UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Suelen Baggio

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

Porto Alegre
2020

CIP - Catalogação na Publicação

Baggio, Suelen
EXPOSIÇÃO EMBRIONÁRIA AO ETANOL INDUZ ALTERAÇÕES COMPORTAMENTAIS E NEUROQUÍMICAS EM ZEBRAFISH ADULTO / Suelen Baggio. -- 2020.
126 f.
Orientador: Diogo Losch de Oliveira.

Coorientador: Eduardo Pacheco Rico.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2020.

1. Síndrome Alcoólica Fetal. 2. Neurodesenvolvimento. 3. Comportamento. 4. Sistema Glutamatérgico. I. de Oliveira, Diogo Losch, orient. II. Rico, Eduardo Pacheco, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Suelen Baggio

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

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.

Porto Alegre
2020

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)

AGRADECIMENTOS

Agradeço primeiramente aos meus pais, que me permitiram escolher meu próprio caminho, que me deixaram seguir minhas próprias escolhas e que sempre estiveram por perto, mesmo de longe, para me amparar e me apoiar sempre que preciso. Ao meu irmão, companheiro de vida, que sempre me apoiou e partilhou momentos de leveza em meio as dificuldades desta caminhada acadêmica.

Ao Ben Hur, meu namorado, meu confidente e meu parceiro de pesquisa e vida. Aquele que sempre me motivou a não desistir, que muito me ensinou, mesmo que a duras penas. Obrigada por tudo!

Meu muito obrigada a pessoas que fizeram dessa jornada algo além dos artigos e experimentos, que trouxeram outros ares à vida de doutoranda: Gio, Fabi, Pati, Jô, Bruna, Jordana, Moni e outros tantos...

Aos colegas de laboratório, a todos com quem convivi ao longo destes 10 anos no Departamento de Bioquímica e aos alunos do Lab 24 F, que dividem o laboratório com muita animação. Às "sobreviventes" do Lab 24 D, que muito me ajudaram, tanto nos experimentos, como nas tarefas de organização e manutenção e nas conversas, risadas e parcerias: Kami, Thai, Gi e Re. A todos que contribuíram ao longo destes anos de experimentos, frustrações e vitórias: colegas de outros laboratórios, pessoal da secretaria, da portaria, da limpeza, das interações, dos congressos e dicas de protocolos. Ao professor Renato, por ser uma inspiração e fonte de sabedoria a todos.

Ao meu co-orientador Eduardo Rico, que desde o mestrado me guia no caminho da pesquisa de zebrafish e etanol. Foste muito importante nesta trajetória toda, obrigada por todo apoio e ensinamentos.

Ao meu orientador Diogo Losch, muito obrigada por me ajudar ao longo destes anos de vida acadêmica, pelas correções precisas e as dicas que ajudaram a conectar as ideias soltas em minha mente confusa. Obrigada por propiciar as condições necessárias para dar continuidade ao meu projeto ao longo do doutorado.

Obrigada ao Departamento de Bioquímica, ao PPG Bioquímica e a UFRGS, pelo ensino de qualidade, pela estrutura de trabalho e pelos aprendizados que mudaram (para melhor) minha concepção do mundo!

E, por fim, muito obrigada aos órgãos de fomento que tornaram este trabalho viável: Capes (obrigada pela bolsa), CNPq, Fapergs e tantos outros.

Que a ciência prevaleça!

SUMÁRIO

RESUMO **2**

ABSTRACT **3**

LISTA DE ABREVIATURAS **4**

1. INTRODUÇÃO **5**

1.1. DESORDENS DO ESPECTRO ALCOÓLICO FETAL 5

1.2. *ZEBRAFISH* COMO MODELO DE FASD 7

1.3. ALTERAÇÕES COMPORTAMENTAIS E NEUROQUÍMICAS OBSERVADAS NA FASD 8

1.4. SISTEMA GLUTAMATÉRGICO 12

1.5. MODULAÇÕES FARMACOLÓGICAS 14

1.5.1. Buspirona *15*

1.5.2. Ceftriaxona *15*

1.6. TÁLAMO E FASD 16

2. OBJETIVOS **18**

2.1. OBJETIVO GERAL 18

2.2. OBJETIVOS ESPECÍFICOS 18

3. CAPÍTULO I **20**

4. CAPÍTULO II **29**

5. CAPÍTULO III **39**

6. CAPÍTULO IV **58**

7. DISCUSSÃO **69**

8. CONCLUSÃO **80**

9. PERSPECTIVAS **81**

10. REFERÊNCIAS GERAIS **82**

11. ANEXOS **96**

11.1. INTRODUÇÃO GERAL 96

11.2. MATERIAL E MÉTODOS GERAL 96

11.3. ANEXO I: AVALIAÇÃO DA ATIVIDADE DA MONOAMINA OXIDASE NO MODELO DE FASD 97

11.4. ANEXO II: AVALIAÇÃO DA ATIVIDADE ENZIMÁTICA DA CHAT E ACHE EM MODELO DE FASD 100

11.5 ANEXO III: GUIA PARA OS AUTORES – NEUROSCIENCE LETTERS 105

11.6 ANEXO IV: CARTA DE ACEITE DO CEUA 119

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 Na^+/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 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 Na^+/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, Na^+/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 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 Na^+/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 impairs 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^+/K^+ 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

GluaA 2: Subunidade receptor de glutamato A2

MRI: Magnetic ressonance 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 teleósteo 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 ºC 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, incialmente 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 hipocampais 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: α-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 Na^{+}/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 Na^{+}/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. Buspirona

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 Buspirona é um ansiolítico não-sedativo bem estabelecido para tratamentos de desordens generalizadas 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 buspirona pode ajudar na redução do comportamento ansioso em alcoolistas com desordens de ansiedade (Lovinger et al., 1989) e possivelmente em pacientes de FASD. Em estudos prévios com *zebrafish*, a buspirona 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 desordens neurológicas. O antibiótico β-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 estruturais 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

Suelen Baggio, Ben Hur Mussulini, Diogo Losch de Oliveira, Robert Gerlai, Eduardo Pacheco Rico.

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.

Behavioural Brain Research 352 (2018) 62–69

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

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

Suelen Baggio[a], Ben Hur Mussulini[a], Diogo Losch de Oliveira[a], Robert Gerlai[c,*], Eduardo Pacheco Rico[b,*]

[a] Programa de Pós-graduação em Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2600-Anexo, 90035-003 Porto Alegre, RS, Brazil
[b] Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense – UNESC, Av. Universitária, 1105, Bairro Universitário, 88806-000 Criciúma, SC, Brazil
[c] Department of Psychology University of Toronto Mississauga 3359 Mississauga Road North, Mississauga, Ontario, L5L 1C6 Canada

ARTICLE INFO

Keywords:
Alcohol
Anxiety
Social preference
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 fisorder; FAS, fetal alcohol syndrome; hpf, hours post-fertilization; EtOH, ethanol

* Corresponding author at: Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense – UNESC, Av. Universitária, 1105, Bairro Universitário, 88806-000 Criciúma, SC, Brazil.
E-mail address: eduprico@gmail.com (E.P. Rico).

http://dx.doi.org/10.1016/j.bbr.2017.08.039
Received 14 June 2017; Received in revised form 26 August 2017; Accepted 28 August 2017
Available online 04 September 2017
0166-4328/

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 cm ± 0.3 in standard length and weighing 0.550 g ± 0.050) were housed in a re-circulating system equipped with mechanical and biological filtration at a temperature of 28 °C, pH of 7.4 and a conductivity of 500 μS (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/ 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 °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 cm × 627.94 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

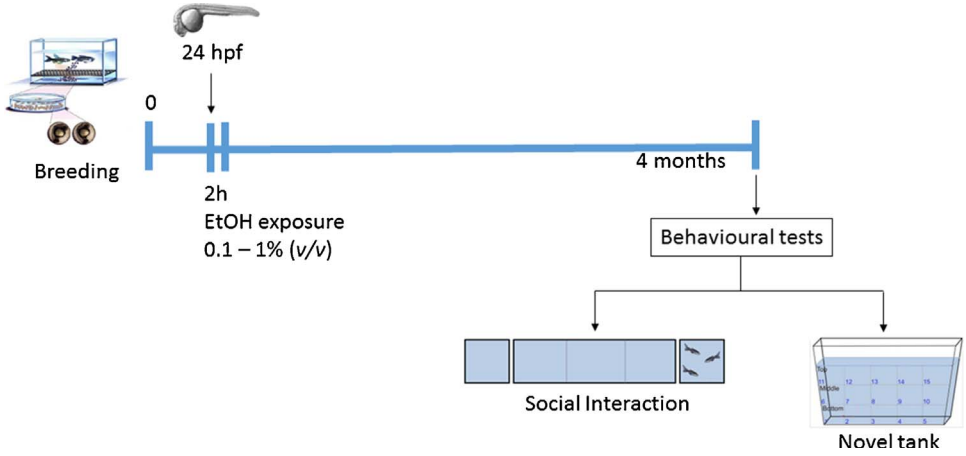


Fig. 1. Experimental design of the methodological time line approach used for the evaluation of the behavior of adult zebrafish FASD model. The experimental protocol consisted of one exposure of embryos 24 hpf during 2 h in ethanol concentrations: 0.00%, 0.01%, 0.25%, 0.50%, or 1.00% (v/v). After 4 months, 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.

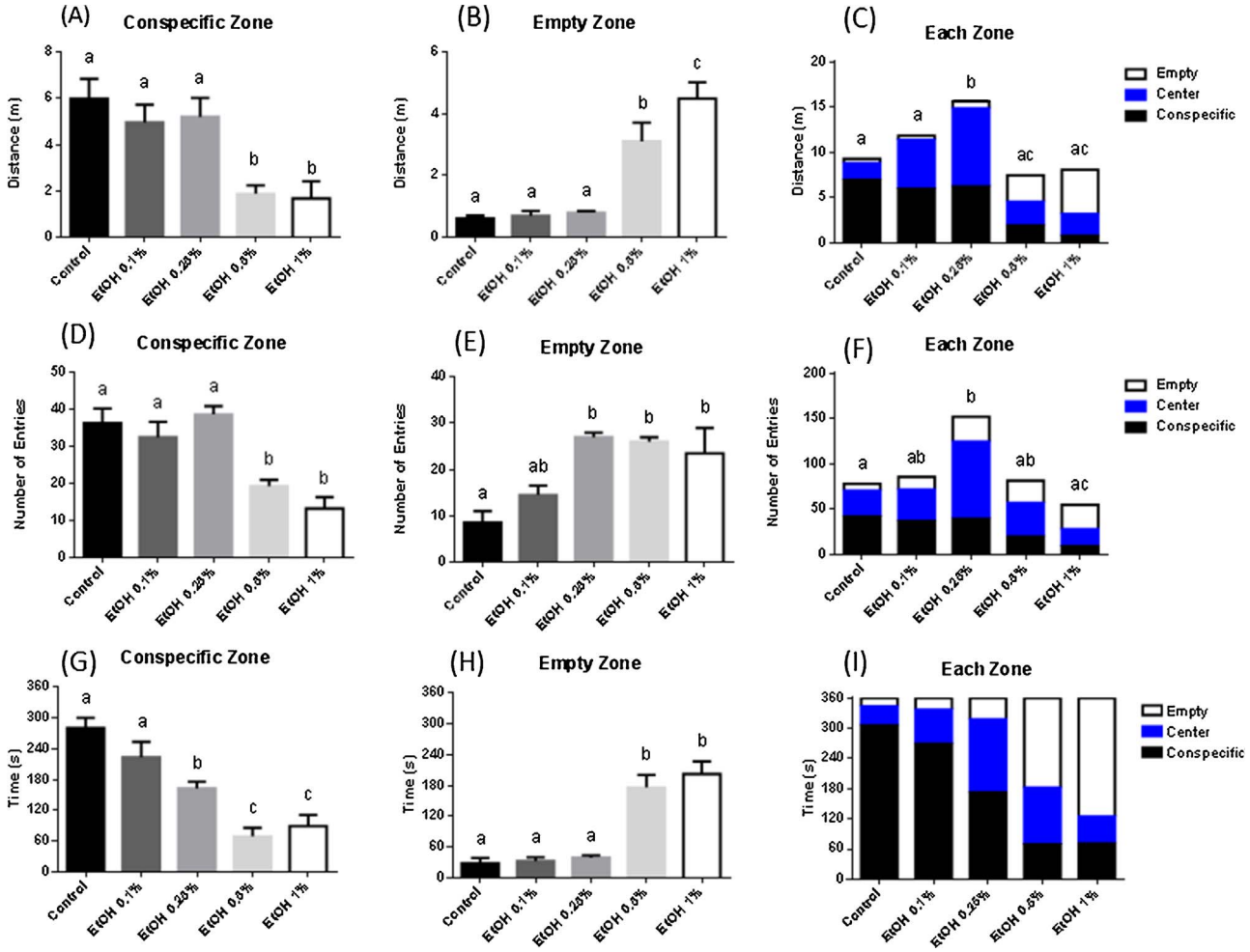


Fig. 2. Behavioral profile of social interaction in adult zebrafish FASD model. The figure shows the distance traveled in each zone: conspecific (A), center (B), empty (C); the number of entries: conspecific (D), center (E), empty (F); and the time spend in each zone: conspecific (G), center (H), empty (I). The data were analyzed by one-way ANOVA followed by Tukey's post hoc test and Kruskal-Wallis test followed by Dunn's multicomparision 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 μL/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 heigh). 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 ± 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 multicomparision 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) (Kruskall-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; Kruskall-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

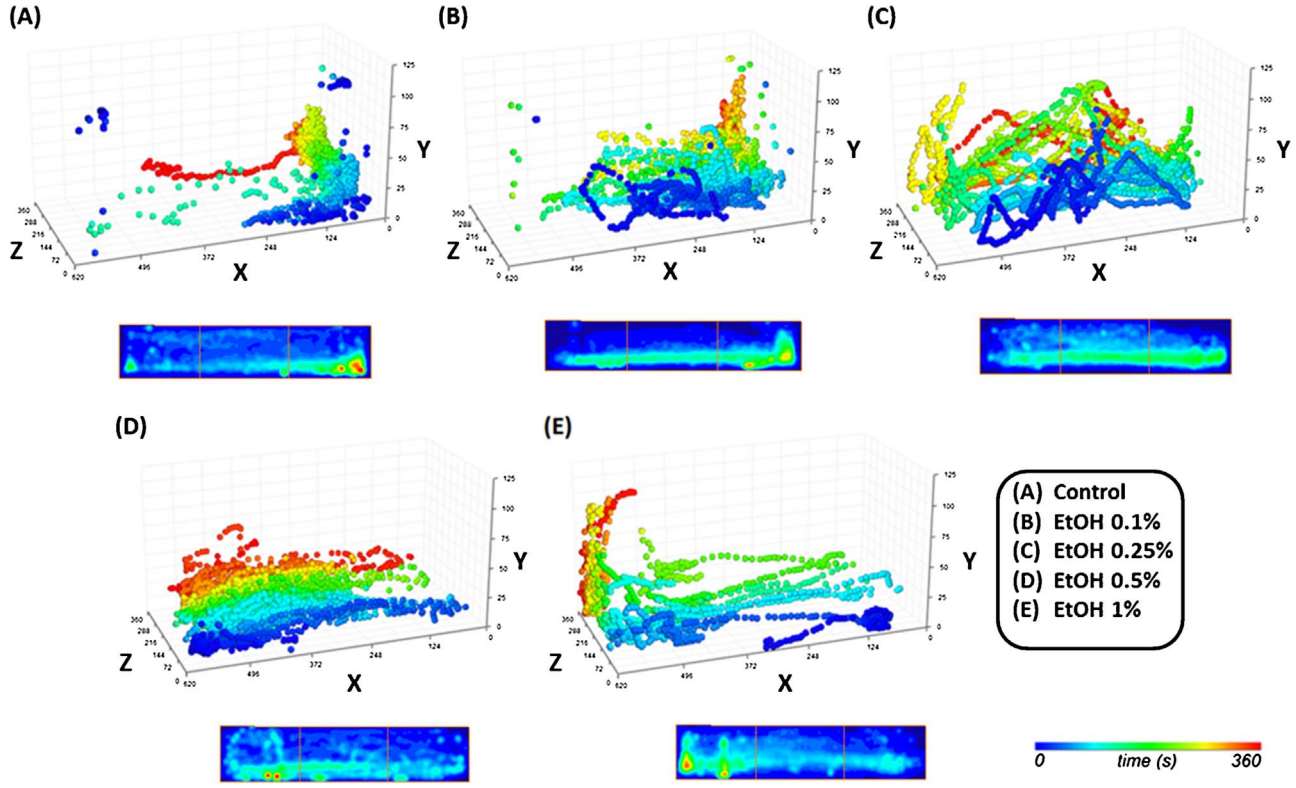


Fig. 3. Comparison of the spatio-temporal behavior of the experimental groups in the social interaction apparatus.
Representative spatio-temporal reconstructions of zebrafish swimming activity during the 6 min of the test were obtained by plotting animal traces (X-axis and Y-axis) over time (Z-axis). 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 apparati [18,26]. The novel tank task is often employed, as it represents an 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

