Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

TESE DE DOUTORADO

Avaliação de parâmetros bioquímicos e comportamentais em peixe-zebra (*Danio rerio*): uma abordagem sobre o sistema purinérgico, colinérgico e efeitos promovidos pela taurina no modelo de exposição aguda ao etanol.

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Porto Alegre, RS, Brasil

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II

"Toda a nossa ciência, comparada com a realidade, é primitiva e infantil

- e, no entanto, é a coisa mais preciosa que temos".

(Albert Einstein)

"O homem que faz coisas comete erros, mas ele nunca comete

o maior erro de todos - não fazer nada".

(Benjamin Franklin)

"Nossas dúvidas são traidoras e nos fazem perder o que, com frequência,

poderíamos ganhar, por simples medo de arriscar".

(William Shakespeare)

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PARTE I

LISTA DE ABREVIATURAS

ACh – acetilcolina

AChE – acetilcolinesterase

ADH – álcool desidrogenase

ADP – adenosina difosfato

ALDH – aldeído desidrogenase

AMP – adenosina monofosfato

ATP – adenosina trifosfato

BuChE – butirilcolinesterase

CAT – catalase

CD73 – cluster de diferenciação da ecto-5'-nucleotidase

CDO – cisteína dioxigenase

CGS 21680 – hidrocloreto de 2-p-(2-carbóxietil)fenilamino-5'-N-etilcarboxaminoadenosina

CHA – N(6)cicloexiladenosina

CSAD – cisteína sulfinato descarboxilase

CYP2E1 – citocromo P450 da família 2E1

DNA – ácido desoxirribonueléico

ecto-ADA – ecto-adenosina deaminase

entpd – nomenclatura do gene codificador das NTPDases

ERN – espécies reativas de nitrogênio

ERO – espécies reativas de oxigênio

GABA – ácido γ-aminobutírico

GDP – guanosina difosfato

GMP – guanosina monofosfato

GPI – glicosilfosfatidilinositol

GPx – glutationa peroxidase

GSH – glutationa reduzida

GTP – guanosina trifosfato

HO – hipotaurina oxigenase

mRNA – ácido ribonucléico mensageiro

NAD^+ – nicotina adenina dinucleotídeo

NMDA – N-metil-D-aspartato

NTPDase – nucleosídeo trifosfato difosfoidrolase

Pi – fosfato inorgânico

SNC – sistema nervoso central

SOD – superóxido dismutase

TAR – *total antioxidant reactivity* (reatividade antioxidante total)

TauT – transportador de taurina

TBARS – *thiobarbituric acid reactive species* (espécies reativas ao ácido tiobarbitúrico)

TNFα – fator de necrose tumoral alfa

TRAP – *total reactive antioxidant potential* (potencial antioxidante reativo total)

tRNA – ácido ribonucléico transportador

VGCC – canais de Ca^{2+} dependentes de voltagem

RESUMO

O peixe-zebra (*Danio rerio*) é um pequeno teleósteo pertencente à família Cyprinidae, o qual vem sendo estudado em diferentes áreas científicas. Essa espécie apresenta genes evolutivamente conservados, diversos sistemas de neurotransmissão já caracterizados e um amplo repertório comportamental, os quais podem ser modulados por diversas drogas. Portanto, a presente tese tem por objetivo estudar parâmetros bioquímicos em peixe-zebra com ênfase nos sistemas de sinalização purinérgico e colinérgico, bem como avaliar o comportamento de animais submetidos à tarefa do *open tank* e investigar os efeitos promovidos pelo tratamento com taurina no modelo de exposição aguda ao etanol. Nós demonstramos que o tratamento agudo (1h) com taurina nas concentrações de 150 e 400 mg/L aumentou a hidrólise de AMP e levou a uma diminuição significativa na desaminação de adenosina em membranas cerebrais, sendo que o último efeito também foi observado nos ensaios *in vitro*. Entretanto, não foram observados efeitos sobre a hidrólise dos nucleotídeos tri e difosfatados, promovida pelas NTPDases. Estudos relacionados à hidrólise dos nucleotídeos ATP e ADP em cérebro, fígado e coração de peixe-zebra demonstraram um perfil diferencial nos respectivos tecidos. O efeito distinto promovido por inibidores apontou que diferentes membros das NTPDases poderiam estar contribuindo para a atividade enzimática detectada, o que foi sugerido por estudos de bioinformática e ensaios de expressão gênica. Além disso, verificamos que, no modelo de exposição aguda ao etanol, a taurina foi capaz de prevenir as alterações promovidas pelo álcool sobre a atividade da AChE e das defesas antioxidantes enzimáticas. Contudo, o pré-tratamento com taurina demonstrou ser mais efetivo na prevenção do estresse oxidativo, visto que, diferentemente do co-tratamento, aumentou o conteúdo total de tióis reduzidos cerebral e diminuiu o dano lipídico. A fim de caracterizar o repertório comportamental espaço-temporal dos animais no teste do *open tank*, nós elaboramos um protocolo que avaliava o efeito do confinamento prévio a estímulos naturalísticos (ambientes claro, escuro e transparente). Os resultados demonstraram que os peixes previamente confinados em um ambiente naturalmente aversivo (claro) apresentaram uma habituação intra-sessão mais rápida em comparação aos grupos escuro e transparente, avaliada pelas transições e tempo de permanência na região superior do aparato. O perfil exploratório espaço-temporal foi analisado através de *plots* representativos, sendo proposto um etograma descritivo para a tarefa do *open tank*. A partir do repertório comportamental determinado, o efeito promovido pelo pré-tratamento com taurina nas alterações comportamentais induzidas pelo etanol foi investigado. Nossos achados demonstraram que o etanol diminuiu a atividade locomotora dos animais, sendo que as concentrações de taurina testadas (42, 150 e 400 mg/L) preveniram essa modificação. Contudo, o pré-tratamento com 150 mg/L de taurina não preveniu as alterações na exploração da área superior do aparato, sugerindo que diferentes mecanismos poderiam estar envolvidos na resposta comportamental observada. Em suma, nossos resultados possibilitam uma maior compreensão das respostas neuroquímicas e comportamentais em peixe-zebra, contribuindo para novas estratégias relacionadas a estudos translacionais.

Palavras chave: peixe-zebra; taurina; bioquímica; comportamento.

ABSTRACT

The zebrafish (*Danio rerio*) is a small teleost fish that belongs to the Cyprinidae family, which has been studied in different scientific areas. This species has evolutively conserved genes, several neurotransmitter systems characterized, and a wide behavioral repertoire, which may be modulated by distinct drugs. Thus, this thesis aims to study biochemical parameters in zebrafish, emphasizing the purinergic and cholinergic signaling, as well as to evaluate the behavior of animals in the open tank task and to investigate the effects of taurine treatment in the acute ethanol exposure model. We showed that acute taurine treatment (1h) at 150 and 400 mg/L increased the AMP hydrolysis and significantly decreased the adenosine deamination in brain membranes. The latter effect was also observed in the enzyme assays performed *in vitro*. However, the tri and diphosphonucleoside hydrolysis promoted by NTPDases was unaffected by taurine. Studies related to ATP and ADP hydrolysis in zebrafish brain, liver, and heart demonstrated a differential profile in the respective tissues. The distinct effect promoted by inhibitors pointed that different NTPDase members could play a role in the enzyme activity detected, as suggested by bioinformatic analysis and gene expression assays. Moreover, we observed that, in the acute ethanol exposure model, taurine prevents the alterations induced by ethanol in AChE and in enzymatic antioxidant defenses activities. However, taurine pretreatment has shown to be more effective in the prevention of oxidative stress, considering that, differently from the cotreatment, it increased the total reduced thiol content and decreased lipid peroxidation in zebrafish brain. To characterize the spatio-temporal behavioral repertoire of animals subjected to the open tank task, we described a protocol which evaluated the effect promoted by the previous confinement using natural stimuli (bright, dark, and transparent environments). The results showed that fish previously confined into an aversive environment (brightly one) presented a faster intra-session habituation response when compared to dark and transparent groups, evaluated by transitions and time spent in the top area of the tank. The spatio-temporal exploratory profile was assessed by representative plots with the proposal of a descriptive ethogram to the behavioral task. Considering these behaviors, the effect promoted by taurine pretreatment on the behavioral changes induced by ethanol was investigated. Our findings demonstrated that ethanol significantly decreased the locomotor activity and that the taurine concentrations tested (42, 150, and 400 mg/L) prevented this modification. Nevertheless, the pretreatment with 150 mg/L taurine did not prevent the alterations on the exploratory profile for the top area of apparatus, suggesting that different mechanisms could be involved in the behavioral response observed. In conclusion, our data help to a better understanding of the neurochemical and behavioral responses in zebrafish, contributing for new strategies related to translational studies.

Key words: zebrafish; taurine; biochemistry; behavior.

APRESENTAÇÃO

Os resultados desta tese de doutorado estão apresentados sob a forma de artigos científicos. As seções Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se nos próprios artigos.

Os itens Introdução, Discussão e Conclusões encontrados nesta tese apresentam interpretações e comentários gerais sobre todos os artigos científicos contidos neste trabalho. As Referências Bibliográficas referem-se somente às citações que aparecem nos itens Introdução e Discussão desta tese.

Detalhes técnicos mais precisos sobre a metodologia empregada em cada um dos trabalhos apresentados podem ser encontrados nos artigos científicos correspondentes.

I.1. INTRODUÇÃO

I.1.a. A utilização do peixe-zebra em pesquisas científicas

O peixe-zebra (*Danio rerio*), também conhecido como "paulistinha" pelos aquariofilistas, é um pequeno teleósteo de 3-4 cm pertencente à família Cyprinidae. No final da década de 60, George Streisinger foi o pioneiro a estudar a biologia dessa espécie através de técnicas de mutagênese sítio dirigidas (Grunwald e Eisen, 2002). A descrição inicial da aplicação em estudos científicos relacionados à biologia do desenvolvimento possibilitou um grande avanço no conhecimento da embriogênese e do ciclo de vida dos vertebrados pela presença de ovos translúcidos, grande prole e desenvolvimento de aproximadamente dois meses (Lele e Krone, 1996; Dahm e Geisler, 2006). Além disso, essas características favorecem a expressão de transgenes fluorescentes tornando-se viável o monitoramento da expressão e atividade de muitos genes através de manipulação via microinjeção (Gulati-Leekha e Goldman, 2006).

Somados a esses aspectos, outros atributos práticos fazem com que o peixe-zebra seja atraente para estudos de laboratório quando comparado a outros modelos tradicionais, tais como a mosca-das-frutas (*Drosophila melanogaster*), o rato (*Rattus norvegicus*) e o camundongo (*Mus musculus*). Algumas vantagens que podem ser destacadas são o pequeno espaço requerido para a manutenção, o baixo custo e a praticidade para triagens em larga escala (Lieschke e Currie, 2007). Pode-se destacar, com isso, que o peixe-zebra é um animal que combina a relevância de ser um vertebrado na escala de um invertebrado (Goldsmith, 2004).

Ao longo da última década, progressos consideráveis na genética e genômica do peixe-zebra vêm sendo observados. Em 2001, o Instituto Sanger começou o sequenciamento do genoma dessa espécie e, paralelamente, a identificação de regiões codificadoras (Vogel, 2000; Stern e Zon, 2003). Foi demonstrado que os genes do peixe-zebra são evolutivamente conservados e apresentam um alto grau de similaridade com os genes de mamíferos (Barbazuk et al., 2000; Lieschke e Currie, 2007). Devido ao crescimento exponencial do número de estudos publicados utilizando esse vertebrado, foi criada uma rede de informações na web sobre o peixe-zebra, o ZFIN (http://zfin.org), na qual laboratórios do mundo inteiro podem depositar um grande número de informações sobre a espécie. Além disso, existe um excelente, compreensivo e frequentemente atualizado manual de manutenção e controle das condições ideais para criação em laboratório (Sprague et al., 2003).

Nos últimos anos, a aplicabilidade do peixe-zebra em estudos científicos vem sendo expandida para outras áreas do conhecimento, tais como bioquímica (Taylor et al., 2004), neurociência (Edwards e Michel, 2002), farmacologia (Goldsmith, 2004) e biologia do comportamento (Gerlai, 2003; Guo, 2004; Egan et al., 2009; Blaser et al., 2010; Cachat et al., 2010; Blaser e Peñalosa, 2011; Gebauer et al., 2011; Maximino et al., 2011; Piato et al., 2011). Até o momento, a maioria das pesquisas na área da biologia do comportamento utiliza somente princípios de validação farmacológica, avaliando o efeito de diferentes compostos, tais como etanol (Mathur et al., 2011), nicotina (Levin et al., 2007), cocaína (López Patiño et al., 2008), cafeína e fluoxetina (Egan et al., 2009). Paralelamente a esses aspectos, estudos recentes sugeriram que a exposição a estímulos naturalísticos aversivos

poderia ser relevante a fim de melhor elucidar os comportamentos relacionados à ansiedade para a espécie e também na possibilidade de descrever o repertório comportamental espaço-temporal dos animais durante a resposta à novidade, os quais ainda permanecem pobremente discutidos e compreendidos (Blaser et al., 2010; Maximino et al., 2010).

Pelo fato do peixe-zebra apresentar um tamanho relativamente pequeno e facilmente absorver compostos adicionados na água, a quantidade dos reagentes a serem testados passa a ser significativamente menor, contribuindo com a otimização do uso de drogas de estudo e também com uma menor produção de resíduos (Goldsmith, 2004). Devido à possibilidade da utilização do organismo em triagens de larga escala, a espécie também desperta o interesse pela oportunidade de acelerar o processo da descoberta de novas drogas que possam servir como estratégias terapêuticas (Stern e Zon, 2003; Rico et al., 2011a). Nesse contexto, a utilização do peixe-zebra como modelo animal em pesquisas biomédicas relacionadas a doenças humanas, tais como crises convulsivas (Hortopan et al., 2010), dano hepático agudo (Hammes et al., 2011) e malformações cardíacas (Chico et al., 2008) vem sendo destacadas na literatura. Além disso, quando comparados aos estudos realizados *in vitro* em culturas de células, as análises em um modelo vertebrado *in vivo* permite o estudo mais detalhado das complexas interações exercidas por diferentes compostos, bem como a relação dos mecanismos envolvidos no efeito promovido por moléculas distintas nos sistemas biológicos (Rico et al., 2011a).

I.1.b. Taurina

A taurina (ácido 2-aminoetano sulfônico) é um β-aminoácido sulfurado, o qual foi inicialmente descoberto como uma molécula biológica no ano de 1826 presente na bile de bovinos (*Bos taurus*), fato que originou sua nomenclatura. Apesar da taurina ser importante na formação se sais biliares, sabe-se que encontra-se presente em diversos tecidos. A biossíntese desse aminoácido ocorre a partir da oxidação da cisteína, tendo a particiação das enzimas cisteína dioxigenase (CDO), cisteína sulfinato descarboxilase (CSAD) e hipotaurina oxigenase (HO) (Banerjee et al., 2008; Vitvitsky et al., 2011). A taurina é abundantemente encontrada em tecidos caracterizados por um alto metabolismo oxidativo, tais como retina, coração, músculo esquelético e cérebro (Huxtable, 1992). No sistema nervoso central (SNC), elevadas concentrações de taurina (cerca de 6-8 mM) podem ser detectadas no meio intracelular, sendo que no meio extracelular a concentração geralmente não ultrapassa a faixa de 80-120 μM (Huxtable, 1992; Oliveira et al., 2010; Vivitsky et al., 2011). Esta discrepância observada entre ambos os compartimentos celulares resulta principalmente de um eficiente mecanismo de transporte através da ação do transportador de taurina (TauT), o qual utiliza os gradientes de Na^+ e Cl^- como força motriz (Junyent et al., 2011), bem como da biossíntese desse aminoácido, a qual pode envolver uma intrínsica cooperação metabólica entre neurônios e células gliais (Tappaz et al., 1994; Banerjee et al., 2008; Vivitski et al., 2011).

Evidências sugerem que a taurina poderia atuar como uma molécula neurotransmissora por apresentar algumas características, tais como: i) a presença de significativas quantidades de taurina já foi detectada no corpo

celular, dendrito e terminais de neurônios, os quais podem expressar enzimas necessárias para sua síntese; ii) a sua liberação pode ocorrer de maneira dependente ou independente de Ca^{2+}; iii) a ação de hiperpolarização da taurina através da ação em canais de Cl^- já foi descrita em estruturas cerebrais de roedores, tais como cerebelo e hipocampo; iv) a existência de um sistema específico de transporte para remoção da taurina extracelular foi comprovada; e v) foi proposta a existência de receptores putativos de taurina (Huxtable, 1992; Lee et al., 1992; Wu e Xu, 2003; Vivitsky et al., 2011);. Contudo, o acúmulo desse aminoácido em vesículas pré-sinápticas ainda não foi demonstrado, bem como a descrição exata do mecanismo de liberação. Dessa forma, a classificação da taurina como uma molécula neurotransmissora ainda é questionável.

Os efeitos da taurina em diferentes modelos experimentais parecem ocorrer pela ativação de receptores $GABA_A$ e de glicina, pois antagonistas seletivos de um ou ambos os receptores podem diminuir as ações promovidas por esse aminoácido (Wang et al., 2007; Bulley e Shen, 2010). Apesar de não existir uma caracterização de receptores específicos, estudos demonstraram que agonistas e antagonistas de receptores de GABA e de glicina podem não influenciar as ações extracelulares promovidas pela taurina, sugerindo que outro receptor poderia estar envolvido nessas respostas. Dessa forma, foi proposta a existência de receptores putativos de taurina, os quais possivelmente possam ser metabotrópicos, apresentando um Kd na faixa nM e um peso molecular aproximado entre 62-100 kDa (Wu et al., 1992; Sung et al., 1996; Wu e Prentice, 2010).

Paralelamente, dados na literatura demonstram que a taurina pode exercer funções importantes na osmorregulação e atuar como um

neuromodulador inibitório, antagonizando os efeitos do glutamato (Wu et al., 2005; Rodríguez-Navarro et al., 2009; Wu e Prentice, 2010). A sinalização mediada por esse neurotransmissor excitatório leva a um aumento dos níveis intracelulares de Ca^{2+} através dos receptores NMDA, via canais de Ca^{2+} dependentes de voltagem (VGCC, dos tipos L, N, P e Q), reversão do trocador Na^+/Ca^{2+}, bem como pela liberação dos estoques de Ca^{2+} intracelulares (Schousboe et al., 1997; Wu et al., 2005; Junyent et al., 2010). O Ca^{2+} atua como um segundo mensageiro em cascatas de sinalização, levando a uma complexa regulação de diferentes enzimas do metabolismo celular (Flavell e Greenberg, 2008). É sabido que o aumento dos níveis de Ca^{2+} intracelular desencadeado por sucessiva estimulação glutamatérgica pode contribuir para um fenômeno conhecido como excitotoxicidade (Mody e MacDonald, 1995; Pivovarova e Andrews, 2010). A captação de glutamato exercida pelos astrócitos possui um importante papel na regulação do tonus glutamatérgico fisiológico, sendo que este mecanismo desencadeia concomitantemente uma série de respostas transientes realizadas pelas células gliais (Danboldt, 2001). Dentre elas, ocorre um aumento na síntese de glutationa reduzida (GSH) a partir da incorporação de cistina e um aumento na concentração de osmólitos, o que culmina no efluxo de taurina dos astrócitos com função de manter o controle osmótico dessas células (Banerjee et al., 2008). Todos esses mecanismos ocorrem com a finalidade de contrabalançear os efeitos celulares resultantes da rápida remoção do glutamato pelos astrócitos, demonstrando uma relação intrínseca da taurina com a sinalização glutamatérgica.

Estudos têm demonstrado que a taurina é capaz de prevenir o acúmulo de Ca^{2+} intracelular, bem como exercer um controle da atividade de enzimas dependentes desse cátion divalente desempenhando, portanto, funções

pleiotrópicas de citoproteção a diversos insultos cerebrais relacionados à excitotoxicidade (Pushpakiran et al., 2005; Wu et al., 2005; Junyent et al., 2010).

Experimentos realizados por David-Watine et al. (1999a; 1999b) caracterizaram a subunidade alfa (α) do receptor de glicina em peixe-zebra (αZ1), a qual revelou um elevado grau de homologia com as isoformas α1, α2 e α3 de mamíferos, além de uma elevada sensibilidade à taurina. Outra subunidade α do receptor de glicina, denominada αZ2, foi identificada em peixe-zebra, apresentando características funcionais semelhantes à subunidade αZ1 e uma expressão gênica específica em cérebro de larvas e adultos (Imboden et al., 2001). Através de um complexo e abrangente atlas molecular da retina de peixe-zebra, foi demonstrado que níveis milimolares de taurina encontram-se amplamente distribuídos no meio intracelular de diferentes células, tais como cones e bastonetes (Marc e Cameron, 2001). Esses dados sugerem que a taurina poderia ter um importante papel para a visão dos teleósteos, similar ao fenômeno observado em outros organismos, tais como salamandra e roedores (Bulley e Shen, 2010). Kozlowski et al. (2008) caracterizaram o transportador TauT durante o desenvolvimento do embrião de peixe-zebra ao nível molecular e funcional. Através desse estudo, foi comprovada uma elevada homologia (cerca de 75-80%) com a proteína TauT de mamíferos, bem como o transporte dependente do gradiente de Na^+ e Cl^-. Testes envolvendo hibridização *in situ* demonstraram a presença do mRNA deste transportador em retina, cérebro, coração, rim e vasos sanguíneos, sendo que o silenciamento da expressão através da técnica de morfolino acarretou em morte de células no SNC e aumentou a mortalidade dos embriões (Kozlowski et al., 2008). O conjunto desses resultados demonstrou

que a regulação dos níveis de taurina poderia desempenhar um papel funcional crítico ao longo do desenvolvimento. Entretanto, a relação da taurina com os diferentes sistemas de neurotransmissão em peixe-zebra ainda carece de maiores informações.

I.1.c. Sistema purinérgico

Além de exercer sua função no metabolismo energético, o ATP é uma importante molécula sinalizadora, sendo que essa ação foi demonstrada através de estudos realizados por Geoffrey Burnstock (Burnstock et al., 1970; Burnstock, 1972).

No SNC e periférico, o ATP age como neurotransmissor excitatório e possivelmente como neuromodulador (Cunha e Ribeiro, 2000; Salgado et al., 2000). O ATP extracelular pode influenciar a atividade sináptica ao interagir com receptores específicos denominados purinoreceptores P2, subdivididos em ionotrópicos (P2X) e metabotrópicos (P2Y), os quais estão amplamente distribuídos em diversos tecidos (North e Barnard, 1997; Ralevic e Bunrnstock, 1998; Burnstock e Knigth, 2004).

A sinalização mediada por nucleotídeos extracelulares necessita de mecanismos eficientes para o controle do nível dos ligantes para os respectivos purinoreceptores (Zimmermann, 1994; Neary e Zimmermann, 2009). Diversos trabalhos evidenciaram a presença de uma variedade de enzimas localizadas na superfície celular, denominadas ectonucleotidases, as quais exercem a hidrólise desses nucleotídeos e, portanto, contribuem para a inativação da sinalização mediada por essas moléculas (Robson et al., 2006; Langer et al., 2008; Knowles, 2011) . Dentre essas enzimas, podemos destacar a família das

nucleosídeo trifosfato difosfoidrolases (NTPDases) e a ecto-5'-nucleotidase. As NTPDases são enzimas que podem estar localizadas tanto na membrana plasmática das células hidrolisando nucleotídeos extracelulares (NTPDases1-3 e 8), bem como ancoradas a organelas citoplasmáticas com o sítio ativo voltado para o lúmen das mesmas (NTPDases4-7) (Lavoie et al., 2004). Os aspectos cinéticos que diferenciam os membros das NTPDases são a preferência por diferentes substratos, hidrolisando nucleotídeos púricos e pirimídicos, e a razão de hidrólise entre nucleosídeos tri e difosfatados (Zimmermann, 2000; Vorhoff et al., 2005). A ecto-5'-nucleotidase, também conhecida como CD73, hidrolisa nucleotídeos 5'-monofosfatatos púricos e pirimídicos ao respectivo nucleosídeo. Essa enzima encontra-se ancorada à membrana plasmática via glicosilfosfatidilinositol (GPI), sendo que formas solúveis podem ser originadas mediante a ação de uma fosfolipase específica (Sträter, 2006). Tanto as NTPDases quanto a ecto-5'-nucleotidase são dependentes de cátions divalentes para suas atividades catalíticas, tais como Ca^{2+} e Mg^{2+}.

A hidrólise extracelular de ATP por essa via resulta na formação de ADP, AMP e adenosina. Esse nucleosídeo pode agir como neuromodularor em receptores metabotrópicos do tipo P1, atuando tanto em mecanismos inibitórios via A_1 e A_3 quanto facilitatórios via A_{2A} e A_{2B}. A inativação da sinalização adenosinérgica ocorre por capatação através de transportadores bidirecionais específicos ou por desaminação até inosina (Fredholm et al., 2005; Rosemberg et al., 2007a). A desaminação de adenosina pode ocorrer tanto no meio intracelular quanto no meio extracelular, por meio de uma adenosina deaminase localizada na membrana plasmática (ecto-ADA) (Franco et al., 1998). Estudos têm demonstrado que a ecto-ADA pode estar co-localizada com

receptores de adenosina do tipo P1 (A_1 e A_{2B}) exercendo um possível papel no controle da sinalização adenosinérgica (Ciruela et al., 1996; Ruiz et al., 2000; Herrera et al., 2001). Além disso, a presença de diferentes membros de enzimas relacionadas à adenosina deaminase, agrupadas como subfamílias das adenil-deaminases, já foi previamente descrita (Maier et al., 2005).

Além dos nucleotídeos de adenina, as ectonucleotidases podem atuar em outros nucleotídos, incluindo os derivados da guanina, hidrolisando GTP, GDP e GMP até guanosina. Estudos têm demonstrado que a guanosina é capaz de alterar parâmetros comportamentais, podendo também exercer um papel anticonvulsivante, amnésico e ansiolítico (Roesler et al., 2000; Vinade et al., 2003). Por atuarem como moduladores de diversos processos intracelulares e extracelulares, foi proposta a existência de um sistema purinérgico dos nucleotídeos da guanina em adição ao sistema adenosinérgico no SNC (Schmidt et al., 2007). Dentre as diversas funções exercidas pela guanosina, pode-se destacar sua importante ação neuroprotetora frente à excitotoxicidade (Thomazi et al., 2008).

A importância da hidrólise de nucleotídeos para a homeostasia em diferentes tecidos tem sido extensivamente descrita na literatura. Estudos realizados por Beldi et al. (2008) demonstraram que a hidrólise de nucleotídeos realizada pela NTPDase1 possui um papel fundamental na proliferação de hepatócitos e células endoteliais em fígado de camundongos, sugerindo que as purinas e seus derivados poderiam exercer um importante papel na regeneração hepática e vascular. Além disso, foi demonstrado que a atividade dessa enzima no coração é capaz de promover proteção às células do miocárdio durante a isquemia e reperfusão (Köhler et al., 2007), sendo que a NTPDase1 já foi caracterizada em ventrículo de ratos (Rücker et al., 2008).

Pelo fato da expressão gênica da *entpd3* ter sido detectada como um fator importante para a prevenção da contratilidade excessiva em cardiomiócitos foi sugerido que diferentes membros dessa família atuam de forma coordenada para a manutenção da homeostasia cardíaca (Barreto-Chaves et al., 2006). Em SNC, alterações na atividade das NTPDases têm sido correlacionadas com mudanças na plasticidade neuronal, aprendizado na esquiva inibitória, convulsões e isquemia (Bonan et al., 2000; Schetinger et al., 2007). É sabido que a regulação dos níveis de nucleotídeos extracelulares exercida pelas ectonucleotidases e da consequente modulação dos ligantes para seus respectivos purinoreceptores ocorre em paralelo à sinalização mediada por outros neurotransmissores. Por ser considerado como uma molécula co-transmissora, o ATP é liberado através de vesículas pré-sinápticaas dependentes de Ca^{2+} juntamente com outros neurotransmissores, tais como glutamato, noradrenalina, serotonina, GABA e acetilcolina (ACh) (Burnstock, 2004; Zimmermann, 2008).

Com relação à sinalização purinérgica em peixe-zebra, a clonagem e caracterização molecular de subtipos dos receptores do tipo P2 demonstraram a existência de um alto grau de similaridade com as sequências de mamíferos conhecidas (Kucenas et al., 2003; Ricatti et al., 2011). A hidrólise de nucleotídeos e a desaminação de adenosina também já foram caracterizadas em frações cerebrais dessa espécie, demonstrando a existência de uma complexa cascata enzimática no controle da sinalização purinérgica (Rico et al., 2003; Senger et al., 2004; Rosemberg et al., 2008).

Ao longo dos anos, estudos vêm sendo realizados utilizando o peixe-zebra a fim de investigar as bases moleculares da neurobiologia, identificando genes envolvidos na formação de circuitos neuronais, no comportamento e nos

mecanismos envolvidos em neuropatogênese (Vascotto et al., 1997; Guo, 2004). Além do sistema purinérgico, outros sistemas de neurotransmissão já foram identificados no peixe-zebra tais como: glutamatérgico, dopaminérgico, serotoninérgico, histaminérgico, GABAérgico e colinérgico (Rico et al., 2011a).

I.1.d. Sinalização colinérgica

A sinalização mediada pela ACh é fundamental para o funcionamento do SNC, representando o sistema neurotransmissor mais antigo do ponto de vista filogenético (Gotti e Clementi, 2004). Além de serem encontrados no SNC, os neurônios colinérgicos inervam a musculatura voluntária e involuntária (Soreq e Seidman, 2001).

