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**EXPOSIÇÃO EMBRIONÁRIA AO ETANOL INDUZ ALTERAÇÕES  
COMPORTAMENTAIS E NEUROQUÍMICAS EM *ZEBRAFISH* ADULTO**

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutora em Bioquímica.

Orientador: Prof. Dr. Diogo Losch de Oliveira.  
Co-orientador: Prof Dr. Eduardo Pacheco Rico.

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Dedico esta tese a todos os profissionais que continuaram trabalhando em atividades essenciais durante esta pandemia. Àqueles que colocaram suas vidas em risco para salvar a de tantos outros. E a todos que confiam na ciência como a maior luz para nos guiar nestes momentos de trevas.

“Os que se encantam com a prática sem a ciência são como os timoneiros que entram no navio sem timão nem bússola, nunca tendo certeza do seu destino”. (Leonardo da Vinci)

“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.” (Arthur Schopenhauer)

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# PARTE I



## RESUMO

As Desordens do Espectro Alcoólico Fetal (*Fetal alcohol spectrum disorders* – FASD) são um conjunto de desordens neuro-desenvolvimentais, que incluem alterações morfológicas, neuroquímicas e comportamentais. Durante a última década, o *zebrafish* têm se tornado um excelente modelo para analisar os efeitos, tanto a curto quanto a longo prazos, das formas severas e brandas desta desordem, incluindo possíveis alterações na morfologia cerebral, déficits comportamentais e modificações nos sistemas de neurotransmissão. O objetivo desta tese foi analisar as possíveis alterações comportamentais e neuroquímicas em *zebrafish* adultos expostos a diferentes concentrações de etanol durante a fase inicial do desenvolvimento. Os embriões (24 horas pós-fertilização) foram expostos, durante duas horas, a diferentes concentrações de etanol (0.1%, 0.25%, 0.5%, e 1%), mimetizando formas brandas da Síndrome alcoólica fetal. Na idade adulta, os animais foram submetidos a análises comportamentais, neuroquímicas e morfológicas. Inicialmente, os animais foram submetidos aos testes comportamentais de exploração ao Tanque novo (*Novel tank test*) e preferência social. Observamos que a exposição ao etanol em fases iniciais do desenvolvimento induz, de forma concentração-dependente, uma redução no tempo gasto na zona coespecífica, próxima ao cardume, e alterações significativas na tarefa de Tanque novo (ex.: aumento do tempo no fundo do aparato), sugerindo elevada ansiedade, enquanto, o tratamento com buspirona mostrou que a redução de ansiedade está relacionada com redução no comportamento de cardume. Após, os animais foram mortos e seus cérebros removidos para análises neuroquímicas e histológicas. Os seguintes parâmetros foram avaliados: *binding* e captação de glutamato, imunoconteúdo de VGLUT2, atividade das enzimas glutamina-sintetase e  $\text{Na}^+/\text{K}^+$  ATPase e respirometria de alta-resolução. Outro grupo de animais foi utilizado para preparação de lâminas histológicas e subsequente avaliação da neurodegeneração na região do tálamo. Quanto aos parâmetros neuroquímicos do sistema glutamatérgico, observamos redução na captação de glutamato dependente de  $\text{Na}^+$  no cérebro de *zebrafish* adulto, a qual pode ser modulada pelo tratamento com ceftriaxona, através do aumento da expressão do transportador EAAT2. Os grupos de maior concentração de etanol, 0,5 e 1%, apresentaram redução na ligação de glutamato em frações enriquecidas de membrana e diminuição na atividade da enzima  $\text{Na}^+/\text{K}^+$  ATPase. Além disso, observamos redução da atividade da enzima glutamina-sintetase no grupo EtOH 1%. A exposição embrionária ao etanol não alterou o imunoconteúdo do transportador vesicular de glutamato VGLUT2, o metabolismo energético mitocondrial, e nem o número de neurônios do tálamo dos animais adultos. Nossos resultados confirmam que a exposição a diferentes concentrações de etanol durante o desenvolvimento leva a alterações comportamentais que persistem até a idade adulta, tais como ansiedade e diminuição da interação social. Quanto aos parâmetros neuroquímicos, a exposição embrionária ao etanol pode levar a alterações na neurotransmissão glutamatérgica no cérebro de *zebrafish* adulto, contudo, sem alterações nos núcleos anterior, ventrolateral e ventromedial do tálamo destes animais. Concluímos que mesmo a exposição embrionária a baixas concentrações de etanol é capaz de induzir alterações comportamentais e neuroquímicas, que persistem ao longo da vida do indivíduo.

## ABSTRACT

Fetal Alcohol Spectrum Disorders (FASD) describe a wide range of ethanol-induced developmental disabilities, including craniofacial dysmorphology, neurochemical and behavioral impairments. Zebrafish has become a popular animal model to evaluate the long-lasting effects of severe and milder forms of Fetal alcohol spectrum disorders, including alterations of neurotransmission, behavior impairments and cerebral structure. Avoidance of social interaction typical of Fetal alcohol spectrum disorders may be the result of increased anxiety and could be correlated with neurochemical alterations. Glutamate is one of the most affected neurotransmitter systems in ethanol-induced developmental disabilities, including decrease of glutamate uptake on adult animal brain. This thesis proposes to assess the behavioral and neurochemical alterations in adult zebrafish after exposure to different concentrations of ethanol in the initial phase of its development. Zebrafish larvae (24 hours post-fertilization) were exposed to ethanol (0.1%, 0.25%, 0.5%, and 1%) for 2 hours, to mimic the most prevalent milder Fetal alcohol syndrome cases. In the adult age (4 - 6 months), the animals were tested in different behavioral tasks: exploration of novel apparatus tank and social interaction. After the behavior analysis, the animals were euthanized and their brains were removed. The following parameters were measured: glutamate uptake, glutamate binding, VGLUT2 immunocontent, glutamine synthetase activity,  $\text{Na}^+/\text{K}^+$  ATPase activity, and high-resolution respirometry. The brains also were processed to histological sections to analyze possible modifications in the number of neurons at the thalamus. We observed an ethanol concentration dependent reduction of time spend in the conspecific zone, near the shoaling, compared to control and significant changes in the novel tank (e.g. increased bottom dwell time, increased distance to top) suggesting elevated anxiety to control, but we also found, using an anxiolytic drug buspirone, that reduction of anxiety is associated with reduced shoaling. Embryonic ethanol exposure reduced  $\text{Na}^+$ -dependent glutamate uptake in the zebrafish brain. This reduction was positively modulated by ceftriaxone treatment, a beta-lactam antibiotic that promotes the expression of the glutamate transporter EAAT2. Moreover, the 0.5 % and 1% ethanol groups demonstrated reduced glutamate binding to brain membranes and decreased  $\text{Na}^+/\text{K}^+$  ATPase activity in adulthood. In addition, ethanol reduced glutamine synthetase activity in the 1% EtOH group. Embryonic ethanol exposure did not alter the immunocontent of the glutamate vesicular transporter VGLUT2, the mitochondrial energetic metabolism of the brain and the number of neurons in the thalamus. Our results confirm that prior exposure to different concentrations of ethanol during the development leads to behavioral changes in adulthood, such as anxiety and decreased social interaction. Embryonic ethanol exposure may cause significant alterations in glutamatergic neurotransmission in the adult zebrafish brain, without alterations on the zebrafish anterior, ventrolateral and ventromedial thalamic nuclei. In conclusion, embryonic exposure even at low ethanol concentrations is able to impair behavior profile and crucial aspects of brain health such as the glutamate metabolism in a long-lasting form.

**LISTA DE ABREVIATURAS**

**FASD:** Fetal Alcohol Spectrum Disorders

**GS:** Glutamina sintetase

**VGLUT:** Transportador vesicular de glutamato

**SNC:** Sistema Nervoso Central

**EtOH:** Etanol

**EAAT:** Transportador de aminoácidos excitatórios

**SAF:** Síndrome alcoólica fetal

**AMPA:** Alfa-amino-3-hidroxi-5-metil-4-isoxazolepropionato

**GABA:** Ácido gama-aminobutírico

**GluR:** Receptores glutamatérgico

**NMDA:** N-metil-D-aspartato

**Na<sup>+</sup>/K<sup>+</sup> ATPase:** Bomba sódio-potássio

**GLT1:** Transportador de glutamato - 1

**5-HT 1A:** Receptor serotoninérgico

**MAO:** Monoamina oxidase

**ChAT:** Colina acetiltransferase

**AChE:** Acetilcolinesterase

**mGluR:** Receptores metabotrópicos de glutamato

**iGluR:** Receptores ionotrópicos de glutamato

**B.O.D.:** Bio Oxygen Demand

**MK-801:** Dizocilpina

**GluA 1:** Subunidade receptor de glutamato A1

**GluA 2:** Subunidade receptor de glutamato A2

**MRI:** Magnetic resonance image/ Imagem por ressonância magnética

## 1. INTRODUÇÃO

A elevada taxa de consumo de bebidas alcoólicas tem levado a números alarmantes de transtornos relacionados ao etanol, desde problemas como cirrose e falência de órgãos até questões de ordem social, como violência doméstica e acidentes de trânsito (World Health Statistics 2019). Além disso, são observadas também desordens de cunho psiquiátrico, como depressão e transtornos de humor (Stephen Rich & Martin, 2014). Estes fatores negativos, relacionados ao consumo abusivo de álcool, levam a incapacidade no trabalho e no convívio social, além de gerar elevados custos aos órgãos de saúde pública anualmente (Subbaraman & Roberts, 2019).

De acordo com a Organização Mundial da Saúde, o consumo de álcool por pessoas acima de 15 anos foi de 6,4 litros (álcool puro) em 2016, com variações dependentes de cada região mundial. O Brasil figura com uma média em torno de 30% superior, com consumo per capita de etanol de 8,7 litros (WHO Global Information System on Alcohol and Health (GISAH)). Este alto consumo está relacionado com uma série de distúrbios de saúde coletiva, segurança, convívio social e mortalidade. Enquanto a conscientização dos malefícios do uso do tabaco e sua regulamentação mais rígida tem prevenido inúmeros desfechos negativos nos últimos anos, o uso do álcool ainda está inserido na sociedade de forma lúdica e com livre acesso, fazendo com que seu consumo não seja moderado e nem reavaliado pela maioria da população (World Health Statistics 2017).

### 1.1. Desordens do Espectro Alcoólico Fetal

De acordo com a hipótese de David Barker na teoria *Developmental Origins of Health and Disease* (DOHaD), os insultos ocasionados ao feto em desenvolvimento podem ser os responsáveis pelo surgimento de doenças crônicas observadas em adultos. Eventos adversos que venham a ocorrer no ambiente uterino, tais como a exposição embrionária ao etanol, estresse materno e diabetes gestacional podem ser a origem de transtornos observados posteriormente na idade adulta (Barker, 2004(a); Barker, 2004(b)). O uso de drogas de abuso, como o álcool, por gestantes leva a alterações no desenvolvimento de órgãos e no crescimento do feto, além de acarretar em alterações nos sistemas fisiológicos metabólico, neurobiológico, cardiovascular e comportamental, o que pode ser relacionado ao conceito de DOHaD (Lunde et al., 2016).

O consumo de álcool por mulheres grávidas, desde pequenas doses até a ingestão frequente, pode levar a inúmeras alterações no desenvolvimento do bebê, as quais são denominadas conjuntamente de Desordens do espectro alcoólico fetal (Fetal alcohol

spectrum disorder –FASD) (Wilhoit et al., 2017). A condição mais grave deste transtorno é denominada como Síndrome alcoólica fetal (SAF), e pode vir a apresentar alterações morfológicas bastante evidentes, que abrangem má formação de órgãos e alterações faciais características – tais como microcefalia, lábio superior fino, maxilar pouco desenvolvido e pregas epicânticas. Neste quadro, o diagnóstico da SAF é de fácil constatação já que apresenta características marcantes, e, mesmo sendo uma condição permanente, o tratamento, quando iniciado precocemente, pode capacitar o indivíduo a condições adequadas de vida (Mukherjee, 2019). A prevalência desta desordem é de 14,6 indivíduos afetados, a cada 10.000 habitantes, quando se consideram suas formas mais severas na população mundial (Popova et al., 2017). Entre os efeitos maléficos ao feto, encontram-se: anormalidades neurológicas, disfunções comportamentais, atrasos no desenvolvimento, deficiências intelectuais, além das alterações morfológicas características (May et al., 2009; Stratton et al., 1996).

Contudo, a exposição embrionária ao etanol tem consequências mais amplas e, por vezes sutis, como observa-se nos portadores de FASD, sigla utilizada para denominar qualquer alteração observada em decorrência desta exposição ao etanol durante o período de desenvolvimento, onde a prevalência é de aproximadamente 77 indivíduos afetados a cada 10.000 (Popova et al., 2017). As alterações comportamentais, tais como ansiedade, dificuldades de interação social, tendência a comportamento violento e uso abusivo de drogas, são observadas nas formas mais brandas da desordem (Denny et al., 2017). Os problemas cognitivos estão relacionados a dificuldades de aprendizagem, baixa concentração, dificuldades de fala e atenção (Popova et al., 2016). Tal quadro pode estar correlacionado com distúrbios neuroquímicos, que tem origem na exposição do feto ao etanol durante o desenvolvimento embrionário. A mortalidade de neurônios, que leva a formação de conexões incorretas, é a causa inicial, podendo resultar em baixa expressão de transportadores e receptores, diminuição na atividade enzimática e reduzida funcionalidade do transporte e captação de neurotransmissores, afetando por fim os processos sinápticos (Valenzuela et al., 2011).

Na ausência de alterações morfológicas evidentes, o diagnóstico torna-se tarefa árdua, por tornar-se necessário um histórico da gestação, que possa comprovar a exposição ao etanol. Desta forma incorre-se em diagnósticos incorretos como déficit de atenção e hiperatividade ou autismo, o que dificulta o tratamento adequado (May et al., 2014; Sampson et al., 1997). Em casos mais brandos ainda, a pessoa pode conviver ao longo de sua vida com pequenas e médias dificuldades de aprendizado, problemas de

conduta e comportamento social, sem ao menos ser diagnosticada com alguma desordem neurocomportamental. Assim surge a importância de se compreender os mecanismos detalhados e as vias de ação do etanol ao longo do desenvolvimento embrionário, o que colabora para a prevenção, tratamento e devido diagnóstico dos danos, já que a FASD não apresenta possibilidade de cura.

A maioria dos estudos de FASD disponíveis na literatura focam justamente no indivíduo jovem (Kalberg et al., 2019; May et al., 2013), contudo, o impacto desta desordem na idade adulta é de extrema relevância, principalmente quando se considera as formas brandas da desordem, que muito provavelmente não receberam o diagnóstico apropriado (Denny et al., 2017). A necessidade de inserção no mercado de trabalho nesta fase da vida pode ser dificultada por problemas cognitivos e de conduta, que não foram devidamente tratados, já que as consequências da FASD são permanentes. Desta forma, é de extrema relevância que, além de se buscar a compreensão dos mecanismos de ação do etanol no indivíduo em desenvolvimento e buscar formas de prevenção, possamos entender seus impactos na vida adulta, buscando melhorias na qualidade de vida e minimização de seus efeitos negativos. Portanto, o foco deve ser a compreensão dos mecanismos de ação do etanol no cérebro em desenvolvimento, unindo as alterações neuroquímicas, com seu possível reflexo no comportamento e aprendizado, que podem ser observados a longo prazo.

## **1.2. Zebrafish como modelo de FASD**

O zebrafish (*Danio rerio*) é um peixe teleosteo da família Cyprinidae amplamente utilizado como espécie ornamental. Possui um tamanho aproximado de 5 cm na idade adulta e vive em média 3 a 5 anos. Com ampla adaptação ao ambiente, é capaz de sobreviver em variações de 18 a 30 °C e é originário da região sudeste do Himalaia (Engeszer et al., 2007). Atualmente, tem se tornado uma importante ferramenta para estudos científicos nas áreas de neurociência e comportamento. Por ser um modelo animal pequeno e necessitar de um menor espaço para sua manutenção, tem ganhado popularidade nos últimos anos em muitos laboratórios. Além disso, apresenta rápido desenvolvimento embrionário e, em um curto período de tempo, atinge sua maturidade sexual, facilitando estudos prospectivos ao longo das gerações. Na perspectiva da pesquisa da FASD, é um excelente modelo, já que a exposição ao etanol é totalmente controlada – tem-se maior precisão na quantidade de etanol que os embriões absorvem, já que estes animais apresentam fertilização externa, seus ovos são tratados de forma

independente, sem qualquer interferência de seus progenitores - , os ovos postos nas soluções alcoólicas absorvem o etanol através de um método não-invasivo comparado aos demais protocolos empregados em outros modelos animais, que é o caso de mamíferos em geral, onde a exposição ao etanol está diretamente ligada ao consumo materno e onde a visualização de alterações ao longo do desenvolvimento acarretaria em cirurgias invasivas de remoção do feto (Seguin et al., 2016).

Devido as características citadas acima, o *zebrafish* tem se destacado nos últimos anos nas pesquisas com etanol, sendo um excelente modelo animal nos estudos de transtornos da FASD (Lovely et al., 2016; Mahabir et al., 2014). Desde o começo dos anos 2000, o *zebrafish* vem sendo utilizado na pesquisa das desordens ocasionadas pela exposição ao etanol na fase embrionária, inicialmente com o uso de altas doses e foco no indivíduo jovem (Bilotta et al., 2004; Carvan et al., 2004) e, posteriormente, com a exposição a baixas concentrações de etanol e o acompanhamento até a fase adulta (Fernandes & Gerlai, 2009). No atual cenário da pesquisa médica, o *zebrafish* tem sido amplamente utilizado, justamente por ser um facilitador na compreensão dos mecanismos de ação de inúmeras drogas de abuso e substâncias nocivas (Müller et al., 2020).

Ao longo dos anos, os protocolos foram modificados e novos estudos foram realizados, variando as concentrações testadas e o tempo de exposição. Mas, sempre reproduzindo adequadamente as características observadas nos demais modelos animais e, até mesmo, o que é observado em humanos (Seguin and Gerlai, 2018). Portanto, além de todas as vantagens de manipulação e criação, é um modelo animal capaz de reproduzir adequadamente as desordens relacionada a FASD, tanto em sua fase larval (juvenil), quanto em sua fase adulta. Por apresentar relativa facilidade de manipulação genética, é uma excelente estratégia para a compreensão dos mecanismos envolvidos na exposição ao etanol, propiciando avanços nas questões ainda não completamente elucidadas (Lockwood et al., 2004).

### **1.3. Alterações comportamentais e neuroquímicas observadas na FASD**

As alterações neurocomportamentais têm importância destacada nos casos mais brandos, quando as alterações morfológicas não são evidentes ou não se tem o histórico do consumo de álcool durante a gestação. Contudo, são características presentes nas mais variadas formas da SAF, inclusive em suas formas mais severas (Denny et al., 2017; Sampson et al., 1997).

Os mais diversos padrões neurocomportamentais são afetados, como cognição, memória, funções executivas, comunicação e comportamento social (Cook et al., 2016). O princípio destas alterações estaria relacionado com déficits no processamento de informações complexas, levando a dificuldades no desempenho de tarefas diárias e socialização, o que se tornaria mais pronunciado na adolescência (Kodituwakku, 2007). Tais alterações são reproduzidas em modelos animais, tanto em roedores, quanto em *zebrafish*, facilitando o estudo da FASD, já que, em humanos, temos a questão ética que nos impossibilita a condução de estudos de ingestão de bebidas alcoólicas por gestantes, além de não haver precisão na quantidade de etanol ao qual o bebê é exposto durante a gestação. (Gerlai, 2015).

O diagnóstico de FASD em humanos é diretamente relacionado com a presença de alterações em no mínimo três domínios do neurodesenvolvimento, como habilidade motora, cognição, aprendizado e habilidades sociais (Cook et al., 2016). Desta forma, os portadores desta desordem costumam apresentar problemas de convívio social, dificuldades de aprendizado e transtornos de ansiedade (Wilhoit et al., 2017). As mesmas alterações são observadas em estudos utilizando roedores, por exemplo. Ratos adultos, expostos ao etanol durante a fase inicial da gestação, apresentam comportamento ansioso no teste de campo aberto (Zhou et al., 2010); também são observados déficits de aprendizado e memória em tarefas correlacionadas com funções hipocámpicas e corticais em modelos de camundongos expostos ao etanol (Brady et al., 2012; Karacay et al., 2015). Alterações nas habilidades sociais são características da FASD, crianças portadoras desta desordem apresentam dificuldades de interação social, na criação de vínculos de amizade e na percepção e resposta adequada a pistas sociais (Bishop et al., 2007; Kully-Martens et al., 2012).

O *zebrafish* é um excelente modelo animal para estudos de comportamentos social, por se tratar de um animal de cardume, a interação social é fator crucial na sua sobrevivência e fácil de ser quantificada por protocolos já bem estabelecidos (Oliveira, 2013). A interação social, que consiste em manter proximidade e interagir com seus coespecíficos, é observada desde cedo, a partir de uma semana de vida (Hinz et al., 2013). O comportamento de cardume, que é mais complexo e precisa de movimentos coordenados em conjunto, é mais facilmente observado nos animais adultos (Miller and Gerlai, 2012). Nos protocolos de exposição embrionária ao etanol, quando se analisa os peixes adultos, observa-se diminuição tanto na interação social, como no comportamento de cardume (Fernandes et al., 2015a). Tal padrão comportamental pode ser observado no



protocolo de indução da forma branda de FASD inicialmente sugerido por Fernandes & Gerlai (2009), onde são utilizadas baixas concentrações de etanol, variando de 0,1% a 1%, em uma exposição de apenas 2 horas. Sem aparentes anormalidades morfológicas, os animais apresentam apenas alterações comportamentais, como diminuição de interação social e comportamento aversivo em relação ao cardume na idade adulta. Na sequência, outros estudos encontraram alterações cognitivas, de aprendizado e memória, além de comportamento do tipo ansioso (Amorim et al., 2017; Cleal & Parker, 2018; Lutte et al., 2018). Estudos posteriores realizados em nosso grupo de pesquisa mostraram que animais expostos previamente a este protocolo de indução de FASD, apresentaram comportamento do tipo ansioso e diminuição de interação social quando analisados na idade adulta (Baggio et al., 2018).

As alterações comportamentais observadas tanto em humanos, como em modelos animais de FASD, não se restringem apenas ao comportamento social. Dificuldades de aprendizado e memória são observadas em *zebrafish* que foram expostos ao etanol na fase embrionária, quando testados no *plus-maze*, os animais são incapazes de associar os estímulos visuais a recompensa de comida (Fernandes et al., 2014). Também se observa efeitos na memória de trabalho espacial, já que *zebrafish* testados na tarefa de *Y-maze* apresentam diminuição na exploração alternada dos braços do aparato (Cleal & Parker, 2018). A ansiedade, apesar de ser característica da FASD, ainda tem resultados controversos no modelo de *zebrafish*. Enquanto Parker et al. (2014) apresenta resultados de aumento no comportamento do tipo ansioso ao realizar o teste de Tanque novo, Seguin et al. (2016) não encontrou alterações de ansiedade frente a novidade e presença de predador.

Normalmente, os transtornos comportamentais têm sua origem em alterações que ocorreram previamente no cérebro em desenvolvimento (Valenzuela et al., 2011). Nesta forma branda de FASD observam-se significativas alterações neuroquímicas, quando se utiliza o *zebrafish* como modelo animal, tais como queda na captação de glutamato, diminuição nos níveis de serotonina e dopamina (Baggio et al., 2017; Buske & Gerlai, 2011; Fernandes et al., 2015a), além de alterações no sistema purinérgico (Lutte et al., 2018). Há estudos que apresentaram alterações na expressão de genes envolvidos no processo de desenvolvimento, formação de tubo neural, cérebro e olhos (*six3b* e *glil*), o que levaria a mudanças no desenvolvimento morfológico dos animais em decorrência da exposição embrionária ao etanol (Loucks & Ahlgren, 2012).

O cérebro de crianças portadoras de FASD tende a ter um menor volume (microcefalia) e apresentar menor número de neurônios ou formação de conexões sinápticas incorretas, o que é ocasionado pela morte celular desencadeada pelo etanol (Valenzuela et al., 2011). Estas alterações podem estar relacionadas com problemas permanentes de aprendizagem e comportamento (Berman & Hannigan, 2000). Entre as ações promovidas pelo consumo excessivo de etanol na idade adulta, está descrito a modificação de vias de transdução de sinal, ocasionadas pela alteração de diferentes sistemas de neurotransmissão, entre eles o glutamatérgico, principal via de sinalização excitatória do SNC e relacionado a eventos de neurodegeneração por excitotoxicidade, que podem ser desencadeados pelo consumo de etanol (Eşel, 2006). As alterações comportamentais, observadas na FASD e já discutidas acima, como hiperatividade, ansiedade e aumento da agressividade, também podem ser relacionadas ao impacto do etanol sobre o SNC, durante o desenvolvimento, por meio da modificação de estruturas, diminuição do volume cerebral e morte neuronal em regiões responsáveis pela execução de tais tarefas comportamentais (Cook et al., 2016; Valenzuela et al., 2011).

A ação do etanol no SNC não é completamente elucidada, ele atravessa facilmente a barreira hemato-encefálica e afeta a atividade cerebral de forma dose-dependente (Eşel & Dinç, 2017). Modula tanto as vias de transdução de sinal excitatórias, quanto inibitórias (Chastain, 2006), diminui a atividade metabólica (Wang et al., 2000), inibe a neurotransmissão glutamatérgica e intensifica as sinapses mediadas por GABA e glicina (Breese et al., 2006; Kash et al., 2017; Roberto & Varodayan, 2017; Söderpalm et al., 2017). Tais alterações na homeostase do SNC são associadas às alterações comportamentais geradas pelo consumo crônico de álcool, como ansiedade (Lovinger & Roberto, 2011), problemas de memória e déficits cognitivos (Mukherjee, 2013).

O mecanismo conhecido pelo qual o álcool causa danos no cérebro é através da formação de conexões neuronais incorretas, em decorrência da morte apoptótica celular (Creeley & Olney, 2013). Essa morte celular é associada com mudanças cerebrais, incluindo reduções na massa encefálica e distúrbios neurocomportamentais de longa duração. Em um estudo utilizando *zebrafish* e exposição embrionária ao etanol, observou-se diminuição no número de neurônios diferenciados e alterações em subpopulações de neurônios motores e sensoriais, ocasionado pelo desbalanço entre proliferação celular e apoptose (Joya et al., 2014). Ainda se especula a sinalização necessária e os mecanismos de fato envolvidos para que tais processos ocorram. A possibilidade mais aceita é o etanol ter como principal alvo o gene ligado ao desenvolvimento Sonic hedgehog (Shh), que

está relacionado diretamente com a diferenciação celular, o que justificaria as alterações no número de neurônios e na diferenciação neuronal (Loucks & Ahlgren, 2009).

A etiologia das desordens do SNC causadas pelo uso de drogas de abuso, como o etanol, é complexa, envolvendo uma combinação de fatores, tais como perfil genético do indivíduo, influência do ambiente, idade e, no caso da FASD, o período gestacional em que ocorreu a exposição ao etanol (Wall et al., 2016). De forma geral, o etanol e as demais drogas de abuso agem de forma a modificar as funções neuronais em níveis moleculares, celulares e de circuitos, gerando alterações fisiológicas e comportamentais (Berridge, 2017; Volkow et al., 2016). Desta forma, compreender os mecanismos e as vias de ação do etanol durante o desenvolvimento pré-natal, conectando estrutura cerebral, aspectos genéticos, alterações comportamentais e análises neuroquímicas é peça chave na prevenção e tratamento de desordens como a FASD, por poder proporcionar a descoberta de novos alvos terapêuticos.

#### **1.4. Sistema Glutamatérgico**

O glutamato é o principal neurotransmissor excitatório do SNC e está correlacionado com patologias do consumo de álcool e abstinência (Most et al., 2014). Sua função como neurotransmissor é crucial para inúmeros processos, especialmente na mediação de neuroplasticidade, aprendizado e memória (Henley & Wilkinson, 2013; Warburton et al., 2013), justamente processos que se encontram alterados em pacientes de FASD em decorrência da exposição embrionária ao etanol. Quando presente em altas concentrações na fenda sináptica, por um longo período, pode ser neurotóxico (Obrenovitch et al., 2000) e sua excitotoxicidade tem sido correlacionada com a patogênese de inúmeros transtornos agudos e crônicos do SNC (Maragakis & Rothstein, 2004).

As diversas ações do glutamato, tanto fisiológicas quanto patológicas, resultam da presença de receptores glutamatérgicos (GluRs), tanto neuronais como gliais (Ozawa et al., 1998). Os GluRs são divididos em duas classes: ionotrópicos (iGluRs) e metabotrópicos (mGluRs). Os iGluRs contêm um canal iônico cátion-específico e são subdivididos em três subtipos:  $\alpha$ -amino-3-hidroxi-5-metil-4-isoxazolepropionato (AMPA), cainato and N-metil-D-aspartato (NMDA). Por outro lado, os mGluRs são acoplados a proteínas G e estão subdivididos em 8 subtipos (mGluR1-8) (Reiner & Levitz, 2018).

