **(Fig. 2B)**.

Schematic representation of TAU and EtOH association effects on shoaling behavior were shown as heat maps **(Fig. 3A)**. RM-ANOVA yielded a significant treatment × time interaction for inter-fish distance ($F_{(8,50)} = 2.336$, $p < 0.05$) and shoal area ($F_{(8,50)} = 2.462$, $p < 0.05$). Analyses across time revealed significant time effects for inter-fish distance ($F_{(2,50)} = 8.867$, $p < 0.001$), shoal area ($F_{(2,50)} = 7.630$, $p < 0.01$), number of interactions ($F_{(2,50)} = 6.708$, $p < 0.01$), and animals in the upper segment ($F_{(2,50)} = 8.346$, $p < 0.001$). Post hoc analyses revealed that the inter-fish distance increased across time in EtOH and TAU/EtOH groups. Although EtOH alone decreased shoal cohesion across time, TAU 42/EtOH and TAU 150/EtOH shoal areas increased in T3 when compared to T1. When compared to their respective controls, EtOH (T2) and TAU/150 (T3) groups showed increased shoal area. Furthermore, EtOH decreased the number of interactions across time with lower values in T2 and T3 compared to their respective controls. TAU 42/EtOH and TAU 400/EtOH groups increased the number of fish in the upper segment across time **(Fig. 3B)**.

3.2. Social preference test

Fig. 4A and **Fig. 5A** show the results of the social preference task. Although TAU alone did not alter the number of transitions and time spent in the conspecific section (S4) (**Fig. 4A**), TAU 400/EtOH showed a decrease of the relative time spent in S4 ($F_{(4,48)} = 2.240$, $p < 0.05$) **(Fig. 5A)**.

3.3. Aversive responses in the predator exposure task

Fig. 4B and **Fig. 5B** depict the behavioral responses observed in the predator exposure task. All TAU groups showed a decrease in the relative number of entries in the predator area ($F_{(3,37)} = 5.210$, $p < 0.01$). Nonetheless, only TAU 150 mg/L reduced the relative time spent in predator area ($F_{(3,37)} = 4.427$, $p < 0.01$). Furthermore, TAU 42, TAU 150, and TAU 400 groups performed fewer risk assessments ($F_{(3,37)} = 12.48$, $p < 0.0001$) without changing the freezing duration **(Fig. 4B)**. EtOH decreased the time spent in the predator area ($F_{(4,52)} = 2.667$, $p < 0.05$), while the number of transitions did not alter. Although freezing duration did not significantly change, risk assessments reduced in EtOH, TAU 42/EtOH, TAU 150/EtOH, and TAU 400/EtOH groups ($F_{(4,52)} = 15.97$, $p < 0.0001$).

4. Discussion

The aim of this study was to examine how TAU modulates EtOH-induced social behavior and fear responses of zebrafish. This was the first study to assess the influence of TAU on EtOH-induced shoaling differences across the entire exposure period, thus giving the potential to uncover temporal effects on social behavior. TAU partially rescued EtOH-induced social interaction deficits, with EtOH decreasing social preference only in fish exposed concurrently to the highest dose (TAU 400). Both TAU and EtOH-exposed fish showed reduced exploration in the predator area and decreased risk assessment episodes, suggesting modulation of the antipredatory response. However, TAU/EtOH co-treated animals did not change the exploration in the predator area but did show reduced risk assessment episodes, indication high-level modulation of EtOH-induced fear-inhibition by TAU.

Temporal analyses of shoaling behavior revealed significant effects of TAU and EtOH alone or concurrently over 30 minutes. These data replicate previously described

alcohol-related effects on zebrafish shoals over 60-minutes exposure (Gerlai et al., 2000). Regarding TAU actions on shoal behavior, TAU 150 and TAU 400 mg/L decreased shoaling. Zebrafish is a prominent species to explore the social behavior domain based on their ability to form larger shoals (Stewart et al., 2014). Thus, shoaling behavior serves as a valuable tool to model normal and pathological social situations (Buske and Gerlai, 2011; Gerlai, 2014), in which four zebrafish in a same test tank rapidly interact with conspecifics (Schmidel et al., 2014). Since the mechanisms involved in zebrafish shoaling are strongly related to the cognitive performance and decision-making strategies (Sporns, 2010), TAU may act on the CNS affecting the group behavior. Importantly, anxiolytic drugs modulate defensive behaviors in zebrafish and decrease shoaling (Hamilton et al., 2017). Considering the anxiolytic-like effects of TAU in zebrafish (Mezzomo et al., 2016), the impaired shoal behavior observed may be directly related to the effects of TAU on anxiety-like behaviors. Additionally, TAU decreased both risk assessments and the number of transitions to the predator area, and fish exposed to 150 mg/L spent less time in predator area. Antipredatory responses serve as valuable tools for translational studies aiming to explore abnormally overstated or misdirected fears in humans. Furthermore, the neurobiological mechanisms underlying antipredatory patterns are predictably related to those involved in abnormal human fear responses (Gerlai, 2010). Thus, we suggest that TAU increases antipredatory behaviors in aversive contexts, exerting a complex role on two behavioral domains (social and defensive behaviors).

Although the observed effects of alcohol consumption on social domain are complex and often contradictory (Monahan and Lannutti, 2000), our data corroborate previous demonstrations that acute EtOH disrupts shoal polarization and reduces shoal cohesion in zebrafish (Gerlai et al., 2000; Miller et al., 2013). The behavioral effects of alcohol are associated with changes in various neurotransmitter systems (Banerjee et al.,

2014), depending on both genetic and environmental factors (Vengeliene et al., 2008). Although the precise mechanisms involved in shoaling behavior are still under debate (Oliveira, 2013), the modulatory effects of EtOH on different neurotransmitter pathways (e.g., GABAergic, serotonergic, dopaminergic and glutamatergic systems) may play a role in this response (Kumar et al., 2010; Roberto et al., 2004; Seo et al., 2008). Furthermore, low EtOH concentrations elicit anxiolytic-like responses in both rodents (Varlinskaya and Spear, 2002) and zebrafish (Baggio et al., 2017; Echevarria et al., 2011; Mathur and Guo, 2011), which may be directly related to reduced social behavior.

Alcohol disinhibits previously punished operant responses through the anxiolytic-like effects of activation of the benzodiazepine/GABA receptors (Koob et al., 1988), and this may decrease awareness of hazardous situations (Spear, 2018). Although the number of risk assessments did not change in alcohol-exposed zebrafish, they spent less time in the predator area, suggesting an increased antipredatory response. Our data confirmed that 0.25% EtOH (v/v) increases defensive responses in dangerous situations, as observed previously where zebrafish increase jumping behavior when exposed to a robotic predator (Gerlai et al., 2000). Importantly, the decreased awareness of risk commonly associated with alcohol effects in humans, is observed following 1% EtOH (v/v) exposure in zebrafish, suggesting a concentration-dependent effect (Gerlai et al., 2000). Thus, EtOH modulates awareness in dangerous situations and social behaviors possibly due to its action on different behavioral domains (Oliveira et al., 2013; Parker et al., 2014; Tran et al., 2016a, b).

Similar to the effects of EtOH, TAU/EtOH co-treatment impaired shoaling depending on the concentration tested, whereas only TAU 400 mg/L decreased social preference. Otherwise, TAU rescued the effects of ethanol in terms of the number of social interactions. Different tasks may elicit distinct behavioral phenotypes, which can

result in various responses at a same behavioral domain depending on the context. For example, the social preference task is related to approach to conspecifics, while the shoaling test measures social interactions and shoal cohesion (Pham et al., 2012). Hence, the distinct behavioral phenotypes related to the social domain we observed here suggests a complex neurobehavioral effect of TAU/EtOH in zebrafish. Although TAU/EtOH groups showed reduced risk assessments, no other behaviors changed in the predator test. Therefore, TAU/EtOH prevented the increase in antipredatory responses following TAU or EtOH exposure alone. TAU displays a concentration-dependent effect on anxiety-like behavior, locomotion, exploration, and aggression following acute EtOH exposure (Fontana et al., 2016; Rosemberg et al., 2012). Moreover, TAU counteracts EtOH-induced neurotoxicity by decreasing brain alcohol levels and preventing locomotor impairments (Rosemberg et al., 2012). For instance, the mechanisms underlying the behavioral responses observed in TAU/EtOH groups are generally attributed to the neuromodulatory role of TAU in the brain as a $GABA_A$ agonist (Ananchaipatana-Auitragoon et al., 2015; Rosemberg et al., 2010; Taranukhin et al., 2009; Taranukhin et al., 2010). Nonetheless, future studies are required to elucidate the mechanisms underlying the behavioral responses observed following TAU/EtOH association.

5. Conclusion

In summary, concurrently administered TAU and EtOH modulates zebrafish social behavior. In the predator test, TAU/EtOH co-treatment did not affect antipredatory behavior, and this may be the results of similar responses observed in fish exposed to TAU or EtOH alone. Our findings also point to a complex relationship between shoal cohesion, social preference, and antipredatory behavior. Therefore, more studies are

necessary to investigate how TAU and EtOH association modulates zebrafish neurobehavioral phenotypes and influence different behavioral domains.

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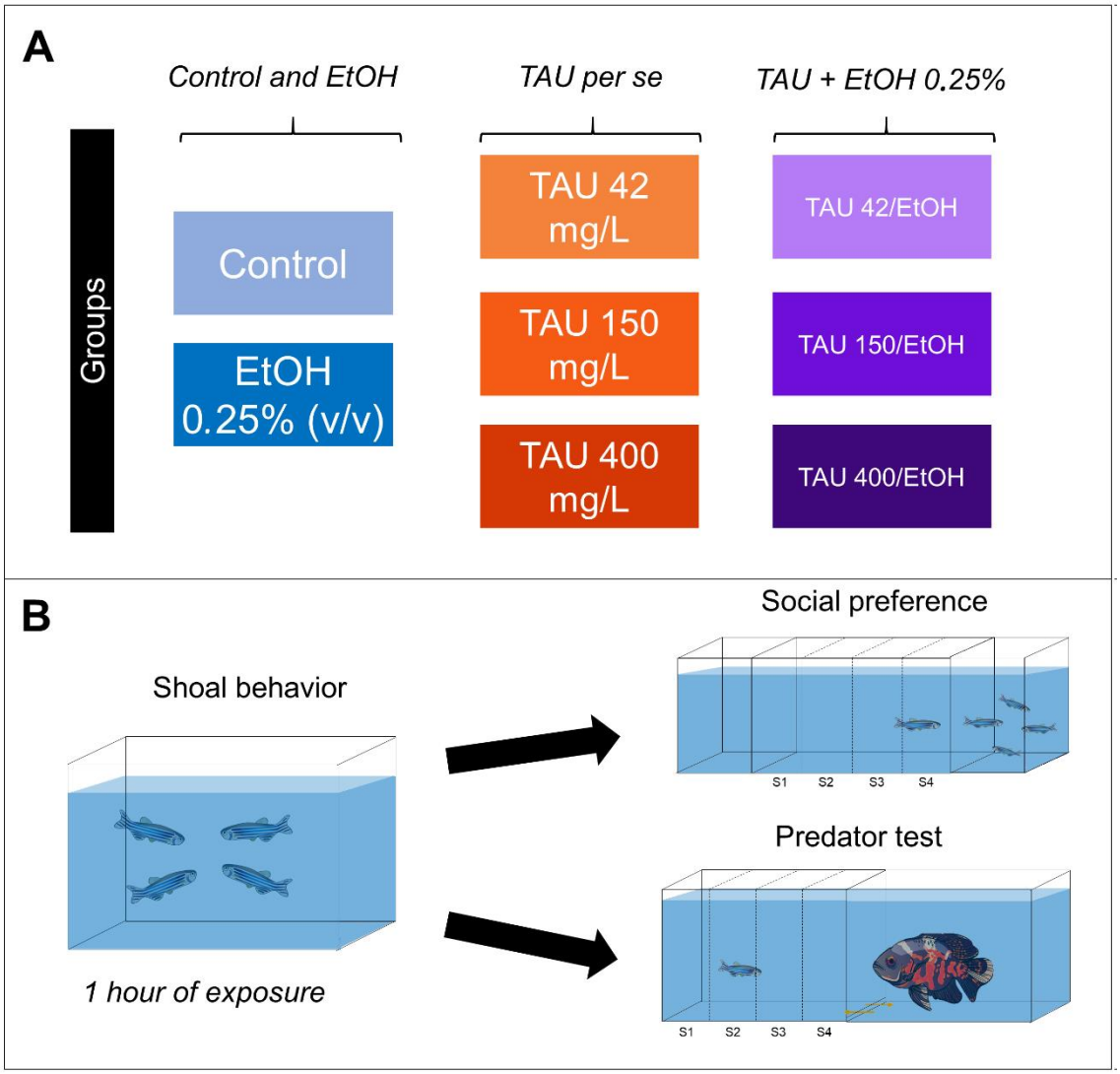

Fig. 1. Experimental design and behavioral tasks. **(A)** Schematic representation of the experimental groups. **(B)** Behavioral tests used to assess shoal behavior during the exposure period, social preference, and antipredatory responses. Four animals were exposed to water (control), 0.25 (v/v) EtOH, and TAU (42, 150, and 400 mg/L) alone or co-treated with TAU and EtOH for 1 h. Shoaling behavior was assessed at different time intervals and, afterwards, two animals were randomly tested in the social preference test while the other fish were submitted to the predator test. S1 is the segment farther from conspecifics/predator, S2 and S3 are transition areas, while S4 is the closest area from conspecifics/predator.