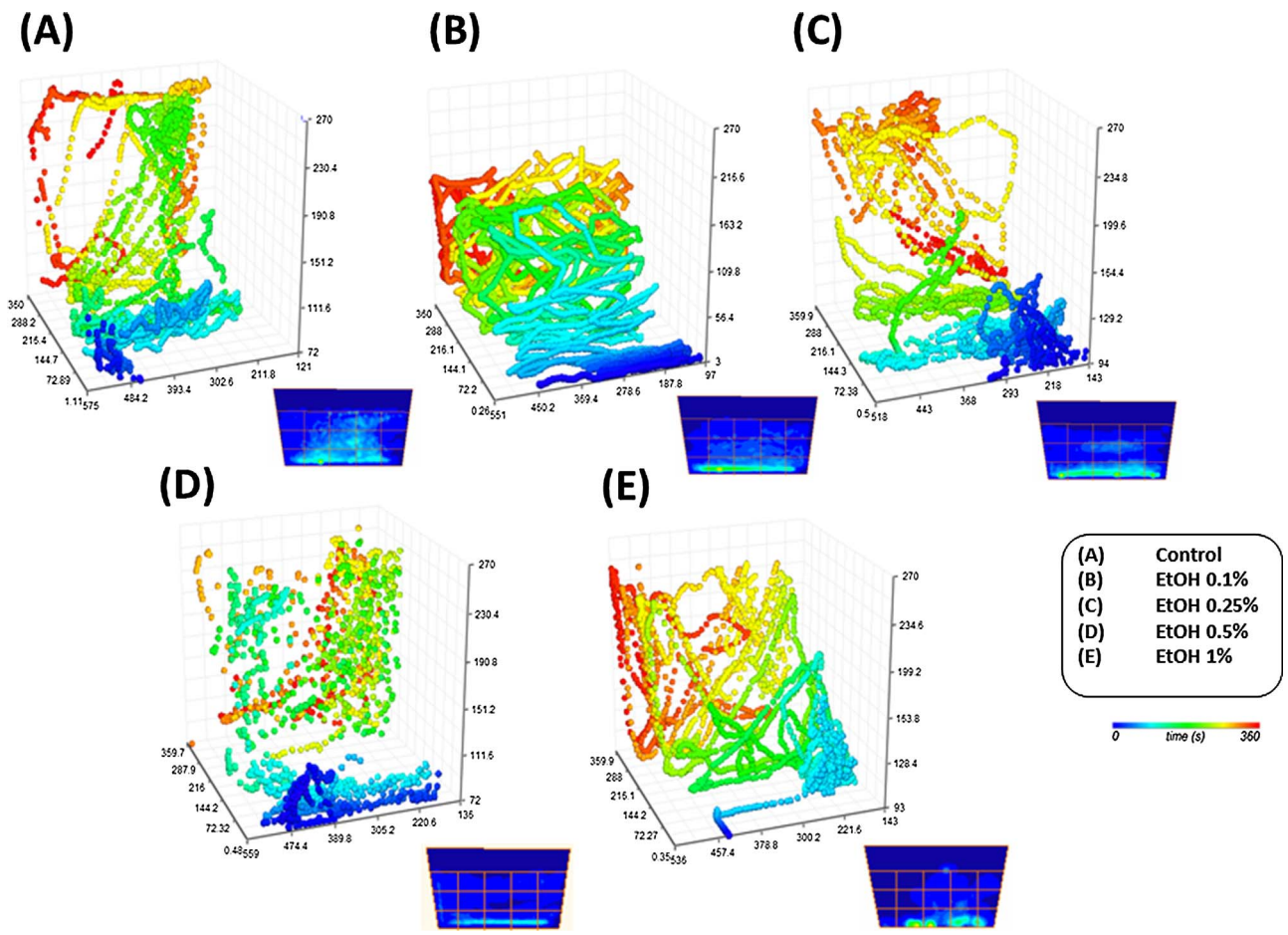


Fig. 4. Occupancy plot of the adult zebrafish FASD model in Novel tank test. Occupancy plot of the control (A), EtOH 0.1% (B), EtOH 0,25% (C), EtOH 0,5% (D) and EtOH 1% (E), displaying specific patterns of time spent in each zone of the apparatus during a 6-min trial. The data were analyzed using video-tracking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

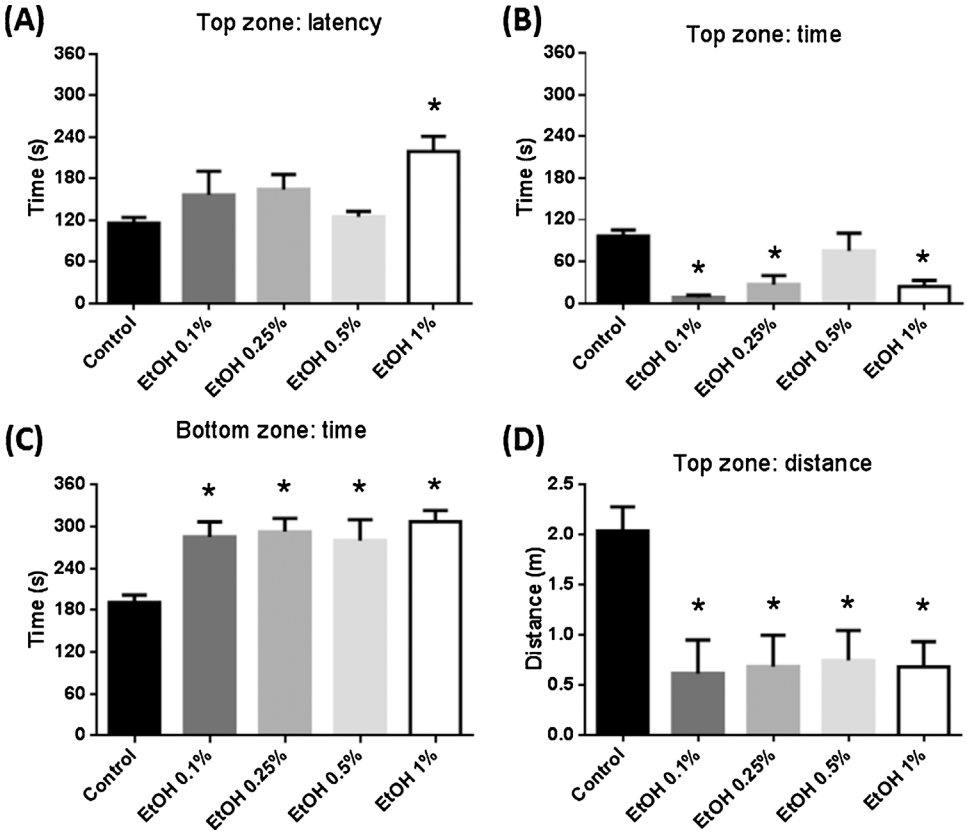


Fig. 5. Classical endpoint analysis for the assessment of anxiolytic-like behavior. The graph shows latency to the top (A), time spent at the top (B), time spent at the top (C) as well as distance travelled at the top area (D) of the control (A), EtOH 0.1% (B), EtOH 0,25% (C), EtOH 0,5% (D) and EtOH 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 (*).

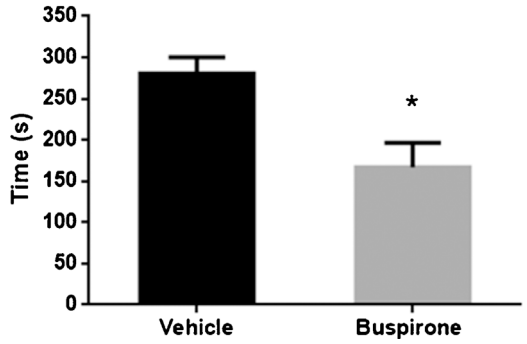


Fig. 6. Buspirone, an anxiolytic drug (positive control) reduces the time spent near the social stimulus. Saline (0.9%) injected (black bar) and buspirone injected (grey bar) fish are compared. The time fish spent in conspecific zone (mean ± S.E.M. are shown). The drug or saline was injected intraperitoneally at a volume of 1 μL/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 and important conclusion: exposure to even small amounts of alcohol and only for a short period of time during 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.

Acknowledgements

The authors thanks technician Diego Del Duca Lima for maintenance of the animals. We are grateful for the financial support provided by, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Financiadora de Estados e Projetos (FINEP). The funders had no role in the study desing, data collection of analysis, decision publish, or preparation manuscript. D.L.O is member of the Zebrafish Neuroscience Research Consortium (ZNRC).

References

[1] P.A. May, J.P. Gossage, W.O. Kalberg, L.K. Robinson, D. Buckley, M. Manning, H.E. Hoyme, Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies, Dev. Disabil. Res. Rev. 15 (2009) 176–192.

[2] P.D. Sampson, A.P. Streissguth, F.L. Bookstein, R.E. Little, S.K. Clarren, P. Dehaene, J.W. Hanson, J.M. Graham Jr., Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder, Teratology 56 (1997) 317–326.

[3] J.Y. Bradfield, J.R. West, S.E. Maier, Uptake and elimination of ethanol by young zebrafish embryos, Neurotoxicol. Teratol. 28 (2006) 629–633.

[4] Y. Fernandes, R.R. Gerlai, Long-term behavioral changes in response to early developmental exposure to ethanol in zebrafish, Alcohol.: Clin. Exp. Res. 33 (2009) 601–609.

[5] S. Mahabir, D. Chatterjee, R. Gerlai, Strain dependent neurochemical changes induced by embryonic alcohol exposure in zebrafish, Neurotoxicol. Teratol. 41

(2013) 1–7.
[6] F.J. Arenzana, M.J. Carvan, J. Aijon, R. Sanchez-Gonzalez, R. Arevalo, A. Porteros, Teratogenic effects of ethanol exposure on zebrafish visual system development, Neurotoxicol. Teratolol. 28 (2006) 342–348.
[7] J. Bilotta, J.A. Barnett, L. Hancock, S. Saszik, Ethanol exposure alters zebrafish development: a novel model of fetal alcohol syndrome, Neurotoxicol. Teratol. 26 (2004) 737–743.
[8] C. Buske, R. Gerlai, Early embryonic ethanol exposure impairs shoaling and the dopaminergic and serotoninergic systems in adult zebrafish, Neurotoxicol. Teratol. 33 (2011) 698–707.
[9] M.J. O'Connor, F. Frankel, B. Paley, A.M. Schonfeld, E. Carpenter, E.A. Laugeson, R. Marquardt, A controlled social skills training for children with fetal alcohol spectrum disorders, J. Consult. Clin. Psychol. 74 (2006) 639–648.
[10] C. Rasmussen, M. Becker, J. McLennan, L. Urichuk, G. Andrew, An evaluation of social skills in children with and without prenatal alcohol exposure, Child Care Health Dev. 37 (2011) 711–718.
[11] D.F. Burton, C. Zhang, O. Boa-Amponsem, S. Mackinnon, G.J. Cole, Long-term behavioral change as a result of acute ethanol exposure in zebrafish: evidence for a role for sonic hedgehog but not retinoic acid signaling, Neurotoxicol. Teratol. 61 (2017) 66–73.
[12] K.K. Caldwell, S. Sheema, R.D. Paz, S.L. Samudio-Ruiz, M.H. Laughlin, N.E. Spence, M.J. Roehlk, S.N. Alcon, A.M. Allan, Fetal alcohol spectrum disorder-associated depression: evidence for reductions in the levels of brain-derived neurotrophic factor in a mouse model, Pharmacol. Biochem. Behav. 90 (2008) 614–624.
[13] T.M. Roebuck, S.N. Mattson, E.P. Riley, Behavioral and psychosocial profiles of alcohol-exposed children, Alcohol. Clin. Exp. Res. 23 (1999) 1070–1076.
[14] A.P. Streissguth, J.M. Aase, S.K. Clarren, S.P. Randels, R.A. LaDue, D.F. Smith, Fetal alcohol syndrome in adolescents and adults, JAMA: J. Am. Med. Assoc. 265 (1991) 1961–1967.
[15] H.C. Steinhausen, J. Willms, C.W. Metzke, H.L. Spohr, Behavioural phenotype in foetal alcohol syndrome and fetal alcohol effects, Dev. Med. Child Neurol. 45 (3) (2003) 179–182.
[16] M. Bourin, Animal models for screening anxiolytic-like drugs: a perspective, Dialogues Clin. Neurosci. 17 (3) (2015) 295–303.
[17] R.E. Blaser, D.B. Rosemberg, Measures of anxiety in zebrafish (Danio rerio): dissociation of black/white preference and novel tank test, PLoS One 7 (2012) e36931.
[18] D.B. Rosemberg, E.P. Rico, B.H. Mussulini, A.L. Piato, M.E. Calcagnotto, C.D. Bonan, E.D. Dias, R.E. Blaser, D.O. Souza, D.L. de Oliveira, Differences in spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments, PLoS One 2 (6) (2011) e19397.
[19] A.M. Stewart, J. Cahcat, J. Green, S. Gaikwad, E. Kyzar, A. Roth, A. Davis, C. Collins, M. Ounsi, M. Pham, A.V. Kalueff, Constructing the habituome for phenotype-driven zebrafish research, Behav. Brain Res. 236 (2013) 110e117.
[20] Y. Fernandes, M. Rampersad, R. Gerlai, Embryonic alcohol exposure impairs the dopaminergic system and social behavioral responses in adult zebrafish, Int. J. Neuropsychopharmacol. 7 (2015) 18(6).
[21] Y. Fernandes, M. Rampersad, R. Gerlai, Impairment of social behaviour persists two years after embryonic alcohol exposure in zebrafish: a model of fetal alcohol spectrum disorders, Behav. Brain Res. 292 (2015) 102–108.
[22] C. Saverino, R. Gerlai, The social zebrafish: behavioral responses to conspecific, heterospecific, and computer animated fish, Behav. Brain Res. 191 (2008) 77–87.
[23] M. Pham, J. Raymond, J. Hester, E. Kyzar, S. Gaikwad, I. Bruce, C. Fryar, S. Chanin, J. Enriquez, S. Bagawandoss, I. Zapolsky, J. Green, A. Stewart, B.D. Robison, A.V. Kalueff, Assessing social behavior phenotypes in adult zebrafish: shoaling, social preference and mirror biting tests. Zebrafish Protocols for Neurobehavioral Research, 66 of the series, Neuromethods (2012) 231–246.
[24] K. Wong, M. Elegante, B. Bartels, S. Elkhayat, D. Tien, S. Roy, J. Goodspeed, C. Suciu, J.L. Tan, C. Grimes, A. Chung, M. Rosenberg, S. Gaikwad, A. Denmark, A. Jackson, F. Kadri, K.M. Chung, A. Stewart, T. Gilder, E. Beeson, I. Zapolsky, N.D. Wu, J. Cachat, A.V. Kalueff, Analyzing habituation responses to novelty in zebrafish (Danio rerio), Behav. Brain Res. 208 (2010) 450e457.
[25] R.J. Egan, C.L. Bergner, P.C. Hart, J.M. Cachat, P.R. Canavello, M.F. Elegante, S.I. Elkhayat, B.K. Bartels, A.K. Tien, D.H. Tien, S. Mohnot, E. Beeson, E. Glasgow, H. Amri, Z. Zukowska, A.V. Kalueff, Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish, Behav. Brain Res. 205 (2009) 38–44.
[26] E.D. Levin, Z. Bencan, D.T. Cerutti, Anxiolytic effects of nicotine in zebrafish, Physiol. Behav. 90 (2007) 54–58.
[27] P. Mathur, S. Guo, Differences of acute versus chronic ethanol exposure on anxiety-like behavioral responses in zebrafish, Behav. Brain Res. 219 (2011) 234–239.
[28] D.B. Rosemberg, M.M. Braga, E.P. Rico, C.M. Loss, S.D. Córdova, B.H. Mussulini, R.E. Blaser, C.E. Leite, M.M. Campos, R.D. Dias, M.E. Calcagnotto, D.L. de Oliveira, D.O. Souza, Behavioral effects of taurine pretreatment in zebrafish acutely exposed to ethanol, Neuropharmacology 63 (4) (2012) 613–623.
[29] M. Ibrahim, B.H. Mussulini, L. Moro, A.M. de Assis, D.B. Rosemberg, D.L. de Oliveira, J.B. Rocha, R.S. Schwab, P.H. Schneider, D.O. Souza, E.P. Rico, Anxiolytic effects of diphenyl diselenide on adult zebrafish in a novelty paradigm, Prog. Neuropsychopharmacol. Biol. Psychiatry. 54 (2014) 187–194.
[30] M. Baiamonte, M.O. Parker, G.P. Vinson, C.H. Brenann, Sustained effects of developmental exposure to ethanol on zebrafish anxiety-like behaviour, Plos One 10 (February (11)) (2016) e0148425.
[31] J. Bilotta, J.A. Barnett, L. Hancock, S. Saszik, Ethanol exposure alters zebrafish development: a novel model of fetal alcohol syndrome, Neurotoxicol. Teratol. 26 (2004) 737–743.
[32] Y. Fernandes, S. Tran, E. Abraham, R. Gerlai, Embryonic alcohol exposure impairs associative learning performance in adult zebrafish, Behav. Brain Res. 265 (2014) 181–187.
[33] M.O. Parker, L.V. Annan, A.H. Kanellopoulos, A.J. Brock, F.J. Combe, M. Baiamonte, M.T. Teh, C.H. Brennan, The utility of zebrafish to study the mechanisms by which ethanol affects social behavior and anxiety during early brain development, Prog. Neuropsychopharmacol. Biol. Psychiatry 3 (December (55)) (2014) 94–100.
[34] D. Seguin, S. Shams, R. Gerlai, Behavioural responses to novelty or to a predator stimulus are not altered in adult zebrafish by early embryonic alcohol exposure, Alcohol.: Clin. Exp. Res. 40 (2016) 2667–2675.
[35] R. Gerlai, S. Prajapati, F. Ahmad, Differences in acute alcohol induced behavioral responses among zebrafish populations, Alcohol.: Clin. Exp. Res. 32 (2008) 1763–1773.
[36] A.P. Streissguth, K. O'Malley, Neuropsychiatric implications and long-term consequences of fetal alcohol spectrum disorders, Semin. Clin. Psychiatry 5 (3) (2000) 177–190.
[37] S.J. Kelly, N. Day, A.P. Streissguth, Effects of prenatal alcohol exposure on social behavior in humans and other species, Neurotoxicol. Teratol. 22 (2) (2000) 143–149.
[38] C.I. Rodriguez, C.M. Magcalas, D. Barto, B.C. Fink, J.P. Rice, C.W. Bird, S. Davies, N.S. Pentkowski, D.D. Savage, D.A. Hamilton, Effects of sex and housing on social, spatial, and motor behavior in adult rats exposed to moderate levels of alcohol during prenatal development, Behav. Brain Res. 15 (October (313)) (2016) 233–243.
[39] M.R. Diaz, S.M. Mooney, E.I. Varlinskaya, Acute prenatal exposure to ethanol on gestational day 12 elicits opposing deficits in social behaviors and anxiety-like behaviors in Sprague Dawley rats, Behav. Brain Res. 310 (2016) 11–19.
[40] K. Marquardt, J.L. Brigman, The impact of prenatal alcohol exposure on social, cognitive and affective behavioral domains: insights from rodent models, Alcohol 51 (2016) 1–15.
[41] S.A. Stevens, J. Dudek, K. Nash, G. Koren, J. Rovet, Social perspective taking and empathy in children with fetal alcohol spectrum disorders, J. Int. Neuropsychol. Soc. 21 (2015) 74–84.
[42] C. Maximino, A.W.B. Silva, A. Gouveia Jr., A.M. Herculano, Pharmacological analysis of zebrafish (Danio rerio) scototaxis, Prog. Neuropsychopharmacol. Biol. Psychiatry 35 (2011) (624e631).
[43] N. Miller, R. Gerlai, Shoaling in zebrafish: what we don't know, Rev. Neurosci. 22 (2011) 17–25.
[44] R. Luca, R. Gerlai, In search of optimal fear inducing stimuli: differential behavioral responses to computer animated images in zebrafish, Behav. Brain Res. 226 (2012) 66–76.
[45] R. Gerlai, Zebrafish antipredatory responses: a future for translational research? Behav. Brain Res. 207 (2010) 223–231.