Uma terceira classe de proteínas, igualmente importante para a função glutamatérgica, são os transportadores de glutamato ou também denominados como transportadores de aminoácidos excitatórios (EAATS). Sua principal função é recaptar o glutamato liberado na fenda sináptica a fim de concluir a transmissão glutamatérgica (Danbolt, 2001). Os transportadores EAAT regulam os níveis de glutamato extracelular de forma sódio-dependente. Por meio de uma associação física com a enzima  $\text{Na}^+/\text{K}^+$  - ATPase, os transportadores captam o glutamato da fenda sináptica, de forma a manter os níveis adequados deste neurotransmissor nos compartimentos sinápticos (Sattler & Rothstein, 2006). O processo de captação do glutamato é contra gradiente, dependendo do transporte ativo da enzima  $\text{Na}^+/\text{K}^+$  - ATPase para formar um gradiente eletroquímico capaz de transportar o glutamato através das membranas de astrócitos e neurônios (Rose et al., 2009). Portanto, a associação dos transportadores com a enzima exerce a função de regulador da neurotransmissão mediada por glutamato.

Além dos transportadores EAAT, o glutamato pode ser armazenado e transportado também pelos transportadores vesiculares, conhecidos como vGluTs. Os transportadores vesiculares de neurotransmissores são proteínas presentes na membrana de vesículas sinápticas, responsáveis pela captação de neurotransmissores específicos, determinando a quantidade destes que será liberada na fenda sináptica (Van Liefferinge et al., 2013). Os transportadores vesiculares de glutamato são responsáveis pelo transporte e posterior armazenamento de glutamato para o interior das vesículas sinápticas, o qual, através de exocitose, é liberado posteriormente na fenda sináptica (Takeda & Ueda, 2017; Ueda, 2016). Existem três isoformas de vGluTs (vGluT1, vGluT2 e vGluT3), que tem sua expressão e distribuição cerebral modificadas ao longo do desenvolvimento (Ueda, 2016).

Em um estudo realizado por nosso grupo de pesquisa, foi observado que *zebrafish* adultos, que haviam sido expostos a baixas concentrações de etanol na fase embrionária, apresentavam diminuição na captação de glutamato cerebral (Baggio et al., 2017). O processo de captação está diretamente ligado com a função dos transportadores, mas ainda pode ser explicado por outros fatores envolvidos com o metabolismo glutamatérgico, como a ação de receptores e de transportadores vesiculares. Alterações nos transportadores vesiculares (vGluTs) são observadas em inúmeros transtornos e patologias, inclusive em modelos de ingestão voluntária de etanol, resultando em redução na expressão do transportador vesicular vGluT2 no córtex pré-frontal de ratos (Pickering et al., 2015).

O processo de apoptose celular induzido pelo etanol está relacionado com o bloqueio dos receptores de glutamato NMDA (Ikonomidou et al., 2000). Receptores estes que tem sua expressão diminuída em ratos adultos que foram expostos ao etanol na fase embrionária (Gerace et al., 2016; Goodfellow et al., 2016). Com a redução na expressão de receptores, pode ocorrer redução nos níveis de glutamato que se liga nas membranas e é captado para dentro das células (Vallés et al., 1995). Assim, menos substrato disponível justificaria a atividade diminuída da enzima glutamina-sintetase – responsável pela conversão de glutamato a glutamina, nos astrócitos - no cérebro de ratos submetidos ao modelo de FASD (Ledig et al., 1989).

As alterações neuroquímicas observadas no modelo de FASD, em especial, no sistema glutamatérgico, podem estar relacionadas com os distúrbios comportamentais característicos desta desordem. O aprendizado social em *zebrafish*, por exemplo, é dependente da sinalização dos receptores de glutamato NMDA, ao passo que tratamentos com MK-801, um antagonista não-competitivo dos receptores NMDA, afeta o comportamento social em modelos animais (Maaswinkel et al., 2013).

Portanto, o consumo de etanol pode afetar a homeostasia glutamatérgica, seja por alterar a expressão e atividade de receptores e transportadores, seja por afetar a atividade de enzimas relacionadas ao seu metabolismo. Desta forma, inúmeras moléculas já foram testadas - MS-153, ceftriaxona e GPI-1046, por exemplo – as quais regulam positivamente a expressão do transportador EAAT2, como possível alvo terapêutico para desordens relacionadas ao álcool (Rao et al., 2015). Tal abordagem evidencia a importância do sistema glutamatérgico como uma estratégia terapêutica nas desordens da síndrome alcoólica fetal, tanto comportamentais quanto neuroquímicas.

### **1.5. Modulações Farmacológicas**

Com o intuito de tentar reverter as alterações neuroquímicas e comportamentais ocasionadas pelo etanol ou como tentativa terapêutica, o reposicionamento de medicamentos já utilizados para outras patologias, parece a escolha mais viável e ágil a ser testada. O reposicionamento de fármacos consiste em utilizar medicamentos que já se sabem seguros para o uso em humanos, com a vantagem de economia de tempo, na pesquisa e desenvolvimento de novos fármacos e com grande economia de recursos monetários (Cha et al., 2018). De outra maneira, o tratamento com fármacos já prescritos para outros fins, pode surgir como uma forma de se compreender os mecanismos de ação do etanol sobre as sinapses glutamatérgicas do cérebro em desenvolvimento. Foi para este

fim que se empregou a ceftriaxona neste estudo, sem fins terapêuticos, mas como mediador no entendimento da ação do etanol no sistema glutamatérgico.

### **1.5.1. Bupirona**

A ansiedade é um dos sintomas mais descritos para FASD e pode estar relacionada com outras alterações comportamentais, como problemas de interação social (Baggio et al., 2018; Lam et al., 2019). O comportamento do tipo ansioso tem relação com desequilíbrios no sistema serotoninérgico, que levaria ao aumento nos níveis de hormônios do estresse e depressão (O'Connor et al., 2006). A exposição embrionária ao etanol é capaz de diminuir a diferenciação, migração e crescimento axonal de neurônios serotoninérgicos, por meio de alterações na comunicação entre células gliais e neuronais (Goodlett et al., 2005). Estes neurônios são responsáveis por controlar funções básicas do organismo, como respiração, batimentos cardíacos e pressão sanguínea.

A Bupirona é um ansiolítico não-sedativo bem estabelecido para tratamentos de distúrbios generalizados de ansiedade em adultos (Strawn et al., 2018). Ela apresenta alta afinidade pelo receptor serotoninérgico 5-HT 1A, ligando-se a ele nos neurônios pré e pós-sinápticos, inibindo a taxa de disparos dos mesmos. O que resulta na supressão do sistema serotoninérgico e aumento do noradrenérgico e dopaminérgico (Loane & Politis, 2012). Já foi demonstrado que esta droga estaria relacionada ao aumento do tempo de abstinência em alcoolistas (Litten et al., 1996; Pettinati, 1996), sugerindo que a bupirona pode ajudar na redução do comportamento ansioso em alcoolistas com distúrbios de ansiedade (Lovinger et al., 1989) e possivelmente em pacientes de FASD. Em estudos prévios com *zebrafish*, a bupirona gerou efeitos ansiolíticos em testes de modelo de ansiedade e interação social (Gebauer et al., 2011), os mesmos resultados foram observados em testes de claro/escuro e Tanque novo (Bencan et al., 2009; Maximino et al., 2013).

### **1.5.2. Ceftriaxona**

O EAAT2 é o transportador de glutamato de maior abundância e importância no sistema nervoso central, tendo sua disfunção associada a distúrbios neurológicos. O antibiótico  $\beta$ -lactâmico ceftriaxona tem ação neuroprotetora, já que é capaz de aumentar a expressão deste transportador (Rothstein et al., 2005). Miller et al. (2008), utilizando roedores como modelo da doença de Huntington, observaram que a administração de ceftriaxona foi capaz de atenuar o fenótipo comportamental associado a esta patologia, além de aumentar os níveis de captação de glutamato, devido a sua ação positiva sobre a expressão de GLT1 (EAAT2). A ceftriaxona tem mostrado efeitos positivos em vários

modelos de desordem, como no estudo de Thöne-Reineke et al. (2008), onde sua administração é capaz de diminuir a mortalidade, aumentar a captação de glutamato e a expressão proteica de neurotrofinas.

Apesar de ser um antibiótico de terceira geração, da classe das cefalosporinas, graças a sua ação neurotóxica, a ceftriaxona tem sido testada e vem apresentado resultados relevantes para uma série de estudos em diferentes patologias. Em ratos expostos ao consumo crônico de etanol, que apresentam o transportador GLT-1 (EAAT2) e a enzima glutamina sintetase com suas atividades diminuídas, a ceftriaxona é capaz de reverter tais resultados (Das et al., 2015). Também apresenta importantes resultados na atenuação do comportamento de dependência em cocaína (Sari et al., 2009), e redução no consumo de álcool tanto em modelos de ratos adultos (Sari et al., 2011), quanto em filhotes (Stennett et al., 2017).

A ceftriaxona tem o potencial de modular a transmissão glutamatérgica e evitar excitotoxicidade através do aumento da captação de glutamato, função primária do transportador EAAT2, justamente o seu alvo de atuação (Lee et al., 2008). Portanto, o uso da ceftriaxona surgiria como uma alternativa de modulação aos resultados de diminuição da captação de glutamato observados previamente no modelo de FASD em *zebrafish*.

### **1.6. Tálamo e FASD**

O tálamo é uma região que recebe *inputs* cerebelares e exerce função central em modular e transferir informações ao córtex cerebral, de forma a otimizar e corrigir os dados fornecidos. Não se trata apenas de uma conexão passiva, e sim de um processador de informações neurais (Sherman, 2016). Em mamíferos e outros vertebrados é uma estrutura multinuclear, localizada no diencéfalo, capaz de regular estados de atenção e alerta, e agir como uma interface entre o isocórtex e estruturas cerebrais mais profundas. (Jones & Rubenstein, 2004). O padrão de mecanismos moleculares do tálamo parece ser altamente conservado entre vertebrados (Scholpp & Lumsden, 2010).

O tálamo tem grande importância na conexão com o córtex cerebral, dependendo de ligações corretas entre as vias aferentes e seus alvos para o funcionamento cerebral. Quando esta conexão é falha ou afetada por eventos como trauma e abuso de drogas, surgem déficits de memória e aprendizado (Armstrong-James et al., 1988). O etanol é capaz de danificar esta via tálamo-cortical, formando conexões incorretas que estariam por trás dos déficits cognitivos observados na FASD (Mooney & Miller, 2010).

A exposição embrionária ao etanol é capaz de gerar danos em várias regiões cerebrais críticas para aprendizado, memória e cognição, como hipocampo e córtex pré-frontal, que formam um circuito funcional com o tálamo (Lawrence et al., 2012; Livy et al., 2003). Ratas que foram expostas ao etanol durante o desenvolvimento, apresentam danos no domínio ventral talâmico, com redução no número de neurônios e no volume das estruturas (Gursky et al., 2019). Em exames de ressonância magnética, crianças e adolescentes diagnosticadas com FASD apresentaram diminuição no volume de estruturas cerebrais, como hipocampo e tálamo (Nardelli et al., 2011). Desta forma, afetando diretamente o tálamo ou as regiões adjacentes que se conectam a ele, o etanol é capaz de levar a danos em circuitos funcionais responsáveis pelo processo de aprendizado ou funções executivas.



## 2. OBJETIVOS

### 2.1. Objetivo geral

Avaliar os mecanismos neuroquímicos responsáveis pelas alterações no perfil comportamental do *zebrafish* adulto exposto a diferentes concentrações de etanol na fase inicial do desenvolvimento embrionário.

### 2.2. Objetivos específicos

**Objetivo específico I:** Analisar as possíveis alterações comportamentais em animais adultos, que foram expostos ao etanol na fase embrionária. Inicialmente, confirmar os dados da literatura que relatam alterações no comportamento social e de cardume de *zebrafish*; como segundo objetivo, analisar se os animais apresentam comportamento do tipo ansioso.

**Objetivo específico II:** Analisar parâmetros relacionados a homeostasia glutamatérgica alterados pelo etanol na fase embrionária, em cérebro de *zebrafish* adulto, os quais seriam responsáveis pela queda na captação de glutamato observada em estudo anterior.

**Objetivo específico III:** Analisar a morfologia cerebral, na região dos núcleos do tálamo, de animais expostos na fase embrionária ao etanol, através da contagem de neurônios e observação de possíveis alterações estruturais.

## **PARTE II**

### 3. CAPÍTULO I

Embryonic alcohol exposure leading to social avoidance and altered anxiety responses in adult zebrafish

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Artigo publicado no periódico Behavioural Brain Research, 2018.

**Tema:** As formas mais brandas de FASD são caracterizadas basicamente por alterações comportamentais, que podem ser reproduzidas em modelos animais, com o objetivo de melhorar a compreensão dos mecanismos celulares envolvidos e buscar possíveis alvos terapêuticos. Alterações de interação social e ansiedade estão entre os distúrbios comportamentais mais relatados.

**Objetivo:** Analisar as possíveis alterações comportamentais em animais adultos, que foram expostos ao etanol na fase embrionária. Inicialmente, confirmar os dados da literatura que relatam alterações no comportamento social e de cardume de *zebrafish*; como segundo objetivo, analisar se os animais apresentam comportamento do tipo ansioso.

**Principal conclusão:** *Zebrafish* adultos, que foram expostos ao etanol na fase embrionária, apresentam diminuição de interação social e comportamento do tipo ansioso. Tais alterações comportamentais podem ser moduladas pelo tratamento com buspirona.

**Contribuição a formação do aluno:** Este artigo possibilitou unir os projetos de mestrado e doutorado, gerando continuidade no meu projeto de pesquisa e abrindo novas ideias para o seguimento do estudo.



## Research report

## Embryonic alcohol exposure leading to social avoidance and altered anxiety responses in adult zebrafish



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Zebrafish

## ABSTRACT

Fetal Alcohol Spectrum Disorders (FASD) is a syndrome characterized by neurological and behavioral impairments. A recently discovered hallmark of FASD is impaired social behavior. Avoidance of social interaction typical of FASD may be the result of increased anxiety. Previously, the zebrafish was successfully used to model embryonic alcohol induced social abnormalities. Here, we analyzed both anxiety and social responses using a zebrafish FASD model, in adult fish. We exposed zebrafish embryos to low concentrations of ethanol (0.1%; 0.25%; 0.5% and 1% v/v) for 2 h at, 24 h post-fertilization, to mimic the most prevalent milder FASD cases, and investigated the ensuing alterations in adult, 4-month-old, zebrafish. We studied social interaction in the social preference task and anxiety in the novel tank task. We observed an ethanol dose dependent reduction of time spend in the conspecific zone compared to control, corroborating prior findings. We also found significant changes in the novel tank (e.g. increased bottom dwell time, increased distance to top) suggesting elevated anxiety to control, but we also found, using an anxiolytic drug buspirone, that reduction of anxiety is associated with reduced shoaling. Our results confirm that embryonic alcohol exposure disrupts social behavior, and also show that its effects on anxiety related phenotypes may be genotype, alcohol administration method, experimental procedure and test-context dependent.

## 1. Introduction

Fetal alcohol spectrum disorders (FASD) result from alcohol exposure during fetal development and range from full “fetal alcohol syndrome” (FAS) to milder forms of the disease. Recent studies suggest the prevalence of FASD to be as high as 5%, a likely underestimate given that the milder forms of the disease may often be misdiagnosed or not diagnosed at all [1,2]. Due to the high prevalence of this disease, and due to the lifelong suffering it causes, it is important to understand the mechanisms of the disease.

Animal experimental models have allowed making important discoveries about how alcohol works in the brain, and how it affects embryonic development. The zebrafish is particularly suited for FASD research. Alcohol can be administered to this fish in a simple and controlled manner by immersion [3–5]. Initially, zebrafish was used

only to study FAS by using higher alcohol concentrations and/or by administering alcohol for prolonged periods resulting in gross abnormalities [6,7]. However, the milder form of FASD is three times more prevalent than the severe forms of the disease [2], and thus [4] decided to attempt to establish a mild embryonic alcohol exposure paradigm using zebrafish. Similar to the human condition, zebrafish exposed to low concentrations of ethanol during embryonic development exhibited reduced social (shoaling) behavior, paralleling the impaired social behaviors found in FASD patients suffering from the milder forms of the disease [4,8–10]. There may be many possible explanations as to the behavioral and neurobiological mechanisms underlying the observed embryonic alcohol induced changes in social behavior of zebrafish. A recent study showed that ethanol modifies the sonic hedgehog signaling pathway and results in long-term behavioral changes, including alterations of anxiety [11]. However, there have

Abbreviations: FASD, fetal alcohol syndrome disorder; FAS, fetal alcohol syndrome; hpf, hours post-fertilization; EtOH, ethanol

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been no studies that directly evaluated the relationship between anxiety responses to social interaction.

Anxiety disorders are among the most commonly reported problems in children and adults with FASD [12–15]. Anxiety is often induced by diffuse, aversive contexts, including the novel nature of a test situation. Novelty is defined as a new or unfamiliar experience and has been found to induce anxiety like responses in humans and other non-human animals including the zebrafish [16]. For the zebrafish, the novel tank diving test has been often utilized to induce and quantify novelty-associated behavioral stress/anxiety-like responses. This test has also been used for investigating pharmacological modulators of anxiety-like phenotypes in adult zebrafish [17–19].

In the current study, we investigate the behavior of adult zebrafish (4-month-old) exposed to alcohol during their embryonic development. We have primarily two goals. One, to confirm or disprove the effect of embryonic alcohol exposure on social behavior reported repeatedly in the literature, and two, to study whether embryonic alcohol exposure alters anxiety-like responses in zebrafish, a highly controversial finding.

## 2. Material and methods

### 2.1. Animals

Adult zebrafish (*Danio rerio*; 10–12 months old, from a genetically uncontrolled and heterogeneous wild-type stock (striped pattern and short-fin phenotype) were obtained from a local commercial supplier (Delphis, RS, Brazil). The fish (mixed male/female, measuring  $3.5 \text{ cm} \pm 0.3$  in standard length and weighing  $0.550 \text{ g} \pm 0.050$ ) were housed in a re-circulating system equipped with mechanical and biological filtration at a temperature of  $28^\circ\text{C}$ , pH of 7.4 and a conductivity of  $500 \mu\text{S}$  (system water). Breeding arrays were used for obtaining fertilized eggs. The zebrafish holding room was illuminated by ceiling-mounted fluorescent lamps on a 14/10 light/dark photoperiod (lights on at 8:00 a.m.). The animals were fed four times a day with a commercial flake fish food (Alcon BASIC, Alcon, Brazil) and nauplii of brine shrimp (*Artemia salina*), and maintained according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011). The Ethics Committee approved all procedures with animal subjects for the Use of Animals – CEUA (number 27725) from the Universidade Federal do Rio Grande do Sul.

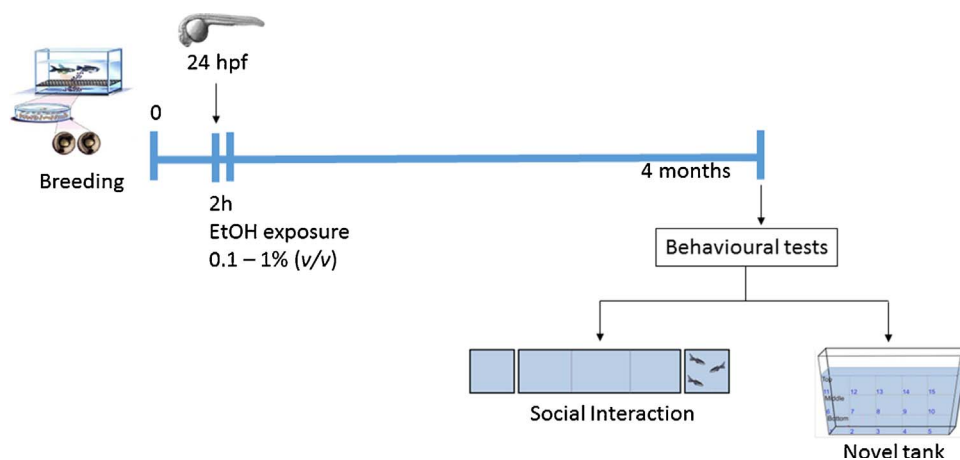
### 2.2. Experimental design

The time line of experimental procedure is represented on (Fig. 1). Eggs of zebrafish were collected 2.5 hpf. Approximately 500 fertilized eggs were randomly selected and divided into 5 equal rearing tanks. At 24 hpf, each group of zebrafish embryos received one of the following concentrations of alcohol (Absolute Ethanol Merck® (CAS number 64-

17-5)) solution: 0.00%, 0.01%, 0.25%, 0.50%, or 1.00% (v/v). The developmental stage of alcohol exposure was chosen to be 24 hpf, because of prior studies showing significant behavioral effects on fish exposed at this stage [4,20,21] and because the stage of neural tube development (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995 also see [http://zfin.org/zf\\_info/zfbook/stages/](http://zfin.org/zf_info/zfbook/stages/) and <http://www.ehd.org/virtual-human-embryo/>). After 2 h of alcohol exposure, the embryos were washed twice with system water. With the above alcohol treatment procedure, we were hoping to induce only mild developmental abnormalities resulting in lack of increased mortality or gross structural aberrations but leading to minor changes detectable at the behavioral level [4]. The embryos were incubated on Biological Oxygen Demand B.O.D. at  $28^\circ\text{C}$  and, once free swimming, were fed twice a day with paramecium during their larval stage. Three weeks later, the fish were moved into 2.8-l rearing tanks (20 fish per tank) of a high-density rack system, which had a multistage filtration and they were fed four times a day with a commercial flake fish food (Alcon BASIC, Alcon, Brazil) and nauplii of brine shrimp (*Artemia salina*). Zebrafish remained in these holding tanks until behavioral experiments, which were conducted after the fish reached 4 months of age (mature young adults, 50–50% male–female). The sample sizes of treated fish were of at least eight per group. One day before the beginning of the behavioral tests, the holding tanks were transferred to the experimental room. The environmental conditions (e.g. temperature and photoperiod) remained the same as during the rearing and housing of the fish. The next day, the lights turned on at 7:00 am, the experimental fish were fed once with *Artemia* as before at 9:00 am, and the behavioral tests commenced subsequently and were conducted between 10:00 am and 4:00 pm. This test schedule remained for subsequent days. The experimental room was illuminated by ceiling-mounted fluorescent lamps, maintaining a 14/10 light/dark photoperiod. Two yellow sheets of paper (standard letter size:  $21.59 \text{ cm} \times 627.94 \text{ cm}$ ) were placed behind the experimental tank to ensure a uniform background for the video analysis. In order to boost the contrast between the background and zebrafish, two 60-watt light bulbs were placed 40 cm behind the yellow screen [18].

### 2.3. Social preference test

The social preference test was conducted in a glass tank divided into three parts: the center area, the empty side and the conspecific side [22]. Adjacent to the conspecific side was another tank that contained three stimulus zebrafish whose size matched the test fish's. The empty side had an empty stimulus tank adjacent to it. The experimental zebrafish was introduced in the center of the tank, and was allowed to explore it for 6 min [23]. After each individual test, the water of the experimental tank was replaced with fresh system water in order to standardize olfactory cues and other water parameters for all experimental subjects. The stimulus fish used for the evaluation of social



**Fig. 1.** Experimental design of the methodological time line approach used for the evaluation of the behavior of adult zebrafish FASD model. The experimental protocol consisted of one exposure of embryos 24 hpf during 2 h in ethanol concentrations: 0.00%, 0.01%, 0.25%, 0.50%, or 1.00% (v/v). After 4 months, the behavior was evaluated during a single 6-min trial in the social behavioral tank and open tank. The tank of social behavior was virtually divided in three horizontal areas and open tank was virtually divided in three vertical areas (bottom, middle, and top), with five sections per area for the evaluation of the behavior.

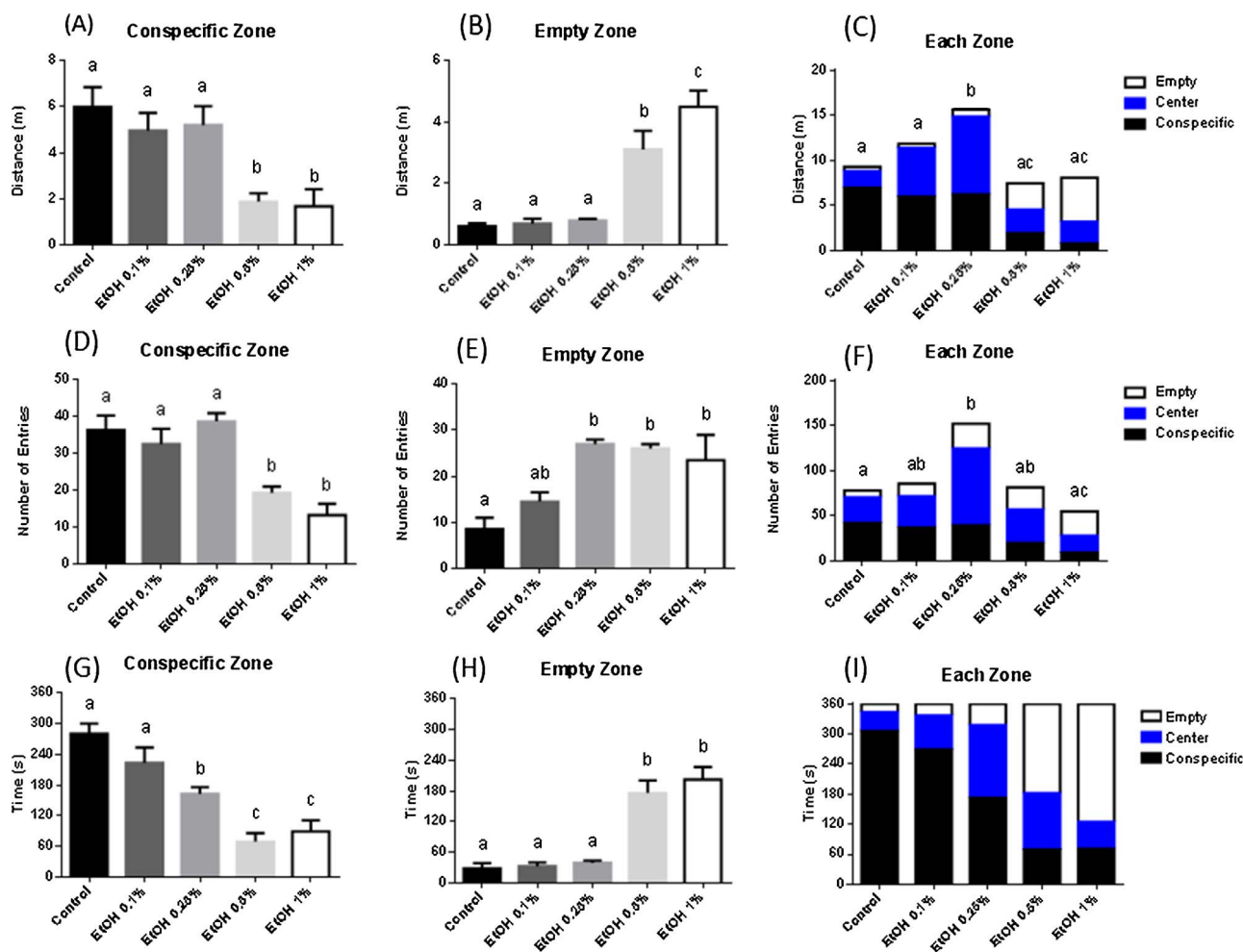


Fig. 2. Behavioral profile of social interaction in adult zebrafish FASD model. The figure shows the distance traveled in each zone: conspecific (A), center (B), empty (C); the number of entries: conspecific (D), center (E), empty (F); and the time spend in each zone: conspecific (G), center (H), empty (I). The data were analyzed by one-way ANOVA followed by Tukey's post hoc test and Kruskal-Wallis test followed by Dunn's multicomparison test, considering  $p < 0.05$  as significant. Different letters indicate significant differences among the groups.

interaction were moved to the experimental room 24 h before the start of experiments to acclimatize them. The experimental fish were gently netted from their home tank, and were individually transferred to the experimental tank. Before and during the experiments all possible environmental distractions (noise, vibration, movement in front of the tank) were kept to a minimum.

In a control experiment, we wanted to demonstrate the effect of an anxiolytic compound on the shoaling response. The anxiolytic drug, positive control, used for this study was buspirone hydrochloride (CAS Number 33386-08-2., Sigma, St. Louis, USA), which we dissolved in saline solution (NaCl 0.9%). The drug was injected intraperitoneally at a volume of 1  $\mu$ L/g, according to the weight of the fish at a dose of 25 mg/kg. Injected fish were returned to their holding tank for a 10 min acclimation period, after which they were subjected to the social preference test (Maximino et al. [42]).

Furthermore, the behavior was recorded at 30 frames/s and quantified using video-tracking ANY-maze® software (Stoelting CO, USA), which allowed us to measure the number of entries and time spent in each of the three areas of the test tank, as well as the total distance travelled in each area. In response to the sight of conspecific stimulus fish, experimental fish are expected to show strong preference toward the side where the stimulus fish are, spending up to 65–70% of their time in the conspecific side.