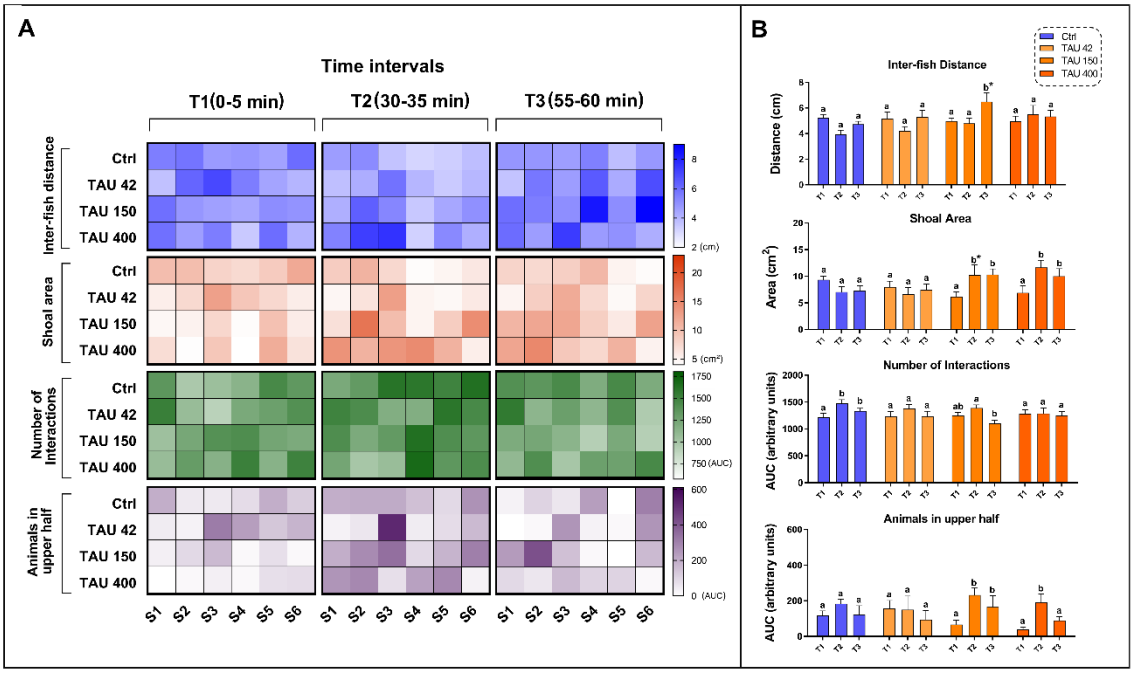

Fig. 2. Effects of TAU on shoaling behavior at different time intervals across 1 h of exposure. **(A)** Representative heat maps that depict the behavioral endpoints measured. A more intense color indicates higher values. **(B)** Behavioral parameters analyzed across time. Data were represented as mean ± S.E.M. and analyzed by repeated measures analysis of variance (RM-ANOVA) followed by Student Newman Keuls multiple comparisons test. RM-ANOVA was performed using treatment and time as factors when different time intervals were analyzed. Different letters indicate statistical differences across time within groups, whereas the asterisks reveal statistical significances of a certain time period compared to its respective control ($p < 0.05$, $n = 6$ per group).

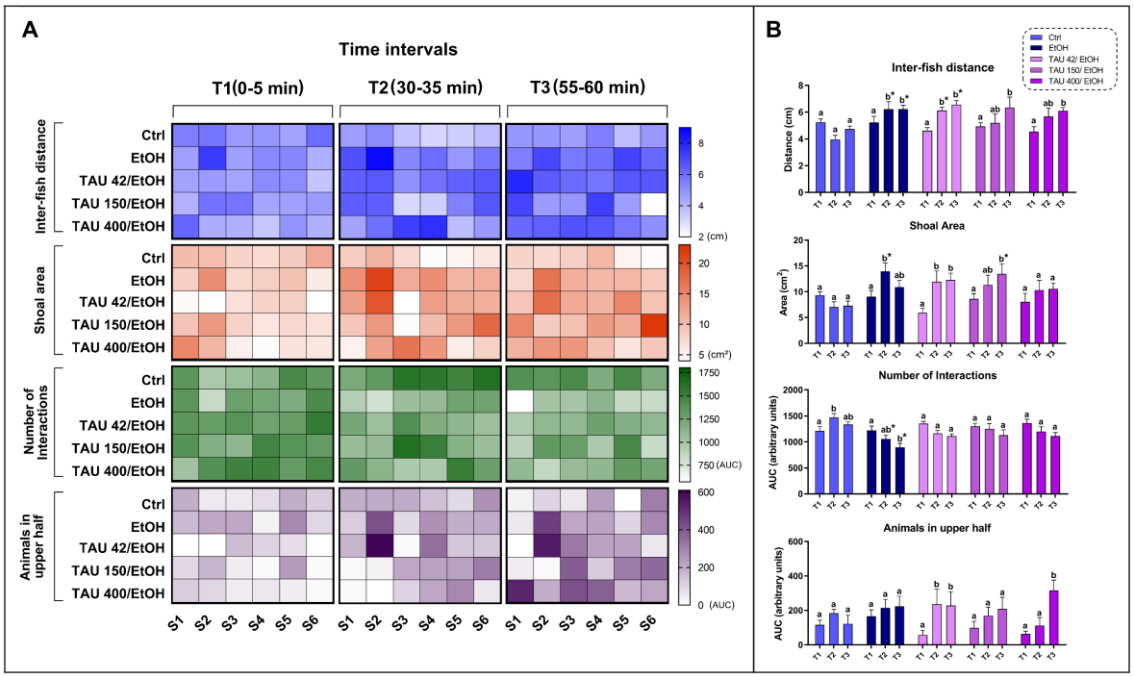

Fig. 3. Effects of EtOH and TAU/EtOH cotreatment on shoaling behavior at different time intervals across 1 h of exposure. (A) Representative heat maps that depict the behavioral endpoints measured. A more intense color indicates higher values. (B) Behavioral parameters analyzed across time. Data were represented as mean ± S.E.M. and analyzed by repeated measures analysis of variance (RM-ANOVA) followed by Student Newman Keuls multiple comparisons test. RM-ANOVA was performed using treatment and time as factors when different time intervals were analyzed. Different letters indicate statistical differences across time within groups, whereas the asterisks reveal statistical significances of a certain time period compared to its respective control ($p < 0.05$, $n = 6$ per group).

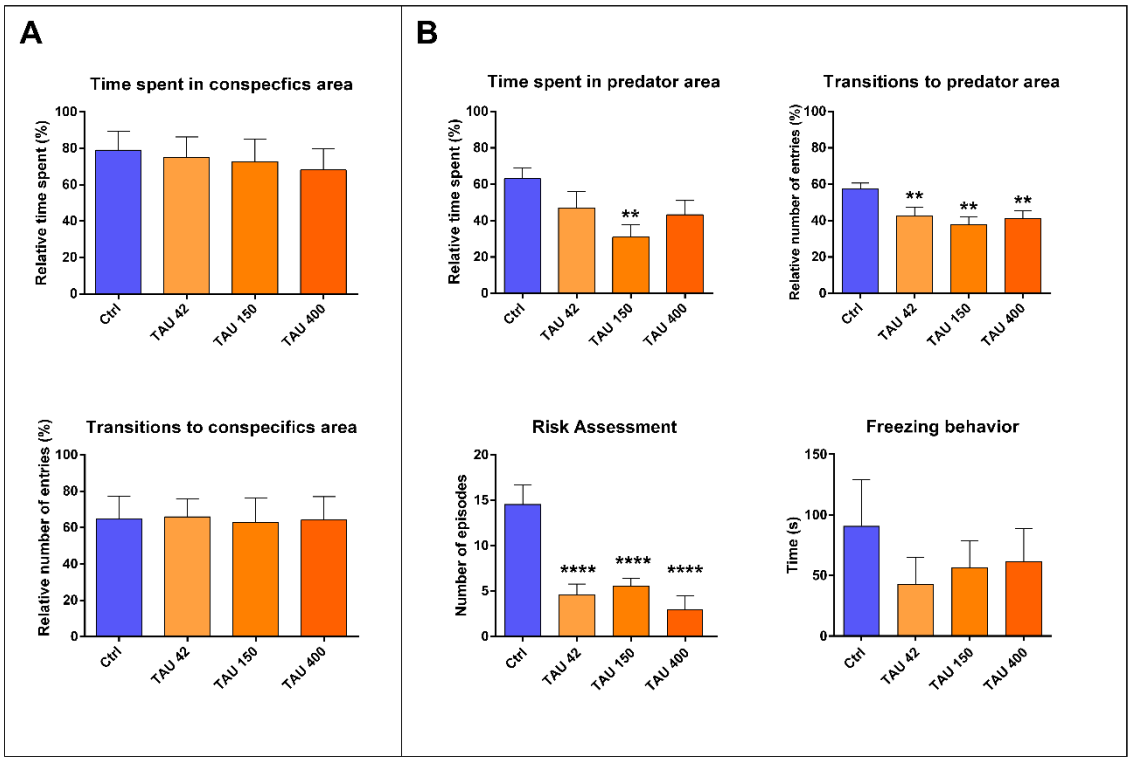

Fig. 4. Effects of TAU on social preference and fear responses of zebrafish exposed to the predator. **(A)** Social preference behaviors. **(B)** Antipredatory-related behavioral endpoints. Data were represented as mean ± S.E.M. and analyzed by one-way ANOVA, following by Student Newman Keuls multiple comparisons test (* $p < 0.05$, n = 10–12 per group).

