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Suelen Baggio

AVALIAÇÃO NEUROQUÍMICA E COMPORTAMENTAL EM PEIXE-ZEBRA ADULTO APÓS A EXPOSIÇÃO AO ETANOL NOS ESTÁGIOS INICIAIS DO DESENVOLVIMENTO

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica, como requisito parcial para obtenção do título de Mestre em Ciências Biológicas – Bioquímica

Orientador: Prof. Dr. Eduardo Pacheco Rico

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“No fim todos os meses planejando e preparando se resumem a 5 minutos de tiroteio.”

Stephen King

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SUMÁRIO

Parte I.

Resumo:

O consumo desenfreado de etanol traz consequências negativas, que envolvem problemas fisiopatológicos e de socialização do indivíduo, além de questões de saúde pública. Seu consumo por mulheres grávidas acarreta em danos ao desenvolvimento e formação cerebral do feto, causando a Síndrome Alcoólica Fetal (SAF). Uma forma mais abrangente deste distúrbio é a FASD (*Fetal Alcohol Spectrum Disorder*), que inclui formas brandas de SAF, bem como qualquer desordem neurológica ou congênita em decorrência do etanol. O objetivo do estudo é avaliar as alterações comportamentais e neuroquímicas em peixe-zebra adulto após a exposição a diferentes concentrações de etanol na fase inicial do seu desenvolvimento. Para a indução desta desordem, utilizamos ovos de peixe-zebra com 24h pós-fertilização, expostos a diferentes concentrações de etanol: 0%, 0,1%, 0,25%, 0,5% e 1%, durante duas horas. Acompanhamos o desenvolvimento destes animais até a fase adulta (4 meses), onde foram realizadas diferentes tarefas comportamentais: exploração do aparato *novel tank* e interação social, além de captação de glutamato, como medida neuroquímica. Como resultados mais relevantes, destacam-se a exploração de todo o aparato pelo grupo controle e uma diminuição concentração-dependente da exploração pelos grupos expostos previamente ao etanol; maior permanência no fundo do *novel tank* nas concentrações elevadas de etanol; uma diminuição concentração-dependente do tempo de permanência próximo ao cardume de acordo com o aumento da concentração de etanol. Quanto às análises bioquímicas, a captação de glutamato mostrou uma diminuição significativa na função de transporte deste neurotransmissor nos animais tratados com concentrações mais elevadas de etanol. A posterior intervenção farmacológica com buspirona reverteu o perfil comportamental previamente observado pelo efeito do etanol. Considerando as análises bioquímicas, a captação de glutamato mostrou uma diminuição significativa na função de transporte deste neurotransmissor nos animais tratados com concentrações intermediárias (0.25 e 0,5%)de etanol. Concluímos que a exposição prévia a diferentes concentrações de etanol na fase embrionária leva a alterações comportamentais na fase adulta, tais como diminuição de exploração frente a novidades, ansiedade e diminuição de interação social.

Abstract:

The excessive consumption of ethanol has negative consequences, which involve pathophysiological and individual socialization issues, and public health issues. Its consumption by pregnant women causes damage to brain development and formation of the fetus, causing fetal alcohol syndrome (FAS). A broader form of this disorder is the FASD (Fetal Alcohol Spectrum Disorder), which includes milder forms of FAS, as well as any neurological disorder or congenital due to ethanol. This project 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. To induce this disorder, we use zebrafish eggs and 24h post-fertilization exposed to different concentrations of ethanol, 0%, 0.1%, 0.25%, 0.5% and 1%, for two hours. We observed the development of these animals to juvenile and adulthood, which will be performed different behavioral tasks: exploration of novel apparatus tank and social interaction, as well as glutamate uptake, as measured neurochemistry. Among the most relevant results, we highlight the exploration of the whole apparatus for the control group and a concentration-dependent decrease of exploration by groups previously exposed to ethanol. It was observed high time spent in the bottom of the *novel tank* in high concentrations of ethanol. Furthermore, elevated spend time close to social interaction chamber was observed, with a dose-dependent decrease of this time in accordance with the increase of ethanol concentration. Further pharmacological intervention with buspirone reversed the behavioral profile previously observed the effect of ethanol. Considering biochemical analysis, glutamate uptake showed a significant decrease in the transport function of this neurotransmitter in animals treated with intermediated doses of ethanol. We conclude that prior exposure to different concentrations of ethanol in infancy leads to behavioral changes in adulthood, such as decreased operating front the news, anxiety and decreased social interaction.

Lista de abreviaturas:

SNC: Sistema nervoso central

SAF: Síndrome alcoólica fetal

FASD: *Fetal alcohol spectrum disorder*

hpf: horas pós-fertilização

dpf: dias pós-fertilização

i.p.: intra-peritonial

NMDA: N-metil D-Aspartato

PKA - proteína cinase A

PKC - proteína cinase C

LTP - Long term potentiation

INTRODUÇÃO

O consumo desenfreado de álcool por parte da população é tema de grande relevância e preocupação para os órgãos de saúde e segurança do mundo todo. No ano de 2012, cerca de 3,3 milhões de pessoas morreram em decorrência deste mal, o que representa 1 em cada 20 óbitos ao redor do mundo (Global status report on alcohol and health 2014). Ao nos determos no panorama do continente americano, a cada 100 mil mortes, 12 poderiam ser evitadas se não houvesse consumo de álcool, é o que mostra uma pesquisa realizada pela Organização Pan-Americana da Saúde (PAHO). Por ano, o álcool aparece na causa de morte de 80 mil pessoas nas Américas, sendo o Brasil dono da quinta maior taxa. Dados do Levantamento Domiciliar sobre o Uso de Drogas Psicotrópicas no Brasil, realizado em 2005, mostram que o alcoolismo é uma doença que atinge 5,8 milhões de pessoas no país (Carlini et al., 2005). O alcoolismo representa tema de grande interesse ao Ministério da Saúde, pois são gastos milhões de reais ao ano em tratamentos e internações de pacientes dependentes. Segundo dados da cartilha do Álcool do ano de 2012, seu consumo por pelo menos uma vez ao ano atinge 52% dos brasileiros, sendo a região sul a situação mais preocupante, onde 11% dos homens bebem diariamente, diferentemente de outras regiões, cujo valor não passa dos 6% (Laranjeira et al., 2012).

Não somente o indivíduo adulto pode ser atingido pelo uso abusivo do álcool, mas também crianças e jovens causando consequências no desenvolvimento do SNC. Seu consumo elevado em mulheres grávidas acarreta em danos ao desenvolvimento e formação cerebral no período de gestação, causando a denominada Síndrome Alcoólica Fetal (SAF), com prevalência de até 5% na

população infantil mundial (Stratton et al., 1996). Entre os efeitos maléficos ao feto, encontram-se: anormalidades neurológicas; disfunções comportamentais; atrasos no desenvolvimento; deficiências intelectuais e determinadas características faciais, como microcefalia, micrognatia e presença de pregas epicânticas (Stratton et al., 1996; May et al., 2009). Entre as ações promovidas pelo consumo excessivo de etanol também 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 parâmetros de neurodegeneração por excitotoxicidade associados ao consumo de etanol (Sampson et al., 1997). Além disso, creditam-se tais alterações comportamentais, entre as quais, hiperatividade, ansiedade e aumento da agressividade ao impacto do etanol durante o desenvolvimento sobre o SNC (Esel, 2006).

Ao longo da história da humanidade, o álcool esteve constantemente presente. Usado em rituais de cura, como tratamento para determinados males, fonte de prazer e indispensável em celebrações e vitórias (Warren, 2015). Demorou-se milhares de anos para se compreender seus possíveis malefícios, apenas em 1724, médicos de Londres começaram a relacionar o aumento no número de doenças e mortes com o alto consumo de bebidas alcoólicas, o que ficou conhecida como *"London Gin Epidemic"* (Warner & Rosett, 1975; Warren & Hewitt, 2009). Quanto ao entendimento da SAF, apenas no começo do século 19, indícios de que o consumo tanto de bebidas destiladas, quanto fermentadas, pelas mães grávidas, poderia estar por trás de certas anomalias vistas em recém nascidos, como nos casos extremos de ciclopia e mortalidade por mal-formação, foram registrados (Sullivan, 1899). Contudo, pouco ou nenhum impacto foi gerado na sociedade, que não diminuiu seu

consumo etílico, nem buscou formas de prevenção para este mal, ao contrário, continuou a indicar altas doses de etanol como forma de evitar partos prematuros. Foi apenas no final da década de 70, que pesquisadores do National Institute on Alcohol Abuse and Alcoholism (NIAAA), unindo pesquisas em humanos e modelos animais, criaram uma série de diretrizes de aconselhamento com o objetivo de estabelecer doses seguras para o consumo de bebidas alcoólicas por mulheres grávidas. Após mais estudos, em 1981, determinou-se que nenhuma quantia de álcool era segura para o consumo de gestantes (U.S. Surgeon General's Advisory on Alcohol and Pregnancy, 1981).

Infelizmente, nos dias atuais, ainda observa-se o descaso perante este grave problema não somente por parte da população, mas também por órgãos públicos que são indiferentes aos diversos estudos e recomendações publicadas nos últimos anos (Warren, 2015). Apenas em fevereiro de 2015, a Grã-Bretanha estabeleceu que se indicasse nos rótulos de bebidas alcoólicas a proibição de consumo para gestantes, mas apenas para aquelas que estão no primeiro trimestre gestacional (Royal College of Obstetricians and Gynecologists, 2015). Nos EUA, de acordo com o Centro para Controle e Prevenção de Doenças, 7,6% das mulheres grávidas bebem ocasionalmente durante a gestação, enquanto no Brasil este índice podem chegar a quase 25%. Tais dados podem não ser um retrato fiel da realidade, já que poucas mulheres admitem que fazem uso de álcool e drogas durante a gravidez, escondendo esta informação inclusive dos profissionais de saúde. Faltam políticas de esclarecimento e prevenção no Brasil, onde a SAF pode ser negligenciada até mesmo pelos médicos que atendem as gestantes e lhes recomendam que o consumo de pequenas doses esporádicas de etanol não são prejudiciais à saúde do bebê. A melhor recomendação, frente aos muitos

estudos, alguns inconclusivos, é a total abstinência durante a gestação, como forma de prevenir qualquer problema morfológico ou neurológico (Morton et al., 2014; Diaz et al., 2014; Fernandes et al., 2015).

A SAF apresenta uma série de variantes, desde formas mais severas, com graves alterações morfológicas e cognitivas, até formas mais brandas, caracterizadas por alterações comportamentais mais sutis. Seus diferentes graus de severidade dependem de quanto, quando, por quanto tempo e com que frequência o feto é exposto ao etanol ao longo de seu desenvolvimento (Cartwright & Smith, 1995; Maier et al., 1997). O conjunto de alterações das formas intermediárias de SAF é denominado de Desordens do Espectro Alcoólico Fetal (FASD), que por não apresentar alterações morfológicas evidentes acabam sendo diagnosticados incorretamente, como casos de autismo ou hiperatividade, ou mesmo não sendo diagnosticados (Sampson et al., 1997; May et al., 2009). Contudo, as mudanças neurológicas e comportamentais persistem através de problemas de interação social, comportamento ansioso e dificuldades de aprendizagem (Buske & Gerlai, 2011; Rasmussen et al., 2011; Fernandes et al., 2015).

Apesar do conhecimento acumulado nas últimas décadas sobre SAF, fatores limitantes impedem maiores avanços. Modelos alternativos em organismos, tais como a *Drosophila* tem sido utilizada como uma excelente ferramenta para estudar desenvolvimento e alterações genéticas, mas estes apresentam menor homologia genética com humanos por se tratarem de invertebrados (Sovik & Barron, 2013). Modelos de roedores que sobrepõem tais limitações são de difícil e custosa varredura genética além de possuírem desenvolvimento intra-uterino, dificultando o acompanhamento de parâmetros do desenvolvimento neural sem procedimentos

invasivos. Além disso, muitos estudos que enfocam a avaliação de alterações durante o desenvolvimento em roedores implicam na eutanásia do animal. Pesquisas com humanos tendem a ser custosas e também muito invasivas, ao passo que requerem um contínuo acompanhamento pré-natal e incorrem na questão de acompanhar mães que bebem, ao passo que se sabe dos muitos malefícios deste ato. Neste contexto, o peixe-zebra mostra-se como excelente modelo animal, ao passo em que reúne a complexidade sistêmica de vertebrados à praticidade e simplicidade de manuseio e manutenção realizada com invertebrados (baixo custo, utilização de pouco espaço, fácil reprodução em laboratório, assim como barata varredura genética) (Fernades & Gerlai, 2009; Gerlai, 2015). Outra característica é o seu desenvolvimento externo e a presença de ovos translúcidos permitindo em seu rápido ciclo de vida acompanhar em detalhes o desenvolvimento embrionário. Esses atributos permitiram através do peixe-zebra novos avanços para a investigação do impacto do etanol sobre o desenvolvimento. Estudos recentes têm utilizado o peixe-zebra como modelo de desenvolvimento de SAF, no qual os ovos são diretamente expostos em solução de etanol nas primeiras horas do desenvolvimento, observando-se uma série de alterações, como microftalmia, ciclopia, comprimento corporal diminuído, anormalidades cardíacas e neurodegeneração (Reimers et al., 2004; Bilotta et al., 2004).