#### 2.4. Novel tank

Adult zebrafish exposed to ethanol during their embryonic development were placed singly in a novel tank. The tank was made of plastic and was trapezoidal (23.9 cm long at the bottom, 28.9 cm long at the top, 15.1 cm high). It was filled with 1.5 L of home tank water. Between each individual test, the water in the novel tank was changed to guarantee the same physical/chemical water conditions. The tank was divided into three equal virtual horizontal areas (bottom, middle, and top), with five sections per area as previously described [18]. A webcam (Microsoft® LifeCam 1.1 with Auto-Focus) was placed in front of the tank to monitor the location and swimming activity of the zebrafish. The webcam was connected to a laptop for recording the videos, and the behavioral parameters were automatically measured using video-tracking software (ANY-maze®, Stoelting CO, USA). We took extra care to minimize handling stress. The locomotor activity of zebrafish was measured using the following behavioral endpoints parameters: 1) the total distance travelled; 2) the mean speed; and 3) the time in the bottom, middle and top area. Zebrafish in the novel tank initially tend to stay close to the bottom and as their fear/anxiety levels subside, they are expected to explore the mid or upper areas, which reflect habituation to the novel environment [18,24]. Increased time spent at the top of the tank has been interpreted as indication of reduced anxiety [25–27]. The exploratory profile of fish was estimated by quantifying the horizontal and vertical movement and location parameters as previously described [18]. We drew an occupancy plot, presented as a heat

map (blue to red), indicating the time the fish spent in each section. If the group shows replicable behavior (i.e., no large interindividual variation), when all the animals are plotted in a single occupancy plot, variation in color (yellow to red) is observed [28,26].

### 2.5. Statistics

Parametric data were expressed as mean  $\pm$  standard error of the mean (S.E.M.) and analyzed by repeated-measures analysis of variance (ANOVA) using Tukey's post hoc test. Differences were considered statistically significant at  $p \leq 0.05$  and Kruskal-Wallis test followed by Dunn's multicomparison test, considering  $p \leq 0.05$  as significant. A one-sample *t*-test was used to investigate whether animals injected with buspirone (positive control) exhibited a significant difference in social interaction.

### 3. Results

There were no physical abnormalities, e.g. reduced eye diameter, delayed eggs hatching, abnormal tail anatomy observed during development in the fish treated with alcohol for any concentration group. These results are in accordance with findings described previously [4].

In the social preference test we analyzed three parameters: distance travelled, number of entries and time in each zone (Fig. 2). The distance travelled in the conspecific zone of the groups EtOH 0.5 and 1% were significantly lower when compared to control group (Fig. 2A) (one-way ANOVA,  $F [4,39] = 7.503$ ,  $p < 0.0001$ ; test  $p < 0.05$ ). In the empty zone, the distance travelled were higher in the groups EtOH 0.5 and 1% and EtOH 1% is the highest (Fig. 2B) (one-way ANOVA,  $F [4,39] = 24.18$ ,  $p < 0.0001$ ; Tukey's test  $p < 0.05$ ). In Fig. 2C we grouped the total distance travelled in the three zones with the EtOH 0.25% showing the highest value. The number of entries in the conspecific zone of the groups EtOH 0.5 and 1% were lower compared to control (Fig. 2D) (Kruskal-Wallis test,  $p < 0.0003$ ; Dunn's Multiple Comparison test,  $p < 0.05$ ), while, the number of entries in the empty zone of the groups EtOH 0.25, 0.5 and 1% were higher compared to control (Fig. 2E; Kruskal-Wallis test,  $p < 0.0001$ ; Dunn's Multiple Comparison test,  $p < 0.05$ ). In Fig. 2F, in relation to the total number of entries in the three zones, the EtOH 0.25% showed the highest value. When observed the conspecific zone, the results showed a significantly reduction of the time spend in a dose-dependent manner among EtOH 0.25, 0.5 and 1%. (Fig. 2G) (one-way ANOVA,  $F [4,39] = 17.10$ ,  $p < 0.0001$ ; Tukey's test  $p < 0.05$ ). The time spend in the empty zone of the groups EtOH 0.5 and 1% were higher compared to control (Fig. 2H) (one-way ANOVA,  $F [4,39] = 26.99$ ,  $p < 0.0001$ ; Tukey's test  $p < 0.05$ ). In Fig. 2I we stratified the time spend in each area with the total task time.

The spatio-temporal analysis of behavior in the social preference test of a representative animal of each group is shown in Fig. 3. The occupancy plot shown in this figure represents a fish of each group. The figure exemplifies that fish of the control group established a home base and spent more time near the conspecific zone (Fig. 3A). Fish of the EtOH 0.1% group also appeared to spend more time near the conspecific zone but its values are somewhat lower compared to control (Fig. 3B). Unlike fish of the control and EtOH 0.1% groups, fish of the EtOH 0.25% group appeared to move in a more dispersed manner, exploring the entire apparatus without any apparent preference for the conspecific or the empty zones (Fig. 3C). The fish of EtOH 0.5% group spent significantly more time near the empty zone reversing the natural preference for conspecifics seen in control fish (Fig. 3D). The fish of the EtOH 1% group appeared to further intensify their preference for the empty zone and spent more time in this zone compared to control fish and fish of all other alcohol treated groups (Fig. 3E).

To evaluate whether the above described differences were associated with alterations in swimming (locomotor) abilities, we analyzed swim speed in the novel tank test. We found no significant differences among fish of any groups in their total distance travelled (one-way

ANOVA,  $F [4,39] = 1.765$ ,  $p < 0.0001$ ; Bonferroni test  $p < 0.05$ ) and mean speed (one-way ANOVA,  $F [4,39] = 0.5253$ ,  $p < 0.0001$ ; Tukey test  $p < 0.05$ ), data not shown. On the other hand, we observed differences between the treated groups and the control in the time spent at the bottom of the apparatus. Control fish (Fig. 4A) showed a preference for the bottom with some exploration of the top replicating a spatio-temporal behavioral profile described previously [29,18]. Fish of the EtOH 0.1% group explored all the apparatus, but showed a preference for the bottom area, whereas the middle was primarily used for vertical transitions (Fig. 4B). Fish of the EtOH 0.25% group explored the top only during the last few minutes of the test, and showed a strong preference for the bottom (Fig. 4C). Fish of the EtOH 0.5% group explored the top only with rapid excursions toward this area returning quickly to the bottom where they stayed immobile for prolonged periods of time (Fig. 4D). Fish of the EtOH 1% group showed strong preference to move near the bottom of apparatus, indicating a reduced vertical but somewhat preserved horizontal exploration profile (Fig. 4E). The above results suggest differences in anxiety levels among fish of the treatment groups, i.e. embryonic alcohol induced modification of anxiety-like behaviors.

The occupancy plot depicts (Fig. 4) the spatial distribution of the experimental fish. Occupancy plot, represented as a heat graph (blue to red), indicates the time that the animals spent in each section, the longer the animal spends in a certain area, the redder the given region. If the group shows replicable behavior, without large variations of locomotion between the individuals and all the animals are plotted in a single occupancy plot, variation in color (yellow to red) is observed [29,18].

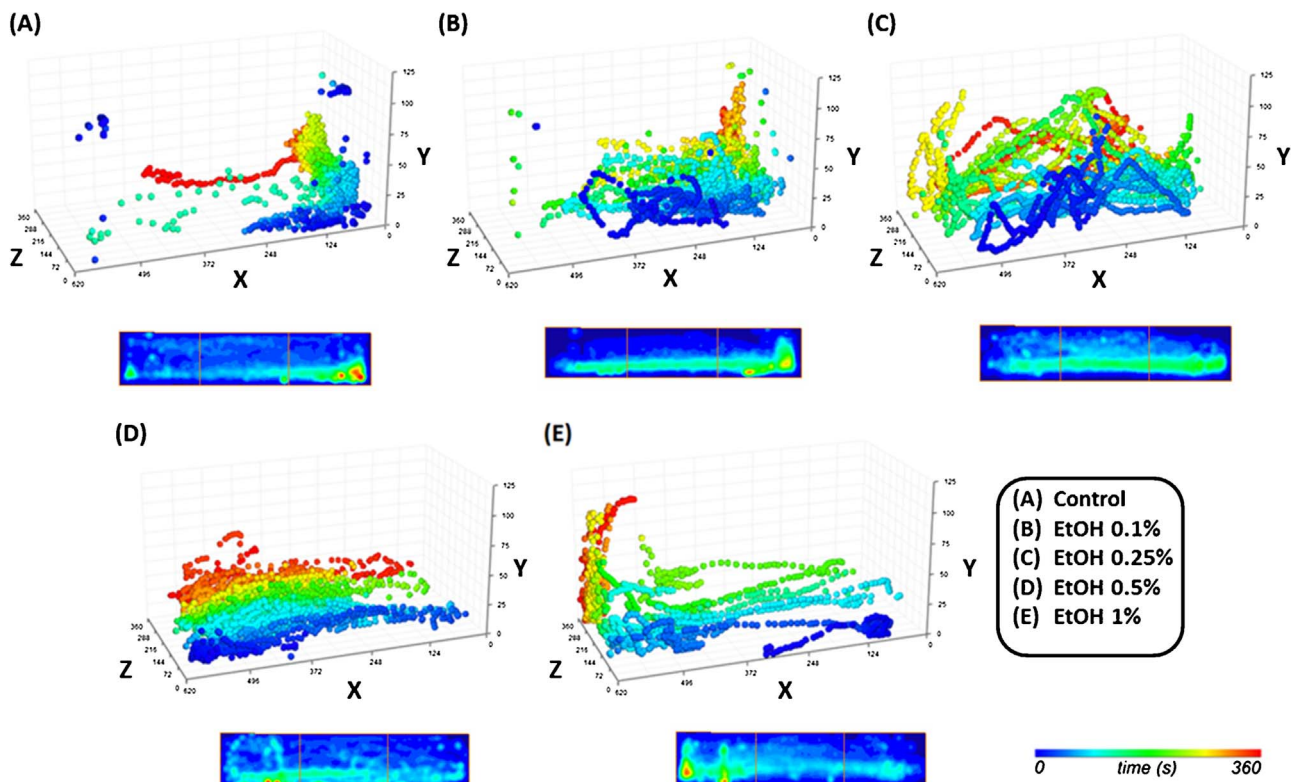
The Fig. 5 shows basic endpoint behaviors in novel tank test. The latency to the top was significant higher in EtOH 1% when compared to control (Fig. 5A) (one-way ANOVA,  $p < 0.0001$ ; Tukey's test  $p < 0.05$ ,  $F [4,35] = 3678$ ). The time spent in the top for the groups EtOH 0.1, 0.25 and 1% were significantly lower when compared to control (Fig. 5B) (one-way ANOVA,  $p < 0.0001$ ; Tukey's test  $p < 0.05$ ,  $F [4,41] = 5934$ ), while in the bottom, of all the groups pretreated with ethanol were significantly higher (Fig. 5C) (one-way ANOVA,  $p < 0.0001$ ; Tukey's test  $p < 0.05$ ,  $F [4,49] = 6564$ ). The distance travelled in the top of all the groups pretreated with ethanol were lower compared to control (Fig. 5D) (one-way ANOVA,  $p < 0.0001$ ; Tukey's test  $p < 0.05$ ,  $F [4,39] = 5623$ ).

In order to verify whether social interaction was modulated by responses related to anxiety, we performed a pharmacological manipulation. We employed the 5-HT1AR partial agonist buspirone as positive control. We found that buspirone, at the 25 mg/kg body weight dose employed, significantly decreased the time experimental fish spent in the conspecific zone compared to saline injected control fish (Fig. 6;  $t = 3244$ ,  $df = 13$ ,  $p = 0.0064$ ).

### 4. Discussion

In the current study, we used low concentrations of ethanol, administered to developing zebrafish embryos at 24 hpf for 2 h, a dosing regimen that is claimed to allow one to model milder forms of FASD in the zebrafish [4]. We measured the effect of this alcohol treatment on social preference and novelty induced anxiety in adult zebrafish. Our goal was, one, to check the validity of previous findings showing impaired shoaling responses induced by embryonic alcohol treatment [4,8,20,21,30], and even more importantly, two, to explore whether this impairment is accompanied or explained by alteration in anxiety.

Recently, the use of zebrafish as an animal model for FASD has increased considerably. After the pioneering work by Billota et al. [31], numerous studies have been conducted to investigate the effect of alcohol and whether it is dependent upon (i) the stage of development at which the animal is exposed to this substance; (ii) the length of time during which the embryos are exposed to ethanol; (iii) the dose of alcohol effects; (iv) and the age of the fish at which the behavioral



**Fig. 3.** Comparison of the spatio-temporal behavior of the experimental groups in the social interaction apparatus.

Representative spatio-temporal reconstructions of zebrafish swimming activity during the 6 min of the test were obtained by plotting animal traces (X-axis and Y-axis) over time (Z-axis). The test segments (0–360 s) are represented by a color scale gradient and are shown in the Z-axis (blue to red). Occupancy plot of the control (A), EtOH 0.1% (B), EtOH 0.25% (C), EtOH 0.5% (D) and EtOH 1% (E) groups displaying specific patterns of time spent in each arm of the apparatus during a 6-min trial. The data were analyzed using video-tracking software (ANY-maze, Stoelting CO, USA). The area on the left side limits with the empty tank. The area on the right side delimits with the tank with conspecifics individuals.

alterations are tested [4,8,21]. In addition to the discovered alterations in social responses, the effects of embryonic alcohol exposure have also been analyzed in the context of other behaviors, including learning, memory, anxiety and aggressive behavior [4,30,32–34]. However, the findings have not always been consistent.

In the current study, in addition to the analysis of shoaling responses, we decided to focus on anxiety related behaviors, because shoaling responses may be dependent upon such behaviors. That is, alterations in the latter may manifest as modified shoaling. Briefly, the main goal of the current study was to investigate whether the embryonic alcohol induced changes in shoaling responses are independent of or accompanied by putative fear/anxiety related alterations.

Anxiety-like behaviors may be induced by, and observed in zebrafish using different behavioral procedures and apparatus [18,26]. The novel tank task is often employed, as it represents a mildly aversive environment known to induce anxiety. Anxiety in this task can be evaluated through measurement of parameters such as time spent and distance travelled in the bottom of the tank, and latency to reach the top. For example, Parker et al. [33] exposed zebrafish between 2 and 9 days post-fertilization to 20 mM (0.1%) ethanol showed an increase of time spent by the alcohol treated fish at the bottom of the novel tank. Using a similar protocol, Baiamonte et al. [30], exposed their fish between 1 and 9 dpf to ethanol at 20 mM and 50 mM dose (0.3%) followed by behavioral analysis in zebrafish larvae 9dpf, 23dpf and adult (6 months). These authors showed a decrease of time spent in the lower part of the apparatus by adult fish exposed to alcohol during their embryonic development, a result opposite to what Parker et al. [33] found. Last, Seguin et al. [34] exposed zebrafish to alcohol at their age of 24 hpf (i.e. one day after fertilization), and found no evidence for altered anxiety or fear in the adult fish analyzed at their age of 6 months.

In the current study, we employed an alcohol dosing procedure whose parameters including timing (developmental stage) and length (2 h) of alcohol exposure as well as the dose of alcohol (0.1% to 1.0%) were identical or very similar to those employed by Fernandes & Gerlai [4] and Seguin et al. [34]. Although we employed a slightly different behavioral set up and also quantified the behavioral responses somewhat differently from those of the latter studies, we found highly similar results with regard to shoaling. We found embryonic alcohol exposure to significantly and dose-dependently reduce shoaling. Furthermore, using a positive control, an anxiolytic drug, buspirone, we demonstrated that reduction of anxiety leads to reduction of the shoaling response. This finding is in-line with the consensus that is based upon a large number of studies [reviewed in [4,8,34,43]] suggesting that the main function of shoaling is predator avoidance and that increased fear or increased anxiety should enhance shoaling. Thus, our finding of reduced/impaired shoaling response in the embryonic alcohol treated adult zebrafish may be explained as a result of diminished fear and/or anxiety.

Notably, however, a recent study by Seguin et al. [34] demonstrated no changes in fear or anxiety induced by embryonic alcohol treatment, despite that the timing, exposure method and length and concentration of alcohol administered were identical to the methods of the current study. It is also notable that in our current study we observed significant increase of anxiety responses in the embryonic alcohol exposed fish when tested at their adult stage of development in the novel tank task. Fish that were administered alcohol during their embryonic development spent increased amount of time at the bottom of the apparatus compared to control when tested at their adult stage, an effect that was alcohol concentration dependent. These results corroborate those of Parker et al. [33] but contradict those found by Seguin et al. [34] and Baiamonte et al. [30]. Our results obtained in our anxiety test paradigm



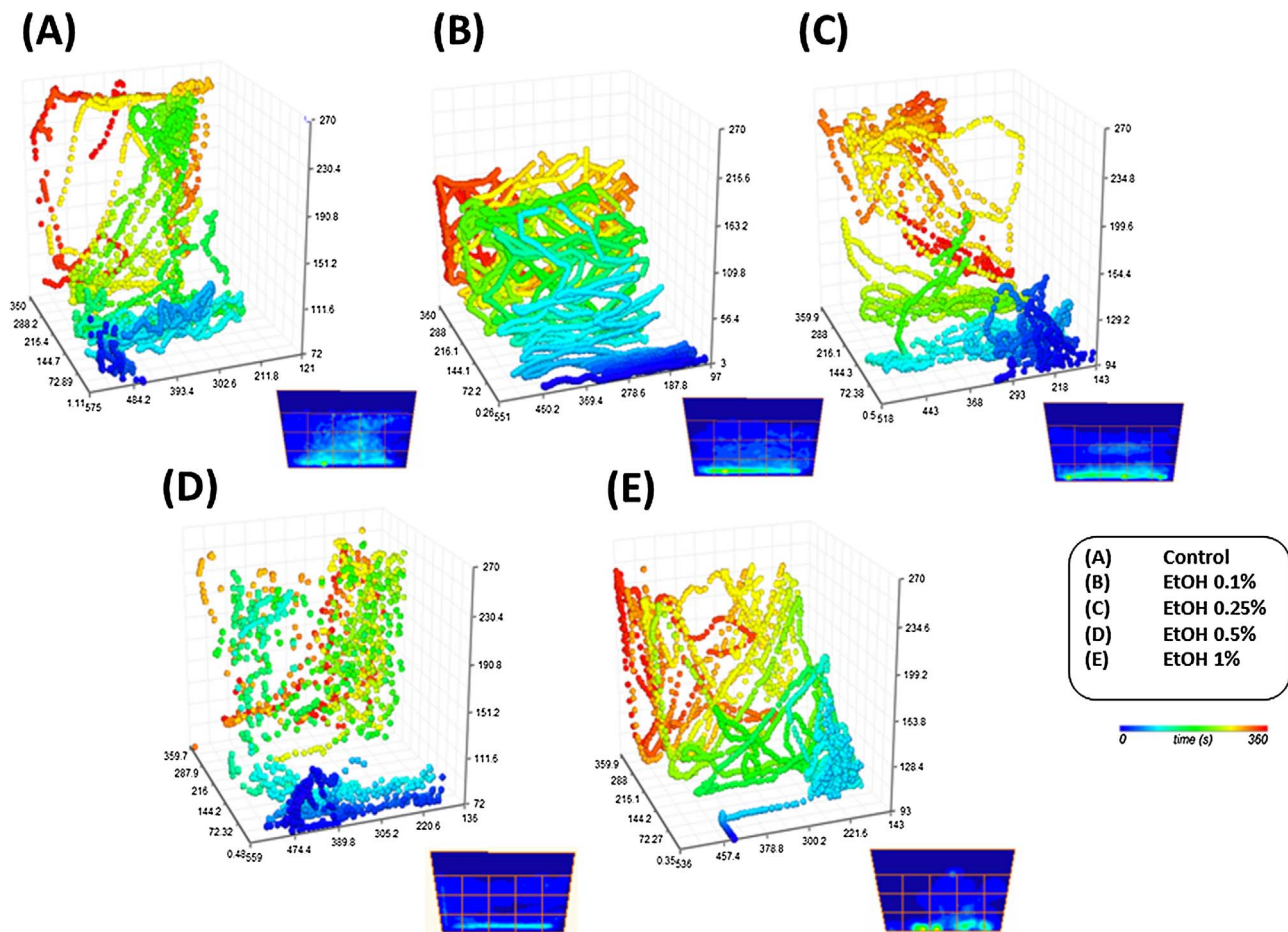


Fig. 4. Occupancy plot of the adult zebrafish FASD model in Novel tank test. Occupancy plot of the control (A), EtOH 0.1% (B), EtOH 0,25% (C), EtOH 0,5% (D) and EtOH 1% (E), displaying specific patterns of time spent in each zone of the apparatus during a 6-min trial. The data were analyzed using video-tracking software (ANY-maze, Stoelting CO, USA).

also contradict what we found with our own positive control. The latter suggested that reduced anxiety (and not increased anxiety) is associated with reduced shoaling.

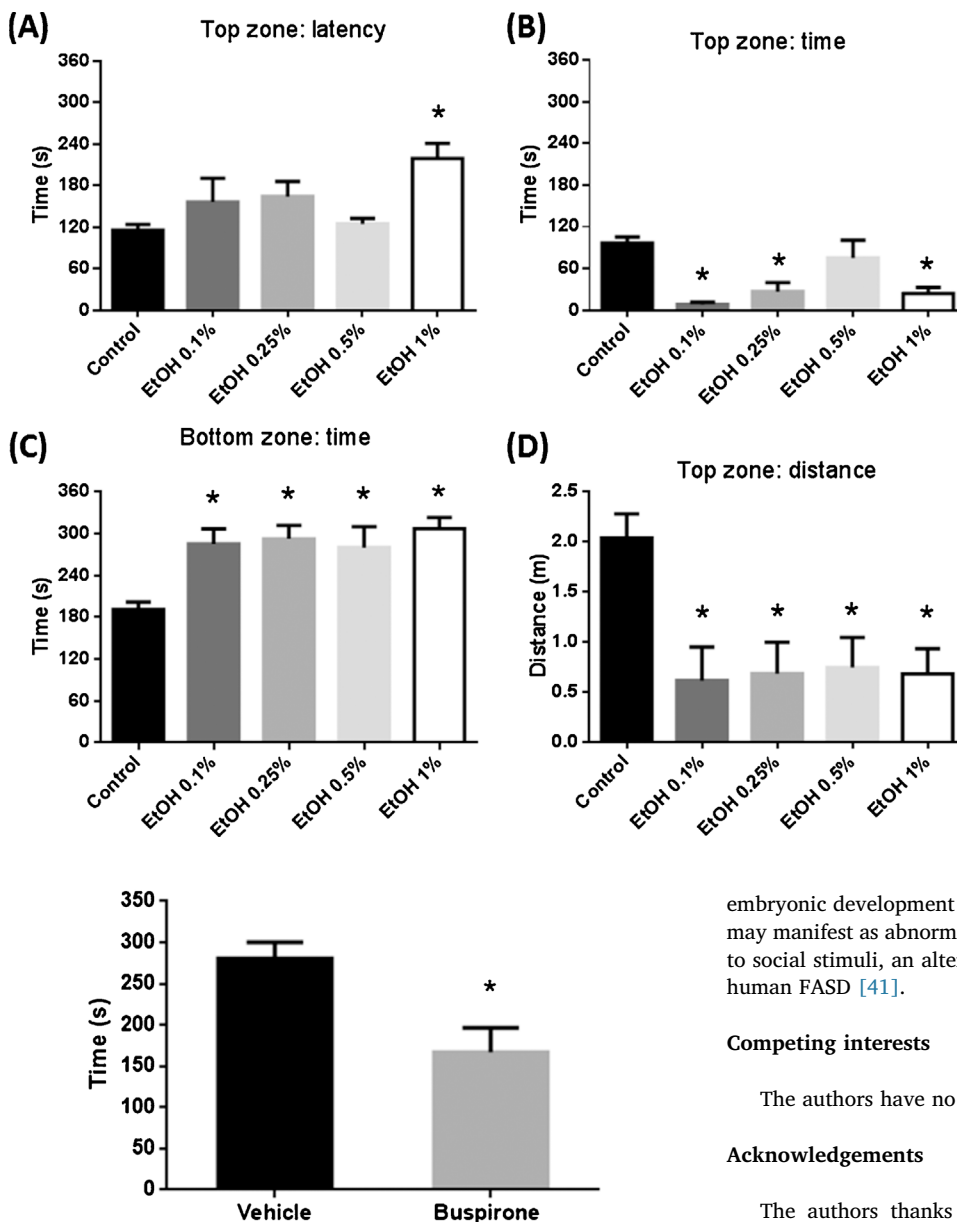
What may be the reason for these discrepancies is not known at this point. However, we note that anxiety responses have been found complex and context dependent [44,45] and thus depending on the experimental set up (shoaling test versus novel tank) different conclusions may be drawn. We also note that the strain/population origin of experimental zebrafish used in our study was different from that of Seguin et al. [34] who employed the genetically well defined, quasi-inbred AB strain, the same strain that was utilized in all studies conducted by Fernandes & Gerlai [4], Buske & Gerlai [8], and Fernandes et al. [20,21]. On the other hand, Baiamonte et al. [30] and Parker et al. [33] both used another genetically defined and genetically fairly homogeneous strain, the Tubingen strain, in their studies. In the current study, we used a genetically undefined, likely highly heterogeneous, population of zebrafish. We emphasize that Mahabir et al. [5] found strain dependent differences in neurochemical changes induced by embryonic alcohol exposure in zebrafish, and that Gerlai et al. [35] detected significant strain differences in behavioral responses of zebrafish to acute alcohol exposure. Thus, genetic effects may explain at least some of the discrepant findings in anxiety related responses of embryonic alcohol exposed zebrafish published in the literature.

Another possibility that may have led to inconsistent results is the different experimental procedures employed by the above cited studies. The timing and method of alcohol exposure in the Parker et al. [33] and Baiamonte et al. [30] studies were different from those employed in our current study and in the Seguin et al. [34] or Fernandes & Gerlai [4] studies. Parker et al. [33] started to expose zebrafish embryos to alcohol

at their age of 48 hpf, while Baiamonte et al. [30] started the exposure as early as 24 hpf, but in both studies the alcohol exposure was continuous. Furthermore, all zebrafish of age 48 hpf or older of these latter studies were dechorionated, and thus were directly immersed in the alcohol solution, certainly leading to a lot higher amount of alcohol reaching the embryonic brain. This procedure is highly different from ours and that of the Seguin et al. [34] and Fernandes & Gerlai [4] studies, which employed a single 2 h long alcohol exposure at 24 hpf after which the embryos were washed. Notably, at this stage the embryos are inside the egg and the chorion protects them, and as a result only about 1/25 of the concentration of alcohol in the bath were found to reach the embryo [4,5]. Last, we also note differences in the analysis of behavior among the above cited studies. For example, Seguin et al. [34] employed a 40-l glass tank as the novel aversive tank to induce anxiety, and also utilized computer animated images of a sympatric predator of zebrafish to induce fear. On the other hand, we utilized a small, 1.5 l, plastic tank as described by Roseberg et al. [18]. Notably, the latter may induce restraint stress, and thus may be more aversive than the large glass tank employed by Seguin et al. [34].

We must emphasize, however, that although controversies do exist with regard to whether embryonic alcohol treatment affects fear and/or anxiety in zebrafish across the cited studies and even within our own results, impairment of social behavior induced by the embryonic alcohol treatment remains a highly consistent finding despite all the methodological differences prevalent in the literature.

Importantly, abnormalities in social behavior have been described in both human FASD patients [36,37] and in rodent models of this syndrome [38–40], whereas the question whether embryonic alcohol treatment alters anxiety remains controversial even in rodent studies.



**Fig. 6.** Buspirone, an anxiolytic drug (positive control) reduces the time spent near the social stimulus. Saline (0.9%) injected (black bar) and buspirone injected (grey bar) fish are compared. The time fish spent in conspecific zone (mean  $\pm$  S.E.M. are shown). The drug or saline was injected intraperitoneally at a volume of 1  $\mu$ L/g, according to the weight of the fish at a dose of 25 mg/kg. The data were analyzed by one-sample *t*-test. \* indicates  $p \leq 0.05$ .

For example, Diaz et al. [39], found alteration of anxiety only in the light/dark behavioral task but not in other tasks.

## 5. Conclusions

Our study confirmed the effect of embryonic alcohol exposure on shoaling, and opened the question of whether this alcohol treatment leads to increased or decreased anxiety or whether it has no effect on it. Our findings highlight the importance of future studies in which the behavioral mechanisms of embryonic alcohol exposure induced changes may be understood, and also demonstrate the complexity of such responses in zebrafish. While the question surrounding the effect of embryonic alcohol exposure on anxiety remains controversial, our study confirms an important conclusion: exposure to even small amounts of alcohol and only for a short period of time during

**Fig. 5.** Classical endpoint analysis for the assessment of anxiolytic-like behavior. The graph shows latency to the top (A), time spent at the top (B), time spent at the top area (D) of the control (A), EtOH 0.1% (B), EtOH 0.25% (C), EtOH 0.5% (D) and EtOH 1% (E). The data were analyzed using video-tracking software (ANY-maze, Stoelting CO, USA). The data were analyzed by one-way ANOVA followed by Tukey post hoc test, considering  $p \leq 0.05$  as significant (\*).

embryonic development can lead to lasting changes in the brain which may manifest as abnormal behavior, in this case as reduced responding to social stimuli, an alteration that now is recognized as a hallmark of human FASD [41].