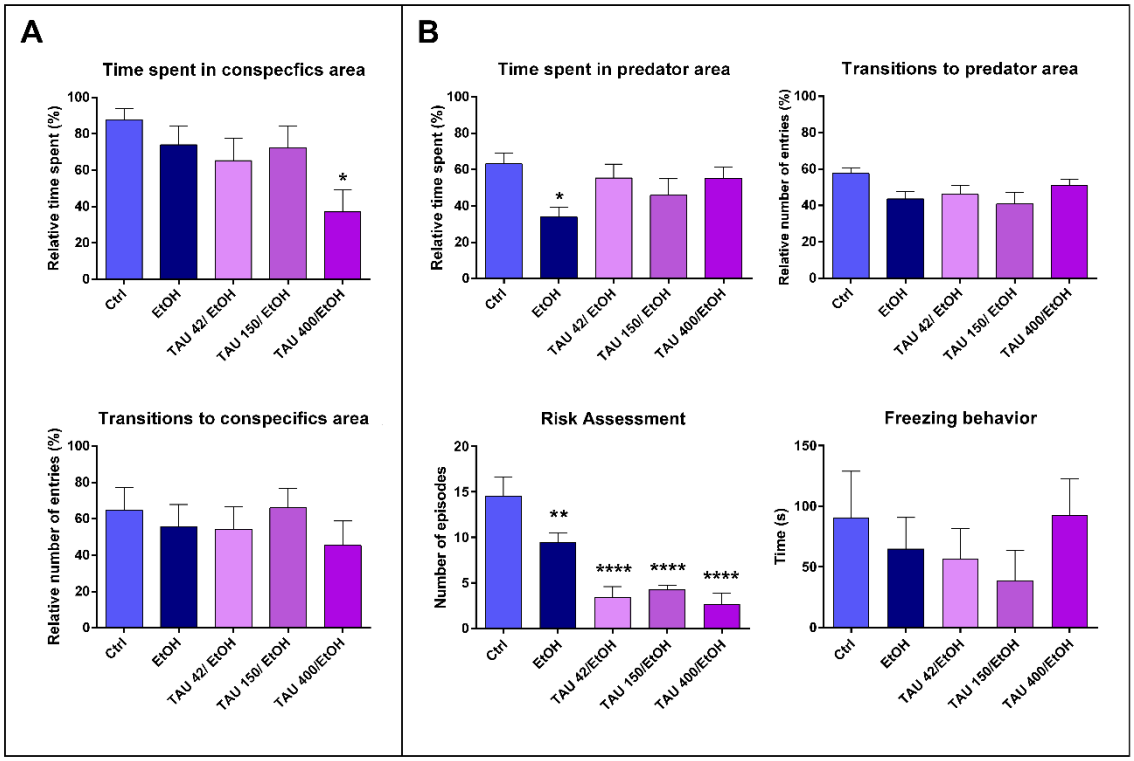

Fig. 5. Effects of EtOH and TAU/EtOH co-treatment on social preference and fear responses of zebrafish exposed to the predator. **(A)** Social preference behaviors. **(B)** Antipredatory-related behavioral endpoints. Data were represented as mean ± S.E.M. and analyzed by one-way ANOVA, following by Student Newman Keuls multiple comparisons test (* $p < 0.05$, n = 10–12 per group).

ARTIGO 3

Taurine protects from pentylenetetrazole-induced behavioral and neurochemical changes in zebrafish

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Taurine Protects from Pentylenetetrazole-Induced Behavioral and Neurochemical Changes in Zebrafish

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Abstract

Epilepsy is a common neurological disorder characterized by recurrent unprovoked seizures, which culminate in various neurobehavioral and neurochemical changes. Taurine (TAU) is an amino sulfonic acid which acts an endogenous inhibitory neuromodulator. Moreover, TAU displays intrinsic antioxidant activity, contributing to its beneficial actions in the CNS. Here, we evaluated whether TAU pretreatment protects from pentylenetetrazole (PTZ)-induced behavioral alterations and oxidative stress-related parameters in zebrafish brain tissue. Fish were pretreated with 42, 150, and 400 mg/L TAU (40 min) and further exposed to 10 mM PTZ (20 min) to analyze the seizure-like behaviors. As a positive control, another group was previously treated with 75 μM diazepam (DZP). Afterwards, biochemical experiments were performed. All TAU concentrations tested decreased seizure intensity in the first 150 s. Importantly, 150 mg/L TAU attenuated seizure-like behavioral scores, decreased seizure intensity, reduced the frequency of clonic-like seizures (score 4), and increased the latency to score 4. TAU (150 mg/L) also prevented oxidative stress in PTZ-challenged fish by decreasing lipid peroxidation and protein carbonylation and preventing changes on nonprotein thiol levels. No significant changes were observed in MTT assay and LDH activity. Differently than observed in DZP group, TAU did not affect the overall swimming activity of fish, suggesting different mechanisms of action. Collectively, we show that TAU attenuates PTZ-induced seizure-like behaviors and brain oxidative stress in zebrafish, suggesting the involvement of antioxidant mechanisms in neuroprotection.

Keywords Epilepsy · Seizure-like behaviors · Neuroprotection · Pentylenetetrazole · Oxidative stress · Zebrafish

Introduction

Epilepsy is a common neurological disorder characterized by recurrent unprovoked seizures that influence behavioral and neurobiological functions [1, 2]. Approximately 65 million people suffer with epilepsy [3], which can lead to psychosocial disorders and even death [4–6]. The occurrence of sudden and abnormal neuronal discharges disrupts cellular metabolism and increases reactive oxygen species (ROS) formation triggering oxidative stress, mechanisms usually associated to neurodegeneration in epileptic patients [7].

Experimental models of epilepsy-related pathogenesis represent interesting strategies to investigate seizure-related phenotypes and their underlying neurochemical mechanisms. Accordingly, the use of neurotoxins (e.g., pentylenetetrazole (PTZ) and kainic acid) represents chemical models that impair GABAergic and glutamatergic neurotransmission, promoting excitotoxicity and seizures [2, 8–11]. PTZ acts in the central nervous system (CNS) as a $GABA_A$ receptor antagonist [12],

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and acute PTZ exposure has been considered a suitable protocol to assess seizure-like behaviors and possible therapeutic approaches [13]. Since one-third of epileptic patients do not respond to antiepileptic drugs (AEDs) and experience important side effects (e.g., cognitive deficit, sedation, increased aggression, and impulsivity) [14, 15], novel pharmacological strategies aiming to attenuate seizures are needed.

Taurine (TAU) is an amino sulfonic acid obtained from the diet and also synthesized in the CNS from sequential cysteine oxidation reactions [16]. This molecule has a pleiotropic role since it regulates Ca^{2+} metabolism, osmoregulation, and membrane potential and acts as antioxidant and inhibitory neuromodulator [17–21]. Although TAU positively modulates $GABA_A$ and glycine receptors, the existence of specific TAU receptors has been postulated [22]. Interestingly, TAU counteracts excitatory synaptic activity in the dentate gyrus of rodents, a CNS region involved in seizure initiation [23–26]. Thus, assessing the neuroprotective effects of TAU may provide important data regarding new alternatives to prevent seizures.

In a translational neuroscience perspective, the use of zebrafish (*Danio rerio*) has grown exponentially during the last decade, offering a real possibility for high-throughput screens [27–30]. Despite the neuroanatomical differences in comparison to the human CNS, zebrafish presents numerous brain areas with homologous functions [31–33]. The evolutionarily conserved physiological responses and the well-characterized behavioral repertoire [29] make zebrafish a suitable model organism to investigate the neurochemical mechanisms underlying the protective roles of TAU in vertebrates [21]. Although the seizure-related behavioral scores following PTZ exposure in adult zebrafish have been characterized previously [2], there are no data regarding the effects of PTZ on oxidative stress-related parameters and a potential protective role of TAU in PTZ-challenged fish. Thus, considering the positive effects of TAU as antioxidant and inhibitory neuromodulator in the CNS, we investigate whether TAU pretreatment prevents PTZ-induced behavioral and neurochemical changes in adult zebrafish.

Materials and Methods

Animals

Subjects were adult *short-fin* zebrafish (*Danio rerio*) (4–6 months old, ~50: 50 male to female ratio, weighing 0.250–0.400 g) obtained from a local distributor (Hobby Aquariums, Santa Maria, RS). Fish were acclimated in the laboratory for 15 days in 50 L tanks with a maximum density of 2 animals/L containing dechlorinated water kept under constant aeration, mechanical and chemical filtration at 25 ± 2 °C, pH = 7.1. The water chemical conditions were monitored using commercial kits for determining pH, nitrite, and ammonia (Alcon Basic®, Alcon, Brazil). Illumination was provided by fluorescent lamp tubes adjusted to a 14/10 h light/dark photoperiod cycle (lights on at 7:00). Fish were fed twice daily with commercial flake food (Alcon Basic®, Alcon, Brazil) and maintained in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. All experimental procedures were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (protocol number 8707070316).

Pharmacological Manipulations

Before PTZ exposure, animals were pretreated with TAU (42, 150, and 400 mg/L) for 40 min. These concentrations were chosen based on previous reports, which showed positive effects of TAU on zebrafish behavior [20, 34–36]. As a positive control, another cohort was exposed to 75 μM diazepam (DZP) for 40 min [2], while the control (CTRL) group was kept in dechlorinated water for the same period. The induction of seizure-like behaviors was further performed using 10 mM PTZ (20 min) in a 1-L tank. Both exposure period and PTZ concentration were chosen to allow the quantification of seizure-related behavioral scores without fish mortality [2]. Figure 1 shows a schematic drawing explaining the pharmacological manipulations and the experimental groups.

Seizure-Like Behavioral Scores

During PTZ exposure, seizure-like behaviors were manually computed by two observers blind to the experimental condition (inter-rater reliability > 0.90) using the scores depicted in Table 1 as described previously [2]. Seizure intensity was estimated by the area under the curve (AUC) of the scores obtained for each cohort across the 20-min exposure period. The latency to reach score 4 represents the time in which animals displayed clonic seizure-like behavior, while score 4 events represent the frequency of these episodes. Behavioral activities were recorded using the ANY-maze™ software (Stoelting CO, USA) at 30 frames/s.

Spatiotemporal Reconstructions of Swimming Behavior

Spatiotemporal reconstructions of the swimming pattern were performed using the spatial coordinates (*x* center and *y* center) across fractions of seconds [20, 34, 37, 38]. For each experimental group, track data were analyzed based on their similarities by two trained observers (inter-rater reliability > 0.85), on a consensus basis [20, 39, 40]. Results were further exported into separated spreadsheets and the representative swimming traces were depicted as scatter plots (Graphis 3D graphing software™). The *x* center (horizontal position), *y* center (vertical position), and time were plotted on the X, Y, and Z axes, respectively. A spectrum of colors (blue to red) was used to represent the position of fish in a 5-min period (0–300 s).

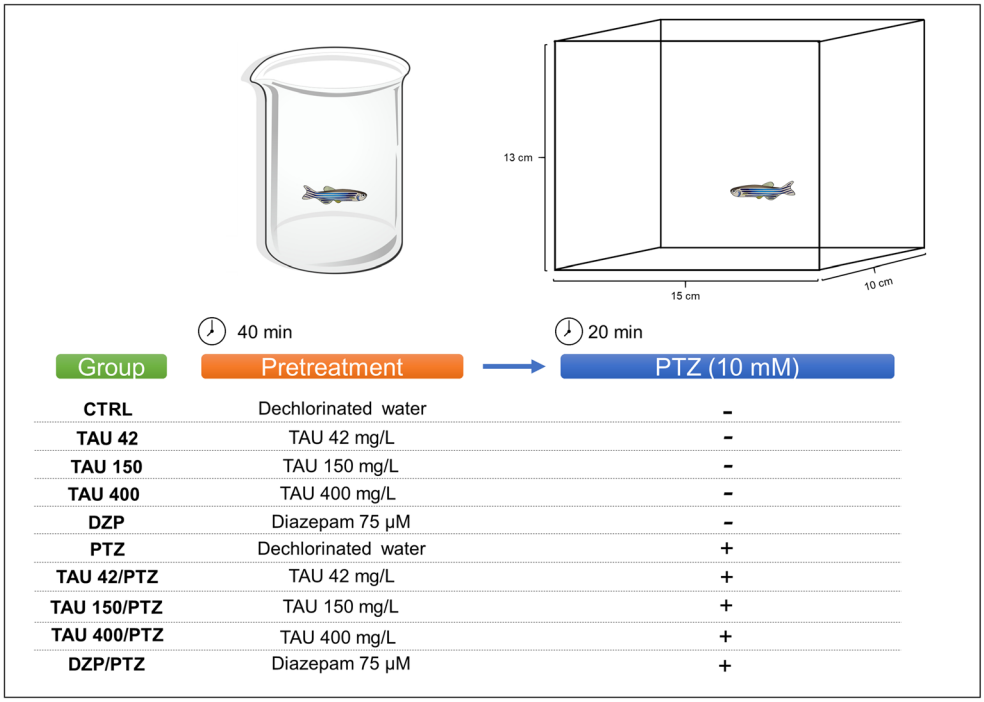

Fig. 1 Representative illustration of the experimental design. Animals were pretreated for 40 min (green and orange column) and further exposed to PTZ (blue column) for 20 min. Seizure-like behavioral scores were evaluated each 30 s

Sample Preparation

To assess enzymatic and non-enzymatic antioxidant defenses and oxidative stress-related parameters, brain samples were prepared as described previously [34]. After PTZ exposure, fish were immediately euthanized, and the brains were further dissected and homogenized in 1 mL of phosphate buffered saline (PBS), pH 7.4, containing 137 mM NaCl, 10.1 mM Na_2HPO_4, and 1.76 mM KH_2PO_4. Four brains were pooled per sample, homogenates were further centrifuged at 700×*g* for 5 min at 4 °C to remove cell debris, and the supernatants were used for the experiments. To determine cell viability and LDH activity, intact brain tissues were transferred to 24-well culture plates containing 0.5 mL of 10 mM HBSS-HEPES buffer (pH 7.4) after euthanasia. All plates were maintained at 37 °C throughout the experiment [41].