Baseando-se em suas diferentes afinidades por agentes que mimetizam a ação da ACh, os receptores colinérgicos são divididos em duas classes distintas: muscarínicos e nicotínicos (Tinsley et al., 2004). Os receptores muscarínicos são metabotrópicos e ligam-se à ACh e à muscarina, um alcalóide presente em certos cogumelos venenosos. Esses receptores são encontrados em gânglios do sistema nervoso periférico e nos órgãos efetores autonômicos, como coração, músculo liso, cérebro e glândulas exócrinas (Sarter e Parikh, 2005). Existem cinco subtipos de receptores muscarínicos (M1-M5) que foram clonados e identificados farmacologicamente, sendo acoplados a distintos tipos de proteínas G, regulando metabolismo do Ca^{2+} intracelular ou a atividade da adenilato ciclase (Andersson, 2011). Por outro lado, os receptores nicotínicos são ionotrópicos, sendo que a ACh e a nicotina são ligantes do mesmo (Dani e De Biasi, 2001). Tais receptores se localizam

no SNC, na medula adrenal, nos gânglios autonômicos e na junção neuromuscular (Sarter e Parikh, 2005).

Após ser liberada na fenda sináptica, a ACh é degradada pela enzima acetilcolinesterase (AChE) em acetato e colina, ambas recaptadas pelo neurônio. O sítio ativo da AChE é composto por uma tríade catalítica que contém resíduos de serina, de histidina e de um grupo ácido (glutamato ou aspartato). O mecanismo de hidrólise envolve o ataque nucleofílico da serina ao grupamento carboxílico da ACh, gerando um intermediário tetraédrico estabilizado por pontes de hidrogênio, o qual libera colina livre e serina acetilada. Ao final, a hidrólise do grupo acetila da serina pela água recupera o sítio catalítico da enzima (Soreq e Seidman, 2001).

Foi demonstrado que o sistema colinérgico possui um papel importante no desenvolvimento das estruturas cerebrais (Zirger et al., 2003). Alterações na neurotramissão colinérgica durante o desenvolvimento produzem danos estruturais com efeitos no comportamento (Bachman et al., 1994; Hohmann, 2003). Da mesma forma, o desbalanço nos níveis de ACh extracelular pode ser detectado como um dos efeitos promovidos por drogas de abuso (Ishibashi et al., 2009).

Estudos realizados por Bertrand et al (2001) demonstraram características funcionais e estruturais da AChE em peixe-zebra, visto que o único gene codificador dessa enzima foi clonado e sequenciado. Além disso, a sequência deduzida de 634 aminoácidos da proteína apresentou 62% de homologia quando comparada à dos mamíferos. Pelo fato deste vertebrado não apresentar um gene que codifique a butirilcolinesterase (BuChE), a qual também é capaz de degradar a ACh, o peixe-zebra é um potencial modelo para a investigação de parâmetros toxicológicos e farmacológicos relacionados à

modulação da AChE (Bertrand et al., 2001; Rico et al., 2011a). Nesse contexto, foi verificado que a atividade dessa enzima em peixe-zebra pode ser alterada após a exposição a diferentes agentes neurotóxicos, tais como o etanol (Rico et al., 2007).

I.1.e. Metabolismo e efeitos do etanol

O etanol é uma substância amplamente consumida, caracterizando um problema de saúde pública em todo o mundo. O crescente consumo dessa droga de abuso está associado com a ocorrência de diversas condições patológicas, como câncer, doenças hepáticas, danos cerebrais e alterações cognitivas (Ferreira e Willoughby, 2008).

O metabolismo do etanol compreende distintas vias. Dentre elas, a principal ocorre através de duas reações enzimáticas que requerem NAD^+ como coenzima. Primeiramente, a enzima álcool desidrogenase (ADH) converte o etanol em acetaldeído, com a concomitante redução de NAD^+ no processo. O segundo passo envolve a metabolização do acetaldeído a acetato pela atividade da aldeído desidrogenase (ALDH), com a redução de NAD^+ (Swift, 2003). Muitos órgãos são capazes de metabolizar o etanol, mas a maior parte do mesmo (aproximadamente 90%) é metabolizada no fígado (Quertemont et al., 2005), sendo que sua eliminação ocorre através de múltiplas reações enzimáticas sequenciais. Além da principal rota de degradação envolvendo ADH e ALDH, há duas menores vias oxidativas para a degradação do etanol em acetaldeído. Nesse contexto, o citocromo P450 (CYP2E1) é responsável por uma pequena parte do total metabolizado e a catalase (CAT) pode transformar etanol em acetaldeído a partir de um radical

peróxido (Swift, 2003). Sabe-se que a participação de cada via de degradação do etanol varia dependendo do tecido. Por exemplo, enquanto no fígado a principal rota de degradação envolve as reações catalisadas pela ADH e ALDH, em cérebro a CAT participa com aproximadamente 60% de importância na conversão do etanol em acetaldeído.

Tanto o acetaldeído quanto o acetato vêm sendo investigados no sentido de esclarecer o envolvimento desses metabólitos em respostas comportamentais e farmacológicas (Israel, et al., 1994; Quertemont, et al., 2005). Estudos têm demonstrado que a toxicidade do acetaldeído está associada a diversos efeitos neuroquímicos e farmacológicos promovidos pelo etanol. Esse metabólito é uma molécula altamente reativa que pode formar aductos com proteínas e outros componentes biológicos (Seitz e Homann, 2007; Yu et al., 2010). O estudo de potenciais estratégias terapêuticas ao abuso de álcool, inclusive com a utilização da taurina, tem demonstrado um efeito protetor frente ao dano oxidativo induzido pela administração de etanol em modelos experimentais de fibrose hepática (Devi e Anuradha, 2010).

Devido ao amplo consumo, o etanol é um agente que promove neurotoxicidade, causada por sua influência em diversos parâmetros neuroquímicos, levando a alterações na cognição e no comportamento (Diana et al., 2003). Dessa maneira, o álcool é capaz de modificar rotas de transdução de sinais mediadas por hormônios e neurotransmissores, bem como promover alterações significativas em parâmetros relacionados ao estresse oxidativo (Hanchar et al., 2005; Belmeguenai et al., 2008; Su et al., 2010). Ao longo da última década, o peixe-zebra tem sido utilizado com sucesso em pesquisas que abordem os efeitos de drogas nas mais diversas respostas comportamentais, dentre elas, o etanol. Foi demonstrado por Gerlai et al.(2000) que a exposição

aguda ao etanol por um período de 1h promove alterações em parâmetros comportamentais, tais como locomoção e preferência social. Adicionalmente, a análise das respostas de linhagens de diferentes genótipos expostas ao etanol serve como uma importante base para estudos genéticos relacionados aos mecanismos envolvidos nos processos da dependência ao álcool (Dlugos e Rabin, 2003). As duas classes de ADH já caracterizadas compartilham similaridade estrutural com as de mamíferos (Reimers et al, 2004), o que sugere que o metabolismo do etanol nessa espécie compreende rotas bioquímicas semelhantes.

Foi demonstrado que o etanol promove alterações morfológicas em peixe-zebra, tais como anormalidades craniofaciais, malformações cardíacas e prejuízos ao longo do desenvolvimento (Bilotta, 2004). Dentre os parâmetros bioquímicos que podem ser influenciados pela exposição ao álcool podemos citar alterações na atividade e expressão gênica das ectonucleotidases em membranas cerebrais após as exposições aguda e crônica (Rico et al., 2008; 2011b), bem como a indução de estresse oxidativo em larvas, a qual é parcialmente atenuada por antioxidantes (Reimers et al., 2006).

I.1.f. Estresse oxidativo e defesas antioxidantes

Estresse oxidativo é o termo utilizado para designar um fenômeno no qual a formação de espécies pró-oxidantes excede a capacidade de transformação dessas moléculas em outras não-oxidantes pelos mecanismos de defesa antioxidante. Dentre as substâncias pró-oxidantes, podem ser destacadas radicais livres, espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN) (Westerblad e Allen, 2011). Denomina-se radical

livre a espécie química que possui um ou mais elétrons desemparelhados no seu orbital mais externo. Pelo fato de apresentarem essa característica, os radicais livres são altamente reativos, tendendo a adquirir ou perder um segundo elétron para este orbital (Halliwell, 2006). Por exemplo, o ânion superóxido ($O_2^{\cdot-}$) é produzido quando uma molécula de oxigênio é reduzida parcialmente, ou seja, quando recebe apenas um elétron ao invés de dois. Caso ocorra uma redução completa, como ocorre no complexo IV da cadeia transportadora de elétrons mitocondrial, existe a formação de H_2O. Excessivas quantidades de $O_2^{\cdot-}$ podem ocasionar danos em diversos sistemas biológicos através da formação do radical hidroxila (OH^-) derivado da reação de Fenton entre a espécie reativa peróxido de hidrogênio (H_2O_2) com os metais de transição Fe^{2+} e Cu^{2+} (Yalfani et al., 2011). Além disso, a reação com o oxido nítrico ($NO^{\cdot}$) pode levar à formação de peroxinitrito ($ONOO^{\cdot}$), o qual, por sua vez, pode favorecer a formação do radical nitrosila ($ONOOH$), cuja decomposição pode aumentar os níveis de OH^-. (Hughes, 1999; Kahn et al., 2000).

Pelo fato das espécies reativas apresentarem a tendência de desencadear reações em cadeia, dano a diferentes estruturas celulares, tais como DNA, carboidratos, proteínas e lipídios são comuns à característica pró-oxidante dessas moléculas (Peng e Jou, 2010). A fim de diminuir a reatividade inerente à natureza dos radicais livres, ERO e ERN, as células apresentam mecanismos de defesa antioxidantes, caracterizados como enzimáticos e não enzimáticos, os quais visam manter a homeostasia do sistema biológico. Dentre os mecanismos de defesa antioxidante enzimáticos podem ser citadas a superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx), enquanto que as defesas não enzimáticas podem ser constituídas por

vitaminas da dieta, tais como as vitaminas C e E. Enquanto a SOD catalisa a dismutação do $O_2^{\cdot-}$ em H_2O_2, a CAT atua sobre o H_2O_2, convertendo-o em H_2O. De maneira similar à CAT, a enzima GPx também age sobre o H_2O_2, porém com um mecanismo distinto que envolve a GSH, atuando em paralelo com a glutationa redutase (Boveris, 1998).

Embora seja sabido que algumas drogas são capazes de alterar parâmetros neuroquímicos e comportamentais em modelos animais alternativos, estudos relacionados a modificações no potencial redox cerebral, bem como potenciais estratégias protetoras nesses organismos ainda são escassos.

I.2. OBJETIVO GERAL

Considerando i) a importância do peixe-zebra como um organismo modelo emergente em estudos de diversas áreas do conhecimento científico; ii) o papel pleiotrópico de citoproteção exercido pela taurina nos sistemas biológicos; iii) a interação existente entre os sistemas de neurotransmissão purinérgico e colinérgico; e iv) os diversos efeitos que o etanol exerce a nível bioquímico e comportamental, o objetivo geral da presente tese de doutorado foi estudar, em peixe-zebra, parâmetros bioquímicos com ênfase nos sistemas de sinalização purinérgico e colinérgico, bem como avaliar o comportamento de animais submetidos à tarefa do *open tank* e os efeitos promovidos pelo tratamento com taurina no modelo de exposição aguda ao etanol.

I.3. OBJETIVOS ESPECÍFICOS

➢ Investigar os efeitos promovidos pela taurina *in vivo* (agudo) e/ou *in vitro* sobre a atividade das NTPDases, ecto-5'-nucleotidase e adenosina deaminase em frações cerebrais.

➢ Caracterizar a atividade, expressão e localização dos membros pertencentes à família das NTPDases em preparações de cérebro, fígado e coração.

➢ Investigar se o tratamento agudo com taurina previne alterações neuroquímicas promovidas pelo etanol em parâmetros da sinalização colinérgica e estresse oxidativo em SNC.

➢ Caracterizar o teste do *open tank* utilizando exposição prévia a estímulos naturalísticos.

➢ Investigar se o tratamento prévio com taurina é capaz de prevenir as alterações comportamentais promovidas pelo etanol na tarefa do *open tank*.

PARTE II

II.1. CAPÍTULO I

Evidence that acute taurine treatment alters extracellular AMP hydrolysis and adenosine deaminase activity in zebrafish brain membranes.

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Evidence that acute taurine treatment alters extracellular AMP hydrolysis and adenosine deaminase activity in zebrafish brain membranes

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ABSTRACT

Taurine is one of the most abundant free amino acids in excitable tissues. In the brain, extracellular taurine may act as an inhibitory neurotransmitter, neuromodulator, and neuroprotector. Nucleotides are ubiquitous signaling molecules that play crucial roles for brain function. The inactivation of nucleotide-mediated signaling is controlled by ectonucleotidases, which include the nucleoside triphosphate diphosphohydrolase (NTPDase) family and ecto-5′-nucleotidase. These enzymes hydrolyze ATP/GTP to adenosine/guanosine, which exert a modulatory role controlling several neurotransmitter systems. The nucleoside adenosine can be inactivated in extracellular or intracellular milieu by adenosine deaminase (ADA). In this report, we tested whether acute taurine treatment at supra-physiological concentrations alters NTPDase, ecto-5′-nucleotidase, and ADA activities in zebrafish brain. Fish were treated with 42, 150, and 400 mg L^{-1} taurine for 1 h, the brains were dissected and the enzyme assays were performed. Although the NTPDase activities were not altered, 150 and 400 mg L^{-1} taurine increased AMP hydrolysis (128 and 153%, respectively) in zebrafish brain membranes and significantly decreased ecto-ADA activity (29 and 38%, respectively). In vitro assays demonstrated that taurine did not change AMP hydrolysis, whereas it promoted a significant decrease in ecto-ADA activity at 150 and 400 mg L^{-1} (24 and 26%, respectively). Altogether, our data provide the first evidence that taurine exposure modulates the ecto-enzymes responsible for controlling extracellular adenosine levels in zebrafish brain. These findings could be relevant to evaluate potential beneficial effects promoted by acute taurine treatment in the central nervous system (CNS) of this species.

Taurine (2-aminoethanosulfonic acid) is a ubiquitous non-protein amino acid abundant in several tissues. In the brain, intracellular taurine concentration ranges from 3 to 9 mM [1,14], while extracellular taurine reaches micromolar range [18]. Previous study demonstrated that high taurine concentrations can be found in astrocytes and also in neurons [28]. This amino acid has been implicated in different cell protecting events, such as osmolarity regulation [7,8], antioxidant properties [21], and membrane stabilization [19]. In addition, extracellular taurine may act as an inhibitory neurotransmitter via GABA$_A$, glycine, and taurine receptors [20,36]. The control of the levels of taurine at synaptic cleft is exerted by a specific transporter, TAUT, whose sequence homology places it within the gene family of Na$^+$- and Cl$^-$-dependent neurotransmitter transporters [6]. Taurine has shown neuroprotective properties against excitotoxic cell death [28,37] mainly by regulating cellular levels of Ca^{2+} and its neuromodulatory role, which influences other neurotransmitter signaling pathways [36].

Nucleotides are ubiquitous signaling molecules that play crucial roles for brain function. ATP is a neurotransmitter that elicits its actions by triggering specific P2 receptors [12]. The inactivation of ATP-mediated neurotransmission is controlled

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by cell-surface enzymes called ectonucleotidases. The NTPDase (nucleoside triphosphate diphosphohydrolase) family hydrolyzes ATP to AMP, whereas an ecto-5′-nucleotidase cleaves AMP to adenosine. This nucleoside is an important neuromodulator of CNS by acting on metabotropic P1 purinoreceptors [8]. Extracellular adenosine can be taken up to the cells through nucleoside transporters and phosphorylated to AMP by adenosine kinase or deaminated to inosine by adenosine deaminase (ADA). These processes are mostly intracellular, but studies showed that ADA is also associated with cell membranes as an ecto-ADA [11]. Because ecto-ADA is colocalized with adenosine A_1 [30] and A_{2B} [13] receptors, adenosine cleavage at synaptic cleft is crucial for controlling P1 signaling. Additionally to the adenine-based purinergic system, it has been proposed a guanine-based purinergic system in the CNS [31]. Like ATP, GTP may be also stored in synaptic vesicles and released after electrical stimuli [27]. In cultured astrocytes, inhibition of ecto-5′-nucleotidase activity significantly reduced accumulation of extracellular guanosine, indicating that, like adenosine, it is to some extent derived from the extracellular metabolism of guanine nucleotides [23]. Moreover, the neuroprotective effects of guanosine have been attributed to modulation of glutamatergic parameters, which prevents brain damage due to excitotoxicity [31,32].

Zebrafish is a promising model vertebrate for neurochemical studies. It has been demonstrated that zebrafish genes are highly conserved sharing a 70–80% homology to those of humans [2]. Recent studies also demonstrated a high degree of similarities between zebrafish and mammalian NTPDase members [26] and TAUT protein [17]. Furthermore, the NTPDase, ecto-5′-nucleotidase, and ADA activities have already been characterized in zebrafish brain [24,25,33] and TAUT expression and functionality have been evaluated during zebrafish development [17]. Considering that the effects of taurine exposure in zebrafish CNS still remains unknown and that purines are important signaling molecules, the goal of the present study was to verify whether acute taurine treatment at supra-physiological concentrations alters ectonucleotidase (NTPDase and ecto-5′-nucleotidase) activities in zebrafish brain membranes. Moreover, the adenosine deamination in both membrane (ecto-ADA activity) and soluble (cytosolic-ADA activity) preparations of zebrafish brain was also studied.

Adult "wild type" (short fin—SF) zebrafish (*Danio rerio*) strain (3–6-month-old, weighing 0.38 ± 0.05 g) of both sexes were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated in a 50-L thermostated aquarium for at least two weeks before the experiments under a 12-h light–dark photoperiod. The aquarium was filled with continuously aerated unchlorinated water at temperature of $26 \pm 2\,°C$ and the animals were fed twice a day to satiety with commercial flake fish food. Before the experiments, the fish were cryoanaesthetized and euthanized by decapitation. Each independent experiment was performed using biological preparations from five animals. The animals were raised and cared for according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

Trizma base, ammonium molybdate, polyvinyl alcohol, Malachite Green, nucleotides, adenosine, EDTA, EGTA, sodium citrate, Coomassie blue G, bovine serum albumin, calcium chloride, and taurine were purchased from Sigma (St. Louis, MO, USA). Magnesium chloride, phenol, and sodium nitroprusside were purchased from Merck (Darmstadt, Germany). All other reagents used were of high analytical grade.

Taking into account the distinct concentrations and types of taurine treatment previously reported and the absence of data related to taurine exposure in zebrafish, in our study, the animals were acutely treated during 1 h by performing a curve using three supraphysiological taurine concentrations (42, 150, and $400\,mg\,L^{-1}$). These concentrations were chosen because represent a range that

has been extensively used in the literature for both *in vivo* and *in vitro* experiments, which vary from 0.33 to 3.2 mM [16,35,37]. Mortality and significant alterations in the fish swimming pattern were not observed during the time of exposure, suggesting that the concentrations of taurine tested in zebrafish could be acceptable for an acute treatment in this species. In order to maintain identical conditions of water from control and taurine-treated groups, the pH was adjusted to 7.0 using a 0.1 mM NaOH solution. For *in vitro* experiments, the same concentrations of taurine were directly added to the reaction medium before incubation with the substrates.

Zebrafish brains were dissected and homogenized in 60 vol. (v/w) of chilled Tris–citrate buffer (50 mM Tris–citrate, 2 mM EDTA, 2 mM EGTA, pH 7.4, adjusted with citric acid) for NTPDase and ecto-5′-nucleotidase assays [24,33]. For ADA activity experiments, brains were homogenized in 20 vol. (v/w) of chilled phosphate buffered saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4 [25]. The preparation of brain membranes was according previously described [3]. Briefly, the homogenates were centrifuged at $800 \times g$ for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at $40\,000 \times g$. The resultant supernatant and the pellet obtained corresponded to the soluble and membrane fractions, respectively. For soluble ADA activity experiments, the supernatant was collected and kept on ice for enzyme assays. The pellets of both membrane preparations were frozen in liquid nitrogen, thawed, resuspended in the respective buffers and centrifuged for 20 min at $40\,000 \times g$. This freeze–thaw-wash procedure was used to ensure the lysis of the brain vesicles membranes. The final pellets were resuspended and used for biochemical analyses. All cellular fractions were maintained at $2–4\,°C$ throughout preparation and they were immediately used for enzyme assays.

The ectonucleotidase activities were determined as previously described [24,33]. Brain membranes (3–5 µg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for NTPDase activities) and 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for ecto-5′-nucleotidase activity) in a final volume of 200 µL. The samples were preincubated for 10 min at 37 °C before starting the reaction with the addition of substrate (ATP, GTP, ADP, GDP, AMP or GMP) to a final concentration of 1 mM. The reactions were terminated after 30 min with the addition of 200 µL of 10% trichloroacetic acid and immediately placed on ice for 10 min. The inorganic phosphate (Pi) released was determined by colorimetric assay [5]. To ensure that the concentration of Pi was within the linear range, aliquots of 15, 25, and 50 µL were diluted to a final volume of 100 µL for assaying the ATP, GTP, and ADP hydrolysis, respectively, whereas aliquots of 100 µL were performed for the other substrates. Each sample was mixed to 250 µL of Malachite Green solution and the nucleotide hydrolysis was measured in a microplate reader at 630 nm after 20 min.

Ecto- and cytosolic-ADA activities were determined as previously reported [25]. The membrane and soluble fractions (5–10 µg protein) were added to the reaction mixture containing 50 mM sodium acetate buffer (pH 5.0) and 50 mM sodium phosphate buffer (pH 7.0), respectively, in a final volume of 200 µL. The samples were preincubated for 10 min at 37 °C and the reaction was initiated with the addition of adenosine to a final concentration of 1.5 mM. After incubated for 120 min (membranes) and 75 min (soluble fraction), the reactions were terminated with 500 µL of phenol–nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/mL). Afterwards, the samples were mixed to 500 µL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH) and vortexed, being incubated at 37 °C for 15 min. The ammonia produced over a fixed time by the Berthelot reaction was spectrophotometrically measured at 635 nm.

Controls with the addition of the enzyme preparation after incubation period were used to correct non-enzymatic hydrolysis of
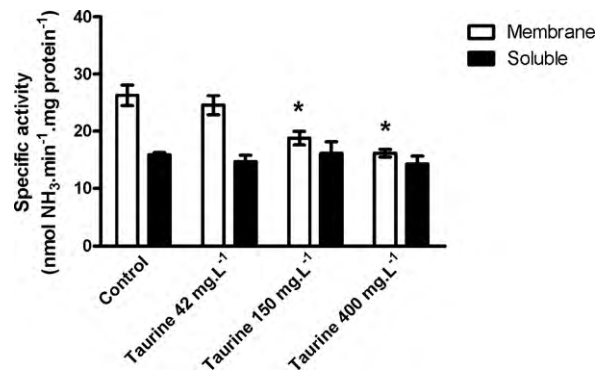
32
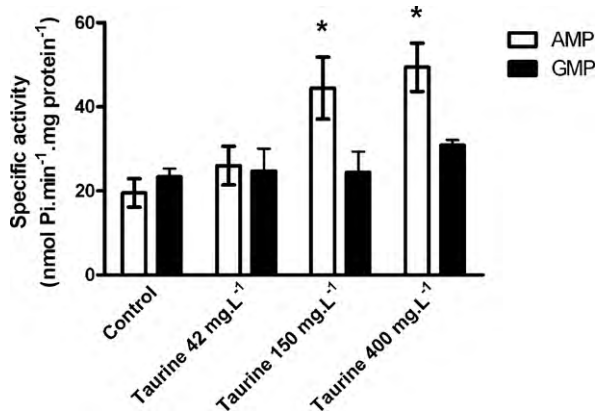
Fig. 1. Ecto-5′-nucleotidase activity in zebrafish brain membranes after acute taurine exposure at supra-physiological concentrations (42, 150, and 400 mg L^{-1}). The AMP and GMP hydrolysis are represented. Data were expressed as means ± S.E.M. of five independent experiments. * Significant difference compared to control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0.05$).

Fig. 2. Ecto- and cytosolic-ADA activities in membrane and soluble preparations of zebrafish brain, respectively, after acute taurine exposure at supra-physiological concentrations (42, 150, and 400 mg L^{-1}). Data were expressed as means ± S.E.M. of four independent experiments. * Significant difference compared to control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0.05$).

substrates. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. The ectonucleotidase and ADA activities were expressed as nmol Pi min^{-1} mg protein^{-1} and nmol NH$_3$ min^{-1} mg protein^{-1}, respectively. Protein concentration was measured by the Coomassie blue method [4], with bovine serum albumin as a protein standard.

All assays were run in triplicate and means ± S.E.M. of at least four independent experiments were presented. Data were analyzed by one-way analysis of variance (ANOVA). Post hoc comparisons were made using Tukey's test considering $P \leq 0.05$ as statistically significant.

The acute taurine treatment did not alter NTPDase activities in zebrafish brain membranes, using ATP/GTP or ADP/GDP as substrates (data not shown). Concerning ecto-5′-nucleotidase activity (Fig. 1), 150 and 400 mg L^{-1} taurine significantly increased AMP hydrolysis (44.4 ± 7.4 nmol Pi min^{-1} mg protein^{-1}, $n = 5$; and 49.4 ± 5.7 nmol Pi min^{-1} mg protein^{-1}, $n = 5$, respectively), when compared to control (19.5 ± 3.4 nmol Pi min^{-1} mg protein^{-1}, $n = 5$), but the GMP hydrolysis was not affected ($n = 5$).

The effect of taurine exposure in adenosine deamination was evaluated in both membrane and soluble fractions of zebrafish brain (Fig. 2). The results showed that 150 and 400 mg L^{-1} taurine significantly decreased ecto-ADA activity (18.8 ± 1.2 nmol NH$_3$ min^{-1} mg protein^{-1}, $n = 4$; and 16.2 ± 0.7 nmol NH$_3$ min^{-1} mg protein^{-1}, $n = 4$, respectively) when compared to control (26.3 ± 1.8 nmol NH$_3$ min^{-1} mg protein^{-1},

$n = 4$). In contrast, the soluble ADA activity was not altered ($n = 4$).

The *in vitro* assays demonstrated that taurine added into the reaction medium did not induce significant changes in AMP hydrolysis ($n = 4$) (Fig. 3A). However, both 150 and 400 mg L^{-1} taurine promoted a significant decrease in ecto-ADA activity (19.4 ± 1.0 nmol NH$_3$ min^{-1} mg protein^{-1}, $n = 4$; and 18.9 ± 1.1 nmol NH$_3$ min^{-1} mg protein^{-1}, $n = 4$, respectively) when compared to control (25.5 ± 1.6 nmol NH$_3$ min^{-1} mg protein^{-1}, $n = 4$) (Fig. 3B).

Studies reported that taurine plays a role in different cellular processes, such as osmolarity regulation and inhibitory neurotransmission [1,20]. It has also been demonstrated that taurine can act as a neuroprotector against excitotoxicity, mainly by preventing glutamate-induced membrane depolarization or even by modulating Ca^{2+} signaling pathways [15,36]. However, the influence of taurine treatment on the central purinergic system still remains unknown. Our results indicate that the enzyme cascade responsible for controlling extracellular nucleotide signaling is altered by acute taurine treatment at supra-physiological concentrations. The *in vivo* experiments demonstrated that even though the concentrations of taurine tested did change neither ATP nor ADP hydrolysis, the AMP hydrolysis was significantly increased at 150 and 400 mg L^{-1} taurine. Interestingly, the acute treatment did not promote any significant changes in NTPDase and ecto-5′-nucleotidase activities when the brain
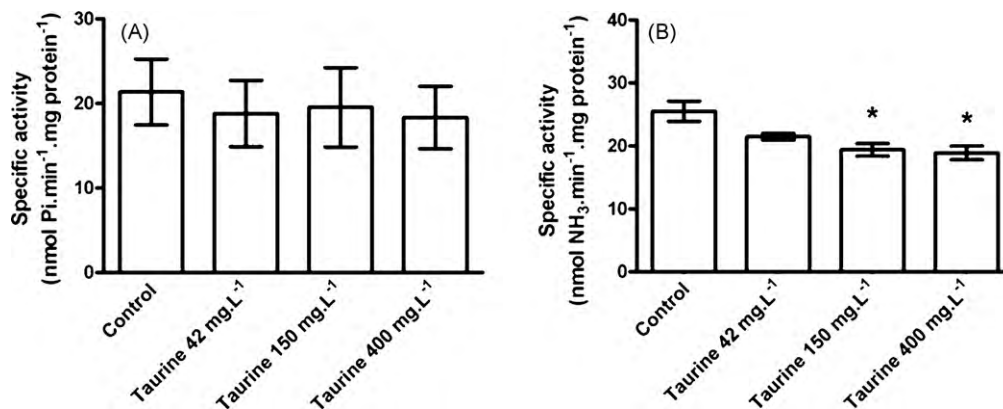
Fig. 3. *In vitro* effect promoted by taurine at supra-physiological concentrations (42, 150, and 400 mg L^{-1}) in AMP hydrolysis (panel A) and adenosine deamination (panel B) in zebrafish brain membranes. Data were expressed as means ± S.E.M. of four independent experiments. * Significant difference compared to control group (one-way ANOVA, followed by Tukey's test as post hoc, $P \leq 0.05$).

membranes were incubated with guanine-based nucleotides. Our results suggest that the increase of AMP hydrolysis in zebrafish brain membranes promoted by acute taurine exposure it is not directly correlated with modulation of ecto-5′-nucleotidase expression because this enzyme is also responsible for cleaving GMP to guanosine [23]. Furthermore, since the concentrations tested did not alter AMP hydrolysis *in vitro*, we propose that the acute taurine treatment did not alter ecto-5′-nucleotidase activity directly, but possibly via signaling transduction mechanisms.