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.

Artigo publicado no periódico Neurotoxicology, 2020.

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.

Neurotoxicology 78 (2020) 152–160

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro

Full Length Article

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

Suelen Baggio[a,*], Kamila Zenki[a], Alberto Martins Silva[a], Thainá Garbino dos Santos[a], Giovana Rech[a], Gabriela Lazzarotto[a], Renato Dutra Dias[a], Ben Hur Mussulini[b,c], Eduardo Pacheco Rico[d], Diogo Losch de Oliveira[a]

[a] Laboratory of Cellular Neurochemistry, Programa De Pós-graduação Em Ciências Biológicas: Bioquímica, Departamento De Bioquímica, Instituto De Ciências Básicas Da Saúde, Universidade Federal Do Rio Grande Do Sul, Porto Alegre, RS, Brazil
[b] Centre of New Technologies, University of Warsaw, Banacha 2C, Warsaw 02-097, Poland
[c] ReMedy International Research Agenda Unit, University of Warsaw, Banacha 2C, Warsaw 02-097, Poland
[d] Programa De Pós-Graduação Em Ciências Da Saúde, Universidade Do Extremo Sul Catarinense - UNESC, Av. Universitária, 1105, Bairro Universitário, 88806-000 Criciúma, SC, Brazil

ARTICLE INFO

Keywords:
Alcohol
Glutamate
Glutamatergic neurotransmission
Zebrafish
FASD
Brain

ABSTRACT

Fetal alcohol spectrum disorders (FASD) describe a wide range of ethanol-induced developmental disabilities, including craniofacial dysmorphology, 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+/K + ATPase activity, and high-resolution respirometry. Embryonic ethanol exposure reduced 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 Na+/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 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 dysmorphology, 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; Facciol 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

* Corresponding author at: Departamento de Bioquímica, ICBS, UFRGS., Rua Ramiro Barcelos 2600 - Anexo., Zip code: 90035-003, Porto Alegre, RS, Brazil.
E-mail addresses: su.baggio02@gmail.com, suelen.baggio@ufrgs.br (S. Baggio).

https://doi.org/10.1016/j.neuro.2020.03.003
Received 5 December 2019; Received in revised form 6 March 2020; Accepted 6 March 2020
Available online 12 March 2020
0161-813X/

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 Na + /K + ATPase activity in zebrafish FASD model. The physical association between Na + /K + ATPase and glutamate transporters regulates the high affinity 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 Na + gradient generated by Na + /K + 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 VGLUTs 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 Na + /K + 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 pair-wise 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 μS/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 euthanatized 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 Na2HPO4; 4.17 NaHCO3; 5.36 KCl; 0.44 KH2PO4; 1.26 CaCl2; 0.41 MgSO4; 0.49 MgCl2 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-1 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-1. mg Prot-1. 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 euthanatized 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 hypoosmotic shock in the polypropylene tube by 1 mL of cold H2O. Then, 3 mL of cold H2O 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 H2O, 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 euthanatized 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+/K+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+/ K + ATPase activity was determined as previously reported Tsakiris and Deliconstantinos (1984). Sample was added to the reaction mixture containing 5 mM MgCl2, 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+/K + 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-1. mg protein-1.

2.7. Glutamine Synthetase activity

The GS enzymatic activity assay was performed as previously described by Petito 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 MgCl2, 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-1. mg prot-1.

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 MgCl2, 20 mM taurine, 10 mM KH2PO4, 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 antimicine 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 mean (S.E.M.). Glutamate uptake data were analyzed by two-way ANOVA followed by Tukey's post hoc test. Data of VGLUT2 immunocontent, glutamate binding, Na+/K + 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.

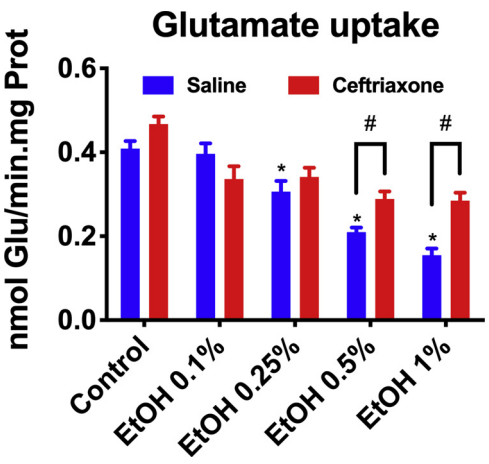


Fig. 1. Effect of ethanol exposure in zebrafish embryos on glutamate uptake of adult zebrafish brain treated or not with ceftriaxone. Data were expressed as mean ± S.E.M. and were analyzed by two-way ANOVA followed by Tukey's post hoc test (# = $p < 0.0001$, * = $p < 0.05$), n = 8 animals/group (a total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

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 immunocontent 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+/K + ATPase to generate ion

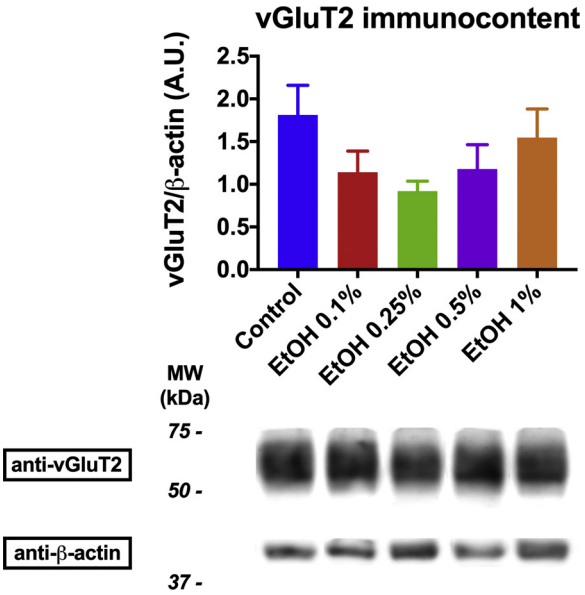


Fig. 2. VGLUT2 immunocontent of brain samples of adult zebrafish submitted to embryonic alcohol exposure. Data were expressed as mean ± S.E.M. of the ratio between VGLUT2 and β-actin band intensities, and were analyzed by one-way ANOVA followed by Tukey's post hoc test, n = 6 pools/group (2 zebrafish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

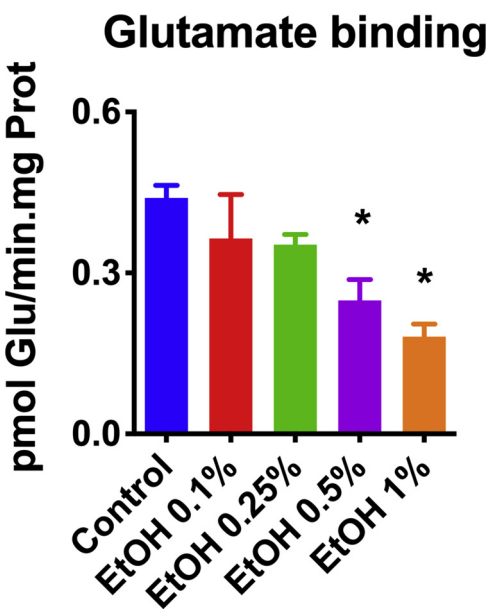


Fig. 3. Effect of ethanol exposure in zebrafish embryos on the plasma membrane glutamate binding in adult brain. Data were expressed as mean ± S.E.M. and were analyzed by one-way ANOVA followed by Tukey's post hoc test (* = $p < 0.05$ when compared to control group), n = 6 pools/group (10 zebrafish brains/pool;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

gradients that drive transmitter uptake. Thus, the Na+/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 Na+/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;

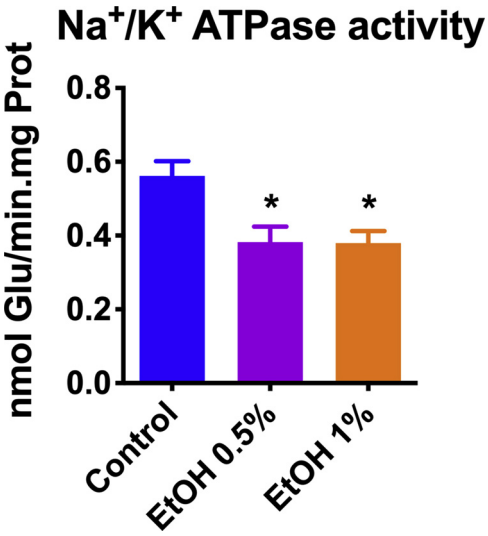


Fig. 4. Enzymatic activity of Na+/K + ATPase in adult zebrafish brains submitted to embryonic alcohol exposure. Data were expressed in mean ± S.E.M. and were analyzed by one-way ANOVA followed by Tukey's post hoc test (* = $p < 0.05$ when compared to control group), n = 10 pools/group (2 zebrafish brains/pool;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

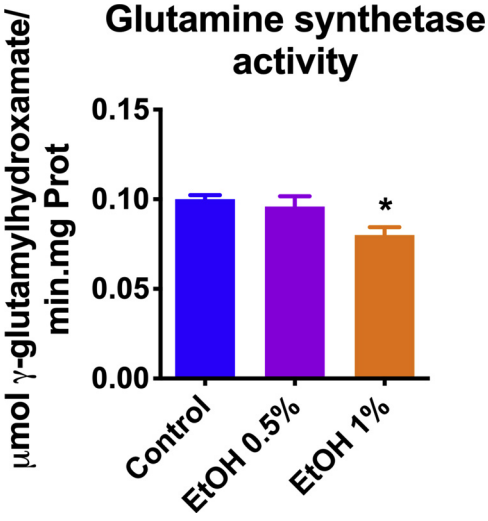


Fig. 5. Enzymatic activity of glutamine synthetase (GS) in adult zebrafish brains submitted to embryonic alcohol exposure. Data were expressed in mean ± S.E.M. and were analyzed by one-way ANOVA followed by Tukey's post hoc test (* = $p < 0.05$ when compared to control group), n = 10 pools/group (2 zebrafish 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 Na+/K + ATPase and GS enzymatic activities, mitochondria 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).

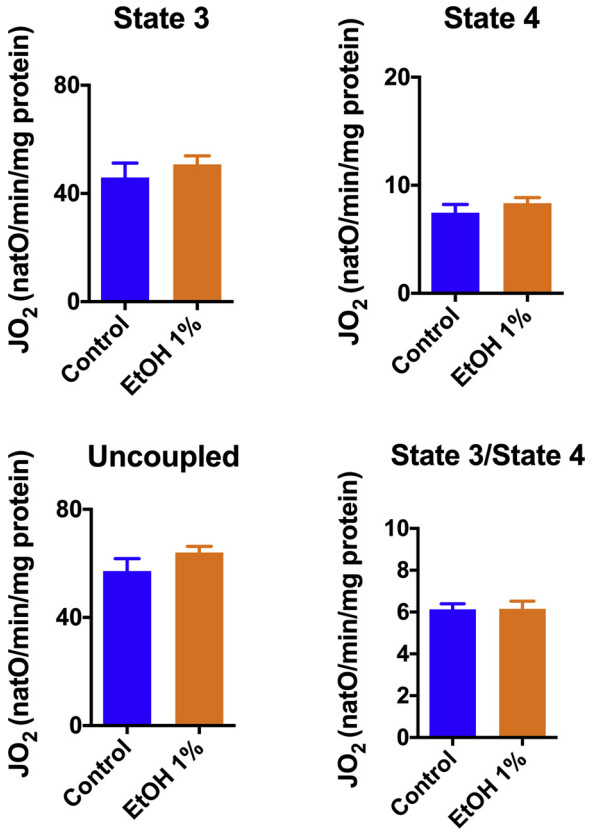


Fig. 6. Mitochondrial respiratory profile of adult zebrafish brains submitted to embryonic alcohol exposure. Oxygen consumption was assessed in the phosphorylating state (state 3) and in the non-phosphorylating state (state 4); maximum OxPHOS capacity was assessed by titration with the uncoupler FCCP; and the respiratory control ratio (RCR) was calculated as state3/state4. Data are expressed as mean ± 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+/K + 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+/K + 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 synapses could be compromised. EAATs were coupled with Na+/K + 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+/K + 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+/K + 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 immunocontent 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 immunocontent 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.

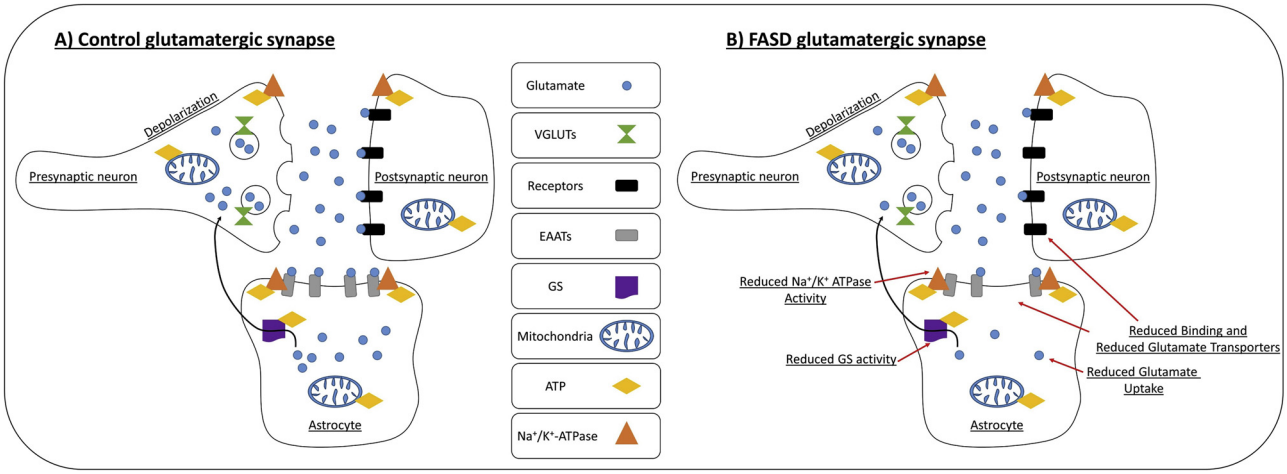


Fig. 7. A) Tripartite glutamatergic synapses. Presynaptic neurons release glutamate, which interacts with receptors in the postsynaptic neuron. Glutamate is mostly taken up by astrocyte excitatory amino acid transporters (EAATs) coupled to Na + /K + ATPase activity. Glutamate uptake is modulated by GS 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, Na + /K + ATPase activity, glutamine synthesis activity impairment, independent of alterations in the immunocontent 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 Na + / K + ATPase to generate ion gradients that drive transmitter uptake (Rose et al., 2009). It has been observed that a reduction in Na + / K + ATPase activity decreases glutamate uptake (Bauer et al., 2012; Illarionova et al., 2014). The modification of Na + /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 Na + /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 treatement (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 Na + /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 Na + /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, Fomal 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.