Table 1 Seizure-like behavioral scores in PTZ-challenged zebrafish

Scores	Behavioral endophenotypes
0	Swimming in the bottom area
1	Increased swimming activity and opercular movements
2	Erratic movements and burst swimming
3	Circular swimming in the top of the tank
4	Clonic seizure-like behavior (abnormal muscular contraction, corkscrew swimming)
5	Tonic seizure-like behavior (loss of body posture in the bottom of the tank)

Table adapted from Mussulini et al. [2]

Determination of Antioxidant Enzyme Activities

Superoxide dismutase (SOD) activity was measured using a colorimetric assay to detect adrenaline oxidation rate at 480 nm [42]. Supernatants from zebrafish brain tissue (20–30 µg protein) were mixed with glycine buffer (50 mM, pH 10.2) following substrate addition (1 mM adrenaline). SOD activity was expressed as U SOD/mg protein [34]. Catalase (CAT) activity was assessed by measuring the decrease in the absorbance of hydrogen peroxide at 240 nm [43]. Results were expressed as µmol/min/mg protein using the conditions described previously for zebrafish (50 mM potassium phosphate buffer, pH 7.0, 0.3 M H_2O_2, and 20–30 µg protein) [34]. Glutathione-S-transferase (GST) activity was determined as reported previously [44]. Supernatants from zebrafish brain tissue (40–60 µg protein) were mixed with potassium phosphate buffer (20 mM, pH 6.5) and reduced glutathione (10 mM). Later, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) diluted in ethanol was added as substrate and samples were read at 340 nm. GST activity was expressed as nmol of S-(2,4-dinitrobenzyl) glutathione (GS-DNB)/min/mg protein.

Non-enzymatic Antioxidant Defenses

Nonprotein thiol levels (NPSH) were measured as described elsewhere [45–47]. Briefly, supernatants from zebrafish brain tissue were mixed with 10% trichloroacetic acid (TCA) and centrifuged at 3000×*g* for 10 min at 4 °C. Supernatants (60–80 µg protein) were then mixed with 10 mM 5,5-dithio-bis-2-

nitrobenzoic acid (DTNB) dissolved in ethanol. The resultant yellow complex was measured at 412 nm after 1 h. Results were expressed as nmol SH/mg protein.

Oxidative Stress-Related Parameters

Lipid peroxidation was evaluated by measuring thiobarbituric reactive substances (TBARS) as described previously [34, 48]. Supernatants from zebrafish brain tissue (80–100 μg protein) were added to 0.16 mL of 15% trichloroacetic acid and further centrifuged at 10,000×*g* for 10 min. Supernatants were further mixed with 0.1 mL of 0.67% thiobarbituric acid and boiled for 30 min. TBARS levels were determined at 532 nm using malondialdehyde (MDA) as standard. Results were expressed as nmol MDA/mg protein. Protein carbonylation was estimated by protein precipitation in the presence of trichloroacetic acid and dinitrophenylhydrazine (DNPH) [49]. Protein samples (200 μL) were mixed with 0.15 mL of 10 mM DNPH and incubated for 1 h. Later, 0.125 mL of SDS (3.0%), 0.5 mL of heptane (99.5%), and 0.5 mL of ethanol (99.8%) were added to samples and mixed for 30 s. Samples were centrifuged at 1000×*g* for 15 min and the supernatant was discarded. Finally, the pellet was homogenized in 0.25 mL of 3% SDS and the amount of carbonylated proteins was determined at 370 nm. Results were expressed as nmol carbonyl/mg protein and calculated using a molar extinction coefficient of 22.000 M/cm.

MTT Assay

MTT assay was measured by the conversion of intracellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to purple formazan by mitochondrial succinate dehydrogenases, which is a standard protocol that predicts cell viability [50, 51]. After euthanasia, whole zebrafish brains were immediately immersed in 0.5 mg/mL of MTT solution in a covered water bath shaker for 20 min at 37° [41, 52]. After the incubation period, 300 μL dimethyl sulfoxide (DMSO) was added per sample and kept overnight under constant homogenization to allow the solubility of formazan crystals in order to quantify the product formation. Lastly, 200 μL of extracted formazan was added to 96-well plates and cell viability was assessed at 560 and 650 nm. Results were expressed as a percentage of control [41].

Lactate Dehydrogenase Activity

Lactate dehydrogenase (LDH) activity, a parameter that estimates cell survival [53], was measured as described elsewhere [41]. Briefly, after incubation of intact zebrafish brain tissues in HBSS-HEPES buffer for 10 min, the medium was removed and used to measure the extracellular conversion of lactate to pyruvate. Thus, 1,10-phenanthroline was dehydrogenated to a colored complex by reacting with NADH, which was measured at 490 nm [41, 54]. Total LDH activity was estimated in lysates of brain tissue. Results were expressed as percentage of total LDH activity per sample.

Protein Quantification

Total protein amount was measured according to Bradford [55] using bovine serum albumin as standard.

Statistics

Normality of data and homogeneity of variances were analyzed by Kolmogorov-Smirnov and Bartlett's tests, respectively. Nonparametric data (seizure scores across 20 min) were expressed as median ± interquartile range, and the area under curve was calculated to estimate seizure intensity. The cumulative frequency was expressed as the percentage of animal that reached each score across the 20-min observation period. Parametric data were expressed as means ± standard error of mean (S.E.M) and further analyzed using one- or two-way analysis of variance (ANOVA), followed by Tukey's post hoc test. The level of significance was set at $p \leq 0.05$.

Results

Seizure Related-Phenotypes

The occurrence of seizure-related behavioral phenotypes and the percentage of animals that reach each score were assessed in a 20-min PTZ exposure period (Fig. 2). PTZ elicited seizure-like behaviors and zebrafish displayed corkscrew swimming and immobility, which closely resemble tonic/clonic seizures (scores 4 and 5). As expected, DZP attenuated PTZ-induced changes in behavior and 150 mg/L TAU showed a similar response (Fig. 2a). Furthermore, while 100% of PTZ-exposed fish reached score 4 after 210 s, the maximum percentage of fish reached score 4 in TAU 150, and DZP groups after 600 and 450 s, respectively (Fig. 2b).

Figure 2c shows the seizure intensity in initial (0–150 s), intermediary (150–300 s), and final (300–1200 s) periods of test estimated by the area under curve. One-way ANOVA yielded significant differences in initial ($F_{4,109} = 28.68$, $p < 0.001$), intermediary ($F_{4,109} = 13.78$, $p < 0.0001$), and final ($F_{4,109} = 11.29$, $p < 0.0001$) periods. In the initial time, all TAU and DZP pretreated groups had decreased seizure intensities when compared to PTZ-exposed fish. However, in the other segments of tests, the seizure was less intense in TAU 150- and DZP-pretreated groups.

Figure 3a shows representative heat maps of the individual scores of zebrafish across the PTZ exposure period. Basically,

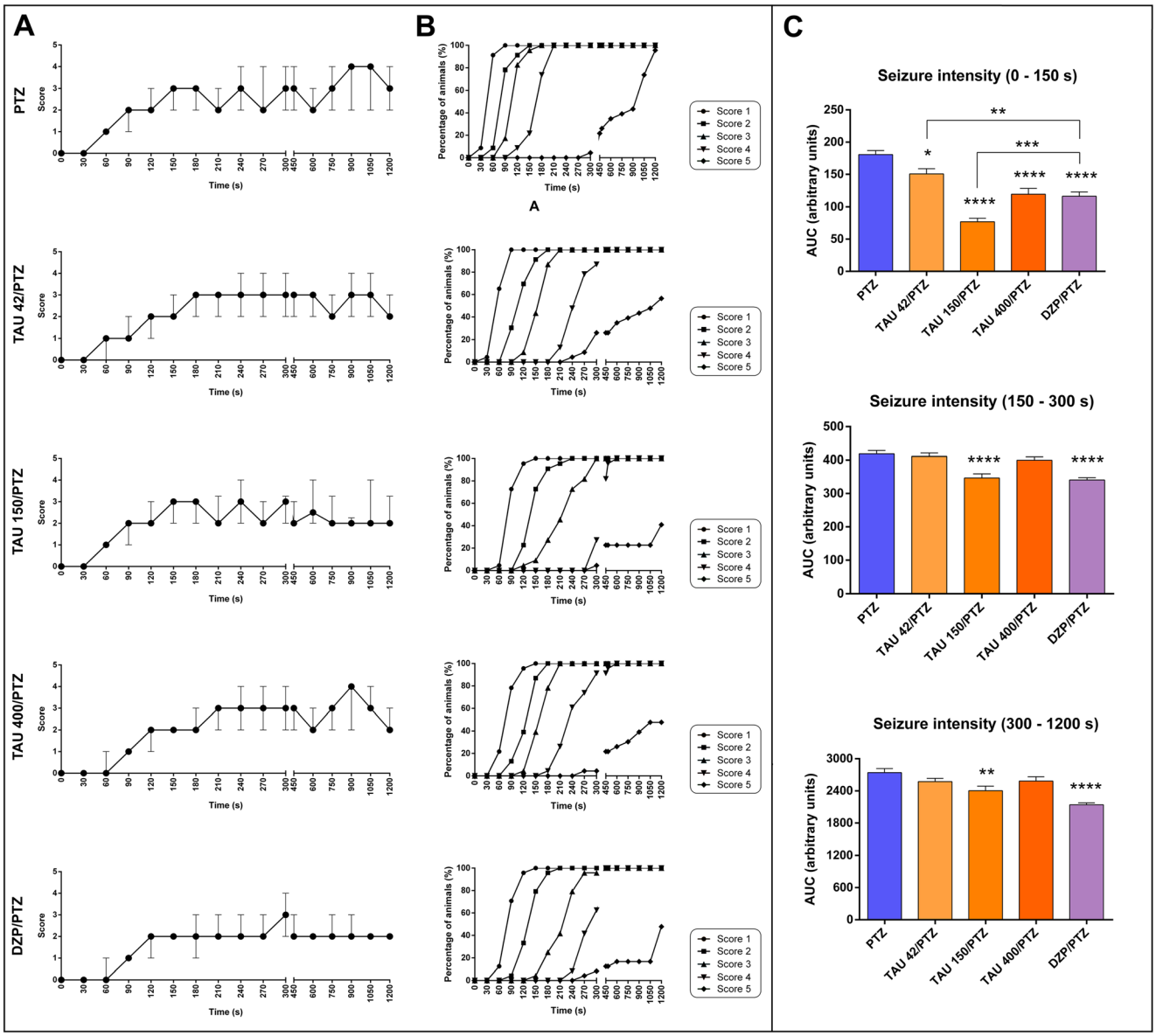

Fig. 2 Effects of TAU and DZP pretreatment in PTZ-challenged zebrafish. **a** Seizure-like behavioral scores across time (data were represented as median ± interquartile range). **b** Percentage of animals (%) that reached each score across time. **c** Seizure intensity at distinct time periods (0–150, 150–300, and 300–1200 s) evaluated by the area under curve (AUC) for each treatment. Seizure intensity was represented as mean ± S.E.M and analyzed by one-way ANOVA followed by Tukey's test. Asterisks above bars express significant differences compared to the PTZ group, while asterisks above brackets indicate statistical differences compared to the DZP group (n = 20–24 animals per group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

the latency to reach score 4 increased in TAU 150-, TAU 400-, and DZP-pretreated groups ($F_{4,109} = 19.17$, $p < 0.001$) (Fig. 3b). Meanwhile, the number of score 4 events was significantly reduced in fish pretreated with 150 mg/L TAU and DZP ($F_{4,109} = 10.32$, $p < 0.001$) (Fig. 3c).