## Competing interests

The authors have no conflicts of interest to declare.

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#### 4. CAPÍTULO II

Fetal alcohol spectrum disorders model alters the functionality of glutamatergic neurotransmission in adult zebrafish.

Suelen Baggio, Kamila Zenki, Alberto Martins Silva, Thainá Garbino dos Santos, Giovana Rech, Gabriela Lazzarotto, Renato Dutra Dias, Ben Hur Mussulini, Eduardo Pacheco Rico, Diogo Losch de Oliveira.

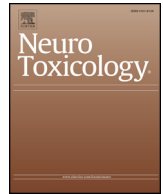
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**Tema:** A queda na captação de glutamato em cérebro de *zebrafish* adulto, exposto ao etanol na fase embrionária, pode estar relacionado a alterações no transporte e metabolismo do glutamato.

**Objetivo:** Analisar parâmetros relacionados a homeostasia glutamatérgica alterados pelo etanol na fase embrionária, em cérebro de *zebrafish* adulto, que poderiam ser responsáveis pela queda na captação de glutamato observada em estudo anterior.

**Principal Conclusão:** Alterações nos transportadores e receptores e diminuição da atividade de enzimas relacionadas com a homeostasia do glutamato, podem ser as razões para a queda na captação de glutamato, observada anteriormente em modelo de FASD em *zebrafish* adulto.

**Contribuição a formação do aluno:** Este trabalho representa o eixo central da tese, portanto foi de grande valia a sua escrita, pois representou uma revisão do tema estudado até então. Também serviu de excelente experiência para ser autora correspondente e responder revisores e editor.



## Full Length Article

## Fetal alcohol spectrum disorders model alters the functionality of glutamatergic neurotransmission in adult zebrafish

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## ABSTRACT

Fetal alcohol spectrum disorders (FASD) describe a wide range of ethanol-induced developmental disabilities, including craniofacial dysmorphism, and neurochemical and behavioral impairments. Zebrafish has become a popular animal model to evaluate the long-lasting effects of, both, severe and milder forms of FASD, including alterations to neurotransmission. Glutamate is one of the most affected neurotransmitter systems in ethanol-induced developmental disabilities. Therefore, the aim of the present study was to evaluate the functionality of the glutamatergic neurotransmitter system in an adult zebrafish FASD model. Zebrafish larvae (24 h post-fertilization) were exposed to ethanol (0.1 %, 0.25 %, 0.5 %, and 1%) for 2 h. After 4 months, the animals were euthanized and their brains were removed. The following variables were measured: glutamate uptake, glutamate binding, glutamine synthetase activity, Na<sup>+</sup>/K<sup>+</sup> + ATPase activity, and high-resolution respirometry. Embryonic ethanol exposure reduced Na<sup>+</sup>-dependent glutamate uptake in the zebrafish brain. This reduction was positively modulated by ceftriaxone treatment, a beta-lactam antibiotic that promotes the expression of the glutamate transporter EAAT2. Moreover, the 0.5 % and 1% ethanol groups demonstrated reduced glutamate binding to brain membranes and decreased Na<sup>+</sup>/K<sup>+</sup> + ATPase activity in adulthood. In addition, ethanol reduced glutamine synthetase activity in the 1% EtOH group. Embryonic ethanol exposure did not alter the immunoccontent of the glutamate vesicular transporter VGLUT2 and the mitochondrial energetic metabolism of the brain in adulthood. Our results suggest that embryonic ethanol exposure may cause significant alterations in glutamatergic neurotransmission in the adult zebrafish brain.

## 1. Introduction

Fetal Alcohol Spectrum Disorders (FASD) result from ethanol (EtOH) consumption during gestation and may affect 1–11 % of children worldwide (Lange et al., 2017). Facial dysmorphism, including short palpebral fissures, low nasal bridge, and microphthalmia are frequently observed in severe forms of FASD (Denny et al., 2017). However, where prenatal alcohol exposure was modest, only behavioral and neurochemical alterations are reported. These milder forms of

FASD are usually classified as Alcohol Related Neurodevelopmental Disorders (ARND) (Roozen et al., 2016).

Currently, zebrafish has been widely used as research organisms to study the severe forms of FASD (Lovely et al., 2016; Faccioli et al., 2019). External fertilization enables precise control in the amount of alcohol administered, without potential confounding maternal factors as has been observed in rodent models (Mahabir et al., 2018). Transparent eggs and a known embryological development map allow easy access to eye morphology alterations 72 h after EtOH exposure, a

**Abbreviations:** FASD, Fetal Alcohol Spectrum Disorders; ARND, Alcohol related neurodevelopmental disorders; GS, Glutamine synthetase; VGLUT, Vesicular glutamate transporter; Hpf, hours post fertilization; CNS, central nervous system; EtOH, ethanol; EAAT, excitatory amino acid transporter; HBSS, Hank's balanced salt solution; SUIT, substrate-uncoupler-inhibitor titration

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common alteration observed in FASD (Peng et al., 2004; Fernandes, Rampersad and Gerlai, 2015). In addition, zebrafish genes and the functionality of encoded proteins are evolutionarily conserved, warranting translational relevance of this model to human diseases (Stewart et al., 2014). As FASD can only be diagnosed through behavioral alterations, researchers developed a protocol to exposure embryo zebrafish to low EtOH concentration for a short period during embryonic development to investigate FASD in adulthood (Fernandes and Gerlai, 2009). This protocol affects the social behavior and a number of neurotransmitters and neuromodulators in the zebrafish central nervous system (CNS), including dopamine and serotonin (Fernandes and Gerlai, 2015).

Another neurotransmitter associated to alcohol disorders and alcohol withdrawal is glutamate. (Most et al., 2014). Glutamate is the major excitatory neurotransmitter of the brain being implied in several physiological processes (Zhou and Danbolt, 2014). Many animal models have demonstrated that prenatal alcohol exposure interacts with glutamatergic neurotransmission (Baculis et al., 2015; Valenzuela et al., 2011). Embryonic EtOH exposure induces widespread neuronal apoptosis through N-Methyl-D-aspartate (NMDA) receptor blocking, which results in reduced brain mass and neurobehavioral disturbances at adulthood (Ikonomidou et al., 2000). Experimenter- or self-administered EtOH decreases glutamate uptake in the cerebral cortex of rats (Schreiber and Freund, 2000) and similar alterations are observed in animals treated with EtOH during gestation until weaning (Brolese et al., 2015). Acute EtOH exposure attenuates glutamate release from presynaptic neurons (Goodwani et al., 2017) and this effect may be attributed to an EtOH-induced downregulation of brain vesicular glutamate transporters (VGLTs) in adult rodents (Zhang et al., 2015). In addition, our group has recently showed that adult zebrafish previously exposed to alcohol during their embryonic development present a dose-dependent reduction of brain glutamate uptake (Baggio et al., 2017). This reduction could be implicated into the increased anxiety-like behaviors and the disrupted social behavior at adulthood in zebrafish FASD model (Baggio et al., 2018). In fact, pharmacological agents, that block glutamate output in the basolateral amygdala neurons, have anxiolytic effects (LeDoux, 1994; Maren, 1996). Selective ligands that activate specific glutamate receptors have beneficial effects in ameliorating innate or pharmacologically-induced deficits in social interaction and social memory as well as in reducing aggression in rodents (Zoicas and Kornhuber, 2019). In addition, genetic alteration of AMPA-type glutamate receptor modulates social interaction and anxiety like behavior in rodents (Vekovischeva et al., 2004). Nevertheless, glutamate uptake is just one of many components of glutamatergic neurotransmission with implications to translational psychiatry disorder investigation (Hasler et al., 2019), components that remain unexplored in zebrafish FASD model.

One cellular mechanism that could be implicated in the reduction of glutamate uptake may be a putative reduction in the  $\text{Na}^+/\text{K}^+ + \text{ATPase}$  activity in zebrafish FASD model. The physical association between  $\text{Na}^+/\text{K}^+ + \text{ATPase}$  and glutamate transporters regulates the high affinity  $\text{Na}^+$ -dependent glutamate uptake in neural cells. After release in the synaptic cleft, glutamate must be taken up by neurons and astrocytes against its concentration gradient. In this sense, the  $\text{Na}^+$  gradient generated by  $\text{Na}^+/\text{K}^+ + \text{ATPase}$  activity is used as an electrochemical driving force to uptake glutamate (Rose et al., 2009). Another potential explanation for a reduction of glutamate uptake in zebrafish FASD model could be the reduction in glutamate binding to the plasma membrane glutamate receptors. Rodent models of FASD showed a decreased glutamate binding to ionotropic and metabotropic glutamate receptors (Gerace et al., 2016; Goodfellow et al., 2016), which may be related to a reduced release of glutamate by pre-synaptic nerve terminals. Presynaptic VGLTs are responsible for concentrating glutamate in the synaptic vesicles prior to its release by nerve terminals. The amount of released glutamate by presynaptic terminals is proportionally to its accumulation in the synaptic vesicles (Ueda, 2016). Thus, a

reduction of VGLUT expression could reduce the total of glutamate accumulated in the synaptic vesicles as well as reduced release.

Another adaptive mechanism putatively implicated in the reduction of glutamate uptake in zebrafish FASD model could be the metabolic fate of this neurotransmitter after its uptake. Glutamine synthetase (GS; E.C. 6.3.1.2) is an important component of the machinery related to the glutamatergic neurotransmission recycling system (Cooper, 2012). Glial cells cultured from cortical brain cells of newborn rat pups with maternal alcohol exposure during the gestational period present a reduction in GS activity (Ledig et al., 1996). GS activity is also affected in the brain of EtOH consuming rat pups (Ledig et al., 1989). Finally, all neurochemical mechanism mentioned above requires high amount of metabolic energy. Glutamatergic synapses are the most abundant synapse in the mammalian brain and consumes 50 % of overall brain energy in the grey matter (Alle et al., 2009; Sibson et al., 1998). Therefore, mitochondria, the major organelle involved in the conversion of organic energy into ATP, is imperative to maintain this neurotransmission system (Osellame et al., 2012; Vos et al., 2010). Mouse models of FASD present impaired mitochondrial development in the CNS (Xu et al., 2005). However, such modulations have not been investigated in zebrafish models of FASD.

Therefore, the aim of this study was to investigate the potential compensatory mechanisms related to the impaired glutamate uptake described by our group in a zebrafish FASD model (Baggio et al., 2017). In the current study, the following parameters were investigated in brain samples of adult zebrafish FASD model: glutamatergic binding, glutamatergic vesicular transport, glutamine synthetase and  $\text{Na}^+/\text{K}^+ + \text{ATPase}$  activity, and brain mitochondria physiology.

## 2. Material and methods

### 2.1. Animals

Adult short-fin heterogeneous wild-type zebrafish (*Danio rerio*), 12–18 months old, were obtained from the zebrafish facility at the Federal University of Rio Grande do Sul. Our animals were derived from two starting stocks, purchased from a local pet store in 2014. Animals were generated by crossing males and females from these two starting stocks. The diploid offspring (F1) were screened for healthy and good looking of embryos and were used to make future generations by pairwise crossing. Our animals have been keeping in this manner since 2015. Fish were housed in an automated recirculating system (Zebtec, Tecniplast, Italy) under mechanical and biological filtration. Water temperature was adjusted to 28 °C, pH to 7.4, and conductivity to 500  $\mu\text{S}/\text{cm}$ . Room illumination was provided by ceiling-mounted fluorescent lamps and animals were kept under a 14/10-h light/dark photoperiod cycle (lights on at 8:00 a.m.). Animals were fed three times a day with commercial flake fish food (Alcon BASIC, Alcon, Brazil) and *Artemia* sp. nauplii. All experimental procedures were performed according to the Brazilian Law for Laboratory Animal Care and Use (Law 11794/2008) and were previously approved by the Committee for Animal Care and Use from Universidade Federal do Rio Grande do Sul protocol number #31,675. All experimental designs were developed in order to minimize the number of fish and their discomfort or suffering.

### 2.2. FASD protocol

A total of 600 adult zebrafish were used in the present work (sex ratio of 50:50 male:female). They were obtained by local breeding through pair-wise crossing. Approximately 700 zebrafish eggs were collected from 2 sets of 3 independent matings (total of 6 independent matings; supplementary Fig. 1). Eggs were collected 1.5 h post fertilization (hpf) and cleaned with methylene blue (10 %) for 10 min. At 24 hpf, embryos were treated with one of the following EtOH concentrations (Merck® - CAS number 64-17-5): 0% (control group), 0.01 %, 0.25 %, 0.5 %, or 1%. Two hours after, embryos were washed twice with

water from the automated recirculating system (with the same water quality conditions as described in Section 2.1.). Animals were maintained on B.O.D. (Bio Oxygen-Demand) incubator at 28 °C and were fed with *Paramecium* sp. infusoria ad libitum. Four weeks later, fish were transferred to 3.5-liter tanks (20 animals per tank) in the automated recirculating system (Zebtec, Tecniplast, Italy) under the same water quality conditions as described above. Growing animals were fed three times a day with a commercial flake fish food (Alcon BASIC, Alcon, Brazil) and *Artemia* sp. nauplii. Mature young adult zebrafish (4 months old; weight 300–400 mg; 50:50 sex ratio) were used for further assays.

### 2.3. Glutamate uptake

Animals were anesthetized using ice-cold water and euthanized by decapitation. The *n* was 8 animals/group. The brains were removed and humidified with Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl; 0.63 Na<sub>2</sub>HPO<sub>4</sub>; 4.17 NaHCO<sub>3</sub>; 5.36 KCl; 0.44 KH<sub>2</sub>PO<sub>4</sub>; 1.26 CaCl<sub>2</sub>; 0.41 MgSO<sub>4</sub>; 0.49 MgCl<sub>2</sub> and 1.11 D-glucose, pH 7.2. Each brain was separated with the help of a magnifying glass and transferred as a whole to two paired 24-well culture plates containing 0.5 mL of HBSS. One plate from each pair was maintained at 37 °C and the other at 4 °C. The brains from the first paired-plate were washed once with 1 ml of 37 °C HBSS. The second paired-plate were washed with 1 ml of ice-cold HBSS containing N-methyl-D-glucamine (4 °C) instead of sodium chloride (for sodium-independent glutamate uptake). Glutamate uptake assay was performed as previously described by Rico et al. (2010). Total glutamate uptake was measured with the addition of 0.33 Ci mL<sup>-1</sup> L-[3 H] glutamate to the incubation medium at 37 °C. Incubation was stopped after 7 min by washing out the remaining glutamate twice with 1 ml ice-cold HBSS. The brains were immediately transferred to 0.5 N NaOH overnight. Intracellular content of L-[2,3-3 H] glutamate was determined by scintillation counting. Sodium-dependent glutamate uptake was determined by the following arithmetic calculation:  $\frac{\text{glutamate uptake for each ethanol + saline groups}}{\text{glutamate uptake for control + saline group}} \times 100$  and  $\frac{\text{glutamate uptake for each ethanol + ceftriaxone groups}}{\text{glutamate uptake for control + ceftriaxone group}} \times 100$ , and were expressed as nmol of Glu.min<sup>-1</sup>. mg Prot<sup>-1</sup>. Protein content was measured according to Peterson et al. (1977).

In order to analyze the role of the excitatory amino acid transporter 2 (EAAT2) in the glutamate uptake, the beta-lactam antibiotic ceftriaxone, a well-known inducer of EAAT2 expression (Miller et al., 2008; Rothstein et al., 2005), was used. Another set of animals were injected i.p. with ceftriaxone sodium salt (300 mg/kg dissolved at NaCl 0.9 %; Sigma, St. Louis, MO, USA) at a volume of 1 µL/g of body weight, once daily for six days. All injections were performed at 9 a.m. Two hours after the last injection, animals were anesthetized with ice-cold water and euthanized by decapitation. The brains were removed and glutamate uptake assay was performed.

### 2.4. Glutamate binding assay

Glutamate binding assay was performed as previously described by Bermejo et al. (2014) with modifications. A total of 300 adult zebrafish were used (*n* = 6 pools/group; 10 brains/pool). Each pool was added to 1 mL of 0.32 M sucrose and completely homogenized using a glass-TEFLON Potter tissue homogenizer. Homogenates were centrifuged at 900 x g for 10 min at 4 °C. The supernatant (S1) was transferred to a new tube and centrifuged at 10,000 x g for 15 min at 4 °C. The supernatant (S2) was removed and the remaining crude synaptosomal fraction pellet (P2) resuspended in 1 mL of 0.32 M HEPES-buffered sucrose solution. Then, another 3 mL of 0.32 M HEPES-buffered sucrose solution was added. The resuspended P2 was centrifuged at 10,000 x g for 15 min at 4 °C. The supernatant was removed and the pellet 3 (P3) was lysed by hyposmotic shock in the polypropylene tube by 1 mL of cold H<sub>2</sub>O. Then, 3 mL of cold H<sub>2</sub>O to the lysate P3 were added, and then it was

transferred to a glass-TEFLON tissue homogenizer and homogenized by hand with 3 strokes. The samples were returned to the polypropylene tube with 16 µL of 1 M HEPES solution and remained under agitation at 4 °C for 30 min to ensure complete lysing. The samples were then transferred to a new tube and centrifuged at 25,000 x g for 20 min at 4 °C. The supernatant (S4) was removed and the pellet (P4) was resuspended in 1 mL of 0.32 M sucrose HEPES-buffered solution. P4 fraction was combined with the sucrose gradient (3 mL of 1.2 M sucrose; 2.5 mL of 1 M sucrose; 2.5 mL of 0.8 M sucrose) and centrifuged at 150,000 x g for 2 h at 4 °C. By using a micropipette, the synaptic plasma membrane fraction (SPM) (localized between layers 1.0 and 1.2 M of sucrose) was withdrawn. The SPM fraction was placed in ultracentrifuge tubes with exactly 3.5 volumes of 4 mM HEPES to adjust the sucrose concentration to 0.32 M. The SPM fraction was centrifuged at 200,000 x g for 30 min at 4 °C. The supernatant obtained was discarded and the synaptic plasma membrane pellet (SPM) was resuspended in 300 µL of Tris-HCl solution.

SPM was moved to falcon tubes, with 10 mL of 5 mM Tris-HCl, and centrifuged at 20,000 x g for 15 min at 4 °C. The pellet was then resuspended again in 10 mL of 5 mM Tris-HCl and centrifuged at 20,000 x g for 15 min at 4 °C. The supernatant was discarded and 1 mL of 5 mM Tris-HCl was added to the pellet at rest for 30 min at 4 °C to undo the vesicles. The protein content was measured by BCA protocol (Smith et al., 1985) and normalized at 1 mg/mL. Membranes were incubated in the reaction mixture with 250 µL of H<sub>2</sub>O, 50 µL of 500 mM Tris-HCl and 100 µL L-[3 H] glutamate. Incubation was carried out at 30 °C for 30 min, and the reaction was stopped by putting the samples in an ice-bath and then centrifuged at 16,000 x g for 10 min at 4 °C. The pellet and the wall of the tube were quickly and carefully washed with ice-cold Milli-Q water. Then 300 µL of NaOH (1 M) with SDS 0,1% solution and scintillation liquid were added to the dry pellet and the radioactivity incorporated was determined after one night. Nonspecific binding was determined by adding 50 µL of 0.5 mM nonradioactive glutamate to the medium in a parallel assay. Specific binding was considered to be the difference between total and nonspecific binding (Soares et al., 2003).

### 2.5. Western blotting

Animals were anesthetized using ice-cold water and euthanized by decapitation. A total of 60 adult zebrafish were used (*n* = 6 pools/group; 2 brains/pool). The brains were homogenized in a lysis buffer (5 mM Tris base, 1 mM EDTA, 0.1 % SDS and protease inhibitor cocktail; pH 7.4), and heated at 95 °C for 10 min. Protein content was normalized to 2 µg/µL. Samples were diluted 1:1 in a sample buffer (0.01 g Bromophenol Blue, 60 mM Tris base, 20 % glycerol, 2% SDS and 5% 2-β-mercaptoethanol; pH 6.8; final protein concentration of 1 µg/µL). Protein separation was resolved by 8% SDS-PAGE gel (Mini-PROTEAN® Tetra System; Bio-Rad Laboratories Inc., USA). After, proteins were electro transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Trans-Blot® SD Semi-Dry Transfer Cell; Bio-Rad Laboratories Inc., USA). Membranes were incubated for 1 h in a blocking solution containing powdered 3% bovine serum albumin (for anti-VGLUT2) or 5% milk (for anti-β-actin) and 0.1 % Tween-20 in Tris-buffered saline TBS; 10 mM Tris base and 30 mM NaCl; pH 7.4. Next, membranes were incubated with a primary antibody anti-VGLUT2 HY-19, Sigma-Aldrich IgG 1:3000 at 4 °C overnight or with anti-β-actin 1:3000, Santa Cruz Biotechnology at 4 °C for 2 h. Finally, membranes were exposed to HRP-conjugated secondary antibody anti-rabbit 1:1000 or anti-mouse 1:1000 for 2 h at 4 °C. The chemiluminescence was detected using ImageQuant™ LAS 4000 (GE Healthcare, USA). Band intensity was analyzed by Image Lab™ software 6.0 (Bio-Rad Laboratories Inc., USA). β-actin was used as a protein loading control and a rat brain samples (5 µg/µL) were used as positive loading control for all antibodies.

## 2.6. Na<sup>+</sup>/K<sup>+</sup>ATPase activity

Animals were anesthetized using ice-cold water and euthanatized by decapitation. A total of 60 adult zebrafish were used (n = 10 pools/group; 2 brains/pool). Each brain pool was homogenized in a 10x v/w of an ice-cold solution containing: 0.32 M sucrose, 5.0 mM HEPES and 0.1 mM EDTA (pH 7.4). Na<sup>+</sup>/K<sup>+</sup>ATPase activity was determined as previously reported Tsakiris and Deliconstantinos (1984). Sample was added to the reaction mixture containing 5 mM MgCl<sub>2</sub>, 80 mM NaCl, 20 mM KCl, and 40 mM Tris-HCl buffer, pH 7.4, for a final volume of 200 μL. The reaction was started by the addition of 20 μL of 30 mM ATP (final concentration of 3 mM) in the presence or absence of ouabain (1 mM). The reaction was stopped after 10 min with addition of 200 μL of trichloroacetic acid 10 %. Na<sup>+</sup>/K<sup>+</sup>ATPase activity was calculated by the difference between the values obtained in the presence and absence of ouabain. The released inorganic phosphate (Pi) was measured by the colorimetric Malachite Green method (Chan et al., 1986) at 630 nm. Results are expressed as nmol Pi.min<sup>-1</sup>.mg protein<sup>-1</sup>.

## 2.7. Glutamine Synthetase activity

The GS enzymatic activity assay was performed as previously described by Petit et al. (1992). Animals were anesthetized using ice-cold water and euthanatized by decapitation. A total of 60 adult zebrafish were used (n = 10 pools/group; 2 brains/pool). Each brain pool was lysed in 150 mM KCl with Triton X-100. Protein content was normalized to 1 μg/μL. Then, 100 μL of each sample volume was incubated in 100 μL of reaction medium (10 mM MgCl<sub>2</sub>, 50 mM L-glu, 10 mM 2-βmercaptoethanol; 50 mM hydroxylamine-HCl; 10 mM ATP and 100 mM imidazole-HCl buffer; pH 7.4) for 15 min at 37 °C. The reaction was stopped with the addition of 400 μL of a solution containing 370 mM ferric chloride, 0.67 N HCl and 3.3 % trichloroacetic acid. After centrifugation 1000 x g for 10 min, absorbance of supernatants was measured at 530 nm. Synthetic γ-glutamylhydroxamate was used as standard. GS activity was expressed as μmol γ-glutamylhydroxamate.h<sup>-1</sup>.mg prot<sup>-1</sup>.

## 2.8. Mitochondrial respiratory assay

A total of 32 adult zebrafish were used (n = 8 pools/group; 2 brains/pool). Each brain pool was homogenized in 200 μL of ice-cold buffer containing 4 mM sucrose, 20 mM Tris, 1,28 mM EDTA, pH 7.1. Homogenization consisted a twenty strokes with a Potter Elvehjem tissue homogenizer (Makrecka-Kuka et al., 2015). Brain homogenates were mixed with mitochondrial respiration buffer MiR05 (110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM MgCl<sub>2</sub>, 20 mM taurine, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM HEPES, pH 7.1 at 30 °C, and 0.1 % BSA essentially fatty acid free) and respiration was measured at 28 °C in an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). Oxygen consumption was assessed in the phosphorylating state with 2 mM malate, 5 mM pyruvate, 10 mM glutamate, 10 mM succinate, and 1 mM ADP (state 3) and in the non-phosphorylating state by adding 2 μg/mL oligomycin (an ATP synthase inhibitor) (state 4) (Pesta and Gnaiger, 2012). Respiration was uncoupled by titration with the uncoupler Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (0.5–1 μM steps) to reach maximal respiration. Finally, 0.5 μM rotenone (a Complex I inhibitor) and 2.5 μM antimycin A (a Complex III inhibitor) were added. The following variables were analyzed: phosphorylating state (State 3), non-phosphorylating state (State 4), the maximum OxPhos capacity, and the respiratory control ratio (RCR). The respiratory control ratio (RCR) was calculated as state3/state4. Only RCR > 5 preparations were used.

## 2.9. Statistical analysis

Parametric data are expressed as mean ± standard error of the

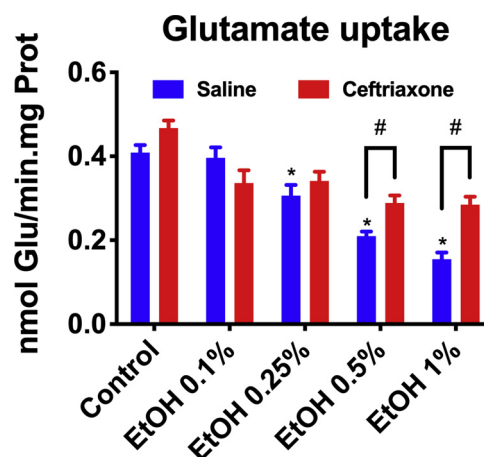


Fig. 1. Effect of ethanol exposure in zebrafish embryos on glutamate uptake of adult zebrafish brain treated or not with ceftriaxone. Data were expressed as mean ± S.E.M. and were analyzed by two-way ANOVA followed by Tukey's post hoc test (# = p < 0.0001, \* = p < 0.05), n = 8 animals/group (a total of 80 animals).

mean (S.E.M.). Glutamate uptake data were analyzed by two-way ANOVA followed by Tukey's post hoc test. Data of VGLUT2 immunoccontent, glutamate binding, Na<sup>+</sup>/K<sup>+</sup>ATPase and GS activities were analyzed by one-way ANOVA followed by Tukey's post hoc test. Mitochondrial respiration data were analyzed by *t*-test for unpaired samples. p < 0.05 was considered significant.

## 3. Results

There was a reduction of 27 %, 49 % and 63 % of glutamate uptake in EtOH + saline groups (0.25 %, 0.5 % and 1%, respectively) when compared to control + saline group (Fig. 1; two-way ANOVA followed by Tukey's post hoc test; F = [4,70]; \* = p < 0.05). In order to investigate if this reduction could be due to a reduced glutamate release induced by EtOH, we evaluated the expression of vesicular glutamate transporter type 2 (VGLUT2), the most abundantly VGLUT expressed in the zebrafish brain (Filippi et al., 2014). There were no alterations in the immunoccontent of VGLUT2 among groups (one-way ANOVA; F [4,23] = 1.891; p = 0.1462) (Fig. 2), suggesting that the reduction of glutamate uptake induced by EtOH was due to other molecular and/or cellular mechanism.

Another possibility for explaining the decreased of glutamate uptake induced by EtOH could be a reduction of glutamate transporter (EAAT) expression in zebrafish brain. Since there are no commercial anti-EAATs antibodies available for zebrafish, we treated animals with ceftriaxone, an antibiotic that increases the expression of the glutamate transporter EAAT2 (Rothstein et al., 2005). Ceftriaxone treatment partially restored the glutamate uptake, which resulted in an increase of 38 % and 86 % in 0.5 % and 1% EtOH + ceftriaxone groups, respectively, when compared to their respective control groups (0.5 % and 1% EtOH + saline) (Fig. 1; two-way ANOVA followed by Tukey's post hoc test; interaction effect, F = [4,70]; # = p < 0.001). The putative EtOH-induced decrease of EAATs expression was corroborated by the reduction in the radioligand binding (Fig. 3), where treatment with 0.5 % and 1% of EtOH reduced glutamate binding about 43 % and 59 % respectively, when compared to control group (one-way ANOVA; F [4, 20] = 5.395; p < 0.05).