In addition to the enhancement of AMP hydrolysis, our results demonstrated that supra-physiological taurine concentrations did not change adenosine deamination in soluble fraction, whereas ecto-ADA activity was significantly decreased by 150 and 400 mg L^{-1} taurine. In relation to *in vitro* assay, both concentrations also decreased ecto-ADA activity, indicating that high concentrations of taurine can alter ecto-ADA activity directly. Taking into consideration that ecto-5′-nucleotidase is attached via a GPI (glycosylphosphatidylinositol) anchor [34] while ecto-ADA is associated to other membrane-bound proteins [11,13,30], a possible explanation for the results obtained after *in vitro* experiments could be related to the differences in membrane anchorage of both enzymes since previous study showed that taurine is able to alter membrane fluidity [19].

Although the acute taurine treatment could potentially induce an increase of extracellular adenosine levels by a modulatory effect on purinergic signaling-related enzymes in zebrafish brain, our results did not allow us to conclude that taurine effectively plays beneficial roles in this species. It is known that many strong antioxidants evaluated *in vitro* could be extremely toxic *in vivo*. For example, it has been reported that the chronic supplementation with vitamin A either at therapeutic (1000 and 2500 IU/kg) or excessive (4500 and 9000 IU/kg) doses induces lipid peroxidation, protein carbonylation, and oxidation of protein thiol groups, as well as changes in CAT and SOD activities and immuno-contents in rat lung [22]. Moreover, vitamin A at therapeutic doses also promoted oxidative/nitrosative stress and mitochondrial impairment on rat heart [9] and the supplementation at clinical doses also increased the levels of molecular markers of oxidative damage in the rat cerebellum [10]. On the other hand, there is several evidence in the literature suggesting that supplementation with other antioxidants, such as taurine, could be beneficial. The dietary taurine supplementation in water (0.625%) protected rats from the lead-induced impairments of synaptic plasticity, demonstrating that it could be a preventive medicine to cure the cognitive deficits induced by lead [38]. It is also important to mention that besides its antioxidant properties, taurine could be important to other cellular functions [14,36]. Since zebrafish TAUT protein shares a high homology to mammalian transporter [17], we suggest that the effects promoted by taurine in vertebrates should be evolutively conserved. Considering that previous study showed that adenosine-mediated signaling regulates taurine efflux from brain stem slices during ischemia [29], we suggest that this amino acid could interact with the purinergic system in zebrafish and that acute supra-physiological taurine supplementation could be relevant to evaluate potential neuroprotective effects promoted by this amino acid in this species.

In summary, our data demonstrate the first evidence that acute taurine treatment at supra-physiological concentrations modulates both AMP hydrolysis and adenosine deamination in zebrafish brain membranes. The mechanisms underlying the modulatory effect promoted by taurine on the ecto-enzymes from purinergic signaling and a possible neuroprotective role in this species still require further investigations.

References

[1] J. Albrecht, A. Schousboe, Taurine interaction with neurotransmitter receptors in the CNS: an update, Neurochem. Res. 30 (2005) 1615–1621.
[2] W.B. Barbazuk, I. Korf, C. Kadavi, J. Heyen, S. Tate, E. Wun, J.A. Bedell, J.D. McPherson, S.L. Johnson, The syntenic relationship of the zebrafish and human genomes, Genome Res. 10 (2000) 1351–1358.
[3] J.M. Barnes, P.A. Murphy, D. Kirkham, J.M. Henley, Interaction of guanine nucleotides with [3H]kainate and 6-[3H]cyano-7-nitroquinoxaline-2,3-dione binding in goldfish brain, J. Neurochem. 61 (1993) 1685–1691.
[4] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[5] K.M. Chan, D. Delfert, K.D. Junger, A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity, Anal. Biochem. 157 (1986) 375–380.
[6] N.H. Chen, M.E. Reith, M.W. Quick, Synaptic uptake and beyond: the sodium- and chloride-dependent neurotransmitter transporter family SLC6, Pflugers Arch. 447 (2004) 519–531.
[7] L. Cubillán, F. Obregón, L. Lima, Effect of medium osmolarity and taurine on neuritic outgrowth from goldfish retinal explants, Adv. Exp. Med. Biol. 643 (2009) 225–231.
[8] R.A. Cunha, Neuroprotection by adenosine in the brain: from A(1) receptor activation to A(2A) receptor blockade, Purinergic Signal. 1 (2005) 111–134.
[9] R.F. Da Rocha, M.R. de Oliveira, P. Schonhofen, C.E. Schnorr, F. Dal Pizzol, J.C. Moreira, Long-term vitamin A supplementation at therapeutic doses induces mitochondrial electrons transfer chain (METC) impairment and increased mitochondrial membrane-enriched fraction (MMEF) 3-nitrotyrosine on rat heart, Free Radic. Res. 44 (2010) 505–512.
[10] M.R. De Oliveira, J.C. Moreira, Impaired redox state and respiratory chain enzyme activities in the cerebellum of vitamin A-treated rats, Toxicology 253 (2008) 125–130.
[11] R. Franco, V. Casadó, F. Ciruela, C. Saura, J. Mallol, E.I. Canela, C. Lluis, Cell surface adenosine deaminase: much more than an ectoenzyme, Prog. Neurobiol. 52 (1997) 283–294.
[12] J. Fu, Q. Yu, W. Guo, C. He, G. Burnstock, Z. Xiang, P2X receptors are expressed on neurons containing luteinizing hormone-releasing hormone in the mouse hypothalamus, Neurosci. Lett. 458 (2009) 32–36.
[13] C. Herrera, V. Casadó, F. Ciruela, P. Schofield, J. Mallol, C. Lluis, R. Franco, Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells, Mol. Pharmacol. 59 (2001) 127–134.
[14] R.J. Huxtable, Physiological actions of taurine, Physiol. Rev. 72 (1992) 101–163.
[15] F. Junyent, R. Romero, L. de Lemos, J. Utrera, A. Camins, M. Pallàs, C. Auladell, Taurine treatment inhibits CaMKII activity and modulates the presence of calbindin D28k, calretinin, and parvalbumin in the brain, J. Neurosci. Res. 88 (2010) 136–142.
[16] W.X. Kong, S.W. Chen, Y.L. Li, Y.J. Zhang, R. Wang, L. Min, X. Mi, Effects of taurine on rat behaviors in three anxiety models, Pharmacol. Biochem. Behav. 83 (2006) 271–276.
[17] D.J. Kozlowski, Z. Chen, L. Zhuang, Y.J. Fei, S. Navarre, V. Ganapathy, Molecular characterization and expression pattern of taurine transporter in zebrafish during embryogenesis, Life Sci. 82 (2008) 1004–1011.
[18] S. Molchanova, S.S. Oja, P. Saransaari, Characteristics of basal taurine release in the rat striatum measured by microdialysis, Amino Acids 27 (2004) 261–268.
[19] J. Moran, P. Salazar, H. Pasantes-Morales, Effect of tocopherol and taurine on membrane fluidity of retinal rod outer segments, Exp. Eye Res. 45 (1987) 769–776.
[20] S.S. Oja, P. Saransaari, Pharmacology of taurine, Proc. West Pharmacol. Soc. 50 (2007) 8–15.
[21] M.W. Oliveira, J.B. Minotto, M.R. De Oliveira, A. Zanotto-Filho, G.A. Behr, R.F. Rocha, J.C. Moreira, F. Klamt, Scavenging and antioxidant potential of physiological taurine concentrations against different reactive oxygen/nitrogen species, Pharmacol. Rep. 62 (2010) 185–193.
[22] M.A. Pasquali, D.P. Gelain, M.R. de Oliveira, G.A. Behr, L.L. da Motta, R.F. da Rocha, F. Klamt, J.C. Moreira, Vitamin A supplementation for different periods alters oxidative parameters in lungs of rats, J. Med. Food 12 (2009) 1375–1380.

[23] M.P. Rathbone, P.J. Middlemiss, J.W. Gysbers, C. Andrew, M.A. Herman, J.K. Reed, R. Ciccarelli, P. Di Iorio, F. Caciagli, Tropic effects of purines in neurons and glial cells, Prog. Neurobiol. 59 (1999) 663–690.

[24] E.P. Rico, M.R. Senger, Mda.G. Fauth, R.D. Dias, M.R. Bogo, C.D. Bonan, ATP and ADP hydrolysis in brain membranes of zebrafish (*Danio rerio*), Life Sci. 73 (2003) 2071–2082.

[25] D.B. Rosemberg, E.P. Rico, M.R. Senger, R.D. Dias, M.R. Bogo, C.D. Bonan, D.O. Souza, Kinetic characterization of adenosine deaminase activity in zebrafish (*Danio rerio*) brain, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 151 (2008) 96–101.

[26] D.B. Rosemberg, E.P. Rico, A.S. Langoni, J.T. Spinelli, T.C. Pereira, R.D. Dias, D.O. Souza, C.D. Bonan, M.R. Bogo, NTPDase family in zebrafish: nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 155 (2010) 230–240.

[27] T.G Santos, D.O. Souza, C.I. Tasca, GTP uptake into rat brain synaptic vesicles, Brain Res. 1070 (2006) 71–76.

[28] P. Saransaari, S.S. Oja, Modulation of taurine release by metabotropic receptors in the developing hippocampus, Adv. Exp. Med. Biol. 483 (2000) 257–264.

[29] P. Saransaari, S.S. Oja, Adenosine receptor agonists affect taurine release from mouse brain stem slices in ischemia, Amino Acids 38 (2010) 1387–1393.

[30] C.A. Saura, J. Mallol, E.I. Canela, C. Lluis, R. Franco, Adenosine deaminase and A1 adenosine receptors internalize together following agonist-induced receptor desensitization, J. Biol. Chem. 273 (1998) 17610–17617.

[31] A.P. Schmidt, D.R. Lara, D.O. Souza, Proposal of a guanine-based purinergic system in the mammalian central nervous system, Pharmacol. Ther. 116 (2007) 401–416.

[32] A.P. Schmidt, A.B. Tort, D.O. Souza, D.R. Lara, Guanosine and its modulatory effects on the glutamatergic system, Eur. Neuropsychopharmacol. 18 (2008) 620–622.

[33] M.R. Senger, E.P. Rico, R.D. Dias, M.R. Bogo, C.D. Bonan, Ecto-5′-nucleotidase activity in brain membranes of zebrafish (*Danio rerio*), Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 139 (2004) 203–207.

[34] N. Sträter, Ecto-5′-nucleotidase: structure function relationships, Purinergic Signal. 2 (2006) 343–350.

[35] R. Suge, N. Hosoe, M. Furube, T. Yamamoto, A. Hirayama, S. Hirano, M. Nomura, Specific timing of taurine supplementation affects learning ability in mice, Life Sci. 81 (2007) 1228–1234.

[36] H. Wu, Y. Jin, J. Wei, H. Jin, D. Sha, J.Y. Wu, Mode of action of taurine as a neuroprotector, Brain Res. 1038 (2005) 123–131.

[37] B. Zhang, X. Yang, X. Gao, Taurine protects against bilirubin-induced neurotoxicity *in vitro*, Brain Res. 1320C (2010) 159–167.

[38] D.M. Zhu, M. Wang, J.Q. She, K. Yu, D.Y. Ruan, Protection by a taurine supplemented diet from lead-induced deficits of long-term potentiation/depotentiation in dentate gyrus of rats in vivo, Neuroscience 134 (2005) 215–224.

II.2. CAPÍTULO II

NTPDase family in zebrafish: Nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart.

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NTPDase family in zebrafish: Nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart

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ARTICLE INFO

ABSTRACT

The nucleoside triphosphate diphosphohydrolase (NTPDase) family cleaves tri- and diphosphonucleosides to monophosphonucleosides and is responsible for terminating purinergic transmission. Since the NTPDase family in zebrafish is poorly understood, here we evaluated the nucleotide hydrolysis in three tissues of adult zebrafish (brain, liver, and heart), confirmed the presence of distinct NTPDase members by a phylogenetic analysis and verified their relative gene expression profiles in the respective tissues. A different profile of ATP and ADP hydrolysis in the brain, liver, and heart as a function of time and protein concentration was observed. Sodium azide (20 mM), ARL 67156 (300 μM) and Suramin (300 μM) differently altered the nucleotide hydrolysis in zebrafish tissues, suggesting the contribution of distinct NTPDase activities. Homology-based searches identified the presence of NTPDase1-6 and NTPDase8 orthologs and the phylogeny also grouped three NTPDase2 and two NTPDase5 paralogs. The deduced amino acid sequences share the apyrase conserved regions, conserved cysteine residues, putative N-glycosylation, phosphorylation, N-acetylation sites, and different numbers of transmembrane domains. RT-PCR experiments revealed the existence of a distinct relative *entpd1-6* and *entpd8* expression profile in brain, liver, and heart. Taken together, these results indicate that several NTPDase members might contribute to a tight regulation of nucleotide hydrolysis in zebrafish tissues.

1. Introduction

The NTPDase family consists of enzymes that hydrolyze tri- and diphosphonucleosides into their monophosphate counterparts and thus plays a crucial role in nucleotide catabolism. After release into the synaptic cleft ATP and other nucleotides undergo rapid enzymatic degradation by ectonucleotidases, which are functionally important because ATP metabolites act as physiological ligands for various purinergic receptors (Burnstock, 2009). In this sense, NTPDase activities not only control the lifetime of nucleotides but, by degrading or interconverting the originally released ligands, they also produce ligands for additional P2 receptors and nucleosides (Zimmermann,

2006). Common features that characterize NTPDase family members are the presence of five apyrase conserved regions (ACRs) and the requirement for divalent cations for enzyme activity (Zimmermann, 2001; Kirley et al., 2006). The eight NTPDase members hydrolyze their substrates with distinct ability and specificity and differ according to their cellular location (Zimmermann, 2001). For example, NTPDases1–3 and 8 are expressed as cell surface-located enzymes being anchored to the plasma membrane via two transmembrane domains, whereas NTPDases4–7 face the lumen of cytoplasmic organelles (Lavoie et al., 2004). While NTPDases4 and 7 contain two transmembrane domains that are entirely intracellular, NTPDases5 and 6 have a single transmembrane domain and undergo secretion after heterologous expression (Murphy-Piedmonte et al., 2005).

The importance of nucleotide hydrolysis for homeostasis in several tissues has been extensively reported. Changes in neuronal plasticity, inhibitory avoidance learning, seizure activity, and cerebral ischemia have been correlated with alterations in NTPDase activities (Bonan

et al., 1998, 2000; Schetinger et al., 2007). It has been shown that NTPDase1 activity coordinates both hepatocyte and endothelial cell proliferation in mouse liver, suggesting that purines are involved in hepatic regeneration and vascular reconstitution (Beldi et al., 2008). Furthermore, NTPDase1 also promotes myocardial protection during cardiac ischemia/reperfusion injury (Köhler et al., 2007) and its activity and expression profile have been characterized in rat heart ventricle (Rücker et al., 2008). The prevention of excessive contractility in cardiomyocytes has been attributed to *entpd3* expression, giving support to the suggestion that distinct NTPDase members act in a coordinated manner to maintain heart homeostasis (Barreto-Chaves et al., 2006).

Zebrafish (*Danio rerio*) have been used as an animal model for biomedical research of numerous pathologies, such as seizures (Baraban, 2007), liver failure (Rekha et al., 2008), and cardiac malfunctions (Chico et al., 2008). The P2X receptor gene family and the nucleotide hydrolysis in brain membranes have already been characterized in zebrafish (Kucenas et al., 2003; Rico et al., 2003; Senger et al., 2004). In addition, the expression of *entpd1*, three *entpd2*, and *entpd3* genes (Rico et al., 2006; Appelbaum et al., 2007) and the immunocytochemical localization of NTPDase1 and 2 in the neural retina of zebrafish (Ricatti et al., 2009) suggest the existence of a compartmentalized regulation of extracellular nucleotide/nucleoside concentration in this teleost.

Since the investigation of NTPDase family members in complementary vertebrate models is relevant to better understand this enzyme group, the study of the NTPDases in zebrafish is imperative and raises the perspective of performing structural and functional comparisons of distinct purinergic signaling parameters between fish and mammals. Although recent studies demonstrated the existence of NTPDase activities in zebrafish (Rico et al., 2006; Ricatti et al., 2009), the identification of cell surface-located and intracellular NTPDase members which might contribute to ATP and ADP hydrolysis in different tissues and the detection of their mRNAs have not yet been reported.

Here we assay the nucleotide hydrolysis and test the influence of distinct inhibitors of ATP and ADP hydrolysis in zebrafish brain, liver, and heart. To specifically verify whether different NTPDase members could be involved in nucleotide catabolism in these respective tissues, we used homology-based searches and a phylogenetic analysis to identify the zebrafish NTPDase sequences. Furthermore, we also evaluated some special features of the deduced amino acid sequence obtained using bioinformatics tools and analyzed the gene expression profile of the *entpd* members in these tissues.

2. Materials and methods

2.1. Animals

Adult males and females of the "wild type" (short fin − SF) zebrafish (*Danio rerio*) strain (3–6 months-old, weighing 0.32 ± 0.06 g) were obtained from a commercial distributor (Delphis, RS, Brazil) and acclimated for at least 2 weeks before the experiments. The animals were maintained in a 50-L thermostated aquarium filled with continuously aerated unchlorinated water at a targeted temperature of 26 ± 2 ºC under a 12-h light-dark photoperiod, and fed twice a day to satiety with commercial flake fish food. Before the experiments, the fish were cryoanaesthetized and euthanized by decapitation. Each independent experiment was performed using biological preparations from five different animals. The animals were raised and cared for according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.2. Chemicals

Trizma Base, ammonium molybdate, polyvinyl alcohol, Malachite Green, nucleotides, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, calcium chloride, ouabain, Ap5A, NEM, orthovanadate, levamisole, oligomycin, sodium azide, ARL 67156 (6-N,N-diethyl-D-β,γ-dibromomethylene adenosine triphosphate) and Suramin were purchased from Sigma Aldrich (St. Louis, MO, USA). TRIzol, SuperScript™ III First-Strand Synthesis SuperMix, Taq Platinum, GelRed and Low DNA Mass Ladder were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents used were of high analytical grade.

2.3. Nucleotide hydrolysis assay

Tissues (brain, liver, and heart) were dissected and added to 1 mL of chilled Tris–citrate buffer (50 mM Tris–citrate, 2 mM EDTA, 2 mM EGTA, pH 7.4, adjusted with citric acid) as described by Barnes et al. (1993). This same buffer has been also used in previous studies (Rico et al., 2003; Senger et al, 2004) to exclude a possible interference of endogenous divalent cations (such as Ca^{2+} and Mg^{2+}) during tissue preparation, which could influence the nucleotidase activities assayed. The samples were homogenized on ice in a motor-driven Teflon-glass homogenizer and centrifuged at 1000 g for 10 min at 4 °C to remove the nuclei and cell debris. The resultant supernatants were kept on ice and used for the enzyme assays.

The optimum conditions for ATP and ADP hydrolysis were determined for each tissue. Brain, liver, and heart samples (3–5 μg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM CaCl₂ in a final volume of 200 μL. The samples were preincubated for 10 min at 37 °C before starting the reaction with the addition of substrate (ATP or ADP) every 10 s to a final concentration of 1 mM. After incubation for 20 min (brain and liver) and 30 min (heart), the reactions were terminated with the addition of 200 μL of 10% trichloroacetic acid every 10 s for each sample to ensure the correct incubation time and immediately placed on ice for 10 min. Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct non-enzymatic hydrolysis of substrates. The inorganic phosphate (Pi) released was measured spectrophotometrically at 630 nm using Malachite Green solution as color reagent (Chan et al., 1986) and KH₂PO₄ as standard after 4-fold dilution to ensure that the concentration of phosphate was within the linear range. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. All enzyme assays were run in triplicate and the specific activity was expressed as nmol Pi min⁻¹ mg protein⁻¹.

2.4. Protein measurement

Protein concentration for the enzyme assays was measured by the Coomassie blue method (Bradford, 1976), with bovine serum albumin as standard.

2.5. In silico confirmation of zebrafish NTPDase sequences

The well-known human and mouse NTPDase protein sequences were used as queries (Table 1) to perform a Basic Local Alignment Search (BLASTP function) via the GenBank, ENSEMBL, and ZFIN *Danio rerio* databases. The NTPDase protein sequences obtained were aligned through the ClustalX program (Thompson et al., 1997) and analyzed using bioinformatics tools available at CBS Prediction Servers (http://www.cbs.dtu.dk/services/). A phylogenetic tree was built with the MEGA 4.0 program (Tamura et al., 2007) and the statistical Neighbor-Joining method (Saitou and Nei, 1987) with proportional (*p*) distance was used. The percent identity and similarity between the zebrafish and mammalian NTPDase proteins were determined by the scoring matrix BLOSUM62 using MatGAT 2.01 software (Campanella et al., 2003). All NTPDase sequences identified are presented in Table 2.

Table 1
Mammalian NTPDase orthologs used in phylogenetic analysis.

Species	GenBank acession number
Human (*Homo sapiens*)	
NTPDase1	NP_001767
NTPDase2	NP_982293
NTPDase3	NM_001248
NTPDase4	BC034477
NTPDase5	NM_001249
NTPDase6	BC025980
NTPDase7	NM_020354
NTPDase8	AAR04374
Mouse (*Mus musculus*)	
NTPDase1	AAH11278
NTPDase2	O55026
NTPDase3	AY376710
NTPDase4	BC043134
NTPDase5	BC015247
NTPDase6	BAE33807
NTPDase7	NM_053103
NTPDase8	AAQ84519

2.6. Primer design and RT-PCR experiments

The *entpd1*, *entpd2_mg*, *entpd2_mq*, and *entpd2_mv* primers were designed as described previously (Rico et al., 2006). Zebrafish DNA sequences encoding each of the NTPDase members retrieved from the GenBank database were aligned using the ClustalX program. Regions with low scores for similarity among the sequences were used to search for specific primers, which were designed using the program Oligos 9.6. The primer specificities were checked by comparing each primer with the zebrafish genome to confirm that it would recognize only its specific target sequence. Thus, the strategy adopted to design the primers avoided cross-amplification. The *β-actin* primers employed were previously described by Chen et al. (2004) and the optimal PCR conditions for *entpd* amplifications were determined (Table 3).

Brain, liver, and heart were dissected under sterile conditions and immediately subjected to a total RNA extraction by the TRIzol method according to the manufacturer's instructions. The purity of the RNA was spectrophotometrically quantified by calculating the ratio between absorbance values at 260 and 280 nm and its integrity was confirmed by electrophoresis through a 1.0% agarose gel. Afterwards, all samples were adjusted to 160 ng/μL and cDNA species were synthesized using SuperScript™ III First-Strand Synthesis SuperMix.

PCR conditions were optimized in order to determine the number of cycles that would allow product detection within the linear phase of band intensities analyzed. The reactions were performed in a final volume of 20 μL, using 0.1 μM primers (Table 2), 0.2 μM dNTP, 2 mM MgCl$_2$ and 0.5 U Taq Platinum for *β-actin* amplification. The same conditions were employed for the *entpd2_mg*, *entpd2_mq*, *entpd2_mv*, *entpd3*, *entpd4*, *entpd5_me*, and *entpd6* genes, with the addition of

Table 3
Primer characteristics for *entpd* and *β-actin* genes.

Gene	Primer sequences (5′-3′)	Annealing	PCR fragment (bp)
entpd1	CCCATGGCACAGGCCGGTTG (forward)	54	380
	GCAGTCTCATGCCAGCCGTG (reverse)		
entpd2_mg*	GGAAGTGTTTGACTCGCCTTGCACG (forward)	62	554
	CAGGACACAAGCCCTTCCGGATC (reverse)		
entpd2_mq*	CCAGCGGATTTAGAGCACGCTG (forward)	62	313
	GAAGAACGGCGGCACGCCAC (reverse)		
entpd2_mv*	GCTCATTTAGAGGACGCTGCTCGTG (forward)	62	263
	GCAACGTTTTCGGCAGGCAGC (reverse)		
entpd3	TACTTTCTTTGGACAGAGCAACCCTG (forward)	62	424
	AAGCATATAGCCCAGGGACCAGG (reverse)		
entpd4	CGTGGTTCTGGAGACTTTGAGCGCTG (forward)	60	387
	TGTCCTGAGGTTCTCATACGCCGCTG (reverse)		
entpd5_ms*	CCGCCAGGTCAACTTCAGTCGCGTC (forward)	57	333
	TCTTTGACCTCATTCAGCAGGGCTTTG (reverse)		
entpd5_me*	TTCTGCTGCTGCTGATTTCTCGGTGC (forward)	60	438
	ATGATCCTGACGCTGTCTGGAGGAACG (reverse)		
entpd6	TGAAGGCCAGCCTTTAGCGGGAC (forward)	60	270
	CCTGTGCGTTCATCAATGAGGCCCAG (reverse)		
entpd8	AGCTTTATTAAGCACACATTTGAGGGCTC (forward)	58	363
	GGGGTTGAAACTGCTTGGTGGGACC (reverse)		
β-actin**	GTCCCTGTACGCCTCTGGTCG (forward)	54	678
	GCCGGACTCATCGTACTCCTG (reverse)		

*Corresponds to the first two amino acid residues of the protein sequence.
**PCR primer sequences previously described (Chen et al., 2004).

1 M betain for PCR experiments on the three *entpd2* genes. The *entpd1* and *entpd5_ms* reactions were performed using 3 mM MgCl$_2$ and 1 M betain was also employed for *entpd1* amplification. PCR assays for the *entpd8* gene were carried out using 2.5 mM MgCl$_2$, 0.2 μM dNTP and 1 U Taq Platinum. All PCR experiments were performed using 1 μL of template cDNA.

The following conditions were used for PCR reactions: 1 min at 94 °C, 1 min at the annealing temperature (see Table 3), 1 min at 72 °C for 30 cycles (for *entpd3*, *entpd4*, *entpd5_me*, and *entpd6*) and for 35 cycles (for *β-actin*, *entpd1*, *entpd2_mg*, *entpd2_mq*, *entpd2_mv*, *entpd5_ms*, and *entpd8*) with a further post-extension cycle at 72 °C

Table 2
Identified members of the NTPDase family in zebrafish.

Gene	Protein	Chromosome	Amino acid residues	ENSEMBL	Accession numbers	
					GenBank	ZFIN ID
entpd1	NTPDase1	12	492	ENSDARP00000066256	AAH78240	ZDB-GENE-040801-58
entpd2_mg [a]	NTPDase2_mg	21	509	Not identified	XP_697600	Not identified
entpd2_mq [a]	NTPDase2_mq	5	497	ENSDARP00000043258	XP_687722	ZDB-GENE-040724-67
entpd2_mv [a]	NTPDase2_mv	5	502	ENSDARP00000051433	AAH78419	ZDB-GENE-040724-187
entpd3	NTPDase3	16	518	ENSDARP00000094926	ABR15509	ZDB-GENE-030131-6186
entpd4	NTPDase4	8	611	ENSDARP00000056073	NP_001002419	ZDB-GENE-040718-116
entpd5_ms [a]	NTPDase5_ms	17	450	Not identified	XP_684862	Not identified
entpd5_me [a]	NTPDase5_me	25	525	Not identified	XP_001339099	Not identified
entpd6	NTPDase6	17	442	ENSDARP00000037620	NP_001017862	ZDB-GENE-050417-412
entpd8	NTPDase8	6	484	ENSDARP00000008913	NP_001002379	ZDB-GENE-040724-142

[a] Correspond to the first two amino acid residues from protein sequences.

for 10 min. A negative control was included for each set of PCR reactions. PCR products were separated on a 1.0% agarose gel with GelRed 10× and visualized with ultraviolet light. The fragment lengths expected for the PCR reactions were confirmed using Low DNA Mass Ladder and β-actin was determined as an internal standard. Band intensities were analyzed by optical densitometry using the software ImageJ 1.37 for Windows after running all PCR products in a single gel.

2.7. Statistical analysis

All assays were performed in triplicate and means ± S.E.M. of at least three independent experiments were presented. Data were analyzed by one-way analysis of variance (ANOVA). Post-hoc comparisons were made using Tukey's HSD test (for gene expression data) or Dunnett's Multiple Comparison Test (for the experiments with inhibitors) and differences were considered statistically significant at a $P \leq 0.05$ level.

3. Results

3.1. Nucleotide hydrolysis in zebrafish tissues

The ability of different zebrafish tissues to hydrolyze both ATP and ADP was tested as a function of time and protein concentration in order to determine the correct assay conditions (Fig. 1). The

incubation with the respective nucleotides (1 mM) showed that the time courses for ATP and ADP hydrolysis were linear up to 40 min for the brain and liver (Fig. 1A and B) and up to 60 min for the heart (Fig. 1C). Furthermore, nucleotide hydrolysis increased as a function of protein concentration and product formation was linear in the range of 3–10 μg protein in the incubation medium for the brain and liver (Fig. 1D and E) and 3–5 μg protein for the heart (Fig. 1F). Concerning nucleotide preference, further experiments using 3–5 μg protein and an incubation time of 20 min (brain and liver) and 30 min (heart) demonstrated that ADP hydrolysis was significantly lower than ATP hydrolysis in the brain (36%) and heart (49%), whereas no significant difference was found in the liver (data not shown).