Uma série de testes metodológicos estão bem descritos na literatura para se avaliar o padrão comportamental de peixe-zebra. Quando se deseja avaliar a interação social do animal alvo com os seus coespecíficos pode-se optar por duas tarefas básicas: comportamento de cardume ou preferência social. No primeiro, o cardume como um todo é objeto da análise, usando-se a distância mantida entre os

animais como medida de interação, geralmente todos os animais fazem parte de um determinado grupo, ou são animais controles ou tratados (Miller & Gerlai, 2007; Saverino & Gerlai, 2008). No segundo caso, um único animal por vez é selecionado para o teste e posto em um aquário central, tendo como opções nadar para próximo do cardume (que está localizado em um aquário lateral) ou em direção ao aquário vazio (posto do outro lado do aquário central), evitando interagir com seus coespecíficos (Pham et al., 2012). Neste teste é possível isolar o comportamento de um animal específico tratado frente a um cardume controle, mimetizando mais fielmente a realidade, onde um indivíduo com limitações ou algum distúrbio tende a se inserir em um meio dito normal. Para análise de comportamento ansioso a tarefa de *novel tank* tem se destacado (Levin et al., 2007; Cachat et al., 2010; Rosemberg et al., 2011; Stewart et al., 2013). Trata-se de um aquário trapezoidal, onde é possível avaliar a locomoção e a exploração do animal tanto vertical quanto horizontalmente, frente a novidade. A tendência natural é que uma situação de novidade seja estressante para o animal, aumentando seu comportamento ansioso, que pode ser mensurado por tais medidas: maior tempo gasto no fundo do aparato e maior latência para adentrar o topo do aparato (Ibrahim et al., 2014). Estas tarefas servem bem ao propósito de analisar os déficits cognitivos e sociais em peixes-zebra expostos ao etanol na fase embrionário do desenvolvimento, já que as alterações comuns a SAF envolvem problemas de interação social e comportamento ansioso.

Os primeiros estudos conduzidos com peixe-zebra como modelo de SAF utilizaram altas doses de etanol por longos períodos de exposição, gerando grandes deformações morfológicas na estrutura óssea, no comprimento da cauda e no sistema visual, com casos extremos de ciclopia, além de problemas cardíacos e de

locomoção (Arenzana et al., 2006; Bilotta et al., 2004; Bilotta et al., 2002; Carvan et al., 2004; Loucks & Carvan, 2004; Reimers et al., 2004). Porém, as taxas de mortalidade eram altíssimas, devido as alterações causadas pelo etanol que impossibilitam a sobrevivência das larvas por um período mais longo, impedindo o acompanhamento do desenvolvimento do animal até a fase adulta. Em 2009, Fernandes & Gerlai publicam um novo protocolo, utilizando baixas concentrações (0.25 – 1% v/v) de etanol, com um período de duas horas de exposição. O resultado é uma forma mais branda da SAF, a FASD, sem alterações físicas perceptíveis, nem problemas locomotores, apenas alterações de perfil comportamental, o que possibilita o acompanhamento destes animais até a fase adulta. Aos seis meses de vida, os peixes adultos são morfologicamente semelhantes aos controles, contudo apresentam diminuição na interação social, além de alterações no sistemas dopaminérgicos e serotoninérgicos (Chatterjee & Gerlai, 2014). Tais alterações estendem-se além, podendo ser observadas até em animais de dois anos de vida (Fernandes et al., 2015).

As alterações comportamentais observadas tanto em humanos portadores da síndrome como em modelos animais estão possivelmente relacionadas a mudanças no SNC. Tanto a expressão quanto a funcionalidade de vários neurotransmissores e seus metabólitos estão alterados em peixes expostos ao etanol, como diminuição nas quantidades de glutamato, aspartato e GABA (Pan et al., 2012). Pequenas concentrações de etanol podem inibir a atividade de receptores glutamatérgicos, como o NMDA, o que está relacionado a problemas cognitivos, perdas neuronais e dificuldades de aprendizado (Gonzalez & Jaworski, 1997). Em modelos de roedores, observou-se aumento na transmissão sináptica e na diferenciação neuronal

glutamatérgica, relacionada com aumento no comportamento ansioso (Baculis & Valenzuela, 2015; Kim et al., 2010). Os prejuízos cognitivos estão relacionados com perda de massa encefálica, ocasionada pela neurodegeneração apoptótica através de dois mecanismos: bloqueio dos receptores de glutamato NMDA e ativação excessiva de receptores de GABA (Olney et al., 2001).

Muitos estudos identificaram os sistemas de neurotransmissão como a base das desordens de neurodesenvolvimento, como é o caso da SAF. Invariavelmente, as perturbações estão relacionadas com comunicação neuronal alterada que pode ser explicada por deficiências no desenvolvimento das sinapses. A exposição ao etanol durante o desenvolvimento causa defeitos tanto na estrutura como na função das sinapses que perduram a longo prazo, o que pode ser causado por conexões incorretas, já que a mortalidade dos neurônios faz com que os demais percam seus alvos e conectem-se incorretamente (Valenzuela et al., 2011). Todos estes processos estão ligados ao efeito básico do etanol: desbalanço entre os sistemas inibitório e excitatório, fazendo com que o próprio SNC busque o equilíbrio por mudanças compensatórias em substratos neuroquímicos.

A exposição embrionário ao etanol, principalmente no primeiro trimestre gestacional, é capaz de diminuir a diferenciação, migração e crescimento axonal de neurônios serotoninérgicos. Os mecanismos por trás destes efeitos possivelmente envolvem 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. Estudos com roedores mostraram a relação entre o etanol e defeitos nos processos de facilitação da respiração dependentes de serotonina em situações de baixo oxigênio,

o que por consequência, pode explicar os casos de morte súbita em crianças com FASD (Kinney et al., 2003; Kervern et al., 2009). O perfil comportamental também é afetado, ao passo que alterações no sistema serotoninérgico estão relacionados com aumento no níveis de hormônios de estresse, ansiedade e depressão (O'Connor et al., 2006).

Para o tratamento destas alterações comportamentais tem se buscado novas intervenções farmacológicas, utilizando medicamentos já estabelecidos para novos fins na pesquisa básica, com o intuito de elucidar seus efeitos e mecanismos. A buspirona é um ansiolítico não-sedativo, que 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. Seu resultado final é a supressão do sistema serotoninérgico e aumento do noradrenérgico e dopaminérgico (Loane & Politis, 2012). Estudos demonstram 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, 1997) e possivelmente em pacientes de FASD. Em estudos prévios com peixe-zebra, 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 (Maximino et al., 2013) e *novel tank* (Bencan et al., 2009; Maximino et al., 2013).

Objetivos:

Esta dissertação de mestrado tem como objetivo focar em animais adultos de um modelo de FASD em peixe-zebra, procurando compreender as manifestações comportamentais e uma breve análise das alterações nos substratos neuroquímicos, ocasionadas pela exposição ao etanol na fase inicial do desenvolvimento, aliado a uma posterior intervenção farmacológica com buspirona, droga utilizada com o objetivo de reverter as alterações comportamentais e neuroquímicas observadas na primeira parte dos experimentos. Utilizando-se animais adultos (4 meses) expostos a distintas concentrações de etanol (0.1; 0.25; 0.5 e 1%) durante a fase embrionária, propomo-nos a realizar os objetivos listados a seguir.

Objetivos específicos:

- Analisar o perfil comportamental de locomoção e exploração de um aparato frente a novidade (comportamento ansioso) – teste *Novel Tank*;
- Analisar o perfil comportamental de interação social, buscando possíveis alterações ocasionadas pelo etanol nas habilidades sociais dos animais, já que se trata de um animal tipicamente social (cardume);
- Realizar a captação de glutamato, com o objetivo de analisar as possíveis consequências no controle do transporte deste neurotransmissor após a exposição prévia ao etanol;
- Avaliar a intervenção farmacológica da buspirona, com o objetivo de verificar seu efeito ansiolítico frente ao possível comportamento ansioso, bem como sua associação com o padrão de interação social.

Parte II.

RESULTADOS:

CAPÍTULO I

Artigo a ser submetido para a revista: Progress in Neuro-Psychopharmacology & Biological Psychiatry

Anxiety-like Behavior is Related to Social Avoidance Phenotype of Adult Zebrafish FASD and is Modulated by Buspirone.

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, Brasil.

[c] Department of Psychology University of Toronto Mississsauga 3359 Mississauga Road North, Mississauga, Ontario, L5L 1C6 Canada.

***Corresponding author:**

- Suelen Baggio, Departamento de Bioquímica, ICBS, Rua Ramiro Barcelos, 2600-Anexo. CEP: 90035-003. Porto Alegre, RS, Brazil. Tel.: +55 51 3316-5555; FAX.: +55 51 3316-5540.

E-mail: suzy-gfbp@hotmail.com

- Eduardo Pacheco Rico, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense – UNESC, Av. Universitária, 1105, Bairro Universitário, 88806-000 Criciúma, SC, Brasil. E-mail: eduprico@gmail.com

Abstract

Fetal Alcohol Spectrum Disorders (FASD) are a syndrome characterized by neurological and behavioral impairments. A recently discovered hallmark of FASD is impaired social behavior. In order to understand whether avoidance of social interaction typical of FASD patients is related to elevated anxiety, we analyzed both anxiety and social responses using a zebrafish FASD model. Furthermore, we also examined whether anxiolytic drug treatment may alter these responses, and pre-treated embryonic alcohol exposed zebrafish with buspirone, an anxiolytic drug. We exposed zebrafish embryos to low concentrations of ethanol (0.1%; 0.25%; 0.5% and 1% v/v) for 2 h at, 24 hours post-fertilization, to mimic the most prevalent milder FASD cases and investigated the ensuing alterations in the adult, 4 month old, zebrafish. We studied social interaction in the social preference task and anxiety in the novel tank task. In the 0.5% ethanol group, we observed a reduction of time spend in the conspecific zone compared to control. This alteration was even more robust in the 1% ethanol group. Even though the 1% ethanol group was the only one to present a higher latency to the top in the novel tank task, all animals treated with ethanol during development presented a higher time in the bottom zone compared to control. Animals from the 1% ethanol group pretreated with buspirone (25 mg/kg) presented a similar profile as control group and no preference for any specific zone in the preference social task. In summary, zebrafish treated with 0.5% or 1.0% ethanol during embryonic development exhibited reduced social preference and increased anxiety-like responses, alterations that were reversed by buspirone treatment at the adult stage. Our

results imply that buspirone may be considered a treatment option for humans suffering from FASD.

Keywords: Fetal Alcohol Syndrome Disorder; zebrafish; anxiety; social preference; buspirone

Abbreviations: FASD (Fetal Alcohol Syndrome Disorder); FAS (Fetal Alcohol Syndrome); hpf (hours post-fertilization); EtOH (ethanol).

Highlights

Fetal Alcohol Syndrome Disorder and anxiety-like behavior in adult zebrafish.

Social Avoidance Phenotype of Adult Zebrafish FASD is Modulated by Buspirone.

Anxiety-like Behavior is Related to Social Avoidance Phenotype of Adult Zebrafish FASD.

1. Introduction

Fetal alcohol spectrum disorders (FASDs) are among the most troubling alcohol related diseases. FASD results from alcohol exposure during fetal development and ranges from full “fetal alcohol syndrome” (FAS) to milder forms of the disease. FAS is associated with observable anatomical abnormalities while the milder cases often present only behavioral alterations. 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 (May et al., 2009; Sampson et al., 1997). Due to the high prevalence of the disease, and due to the lifelong suffering it causes, it is important to understand the long-term impact of embryonic alcohol exposure and how adults with the disease may be treated, and how the quality of their life may be improved. These goals may be achieve by studying the biological consequences of fetal alcohol exposure.

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 added in this fish in a simple and controlled manner. The developing embryo inside the egg may be exposed precisely to a concentration of alcohol by immersing the eggs into the alcohol solution for a specific period of time and at any desired developmental stage (Bradfield et al., 2006; Fernandes & Gerlai, 2009; Mahabir et al., 2013). The immersion may be performed using a large number of subjects in a uniform manner at the same time. Briefly, the zebrafish represents a reductionist approach that allows investigating the effects of alcohol on

embryonic development and later in adult without the complicating aspects of mammalian maternal physiology and parental care.