As ceftriaxone treatment was able to rescue glutamate uptake impairment observed only in the group 0.25 % EtOH compared to control + saline level (two-way ANOVA followed by Tukey's post hoc test; F = [4,70]; p < 0.05), other cellular mechanisms could also be implicated. Glutamate transporters are sodium-dependent proteins that putatively rely indirectly on Na<sup>+</sup>/K<sup>+</sup>ATPase to generate ion



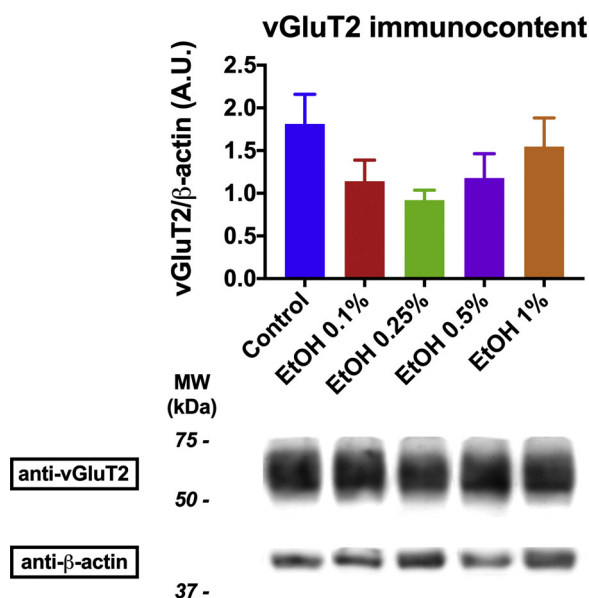


Fig. 2. VGLUT2 immunocontent of brain samples of adult zebrafish submitted to embryonic alcohol exposure. Data were expressed as mean  $\pm$  S.E.M. of the ratio between VGLUT2 and  $\beta$ -actin band intensities, and were analyzed by one-way ANOVA followed by Tukey's post hoc test,  $n = 6$  pools/group (2 zebrafish brains/pool; a total of 60 animals).

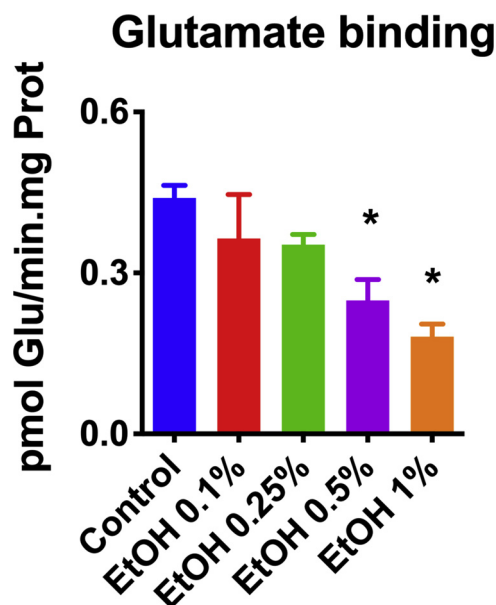


Fig. 3. Effect of ethanol exposure in zebrafish embryos on the plasma membrane glutamate binding in adult brain. Data were expressed as mean  $\pm$  S.E.M. and were analyzed by one-way ANOVA followed by Tukey's post hoc test (\* =  $p < 0.05$  when compared to control group),  $n = 6$  pools/group (10 zebrafish brains/pool; a total of 300 animals).

gradients that drive transmitter uptake. Thus, the  $\text{Na}^+/\text{K}^+$  ATPase activity was evaluated (only 0.5 % and 1% treated groups were used, since they presented the major glutamate uptake reductions). A reduction of 32 % in the  $\text{Na}^+/\text{K}^+$  ATPase activity was observed in 0.5 % and 1% EtOH-treated fish when compared to control group (one-way ANOVA;  $F [2, 25] = 7.315$ ;  $p < 0.05$ ) (Fig. 4). Another important enzyme coupled to glutamate uptake is the GS. After astrocytic uptake, glutamate is destined to be converted to glutamine and return to neurons via GS activity. Alterations in the activity to this enzyme are normally related to glutamate uptake impairments (Rose et al., 2013;

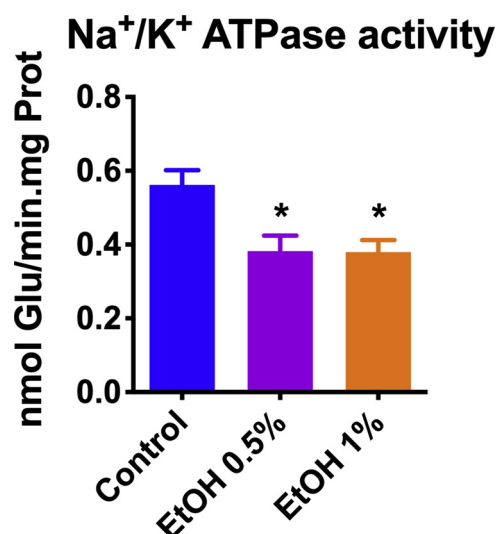


Fig. 4. Enzymatic activity of  $\text{Na}^+/\text{K}^+$  ATPase in adult zebrafish brains submitted to embryonic alcohol exposure. Data were expressed in mean  $\pm$  S.E.M. and were analyzed by one-way ANOVA followed by Tukey's post hoc test (\* =  $p < 0.05$  when compared to control group),  $n = 10$  pools/group (2 zebrafish brains/pool; a total of 60 animals).

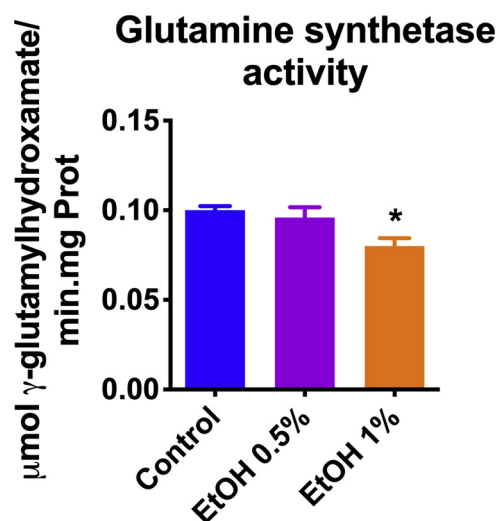


Fig. 5. Enzymatic activity of glutamine synthetase (GS) in adult zebrafish brains submitted to embryonic alcohol exposure. Data were expressed in mean  $\pm$  S.E.M. and were analyzed by one-way ANOVA followed by Tukey's post hoc test (\* =  $p < 0.05$  when compared to control group),  $n = 10$  pools/group (2 zebrafish brains/pool; a total of 60 animals).

Tiburcio-Félix et al., 2018). A reduction of 20 % in GS activity was detected only in the 1% group compared to control (one-way ANOVA;  $F [4, 51] = 6.301$ ;  $p < 0.05$ ) (Fig. 5).

Because the energetic cost of glutamatergic synapses comprises 50 % of ATP consumption of the brain (Alle et al., 2009; Sibson et al., 1998), mostly related  $\text{Na}^+/\text{K}^+$  ATPase and GS enzymatic activities, mitochondrial physiology was further investigated in the group EtOH 1% which presented alteration in both parameters. High-resolution respiratory parameters indicated no difference in mitochondrial bioenergetics between control and EtOH 1% group when analyzed as follow: Oxygen consumption was assessed in phosphorylating state – State 3, non-phosphorylating state – State 4, the maximum OxPHOS capacity, and the respiratory control ratio (RCR) were calculated as state3/state4 (Fig. 6).

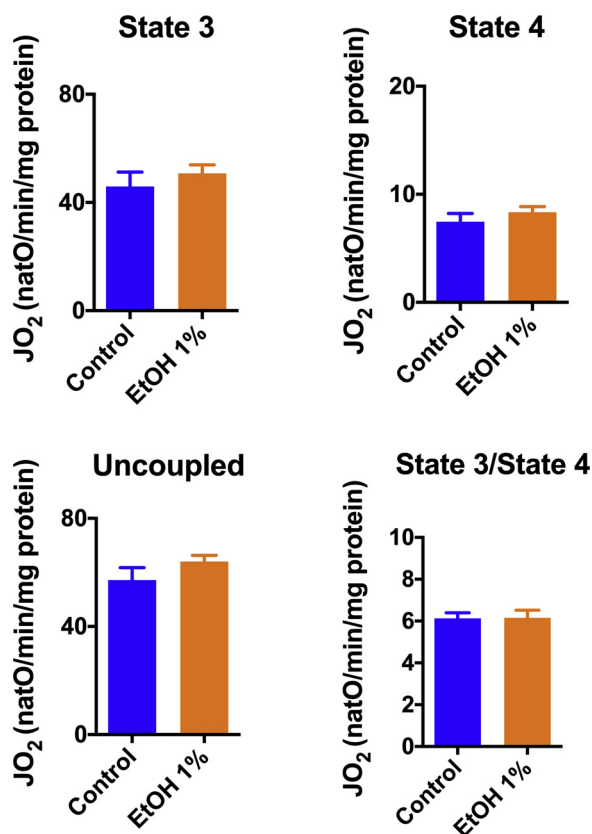


Fig. 6. Mitochondrial respiratory profile of adult zebrafish brains submitted to embryonic alcohol exposure. Oxygen consumption was assessed in the phosphorylating state (state 3) and in the non-phosphorylating state (state 4); maximum OxPHOS capacity was assessed by titration with the uncoupler FCCP; and the respiratory control ratio (RCR) was calculated as state3/state4. Data are expressed as mean  $\pm$  S.E.M. and were analyzed by *t*-test for unpaired samples,  $n = 8$  pools/group (2 zebrafish brains/pool; a total of 32 animals).

#### 4. Discussion

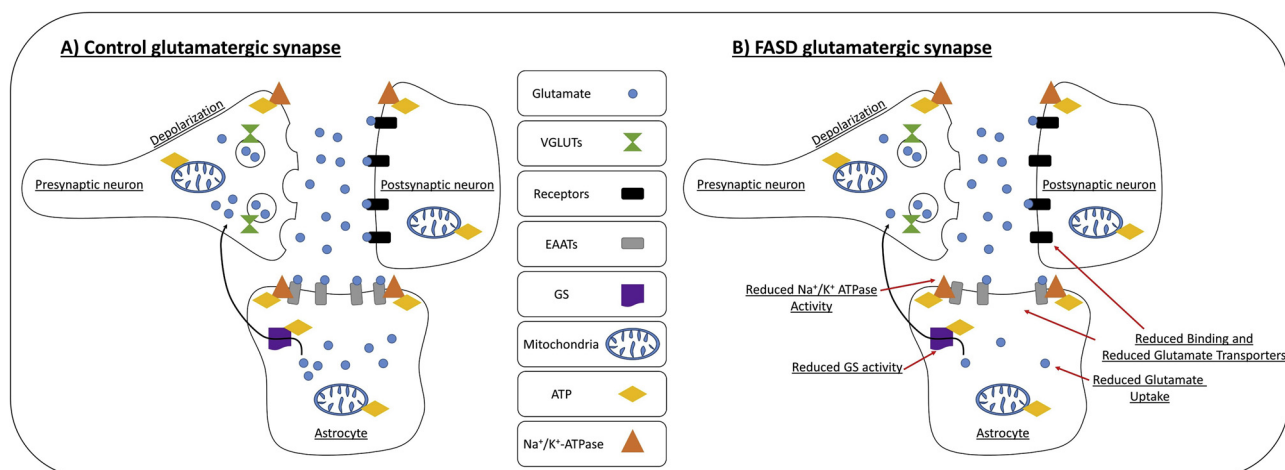
Social consumption of EtOH during unaware pregnancy is the main cause of FASD (McHugh et al., 2014). Here, the zebrafish brain glutamatergic neurotransmission system was investigated using a mild form of the zebrafish FASD model. Glutamatergic tripartite synapses are formed by presynaptic glutamatergic neurons, postsynaptic neurons with glutamatergic receptors, and astrocytes (Danbolt, 2001). As the pre-synaptic glutamatergic neuron is depolarized, vesiculated glutamate is released in the synapse cleft acting on receptors present at the postsynaptic neurons. Meanwhile, glutamate is continuously taken up by astrocytes. Neuronal repolarization and glutamate uptake rely on Na<sup>+</sup>/K<sup>+</sup> + ATPase activity (Matchkov and Krivoi, 2016). Glutamate is recycled by GS after uptake and returns to the presynaptic neurons from astrocytes (Kvamme, 1998). Both Na<sup>+</sup>/K<sup>+</sup> + ATPase and GS activities demand high ATP consumption, which is closely dependent on proper mitochondrial physiology (Fig. 7A).

In this study, no impairment of glutamate vesiculation from the perspective of VGLUT2 expression was observed in the zebrafish FASD model. A previous report shows no alteration in glutamate concentration at the synaptic cleft in the model (Mahabir et al., 2018), suggesting no impairment of neurotransmitter release by presynaptic glutamatergic neurons. Thus, glutamate uptake impairment could be related to other components of the synapsis, like EAATs expression decrease. Ceftriaxone treatment corroborates this hypothesis; a complete rescue of glutamate uptake was observed in the 0.25 % EtOH + ceftriaxone group and a partial recovery in 0.5 % and 1% EtOH + ceftriaxone. In addition, EAATs are proteins with high affinity to glutamate. If their

expression was reduced, glutamate binding to brain cell membranes could decrease, which was observed in the 0.5 % and 1% EtOH groups. As full recovery of glutamate uptake was not observed in the 0.5 % and 1% EtOH + ceftriaxone groups, additional components of the synapsis could be compromised. EAATs were coupled with Na<sup>+</sup>/K<sup>+</sup> + ATPase activity, and this parameter was equally decreased in the 0.5 % and 1% EtOH groups. Nevertheless, group 1% EtOH presented a more pronounced glutamate uptake reduction compared to the 0.5 % EtOH group. As GS activity modulates glutamate clearance of the synaptic cleft (Shaked et al., 2002), GS was further investigated and only group, 1% EtOH, presented a reduction in this parameter. As mitochondrial physiology impairment may compromise Na<sup>+</sup>/K<sup>+</sup> + ATPase and GS activities (Bogdanova et al., 2016; Chen et al., 2018), mitochondrial physiology was further analyzed without any alteration in the 1% EtOH group compared to the control (Fig. 7B). Therefore, EtOH exposure during zebrafish development impaired adult brain glutamate uptake, which is associated with the following parameters: EAATs expression in the groups 0.25 %, 0.5 %, and 1% EtOH; glutamate binding and Na<sup>+</sup>/K<sup>+</sup> + ATPase activity in the groups 0.5 % and 1% EtOH; and GS activity in the group 1% EtOH. In contrast, there were no alterations in the immuncontent of VGLUT2 and mitochondrial OxPHOS physiology in any of the groups.

Exposure to EtOH during development produces long-lasting alterations to the glutamate synapse not restricted to EAATs in the zebrafish brain. Acute EtOH exposure is known to attenuate glutamate release from presynaptic neurons (Goodwani et al., 2017). Expression of VGLUT2, an important component of the glutamate presynaptic release machinery, is downregulated in the hippocampus of adult mice that were previously exposed to ethanol at an early stage of development (Zhang et al., 2015). In contrast, in the present study, no alteration was observed in the immuncontent of VGLUT2, suggesting that EtOH treatment did not alter glutamate release from presynaptic neurons. In this context, Wistar rats exposed to ethanol during development presented no alterations in VGLUT2 expression in the nucleus accumbens (Vrettou et al., 2017). However, the same study demonstrated alterations in VGLUT2 expression in the ventral tegmental area only, indicating that vesicular glutamate transporters show different responses to EtOH exposure. In the case of this investigation, no particular alterations were observed.

EtOH exposure during the early period of embryonic development promotes a concentration-dependent reduction in glutamate uptake in the adult zebrafish brain (Baggio et al., 2017). Ceftriaxone enhances glutamate transporter EAAT2 expression (Rothstein et al., 2005), and was used in this study to verify if the decreased glutamate uptake could be associated with a reduction in glutamate transporter expression. Ceftriaxone ameliorated the decrease in glutamate uptake, which suggests that EAAT2 may be a possible target of EtOH brain toxicity in the zebrafish FASD model. Notably, the 0.25 % EtOH + ceftriaxone group showed a complete recovery of glutamate uptake impairment. In the literature, the EtOH group (0.25 %) is depicted as a threshold between control and major alterations observed in the 0.5 % and 1% EtOH groups. Neurochemical analysis showed no alteration in the overall brain glutamate, GABA, and taurine concentrations in the 0.25 % EtOH group while a decrease in the remaining groups was observed (Mahabir et al., 2018). In addition, an EtOH dose-dependent decrease was observed in the dopamine, serotonin, DOPAC, and 5HIAA brain levels when all the groups were analyzed (Buske and Gerlai, 2011; Mahabir et al., 2014). Regarding behavior, EtOH 0.25 % displayed minor alterations in parameters associated with social interaction, associative learning performance, and anxiety-like behavior compared to EtOH 1%, which presented major alterations in the same behavioral tasks (Fernandes and Gerlai, 2009; Fernandes et al., 2014; Baggio et al., 2018). In the present study, glutamate uptake impairment in the group EtOH 0.25 % seems to be only related to EAAT expression, which is the reason why this group recovered fully by ceftriaxone treatment while the same was not observed in the other EtOH groups.



**Fig. 7. A) Tripartite glutamatergic synapses.** Presynaptic neurons release glutamate, which interacts with receptors in the postsynaptic neuron. Glutamate is mostly taken up by astrocyte excitatory amino acid transporters (EAATs) coupled to  $\text{Na}^+/\text{K}^+$  ATPase activity. Glutamate uptake is modulated by GS activity and relies on mitochondrial physiology due to energy costs. **B) FASD glutamatergic synapses.** Ethanol exposure during zebrafish development impaired glutamate uptake in adulthood. It is related to glutamate binding,  $\text{Na}^+/\text{K}^+$  ATPase activity, glutamine synthesis activity impairment, independent of alterations in the immunoccontent of VGLUT2, and mitochondrial physiology.

Prenatal EtOH exposure leads to alterations in learning and memory, likely linked to permanent damage of the hippocampus, mediated in part by changes in glutamatergic transmission (Mameli et al., 2005). The receptor subunits GluA1, GluA2 AMPA and GluN2A NMDA show a significant reduction in the expression in immature hippocampal slices after exposure to 150 mM EtOH for 7 days (Gerace et al., 2016). These results suggest that incubation with EtOH impairs the structure and function of the excitatory pre- and postsynaptic compartments. Postnatal attenuation of AMPA receptor-mediated synaptic transmission in CA1 pyramidal cells is also observed after prenatal EtOH exposure (Wijayawardhane et al., 2007). This type of synaptic transmission is associated with alterations in the NMDA receptor subunit composition in the dentate gyrus (Brady et al., 2013). These effects of EtOH lead to incorrect formation of neuronal circuits and compromised synaptic transmission in the developing brain. This compromised synaptic transmission could be linked to the observed reduction in glutamate binding in brain membranes evoked by FASD in this study.

Glutamate transporters are sodium-dependent proteins that rely on  $\text{Na}^+/\text{K}^+$  ATPase to generate ion gradients that drive transmitter uptake (Rose et al., 2009). It has been observed that a reduction in  $\text{Na}^+/\text{K}^+$  ATPase activity decreases glutamate uptake (Bauer et al., 2012; Illarionova et al., 2014). The modification of  $\text{Na}^+/\text{K}^+$  ATPase activity induced by EtOH exposure during gestation may result from a direct effect of ethanol on the lipoprotein structure of the enzyme (Ledig et al., 1985). Changes in membrane permeability due to concomitant modification of ion and amino acid transport have also been implicated (Heitman et al., 1987). Therefore, the glutamate uptake impairment and the decrease in  $\text{Na}^+/\text{K}^+$  ATPase activity observed in the 0.5 % and 1% groups could be directly linked.

Alcohol also causes changes in glutamate synthesis and oxidation pathways (Bell et al., 2016; Miguel-Hidalgo, 2006). Glutamate is released by neurons in the synaptic cleft and taken up by astrocytes, then rapidly converted into glutamine by GS in order to recycle it for neuronal use (Hertz and Zielke, 2004). Alcohol exposure impairs this process, leading to neurotoxicity. Chronic EtOH exposure has been shown to decrease GS (Cesconetto et al., 2016). A postmortem study indicated that GS was downregulated in the hippocampus of alcoholics without hepatic pathology (Matsumoto, 2009). In addition, in cell culture, EtOH exposure produced a reduction in GS activity (Davies and Vernadakis, 1984). Our study showed a decrease in GS activity in the EtOH 1% group only. Decreased GS activity in the brains of animal

models of FASD may reflect impaired ammonia elimination, which may add to the toxicity of EtOH and acetaldehyde (Davies and Vernadakis, 1984). This enzymatic activity impairment could be related to the negative developmental effects of prenatal alcohol exposure, such as acidemia and alterations in fetal brain blood flow (Sawant et al., 2015).

Lastly, phosphorylation, non-phosphorylation, and uncoupled mitochondrial activity showed no impairments in the adult zebrafish brain previously exposed to EtOH during development. In another study, exposure to neither alcohol nor nicotine affected the activity of Complex I or IV in the cerebellum of six weeks old rats exposed to the same treatment (Bhattacharya et al., 2018). However, mitochondrial function could be altered by EtOH. Girault et al. (2017) observed EtOH-related damage affiliated with impaired autophagy in microvessel endothelial cells in the developing mouse brain. These results demonstrated that EtOH-induced cell death is likely to be mediated by decreased mitochondrial integrity. This finding was supported by the observation that alcohol exposure during prenatal development results in impaired mitochondrial morphology and function (Devi et al., 1994). This range of distinct results could be explained by the fact that fetal mitochondrial vulnerability to alcohol is tissue-specific (Bukiya, 2019). Marin-Garcia et al. (1996) observed that while liver and brain mitochondria exhibit reduced ATP synthase activity; only the liver is characterized by decreased complex III activity. Therefore, glutamatergic synapses are impaired in FASD because of alterations in glutamate transporter expression and functionality related to  $\text{Na}^+/\text{K}^+$  ATPase and GS activities rather than a potential mitochondrial impairment, which could compromise the energetic balance of this system.

## 5. Conclusion

The findings of this study demonstrate the effects of EtOH exposure during embryonic development on the glutamatergic system of the adult zebrafish brain. Crucial aspects of brain health such as glutamate uptake, glutamate binding, and  $\text{Na}^+/\text{K}^+$  ATPase and GS activities were impaired 4 months after EtOH exposure. However, EtOH had no impact on mitochondrial bioenergetics. These findings are important to increase knowledge about the neural mechanisms implicated in the behavioral impairments associated to milder forms of FASD, which are crucial for our understanding of the pathology as well as the search for new treatment strategies.

## CRedit authorship contribution statement

**Suelen Baggio:** Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft. **Kamila Zenki:** Formal analysis, Investigation, Methodology. **Alberto Martins Silva:** Methodology. **Thainá Garbino dos Santos:** Methodology, Formal analysis. **Giovana Rech:** Methodology, Formal analysis. **Gabriela Lazzarotto:** Methodology. **Renato Dutra Dias:** Validation, Visualization. **Ben Hur Mussulini:** Investigation, Writing - review & editing, Funding acquisition. **Eduardo Pacheco Rico:** Project administration, Funding acquisition. **Diogo Losch de Oliveira:** Project administration, Funding acquisition, Supervision, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence work reported in this paper.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.neuro.2020.03.003>.

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## 5. CAPÍTULO III

Evaluation of thalamic neurodegeneration in zebrafish FASD model

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Günther Gehlen, Diogo Losch de Oliveira.

Manuscrito a ser submetido ao periódico Neuroscience Letters.

**Tema:** O tálamo tem grande importância na conexão com o córtex cerebral. Quando esta conexão é falha ou afetada por eventos como trauma e abuso de drogas, surgem déficits de memória e aprendizado. O etanol é capaz de danificar esta via tálamo cortical, formando conexões incorretas que estariam por trás dos déficits cognitivos observados na FASD.

**Objetivo:** Analisar a morfologia cerebral, na região dos núcleos do tálamo, de animais expostos na fase embrionária ao etanol, através da contagem de neurônios e possíveis alterações estruturais.

**Principal conclusão:** Os resultados demonstram a ausência de efeitos da exposição embrionária ao etanol na estrutura e na contagem de células do tálamo de zebrafish adultos. Tais achados corroboram para a ideia de que as alterações observadas na morfologia de estruturas cerebrais dependem do momento da exposição ao etanol e do período da vida dos animais em que as análises são realizadas.

**Contribuição a formação do aluno:** Permitiu a colaboração com um grupo de pesquisa de outra instituição, fortalecendo interações que são tão positivas para a ciência. Além de proporcionar o aprendizado de novas técnicas e conhecimentos não explorados em nosso laboratório.

**Evaluation of thalamic neurodegeneration in zebrafish FASD model**

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**Abstract**

Fetal alcohol spectrum disorders (FASD) describe a wide range of ethanol-induced developmental disabilities, including craniofacial dysmorphology, neurochemical and behavioral impairments. Zebrafish has become a popular animal model to evaluate the long-lasting effects of FASD, including alterations to neurotransmission and cerebral structure. Therefore, the aim of the present study was to evaluate the possible alterations in the number of neurons in the anterior, ventrolateral and ventromedial thalamic nuclei of an adult zebrafish FASD model. Zebrafish larvae (24 hours post-fertilization) were exposed to ethanol (0.5% and 1%) for 2 hours. After 4 months, the animals were euthanized; their brains were removed and processed to histological sections by Nissl staining. Embryonic ethanol exposure did not alter the number of neurons in the thalamus. There is just a trend of decrease in the EtOH groups, but without significant difference when compared to control. Our results demonstrate the absence of effects of EtOH exposure during embryonic development on the zebrafish anterior, ventrolateral and ventromedial thalamic nuclei. According with other studies, our findings corroborate that the brain structure alterations may depend on the stage of ethanol exposure and also the life period of investigation.

**Keywords:** zebrafish; thalamus; ethanol; FASD; brain.

**Highlights**

- Thalamus is not affected by embryonic ethanol exposure;
- There is no difference in the number of thalamic neurons between FASD and control;

**Abbreviations**

FASD: Fetal Alcohol Spectrum Disorders

Hpf: Hours post fertilization

CNS: Central Nervous System

EtOH: Ethanol

MRI: Magnetic Resonance Image

NF: Neurofilament

PEE: Prenatal Ethanol Exposure

GABA: Gamma-aminobutyric acid

NMDA: N-methyl-D-aspartate



## 1. Introduction

The embryonic ethanol exposure is a public health issue. It can lead to morphological alterations, impaired development, behavior and cognitive deficits (Christopher Glantz, 1997). The term FASD or Fetal Alcohol Spectrum Disorder describes all the possible impairments in the mild forms of this disorder, once the focus is the behavior impairments without gross morphological alterations (Roozen et al., 2016). At this case, it is crucial to understand the neurochemical and brain alterations that occur during the gestational period and their long-term consequences to facilitate diagnosis when no physical evidences are observed.

The thalamus is a brain region that receives cerebellar inputs and plays a central role in modulate and transfer information to the cerebral cortex, in order to optimize and correct the data provided. The thalamus is not just a passive connection, but a processor of neural information (Sherman, 2016). It is a multinuclear structure in mammals and other vertebrates, located in the diencephalon, which is able of regulate states of attention and alertness, and acting as an interface between the isocortex and deeper brain structures. It has great relevance in human brain pathologies and it is functionally subdivided into four types of nuclei: sensory, motor, associative and limbic (Jones and Rubenstein, 2004).

In zebrafish, the thalamus is a less prominent structure, because the teleost present the preglomerular complex that serves as a sensory relay station prevalent in the diencephalon, absent in mammals (Northcutt, 2008; Yamamoto and Ito, 2008). The thalamus in zebrafish is subdivided into the anterior thalamic nucleus, the dorsal posterior thalamic nucleus, the central posterior thalamic nucleus, and the ventral and dorsal habenular nuclei (Amo et al., 2010). Some prethalamic structures are present in zebrafish, but absent in mammalian brain (Mueller, 2012). The zebrafish thalamus is correlated with light stimulus and circadian behavior, the connections between the thalamus and the tectum enhances the probability of escape behavior and powerfully influences the direction of these escapes (Heap et al., 2018). Therefore, impairments in escape behavior and predator avoid in zebrafish could be related to thalamus damage.

The connection between the thalamus and the cerebral cortex is extremely important to optimize the neuronal function and to ensure the correct brain activity. When this connection is impaired by trauma or drugs abuse, for example, memory and learning deficits arise (Armstrong-James et al., 1988). The ethanol is able to damage this thalamus-cortical pathway, forming incorrect neuronal connections that would be responsible for the cognitive deficits observed in FASD, such as memory and learning impairments

(Mooney and Miller, 2010). Zebrafish have been shown to recapitulate some of these deficits, with decrease in the social interaction, elevated anxiety and impaired associative learning (Baggio et al., 2018; Carvan et al., 2004).