Acknowledegments

We are grateful for the financial support provided by Coordenação de Apeiçoamento de Pessoal de Nível Superior (CAPES PROEX 88882.345556/2019-01; AUXPE PROEX N. 23038.027251/2016-85), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Fundação de Amparo à Pesquisa do Estado de Santa Catarina (FAPESC), E.P.R. was recipient of fellowships from CNPq research grant (Universal 429302/2018-5), Brazil. Also, the work was funded by "Regenerative Mechanisms for Health" project MAB/2017/2, carried out within the International Research Agendas programme of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund.

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.

References

Baggio, S., Mussulini, B.H., de Oliveira, D.L., Zenki, K.C., Santos da Silva, E., Rico, E.P., 2017. Embryonic alcohol exposure promotes long-term effects on cerebral glutamate transport of adult zebrafish. Neurosci. Lett. 636, 265–269. https://doi.org/10.1016/j.neulet.2016.11.016.

Baggio, S., Mussulini, B.H., de Oliveira, D.L., Gerlai, R., Rico, E.P., 2018. Embryonic alcohol exposure leading to social avoidance and altered anxiety responses in adult zebrafish. Behav. Brain Res. 352, 62–69. https://doi.org/10.1016/j.bbr.2017.08.039.

Bauer, D.E., Jackson, J.G., Genda, E.N., Montoya, M.M., Yudkoff, M., Robinson, M.B., 2012. The glutamate transporter, GLAST, participates in a macromolecular complex that supports glutamate metabolism. Neurochem. Int. 61, 566–574. https://doi.org/10.1016/j.neuint.2012.01.013.

Bell, R.L., Hauser, S.R., McClintick, J., Rahman, S., Edenberg, H.J., Szumlinski, K.K., McBride, W.J., 2016. Ethanol-associated changes in glutamate reward neurocircuitry: a minireview of clinical and preclinical genetic findings. Progress in Molecular Biology and Translational Science. pp. 41–85. https://doi.org/10.1016/bs.pmbts.2015.10.018.

Bermejo, M.K., Milenkovic, M., Salahpour, A., Ramsey, A.J., 2014. Preparation of synaptic plasma membrane and postsynaptic density proteins using a discontinuous sucrose gradient. J. Vis. Exp. 91, 1–8. https://doi.org/10.3791/51896.

Bhattacharya, D., Majrashi, M., Ramesh, S., Govindarajulu, M., Bloemer, J., Fujihashi, A., Crump, B.R., Hightower, H., Bhattacharya, S., Moore, T., Suppiramaniam, V., Dhanasekaran, M., 2018. Assessment of the cerebellar neurotoxic effects of nicotine in prenatal alcohol exposure in rats. Life Sci. 194, 177–184. https://doi.org/10.1016/j.lfs.2017.12.010.

Bogdanova, A., Petrushanko, I.Y., Hernansanz-Agustín, P., Martínez-Ruiz, A., 2016. "Oxygen sensing" by Na,K-ATPase: these miraculous thiols. Front. Physiol. https://doi.org/10.3389/fphys.2016.00314.

Brady, M.L., Diaz, M.R., Iuso, A., Everett, J.C., Valenzuela, C.F., Caldwell, K.K., 2013. Moderate prenatal alcohol exposure reduces plasticity and alters NMDA receptor subunit composition in the dentate gyrus. J. Neurosci. 33, 1062–1067. https://doi.org/10.1523/JNEUROSCI.1217-12.2013.

Brolese, G., Lunardi, P., De Souza, D.F., Lopes, F.M., Leite, M.C., Gonçalves, C.A., 2015. Pre-and postnatal exposure to moderate levels of ethanol can have long-lasting effects on hippocampal glutamate uptake in adolescent offspring. PLoS One. https://doi.org/10.1371/journal.pone.0127845.

Bukiya, A.N., 2019. Fetal cerebral artery mitochondrion as target of prenatal alcohol exposure. Int. J. Environ. Res. Public Health 16, 1–16. https://doi.org/10.3390/ijerph16091586.

Cesconetto, P.A., Andrade, C.M., Cattani, D., Domingues, J.T., Parisotto, E.B., Filho, D.W., Zamoner, A., 2016. Maternal exposure to ethanol during pregnancy and lactation affects glutamatergic system and induces oxidative stress in offspring Hippocampus. Alcohol. Clin. Exp. Res. 40, 52–61. https://doi.org/10.1111/acer.12917.

Chan, K.M., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca2+-stimulated ATPase activity. Anal. Biochem. 157, 375–380. https://doi.org/10.1016/0003-2697(86)90640-8.

Chen, Q., Kirk, K., Shurubor, Y.I., Zhao, D., Arreguin, A.J., Shahi, I., Valsecchi, F., Primiano, G., Calder, E.L., Carelli, V., Denton, T.T., Beal, M.F., Gross, S.S., Manfredi, G., D'Aurelio, M., 2018. Rewiring of glutamine metabolism is a bioenergetic adaptation of human cells with mitochondrial DNA mutations. Cell Metab. https://doi.org/10.1016/j.cmet.2018.03.002.

Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. https://doi.org/10.1016/S0301-0082(00)00067-8.

Davies, D.L., Vernadakis, A., 1984. Effects of ethanol on cultured glial cells: proliferation and glutamine synthetase activity. Dev. Brain Res. 16, 27–35. https://doi.org/10.1016/0165-3806(84)90059-2.

Denny, L.A., Coles, S., Blitz, R., 2017. Fetal alcohol syndrome and fetal alcohol Spectrum disorders. Am. Fam. Physician 96, 515–522.

Devi, B.G., Henderson, G.I., Frosto, T.A., Schenker, S., 1994. Effect of acute ethanol exposure on cultured fetal rat hepatocytes: relation to mitochondrial function. Alcohol. Clin. Exp. Res. 18, 1436–1442. https://doi.org/10.1111/j.1530-0277.1994.tb01447.x.

Filippi, A., Mueller, T., Driever, W., 2014. vglut2 and gad expression reveal distinct patterns of dual GABAergic versus glutamatergic cotransmitter phenotypes of dopaminergic and noradrenergic neurons in the zebrafish brain. J. Comp. Neurol. 522, 2019–2037. https://doi.org/10.1002/cne.23524.

Gerace, E., Landucci, E., Totti, A., Bani, D., Guasti, D., Baronti, R., Moroni, F., Mannaioni, G., Pellegrini-Giampietro, D.E., 2016. Ethanol toxicity during brain development: alterations of excitatory synaptic transmission in immature organotypic hippocampal slice cultures. Alcohol. Clin. Exp. Res. 40, 706–716. https://doi.org/10.1111/acer.13006.

Gerlai, R., 2015. Embryonic alcohol exposure: towards the development of a zebrafish model of fetal alcohol spectrum disorders. Dev. Psychobiol. 57, 787–798. https://doi.org/10.1002/dev.21318.

Girault, V., Gilard, V., Marguet, F., Lesueur, C., Hauchecorne, M., Ramdani, Y., Laquerrière, A., Marret, S., Jégou, S., Gonzalez, B.J., Brasse-Lagnel, C., Bekri, S., 2017. Prenatal alcohol exposure impairs autophagy in neonatal brain cortical microvessels. Cell Death Dis. 8. https://doi.org/10.1038/cddis.2017.29.

Goodwani, S., Saternos, H., Alasmari, F., Sari, Y., 2017. Metabotropic and ionotropic glutamate receptors as potential targets for the treatment of alcohol use disorder. Neurosci. Biobehav. Rev. 77, 14–31. https://doi.org/10.1016/j.neubiorev.2017.02.024.

Hasler, G., Buchmann, A., Haynes, M., Müller, S.T., Ghisleni, C., Brechbühl, S., Tuura, R., 2019. Association between prefrontal glutamine levels and neuroticism determined using proton magnetic resonance spectroscopy. Transl. Psychiatry. https://doi.org/10.1038/s41398-019-0500-z.

Heitman, D.W., Frosto, T.A., Schenker, S., Henderson, G.I., 1987. Stimulatory effects of ethanol on amino acid transport by rat fetal hepatocytes. Hepatology 7, 307–314. https://doi.org/10.1002/hep.1840070216.

Hertz, L., Zielke, H.R., 2004. Astrocytic control of glutamatergic activity: astrocytes as stars of the show. Trends Neurosci. 27, 735–743. https://doi.org/10.1016/j.tins.2004.10.008.

Ikonomidou, C., Bittigau, P., Ishimaru, M.J., Wozniak, D.F., Koch, C., Genz, K., Price, M.T., Stefovska, V., Hörster, F., Tenkova, T., Dikranian, K., Olney, J.W., 2000. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 287, 1056–1060. https://doi.org/10.1126/science.287.5455.1056.

Illarionova, N.B., Illarionava, N.B., Brismar, H., Aperia, A., Gunnarson, E., 2014. Role of Na,K-ATPase α1 and α2 isoforms in the support of astrocyte glutamate uptake. PLoS One 9, e98469. https://doi.org/10.1371/journal.pone.0098469.

Kvamme, E., 1998. Synthesis of glutamate and its regulation. Prog. Brain Res. https://doi.org/10.1016/s0079-6123(08)60431-8.

Lange, S., Probst, C., Gmel, G., Rehm, J., Burd, L., Popova, S., 2017. Global prevalence of fetal alcohol spectrum disorder among children and youth: a systematic review and meta-analysis. JAMA Pediatr. 171, 948–956. https://doi.org/10.1001/jamapediatrics.2017.1919.

Ledig, M., Kopp, P., Mandel, P., 1985. Effect of ethanol on adenosine triphosphatase and enolase activities in rat brain and in cultured nerve cells. Neurochem. Res. 10, 1311–1324. https://doi.org/10.1007/BF00964849.

LeDoux, J.E., 1994. Emotion, memory and the brain. Sci. Am. 270, 50–57. https://doi.org/10.1038/scientificamerican0694-50.

Mahabir, S., Chatterjee, D., Gerlai, R., 2018. Short exposure to low concentrations of alcohol during embryonic development has only subtle and strain- dependent effect on the levels of five amino acid neurotransmitters in zebrafish. Neurotoxicol. Teratol. https://doi.org/10.1016/j.ntt.2018.05.005.

Makrecka-Kuka, M., Krumschnabel, G., Gnaiger, E., 2015. High-resolution respirometry for simultaneous measurement of oxygen and hydrogen peroxide fluxes in permeabilized cells, tissue homogenate and isolated mitochondria. Biomolecules 5, 1319–1338. https://doi.org/10.3390/biom5031319.

Mameli, M., Zamudio, P.A., Carta, M., Valenzuela, C.F., 2005. Developmentally regulated

actions of alcohol on hippocampal glutamatergic transmission. J. Neurosci. 25, 8027–8035. https://doi.org/10.1523/JNEUROSCI.2434-05.2005.
Maren, S., 1996. Synaptic transmission and plasticity in the amygdala. An emerging physiology of fear conditioning circuits. Mol. Neurobiol. 13, 1–22. https://doi.org/10.1007/BF02740749.
Marin-Garcia, J., Ananthakrishnan, R., Goldenthal, M.J., 1996. Mitochondrial dysfunction after fetal alcohol exposure. Alcohol. Clin. Exp. Res. 20, 1029–1032. https://doi.org/10.1111/j.1530-0277.1996.tb01942.x.
Matchkov, V.V., Krivoi, I.I., 2016. Specialized functional diversity and interactions of the Na,K-ATPase. Front. Physiol. https://doi.org/10.3389/fphys.2016.00179.
Matsumoto, I., 2009. Proteomics approach in the study of the pathophysiology of alcohol-related brain damage. Alcohol Alcohol. 44, 171–176. https://doi.org/10.1093/alcalc/agn104.
McHugh, R.K., Wigderson, S., Greenfield, S.F., 2014. Epidemiology of substance use in reproductive-age women. Obstet. Gynecol. Clin. North Am. 41, 177–189. https://doi.org/10.1016/j.ogc.2014.02.001.
Miguel-Hidalgo, J.J., 2006. Withdrawal from free-choice ethanol consumption results in increased packing density of glutamine synthetase-immunoreactive astrocytes in the prelimbic cortex of alcohol-preferring rats. Alcohol Alcohol. 41, 379–385. https://doi.org/10.1093/alcalc/agl006.
Miller, B.R., Dorner, J.L., Shou, M., Sari, Y., Barton, S.J., Sengelaub, D.R., Kennedy, R.T., Rebec, G.V., 2008. Up-regulation of GLT1 expression increases glutamate uptake and attenuates the Huntington's disease phenotype in the R6/2 mouse. Neuroscience 153, 329–337. https://doi.org/10.1016/j.neuroscience.2008.02.004.
Pesta, D., Gnaiger, E., 2012. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. In: Palmeira, C.M., Moreno, A.J. (Eds.), Methods in Molecular Biology. Springer, pp. 25–58. https://doi.org/10.1007/978-1-61779-382-0_3.
Peterson, G.L., 1977. A simplification of the protein assay method of Lowry et al. Which is more generally applicable. Anal. Biochem. 83, 346–356. https://doi.org/10.1016/0003-2697(77)90043-4.
Petito, C.K., Chung, M.C., Verkhovsky, L.M., Cooper, A.J.L., 1992. Brain glutamine synthetase increases following cerebral ischemia in the rat. Brain Res. 569, 275–280. https://doi.org/10.1016/0006-8993(92)90639-Q.
Rico, E.P., de Oliveira, D.L., Rosemberg, D.B., Mussulini, B.H., Bonan, C.D., Dias, R.D., Wofchuk, S., Souza, D.O., Bogo, M.R., 2010. Expression and functional analysis of Na + -dependent glutamate transporters from zebrafish brain. Brain Res. Bull. 81, 517–523. https://doi.org/10.1016/j.brainresbull.2009.11.011.
Roozen, S., Peters, G.J.Y., Kok, G., Townend, D., Nijhuis, J., Curfs, L., 2016. Worldwide prevalence of fetal alcohol Spectrum disorders: a systematic literature review including meta-analysis. Alcohol. Clin. Exp. Res. 40, 18–32. https://doi.org/10.1111/acer.12939.
Rose, E.M., Koo, J.C.P., Antflick, J.E., Ahmed, S.M., Angers, S., Hampson, D.R., 2009. Glutamate transporter coupling to Na,K-ATPase. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.1081-09.2009.
Rose, C.F., Verkhratsky, A., Parpura, V., 2013. Astrocyte glutamine synthetase: pivotal in health and disease. Biochem. Soc. Trans. 41, 1518–1524. https://doi.org/10.1042/BST20130237.
Rothstein, J.D., Patel, S., Regan, M.R., Haenggeli, C., Huang, Y.H., Bergles, D.E., Jin, L., Hoberg, M.D., Vidensky, S., Chung, D.S., Shuy, V.T., Bruijn, L.I., Su, Z.Z., Gupta, P., Fisher, P.B., 2005. β-Lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature 433, 73–77. https://doi.org/10.1038/nature03180.
Sawant, O.B., Wu, G., Washburn, S.E., 2015. Maternal L-glutamine supplementation prevents prenatal alcohol exposure-induced fetal growth restriction in an ovine model. Amino Acids 47, 1183–1192. https://doi.org/10.1007/s00726-015-1945-x.
Schreiber, R., Freund, W.D., 2000. Glutamate transport is downregulated in the cerebral cortex of alcohol-preferring rats. Med. Sci. Monit. 6, 649–652.
Shaked, I., Ben-Dror, I., Vardimon, L., 2002. Glutamine synthetase enhances the clearance of extracellular glutamate by the neural retina. J. Neurochem. https://doi.org/10.1046/j.1471-4159.2002.01168.x.
Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85. https://doi.org/10.1016/0003-2697(85)90442-7.
Soares, F.A., Farina, M., Santos, F.W., Souza, D., Rocha, J.B.T., Nogueira, C.W., 2003. Interaction between metals and chelating agents affects glutamate binding on brain synaptic membranes. Neurochem. Res. 28, 1859–1865. https://doi.org/10.1023/A:1026175825871.
Tiburcio-Félix, R., Escalante-López, M., López-Bayghen, B., Martínez, D., Hernández-Kelly, L.C., Zinker, S., Hernández-Melchor, D., López-Bayghen, E., Olivares-Bañuelos, T.N., Ortega, A., 2018. Glutamate-dependent translational control of glutamine synthetase in Bergmann Glia Cells. Mol. Neurobiol. 55, 5202–5209. https://doi.org/10.1007/s12035-017-0756-3.
Tsakiris, S., Deliconstantinos, G., 1984. Influence of phosphatidylserine on (Na + + K +)-stimulated ATPase and acetylcholinesterase activities of dog brain synaptosomal plasma membranes. Biochem. J. 220, 301–307. https://doi.org/10.1042/bj2200301.
Vekovischeva, O.Y., Aitta-Aho, T., Echenko, O., Kankaanpää, A., Seppälä, T., Honkanen, A., Sprengel, R., Korpi, E.R., 2004. Reduced aggression in AMPA-type glutamate receptor GluR-A subunit-deficient mice. Genes Brain Behav. https://doi.org/10.1111/j.1601-1848.2004.00075.x.
Vrettou, M., Granholm, L., Todkar, A., Nilsson, K.W., Wallén-Mackenzie, Å., Nylander, I., Comasco, E., 2017. Ethanol affects limbic and striatal presynaptic glutamatergic and DNA methylation gene expression in outbred rats exposed to early-life stress. Addict. Biol. 22, 369–380. https://doi.org/10.1111/adb.12331.
Wijayawardhane, N., Shonesy, B.C., Vaglenova, J., Vaithianathan, T., Carpenter, M., Breese, C.R., Dityatev, A., Suppiramaniam, V., 2007. Postnatal aniracetam treatment improves prenatal ethanol induced attenuation of AMPA receptor-mediated synaptic transmission. Neurobiol. Dis. 26, 696–706. https://doi.org/10.1016/j.nbd.2007.03.009.
Zhang, C.R., Ho, M.F., Vega, M.C.S., Burne, T.H.J., Chong, S., 2015. Prenatal ethanol exposure alters adult hippocampal VGLUT2 expression with concomitant changes in promoter DNA methylation, H3K4 trimethylation and miR-467b-5p levels. Epigenetics Chromatin 8, 1–12. https://doi.org/10.1186/s13072-015-0032-6.
Zhou, Y., Danbolt, N.C., 2014. Glutamate as a neurotransmitter in the healthy brain. J. Neural Transm. 121, 799–817. https://doi.org/10.1007/s00702-014-1180-8.
Zoicas, I., Kornhuber, J., 2019. The role of metabotropic glutamate receptors in social behavior in rodents. Int. J. Mol. Sci. 20. https://doi.org/10.3390/ijms20061412.