Initially, zebrafish was used only to study FAS by using higher alcohol concentrations and/or by administering alcohol for prolonged periods. The resulting abnormalities included robust anatomical deformities, cyclopia, heart defects, and/or increased mortality; alcohol induced developmental abnormalities that recapitulated human FAS, the most severe form of FASD (Arenzana et al., 2006; Bilotta et al., 2004; Bilotta et al., 2002; Carvan et al., 2004; Loucks & Carvan, 2004; Reimers et al., 2004). However, the milder form of FASD is three times more prevalent than the severe forms of the disease (Sampson et al., 1997). Thus, it is crucial to model the milder forms of the disease. In this context, subsequent studies the developing eggs of zebrafish were exposed to smaller doses of alcohol (0.25 – 1.00%), and only for 2 h. For example Fernandes & Gerlai (2009) and Buske & Gerlai (2011) used the above dosing regimen at 24 h hpf, a developmental stage when the zebrafish brain undergoes major development. Importantly, in these latter studies, fish from even the highest alcohol concentration group appeared healthy, and had no clearly observable anatomical abnormalities. Nevertheless, a significantly abnormal behavioral alteration was detected. Similarly to the human condition, zebrafish exposed to low concentrations of ethanol during embryonic development exhibited reduced shoaling behavior, paralleling the impaired social behaviors found in FASD patients suffering from the milder forms of the disease (Buske & Gerlai, 2011; O'Connor et al., 2006; Rasmussen et al. 2011; Fernandes et al. 2015). 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. We hypothesized that one of the reasons for impaired social behavior is altered anxiety. We also hypothesized that if altered anxiety explains the impaired social behavior, then anxiolytic drugs may alleviate the observed social impairment in this zebrafish model of FASD.

Anxiety disorders are among the most commonly reported problems in children and adults with FASD (Caldwell et al., 2008; Roebuck et al., 1999; Streissguth & O'Malley, 2000; Steinhausen et al., 2003). Anxiety is often induced by diffuse, aversive contexts, including the novel nature of a test stuation. Novelty is defined as a new or unfamiliar experience. Due to the lack of experience in the new situations, the brain identifies novel situations as stressful and this induces anxiety-like behaviors observable in humans and other non-human animals including the zebrafish (ref). For the zebrafish, the novel tank diving test has been often utilized to induce and quantify novelty-associated behavioral stress/anxiety-like responses. The task is based on the initial tendency of the fish to dive to the bottom and gradually swim to the upper areas of the new tank. This test can be used for investigating pharmacological modulators of anxiety-like phenotypes in adult zebrafish (Bencan et al., 2009; Blaser & Rosemberg, 2012; Rosemberg et al., 2011). Moreover, the novel tank test can be used to assess the complete exploratory and locomotor behavior of zebrafish. This perspective allows for the assessment substances that promote anxiogenic-like effects. It has been employed to investigate the anxiolytic- effect at low doses and sedative effect at higher concentrations of ethanol in adult zebrafish (Blaser & Rosemberg, 2012; Stewart et al., 2013; Wong et al., 2010). Importantly, anxiety related phenotypical responses have not been thoroughly investigated in the zebrafish FASD model of Fernandes & Gerlai (2009).

A possible way to alter anxiety-like responses is to employ a pharmacological tool. Buspirone is an anxiolytic drug that has been found to be efficacious in a range of human and nonhuman species, including the zebrafish. In the latter, its effects have been demonstrated in geotaxis and scototaxis tests (Maximino et al., 2013). Buspirone's anxiolytic effect has been recognized in treating disorders associated with ethanol abuse (Leggio et al., 2014; Gebauer et al., 2011).

In the current study, we investigate whether the mild FASD zebrafish model is associated with altered anxiety, and whether behavioral alterations seen in this zebrafish model, including the embryonic alcohol exposure induced impairment of social behavior, may be reversed by treatment with the anxiolytic drug, buspirone.

2. Material and methods

2.1. Animals

Adult zebrafish (*Danio rerio*; 10 to 12 months old, from a heterogeneous wild-type stock (standard short-fin phenotype) were obtained from a local commercial supplier (Delphis, RS, Brazil). The fish (mixed male/female) were housed in a re-circulating system maintained with mechanical and biological filtration at a temperature of 28 °C, pH of 7.4 and a conductivity of 500µS (system water). Breeding arrays were used for obtaining fertilized eggs. The 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 Figure 1. Eggs of zebrafish were collected 2.5 hpf. Approximately 500 fertilized eggs were randomly selected and were 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 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 hours 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 (Fernandes & Gerlai, 2009). The embryos were incubated on Biological Oxygen Demand B.O.D. at 28^{o}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 artemia.

Zebrafish remained in these holding tanks until behavioral experiments, which were conducted after the fish reached 4 months of age (mature young adults, 50 to 50% male–female). The sample sizes of treated fish were of at least eight animals per group.

2.3. Buspirone

Buspirone hydrochloride (CAS Number 33386-08-2., Sigma, St. Louis, USA) was dissolved in saline solution (NaCl 0,9%). The drug was injected intraperitoneally at a volume of 1µL/g, according to the weight of the fish at a dose of 25mg/kg. Injected fish were returned to their holding tank for a 10 min acclimation period, after which they were subjected to one of two behavioral tests as described below. Briefly, 50 fish were tested in the open tank test and another 50 fish in the social preference test (Maximino et al., 2013).

2.4. 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. Adjacent to the conspecific side was another tank that contained five 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 (Pham et al., 2012). Furthermore, behavior was quantified at a rate of 30 frames/s, using video-tracking software (ANY-maze® software (Stoelting CO, USA), that 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. Experimental fish are also expected to exhibit increased number of entries to the conspecific side. This social preference could be altered by anxiety and has been shown to be impaired by embryonic alcohol exposure.

2.5. 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. The tank was divided into three equal virtual horizontal areas (bottom, middle, and top), with five sections per area as previously described (Rosemberg et al., 2011). 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 traveled; 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 (Rosemberg et al., 2011; Wong et al., 2010). Increased time spent at the top of the tank has been interpreted as indication of reduced anxiety (Egan et al., 2009; Levin et al, 2007;

Mathur & Guo, 2011). The exploratory profile of fish was estimated by quantifying the horizontal and vertical movement and location parameters as described by Rosemberg et al (2011). 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 (Rosemberg et al., 2011, 2012).

2.6. Statistics

Parametric data were expressed as the means ± standard error of the mean (S.E.M.) and analyzed by repeated-measures analysis of variance (ANOVA) using Bonferroni's post hoc test. Differences were considered statistically significant at $p \leq 0.05$. Experiments related to buspirone it was analyzed by two-way (ANOVA) using Bonferroni's post hoc test.

3. Results

There were no physical abnormalities, e.g. reduced eye diameter, delayed eggs hatching abnormal tail anatomy observed during the developmental in the fish treated with alcohol for any concentration group. These results are in accordance with findings described previously by Fernandes & Gerlai (2009) who found no physical abnormalities in the developing or in the adult fish exposed embryonically to concentrations of alcohol similar to those employed in the current study.

To evaluate the behavioral profile of the animals in the social preference test we analyzed three parameters: distance travelled, number of entries and time in

each zone (**Fig.2**). The distance experimental fish of the groups EtOH 0.5 and 1% travelled in the conspecific zone was significantly lower compared to that of fish of the control group (**Fig. 2A**) (one-way ANOVA, $F_{[4, 39]} = 7.503$, $p<0.0001$; Bonferroni test $p<0.05$). The distance travelled in the center zone of the groups EtOH 0.1 and 0.25% were higher compared to control and EtOH 0.25% is the highest (**Fig. 2B**) (one-way ANOVA, $F_{[4,39]}= 26.01$, $p<0.0001$; Bonferroni 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. 2C**) (one-way ANOVA, $F_{[4, 39]} = 24.18$, $p<0.0001$; Bonferroni test $p<0.05$). **Figure 2D** depicts total distance travelled pooled for 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. 2E**) (Kruskall-Wallis test, p 0.0003; Dunn's Multiple Comparison test, $p<0.05$). The number of entries in the center zone of the group EtOH 0.25% was higher compared to control (**Fig. 2F**)) (Kruskall-Wallis test, p 0.0010; 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. 2G**)) (Kruskall-Wallis test, $p<0.0001$; Dunn's Multiple Comparison test, $p<0.05$). **Figure 2H** shows that fish of the EtOH 0.25% exhibited the highest value. The results showed a significant alcohol concentration dependent reduction of time spent in the conspecific zone for the EtOH 0.25, 0.5 and 1%. Groups (**Fig. 2I**) (one-way ANOVA, $F_{[4,39]}= 17.10$, $p<0.0001$; Bonferroni test $p<0.05$). The time spend in the center zone by fish of groups EtOH 0.25 and 0.5% was significantly higher compared to control (**Fig. 2J**) (one-way ANOVA, $F_{[4, 39]} = 6.154$, $p<0.0001$; Bonferroni test $p<0.05$). The time spend in the empty zone by fish of the groups EtOH 0.5 and 1% was

significantly higher compared to control (**Fig. 2K**) (one-way ANOVA, $F_{[4, 39]}$ = 26.99, $p<0.0001$; Bonferroni test $p<0.05$). In **Figure 2L** we depict the data stratified according to the time spend in each area. The average speed of fish of the group EtOH 0.25% was highest as compared to fish of all other groups (**Fig. 2M**) (one-way ANOVA, $F_{[4, 39]}$ = 5.963, $p<0.0001$; Bonferroni test $p<0.05$).

The spatio-temporal analysis of behavior in the social preference test of a representative animal of each group is shown in figure 3. The occupancy plot shown in this figure represents fish of each group. The results suggest that fish of the control group established a homebase and spent more time near to the conspecific zone (**Fig. 3A**). Fish of the EtOH 0.1% group also appeared to spend more time near the conspecific zone but their 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 more dispersed, 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 intensified 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 (**Fig. 4A**) (one-way ANOVA, $F_{[4, 39]}$ = 1.765, $p<0.0001$; Bonferroni test $p<0.05$) and mean speed (**Fig. 4B**) (one-way ANOVA, $F_{[4, 39]}$ = 0.5253, $p<0.0001$; Bonferroni test $p<0.05$). It related that the analysis of

classical endpoint parameters is relevant to evaluate and identify a display of like-anxiety behavior in this apparatus. Control fish (**Fig. 5A**) showed a preference for the bottom with some exploration of the top replicating the same spatio-temporal behavioral profile as previously described (Ibrahim et al., 2014; Rosemberg et al., 2011). 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. 5B**). 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. 5C**). 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. 5D**). 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. 5E**). The above results suggest differences in anxiety levels among fish of the treatment groups, i.e. embryonic alcohol induced modification of anxiety-like behaviors. To explore the possibility that the anxiety-like responses may be modified, ameliorated, we decided to employ a pharmacological approach. We chose buspirone, an anxiolytic drug, which has previously been demonstrated to be efficacious in adult zebrafish (Maximino et al., 2013; Gebauer et al., 2011).

Fish of the EtOH 1% group took significantly longer to enter the top segment of the tank as compared to control fish (**Fig. 6A**) (two-way ANOVA, Column factor $F_{[1, 60]} = 177.5$, $p<0.0001$; Bonferroni test $p<0.05$). Also, fish of the EtOH 0.1, 0.25 and 1% groups spent significantly shorter duration of time in the top segment compared to control fish compared to control (**Fig. 6B**) (two-way ANOVA, Column factor $F_{[1, 68]} = 103.3$, $p<0.0001$; Bonferroni test $p<0.05$).

Conversely, fish exposed to alcohol during their embryonic development remained in the bottom segment of the tank for significantly longer duration of time compared to control (**Fig. 6D**) (two-way ANOVA, Column factor $F_{[1, 63]}$ = 35.22, $p<0.0001$; Bonferroni test $p<0.05$). The distance travelled in the top of all the groups pretreated with ethanol were lower compared to control (**Fig. 6C**) (two-way ANOVA, Column factor $F_{[1, 78]}$ = 188.2, $p<0.0001$; Bonferroni test $p<0.05$). Importantly, buspirone was found to significantly reverse the embryonic alcohol exposure induced behavioral changes (**Fig. 6**). For example, fish of the 1,0% EtOH group exhibited a significant decrease of time spent in top area. In this parameter, this group when receive buspirone, presented a differentiated profile manifesting a similar aspect to control group. (**Fig. 6A**). The occupancy plot depicts (**Fig. 7**) the spatial distribution of the experimental fish.

In order to investigate whether social behavior was diminished by high doses of ethanol and whether this change was associated with anxiety, the effects of buspirone was examined on fish exposed to alcohol during their embryonic development. The results shows that the profile of social interaction previously observed was modified (**Fig.8 A-C**) (one-way ANOVA, $F_{[4, 39]}$ = 0.5823 (A), $F_{[4, 39]}$ = 0.1146 (B), $F_{[4, 39]}$ = 0.7277 (C), $p<0.0001$; Bonferroni test $p<0.05$). The occupancy plot elucidate the distribution of explorations in apparatus for all concentration tested (**Fig.8 D-H**). These findings show that there is no significant difference in time spent in each compartment.