Studies demonstrated that development ethanol exposure produces long-term changes in the glutamate NMDA receptor levels in several brain regions (Berman and Hannigan, 2000; Costa et al., 2000). The ethanol acts, in part, by inhibiting NMDA receptor in mature and immature neurons. This kind of receptor is involved in cellular mechanisms that are thought to be important for learning and memory – normally impaired in FASD (Valenzuela et al., 2011). Thus, the glutamate homeostasis is impaired with the inhibition of receptors. Glutamate uptake does not occur properly, leading to neurotransmitter accumulation in the synaptic cleft and then to a potential situation of glutamate excitotoxicity and neuronal death (Blaker et al., 2019). Therefore, the glutamatergic system impaired caused by ethanol could be responsible for the neurochemical and behavioral alterations observed in FASD and for the incorrect neuron connection in the thalamus-cortex pathway (Baggio et al., 2020).

In this context, it is important to investigate the possible thalamic neurodegeneration using the zebrafish FASD model to better understand the relationship between this structure and the behavior impairments, commonly observed in the mild forms of FASD. Therefore, the aim of this study was to analyze neurodegeneration of adult zebrafish thalamus of animals that were exposed to ethanol during the embryonic period.

## 2. Material and methods

### 2.1. Animals

Adult short-fin heterogeneous wild-type zebrafish (*Danio rerio*), 12 to 18 months old, were obtained from the zebrafish facility at the Federal University of Rio Grande do Sul. Our animals were derived from two starting stocks, purchased from a local pet store in 2014. Animals were generated by crossing males and females from these two starting stocks. The diploid offspring (F1) were screened for healthy and good looking of embryos and were used to make future generations by pair-wise crossing. Fish were housed in an automated recirculating system (Zebtec, Tecniplast, Italy) under mechanical and biological filtration. Water temperature was adjusted to 28 °C, pH to 7.4, and conductivity to 500 µS/cm. Room illumination was provided by ceiling-mounted fluorescent lamps and animals were kept under a 14/10-hour light/dark photoperiod cycle (lights on at 8:00 a.m.). Animals were fed three times a day with commercial flake fish food (Alcon BASIC, Alcon, Brazil) and *Artemia sp.* nauplii. All experimental procedures were performed according to the Brazilian Law for Laboratory Animal Care and Use (Law 11794/2008) and were previously approved by the Committee for Animal Care and Use from Universidade Federal do Rio Grande do Sul (protocol number #31675). All experimental designs were developed in order to minimize the number of fish and their discomfort or suffering.

### 2.2. FASD protocol

The adult zebrafish (sex ratio of 50:50 male:female) were obtained by local breeding through pair-wise crossing. Eggs were collected 1.5 hours post fertilization (hpf) and cleaned with methylene blue (10%) for 10 minutes. At 24 hpf, embryos were treated with one of the following EtOH concentrations (Merck® - CAS number 64-17-5): 0% (control group), 0.5%, or 1%. Two hours after, embryos were washed twice with water from the automated recirculating system (with the same water quality conditions as described in section 2.1.). Animals were maintained on B.O.D. (Bio Oxygen-Demand) incubator at 28 °C and were fed with *Paramecium sp.* infusoria *ad libitum*. Four weeks later, fish were transferred to 3.5-liter tanks (20 animals per tank) in the automated recirculating system (Zebtec, Tecniplast, Italy) under the same water quality conditions as described above. Growing animals were fed three times a day with a commercial flake fish food (Alcon BASIC, Alcon, Brazil) and *Artemia sp.* nauplii. Mature young adult

zebrafish (4 months old; weight 300-400 mg; 50:50 sex ratio) were used for further assays.

### **2.3. Histological procedure**

Animals were anesthetized using ice-cold water and euthanatized by decapitation. The n was 3-5 animals/group. The brains were removed. The samples were fixed in 4% Formol (Synth) for 24 hours. Slides preparation followed the protocol described by Dalzochio et al. (2016). Briefly, samples were dehydrated, embedded in paraffin, sectioned in a rotatory microtome (5mm) (Leica®, Germany) and were stained with the Nissl technique (Magalhães Horn and Rasia-Filho, 2018) for further analysis. The analysis consisted of the observation with the camera (CCD 3.0M) attached to microscope (Olympus CX 41), through magnification of 400x. For the histological analysis of the thalamus, we were guided by the atlas of neuroanatomy of zebrafish elaborated by Wullimann et al. (1996), the location of the cuts is in the section 136 that corresponding to the brain of the animals, where the analyzed region belonged to the zebrafish anterior, ventrolateral and ventromedial thalamic nuclei. The cellular quantification was in 5 interest areas with 2 µm/2 µm size, defined by two inclusion borders (left and inferior) and two exclusion edges (right and superior), to avoid overlap, as described by Xavier et al. (2005).

### **2.4. Statistical analysis**

Statistical analysis for the evaluation of neuronal quantification by Kruskal-Wallis test, followed by Dunn post-hoc test, to a non-parametric sample. The data were expressed as means and standard deviation.  $p < 0.05$  was considered significant.

### 3. Results

In this study, we verified the effects of embryonic ethanol treatment in the number of neurons of the region belonged to the zebrafish anterior, ventrolateral and ventromedial thalamic nuclei. To evaluate the long-term effect of ethanol during development process in adult thalamus, we analyzed the number of neurons in the histological section 136 of the zebrafish brain, according to the atlas of neuroanatomy of zebrafish elaborated by Wullimann et al. (1996). The representative images are depicted in the **Figure 1**.

There is no difference in the number of neurons recorded in the thalamus of adult zebrafish previously exposed to ethanol during the embryonic development. According the non-parametric test of Kruskal-Wallis the difference is not significant ( $H= 1.245$   $p = 0.5754$ ) (**Figure 2**).

#### 4. Discussion

The social consumption of ethanol during the pregnancy is the main cause of preventable cognitive disabilities in children committed by FASD (Streissguth and O'Malley, 2000). Here, the zebrafish thalamus was investigated using a mild form of the zebrafish FASD model. No differences were observed when compared the 0.5% and 1% EtOH groups with the control group in the number of neurons recorded in the zebrafish anterior, ventrolateral and ventromedial thalamic nuclei, through histological sections at microscopically observation.

Despite the results of this study showing no significant difference, the literature presents some studies with alterations in the thalamus as a result of embryonic ethanol exposure, which is able to damage brain regions responsible by learning, memory and cognitive functions, such as hippocampus and prefrontal cortex that form a circuit with the thalamus (Lawrence et al., 2012; Livy et al., 2003). Female rats exposed to ethanol during the development presents damages in the thalamic ventral domain, with reduction in the number of neurons and in the volume of structures (Gursky et al., 2019). Through Magnetic Resonance Image (MRI) tests, diagnosed FASD children and teenagers showed decrease in the volume of cerebral structures, such as hippocampus and thalamus (Nardelli et al., 2011). Therefore, the ethanol is able to damage the functional pathways and circuits relate to learning and cognition by affecting different brain regions, including the thalamus or adjacent regions that connect to it.

Nguyen et al. (2020) investigated the effects of early moderate prenatal ethanol exposure (PEE) on the brain in a mouse model, through anatomical and diffusion MRI performed in vivo at postnatal day 28 (P28, adolescence) and P80 (adulthood). The PEE animals exhibited a significantly bigger thalamus, ventricles and hypothalamus compared to control animals, also reduced Neurofilament (NF) staining in the striatum and thalamus at P28. However, no significant differences were detected in any structure including the thalamus at P80. Therefore, the thalamus impairment of PEE depends on the life stage of the animal model in which the testes are performed in each study, the structural alterations could be permanent or not detectable at later stages. This explanation could be a reason for our negative results, because the animals here analyzed are in the adulthood and possible the brain and the thalamus has adapted over time to the new condition. Another study analyzed at the adolescence (P28) and adulthood (P64) the brains of male offspring which were scanned ex vivo using ultra-high field MRI and diffusion tensor imaging, after ethanol exposure in the initial stages of pregnancy - to mimic when pregnancy is not

typically recognized. Ethanol exposure had no substantial impact on white matter organization in the anterior commissure, corpus callosum, hippocampal commissure, internal capsule, optic tract or thalamus (Zhang et al., 2019). This is another characteristic of our study, just a unique low ethanol exposure in the beginning of the development, and no significant impairments are observed at the thalamus.

The pattern of thalamus molecular mechanisms seems to be conserved among vertebrates (Scholpp and Lumsden, 2010). During the early stages of development, the zebrafish thalamus splits in two different regions: rostral and caudal, as it does in mammals. Due to the distinct gene expression, the rostral domain gives rise to GABAergic neurons while the caudal domain is responsible for the glutamatergic neurons (Peukert et al., 2011; Scholpp et al., 2009). Both neurotransmitters could be impaired by the embryonic ethanol exposure. The relative proportion of the different populations of GABAergic interneurons can be altered and the GABAergic neurotransmission could be potentially evoked in a number of different brain regions when exposed to ethanol (Granato and Dering, 2018; Weiner and Valenzuela, 2006). Two recent studies of our group showed alterations in the glutamatergic neurotransmission at the adult brain of zebrafish FASD model, with decrease in the glutamate uptake, glutamate binding, glutamine synthetase activity and  $\text{Na}^+/\text{K}^+ - \text{ATPase}$  activity (Baggio et al., 2020, 2017). Apparently the alterations related above are limited to the expression and activity of the receptors, transporters and enzymes, and not because of structural alterations, since the FASD induction protocol used is the same here.

Ethanol exerts its apoptogenic action by a dual mechanism that consists of the blockade of NMDA glutamate receptors and hyperactivation of GABA(A) receptors (Harris et al., 1995; Lovinger et al., 1989). Through these pathways the ethanol is able to induce apoptosis in the brain cells and induce incorrect connections. The disruption of brain development and consequent neurobehavioral disturbances are the most debilitating effects of ethanol on the developing fetus (Olney et al., 2001). It is interesting to note that both the neurons are present in the thalamus. Thus, this brain structure is important to broaden understanding the mechanisms not fully elucidated of the ethanol action in the developing brain. Therefore, new studies around the FASD and thalamus are necessary to solve some doubts about the correlation between the absence of structure alterations and presence of neurochemical and behavior impairments, observed after the ethanol embryonic exposure, including differentiate the neuronal population with co-staining. Also important is understand the different results that could be observed in distinct life

stages and why this occurs. In this scenario, we suggest that our findings in this FASD model could be important to stimulate future studies and encourages us to continue researching the changes caused by ethanol in the brain.



## **5. Conclusion**

The findings of this study demonstrate the absence of effects of EtOH exposure during embryonic development on the zebrafish anterior, ventrolateral and ventromedial thalamic neuronal population. No significant differences in the number of neurons in EtOH groups were observed in the thalamus of adult zebrafish compared to control. According with other studies, our findings corroborate that brain structure alterations may depend on the stage of ethanol exposure and also the life period of investigation.

## **6. Acknowledgments**

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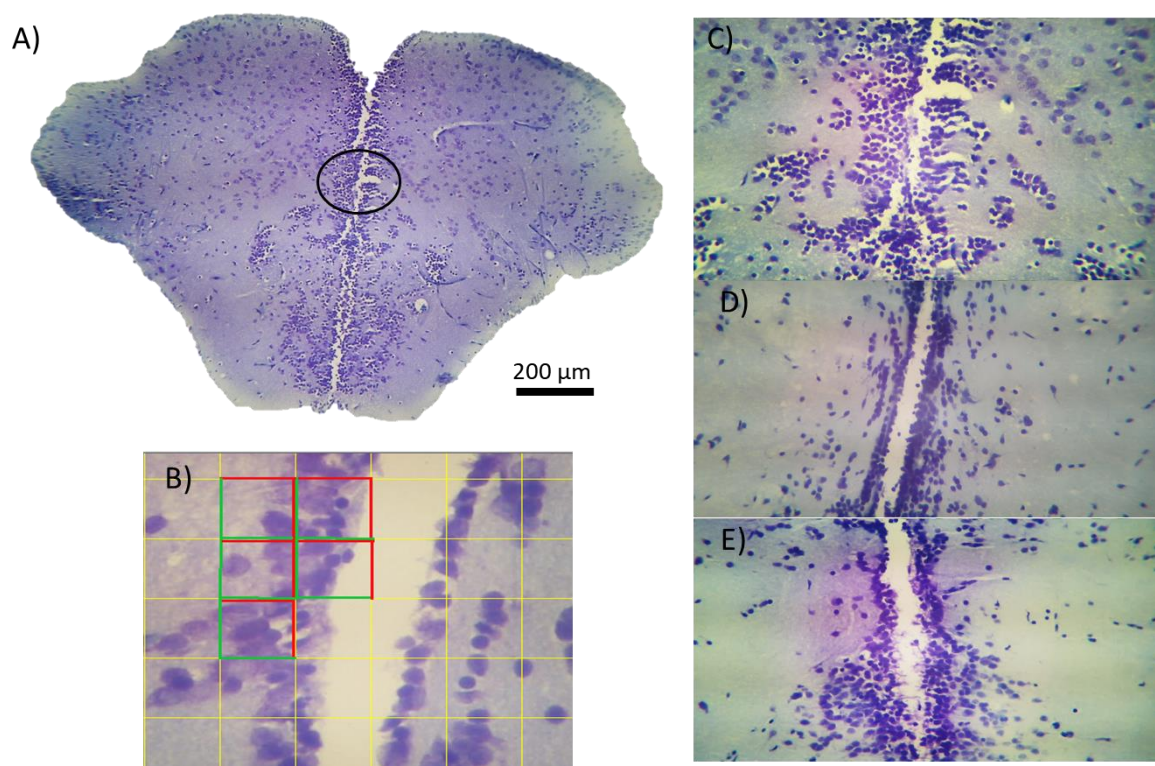
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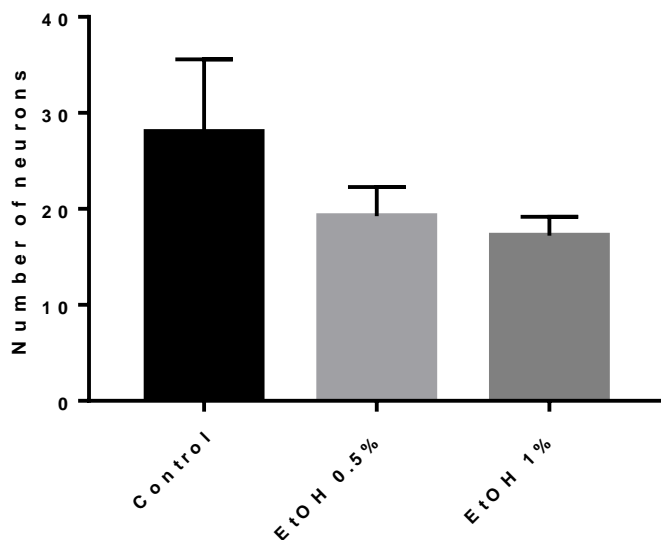
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## FIGURES AND LEGENDS



**Figure 1: Representative histological sections of zebrafish adult brain in a FASD model.**

**A)** Histological section of the zebrafish brain evidencing the studied region (100x of magnification). **B)** Thalamus at higher magnification (400x), depicting the analysis quadrants for neuron count, with red exclusion borders and green inclusion, representing an animal from control group. **C)** Thalamus at higher magnification (400x) of a representative animal from control group. **D)** Thalamus at higher magnification (400x) without apparent reduction in the number of neurons, representing an animal exposed to EtOH 0.5% during the development. **E)** Thalamus at higher magnification (400x) without apparent reduction in the number of neurons, representing an animal exposed to EtOH 1% during the development



**Figure 2: Effect of ethanol exposure in zebrafish embryos on the number of thalamic neurons in adult brain.** Number of neurons recorded in thalamus of zebrafish exposed to different EtOH concentrations during the embryonic development. Data are expressed as mean  $\pm$  S.E.M. and were analyzed by Kruskal Wallis test, followed by Dunn post-hoc test for unpaired samples (n = 3-5 animals/group).



## 6. CAPÍTULO IV

### **RESULTADOS EXTRAS: Efeito da Buspirona sobre as alterações comportamentais induzidas pela FASD**

Os resultados apresentados neste Capítulo IV são complementares ao Capítulo I, onde foram abordadas as alterações comportamentais observadas em *zebrafish* adultos submetidos ao modelo de FASD. Aqui são apresentadas as demais análises realizadas, as quais não foram utilizadas no artigo publicado. Nos testes aqui apresentados, utilizamos o ansiolítico buspirona como modulador do perfil comportamental, com o intuito de diminuir o comportamento do tipo ansioso apresentado pelos animais expostos ao etanol na fase embrionária.

## INTRODUÇÃO

Os animais submetidos a FASD tendem a apresentar alterações comportamentais que se prolongam até a fase adulta, tais como comportamento do tipo ansioso e diminuição de interação social. Permanecer por longos períodos no fundo do aparato Tanque novo, além de evitar períodos de interação com o cardume no aparato de preferência social são exemplos de resultados observados em testes comportamentais neste modelo de FASD em *zebrafish* adulto (Baggio et al., 2018). Tais déficits comportamentais estão de acordo com o que é descrito na literatura e corroboram para a possível ideia de que a exposição embrionária ao etanol é capaz de alterar parâmetros do SNC permanentemente.

Uma vez que a desordem é constatada, inicia-se a busca por medidas que possam diminuir as dificuldades inerentes a ela. Portanto, se o comportamento do tipo ansiedade é característica dos pacientes de FASD e observado em estudos com modelos animais, o uso de ansiolíticos já consolidados surge como uma boa alternativa de estudo. A buspirona age nos receptores de serotonina, sendo amplamente utilizada na clínica para diferentes tipos de ansiedade, agindo também em modelos animais de roedores e *zebrafish* (Loane & Politis, 2012; Maximino et al., 2013). Contudo, ao se utilizar a dose já descrita na literatura para *zebrafish* (25mg/kg), observamos que não apenas os animais com FASD alteravam seu comportamento, mas também os animais do grupo controle, levando à diminuição de interação social com o cardume e aumento no tempo de exploração do topo dos aparatos comportamentais. Criou-se, portanto, a necessidade de buscar doses que tenham efeito mínimo em animais controles, mas que possam reverter os déficits comportamentais em animais portadores de desordens de ansiedade.

## OBJETIVO

Modular farmacologicamente o comportamento do tipo ansioso e a diminuição na interação social, alterações comportamentais observadas anteriormente no modelo de FASD, por meio de tratamento com o ansiolítico buspirona, em diferentes doses testadas.

## MATERIAL E MÉTODOS

**Protocolo de indução de FASD:** *Zebrafish* adultos foram colocados em uma proporção de 2 machos: 1 fêmea nos aquários para reprodução. Os ovos foram coletados 1,5 horas após a fertilização (hpf) e lavados com uma solução de azul de metileno 10%, por 10 minutos. Com 24 hpf, os embriões foram tratados com diferentes concentrações de etanol: 0,1%; 0,25%; 0,5% e 1%. Após duas horas de tratamento, os embriões foram removidos e lavados duas vezes em água do sistema de recirculação, onde permanecem os aquários moradia. Os animais foram mantidos na incubadora B.O.D. (Bio Oxygen-Demand) a 28 °C e alimentados com *Paramecium sp.* até aproximadamente duas semanas de vida, onde passam a receber náuplios de *Artemia sp.* uma vez ao dia. Com um mês de vida, os alevinos foram transferidos para aquários de 3,5 litros de capacidade (20 animais por tanque) em um sistema de recirculação automática (Zebtec, Tecniplast, Italy). Os animais em crescimento foram alimentados três vezes ao dia com ração flocada comercial (Alcon BASIC, Alcon, Brazil) e náuplios de *Artemia s.* *Zebrafish* adultos (4 meses de idade; peso 300-400 mg; 50%:50% proporção de machos e fêmeas) foram usados para os testes realizados.

**Tratamento farmacológico:** Os animais foram tratados com um ansiolítico utilizado comercialmente, com a finalidade de reverter o comportamento ansioso observado previamente. Através de injeção intraperitoneal, os animais foram tratados com buspirona nas doses de 12,5 e 6,25 mg/kg (Maximino et al., 2011), em dose única 10 minutos antes das tarefas comportamentais. Na sequência, os animais foram submetidos aos protocolos de comportamento.

**Tarefa comportamental - Tanque novo:** A tarefa do *Tanque novo* se propõe a avaliar basicamente a locomoção/exploração vertical do *zebrafish*, bem como comportamentos relacionados à resposta a novidade (Rosemberg et al., 2011; Cachat et al., 2010). Os animais são postos em um aparato, virtualmente dividido em topo, meio e fundo, observando-se a exploração ao longo de seis minutos de teste. São quantificados parâmetros de distância percorrida, tempo gasto em cada zona e número de entradas.

**Tarefa Comportamental – Interação social:** Consiste em um aquário central de vidro, dividido virtualmente em três regiões: uma área central, onde o animal teste é colocado; área coespecífica, próxima a um aquário lateral contendo um cardume de 3 peixes; área oposta, próxima a um aquário contendo apenas água (Pham et al., 2012). O teste tem duração de 6 minutos, onde é observada a exploração do aparato realizada pelo animal e sua interação com o cardume.

## RESULTADOS

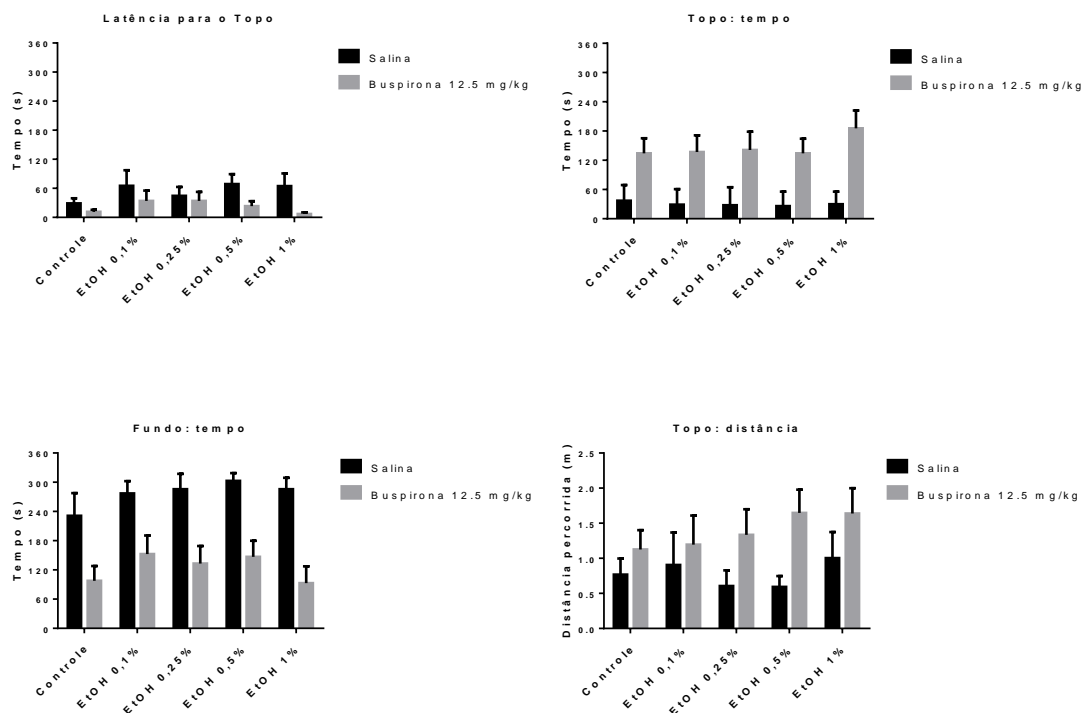
Não foram observadas diferenças estatísticas entre os grupos tratados com etanol e injetados com bupirona, devido à grande variação de valores dentro dos grupos. Os animais tratados com bupirona apresentam menor tempo para atingir o topo do aparato (**Fig. 1.A**), mais tempo (**Fig. 1.B**) e maior distância percorrida nesta região (**Fig. 1.D**), além de menos tempo gasto no fundo (**Fig. 1.C**), contudo sem diferenças significativas apontadas pela ANOVA de duas vias, seguida de pós-teste de Bonferroni.

A utilização de uma menor dose de bupirona visava a não alteração do perfil comportamental dos animais controles, contudo em decorrência da não-replicação dos dados do modelo de FASD, não se observou alterações significativas no comportamento dos diferentes grupos nos parâmetros de latência para o topo (**Fig. 2.A**), tempo no topo (**Fig. 2.B**), tempo no fundo (**Fig. 2.C**) e distância percorrida no topo do aparato (**Fig. 2.D**). Dados analisados pela ANOVA de duas vias, seguido de pós-teste de Bonferroni.

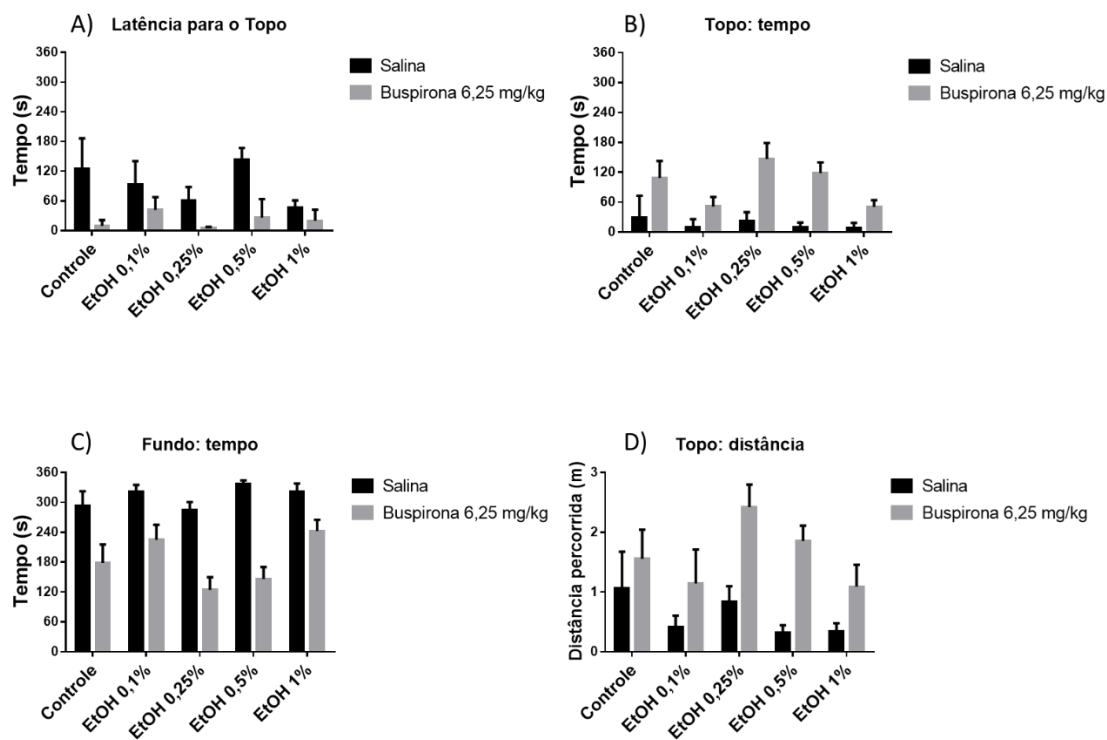
A **Figura 3** apresenta os resultados da tarefa de interação social dos animais tratados com bupirona 12,5mg/kg. Em **A e B**, temos o número de entradas em cada zona para salina e bupirona, respectivamente; as figuras **C e D** apresentam o tempo gasto em cada zona; e em **E e F**, temos a distância percorrida pelos grupos salina e bupirona respectivamente. Observou-se diferença significativa no número de entradas na zona oposta no grupo EtOH 0,5% em comparação ao EtOH 0,1% e controle (**Fig. 3.A**); e entre os grupos EtOH 0,5% e EtOH 1% salina quando comparados aos mesmos grupos tratados com bupirona (**Fig. 3.B**) (ANOVA de duas vias, Column factor  $F(1,79) = 39,98$ ,  $p < 0,0001$ ; Bonferroni test  $p < 0,05$ ). Quanto ao número de entradas na zona central, observou-se diferença novamente nos grupos EtOH 0,5% e 1% salina quando comparados aos mesmos grupos tratados com bupirona (**Fig. 3. B**) (ANOVA de duas vias, Column factor  $F(1,79) = 45,47$ ,  $p < 0,0001$ ; Bonferroni test  $p < 0,05$ ). Quando analisou-se o tempo gasto no centro, observou-se diferença no grupo EtOH 1% salina comparado ao EtOH 1% bupirona (**Fig. 3.D**) (ANOVA de duas vias, Column factor  $F(1,79) = 42,49$ ,  $p < 0,0001$ ; Bonferroni test  $p < 0,05$ ). Por fim, a análise das distâncias percorridas, apresentou diferença estatística apenas na região central, com os grupos EtOH 0,5% e 1% salina quando comparados aos seus respectivos tratamentos com bupirona (**Fig. 3.F**) (ANOVA de duas vias, Column factor  $F(1,79) = 448,45$ ,  $p < 0,0001$ ; Bonferroni test  $p < 0,05$ ).