5. CAPÍTULO III

Evaluation of thalamic neurodegeneration in zebrafish FASD model

Suelen Baggio, Gabriela Zimmermann Prado Rodrigues, Thainá Garbino dos Santos, 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 o 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.

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.

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 especificas, 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+/K+ 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 neurotrasmissão. 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 β-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 imunoconteúdo 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 Na^+/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

Na^+/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 glutamátergica.

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 glutamatérgica, como a Na^+/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 Bliek 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 Na^+/ 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.

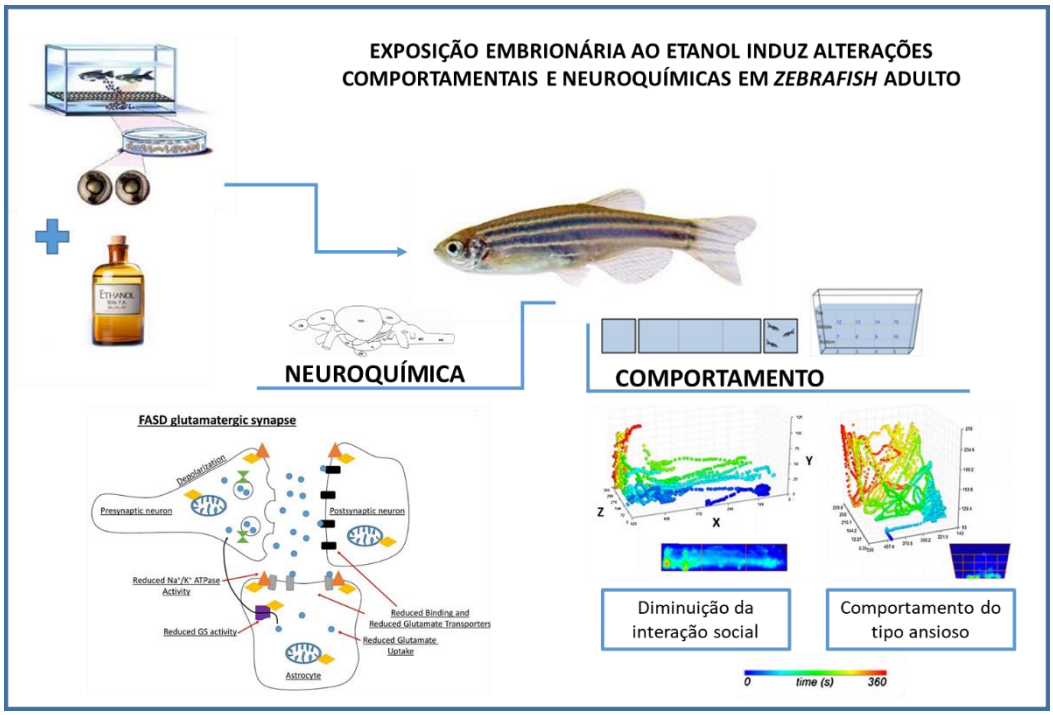
O glutamato, principal neurotransmissor excitatório do SNC, é responsável por inúmeras funções cognitivas e comportamentais, que frequentemente encontram-se alteradas na FASD e em demais desordens relacionadas com o consumo de etanol (Danbolt, 2001; Valenzuela et al., 2011). A comprovação de que a queda 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 Na^+/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.

10. REFERÊNCIAS GERAIS

Amorim, R.R., Silva, P.F., Luchiari, A.C., 2017. Effects of Alcohol on Inhibitory Avoidance Learning in Zebrafish (Danio rerio). Zebrafish. https://doi.org/10.1089/zeb.2017.1438

Anglade, P., Larabi-Godinot, Y., 2010. Historical landmarks in the histochemistry of the cholinergic synapse: Perspectives for future researches. Biomed. Res. https://doi.org/10.2220/biomedres.31.1

Antyborzec, I., O'Leary, V.B., Dolly, J.O., Ovsepian, S. V., 2016. Low-Affinity Neurotrophin Receptor p75 Promotes the Transduction of Targeted Lentiviral Vectors to Cholinergic Neurons of Rat Basal Forebrain. Neurotherapeutics. https://doi.org/10.1007/s13311-016-0445-3

Arendt, T., Hennig, D., Gray, J.A., Marchbanks, R., 1988. Loss of neurons in the rat basal forebrain cholinergic projection system after prolonged intake of ethanol. Brain Res. Bull. https://doi.org/10.1016/0361-9230(88)90193-1

Armstrong-James, M., Ross, D.T., Chen, F., Ebner, F.F., 1988. The effect of thiamine deficiency on the structure and physiology of the rat forebrain. Metab. Brain Dis. https://doi.org/10.1007/BF01001012

Baculis, B.C., Diaz, M.R., Fernando Valenzuela, C., 2015. Third trimester-equivalent ethanol exposure increases anxiety-like behavior and glutamatergic transmission in the basolateral amygdala. Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2015.08.009

Baggio, S., Mussulini, B.H., de Oliveira, D.L., Gerlai, R., Rico, E.P., 2018. Embryonic alcohol exposure leading to social avoidance and altered anxiety responses in adult zebrafish. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2017.08.039

Baggio, S., Mussulini, B.H., de Oliveira, D.L., Zenki, K.C., Santos da Silva, E., Rico, E.P., 2017. Embryonic alcohol exposure promotes long-term effects on cerebral glutamate transport of adult zebrafish. Neurosci. Lett. https://doi.org/10.1016/j.neulet.2016.11.016

Baggio, S., Zenki, K., Martins Silva, A., dos Santos, T.G., Rech, G., Lazzarotto, G., Dutra Dias, R., Hur Mussulini, B., Pacheco Rico, E., de Oliveira, D.L., 2020. Fetal alcohol spectrum disorders model alters the functionality of glutamatergic neurotransmission in adult zebrafish. Neurotoxicology. https://doi.org/10.1016/j.neuro.2020.03.003

Baiamonte, M., Parker, M.O., Vinson, G.P., Brennan, C.H., 2016. Sustained effects of developmental exposure to ethanol on Zebrafish anxiety-like behaviour. PLoS One. https://doi.org/10.1371/journal.pone.0148425

Barker, D J P, 2004. Developmental origins of adult health and disease. J. Epidemiol. Community Heal. https://doi.org/10.1136/jech.58.2.114

Barker, D. J.P., 2004. The Developmental Origins of Adult Disease. J. Am. Coll. Nutr. https://doi.org/10.1080/07315724.2004.10719428

Barker, G.R.I., Warburton, E.C., 2009. Critical role of the cholinergic system for object-in-place associative recognition memory. Learn. Mem. https://doi.org/10.1101/lm.1121309

Bauer, D.E., Jackson, J.G., Genda, E.N., Montoya, M.M., Yudkoff, M., Robinson, M.B.,

2012. The glutamate transporter, GLAST, participates in a macromolecular complex that supports glutamate metabolism. Neurochem. Int. https://doi.org/10.1016/j.neuint.2012.01.013

Bencan, Z., Sledge, D., Levin, E.D., 2009. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2009.07.009

Berman, R.F., Hannigan, J.H., 2000. Effects of prenatal alcohol exposure on the hippocampus: Spatial behavior, electrophysiology, and neuroanatomy. Hippocampus. https://doi.org/10.1002/(SICI)1098-1063(2000)10:1<94::AID-HIPO11>3.0.CO;2-T

Berridge, K.C., 2017. Is Addiction a Brain Disease? Neuroethics. https://doi.org/10.1007/s12152-016-9286-3

Bilotta, J., Barnett, J.A., Hancock, L., Saszik, S., 2004. Ethanol exposure alters zebrafish development: A novel model of fetal alcohol syndrome, in: Neurotoxicology and Teratology. https://doi.org/10.1016/j.ntt.2004.06.011

Bishop, S., Gahagan, S., Lord, C., 2007. Re-examining the core features of autism: A comparison of autism spectrum disorder and fetal alcohol spectrum disorder. J. Child Psychol. Psychiatry Allied Discip. https://doi.org/10.1111/j.1469-7610.2007.01782.x

Bliss, T.V.P., Collingridge, G.L., 1993. A synaptic model of memory: Long-term potentiation in the hippocampus. Nature. https://doi.org/10.1038/361031a0

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. https://doi.org/10.1016/0003-2697(76)90527-3

Brady, M.L., Allan, A.M., Caldwell, K.K., 2012. A Limited Access Mouse Model of Prenatal Alcohol Exposure that Produces Long-Lasting Deficits in Hippocampal-Dependent Learning and Memory. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/j.1530-0277.2011.01644.x

Brady, M.L., Diaz, M.R., Iuso, A., Everett, J.C., Valenzuela, C.F., Caldwell, K.K., 2013. Moderate prenatal alcohol exposure reduces plasticity and alters NMDA receptor subunit composition in the dentate gyrus. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.1217-12.2013

Breese, G.R., Criswell, H.E., Carta, M., Dodson, P.D., Hanchar, H.J., Khisti, R.T., Mameli, M., Ming, Z., Morrow, A.L., Olsen, R.W., Otis, T.S., Parsons, L.H., Penland, S.N., Roberto, M., Siggins, G.R., Valenzuela, C.F., Wallner, M., 2006. Basis of the gabamimetic profile of ethanol, in: Alcoholism: Clinical and Experimental Research. https://doi.org/10.1111/j.0145-6008.2006.00086.x

Brolese, G., Lunardi, P., De Souza, D.F., Lopes, F.M., Leite, M.C., Gonçalves, C.A., 2015. Pre-and postnatal exposure to moderate levels of ethanol can have long-lasting effects on hippocampal glutamate uptake in adolescent offspring. PLoS One. https://doi.org/10.1371/journal.pone.0127845

Buske, C., Gerlai, R., 2011. Early embryonic ethanol exposure impairs shoaling and the dopaminergic and serotoninergic systems in adult zebrafish. Neurotoxicol. Teratol. https://doi.org/10.1016/j.ntt.2011.05.009

Carvan, M.J., Loucks, E., Weber, D.N., Williams, F.E., 2004. Ethanol effects on the

developing zebrafish: Neurobehavior and skeletal morphogenesis, in: Neurotoxicology and Teratology. https://doi.org/10.1016/j.ntt.2004.06.016

Cesconetto, P.A., Andrade, C.M., Cattani, D., Domingues, J.T., Parisotto, E.B., Filho, D.W., Zamoner, A., 2016. Maternal Exposure to Ethanol During Pregnancy and Lactation Affects Glutamatergic System and Induces Oxidative Stress in Offspring Hippocampus. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/acer.12917

Cha, Y., Erez, T., Reynolds, I.J., Kumar, D., Ross, J., Koytiger, G., Kusko, R., Zeskind, B., Risso, S., Kagan, E., Papapetropoulos, S., Grossman, I., Laifenfeld, D., 2018. Drug repurposing from the perspective of pharmaceutical companies. Br. J. Pharmacol. https://doi.org/10.1111/bph.13798

Chao, L. -P, Wolfgram, F., 1973. PURIFICATION AND SOME PROPERTIES OF CHOLINE ACETYLTRANSFERASE (EC 2.3.1.6) FROM BOVINE BRAIN. J. Neurochem. https://doi.org/10.1111/j.1471-4159.1973.tb00078.x

Chastain, G., 2006. Alcohol, neurotransmitter systems, and behavior. J. Gen. Psychol. https://doi.org/10.3200/GENP.133.4.329-335

Cleal, M., Parker, M.O., 2018. Moderate developmental alcohol exposure reduces repetitive alternation in a zebrafish model of fetal alcohol spectrum disorders. Neurotoxicol. Teratol. https://doi.org/10.1016/j.ntt.2018.09.001

Cook, J.L., Green, C.R., Lilley, C.M., Anderson, S.M., Baldwin, M.E., Chudley, A.E., Conry, J.L., LeBlanc, N., Loock, C.A., Lutke, J., Mallon, B.F., McFarlane, A.A., Temple, V.K., Rosales, T., 2016. Fetal alcohol spectrum disorder: A guideline for diagnosis across the lifespan. CMAJ. https://doi.org/10.1503/cmaj.141593

Creeley, C.E., Olney, J.W., 2013. Drug-induced apoptosis: Mechanism by which alcohol and many other drugs can disrupt brain development. Brain Sci. https://doi.org/10.3390/brainsci3031153

Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. https://doi.org/10.1016/S0301-0082(00)00067-8

Das, S.C., Yamamoto, B.K., Hristov, A.M., Sari, Y., 2015. Ceftriaxone attenuates ethanol drinking and restores extracellular glutamate concentration through normalization of GLT-1 in nucleus accumbens of male alcohol-preferring rats. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2015.05.009

Davies, D.L., Vernadakis, A., 1984. Effects of ethanol on cultured glial cells: proliferation and glutamine synthetase activity. Dev. Brain Res. https://doi.org/10.1016/0165-3806(84)90059-2

Denny, L.A., Coles, S., Blitz, R., 2017. Fetal Alcohol Syndrome and Fetal Alcohol Spectrum Disorders. Am. Fam. Physician.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. https://doi.org/10.1016/0006-2952(61)90145-9

Engeszer, R.E., Patterson, L.B., Rao, A.A., Parichy, D.M., 2007. Zebrafish in the wild: A review of natural history and new notes from the field. Zebrafish. https://doi.org/10.1089/zeb.2006.9997

Eşel, E., 2006. Neurobiology of alcohol withdrawal: Inhibitory and excitatory neurotransmitters. Turk Psikiyatr. Derg.

Eşel, E., Dinç, K., 2017. Neurobiology of alcohol dependence and implications on treatment. Turk Psikiyatr. Derg. https://doi.org/10.5080/u14894

Fernandes, Y., Gerlai, R., 2009. Long-term behavioral changes in response to early developmental exposure to ethanol in Zebrafish. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/j.1530-0277.2008.00874.x

Fernandes, Y., Rampersad, M., Gerlai, R., 2015a. Embryonic alcohol exposure impairs the dopaminergic system and social behavioral responses in adult Zebrafish. Int. J. Neuropsychopharmacol. https://doi.org/10.1093/ijnp/pyu089

Fernandes, Y., Rampersad, M., Gerlai, R., 2015b. Impairment of social behaviour persists two years after embryonic alcohol exposure in zebrafish: A model of fetal alcohol spectrum disorders. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2015.05.060

Fernandes, Y., Tran, S., Abraham, E., Gerlai, R., 2014. Embryonic alcohol exposure impairs associative learning performance in adult zebrafish. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2014.02.035

Gebauer, D.L., Pagnussat, N., Piato, Â.L., Schaefer, I.C., Bonan, C.D., Lara, D.R., 2011. Effects of anxiolytics in zebrafish: Similarities and differences between benzodiazepines, buspirone and ethanol. Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2011.04.021

Gemberling, M., Bailey, T.J., Hyde, D.R., Poss, K.D., 2013. The zebrafish as a model for complex tissue regeneration. Trends Genet. https://doi.org/10.1016/j.tig.2013.07.003

Gerace, E., Landucci, E., Totti, A., Bani, D., Guasti, D., Baronti, R., Moroni, F., Mannaioni, G., Pellegrini-Giampietro, D.E., 2016. Ethanol Toxicity During Brain Development: Alterations of Excitatory Synaptic Transmission in Immature Organotypic Hippocampal Slice Cultures. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/acer.13006

Gerlai, R., 2015. Embryonic alcohol exposure: Towards the development of a zebrafish model of fetal alcohol spectrum disorders. Dev. Psychobiol. https://doi.org/10.1002/dev.21318

Giovannini, M.G., Lana, D., Pepeu, G., 2015. The integrated role of ACh, ERK and mTOR in the mechanisms of hippocampal inhibitory avoidance memory. Neurobiol. Learn. Mem. https://doi.org/10.1016/j.nlm.2014.12.014

Goodfellow, M.J., Abdulla, K.A., Lindquist, D.H., 2016. Neonatal Ethanol Exposure Impairs Trace Fear Conditioning and Alters NMDA Receptor Subunit Expression in Adult Male and Female Rats. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/acer.12958

Goodlett, C.R., Horn, K.H., Zhou, F.C., 2005. Alcohol teratogenesis: Mechanisms of damage and strategies for intervention, in: Experimental Biology and Medicine. https://doi.org/10.1177/15353702-0323006-07

Goodwani, S., Saternos, H., Alasmari, F., Sari, Y., 2017. Metabotropic and ionotropic glutamate receptors as potential targets for the treatment of alcohol use disorder. Neurosci. Biobehav. Rev. https://doi.org/10.1016/j.neubiorev.2017.02.024

Gursky, Z.H., Savage, L.M., Klintsova, A.Y., 2019. Nucleus reuniens of the midline thalamus of a rat is specifically damaged after early postnatal alcohol exposure. Neuroreport. https://doi.org/10.1097/WNR.0000000000001270

Harris, R.A., Proctor, W.R., McQuilkin, S.J., Klein, R.L., Mascia, M.P., Whatley, V., Whiting, P.J., Dunwiddie, T. V., 1995. Ethanol Increases GABAA Responses in Cells Stably Transfected with Receptor Subunits. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/j.1530-0277.1995.tb01496.x

Hellemans, K.G.C., Sliwowska, J.H., Verma, P., Weinberg, J., 2010. Prenatal alcohol exposure: Fetal programming and later life vulnerability to stress, depression and anxiety disorders. Neurosci. Biobehav. Rev. https://doi.org/10.1016/j.neubiorev.2009.06.004

Henley, J.M., Wilkinson, K.A., 2013. AMPA receptor trafficking and the mechanisms underlying synaptic plasticity and cognitive aging. Dialogues Clin. Neurosci.

