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ALINE HAAB LUTTE

AVALIAÇÃO DA PARTICIPAÇÃO DO METABOLISMO DA ADENOSINA NOS EFEITOS TARDIOS DA EXPOSIÇÃO EMBRIONÁRIA AO ETANOL EM PEIXE-ZEBRA (*Danio rerio*)

Porto Alegre
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Orientadora: Profª Drª ROSANE SOUZA DA SILVA

Tese apresentada como requisito para obtenção do grau de Doutor pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Escola de Ciências da Pontifícia Universidade Católica do Rio Grande do Sul.

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Aprovada em: ____de_____de_____.

BANCA EXAMINADORA:

_____

Ângelo Luis Stapassoli Piato - UFRGS

_____

Dênis Broock Rosemberg - UFSM

_____

Carla Denise Bonan - PUCRS

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2018

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“Estou na situação de uma criancinha que entra em uma imensa biblioteca, repleta de livros em muitas línguas. A criança sabe que alguém deve ter escrito aqueles livros, mas não sabe como. Não compreende as línguas em que foram escritos. Tem uma pálida suspeita de que a disposição dos livros obedece a uma ordem misteriosa, mas não sabe qual é...” (Albert Einstein).

Porém, meu empenho em aprender a leitura aumenta a cada dia, hoje apresento apenas as letras do alfabeto, continuarei, no entanto, tentando montar palavras...

Resumo

O etanol é uma das substâncias psicoativas com propriedades que causam dependência mais comumente utilizadas no mundo. Uma das consequências do uso do etanol é a Síndrome Alcoólica Fetal (SAF), caracterizada por um conjunto de alterações no desenvolvimento de crianças cujas mães ingeriram etanol durante a gestação. O etanol afeta vários sistemas de neurotransmissão, incluindo o sistema purinérgico. O período de desenvolvimento embrionário é uma fase de grande suscetibilidade a agentes exógenos e endógenos e as perturbações capazes de alterar a sinalização adenosinérgica durante a fase embrionária podem estar relacionadas com alterações morfológicas, bioquímicas e comportamentais que vão do nascimento à vida adulta. Nos últimos anos, um substancial conjunto de evidências têm surgido, demonstrando que a síntese e o metabolismo das purinas e pirimidinas desempenham papéis importantes no controle do desenvolvimento embrionário e fetal e da organogênese. Disfunções na homeostase normal da adenosina durante o desenvolvimento inicial do cérebro podem ter consequências importantes na formação de circuitos neuronais, contribuindo assim para as alterações do neurodesenvolvimento.

Nos capítulos que seguem nesta tese foram abordadas diferentes alterações mnemônicas, bioquímicas e comportamentais em peixes-zebra expostos ao etanol em dois estágios distintos de desenvolvimento e o possível papel da adenosina em tais alterações. No primeiro capítulo foi analisado o prejuízo causado pelo etanol nos estágios de gástrula / segmentação e faríngula em parâmetros mnemônicos, e sugere que alterações no controle dos níveis de adenosina causadas pelo etanol poderiam alterar a neuromodulação de componentes importantes na formação da memória, como os neurotransmissores. Foi mostrado que o ajuste dos níveis de adenosina pela inibição da ecto-5'-nucleotidase parece ser eficaz na restauração da aquisição de memória em animais expostos ao etanol durante a faríngula. Foi detectada uma diminuição no tamanho corporal dos animais e nas proporções do telencéfalo / encéfalo e cerebelo / encéfalo em ambos os estágios, quando comparado com os controles.

No segundo capítulo foram avaliadas as alterações causadas pelo etanol nos estágios de gástrula / segmentação e faríngula na atividade enzimática das enzimas ecto-5´-nucleotidase e adenosina deaminase e expressão gênica da enzima ecto-5´-nucleotidase, em parâmetros morfológicos a na quantificação da adenosina em encéfalo total de peixe-zebra. A exposição ao etanol 1% não promoveu efeitos morfológicos severos, porém foi percebido uma dimunição no comprimento corporal. A atividade enzimática da ecto-5´-nucleotidase foi

aumentada em animais adultos expostos ao etanol no estágio de gástrula. A expressão gênica da ecto-5´-nucleotidase e a atividade enzimática da adenosina deaminase não sofreram alterações significativas em ambos os estágios de desenvolvimento. A quantificação da adenosina não mostrou diferenças na concentração do nucleosídeo em encéfalo total de animais adultos expostos ao etanol no início do desenvolvimento.

No terceiro capítulo foram avaliados os parâmetros comportamentais de locomoção, ansiedade, agressividade e interação social em animais de 3 e 12 meses pós fertilização (mpf) que foram expostos ao etanol 1% durante os estágios de desenvolvimento da gástrula / segmentação e faríngula. Adicionalmente, avaliou-se o uso de inibidores das enzimas ecto-5´-nucleotidase (3 e 12 mpf) e adenosina deaminase (3 mpf) sobre as alterações comportamentais investigadas. Não houve alterações significativas nos parâmetros locomotores. Um perfil ansiolítico foi detectado aos 3 mpf nos animais expostos ao etanol em ambos estágios de desenvolvimento, porém, este perfil não se manteve aos 12 mpf e o uso dos inibidores não gerou efeitos significativos neste parâmetro. A agressividade teve um aumento significativo em animais de 3 mpf expostos ao etanol no estágio de faríngula e manteve-se aumentada aos 12 mpf, sendo recuperada com o uso do inibidor da ecto-5´-nucleotidase. A interação social diminuiu nos animais de 3 mpf expostos ao etanol em ambos os estágios de desenvolvimento, sendo recuperada pelo uso do inibidor da ecto-5´-nucleotidase naqueles animais expostos ao etanol na fase de faríngula. Aos 12 mpf não houve alterações significativas.

Esses resultados estão de acordo com uma série de estudos que reportam a importância da sinalização adenosinérgica durante o desenvolvimento, bem como os efeitos deletérios provenientes de perturbações nessa via de sinalização. Os resultados desta tese, em contribuição ao que há na literatura, indicam que a modulação da sinalização adenosinérgica, em especial ajustes compensatórios entre ecto-5´-nucleotidase e transportadores de nucleosídeos, podem ser alvos importantes da exposição gestacional ao etanol.

Palavras-chave: Adenosina, Ecto-5´-nucleotidase, Etanol, Desenvolvimento, Peixe-zebra.

Abstract

Ethanol is one of the most commonly used psychoactive substances with addictive properties in the world. One of the consequences to ethanol use is the Fetal Alcohol Syndrome (FAS), characterized by a set of changes in the development of children whose mothers ingested ethanol during gestation. Ethanol affects several neurotransmission systems, including the purinergic system. The embryonic development period is a phase of great susceptibility to exogenous and endogenous agents and the perturbations capable of altering the adenosinergic signaling during the embryonic phase may be related to morphological, biochemical and behavioral changes that go from birth to adult life. In recent years, a substantial number of evidence has emerged demonstrating that the synthesis and metabolism of purines and pyrimidines play important roles in controlling embryonic and fetal development and organogenesis. Dysfunctions in normal adenosine homeostasis during early brain development may have important consequences in the formation of neuronal circuits, thus contributing to neurodevelopmental changes.

In the chapters that follow this thesis different mnemonic, biochemical and behavioral alterations in zebrafish exposed to ethanol were discussed in two distinct stages of development and the possible role of adenosine in such alterations.
In the first chapter, the damage caused by ethanol in the gastrula / segmentation and pharyngula stages in the mnemonic parameters was analyzed, and suggests that the changes in the control of adenosine levels caused by ethanol could alter the neuromodulation of important components in memory formation, such as neurotransmitters. It was shown that adjustment of adenosine levels by the inhibition of ecto-5'-nucleotidase appears to be effective in restoring normal adenosine levels and memory acquisition in animals exposed to ethanol during pharyngula stage. A decrease in the body size of the animals in the proportional analysis of the telencephalon / encephalon and cerebellum / encephalon at both stages was detected when compared to controls.

In the second chapter were evaluated the changes caused by ethanol exposition in the gastrula / segmentation and pharyngula stages in the ecto-5'-nucleotidase and adenosine deaminase enzymatic activity, gene expression of the enzyme ecto-5'-nucleotidase, morphological parameters and in the quantification of adenosine in zebrafish encephalon. Exposure to 1% ethanol did not promote severe morphological effects, but a decrease in body length was observed. The enzymatic activity of the ecto-5'-nucleotidase was increased in adult

animals exposed to ethanol in the gastrula / segmentation stage. The ecto-5'-nucleotidase gene expression and the enzymatic activity of adenosine deaminase did not change significantly at both stages of development. The adenosine quantification did not show differences in nucleoside concentration in total encephalon of adult animals exposed to ethanol at early development.

In the third chapter, the behavioral parameters of locomotion, anxiety, aggressiveness and social interaction were evaluated in animals at 3 and 12 months after fertilization (mpf) that were exposed to1% ethanol during gastrula / segmentation and pharyngula stages. In addition, was evaluated the use of inhibitors of the enzymes ecto-5'-nucleotidase (3 and 12 mpf) and adenosine deaminase (3 mpf) on the behavioral changes investigated. There were no significant changes in locomotor parameters. An anxiolytic profile was detected at 3 mpf in the ethanol exposed animals at both stages of development, however, this profile did not remain at 12 mpf and the use of the inhibitors did not generate significant effects in this parameter. The aggressiveness had a significant increase in ethanol exposed animals at the pharyngula stage at 3 mpf and remained increased at 12 mpf, being recovered with the use of the ecto-5'-nucleotidase inhibitor. The social interaction decreased in the ethanol exposed animals in both stages of development at 3 mpf, being recovered by the use of the ecto-5'-nucleotidase inhibitor in those animails exposed to ethanol in the pharyngula stage. At 12 mpf there were no significant changes.

These results are in agreement with a series of studies that show the importance of adenosinergic signaling during development, as well as the deleterious effects of disturbances in this signaling pathway. The results of this thesis, in contribution to what is in the literature, indicate that the modulation of adenosinergic signaling, especially compensatory adjustments between ecto-5'-nucleotidase and nucleoside transporters, may be important targets of gestational exposure to ethanol.

Key –words: Adenosine, Ecto-5'-nucleotidase, Ethanol, Development, Zebrafish.

LISTA DE FIGURAS

LISTA DE SIGLAS E ABREVIATURAS

ADA – Adenosina Deaminase

ADAL - Adenosine Deaminase-Like

ADH - Álcool Desidrogenase

ALDH - Aldeído Desidrogenase

AMP - Adenosina Monofostato

AMP_c - Adenosina Monofostato Cíclica

AOPCP - αβ-metileno-adenosina-5'-difosfato

ATP – Adenosina Trifosfato

CNT - Transportadores de Nucleosídeos Concentrativos

CYP2E1 - Citocromo P450 2E1

DARPP - Fosfoproteína Regulada pela Dopamina

DOPAC - Ácido 3,4-diidroxifenilacético

DPCPX - 8-Ciclopentil-1,3-dipropilxantina

Dpf – Dias pós-fertilização

DSM-5 - Manual Diagnóstico e Estatístico de Transtornos Mentais

EA-PA - Transtorno Neurocomportamental Associado à Exposição Pré-Natal ao Álcool

E-NPP - Ectonucleotídeo pirofosfatase/fosfodiesterases

ENT 1 - Transportadores de nucleosídeos equilibrativos do tipo 1

ENT 2 - Transportadores de nucleosídeos equilibrativos do tipo 2

E-NTPDase - Ectonucleosídeo trifosfato difosfohidrolases

GABA: Ácido gama-aminobutírico

GMP - Guanosina monofosfato

GPI - Fosfatidil-inositol-glicano

HPA - Hipotálamo – Hipófise –Adrenal

Hpf – Horas pós-fertilização

IPH - Hipotálamo – Hipófise – Interrenal

NGF - Fator de Crescimento Neuronal

NMDA - N-metil D-Aspartato

OMS – Organização Mundial da Saúde

QI – Quociente de Inteligência

RNAm- Ácido Ribonucleico Mensageiro

SAF - Síndrome Alcoólica Fetal

SEMO - Sistema de Enzimas Microssomais Oxidativas

SNC - Sistema Nervoso Central

UMP – Uridina Monofosfato

HPLC – Cromatografia Líquida de Alta Eficiência

SUMÁRIO

1. INTRODUÇÃO

1.1 Etanol

O etanol (C_2H_6O) também conhecido como álcool ou álcool etílico é uma substância psicoativa com propriedades que causam dependência, sendo obtido da fermentação de açúcares, hidratação do etileno ou redução de acetaldeídos (Marek e Kraft, 2014). A Organização Mundial da Saúde (OMS) divulgou dados apontando para um aumento de 43,5% no consumo de álcool no Brasil, no decorrer da última década. A taxa anual per capita elevou-se de 6,2 para 8,9 litros de álcool puro, fazendo o Brasil ocupar a posição de número 49 entre os 193 países avaliados – a média mundial é de 6,4 litros ao ano por habitante com 15 anos ou mais (OMS, 2017). Os estudos realizados indicam que 5,9% de todas as mortes no mundo estejam relacionadas ao consumo de álcool e também que, na população em idade fértil entre 20 e 39 anos, esse percentual sobe para 25% (OMS, 2017). No Brasil, estudos realizados com diferentes metodologias entre 2001 e 2014, estimam a frequência de consumo de álcool entre gestantes em torno de 10 a 40% (Baptista et al., 2017).

A concentração sanguínea de etanol depende da quantidade ingerida e absorvida pelo trato gastrointestinal, do volume de distribuição no organismo e da razão de eliminação. Menos de 10% do etanol é excretado no suor, respiração e urina (Marek e Kraft, 2014). A maioria dos tecidos do organismo contém enzimas capazes de metabolizar o etanol, mas a atividade mais significativa dessas enzimas ocorre no fígado e, em menor extensão, no estômago (Cassini e Linden, 2011; Chaudhuri, 2000). A maior fração do etanol é metabolizada no fígado pela ação das enzimas *álcool desidrogenase* (ADH) *e aldeído desidrogenase* (ALDH). Em humanos, a enzima ADH, mais precisamente da classe 1, converte o etanol em acetaldeído, que então sofre a ação da enzima ALDH e é transformado em acetato. O acetato formado no fígado destina-se à corrente sanguínea onde será captado e utilizado em outros tecidos (Tran et al., 2015; Plawecki e Crabb, 2014) e / ou convertido em acetil-Coenzima A para produzir dióxido de carbono e água (Marek e Kraft, 2014). O acetaldeído antes de se tornar acetato, é uma molécula tóxica e reativa e pode estar envolvido na neurotoxicidade induzida pelo etanol *in vivo* através da formação de aductos com proteínas e macromoléculas cerebrais (Rintala et al., 2000). O acúmulo de acetaldeído devido ao consumo excessivo de etanol pode levar ao aumento da interação desse aldeído com biomoléculas e várias proteínas como albumina, tubulina, lipoproteínas, colágenos e proteínas da membrana eritrocitária servem como alvos para a formação de aductos com esse aldeído (Sapkota e Wyatt, 2015).

O metabolismo do etanol também ocorre pela ação do Sistema de Enzimas Microssomais Oxidativas (SEMO) que utiliza as enzimas do grupo citocromo P450, mais especificamente a enzima CYP2E1. A CYP2E1 tem um Km elevado para o etanol, portanto, tem um papel mais significativo quando os níveis de etanol no sangue são elevados. CYP2E1 facilmente gera espécies reativas de oxigênio, incluindo radical hidroxila (OH^-), ânion superóxido (O_2^-) e peróxido de hidrogênio (H_2O_2) (Plawecki e Crabb, 2014).

Uma terceira forma de metabolização do etanol ocorre através da catalase. A catalase é uma enzima antioxidante que degrada peróxido de hidrogênio em água e oxigênio. Ela pode oxidar uma série de componentes através do uso de peróxido de hidrogênio, incluindo o etanol. A via da catalase é responsável por cerca de 2% do metabolismo do etanol (Marek e Kraft, 2014).

1.1.1 Transtornos do Espectro Alcoólico Fetal

A exposição fetal ocorre quando uma mulher ingere etanol durante a gravidez. O etanol pode atrapalhar o desenvolvimento fetal em qualquer estágio, incluindo nos estágios iniciais quando a mulher pode ainda não saber que está grávida (DMS-5, 2013).

Uma das consequências do uso do etanol, que faz parte dos transtornos do espectro alcoólico fetal, é a Síndrome Alcoólica Fetal (SAF), caracterizada por um conjunto de alterações no desenvolvimento de crianças cujas mães ingeriram etanol durante a gestação (DSM-5, 2013; Momino, Sanseverino e Schüler–Faccini, 2008; Stratton et al., 1996). A SAF é caracterizada por anormalidades faciais, retardo do crescimento, deficiências do Sistema Nervoso Central (SNC), entre outras características (Momino, Sanseverino e Schüler–Faccini, 2008) (Figura 1).

A exposição ao etanol pode causar alterações em níveis diferentes de gravidade, dependendo do tempo e dose de contato. As crianças expostas ao etanol de forma branda e moderada podem ser diagnosticadas com SAF parcial (Stratton et al., 1996), sem alterações morfológicas, mas com alterações neurológicas que se refletem em parâmetros comportamentais, mnemônicos e de aprendizado (Momino, Sanseverino e Schüler–Faccini, 2008; Stratton et al., 1996). Déficits no comportamento social têm sido associados com a exposição pré-natal ao etanol em adolescentes e adultos sem o diagnóstico completo de SAF,

ou seja, sem que apresentem todas as caracteristicas da síndrome (Streissguth et al., 1991; Streissguth et al., 1997).

A Associação Americana de Psiquiatria, através do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-5) caracterizou também o Transtorno Neurocomportamental Associado à Exposição Pré-Natal ao Álcool (EA-PA), sendo esse, um novo diagnóstico psiquiátrico que exige evidências de exposição pré-natal ao etanol e envolvimento do SNC, indicado por deficiências em três áreas: cognição, auto-regulação e funcionamento adaptativo (DSM-5, 2013). Este novo diagnóstico tem por objetivo melhorar a compreensão dos déficits comportamentais multifacetados observados em algumas pessoas expostas ao etanol durante o desenvolvimento e facilitar o tratamento desses indivíduos (DSM-5, 2013).

O comportamento social em um indivíduo adulto é o resultado de uma complexa interação de fatores genéticos, desenvolvimento do cérebro, experiências sociais na primeira infância e de aprendizagem ao longo da vida. Claramente, as mudanças no comportamento social observadas em adultos com SAF parcial poderiam ser consequência de vários fatores e, dentre estes, estão as alterações induzidas pelo etanol em estruturas cerebrais envolvidas no comportamento social (Kelly, Day e Streissguth, 2000). Do ponto de vista social, as pessoas com SAF têm um alto grau de inadimplência e / ou criminalidade e alguns destes problemas podem surgir a partir de dificuldades de comportamento em geral (Streissguth et al., 1997).

Recém-nascidos com SAF ou crianças expostas ao etanol durante a gravidez apresentam elevados níveis de irritabilidade (Coles e Platzman, 1993), o que possivelmente desenvolverá um temperamento difícil e problemas de comportamento mais tarde. Adolescentes e adultos com SAF ou SAF parcial (com idade média de 17 anos) mostram falta de resposta aos sinais sociais, falta de amizades recíprocas e dificuldades em cooperar com os colegas (LaDue, Streissguth e Randels, 1992; Streissguth et al., 1991). Mesmo os adultos com SAF que apresentam um QI normal demostram problemas no domínio social (Streissguth et al., 1991).

Quando se tratam de efeitos cognitivos causados pela exposição embrionária ao etanol, déficits de aprendizagem e memória são os efeitos mais relatados em crianças. A exposição pré-natal ao etanol está associada a imparidades tanto na recordação de informações quanto no reconhecimento da memória verbal (Mattson et al., 1996; Mattson e Riley, 1999). Também há estudos que relatam déficits na memória auditiva e memória espacial (Carmichael–Olson et al., 1992; Uecker e Nadel, 1996). Willford et al. (2004) realizaram um estudo incluindo

580 crianças e suas mães, sendo que, as mulheres foram avaliadas durante cada trimestre de gravidez e seus filhos desde o nascimento até os 16 anos de idade, com exposição ao etanol de leve a moderada, durante o primeiro trimestre de gravidez. Aos 14 anos, a função de memória foi avaliada e a exposição pré-natal ao etanol foi associada com um déficit generalizado na aprendizagem e memória. Os resultados deste estudo demonstraram que a exposição pré-natal ao etanol afetou parâmetros que exigiam recordar informações aprendidas (Willford et al., 2004). Déficits semelhantes no aprendizado e memória foram relatados em outro estudo realizado em 2002 por Richardson et al. Aos 10 anos, crianças cujas mães utilizaram etanol durante o período pré-natal, tiveram o desempenho no aprendizado e na memória verbal e não-verbal prejudicado (Richardson et al., 2002).