4. Discussion

In the current study we used low concentrations of ethanol, employed at 24 hpf for 2 h, to model a milder form of human FAS in the zebrafish. We

measured the impact of this alcohol treatment on social preference and novelty induced anxiety in the adult fish. Furthermore, to investigate whether altered anxiety may underlie the observed embryonic alcohol induced behavioral changes, we took a pharmacology approach and employed a known anxiolytic drug, buspirone.

Our results replicated those of previous studies showing significant impairment of social behavioral responses to conspecifics induced by the embryonic alcohol treatment. Furthermore, they also suggested significant increases in anxiety-like responses in the alcohol treated fish. Most importantly, our results also demonstrated a significant buspirone effect that was in the opposite direction to that of embryonic alcohol.

The interpretation of these results, however, are somewhat complex. The buspirone effects were embryonic alcohol dose independent in on the anxiety-like behavioral responses tested in the novel tank. These latter results imply that buspirone may not have specifically counteracted the alcohol effects, but rather had a general performance altering function. While this effect may be explained by generalized reduction of anxiety, which affected the control fish as much as the alcohol treated fish, it may also be explained by an overall alteration of some non-anxiety related performance feature. For example, buspirone is known to engage the serotoninergic neurotransmitter system and serotonin receptors have been known to mediate swim blader function. Altered serotoninergic activity induced by buspirone thus may have affected the buoyancy of the experimental fish.

However, the effects of buspirone found in the social behavior test are more difficult to explain by such trivial performance alteration. Here, buspirone

was found to abolish the alcohol induced reduction of social preference, i.e. a significant interaction between buspirone treatment and embryonic alcohol concentration was found. Such interaction may not result from altered vertical location of the fish and may be explained more parsimoniously by the known anxiolytic effect of the drug.

The first point to take any serious conclusion in science is to be sure of a proper animal model. In Ali S. et al. (2011), the authors summarize many ethanol interventions during zebrafish embryo development in order to study FAS and FASD. In despite of the critical alterations promoted by FAS and its importance to study to human society, FAS zebrafish model are high deleterious, involving visual and possible muscles abnormalities (Billota et al., 2002; Carvan et al., 2004; Dlugos & Rabin, 2007; Loucks et al., 2007). Such alterations make it impossible to investigate our aim, thus we needed a milder form of FAS. In this sense, exposing zebrafish eggs during the neural tube formation would resemble FASD, because it corresponds approximately to the late first trimester or early second trimester of the human (Kimmel et al., 1995. At this stage, the brain has started to develop, for example, neuronal progenitor cells have started to specialize and develop into neurons, and have begun to migrate and establish connections. Furthermore, such exposure do not affects anatomical formation, allowing us to test our hypothesis (Gerlai, 2015). Animals treated in this study did not present any morphological alterations allowing us to perform further behavior analysis.

A crucial point to take any behavior conclusion is wisely choose the task that the animal are going to be exposure, as well as the metrics that define it. The social interaction can be measured by many ways regarding zebrafish. However,

two tasks are often use, the shoaling behavior and the social preference task. The first consist of an open tank filmed from above, with a free-swimming group of animals. In despite of the presence of all animals in a single compartment, exchanging hormones and contact, usually this behavior is of hard and confusing analysis. Furthermore, the test are made with animals of the same group, which does not reflect the interaction of an “altered” animal and normal individuals, thus it does not reflect the human condition. In another hand, the social preference task allows to measure exactly it. One animal is placed in a central tank, which in virtual divided in three zones, one near of another tank with control animals (conspecific zone), a central zone, and one near an empty tank (empty zone) (Pham et al., 2012). The **Figure 2** reflect the classical measurements of the social preference test, indicating an transition of more time, distance and entries in the conspecific zone from the control to a preference in the empty zone seen in animals treated with ethanol 1%. In prior studies published in the field, it was suggested that animals treated with ethanol 0.5% and 1% could present a lower level of motivation, which could reflect in animals without motivation to explore the apparatus, thus reducing interaction (Gerlai, 2015). However, something called our attention during the experiments. As can be seen in the spatio-temporal analysis in **Figure 3**, animals treated with ethanol 0.5% presented a behavior similar to risk assessment from plus-maze for rodent models. The animals across the time go from the empty zone to the central zone and choose to return to the empty zone. The animals are avoiding interacting with the conspecific, but it goes to the central zone staying in this zone even more time than control group. It looks like the animals went to the central zone, saw the animals in the tank, and returned to the empty zone. It was even clearer in the group treated with ethanol

1%. The animals seldom goes to the conspecific zone and after they quickly go to the empty zone. Thus, it could not be just a matter of motivation. We than, questioned ourselves, what kind of behavior could influenciate social preference in such level. Two possible explanations seems to be possible. The animals could be afraid of interaction, however, fear usually makes zebrafish to go near to the conspecific and to present other behaviors, like freezing and high opercula movements, which was not detected in our study. The other could be anxiety, because as the animals was exposed to a new group of animals, in a new tank it could choose to avoid the new group, define a secure homebase to explore the apparatus, and only than to make swimming strategies, without present peculiar fear behavior of zebrafish. The change in the homebase formation (a safe zone were the animals always returns to make a new exploration) seen in red on the occupancies plots of the **Figure 3**, suggests it. Nevertheless, there is a proper task to make such inference.

The novel tank diving test is highly used to measure anxiety-like behavior manifestation in zebrafish in front of a new situation. The first point to make any inference in this test is to measure the swimming activity of the animals. In despite of the animals treated with ethanol 0.25% present a higher mean speed in the social preference test (**Figure 2M**), we have seen no alteration in the novel tank task in any group (**Figure 4**). During the experiment, we saw the group ethanol 0.5% choosing to avoid the top. As can be seen in **Figure 5**, the animals goes to the top and always returns to the bottom and a similar behavior can be seen in the animals of the group ethanol 1%. It is like the animals of these groups in an unfamiliar situations defines a safe zone and quickly return to this zone after exploring regions of potential risk, like the top of the novel tank. The classical

analysis of this behavior paradigm shows a progression of anxiety in all groups, from a higher time spend in the bottom, low distance travelled in the top zone and culminating in a higher latency to the top zone in the group ethanol 1% (**Figure 6**).

The neurotransmitter systems are the basis of neurodevelopmental disorders, including autism, Down syndrome, and Fetal Alcohol Spectrum Disorders. Although these conditions have different causes, they are all characterized by altered neuronal communication correlated with problems in synapse development. For example, the disorders caused by exposure of ethanol during development are related with long-lasting defects in both the structure and function of synapses (Valenzuela et al., 2011). Few studies have shown the behavior impairment observed in zebrafish FASD model accompanied by neurochemical changes, like decreased in levels of dopamine, serotonin and their metabolites (Buske & Gerlai, 2011; Mahabir et al., 2013). In order to elucidate if anxiety was behind the social interaction impairment and in front of such neural change we choose to pharmacology modulate anxiety by interfering upon serotoninergic system. Some evidence indicates that buspirone—an agent that binds to the 5-HT1A receptor and which is used as an anxiety reducing (i.e., anxiolytic) medication—also increases the time of abstinence from heavy drinking (Litten et al., 1996; Pettinati 1996). These findings suggest that buspirone may help reduce anxiety in alcoholics with anxiety disorders, thereby possibly improving their compliance with therapeutic regimens (Lovinger, 1997). Previous studies with zebrafish showed different consequences due to the use of buspirone in relation on the content of its receptor, in a behavior task model of anxiety and social interaction the 5-HT1AR partial agonist buspirone produces an

anxiolytic-like effect (Gebauer et al., 2011). The same drug effect was observed in the light/dark test (Maximino et al., 2011; Steenbergen et al., 2011) and in the novel tank diving tests (Bencan et al., 2009; Maximino et al., 2013).

Unfortunately, only the study of Maximino et al., (2013) used buspirone by intra-peritonial injection. Because many drug compounds can interact and alter visual system, we choose to work with buspirone 25 mg/kg as pretreatment to the experiments by intra-peritonial injection. It was able to reverse the behavior seen in all animals treated with ethanol during development in the novel tank task. The animals presented almost no latency to the top zone; many of them did not went to the bottom zone (**Figure 6**), which can be easily seen in the occupancy plot presented in the **Figure 7**. Regarding the social preference task, all the animals groups pretreated with buspirone 25 mg/kg presented a similar behavior profile with 50% of the time spend in the conspecific zone, and with clear preference for this zone seen in the occupancy plot analysis (**Figure 8**).

In despite of one prior study indicates that social interaction and anxiety are related in adult zebrafish FASD model we need to show clearly the differences with our study. Parker et al. (2014) opted for a chronic exposure of one single dose (0.12%) by 7 days from 48 hpf to 96 hpf without chorion protection. Such exposure is more reliable with the third gestational trimester, which is far away from the beginning of the brain formation, the focus on our study. In addition, any behavioral are more than one single measure. Unfortunately in the social paradigm of the study the animals does not have a big area to choose to avoid interaction, as well in the anxiety-like paradigm the authors analysis only time in the bottom, and used a similar tank were the animals are created. Thus, we present our hypothesis in front of a more reliable FASD

model, with a more profound behavioral analysis, and a proper behavior apparatus and used a spectrum of ethanol exposure. Furthermore, we show the possible relationship between such behaviors by using a pharmacological approach.

5. Conclusion

In this study, we showed by the first time that zebrafish embryos exposed to ethanol in early development present a social impairment due to a higher level of anxiety. Furthermore, we show that buspirone should be used more often to human FASD patients treatment.

Competing interests

The authors have no conflicts of interest to declare.

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Figures

Figura 1

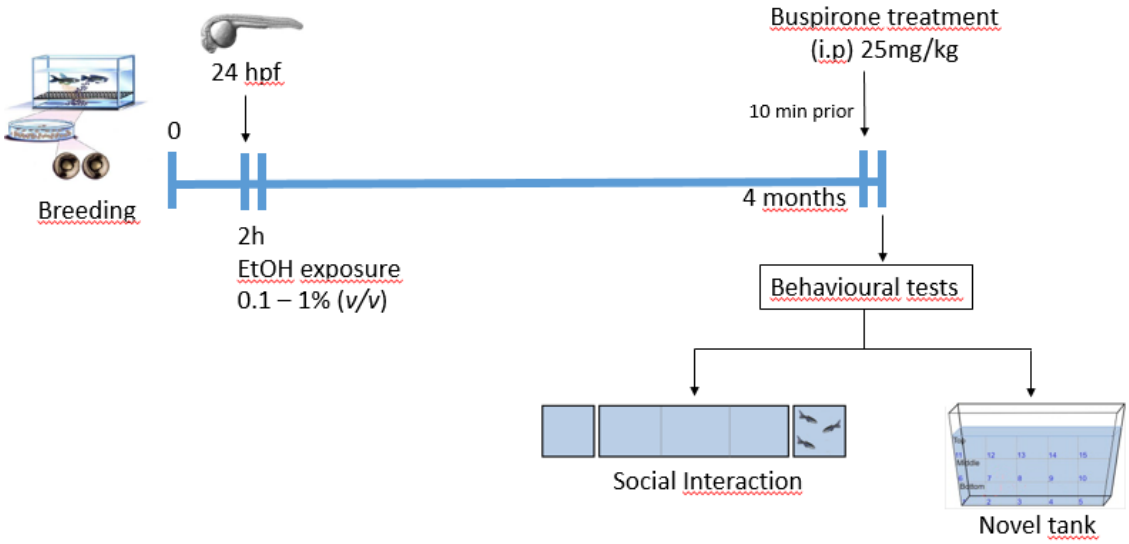

Figura 2

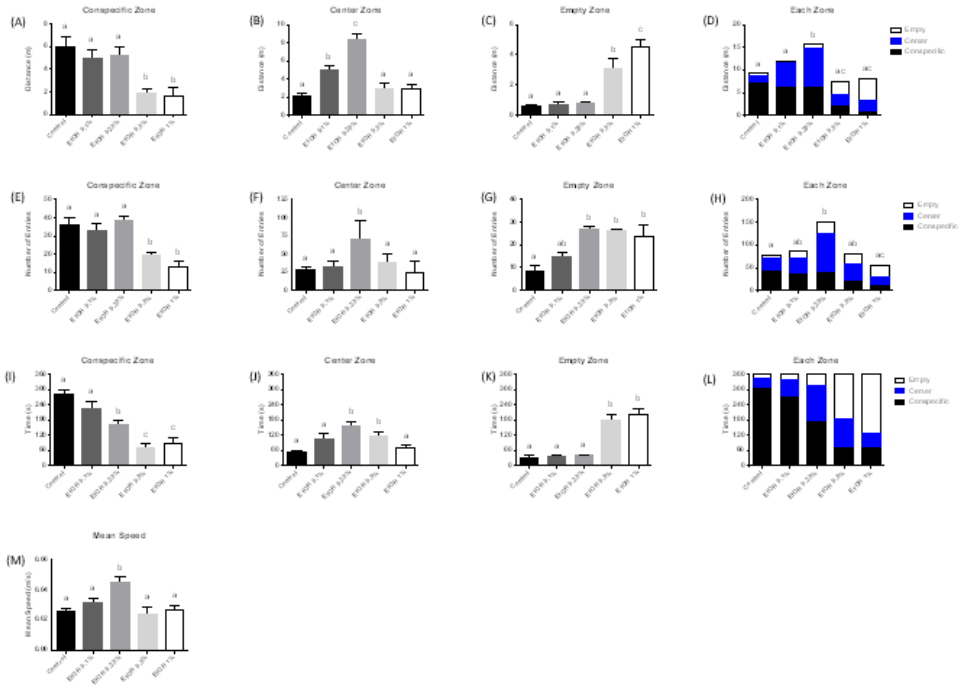
(A)
Conspecific Zone
(B)
Center Zone
(C)
Empty Zone
(D)
Each Zone
Empty
Center
Conspecific
(E)
Conspecific Zone
(F)
Center Zone
(G)
Empty Zone
(H)
Each Zone
(I)
Conspecific Zone
(J)
Center Zone
(K)
Empty Zone
(L)
Each Zone
(M)
Mean Speed