Reconstruction of Zebrafish Swimming Traces

Figure 4 shows the swimming traces across the first 5 min of exposure, a period in which animals reached score 4. Representative spatiotemporal reconstructions of behavior depict different patterns of activity, where the PTZ group showed aberrant swimming in the surface of the tank. Both TAU 42/PTZ and TAU 150/PTZ groups had a similar profile in comparison to CTRL, showing an initial swimming in the bottom area followed by increased activity in the top. TAU 400/PTZ group showed a distinct pattern of activity, exploring the bottom and top area proportionally across the test. Additionally, DZP-pretreated group presented different swimming traces, since animals swam preferentially in the bottom area of the tank.

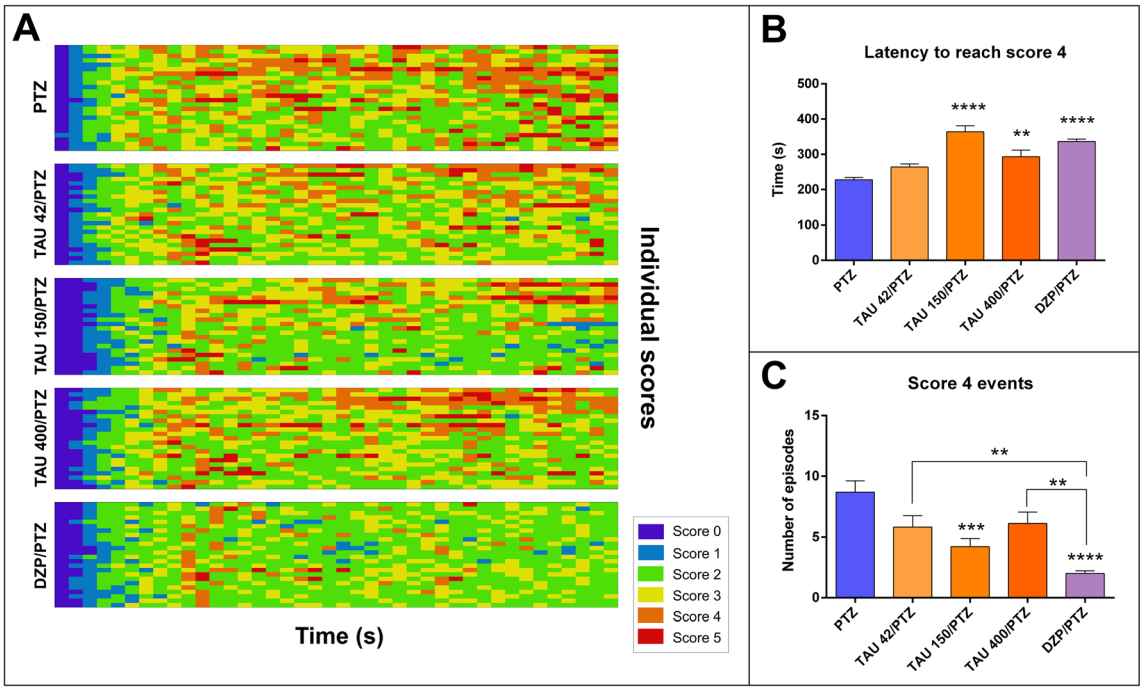

Fig. 3 TAU and DZP attenuate PTZ-induced changes in seizure-like behavioral scores. **a** Representative heat maps showing the individual scores every 30 s across the 20-min exposure period (X axis) of each individual fish (Y axis). **b** Latency to reach score 4. **c** Frequency of score 4. Latency and frequency of score 4 were represented as mean ± S.E.M and analyzed by one-way ANOVA followed by Tukey's test. Asterisks above bars express significant differences compared to the PTZ group, while asterisks above brackets indicate statistical differences compared to the DZP group (n = 20–24 animals per group; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$)

Enzymatic and Non-enzymatic Antioxidant Defenses

Figure 5 shows the effects of PTZ and TAU on enzymatic and non-enzymatic antioxidant defenses. Concerning the enzymatic antioxidant defenses, no significant differences were observed in SOD and GST assays. Regarding CAT activity, two-way ANOVA using treatment and PTZ as factors showed significant effects of treatment ($F_{4,54} = 18.82$, $p < 0.001$). Basically, PTZ increased CAT activity similarly to TAU 42 and TAU 150 alone and TAU 42/PTZ groups. Moreover, two-way ANOVA showed a significant effect of interaction ($F_{4,54} = 3.69$ $p < 0.0099$), where all PTZ-exposed groups had decreased NPSH levels, except TAU 150/PTZ.

Oxidative Damage and Cell Viability-Related Parameters

Figure 6 depicts the effects of TAU and PTZ on oxidative damage- and cell viability-related parameters. Concerning lipid peroxidation, two-way ANOVA showed a significant effect of treatment ($F_{4,54} = 91.04$, $p < 0.0001$) and a treatment x PTZ interaction ($F_{4,54} = 4.21$, $p < 0.0052$). PTZ increased TBARS levels, whereas pretreatment with 42 mg/L TAU, 150 mg/L TAU, and DZP prevented this effect. PTZ increased carbonylated protein levels and a preventive effect was observed in TAU 150/PTZ and DZP/PTZ groups ($F_{4,46} = 8.69$, $p < 0.0001$ for interaction term, and $F_{1,46} = 10.63$, $p < 0.0001$ for treatment, respectively). No significant differences were observed in MTT assay and LDH activity among groups.

Discussion

In this study, we report for the first time a preventive effect of TAU on neurobehavioral parameters in PTZ-exposed zebrafish. Our data showed that similarly to DZP, TAU attenuates seizure-like behaviors since TAU pretreatment increases the latency to reach score 4 (clonic-like behaviors) and decreases seizure frequency and intensity. Moreover, depending on the concentration tested, TAU abolished the effects of PTZ on NPSH levels, lipid peroxidation, and protein carbonylation. Overall, we suggest a protective role of TAU against PTZ-induced behavioral and neurochemical changes in zebrafish.

The occurrence of seizure-like behaviors after a single PTZ exposure has been extensively described in zebrafish [2, 3, 6, 9, 56, 57]. Exposure to 10 mM PTZ for 20 min elicits all seizure-like behavioral phenotypes, in which 95% animals reached score 5 and frequently exhibited tonic/clonic seizure scores [2]. In the CNS, PTZ antagonizes $GABA_A$ receptors, thereby modifying excitatory/inhibitory tonus, which culminates in acute seizures [58–60]. Importantly, corkscrew swimming (a behavioral phenotype observed in score 4) occurs simultaneously to electroencephalogram (EEG) abnormal discharges in zebrafish exposed to 10 mM PTZ [6].

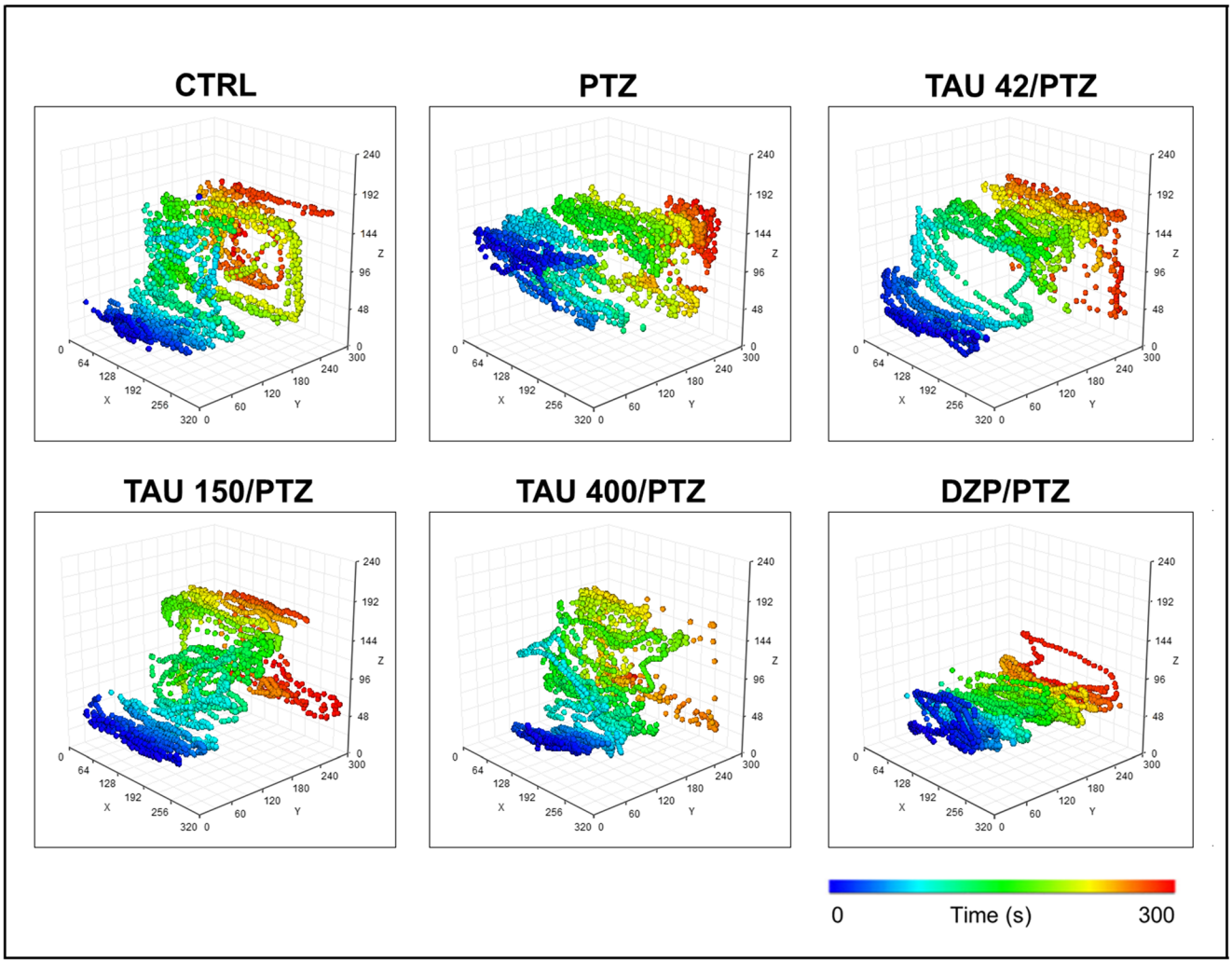

Fig. 4 Representative swimming traces of the experimental groups during the initial periods of PTZ exposure (5 min). Reconstructions were obtained by plotting animal coordinates (X and Z axes) across time (Y axis). The segments of test (0–300 s) were represented by a color scale gradient, indicating the beginning (blue) and the end of trial (red)

As an endogenous neuromodulator, TAU plays a role in controlling the inhibitory/excitatory synapses since it may act as a $GABA_A$ receptor agonist [61–63]. TAU can also prevent excitotoxicity by inhibiting the reverse mode of Na^+–Ca^{2+} exchanger decreasing intracellular Ca^{2+} levels, suggesting an interaction between TAU and N-methyl-D-aspartate (NMDA) receptor [64, 65]. All TAU concentrations tested decreased the seizure intensity at the first 150 s, whereas only 150 mg/L TAU showed a protective effect from 150 to 1200 s, similarly to DZP. Additionally, 150 mg/L TAU pretreatment increased the latency to reach stage 4 and decreased the frequency of clonic-like behaviors. Since TAU promotes hyperpolarization and inhibits neuronal firing [66], these data could be associated to a modulatory role in the CNS. Interestingly, TAU levels increase in the brain following seizure episodes in both animal models [67] and human serum [68–70]. Thus, TAU may counteract glutamatergic excitotoxicity and attenuate seizure episodes in PTZ-challenged animals, which is in accordance with the individual heat maps of scores and the representative spatiotemporal reconstructions of behavior depicted here.