The correlation between NTPDase activities with ATPases and alkaline phosphatase was also evaluated. The classical inhibitors of Na$^+$- K$^+$-ATPase (ouabain and orthovanadate), Ca^{2+}, Mg^{2+}-ATPase (NEM), alkaline phosphatase (levamisole), and adenylate kinase (P^1, P^5-di (adenosine 5′-)pentaphosphate, Ap$_5$A) did not induce significant changes in nucleotide hydrolysis in zebrafish tissues (data not shown). However, some compounds associated with an inhibitory effect on NTPDase activities did alter the nucleotide hydrolysis in brain (Fig. 2A), liver (Fig. 2B) and heart (Fig. 2C). Sodium azide (20 mM), which has been considered an inhibitor of ATP diphosphohydrolase from several sources, significantly inhibited ATP and ADP hydrolysis in brain (34% and 63%, respectively), liver (34% and 30%, respectively), and heart (45% and 40%,
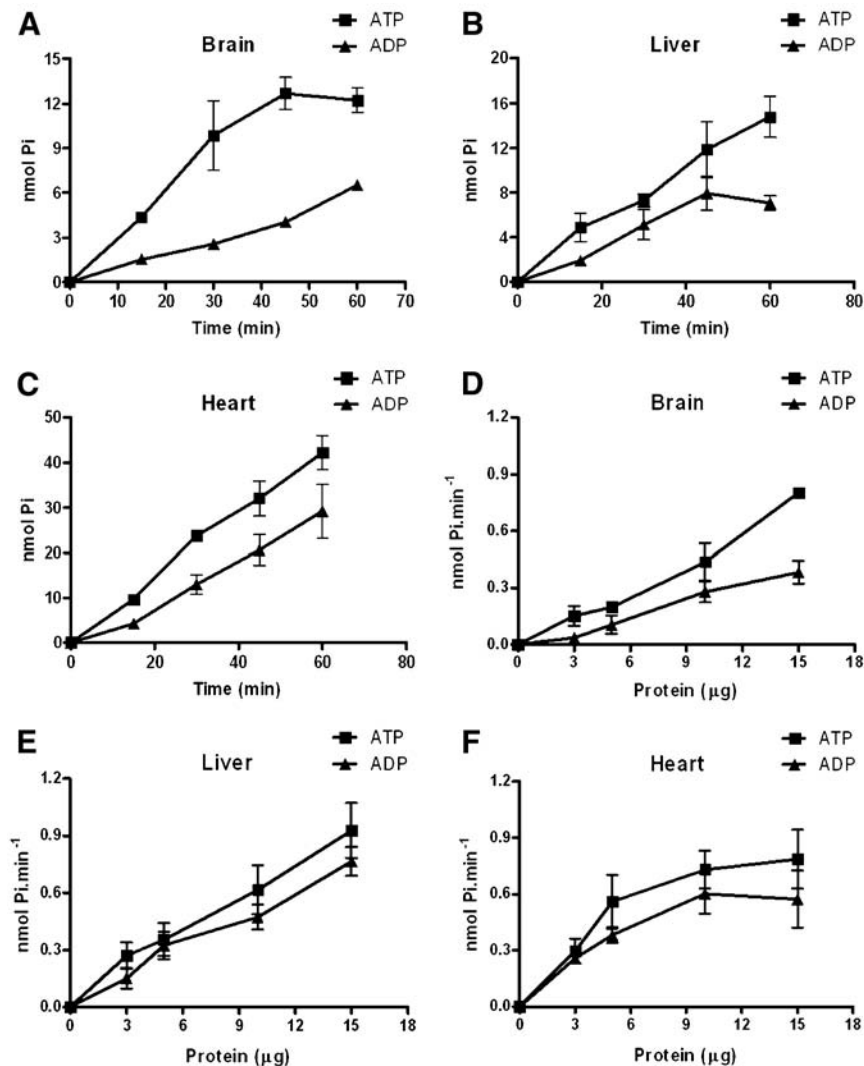
Fig. 1. Time courses (A–C) for Ca^{2+}-ATP and Ca^{2+}-ADP hydrolysis in 3–5 μg fractions of zebrafish brain (A), liver (B), and heart (C). Effect of different protein concentrations (D–F) on Ca^{2+}-ATP and Ca^{2+}-ADP hydrolysis in zebrafish brain (D), liver (E), and heart (F). Data represent means ± S.E.M of four different experiments, each in duplicate.
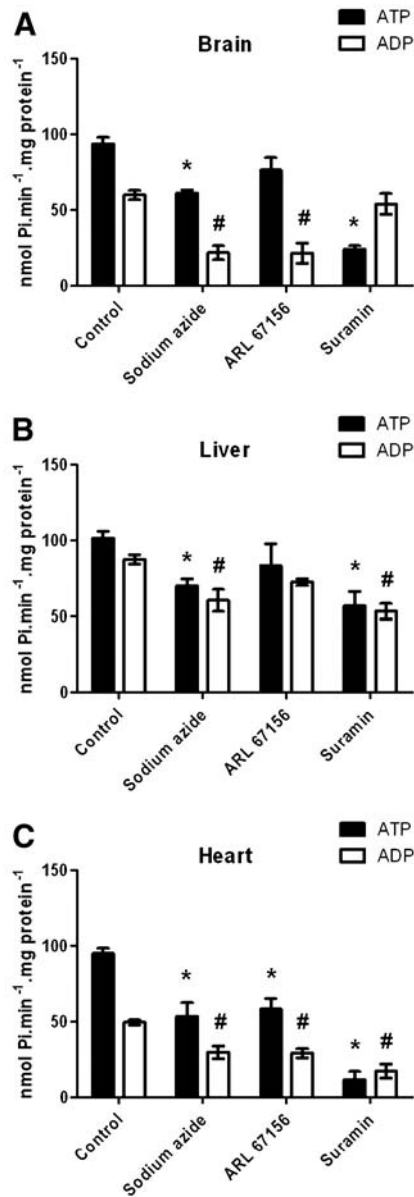
Fig. 2. Effect of putative NTPDase inhibitors (sodium azide 20 mM, ARL 67156 300 μM and Suramin 300 μM) on ATP (black bars) and ADP (white bars) hydrolysis in the brain (A), liver (A), and heart (C) of zebrafish. Data represent the means ± S.E.M of three different experiments. The asterisks (*) represent significant differences in ATP hydrolysis when compared to control and the symbol (#) represents significant differences in ADP hydrolysis when compared to control (ANOVA, followed by Dunnett's Multiple Comparison Test as post-hoc, $P \leq 0.05$).

respectively). Moreover, treatment with two ATPDase inhibitors (300 μM ARL 67156 and 300 μM Suramin) differentially altered the nucleotide hydrolysis. ARL 67156 inhibited ADP hydrolysis in brain (18%) and also caused a significant decrease in ATP (39%) and ADP (41%) hydrolysis in heart. Suramin strongly inhibited ATP hydrolysis in the brain (74%) and hydrolysis of both nucleotides in the heart (88% and 65% for ATP and ADP, respectively). A weaker inhibitory effect was observed for Suramin against ATP and ADP hydrolysis in liver (44% and 39%, respectively). These results strongly suggest the presence of different NTPDase activities in the brain, liver, and heart of zebrafish.

3.2. Phylogenetic analysis of zebrafish NTPDase family

Considering that ATP and ADP hydrolysis in different tissues involves the contribution of several enzyme activities, the presence of

distinct zebrafish NTPDase family members was investigated. The well-known *Homo sapiens* and *Mus musculus* NTPDase proteins were used as queries to obtain the zebrafish NTPDase orthologs. Searches in GenBank, ENSEMBL, and ZFIN databases lead to the identification of ten putative NTPDase sequences (Table 2). The phylogeny constructed resulted in eight well-resolved terminal clades confirming the presence of ten NTPDase orthologs for zebrafish. The graphic depiction of a multiple sequence alignment of zebrafish and mammalian members of the NTPDase family illustrates the subdivision of the family into surface-located and intracellular enzymes (Fig. 3). Among all identified sequences, five members (NTPDase1, three NTPDase2 paralogs, and NTPDase3) had been previously shown to exist (Rico et al., 2006; Appelbaum et al., 2007). However, two NTPDase5 paralogs (NTPDase5_ms and NTPDase5_me), NTPDase6, and NTPDase8 orthologs were identified by phylogenetic analysis for the first time. The putative NTPDase8 sequence was obtained only when mammalian orthologs were compared to the zebrafish protein database at ENSEMBL. The searches performed did not allow the identification of a zebrafish NTPDase7 sequence.

3.3. Properties of the NTPDase members in zebrafish

The chromosome location, accession numbers (GenBank, ENSEMBL, and ZFIN), and number of amino acid residues of the distinct zebrafish NTPDase members are presented in Table 2. In order to compare the zebrafish proteins with their mammalian orthologs, the deduced amino acid sequences were aligned and the percent identity and similarity were determined.

The relatedness between the surface-located and intracellular NTPDase members is shown in Table 4A and 4B, respectively. Zebrafish sequences generally have a percent identity of around 50% when compared to mouse and human NTPDase orthologs. Nevertheless, NTPDase4 exhibits a percent identity of around 70% when compared to the respective mouse and human orthologs. The high percent identity and similarity among vertebrate NTPDase proteins indicate that this enzyme family is highly conserved within organisms. The deduced amino acid alignment among zebrafish NTPDase sequences allowed the identification of the five apyrase conserved regions (ACRs), conserved cysteine residues, transmembrane domains, possible signal peptides, and putative regulatory sites related to posttranslational modifications (Fig. 4).

The presence of two hydrophobic domains was predicted for most zebrafish NTPDase ortholog sequences. However, NTPDase2_mg presented a single transmembrane domain at the C-terminal region. Meanwhile, analysis of zebrafish NTPDase5_ms, NTPDase6 and NTPDase8 showed the presence of one transmembrane domain at the N-terminal region, whereas the predicted NTPDase5_me sequence did not contain a characteristic hydrophobic domain at all. Several predicted cysteine residues and putative posttranslational modification sites were aligned consistently among the enzymes, suggesting a possible conservation of these amino acid residues in the zebrafish NTPDase family (Fig. 4).

3.4. Transcriptional analysis of entpd genes

Considering the presence of NTPDase activity in zebrafish tissues and the identification of distinct members of this enzyme family in this species, the relative *entpd* gene expression profile in the brain, liver, and heart was determined by RT-PCR experiments (Fig. 5). The PCR products obtained for each *entpd* primer were specific, displayed the expected fragment sizes and were identical to their respective sequences retrieved from databases. Higher amounts of *entpd1* and *entpd3* transcripts were detected in brain, with an intermediate expression level in liver, while less abundant mRNA was found in heart. Concerning the three *entpd2* paralogs, it was found that all these genes are differently expressed in zebrafish tissues. The
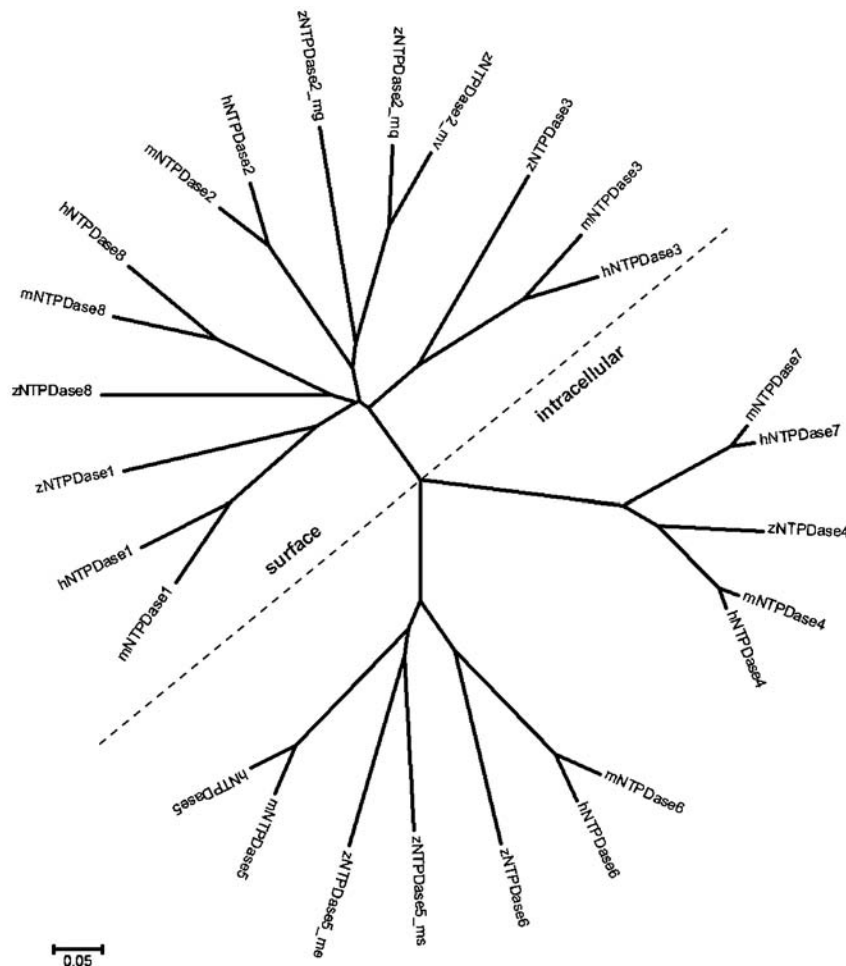
Fig. 3. Phylogenetic analysis of NTPDase family members in zebrafish (z) showing the surface-located and intracellular enzymes consistently grouped with their respective human (h) and mouse (m) orthologs. The deduced amino acid sequences were aligned with the ClustalX program and the phylogenetic tree was constructed using the Neighbor-Joining method and proportional (p) distance with the MEGA 4.0 program.

entpd2_mg and *entpd2_mq* genes were less expressed in liver when compared to brain, and large amounts of transcripts of *entpd2* paralogs were detected in heart. Brain and liver had similar levels of *entpd4* transcripts, whereas a lower *entpd4* expression was detected in heart. In relation to *entpd5* expression, the *entpd5_ms* was highly expressed in brain and less expressed in heart. The expression of the *entpd5_me* gene was also investigated and the results demonstrated that a similar amount of mRNA was present in brain and liver, with a lower abundance in heart. Expression of the *entpd6* gene was similar in the three tissues tested, while a lower mRNA level for *entpd8* was detected in brain when compared to liver and heart. Representative RT-PCR experiments demonstrating the *β-actin* amplification (Fig. 5A) and *entpd* expression profile of the surface-located enzymes (Fig. 5B) and intracellular NTPDase members (Fig. 5C) are shown.

4. Discussion

In the present report we describe some biochemical and molecular features of the NTPDase family in zebrafish. Although the presence of ATP and/or ADP hydrolyzing activity had been described for several decades in distinct cell types, the molecular identity of the first member of the NTPDase family (*entpd1*) remained unknown until the mid-1990 s (Robson et al., 2006). Since the structural and functional characterization of the ionotropic P2X receptor from zebrafish suggested that ATP-mediated responses were selected early in the vertebrate phylogeny (Boué-Grabot et al., 2000), several studies have shown the importance of purinergic signaling in this species (Kucenas

et al., 2003; Rico et al., 2003; Senger et al., 2004; Ricatti et al., 2009). However, besides the identification and confirmation of different NTPDase sequences by a phylogenetic analysis, this is the first study to demonstrate the mRNA expression profile of all the *entpd* family members identified so far in the brain, liver, and heart of zebrafish. Thus, it appears that these tissues are able to hydrolyze both ATP and ADP in a divalent cation-dependent manner.

Here we demonstrate an easy and rapid tissue preparation useful for studying the nucleotide hydrolysis and the influence of several inhibitors on NTPDase activities in different zebrafish organs that require a small amount of protein for enzyme assays (3–5 µg). The distinct ATPase inhibitors tested did not cause any significant changes in ATP and ADP hydrolysis in zebrafish brain, liver, and heart. Therefore, a possible contamination with classical ATPases in our fraction did not significantly influence nucleotide hydrolysis in the tissues analyzed. Moreover, Ca^{2+}-ATP was chosen as the substrate in this study because it can also eliminate interference by P-type ATPases, which are not activated by Ca^{2+} alone. Sodium azide is a well-known inhibitor of the mitochondrial ATPase, acting on both F_oF_1 and soluble F_1, indicating that the inhibitory site for azide is on F_1, with an I_{50} of 0.04 mM (Pullman et al., 1960). However, it has been reported that high concentrations of this compound are able to inhibit ATP diphosphohydrolase activity from several sources, including the ATP and ADP hydrolysis in zebrafish brain membranes (Plesner, 1995; Rico et al., 2003; Rücker et al., 2008). In our experiments, 20 mM sodium azide significantly decreased both ATP and ADP hydrolysis in the brain, liver, and heart. Meanwhile, ARL 67156 and Suramin, two

Table 4
Relatedness between the *Danio rerio* (Dr) NTPDase (D) proteins and their *Homo sapiens* (Hs) and *Mus musculus* (Mm) orthologs.

A

	DrD1	HsD1	MmD1	DrD2_mg	DrD2_mq	DrD2_mv	HsD2	MmD2	DrD3	HsD3	MmD3	DrD8	HsD8	MmD8
DrD1	.....	51.8	51.4	38.4	42.2	42.5	42.1	42.4	39.1	41.0	38.9	43.1	40.5	40.7
HsD1	68.8	.....	75.8	37.0	40.5	40.8	38.6	41.0	38.5	36.7	36.4	39.7	42.6	43.2
MmD1	67.6	86.5	.....	38.1	41.1	40.5	39.5	42.0	39.0	37.7	36.8	39.8	39.7	41.9
DrD2_mg	57.9	58.3	58.1	.....	50.7	51.4	45.7	47.0	35.2	38.4	35.8	38.7	34.8	36.2
DrD2_mq	61.0	61.6	61.2	69.1	.....	75.2	49.0	49.4	37.2	38.9	36.7	40.1	39.5	39.1
DrD2_mv	60.0	59.8	58.8	73.0	83.1	.....	48.4	48.9	37.2	37.6	37.6	41.5	37.9	38.5
HsD2	58.6	57.5	58.6	63.0	67.4	66.5	.....	83.4	34.4	38.5	39.3	42.9	42.7	42.2
MmD2	60.0	60.2	60.4	65.0	67.8	68.7	90.3	.....	35.0	38.5	39.7	43.3	43.3	43.4
DrD3	58.1	59.5	58.9	57.7	59.1	58.5	55.8	57.3	.....	48.8	45.1	37.8	35.1	35.6
HsD3	57.7	56.5	56.3	57.7	57.8	56.9	56.0	58.4	65.8	.....	81.1	37.6	38.4	37.9
MmD3	57.3	53.9	55.0	54.8	56.9	58.0	56.9	58.4	64.5	89.6	.....	37.9	38.2	38.5
DrD8	61.6	58.4	59.2	57.9	61.6	59.8	60.6	62.8	56.9	54.3	53.9	.....	44.2	45.6
HsD8	58.4	61.0	57.5	55.4	59.4	57.8	60.8	59.0	53.9	55.0	55.8	61.4	.....	74.1
MmD8	59.8	61.6	60.2	56.4	60.8	59.2	60.6	60.8	56.9	54.6	56.9	62.4	85.9	.....

B

	DrD4	HsD4	MmD4	DrD5_ms	DrD5_me	HsD5	MmD5	DrD6	HsD6	MmD6
DrD4	.....	64.8	70.1	20.1	18.8	20.2	20.6	20.1	21.0	21.2
HsD4	77.3	.....	83.0	22.0	19.7	21.2	20.5	22.4	22.1	22.6
MmD4	84.3	88.3	.....	21.5	19.2	20.6	19.9	21.0	21.4	22.7
DrD5_ms	36.0	38.4	34.7	.....	48.0	52.2	51.1	43.6	42.7	45.7
DrD5_me	37.5	38.7	36.2	63.5	.....	43.4	42.8	36.2	37.6	38.1
HsD5	34.2	36.8	33.9	68.5	56.6	.....	88.1	43.4	44.7	46.8
MmD5	34.7	36.6	33.8	67.7	55.9	93.7	.....	43.7	44.5	47.4
DrD6	32.7	36.4	34.6	60.3	51.4	60.4	59.5	.....	49.6	52.1
HsD6	35.5	37.7	35.1	60.5	55.5	57.8	58.8	62.7	.....	82.6
MmD6	35.4	37.7	35.7	62.9	53.4	59.1	61.3	66.4	87.8	.....

Note: The percentage of amino acid identity (black boxes) and similarity (gray boxes) between the proteins was determined by the scoring matrix BLOSUM62 using MatGAT 2.01 software.
A — Comparison between surface-located NTPDase members.
B — Comparison between intracellular NTPDase members.

ATPDase inhibitors, differentially altered the nucleotide hydrolysis assayed. In comparison to ARL 67156, Suramin was a more potent inhibitor of nucleotide hydrolysis in zebrafish tissues even though ARL 67156 had a partial, but significant inhibitory effect on ADP hydrolysis in brain. It has been recently reported that ARL 67156 is a weak competitive inhibitor of human NTPDase1 and NTPDase3, and partially inhibits the mouse and human forms of these enzymes in the micromolar range, being less effective against NTPDase2 and NTPDase8 (Lévesque et al., 2007). In another work Suramin was also observed to strongly inhibit both ecto-ATPase and -ADPase hydrolytic activities in recombinant *Trypanosoma cruzi* NTPDase-1 and in live trypomastigotes (Santos et al., 2009) at the same concentrations tested in our study. We cannot exclude the possibility that the inhibitors used here had an additional action on other molecular targets such as P2 receptors, which have been shown to be susceptible to blockade by Suramin (von Kügelgen, 2006), or even that the high substrate concentrations used in the enzyme assays might have partially overcome the blockade effected by ARL 67156. Although more selective NTPDase inhibitors, such as sodium polyoxotungstate (Wall et al., 2008) were not used in this study, our results suggest that the distinct profile of ATP and ADP hydrolysis observed in the range of time and protein concentration could be attributed to the existence of different NTPDase members cleaving tri- and diphosphonucleosides in brain, liver, and heart of zebrafish.

Ten zebrafish NTPDase orthologs (NTPDase1–6 and 8) were found to be present by phylogenetic analysis. A previous study from our laboratory had already identified the presence of three independent *entpd2* genes in zebrafish (*entpd2_mg*, *entpd2_mq*, and *entpd2_mv*) (Rico et al., 2006); however, here, for the first time, two distinct *entpd5* (*entpd5_ms* and *entpd5_me*), *entpd6*, and *entpd8* sequences were also identified in the zebrafish genome. It is tempting to speculate that the presence of three NTPDase2 and two NTPDase5

paralog proteins in zebrafish could be a consequence of genome-wide duplication in the teleost radiation, which has been previously reported in the literature (Amores et al., 1998). In fact, several studies have demonstrated the existence of duplicated genes in zebrafish (Amores et al., 1998; Rosemberg et al., 2007). Concerning purinergic signaling, it has been recently reported that the zebrafish family of ionotropic P2X receptors is comprised of nine members with two paralog copies of P2X$_3$, P2X$_4$, and P2X$_5$ (Low et al., 2008). The NTPDase7 ortholog was not detected in zebrafish, which lead us to hypothesize that its respective gene might not yet have been characterized or even that this member might have been lost from the zebrafish genome during evolution. Interestingly, Low et al. (2008) did not find a gene encoding P2X$_6$ in the zebrafish genome, which shows that nucleotide-mediated signaling in this teleost could differ from mammals and be more complex than previously thought. The identification of three NTPDase2 and two NTPDase5 paralogs in zebrafish suggests that they might be important for a differential regulation of nucleotide signaling in this species.

The phylogeny also showed that zebrafish NTPDase4, NTPDases5 and 6 are separated into two different subfamilies. This separation is probably due to their biochemical properties, which may be similar to the intracellular NTPDase members previously characterized in mammals (Robson et al., 2006). Using bioinformatics tools we verified that zebrafish NTPDase4 presents two transmembrane domains, whereas different hydrophobic domain numbers were identified for NTPDase5 paralogs and NTPDase6. Moreover, sequence analysis of NTPDase2_mg and NTPDase8 showed the presence of a single transmembrane domain for both proteins. Considering that transmembrane domain interactions can modify the stability of the extracellular portion of the human NTPDase2 (Chiang and Knowles, 2008), the difference in the number of hydrophobic domains observed among zebrafish NTPDase sequences might contribute to a distinct