Figura 3

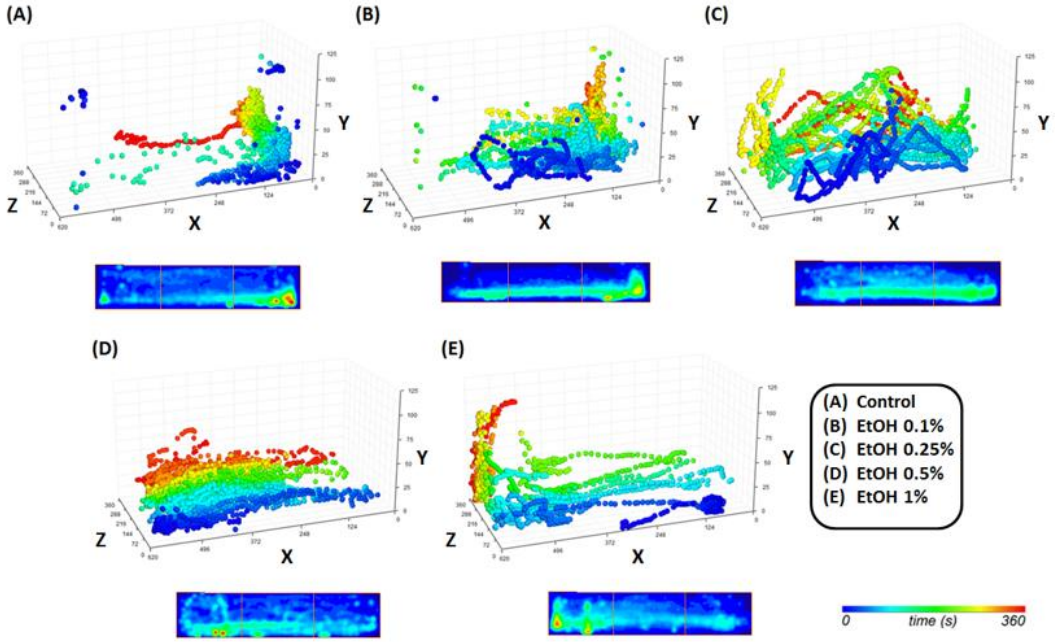
(A)
(B)
(C)
(D)
(E)
X
Y
Z
(A) Control
(B) EtOH 0.1%
(C) EtOH 0.25%
(D) EtOH 0.5%
(E) EtOH 1%
0
time (s)
360

Figura 4

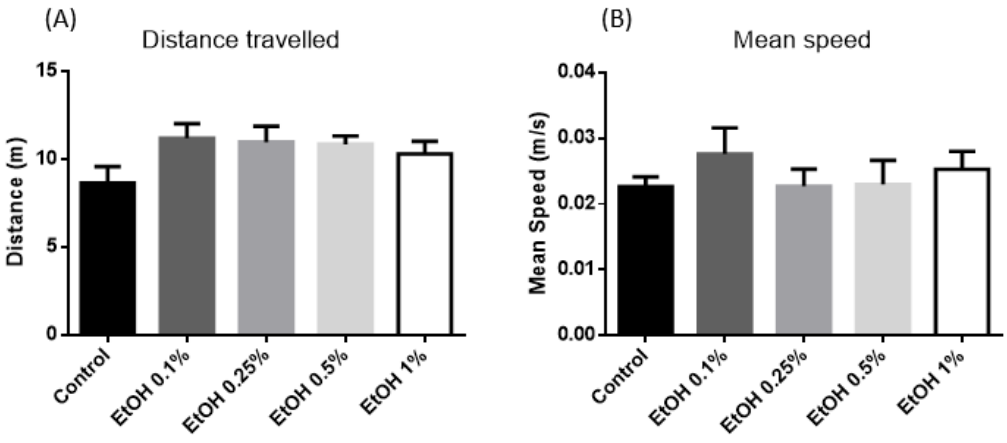
(A)
Distance travelled
Distance (m)
15
10
5
0
Control
EtOH 0.1%
EtOH 0.25%
EtOH 0.5%
EtOH 1%
(B)
Mean speed
Mean Speed (m/s)
0.04
0.03
0.02
0.01
0.00
Control
EtOH 0.1%
EtOH 0.25%
EtOH 0.5%
EtOH 1%

Figura 5

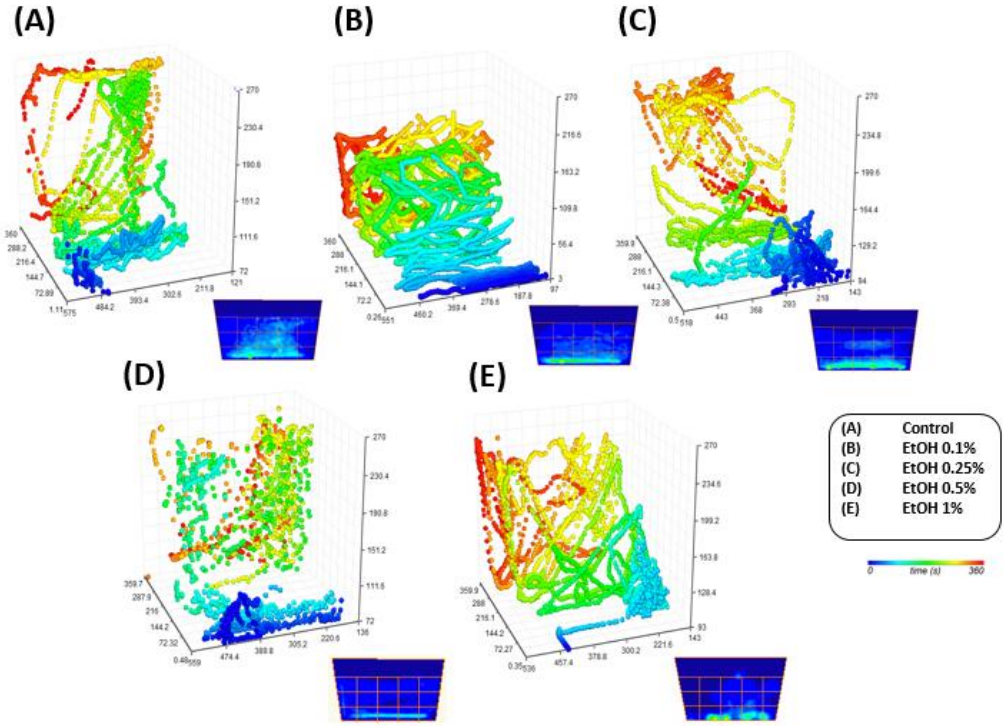
(A)
(B)
(C)
(D)
(E)
(A) Control
(B) EtOH 0.1%
(C) EtOH 0.25%
(D) EtOH 0.5%
(E) EtOH 1%
0
time (s)
360

Figura 6

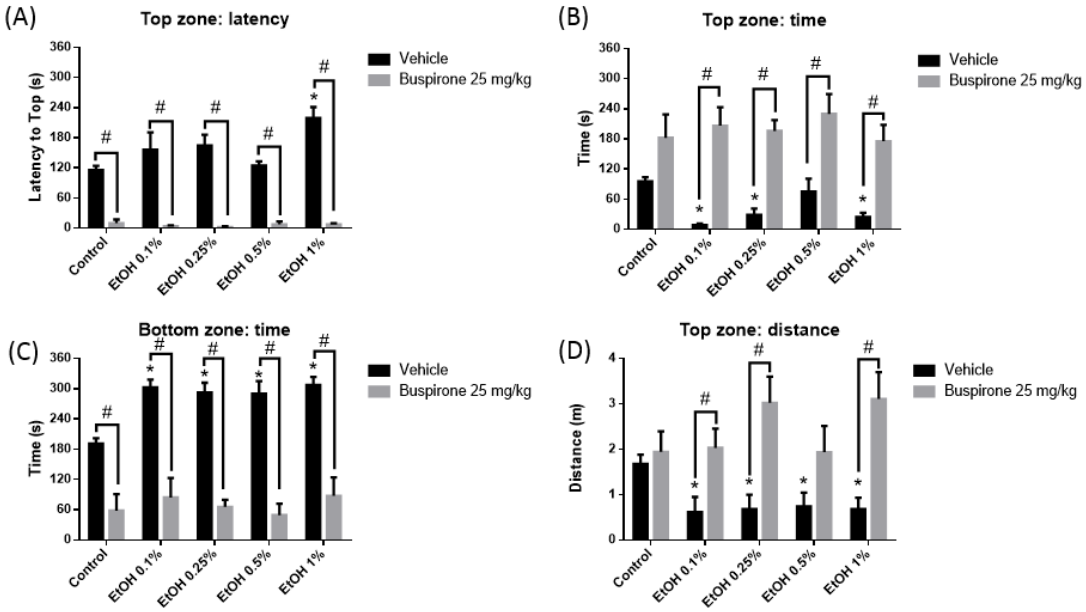
(A)
Top zone: latency
Latency to Top (s)
Vehicle
Buspirone 25 mg/kg
Control
EtOH 0.1%
EtOH 0.25%
EtOH 0.5%
EtOH 1%
(B)
Top zone: time
Time (s)
(C)
Bottom zone: time
(D)
Top zone: distance
Distance (m)

Figura 7

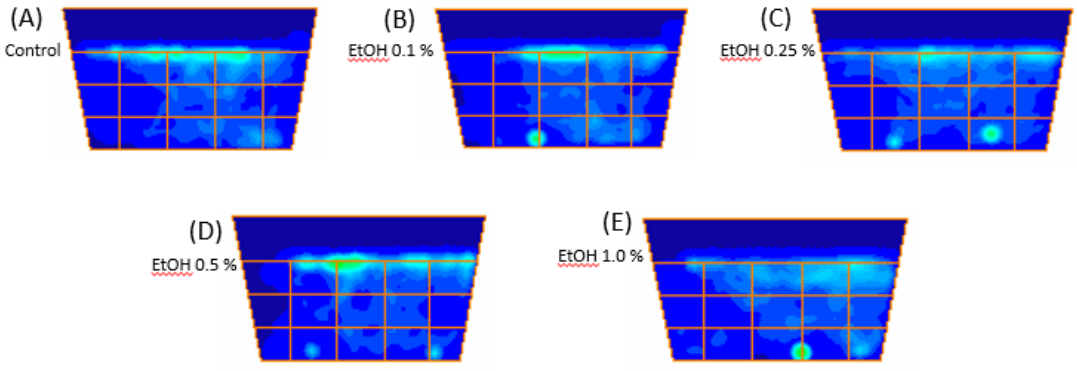

Figura 8

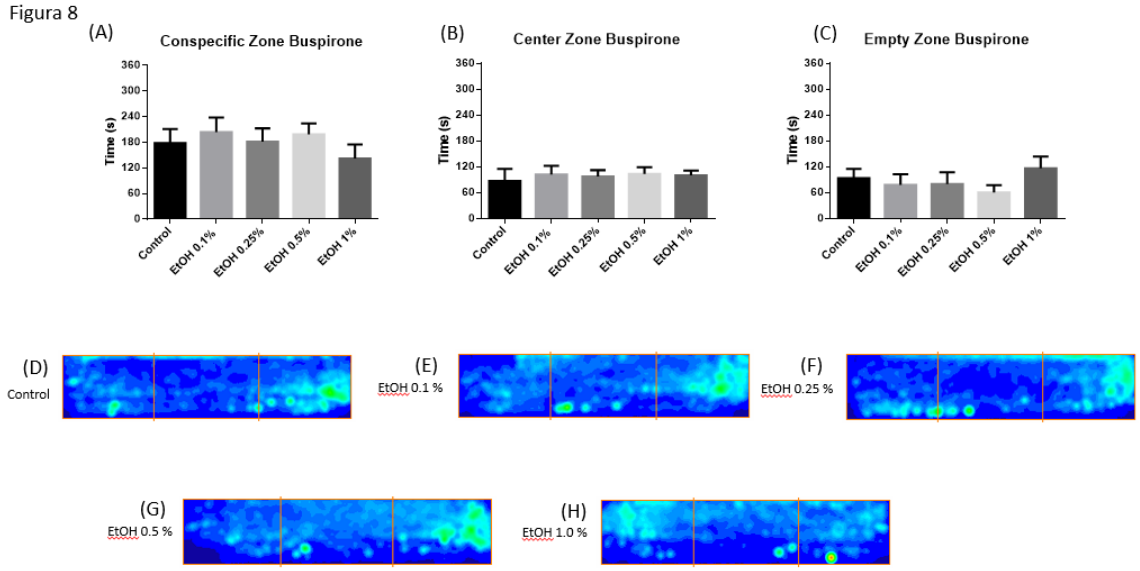
(A)
Conspecific Zone Buspirone
Time (s)
360
300
240
180
120
60
0
Control
EtOH 0.1%
EtOH 0.25%
EtOH 0.5%
EtOH 1%
(B)
Center Zone Buspirone
Time (s)
360
300
240
180
120
60
0
Control
EtOH 0.1%
EtOH 0.25%
EtOH 0.5%
EtOH 1%
(C)
Empty Zone Buspirone
Time (s)
360
300
240
180
120
60
0
Control
EtOH 0.1%
EtOH 0.25%
EtOH 0.5%
EtOH 1%
(D)
Control
(E)
EtOH 0.1 %
(F)
EtOH 0.25 %
(G)
EtOH 0.5 %
(H)
EtOH 1.0 %

Figure legends

Figure 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 hour hpf during 2h 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 behavioural 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.