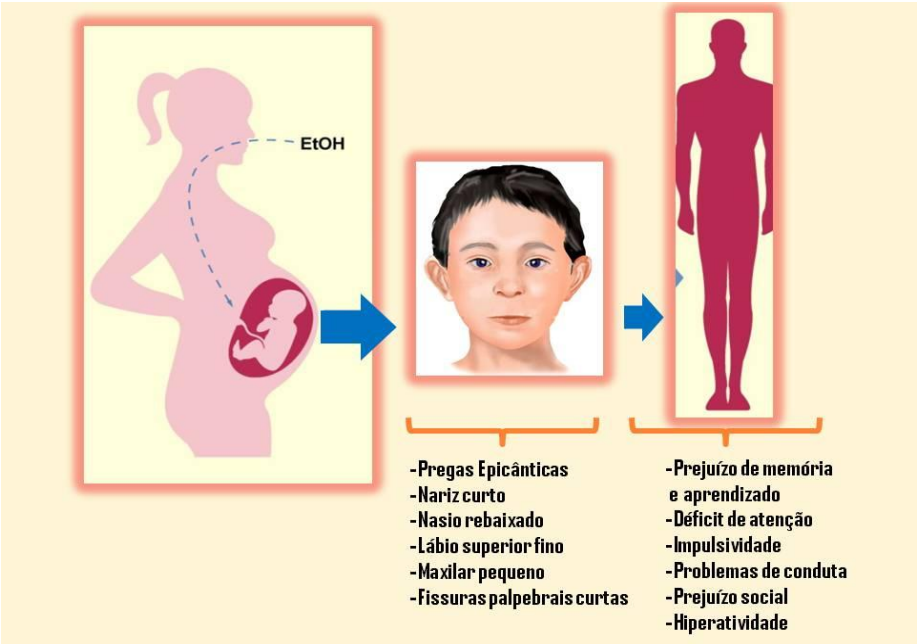

FIGURA 1: Resumo das principais características evidenciadas após exposição embrionária ao etanol em humanos. Produzido pelo autor. Informações baseadas em Mattson et al., 1996 e Momino, Sanseverino e Schüler–Faccini, 2008.

1.1.2 Mecanismos de Ação do Etanol

Os alvos moleculares que dão subsídios aos efeitos morfológicos e cognitivos da exposição ao etanol não são plenamente identificados. Os déficits cognitivos associados à SAF parecem ser consequência de alterações moleculares que prejudicam a plasticidade neuronal, que é a capacidade do cérebro de se moldar e estabelecer conexões essenciais para os processos de aprendizado e de memória. O etanol afeta vários sistemas de

neurotransmissão, incluindo os sistemas serotoninérgico, dopaminérgico, gabaérgico, glutamatérgico e purinérgico (Bliss, Collingridge e Morris, 2014; Choi et al., 2004; Howard et al., 2011; Rao et al., 2015; Trudell et al., 2014). Já foi demonstrado que o etanol pode remodelar membranas em roedores e peixe-zebra (Podechard et al., 2017; Tang et al., 2011). Devido a sua natureza anfifílica, o etanol pode interagir com moléculas polares e igualmente com regiões hidrofóbicas, presentes em lipídios e proteínas, podendo regular indiretamente a função de canais iônicos alterando o processamento pós-tradução e / ou o tráfico de proteínas. Por exemplo, o etanol pode alterar a função das proteínas tirosina-quinases *Src* e *Fyn*, que modulam os receptores NMDA e a plasticidade sináptica (Trudell et al., 2014).

Em ratos, o consumo de etanol causa um aumento da inibição e atenuação da neurotrasmissão excitatória (Basavarajappa et al., 2008; Leriche et al., 2008; Roberto et al., 2003). A exposição ao etanol regula negativamente os receptores NMDA e toda a cascata posterior (onde participam segundos mensageiros como o cálcio e o AMP cíclico), que culmina na ativação da transcrição de genes relacionados à plasticidade neuronal (Medina, 2011). A DARPP-32, localizada nos neurônios que contêm receptores de dopamina é um potente inibidor da proteína fosfatase 1 e também parece estar envolvida nas respostas ao etanol, agindo na regulação da capacidade do etanol de inibir a função dos receptores NMDA (Svenningsson, Nairn e Greengard, 2005).

O prejuízo do desenvolvimento cerebral intrauterino provocado pelo etanol pode ser evidenciado pelo volume diminuído do cérebro / estruturas cerebrais e alterações na sua constituição (Duffy et al., 1991; Miki et al., 2008). De fato, o giro denteado, parte do hipocampo, região cerebral crucial para memória, aprendizado e atenção, tem o seu desenvolvimento diminuído pelo etanol (Medina, 2011).

O etanol também induz ao estresse oxidativo, e o SNC, por conter baixos níveis de antioxidantes, alta concentração de lipídeos e consumir muito oxigênio, torna-se suscetível ao dano (Li e Wang, 2004). Exposições de longo período ao etanol levam à perda desproporcional de substância branca cerebral e deficiências nas funções executivas (de la Monte e Kril, 2014). O etanol prejudica a função de neurônios e células da glia, interrompendo uma ampla gama de funções incluindo a sobrevivência neuronal, a migração e a diferenciação celular (de la Monte e Kril, 2014). Os oligodendrócitos e terminais sinápticos são os principais alvos do etanol no sistema nervoso, quando afetados levam à inflamação, toxicidade neural e deficiências na sinaptogênese (de la Monte e Kril, 2014). Em geral, ocorre

a ativação de vias de indução de morte celular pela formação de moléculas tóxicas (Goodlett, Horn e Zhou, 2005). A exposição ao etanol leva ao prejuízo do desenvolvimento neuronal por inibição de fatores neurotróficos e déficit de energia celular por alterar o transporte e a utilização de glicose, provocando prejuízo no neurodesenvolvimento (Goodlett, Horn e Zhou, 2005).

Adicionalmente, em camundongos, tem sido proposto que o etanol eleva os níveis extracelulares de adenosina, um potente neuromodulador, estimulando de forma indireta os receptores de adenosina (Choi et al., 2004). Estudos com diferentes preparações biológicas, indicam que a elevação da concentração extracelular de adenosina na presença de etanol parece estar envolvida com a inibição dos transportadores de nucleosídeos equilibrativos do tipo 1 (ENT 1) e aumento da hidrólise de AMP, o que leva a um acúmulo de adenosina extracelular (Choi, 2004; Dunwiddie e Masino, 2001; Lutte et al., 2015).

Considerando que o nucleosídeo adenosina tem um papel essencial na modulação de diversos sistemas de neurotransmissão é possível que os efeitos do etanol sobre o metabolismo e ação da adenosina exerçam papel importante nas alterações relacionadas à exposição ao etanol.

1.2 Adenosina

A adenosina é um nucleosídeo presente no meio intra e extracelular. No meio intracelular, a adenosina exerce um papel homeostático participando do balanço energético celular (Dunwiddie e Masino, 2001). No meio extracelular, a adenosina exerce seus efeitos neuromodulatórios através da ativação de receptores específicos localizados na membrana celular, denominados receptores purinérgicos P1 (A_1, A_{2A}, A_{2B} e A_3) (Fredholm et al., 2005) (Figura 2). Estudos em humanos e roedores indicam que esses receptores são diferentes quanto a afinidade pelo ligante, distribuição tecidual, via de sinalização e perfil farmacológico (Dunwiddie e Masino, 2001; Fredholm et al., 2001). Os receptores A_1 e A_3 são de caráter inibidor, e inibem a enzima adenil ciclase e os receptores A_{2A} e A_{2B} são facilitadores e estimulam a adenil ciclase (Fredholm et al., 2005). Os receptores A_1 e A_{2A} apresentam alta afinidade pelo ligante, enquanto que os receptores A_{2B} e A_3 apresentam menor afinidade (Sebastião e Ribeiro, 2000).

Os receptores A_1 atuam na inibição da adenil ciclase através da ativação da proteína G_i e estão localizados, em mamíferos, em várias regiões do cérebro, como hipocampo, tronco cerebral, medula espinhal e gânglios basais (Guillén-Gómez et al., 2004; Rathbone et al., 1999). Os seguintes efetores também estão envolvidos na ativação dos receptores A_1: guanilil ciclase, canais de K^+, canais de cálcio dependentes de voltagem, a fosfolipase C e a fosfolipase A_2 (Guillén-Gómez et al., 2004; Scheimann e Hicks, 1991). Diferentemente dos receptores A_1, os receptores A_2 estimulam a adenil ciclase acoplando-se à proteína G_s e provocando o aumento dos níveis de AMP_c (Olah e Stiles, 1990; Ruby et al., 2010). A ativação dos receptores A_{2A} medeia ações excitatórias através de um aumento na liberação de neurotransmissores nos terminais pré-sinápticos (Li e Henry, 1998).

Para manter os níveis intra e extracelulares da adenosina em equilíbrio, a adenosina que é formada intracelularmente pode se difundir através da membrana celular por meio de transportadores de nucleosídeos equilibrativos (ENT1 e ENT2) para o espaço extracelular (Nagai, Nagasawa e Fujimoto, 2005). Transportadores de nucleosídeos concentrativos (CNT) também são encontrados no sistema nervoso central e transportam a adenosina de forma acoplada ao gradiente de Na^+ (Fredholm et al., 2005).

No meio extracelular, a adenosina pode ser produzida a partir da hidrólise sequencial do ATP, exercida pela cascata das ectonucleotidases (Zimmermann, 2001). A cascata das ectonucleotidases é composta pelas famílias de enzimas ectonucleosídeo trifosfato difosfohidrolases (E-NTPDase), ectonucleotídeo pirofosfatase / fosfodiesterases (E-NPP), fosfatase alcalina e a ecto-5'- nucleotidase (Zimmermann, 2001; Zimmermann, 1996; Cunha, 2001).

A hidrólise seqüencial dos nucleotídeos 5'- trifosfatados até adenosina tem a ecto-5´-nucleotidase como enzima marcapasso do processo (Fredholm et al., 2001) (Figura 2). A ecto-5´-nucleotidase é uma proteína de membrana ancorada à superfície celular por um fosfatidil-inositol-glicano (GPI) (Zimmermann, 1994). A atividade enzimática das ecto-5´-nucleotidases é dependente de cátions divalentes, como cálcio e magnésio. A enzima encontra-se presente na maioria dos tecidos e seus principais substratos são nucleotídeos monofosfatados extracelulares, tais como AMP, GMP ou UMP, sendo o AMP o nucleotídeo hidrolisado com maior eficiência (Zimmermann, 1996).

A ausência da ecto-5'-nucleotidase durante o desenvolvimento do sistema nervoso impede a diferenciação neuronal e leva a morte celular. Segundo Zimmermann (1996), o

contato de neurônios em migração ou crescimento com outras células expressando a ecto-5'-nucleotidase leva a um aumento significativo na duração dos processos neuríticos induzidos por NGF (fator de crescimento neuronal) e sugere que a ecto-5'-nucleotidase pode ter um efeito trófico sobre a extensão neurítica. Já a aplicação de anticorpos que inibem a atividade da ecto-5'-nucleotidase ou a aplicação de AOPCP (αβ-metileno-adenosina-5'-difosfato), um inibidor competitivo da atividade da ecto-5'-nucleotidase, resulta em uma diminuição da formação de neuritos induzidos pelo NGF (Zimmermann, 1996).

A degradação da adenosina pode ocorrer pela ação da adenosina deaminase (ADA) (Figura 2) que catalisa a conversão da adenosina e da deoxiadenosina à inosina e deoxiinosina, respectivamente (Zavialov e Engström, 2005). A ADA pode ser encontrada como uma enzima citosólica e também pode ser expressa na superfície celular como uma ectoenzima. Dois membros desta família já foram descritos, sendo eles denominados como ADA1 e ADA2 (Hirschhorn e Ratech, 1980; Zavialov e Engström, 2005), além de um grupo similar desta família de proteínas denominado ADAL (adenosine deaminase-like) (Maier, Galellis e McDermid, 2005). Todos estes membros foram classificados como subfamílias pertencentes ao grupo das adenil-desaminases. Por apresentar sítios de aminoácidos importantes relacionados à desaminação de adenosina e motivos conservados entre as subfamílias da ADA, é possível que a ADAL também realize a desaminação hidrolítica de adenosina à inosina (Maier, Galellis e McDermid, 2005).

No SNC, a atuação da ecto-5'-nucleotidase, da ADA e dos transportadores de adenosina sobre a produção de adenosina impacta diretamente a neurotransmissão, visto que, através da ativação dos receptores específicos, a adenosina atua como neuromodulador endógeno e apresenta forte ação depressora sobre a atividade neuronal, principalmente através da ativação de receptores A_1 (Cunha, 2005; Fredholm et al., 2005).

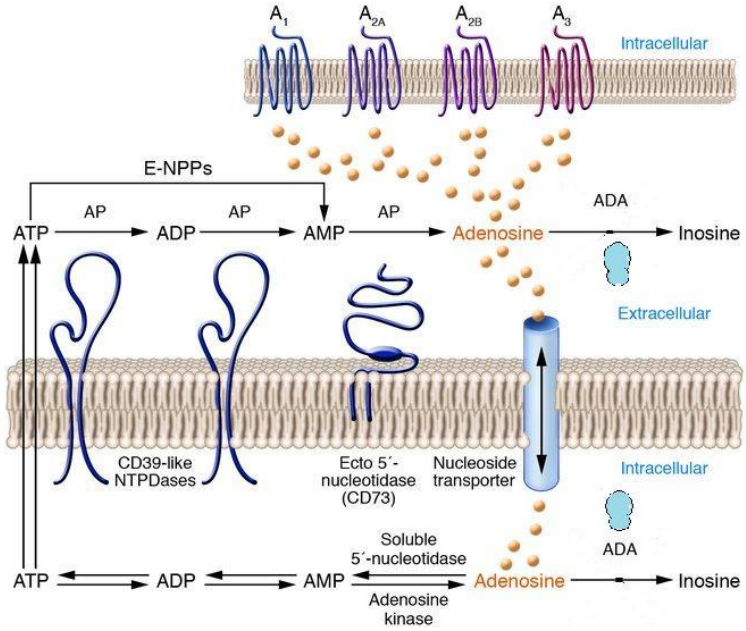

FIGURA 2 - Mecanismos envolvidos na concentração de adenosina. Modificado de Tilley e Boucher (Tilley e Boucher, 2005)**.**

1.2.1 Adenosina e etanol

A adenosina parece estar envolvida nas respostas neuroquímicas e comportamentais causadas pela exposição aguda e crônica ao etanol. Embora a adenosina não seja a única responsável por estas alterações, ela tem um papel neuromodulador que afeta as funções de muitos neurotransmissores que também estão envolvidos nas respostas à exposição ao etanol (Dohrman, Diamond e Gordon, 1997).

Como um neuromodulador, uma das funções da adenosina é inibir a liberação de glutamato. Uma vez que os níveis de adenosina são aumentados em resposta à exposição aguda ao etanol, a inibição da liberação de glutamato regulada pela adenosina pode ser parcialmente responsável pelos efeitos intoxicantes do etanol (Nam et al., 2012; Dunwiddie e Masino, 2001). Além disso, estudos demonstram que a deleção ou inibição do transportador de nucleosídio equilíbrativo tipo 1 (ENT1) provoca uma alteração nos níveis extracelulares de adenosina e regula negativamente um dos principais transportadores de glutamato, consequentemente, aumentando os níveis de glutamato sináptico (Holmes, 2011; Nam et al., 2011; Wu et al., 2011).

Exposições agudas e crônicas ao etanol através de diversas metodologias indicam alterações nos níveis extracelulares de adenosina. O efeito sedativo do etanol, por exemplo, que ocorre depois da ingestão, quando os níveis de etanol no sangue estão declinando, é

regulado pelo aumento de adenosina (Sharma et al., 2010). Quando o etanol é consumido, ocorre o bloqueio da recaptação da adenosina, levando ao aumento da sua concentração extracelular (Choi et al., 2004; Krauss et al., 1993; Marczinski, 2014; Sharma et al., 2010). A exposição aguda ao etanol também resulta no aumento dos níveis intracelulares de AMP_c dependente de adenosina que é mediado pela ativação dos receptores $A_{2A,}$ embora, os receptores A_{2A} tornem-se desensibilizados com a exposição prolongada ao etanol (Tracy e Mark, 2012; Jarvis e Becker, 1998). O transportador de nucleosídeos ENT1 parece desempenhar papel importante no que se refere aos efeitos motores do etanol, visto que, animais sem a expressão destes possuem respostas hipnóticas e de ataxia reduzidas após a exposição ao etanol (Choi et al., 2004; Marczinski, 2014).

Os receptores de adenosina A_1 medeiam vários efeitos do etanol e antagonistas dos receptores A_1 atenuam a descoordenação motora induzida pelo etanol, indicando que a sinalização mediada por receptores A_1 está envolvida nos efeitos de ataxia causados pelo etanol (Dohrman, Diamond e Gordon, 1997). Já os agonistas de receptores A_1 diminuem efeitos como ansiedade, tremor e convulsões durante a retirada aguda do etanol (Ruby et al., 2010). Assim, respostas ansiolíticas a exposição aguda ao etanol são também alteradas por agonistas de receptores de adenosina (Prediger, Batista e Takahashi, 2004).

Em ratos, a exposição aguda ao etanol diminui o estado de vigília e aumenta o movimento dos olhos durante o sono. Esses efeitos são revertidos pela administração de um antagonista de receptores A_1 (DPCPX) (Thakkar et al., 2010). Também já foi visto que antagonistas dos receptores A_{2A} diminuem o nível de consumo de etanol em ratos que preferiam etanol anteriormente (Adams et al., 2008).

Quanto aos efeitos morfológicos, um estudo utilizando peixe-zebra, demonstrou que a exposição ao etanol 2% causa alterações morfológicas acompanhadas de um aumento na hidrólise do AMP, devido a um aumento da atividade da enzima ecto-5´-nucleotidase (Lutte et al., 2015). O AOPCP, inibidor da ecto-5´-nucleotidase não é capaz de prevenir plenamente as alterações morfológicas causadas pelo etanol. Neste mesmo estudo, a inibição do transporte de nucleosídeos com o uso do dipiridamol promoveu uma piora nas alterações morfológicas causadas pelo etanol no peixe-zebra (Lutte et al., 2015). Desta forma, a atividade da ecto-5´-nucleotidase parece ter uma contribuição nas alterações morfológicas causadas pelo etanol, enquanto os transportadores de nucleosídeos parecem estar fortemente relacionados, como já descrito anteriormente (Choi et al., 2004, Lutte et al., 2015).

1.2.2 Adenosina e desenvolvimento

Um grande número de evidências indica que o metabolismo da adenosina tem grande papel no controle do desenvolvimento embrionário e fetal. O período de desenvolvimento embrionário é uma fase de grande suscetibilidade a agentes exógenos e endógenos e as perturbações capazes de alterar a sinalização adenosinérgica durante a fase embrionária podem estar relacionadas com alterações morfológicas e comportamentais que vão do nascimento à vida adulta. Nos últimos anos, se tem demonstrado que a síntese e o metabolismo das purinas e pirimidinas desempenham papéis importantes no controle do desenvolvimento embrionário e fetal e da organogênese (Fumagalli et al., 2017). Mudanças dinâmicas e dependentes do tempo na expressão de enzimas que metabolizam purinas (como ectonucleotidases e adenosina desaminase) representam um ponto de verificação chave para a geração correta das diferentes moléculas sinalizadoras (Fumagalli et al., 2017).

A adenosina está envolvida na eliminação de membranas interdigitais, na apoptose de linfócitos tímicos e no crescimento morfogenético de membros nos vertebrados (Jacobson et al., 1999). Além disso, há indícios de que o desenvolvimento de tecidos e órgãos é altamente sensível ao aumento na concentração de adenosina, uma vez que a adenosina deaminase (ADA), enzima que desamina adenosina em inosina, é expressa em níveis elevados na placenta e sua inibição farmacológica interrompe o desenvolvimento fetal (Knudsen et al., 1992). Deficiências nas funções da ADA ligadas a mutações recessivas autossômicas no gene ADA (20q13.12) têm sido ligadas a severa imunodeficiência e comportamento autista (Bottini et al., 2001; Micheli et al., 2011).