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NTPDase1     ------------------------------------------------MEVKVKNPWHRPVVIFLMAVVAVGIVIMVSISVVQHKPL----------------  39
NTPDase2_mg  ----------------------------------------------MGAFTRFFWEFDLHECVIEQQ--VHANPRELWLTEHGC----------------  36
NTPDase2_mq  --------------------------------------------MOLQTCTIIAG--LFVSFVGILLLVVNTE------------------  26
NTPDase2_mv  -----------------------------------------MVSTGNRQQCQIITATGALFLAVGGILLLVLPSE-----------------  33
NTPDase3     -----------------------------------MINKESQSSDHLTVASRVMAKVAVVLAAAFMLASIAVIISIAVVCQTN----------------  50
NTPDase4     -----------------MGRISISCLFPASWHFSLSPVVLPRFLLPSFRQLLLLGLLLAGVMGLLYLLLVTGKGQYSWLREDRHIHTRLG----------  72
NTPDase5_ms  ------------MSQQMLHLTLVSMWLFAG--YFLAEATYYNHHMYNRYR----AINHNANMDN---QLPPEEPVVMEPVAP-------------------  61
NTPDase5_me  MEAGGSALPVEDLQGSSPSLRRRDQRMFGGSWLLLLRGSGLTGGRTLKLRPACGAPDRDQTADSSRDLRITPACAQITDPSTARMVMKMMMFWLLPVWTL  100
NTPDase6     ----------MRFPKLAFIFMFVG--CLVVYLTYVKRHYD-------DSKLSVPE---QLPQYRTDTRKHPTS----------------  51
NTPDase8     ----------------------------MAAVAVTTIIALILALANRQTL------------------  22
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                 *   * **                                              * * *
NTPDase1     -----------------PQKY-----KYGIVLDAGSSHTSVFIYKWPAEKENNTGMVQQHHTCNVKGK--------GISSY---F    91
NTPDase2_mg  -----------------SHVHVCLSLQYGIVLDAGSSHTALYVVYKWPADKLNGTGVVTQHSECHVKGG--------GISSGY---A    93
NTPDase2_mq  --------------------DFKEPAQLMYGIVLDAGSSHTSMFIYKWPADKQNGTGIVSQHSECRVKGG--------GISSY---A    83
NTPDase2_mv  -------------------DIKGSPGFMYGIVLDAGSSHTSMFIYKWPADKQNGTGIVSQHSECHAKGG--------GISSY---A    90
NTPDase3     ----------------KKTYVSRGLKYGIVLDAGSSRTTVYLYEWPAEKENNTGVVSCQTTKCLVKGP--------GISDLGLDG   110
NTPDase4     ----------------RVPDMEATDTNNPNLNYGLVVDCGSSGSRVFVYTWPRHNGNLHELLDIKQMRDKHRKPVMKIKPGISEY---A   143
NTPDase5_ms  ----------------RQVNFSRVFYGIMFDAGSSHTRIHIYKFIQKDPVELPVLDNEMYHAVKP--------GLSAY---A   116
NTPDase5_me  LLLSSSASSSSPPSSSAAADFSVRFGHTLPSLSRPANSTQIFYGIMFDAGSTGTRIHVYTFIQREPDGLPVLDNEMFHSMKP--------GLSAY---A   188
NTPDase6     ----------------GQVVSENFQYGIMLDAGSTGTRIHIFKF-QMEPNEAPKLAHETFRAIKP--------GLSAY---A   105
NTPDase8     ----------------DQPYST---QFGIVFDAGSSHTALFLYQWQGNKENNTGIVSQKQSCDVDGD--------GISSY---V    76
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              *   **    **** * *
NTPDase1     DKPHGAGASLEEMCKEAKEKIPAHRHSETPVYLGATAGMRLLKMEDEMASEKVLTSVAHSLKTYPFSYQG--ARILSGQEEGAFGWITVNYLSENLRKP-  188
NTPDase2_mg  GQRGAAGRSLELCLKQAVLDIPPHRHHLTPVYLGATAGMRLLKLSNPYKASQVLQEVKETIKSPFFSFRG--AVILSSQDEGVYGWVTVNYLLENFIKYG  191
NTPDase2_mq  GIKGAAARSLEECMDKATKSIPKSRQRITPVYLGATAGMRLLNTSKPKESAEVLKEVGELKSYPFSFKG--ASILTGQDEGAYGWITVNYLLENFIKYG  181
NTPDase2_mv  GVKGGAARSLKECMDKAMREIFTVRYKHTPVYLGATAGMRLLNTSKPKESAEVLKEVGELKSYPFSFKG--ASILTGQDEGAYGWITVNYLLENYIKYG  183
NTPDase3     DQDQNTWYSLKECMKKMTEAIPSHQHNSTPVFLGATAGMRLLHMTNEGRSNDVLTQMKNYLSSLPFKFQ--NASIISGQEEGLYGWITVNYLKGNFLEKN  208
NTPDase4     STPDKASDYISPLLSFAAQHIPKNKHQETPLYILCTAGMRVLPESQ--QEALLEDLRTDIPVNFNFLFSDSHVEVISGKQEGVYAWIGINFVLGRFNHVE  241
NTPDase5_ms  DMPEKGGGESIVVLKKFVLPMEGWIQTPVVLKATAGLRLLPEE--KAKALLNEKVEVFDSPFFVPNNSVTIMNGINEGVLAWVTVNFLTGHLYSR-  212
NTPDase5_me  DMPEIAGHTVRQLLRVAKKTVPPLEWKRTPLVFRATAGLRLLPAA---KAHALLEEVQLVIRKG-CILDYNNPGLLMVFLTGILAWVTVNFLTGRLERN-  283
NTPDase6     DEPEKSKEGILELLNVAKDTVPEVLWSSTPLMLRATAGLRLLPGE---KATILLDKVREVFSESLFLYKPDSVSIMDGTDEGMSAWITINFLIGALHGT-  201
NTPDase8     QNPAGAGESLKKCLDIAKAAIPTEQQKSTPVYLGATAGMRLLNLQNQTQSDSILAEVAKTICTYPFDFRG--ARILTGMEEGAYGWITTNNYLLESFIKHT  174
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                    *  ** **  ****
NTPDase1     --------------------AG-TLGALDLGGASTQITFVPQQI-------IESSDNSIDFRLYGNDYHLYTHSFLCYGKDQALKLAMAEKLRSTPDKT  259
NTPDase2_mg  YVGRWLNS-----------GRKTVGALDLGGASTQITFETPER-------VESELNHMTLRLFGHEYSLYTHSYLCYGKEEALRMILAYLITSQDYTS  271
NTPDase2_mq  FVGQWITP-----------GRDTVGALDFGGASTQITFVTKQT-------VENKDDLMKLRLYGRDYQITYTHSFLCYGQDQALLRLLAHLIKTQG--S  259
NTPDase2_mv  FVGQWITP-----------GRDTVGALDFGGASTQITFVTKQT-------VENKDDLMKLRLYGRDYQIYTHSFLCYGRDQVLLRLLAHLITIQG--S  266
NTPDase3     LWNAWVHPH-----------GAKTVGSLDLGGASTQIAFATSDD-------AKGED-IIKVSLYGYEYNIYTHSFLCYGKNEAEKRVLAKLAKEPISWA  288
NTPDase4     DEHDAVVEVQVPGSDQQETLLRKRTAGVLDMGGVSTQIAYEVPKTEEIAKNLLAEFNLGCDAHRTEHVVRVVYSTFLGFGGNAARQRYEENLISSTQIQN  341
NTPDase5_ms  ---------------TKRTVGILDLGGGSTQITFPRLKKTVQ---SAPSDYIAKINMFNSTYELYTHSYLGNGVFASRLATLGALG--ADGLE  286
NTPDase5_me  ---------------TERTVGILDLGGGSTQITFPKSKKTIE---AAPPDYISRFCNCSYQLYTHSYLGNGIKAARLSALGALN--ADGLE  357
NTPDase6     ---------------ETPTVGMLDLGGGSSTQITFALQDEITIQ---TSPIDYVTSFQMFNISHALYSHSYLGLGLMSARLAVLGGGIE--GQPLA  275
NTPDase8     FEGSWIHPK-----------AGKIIGALDLGGGASTQISFTPKDP-------VRDPNSAFNLQLYGYKYELYTYSYLCYGKDQALKKLQAYLHKNAGSSS  255
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                                            *                                    *
NTPDase1     DAILLRDPCGHPGYNTTKTLESVN-TPCMK--PLK--MPKEQFSHVGLGNMSCCQESIRKVFNTSHCPYS-GCSFNGVFQPTVEGKFGAFSAFFFVMDFL  353
NTPDase2_mg  TSK-MYHPCYPSDFINAFRLKEVFDSPCTASWRPKPYNPHSHITVQGTGDYQSCLGNTSKIFSFKSCSFS-GCAFNGVFQPNITGNFMAFSAYYFTHSFL  369
NTPDase2_mq  DTS-IVHPCYPADYSSSIKLSSVFDTPCVK--RQTPYKPDDELQIKGTGNYNQCLGNVSQLFSFSSCSYS-KCSFDGVFQPNITGNFMAFSAFYYIHQFL  355
NTPDase2_mv  DTS-IIHPCYPADYSSSIKLSSVFGTPCVE--KQTSYNPDGELQIKGTGNYQCCLGNVSQLFSFSSCSYS-KCSFDGVFQPNITGNFMAFSAFFYTHSFL  362
NTPDase3     N---VKHPCFPTGYNTSIPAEEIFGSECTKNNVPSRYNPKRLITFFGQSNPECCKALVRSIFDLTSCQGTENGCFDGVYQPPLNGDFTAYAGFYWTALAL  385
NTPDase4     KLLNQHQGESADSPILDPCLPVDLQDELGP--------PEQRLHLRGSGDFPRCRLLQPFLNRTNDTNTSLNGVYQPPIDFHNSQFYGFSEFYYCTEDV  433
NTPDase5_ms  W-KVPRSCLPKKFSEEWSFGGLTYKVSGT---PDEFPTTMGLVKRFMQGYKLCYQEVLQVVKG--------IVHQPFEVKGSSIFYAFSYYYDRAVES  373
NTPDase5_me  K-KLFRSSCLSRSHTAEFSFGGVSYHVSG-----------DISGVR---AAPPDYISRFQMFNCSYQLYTHSYLGNGIKAARLSALGALN--ADGLE  433
NTPDase6     GPQELVSECLAPDYSGQWEHAEVLYTVKG----------QKPGEP----IYESCLRKVEKMIYK--------QVRKAEEVKN-MDFYAFSYYYDRAVDL  351
NTPDase8     S---ISHPCYHKGYNLNTLTAELYNSPCVV--PPSSFNPKDTILFKGTGNSSLCLSLIENIVNLTGCALSPDCGFNGIYQPPVNGEFFAFSAYFYTFDFL  350
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                                  *                      *                 *               * **
NTPDase1     NLK-NDSLDK-------TKQRLAMYCSTPWQKIVQDHPK------VKEKYLSEYCFSATYILTLLEHGYNETSDNWNDIKFIKKIGDSDAGWTLGYMLNLTN  441
NTPDase2_mg  QQITEMKISTYAQ--LEEATQAVCNMTITEMTMKAP-------DLKKYLRDYCAVAVYIQVLMRLGRYNFDENSFQNVAFQKKAGEASVGWALGYILSVSS  460
NTPDase2_mq  QKAAGITITSPAD--LEHAAQFVCNMSFQQMQNKFP-------TEGDHLRDYCADSVLMRVLLINGYGFSDLSFPHISFQEKVEDTSVGWSLGYMLSLSN  446
NTPDase2_mv  QEATGIKIWTPAH--LEDAARVICNSLQEMGKKSQ-------DNESRLKDYCAVSVFVRALLVNGYSFNDRTFPQISFQKKAGDTSVGWSLGYMLSLSN  453
NTPDase3     KVNGSNSMEK-------FNTNMKLFCSNDWKNLKQNYE------YLSANYVHTILADLYKFNTDNWENLEFSKEVNNTSIAWSLGYMLALSN  473
NTPDase4     LRMGGDYNSTK----YTNAAKSYCATQWKTLKERFDRGLYASHADQHRLKYCCFKSAWMHEVLHSGFAFP--AAYENLRTALLVYDKEVQWTLGAILYRTA  528
NTPDase5_ms  GLIDGSRGGTVEVRDFKKKAKEVCNRMTK-YRPISP-------------YLCMDMTYITCLLKDGFGFKDSTV--LQLAKKVNNVETSWALGAIFDHFH  456
NTPDase5_me  GLIDEQRGGAVKVRDFRKTARQVCNRPAR-HTQLNQ-------------FLCMDLTYITCLLKDGFGFKESTV--LQLTRKVKNVNKGFKEST-WALGAAIDHFQ  516
NTPDase6     GLIDERTGGTVRVSDYIEGAKKVCNNMSAGGIKESP-------------FLCLDLTYISVLLQE-LDFPPDKE--LKLARQINNAETSWALGAAFHCIE  434
NTPDase8     GLTPKAPLTK-----VLSTIDTLCSKNWTALTAENPS------IKPKYLRDYCASGHYITTILLKGYKFN-NTWDQISFVKQVADTDIGWTLGYMLNLTN  438
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NTPDase1     MIPAE-DPDKPLMPHG---------GYVTFMILFSLLILVLIIMAYIYFRRFTKTAQKDII----------------------  492
NTPDase2_mg  LLPEESAAIRKGLCPA---------AWTGLMFLISVLLTATFCYMVLLVIQRRKSKEDVS----------------------  511
NTPDase2_mq  LLDAENAMVRKALRPD---------VWRAAVLLFSVLVIASLFFLLRSYQK--NGTKLCCYI----------------------  497
NTPDase2_mv  LLPAENVALKKSLR---------SGAWSFLIFLFSILLTSALIFLFRAICH--KNKDDVV----------------------  502
NTPDase3     MIPAEBGKIIKLPINN---------VLFTGLLLLFSVLTIITLTYLVIALVRYCY----------------------  518
NTPDase4     FLPLRDIQQESLKASHSHWQHSFSFMNNHYLFLVCFLVVVLSILLYLLRLRRIHNRSMLHRSASASVQWLEEGLGSPDLPVTL  611
NTPDase5_ms  NFSIQ-----------------------------------------------  461
NTPDase5_me  KSRIH-----------------------------------------------  521
NTPDase6     SFRKHGTC---------------------------------------------  442
NTPDase8     MIPSERSSAVTGVPHS---------QWAAEIFFIAFALFLTLLIIVILSVRDITK----------------------  484
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Fig. 4. Alignment of deduced amino acid sequences for the zebrafish NTPDase family members. The conserved apyrase domains (ACRs) are represented in the boxes. The transmembrane domains are underlined and the signal peptide from NTPDase5_ms is in bold face. Potential *N*-glycosylation and N-acetylation sites are shown by dotted lines and double underline, respectively. Conserved cysteine residues are bold face in black boxes. Putative PKA and PKC phosphorylation sites are marked in gray boxes with white and black letters, respectively. The asterisks (*) indicate similarities among all zebrafish NTPDase members after ClustalX alignment.

anchorage stability and enzyme activity. Although further studies are still required to clarify the involvement of hydrophobic domains in zebrafish NTPDase activities it is possible to speculate that NTPDase5_me might act as a soluble enzyme or even possess another membrane anchorage mechanism since its sequence did not present a characteristic transmembrane domain.

Posttranslational processes are responsible for regulating several enzyme activities and modulate metabolic pathways. Although there

45
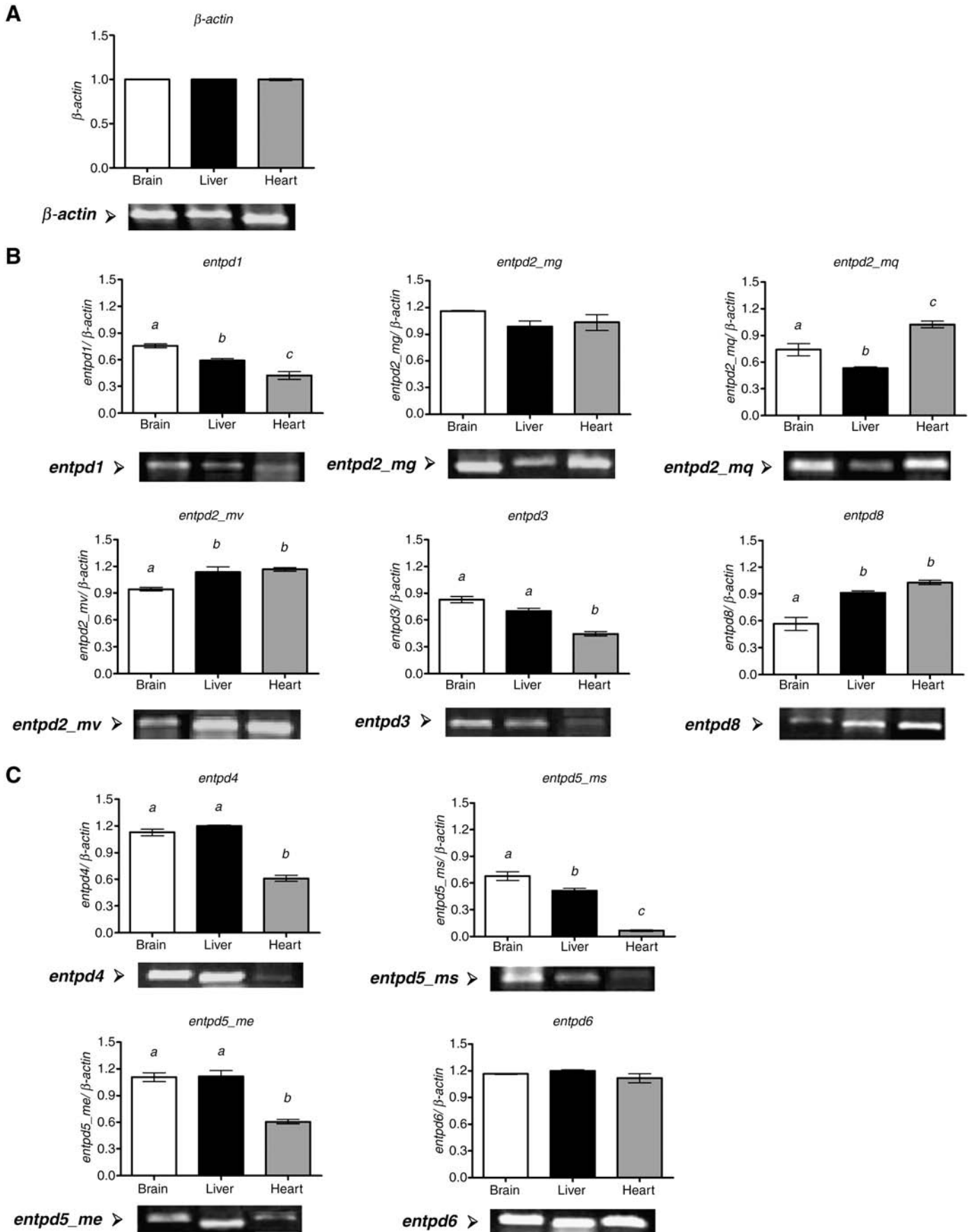
are no experimental data for phosphorylation mechanisms on ectonucleotidases so far, PKC target residues for zebrafish NTPDase members were also aligned with other putative PKC regulatory sites on mammalian orthologs, such as human Lys62 and Asn64 from the NTPDase2 sequence (data not shown). Given that both regions have been described as essential structural elements for the enzyme (Javed et al., 2007) the conservation of these phosphorylation sites could indicate that PKC activity may be important for the mediation of posttranslational events in vertebrate NTPDase members. In the same way, a previous study reported that N-termini of NTPDases also contain consensus sequences for protein phosphorylation by PKC (Kegel et al., 1997).

Here we also verified that conserved asparagine residues from zebrafish NTPDase1, NTPDase2_mg, NTPDase2_mq, NTPDase2_mv, NTPDase3, and NTPDase8 (Asn69, Asn71, Asn61, Asn68, Asn85, and Asn 54, respectively) were predicted as putative N-glycosylation sites. A recent study showing asparagine-to-glutamine mutation at six individual potential N-linked glycosylation sites from human NTPDase2 established the importance for protein function and expression of Asn64, which is conserved in all cell surface NTPDases (Javed et al., 2007). The proximity of these respective residues with Asn64 from human NTPDase2 obtained by alignment of the sequences (data not shown), associated with the separation of NTPDase1-3 and 8 from NTPDase4-6 clades corroborated by our phylogenetic analysis, strongly supports the idea that these members act as ecto-enzymes in zebrafish.

The RT-PCR assays in the present study showed a distinct expression profile for the three entpd2 paralogs (entpd2_mg, mq, and mv) in zebrafish brain, as previously reported (Rico et al., 2006, 2008). Despite the lower entpd5_ms expression in heart, large amounts of entpd2 paralogs and entpd6 transcripts could be detected in this tissue. These data are in accordance with the literature, since NTPDase activity in rat left ventricle is associated with a high entpd2 expression and NTPDase6 may participate in homeostasis by playing a role in ADP hydrolysis in heart (Yeung et al., 2000; Rücker et al., 2008). Interestingly, in contrast to mammals, entpd8 transcripts were detected in zebrafish brain, even though at lower levels than in the liver and heart. The high entpd8 expression level detected in liver could indicate a possible relevance of this enzyme to the hepatic physiology of zebrafish since the main hepatic ecto-ATPase activity in mammals has been attributed to the canalicular entpd8 expression, which is involved in the regulation of bile secretion and/or nucleoside salvage (Fausther et al., 2007). In addition, the differential entpd1, entpd2, and entpd3 expression observed in zebrafish liver could be important for the regulation of several metabolic pathways since there is evidence that extracellular nucleotides play a role in the control of glycogenolysis and in the activation of JNK signaling (Keppens et al., 1989; Thevananther et al., 2004; Robson et al., 2006). Furthermore, the presence of at least two members of the surface-located NTPDase family has been observed in intact goldfish hepatocytes, suggesting that distinct enzymes may account for the hydrolysis of extracellular nucleotides in the liver of this teleost (Alleva et al., 2002).

Despite the identification of ten distinct entpd transcripts, the possibility remains that splice variants exist for the NTPDase members, since transcript variants from NTPDases have already been demonstrated in different organisms (Vlajkovic et al., 1999; Biederbick et al., 2000; Mateo et al., 2003). Thus, the involvement of possible NTPDase isoforms in nucleotide hydrolysis in zebrafish tissues still requires further investigation.

In conclusion, our findings demonstrate that several NTPDase members may hydrolyze tri- and diphosphonucleosides in zebrafish tissues. Our data reinforce the idea that zebrafish represent a useful vertebrate model for studying distinct purinergic signaling parameters. The elucidation of zebrafish NTPDase members should certainly contribute to better understanding of this enzyme family in this species.

References

Alleva, K.E., Espelt, M.V., Krumschnabel, G., Schwarzbaum, P.J., 2002. Identification of two distinct E-NTPDases in liver of goldfish (Carassius auratus L.). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 131, 725–731.

Amores, A., Force, A., Yan, Y.L., Joly, L., Amemiya, C., Fritz, A., Ho, R.K., Langeland, J., Prince, V., Wang, Y.L., Westerfield, M., Ekker, M., Postlethwait, J.H., 1998. Zebrafish hox clusters and vertebrate genome evolution. Science 282, 1711–1714.

Appelbaum, L., Skariah, G., Mourrain, P., Mignot, E., 2007. Comparative expression of P2X receptors and ecto-nucleoside triphosphate diphosphohydrolase 3 in hypocretin and sensory neurons in zebrafish. Brain Res. 1174, 66–75.

Baraban, S.C., 2007. Emerging epilepsy models: insights from mice, flies, worms and fish. Curr. Opin. Neurol. 20, 164–168.

Barnes, J.M., Murphy, P.A., Kirkham, D., Henley, J.M., 1993. Interaction of guanine nucleotides with [3H]kainate and 6-[3H]cyano-7-nitroquinoxaline-2, 3-dione binding in goldfish brain. J. Neurochem. 61, 1685–1691.

Barreto-Chaves, M.L., Carneiro-Ramos, M.S., Cotomacci, G., Júnior, M.B., Sarkis, J.J., 2006. E-NTPDase 3 (ATP diphosphohydrolase) from cardiomyocytes, activity and expression are modulated by thyroid hormone. Mol. Cell. Endocrinol. 251, 49–55.

Beldi, G., Wu, Y., Sun, X., Imai, M., Enjyoji, K., Csizmadia, E., Candinas, D., Erb, L., Robson, S.C., 2008. Regulated catalysis of extracellular nucleotides by vascular CD39/ENTPD1 is required for liver regeneration. Gastroenterology 135, 1751–1760.

Biederbick, A., Kosan, C., Kunz, J., Elsässer, H.P., 2000. First apyrase splice variants have different enzymatic properties. J. Biol. Chem. 275, 19018–19024.

Bonan, C.D., Dias, M.M., Battastini, A.M., Dias, R.D., Sarkis, J.J., 1998. Inhibitory avoidance learning inhibits ectonucleotidases activities in hippocampal synaptosomes of adult rats. Neurochem. Res. 23, 977–982.

Bonan, C.D., Amaral, O.B., Rockenbach, I.C., Walz, R., Battastini, A.M., Izquierdo, I., Sarkis, J.J., 2000. Altered ATP hydrolysis induced by pentylenetetrazol kindling in rat brain synaptosomes. Neurochem. Res. 25, 775–779.

Boué-Grabot, E., Akimenko, M.A., Séguéla, P., 2000. Unique functional properties of a sensory neuronal P2X ATP-gated channel from zebrafish. J. Neurochem. 75, 1600–1607.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 218–254.

Burnstock, G., 2009. Purinergic cotransmission. Exp. Physiol. 94, 20–24.

Campanella, J.J., Bitincka, L., Smalley, J., 2003. MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences. BMC Bioinformatics 4, 29.

Chan, K.M., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity. Anal. Biochem. 157, 375–380.

Chen, W.-Y., John, J.A.C., Lin, C.-H., Lin, H.-F., Wu, S.-C., Lin, C.-H., Chang, C.-Y., 2004. Expression of metallothionen gene during embryonic and early larval development in zebrafish. Aquat. Toxicol. 69, 215–227.

Chiang, W.C., Knowles, A.F., 2008. Transmembrane domain interactions affect the stability of the extracellular domain of the human NTPDase 2. Arch. Biochem. Biophys. 472, 89–99.

Chico, T.J., Ingham, P.W., Crossman, D.C., 2008. Modeling cardiovascular disease in the zebrafish. Trends Cardiovasc. Med. 18, 150–155.

Fausther, M., Lecka, J., Kukulski, F., Lévesque, S.A., Pelletier, J., Zimmermann, H., Dranoff, J.A., Sévigny, J., 2007. Cloning, purification, and identification of the liver canalicular

Fig. 5. Relative expression profiles of the entpd genes in different adult zebrafish tissues. The figure shows representative RT-PCR experiments and the optical densitometry analysis for β-actin (A) and entpd members (surface-located enzymes (B) and intracellular NTPDases (C)). Four independent experiments were performed, with entirely consistent results (ANOVA, followed by Tukey HSD test as post-hoc, $P \leq 0.05$).

ecto-ATPase as NTPDase8. Am. J. Physiol. Gastrointest. Liver Physiol. 292, G785–G795.

Javed, R., Yarimizu, K., Pelletier, N., Li, C., Knowles, A.F., 2007. Mutagenesis of lysine 62, asparagine 64, and conserved region 1 reduces the activity of human ecto-ATPase (NTPDase 2). Biochemistry 46, 6617–6627.

Kegel, B., Braun, N., Heine, P., Maliszewski, C.R., Zimmermann, H., 1997. An ecto-ATPase and an ecto-ATP diphosphohydrolase are expressed in rat brain. Neuropharmacology 36, 1189–1200.

Keppens, S., VandeKerckhove, J., Wulf, H.De., 1989. Characterization of purinoceptors present on human liver plasma membranes. FEBS Lett. 248, 137–140.

Kirley, T.L., Crawford, P.A., Smith, T.M., 2006. The structure of the nucleoside triphosphate diphosphohydrolases (NTPDases) as revealed by mutagenic and computational modeling analyses. Purinergic Signal. 2, 379–389.

Köhler, D., Eckle, T., Faigle, M., Grenz, A., Mittelbronn, M., Laucher, S., Hart, M.L., Robson, S.C., Müller, C.E., Eltzschig, H.K., 2007. CD39/ectonucleoside triphosphate diphosphohydrolase 1 provides myocardial protection during cardiac ischemia/reperfusion injury. Circulation 116, 1784–1794.

Kucenas, S., Li, Z., Cox, J.A., Egan, T.M., Voigt, M.M., 2003. Molecular characterization of the zebrafish P2X receptor subunit gene family. Neuroscience 121, 935–945.

Lavoie, E.G., Kukulski, F., Lévesque, S.A., Lecka, J., Sévigny, J., 2004. Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-3. Biochem. Pharmacol. 67, 1917–1926.

Lévesque, S.A., Lavoie, E.G., Lecka, J., Bigonnesse, F., Sévigny, J., 2007. Specificity of the ecto-ATPase inhibitor ARL 67156 on human and mouse ectonucleotidases. Br. J. Pharmacol. 152, 141–150.

Low, S.E., Kuwanda, J.Y., Hume, R.I., 2008. Amino acid variations resulting in functional and nonfunctional zebrafish P2X$_1$ and P2X$_{5.1}$ receptors. Purinergic Signal 4, 383–392.

Mateo, J., Kreda, S., Henry, C.E., Harden, T.K., Boyer, J.L., 2003. Requirement of Cys399 for processing of the human ecto-ATPase (NTPDase2) and its implications for determination of the activities of splice variants of the enzyme. J. Biol. Chem. 278, 39960–39968.

Murphy-Piedmonte, D.M., Crawford, P.A., Kirley, T.L., 2005. Bacterial expression, folding, purification and characterization of soluble NTPDase5 (CD39L4) ecto-nucleotidase. Biochim. Biophys. Acta 1747 (2), 251–259.

Plesner, L., 1995. Ecto-ATPases: identities and functions. Int. Rev. Cytol. 158, 141–214.

Pullman, M.E., Penefsky, H.S., Datta, A., Racker, E., 1960. Partial resolution of the enzymes catalyzing oxidative phosphorylation. I. Purification and properties of soluble dinitrophenol-stimulated adenosine triphosphatase. J. Biol. Chem. 235, 3322–3329.

Rekha, R.D., Amali, A.A., Her, G.M., Yeh, Y.H., Gong, H.Y., Hu, S.Y., Lin, G.H., Wu, J.L., 2008. Thioacetamide accelerates steatohepatitis, cirrhosis and HCC by expressing HCV core protein in transgenic zebrafish *Danio rerio*. Toxicology 243, 11–22.

Ricatti, M.J., Alfie, L.D., Lavoie, E.G., Sévigny, J., Schwarzbaum, P.J., Faillace, M.P., 2009. Immunocytochemical localization of NTPDases1 and 2 in the neural retina of mouse and zebrafish. Synapse 63 (4), 291–307.

Rico, E.P., Senger, M.R., Fauth, Mda.G., Dias, R.D., Bogo, M.R., Bonan, C.D., 2003. ATP and ADP hydrolysis in brain membranes of zebrafish (*Danio rerio*). Life Sci. 73, 2071–2082.

Rico, E.P., Rosemberg, D.B., Senger, M.R., Arizi, Mde.B., Bernardi, G.F., Dias, R.D., Bogo, M.R., Bonan, C.D., 2006. Methanol alters ecto-nucleotidases and acetylcholinesterase in zebrafish brain. Neurotoxicol. Teratol. 28, 489–496.

Rico, E.P., Rosemberg, D.B., Senger, M.R., Arizi, M., de Bem Dias, R.D., Souto, A.A., Bogo, M.R., Bonan, C.D., 2008. Ethanol and acetaldehyde alter NTPDase and 5′-nucleotidase from zebrafish brain membranes. Neurochem. Int. 52, 290–296.

Robson, S.C., Sévigny, J., Zimmermann, H., 2006. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal 2, 409–430.

Rosemberg, D.B., Rico, E.P., Guidoti, M.R., Dias, R.D., Souza, D.O., Bonan, C.D., Bogo, M.R., 2007. Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (*Danio rerio*). Life Sci. 81, 1526–1534.

Rücker, B., Almeida, M.E., Libermann, T.A., Zerbini, L.F., Wink, M.R., Sarkis, J.J., 2008. E-NTPDases and ecto-5′-nucleotidase expression profile in rat heart left ventricle and the extracellular nucleotide hydrolysis by their nerve terminal endings. Life Sci. 82, 477–486.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Santos, R.F., Pôssa, M.A., Bastos, M.S., Guedes, P.M., Almeida, M.R., Demarco, R., Verjovski-Almeida, S., Bahia, M.T., Fietto, J.L., 2009. Influence of ecto-nucleoside triphosphate diphosphohydrolase activity on *Trypanosoma cruzi* infectivity and virulence. PLoS Negl. Trop. Dis. 3 (3), e387.

Schetinger, M.R., Morsch, V.M., Bonan, C.D., Wyse, A.T., 2007. NTPDase and 5′-nucleotidase activities in physiological and disease conditions: new perspectives for human health. Biofactors 31, 77–98.

Senger, M.R., Rico, E.P., Dias, R.D., Bogo, M.R., Bonan, C.D., 2004. Ecto-5′-nucleotidase activity in brain membranes of zebrafish (*Danio rerio*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 139, 203–207.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Thevananther, S., Sun, H., Li, D., Arjunan, V., Awad, S.S., Wyllie, S., Zimmerman, T.L., Goss, J.A., Karpen, S.J., 2004. Extracellular ATP activates c-Jun N-terminal kinase signalling and cell cycle progression in hepatocytes. Hepatology 39, 393–402.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Vlajkovic, S.M., Housley, G.D., Greenwood, D., Thorne, P.R., 1999. Evidence for alternative splicing of ecto-ATPase associated with termination of purinergic transmission. Brain Res. Mol. Brain Res. 73, 85–92.

von Kügelgen, I., 2006. Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. Pharmacol. Ther. 110, 415–432.

Wall, M.J., Wigmore, G., Lopatár, J., Frenguelli, B.G., Dale, N., 2008. The novel NTPDase inhibitor sodium polyoxotungstate (POM-1) inhibits ATP breakdown but also blocks central synaptic transmission, an action independent of NTPDase inhibition. Neuropharmacology 55, 1251–1258.

Yeung, G., Mulero, J.J., McGowan, D.W., Bajwa, S.S., Ford, J.E., 2000. CD39L2, a gene encoding a human nucleoside diphosphatase, predominantly expressed in the heart. Biochemistry 39, 12916–12923.

Zimmermann, H., 2001. Ectonucleotidases: some recent developments and a note on nomenclature. Drug Dev. Res. 52, 44–56.

Zimmermann, H., 2006. Ectonucleotidases in the nervous system. Novartis Found. Symp. 276, 113–128 discussion 128–130, 233–237, 275–281.

II.3. CAPÍTULO III

Taurine prevents enhancement of acetylcholinesterase activity induced by acute ethanol exposure and decreases the level of markers of oxidative stress in zebrafish brain.