Figure 2: Behavioral profile of social interaction in adult zebrafish FASD model. The figures shows the distance traveled in the conspecific zone (A), center zone (B) empty zone (C) and all the distances traveled in each zone (D); number of entries in the conspecific zone (E), center zone (F), empty zone (G) and all the entries in each zone (H); time spend in the conspecific zone (I), center zone (J), empty zone (K) and all the time spend in each zone (L); and mean speed (M). The data were analyzed by one-way ANOVA followed by Bonferroni´s post hoc test and Kruskal-Wallis test followed by Dunn's multicomparition test, considering $p \leq 0.05$ as significant. Different letters indicate significant differences among the groups.

Figure 3: Comparison of the spatio-temporal behavior and the occupancy plot of the adult zebrafish FASD model.

Representative spatio-temporal reconstructions of zebrafish swimming activity during the 6 minutes 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), 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). The area on the left side limits with the empty tank. The area on the right side delimits with the tank with conspecifics individuals.

Figure 4: Basic endpoint behaviors in novel tank. The graph shows the distance travelled (A), mean speed (B). The data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test, considering $p \leq 0.05$ as significant.

Figure 5: 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 minutes 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).

Figure 6: Comparison of the spatio-temporal behavior of the adult zebrafish FASD model and acutely treated with buspirone.

The figures shows the time spend in the top (A); distance traveled in the top (B); latency to reach the top (C); and time spend in the bottom (D). The data were analyzed by one-way ANOVA followed by Bonferroni´s post hoc test and Kruskal-Wallis test followed by Dunn's multicomparition test, considering $p \leq 0.05$ as significant. Symbols indicate significant differences among the groups vehicle (*), and between buspirone 25mg/kg and vehicle (#).

Figure 7: Occupancy plot of the adult zebrafish FASD model pretreated with buspirone 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).

Figure 8: Behavioural profile of social interaction in adult zebrafish FASD model pretreated with buspirone. Time spend in the conspecific zone (A), center zone (B), empty zone (C). The data were analyzed by one-way ANOVA followed by Bonferroni´s post hoc test and Kruskal-Wallis test followed by Dunn's multicomparition test, considering $p \leq 0.05$ as significant. Occupancy plot of the control (D), EtOH 0.1% (E), EtOH 0,25% (F), EtOH 0,5% (G) and EtOH 1% (H), 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).

CAPÍTULO II

Artigo a ser submetido para a revista: Neuroscience Letters

Embryonic Alcohol Exposure promotes long-term effects on cerebral glutamate transport of adult zebrafish

Suelen Baggio[a], Ben Hur Mussulini[a], Diogo Losch de Oliveira[a], Kamila Cagliari Zenki[a], Emerson Santos da Silva[a], 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, Brasil.

*Corresponding author:

Eduardo Pacheco Rico, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense – UNESC, Av. Universitária, 1105, Bairro Universitário, 88806-000 Criciúma, SC, Brasil.

E-mail: eduprico@gmail.com

Abstract

Ethanol is a widely consumed substance throughout the world. During pregnancy can substantially damage the human fetus, and the developing brain is particularly vulnerable. The brain damage induced by prenatal alcohol exposure can lead to a variety of long-lasting behavioral and neurochemical problems. However, there are no data concerning the effects of developmental ethanol exposure on the glutamatergic system, where extracellular glutamate acts as signaling molecule. Here we investigated the effect of ethanol exposure for 2 hours (concentrations of 0.0%, 0.01%, 0.25%, 0.50%, and 1.00%) in embryos at 24 hour post-fertilization (hpf) by measuring the functionality of glutamate transporters brain of adult (4 months) zebrafish. Ethanol 0.01%, was not able to modify glutamate uptake. However, 0.25% and 0.50% of ethanol decreased transport of glutamate (58.06% and 43.54%, respectively). Interestingly, 1.00% was able to inhibit the transport activity (69.35%), however to a lesser degree when compared with 0.5%. In response to the embryonic alcohol exposure, we found impairment in the function of glutamate transport in cerebral adult fish, contributing to modify in long-term the homeostasis glutamatergic signaling.

1. Introduction

Alcohol abuse in pregnancy resulted in economic costs to society. In the United States, the lifetime cost for an individual suffering from FAS may be as high as $2 million. The majority of these costs are required for special education, medical, and mental health treatment [21]. Prenatal ethanol exposure is the cause of fetal alcohol syndrome (FAS), a clinical condition characterized by a variety of brain and physical malformations. Microcephaly and microphtalmia are common features of fully developed FAS. Children thus affected exhibit a typical physical phenotype and varying de grees of CNS dysfunction, ranging from mild cognitive disorders to profound mental retardation [22]. On the other hand, last decade a growing number of studies have failed to detect adverse neurodevelopmental effects of mild--to moderate maternal drinking in the exposed child and recent experimental study, mimicking conditions of mild drinking in pregnancy, provides powerful evidence that there are serious lifelong risks to fetal exposure to alcohol [9].

During the development, ethanol consumption affects a number of neurotransmitters and neuromodulators in the CNS, including dopamine, serotonin and GABA. Importantly, research findings of changes in glutamatergic neurotransmission induced by alcohol self- or experimenter-administration have resulted in a focus on therapies targeting glutamatergic receptors and normalization of glutamatergic neurotransmission [29]. Glutamate is the major excitatory amino acid in the mammalian CNS, being implicated in several physiological processes. Termination of excitatory activity is mediated by high-affinity Na+-dependent glutamate transporters (EAAT), principally located in glial cells surrounding synapses and in post-synaptic neurons. A family of Na+-dependent transporters is of prominent importance for glutamate uptake and for regulating homeostasis in the CNS [3,35]. The EAATs represent a protein family that displays considerable homology (50–60% at the amino acid level) [4]. To date, five structurally

distinct subtypes of excitatory amino acid transporters have been identified and characterized in the mammalian brain: EAAT1 [34], EAAT2 [28], EAAT3 [18] EAAT4 [13] and EAAT5 [2]. In order to maintain extracellular glutamatergic tonus, the activity of Na+-dependent excitatory amino acid transporters (EAAT) plays a key role in the clearance of neurotransmitter from synaptic cleft [1,32]. Concerning glutamatergic signaling parameters, our group has recently identified and characterized the presence and function of five glutamate transporter members in zebrafish brain [30]. Furthermore, we studied the *in vitro* influence of ethanol and acetaldehyde on glutamate uptake and on toxicity-related parameters [38].

Rat [17], mouse [24] and chicken [11] embryo experimental models have been used to characterize the nature of the alcohol-induced brain damage. However, the advances in translational research linking human beings and animal work are imperative in order to paint a vivid picture of damage caused by prenatal ethanol exposure (PNEE) [25]. Studies utilizing the zebrafish or clawed frog as models for PNEE have shown that ethanol exposure during development can cause growth retardation including reduced body length, microcephaly, skeletal deficits, and eye malformation [20,26] as well as cognitive dysfunction in simple behavioral tasks such as visual acuity tests (Bilotta et al., 2004), associative learning [15], and social behavior [14], which were apparent even in the absence of physical malformations. Furthermore, these deficits were also accompanied by changes in gene expression [26].

Thus, considering that (i) ethanol mediates its actions through several excitatory or inhibitory neurotransmitter systems in development; (ii) glutamatergic receptors play a role in mediating cellular and behavioral effects of ethanol; (iii) ethanol activates signal transduction pathways during the development, leading to changes in neuronal function in adult, the present study we evaluated glutamate transport in cerebral tissue of adulthood zebrafish submitted previously with ethanol in embryonic period.

2. Material and methods

2.1. Animals

Adult zebrafish (*Danio rerio)*; 10 to 12 months old, from a heterogeneous wild-type stock (standard short-fin phenotype) were obtained from a local commercial supplier (Delphis, RS, Brazil). The fish (mixed male/female) were housed in a re-circulating system maintained with mechanical and biological filtration at 28 °C, pH of 7.4 and a conductivity of 500µS (system water). Breeding arrays were used for obtaining fertilized eggs. The 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). All procedures with animal subjects were approved by the Ethics Committee for the Use of Animals- CEUA (number 27725) from the Universidade Federal do Rio Grande do Sul.

2.2. Experimental design

Eggs of zebrafish short fin were collected 2.5 hour hpf. Approximately 500 fertilized eggs were randomly selected and were divided into 5 equal rearing tanks. At 24 hour post-fertilization (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 and because the stage of neural tube development, and represents the end of the segmentation and the beginning of the pharyngula stage of zebrafish development, which corresponds approximately to the late 1st trimester or early second trimester of human fetal 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 hours 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 [14]. The embryos were maintained on B.O.D. Incubator in system water at 28^{o}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 artemia. Zebrafish remained in these holding tanks until behavioral experiments, which were conducted after the fish reached 4 months of age (mature young adults, 50 to 50% male–female). The sample sizes of treated fish were of at least eight animals per group.

2.3. Glutamate uptake

2.3.1. Tissue preparation

The animals were anesthetized using ice-cold water and euthanatized by decapitation to remove the brain. The structure was humidified with Hank's balanced salt solution (HBSS) containing (in mM): 137 NaCl; 0.63 Na_2HPO_4; 4.17 $NaHCO_3$; 5.36 KCl; 0.44 KH_2PO_4; 1.26 $CaCl_2$; 0.41 $MgSO_4$; 0.49 $MgCl_2$ and 1.11 glucose, pH 7.2. Each brain was separated with the help of a magnifying glass and entirely transferred to paired 24-well culture plates containing 0.5 ml of HBSS. One plate from each pair was maintained at 37 ^{o}C and the other at 4 ^{o}C. The brains from the first plate were washed once with 1 ml of 37^{o}C HBSS and those of the second were washed with 1 ml of ice-cold HBSS containing N-methyl-D-glucamine (4^{o}C) instead of sodium chloride.

2.3.2. Uptake assay

The glutamate uptake assay was performed as previously described by Rico et al. (2010) [30], using a total of 36 animals for the experiment (n = 6 per group). Total glutamate uptake was measured with the addition of 0.33 µCi mL^{-1} L-[^{3}H] glutamate to the incubation medium at 37 ^{o}C. Incubations were stopped after 7 min by washing out the glutamate remaining in the incubating medium followed by two washes with l ml ice-cold HBSS. The brains were immediately transferred to 0.5N NaOH and incubated overnight, resulting in a homogenate. Protein content was measured using aliquots of homogenate (10 µl) following the method described by Peterson et al., 1977. Samples were taken for determination of the intracellular content of L-[2,3–3H] glutamate by scintillation counting. Sodium-independent uptake was determined by using ice-cold (4 ^{o}C) HBSS containing N-methyl-D-glucamine instead of sodium chloride. The results were subtracted from the total uptake to obtain the sodium-dependent uptake.

2.4. Statistical analysis

The glutamate uptake was expressed as nmol glutamate min^{-1} mg $protein^{-1}$. All parameters were analyzed using one-way ANOVA for multiple group comparison followed by *post hoc* analysis carried out by Duncan's multiple range, considering $p \leq 0.05$ as significant.

3. Results.

In this study, we verified the effects of embryonic ethanol treatment on the functionality of glutamate transporter, responsible for regulating the extracellular concentrations of this excitatory neurotransmitter. To evaluate the long-term effect of ethanol during development process on adult on glutamatergic signaling, embryos (24hpf) were exposed to ethanol at concentrations of 0.1, 0.25, 0.5 and 1.0% (v/v) for 2 hours. After treatment with ethanol, glutamate uptake of adult zebrafish brains with 4

months old was verified. The results shown a significant decrease in glutamate uptake at 0.25% (58.06%; = 0.00339), whereas group 0.5%, uptake was inhibited treatment (43.54%; p = 0.000255) compared to control group (Fig. 1). In contrast, glutamate uptake for the group of embryos subjected at ethanol 1% showed a significant decrease compared to the control group (69.35%; p=0.00083). In contrast, when compared with the group 0.5, it was observed an increase of 61,27% on glutamate uptake. The group 0.1% of ethanol exposure did not significantly the functionality of transporters.