Hertz, L., Zielke, H.R., 2004. Astrocytic control of glutamatergic activity: Astrocytes as stars of the show. Trends Neurosci. https://doi.org/10.1016/j.tins.2004.10.008

Hinz, F.I., Aizenberg, M., Tushev, G., Schuman, E.M., 2013. Protein synthesis-dependent associative long-term memory in larval zebrafish. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.0560-13.2013

Ikonomidou, C., Bittigau, P., Ishimaru, M.J., Wozniak, D.F., Koch, C., Genz, K., Price, M.T., Stefovska, V., Hörster, F., Tenkova, T., Dikranian, K., Olney, J.W., 2000. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 287, 1056–60. https://doi.org/10.1126/science.287.5455.1056

Illarionava, N.B., Brismar, H., Aperia, A., Gunnarson, E., 2014. Role of Na,K-ATPase α1 and α2 isoforms in the support of astrocyte glutamate uptake. PLoS One. https://doi.org/10.1371/journal.pone.0098469

Jones, E.G., Rubenstein, J.L.R., 2004. Expression of regulatory genes during differentiation of thalamic nuclei in mouse and monkey. J. Comp. Neurol. https://doi.org/10.1002/cne.20234

Jopling, C., Sleep, E., Raya, M., Martí, M., Raya, A., Belmonte, J.C.I., 2010. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. Nature. https://doi.org/10.1038/nature08899

Joya, X., Garcia-Algar, O., Vall, O., Pujades, C., 2014. Transient exposure to ethanol during zebrafish embryogenesis results in defects in neuronal differentiation: An alternative model system to study fasd. PLoS One. https://doi.org/10.1371/journal.pone.0112851

Kalberg, W.O., May, P.A., Buckley, D., Hasken, J.M., Marais, A.S., De Vries, M.M., Bezuidenhout, H., Manning, M.A., Robinson, L.K., Adam, M.P., Hoyme, D.B., Parry, C.D.H., Seedat, S., Elliott, A.J., Hoyme, H.E., 2019. Early-life predictors of fetal alcohol spectrum disorders. Pediatrics. https://doi.org/10.1542/peds.2018-2141

Karacay, B., Bonthius, N.E., Plume, J., Bonthius, D.J., 2015. Genetic Absence of nNOS Worsens Fetal Alcohol Effects in Mice. I: Behavioral Deficits. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/acer.12616

Kash, T., Hwa, L., Besheer, J., 2017. Glutamate plasticity woven through the progression to alcohol use disorder: A multi-circuit perspective. F1000Research. https://doi.org/10.12688/f1000research.9609.1

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. https://doi.org/10.1002/aja.1002030302

Kodituwakku, P.W., 2007. Defining the behavioral phenotype in children with fetal alcohol spectrum disorders: A review. Neurosci. Biobehav. Rev. https://doi.org/10.1016/j.neubiorev.2006.06.020

Kully-Martens, K., Denys, K., Treit, S., Tamana, S., Rasmussen, C., 2012. A Review of Social Skills Deficits in Individuals with Fetal Alcohol Spectrum Disorders and Prenatal Alcohol Exposure: Profiles, Mechanisms, and Interventions. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/j.1530-0277.2011.01661.x

Kumar, R., Långström, B., Darreh-Shori, T., 2016. Novel ligands of Choline Acetyltransferase designed by in silico molecular docking, hologram QSAR and lead optimization. Sci. Rep. https://doi.org/10.1038/srep31247

Lam, V.Y.Y., Raineki, C., Wang, L.Y., Chiu, M., Lee, G., Ellis, L., Yu, W., Weinberg, J., 2019. Role of corticosterone in anxiety- and depressive-like behavior and HPA regulation following prenatal alcohol exposure. Prog. Neuro-Psychopharmacology Biol. Psychiatry. https://doi.org/10.1016/j.pnpbp.2018.10.008

Lawrence, R.C., Otero, N.K.H., Kelly, S.J., 2012. Selective effects of perinatal ethanol exposure in medial prefrontal cortex and nucleus accumbens. Neurotoxicol. Teratol. https://doi.org/10.1016/j.ntt.2011.08.002

Ledig, M., Kopp, P., Mandel, P., 1985. Effect of ethanol on adenosine triphosphatase and enolase activities in rat brain and in cultured nerve cells. Neurochem. Res. https://doi.org/10.1007/BF00964849

Ledig, M., Megias-megias, L., Tholey, G., 1991. Maternal alcohol exposure before and during pregnancy: Effect on development of neurons and glial cells in culture. Alcohol Alcohol. https://doi.org/10.1093/oxfordjournals.alcalc.a045098

Ledig, M., Tholey, G., Kopp, P., Mandel, P., 1989. An experimental study of fetal alcohol syndrome in the rat: Biochemical modifications in brain and liver. Alcohol Alcohol.

Lee, S.G., Su, Z.Z., Emdad, L., Gupta, P., Sarkar, D., Borjabad, A., Volsky, D.J., Fisher, P.B., 2008. Mechanism of ceftriaxone induction of excitatory amino acid transporter-2 expression and glutamate uptake in primary human astrocytes. J. Biol. Chem. https://doi.org/10.1074/jbc.M707697200

Litten, R.Z., Allen, J., Fertig, J., 1996. Pharmacotherapies for alcohol problems: A review of research with focus on developments since 1991. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/j.1530-0277.1996.tb05264.x

Livy, D.J., Miller, E.K., Maier, S.E., West, J.R., 2003. Fetal alcohol exposure and temporal vulnerability: Effects of binge-like alcohol exposure on the developing rat hippocampus. Neurotoxicol. Teratol. https://doi.org/10.1016/S0892-0362(03)00030-8

Loane, C., Politis, M., 2012. Buspirone: What is it all about? Brain Res. https://doi.org/10.1016/j.brainres.2012.04.032

Lockwood, B., Bjerke, S., Kobayashi, K., Guo, S., 2004. Acute effects of alcohol on larval zebrafish: A genetic system for large-scale screening. Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2004.01.003

Loucks, E., Ahlgren, S., 2012. Assessing teratogenic changes in a zebrafish model of fetal alcohol exposure. J. Vis. Exp. https://doi.org/10.3791/3704

Loucks, E.J., Ahlgren, S.C., 2009. Deciphering the role of Shh signaling in axial defects

produced by ethanol exposure. Birth Defects Res. Part A - Clin. Mol. Teratol. https://doi.org/10.1002/bdra.20564

Lovely, C. Ben, Fernandes, Y., Eberhart, J.K., 2016. Fishing for Fetal Alcohol Spectrum Disorders: Zebrafish as a Model for Ethanol Teratogenesis. Zebrafish. https://doi.org/10.1089/zeb.2016.1270

Lovinger, D.M., Roberto, M., 2011. Synaptic effects induced by alcohol. Curr. Top. Behav. Neurosci. https://doi.org/10.1007/7854_2011_143

Lovinger, D.M., White, G., Weight, F.F., 1990. NMDA receptor-mediated synaptic excitation selectively inhibited by ethanol in hippocampal slice from adult rat. J. Neurosci. https://doi.org/10.1523/jneurosci.10-04-01372.1990

Lovinger, D.M., White, G., Weight, F.F., 1989. Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science (80-.). https://doi.org/10.1126/science.2467382

Lunde, E.R., Washburn, S.E., Golding, M.C., Bake, S., Miranda, R.C., Ramadoss, J., 2016. Alcohol-Induced Developmental Origins of Adult-Onset Diseases. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/acer.13114

Lutte, A.H., Nazario, L.R., Majolo, J.H., Pereira, T.C.B., Altenhofen, S., Dadda, A. da S., Bogo, M.R., Da Silva, R.S., 2018. Persistent increase in ecto-5′-nucleotidase activity from encephala of adult zebrafish exposed to ethanol during early development. Neurotoxicol. Teratol. https://doi.org/10.1016/j.ntt.2018.10.004

Maaswinkel, H., Zhu, L., Weng, W., 2013. Assessing Social Engagement in Heterogeneous Groups of Zebrafish: A New Paradigm for Autism-Like Behavioral Responses. PLoS One. https://doi.org/10.1371/journal.pone.0075955

Mahabir, S., Chatterjee, D., Gerlai, R., 2018. Short exposure to low concentrations of alcohol during embryonic development has only subtle and strain- dependent effect on the levels of five amino acid neurotransmitters in zebrafish. Neurotoxicol. Teratol. https://doi.org/10.1016/j.ntt.2018.05.005

Mahabir, S., Chatterjee, D., Gerlai, R., 2014. Strain dependent neurochemical changes induced by embryonic alcohol exposure in zebrafish. Neurotoxicol. Teratol. https://doi.org/10.1016/j.ntt.2013.11.001

Mameli, M., Zamudio, P.A., Carta, M., Valenzuela, C.F., 2005. Developmentally regulated actions of alcohol on hippocampal glutamatergic transmission. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.2434-05.2005

Maragakis, N.J., Rothstein, J.D., 2004. Glutamate transporters: Animal models to neurologic disease. Neurobiol. Dis. https://doi.org/10.1016/j.nbd.2003.12.007

Marin-Garcia, J., Ananthakrishnan, R., Goldenthal, M.J., 1996. Mitochondrial dysfunction after fetal alcohol exposure. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/j.1530-0277.1996.tb01942.x

Matsumoto, I., 2009. Proteomics approach in the study of the pathophysiology of alcohol-related brain damage. Alcohol Alcohol. https://doi.org/10.1093/alcalc/agn104

Maximino, C., Puty, B., Benzecry, R., Araújo, J., Lima, M.G., De Jesus Oliveira Batista, E., Renata De Matos Oliveira, K., Crespo-Lopez, M.E., Herculano, A.M., 2013. Role of serotonin in zebrafish (Danio rerio) anxiety: Relationship with serotonin levels and effect of buspirone, WAY 100635, SB 224289, fluoxetine and para-

chlorophenylalanine (pCPA) in two behavioral models. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2013.03.006

May, P.A., Baete, A., Russo, J., Elliott, A.J., Blankenship, J., Kalberg, W.O., Buckley, D., Brooks, M., Hasken, J., Abdul-Rahman, O., Adam, M.P., Robinson, L.K., Manning, M., Hoyme, H.E., 2014. Prevalence and characteristics of fetal alcohol spectrum disorders. Pediatrics. https://doi.org/10.1542/peds.2013-3319

May, P.A., Blankenship, J., Marais, A.S., Gossage, J.P., Kalberg, W.O., Joubert, B., Cloete, M., Barnard, R., De Vries, M., Hasken, J., Robinson, L.K., Adnams, C.M., Buckley, D., Manning, M., Parry, C.D.H., Hoyme, H.E., Tabachnick, B., Seedat, S., 2013. Maternal alcohol consumption producing fetal alcohol spectrum disorders (FASD): Quantity, frequency, and timing of drinking. Drug Alcohol Depend. https://doi.org/10.1016/j.drugalcdep.2013.07.013

May, P.A., Gossage, J.P., Kalberg, W.O., Robinson, L.K., Buckley, D., Manning, M., Hoyme, H.E., 2009. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. Dev. Disabil. Res. Rev. https://doi.org/10.1002/ddrr.68

McHugh, R.K., Wigderson, S., Greenfield, S.F., 2014. Epidemiology of substance use in reproductive-age women. Obstet. Gynecol. Clin. North Am. https://doi.org/10.1016/j.ogc.2014.02.001

Miller, B.R., Dorner, J.L., Shou, M., Sari, Y., Barton, S.J., Sengelaub, D.R., Kennedy, R.T., Rebec, G. V., 2008. Up-regulation of GLT1 expression increases glutamate uptake and attenuates the Huntington's disease phenotype in the R6/2 mouse. Neuroscience. https://doi.org/10.1016/j.neuroscience.2008.02.004

Miller, N., Gerlai, R., 2012. From Schooling to Shoaling: Patterns of Collective Motion in Zebrafish (Danio rerio). PLoS One. https://doi.org/10.1371/journal.pone.0048865

Miller, N.Y., Gerlai, R., 2011. Shoaling in zebrafish: What we don't know. Rev. Neurosci. https://doi.org/10.1515/RNS.2011.004

Mokalled, M.H., Patra, C., Dickson, A.L., Endo, T., Stainier, D.Y.R., Poss, K.D., 2016. Injury-induced ctgfa directs glial bridging and spinal cord regeneration in zebrafish. Science (80-.). https://doi.org/10.1126/science.aaf2679

Mooney, S.M., Miller, M.W., 2010. Prenatal exposure to ethanol affects postnatal neurogenesis in thalamus. Exp. Neurol. https://doi.org/10.1016/j.expneurol.2010.02.003

Mooney, S.M., Miller, M.W., 2001. Effects of prenatal exposure to ethanol on the expression of bcl-2, bax and caspase 3 in the developing rat cerebral cortex and thalamus. Brain Res. https://doi.org/10.1016/S0006-8993(01)02718-4

Most, D., Ferguson, L., Harris, R.A., 2014. Molecular basis of alcoholism, in: Handbook of Clinical Neurology. https://doi.org/10.1016/B978-0-444-62619-6.00006-9

Mukherjee, R.A.S., 2019. Diagnosis and management of foetal alcohol specturm disorder. Curr. Opin. Psychiatry. https://doi.org/10.1097/YCO.0000000000000479

Mukherjee, S., 2013. Alcoholism and its Effects on the Central Nervous System. Curr. Neurovasc. Res. https://doi.org/10.2174/15672026113109990004

Müller, T.E., Fontana, B.D., Bertoncello, K.T., Franscescon, F., Mezzomo, N.J., Canzian, J., Stefanello, F. V., Parker, M.O., Gerlai, R., Rosemberg, D.B., 2020.

Understanding the neurobiological effects of drug abuse: Lessons from zebrafish models. Prog. Neuro-Psychopharmacology Biol. Psychiatry. https://doi.org/10.1016/j.pnpbp.2020.109873

Nardelli, A., Lebel, C., Rasmussen, C., Andrew, G., Beaulieu, C., 2011. Extensive deep gray matter volume reductions in children and adolescents with fetal alcohol spectrum disorders. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/j.1530-0277.2011.01476.x

Nguyen, V.T., Tieng, Q.M., Mardon, K., Zhang, C., Chong, S., Galloway, G.J., Kurniawan, N.D., 2020. Magnetic Resonance Imaging and Micro-Computed Tomography reveal brain morphological abnormalities in a mouse model of early moderate prenatal ethanol exposure. Neurotoxicol. Teratol. https://doi.org/10.1016/j.ntt.2019.106849

Nyquist-Battie, C., Freter, M., 1988. Cardiac Mitochondrial Abnormalities in a Mouse Model of the Fetal Alcohol Syndrome. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/j.1530-0277.1988.tb00192.x

O'Connor, M.J., Frankel, F., Paley, B., Schonfeld, A.M., Carpenter, E., Laugeson, E.A., Marquardt, R., 2006. A controlled social skills training for children with fetal alcohol spectrum disorders. J. Consult. Clin. Psychol. https://doi.org/10.1037/0022-006X.74.4.639

Obrenovitch, T.P., Urenjak, J., Zilkha, E., Jay, T.M., 2000. Excitotoxicity in neurological disorders - The glutamate paradox, in: International Journal of Developmental Neuroscience. https://doi.org/10.1016/S0736-5748(99)00096-9

Oliveira, R.F., 2013. Mind the fish: Zebrafish as a model in cognitive social neuroscience. Front. Neural Circuits. https://doi.org/10.3389/fncir.2013.00131

Olney, J.W., Wozniak, D.F., Jevtovic-Todorovic, V., Ikonomidou, C., 2001. Glutamate signaling and the fetal alcohol syndrome. Ment. Retard. Dev. Disabil. Res. Rev. https://doi.org/10.1002/mrdd.1037

Ozawa, S., Kamiya, H., Tsuzuki, K., 1998. Glutamate receptors in the mammalian central nervous system. Prog. Neurobiol. https://doi.org/10.1016/S0301-0082(97)00085-3

Parker, M.O., Annan, L. V., Kanellopoulos, A.H., Brock, A.J., Combe, F.J., Baiamonte, M., Teh, M.T., Brennan, C.H., 2014. The utility of zebrafish to study the mechanisms by which ethanol affects social behavior and anxiety during early brain development. Prog. Neuro-Psychopharmacology Biol. Psychiatry. https://doi.org/10.1016/j.pnpbp.2014.03.011

Pettinati, H.M., 1996. Use of Serotonin Selective Pharmacotherapy in the Treatment of Alcohol Dependence. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/j.1530-0277.1996.tb01186.x