Spatiotemporal reconstructions of swimming activity serve as useful tools for assessing the behavioral neurophenotypes of zebrafish after pharmacological treatments [39, 40]. In general, zebrafish has a tendency to swim in the bottom section and gradually increase the activity in upper sections of a tank [29, 71, 72]. PTZ-exposed animals swam mainly in the top area, showing a disrupted swimming activity. These changes could be a consequence of impaired neurochemical signaling pathways, with culmination in aberrant swimming activity [2, 73]. Interestingly, TAU 42/PTZ and TAU 150/PTZ exhibited similar swimming traces when compared to CTRL group. These data may reflect protective effects of TAU in attenuating seizure-like behaviors, since TAU alone does not affect swimming activity [20, 35, 36]. Conversely, DZP group spent more time in the bottom area, which could reflect sedative effects due

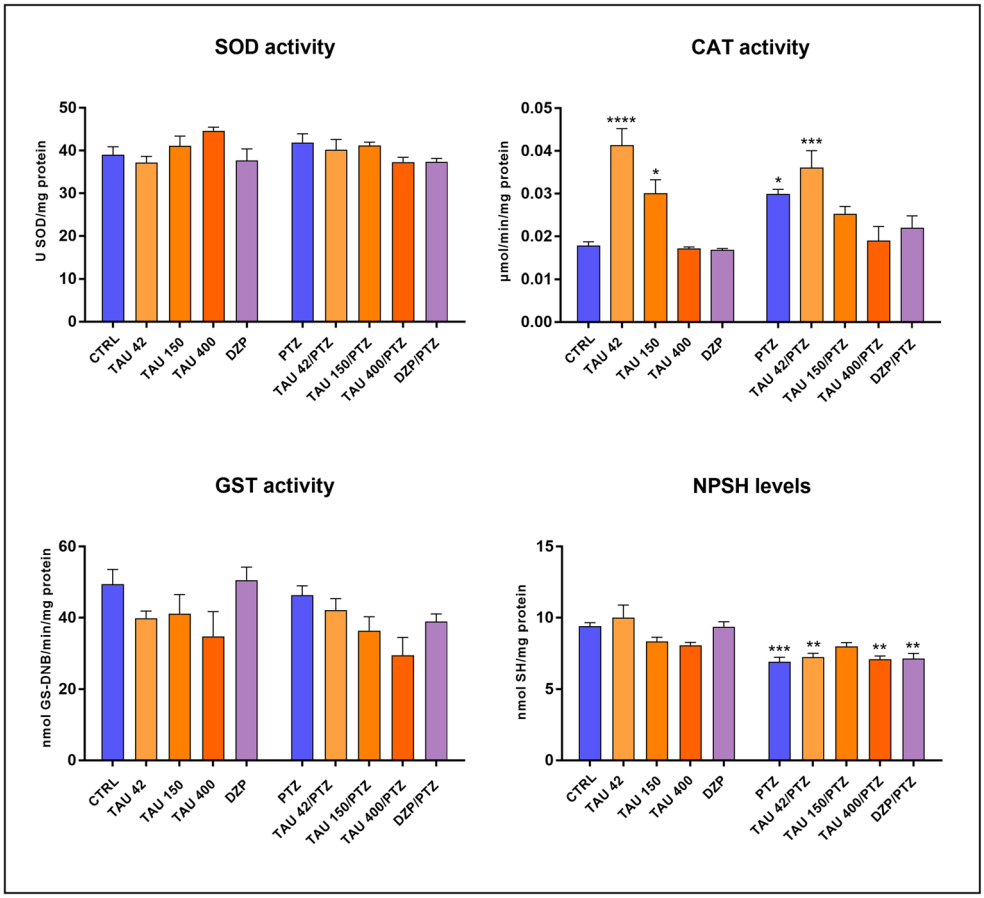

Fig. 5 Enzymatic and non-enzymatic antioxidant defenses in zebrafish brain. Data were expressed as mean ± SEM and analyzed by two-way ANOVA followed by Tukey's test using treatment and PTZ as factors. Asterisks express significant differences compared to control (CTRL) ($n = 6$ biological preparations per group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$)

to its interaction with $GABA_A$ receptors [74, 75]. This sedative side effect is common in epileptic patients and impairs several daily activities [76, 77]. Moreover, AEDs, such DZP, are not effective for many epileptic conditions, in which 5–10% of patients are resistant for first- and second-generation of AEDs [78]. These data highlight the importance for screening new therapeutic molecules to prevent seizures. Due to its pleiotropic role in the CNS [17, 21, 64, 79], TAU probably attenuates seizures via different molecular pathways compared to DZP, modulating seizure-like behaviors without altering the overall swimming pattern.

Seizures may lead to brain insults due to excitotoxicity, which culminates in an increased production of reactive oxygen/nitrogen species (ROS/RNS) [80]. ROS and RNS play a role in seizure-induced neurodegeneration [81–83]. PTZ may induce oxidative stress by increasing free radical production [84] and/or decreasing antioxidant defenses [85]. Since the brain has an increased demand for oxygen consumption and is enriched with polyunsaturated fatty acids, it is more susceptible to lipid peroxidation and protein carbonylation [7]. PTZ-exposed fish showed decreased NPSH content, as well as increased TBARS levels and protein carbonylation. We observed that 150 mg/L TAU prevented PTZ-induced changes in NPSH, TBARS, and carbonylated protein levels suggesting antioxidant activity. TAU may protect against glutamate excitotoxicity by inhibiting extracellular calcium influx and calcium release from the internal pools, which reflect putative mechanisms of TAU antioxidant activity [64, 86]. Furthermore, TAU has antioxidant properties by modulating oxidative stress-related parameters in vivo [34, 87]. Despite the neuromodulatory role of TAU in extracellular milieu, TAU may interact directly with some oxidant radicals that cause lipid peroxidation, acting as scavenger at physiological intracellular concentrations [88]. Interestingly, CAT activity increased substantially in both TAU 42 and TAU 42/PTZ groups, reflecting a complex modulatory effect of TAU on enzymatic antioxidant defenses. Vasodilator molecules may increase CAT activity, which represents a possible mechanism of protection [89]. Since TAU may decrease blood pressure in experimental models [21], the increased CAT activity could reflect a vasodilator action, not necessarily indicating alterations in a specific response to oxidative stress. Conversely, the increased CAT activity in PTZ-exposed animals could reflect a compensatory mechanism to stimulate enzymatic antioxidant defenses in PTZ-exposed fish. Thus, although CAT activity in both TAU 42 and PTZ groups showed a similar result, they may indicate different physiological responses. Additionally, DZP showed protective effects against PTZ-induced lipid peroxidation and

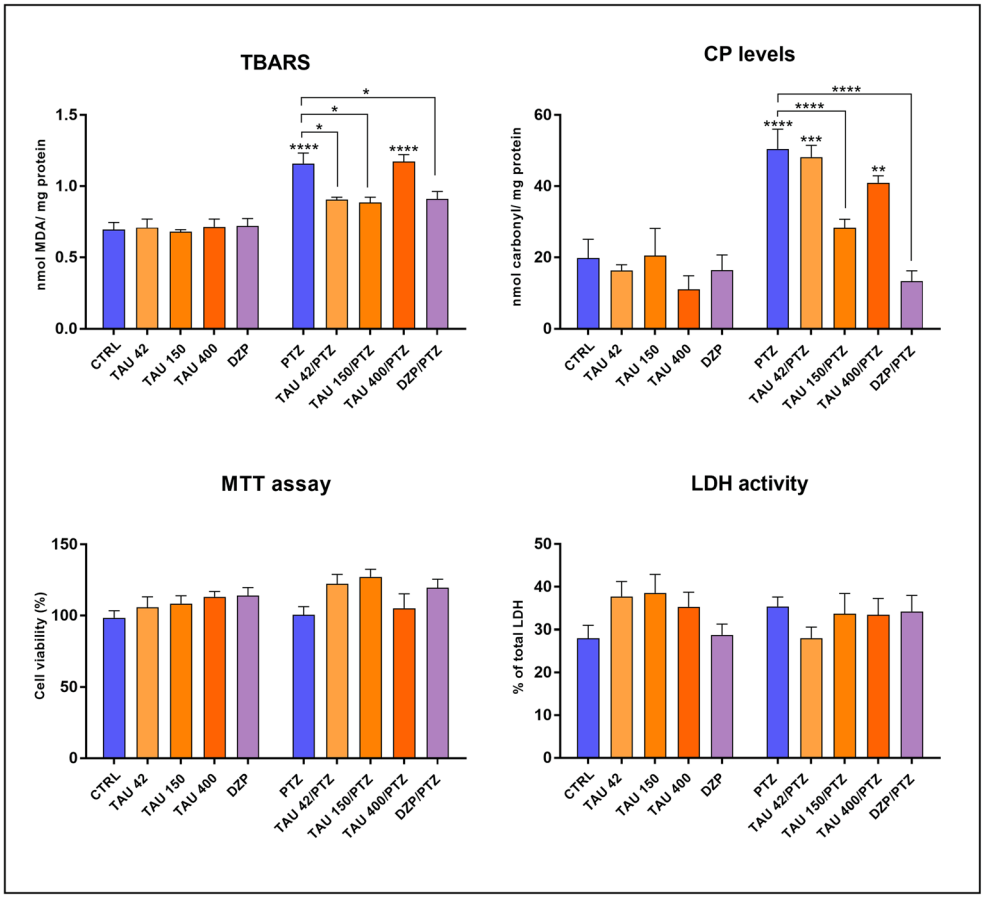

Fig. 6 Effects of TAU and DZP pretreatment on TBARS, carbonylated protein (CP) levels, and cell death-related parameters in brain tissue of PTZ-challenged zebrafish. Data were expressed as mean ± SEM and analyzed by two-way ANOVA followed by Tukey's test using treatment and PTZ as factors. Asterisks above bars express significant differences compared to control (CTRL), while asterisks above brackets indicate statistical differences compared to the PTZ group ($n = 6$ biological preparations per group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

protein carbonylation. Accordingly, previous studies showed that enhanced GABAergic neurotransmission decreases oxidative stress by counteracting glutamatergic excitotoxicity in mice [90]. We did not verify significant effects on MTT and LDH assays probably due to the lack of seizure recurrence in the acute PTZ exposure model [91]. Notably, the effects of TAU on PTZ-induced toxicity seem not to be concentration-dependent in biochemical or behavioral analyses. Although the precise mechanisms underlying TAU actions in zebrafish brain have not been fully elucidated, this biphasic response suggests that TAU may act on different receptors or signaling transduction pathways in the CNS [21, 92]. Nonetheless, our data show positive effects of TAU in attenuating seizure-like behaviors and preventing oxidative stress in PTZ-challenged zebrafish.

Conclusion

In summary, our novel data show a protective role of TAU against PTZ-induced behavioral changes and oxidative stress in zebrafish. TAU prevents lipid peroxidation and protein carbonylation following PTZ exposure in zebrafish brain, suggesting that antioxidant mechanisms are involved in neuroprotection. Importantly, TAU does not affect swimming activity, a common effect observed after DZP treatment. Since TAU presents beneficial effects in the CNS, further studies are needed to elucidate the mechanisms underlying neuroprotection, as well as its long-term efficacy in recurrent seizure episodes.

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Compliance with Ethical Standards

All experimental procedures were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (protocol number 8707070316).