4. Discussion

Although many reports of adverse effects related to prenatal exposure involve heavier drinking [22], recent research documenting deleterious outcomes for children prenatally exposed to small amounts of alcohol [33] had led to recognition that a threshold has not been adequately identified. In this sense, our study proposes to evaluate low doses of ethanol in zebrafish embryos. This moderate levels in the early development phase enables the study of mild changes compared with significant changes promoted by high doses of ethanol. Moreover, the consumption of moderate amounts and regular social drinking, generally with meals, is the modal pattern of alcohol consumption among females; but drinking frequency and specific levels of fetal alcohol exposure are not adequately understood of ethanol occurs in women who consume socially, sometimes unaware that they are pregnant. The present results provide evidence that functionality of cerebral glutamate transporters of adult zebrafish was affected by ethanol consumption in the initial period of embryonic development.

Alcohol is a known teratogen, with effects that may be more pronounced during organogenesis [36]. Ethanol has well-known teratogenic effects by mechanisms including induction of apoptosis and inhibition of proliferation, migration, differentiation, and other cellular functions during developmental period [16]. Recent evidence indicates that ethanol modulates the function of specific intracellular signaling cascades, including

those that contain cyclic adenosine 3',5'-monophosphate (cAMP)-dependent, protein kinase A (PKA) and protein kinase C (PKC) [23]. A number of studies have shown, without having pin-pointed the exact mechanism, that protein kinases are able to modulate the transport activities for glutamate, including PKA and PKC [37]. In this context, the adult brain glutamatergic system zebrafish could be altered through the premature insult occurred in the embryonic stage. Furthermore, PKC phosphorylates numerous proteins, including transcription factors, which regulate the activity of many genes in the cell nucleus [12]. Neural stem/progenitor cells are self-renewable cells in the CNS. These cells are able to differentiate into specific cell types including neuron during the brain developmental period by its multi-potent capacity. Moreover, there is a group of genes sensitives to ethanol (*Pax6, Ngn2,* and *NeuroD*) that are crucial for neuronal differentiation process permitting the imbalance between glutamatergic and GABAergic neuronal differentiation [19]. Thus, Increasing the numerical or functional balance of excitatory vs. inhibitory cells can lead to a hyper-excitable state, which might be an underlying neurobiological feature in the manifestation of neurological abnormalities such as hyperactivity symptoms of FASD [19]. Therefore its possible suggest that prenatal ethanol in zebrafish embryo could affects overall architecture and size of the brain by influencing the proliferation and differentiation compromising the excitatory (glutamatergic) function though the functionality of glutamatergic transport in adult brain.

Early stages of alcohol/drug dependence are also marked by increased glutamatergic activity within the extended amygdala and the presence of neuroplastic changes in the mesocorticolimbic system. Dependence is associated with the development of tolerance and attendant decreases, as a result of neuroplastic changes, in positive reinforcement (e.g., mesocorticolimbic dopaminergic activity). Dependence is also associated with withdrawal, which is correlated with a number of symptoms linked to increased glutamatergic/excitatory neuronal activity and decreased GABAergic/inhibitory neuronal

activity. The extended amygdala mediates, at least in part, withdrawal symptoms related to anxiety and irritability as well as accompanying heightened physiological and autonomic responses. These heightened responses are mediated not only by increased glutamatergic (as opposed to GABAergic) activity but also increased anxiogenic activity within the extended amygdala, particularly the AMYG and BNST [31]. Our results may suggest that the changes in the control of glutamate levels may be related to possible behavioral responses mediated by ethanol in zebrafish.

Dose-response relationships that display an inverted U- or U-shaped curve dependent on the endpoint measured are known as examples of hormesis, which is a common phenomenon in neuroscience and pharmacology [7]. The biphasic dose-responses often involve a complex interaction of molecules with a certain receptor or even a regulatory mechanism that recruits different neurochemical pathways [8]. The classical inverted U-shaped response promoted by ethanol is correlated with alterations of neurotransmitter systems, cell signaling transduction machinery, and on enzyme activities. In this regard, most studies have called attention to the modulatory effects of ethanol on glutamatergic, GABAergic, dopaminergic, and serotonergic systems [6]. These data are roughly in line with patterns of results from previous studies that showed an inverted U-shaped response in glutamate uptake after zebrafish single ethanol exposure 24hpf, in which low to intermediate doses result in reduction of activity compared to higher dose, which cause causes a less robust inhibition. This dose response curve profile is observed in short fin population of zebrafish acutely exposed to ethanol in dopamine, GABA and glutamate levels [10].

Conclusion

These findings demonstrate the actions induced by ethanol in embryos and on glutamate uptake in adult zebrafish brain. This investigation evaluated the relationship between ethanol, recognized for acting in neurotransmission, and the proteins responsible for the control of physiological levels of glutamate in synapse. According with other animal models, our findings corroborate that the glutamatergic system is an interesting field to understand the neurochemical mechanisms related to FASD in adult individual.

Conflict of interest

The authors have no conflicts of interest to declare.

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Figures

Figura 1

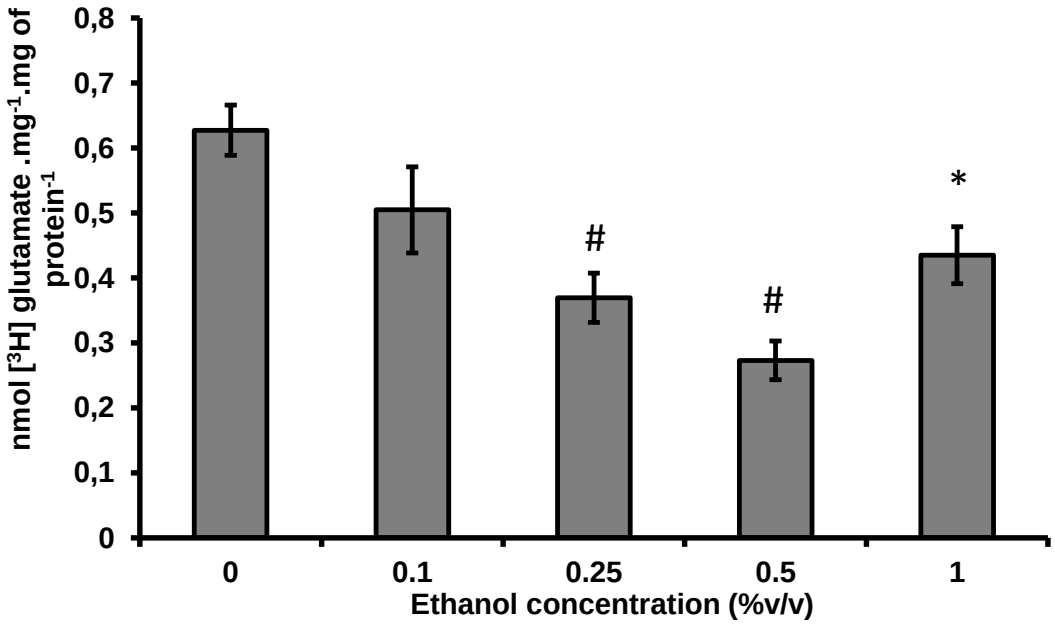

Figure Legends

Fig. 1.. Effect of ethanol exposure in zebrafish embryos in glutamate uptake in adult brain. Bars represent the mean S.D. of at least six different experiments. Data were analyzed by ANOVA followed by Duncan's post hoc test ($p \leq 0.05$, when compared to control group). *Significantly different from control. # Significantly different from control and 1.0%.

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Parte III.

DISCUSSÃO

O modelo de indução de SAF utilizado nesta dissertação, consistiu em estudar uma forma mais branda da síndrome, a denominada FASD, que pode ser utilizada como um termo guarda-chuva que abrange todas as pequenas alterações ocasionadas pela exposição ao etanol durante a fase de desenvolvimento embrionário. 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 um perfil comportamental fortemente alterado. A importância de focar nesta forma mais branda de SAF é justificada pelo seu difícil reconhecimento, o que faz com que seus portadores sejam negligenciados com diagnósticos incorretos e sem perspectivas de uma melhor qualidade de vida. Portanto, compreender suas características mais sutis, como as alterações comportamentais, que podem passar despercebidas, é o primeiro passo para facilitar o entendimento da FASD e auxiliar em seu diagnóstico.

Os resultados aqui observados assemelham-se aos já descritos na literatura, que mostram diminuição na interação social (Fernandes & Gerlai, 2009), e acrescentam o fator do aumento do comportamento ansioso, o que pode ser uma justificativa para as demais alterações comportamentais. A intervenção farmacológica com buspirona, que apresentou uma reversão dos resultados previamente observados, também surge como uma novidade, aliada a estudos que já consideram tratamentos ansiolíticos para pacientes dependentes de álcool, como forma de diminuir a ansiedade gerada pela abstenção desta droga (Lovinger, 1997). E, por fim, a busca por explicações neuroquímicas que possam estar por trás das mudanças comportamentais observadas, em uma tentativa de integrar e

compreender as alterações ocasionadas pelo consumo de álcool durante a gestação.

A SAF é caracterizada por uma série de disfunções que incluem dificuldades de aprendizagem, déficit de atenção, problemas motores e distúrbios comportamentais e de conduta; em seus casos mais graves, alterações morfológicas faciais são observadas. Alguns problemas são mais severos e podem mesmo levar os recém nascidos ou crianças na primeira infância a óbito, como alterações cardíacas, disfunções de certos órgãos e epilepsia (Gerlai, 2015). Tais situações se reproduzem nos modelos animais, fazendo com que seja impossível acompanhar o seu desenvolvimento, já que no caso do peixe-zebra, a utilização de altas doses de etanol gera malformações que impossibilitam a sobrevivência do animal, levando-o a óbito ainda na fase larval (Billota et al., 2004).

Assim, a busca por uma forma mais branda da desordem mostra-se como a melhor opção para um estudo a longo prazo, que permita acompanhar o desenvolvimento até o início da fase adulta. Questão que merece mais atenção dos pesquisadores, devido à escassez de trabalhos que abordem a SAF nesta fase da vida, quando a pessoa precisará inserir-se em uma sociedade despreparada para atender às suas limitações. O protocolo de Fernandes & Gerlai (2009) utiliza baixas concentrações (0.1 – 1.0%) durante duas horas de exposição, 24h pós-fertilização, gerando uma forma tênue da síndrome, com animais sem alterações morfológicas. Modelo que mimetiza um consumo de doses moderadas de etanol por uma gestante durante o primeiro trimestre de gestação (Kimmel et al., 1995 also see http://zfin.org/zf_info/zfbook/stages/ and http://www.ehd.org/virtual-humanembryo/). Como primeiro passo desta dissertação, conseguiu-se reproduzir

adequadamente o modelo citado acima, obtendo-se animais morfologicamente semelhantes aos controles, que atingiram, com baixa taxa de mortalidade, a fase adulta, permitindo então que de se desse prosseguimento aos testes comportamentais e neuroquímicos.

Na **Figura 2** temos um perfil comportamental de cada grupo ao longo da tarefa de preferência social. Por se tratar de um animal social, que costuma deslocar-se em cardumes, o peixe-zebra, quando possível, tende a permanecer perto de seus coespecíficos (Wright & Krause, 2006; Miller & Gerlai, 2007). Certas situações podem alterar este padrão, como casos de ansiedade ou tratamentos com determinadas drogas (Kurta & Palestis, 2010; Ward et al., 2008). Neste modelo de SAF, observou-se que conforme aumenta-se a concentração de etanol utilizada, o animal tende a interagir menos com o cardume. De acordo com o que se pode ver na **Figura 2.A**, onde ocorre uma diminuição gradativa da distância percorrida na zona coespecífica, com consequente aumento da distância percorrida na zona oposta (vazia) (**Figura 2.C**). Este distanciamento é reforçado pelos demais resultados, que mostram aumento no número de entradas na zona oposta (**Figura 2.G**) e aumento no tempo gasto nesta mesma zona (**Figura 2.K**) conforme aumenta-se a concentração de etanol. Os grupos EtOH 0.5% e 1% são os que apresentam os resultados mais expressivos, chegando ao extremos de inverter totalmente a preferência social, como se pode observar na representação espaço-temporal do deslocamento dos animais ao longo dos seis minutos de teste (**Figura 3**). Enquanto o grupo controle gasta seu tempo próximo ao aquário com o cardume (**Figura 3.A**), os grupos pré-expostos ao etanol na fase embrionária tendem de forma concentração-dependente a afastar-se de seus coespecíficos, até que

passam a gastar mais tempo na zona vazia (**Figura 3.E**). Tais resultados estão de acordo com o que é descrito para humanos, onde portadores de SAF tendem a ter suas habilidades sociais afetadas, com graves problemas de interação e socialização (Mattson et al., 2011; Schonfeld et al., 2006).