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TAURINE PREVENTS ENHANCEMENT OF ACETYLCHOLINESTERASE ACTIVITY INDUCED BY ACUTE ETHANOL EXPOSURE AND DECREASES THE LEVEL OF MARKERS OF OXIDATIVE STRESS IN ZEBRAFISH BRAIN

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Abstract—Ethanol (EtOH) is a drug widely consumed throughout the world that promotes several neurochemical disorders. Its deleterious effects are generally associated with modifications in oxidative stress parameters, signaling transduction pathways, and neurotransmitter systems, leading to distinct behavioral changes. Taurine (2-aminoethanesulfonic acid) is a β-amino acid not incorporated into proteins found in mM range in the central nervous system (CNS). The actions of taurine as an inhibitory neurotransmitter, neuromodulator, and antioxidant make it attractive for studying a potential protective role against EtOH-mediated neurotoxicity. In this study, we investigated whether acute taurine cotreatment or pretreatment (1 h) prevent EtOH-induced changes in acetylcholinesterase (AChE) activity and in oxidative stress parameters in zebrafish brain. The results showed that EtOH exposure (1% in volume) during 1 h increased AChE activity, whereas the cotreatment with 400

mg·L^{-1} taurine prevented this enhancement. A similar protective effect of 150 and 400 mg·L^{-1} taurine was also observed when the animals were pretreated with this amino acid. Taurine treatments also prevented the alterations promoted in superoxide dismutase and catalase activities by EtOH, suggesting a modulatory role in enzymatic antioxidant defenses. The pretreatment with 150 and 400 mg·L^{-1} taurine significantly increased the sulfydryl levels as compared to control and EtOH groups. Moreover, 150 and 400 mg·L^{-1} taurine significantly decreased thiobarbituric acid reactive species (TBARS) levels, but the cotreatment with EtOH plus 400 mg·L^{-1} taurine did not prevent the EtOH-induced lipoperoxidation. In contrast, the pretreatment with 150 and 400 mg·L^{-1} taurine prevented the TBARS increase besides decreased the basal levels of lipid peroxides. Altogether, our data showed for the first time that EtOH induced oxidative stress in adult zebrafish brain and reinforce the idea that this vertebrate is an attractive alternative model to evaluate the beneficial effect of taurine against acute EtOH exposure. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: taurine, ethanol, acetylcholinesterase, oxidative stress parameters, zebrafish, brain.

The zebrafish (*Danio rerio*) is emerging as a promising model organism for experimental studies in different biomedical areas. As a relatively simple vertebrate species, zebrafish is an ideal animal model for laboratory research because they are inexpensive, low-maintenance, and abundantly produce offspring (Gerlai et al., 2006; Egan et al., 2009). Because zebrafish genes are highly conserved sharing a 70−80% homology to those of humans (Barbazuk et al., 2000), it is a tempting vertebrate model for modeling behavioral and functional parameters related to human pathogenesis and for clinical treatments screening, including alcohol abuse and therapeutic strategies.

Ethanol (EtOH) is a drug widely consumed throughout the world. Alcoholic consumption is linked to the occurrence of several pathological conditions such as various forms of cancer, liver failure, brain damage, and fetal injuries (Quertemont et al., 2005; Dalitz et al., 2008). The cerebral effects of acute EtOH exposure in central nervous system (CNS) lead to an impairment of motor coordination, sensory perception and cognition, which can be correlated to oxidative stress and modifications of neurotransmitter systems and intricate signaling pathways (Hanchar et al., 2005; Belmeguenai et al., 2008).

Acetylcholine (ACh) is a neurotransmitter that elicits its effects through nicotinic and muscarinic receptors. In the extracellular space, acetylcholinesterase (EC 3.1.1.7;

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; CAT, catalase; CNS, central nervous system; DTNB, 5,5′-dithionitrobis 2-nitrobenzoic acid; EtOH, ethanol; GSH, reduced glutathione; ROS, reactive oxygen species; SOD, superoxide dismutase; TAR, total antioxidant reactivity; TBARS, thiobarbituric acid reactive species; TRAP, total antioxidant potential.

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AChE) is the enzyme responsible for terminating the cholinergic transmission by degradation of ACh into choline and acetate. Since the cholinergic system can also be involved in the modulation of behavioral and cognitive functions (Sarter and Bruno, 2004; Furey et al., 2008) the levels of ACh in extracellular milieu must be tightly regulated and the screening for molecules able to modulate directly and/or indirectly AChE activity could be a pharmacological strategy to lead the maintenance of brain homeostasis.

Taurine (2-aminoethanesulfonic acid) is a simple sulfur-containing β-amino acid, which is not incorporated into proteins and is found free in virtually all animal cells. In particular, high concentrations of taurine are detected in electrically excitable tissues such as brain, retina, heart, and skeletal muscles (Huxtable, 1992; Saransaari and Oja, 2000; Oja and Saransaari, 2007). In the CNS, taurine plays a critical role for brain function, being implicated in cell volume regulation and also in neuromodulation or inhibitory neurotransmission (Banerjee et al., 2008). In certain tissues such as brain, intracellular taurine concentrations can range up to 50 mM, whereas extracellular concentrations are in the micromolar range (Huxtable, 1992). The intracellular taurine accumulation results primarily from uptake by an efficient transport system (TauT protein) in the plasma membrane which utilizes transmembrane gradients of Na^+ and Cl^- as the driving force, as well from intracellular biosynthesis of taurine (Kozlowski et al., 2008; Kang, 2009). Several roles of taurine have been reported, including its trophic actions during the CNS development; antioxidant functions, the ability for modifying protein phosphorylation, maintenance of calcium homeostasis, and membrane integrity (Wu et al., 2005; Oliveira et al., 2010; Junyent et al., 2010). Although the mechanisms involved in taurine actions still remains poorly understood, it is conceivable that its extracellular effects are mediated by opening the chloride channels through the interaction with $GABA_A$ receptors, glycine receptors, or putative taurine receptors (Albrecht and Schousboe, 2005). Due to its biochemical properties, taurine interacts with other transmitter systems and acts as a neuroprotector against various types of injury, including alcohol abuse (Oja and Saransaari, 2007; Chen et al., 2009).

The teratogenic properties of EtOH have been previously established in zebrafish (Dlugos and Rabin, 2003; Reimers et al., 2006). It has been shown that EtOH modulates distinct behavioral parameters in this species such as swimming activity, aggression, group preference, and pigment response, possibly through alterations in neurotransmitter systems and in cell signaling cascades (Gerlai et al., 2000; Rico et al., 2007; Peng et al., 2009). Concerning the cholinergic signaling, zebrafish presents a unique situation among vertebrates because its genome does not encode a functional butyrylcholinesterase, being AChE the only ACh-hydrolyzing enzyme in this organism (Behra et al., 2004). It was reported that brain AChE activity in zebrafish is altered after acute EtOH exposure, suggesting an involvement of cholinergic parameters on EtOH-mediated responses (Rico et al., 2007). Moreover, previous

study characterized the expression profile of TauT in zebrafish during embryogenesis, showing a high degree of homology to mammalian taurine transporter (Kozlowski et al., 2008). Although it has been previously reported that the EtOH-mediated toxicity in zebrafish embryos can be partially attenuated by antioxidants (Reimers et al., 2006), little is known about the effects of EtOH and taurine on the brain function of adult zebrafish. In this context, the influence of acute EtOH exposure on oxidative stress parameters and the beneficial actions of taurine in this species still remain obscure. Therefore, in the present study we focused for the first time our attention on the potential neuroprotective effects of taurine in zebrafish brain. First, we evaluated whether acute cotreatment or pretreatment with taurine prevent EtOH-induced enhancement of AChE activity. In addition, the effect of acute EtOH exposure on oxidative stress parameters and the influence of both taurine treatments on neurochemical redox profile were also studied.

EXPERIMENTAL PROCEDURES

Animals

Adult males and females of the "wild type" (short fin—SF) zebrafish (*Danio rerio*) strain (3–6 months-old, weighing 0.43 ± 0.07 g) were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated for at least 2 weeks in 50-L thermostated aquarium before the experiments. All tanks were filled with unchlorinated water previously treated with 132 μL.L^{-1} AquaSafe® (Tetra, USA) and kept under mechanical and chemical filtration at a targeted temperature of 26 ± 2 °C and water pH and conductivity at $7.0-8.0$ and $1,500-1,600$ μS.cm^{-1}, respectively. Illumination was provided by ceiling-mounted fluorescent light tubes on a 12-h light-dark photoperiod (on 7:00 h; off 19:00 h). Fish were fed twice a day to satiety with commercial alcon BASIC® (Alcon, Brazil) as flake fish food. For the experiments, fish were euthanized by decapitation and the brains were quickly dissected. Each independent experiment was performed using biological preparations from a pool of three animals for AChE experiments and 10 animals for oxidative stress evaluation. All animals used were naive being healthy and free of any signs of disease and maintained in accordance to the National Institute of Health Guide for Care and Use of Laboratory Animals. The protocols were approved by the Ethical Committee of the Federal University of Rio Grande do Sul under the number 2007950.

Chemicals

Ethanol (C_2H_6O; CAS number 64-17-5) was purchased from Merck (Darmstadt, Germany). All other reagents used were purchased from Sigma (St. Louis, MO, USA).

Experimental design

For acute EtOH exposure, fish were placed in 3-L aquarium and kept in a solution of 1% EtOH in volume during 1 h. The same time of exposure and EtOH concentration have been successfully tested in adult zebrafish (Gerlai et al., 2000; Dlugos and Rabin, 2003; Rico et al., 2007) leading to alterations in behavioral and cholinergic signaling parameters of this species. In addition, the blood alcohol levels achieved with it are expected to be in the range seen in the human clinic after mild to moderate acute alcohol consumption (Gerlai et al., 2008). Acute taurine treatments were performed using distinct concentrations (42, 150, and 400 mg·L^{-1}) during 1 h, the same time used for EtOH exposure. Taurine chosen concentrations correspond to a range previously

used in other studies related to taurine effects on several biochemical parameters, which vary from 0.33 to 3.2 mM (Wu et al., 2005; Kong et al., 2006; Rosemberg et al., 2010b). Two treatment protocols with taurine were performed: (i) concomitant treatment with EtOH; and (ii) a pretreatment during 1 h before EtOH exposure. Importantly, the acute treatments procedure (e.g., the origin and quality of the system water and the timing of taurine delivery, etc.) was identical for all fish. Different experimental groups were analyzed: (1) control (1 h into water); (2) EtOH-exposed during 1 h; (3) taurine-treated during 1 h; (4) concomitant taurine treatment with EtOH during 1 h (cotreatment); (5) 1 h into water before transference to another aquaria+1 h into water (pretreatment—control group unexposed to taurine); (6) 1 h into water before transference to another aquaria+EtOH exposure during 1 h (pretreatment—EtOH group unexposed to taurine); (7) taurine-treated during 1 h before transference to another aquaria+EtOH exposure during 1 h (pretreatment—experimental group); and (8) taurine-treated during 1 h before transference to another aquaria+1 h into water (pretreatment—control group treated with taurine).

Determination of AChE activity

The brains were homogenized on ice in 60 volumes (v/w) of Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) using a Potter–Elvehjen-type glass homogenizer. The rate of hydrolysis of acetylthiocholine iodide (0.88 mM) was determined in a final volume of 300 μL, with 33 μL of 100 mM phosphate buffer, pH 7.5 mixed to 2.0 mM 5,5'-dithionitrobis 2-nitrobenzoic acid (DTNB). Samples containing 5 μg protein and the reaction medium specified above were preincubated for 10 min at 25 °C. The hydrolysis of acetylthiocholine iodide was monitored in a microplate reader by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) (Ellman et al., 1961). Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of the substrate. AChE activity was expressed as μmol thiocholine (SCh). h^{-1}. mg protein^{-1}. All experiments were performed in quadruplicate.

Oxidative stress analyses

Zebrafish brains were dissected out in ice immediately after the fish were euthanized and homogenized in 1.0 mL phosphate buffer saline (PBS) pH 7.4, containing in mM: 137 NaCl, 10.1 Na_2HPO_4, and 1.76 KH_2PO_4. The homogenates were centrifuged ($700 \times g$, 5 min) to remove cellular debris. Supernatants were collected and used to all biochemical assays described herein.

Antioxidant enzyme activities determination

Superoxide dismutase (EC 1.15.1.1, SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation at 480 nm (Misra and Fridovich, 1972), and the results were expressed as Units SOD. mg protein^{-1}. Catalase (EC 1.11.1.6; CAT) activity was assessed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm (Aebi, 1984), and the results were expressed as Units CAT. mg protein^{-1}. For SOD assay, protein amounts ranged from 15–60 μg, while CAT activity was determined using 30–50 μg protein. To better understand the effect promoted by the treatments upon these two oxidant-detoxifying enzymes that work in sequence converting superoxide anion to water, a ratio SOD/CAT activities was calculated. An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which could culminate in oxidative stress.

Non-enzymatic antioxidant defenses

The non-enzymatic antioxidant potential of zebrafish brains was estimated by the total antioxidant potential (TRAP) and total an-

tioxidant reactivity (TAR) (Lissi et al., 1995). The reaction was initiated by adding luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione, 4 mM)—an external probe to monitoring radical production—and AAPH (2,2'-azobis[2-methylpropionamidine]dihydrochloride, 10 mM)—a free radical source that produces peroxyl radical at a constant rate—in glycine buffer (0.1 M) pH 8.6 at room temperature, resulting in a steady luminescence emission (system counts). Chemiluminescence was read in a liquid scintillation counter (Wallace, 1409) as counts per minutes. Sample addition decreases the luminescence proportionately to its antioxidant potential. The luminescence emission was followed for 40 min after the addition of the sample (10 μg protein) in a TRAP protocol, and the area under the curve was quantified. In the TAR protocol, results were calculated as percentage of radical production (system counts considered as 100% of radical production).

Total reduced thiol content

Oxidative alterations in proteins can be evaluated by the level of protein thiol content in samples. Briefly, samples (40 μg protein) were mixed to 35 μL of 0.2 mM EDTA, 100 mM boric acid buffer, pH 8.5. DTNB (0.01 M dissolved in ethanol) was added and the intense yellow color developed was measured at 412 nm after 1 h (Ellman, 1959). Total reduced sulfydryl content was estimated in a final volume of 210 μL and the results were expressed as μmol SH. mg protein^{-1}.

Thiobarbituric acid reactive species (TBARS)

The formation of TBARS during an acid-heating reaction, which is widely performed for measurement of lipid redox state (Draper and Hadley, 1990), was used as an index of lipid peroxidation. Briefly, 300 μL of samples (80–100 μg protein) were mixed with 600 μL of 15% trichloroacetic acid (TCA) and centrifuged ($10.000 \times g$, 10 min). Supernatants (100 μL) were mixed with 100 μL of 0.67% thiobarbituric acid (TBA, 4,6-Dihydroxypyrimidine-2-thiol) and heated at 100 °C for 30 min. TBARS levels were determined by the absorbance at 532 nm using 1,1,3,3-tetramethoxypropane (TMP) as standard. Results were expressed as nmol TBARS. mg protein^{-1}.

Protein quantification

For AChE activity assays, the protein was measured by the Coomassie Blue method (Bradford, 1976). For oxidative stress analyses, the protein concentration was determined according to Peterson (1977). Bovine serum albumin was used as a protein standard.

Statistics

Data were expressed as mean $\pm$ standard error of the mean (SEM) and P-values were considered significant for $P \leq 0.05$. Differences within the experimental groups were determined by one or two-way analysis of variance (ANOVA). Comparison among means was carried out using Bonferroni's multiple comparison test as post hoc. All experiments were performed at least in triplicate.

RESULTS

Taurine prevents alterations in AChE activity promoted by acute EtOH exposure

As previously reported by our group (Rico et al., 2007), we demonstrate that acute EtOH exposure (1% in volume) increased AChE activity in zebrafish brain (37%, $n=6$) when compared to control group (Fig. 1). When the animals were treated concomitantly with taurine (42 and 150
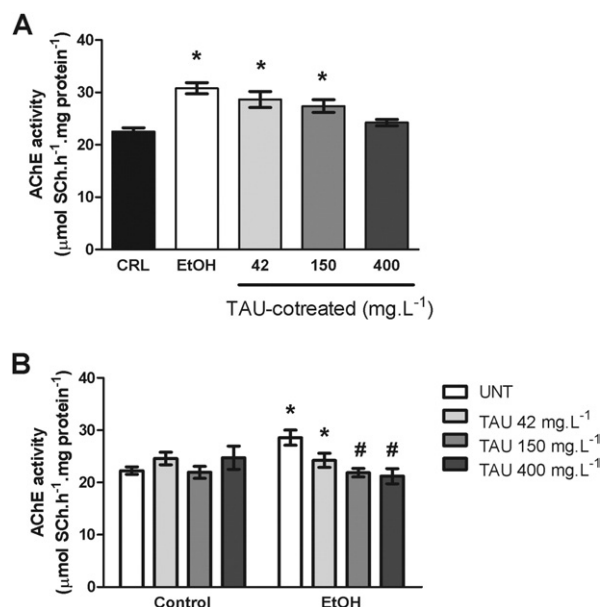
Fig. 1. Effect of taurine on the EtOH-induced enhancement of AChE activity after acute exposure. (A) Cotreatment with taurine and EtOH. The groups were represented as control (CRL), ethanol (EtOH), taurine-cotreated animals. Data represent mean±SEM of at least three independent experiments. * Significant difference compared to control (one-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$). (B) Pretreatment with taurine followed by water (control group) or EtOH exposure. The treatments were represented as taurine-untreated (UNT) and taurine-treated (TAU). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to UNT from control group. # Significant difference compared to UNT from EtOH group (two-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$).

$mg \cdot L^{-1}$) and EtOH, the AChE activity did not change in comparison to EtOH group. However, taurine cotreatment at 400 $mg \cdot L^{-1}$ prevented zebrafish brain from EtOH-induced enhancement of AChE activity ($n=6$) (Fig. 1A).

Next, we evaluated the effect of taurine pretreatment on EtOH-induced AChE stimulation (Fig. 1B). A two-way ANOVA revealed a significant effect of taurine pretreatment×EtOH exposure interaction ($F(3,32)=3.63$, $P<0.05$). Post hoc analyses indicated that pretreatment with 150 and 400 $mg \cdot L^{-1}$ taurine prevented the effects promoted by EtOH in AChE activity.

To verify whether taurine alters AChE activity, the animals were treated with the same concentrations tested (42, 150, and 400 $mg \cdot L^{-1}$). The results demonstrated that AChE activity remained similar to control group ($n=4$).

Taurine prevents changes in antioxidant enzyme activities induced by EtOH

SOD and CAT play a key role in the control of reactive oxygen species (ROS) levels and in the prevention of oxidative damage. Acute exposure to EtOH significantly decreased SOD activity (49%, $n=3$) (Fig. 2) and concomitantly increased CAT activity (89%, $n=4$) (Fig. 3). The cotreatment with 400 $mg \cdot L^{-1}$ taurine prevented the EtOH-induced changes in SOD (Fig. 2A) and CAT activities (Fig. 3A) ($n=4$).

The influence of taurine pretreatment on the effects of EtOH in SOD (Fig. 2B) and CAT activities (Fig. 3B) was also evaluated. A two-way ANOVA revealed a significant effect of taurine pretreatment ($F(2,28)=6.15$, $P<0.05$) and EtOH exposure ($F(1,28)=4.5$, $P<0.05$). Post hoc analyses indicated that pretreatment with 150 and 400 $mg \cdot L^{-1}$ taurine prevented the EtOH-induced decrease in SOD activity. In relation to CAT activity, a two-way ANOVA revealed a significant effect of taurine pretreatment×EtOH exposure interaction ($F(2,19)=10.37$, $P<0.05$) and EtOH exposure ($F(1,19)=8.79$, $P<0.05$). Post hoc analyses indicated that pretreatment with 150 and 400 $mg \cdot L^{-1}$ taurine also prevented the EtOH-induced increase in CAT activity. Both taurine concentrations *per se* did not significantly alter SOD and CAT activities in zebrafish brain.
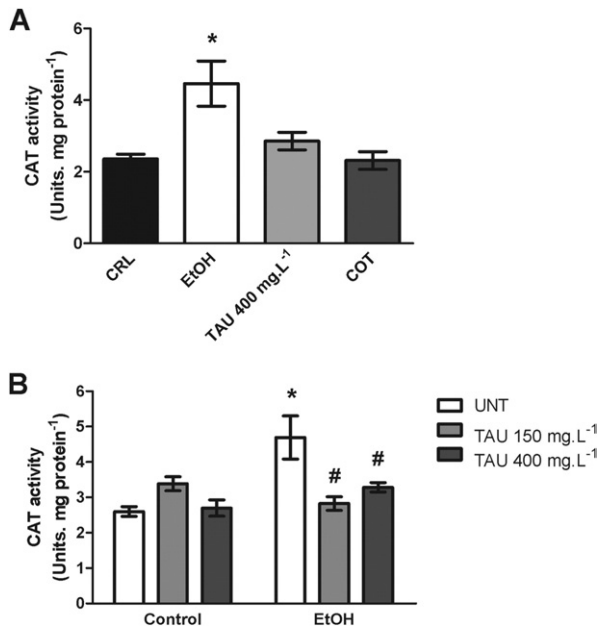
As depicted in Fig. 4, EtOH induced an imbalance in the SOD/CAT ratio ($n=4$; $P<0.05$). Both cotreatment (400 $mg \cdot L^{-1}$) (Fig. 4A) and pretreatment (150 and 400 $mg \cdot L^{-1}$) (Fig. 4B) with taurine reversed SOD/CAT ratio to the basal level ($n=5$).

Non-enzymatic antioxidant defenses are altered neither by EtOH exposure nor taurine treatments

The effects promoted by EtOH, taurine and both cotreatment and pretreatment on non-enzymatic antioxidant
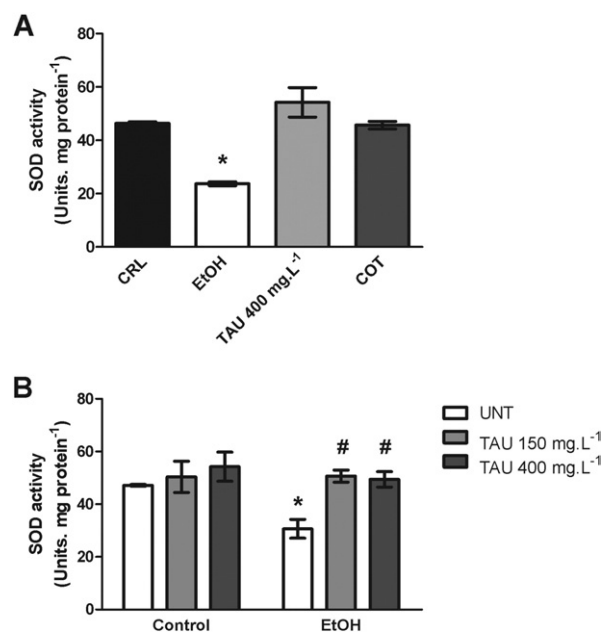
Fig. 2. Effect of EtOH and taurine treatments in SOD activity. (A) Cotreatment with taurine and EtOH. The groups were represented as control (CRL), ethanol (EtOH), taurine-treated (TAU) and taurine-cotreated animals (COT). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to control (one-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$). (B) Pretreatment with taurine followed by water (control group) or EtOH exposure. The treatments were represented as taurine-untreated (UNT) and taurine-treated (TAU). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to UNT from control group. # Significant difference compared to UNT from EtOH group (two-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$).

Fig. 3. Effect of EtOH and taurine treatments in CAT activity. (A) Cotreatment with taurine and EtOH. The groups were represented as control (CRL), ethanol (EtOH), taurine-treated (TAU) and taurine-co-treated animals (COT). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to control (one-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$). (B) Pretreatment with taurine followed by water (control group) or EtOH exposure. The treatments were represented as taurine-untreated (UNT) and taurine-treated (TAU). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to UNT from control group. # Significant difference compared to UNT from EtOH group (two-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$).

defenses were also evaluated. Neither EtOH exposure nor taurine treatments significantly changed TRAP (according to decreased radical production during TRAP experiment) and TAR in zebrafish brain ($n=3$) (data not shown).

Effect of taurine treatments on total reduced thiol content

EtOH, at the concentration and time of exposure tested, did not induce any significant changes in the total reduced thiol content of zebrafish brain (Fig. 5). Similarly, 150 and 400 mg·L^{-1} taurine did not alter total thiol content. Furthermore, the cotreatment with 400 mg·L^{-1} taurine did not significantly change the basal total reduced thiol content as compared to control and EtOH-treated groups ($n=4$) (Fig. 5A). However, a two-way ANOVA revealed a significant effect of EtOH exposure ($F_{(1,20)}=12.47$, $P<0.05$) and taurine pretreatment ($F_{(2,20)}=5.27$, $P<0.05$). Post hoc analyses indicated that pretreatment with 150 and 400 mg·L^{-1} taurine followed by EtOH exposure induced a significant increase in total reduced sulfydryl content (52% and 64%, respectively, $n=4$) as compared to control and EtOH-treated groups ($n=5$) (Fig. 5B).

Protective effect of taurine against EtOH-induced lipid peroxidation

To evaluate whether EtOH exposure alters lipid peroxidation in zebrafish brain, we assessed the formation of TBARS during an acid-heating reaction. Acute EtOH exposure significantly increased by 40% ($n=3$) the lipid peroxidation (Fig. 6). When the animals were treated with 150 and 400 mg·L^{-1} taurine, there was a significant decrease of TBARS levels (30% and 50%, respectively) as compared to the control ($n=3$). Interestingly, the cotreatment with taurine (400 mg·L^{-1}) did not prevent the increase of TBARS levels induced by EtOH ($n=5$) (Fig. 6A).

Regarding the taurine pretreatment experiment (Fig. 6B), a two-way ANOVA revealed significant effects of taurine pretreatment ($F_{(2,23)}=74.45$, $P<0.05$), taurine pretreatment×EtOH exposure interaction ($F_{(2,23)}=6.27$, $P<0.05$) and EtOH exposure ($F_{(1,23)}=4.75$, $P<0.05$). Post hoc analyses indicated that pretreatment with 150 and 400 mg·L^{-1} taurine prevented EtOH-induced enhancement of TBARS formation and the lipid peroxidation levels were statistically lower than that determined in control group (34% and 41%, respectively, $n=3$).
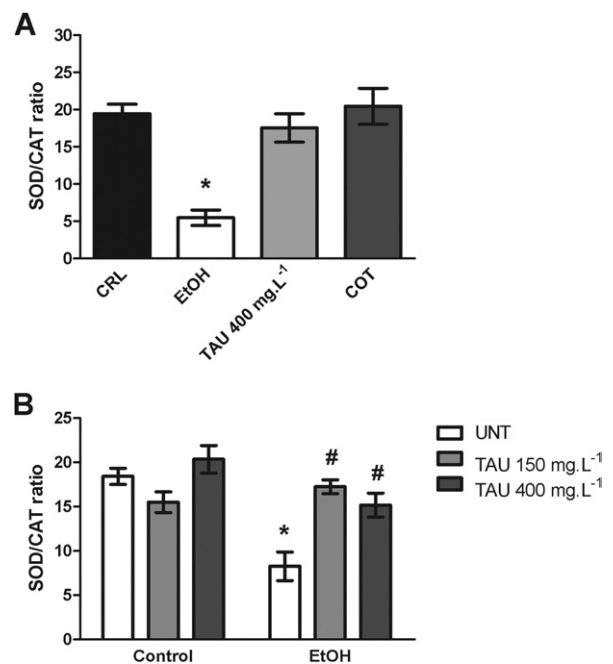
Fig. 4. Protective effect of taurine in SOD/CAT ratio. (A) Cotreatment with taurine and EtOH. The groups were represented as control (CRL), ethanol (EtOH), taurine-treated (TAU) and taurine-cotreated animals (COT). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to control (one-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$). (B) Pretreatment with taurine followed by water (control group) or EtOH exposure. The treatments were represented as taurine-untreated (UNT) and taurine-treated (TAU). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to UNT from control group. # Significant difference compared to UNT from EtOH group (two-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$).
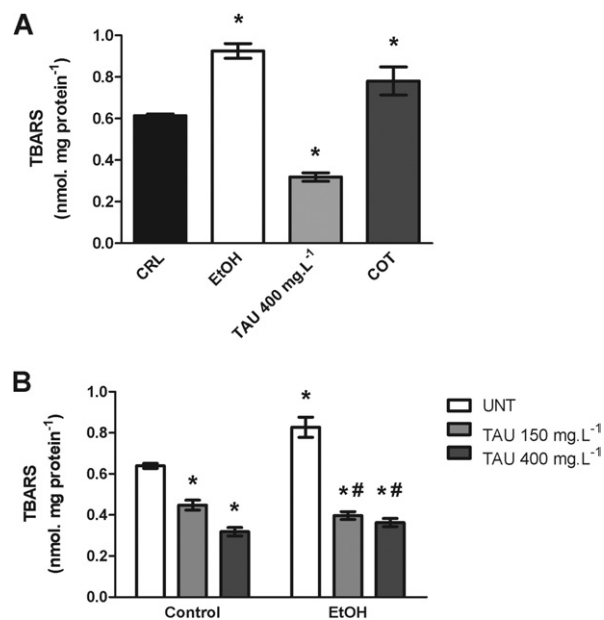
Fig. 5. Effect of EtOH and taurine on total thiol content in zebrafish brain. (A) Cotreatment with taurine and EtOH. The groups were represented as control (CRL), ethanol (EtOH), taurine-treated (TAU) and taurine-cotreated animals (COT). Data represent mean±SEM of at least three independent experiments. (B) Pretreatment with taurine followed by water (control group) or EtOH exposure. The treatments were represented as taurine-untreated (UNT) and taurine-treated (TAU). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to UNT from control group. # Significant difference compared to UNT from EtOH group (two-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$).