Já a adenosina quinase, enzima que realiza a refosforilação da adenosina em nucleotídeo, exibe alterações ontogenéticas, sendo principalmente expressa em neurônios no início do desenvolvimento e principalmente em astrócitos na vida adulta (Studer et al., 2006). Estes dados aumentam os indícios de que um controle rigoroso nos níveis de adenosina no cérebro é fundamental para o desenvolvimento embrionário e a plasticidade neural (Boison et al., 2012).

Disfunções na homeostase normal da adenosina durante o desenvolvimento inicial do SNC podem ter consequências importantes na formação de circuitos neuronais, contribuindo assim para as alterações do neurodesenvolvimento (Lara e Souza, 2000; Lara et al., 2006). A adenosina também parece ter um papel no crescimento dos neuritos através de sua ação pelos receptores de adenosina A_{2A} (Heine, Sygnecka e Franke, 2016).

Alterações nas funções dos receptores de adenosina A_1 durante a embriogênese também parecem estar criticamente ligadas a prejuízos, principalmente cardíacos, na vida adulta. Camundongos *knockout* para os receptores de adenosina A_1 apresentam retardo do crescimento após hipóxia intrauterina (Rivkees e Wendler, 2012). A adenosina parece atuar via receptores A_1 desempenhando um papel essencial na proteção do embrião contra estresses (Rivkees e Wendler, 2012). Já a exposição durante o início do desenvolvimento aos antagonistas de receptores de adenosina, incluindo a cafeína, parece promover disfunção cardíaca (Rivkees e Wendler, 2012). Dados também mostram que a ativação de receptores A_1 pode prejudicar a formação do cérebro levando a reduções no volume da substância branca subcortical e hipocampal e perdas em axônios (Rivkees et al., 2001).

A adenosina está envolvida na regulação de várias vias de sinalização no SNC (Boison, 2008), atuando como um neuromodulador através de múltiplos mecanismos, incluindo o controle da liberação de neurotransmissores, ou via efeitos regulatórios nas células da glia (Boison, Chen e Fredholm, 2010; Boison et al., 2012). Já foi demostrado que a ativação dos receptores A_1 inibe a liberação de neurotransmissores, como a dopamina e o glutamato, e diminui a excitabilidade neural pela indução da hiperpolarização pós-sináptica e, por outro lado, os receptores A_{2A} promovem a liberação de neurotransmissores (Fredholm et al., 2005). Com base nisso, e nas interações do sistema purinérgico com a neurotransmissão glutamatérgica e dopaminérgica, a adenosina tem sido ligada a defeitos no desenvolvimento neurológico e envolvimento em patologias como a esquizofrenia (Lara e Souza, 2000; Lewis e Levitt, 2002), epilepsia (Boison, 2016; Menezes e Da Silva, 2017) doença de Parkinson e processos cognitivos (Soliman et al., 2018).

1.3 Modelos de SAF e SAF parcial em peixe-zebra

O peixe-zebra (*Danio rerio*) é um pequeno teleósteo da família Cyprinidae, de aproximadamente 4 cm de comprimento (Parichy, 2006). O peixe-zebra, além de ser um organismo vertebrado, possui um genoma com alto grau de homologia em relação ao genoma humano (Howe et al., 2013). O peixe-zebra é uma espécie com comportamento de cardume, que procura preferencialmente por interação social com o maior número de indivíduos possível (Buske e Gerlai, 2011; Spence et al., 2008). Muito do repertório de respostas comportamentais do peixe-zebra pode ser visto em testes de memória (Yu et al., 2006),

aprendizado, interação social (Colwill et al., 2005; Sison e Gerlai, 2010) e preferência por ambientes (Blaser e Peñalosa, 2011).

A utilização do peixe-zebra em estudos em estágios iniciais do desenvolvimento está em ascensão, pois ele oferece uma série de vantagens. Seu tamanho e a forma de criação permitem a manutenção de uma grande quantidade de peixes em um espaço relativamente pequeno. O cruzamento resulta em um grande número de ovos e o desenvolvimento ocorre rapidamente e progride através de etapas bem definidas (Kimmel et al., 1995; Westerfield, 2000). Estudos recentes têm empregado o peixe-zebra como modelo para a síndrome alcoólica fetal e demonstrado que a exposição embrionária ao etanol resulta em fenótipos comparáveis com aqueles observados em outros modelos vertebrados, o que sugere que o peixe-zebra é um modelo relevante para entender os danos causados pelo etanol ao longo do desenvolvimento (Howarth, Passeri e Sadler, 2011).

Alguns dos danos causados pela exposição embrionária ao etanol em altas concentrações (entre 1.5 e 3%) no peixe-zebra são: microftalmia (Kashyap, Frederickson e Stenkamp, 2007; Lutte et al., 2015), ciclopia, aumento da distância ocular, edema pericardial, má formação axial (Arenzana et al., 2006; Lutte et al., 2015) e aumento da morte celular na parte posterior do cérebro (Loucks e Carvan, 2004). Já quando se usam concentrações mais baixas de etanol (entre 0.1 e 1%), raramente as alterações morfológicas se mostram evidentes, em contrapartida, podem ser observadas alterações na locomoção, no comportamento, no aprendizado e no *shoaling* (Carvan et al., 2004; Lockwood et al., 2004; Fernandes e Gerlai, 2009; Willford et al., 2004; Richardson et al., 2002; Baggio et al., 2018).

No peixe-zebra, a exposição ao etanol provoca alterações no eixo hipotálamo – hipófise – interrenal (HPI) (o equivalente ao hipotálamo – hipófise –adrenal (HPA) no ser humano), após exposição ao etanol do 1º ao 9º dia pós-fertilização (dpf), reduzindo de forma duradoura a resposta do cortisol (Baiamonte, Brennan e Vinson, 2015). Teorias sugerem que adaptações no eixo HPA, podem ter um papel importante nas respostas comportamentais e fisiológicas causadas por agentes aditivos (Baiamonte, Brennan e Vinson, 2015).

Estudos indicam que um dos fatores que pode estar envolvido nas alterações de *shoaling* é a alteração no sistema dopaminérgico (Buske e Gerlai, 2011). Sabe-se que a quantidade de dopamina e do seu metabólito, DOPAC, é significativamente reduzida em encéfalos de peixes-zebra adultos que foram expostos ao etanol durante o desenvolvimento embrionário. Tem-se mostrado que testes com imagens animadas específicas induzem a uma

rápida elevação dos níveis de dopamina e DOPAC no encéfalo de peixe-zebra (Chatterjee, Buske e Gerlai, 2013), mas não em peixes-zebra que foram expostos ao etanol durante o desenvolvimento embrionário (Fernandes, Rampersad e Gerlai, 2015).

No que se refere ao estudo do sistema purinérgico e, em especial, ao metabolismo e função da adenosina, a presença da atividade de hidrólise de AMP, bem como a expressão gênica da ecto-5´-nucleotidase já foram demonstradas em peixe-zebra (Senger et al., 2004; Rico et al., 2008), assim como a presença dos receptores de adenosina A_1, A_{2A} e A_{2B} (Capiotti et al., 2011; Boehmler et al., 2009). Mais recentemente, o bloqueio temporário da tradução desses receptores em peixe-zebra em fase embrionária promoveu alterações na morfologia e sobrevivência dos animais (Menezes et al., 2018). O mapeamento do padrão de expressão de genes relacionados à adenosina deaminase também foi realizado em vários tecidos de peixe-zebra (Rosemberg et al., 2007), bem como, a caracterização cinética da atividade da ADA em encéfalo de peixe-zebra (Rosemberg et al., 2008).

Esses achados indicam que a neuromodulação exercida pela adenosina pode estar fortemente envolvida nos traços alterados pela exposição ao etanol nas fases críticas do desenvolvimento neurológico. O uso do peixe-zebra nesta abordagem é interessante visto que durante o seu desenvolvimento, os estágios de gástrula / segmentação e faríngula representam períodos importantes no desenvolvimento do sistema nervoso, e por isso, esses períodos se tornam fundamentais na avaliação dos danos causados pela exposição ao etanol e a avaliação acerca da persistência dos efeitos desta droga até a idade adulta, além disto, existe um vasto repertório de análises de parâmetros locomotores, comportamentais, mnemônicos e bioquímicos aplicáveis neste estudo.

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar o efeito da exposição embrionária ao etanol nos estágios de Gástrula / Segmentação e Faríngula sobre parâmetros comportamentais, locomotores e mnemônicos de adultos de peixe-zebra (*Danio rerio*) e correlacioná-los ao estudo da neuromodulação adenosinérgica.

2.2 Objetivos Específicos

- Avaliar a atividade enzimática e a expressão gênica da ecto-5´-nucleotidase e da adenosina deaminase em encéfalo de adultos de peixe-zebra de 3 mpf após a exposição ao etanol.

- Avaliar parâmetros comportamentais e locomotores de adultos de 3 e 12 mpf após a exposição ao etanol.

- Avaliar parâmetros de memória aversiva em adultos de 3 mpf após a exposição ao etanol.

- Avaliar o efeito de um inibidor da ecto-5´-nucleotidase e de um inibidor da adenosina deaminase sobre parâmetros locomotores, comportamentais e de memória aversiva em adultos de 3 mpf após a exposição ao etanol.

- Avaliar o efeito de um inibidor da ecto-5´-nucleotidase sobre parâmetros locomotores e comportamentais em adultos de 12 mpf após a exposição ao etanol.

- Avaliar o conteúdo de adenosina em encéfalo de adultos de peixe-zebra de 3 mpf após a exposição ao etanol.

3. RESULTADOS

3.1 Capítulo I

Artigo Científico Original

Early exposure to ethanol is able to affect the memory of adult zebrafish: Possible role of adenosine.

Autores: Aline Haab Lutte, Júlia Huppes Majolo, Luiza Reali Nazario, Rosane Souza Da Silva.

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Full Length Article

Early exposure to ethanol is able to affect the memory of adult zebrafish: Possible role of adenosine

Aline Haab Lutte, Júlia Huppes Majolo, Luiza Reali Nazario, Rosane Souza Da Silva*

Laboratório de Neuroquímica e Psicofarmacologia, Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

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ABSTRACT

Ethanol is one of the most widely consumed drugs in the world, and the effects of ethanol during early development include morphological and cognitive problems. The regulation of adenosine levels is essential for the proper function of major neurotransmitter systems in the brain, particularly glutamate and dopamine; thus, the investigation of the relation of adenosine and memory after early ethanol exposure becomes relevant. Embryos of zebrafish were exposed to 1% ethanol during two distinct developmental stages: gastrula/segmentation or pharyngula. The evaluation of memory, morphology, and locomotor parameters was performed when fish were 3 months old. The effect of ecto-5′-nucleotidase and adenosine deaminase inhibition on the consequences of ethanol exposure with regard to memory formation was observed. Morphological evaluation showed decreases in body length and the relative telencephalic and cerebellar areas in ethanol exposed animals. The locomotor parameters evaluated were not affected by ethanol. In the inhibitory avoidance paradigm, ethanol exposure during the gastrula/segmentation and pharyngula stages decreased zebrafish memory retention. When ethanol was given in the pharyngula stage, the inhibition of ecto-5′-nucleotidase in the acquisition phase of memory tests was able to revert the effects of ethanol on the memory of adults. These findings suggest that the increased adenosine levels caused by ethanol could alter the neuromodulation of important components of memory formation, such as neurotransmitters. The adjustment of adenosine levels through ecto-5′-nucleotidase inhibition appears to be effective at restoring normal adenosine levels and the acquisition of memory in animals exposed to ethanol during the pharyngula stage.

1. Introduction

Ethanol is one of the most widely consumed drugs in the world, and the fetal effects of ethanol are the cause of fetal alcohol syndrome (FAS) (Cudd, 2005). FAS consists of central nervous system (CNS) dysfunction (Clarren and Smith, 1978); intrauterine growth deficiency (Haghighi et al., 2014); facial abnormalities; and heart, skeleton, and muscle anomalies (Sei et al., 2003; Garriga et al., 2000; Mattson and Furukawa, 1998). Symptoms and characteristics include mental retardation, hypotonia, memory and behavioral problems, microcephaly, reduced cerebral white matter volume, and abnormalities of the corpus callosum and cerebellar vermis (Archibald et al., 2001; Mattson et al., 1996; Olson et al., 1998).

Long-term follow-up of children exposed to ethanol demonstrates that mental retardation, abnormal behavior, and facial dimorphism persist into adulthood (Lemoine, 1992). It is also evident that cognitive and behavioral abnormalities can occur in the absence of dimorphism, so-called "alcohol-related neurodevelopmental disorder" (ARND) or "fetal alcohol spectrum disorder" (FASD) (O'Callaghan et al., 2007). Ethanol can cause learning impairments, amnesia, or impaired retrieval of information (López-Cruz et al., 2013). Ethanol clearly interacts with a variety of neurotransmitters and neuromodulators, including adenosine (Vasconcelos et al., 2012; López-Cruz et al., 2016; Oliveros et al., 2017), affecting different functional and anatomical systems to varying degrees (Paule, 2006).

In general, long-term potentiation (LTP) of excitatory synaptic transmission seems to require activation of glutamate receptors and inhibition of Gamma-AminoButyric Acid A ($GABA_A$) receptors. The ethanol might inhibit LTP because short-term ethanol exposure inhibits glutamate receptor function (Lovinger et al., 1990; Bliss and Collingridge, 1993) and stimulates $GABA_A$ receptor function in the hippocampus (Weiner et al., 1994). Whereas the regulation of adenosine levels is essential for the proper function of major neurotransmitter systems in the brain, particularly glutamate (Ruby et al., 2010), the investigation of the relation of adenosine and memory after early ethanol exposure becomes relevant. In fact, the main known

* Corresponding author at: Escola de Ciências, PUCRS Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil.
E-mail address: rosane.silva@pucrs.br (R.S. Da Silva).

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physiological action of adenosine on the CNS is the inhibition of neurotransmitter release by reducing neuronal excitability (Burnstock, 2008; Fredholm et al., 2005). The extracellular levels of this neuromodulator are affected by the sequential hydrolysis of ATP exerted by the cascade of ectonucleotidases, including ecto-5′-nucleotidase (Dunwiddie et al., 1997), and their conversion to the inactive metabolite inosine by ecto-adenosine deaminase (Dunwiddie et al., 1997).

Ethanol appears to affect adenosine tonus through the inhibition of adenosine reuptake exerted by type I equilibrative nucleoside transporters and an increase in ecto-5′-nucleotidase activity, which seems to promote activation of A_2 adenosine receptors (Diamond et al., 1991; Lutte et al., 2015; Nagy et al., 1990). However, there is a lack of knowledge about the long-term consequences of ethanol disturbance in early development over behavioral and mnemonic events. In this context, the objective of this research was to evaluate the memory parameters in adult zebrafish that were exposed to ethanol during early development, and the participation of adenosine in the consequences of early ethanol exposure on young/adult memory.

2. Material and methods

2.1. Animal maintenance

Wild type adult zebrafish (*Danio rerio*) (Tübingen background; 3–5 cm) maintained in an automated recirculating tank system were used for obtaining fertilized eggs. Male and female (3:1 ratio) zebrafish were separated at dusk. At the next dawn, zebrafish were allowed to mate in a 3.5 L aquarium. After mating, eggs were collected and kept in water for maintenance (water from reverse osmosis reconstituted with marine salt [Instant Ocean, Blacksburg, VA] 0.4 parts per thousand) in an incubator at 28.5 °C on a 14:10 light/dark cycle. After the beginning of the swimming phase, larval zebrafish were transferred to aquariums and kept until 3 months in proper fish densities (6–9 dpf [days post-fertilization]: 16 fish/L; 18–22 dpf: 10 fish/L; 45 dpf or more: 5 fish/L). Animals were fed three times a day with commercial flakes (TetraMin™, NC, USA) and supplemented with live brine shrimp.

At 90 dpf, animals received locomotor, mnemonic, and morphological evaluations. After evaluations, adult animals were cryoanesthetized using flocked ice immersion with water with controlled physicochemical parameters and temperature of about 2 °C, followed by euthanasia through decapitation.

All procedures were in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and were approved by the Institutional Animal Care Committee (15/00468 — CEUA PUCRS).

2.2. Ethanol exposure

Embryos of zebrafish were exposed to ethanol in two distinct developmental stages: gastrula/segmentation (5–24 hpf [hours post-fertilization]) or pharyngula (24–48 hpf), where the CNS begins to be formed followed by further subdivision of encephalic regions (Kimmel et al., 1995). The animals were exposed to 1% (v/v) ethanol (diluted in the water of animal maintenance) in groups of 100 embryos kept in Petri dishes during the gastrula/segmentation or pharyngula stage. Such a dose is representative of doses used in several studies to induce behavioral changes (Fernandes and Gerlai, 2009; Mathur and Guo, 2011).

After ethanol treatment, animals were kept 3 months at proper densities in aquariums (5 L; 27 cm × 17 cm × 12 cm; width [w] × height [h] × depth [d]) with drug-free water under biological and mechanical filtration and aeration (7.20 mg O_2/L) and controlled temperatures (28 ± 2 °C). At the end of 3 months, the evaluation of memory, locomotor, and morphological parameters was performed.

2.3. Exposure to α,β-Methyleneadenosine 5′-diphosphate (AMPCP) or erythro-9-(2-Hydroxy-3-nonyl)adenine hydrochloride (EHNA)

To evaluate the effects of an inhibitor of ecto-5′-nucleotidase or an inhibitor of adenosine deaminase on the consequences observed by ethanol exposure, we used adenosine 5′-(α,β-methylene)diphosphate (AMPCP; 150 mg/kg) or erythro-9-Amino-β-hexylα-methyl-9H-purine-9-ethanol hydrochloride (EHNA; 100 mg/kg), respectively. The pharmacological treatments in adults were performed by intraperitoneal (ip) injection with the use of an insulin syringe (Ultra-Fine™ Short Insulin Syringe 8 mm [5/16″]) in a volume of 10 µL per animal (20 mL/kg). Prior to the injections, the adult animals were anesthetized in tricaine solution (MS-222; 100 mg/L) (Siebel et al., 2015). Locomotion assessment was performed 30 min after drug exposure (Siebel et al., 2015). In the experiments of inhibitory avoidance, the drugs were administered 30 min before or after the training session (Blank et al., 2009).

2.4. Morphological assessment

For the purpose of monitoring body and brain development after ethanol exposure, we measured the body length (cm), encephalic surface area (mm^2), and two areas of interest, the telencephalon (mm^2) and cerebellum (mm^2), of animals based on Naslund (2014). The adult animals were evaluated under stereomicroscopy (3×) (Nikon SMZ1500, Melville, USA). Animals were anesthetized in tricaine solution (MS-222; 100 mg/L) (Siebel et al., 2015). The measurement was performed by photographical registration, followed by analysis using the software NIS-Elements D 3.2 for Windows supplied by Nikon Instruments, Inc. (Melville, USA). Encephalic surface area (mm^2) was assumed as the sum of the three areas (cerebellum, optic tectum, and telencephalon), and measurements of the telencephalon (mm^2) and cerebellum (mm^2) areas were performed according to the delimitations showed in Fig. 1. The body length was assumed as the distance from the center of an eye to the tip of the tail bud (Lutte et al., 2015).

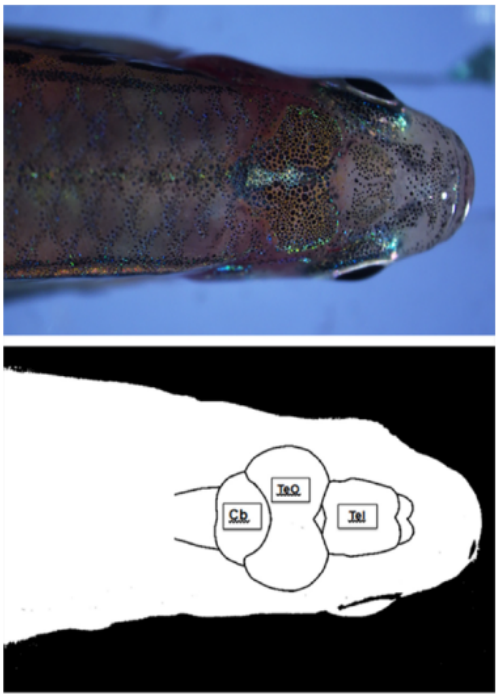


Fig. 1. Representative morphological measures of 90 dpf zebrafish control. Cb: cerebellum; TeO: optic tectum; Tel: telencephalon. Optical zoom 3×.