Os resultados observados na tarefa de preferência social levantaram o seguinte questionamento: estariam os animais que foram expostos ao etanol na fase embrionária com algum problema de locomoção ou exploração em decorrência da ação desta droga? Sendo assim, partiu-se para uma segunda tarefa comportamental, *o novel Tank*, que além de mostrar o comportamento do animal frente a um novo ambiente, é capaz de gerar um padrão locomotor e exploratório do peixe-zebra ao longo do aparato (Rosemberg et al., 2011). A resposta pode ser inferida a partir da **Figura 4**, que mostra a distância percorrida e a velocidade média de todos os grupos na tarefa de *novel tank* sem diferenças estatisticamente significativas encontradas entre os mesmos. Portanto, pode-se deduzir que os animais dos grupos submetidos a exposição com etanol na fase embrionária não apresentam problemas de locomoção e exploração, desempenhando adequadamente as tarefas comportamentais propostas. Sendo assim, as alterações encontradas nos demais resultados comportamentais não são causadas por problemas físicos ou locomotores.

Apesar de não existirem diferenças estatísticas significativas na velocidade média e na distância percorrida dos animais, cada grupo apresenta um padrão de exploração do aparato distinto. O grupo controle apresenta preferência inicial pelo fundo do aquário, mas com o passar do tempo, tende a explorar todo o aparato, utilizando a região central como uma área de transição para exploração do topo

(**Figura 5.A**), perfil este que está de acordo com o que já foi descrito na literatura (Ibrahim et al., 2014; Rosemberg et al., 2011). O grupo EtOH 0.1% possui um perfil semelhante ao controle, apenas com uma considerável diminuição do tempo gasto explorando a região superiora do aparato (**Figura 5.B**). Os animais do grupo EtOH 0.25% exploraram o topo apenas nos minutos finais do teste, com intensa preferência pelo fundo (**Figura 5.C**). É no grupo EtOH 0.5% que a exploração do aparato diminui de intensidade, os animais permanecem a maior parte do tempo da tarefa no fundo do aquário, com pequenos acessos ao topo e imediato retorno à área de origem, permanecendo imóveis (**Figura 5.D**). Por fim, o grupo EtOH 1.0% apresentou forte preferência pelo fundo do aparato, sem exploração vertical do aquário, mantendo apenas a locomoção horizontal (**Figura 5.E**).

Ao se analisar o padrão exploratório dos grupos etanol comparados ao controle, a permanência no fundo do aparato torna-se o resultado mais distinto. Este dado somado a latência para o topo e o tempo gasto nesta zona permite inferir que os animais submetidos ao modelo de FASD estão apresentando comportamento ansioso (**Figura 6**). As respostas de ansiedade em peixe-zebra podem estar por trás de uma série de fatores, como medo e aversão ou devido algum fator físico ou ambiental (Mathur & Guo, 2011; Maaswinkel et al., 2013). Portanto para que se possa indicar que realmente trata-se de um comportamento ansioso, é necessário algum teste adicional. Neste estudo, optou-se por uma intervenção farmacológica que pudesse auxiliar a solucionar as dúvidas decorrentes do comportamento observado na tarefa de *Novel tank* e sua influência na tarefa de Preferência social. O fármaco escolhido foi a buspirona, um ansiolítico utilizado para desordens de ansiedade e quadros depressivos (Loane & Politis, 2012), antagonista parcial do

receptor de serotonina 5-HT1A. Este medicamento já apresentou resultados ansiolíticos em estudos prévios com peixe-zebra (Gebauer et al., 2011; Maximino et al., 2013).

Repetindo-se as tarefas comportamentais, agora com animais que receberam uma injeção i.p. de buspirona 25mg/kg, os resultados encontrados foram bem distintos dos anteriores. De uma forma geral, a buspirona tende a equilibrar os grupos, fazendo com que os mesmos apresentem um comportamento bem semelhante entre si, sem diferenças estatísticas (**Figura 6** – quando comparados apenas os grupos buspirona). Mas, quando comparado aos grupos que não receberam o fármaco, todas as medidas de ansiedade tem seus valores diminuídos. A latência para o topo que apresentava médias em torno de 150s, cai para menos de 5s, os animais dos grupos buspirona quando postos no aquário subiam imediatamente (**Figura 6.A**). O tempo gasto no topo do aparato triplica (**Figura 6.B**), enquanto o tempo gasto no fundo cai a menos de um terço dos valores anteriores (**Figura 6.C**). A distância percorrida no topo também aumenta nos grupos administrados com buspirona, ao passo que permanecem mais tempo na área superior, os animais tendem a explorar esta região, deslocando-se horizontalmente; apenas os grupos controle não apresentam diferença significativa entre si, já que este grupo tende a explorar mais esta zona independentemente do tratamento com o ansiolítico (**Figura 6.D**). A representação espaço- temporal confirma os dados acima citados, os animais tratados com buspirona, independente de grupo, permanecem a maior parte da tarefa no topo no aparato, com pequenas incursões para o fundo; é possível também observar que apesar da preferência pela região superior, todo o aquário é explorado (**Figura 7**).

O padrão de comportamento ansioso influenciando na exploração e locomoção do peixe-zebra em *Novel tank* já foi discutido na literatura (Levin et al., 2007; Maximino et al., 2010; Blaser & Rosemberg, 2012; Mezzomo et al., 2016). Barba-Escobeda & Gold, 2012 e Maaswinkel et al., 2013 já relacionaram comportamento social com ansiedade em peixe-zebra, mas sem relação de causa-consequência. Contudo, analisando os resultados desta dissertação pode-se inferir que o aumento da ansiedade pode estar diretamente relacionado com a diminuição na preferência social e busca pelo isolamento. Os animais do modelo de FASD sofrem de um distúrbio de ansiedade que impede que os mesmos aproximem-se de seus coespecíficos, mas, ao receberem uma injeção i.p. de buspirona, restabelecem-se os valores de controle, permanecendo todos os grupos mais tempo na zona coespecífica e diminuindo o tempo de exploração da zona oposta (**Figura 8. A e C**). Novamente, os valores de cada grupo não apresentam diferença estatística entre si, apenas se comparados com os animais que não receberam o fármaco. Pode-se inferir que ao passo que a buspirona produz um efeito ansiolítico, ela permite que os animais sintam-se mais seguros para interagir com o cardume presente no aquário lateral, o que pode ser observado na **Figura 8.D-H**, mostrando que ocorre um aumento na exploração da zona coespecífica, mesmo nas maiores doses de etanol, que anteriormente apresentavam preferência pela zona oposta.

Tais mudanças comportamentais observadas nos animais expostos ao etanol na fase embrionária do desenvolvimento podem estar envolvidas com alterações no SNC. Estudos prévios já mostraram que o álcool pode afetar neurotransmissores específicos, alterando a estrutura e função de seus receptores, o que pode estar por trás das alterações comportamentais (Lovinger, 1989). Em seu princípio básico,

o etanol tende a inibir a ação do sistema inibitório, aumentando a atividade do sistema excitatório. Contudo, um curto período de exposição pode inibir a função dos receptores de glutamato (Lovinger et al. 1990) e estimular a função de receptores GABA no hipocampo (Weiner et al.1994), tais modificações neuroquímicas estão diretamente relacionadas com problemas de memória e aprendizagem, impedindo a formação de LTP, processo essencial para formação de memória (Bliss and Collingridge 1993).

Os sistemas de neurotransmissores são indicados como os principais substratos para o surgimento de desordens de neurodesenvolvimento, incluindo autismo, síndrome de Down e FASD. Apesar das diferentes causas, todas estas desordens estão relacionadas a problemas na comunicação neural, em decorrência de sinapses defeituosas (Valenzuela et al., 2011). Para o funcionamento adequado das conexões estabelecidas é necessária a estabilização das sinapses funcionais e a poda das sinapses desnecessárias. Este processo de refinamento requer a homeostasia dos sistemas excitatório e inibitório (Ramocki and Zoghbi 2008), que pode estar alterado em decorrência do etanol. Em resposta a perturbações, os neurônios em desenvolvimento tentam reestabelecer o equilíbrio com mudanças compensatórias como o aumento ou diminuição da função dos receptores envolvidos na neurotransmissão. Tais mudanças compensatórias nem sempre são eficazes, podendo modificar ainda mais o processo de desenvolvimento do SNC, resultando em consequências que se estendem a longo prazo (Valenzuela et al., 2011).

O glutamato, principal neurotransmissor excitatório do SNC, também é criticamente afetado pelo consumo de etanol. Mesmo pequenas doses podem inibir a atividade

de seus receptores NMDA, o que pode ser um dos motivos do desenvolvimento da Síndrome alcoólica fetal e de outras desordens (Gonzales & Jaworski, 1997). Alguns estudos utilizando fatias de cérebro indicam que o álcool induz a diminuição na liberação induzida por NMDA de neurotransmissores, como dopamina e acetilcolina (Göthert and Fink 1989; Woodward and Gonzales 1990); além de reduzir o sinal elétrico excitatório evocado por NMDA (Leslie and Weaver 1993; Tsai et al., 1995). Sua direta relação com mudanças comportamentais ainda não é tão clara, mas possivelmente esteja relacionada com a excitotoxicidade provocada pelo consumo de etanol e responsável pelos comportamentos de dependência e crises de abstinência observadas em alcoolistas, além dos déficits cognitivos (Gonzales & Jaworski, 1997).

O etanol como uma droga teratogênica é capaz de afetar a organogênese do indivíduo, modulando a função de cascatas de sinalização intracelular específicas, como as que contém adenosina cíclica 3',5' -monofosfato (cAMP)-dependentes, proteína cinase A (PKA) e proteína cinase C (PKC) (Newton and Messing, 2006). Estas proteínas cinases estão relacionadas com a modulação do transporte de glutamato (Zhang et al., 2014). Neste contexto, é possível inferir que o sistema glutamatérgico no cérebro do peixe-zebra adulto pode ter sido alterado por um insulto prematuro, ocorrido no estágio embrionário, justamente o que acontece no modelo de indução de FASD utilizado nesta dissertação. Unindo a literatura já publicada com algumas possíveis especulações em torno do tema, este trabalho propôs a realização da captação de glutamato como uma forma de inferir de forma neuroquímica as alterações decorrentes da exposição ao etanol. De acordo com a **Figura 1** do Capítulo II dos resultados, podemos observar uma diminuição na

captação no grupo EtOH 0.25% e inibição da captação no grupo EtOH 0.5%. No grupo EtOH 1.0% pode-se observar diminuição quando comparado ao controle, mas aumento em relação ao grupo 0.5%, quanto ao grupo EtOH 0.1% não observou-se diferença estatística em relação ao controle.

Nosso resultado de captação de glutamato apresenta-se com uma curva concentração-dependente na forma de U (*U-shaped curve*). Esta formação bifásica de dose-resposta envolve uma complexa interação de moléculas com receptores e mecanismos regulatórios que recrutam diferentes vias neuroquímicas (Calabrese and Baldwin, 2001). Esta clássica resposta promovida pelo etanol é correlacionada com alterações nos sistemas de neurotransmissores, maquinário de sinalização celular e atividade enzimática, o que já foi observado em outro sistemas (Camarini et al., 2010). Aumento de atividade excitatória ou inibitória pode levar a um estado de hiper-excitabilidade, o que pode estar por trás da manifestação de anormalidades neurológicas, como os sintomas de hiperatividade em FASD (Kim et al., 2010). Desta maneira, é possível sugerir que a exposição ao etanol na fase embrionária pode afetar a arquitetura e o tamanho do cérebro, influenciando na proliferação e comprometendo a diferenciação da função excitatória (glutamatérgica) através da funcionalidade dos transportadores de glutamato no cérebro adulto.

CONCLUSÃO

Este trabalho induziu um modelo de FASD em peixe-zebra, acompanhando o animal até a fase adulta para compreender as alterações provocadas pelo etanol no perfil comportamental e neuroquímico. Após os resultados obtidos, pode-se concluir que o etanol, mesmo em pequenas doses, pode ser muito nocivo para o desenvolvimento dos indivíduos, seja em modelo animal ou em humanos. Esta droga tem a capacidade de alterar o perfil comportamental dos animais, aumentando o comportamento ansioso e diminuindo a interação social, além de modificar os substratos neuroquímicos, neste caso, interferindo na atividade de captação glutamatérgica. Assim, pode-se inferir que tais mudanças estejam relacionadas, o que é alterado na fase embrionária no SNC persiste até a idade adulta, gerando consequências no comportamento e desenvolvimento dos animais. A buspirona, auxiliou através da sua função ansiolítica na reversão do comportamento ansioso dos animais do modelo de FASD, porém mais estudos são necessários para a total compreensão destes resultados.

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