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

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5. DISCUSSÃO

As doenças neurológicas e neuropsiquiátricas afetam grande parte da população mundial desde os primórdios, constituindo um problema de saúde pública (VIGO et al., 2016). Nesse contexto, podemos destacar três doenças neurológicas, tais como epilepsias, doença de Parkinson e doença de Alzheimer e três doenças neuropsiquiátricas, tais como as doenças do espectro ansioso, doenças de humor/depressão e esquizofrenia. Durante décadas, estudos relacionados a estas doenças utilizaram exclusivamente modelos mamíferos, porém recentemente o uso de organismos modelo alternativos como a mosca da fruta (*Drosophila melanogaster*), o nematódeo *Caenorhabditis. elegans* e o peixe-zebra (*Danio rerio*) têm ganhado grande destaque gerando resultados de alta confiabilidade para comunidade científica (McGONIGLE e RUGERRI, 2014; ELLENBROEK e YOUN, 2016; FREIRES et al., 2017). Assim, no nosso primeiro artigo apresentado nessa dissertação, discutimos as perspectivas do uso do peixe-zebra como organismo modelo para estudos de doenças do SNC. Primeiramente, o trabalho abordou as vantagens e limitações no uso do peixe-zebra em pesquisas translacionais. Dentre as vantagens, destacamos o seu tamanho diminuto, a fácil manutenção e reprodução, o baixo custo e a presença de embriões translucidos (PARKER et al., 2012; RICO et al., 2011; STEWART et al., 2014; STEWART et al., 2015). Apesar da adição de drogas na água ser uma limitação pelo fato da dificuldade de estimar as concentrações exatas que são absorvidas e exercem efeitos farmacológicos nos animais (ROSEMBERG et al., 2012; TRAN et al., 2015), a quantidade que atinge o SNC pode ser determinada por vários métodos quimioanalíticos, tais como espectroscopia de massa ou cromatografia líquida de alta performance (CHATTERJEE AND GERLAI, 2009; KOLESNIKOVA et al., 2017; ROSEMBERG et al., 2012). Posteriormente, analisamos as vantagens e limitações do uso do peixe-zebra em diferentes doenças relacionadas ao SNC, discutindo as principais ferramentas químicas, moleculares e genéticas utilizadas como estratégias para modelar patologias distintas. Tendo em vista a consolidação do peixe-zebra dentro da neurociência e o potencial protetor da TAU como molécula protetora discutido previamente, os seguintes trabalhos utilizaram um modelo neuropsiquiátrico que mimetiza alterações comportamentais induzidas pelo abuso de álcool e um importante modelo químico para a avaliação das crises convulsivas através da exposição ao PTZ.

Os principais achados do segundo e terceiro artigo incluem a atividade da TAU, dependendo da concentração, na modulação de alterações comportamentais induzidas pelo EtOH em peixe-zebra. Nossos resultados apontam uma relação complexa de distintos domínios comportamentais, onde a TAU e o EtOH exercem papeis distintos sobre a agressividade, o comportamento social e o comportamento aversivo. Sabe-se que os mecanismos neurais envolvidos no aumento de agressividade envolvem áreas cerebrais relacionadas à deficiência de comportamentos sociais e um aumento na impulsividade (NELSON e TRAINOR, 2007). Ademais, respostas aversivas estão fortemente relacionadas ao medo (GERLAI, 2010), onde um déficit nestas respostas pode ser associado ao excesso de impulsividade em seres humanos (FRIDJA et al., 2014). Assim, a TAU diminuiu o número de avaliações de risco no teste do predador enquanto aumentou os episódios agressivos nas concentrações de 42 e 400 mg/L. Essas respostas indicam que a TAU pode modular a impulsividade em peixe-zebra, diminuindo suas respostas aversivas e aumentando a agressividade frente ao oponente. A TAU na concentração de 150 mg/L também diminuiu o número de avaliações de risco, além de reduzir o tempo de permanência na área próxima ao predador sem modular a agressividade. Interessantemente, os grupos TAU/EtOH mantiveram uma agressividade aumentada e uma avaliação de risco diminuída nas concentrações de 42 e 400 mg/L, onde somente no grupo TAU 150 observamos uma proteção frente aos efeitos do EtOH nestes comportamentos. Logo, a TAU modulou os comportamentos agonísticos de forma bifásica, onde TAU nas concentrações de 42 e 400 mg/L modularam positivamente, enquanto TAU 150 mg/L diminuiu as alterações comportamentais induzidas pelo EtOH. Estudos anteriores demonstraram este efeito dualístico em peixe-zebra, onde 42, 150 e 400 mg/L de TAU preveniram a redução na atividade locomotora, mas somente TAU 42 e 400 mg/L preveniram a diminuição na exploração vertical após exposição ao EtOH 1% (v/v) (ROSEMBERG et al., 2012). Esta resposta bifásica sugere que as ações da TAU na exploração não estão diretamente ligadas às alterações na atividade locomotora, mas que podem estar associadas a aspectos motivacionais do comportamento. Pela primeira vez, desenvolvemos uma estratégia para a avaliação do componente agressivo através de um cálculo de escore de agressividade para peixe-zebra utilizando diversas variáveis, o que permite uma análise completa do componente agressivo em peixe-zebra. Através dos escores de agressividade, foi confirmado que a TAU *per se* e associada ao EtOH aumentam a agressividade em concentrações de 42 e 400 mg/L, enquanto o tratamento com TAU 150 mg/L protegeu contra o aumento na agressividade causado pelo EtOH.

Para completa visualização dos fenótipos comportamentais, fizemos a reconstrução do comportamento através de etogramas, os quais são importantes ferramentas dentro da etologia para a caracterização de comportamentos complexos (GOMEZ-MARIN et al., 2014). Assim, embora os escores de agressividade não indiquem diferença entre o aumento de agressividade gerada pela TAU 42/EtOH e TAU 400/EtOH, os etogramas relevaram padrões distintos relacionados ao ataque ao oponente. A diferente resposta no padrão de agressividade indica que os parâmetros relacionados à exploração podem afetar a agressividade em peixe-zebra, e que TAU e EtOH podem não necessariamente apresentar mecanismos de ação similares. Assim, as alterações nos comportamentos de agressividade e nas respostas antipredatórias parecem apresentar padrões complexos, que vão além do aumento ou diminuição nos parâmetros comportamentais avaliados. A fim de verificar a associação da agressividade e das respostas pigmentares, a quantificação da intensidade de cor dos animais foi realizada após os testes comportamentais. TAU *per se* alterou a agressividade em 42 e 400 mg/L sem alterar a resposta pigmentar, enquanto que todas as concentrações de TAU associadas ao EtOH aumentaram a resposta pigmentar em relação aos grupos controle ee EtOH. A modulação da resposta pigmentar está associada a mudanças nas alterações na iluminação/condições ambiente (resposta de camuflagem), estresse ou à exposição a diversos fármacos (KALUEFF et al., 2013; PRICE et al., 2008; WAGLE et al.,2011). Além disso, sabe que o aumento pigmentar induzido pelo EtOH e pelas alterações as alterações de iluminação envolvem a liberação do fator de corticotropina (CRF). O aumento nos níveis do hormônio adrenocorticotrópico (ACTH) é um mecanismo relacionado a respostas de estresse e agentes estressores podem modular a agregação dos melanossomos em peixe-zebra, culminando em alterações na resposta pigmentar (WAGLE et al., 2011). Assim, nossos dados indicam que a TAU e o EtOH modulam comportamentos defensivos de forma distinta à modulação pigmentar. Apesar dos mecanismos envolvidos nas respostas da associação da TAU com EtOH ainda não estarem elucidados, as respostas encontradas nos grupos TAU/EtOH podem estar relacionadas a um aumento nos níveis de CRF e à ativação do eixo hipotálamo-hipófise-adrenal (HPA).

Mecanismos envolvidos na formação de cardume em peixe-zebra estão fortemente relacionados ao desempenho cognitivo e às estratégias de tomada de decisão (SPORNS, 2010). Nossos dados mostraram através da análise temporal que tanto a TAU quanto o EtOH exercem efeitos significativos sobre o comportamento social a partir de

30 minutos. Embora os efeitos associados ao consumo de álcool sejam complexos e muitas vezes contraditórios (MONAHAN e LANUTTI, 2000), os resultados apresentados no terceiro artigo científico corroboraram com estudos anteriores, onde a exposição aguda ao EtOH altera a polarização dos cardumes, reduzindo sua coesão (GERLAI et al., 2000; MILLER et al., 2013). Semelhante aos efeitos do EtOH, a associação da TAU com o EtOH induziu modificações no comportamento em cardume dependendo da concentração testada, enquanto que o grupo TAU 400/EtOH também apresentou redução na preferência social. Todavia, TAU protegeu contra os efeitos do EtOH em relação ao número de interações socais. Sabe-se que diferentes fenótipos comportamentais podem ser observados dependendo do contexto, o que pode resultar em diversas respostas dentro de um mesmo domínio comportamental. Por exemplo, a tarefa de preferência social está relacionada à abordagem de co-específicos, enquanto o teste do cardume mede as interações sociais e a coesão dos peixes (PHAM et al., 2012). Assim, por mais que a TAU resgate os fenótipos comportamentais no teste do cardume, TAU 400/EtOH diminuiu a busca por coespecíficos no teste de preferência social, indicando um efeito complexo da exposição a TAU com EtOH em peixe-zebra. É importante também ressaltar que os ansiolíticos modulam comportamentos sociais em peixe-zebra e diminuem a formação de cardume (HAMILTON et al., 2017). Considerando os efeitos ansiolíticos da TAU em peixe-zebra (MEZZOMO et al., 2016), as respostas comportamentais observadas no teste do cardume parecem ter forte relação com o caráter ansiolítico desta molécula. Logo, a TAU parece atuar de forma distinta nos domínios comportamentais, possuindo uma resposta bifásica na agressividade e na resposta antipredatória, e um padrão dependente de concentração nos testes de comportamento social.

No quarto artigo desta dissertação, avaliamos o potencial papel protetor da TAU frente às crises convulsivas induzidas pelo PTZ. Nesse estudo, relatamos pela primeira vez um efeito preventivo da TAU frente a crises convulsivas em peixe-zebra. Nossos dados mostraram que, da mesma forma que o DZP, a TAU atenuou os escores de crise convulsiva, aumentando a latência para o escore 4 (comportamento convulsivo do tipo clônico), diminuindo a frequência e a intensidade das crises convulsivas. Como a TAU promove hiperpolarização e inibe a ativação neuronal (SARANSAARI e OJA, 2008), o efeito neuroprotetor da TAU pode estar associado a sua ação moduladora no SNC. Interessantemente, os níveis de TAU aumentam no cérebro após os episódios de crises