2.5. Locomotor assessment

The locomotion of adult fish (90 dpf) was evaluated in the groups treated with ethanol in the gastrula/segmentation or pharyngula stage, control (no ethanol added), and after 30 min of exposure to the inhibitors of AMPCP and EHNA. The adult zebrafish were placed individually in a small tank (10 cm × 15 cm × 30 cm; w × h × d). After 30 s of adaptation, the locomotor activity was recorded for 5 min and analyzed using the video-tracking system Ethovision® XT8 (Noldus, Netherlands) (Menezes et al., 2015). The locomotor parameters analyzed were distance traveled (m) and mean speed (m/s).

2.6. Memory assessment task

The protocol of inhibitory avoidance followed Blank et al. (2009) and represents a valuable tool to characterize robust and stable events of vertebrate memory formation. Briefly, the animals were individually trained and tested in a tank (18 cm × 9 cm × 7 cm; w × h × d) subdivided into white and dark chambers apart from a sliding wall. In each session, the animals were gently placed in the white tank compartment while the sliding wall was closed. After 1 min of habituation and orientation, the wall was raised, allowing the fish to cross to the dark side of the tank through a 1 cm high opening. In a training session, immediately after crossing to the dark compartment, the sliding partition was closed and a pulsed electric shock of 3 ± 0.2 V was administered for 5 s, after which animals were removed from the apparatus. Twenty-four hours after training, the animals were submitted to a test session that repeated the training protocol, except that no shock was administered and the sliding wall was kept open, allowing the animals to freely explore the apparatus. Fish that would not enter the black compartment within 180 s were excluded from further experimentation. The latency to enter the dark compartment was measured in the training and test sessions and was used as an index of memory retention. AMPCP and EHNA were administered 30 min before or after training sessions. To avoid the stress or interference caused by the handling of the animals during the experiments, different groups were used for the evaluations of locomotion and memory, and these experiments were repeated three times with 10 animals per group (Scheme 1).

2.7. Statistical analysis

Morphological parameters were compared by one-way Analysis of Variance (ANOVA), followed by Tukey's multiple comparison test. For locomotion assessment, two-way ANOVA was used to compare groups exposed to ethanol and AMPCP or EHNA, followed by Sidak's multiple comparison test. For memory assessment, as a cutoff point was introduced, the comparison of latencies in training versus test sessions (within group's comparison) was performed using the unpaired T-Test, while the One-Way ANOVA was used to compare the latency between groups (between group's comparison), followed by Tukey's multiple comparison test. The significance levels were attributed at $p < 0.05$. Data were expressed as mean ± standard error of mean.

3. Results

3.1. Long-term ethanol effects

Animals treated with ethanol did not demonstrate gross body malformations, and no deaths occurred through the experiments. Body length of control animals was 2.67 ± 0.03 cm, while animals treated with ethanol at the gastrula/segmentation stage and pharyngula stage were 2.21 ± 0.07 cm and 2.08 ± 0.04 cm ($p < 0.001$; $F_{(2;68)} = 40.74$), respectively. In accordance with that, the encephalic surface area of ethanol-treated animals (gastrula/segmentation: 2.61 ± 0.06 mm^2; pharyngula: 2.48 ± 0.07 mm^2) was also smaller than control animals (3.13 ± 0.07 mm^2) ($p < 0.001$; $F_{(2;68)} = 24.20$). The analysis of the proportional area of telencephalon/encephalon and cerebellum/encephalon also detected significant differences between control and ethanol-treated animals. Control animals showed a proportional telencephalic area of 0.24 (24%) and cerebellar area of 0.17 (17%), while animals treated during gastrula/segmentation presented with a telencephalic area of 0.20 (20%) and those treated during pharyngula presented with a telencephalic area of 0.22 (22%) ($p < 0.01$; $F_{(2;68)} = 6.283$). Animals treated during gastrula/segmentation presented with a cerebellar area of 0.14 (14%), while animals treated during pharyngula presented with a cerebellar area of 0.15 (15%) ($p < 0.001$; $F_{(2;68)} = 13.67$).

The locomotor parameters evaluated (distance traveled and mean speed) were not affected by exposure to ethanol during the early phases of development (Fig. 2).

In the inhibitory avoidance paradigm, ethanol exposure during the gastrula/segmentation stage decreased zebrafish latencies between 46 and 70% (Fig. 3B and A, respectively), while during the pharyngula stage, ethanol decreased the latencies of adults in the avoidance paradigm between 47 and 63% (Fig. 3B and A, respectively), when both were compared to the control/saline group ($p < 0.001$).

3.2. Effects of ecto-5′-nucleotidase and adenosine deaminase inhibition

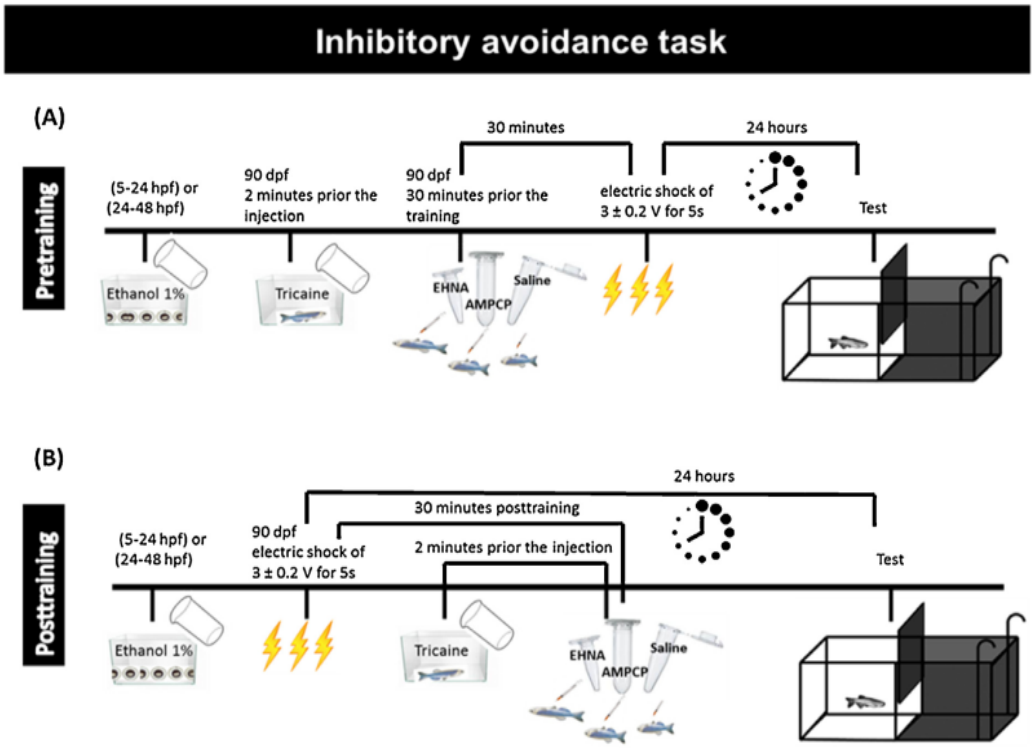
AMPCP alone did not generate a significant effect on locomotor and mnemonic parameters when compared to the control/saline group. The decrease in latency in the test sessions, which correlates with the impairment on memory, exerted by 1% ethanol in the gastrula/segmentation stage was not affected by the administration of AMPCP 30 min before the training session (pretraining) or 30 min after the training session (posttraining) (Fig. 3A and B). However, when ethanol was given in the pharyngula stage, the administration of AMPCP during pretraining was able to revert the decrease of latency caused by ethanol (Fig. 3A). The administration of AMPCP during posttraining, in animals exposed to ethanol in the pharyngula stage, did not change ethanol's effects on memory (Fig. 3B).

The use of EHNA alone, an inhibitor of adenosine deaminase, decreased the latency to 76% of the control/saline group ($p < 0.001$), which interrupted further analysis (data not shown).

4. Discussion

The exposure period appears to be as important as the dose of ethanol, displaying different consequences (Joya et al., 2014; Gerlai, 2015). In fish, the morphogenesis and primary organogenesis occurs at the gastrula/segmentation stage, while in the pharyngula stage the brain starts to develop and the early touch reflex and spontaneous movements appear (Kimmel et al., 1995). In this sense, severe morphological consequences of ethanol in fish that resemble FAS are more expected during the gastrula/segmentation period and at higher ethanol doses (Joya et al., 2014), while the pharyngula stage is more suitable to effects on neural refinement and can be correlated with ARND (Gerlai, 2015).

Here, we had no visible gross body malformation in 1% ethanol-treated animals. However, reduced body length and reduced proportional telencephalic and cerebellar areas were registered in both groups with ethanol exposure, which could be related to mnemonic consequences. As the normal swimming performance of fish is a mandatory requirement to access memory, we initially evaluated their locomotion. Locomotion is controlled by a number of different regulatory neuronal components in zebrafish, but it is the sensory signals that are sent to supraspinal structures, including the cerebellum, that are used to correct and adapt the locomotor behavior (Aizenberg and Schuman, 2011; Kiehn and Dougherty, 2013). Although the body length was reduced by ethanol exposure, the locomotor parameters evaluated were not affected by exposure to ethanol during the early phases of development,



Scheme 1. Experimental design of the inhibitory avoidance task. Ethanol treatment: The animals were treated with ethanol in two distinct developmental stages: gastrula/segmentation (5–24 hpf) or pharyngula (24–48 hpf). Ninety days after this, the animals were anesthetized in tricaine solution (MS-222; 100 mg/L) for 2 min and removed from the tricaine. One group was injected with EHNA, another group was injected with AMPCP, and the control group was injected with saline solution, 30 min before (A) (pretraining) or 30 min after a training session (B) (posttraining). In a training session, immediately after crossing to the dark compartment, the sliding partition was closed and a pulsed electric shock of 3 ± 0.2 V was administered for 5 s. Twenty-four hours after training, the same animals were submitted to a test session that repeated the training protocol, except no shock was administered. The latency to enter the dark compartment was measured in all sessions, and the test latency was used as an index of memory retention. This procedure was repeated three times with different sets of 10 animals.

excluding that as a confounding factor on memory assessment.

In the inhibitory avoidance paradigm, ethanol exposure during the gastrula/segmentation and pharyngula stages decreased zebrafish latencies when compared to the control/saline group, which correlates with the impairment of memory formation. In teleost fish, the dorsal telencephalic areais currently considered to be the homolog of the mammalian hippocampus (Rodríguez et al., 2002; Wullimann and Mueller, 2004) that is required for the formation of several kinds of memories (Vargas et al., 2006; Lombroso, 2004). An increasing number of studies are dedicating efforts to contribute to the identification of targets of ethanol that underline long-term cognitive impairments associated with ARND. Adenosine is a modulator of several neurotransmitter systems, including dopamine, glutamate, and acetylcholine (Ferre et al.,1992; Okada et al.,1996; Cunha, 2001; Quarta et al., 2004). Adenosine is ubiquitous in the brain and is released by both neurons and glial cells via constitutive and activity-dependent mechanisms. Adenosine equilibrates neuronal activity and sets the stage for synaptic plasticity. Through A_1 and A_{2A} receptor activation, it modulates neuronal homeostasis and tunes the ability of synapses to undergo and/or sustain plasticity, an essential process in memory formation (Dias et al., 2013). Adenosine influences neurotransmitter function through hyperpolarization of postsynaptic membranes, reduction of vesicle release, and receptor-receptor interactions (Ciruela et al., 2011; Cunha, 2001). The use of AMPCP in an acute approach is intended to reduce adenosine viability through inhibition of ecto-5′-nucleotidase. The effects *per se* of this drug do not affect the memory index, indicating that adenosine levels are kept by other sources, such as nucleoside transporters. However, when an adenosine deaminase inhibitor is added, the degradation of adenosine decreases, which increases adenosine availability and is enough to affect *per se* memory processes.

Adenosine signaling could be participating in the long-lasting effects of ethanol in several ways. Glutamate, as an important player in LTP, and dopamine, through its role in the interconnection of the striatum and prefrontal cortex, are some of the important neurotransmitters in the memory process that could be targets of impaired adenosine signaling (Bliss and Collingridge, 1993; Olvera-Cortés et al., 2008). Studies of early exposure to ethanol using zebrafish demonstrated impairment in the function of cerebral glutamate transport in adult fish (Baggio et al., 2017). Additionally, it was already demonstrated that 3,4-Dihydroxyphenylacetic acid (DOPAC) levels are unresponsive to behavioral challenges in adult zebrafish exposed during early life to ethanol (Fernandes et al., 2015). In this way, a hypothesis to be raised and investigated is the possibility of increased adenosine levels contributing to the reduction of dopaminergic and glutamatergic activities.

In accordance with the notion that synaptic plasticity is the basis for learning and memory in different brain areas, adenosine correspondingly modulates behavior in various learning and memory paradigms (Jay, 2003). In zebrafish, the regions responsible for memory have already been formed in the segmentation stage (Kimmel et al., 1995) and are less susceptible to damage caused by ethanol. The telencephalon and cerebellum are formed during the first half of the segmentation period, with the cerebellum becoming distinctive late in the segmentation period. With the rapid cerebellar morphogenesis of the metencephalon, just preceding the pharyngula period, the brain is sculptured into five lobes (Kimmel et al., 1995). Our results showed that the memory impairment detected in adults when ethanol was given in the pharyngula stage was reverted by the acute inhibition of ecto-5′-nucleotidase by AMPCP administered during pretraining sessions. Thus,

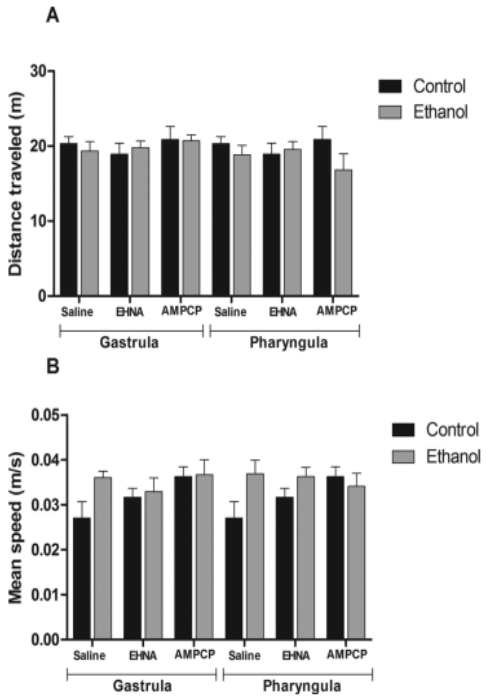


Fig. 2. The locomotor parameters evaluated, distance traveled (m) (A) and mean speed (m/s) (B), were not affected by 1% ethanol during the gastrula/segmentation and pharyngula stages (n = 3). For locomotion assessment, two-way ANOVA was used to compare groups exposed to ethanol and AMPCP or EHNA, followed by Sidak's multiple comparison test.

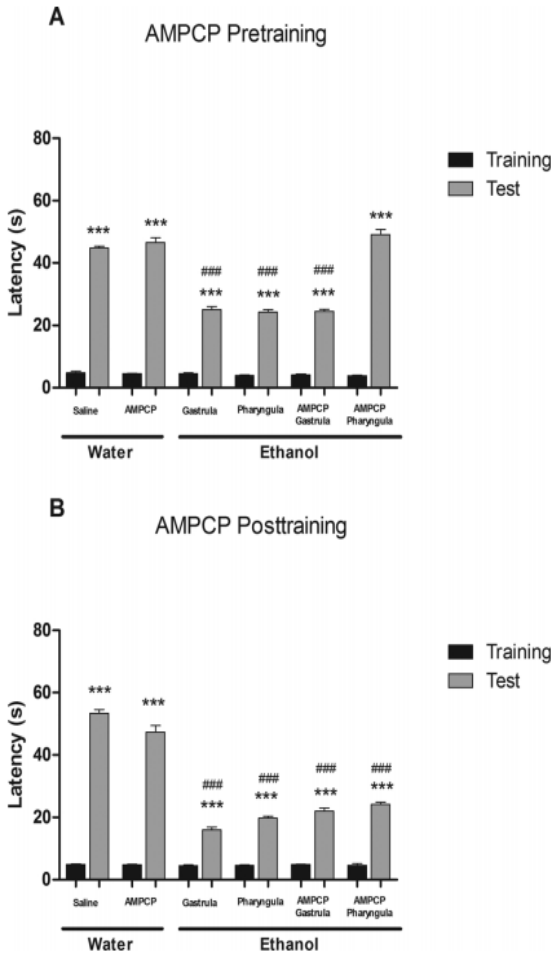


Fig. 3. Treatment with 1% ethanol during the gastrula/segmentation and pharyngula stages had long-term effects on memory acquisition (A) and formation (B) in a one-trial inhibitory avoidance task. The inhibitor of ecto-5′-nucleotidase (AMPCP) given in the adult animals treated at the pharyngula stage was able to revert the decrease of latency caused by ethanol (3 different experiments were performed with 10 animals as replicates). Latency (in seconds) to cross to the dark compartment is depicted in the pretraining (A) and posttraining (B) test sessions in the animals exposed to ethanol during the gastrula/segmentation and pharyngula stages. AMPCP was given 30 min prior to the training session or 30 min after the training session. Latencies in training versus test sessions (within group's comparison) were compared using the T-Test, while the One-Way ANOVA was used to compare the latencies between groups (between group's comparisons), followed by Tukey's multiple comparison test. The significance levels were attributed at $p < 0.05$. Data were expressed as mean ± standard error of mean. * represents difference from respectively group at Training session; # represents difference from saline group. Three * or # represent significance level at $p < 0.001$.

the inhibition of ecto-5′-nucleotidase, at least in the memory acquisition phase, may alter adenosine levels sufficiently to recover adequate levels of neurotransmitters (e.g., dopamine and glutamate), reflecting the recovery of memory mechanisms. Adenosine levels receive contribution, at least, from a triplet of components-ecto-5′-nucleotidase, ecto-adenosine deaminase, and nucleoside transporters— which can be regulated by transcriptional adjustments, changes in kinetic properties, allosteric effects, and stoichiometric effects (Frick et al., 1986; Heyliger et al., 1981; Bianchi and Spychala, 2003). While no data is available from zebrafish studies, in rodents, the knockout of ecto-5′-nucleotidase improved short-term spatial working memory performance (Zlomuzica et al., 2013).

The final consequence of an adjustment of these components is that the adenosine levels in the extracellular medium, a compensatory arrangement between ecto-5′-nucleotidase, ecto-adenosine deaminase, and nucleoside transporters, could reflect in the specific differences between developmental stages and the dependence of adenosine in the memory retention process. Taken together, these findings suggest that the control of adenosine levels appear to be involved in the long-lasting mnemonic effects of early exposure to ethanol. While the ecto-5′-nucleotidase could be a target for the effects seemed here, further studies could be performed to evaluate the actual adenosine concentration in this context of ethanol early exposure.

Conflict of interest

The authors declare no conflict of interest.

Author's contribution

AHL and JHM contributed to the breeding and maintenance of animals. AHL, LRN, and JHM were responsible for memory assessment tasks. AHL was responsible for the assessment of morphological and locomotor parameters. AHL and RSS assisted with data analysis and interpretation of the findings. AHL and RSS did the study design and drafted the manuscript. All authors critically reviewed content and approved the final version for publication.