Peukert, D., Weber, S., Lumsden, A., Scholpp, S., 2011. Lhx2 and Lhx9 determine neuronal differentiation and compartition in the caudal forebrain by regulating Wnt signaling. PLoS Biol. https://doi.org/10.1371/journal.pbio.1001218

Pickering, C., Alsiö, J., Morud, J., Ericson, M., Robbins, T.W., Söderpalm, B., 2015. Ethanol impairment of spontaneous alternation behaviour and associated changes in medial prefrontal glutamatergic gene expression precede putative markers of dependence. Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2015.02.021

Popova, S., Lange, S., Probst, C., Gmel, G., Rehm, J., 2017. Estimation of national, regional, and global prevalence of alcohol use during pregnancy and fetal alcohol syndrome: a systematic review and meta-analysis. Lancet Glob. Heal. https://doi.org/10.1016/S2214-109X(17)30021-9

Popova, S., Lange, S., Shield, K., Mihic, A., Chudley, A.E., Mukherjee, R.A.S., Bekmuradov, D., Rehm, J., 2016. Comorbidity of fetal alcohol spectrum disorder: A systematic review and meta-analysis. Lancet. https://doi.org/10.1016/S0140-6736(15)01345-8

Ramocki, M.B., Zoghbi, H.Y., 2008. Failure of neuronal homeostasis results in common neuropsychiatric phenotypes. Nature. https://doi.org/10.1038/nature07457

Rao, P.S.S., Bell, R.L., Engleman, E.A., Sari, Y., 2015. Targeting glutamate uptake to treat alcohol use disorders. Front. Neurosci. https://doi.org/10.3389/fnins.2015.00144

Reiner, A., Levitz, J., 2018. Glutamatergic Signaling in the Central Nervous System: Ionotropic and Metabotropic Receptors in Concert. Neuron. https://doi.org/10.1016/j.neuron.2018.05.018

Roberto, M., Varodayan, F.P., 2017. Synaptic targets: Chronic alcohol actions. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2017.01.013

Roozen, S., Peters, G.J.Y., Kok, G., Townend, D., Nijhuis, J., Curfs, L., 2016. Worldwide Prevalence of Fetal Alcohol Spectrum Disorders: A Systematic Literature Review Including Meta-Analysis. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/acer.12939

Rose, E.M., Koo, J.C.P., Antflick, J.E., Ahmed, S.M., Angers, S., Hampson, D.R., 2009. Glutamate transporter coupling to Na,K-ATPase. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.1081-09.2009

Rosemberg, D.B., Rico, E.P., Mussulini, B.H.M., Piato, Â.L., Calcagnotto, M.E., Bonan, C.D., Dias, R.D., Blaser, R.E., Souza, D.O., de Oliveira, D.L., 2011. Differences in spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments. PLoS One. https://doi.org/10.1371/journal.pone.0019397

Rothstein, J.D., Patel, S., Regan, M.R., Haenggeli, C., Huang, Y.H., Bergles, D.E., Jin, L., Hoberg, M.D., Vidensky, S., Chung, D.S., Shuy, V.T., Bruijn, L.I., Su, Z.Z., Gupta, P., Fisher, P.B., 2005. β-Lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature. https://doi.org/10.1038/nature03180

Sampson, P.D., Streissguth, A.P., Bookstein, F.L., Little, R.E., Clarren, S.K., Dehaene, P., Hanson, J.W., Graham, J.M., 1997. Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. Teratology. https://doi.org/10.1002/(SICI)1096-9926(199711)56:5<317::AID-TERA5>3.0.CO;2-U

Sari, Y., Sakai, M., Weedman, J.M., Rebec, G. V., Bell, R.L., 2011. Ceftriaxone, a beta-lactam antibiotic, reduces ethanol consumption in alcohol-preferring rats. Alcohol Alcohol. https://doi.org/10.1093/alcalc/agr023

Sari, Y., Smith, K.D., Ali, P.K., Rebec, G. V., 2009. Upregulation of Glt1 attenuates cue-induced reinstatement of cocaine-seeking behavior in rats. J. Neurosci.

https://doi.org/10.1523/JNEUROSCI.1746-09.2009

Sattler, R., Rothstein, J.D., 2006. Regulation and dysregulation of glutamate transporters. Handb. Exp. Pharmacol. https://doi.org/10.1007/3-540-29784-7-14

Sawant, O.B., Wu, G., Washburn, S.E., 2015. Maternal L-glutamine supplementation prevents prenatal alcohol exposure-induced fetal growth restriction in an ovine model. Amino Acids. https://doi.org/10.1007/s00726-015-1945-x

Scholpp, S., Delogu, A., Gilthorpe, J., Peukert, D., Schindler, S., Lumsden, A., 2009. Her6 regulates the neurogenetic gradient and neuronal identity in the thalamus. Proc. Natl. Acad. Sci. U. S. A. https://doi.org/10.1073/pnas.0910894106

Scholpp, S., Lumsden, A., 2010. Building a bridal chamber: Development of the thalamus. Trends Neurosci. https://doi.org/10.1016/j.tins.2010.05.003

Seguin, D., Gerlai, R., 2018. Fetal alcohol spectrum disorders: Zebrafish in the analysis of the milder and more prevalent form of the disease. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2017.10.005

Seguin, D., Shams, S., Gerlai, R., 2016. Behavioral Responses to Novelty or to a Predator Stimulus Are Not Altered in Adult Zebrafish by Early Embryonic Alcohol Exposure. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/acer.13249

Sherman, S.M., 2016. Thalamus plays a central role in ongoing cortical functioning. Nat. Neurosci. https://doi.org/10.1038/nn.4269

Söderpalm, B., Lidö, H.H., Ericson, M., 2017. The Glycine Receptor—A Functionally Important Primary Brain Target of Ethanol. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/acer.13483

Stennett, B.A., Frankowski, J.C., Peris, J., Knackstedt, L.A., 2017. Ceftriaxone reduces alcohol intake in outbred rats while upregulating xCT in the nucleus accumbens core. Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2017.07.001

Stephen Rich, J., Martin, P.R., 2014. Co-occurring psychiatric disorders and alcoholism, in: Handbook of Clinical Neurology. https://doi.org/10.1016/B978-0-444-62619-6.00033-1

Stratton KR, Howe CJ, Battaglia, 1996. FC.Institute of Medicine (U.S.). Division of Biobehavioral Sciences and Mental Disorders. Committee to Study Fetal Alcohol Syndrome, National Institute on Alcohol Abuse and Alcoholism (U.S.) Fetal alcohol syndrome: diagnosis, epidemiology, prevention, and treatment. Washington DC: National Academy Press.

Strawn, J.R., Mills, J.A., Cornwall, G.J., Mossman, S.A., Varney, S.T., Keeshin, B.R., Croarkin, P.E., 2018. Buspirone in Children and Adolescents with Anxiety: A Review and Bayesian Analysis of Abandoned Randomized Controlled Trials. J. Child Adolesc. Psychopharmacol. https://doi.org/10.1089/cap.2017.0060

Subbaraman, M.S., Roberts, S.C.M., 2019. Costs associated with policies regarding alcohol use during pregnancy: Results from 1972-2015 vital statistics. PLoS One. https://doi.org/10.1371/journal.pone.0215670

Takeda, K., Ueda, T., 2017. Effective Mechanism for Synthesis of Neurotransmitter Glutamate and its Loading into Synaptic Vesicles. Neurochem. Res. https://doi.org/10.1007/s11064-016-2037-3

Thöne-Reineke, C., Neumann, C., Namsolleck, P., Schmerbach, K., Krikov, M., Schefe,

J.H., Lucht, K., Hörtnagl, H., Godes, M., Müller, S., Rumschüssel, K., Funke-Kaiser, H., Villringer, A., Steckelings, U.M., Unger, T., 2008. The β-lactam antibiotic, ceftriaxone, dramatically improves survival, increases glutamate uptake and induces neurotrophins in stroke. J. Hypertens. https://doi.org/10.1097/HJH.0b013e328313e403

Ueda, T., 2016. Vesicular Glutamate Uptake. Adv. Neurobiol. https://doi.org/10.1007/978-3-319-45096-4_7

Valenzuela, C.F., Puglia, M.P., Zucca, S., 2011. Focus on: Neurotransmitter systems. Alcohol Res. Heal.

Vallés, S., Felipo, V., Montoliu, C., Guerri, C., 1995. Alcohol exposure during brain development reduces 3H-MK-801 binding and enhances metabotropic-glutamate receptor-stimulated phosphoinositide hydrolysis in rat hippocampus. Life Sci. https://doi.org/10.1016/0024-3205(95)00101-8

Van Der Bliek, A.M., Sedensky, M.M., Morgan, P.G., 2017. Cell biology of the mitochondrion. Genetics. https://doi.org/10.1534/genetics.117.300262

Van Liefferinge, J., Massie, A., Portelli, J., Di Giovanni, G., Smolders, I., 2013. Are vesicular neurotransmitter transporters potential treatment targets for temporal lobe epilepsy? Front. Cell. Neurosci. https://doi.org/10.3389/fncel.2013.00139

Volkow, N.D., Koob, G.F., McLellan, A.T., 2016. Neurobiologic advances from the brain disease model of addiction. N. Engl. J. Med. https://doi.org/10.1056/NEJMra1511480

Wall, T.L., Luczak, S.E., Hiller-Sturmhöfel, S., 2016. Biology, genetics, and environment: Underlying factors influencing alcohol metabolism. Alcohol Res. Curr. Rev.

Wan, J., Goldman, D., 2016. Retina regeneration in zebrafish. Curr. Opin. Genet. Dev. https://doi.org/10.1016/j.gde.2016.05.009

Wang, G.J., Volkow, N.D., Franceschi, D., Fowler, J.S., Thanos, P.K., Scherbaum, N., Pappas, N., Wong, C.T., Hitzemann, R.J., Felder, C.A., 2000. Regional brain metabolism during alcohol intoxication. Alcohol. Clin. Exp. Res. https://doi.org/10.1111/j.1530-0277.2000.tb02061.x

Warburton, E.C., Barker, G.R.I., Brown, M.W., 2013. Investigations into the involvement of NMDA mechanisms in recognition memory. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2013.04.013

Weiner, J.L., Zhang, L., Carlen, P.L., 1994. Potentiation of GABA(A)-mediated synaptic current by ethanol in hippocampal CA1 neurons: Possible role of protein kinase C. J. Pharmacol. Exp. Ther.

WHO Global Information System on Alcohol and Health (GISAH) [online database]. Geneva: World Health Organization (http://www.who.int/gho/alcohol/en/)

World health statistics 2017: monitoring health for the SDGs, Sustainable Development Goals. Geneva: World Health Organization; 2017. Licence: CC BY-NC-SA 3.0 IGO.

World Health Statistics 2019: Monitoring health for the SDGs (Sustainable Development goals). WHO - World Health Organization. p. 31-35.

Wijayawardhane, N., Shonesy, B.C., Vaglenova, J., Vaithianathan, T., Carpenter, M.,

Breese, C.R., Dityatev, A., Suppiramaniam, V., 2007. Postnatal aniracetam treatment improves prenatal ethanol induced attenuation of AMPA receptor-mediated synaptic transmission. Neurobiol. Dis. https://doi.org/10.1016/j.nbd.2007.03.009

Wilhoit, L.F., Scott, D.A., Simecka, B.A., 2017. Fetal Alcohol Spectrum Disorders: Characteristics, Complications, and Treatment. Community Ment. Health J. https://doi.org/10.1007/s10597-017-0104-0

Windisch, K.A., Czachowski, C.L., 2018. Effects of group II metabotropic glutamate receptor modulation on ethanol- and sucrose-seeking and consumption in the rat. Alcohol. https://doi.org/10.1016/j.alcohol.2017.07.011

Wozniak, J.R., Fuglestad, A.J., Eckerle, J.K., Fink, B.A., Hoecker, H.L., Boys, C.J., Radke, J.P., Kroupina, M.G., Miller, N.C., Brearley, A.M., Zeisel, S.H., Georgieff, M.K., 2015. Choline supplementation in children with fetal alcohol spectrum disorders: A randomized, double-blind, placebo-controlled trial. Am. J. Clin. Nutr. https://doi.org/10.3945/ajcn.114.099168

Xu, Y.J., Liu, P., Li, Y., 2005. Effect of alcohol on brain mitochondria development of mouse embryos. Wei Sheng Yan Jiu.

Zhou, R., Wang, S., Zhu, X., 2010. Prenatal ethanol exposure attenuates GABAergic inhibition in basolateral amygdala leading to neuronal hyperexcitability and anxiety-like behavior of adult rat offspring. Neuroscience. https://doi.org/10.1016/j.neuroscience.2010.07.055

Zirger, J.M., Beattie, C.E., McKay, D.B., Boyd, R.T., 2003. Cloning and expression of zebrafish neuronal nicotinic acetylcholine receptors. Gene Expr. Patterns. https://doi.org/10.1016/S1567-133X(03)00126-1

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 homogeinizados e incubados com o substrato tiramina para uma leitura cinética de duas horas, de acordo com Anichtchik et al., 2006.

RESULTADOS

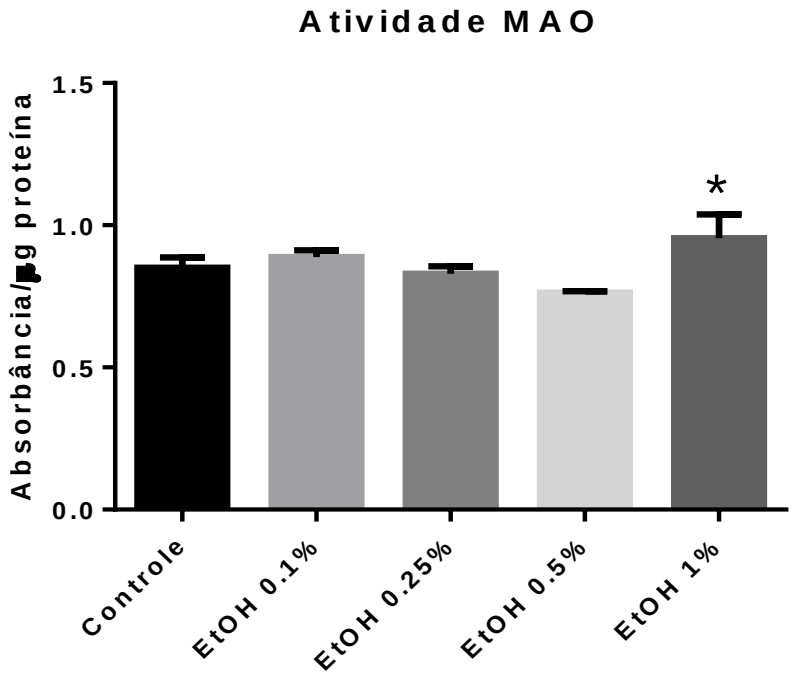


Figura 1: Atividade enzimática da Monoamina oxidase em homogeneizado de cérebro total de *zebrafish* adulto exposto ao etanol na fase embrionária. Dados expressos por média ± S.E.M e analisados pela ANOVA de uma via, seguido pelo pós-teste de Tukey, considerando $p \leq 0,05$ como significante. N= 6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

REFERÊNCIAS

Anichtchik O, Sallinen V, Peitsaro N, Panula P. (2006) Distinct structure and activity of monoamine oxidase in the brain of zebrafish (Danio rerio). J Comp Neurol. Oct 10;498(5):593-610.

Chuhma N, Choi WY, Mingote S, Rayport S. (2009) Dopamine neuron glutamate cotransmission: frequency-dependent modulation in the mesoventromedial projection. Neuroscience. Dec 15;164(3):1068-83.

Filippi A, Mueller T, and Driever W. (2014) vglut2 and gad expression reveal distinct patterns of dual GABAergic versus glutamatergic cotransmitter phenotypes of dopaminergic and noradrenergic neurons in the zebrafish brain. J Comp Neurol. Jun 15; 522(9): 2019–2037.

Hnasko TS, Edwards RH. (2012) Neurotransmitter corelease: mechanism and physiological role. Annu Rev Physiol. 74:225–243.

Johnson MD. Synaptic glutamate release by postnatal rat serotonergic neurons in microculture. (1994) Neuron. 12:433–442.

Pan Y, Kaiguo M, Razak Z, Westwood TJ, Gerlai R (2012). Chronic Alcohol Exposure Induced Gene Expression Changes in the Zebrafish Brain. Behavioural Brain Research, 1; 216(1): 66–76.

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 x 104 $M^{-1}cm^{-1}$, e estão expressos em $nmol.min^{1}$ .mg de $proteína^{-1}$. 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 μg 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 μmol de $AcSCh.min^{-1}$.mg de $proteína^{-1}$.

RESULTADOS

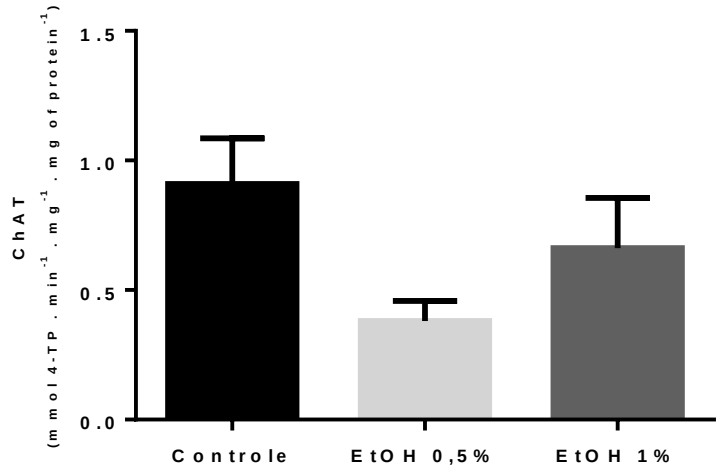


Figura 1: Atividade enzimática da ChAT em homogeneizado de cérebro total de *zebrafish* adulto exposto ao etanol na fase embrionária. Dados expressos por média ± S.E.M e analisados pela ANOVA de uma via, seguido pelo pós-teste de Tukey, considerando $p \leq 0,05$ como significante. N= 5.