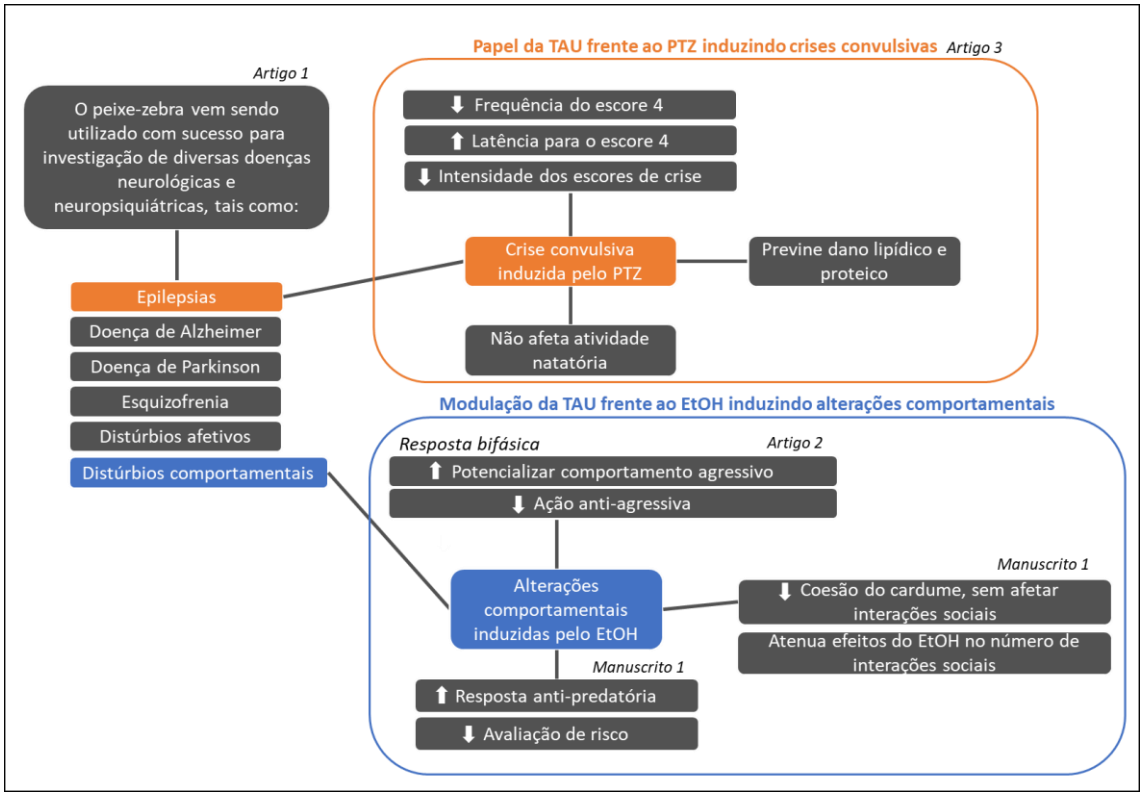
epilépticas em modelos animais (VEZZANI e SCHWARCZ, 1985) e em soro humano (WILSON et al., 1996; GABY, 2007). Assim, a TAU pode proteger contra a excitotoxicidade glutamatérgica e atenuar a crise convulsiva em animais expostos ao PTZ, o que está representado pelos escores individuais dos animais ao longo de 20 minutos representados nos mapas de calor. Curiosamente, os grupos TAU 42/PTZ e TAU 150/PTZ exibiram perfis de exploração semelhantes quando comparados ao controle. Esses dados podem refletir um importante papel da TAU na atenuação de comportamentos do tipo convulsivo, uma vez que a TAU *per se* não afeta a atividade natatória dos animais (ROSEMBERG et al., 2012; MEZZOMO et al., 2016). Por outro lado, o grupo DZP passou mais tempo no fundo do tanque, refletindo um efeito sedativo, provavelmente relacionados a sua interação com os receptores $GABA_A$ (PRINGLE et al., 2016; TRAN et al., 2016). Esse efeito é comum em pacientes epilépticos e prejudica suas atividades diárias (BALDWIN, 2013; WEINTRAUB, 2017). Além disso, fármacos antiepilépticos como o DZP, não são eficazes para muitas condições epilépticas, nas quais 5-10% dos pacientes são resistentes à primeira e segunda geração desses medicamentos (JUNYENT et al., 2009). De modo geral, esses dados destacam a importância da busca por novas moléculas terapêuticas para prevenir convulsões. Devido ao seu papel pleiotrópico no SNC (HUXTABLE, 1992; OJA e SARANSAARI, 2007; MEZZOMO et al., 2017; WU et al., 2005), a TAU diminui as convulsões através de diferentes vias neuronais comparadas ao DZP, atenuando crises convulsivas sem causar mudanças significativas no padrão de nado. Além disso, dependendo da concentração testada, a TAU aboliu os efeitos do PTZ relacionados à redução dos níveis de tióis não proteicos (NPSH), ao aumento da peroxidação lipídica e da carbonilação de proteínas. Sabe-se que a TAU pode proteger contra a excitotoxicidade do glutamato, inibindo o influxo de cálcio extracelular e a liberação de cálcio dos estoques internos, os quais refletem mecanismos putativos da sua atividade antioxidante (WU et al., 2005; CHEN et al., 2001). Ademais, a TAU pode interagir diretamente com alguns radicais livres que causam a peroxidação lipídica, atuando como sequestrador nas concentrações intracelulares fisiológicas (OLIVEIRA et al., 2010). A atividade da CAT aumentou substancialmente nos grupos TAU 42 e TAU 42 PTZ, refletindo um papel complexo da TAU na modulação das defesas antioxidantes enzimáticas. Evidências demonstram que moléculas vasodilatadoras podem aumentar a atividade da CAT, o que representa um possível mecanismo de proteção (FURIAN et al., 2009). Como a TAU pode diminuir a pressão arterial em modelos experimentais (MEZZOMO et al., 2017), o aumento da atividade da CAT poderia refletir

uma ação vasodilatadora, não necessariamente indicando alterações em uma resposta específica ao estresse oxidativo. Por outro lado, o aumento da atividade da CAT em animais expostos ao PTZ poderia refletir um mecanismo compensatório para estimular as defesas antioxidantes enzimáticas em peixes expostos a PTZ. Assim, embora a atividade da CAT nos grupos TAU 42 e PTZ tenha apresentado um resultado semelhante, elas podem estar relacionadas a diferentes respostas fisiológicas. Em suma, os dados mostram um papel protetor da TAU frente as alterações comportamentais e neuroquímicas induzidas pelo PTZ em peixe-zebra.

6. CONCLUSÃO

A presente dissertação mostrou a relevância do uso de peixe-zebra em pesquisas de neurociência translacional e demonstrou que a TAU é capaz de modular alterações de sociabilidade induzidas pelo EtOH, bem como exercer um papel neuroprotetor frente ao PTZ por atenuar as crises convulsivas. Essa conclusão pode ser sustentada pelos achados que estão representados na figura abaixo.

Figura 4. Diagrama representativo mostrando os principais achados desta dissertação.



Fonte: Do autor.

7. PERSPECTIVAS

Este trabalho mostrou que a TAU possui importante papel protetor no SNC tanto em modelos de exposição aguda ao EtOH quanto em modelos de crises convulsivas induzidas pelo PTZ. Dessa maneira, as perspectivas desse estudo são:

- Investigar o papel da TAU associada ao EtOH em parâmetros relacionados ao medo e à ansiedade, a fim de buscar uma melhor compreensão sobre a ação modulatória da associação das mesmas em outros domínios comportamentais.

- Avaliar se a TAU é capaz de manter seu papel neuroprotetor frente a altas concentrações de EtOH que causam efeitos sedativos em peixe-zebra.

- Explorar se os mecanismos associados aos efeitos comportamentais da TAU e EtOH envolvem a ativação de receptores de GABA e de glicina em peixe-zebra.

- Investigar as alterações nos níveis de neurotransmissores, tais como GABA, glutamato, dopamina e serotonina causados pela associação de TAU e EtOH no cérebro de peixe-zebra.

- Avaliar se TAU protege contra a indução de crise convulsiva em outros modelos químicos, tais como as crises induzidas por ácido caínico.

- Analisar se a TAU é capaz de alterar registros eletroencefalográficos em diferentes modelos de crise convulsiva.

- Verificar os efeitos da TAU em modelos de crises convulsivas repetidas em peixe-zebra, bem como o seu envolvimento na modulação de parâmetros antioxidantes e inflamatórios no tecido cerebral.

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ANEXO A – CARTA DE APROVAÇÃO DO CEUA – ARTIGOS 2 e 3

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Avaliação neuroquímica e comportamental dos efeitos promovidos pela taurina em peixe zebra expostos ao etanol: uma abordagem em modelos de exposição aguda e crônica".

Número do Parecer: 026/2014

Pesquisador Responsável: Prof. Drº Denis Broock Rosemberg

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO: 08/05/2014.

Santa Maria, 08 de Maio de 2014.

Profª Drª Vania Lucia Loro
Vice - Coordenadora da Comissão de Ética no Uso de Animais- UFSM

Comissão de Ética no Uso de Animais - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362

ANEXO B – CARTA DE APROVAÇÃO DO CEUA – ARTIGO 4

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que o Projeto intitulado "Investigação dos efeitos da taurina sobre a crise convulsiva induzida pelo pentilenotetrazol em peixe zebra (Danio rerio) ", protocolado sob o CEUA nº 8707070316, sob a responsabilidade de **Denis Broock Rosemberg** *e equipe; Fernanda Dias Guilherme; Julia Canzian Marion; Paola Rampelotto Ziani* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei 11.794, de 8 de outubro de 2008, com o Decreto 6.899, de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) em reunião de 14/04/2016.

We certify that the proposal "Evaluation of the effects of taurine against PTZ-induced seizures in zebrafish (Danio rerio)", utilizing 290 Fishes (males and females), protocol number CEUA 8707070316, under the responsibility of **Denis Broock Rosemberg** *and team; Fernanda Dias Guilherme; Julia Canzian Marion; Paola Rampelotto Ziani* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes (or teaching) - it's in accordance with Law 11.794, of October 8 2008, Decree 6899, of July 15, 2009, with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 04/14/2016.

Vigência da Proposta: de 06/2016 a 05/2018 Área: Bioquímica E Biologia Molecular

Procedência:	Não aplicável biotério				
Espécie:	Peixes	sexo: Machos e Fêmeas	idade:	4 a 6 meses	N: 290
Linhagem:	Danio rerio / wild type (WT/SF)		Peso:	0250 a 0280 g	

Resumo: A epilepsia é uma desordem neurológica caracterizada pelas crises epiléticas espontâneas e recorrentes associadas com alterações neurobiológicas e comportamentais. Um dos métodos mais utilizados para investigar a ocorrência de crises convulsivas consiste na análise do perfil comportamental em modelos animais por meio de escores fenotípicos. Existem diversos agentes químicos que são capazes de induzir convulsões através da alteração do tônus GABAérgico/glutamatérgico no sistema nervoso central (SNC). O composto pentilenotetrazol (PTZ) é um fármaco que inicialmente foi utilizado como estimulante circulatório e respiratório. O mecanismo de ação do PTZ acontece pelo antagonismo do receptor GABAA, o que pode causar convulsões em modelos animais. Desta maneira, diversos estudos vêm sendo desenvolvidos em busca de moléculas neuroprotetoras que possam prevenir os efeitos convulsivos causados pelo PTZ. A taurina (TAU) é um ?-aminoácido derivado da oxidação da cisteína que desempenha uma ação pleiotrópica no SNC. Dentre elas, destaca-se seu potencial efeito neuroprotetor frente à excitotoxicidade glutamatérgica, atuando como um neuromodulador inibitório através da ligação em receptores do tipo GABAA e glicina. Fisiologicamente, a TAU possui ação osmorreguladora, antioxidante e atua na modulação do metabolismo do Ca2+, o que a classifica como uma molécula candidata para estudos em modelos animais. O peixe zebra (Danio rerio) é um organismo teste emergente para estudos neurocomportamentais. Dentre os fatores, pode-se destacar o elevado grau de homologia genética em comparação aos genes humanos (cerca de 70%), a conservação de estruturas análogas no SNC e a similaridade de função dos sistemas de neurotransmissores. Além disso, este modelo possui um baixo custo de manutenção e alta reprodutibilidade, sendo um atrativo para triagens de médio/alto rendimento. Logo, em vista do peixe zebra ser um bom modelo animal para estudos neurocomportamentais e considerando o potencial papel neuroprotetor da TAU na excitotoxicidade glutamatérgica, este trabalho busca avaliar os efeitos da TAU como molécula protetora frente à exposição ao PTZ em peixe zebra.

Santa Maria, 14 de abril de 2016

Profa. Dra. Daniela Bitencourt Rosa Leal
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Denis Broock Rosemberg
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

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CEUA N 8707070316

ANEXO C – COMPROVANTE DE SUBMISSÃO – ARTIGO 3

Denis Rosemberg ▾ | My Journals ▾ | Log Out | Help EVISE

JOURNAL OF PSYCHIATRIC RESEARCH

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Taurine modulates acute ethanol-induced social behavioral deficits and fear responses in adult zebrafish

Current status: Under Review (04/Apr/2018)

JPSYCHIATRRES_2018_332
Editor: Alan Schatzberg
Article Type: Original article
Initial submission : 23/Mar/2018

ANEXO D – LISTA DOS TRABALHOS COLABORATIVOS DESENVOLVIDOS DURANTE O MESTRADO

1. Maximino C, Meinerz DL, **Fontana BD**, Mezzomo NJ, Stefanello FV, de S Prestes A, Batista CB, Rubin MA, Barbosa NV, Rocha JBT, Lima MG, Rosemberg DB. Extending the analysis of zebrafish behavioral endophenotypes for modeling psychiatric disorders: Fear conditioning to conspecific alarm response. **Behav Processes.** 2018 Apr;149:35-42. doi: 10.1016/j.beproc.2018.01.020.

2. Rosa LV, Ardais AP, Costa FV, **Fontana BD**, Quadros VA, Porciúncula LO, Rosemberg DB. Different effects of caffeine on behavioral neurophenotypes of two zebrafish populations. **Pharmacol Biochem Behav.** 2018 Feb;165:1-8. doi: 10.1016/j.pbb.2017.12.002.

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