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3.2 Capítulo II

Artigo Científico Original

Persistent increase in ecto-5'-nucleotidase activity from encephala of adult zebrafish exposed to ethanol during early development

Autores: Aline Haab Lutte, Luiza Reali Nazario, Júlia Huppes Majolo, Talita Carneiro Brandão Pereira, Stefani Altenhofen, Adilio da Silva Dadda, Maurício Reis Bogo, Rosane Souza Da Silva

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Persistent increase in ecto-5′-nucleotidase activity from encephala of adult zebrafish exposed to ethanol during early development

Aline Haab Lutte[a], Luiza Reali Nazario[a], Júlia Huppes Majolo[a], Talita Carneiro Brandão Pereira[b], Stefani Altenhofen[a], Adilio da Silva Dadda[c], Maurício Reis Bogo[b], Rosane Souza Da Silva[a,*]

[a] Laboratório de Neuroquímica e Psicofarmacologia, Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Laboratório de Biologia Genômica e Molecular, Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil
[c] Instituto Nacional de Ciência e Tecnologia em Tuberculose, Centro de Pesquisas em Biologia Molecular e Funcional, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

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ABSTRACT

Prenatal alcohol exposure causes alterations to the brain and can lead to numerous cognitive and behavioral outcomes. Long-lasting effects of early ethanol exposure have been registered in glutamatergic and dopaminergic systems. The purinergic system has been registered as an additional target of ethanol exposure. The objective of this research was to evaluate if the ecto-5′-nucleotidase and adenosine deaminase activities and gene expression of adult zebrafish exposed to 1% ethanol during early development could be part of the long-lasting targets of ethanol. Zebrafish embryos were exposed to 1% ethanol in two distinct developmental phases: gastrula/segmentation (5–24 h post-fertilization) or pharyngula (24–48 h post-fertilization). At the end of three months, after checking for morphological outcomes, the evaluation of enzymatic activity and gene expression was performed. Exposure to ethanol did not promote gross morphological defects; however, a significant decrease in the body length was observed (17% in the gastrula and 22% in the pharyngula stage, $p < 0.0001$). Ethanol exposure during the gastrula/segmentation stage promoted an increase in ecto-5′-nucleotidase activity (39.5%) when compared to the control/saline group ($p < 0.0001$). The ecto-5′-nucleotidase gene expression and the deamination of adenosine exerted by ecto and cytosolic adenosine deaminase were not affected by exposure to ethanol in both developmental stages. HPLC experiments did not identify differences in adenosine concentration on the whole encephala of adult animals exposed to ethanol during the gastrula stage or on control animals ($p > 0.05$). Although the mechanism underlying these findings requires further investigation, these results indicate that ethanol exposure during restricted periods of brain development can have long-term consequences on ecto-5′-nucleotidase activity, which could have an impact on subtle sequels of ethanol early exposure.

1. Introduction

The negative effects of prenatal ethanol exposure on development are well documented and include a wide range of physical anomalies and neurocognitive deficits (Jones and Smith, 1973). Depending on how much, how long, and how frequently ethanol is consumed during pregnancy, fetal alcohol syndrome (FAS) may manifest with differing severity (Cartwright and Smith, 1995). This diagnosis is considered when there is pre- or post-natal growth deficiency, cranio-facial abnormalities, and evidence of central nervous system (CNS) dysfunction (Jones and Smith, 1973). When the prenatal ethanol exposure is associated with cognitive and behavioral deficits in the absence of the facial features and growth deficiency, this profile is known as fetal alcohol spectrum disorders (FASDs) (Popova et al., 2013).

Neurobehavioral deficits are reported relatively frequently in FAS, for example, hyperactivity and attention deficits, deficits in motor coordination, lack of regulation of social behavior or poor psychosocial functioning, and deficits in verbal learning and memory (Sei et al., 2003; Garriga et al., 2000; Mattson and Furukawa, 1998; Mattson et al., 1996). The mechanisms behind the long-term neural outcomes of ethanol exposure are difficult to define. Ethanol can interact or functionally alter many target molecules, including receptors and ion channels, cell signaling proteins, metabolizing enzymes, and lipids. Ethanol can alter glucose utilization and transport (Fattoretti et al., 2003), suppress protein and DNA synthesis (Shibley and Pennington, 1997), alter cell cycle, impair neurogenesis, migration, neurite

* Corresponding author at: Escola de Ciências, PUCRS, Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil.
E-mail address: rosane.silva@pucrs.br (R.S. Da Silva).

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outgrowth, synaptogenesis, and myelination (Miller, 1996; Liesi, 1997).

Long-lasting effects of early ethanol exposure have been registered in glutamatergic and dopaminergic systems (Baggio et al., 2017; Melis et al., 2009). The purinergic system has also been shown to be affected by ethanol exposure (Vasconcelos et al., 2012), but the effects in adulthood of ethanol embryonic exposure on the purinergic system has not been reported. Considering acute exposure, ethanol appears to affect adenosine tonus through inhibition of adenosine reuptake exerted by type I equilibrative nucleoside transporters (ENT1) and an increase in ecto-5′-nucleotidase activity, which seems to promote activation of A_2 adenosine receptors (Diamond et al., 1991; Nagy et al., 1990). Adenosine is related to ethanol intoxication and behavioral responses. In an in vivo study, ENT1-null mice exhibited decreased ethanol-mediated behaviors and increased ethanol consumption associated with decreased A_1 adenosine receptor function (Choi et al., 2004). Additionally, striatal adenosine A_{2A} receptors appeared to be critically involved in ethanol-induced behavior (Nam et al., 2013).

In the CNS, adenosine influences neurotransmitter release (Burnstock, 2008; Fredholm et al., 2005). The extracellular levels of adenosine are affected by the sequential hydrolysis of ATP exerted by the cascade of ectonucleotidases, including an ecto-5′-nucleotidase (Dunwiddie et al., 1997; Cunha, 2016), and the conversion to the inactive metabolite inosine by ecto-adenosine deaminase (Dunwiddie et al., 1997; Franco et al., 1997). An altered adenosinergic tonus at the beginning of development can have long-lasting effects on locomotor and behavioral activities (Ajarem and Brain, 1993). Recently, we found increased ecto-5′-nucleotidase activity in encephala of zebrafish larvae exposed to ethanol in the beginning of their development (Lutte et al., 2015). The zebrafish have been proposed as a model organism for exploring the mechanisms of FASD (Gerlai et al., 2009; Gerlai, 2015). The developing embryo inside the egg may be exposed to a concentration of ethanol by immersing the eggs in an ethanol solution for a specific period of time at any desired developmental stage (Fernandes and Gerlai, 2009; Mahabir et al., 2014). The CNS of zebrafish begins to form, followed by further subdivision of encephalic regions in the gastrula/segmentation (5–24 hpf, hours post-fertilization) and pharyngula (24–48 hpf) stages (Kimmel et al., 1995). These periods of zebrafish development combined with different ethanol concentrations have been used to model ethanol effects on morphology and behavior (Carvan et al., 2004; Joya et al., 2014).

While we detected increased ecto-5′-nucleotidase activity in larval zebrafish exposed to ethanol, there was no evidence for the persistence of altered ecto-5′-nucleotidase activity until adulthood, which could underly the long-lasting neurocognitive outcomes of ethanol even in doses not related to morphological outcomes. In this context, the rationale of this research was to evaluate if exposure to 1% ethanol during early development promotes consequences to ecto-5'-nucleotidase and adenosine deaminase (ADA) enzymatic activities and gene expression in adult zebrafish. Additionally, morphological outcomes were assessed in the adult animal.

2. Material and methods

2.1. Chemicals

Ethanol (99.5%) was purchased from Merck KGaA (Darmstadt, Germany). Instant Ocean (marine salt) was purchased from Marine Enterprises International (Maryland, USA). Tris, EDTA, EGTA, and AMP were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents used were of analytical grade.

2.2. Animal maintenance

The progenitor animals used in this study were generated from the mating of fish from commercial suppliers (Delphis LTDA, Porto Alegre, Brazil). Adult wild type zebrafish (*Danio rerio*) (Tübingen background, 3–5 cm) from our breeding colony were maintained in an automated recirculating tank system. For egg production, male and female zebrafish were separated at dusk to mate during the following dawn. The fish were placed in a ratio of two males to each female, totaling 3 animals per 3.5 L aquarium. After mating, eggs were collected and kept in water for maintenance (water from reverse osmosis reconstituted with marine salt, 0.4 parts per thousand) in an incubator at 28.5 °C on a 14:10 light/dark cycle. After the beginning of the swimming phase, larval zebrafish were transferred to aquariums and kept until 3 months in proper fish density (6–9 dpf: 16 fish/l; 18–22 dpf: 10 fish/l; 45 dpf or more: 5 fish/l). The experiments were conducted with adult animals. Zebrafish reach sexual maturity around 90 dpf (days post-fertilization) when development is complete (Van der Heyden and Huysseune, 2000; Spence et al., 2008; Kaslin et al., 2013). To obtain brain samples, zebrafish were euthanized by hypothermal shock, and the brains were removed by dissection (Matthews and Varga, 2012; Wilson et al., 2009).

2.3. Ethanol exposure

Zebrafish embryos were exposed to ethanol in two distinct developmental phases: gastrula/segmentation (5–24 hpf) or pharyngula (24–48 hpf). The animals were exposed to 1% (v/v) ethanol (diluted with water). Such dose is representative of the doses used in several studies to induce behavioral changes (Fernandes and Gerlai, 2009; Mathur and Guo, 2011).

After ethanol treatment, animals were kept for up to three months in an aquarium (5 L, 27 × 17 × 12 cm, width × height × depth) with drug-free water under biological and mechanical filtration and aeration (7.20 mg O_2/L) and controlled temperature (28 ± 2 °C). At the end of three months, the evaluation of enzymatic activity, gene expression, and morphology was performed.

2.4. Morphological assessment

The adult animals were evaluated under stereomicroscopy (3×) (Nikon SMZ1500, Melville, USA). Animals were anesthetized by immersion in tricaine solution (MS-222, 100 mg/L) (Siebel et al., 2015). Searching for body deformities (e.g., axial and craniofacial malformations) and measurement of body length through general visualization were performed by photographical registration, followed by analysis using the software NIS-Elements D 3.2 for Windows, supplied by Nikon Instruments Inc. (Melville, USA). The body length was assumed from the tip of the snout to the end of the spine (Näslund, 2014).

2.5. Preparation of soluble and membrane fractions

Adult zebrafish were euthanized and their whole brains (five brains for each sample) were dissected and homogenized in tris-citrate buffer for the ecto-5′-nucleotidase assay. For ADA experiments, the brains were homogenized in phosphate buffered saline (PBS). In brief, the homogenates were centrifuged at 800 ×*g* for 10 min, and the supernatant fraction was subsequently centrifuged for 25 min at 40,000 × g. For soluble ADA activity assays, the supernatant was collected and kept on ice for enzyme assays. The pellets of membrane preparations were frozen in liquid nitrogen, thawed, resuspended in the respective buffers, and centrifuged for 20 min at 40,000 × g. This freeze-thaw-wash procedure was used to ensure the lysis of the brain vesicle membranes. The final pellets were resuspended and used for enzyme assays. Protein concentration was measured by the Coomassie blue method with bovine serum albumin as a protein standard (Bradford, 1976).

2.6. Ecto-5'-nucleotidase assays

The ecto-5′-nucleotidase activity was determined as previously described (Rico et al., 2003; Senger et al., 2004). Briefly, brain membranes of adult zebrafish were added to the reaction medium containing

50 mM tris–HCl (pH 7.2) and 5 mM $MgCl_2$ at a total volume of 200 µL. The samples were preincubated and the reaction was initiated by the addition of substrate (AMP). After 30 min, the reaction was stopped by the addition of 200 µL 10% trichloroacetic acid, and the samples were kept on ice for 10 min. To determine the inorganic phosphate released, 1 mL of a colorimetric reagent was added to the samples for 20 min. The quantification of inorganic phosphate released was determined spectrophotometrically at 630 nm, and the specific activity was expressed as nanomoles of inorganic phosphate released per minute per milligram of protein. All enzyme assays were performed in triplicate.

2.7. Adenosine deaminase assays

Ecto- and cytosolic-ADA activities were determined as previously described (Rosemberg et al., 2008). The brain fractions were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for soluble and membrane fractions, respectively, in a final volume of 200 µL. The samples were preincubated and the reaction was initiated by the addition of substrate (ADO). The reaction was stopped after 75 min (soluble fraction) and 120 min (membrane fraction) by the addition of 500 µL phenol-nitroprusside reagent. ADA activity was determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction, as previously reported (Weisman et al., 1988). The reaction mixtures were immediately mixed with 500 µL of alkaline-hypochlorite reagent and vortexed. The samples were incubated, and the colorimetric assay was carried out at 635 nm. The ADA activity was expressed as nanomoles of ammonia released per minute per milligram of protein. All enzyme assays were performed in triplicate.

2.8. Ecto-5′-nucleotidase gene expression analysis

Gene expression analysis was performed using pools of 5 adult brains (n = 4/group) as previously described elsewhere (Capiotti et al., 2013) and in accordance with Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines (Bustin et al., 2009). Briefly, the total RNA was isolated using TRIzol® (Invitrogen, USA) and quantified by spectrophotometry using NanoDrop Lite (ThermoScientific, USA) after DNase treatment (DNase I, Amplification Grade, Invitrogen). The cDNA synthesis was performed from 1 µg of RNA-treated samples using *ImProm-II™ Reverse Transcription System* (Promega, USA). Quantitative PCR reactions were performed in 25 µL under the following cycling conditions: 5 min at 95 °C and 40 cycles of 15 s at 95 °C, 35 s at 60 °C, and 15 s at 72 °C, in addition to a melting curve analysis with fluorescence measures from 60 to 99 °C in a 7500 Real-Time System Software v.2.0.5 (Applied Biosystems). Relative expression levels for the ecto-5′-nucleotidase-related gene (*cd73*) (Rico et al., 2003) were evaluated using *β-actin, ef1α,* and *rpl13α* as reference genes (Table 1) (Tang et al., 2007) by the $2^{-\Delta\Delta Cq}$ method and considering the individual sample calculated with LinRegPCR Software v2016.1 (http://LinRegPCR.nl).

2.9. Adenosine quantification in encephala

To obtain brain samples, zebrafish were euthanized by hypothermal shock and brains were removed by dissection. Each independent experiment was performed using biological preparations consisting of a pool of 3 brains for endogenous adenosine analysis. Samples were homogenized on ice with an Ultra-Turrax (T10 basic IKA®) in 500 µL of ice-cold 6% perchloric acid and were centrifuged at 16,000 *g* for 15 min at 4 °C. The supernatants were neutralized with 100 µL of 4 M K_2CO_3 and centrifuged again at 16,000 *g* for 10 min at 4 °C. The supernatants were filtered (0.22 µM filter) and stored at −80 °C until the high performance liquid chromatography (HPLC) analysis (Maccormack et al., 2006). An HPLC system equipped with a diode array detector (DAD) and an autosampler system was used (Dionex Ultimate 3000). Aliquots of 20 µL were injected to the HPLC system and chromatographic separations were performed using a reverse-phase column C-18 (RP), 250/4.6 mm, 5 µm (Macherey-Nagel, Bethlehem, PA, USA). The flow rate of the mobile phase (60 mM KH_2PO_4, 5 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol) was 0.6 mL/min (isocratic). The autosampler and column temperatures were maintained at 6 and 16 °C respectively. The mobile phase was prepared daily due to low stability. Absorbance was monitored at 260 nm with a DAD. The peak of adenosine was identified by its retention time and quantified by comparison with a standard. The results are expressed as micromolar of adenosine.

2.10. Statistical analysis

Morphological parameters were expressed as the mean ± S.E.M from at least 20 animals per group. Biochemical assays were run in triplicate and expressed as the mean ± S.E.M. The data was analyzed by one-way analysis of variance (ANOVA). Post hoc comparisons were made using Tukey's test. Gene expression data is shown as the mean ± 95% CI, after Shapiro-Wilk normality test, followed by one-way ANOVA and for analysis of endogenous adenosine (HPLC), was used Unpaired *t*-test considering $p < 0.05$ as statistically different.

3. Results

Exposure to 1% ethanol during gastrula/segmentation or pharyngula stages of zebrafish development did not promote gross morphological defects assessed during adult life. The 1% ethanol dose was chosen since it does not cause apparent and serious morphological deformities (Fernandes and Gerlai, 2009) that would hamper the growth and development of animals until adulthood. However, a significant decrease in the body length and weight of adult animals occurred in animals treated with ethanol during both developmental stages (body length: 18% reduction in the gastrula stage group and 22% in the pharyngula stage group, in relation to control group; weight: 45%

Table 1
Primer sequences for RT-qPCR experiments included in the study.

Gene	Primer sequences (5′-3′)	Accession number (mRNA)	Amplicon size (bp)
β-actin[a]	F-CGAGCTGTCTTCCCATCCA R-TCACCAACGTAGCTGTCTTTCTG	ENSDART00000055194	86
Rpl13α[a]	F-TCTGGAGGACTGTAAGAGGTATGC R-AGACGCACAATCTTGAGAGCAG	NM_212784	147
EF1α[a]	F-CTGGAGGCCAGCTCAAACAT R-ATCAAGAAGAGTAGTACCGCTAGCATTAC	ENSDART 00000023156	86
ecto-5′-nucleotidase[b]	F-TGGACGGAGGAGACGGATTCACC R-GGAGCTGCTGAACTGGAAGCGTC	BC055243.1	149

[a] According to Tang et al., 2007.
[b] According to Capiotti et al., 2013.

Table 2
Morphological evaluation (body length and weight) of 90 days post-fertilization zebrafish exposed to 1% ethanol during gastrula/segmentation or pharyngula. N: number of animals; Min: lowest value found; Max: highest value found; Mean: mean of values; SD: standard deviation. [a]Difference from control group, $p < 0.0001$.

	Body Length (cm)					Weight (g)				
	N	Min	Max	Mean	SD	N	Min	Max	Mean	SD
Control	24	2.5	3.0	2.67	± 0.18	24	0.160	0.357	0.248	± 0.04
Gastrula/Segmentation	20	1.8	2.6	2.19[a]	± 0.31	20	0.107	0.208	0.160[a]	± 0.03
Pharyngula	28	1.6	2.6	2.08[a]	± 0.22	28	0.100	0.178	0.126[a]	± 0.03

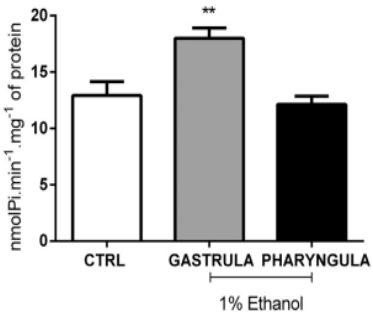

Fig. 1. Long-term effects of 1% ethanol treatment during gastrula/segmentation or pharyngula phases on ecto-5′-nucleotidase enzyme activity from encephala of adult zebrafish. Data is expressed as nmol Pi min^{-1} mg^{-1} of protein. Bars represent mean ± SEM of at least five experiments performed in triplicate. The data was analyzed by one-way analysis of variance (ANOVA). Post hoc comparisons were made using Tukey's test. The asterisks represent a significant difference from the control group at $p < 0.001$. "ns" means not significant from the control group.

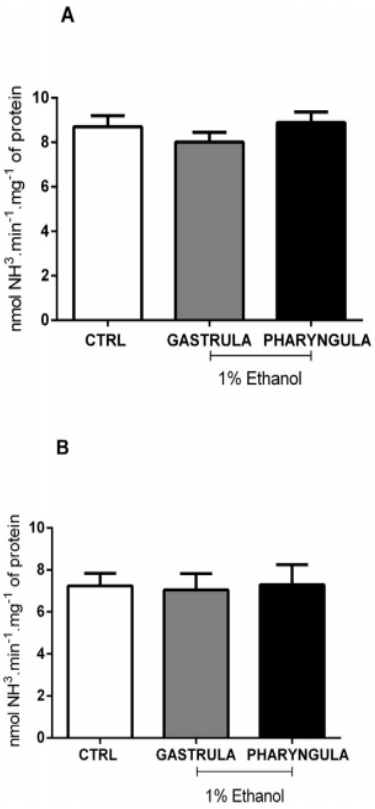

Fig. 2. Long-term effects of 1% ethanol treatment during gastrula/segmentation or pharyngula phases on (A) cytosolic-ADA and (B) Ecto-ADA activities from encephala of adult zebrafish. Data is expressed as nmol NH^3 min^{-1} mg^{-1} of protein. Bars represent mean ± SEM of at least five experiments performed in triplicate. The data was analyzed by one-way analysis of variance (ANOVA). Post hoc comparisons were made using Tukey's test. "ns" means not significant from the control group.

reduction in the gastrula stage group and 49% in the pharyngula stage group, in relation to control group) ($p < 0.0001$) (Table 2). No deaths occurred during the experiments.

The hydrolysis of AMP from whole encephala of adult zebrafish was persistently affected by early ethanol exposure. Ethanol exposure during the gastrula/segmentation stage promoted a 39.5% increase in ecto-5′-nucleotidase activity from the encephala of 3-month-old zebrafish when compared to the control group ($p < 0.001$) (Fig. 1). When the ethanol was given during the pharyngula stage, the ecto-5′-nucleotidase activity from encephala of adult animals was not affected (Fig. 1). The profile of AMP hydrolysis from encephala of adult zebrafish treated during gastrula/segmentation or pharyngula stages with ethanol was not accompanied by an alteration in ecto-5′-nucleotidase gene expression (Fig. 3). While one of the most important sources of adenosine formation was affected by early ethanol exposure, the deamination of adenosine exerted by ecto and cytosolic adenosine deaminase was not affected by exposure to ethanol in both developmental stages studied (Fig. 2). In fact, HPLC indicated that the overall concentration of adenosine was similar between the control and ethanol-exposed group ($p > 0.05$) (Fig. 4).