DISCUSSION

Studies have demonstrated that the behavioral changes induced by acute EtOH exposure in mammalian models are generally associated to its influence on several neurotransmitter systems (Hanchar et al., 2005; Belmeguenai et al., 2008). Due to the susceptibility of zebrafish to environmental and pharmacological manipulations and the characterization of distinct neurotransmitter parameters in this species, it is rapidly becoming a popular vertebrate model in behavioral and neuroscience research (Egan et al., 2009; Rico et al., 2010; Rosemberg et al., 2010a). It has been shown that acute EtOH exposure during 1 h in the tank water induced significant alterations in behavioral tasks of zebrafish and also increased the levels of dopamine, serotonin, and its metabolite 5-HIAA in whole brain extracts (Chatterjee and Gerlai, 2009). Zebrafish may be especially suitable for modeling the effects of EtOH because of the simplicity of alcohol delivery (Gerlai et al., 2000). The EtOH mixed in their environment is absorbed by the blood vessels of the gill and the skin of the fish so that blood alcohol levels reach equilibrium with the external alcohol concentration quickly (Gerlai et al., 2000; Dlugos and Rabin, 2003; Chatterjee and Gerlai, 2009). Despite to the relative differences in comparison to mammalian metabolism of EtOH ingestion, the alcohol mixed to the fish tank is rapidly diffused through systemic circulation and reaches distinct tissues. There is evidence suggesting that hepatic alcohol dehydrogenase and aldehyde dehydrogenase play a role in alcohol metabolism in fish, since 32 h of continuous 2% EtOH exposure caused characteristics signs of acute alcoholic liver disease in zebrafish larvae, including hepatomegaly, steatosis and changes in hepatic gene expression (Passeri et al., 2009). Furthermore, it has been shown that significant brain alcohol levels can be detected 15 min after exposure to 0.5% (v/v) EtOH in adult zebrafish brain, reaching a steady-state level that is maintained for at least 24 h without significant difference among distinct strains (Dlugos and Rabin, 2003). These data are in accordance with studies involving other fish species (Ryback et al., 1969; Galizio et al., 1985) in that, following a few hours of exposure, an equilibrium between the level of alcohol in the tank and EtOH content in brain was reached. Previous report demonstrated that the brain alcohol level of the zebrafish was achieved when brain alcohol levels were approximately 90% of the tank alcohol level (Dlugos and Rabin, 2003; Gerlai et al., 2006). Thus, it is comprehensive that the physiological responses promoted by acute EtOH exposure might involve alterations in neurotransmitter systems and also in oxidative stress parameters of zebrafish, suggesting that studies which comprise both approaches are tempting for the

Fig. 6. Effect of EtOH and taurine on lipid peroxidation measured as TBARS formation in zebrafish brain. (A) Cotreatment with taurine and EtOH. The groups were represented as control (CRL), ethanol (EtOH), taurine-treated (TAU) and taurine-cotreated animals (COT). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to control (one-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$). (B) Pretreatment with taurine followed by water (control group) or EtOH exposure. The treatments were represented as taurine-untreated (UNT) and taurine-treated (TAU). Data represent mean±SEM of at least three independent experiments. * Significant difference compared to UNT from control group. # Significant difference compared to UNT from EtOH group (two-way ANOVA followed by Bonferroni's test as post hoc, $P \leq 0.05$).

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assessment of potential beneficial effects against EtOH-mediated neurotoxicity.

In order to determine whether taurine could prevent the enhancement of AChE activity in zebrafish brain, we performed a cotreatment and pretreatment using different taurine concentrations during 1 h. The results demonstrated that only 400 mg·L^{-1} taurine prevented the increase of AChE activity when cotreated with EtOH. However, we further verified that the EtOH-induced enhancement of AChE activity was prevented by the pretreatment with 150 and 400 mg·L^{-1} taurine. Probably, the increase in AChE activity promoted by EtOH and the protective effect of taurine are not due to a direct mechanism, since both compounds did not alter the enzyme activity *in vitro* (data not shown). In this sense, the investigations related to the effects of taurine against EtOH-induced modifications in oxidative stress parameters were performed using the amino acid concentrations which prevented the enhancement of AChE activity for each treatment.

Previous studies have shown that ROS are products of normal cellular metabolism that act as important signaling molecules at low/moderate concentrations (Avshalumov et al., 2007; Chiarugi and Fiaschi, 2007). In this context, SOD and CAT activities normally have a synergism under physiological situations playing a key role in the fine-tuning regulation of superoxide anion (O$_2$ $^-$) and H$_2$O$_2$ levels. Our results demonstrated that even though EtOH did not induce significant changes in TRAP and TAR, the acute EtOH exposure significantly decreased SOD activity and increased CAT activity from zebrafish brain. Although these data seem contradictory, there are some possible explanations for these effects and even potential consequences. It is important to emphasize that EtOH is an exogenous drug that impairs distinct neurotransmitter systems and also promotes oxidative stress in several organisms. Hence, we suggest that an imbalance between oxidants and enzymatic/non-enzymatic antioxidant defenses might contribute to an increase of ROS and its harmful effects on living cells. Previous studies demonstrated that i.p. injections of EtOH led to an inhibition of SOD activity in rat brain (Ledig et al., 1981) and also that 400 mg.dL^{-1} EtOH reduced SOD activity in cerebellar granule cells (Siler-Marsiglio et al., 2004). Therefore, the significant decrease in SOD activity could be due to a direct effect of EtOH or an indirect effect mediated by its metabolite acetaldehyde. As a consequence, it is possible to suggest an increase of O$_2$ $^-$ after EtOH exposure which could mediate toxic effects by itself or via another ROS such as nitric oxide (NO), peroxynitrite (ONOO$^-$) and the radical OH (Halliwell and Gutteridge, 1984; Radi et al., 2002). Concerning CAT activity, it has been demonstrated that EtOH increased this enzyme activity in cerebellar granule cells (Siler-Marsiglio et al., 2004). In the same way, another study showed that EtOH orally administered in rats (2, 4, and 6 g.kg^{-1}) also promoted a significant increase of CAT activity in rat brain subcellular fractions (Reddy et al., 1999). There is evidence that CAT is the major enzyme responsible for oxidizing brain EtOH to acetaldehyde after alcohol consumption (Cohen et al., 1980; Swift, 2003).

This metabolite has been implicated to mediate central effects that follow EtOH exposure and also plays a key role in the mediation of psychopharmacological effects of EtOH (Pastor et al., 2002). Moreover, acetaldehyde is highly reactive and its neurotoxicity has been associated to the ability to form protein–acetaldehyde adducts or to induce structural chromosomal changes (Nakamura et al., 2003; Kayani and Parry, 2010). Besides the EtOH-induced increase in CAT activity initially appears to be protective, this alcohol also serves as a substrate of CAT, forming the toxic metabolite, acetaldehyde. Thus, CAT activity in the presence of EtOH could be a double-edged sword, ridding the cell of H$_2$O$_2$ while producing acetaldehyde. Finally, we demonstrated that SOD/CAT ratio was altered after acute EtOH exposure which suggests that EtOH induces oxidative damage in zebrafish brain. In contrast, both taurine cotreatment and pretreatment maintained SOD/CAT ratio at control levels, suggesting that this amino acid might exert a neuroprotective role by modulating enzymatic antioxidant defenses, which could be important for controlling the levels of O$_2$ $^-$, H$_2$O$_2$, and acetaldehyde in the CNS.

Lipid peroxidation is one important cause of neuronal damage and the major consequence of enhanced lipid peroxidation is attributed to the oxidative deterioration of cellular membranes (Kaizer et al., 2005). Our data showed that EtOH enhanced lipid peroxides, as measured by TBARS formation. Taurine, at 150 and 400 mg·L^{-1}, promoted a significant decrease in TBARS levels in a concentration-dependent fashion. When cotreated with EtOH, 400 mg·L^{-1} taurine did not reduce the EtOH-induced lipid peroxidation, whereas the pretreatment with 150 and 400 mg·L^{-1} taurine prevented the increase of TBARS maintaining the levels of lipid peroxidation significantly lower than the control group. Considering these data, it is possible to suggest that the decrease of lipid peroxidation could be correlated to the increase in total reduced thiol content detected after taurine pretreatment and alcohol exposure. Studies reported that taurine is known to attenuate tissue lipid peroxidation either by scavenging or quenching oxygen-derived free radicals, H$_2$O$_2$ or hypochlorous acid directly or by binding free metal ion species like Fe^{2+} or Cu^{2+} by its sulfonic acid group (Franconi et al., 2004; Hagar, 2004). In addition, it has been demonstrated that taurine treatment has been found to increase reduced glutathione (GSH) levels by directing cysteine into the GSH synthesis pathway (Schaffer et al., 2003; Hagar, 2004), which could result in the alterations observed in the reduced sulfhydryl content after the pretreatment with taurine and EtOH exposure.

Because AChE is anchored to the plasma membrane, there is evidence that lipid peroxidation can alter its activity (Flora et al., 2003; Kaizer et al., 2005). Previous study demonstrated that AChE activation could be correlated with an enhancement of lipid peroxidation (Kaizer et al., 2005). Similarly, our results showed that acute EtOH exposure increased AChE activity and lipid peroxides in zebrafish brain. However, lipid peroxidation is probably not the only explanation for the modulation of AChE activity, considering that the cotreatment with EtOH prevented the

enzyme activation but did not reduce TBARS levels. Our recent study showed that taurine increases AMP hydrolysis and concomitantly decreases adenosine deaminase activity in zebrafish brain membranes, suggesting a potential role of adenosine in taurine-mediated effects (Rosemberg et al., 2010b). Furthermore, it is known that taurine also modulates Ca^{2+} signaling pathways (Wu et al., 2005; Junyent et al., 2010), which are potential targets of ROS (Turrens, 2003). Although speculating about the physiological relevance of our data and also about the putative mechanisms involved in the potential neuroprotective of taurine in zebrafish are tempting, it must be acknowledged that both EtOH and taurine act through numerous neurotransmitters, second messenger systems and other molecular targets. Therefore, our speculations are only correlative, and conclusions regarding the precise molecular mechanism cannot be drawn at this moment. Briefly, the manipulation and/or the systemic assay of all possible neurotransmitter systems and also the exploration of other molecular changes, perhaps at gene expression level and/or transduction signaling pathways, could be interesting strategies to better understand the mechanistic details of the actions promoted by alcohol and taurine in zebrafish brain.

CONCLUSION

In conclusion, this is the first study demonstrating that acute EtOH exposure induced oxidative damage in adult zebrafish brain, which could be correlated to the EtOH-induced modifications in cholinergic signaling parameters and in behavioral tasks in this species. The impairment of the physiological synergism of SOD and CAT activities associated to an increase of lipid peroxides suggests an imbalance between O_2^- and H_2O_2 levels and that acetaldehyde could also play a role in mediating EtOH effects in zebrafish CNS. Moreover, our data report the first evidence that two different acute taurine treatments (cotreatment and pretreatment) prevented AChE activation, maintained SOD/CAT ratio at basal levels and differently changed total reduced sulfydryl content and TBARS formation. Therefore, we suggest that free radicals could in part be involved in the effects of alcohol on zebrafish brain function. There is certainly value in further examining the neural basis of the effects promoted by EtOH and taurine in this species—not only in terms of its face validity (in producing behavioral symptoms related to alcohol consumption) but also its construct validity in relation to modeling the underlying mechanisms related to the alcohol-mediated responses and also to the potential neuroprotective effect of taurine in adult zebrafish brain. In this sense, it is important to reinforce the idea that our data also provide implications for future studies. First, they open the possibility of analyzing the effects EtOH and taurine in oxidative stress parameters of distinct zebrafish strains. Furthermore, one would need to assess a potential functional role of taurine treatments against the neurochemical changes promoted by alcohol exposure in zebrafish by performing additional pharmacokinetic assays and testing

whether this amino acid can prevent/attenuate or even reverse EtOH-induced changes in different behavioral paradigms of this vertebrate. Nevertheless, our zebrafish assay may therefore complement existing approaches, bringing new insights related to black box screening and rational experimental design. The current paper also demonstrates that researchers now have yet another available tool which may help in solving the puzzle related to EtOH and taurine effects in zebrafish brain and allows that other questions might be addressed.

REFERENCES

Aebi H (1984) Catalase *in vitro*. Methods Enzymol 105:121–126.

Albrecht J, Schousboe A (2005) Taurine interaction with neurotransmitter receptors in the CNS: an update. Neurochem Res 30: 1615–1621.

Avshalumov MV, Bao L, Patel JC, Rice ME (2007) H2O2 signaling in the nigrostriatal dopamine pathway via ATP-sensitive potassium channels: issues and answers. Antioxid Redox Signal 9:219–231.

Banerjee R, Vitvitsky V, Garg SK (2008) The undertow of sulfur metabolism on glutamatergic neurotransmission. Trends Biochem Sci 33:413–419.

Barbazuk WB, Korf I, Kadavi C, Heyen J, Tate S, Wun E, Bedell JA, McPherson JD, Johnson SL (2000) The syntenic relationship of the zebrafish and human genomes. Genome Res 10:1351–1358.

Behra M, Etard C, Cousin X, Strähle U (2004) The use of zebrafish mutants to identify secondary target effects of acetylcholine esterase inhibitors. Toxicol Sci 77:325–333.

Belmeguenai A, Botta P, Weber JT, Carta M, De Ruiter M, De Zeeuw CI, Valenzuela CF, Hansel C (2008) Alcohol impairs long-term depression at the cerebellar parallel fiber-Purkinje cell synapse. J Neurophysiol 100:3167–3174.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Chatterjee D, Gerlai R (2009) High precision liquid chromatography analysis of dopaminergic and serotoninergic responses to acute alcohol exposure in zebrafish. Behav Brain Res 200:208–213.

Chen X, Sebastian BM, Tang H, McMullen MM, Axhemi A, Jacobsen DW, Nagy LE (2009) Taurine supplementation prevents ethanol-induced decrease in serum adiponectin and reduces hepatic steatosis in rats. Hepatology 49:1554–1562.

Chiarugi P, Fiaschi T (2007) Redox signalling in anchorage-dependent cell growth. Cell Signal 19:672–682.

Cohen G, Sinet PM, Heikkila R (1980) Ethanol oxidation by rat brain *in vivo*. Alcohol Clin Exp Res 4:366–370.

Dalitz P, Cock M, Harding R, Rees S (2008) Injurious effects of acute ethanol exposure during late gestation on developing white matter in fetal sheep. Int J Dev Neurosci 26:391–399.

Dlugos CA, Rabin RA (2003) Ethanol effects on three strains of zebrafish: model system for genetic investigations. Pharmacol Biochem Behav 74:471–480.

Draper HH, Hadley M (1990) Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol 186:421–431.

Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, Kalueff AV (2009) Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res 205:38–44.

Ellman GL (1959) Tissue sulphydryl groups. Arch Biochem Biophys 82:70–77.

Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95.

Flora SJ, Mehta A, Satsangi K, Kannan GM, Gupta M (2003) Aluminum-induced oxidative stress in rat brain: response to combined administration of citric acid and HEDTA. Comp Biochem Physiol C Toxicol Pharmacol 134:319–328.

Franconi F, Di Leo MA, Bennardini F, Ghirlanda G (2004) Is taurine beneficial in reducing risk factors for diabetes mellitus? Neurochem Res 29:143–150.

Furey ML, Ricciardi E, Schapiro MB, Rapoport SI, Pietrini P (2008) Cholinergic enhancement eliminates modulation of neural activity by task difficulty in the prefrontal cortex during working memory. J Cogn Neurosci 20:1342–1353.

Galizio M, Woodard RL, Keith J (1985) Effects of ethanol and naltrexone on aggressive display in the Siamese fighting fish, *Betta splendens*. Alcohol 2:637–640.

Gerlai R, Lahav M, Guo S, Rosenthal A (2000) Drinks like a fish: zebrafish (*Danio rerio*) as a behavior genetic model to study alcohol effects. Pharmacol Biochem Behav 67:773–782.

Gerlai R, Lee V, Blaser R (2006) Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (*Danio rerio*). Pharmacol Biochem Behav 85:752–761.

Gerlai R, Ahmad F, Prajapati S (2008) Differences in acute alcohol-induced behavioral responses among zebrafish populations. Alcohol Clin Exp Res 32:1763–1773.

Hagar HH (2004) The protective effect of taurine against cyclosporine A-induced oxidative stress and hepatotoxicity in rats. Toxicol Lett 151:335–343.

Halliwell B, Gutteridge MC (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1–14.

Hanchar HJ, Dodson PD, Olsen RW, Otis TS, Wallner M (2005) Alcohol-induced motor impairment caused by increased extrasynaptic GABA(A) receptor activity. Nat Neurosci 8:339–345.

Huxtable RJ (1992) Physiological actions of taurine. Physiol Rev 72:101–163.

Junyent F, Romero R, de Lemos L, Utrera J, Camins A, Pallàs M, Auladell C (2010) Taurine treatment inhibits CaMKII activity and modulates the presence of calbindin D28k, calretinin, and parvalbumin in the brain. J Neurosci Res 88:136–142.

Kaizer RR, Corrêa MC, Spanevello RM, Morsch VM, Mazzanti CM, Gonçalves JF, Schetinger MR (2005) Acetylcholinesterase activation and enhanced lipid peroxidation after long-term exposure to low levels of aluminum on different mouse brain regions. J Inorg Biochem 99:1865–1870.

Kang YS (2009) Downregulation of taurine transport by calcium blockers in osteoblast cells. Adv Exp Med Biol 643:513–521.

Kayani MA, Parry JM (2010) The *in vitro* genotoxicity of ethanol and acetaldehyde. Toxicol In Vitro 24:56–60.

Kong WX, Chen SW, Li YL, Zhang YJ, Wang R, Min L, Mi X (2006) Effects of taurine on rat behaviors in three anxiety models. Pharmacol Biochem Behav 83:271–276.

Kozlowski DJ, Chen Z, Zhuang L, Fei YJ, Navarre S, Ganapathy V (2008) Molecular characterization and expression pattern of taurine transporter in zebrafish during embryogenesis. Life Sci 82:1004–1011.

Ledig M, M'Paria JR, Mandel P (1981) Superoxide dismutase activity in rat brain during acute and chronic alcohol intoxication. Neurochem Res 6:385–390.

Lissi E, Salim-Hanna M, Pascual C, del Castillo MD (1995) Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity

from luminol-enhanced chemiluminescence measurements. Free Radic Biol Med 18:153–158.

Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247:3170–3175.

Nakamura K, Iwahashi K, Furukawa A, Ameno K, Kinoshita H, Ijiri I, Sekine Y, Suzuki K, Iwata Y, Minabe Y, Mori N (2003) Acetaldehyde adducts in the brain of alcoholics. Arch Toxicol 77:591–593.

Oja SS, Saransaari P (2007) Pharmacology of taurine. Proc West Pharmacol Soc 50:8–15.

Oliveira MW, Minotto JB, De Oliveira MR, Zanotto-Filho A, Behr GA, Rocha RF, Moreira JC, Klamt F (2010) Scavenging and antioxidant potential of physiological taurine concentrations against different reactive oxygen/nitrogen species. Pharmacol Rep 62:192–200.

Passeri MJ, Cinaroglu A, Gao C, Sadler KC (2009) Hepatic steatosis in response to acute alcohol exposure in zebrafish requires sterol regulatory element binding protein activation. Hepatology 49:443–452.

Pastor R, Sanchis-Segura C, Aragon CM (2002) Ethanol-stimulated behaviour in mice is modulated by brain catalase activity and H2O2 rate of production. Psychopharmacology (Berl) 165:51–59.

Peng J, Wagle M, Mueller T, Mathur P, Lockwood BL, Bretaud S, Guo S (2009) Ethanol-modulated camouflage response screen in zebrafish uncovers a novel role for cAMP and extracellular signal-regulated kinase signaling in behavioral sensitivity to ethanol. J Neurosci 29:8408–8418.

Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356.

Quertemont E, Grant KA, Correa M, Arizzi MN, Salamone JD, Tambour S, Aragon CM, McBride WJ, Rodd ZA, Goldstein A, Zaffaroni A, Li TK, Pisano M, Diana M (2005) The role of acetaldehyde in the central effects of ethanol. Alcohol Clin Exp Res 29:221–234.

Radi R, Cassina A, Hodara R, Quijano C, Castro L (2002) Peroxynitrite reactions and formation in mitochondria. Free Radic Biol Med 33:1451–1464.

Reddy SK, Husain K, Schlorff EC, Scott RB, Somani SM (1999) Dose response of ethanol ingestion on antioxidant defense system in rat brain subcellular fractions. Neurotoxicology 20:977–987.

Reimers MJ, La Du JK, Periera CB, Giovanini J, Tanguay RL (2006) Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants. Neurotoxicol Teratol 28:497–508.

Rico EP, de Oliveira DL, Rosemberg DB, Mussulini BH, Bonan CD, Dias RD, Wofchuk S, Souza DO, Bogo MR (2010) Expression and functional analysis of Na(+)-dependent glutamate transporters from zebrafish brain. Brain Res Bull 81:517–523.

Rico EP, Rosemberg DB, Dias RD, Bogo MR, Bonan CD (2007) Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain. Toxicol Lett 174:25–30.

Rosemberg DB, Rico EP, Langoni AS, Spinelli JT, Pereira TC, Dias RD, Souza DO, Bonan CD, Bogo MR (2010a) NTPDase family in zebrafish: nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart. Comp Biochem Physiol B Biochem Mol Biol 155:230–240.

Rosemberg DB, Kist LW, Etchart RJ, Rico EP, Langoni AS, Dias RD, Bogo MR, Bonan CD, Souza DO (2010b) Evidence that acute taurine treatment alters extracellular AMP hydrolysis and adenosine deaminase activity in zebrafish brain membranes. Neurosci Lett 481:105–109.

Ryback R, Percarpio B, Vitale J (1969) Equilibration and metabolism of ethanol in the goldfish. Nature 222:1068–1070.

Saransaari P, Oja SS (2000) Modulation of taurine release by metabotropic receptors in the developing hippocampus. Adv Exp Med Biol 483:257–264.

Sarter M, Bruno JP (2004) Developmental origins of the age-related decline in cortical cholinergic function and associated cognitive abilities. Neurobiol Aging 25:1127–1139.

Schaffer S, Azuma J, Takahashi K, Mozaffari M (2003) Why is taurine cytoprotective? Adv Exp Med Biol 526:307–321.

Siler-Marsiglio KI, Paiva M, Madorsky I, Serrano Y, Neeley A, Heaton MB (2004) Protective mechanisms of pycnogenol in ethanol-insulted cerebellar granule cells. J Neurobiol 61:267–276.

Swift R (2003) Direct measurement of alcohol and its metabolites. Addiction 98:73–80.

Turrens JF (2003) Mitochondrial formation of reactive oxygen species. J Physiol 552:335–344.

Wu H, Jin Y, Wei J, Jin H, Sha D, Wu JY (2005) Mode of action of taurine as a neuroprotector. Brain Res 1038:123–131.

II.4. CAPÍTULO IV

Differences in spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments.

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Differences in Spatio-Temporal Behavior of Zebrafish in the Open Tank Paradigm after a Short-Period Confinement into Dark and Bright Environments

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Abstract

The open tank paradigm, also known as novel tank diving test, is a protocol used to evaluate the zebrafish behavior. Several characteristics have been described for this species, including scototaxis, which is the natural preference for dark environments in detriment of bright ones. However, there is no evidence regarding the influence of ''natural stimuli'' in zebrafish subjected to novelty-based paradigms. In this report, we evaluated the spatio-temporal exploratory activity of the short-fin zebrafish phenotype in the open tank after a short-period confinement into dark/bright environments. A total of 44 animals were individually confined during a 10-min single session into one of three environments: black-painted, white-painted, and transparent cylinders (dark, bright, and transparent groups). Fish were further subjected to the novel tank test and their exploratory profile was recorded during a 15-min trial. The results demonstrated that zebrafish increased their vertical exploratory activity during the first 6-min, where the bright group spent more time and travelled a higher distance in the top area. Interestingly, all behavioral parameters measured for the dark group were similar to the transparent one. These data were confirmed by automated analysis of track and occupancy plots and also demonstrated that zebrafish display a classical homebase formation in the bottom area of the tank. A detailed spatio-temporal study of zebrafish exploratory behavior and the construction of representative ethograms showed that the experimental groups presented significant differences in the first 3-min vs. last 3-min of test. Although the main factors involved in these behavioral responses still remain ambiguous and require further investigation, the current report describes an alternative methodological approach for assessing the zebrafish behavior after a forced exposure to different environments. Additionally, the analysis of ethologically-relevant patterns across time could be a potential phenotyping tool to evaluate the zebrafish exploratory profile in the open tank task.

Introduction

The open field is the most used test for animal psychology studies in basic sciences. It consists basically of introducing an animal into a plain arena to observe its behavior across a specific range of time [1]. This test, usually performed with experimental rats or mice, provides an index of general behavior [2,3], and in particular, exploratory activity, which is a crucial response to novelty [4–6]. The initial responses to the open field test of adult rats consist in thigmotaxis and increased exploratory activity, which substantially decreases during the trial, reflecting an intra-session state of habituation [7–9]. The intra-session habituation involves spatial working memory and may also represent deeper neurobiological constructs, such as adaptive processing of sensory information and development of a cognitive map [10–12]. Furthermore, animals tend to establish during the test a key location (homebase), characterized as a ''safe'' place to which they repeatedly return after exploring the environment and spend more time during the trial [13,14]. Thus, the open field test offers a valuable and reliable test of activity and sequential (spatio-temporal) structure of the exploratory behavior [3,15], which emerges as an interesting tool that reveals the animal's interaction with a novel environment [16,17].

Zebrafish is becoming a popular animal model for behavioral neuroscience studies [18–20]. Although the use of zebrafish in behavioral research is increasing rapidly, the full potential offered by its use in these studies still needs further elucidation. Similar to the open field used for rodents, the novel tank diving test – also

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known as open tank paradigm – is emerging as a task for behavioral analysis in zebrafish. This test fundamentally consists in evaluate its vertical exploratory activity based on the tendency of this species to initially dive to the bottom and gradually swim to upper areas of the tank [18]. Several reports have been undertaken in order to characterize the zebrafish responses to novelty-based paradigms [16,21–24]. Recent data showed that zebrafish display a robust habituation response to novelty [12] and the establishment of a homebase [25]. Furthermore, pharmacological studies have demonstrated that anxiogenic and anxiolytic drugs can influence the habituation response to the novel tank test and induce changes in some endpoint behaviors, such as freezing, erratic movements, hyperactivity, and bottom-dwelling (or diving) [12,16,19,21–24,26,27]. However, due to the complexity of the behavioral repertoire displayed by adult zebrafish, the behaviors themselves still remain poorly understood [28].

A straightforward approach in the validation of behavioral measures for this species was performed using the bright/dark apparatus [28–31]. This task is characterized by the natural preference of zebrafish for dark environments (scototaxis), an innate feature previously suggested for its usefulness for the development of behavioral paradigms [32]. In fact, the bright/dark test allowed the organization of ethograms, which show relevant dimensions of defensive behavior [29,31]. This advance was taken for the first time by Blaser et al. [28], using the dark/bright tank in zebrafish. The confinement to each environment demonstrated that animals with a high avoidance of the bright side displayed substantial amount of freezing behavior when forcefully exposed to the bright chamber. Moreover, a recent study using a different protocol also demonstrated that animals forcefully exposed to the white compartment three consecutive times presented substantial differences in the behavioral repertoire observed in the light/dark tank [29]. Although these reports strongly suggest the aversion of zebrafish to bright environments, there is no data evaluating the effect of "natural stimuli" on the zebrafish behavior in the novel tank. Thus, it may be equally interesting to determine how the forced short-period exposure in two distinct preferred environments (dark vs. bright) affect their spatio-temporal exploratory activity [28,29,32].

Therefore, the aim of the current study was to investigate the spatio-temporal exploratory activity of the short-fin zebrafish phenotype in the novel tank test after a short-period confinement into dark and bright environments. The purpose to map the behavioral repertoire typically employed by the species in the open tank task lead us to suggest a standard exploratory profile for the confined groups.

Methods

Ethics statement

All procedures with animal subjects have been approved by the Ethics Committee for Use of Animals – CEUA from Universidade Federal do Rio Grande do Sul (protocol number 2008058).

Animals

Adult male and female zebrafish (*Danio rerio*) (4–6 months-old, ~50:50 male:female ratio) of heterogeneous wild-type stock (standard short-fin phenotype) were obtained from a local commercial supplier (Delphis, RS, Brazil). Fish were housed in 50-L aquariums (80–100 fish per aquarium) for at least 2 weeks prior to the experiments in order to acclimate to the animal facility. All tanks were filled with unchlorinated water previously treated with 132 μL.L^{-1} AquaSafe® (Tetra, VA, USA) and kept under mechanical and chemical filtration at a targeted tempera-

ture of $26\pm2°$C and water pH at 7.0–8.0. The room illumination was provided by ceiling-mounted fluorescent light tubes on a 12/12 light/dark photoperiod cycle (lights on at 7:00 am). Animals were fed twice a day until satiety with a commercial flake fish food (alcon BASIC®, Alcon, Brazil). All animals used in this study were experimentally naive, healthy and free of any signs of disease. They were maintained according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

Apparatuses and experimental procedures

The behavioral test was performed during the same time frame each day (between 10:00 am and 4:00 pm). All apparatuses were filled with water adjusted to home tanks conditions and the experimental procedures were performed in a stable surface with all environmental distractions kept to a minimum. A total of 44 animals obtained in five separate batches were used for independent behavioral experiments. The forced exposures to the different environments were performed during a 10-min period. Animals were randomly handled from their home tanks and individually transferred to the confinement cylinder (7.5 cm diameter×12.5 cm high) filled with 0.5 L of aquarium-treated water. Fish from both sexes (~50:50 male:female ratio) were used for each experimental group. For the bright confinement (white group, $n = 16$), fish were placed in a white-painted cylinder, whereas the dark confinement (black group, $n = 16$) was performed in a black-painted cylinder. Another group of fish was confined in a transparent cylinder (transparent group, $n = 12$), which closely resembled the home tanks. After the forced exposure period, animals were carefully removed from their respective confinement cylinder and placed in the novel tank where their behavioral activity was recorded. This apparatus consisted in a trapezoidal plastic tank (23.9 cm along the bottom×28.9 cm at the top×15.1 cm high and 15.9 cm along the diagonal side. It was 7.4 cm wide at the top, and tapered to 6.1 cm at the bottom) (**Figure 1A**) filled with 1.5 L of aquarium treated water. The dimension of the apparatus was similar to those previously described for the zebrafish novel tank test [12,16,21–24]. A webcam (Microsoft® LifeCam 1.1 with Auto-Focus) was placed 40 cm from the testing tank to ensure that the apparatus was within the camera vision range and it was used to monitor the location and swimming activity of the fish. Two yellow sheets of paper (standard letter size: 21.59 cm×27.94 cm) were placed 4.3 cm behind the tank to ensure a uniform background for the video analysis. In order to boost the contrast between the background and zebrafish, two 60-watts light bulbs were placed 40 cm behind the yellow screen. The webcam was plugged to a computer to record and analyze the videos using appropriate automated video-tracking software.