Dando seguimento aos resultados da Interação social, a **Figura 4** traz os valores agrupados para animais salina e injetados i.p. com bupirona 6,25mg/kg. Não se observou alterações significativas nos padrões de exploração das três zonas do aparato, tanto para

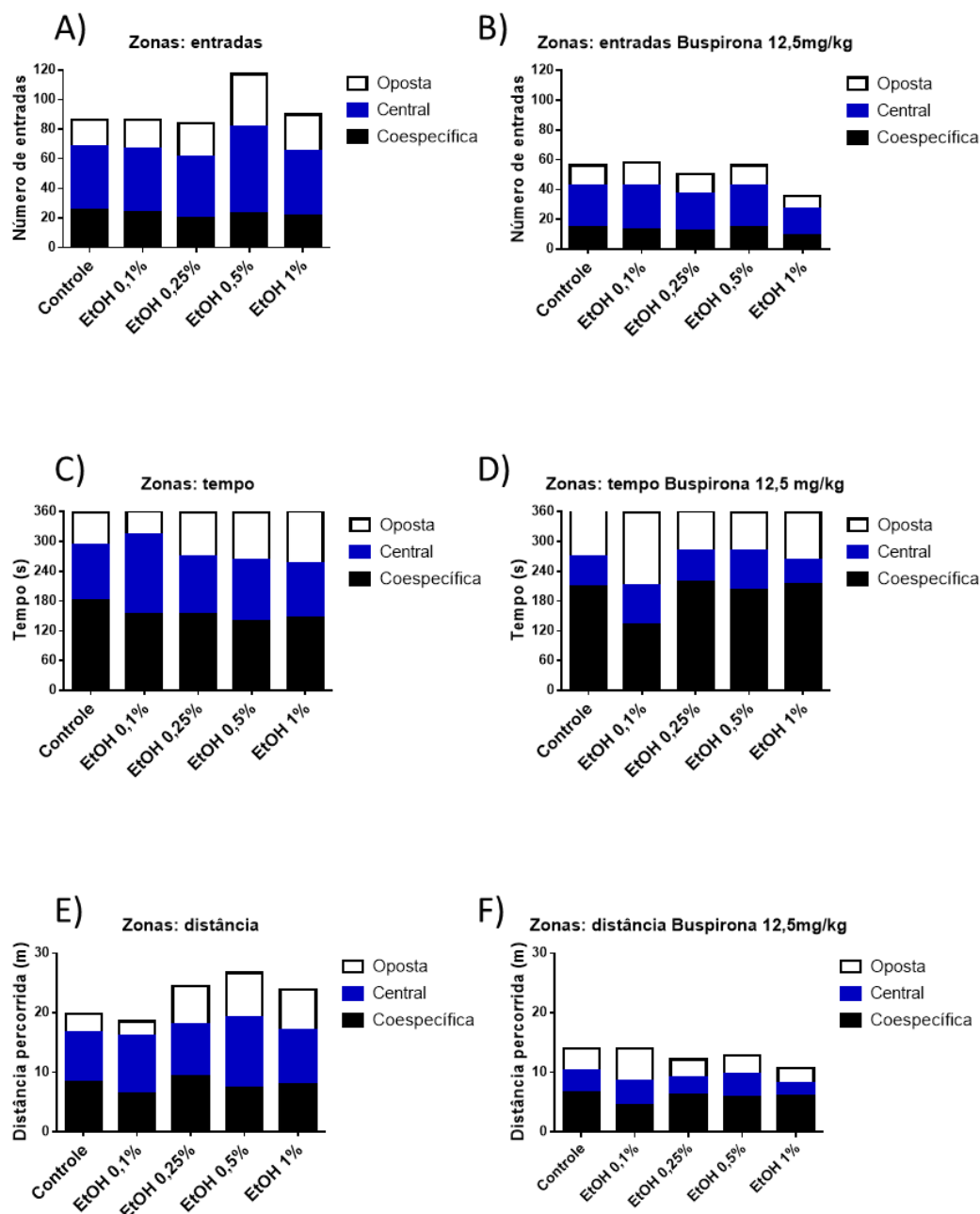
animais salina, quanto para os tratados com buspirona, exceto pelo número de entradas na zona coespecífica, onde o grupo EtOH 0,5% salina difere do EtOH 0,5% buspirona (**Fig. 4.B**) (ANOVA de duas vias, Column factor  $F(1,55) = 16,21$ ,  $p = 0,0002$ ; Bonferroni test  $p < 0,05$ ). Perfil comportamental semelhante é observado no número de entradas por zona (**Figuras 4.A e 4.B**), tempo gasto (**Figuras 4.C e 4.D**) e distância percorrida em cada região do aparato (**Figuras 4.E e 4.F**).



**Figura 1: Perfil comportamental da tarefa de Tanque novo em *zebrafish* adulto pré-tratado com buspirona 12,5 mg/kg.** Latência para atingir o topo do aparato (A), tempo gasto no topo (B), tempo gasto no fundo (C) e distância percorrida no topo (D). Dados expressos por média  $\pm$  S.E.M e analisados pela ANOVA de duas vias, seguida de pós-teste de Bonferroni. N= 7(salina)/12(buspirona).



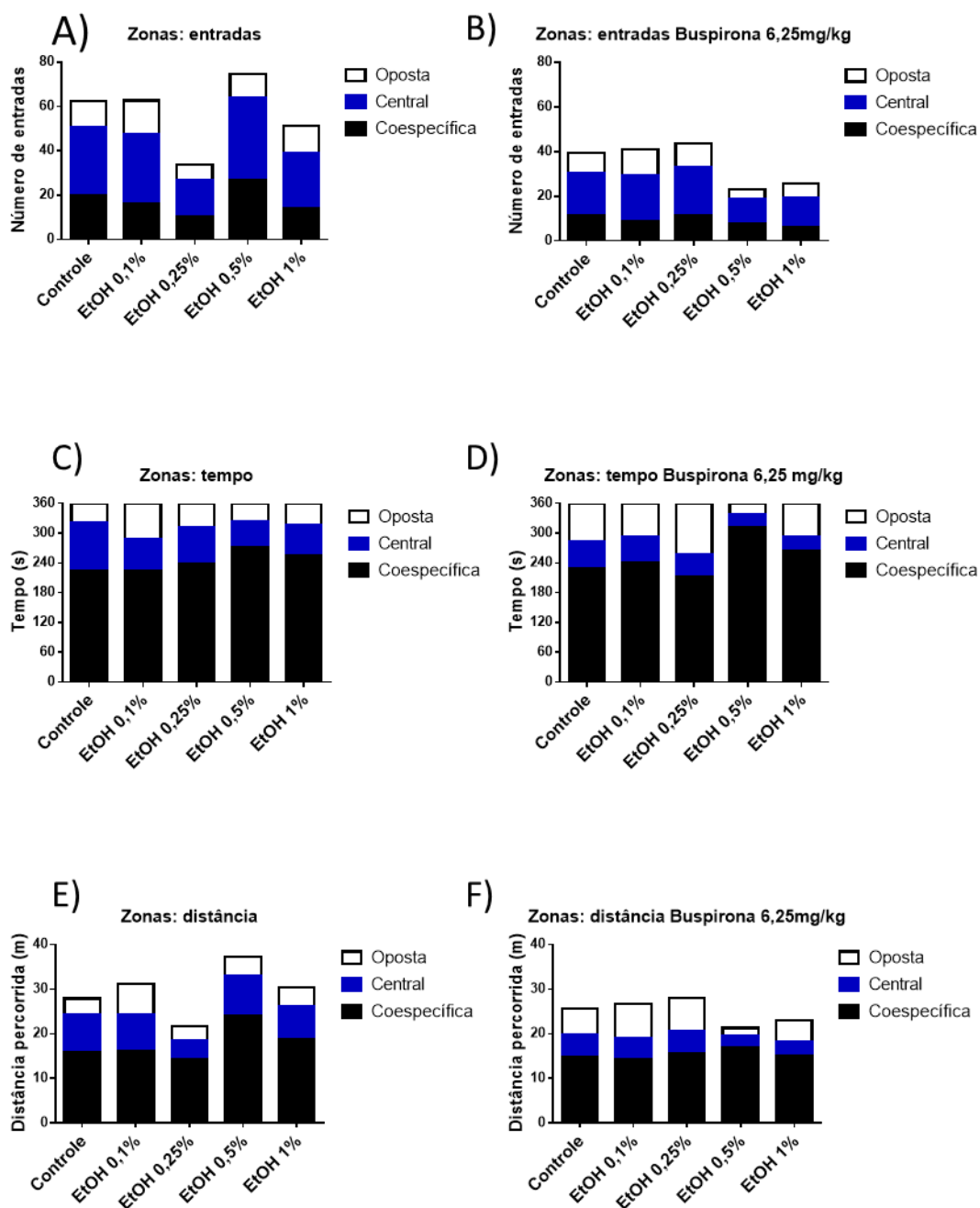
**Figura 2: Perfil comportamental da tarefa de Tanque novo em zebrafish adulto pré-tratado com buspirona 6,25 mg/kg.** Latência para atingir o topo do aparato (A), tempo gasto no topo (B), tempo gasto no fundo (C) e distância percorrida no topo (D). Dados expressos por média  $\pm$  S.E.M e analisados pela ANOVA de duas vias, seguida de pós-teste de Bonferroni. N=8 animais por grupo.



**Figura 3: Perfil comportamental da tarefa de Interação social em *zebrafish* adulto pré-tratado com buspirona 12,5 mg/kg quando comparado ao perfil dos controles.**

Número de entradas total estratificado por cores em cada zona para animais injetados com salina (A) e buspirona (B), tempo total estratificado por cores em cada zona para animais salina (C) e buspirona (D), distância total percorrida estratificada por cores em cada zona para animais salina (E) e buspirona (F). Dados analisados pela ANOVA de duas vias, seguido de pós-teste de Bonferroni. N= 7 (salina)/12 (buspirona). # diferença entre os grupos salina; \* diferença na comparação entre grupos salina e seus respectivos grupos buspirona.





**Figura 4: Perfil comportamental da tarefa de Interação social em *zebrafish* adulto pré-tratado com buspirona 6,25 mg/kg quando comparado ao perfil dos controles.**

Número de entradas total estratificado em cada zona para animais injetados com salina (A) e buspirona (B), tempo total estratificado em cada zona para animais salina (C) e buspirona (D), distância total percorrida estratificada em cada zona para animais salina (E) e buspirona (F). Dados analisados pela ANOVA de duas vias. N= 6(salina)/8(buspirona). \* = diferença na comparação entre os grupos salina e seus respectivos grupos buspirona.

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# PARTE III

## 7. DISCUSSÃO

O consumo social de etanol durante a gestação é a principal causa de distúrbios cognitivos evitáveis observados em crianças (McHugh et al., 2014). A exposição embrionária ao etanol gera prejuízos comportamentais e cognitivos, além de poder causar alterações morfológicas nos casos mais graves. Este conjunto de alterações é denominado de FASD (*Fetal alcohol spectrum disorder* – Desordens do espectro alcoólico fetal), condição congênita e persistente ao longo da vida. Em decorrência da ausência de alterações faciais ou de características mais específicas, as formas mais brandas deste transtorno são de difícil diagnóstico, ao passo que o portador pode apresentar apenas distúrbios comportamentais e de aprendizado, recebendo o diagnóstico incorreto de autismo ou transtorno de déficit de atenção na adolescência ou vida adulta (Roozen et al., 2016). Desta forma, é relevante que se compreenda as alterações neuroquímicas ocasionadas pelo etanol e que se possa correlacionar com as alterações comportamentais observadas na FASD, facilitando tanto o diagnóstico, como o surgimento de possíveis alvos terapêuticos.

Nesta tese de doutorado, utilizamos o *zebrafish* como modelo animal para indução de FASD. O protocolo utilizado neste estudo é de Fernandes & Gerlai, (2009), onde os embriões de *zebrafish* são tratados por duas horas com concentrações de etanol que variam de 0,1 a 1%. O estágio de desenvolvimento escolhido para a exposição é de 24 hpf, o que seria o equivalente ao fim do primeiro trimestre de gestação em humanos e ao desenvolvimento do tubo neural (Kimmel et al., 1995). Portanto, trata-se de um momento crítico, em que a exposição ao etanol é capaz de induzir alterações neurocomportamentais, como as relatadas nesta tese e em outros estudos que utilizaram o mesmo protocolo (Buske & Gerlai, 2011; Fernandes et al., 2015a).

A busca por uma forma mais branda da desordem mostra-se como a melhor opção para um estudo a longo prazo, permitindo acompanhar o desenvolvimento até a fase adulta. No caso do *zebrafish*, a utilização de altas doses de etanol gera malformações que inviabilizam a sobrevivência do animal, levando-o a óbito ainda na fase larval, impossibilitando acompanhar o seu desenvolvimento (Bilotta et al., 2004). O que deve ser levado em consideração também são outros parâmetros do protocolo de indução do modelo de FASD, como o tempo de exposição ao etanol, o estágio do desenvolvimento em que ocorre a exposição e a idade em que os animais são analisados posteriormente (Fernandes et al., 2015a; Fernandes & Gerlai, 2009). Os resultados observados em cada

estudo são influenciados por estes parâmetros, que devem ser controlados para permitir a replicabilidade e comparação dos estudos.

Ao longo deste trabalho, avaliamos as alterações comportamentais, observando diminuição na interação social e comportamento do tipo ansioso; alterações no sistema glutamatérgico, com diminuição na captação e ligação de glutamato, e diminuição na atividade de enzimas relacionadas a homeostase de glutamato – Glutamina sintetase e Na<sup>+</sup>/K<sup>+</sup> ATPase; além de avaliação do número de neurônios no tálamo dos animais. Todas as análises foram realizadas em animais adultos, que haviam sido expostos a baixas concentrações de etanol durante o desenvolvimento (24 hpf), por apenas duas horas. A utilização de baixas concentrações de etanol aliada a um curto tempo de exposição resulta em animais aparentemente normais, sem alterações morfológicas visíveis, mas com perfil comportamental e neuroquímico alterados.

As alterações no comportamento social e na formação de cardume já eram relatadas na literatura por estudos que utilizaram este mesmo protocolo de indução de FASD (Fernandes et al., 2015b; Fernandes & Gerlai, 2009). Por se tratar de um animal extremamente sociável e viver em cardume a maior parte do tempo, o *zebrafish* é um excelente modelo para o estudo de alterações na interação e preferência social, fatores que normalmente encontram-se alterados nos pacientes de FASD (Cook et al., 2016; Wilhoit et al., 2017). Os resultados aqui observados corroboram para o relato de diminuição da interação social, dessa vez aliado ao comportamento do tipo ansioso, que ainda é alvo de muita discussão em estudos com *zebrafish* (Fernandes et al., 2015a; Fernandes and Gerlai, 2009). Desta forma, o objetivo do Capítulo I foi analisar se as alterações no comportamento social eram independentes ou acompanhadas por comportamento do tipo ansioso e se tais padrões estariam relacionados entre si.

A ansiedade em *zebrafish* pode ser analisada através de suas respostas frente a novidade, como é o teste do Tanque novo (*Novel tank*), onde o animal é exposto a um ambiente desconhecido, um aquário de formato trapezoidal, distinto do qual ele está habituado, permitindo que ele explore o aparato ao longo de 6 minutos (Rosemberg et al., 2011). O comportamento do tipo ansioso pode ser avaliado nos animais adultos por meio de parâmetros pré-definidos, como o tempo gasto e a distância percorrida no fundo e a latência para atingir o topo do aquário. De acordo com nossos resultados, o aumento na concentração de etanol ao qual os animais foram expostos na fase embrionária está relacionado com o aumento na resposta do tipo ansiedade nos animais avaliados. O grupo EtOH 1%, a maior concentração empregada no estudo, apresenta uma latência maior para

atingir o topo do aparato, além de gastar a maior parte do tempo do teste apenas no fundo do aquário. Os demais grupos EtOH também apresentaram diferenças significativas de tempo e distância quando comparados ao grupo controle. Em geral, estes grupos gastam mais tempo no fundo do aparato e menos tempo no topo, além de percorrerem menor distância na área superior do aparato, como podemos observar nos resultados do Capítulo I desta tese.

Diferentes protocolos de exposição embrionária ao etanol também observaram comportamento do tipo ansioso em *zebrafish*. Parker et al. (2014) expôs os animais ao EtOH 0,1% do 2º ao 9º dia pós-fertilização, observando posteriormente que os animais permaneceram mais tempo no fundo do Tanque novo. Utilizando um protocolo semelhante, Baiamonte et al. (2016) expuseram os animais a uma concentração de 0,3% de etanol, seguido de análise comportamental em larvas e adultos, e observaram uma diminuição no tempo gasto no fundo do aparato pelos animais tratados com etanol. Este resultado conflita com o anterior encontrado por Parker et al. (2014). Seguin et al. (2016), utilizando o mesmo protocolo empregado nas análises desta tese, não observou indícios de ansiedade ou medo em animais adultos (6 meses de idade) que foram expostos ao etanol durante o período embrionário (24 hpf), contudo, cabe ressaltar que o aparato utilizado neste estudo consistia em um aquário maior e de formato distinto ao empregado nas análises desta tese. Desta forma, o emprego de diferentes aparatos para os testes comportamentais pode refletir em resultados conflitantes, mesmo quando se utiliza um mesmo protocolo de indução de FASD.

Apesar dos resultados controversos observados nos estudos envolvendo *zebrafish* e FASD, os transtornos de ansiedade são características marcantes das desordens relacionadas com a exposição pré-natal ao etanol (Baculis et al., 2015; Hellemans et al., 2010; Lam et al., 2019). Como nos testes comportamentais realizados neste estudo, o comportamento do tipo ansiedade mostrou-se característico dos animais tratados com etanol na fase embrionária, sugerimos na sequência das análises que a utilização de buspirona- um ansiolítico já consolidado para tratar transtornos de ansiedade- poderia ser uma tentativa interessante de modular os resultados e observar se existe uma conexão entre os comportamentos testados, ou seja, se a diminuição do comportamento do tipo ansioso teria alguma influência nos resultados de comportamento social. De acordo com a literatura, a buspirona seria capaz de diminuir o tempo gasto pelos animais no fundo do aquário, levando a um aumento da exploração do topo, sem alterar a velocidade de nado dos mesmos (Bencan et al., 2009; Gebauer et al., 2011). Em nosso estudo, os animais

tratados com buspirona apresentaram diminuição na preferência social, ou seja, diminuição da resposta de cardume, exemplificando a relação que existe entre os dois comportamentos. Este resultado está amparado pela literatura, que justamente apresenta o comportamento de cardume como uma forma de proteção contra predadores, assim o medo e a ansiedade seriam capazes de aumentar esse comportamento para proteção e preservação do indivíduo (Buske & Gerlai, 2011; Miller & Gerlai, 2011; Seguin et al., 2016). Desta forma, o aumento de ansiedade observado nos animais FASD no teste do Tanque novo aliado a diminuição na preferência social também se contrapõe ao resultado de que o uso de um ansiolítico é capaz de diminuir a interação social. Tais resultados conflitantes, dentro do Capítulo I desta tese, não são de fácil elucidação, porém pode-se inferir que o comportamento do tipo ansioso é muito complexo para ser medido através de poucos fatores e que ainda precisa ser melhor explorado em *zebrafish*. Principalmente, do ponto de vista da utilização de distintas linhagens para os estudos realizados, o que reforça o efeito genético como uma variável importante no momento de se comparar diferentes trabalhos (Mahabir et al., 2014).

As alterações comportamentais observadas tanto em humanos, quanto em modelos animais de FASD estão relacionadas com modificações ocasionadas pelo etanol diretamente no SNC. O álcool é capaz de afetar neurotransmissores específicos, por meio da alteração da função e expressão de seus receptores (Lovinger et al., 1989). A exposição ao etanol durante o desenvolvimento leva a inibição na função de receptores de glutamato (Lovinger et al., 1990) e ao aumento de atividade dos receptores de GABA no hipocampo (Weiner et al., 1994), o que pode ter relação com problemas de memória e aprendizado (Mukherjee, 2013). Outro mecanismo pelo qual o etanol pode gerar déficits no SNC é a indução de apoptose neuronal, através da formação de conexões sinápticas incorretas (Ikonomidou et al., 2000). Para o funcionamento adequado das conexões cerebrais estabelecidas ao longo do desenvolvimento é necessária a estabilização das sinapses funcionais e a remoção das sinapses desnecessárias. Este processo de refinamento requer a homeostasia dos sistemas excitatório e inibitório (Ramocki & Zoghbi, 2008), que pode estar alterado em função da exposição ao etanol. Em resposta às perturbações, os neurônios em desenvolvimento tentam reestabelecer o equilíbrio por meio de mudanças compensatórias, alterando a função ou até mesmo a expressão de receptores e/ou transportadores envolvidos na neurotransmissão. Tais mudanças nem sempre são eficazes, podendo por vezes, gerar ainda mais danos ao SNC, com consequências a longo prazo (Valenzuela et al., 2011).

O sistema glutamatérgico é diretamente afetado pelo etanol, tanto pela inibição dos receptores NMDA, quanto por alterações na liberação de glutamato na fenda sináptica e sua recaptação pelos astrócitos (Brolese et al., 2015; Goodwani et al., 2017). Também é associado a desordens relacionadas ao abuso de álcool e pelos comportamentos de dependência e abstinência em alcoolistas (Most et al., 2014). Além disso, apresenta ação direta em funções neurocomportamentais que se encontram alteradas na FASD, como memória e aprendizado (Bliss & Collingridge, 1993). Devido a relevância do sistema glutamatérgico na conexão de alterações comportamentais e neuroquímicas, o Capítulo II desta tese teve como objetivo compreender quais mecanismos da transmissão glutamatérgica estariam envolvidos na queda de captação de glutamato em cérebros de *zebrafish* adultos expostos ao etanol na fase embrionária, resultados estes observados previamente em estudos do nosso grupo (Baggio et al., 2017).

Em nosso segundo estudo não foi observado alteração no transporte vesicular de glutamato, do ponto de vista da expressão do transportador vGluT2. Utilizando o mesmo protocolo de indução de FASD, Mahabir et al. (2018) não encontrou diferenças na quantidade de glutamato no cérebro de *zebrafish* adulto, exposto na fase embrionária ao etanol, quando comparado ao grupo controle. Desta forma, a vesiculação e a liberação de glutamato pelo neurônio pré-sináptico parecem não estar alteradas neste modelo. Portanto, a captação de glutamato alterada, neste estudo, não seria explicada por uma possível excitotoxicidade por excesso de glutamato na fenda, ocasionada pelo etanol, de modo que não haveria diminuição no transporte vesicular e nem acúmulo de glutamato na fenda.

O processo de captação de glutamato é promovido pela atividade de transportadores dependentes de sódio (Danbolt, 2001). Dentre eles, o EAAT2 é o mais abundante e pode ter sua expressão afetada pela exposição ao etanol (Zhou & Danbolt, 2013). Para confirmar a hipótese de que os transportadores estariam com sua função ou expressão alteradas, utilizamos a ceftriaxona, um antibiótico  $\beta$ -lactâmico já descrito na literatura como capaz de aumentar a expressão do transportador EAAT2 (Rothstein et al., 2005). Nos animais tratados com ceftriaxona, obtivemos uma reversão parcial da queda observada na captação de glutamato nos grupos de maior concentração de etanol, sugerindo que o transportador EAAT2 esteja de fato alterado em função da exposição embrionária ao etanol. A confirmação de que a expressão do transportador estaria diminuída por meio do imunocontéudo proteico, via Western blotting, não foi possível



devido à dificuldade em se encontrar anticorpos que reconheçam adequadamente a isoformas do transportador expressas em *zebrafish*.

Como a modulação na captação de glutamato por meio do tratamento com ceftriaxona não eleva os níveis de captação ao nível do controle, supusemos que existam outros componentes do sistema de transmissão glutamatérgica que estariam alterados. Através da análise da ligação de glutamato em frações enriquecidas de membrana plasmática de cérebro de animais adultos, observamos diminuição na ligação de glutamato, novamente nos grupos de maior concentração de etanol. Tais dados corroboram com a literatura que mostra claramente a ação do etanol nos receptores de glutamato, como a redução na expressão de algumas subunidades – GluA1 e GluA2 – em fatias de hipocampo de ratos expostos a 150 mM de etanol por 7 dias (Gerace et al., 2016). A exposição embrionária ao etanol leva também à diminuição na transmissão sináptica em células piramidais da região CA1, por afetar a atividade de receptores AMPA (Wijayawardhane et al., 2007), além de alterar a composição das subunidades dos receptores NMDA no giro denteado do hipocampo de roedores (Brady et al., 2013). Tais danos no hipocampo destes animais modelos de FASD podem ser os responsáveis pelas alterações comportamentais, tais como déficits de memória e aprendizado, já que se tratam de funções mediadas pela transmissão glutamatérgica (Mameli et al., 2005). Outra evidência da relação do sistema glutamatérgico com desordens relacionadas ao consumo de etanol é a ação dos receptores metabotrópicos do grupo II (mGluR2/3), por exemplo, que estão envolvidos na regulação do comportamento de dependência e consumo de etanol (Windisch & Czachowski, 2018). Portanto, os dados disponíveis na literatura reforçam a conexão do sistema glutamatérgico com os transtornos observados em função do abuso de álcool.

O transporte de alta afinidade de glutamato é dependente de sódio, portanto está diretamente relacionado com a atividade da enzima  $\text{Na}^+/\text{K}^+$  ATPase, a qual gera um gradiente iônico na membrana celular como força motriz para a captação de glutamato (Rose et al., 2009). Como observado no Capítulo II desta tese, o tratamento com EtOH 0,5% e 1% diminui a atividade da enzima em 32%, em ambos os grupos, o que pode ser mais um fator responsável pela diminuição na captação de glutamato. O etanol seria capaz de agir diretamente na estrutura lipoproteica da enzima, o que justificaria a alteração de função (Ledig et al., 1985). Ao passo que o grupo EtOH 0,25% apresenta, a partir do tratamento com ceftriaxona, o retorno completo dos valores de captação para níveis de controle, e não mostra alteração nos demais fatores, entre eles a atividade da enzima

$\text{Na}^+/\text{K}^+$  ATPase, podemos concluir que em concentrações menores de etanol, a alteração possa estar mais restrita à função dos transportadores. Contudo, com o aumento na concentração de etanol a que os animais foram expostos, surgem novos padrões alterados na transmissão sináptica glutamatergica.

O glutamato, após ser liberado na fenda sináptica, é captado pelos astrócitos e convertido pela enzima glutamina sintetase (GS) em glutamina, dando seguimento ao ciclo de síntese e oxidação do neurotransmissor (Hertz & Zielke, 2004). O álcool é capaz de afetar este ciclo, interferindo na atividade da enzima, com diminuição da atividade em modelo animal e cultura celular exposta ao etanol (Cesconetto et al., 2016; Davies and Vernadakis, 1984), e diminuição da expressão em estudos *post mortem* de hipocampo de alcoolistas (Matsumoto, 2009). Apenas o grupo EtOH 1% apresentou diminuição da atividade de GS em nosso estudo, o que poderia ser justificado por uma menor disponibilidade de substrato, no caso o glutamato, já que este grupo apresenta justamente a maior redução na captação de glutamato. A diminuição na atividade desta enzima pode também estar relacionada com déficits na eliminação de amônia, o que contribuiria com a toxicidade do etanol e seus metabólitos (Ledig et al., 1991), além de efeitos negativos da exposição embrionária ao etanol, como acidemia e alterações no fluxo sanguíneo cerebral do feto (Sawant et al., 2015).

A diminuição da atividade de enzimas ligadas a transmissão glutamatergica, como a  $\text{Na}^+/\text{K}^+$  ATPase, poderia ter relação com a ocorrência de déficit energético no cérebro dos animais expostos ao etanol na fase embrionária. Desta forma, decidimos avaliar a função mitocondrial, por se tratar de uma organela responsável pelo fornecimento de energia às células (Van Der Blik et al., 2017), por meio da respirometria. Em um estudo com camundongos, observou-se no dia gestacional 18, déficits na proliferação e diferenciação mitocondrial cerebral, após a exposição ao etanol do 6º ao 15º dia, além de redução de atividade nos complexos I e IV da cadeia respiratória (Xu et al., 2005). Também se observa disfunção mitocondrial em cardiomiócitos, o que estaria por trás do desenvolvimento de patologias cardíacas nos casos mais severos de exposição embrionária ao etanol (Nyquist-Battie & Freter, 1988). Marin-Garcia et al. (1996) observou redução na atividade da ATP sintase em fígado e cérebro de ratos com um dia pós-natal, mas apenas o fígado apresentou redução de atividade do complexo III, enquanto no coração, nenhuma enzima mitocondrial apresentou alteração. Desta forma, a vulnerabilidade mitocondrial ao etanol é tecido-específica. Contudo, em nosso modelo de FASD não encontramos alterações nos fatores analisados, quanto a respiração

mitocondrial em cérebro de *zebrafish* adulto. Portanto, as alterações do SNC observadas neste modelo não estão relacionadas com potenciais déficits mitocondriais, que poderiam comprometer o balanço energético do sistema. Outro fator que se pode considerar é alta taxa de regeneração apresentada pelo *zebrafish*, o que nos leva a supor que os animais seriam capazes de se adaptar às alterações induzidas pelo etanol e gerar um novo balanço energético (Gemberling et al., 2013).