4. Discussion

The activation of A_1 and A_{2A} adenosine receptors can balance neuronal activity and modulate homeostasis, as well as improve the ability of synapses to undergo and/or keep plasticity mechanisms (Dias et al., 2013). Alteration in the adenosinergic tonus can be triggered by several external factors, including exposure to ethanol. Ethanol, when given acutely, can alter adenosine levels in the brain, and several mechanisms have been proposed (Nagy et al., 1990; Lutte et al., 2015; Sharma et al., 2010; Carmichael et al., 1991; Cullen and Carlen, 1992; Carmichael et al., 1993; Allen-Gipson et al., 2009; Kim et al., 2011). Here, we investigated if early exposure to ethanol was able to permanently affect adenosine production through the ecto-5′-nucleotidase. While the cleavage of AMP to produce adenosine is a major implication of catalytic action of ecto-5′-nucleotidase, this enzyme also appears to have non-catalytic properties, such as acting in intercellular adhesion, signaling, growth, proliferation, and cell migration processes, what can generate impacts on neural development (Bianchi and Spychala, 2003; Neary and Burnstock, 1996; Zimmermann et al., 1998; Vogel et al., 1991).

The developmental stage and the concentration of ethanol are important factors to define the extent of ethanol consequences on normal development, from strong morphological impacts to subtle cognitive outcomes (Jones and Smith, 1973; Khoury et al., 2018). The ethanol concentration used in this study did not promote gross morphological outcomes but reinforced one of the consequences already associated

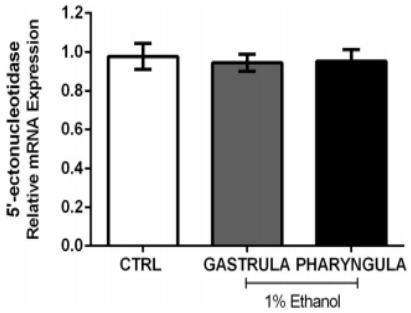

Fig. 3. Ecto-5′-nucleotidase-related gene expression analysis from encephala of adult zebrafish after 1% ethanol exposure during gastrula/segmentation or pharyngula phases. RT-qPCR analyses were performed with *β-actin*, *ef1a*, and *rlp13a* as reference genes using the $2^{-\Delta\Delta Cq}$ method. Data is expressed as mean ± 95% CI after performing the Shapiro-Wilk normality test and one-way analysis of variance (ANOVA), considering $p < 0.05$ as statistically significant (n = 4/group). "ns" means not significant from the control group.

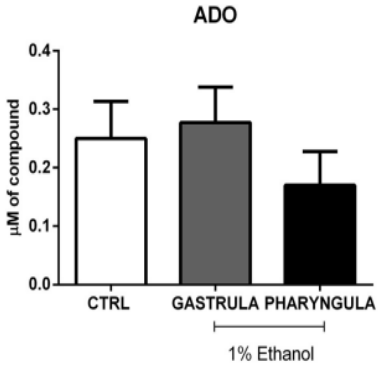

Fig. 4. Quantification of endogenous adenosine from whole encephala of control and ethanol-treated animals. HPLC analysis was performed with at least three pools of four encephala from each group. The data represents the mean ± SEM.

with embryonic exposure to ethanol in larval zebrafish, the decrease in body length and, consequently, the weight (Joya et al., 2014; Ramlan et al., 2017; Muralidharan et al., 2017). The correct formation of the body axis in vertebrates usually depends on the development of the notochord and somites (Stemple, 2005). Zebrafish embryos start this process in the gastrula stage, where formation of the axis and neural plate, brain, and notochord rudiments begin (https://zfin.org/zf_info/zfbook/stages/). The role of notochord includes cells convergence and lengthening to settle the anterior/posterior extent of the body axis, and it serves as a signaling center during development through the secretion of proteins named hedgehog (Stemple, 2005; Fernandes and Gerlai, 2009; Warga and Kimmel, 1990). The suppression of the Sonic hedgehog signaling gene (*Shh-s*) has been suggested to support the morphological effects of ethanol in zebrafish (Loucks and Ahlgren, 2009; Hammerschmidt et al., 1996) and can be related to the body length and weight decrease registered here.

Several targets can be identified as contributors of subtle effects of ethanol early exposure, including neurotransmitters and neuromodulators, such as adenosine. Lutte et al. (2015) showed that 7-day old zebrafish larvae exposed acutely and chronically to 2% ethanol during early development exhibited a 28% and 58% increase in ecto-5′-nucleotidase activity, respectively. Here, we demonstrated that this effect persists in adulthood when exposure to 1% ethanol occurs during the gastrula/segmentation stage, but not during pharyngula stage. The 1% ethanol is a high concentration (171 mM) in comparison to human levels, which often reach 66 mM (Burd et al., 2012). The egg chorion is an important barrier, in fact some studies indicate that the concentration in the actual embryo exposed to 1% ethanol from 6 to 24 hpf reaches 2.7–4.2-fold lower than ethanol media levels, while embryos exposed at 48 hpf were 5.7–6.2-fold lower (Fernandes and Gerlai, 2009; Ali et al., 2011; Lovely et al., 2014). Several investigators consider this ethanol concentration suitable to the study of long-lasting effects of ethanol on brain activity (Baggio et al., 2017; Fernandes and Gerlai, 2009).

The persistence of increased ecto-5′-nucleotidase in to adulthood without an alteration to gene expression of ecto-5′-nucleotidase raises the concept that responses at the mRNA level did not have direct impacts at the protein level or at the level of the active enzyme (Glanemann et al., 2003) and could indicate that several types of ecto-5′-nucleotidase posttranslational regulations could be involved. This enzyme is controlled in many levels, such as the allosteric control performed by ADP, ATP, and H^+ (Heylige et al., 1981). Also, analysis from computational tools indicates that the ecto-5′-nucleotidase from zebrafish presents several predictive phosphorylation sites, which could also contribute to the increased activity (NetPhos 3.1 Server, University of Denmark). In fact, the control of ecto-5′-nucleotidase by protein kinase C was already shown in a canine heart (Node et al., 1997). Another possibility of enzymatic control that should be further evaluated in the context presented here is the different levels of *N*-glycosylation. Ecto-5′-nucleotidase is a glycosylphosphatidylinositol-anchored raft-associated enzyme that suffers regulation from Ca^{2+}-dependent mechanisms, annexin-mediated stabilization of membrane rafts, which is a major candidate to contribute for long-term effects (Babiychuk and Draeger, 2006). In fact, ethanol can remodel membranes in rodents and zebrafish (Podechard et al., 2017; Tang et al., 2011).

Shan et al. (2015), showed that ethanol exposure during gastrulation can alter neuronal morphology and behavior in zebrafish, mainly by affecting excitatory synaptic and morphology associated with mauthner cells. Here, the gastrula period appeared to be a more vulnerable period, and this could be a reflection of the large number of cell migrations and the modeling of neural structures occurring during this period (Kimmel et al., 1995). Acute and chronic exposure to ethanol can affect expression of several cellular targets related to neurotransmission, such as dopamine synthesis and glutamate receptor function (Fernandes and Gerlai, 2009; Marszalek-Grabska et al., 2018). Here, the persistent altered ecto-5′-nucleotidase could contribute to the deregulation of adenosine signaling and, at least in part, be implicated in the long-term effects on brain function associated with fetal alcohol spectrum disorders. However, impaired memory of adult zebrafish treated with ethanol during pharyngula phase is restored with ecto-5′-nucleotidase inhibitor (Lutte et al., 2018).

Curiously, HPLC experiments identified similar adenosine concentration from encephala of adult animals exposed to ethanol during the gastrula and pharyngula stages and control animals. These data are not expected with the altered ecto-5′-nucleotidase activity and some technical limitations could contribute to that. One of the technical constraints is the fact that the whole adenosine content was measured (intra and extracellular). On the other hand, this same adenosine content is in accordance with the absence of alteration on the activity of adenosine deaminase. However, the analysis of whole brain adenosine content could mask some regional adenosine tonus and actions, since it has been demonstrated in several cellular sources that one of the impacts of ecto-5′-nucleotidase on neuromodulation is related to the channeling of adenosine to A_{2A} adenosine receptors, while the diffusion of adenosine from transporters mainly acts on A_1 adenosine receptors (Boeck et al., 2007; Cunha et al., 1996).

These findings raise the question of whether the purinergic system could be a potential long-lasting target of ethanol exposure. However, several other aspects of the purinergic system, such as adenosine release, preferential activation, sensitization, density, and transduction efficiency of the adenosine receptors, should be analyzed under this

context of early exposure to ethanol.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Compliance with ethical standards

All procedures performed in studies involving animals were in accordance with the ethical standards of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the Institutional Animal Care Committee (15/00468, CEUA/PUCRS).

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3.3 Capítulo III

Artigo Científico Original

Modulation of adenosine metabolism is able to reverse long-term behavioral effects of early ethanol exposure in zebrafish (*Danio rerio*)

Autores: Aline Haab Lutte, Júlia Huppes Majolo, Rosane Souza Da Silva

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Title page

Title: **Modulation of adenosine metabolism is able to reverse long-term behavioral effects of early ethanol exposure in zebrafish (*Danio rerio*)**

Authors: Aline Haab Lutte, Júlia Huppes Majolo, Rosane Souza Da Silva*

Laboratório de Neuroquímica e Psicofarmacologia, Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

*Corresponding author:

Rosane Souza Da Silva

Escola de Ciências, PUCRS

Avenida Ipiranga, 6681, 90619-900

Porto Alegre, RS, Brazil

Phone/ Fax: + 55 51 3320 3500 Ext. 4158/+ 55 51 3320 3612

E-mail: rosane.silva@pucrs.br

ABSTRACT

The behavioral impacts of prenatal exposure to ethanol include lowering of IQ, learning problems, anxiety and conduct disorders. The purinergic system has been investigated as one of the targets of ethanol. We used zebrafish as a model to evaluate the role of adenosine metabolism in the long-lasting behavioral effects of ethanol. Embryos of zebrafish were exposed to 1% (v/v) ethanol in two distinct developmental stages: gastrula / segmentation (from 5 to 24 hpf [hours post-fertilization]) or pharyngula (from 24 to 48 hpf). Ethanol-treated animals did not show alteration on morphological or locomotor outcomes when evaluated at 3 or 12 months post-fertilization (mpf). However, ethanol-treated animals had long-lasting behavioral effects. At adult life, the manipulation of purinergic system was reached by pharmacological inhibition of ecto-5'-nucleotidase or adenosine deaminase using AMPCP (150 mg/kg) or EHNA (100 mg/kg), respectively, by intraperitoneal injection of adults. At 3 mpf, but not at 12 mpf, ethanol-treated animals from both groups exhibited anxiolytic effect and AMPCP and EHNA did not counteract this effect. The exposure to 1% ethanol during gastrula / segmentation did not alter aggressiveness when this behavior was evaluated at 3 or 12 mpf. However, animals exposed to 1% ethanol at pharyngula stage increased the aggressiveness at 3 and 12 mpf and this effect was reversed by AMPCP at 3 and 12 mpf, but not by EHNA. Social interaction was decreased in animals at 3 mpf that received 1% ethanol during gastrula / segmentation or pharyngula stages. These alterations were recovered by EHNA and AMPCP in those animals treated with 1% ethanol at gastrula / segmentation stage, while only AMPCP recovered this parameter in those animals treated with 1% ethanol during pharyngula stage. Ethanol-treated animals had no significant alterations in social interaction at 12 mpf. These results suggest that ethanol could have adenosine metabolism as a target during early life with long-lasting behavioral effects.

Keywords: Alcohol, Development, Purinergic system, Neurodevelopment, Nucleosides.

1 INTRODUCTION

The worldwide consume of ethanol is estimated around 10% of pregnant women (WHO, 2017). The term alcohol-related neurodevelopmental disorder (ARND) describes a particular pattern of disordered behavior and impaired cognitive development in children and young who did not develop physical and morphological impacts of alcohol exposure (Comasco *et al*., 2018). The behavioral impacts of prenatal exposure to ethanol include lowering of IQ, learning problems, anxiety and conduct disorders (Streissguth *et al*., 1990; Khoury *et al*., 2018). Several animal models were developed to contribute to a trustworthy picture of the neurobiology of behavior sequels of ARND (Brady *et al*., 2012; Hellemans *et al*., 2010). Several cellular targets have been related to long-lasting neurological outcomes of ethanol exposure including components of serotoninergic, glutamatergic and dopaminergic systems (Savage *et al*., 2002; Samúdio-Ruiz *et al*., 2009; Sliwowska *et al*., 2014).

The purinergic system is also one of the targets of ethanol exposure but data on the relationship of purinergic signaling and behavioral effects of ethanol are limited. Besides the activity of adenosine into energy metabolism, epigenetic mechanisms, and the genetic transmission of information, this nucleoside exerts important neuromodulatory role (Dunwiddie *et al*., 1997; Rivkees and Wendler, 2017). The extracellular levels of adenosine is a result of several enzymatic activities, such as ecto-5'nucleotidase and ecto-adenosine deaminase, and equilibrative and concentrative transport, controlling the availability of adenosine to the P1 receptors and, consequently, the neuromodulation of major neurotransmitter systems (Zimmermann and Braun 1999; Fredholm *et al*., 2005).

Acute and chronic ethanol exposure can affect adenosine metabolism and action, through production of AMP and, consequently, adenosine during ethanol metabolism (Carmichael *et al*., 1991), reduction of adenosine reuptake by inhibition of bidirectional nucleoside transporters (Krauss *et al*., 1993), and facilitation of the receptor-mediated activation of adenylyl cyclase by hormones and neurotransmitters, enhancing the action of intracellular cascade of adenosine receptors (Rabin and Molinoff, 1981). However, little is known about the long-lasting consequences of altered metabolism and action of adenosine induced by ethanol exposure during early brain development.

Behavior impairments caused by ethanol has been widely studied over the years in zebrafish (*Danio rerio*) model (Gerlai, Lee and Blaser 2006; Echevarria, Toms and Jouandot, 2011; Sterling *et al*., 2016). The

embryonic zebrafish has some advantages as a model of early ethanol exposure, for example, the easy manipulation and clear differentiation of the developmental stages through a transparent egg (Kimmel *et al*., 1995). Zebrafish neuroanatomy has many similarities to mammals, especially in areas such as the spinal cord and hindbrain, also zebrafish shares major neurotransmitter pathways (Cheng *et al*., 2014). An increasing number of studies have demonstrated that ethanol exposure affects a variety of zebrafish behaviors as anxiety (Egan *et al*., 2009), aggressiveness (Gerlai *et al*., 2000; Gerlai, 2003), memory (Lutte *et al*., 2018) and social interaction (Baggio *et al*., 2018), and some of these effects are long-lasting (Baggio *et al*., 2018; Fernandes and Gerlai, 2009; Lutte *et al*., 2018).

Because adenosine is a potent neuromodulator and ethanol is able to affect adenosinergic tonus, the objective of this research was to investigate and understand the possible association of adenosine in the long-lasting behavioral effects caused by ethanol exposure in different stages of early development.

2 MATERIAL AND METHODS

2.1 Animals

Adult zebrafish (*Danio rerio)* (09–12 months-old; measuring 3.4 ± 0.5 cm; 2:1 male/female ratio) were used for obtaining fertilized eggs. Animals were housed in a re-circulating system equipped with mechanical and biological filtration at a temperature of 28 °C and pH of 7.4. After mating, eggs were collected and kept in water for maintenance (water from reverse osmosis reconstituted with marine salt [Instant Ocean, Blacksburg, VA] 0.4 parts *per* thousand) on a 14:10 light / dark cycle. After the beginning of the swimming phase, larval zebrafish were transferred to aquariums and kept until 3 months or 12 months in proper fish densities (6–9 dpf [days post-fertilization]: 16 fish / L; 18–22 dpf: 10 fish / L; 45 dpf or more: 5 fish / L). Animals were fed three times a day with commercial flakes (TetraMin™, NC, USA) and supplemented with live brine shrimp.

Animals were cryoanesthetized using flocked ice immersion with water with controlled physicochemical parameters and temperature of about 2 °C, followed by euthanasia through decapitation. All procedures were in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and were approved by the Institutional Animal Care Committee (15/00468 — CEUA PUCRS).

2.2 Ethanol treatment

Embryos of zebrafish were exposed to 1% (v/v) ethanol (diluted in the maintenance water) in two distinct developmental stages: gastrula / segmentation (from 5 to 24 hpf [hours post-fertilization]) or pharyngula (from 24 to 48 hpf). The animals were exposed in groups of 50 embryos kept in Petri dishes. After the gastrula / segmentation or pharyngula stages, animals were maintained in drug-free water until the end of the experiments (3 or 12 months post-fertilization (mpf)). The proper fish densities were adjusted all period long (6–9 dpf [days post-fertilization]: 16 fish / L; 18–22 dpf: 10 fish / L; 45 dpf or more: 5 fish / L).

2.3 Exposure to α,β-Methyleneadenosine 5′-diphosphate (AMPCP) or erythro-9-(2-Hydroxy-3-nonyl)-adenine hydrochloride (EHNA)

The pharmacological treatments (AMPCP; 150 mg/kg or EHNA; 100 mg/kg) of adults were performed by intraperitoneal (ip) injection with the use of a Hamilton microliter syringe in a volume of 10 μL *per* animal (20 mL/kg). Prior to the injection, the adult animals were anesthetized in tricaine solution (MS-222; 100 mg/L) (Siebel *et al*., 2015). The behavior analysis was performed 30 min after drug exposure. Control animals received saline.

2.4 Locomotion and anxiety assessment

At 3 or 12 mpf, after 30 minutes of exposure to AMPCP, EHNA or saline, the locomotor and anxiety assessments were performed. Zebrafish were placed individually in a tank (30 × 15 × 10 cm length × height × width). After 30 seconds of adaptation, the zebrafish activity was recorded for 5 minutes and analyzed using the video-tracking system Ethovision® XT8 (Noldus, Netherlands). The locomotor parameters analyzed were distance traveled (m) and mean speed (m/s) (assessed only during moving time). Exploratory behavior, an indicator of anxiety, was assessed as the time spent in the top section of tank, virtually divided into two horizontal lines (lower and top section) (Egan *et al*., 2009). The locomotion and anxiety were evaluated individually for each drug (AMPCP or EHNA) in each stage (gastrula / segmentation or pharyngula) and for each control (no ethanol added) with 10 animals per group.

2.5 Aggressiveness assessment

At 3 or 12 mpf, aggressive behavior was analyzed in control and ethanol-treated animal 30 minutes after injection of AMPCP, EHNA or saline. Individually, the fishes were placed in a single tank (30 × 15 × 10 cm length × height × width) with a mirror (22 cm) positioned in the back of the tank forming a 22.5° angle (Way *et al*., 2015; Gerlai *et al*., 2000). The fish were habituated for 30 seconds, after which the video was recorded for 8 minutes (Way *et al*., 2015). The tank was virtually divided into four equal sessions from which the time spent in each zone was analyzed, with the time spent in the zone near the mirror being the sign of aggressive behavior. The activity was analyzed using the video-tracking system Ethovision® XT8 (Noldus, Netherlands). Additionally, was made the observation of aggressive behavior like biting, sprinting, and changes in color pattern. Usually, attack behavior is a characteristic short bout of fast swimming directed towards the opponent and is sometimes accompanied by opening the mouth and biting (Gerlai *et al*., 2000) (n = 10).

2.6 Social interaction assessment

At 3 or 12 mpf, social interaction behavior from control and ethanol-treated animals was analyzed 30 minutes after AMPCP, EHNA, and saline injections. Three tanks (30 × 15 × 10 cm length × height × width) were placed side by side, the far left being empty, the one in the middle with the test animals, and the far right with 15 stimulus conspecific fishes (Gerlai *et al*., 2000). Zebrafish were placed individually in the test aquarium and after 5 minutes habituation, the social interaction was recorded for 10 minutes. The tank with the test fish was separated into two equal sessions, with the social interaction indicator being the time spent in the side with the stimulus fishes. All data were analyzed using the video-tracking system Ethovision® XT8 (Noldus, Netherlands) (n = 10).

2.8 Statistical analysis

The normality of data was checked by D'Agostino & Pearson Omnibus normality test. Two-way Analysis of Variance (ANOVA) was used to compare groups exposed to ethanol (during gastrula or pharyngula stages) and AMPCP or EHNA followed by Tukey's multiple comparisons test in behavior and locomotors parameters. The significance levels were attributed at $p < 0.05$. Data were expressed as mean ± standard deviation.