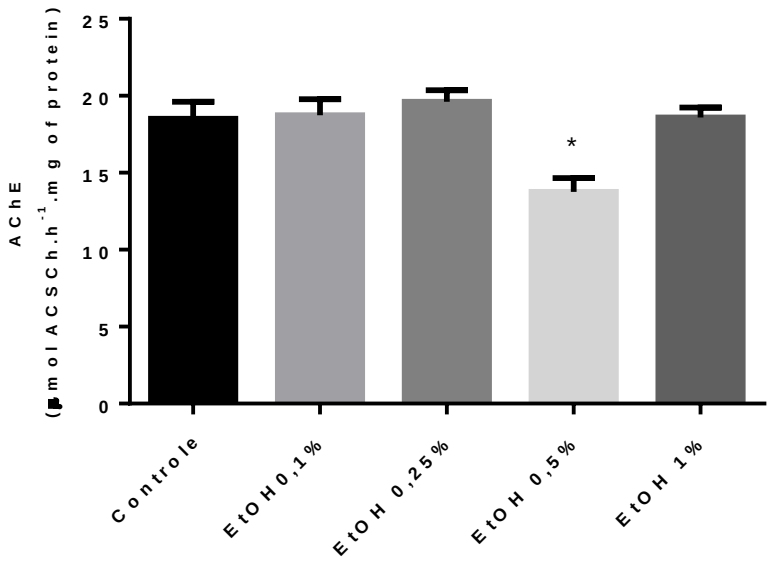


Figura 2: Atividade enzimática da AChE em homogeneizado de cérebro total de *zebrafish* adulto exposto ao etanol na fase embrionária. Dados expressos por média ± S.E.M e analisados pela ANOVA de uma via, seguido pelo pós-teste de Tukey, considerando $p \leq 0,05$ como significante. N= 5 * Diferença significativa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

REFERÊNCIAS

Anglade, P., Larabi-Godinot, Y., 2010. Historical landmarks in the histochemistry of the cholinergic synapse: Perspectives for future researches. Biomed. Res. https://doi.org/10.2220/biomedres.31.1

Antyborzec, I., O'Leary, V.B., Dolly, J.O., Ovsepian, S. V., 2016. Low-Affinity Neurotrophin Receptor p75 Promotes the Transduction of Targeted Lentiviral Vectors to Cholinergic Neurons of Rat Basal Forebrain. Neurotherapeutics. https://doi.org/10.1007/s13311-016-0445-3

Arendt, T., Hennig, D., Gray, J.A., Marchbanks, R., 1988. Loss of neurons in the rat basal forebrain cholinergic projection system after prolonged intake of ethanol. Brain Res. Bull. https://doi.org/10.1016/0361-9230(88)90193-1

Barker, G.R.I., Warburton, E.C., 2009. Critical role of the cholinergic system for object-in-place associative recognition memory. Learn. Mem. https://doi.org/10.1101/lm.1121309

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. https://doi.org/10.1016/0003-2697(76)90527-3

Chao, L. -P, Wolfgram, F., 1973. PURIFICATION AND SOME PROPERTIES OF CHOLINE ACETYLTRANSFERASE (EC 2.3.1.6) FROM BOVINE BRAIN. J. Neurochem. https://doi.org/10.1111/j.1471-4159.1973.tb00078.x

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. https://doi.org/10.1016/0006-2952(61)90145-9

Giovannini, M.G., Lana, D., Pepeu, G., 2015. The integrated role of ACh, ERK and mTOR in the mechanisms of hippocampal inhibitory avoidance memory. Neurobiol. Learn. Mem. https://doi.org/10.1016/j.nlm.2014.12.014

Kumar, R., Långström, B., Darreh-Shori, T., 2016. Novel ligands of Choline Acetyltransferase designed by in silico molecular docking, hologram QSAR and lead optimization. Sci. Rep. https://doi.org/10.1038/srep31247

Wozniak, J.R., Fuglestad, A.J., Eckerle, J.K., Fink, B.A., Hoecker, H.L., Boys, C.J., Radke, J.P., Kroupina, M.G., Miller, N.C., Brearley, A.M., Zeisel, S.H., Georgieff, M.K., 2015. Choline supplementation in children with fetal alcohol spectrum disorders: A randomized, double-blind, placebo-controlled trial. Am. J. Clin. Nutr. https://doi.org/10.3945/ajcn.114.099168

Zirger, J.M., Beattie, C.E., McKay, D.B., Boyd, R.T., 2003. Cloning and expression of zebrafish neuronal nicotinic acetylcholine receptors. Gene Expr. Patterns. https://doi.org/10.1016/S1567-133X(03)00126-1

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.

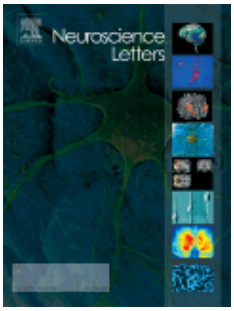
NEUROSCIENCE LETTERS

The rapid communication journal for the neurosciences.

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

- **Description** **p.1**
- **Audience** **p.1**
- **Impact Factor** **p.1**
- **Abstracting and Indexing** **p.2**
- **Editorial Board** **p.2**
- **Guide for Authors** **p.4**



ISSN: 0304-3940

DESCRIPTION

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.

Neuroscience Letters does not publish case reports; papers that are primarily devoted to psychological or philosophical questions, papers that use unvalidated methodology, or that fall outside of the realm of neuroscience will also not be published. In addition, please note:

Clinical research will not be considered, unless the study includes new experimental findings of interest to neuroscientists in general. Clinical studies will not be considered unless they teach a lesson about organization or function of the nervous system. Research on natural products, alternative medicine (acupuncture, etc.) and "traditional medicine" are not appropriate for Neuroscience Letters and will not be considered. Research that provides descriptive data but without a mechanistic link which is tested rigorously with experimental data, will not be considered. Research using only cell lines, for example PC12 cells, will not be considered.

AUDIENCE

Neuroscientists, neurologists

IMPACT FACTOR

2018: 2.173 © Clarivate Analytics Journal Citation Reports 2019

ABSTRACTING AND INDEXING

BIOSIS Citation Index
Chemical Abstracts
Current Contents - Life Sciences
Embase
PubMed/Medline
Pascal Francis
Reference Update
Science Citation Index
Elsevier BIOBASE
Scopus

EDITORIAL BOARD

GUIDE FOR AUTHORS

INTRODUCTION

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.

Neuroscience Letters does not publish case reports; papers that are primarily devoted to psychological or philosophical questions, papers that use unvalidated methodology, or that fall outside of the realm of neuroscience will also not be published. In addition, please note: Clinical research will not be considered, unless the study includes new experimental findings of interest to neuroscientists in general. Clinical studies will not be considered unless they teach a lesson about organization or function of the nervous system. Research on natural products, alternative medicine (acupuncture, etc.) and "traditional medicine" are not appropriate for Neuroscience Letters and will not be considered. Research that provides descriptive data but without a mechanistic link which is tested rigorously with experimental data, will not be considered. Research using only cell lines, for example PC12 cells, will not be considered.

Types of Article

Neuroscience Letters will publish only the following article type:

Research Article: A report of original research which assesses the contribution of the research outcomes to the body of knowledge in a given area.

Mini-Reviews: Reviews are typically invitation-only and are normally published in thematic special issues.
Length of manuscripts should be no more than 5000 words (approximately 6 printed pages). As an approximate guide to authors for judging the length of their paper, the following estimation may be used: heading + abstract = 0.5-0.6 pages; 3 type-written (double-spaced) pages = 1 printed page; (when using a word-processor) 850 words or 5300 characters = 1 printed page; 3 single-column wide or 2 double-column wide figures plus legends = 1 printed page; 3 single-column wide or 2 double-column wide tables = 1 printed page; 17 references = 0.5 printed page.
Authors of invited Special Issue review articles may exceed the 5000 word limit if required, although please check with the issues Guest Editor(s) prior to submission.

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:
- E-mail address
- Full postal address

All necessary files have been uploaded:
Manuscript:
- Include keywords
- All figures (include relevant captions)
- All tables (including titles, description, footnotes)
- Ensure all figure and table citations in the text match the files provided
- Indicate clearly if color should be used for any figures in print

Graphical Abstracts / Highlights files (where applicable)
Supplemental files (where applicable)

Further considerations
- Manuscript has been 'spell checked' and 'grammar checked'
- All references mentioned in the Reference List are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Internet)
• A competing interests statement is provided, even if the authors have no competing interests to declare
• Journal policies detailed in this guide have been reviewed
• Referee suggestions and contact details provided, based on journal requirements

For further information, visit our Support Center.

BEFORE YOU BEGIN

Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

Studies in humans and animals

If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The manuscript should be in line with the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals and aim for the inclusion of representative human populations (sex, age and ethnicity) as per those recommendations. The terms sex and gender should be used correctly.

Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed. The sex of animals must be indicated, and where appropriate, the influence (or association) of sex on the results of the study.

Policy and ethics

The work described in your article must have been carried out in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans* http://www.wma.net/en/30publications/10policies/b3/index.html; *EC Directive 86/609/EEC for animal experiments* http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm; *Uniform Requirements for manuscripts submitted to Biomedical journals* http://www.icmje.org. This must be stated at an appropriate point in the article.

For other policy issues, authors are referred to the policy guidelines of the Society for Neuroscience (see their website http://www.jneurosci.org/misc/itoa.shtml).

Declaration of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Authors must disclose any interests in two places: 1. A summary declaration of interest statement in the title page file (if double-blind) or the manuscript file (if single-blind). If there are no interests to declare then please state this: 'Declarations of interest: none'. This summary statement will be ultimately published if the article is accepted. 2. Detailed disclosures as part of a separate Declaration of Interest form, which forms part of the journal's official records. It is important for potential interests to be declared in both places and that the information matches. More information.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic thesis, see 'Multiple, redundant or concurrent publication' for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in

English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service Crossref Similarity Check.

Preprints
Please note that preprints can be shared anywhere at any time, in line with Elsevier's sharing policy. Sharing your preprints e.g. on a preprint server will not count as prior publication (see 'Multiple, redundant or concurrent publication' for more information).

Use of inclusive language
Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Articles should make no assumptions about the beliefs or commitments of any reader, should contain nothing which might imply that one individual is superior to another on the grounds of race, sex, culture or any other characteristic, and should use inclusive language throughout. Authors should ensure that writing is free from bias, for instance by using 'he or she', 'his/her' instead of 'he' or 'his', and by making use of job titles that are free of stereotyping (e.g. 'chairperson' instead of 'chairman' and 'flight attendant' instead of 'stewardess').

Author contributions
For transparency, we encourage authors to submit an author statement file outlining their individual contributions to the paper using the relevant CRediT roles: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. Authorship statements should be formatted with the names of authors first and CRediT role(s) following. More details and an example

Changes to authorship
Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.
Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

Copyright
Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (see more information on this). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations. If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases.

For gold open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (more information). Permitted third party reuse of gold open access articles is determined by the author's choice of user license.

Author rights
As an author you (or your employer or institution) have certain rights to reuse your work. More information.

Elsevier supports responsible sharing
Find out how you can share your research published in Elsevier journals.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Open access

Please visit our Open Access page for more information.

Elsevier Researcher Academy

Researcher Academy is a free e-learning platform designed to support early and mid-career researchers throughout their research journey. The "Learn" environment at Researcher Academy offers several interactive modules, webinars, downloadable guides and resources to guide you through the process of writing for research and going through peer review. Feel free to use these free resources to improve your submission and navigate the publication process with ease.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's Author Services.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Submit your article

Please submit your article via http://ees.elsevier.com/nsl/
For submission queries, please contact the Editorial Office (NSL@elsevier.com).

Referees

Authors must send the names, addresses and email addresses for 8-10 potential referees that meet the following criteria: potential referees must be experts or active workers in the field, must not be current or prior mentors or collaborators, and must have institutional email addresses (e.g., xx.yy@zz.edu) and not generic email addresses (e.g., xx.yy@163.com or xx.yy@gmail.com). Although the journal does not guarantee these reviewers will be used, the Editors take these suggestions under consideration. These recommendations help the journal speed the editorial process.

PREPARATION

Peer review

This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. More information on types of peer review.

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Subdivision
Divide your article into clearly defined sections. Each subsection is given a brief heading. Each heading should appear on its own separate line. Subsections should be used as much as possible when cross-referencing text: refer to the subsection by heading as opposed to simply " the text" .

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is in very occasional cases appropriate, although in general, Results and Discussion should be presented as distinct sections of the manuscript. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Highlights

Highlights are mandatory for this journal as they help increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the examples here: example Highlights.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Graphical abstract

Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site.

Authors can make use of Elsevier's Illustration Services to ensure the best presentation of their images and in accordance with all technical requirements.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Artwork

Electronic artwork

General points

- Make sure you use uniform lettering and sizing of your original artwork.
- Embed the used fonts if the application provides that option.
- Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.

• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.
• Ensure that color images are accessible to all, including those with impaired color vision.

A detailed guide on electronic artwork is available.
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.
Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.
Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF) or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) in addition to color reproduction in print. Further information on the preparation of electronic artwork.

Figure captions
Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables
Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

References
Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references
As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Data references
This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software
Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley. Using citation plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript. More information on how to remove field codes from different reference management software.

Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following link:
http://open.mendeley.com/use-citation-style/neuroscience-letters
When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice.

Reference style
Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:
Reference to a journal publication:
[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.

Journal abbreviations source
Journal names should be abbreviated according to the List of Title Word Abbreviations.

Video

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly

relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the file in one of our recommended file formats with a preferred maximum size of 150 MB per file, 1 GB in total. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Data visualization

Include interactive data visualizations in your publication and let your readers interact and engage more closely with your research. Follow the instructions here to find out about available data visualization options and how to include them with your article.

Supplementary material

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

Research data

This journal encourages and enables you to share data that supports your research publication where appropriate, and enables you to interlink the data with your published articles. Research data refers to the results of observations or experimentation that validate research findings. To facilitate reproducibility and data reuse, this journal also encourages you to share your software, code, models, algorithms, protocols, methods and other useful materials related to the project.

Below are a number of ways in which you can associate data with your article or make a statement about the availability of your data when submitting your manuscript. If you are sharing data in one of these ways, you are encouraged to cite the data in your manuscript and reference list. Please refer to the "References" section for more information about data citation. For more information on depositing, sharing and using research data and other relevant research materials, visit the research data page.

Data linking

If you have made your research data available in a data repository, you can link your article directly to the dataset. Elsevier collaborates with a number of repositories to link articles on ScienceDirect with relevant repositories, giving readers access to underlying data that gives them a better understanding of the research described.

There are different ways to link your datasets to your article. When available, you can directly link your dataset to your article by providing the relevant information in the submission system. For more information, visit the database linking page.

For supported data repositories a repository banner will automatically appear next to your published article on ScienceDirect.

In addition, you can link to relevant data or entities through identifiers within the text of your manuscript, using the following format: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN).

Mendeley Data

This journal supports Mendeley Data, enabling you to deposit any research data (including raw and processed data, video, code, software, algorithms, protocols, and methods) associated with your manuscript in a free-to-use, open access repository. During the submission process, after uploading your manuscript, you will have the opportunity to upload your relevant datasets directly to *Mendeley Data*. The datasets will be listed and directly accessible to readers next to your published article online.

For more information, visit the Mendeley Data for journals page.

Data in Brief
You have the option of converting any or all parts of your supplementary or additional raw data into one or multiple data articles, a new kind of article that houses and describes your data. Data articles ensure that your data is actively reviewed, curated, formatted, indexed, given a DOI and publicly available to all upon publication. You are encouraged to submit your article for *Data in Brief* as an additional item directly alongside the revised version of your manuscript. If your research article is accepted, your data article will automatically be transferred over to *Data in Brief* where it will be editorially reviewed and published in the open access data journal, *Data in Brief*. Please note an open access fee of 600 USD is payable for publication in *Data in Brief*. Full details can be found on the Data in Brief website. Please use this template to write your Data in Brief.

Data statement
To foster transparency, we encourage you to state the availability of your data in your submission. This may be a requirement of your funding body or institution. If your data is unavailable to access or unsuitable to post, you will have the opportunity to indicate why during the submission process, for example by stating that the research data is confidential. The statement will appear with your published article on ScienceDirect. For more information, visit the Data Statement page.

AFTER ACCEPTANCE

Online proof correction
To ensure a fast publication process of the article, we kindly ask authors to provide us with their proof corrections within two days. Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.
If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.
We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Offprints
The corresponding author will, at no cost, receive a customized Share Link providing 50 days free access to the final published version of the article on ScienceDirect. The Share Link can be used for sharing the article via any communication channel, including email and social media. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's Author Services. Corresponding authors who have published their article gold open access do not receive a Share Link as their final published version of the article is available open access on ScienceDirect and can be shared through the article DOI link.

AUTHOR INQUIRIES
Visit the Elsevier Support Center to find the answers you need. Here you will find everything from Frequently Asked Questions to ways to get in touch.
You can also check the status of your submitted article or find out when your accepted article will be published.

11.6 ANEXO IV: Carta de aceite do CEUA

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

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