No último capítulo desta tese buscamos unir as características já discutidas para o modelo animal e a desordem estudada. Portanto, a ideia central do capítulo III consistiu em observar possíveis modificações morfológicas no cérebro dos animais adultos submetidos ao modelo de FASD. O tálamo foi a região escolhida para as análises por se tratar de uma estrutura responsável por conectar diferentes regiões cerebrais, modulando e transmitindo as informações recebidas, além de apresentar projeções de neurônios glutamatérgicos (Sherman, 2016), que seriam o foco deste estudo. O etanol seria capaz de danificar a conexão do tálamo com o córtex cerebral, levando a formação de conexões incorretas, as quais poderiam ser responsáveis pelos déficits cognitivos e comportamentais observados no modelo de FASD (Mooney & Miller, 2010). Contudo, neste estudo não observamos alterações significativas, o que nos leva a inferir algumas justificativas inerentes ao modelo e ao protocolo empregados. O *zebrafish* é utilizado como modelo vertebrado de regeneração graças a sua ampla capacidade de recuperar tecidos, desde caudas amputadas a outras estruturas mais complexas, como retina, coração e cérebro lesionados (Jopling et al., 2010; Mokalled et al., 2016; Wan & Goldman, 2016). Unindo tal característica com o protocolo utilizado de exposição a baixas concentrações de etanol, onde não são observadas alterações morfológicas aparentes, o número de neurônios contabilizados não apresentar diferença significativa quando se compara os grupos EtOH ao grupo controle, é um resultado compreensível.

Mooney & Miller (2010) também não encontraram diferenças no número de neurônios no complexo ventrobasal do tálamo de ratos adolescentes expostos no período pré-natal ao etanol, apesar de observarem alterações no número de neurônios em demais regiões cerebrais ao longo do tempo e um padrão alterado de neurogênese. Desta forma, a resposta ao etanol nesta região seria diferente que a observada nas demais regiões do cérebro, o efeito seria apenas na proliferação celular pós-natal, com ausência de indicadores bioquímicos de morte celular no tálamo maduro (Mooney & Miller, 2001).

Contudo, apesar dos resultados negativos relatados, a literatura apresenta trabalhos que mostram alterações no tálamo, tanto em modelos animais, quanto em humanos, após

a exposição ao etanol na fase embrionária, o qual seria capaz de causar danos em áreas responsáveis pelo aprendizado, memória e funções cognitivas, como hipocampo e córtex pré-frontal, regiões que formam circuitos com o tálamo (Lawrence et al., 2012; Livy et al., 2003). Ratas adultas, expostas ao etanol durante o desenvolvimento, apresentaram redução no domínio ventral talâmico, com diminuição no volume das estruturas e no número de neurônios (Gursky et al., 2019). Já através de análises de MRI, crianças e adolescentes diagnosticadas com FASD apresentaram diminuição no volume de estruturas cerebrais, como hipocampo e tálamo (Nardelli et al., 2011). As modificações observadas podem ter relação com a idade em que é realizada a análise, além do protocolo utilizado. Análises anatômicas e exames de MRI feitas em camundongos na adolescência (P28) e na idade adulta (P80), previamente expostos ao etanol na fase pré-natal, apresentaram resultados distintos quanto a modificações estruturais e neuronais nas diferentes idades. Os camundongos adolescentes apresentaram tálamo e hipotálamo maior, além de menor marcação de neurofilamentos, quando comparados ao grupo controle; mudanças estas que não foram observadas nos camundongos adultos (Nguyen et al., 2020). De acordo com este número considerável de estudos, que apresentam alterações em estruturas cerebrais em decorrência da exposição embrionária ao etanol, seria de extrema relevância a realização de análises em demais regiões cerebrais como perspectiva de estudos futuros, dando continuidade aos resultados apresentados nesta tese.

Nossas análises foram realizadas apenas na idade adulta, após uma única exposição ao etanol na fase embrionária, o que pode corroborar para os resultados negativos observados no tálamo dos animais. Também é possível que as modificações a nível cerebral sofram modulações ao longo do desenvolvimento, gerando novos padrões de equilíbrio, que só possam ser identificados por meio de análises neuroquímicas, como as realizadas no Capítulo II. Porém, cabe ressaltar, que por se tratar de uma estrutura diminuta no cérebro do *zebrafish*, a técnica histológica para observação do tálamo é difícil de ser executada, assim o N empregado neste estudo não foi o inicialmente planejado, por perdas ao longo da confecção das lâminas. Como perspectiva, a realização de novas análises da região poderia colaborar para a obtenção de melhores conclusões acerca dos resultados observados.

O padrão de mecanismos moleculares do tálamo é bem conservado entre os vertebrados ao longo da evolução (Scholpp & Lumsden, 2010). Nos estágios iniciais do desenvolvimento, o tálamo, tanto em *zebrafish*, quanto em mamíferos, divide-se em dois

domínios: o rostral, que dará origem a neurônios gabaérgicos, e o domínio caudal, que originará neurônios glutamatérgicos (Peukert et al., 2011; Scholpp et al., 2009). Ambos os neurotransmissores podem ser afetados pela exposição embrionária ao etanol (Valenzuela et al., 2011). Como observado em trabalho anterior do grupo e no Capítulo II desta tese, o sistema glutamatérgico é amplamente alterado, apresentando queda na captação e ligação de glutamato e diminuição na atividade das enzimas  $\text{Na}^+/\text{K}^+$  - ATPase e glutamina sintetase (Baggio et al., 2020, 2017). Desta forma, as alterações observadas estão limitadas a modificações na função e expressão dos transportadores, receptores e enzimas do metabolismo do glutamato, e não devido a alterações estruturais do tálamo, já que o protocolo de indução de FASD empregado em ambos os estudos foi o mesmo.

A ação básica do etanol através do bloqueio dos receptores de glutamato NMDA e da hiperativação de receptores de GABA, ambos sistemas com neurônios presentes no tálamo, faz dessa estrutura um interessante alvo de maiores investigações (Harris et al., 1995; Lovinger et al., 1989). Acrescenta-se também a questão comportamental, que é fortemente influenciada por distúrbios do tálamo ou de suas regiões conectadas, como córtex cerebral, e que representa o foco da maioria das desordens mais brandas induzidas pela exposição ao etanol durante o desenvolvimento (Olney et al., 2001). É através da análise das vias de neurotransmissão de forma detalhada e simplificada que se possibilita a compreensão dos mecanismos de atuação do etanol no SNC de forma geral, ampliando nossa compreensão e capacidade de conectar a questão neuroquímica e comportamental, de forma a facilitar o diagnóstico e a procura de alvos terapêuticos.

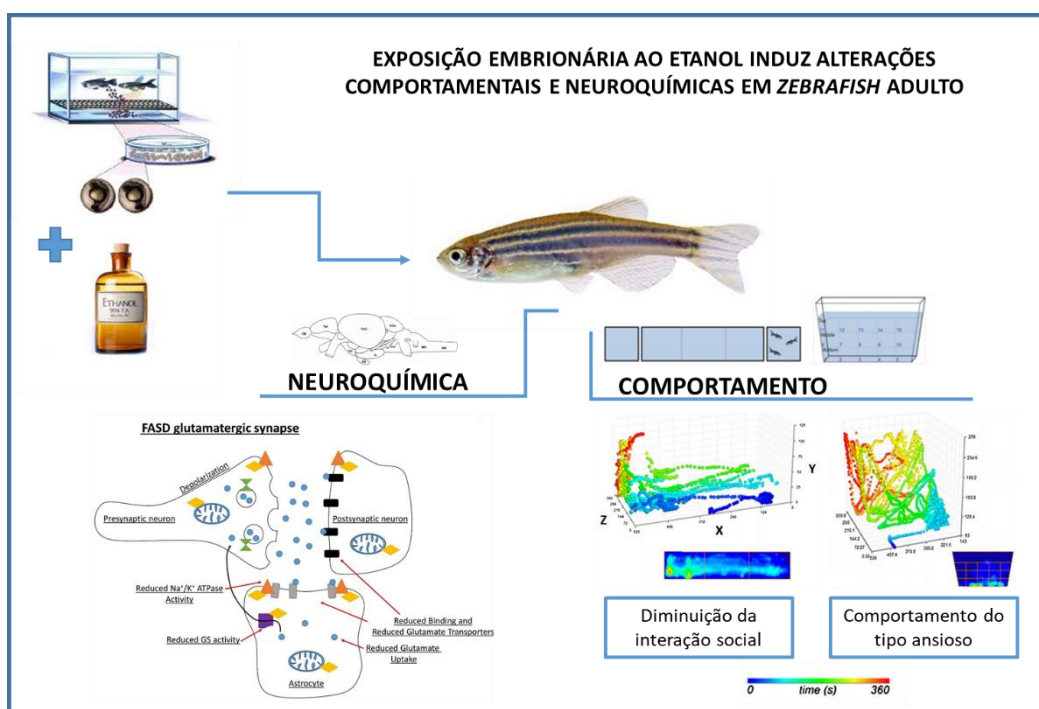
O glutamato, principal neurotransmissor excitatório do SNC, é responsável por inúmeras funções cognitivas e comportamentais, que frequentemente encontram-se alteradas na FASD e em demais desordens relacionadas com o consumo de etanol (Danbolt, 2001; Valenzuela et al., 2011). A comprovação de que a queda na captação de glutamato, no cérebro de animais adultos expostos ao etanol na fase embrionária, está relacionada com alterações em inúmeros fatores da transmissão sináptica, mostra como o etanol é capaz de afetar diversas vias neurais, tendo ação ampla sobre o SNC. Também podemos observar que as alterações comportamentais descritas estão provavelmente conectadas com as mudanças neuroquímicas relatadas em nossos estudos, e que ambas – alterações comportamentais e neuroquímicas – podem ser moduladas por fármacos amplamente utilizados para outras desordens, como por exemplo a buspirona, utilizada para o tratamento de transtornos de ansiedade, e a ceftriaxona, que é capaz de controlar a excitotoxicidade glutamatérgica pelo aumento da expressão do transportador EAAT2. Por

fim, certas estruturas cerebrais e parâmetros neuroquímicos tem a capacidade de gerar um novo estado de equilíbrio e não apresentar diferenças significativas, quando analisados na idade adulta, seja por funções adaptativas ou em decorrência das baixas concentrações de etanol que foram utilizadas no protocolo.

## 8. CONCLUSÃO

O *zebrafish* é um excelente modelo para o estudo dos transtornos ocasionadas pela exposição embrionária ao etanol. Utilizando-se deste modelo animal, demonstramos as alterações comportamentais e neuroquímicas, com foco no sistema glutamatérgico, em animais adultos, tratados com baixas concentrações de etanol durante o desenvolvimento. É notável que mesmo uma única exposição a baixas concentrações de etanol seja capaz de produzir alterações em longo prazo e que estas, possivelmente, estejam correlacionadas entre si.

Após a indução de uma forma branda de FASD, observamos no cérebro dos animais adultos queda na captação e ligação de glutamato, além de diminuição da atividade das enzimas  $\text{Na}^+/\text{K}^+$  ATPase e glutamina sintetase. Tais alterações na homeostase do sistema glutamatérgico podem ser as responsáveis pelas alterações comportamentais observadas, tais como diminuição na preferência e interação social e comportamento do tipo ansioso. As modificações encontradas nesta tese não têm relação com alterações estruturais e de número de neurônios do tálamo, estrutura cerebral relacionada com funções cognitivas e comportamentais, que frequentemente encontram-se alteradas nos portadores da FASD. Desta forma, concluímos que o sistema glutamatérgico é amplamente afetado pela exposição ao etanol durante o desenvolvimento, e que mesmo baixas concentrações de etanol são capazes de induzir alterações neuroquímicas e comportamentais, que podem ser observadas a longo prazo.



## 9. PERSPECTIVAS

- Avaliar a expressão dos receptores e transportadores de glutamato no cérebro de *zebrafish* adulto exposto ao etanol na fase embrionária (24hpf), com foco no transportador EAAT2;
- Modular as alterações comportamentais por meio da reversão da queda de captação de glutamato observada em *zebrafish* adultos expostos ao etanol na fase embrionária (24hpf), através do tratamento com ceftriaxona;
- Ampliar as análises do sistema colinérgico, avaliando o imunoconteúdo proteico das enzimas colina-acetiltransferase (ChAT) e acetilcolinesterase (AChE), em cérebro de *zebrafish* adulto exposto ao etanol na fase embrionária (24hpf);
- Ampliar as análises histológicas do tálamo, aumentando o N de cada grupo avaliado, além da análise de outras estruturas cerebrais, como palium e distintas áreas talâmicas;
- Estudo de bioinformática, com foco na procura de possíveis proteínas e genes responsáveis pelas alterações descritas nas desordens da FASD, os quais poderiam ser considerados marcadores do transtorno.



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# **ANEXOS**

## 11. ANEXOS

### 11.1. INTRODUÇÃO GERAL

Os anexos apresentados nesta tese fazem parte de trabalhos em andamento ou resultados complementares aos capítulos anteriormente mostrados. Os animais utilizados nestes experimentos foram submetidos ao mesmo protocolo de indução de uma forma branda de FASD e analisados posteriormente na idade adulta.

### 11.2. MATERIAL E MÉTODOS GERAL

**Protocolo de indução de FASD:** *Zebrafish* adultos foram colocados em uma proporção de 2 machos: 1 fêmea nos aquários para reprodução. Os ovos foram coletados 1,5 horas após a fertilização (hpf) e lavados com uma solução de azul de metileno 10%, por 10 minutos. Com 24 hpf, os embriões foram tratados com diferentes concentrações de etanol: 0,1%; 0,25%; 0,5% e 1%. Após duas horas de tratamento, os embriões foram removidos e lavados duas vezes em água do sistema de recirculação, onde permanecem os aquários moradia. Os animais foram mantidos na incubadora B.O.D. (Bio Oxygen-Demand) a 28 °C e alimentados com *Paramecium sp.* até aproximadamente duas semanas de vida, onde passam a receber náuplios de *Artemia sp.* uma vez ao dia. Com um mês de vida, os alevinos foram transferidos para aquários de 3,5 litros de capacidade (20 animais por tanque) em um sistema de recirculação automática (Zebtec, Tecniplast, Italy). Os animais em crescimento foram alimentados três vezes ao dia com ração flocada comercial (Alcon BASIC, Alcon, Brazil) e náuplios de *Artemia s.* *Zebrafish* adultos (4 meses de idade; peso 300-400 mg; 50%:50% proporção de machos e fêmeas) foram usados para os testes realizados.

**Eutanásia dos animais para testes neuroquímicos:** *Zebrafish* adultos, em uma proporção de machos e fêmeas de 50%:50%, foram anestesiados em água gelada, com posterior dissecação total do cérebro, que foi acondicionado e processado de acordo com cada protocolo realizado (Anexos I e II).

### **11.3. ANEXO I: Avaliação da atividade da Monoamina Oxidase no modelo de FASD**

#### **INTRODUÇÃO**

Estudos recentes mostram que a maioria dos neurônios se comunica com mais de um neurotransmissor (Hnasko and Edwards, 2012) e que essa cotransmissão é crucial para neurônios que usam monoaminas como dopamina ou noradrenalina como sua fonte primária (Chuhma et al., 2009). Esse dualismo na transmissão sináptica parece ser um mecanismo conservado em neurônios dopaminérgicos e constatado também em estudos com *zebrafish*. Em Filippi et al., 2014, foi observado que a coexpressão de GABA ou glutamato como um segundo neurotransmissor é característica fundamental de neurônios catecolaminérgicos de *zebrafish*. Portanto, existe uma clara ligação entre os diferentes neurotransmissores ao longo dos processos de transmissão sináptica e as diferenças observadas em estudos glutamatérgicos podem ter influência de outros sistemas, como o dopaminérgico ou serotoninérgico. Uma série de estudos tem constatado que neurônios que liberam monoaminas neuromodulatórias, possuem a capacidade de liberar também o neurotransmissor excitatório glutamato (Johnson, 1994; Chuhma et al., 2009).

A enzima Monoamina oxidase (MAO), que degrada uma série de neurotransmissores tem sido implicada no alcoolismo, além de ser diferentemente expressa em resposta ao tratamento crônico com etanol em *zebrafish* (Pan et al., 2012). Através da atividade enzimática da MAO, pode-se checar se há alguma diferença entre os animais expostos na fase embrionária ao etanol e seus controles, quanta a oxidação de monoaminas, que agem como importantes neurotransmissores (Anichtchik et al., 2006).

#### **OBJETIVO**

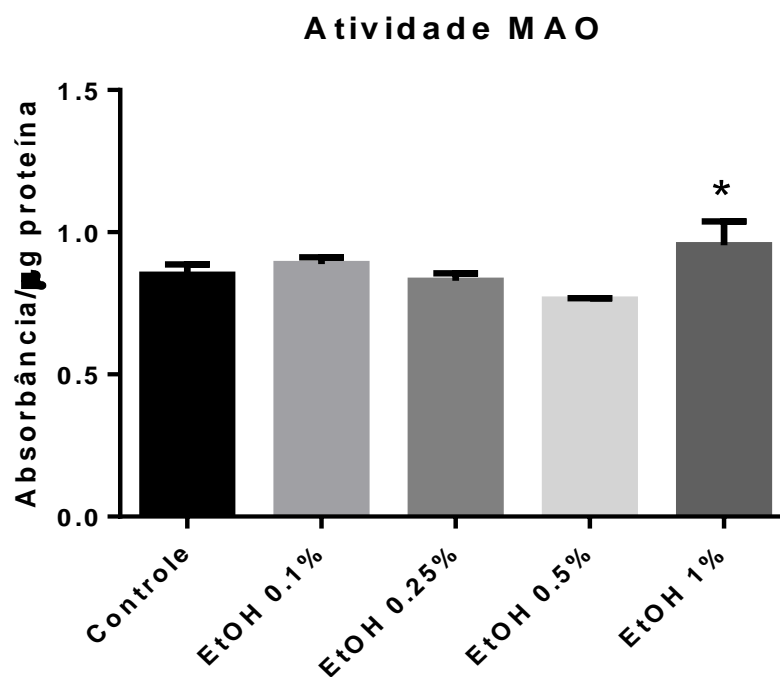
Quantificar a atividade enzimática da Monoamina Oxidase em homogeneizado de cérebro total de *zebrafish* adulto, exposto na fase embrionária ao etanol.

#### **MATERIAL E MÉTODOS**

**Atividade enzimática da Monoamina Oxidase:** Para analisar a oxidação de monoaminas por meio da atividade enzimática da Monoamina Oxidase, utilizou-se cérebros totais homogeneizados e incubados com o substrato tiramina para uma leitura cinética de duas horas, de acordo com Anichtchik et al., 2006.



## RESULTADOS



**Figura 1:** Atividade enzimática da Monoamina oxidase em homogeneizado de cérebro total de *zebrafish* adulto exposto ao etanol na fase embrionária. Dados expressos por média  $\pm$  S.E.M e analisados pela ANOVA de uma via, seguido pelo pós-teste de Tukey, considerando  $p \leq 0,05$  como significativo. N= 6 \* Diferença significativa entre o grupo controle e os grupos tratados com etanol.

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#### 11.4. ANEXO II: Avaliação da atividade enzimática da ChAT e AChE em modelo de FASD

##### INTRODUÇÃO

O sistema colinérgico desempenha funções essenciais no SNC, como desenvolvimento de estruturas cerebrais (Zirger et al., 2003), processos de aprendizagem e memória (Antyborzec et al., 2016; Barker and Warburton, 2009) e funções sensoriais (Giovannini et al., 2015) através do controle de fluxo sanguíneo cerebral (Anglade and Larabi-Godinot, 2010). A síntese de acetilcolina (ACh) ocorre nos terminais nervosos, a partir de dois precursores, colina e acetil-coenzima A. A colina acetil-transferase (ChAT) catalisa a síntese de acetilcolina, que pode interagir com receptores pré e pós-sinápticos. A acetilcolinesterase (AChE), localizada na fenda sináptica, hidroliza a acetilcolina em colina e acetato (Zirger et al., 2003). O etanol é capaz de afetar o sistema colinérgico, levando a perda de neurônios em várias regiões cerebrais, além da redução no conteúdo de acetilcolina e diminuição das atividades das enzimas ChAT e AChE (Arendt et al., 1988). A atividades destas enzimas é utilizada como marcador específico da funcionalidade dos neurônios colinérgicos, portanto analisa-se tal resultado em estudos de doenças relacionadas a prejuízos cognitivos e exposição ao etanol, como é o caso da FASD, onde a suplementação de colina é capaz de reverter os danos cognitivos observados (Kumar et al., 2016; Wozniak et al., 2015).

##### OBJETIVO

Quantificar a atividade das enzimas acetilcolinestaserase (AChE) e acetiltransferase (ChAT) em homogeneizado de cérebro total de *zebrafish* adulto, exposto na fase embrionária ao etanol.

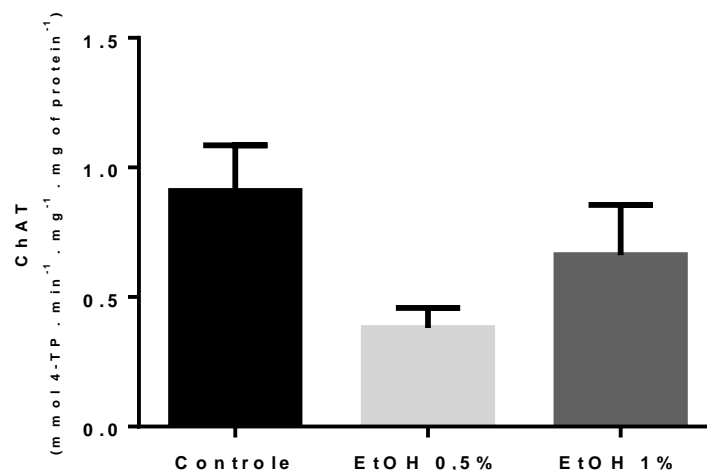
##### MATERIAL E MÉTODOS

**Atividade da ChAT:** A atividade da enzima ChAT foi determinada conforme Chao and Wolfgram (1973) em cérebro total de *zebrafish* adulto. As amostras foram incubadas no meio de reação contendo tampão fosfato de sódio 0,5 M (pH 7,2), 6,2 mM de acetil-CoA, 1 M de cloreto de colina, 0,76 mM de sulfato de neostigmina 0,76 mM, 3 M de cloreto de sódio e 1,1 mM de ácido etilenodiaminotetra-acético (EDTA). Após, foi adicionado 1 mM de 4,4'-ditiodipiridina (4-PDS) e a absorvância foi lida a 324 nm por 20 minutos em um leitor de microplaca SpectraMax® (Molecular Devices® Califórnia,USA). A atividade foi medida pela formação do conjugado 4-tiopiridona (4-TP), produto resultante da ligação do CoA com o 4-PDS. Os resultados foram calculados

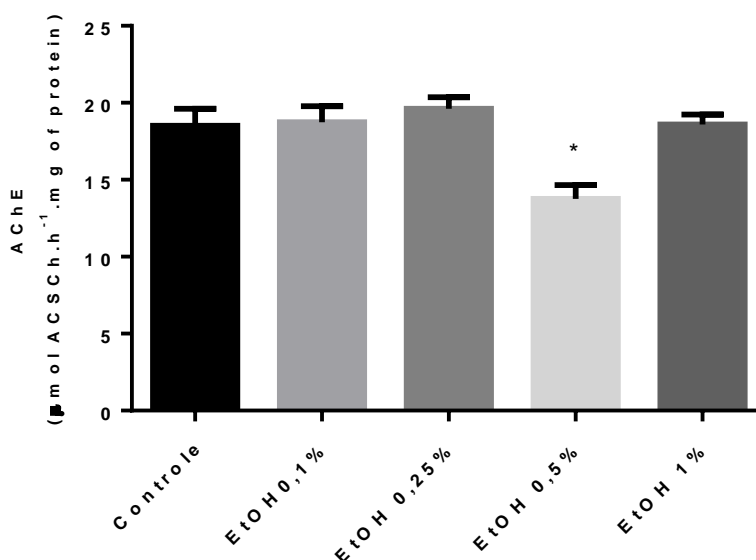
utilizando-se o coeficiente de extinção molar do 4-TP,  $1,98 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ , e estão expressos em  $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}$  de proteína<sup>-1</sup>. A proteína foi quantificada pelo método de Bradford (1976).

**Atividade da AChE:** A análise da atividade da enzima AChE foi realizada em cérebro total de *zebrafish* adulto de acordo com o método descrito por Ellman et al. (1961). A mistura de reação (volume final 2 mL) continha tampão fosfato de potássio 150 mM (pH 7,5) e ácido 5,5'-ditio-bis-(2-nitrobenzóico) (DTNB) 10 mM. Posteriormente a enzima (10  $\mu\text{g}$  de proteína) foi pré-incubada durante 3 minutos. A reação foi iniciada pela adição de 8 mM de iodeto de acetiltiocolina (AcSCh). Todas as amostras foram testadas em duplicata e a atividade enzimática foi expressa em  $\mu\text{mol}$  de AcSCh. $\text{min}^{-1}\cdot\text{mg}$  de proteína<sup>-1</sup>.

## RESULTADOS



**Figura 1: Atividade enzimática da ChAT em homogeneizado de cérebro total de zebrafish adulto exposto ao etanol na fase embrionária.** Dados expressos por média  $\pm$  S.E.M e analisados pela ANOVA de uma via, seguido pelo pós-teste de Tukey, considerando  $p \leq 0,05$  como significativo. N= 5.



**Figura 2: Atividade enzimática da AChE em homogeneizado de cérebro total de zebrafish adulto exposto ao etanol na fase embrionária.** Dados expressos por média  $\pm$  S.E.M e analisados pela ANOVA de uma via, seguido pelo pós-teste de Tukey, considerando  $p \leq 0,05$  como significativo. N= 5 \* Diferença significativa entre o grupo controle e os grupos tratados com etanol.

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### **11.5 ANEXO III: Guia para os autores – Neuroscience Letters**

Guia de orientação dos autores para submissão do manuscrito do Capítulo III, na revista Neuroscience Letters.





# NEUROSCIENCE LETTERS

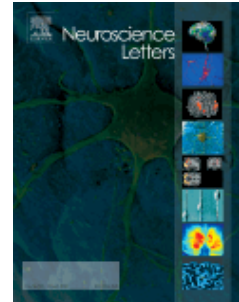
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*Neuroscience Letters* is devoted to the rapid publication of short, high-quality papers of interest to the broad community of neuroscientists. Papers in all areas of neuroscience - molecular, cellular, developmental, systems, behavioral, cognitive, and computational - will be considered for publication. Submission of laboratory investigations that shed light on disease mechanisms is encouraged.

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[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, *J. Sci. Commun.* 163 (2010) 51–59. <https://doi.org/10.1016/j.Sc.2010.00372>.

Reference to a journal publication with an article number:

[2] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, 2018. The art of writing a scientific article. *Heliyon.* 19, e00205. <https://doi.org/10.1016/j.heliyon.2018.e00205>.

Reference to a book:

[3] W. Strunk Jr., E.B. White, *The Elements of Style*, fourth ed., Longman, New York, 2000.

Reference to a chapter in an edited book:

[4] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), *Introduction to the Electronic Age*, E-Publishing Inc., New York, 2009, pp. 281–304.

Reference to a website:

[5] Cancer Research UK, Cancer statistics reports for the UK. <http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/>, 2003 (accessed 13 March 2003).

Reference to a dataset:

[dataset] [6] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, *Mendeley Data*, v1, 2015. <https://doi.org/10.17632/xwj98nb39r.1>.

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**11.6 ANEXO IV: Carta de aceite do CEUA**



## CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 31675

Título:

AVALIAÇÃO DOS MECANISMOS NEUROQUÍMICOS RESPONSÁVEIS PELAS ALTERAÇÕES NO PERFIL COMPORTAMENTAL DE PEIXE-ZEBRA ADULTO EXPOSTO A DIFERENTES CONCENTRAÇÕES DE ETANOL NA FASE INICIAL DO DESENVOLVIMENTO: ENF

Vigência: 01/09/2016 à 01/03/2020

Pesquisadores:

Equipe UFRGS:

DIOGO LOSCH DE OLIVEIRA - coordenador desde 01/09/2016  
Ben Hur Marins Mussulini - Aluno de Doutorado desde 01/09/2016  
SUELEN BAGGIO - Aluno de Doutorado desde 01/09/2016  
EMERSON SANTOS DA SILVA - Aluno de Mestrado desde 01/09/2016  
THAINÁ GARBINO DOS SANTOS - Aluno de Mestrado desde 01/09/2016

Equipe Externa:

Eduardo Pacheco Rico - pesquisador desde 01/09/2016

**Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 03/10/2016 - NA SALA 330 DO ANEXO I - PRÉDIO DA REITORIA DA UFRGS/CAMPUS CENTRO, em seus aspectos éticos e metodológicos, para a utilização de 30 peixes-zebra (*Danio rerio*) wild-type, a partir da sexta geração, sendo 20 fêmeas e 10 machos, provenientes do Biotério de peixe-zebra do Departamento de Bioquímica, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.**

Porto Alegre, Sexta-Feira, 14 de Outubro de 2016.

ALEXANDRE TAVARES DUARTE DE OLIVEIRA  
Vice Coordenador da comissão de ética