3 RESULTS

Animals treated with 1% ethanol during gastrula / segmentation or pharyngula stages did not show morphological (data not shown) or locomotors (distance traveled and mean speed) altered outcomes when evaluated at 3 or 12 mpf (Figure 1 and 2). The inhibitors of ecto-5'-nucleotidase (AMPCP) and adenosine deaminase (EHNA) alone did not generate a significant effect on any locomotor parameter when compared to the control / saline group (Figure 1 and 2). However, EHNA gave to 3 mpf animals treated with ethanol at pharyngula stage reduced significantly the mean speed (Figure 2A).

Zebrafish treated with 1% ethanol during gastrula / segmentation or pharyngula stages had long-lasting behavioral effects. As EHNA gave to zebrafish at 3 mpf had negative effects to locomotor activity (Figure 2A) and to the majority of behavioral parameter altered in animals exposed to ethanol (Figures 3A, 4A, and 5A), this inhibitor was not assessed at 12 mpf. To check the behavior related to anxiety, we have analyzed the time of zebrafish in the upper zone of the aquarium. At 3 mpf, ethanol treated animals from both groups exhibited anxiolytic effect, since the animals increased their time on upper zone (61% in gastrula / segmentation group and 92% in pharyngula group in comparison to control group) ($p=0.0052$ and $p=0.0004$) (Figure 3). AMPCP and EHNA did not alter the anxiolytic effect detected in 3mpf animals exposed to ethanol during early stages of development (Figure 3A). At 12 mpf, there is no alteration on time in upper zone between experimental groups (Figure 3B).

Data analysis from aggressiveness indicated interaction between the factors treatment and developmental stage of ethanol exposure [$F_{(5;108)}=5.114$; $p=0.0003$; for 3 mpf animals; $F_{(3;64)}=3.149$; $p=0.003$, for 12 mpf animals]. The exposure to 1% ethanol during gastrula / segmentation did not alter aggressiveness when this behavior was evaluated at 3 or 12 mpf (Figures 4AB). However, animals exposed to 1% ethanol at pharyngula stage increased the aggressiveness at 3 [$p=0.0338$] and 12 mpf [$p=0.0051$] (76% and 137%, respectively in comparison to control group) (Figures 4AB). This increased aggressiveness was reduced by AMPCP at 3 and 12 mpf (4AB), but not by EHNA (Figure 4A).

Social interaction was decreased in animals at 3 mpf that received 1% ethanol during gastrula / segmentation or pharyngula stages (Figure 5A). Animals exposed to ethanol at gastrula / segmentation stage decreased in 20% the time in the stimulus side of the aquarium when the animals were at 3mpf ($p=0.0079$) (Figure 5A). Ethanol gave to animals at pharyngula stage decreased in 33% the time in the stimulus side of the aquarium when the animals were at 3mpf ($p<0.0001$) (Figure 5A). These alterations were recovered by EHNA

and AMPCP in those animals treated with 1% ethanol at gastrula / segmentation period, while only AMPCP recovered this parameter in those animals treated with 1% ethanol during pharyngula stage (Figure 5A). While the factor treatment was detected as a source of variation [$F_{(1;72)}$=9.659; p=0.0027], the multiple comparison between groups indicated that ethanol-treated animals had no significant alterations in social interaction at 12 mpf (Figure 5B).

4 DISCUSSION

Zebrafish have been emerged as an excellent animal model to evaluate long-term effect of early exposure to ethanol (Fernandes and Gerlai, 2009; Buske and Gerlai, 2011; Baggio et al., 2018; Lutte et al., 2018). Here, we evaluated the possible role of adenosine in long-term behavioral effects of ethanol.

Exposure to 1% ethanol during gastrula / segmentation or pharyngula stages did not affect locomotor activity of animals at adult stages in agreement with the literature (Fernandes et al., 2015ab; Baggio et al., 2018; Lutte et al., 2018). While the methods of exposure to ethanol are sensitive variable in the literature, these data suggest that none of the long-term behavioral outcomes was affected by locomotor impairment.

Here, the results suggest an anxiolytic effect of ethanol. Ethanol exposure in the gastrula / segmentation and pharyngula stages caused an increased time in the upper zone of the tank, indicating an increase of exploratory behavior at 3 mpf, which was not persistent until 12 mpf. Recently, the exposure to 1% ethanol during 2h at 24 hpf embryos of zebrafish also revealed to be able to promote anxiolytic-like behavior in adult zebrafish (Baggio et al., 2018). This behavior could be related to the decrease of cortisol levels since, in zebrafish the hypothalamic–pituitary–interrenal axis is fundamental to stress and anxiety responses, and involves the cortisol hormone (Egan et al., 2009). In fact, zebrafish exposed to low doses of ethanol during development has the cortisol levels reduced when evaluated at 6-months of age (Baiamonte et al., 2015). When the homeostasis is altered by ethanol, zebrafish can respond adaptively by altering their metabolism, physiology and behavior. When homeostasis is threatened by a stressor, as ethanol, a diverse suite of neuronal, endocrine, and autonomic response mechanisms can be utilized to reestablish homeostasis (Clark, Boczek and Ekker, 2011). A normal stress response is required for healthy living. However, intense acute, repetitive, or chronic stress may overwhelm the ability to regain homeostasis leading to overloaded or imbalanced stress pathways (Clark, Boczek and Ekker, 2011). The results presented here could suggest that there are adaptations to ethanol exposure and

development alterations able to reduce the sensibility to effects of ethanol permitting that anxiolytic effects appear in young but not adult animals.

Zebrafish is a social species and the preference for being close to a conspecific may be a result of aggressive behavior, but it could also be due to social interaction tendencies, both of which may be influenced by ethanol (Gerlai *et al*, 2000; Gerlai, 2003). To differentiate the behaviors, additionally to mirror test, was made the observation of aggressive behavior like biting, sprinting, attack behavior and changes in color pattern. Here, the social interaction was decreased, while aggressiveness was increased, at young adult phase of animals exposed to ethanol during early phases of development. While the social interaction reduction was not persistent until 12 mpf, the aggressive behavior persisted until 12 mpf in the animals exposed to ethanol during pharyngula stage.

Thought in the morphological abnormalities, ethanol exposure during the first 24 hours of development seems affected the zebrafish more than ethanol exposure during any other 24-hour period (Bilotta *et al*., 2004; Dlugos and Rabin, 2010), our results showed that at least in behavior alterations, the pharyngula stage seems to be the period more affected by ethanol exposition. In the pharyngula stage, the heartbeat, vascularization and circulation in the yolk occur (Kimmel *et al*., 1995). Studies performed on mice and zebrafish have demonstrated that ethanol can affect vasculogenesis and angiogenesis development that can affect neuronal activity, a necessary prerequisite to growth and behavior development (Radek *et al*., 2005; Radek *et al*., 2008; Qian *et al*., 2005).

Embryonic ethanol exposure seems alter social behavior in zebrafish across strains (AB and TU) and likely throughout adulthood, at least from 4 months (Parker *et al*., 2016) up to 24 months (Fernandes, Rampersad and Gerlai 2015b). Although in our results the social interaction reduction was not persistent until 12 mpf, studies demonstrated that zebrafish embryos that were exposed to 1% ethanol for two hours, 24 hours after fertilization, has an impaired social interaction response with twenty four-month-old, not due to altered motor function or visual perception, but likely to a Central Nervous System alteration affecting social behavior itself (Fernandes, Rampersad and Gerlai, 2015b). The dopaminergic system appears to be one of the systems impaired in adult fishes by embryonic exposure to ethanol under the above experimental conditions (Fernandes, Rampersad and Gerlai, 2015a).

Mahabir, Chatterjee and Gerlai, (2018) found that embryonic ethanol exposure do not have any significant effect on the levels of glutamate, aspartate, glycine and GABA in both AB and TU zebrafish. These and other results available from literature suggest that behavioral abnormalities resulting from embryonic ethanol exposure may primarily be due to altered dopaminergic and serotoninergic mechanisms (Fernandes, Rampersad and Gerlai, 2015a; Mahabir, Chatterjee and Gerlai, 2018).

Baggio et al. (2018) showed another explanation for changes in social interaction, using a positive control, the anxiolytic drug buspirone, they showed a reduction of anxiety leads to reduction of the social interaction response, thus, our finding of impaired social interaction response in the embryonic ethanol treated adult zebrafish could be explained too as a result of diminished anxiety.

Studies suggest that adenosinergic systen can be involved on aggressiveness behavior. Behavioral assessment of mice lacking adenosine A_1 receptors showed increased aggressiveness (Giménez-Llort *et al.*, 2002). Others data show that dipyridamol, an inhibitor of nucleoside transporter, antagonized this aggressiveness behavior in mice, implying that endogenous adenosine seem to play a role in the brain in influencing the aggressive behavior (Ushijima *et al.*, 1984). Our results show that adenosine could be related to persistent aggressiveness behavior, because the AMPCP inhibitor was able to reverse this behavior in 3 or 12 mpf. There are discrepant effects of adenosine metabolism in aggressive responses and the neuronal pathways involved in aggressive behavior clearly includes different mechanisms. The persistence of the effect in adulthood suggests that a profound change has occurred during development in the treated animals, and this must be linked to irreversible functional changes, presumably in the adenosine metabolism, while social interaction seems to be temporary and linked to adenosine metabolism in the pharyngula stage, when is reverted by AMPCP.

These results suggest that ethanol could have adenosine metabolism as a target during early life with long-lasting effect, which could be implicated in behavioral consequences. While ecto-5'-nucleotidase modulation appears to be implicated in this process, the nucleoside transporter, another major target of ethanol, must be evaluated regard to these late consequences.

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FIGURES

Figure 1AB:

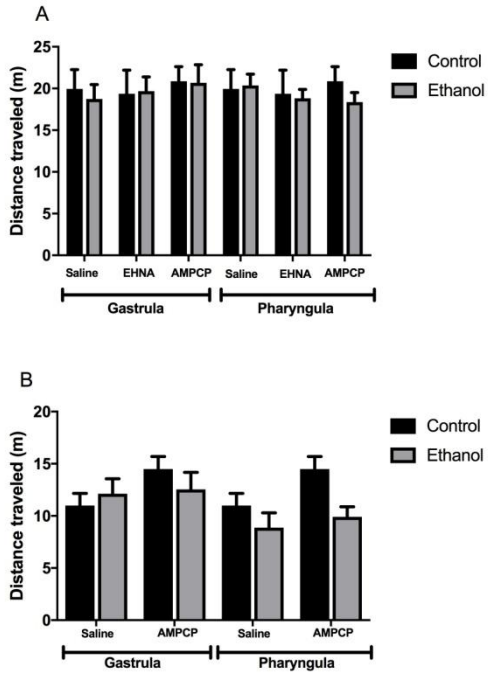


Figure 2AB:

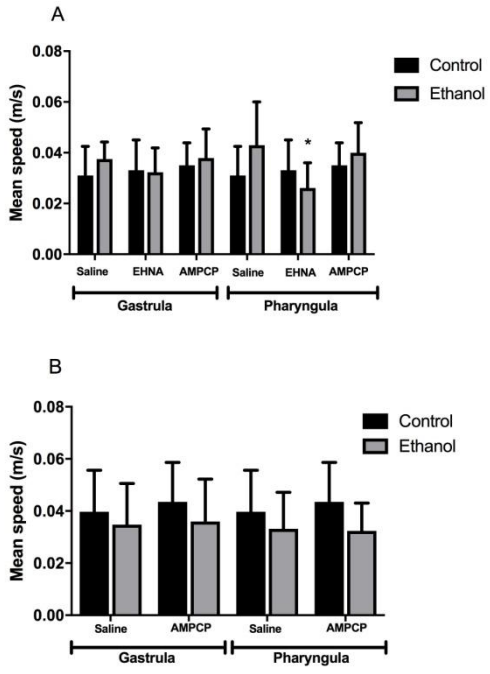
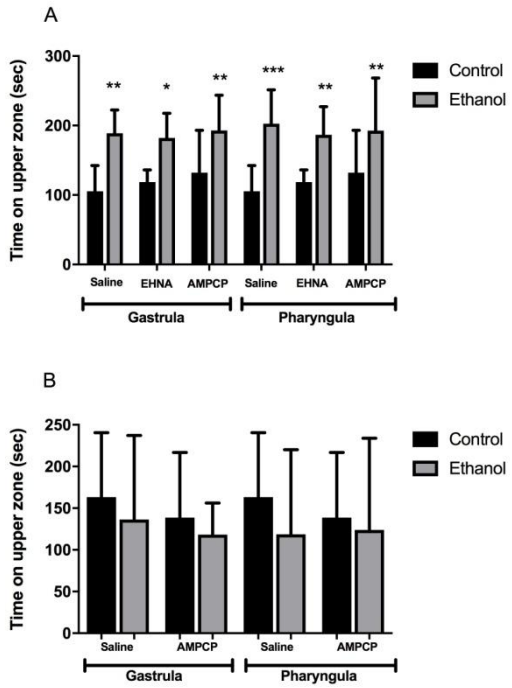


Figure 3AB:



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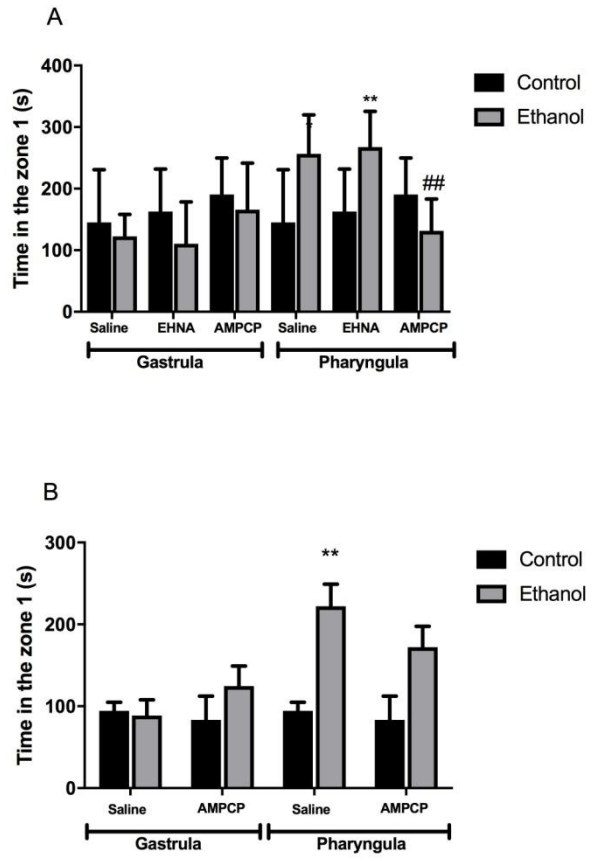
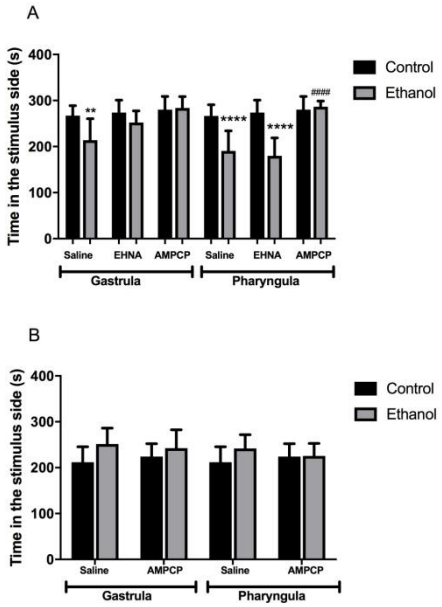


Figure 5AB:



LEGENDS:

Fig. 1AB: The locomotor parameter distance traveled (m) evaluated at 3 mpf (A) and 12 mpf (B) was not affected by 1% ethanol during the gastrula/segmentation and pharyngula stages. AMPCP or EHNA inhibitors alone did not generate significant effects on any parameter or period of ethanol exposure when compared to the control/saline group. Data were expressed as mean ± S.D. and analyzed by Two-Way Analysis of Variance (ANOVA), used to compare control and ethanol-treated animal during gastrula and pharyngula when exposed to AMPCP or EHNA. Multiple analysis of group was performed using Tukey's test (n = 10 animals per group per experiments/3 experiments).

Fig. 2AB: The locomotor parameter mean speed (m/s) evaluated at 3 mpf (A) and 12 mpf (B) was not affected by 1% ethanol during the gastrula/segmentation and pharyngula stages. AMPCP and EHNA inhibitors alone did not generate a significant effect on any parameter or period of ethanol exposure when compared to the control/saline group. EHNA inhibitors when used with ethanol treatment at 3 mpf in the pharyngula stage (A) decreased the mean speed when compared to specific control ($p < 0.05$). Data were expressed as mean ± S.D. analyzed by Two-Way Analysis of Variance (ANOVA), used to compare control and ethanol-treated animal during gastrula and pharyngula when exposed to AMPCP or EHNA. Multiple analysis of group was performed using Tukey's test (n = 10 animals per group per experiments/3 experiments).

Fig. 3AB: Long-term effects of 1% ethanol treatment during gastrula/segmentation or pharyngula stages on time in the upper zone (seconds) at 3 mpf (A) and 12 mpf (B). AMPCP or EHNA inhibitors alone did not generate a significant effect on any parameter or period of ethanol exposure when compared to the control/saline group. At 3 mpf (A) the ethanol increased the time in the upper zone in all groups. Time spent in the upper zone was registered for 5 minutes by video recording in the tank diving behavioral test. Data were expressed as mean ± S.D. and analyzed by Two-Way Analysis of Variance (ANOVA) used to compare control and ethanol-treated animal during gastrula and pharyngula when exposed to AMPCP or EHNA. Multiple analysis of group was performed using Tukey's test. The asterisks represent a significant difference from the control group at * $p<0.01$; ** $p<0.001$; ***$p<0.0001$ (n = 10 animals per group per experiments/3 experiments).

Fig. 4AB: Long-term effects of 1% ethanol treatment during gastrula/segmentation or pharyngula stages on aggressiveness behavior at 3 mpf (A) and 12 mpf (B). The treatment during gastrula/segmentation stage and the use of AMPCP or EHNA inhibitors alone did not generate a significant effect on any parameter or time when compared to the control/saline group. In the pharyngula stage, 1% ethanol treatment caused an increase in aggressiveness behavior that was recovered by AMPCP at 3 mpf (A) and 12 mpf (B). Data were expressed as mean ± S.D. and analyzed by Two-Way Analysis of Variance (ANOVA) used to compare control and ethanol-treated animal during gastrula and pharyngula when exposed to AMPCP or EHNA. Multiple analysis of group was performed using Tukey's test. The asterisks represent a significant difference from the control group at * $p<0.05$ and ** $p<0.01$. The hashtag represent a significant difference from the ethanol/saline group at $^{\#}$ $p<0.05$ (n = 9-10 animals per group per experiments/3 experiments).

Fig. 5AB: Long-term effects of 1% ethanol treatment during gastrula/segmentation or pharyngula stages on social interaction behavior at 3 mpf (A) and 12 mpf (B). The use of AMPCP or EHNA inhibitors alone did not generate a significant effect on any parameter or period of ethanol exposure when compared to the control/saline group. The treatment during gastrula/segmentation and pharyngula stage decreased the social interaction that was recovered by AMPCP and EHNA in the gastrula/segmentation stage and by AMPCP in the pharyngula stage at 3 mpf (A). Data were expressed as mean ± S.D. and analyzed by Two-Way Analysis of Variance (ANOVA) used to compare control and ethanol-treated animal during gastrula and pharyngula when exposed to AMPCP or EHNA. Multiple analysis of group was performed using Tukey's test. The asterisks represent a significant difference from the control group at ** $p<0.01$; ****$p<0.0001$. The hashtag represent a significant difference from the ethanol / saline group at $^{\#\#\#\#}$ $p<0.0001$ (n = 9-10 animals per group per experiments/3 experiments).

4. DISCUSSÃO E CONCLUSÃO

Os transtornos do espectro alcoólico fetal, apesar de totalmente preveníveis, são a causa mais comum de retardo mental não congênito no mundo (OMS, 2017). Na fisiopatologia da síndrome, durante a gestação, ocorre uma diminuição da transferência placentária de aminoácidos essenciais, vasoconstrição placentária causando hipóxia fetal crônica, proliferação celular indiferenciada do sistema nervoso central, disfunções hormonais e disfunções nos sistemas de neurotransmissão (Segre, Rego e Cardoso, 2017).

Embora não esteja totalmente claro o envolvimento da adenosina nos efeitos causados pela exposição ao etanol durante o desenvolvimento, alguns trabalhos já mostraram que a adenosina é um componente importante e essencial no controle do desenvolvimento embrionário, fetal e da organogênese (Fumagalli et al., 2017). A relação das perturbações na sinalização adenosinérgica e a exposição ao etanol durante as fases inicias do desenvolvimento do sistema nervoso tem sido alvo crescente na pesquisa no decorrer dos últimos anos (Tchekalarova, Kubová e Mareš, 2014; Rivkees e Wendler, 2017).

No primeiro capítulo desta tese, uma possível alteração no tônus adenosinérgico causado pela exposição embrionária ao etanol, poderia alterar a neuromodulação de componentes importantes na formação da memória, uma vez que, foi mostrado que o ajuste nos níveis de adenosina através da inibição da enzima ecto-5´-nucleotidase parece restaurar a aquisição de memória em animais adultos, expostos ao etanol no estágio da faríngula.

Em humanos, em qualquer período gestacional, o etanol pode causar efeitos no sistema nervoso central (SNC) fetal, sendo esses, possivelmente mais graves nas primeiras cinco semanas (Maier e West, 2001; Jones e Bass, 2003). Em peixe-zebra, pode-se perceber que alterações morfológicas severas, embora possam ocorrer em ambos os estágios, parecem estar mais suscetíveis a acontecer quando a exposição ao etanol ocorre no estágio de gástrula / segmentação, quando acontece a organogênese primária e morfogênese (Arenzana et al., 2006; Kimmel et al., 1995). Enquanto que concentrações menores de etanol, como neste estudo, parecem ter mais efeitos no desenvolvimento do cérebro e refinamento neural que ocorrem no estágio de faríngula (Carvan et al., 2004; Kimmel et al., 1995).

Pesquisas já mostraram que os efeitos estruturais de altas concentrações de etanol no SNC incluem a atrofia cerebral com microcefalia, alterações estruturais de forma e tamanho do corpo caloso, incluindo agenesia, diminuição do volume dos gânglios basais e hipoplasia do cerebelo e do hipocampo (Medina, 2011) e pode ocorrer microcefalia e / ou microencefalia consequentes à diminuição do crescimento cerebral (Jones e Bass, 2003). Embora não tenha sido observada má-formação corporal severa em animais expostos tanto no estágio de gástrula / segmentação quanto no estágio de faríngula, foi verificado que o comprimento corporal e a área encefálica foram menores nos animais tratados, comparando com os controles, e, além disso, proporcionalmente ao tamanho total do encéfalo, estruturas como telencéfalo e cerebelo sofreram uma diminuição no tamanho.

Para evitar que qualquer consequência da exposição ao etanol pudesse afetar os parâmetros comportamentais avaliados, foi analisada a locomoção dos animais. Os dados locomotores atestaram a não manifestação de qualquer prejuízo capaz de interferir na avaliação dos parâmetros mnemônicos.

Este trabalho, além de mostrar que a exposição embrionária ao etanol pode causar prejuízos na memória durante a vida adulta, sugere que os mecanismos de modulação dos níveis de adenosina estão envolvidos nestes efeitos em longo prazo, sendo que a modulação da enzima ecto-5´-nucleotidase parece exercer um papel chave neste processo.

Estudos confirmaram a proximidade da ecto-5´-nucleotidase e dos receptores de adenosina A_{2A} no estriado de roedores e mostram que a formação de adenosina extracelular mediada pela ecto-5´-nucleotidase é a principal responsável pela ativação da função dos receptores de adenosina A_{2A} do estriado (Augusto et al., 2013). Já foi demonstrado que a ativação dos receptores de adenosina A_{2A} parece estar associada a prejuízos na memória, em especial na aquisição da memória (Kim e Ruy, 2008; Pereira et al., 2005). Em todos os vertebrados, o estriado é uma das subdivisões do subpalio que faz parte do telencéfalo. O subpalio é umas das regiões de controle do comportamento e cognição e estudos relatam a região do telencéfalo como área essencial para aquisição de memória aversiva em peixe-zebra (Rink e Wullimann, 2001; Lal, 2013).

Juntos, esses achados dão suporte para uma possível via pela qual o metabolismo da adenosina pode atuar sobre as alterações mnemônicas encontradas no nosso estudo, onde o

uso do inibidor da ecto-5´-nucleotidase, possivelmente causou a diminuição dos níveis de adenosina, consequentemente diminuindo a ativação dos receptores A_{2A} e recuperando o déficit de memória causado pelo aumento da adenosina extracelular em consequência da exposição embrionária ao etanol. Nossos resultados corroboram com Kim e Ruy (2008) que relatam que a ativação dos receptores A_{2A} causam prejuizo na aquisição da memória, mas não na consolidação, assim como nossos resultados mostraram que o uso do inibidor da ecto-5´-nucleotidase foi capaz de recuperar a aquisição da memória, mas não a consolidação.

No segundo capítulo, seguindo o raciocínio de que a enzima ecto-5´-nucleotidase, parece estar envolvida nas alterações causadas pelo etanol, e com base no fato de que já havíamos constatado aumento nos níveis desta enzima aos 7 dpf de larvas expostas ao etanol (2%) durante o desenvolvimento (Lutte et al., 2015), tivemos por objetivo verificar se a exposição ao etanol (1%) e em dois estágios distintos do desenvolvimento causariam um aumento na atividade da enzima persistente até a vida adulta, quando muitas alterações comportamentais são manifestadas. Como resultado, obtivemos o persistente aumento na atividade da enzima nos animais expostos ao etanol 1% no estágio de gástrula / segmentação, no entanto, sem alteração na expressão gênica da mesma, o que corrobora com pesquisas que levantam a hipótese de que o aumento nos níveis de RNAm podem não ter impacto direto nos níveis da enzima ativa (Glanemann et al., 2003) e que outras formas de regulação pós-traducionais poderiam estar envolvidas.

A realização da quantificação da adenosina por HPLC aos 3 mpf não mostrou diferenças significativas nos níveis de adenosina endógena entre controles e animais expostos ao etanol, embora a atividade da enzima ecto-5´-nucleotidase mostrou-se elevada nos animais expostos ao etanol no estágio de gástrula / segmentação, o que poderia levar ao aumento da adenosina extracelular. Entretanto, este resultado está de acordo com a ausência de alteração na atividade da enzima adenosina deaminase e deve-se levar em conta que a adenosina quantificada corresponde a adenosina endógena total (intra e extracelular) o que poderia mascarar ações regionais da adenosina. Além disso, estudos mostram que o aumento da atividade da ecto-5´-nucleotidase, pode ter como destino final, outras funções que não a clivagem do AMP em adenosina, como por exemplo, funções não catalíticas como adesão intracelular, sinalização, crescimento, proliferação e migração celular que podem impactar no desenvolvimento neural (Bianchi e Spychala, 2003; Neary and Burnstock, 1996; Zimmermann et al., 1998; Vogel et al., 1991).

No terceiro capítulo, foram realizados experimentos comportamentais com peixe-zebra aos 3 e 12 mpf, após exposição ao etanol nos estágios de gástrula / segmentação e faríngula. Foi constatado que a locomoção não sofre alterações significativas, enquanto o parâmetro ansiedade é alterado aos 3 mpf, tanto em animais expostos ao etanol no estágio de gástrula / segmentação, quanto no estágio de faríngula, porém este efeito ansiolítico não se manteve até os 12 mpf. O uso de inibidores das enzimas ecto-5´-nucleotidase e adenosina deaminase não causou nenhum efeito significativo sobre os parâmetros de ansiedade dos animais. Estudos realizados por Baggio et al. (2018) também demonstraram um efeito ansiolítico em animais adultos com 4 mpf, causado pela exposição ao etanol por 2 horas, 24 hpf, um período que abrange o fim do estágio de gástrula / segmentação e início do estágio de faríngula. Em resultados anteriores, com o mesmo protocolo de exposição e idade de avaliação, Baggio et al. (2017) encontraram um prejuízo no transporte de glutamato que poderia estar ligado as alterações nos parâmetros de ansiedade.

Segundo estudos de Egan et al. (2009), a diminuição dos níveis de cortisol causada pela exposição ao etanol também pode ser um dos mecanismos envolvidos neste efeito ansiolítico, já que níveis elevados de cortisol estão relacionados ao estresse. A exposição pré-natal ao etanol em humanos e ratos parece induzir a adaptações duradouras em múltiplos níveis dentro do eixo HPA (Hellemans et al., 2010; Glavas et al., 2007), e em peixe-zebra, o efeito ansiolítico da exposição ao etanol durante o desenvolvimento tem sido mostrado como duradouro até o 6 mpf (Baiamonte et al., 2016).

O efeito ansiolítico registrado aos 3 mpf em animais expostos ao etanol no início do desenvolvimento não persistiu aos 12 mpf, isso pode ser devido a uma série de fatores que precisam ser aprofundados. Possivelmente estes fatores envolvam o próprio desenvolvimento dos animais, o ambiente de crescimento e a diminuição na manipulação, visto que recentemente foi mostrado que o ambiente enriquecido, ou seja, o fato de haver uma melhora no ambiente é capaz de modular e reduzir a vulnerabilidade ao estresse no peixe-zebra (Marcon et al., 2018). O sistema dopaminérgico também tem sido ligado a estados de ansiedade em peixes-zebra jovens e adultos e pesquisas recentes mostram que o envelhecimento do peixe-zebra pode estar associado a déficits nos transportadores de dopamina e a um estado de ansiedade (Kacprzak et al., 2017), o que colabora com o fato de que o efeito ansiolítico do etanol pode ser diminuído pelo envelhecimento.

Os reflexos sobre o comportamento de agressividade foram diferentes quanto a fase de exposição ao etanol. O tempo na zona próxima ao espelho, utilizado como indicador de agressividade, foi aumentado apenas nos animais expostos ao etanol no estágio de faríngula tanto aos 3 quanto aos 12 mpf. Em ambas as idades este efeito foi revertido com a inibição da ecto-5´-nucleotidase, embora a potência deste efeito seja diferente em cada estágio de desenvolvimento. O estágio da faríngula, mais uma vez se mostrou o mais suscetível para levar a alterações comportamentais quando se usam concentrações baixas de etanol. Embora o sistema purinérgico não seja extensivamente estudado quando se trata de comportamento agressivo, diferente do que observamos no parâmetro ansiolítico, as alterações na agressividade parecem estar de alguma forma ligadas as interações com o metabolismo da adenosina, visto que a possível alteração dos seus níveis parece reverter o comportamento agressivo causado pela exposição ao etanol no estágio da faríngula, porém ainda são necessários mais estudos para aprofundar o conhecimento acerca dos mecanismos envolvidos neste processo. Já há dados bem consolidados que mostram alterações na expressão de receptores de adenosina e aumento da agressividade, como por exemplo, em camundongos *knockout* para os receptores A_1 que apresentam um comportamento mais agressivo (Giménez-Llort *et al*., 2002). Além disso, a adenosina pode interagir e participar de diversos mecanismos com diferentes sistemas de neurotransmissão, entre eles os sistemas dopaminérgico, gabaérgico e serotoninérgico (Werner e Coveñas, 2017), que segundo uma recente revisão de Gutwinski, Heinz e Heinz (2018) estão ligados ao comportamento agressivo causado pelo consumo do etanol.

Na avaliação da interação social, a exposição ao etanol levou a uma diminuição deste parâmetro quando analisado em ambos os estágios de desenvolvimento analisados. Entretanto, este parâmetro foi revertido no estágio de gástrula / segmentação tanto pela inibição da ecto-5´-nucleotidase quanto pela inibição da adenosina deaminase, enquanto no estágio de faríngula, apenas a inibição da ecto-5´-nucleotidase reverteu esse parâmetro aos 3 mpf. As alterações na interação social não foram persistentes até os 12 mpf. O peixe-zebra é conhecido por naturalmente formar grupos, um comportamento chamado *shoaling* (Miller e Gerlai, 2011) que é alterado pela exposição ao etanol, mesmo em baixas concentrações, sendo essa alteração persistente até a vida adulta (Fernandes, Rampersad e Gerlai, 2015). Nossos resultados mostram que tanto a inibição da ecto-5´-nucleotidase, a qual pode reduzir os níveis de adenosina extracelular, quanto à inibição da adenosina deaminase, que pode aumentar os

níveis de adenosina extracelular reverteram as alterações na interação social, pelo menos nos animais expostos no estágio de gástrula / segmentação. Tal resultado intrigante, nos leva a crer que outros mecanismos compensatórios possam estar agindo na regulação dos níveis extracelulares de adenosina, como os transportadores de adenosina ou que outros sistemas de neurotransmissão estejam envolvidos no processo.

Sabe-se que imagens de coespecíficos induzem a uma rápida elevação dos níveis de dopamina e DOPAC no cérebro do peixe-zebra, mas isso parece não acontecer em animais que foram expostos ao etanol durante o desenvolvimento embrionário (Fernandes, Rampersad e Gerlai, 2015). Embora Fernandes, Rampersad e Gerlai (2015) mostrem um efeito persistente na diminuição da interação social até os 2 anos após a exposição ao etanol, nas nossas condições de exposição, a diminuição na interação social não foi persistente até os 12 mpf, isso pode ter ocorrido devido às diferenças nos estágios e tempo de exposição ao etanol.

No presente estudo foi possível observar que as intervenções na sinalização adenosinérgica na fase embrionária resultaram em alterações no sistema nervoso capazes de causar, no peixe-zebra, alterações mnemônicas, bioquímicas e comportamentais induzidas pela exposição ao etanol, mesmo em um longo período após a interrupção da exposição. Além de ter efeitos por si só, a capacidade da adenosina de interferir na liberação e sinalização de outros neurotransmissores como GABA, glutamato e dopamina, faz com que perturbações em suas concentrações possam causar alterações significativas na sinalização exercida por esses neurotransmissores, principalmente na fase inicial do desenvolvimento (Delic e Zimmermann, 2010; Ferreira et al., 2014; Fredholm e Svenningsson, 2003; Popoli et al., 2003).

Com base em todas as questões discutidas nesse trabalho, fica evidente que a maioria das alterações podem ocorrer em ambas as fases de exposição ao etanol e que a adenosina tem um importante papel na formação da rede neural e alterações na sua sinalização durante esse processo são altamente danosas e duradouras. Além disto, a participação do controle da ecto-5’-nucleotidase parece ser um requisito importante para restauração de parâmetros mnemônicos e comportamentais afetados pelo etanol. O processo de aquisição de memória, mas não a consolidação, parece estar mais ligado ao metabolismo da adenosina, uma vez que o uso do AMPCP foi capaz de recuperar o prejuízo causado pela exposição ao etanol na fase da faríngula. Como o aumento da atividade da ecto-5´-nucleotidase foi visto apenas na

gástrula, possivelmente, neste parâmetro também estejam envolvidas alterações nos transportadores de adenosina, pois o controle do tônus adenosinérgico não é atribuição exclusiva desta enzima e a participação de transportadores de nucleosídeos neste contexto de efeitos em longo prazo do etanol deve ainda ser explorado.

Tais dados poderão contribuir para o embasamento acerca da suscetibilidade do cérebro em desenvolvimento a alterações no tônus adenosinérgico, as quais podem ocorrer em situações que ainda são comuns, como a exposição ao etanol durante a gestação. Dados de estudos de ciência básica acerca da exposição gestacional ao etanol são subsídios importantes para a conscientização de gestantes sobre os potenciais riscos da exposição ao etanol mesmo que em baixas concentrações, além de permitir avançar nos estudos em busca de um manejo adequado das sequelas da exposição embrionária ao etanol.

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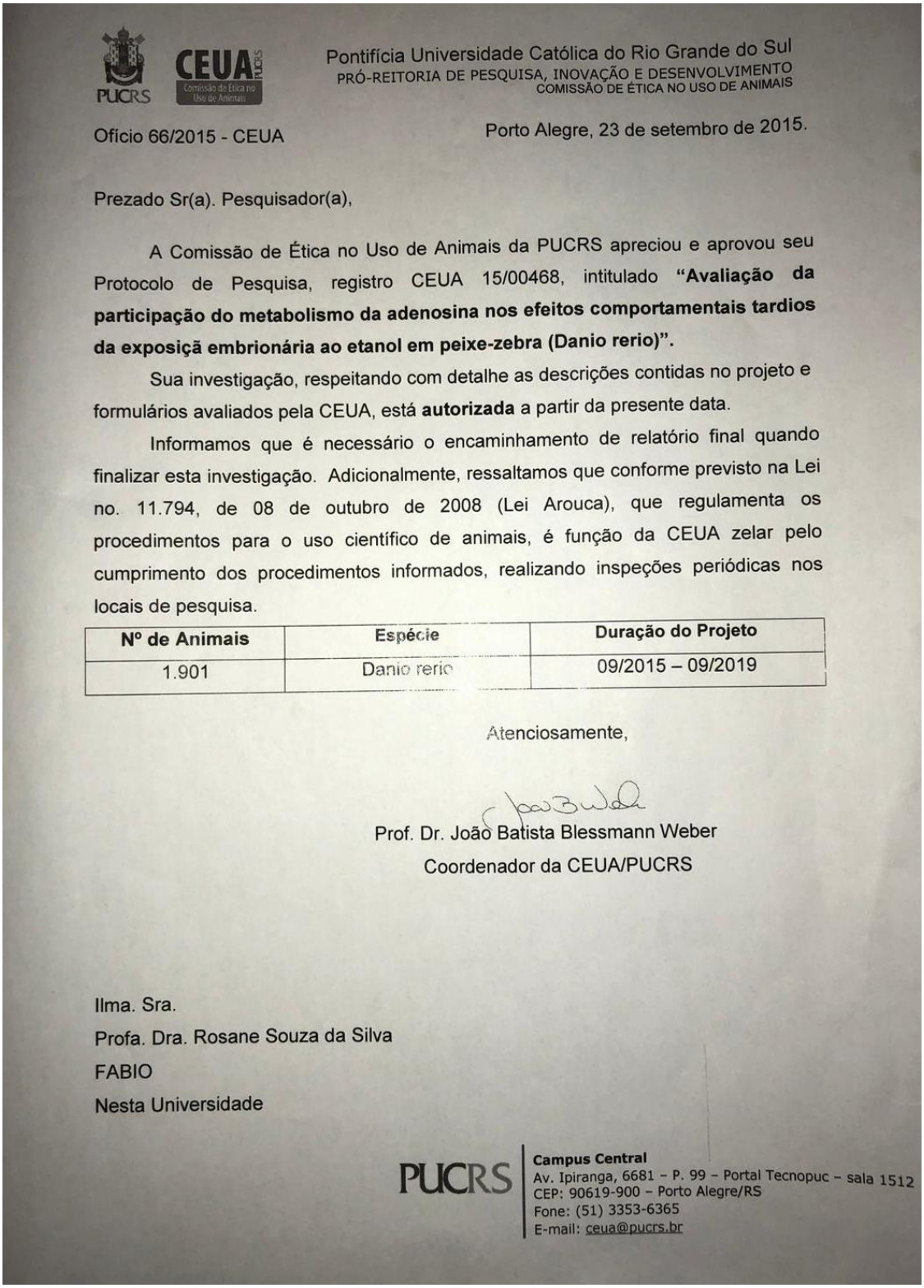
ANEXO A - APROVAÇÃO DA COMISSÃO DE ÉTICA



PUCRS CEUA Comissão de Ética no Uso de Animais

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 66/2015 - CEUA

Porto Alegre, 23 de setembro de 2015.

Prezado Sr(a). Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 15/00468, intitulado **"Avaliação da participação do metabolismo da adenosina nos efeitos comportamentais tardios da exposiçã embrionária ao etanol em peixe-zebra (Danio rerio)".**

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está **autorizada** a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Nº de Animais	Espécie	Duração do Projeto
1.901	Danio rerio	09/2015 – 09/2019

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

Ilma. Sra.
Profa. Dra. Rosane Souza da Silva
FABIO
Nesta Universidade

PUCRS
Campus Central
Av. Ipiranga, 6681 - P. 99 - Portal Tecnopuc - sala 1512
CEP: 90619-900 - Porto Alegre/RS
Fone: (51) 3353-6365
E-mail: ceua@pucrs.br

Pontifícia Universidade Católica do Rio Grande do Sul
Pró-Reitoria de Graduação
Av. Ipiranga, 6681 - Prédio 1 - 3º. andar
Porto Alegre - RS - Brasil
Fone: (51) 3320-3500 - Fax: (51) 3339-1564
E-mail: prograd@pucrs.br
Site: www.pucrs.br