The trapezoidal tank was virtually divided into three equally horizontal areas (bottom, middle, and top) in order to evaluate vertical exploratory activity. To analyze the horizontal exploratory activity, the tank was also virtually divided into fifteen sections, with five sections per area as demonstrated in **Figure 1B**. Once the animals were placed in the novel test tank, the recording was started. Each subject was observed individually in a single session and the behavior was recorded over a period of 15 min. Before and after the test, oxygen levels in water of the apparatuses were measured and remained adequate during the experiment (8 ppm, Labcom Test®, SC, Brazil).

Behavioral analysis

The behavioral analysis was performed in a laptop computer using ANY-maze® software (Stoelting CO, USA) to track the swimming activity of the animals at a rate of 30 frames/sec. The video-tracking data were then used to determine relevant measures of vertical exploration across time, such as time spent per area and

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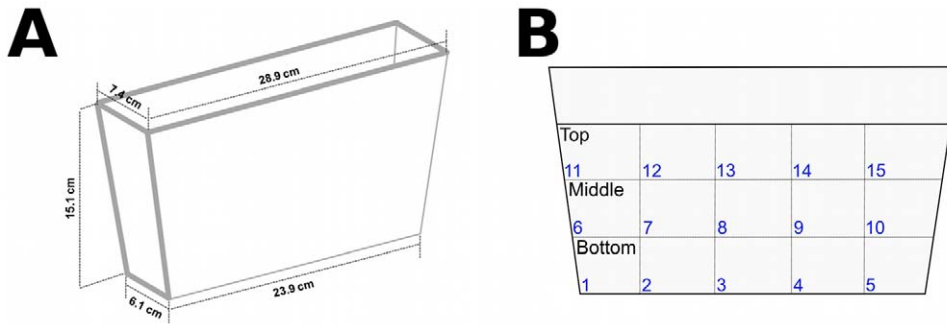
Figure 1. The novel tank. (A) The apparatus consisted in a trapezoidal plastic tank with the specific dimensions described above. (B) Virtual divisions were used for evaluation of zebrafish swimming activity in the novel tank diving test, with three vertical areas (bottom, middle, and top) and fifteen horizontal sections (1–15), with five sections per area.
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transitions to each area. Moreover, some endpoint behaviors were measured during the test, including distance travelled, absolute turn angle, meandering, average speed, and time mobile. The absolute turn angle represents the sum of all vectors angle of movements created from one position to animal's center point to the next. The anti-clockwise movement was considered negative and clockwise movement positive ($-180°$ to $180°$C). From this measure we calculated the meandering, which is the result of the absolute turn angle divided by the total distance travelled. In addition to the time spent, number of transitions, and the latency to middle and top area transitions, the vertical exploratory activity was assessed by measuring, in each area, the distance travelled, absolute turn angle, and meandering. The evaluation of the horizontal exploratory activity of zebrafish was performed by determining the time spent in each section per area and the number of transitions between sections per area. The ratio between the number of transitions per section and number of transitions per area was calculated to estimate the exploratory profile of fish considering both horizontal and vertical parameters (≤ 1 values predominantly characterize vertical exploration in each section, whereas >1 values indicate the predominance of horizontal exploration in the respective section). The distribution of the animals during the novel tank test was also evaluated by representative tracks, occupancy plots, and 3D reconstruction graphs. To establish a general profile of the exploratory activity, we created representative ethograms from each confined group by analyzing the 6-min behavioral responses. These ethograms were analyzed more specifically by comparing the first 3-min vs. last 3-min of test, which allowed a detailed evaluation of the exploratory activity of zebrafish during the intra-session habituation period [12].

3D Track reconstruction across time

The spatio-temporal analysis of zebrafish behavior in the novel tank diving test was also performed using track reconstruction across time as described previously [16,24]. Briefly, the videos were analyzed using the ANY-maze® software with the coordinates of the experimental tank properly calibrated. The track data for each fish was exported as raw data into separate spreadsheets, providing spatial coordinates (x center and y center) across a time scale broken down into fractions of a second. The exported traces were analyzed based on similarity to each other by two trained observers (inter-rater reliability >0.85), on a consensus basis. The middle trace was selected as representative for the group, to illustrate the pattern of exploration (first 3-min vs. last 3-min of test). Spatio-temporal 3D reconstructions were created with Graphis 3D graphing software® in which the x center (horizontal

distribution), y center (vertical distribution), and time were plotted on the X-, Z- and Y-axis, respectively.

Statistics

Data were expressed as mean $\pm$ standard error of the mean (S.E.M.) and p-values were considered significant for $p \leq 0.05$. All behavioral parameters evaluated across time were analyzed by repeated-measures analysis of variance (ANOVA). The endpoint behavioral measures for vertical and horizontal exploration, homebase parameters, and the exploratory profile (transitions ratio) were analyzed by two-way ANOVA. Comparison among means was carried out using Bonferroni's test as post hoc. The basic data of general locomotor activity (distance travelled, average speed, absolute turn angle, meandering, and time mobile) and the comparison of homebase parameters between the experimental groups were analyzed by one-way ANOVA, followed by Bonferroni's test as post hoc.

Results

Vertical exploration

In the 15-min novel tank test (**Figure 2**), the black, white, and transparent cylinder-confined groups showed a characteristic pattern of duration in the different areas (bottom, middle and top) and in transitions between these vertical areas across time. A 3×15 (Color$\times$Time) repeated-measures ANOVA was used to analyze the duration in each of the three vertical areas. We observed that the duration in the bottom decreased across the 15-min test (F [14,660] = 9.18, $p < 0.0001$) and both black and transparent-confined groups spent significantly more time in the bottom than the white-confined group (F [2,660] = 7.33, $p < 0.005$). Moreover, the time spent in the bottom area dropped faster in the white-confined group than in the other experimental groups (F [28,660] = 2.12, $p < 0.05$). Although there was no significant effects of any variable on time in the middle area, the duration in the top increased significantly across the 15-min test (F [14,660] = 10.23, $p < 0.0001$). Both black and transparent-confined groups spent significantly less time in the top than the white-confined group (F [2,660] = 5.83, $p < 0.01$). Finally, the time spent in the top increased faster in the white-confined group than in the black and transparent-confined groups (F [28,660] = 2.12, $p < 0.05$). Regarding the number of transitions between the three vertical areas, the animals displayed few entries to the middle and top areas (F [14,660] = 4.25, $p < 0.0001$; and F [14,660] = 4.12, $p < 0.0001$; respectively) in the first minute when compared to subsequent minutes of test.
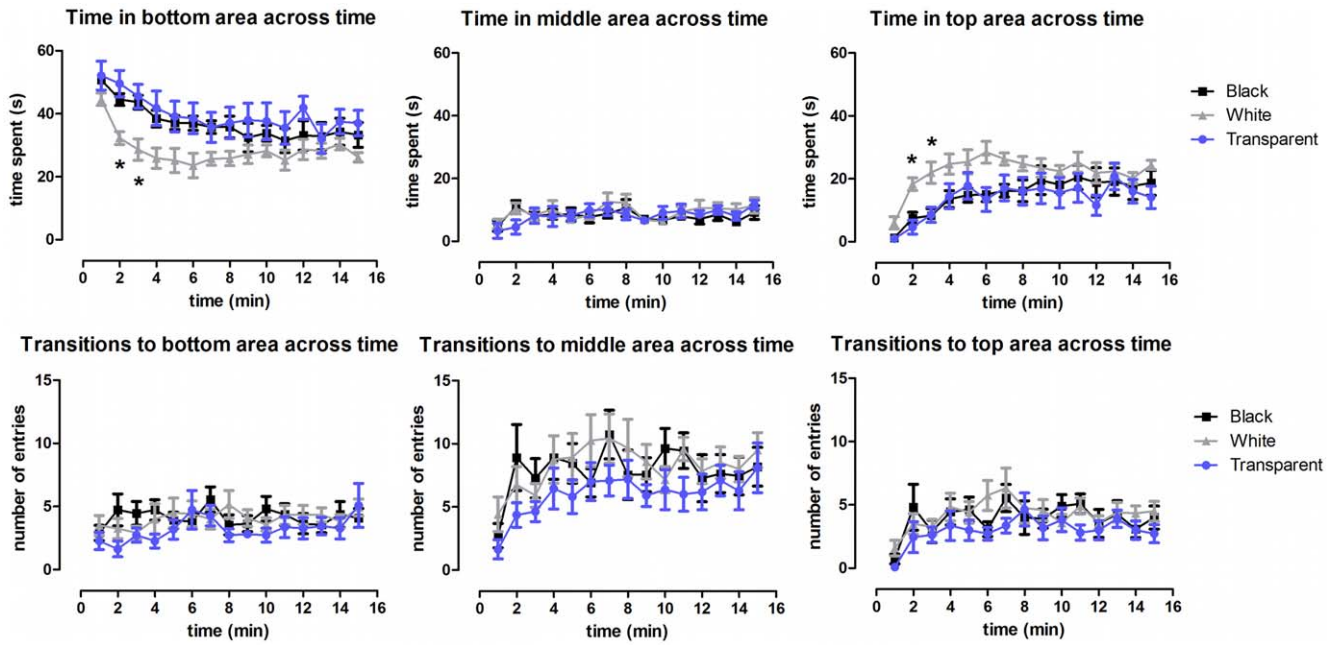
Figure 2. Vertical exploration of zebrafish previously confined into dark, bright, and transparent environments. The exploratory activity in each vertical area (bottom, middle, and top) was assessed during a 15-min trial and the time spent and number of transitions per area were shown. * Significant difference between black/transparent and white cylinder-confined groups (repeated-measures ANOVA followed by Bonferroni's test as post hoc, $p \leq 0.05$).
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Horizontal exploration

Since the time spent in bottom and top areas for animals previously exposed to dark, bright, and transparent environments reached a plateau after 7 min, further behaviors were assessed using the initial 6-min period. The horizontal exploratory activity was analyzed by two-way ANOVA using the duration of time spent and the transitions between each horizontal section (**Figure 3**). In general, animals spent more time in central sections of the bottom area (2, 3 and 4), while this preference for the center was less evident in the middle and top areas.
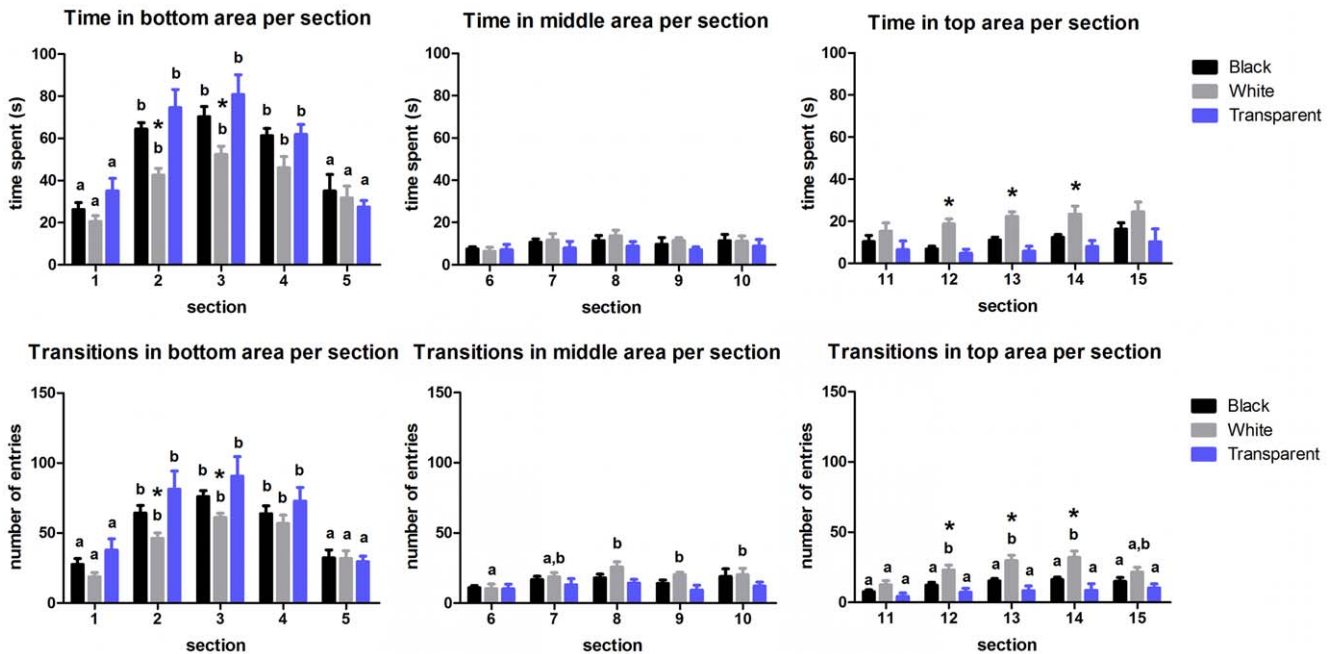
Figure 3. Effect of the confinement in the horizontal exploratory activity of zebrafish in the open tank. * Significant difference between black/transparent and white cylinder-confined groups. Distinct letters mean statistically significant differences within groups (two-way ANOVA followed by Bonferroni's test as post hoc, $p \leq 0.05$).
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Additionally, both black and transparent-confined fish spent significantly more time in the central sections of the bottom area than did white-confined animals, while the white-confined fish spent significantly more time in the central sections of the top area. The same pattern of results was observed for transitions between sections in each area.

Representative occupancy plots across time were constructed (**Figure 4A**), as well as a detailed 3D reconstruction of behavior (**Figure 4B**), which illustrate the differences between the three groups in terms of both lateral and vertical exploration (**see video S1**).

Endpoint behaviors

The general basic behaviors, such as total distance travelled, absolute turn angle, meandering, and average speed did not significantly differ between the experimental groups (**Figure 5A**). It is interesting to mention that animals did not freeze at all during the novel tank test; they explored the apparatus during the entire 15-min trial and travelled a constant distance across time.

These endpoint behaviors were then filtered by vertical location and analyzed using two-way ANOVA (**Figure 5B**). Total swim distance, absolute turn angle, and duration in each area showed identical patterns of results: they were significantly higher in the bottom than in the middle or top areas. Additionally, they were significantly higher in the bottom area for black and transparent-confined animals, and significantly higher in the top area for white-confined animals. Meandering and transition frequency did not change between groups. However, latency to enter in the top was significantly shorter in the white-confined group than in the black and transparent-confined groups.

Homebase formation

Representative endpoint data (**Figure 6A**) illustrate the differences in the swimming traces among the areas and sections and also show that all groups spent significantly more time in the bottom area than the middle or top. These data allowed us to identify a classical homebase formation for the short-fin zebrafish strain in the novel tank test (middle sections of the bottom area). In this place, the animals travelled a greater distance, spent the most part of the test, and also performed a considerable number of entries. Fish confined into the white cylinder exhibited a significant decrease in all homebase parameters as compared to the groups forcefully exposed to the black and transparent cylinders (**Figure 6B**).

The analysis of the homebase parameters across time by a 3×6 (Color$\times$Time) repeated-measures ANOVA showed that the duration and distance travelled in the homebase decreased across the 6-min test (F [5,264] = 11.25, $p<0.0001$ and F [5,264] = 4.66, $p<0.001$), respectively. The confinement into distinct environments also promoted significant differences in the time spent (F [2,264] = 4.35, $p<0.05$) and distance travelled in the homebase (F [2,264] = 4.90, $p<0.05$), which were significantly lower for the white cylinder-confined group during the 5th and 6th minutes of test. However, no significant differences in the number of homebase transitions between groups (**Figure 6C**) were observed. In the 15-min test, all homebase parameters remained similar to those observed in the final of the 6-min analysis (data not shown).
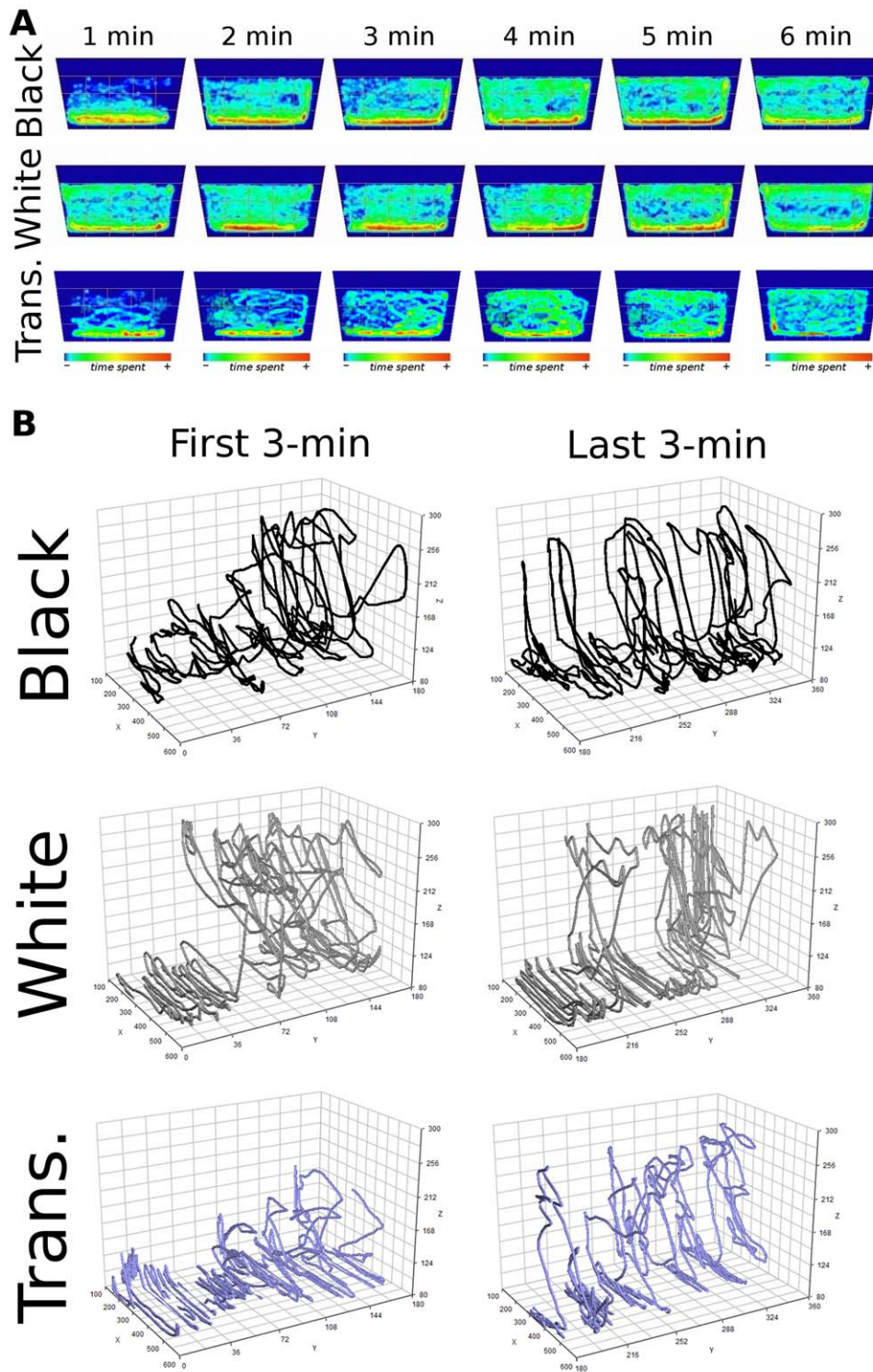
Spatio-temporal patterns of behavior

The relative exploratory activity across both dimensions of the novel tank was estimated by calculating the ratio of transitions between horizontal sections to transitions between vertical areas (**Figure 7A**). These ratios, analyzed across time (**Figure S1**), were then used to create representative visual diagrams (ethograms) that reflect frequencies and transitions between each individual behavioral activity [24,33–35] and to characterize the overall spatio-temporal exploratory pattern during the trial. Ethograms for the black, white, and transparent cylinder-confined groups were generated for the novel tank test during the first 3-min and last 3-min of the test (**Figure 7B**). The diameter of each circle corresponded to the frequency of each individual behavioral activity, while the arrow width and direction reflected the frequency of transitions between these behaviors. This ethological analysis allowed us to define the differences in the main behaviors presented by the experimental groups during the intra-session habituation period to the open tank, such as homebase swimming, lateral exploration, and transition swimming between bottom and top areas (**see video S1**).

Discussion

The main finding of this study is that a short-period confinement into dark and bright environments induces differences in the spatio-temporal structure of zebrafish behavior in the open tank paradigm. Previous studies demonstrated the usefulness of the novel tank test to evaluate the vertical exploration of fish after exposure to several drugs [21,23,24,27]. However, since adult zebrafish has been consolidated as an emergent vertebrate model in behavioral neuroscience research [28–31], it becomes reasonable to evaluate the effect promoted by "natural stimuli" in the behavioral repertoire of fish subjected to the open tank paradigm. The protocol consisted in confining the animals into a black or a white cylinder (dark vs. bright environments) during 10 min prior to the novel tank test. Additionally, another group of fish was confined into a transparent cylinder (transparent environment), which closely resembled the home tanks. Our results showed that all groups steadily increased their vertical exploratory activity within the first 6-min of the test, reaching a plateau after the 7th minute. These data corroborate with previous findings which demonstrated a rapid habituation response of zebrafish in the novel tank test [12,23,36]. Our results support the hypothesis that the behavioral manifestation of habituation responses to novelty in zebrafish is different from that of rodents [12]. Instead of a reduced locomotion when rodents become familiar with the novel environment [37,38], zebrafish appears to do the opposite. Furthermore, motor and posture patterns that are known to be exhibited in the open tank trial, such as freezing and erratic movements [12,16,18,19], were absent during our test. Studies demonstrated that both behaviors may significantly decrease over the habituation course to the novel tank [12,24], or even occur with an extremely low frequency during the trial [18,19,39]. It is likely that several factors can explain these discrepancies, including differences in testing apparatuses (e.g. light intensity for the tracking) and in the protocol used (e.g. isolating the fish before the behavioral test). Thus, our data suggest that the time spent in the top and the number of transitions to top area across time may be better behavioral indicators of habituation response to the open tank.

Although we observed a rapid habituation response to novel tank test in all experimental groups, the confinement into bright environments altered the intra-session habituation response. Such as many teleosts, zebrafish displays a natural preference for dark environments in opposition to brightly lit ones, named scototaxis [29,40]. Studies have been suggested that it represents a typical defensive pattern of species that exploits crypsis with the substratum as a strategy for predator avoidance [31,40]. This task has already been validated at construct level for zebrafish [29–31] and recent pharmacological data also give a robust

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Figure 4. Comparison of the spatio-temporal behavior of the experimental groups in the novel tank test. (A) Representative occupancy plots of black, white, and transparent-confined groups displaying the specific patterns of time spent in each segment of the apparatus across time. Data were analyzed using video-tracking software (ANY-maze®, Stoelting CO, USA). (B) Representative 3D reconstructions of zebrafish swimming activity during the first 3-min vs. last 3-min of test obtained by plotting animal traces across the time. The X-, Z- and Y-axis represent the horizontal distribution, vertical distribution, and time, respectively.
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support for its predictive validity [41]. Additionally, Lau et al. [42] demonstrated that fish that highly avoided a bright image presented a significant activation of the medial zone of the dorsal telencephalic region (Dm) and the dorsal nucleus of the ventral telencephalic area (Vd), which is anatomically homolog to the mammalian amygdala and striatum, respectively. It has been

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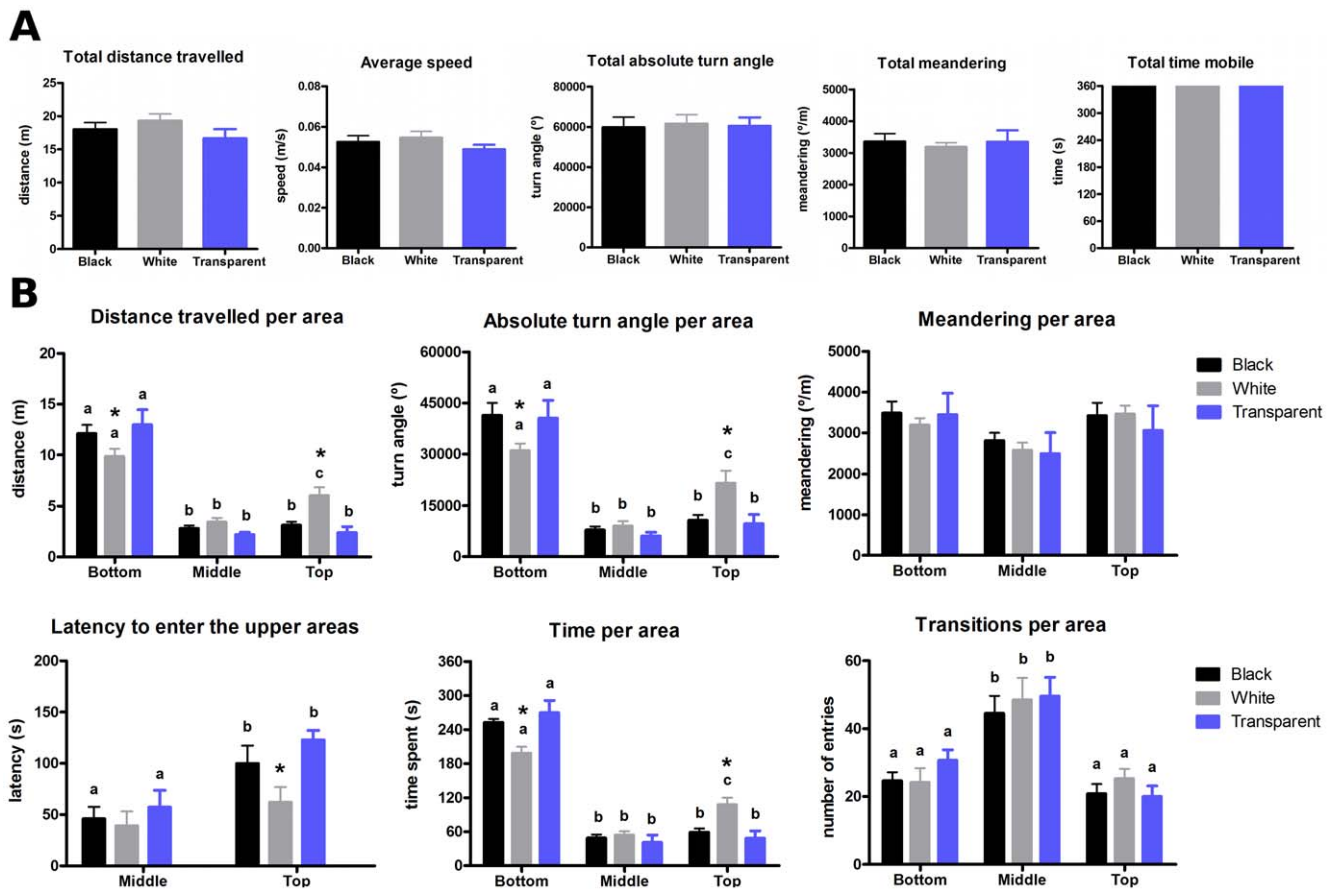
Figure 5. Basic endpoint behaviors of the experimental groups in the novel tank test during 6 min. (A) The graph shows total distance travelled, average speed, absolute turn angle, meandering, and time mobile. Data were analyzed by one-way ANOVA followed by Bonferroni's test as post hoc, considering $p \leq 0.05$ as significant. (B) Endpoint parameters of zebrafish behavior filtered by each vertical area (bottom, middle, and top) of the novel tank. * Significant difference between black/transparent and white cylinder-confined groups. Distinct letters mean statistically significant differences within groups (two-way ANOVA followed by Bonferroni's test as post hoc, $p \leq 0.05$).
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shown that the scototaxis test did not present intra- or inter-session habituation of white avoidance [29], even though the authors could not reliably record the vertical distribution of zebrafish in the apparatus due to technical difficulties (e.g. in this task recordings must be made from top). Since zebrafish display a natural preference for dark environments, it is interesting that animals confined to the white cylinder habituate to the novel tank more rapidly than those confined to the black and the transparent cylinders. On the assumption that the white chamber is aversive, the obvious prediction is that white-confined fish should habituate less readily to the novel tank – a prediction which is inconsistent with our data. The faster habituation of the white-confined group is difficult to interpret, and highlights the need for a clearer understanding of the interaction between motivational state and vertical exploratory behavior in zebrafish. In the open tank trial, the total distance travelled, absolute turn angle, meandering, average speed, and time mobile did not change between the experimental groups, which indicate that the general locomotor activity of fish remained unaltered after the forced exposure to distinct environments. To better understand the nature of this effect, a more detailed evaluation of the spatio-temporal exploratory behavior across the intra-session habituation period was undertaken.

The sub-division of the novel tank in different sections allowed the estimation of the exploratory profile of the dark, bright, and

transparent groups by considering the exploration of fish in both dimensions of the apparatus. In all three groups, fish show significantly more horizontal (lateral) exploratory activity in the central sections of bottom, whereas the middle area was mainly used for vertical transitions, in which animals practically did not explore. However, the top area ratio suggests that white-confined fish showed more lateral exploration in the upper portion of the tank than black and transparent-confined fish. These data were confirmed by representative track and occupancy plots, and suggest a characteristic homebase formation in the open tank paradigm by all three groups. The homebase is defined as a place in the field for which the experimental animal shows a preference across time, both in terms of occupancy and as a starting and ending point of exploratory excursions [14]. It has been shown that similarly to rodent behavior, zebrafish display a typical homebase formation in novelty-based paradigms [25]. Our 6-min observation period demonstrated that the white-confined group differed significantly on all homebase parameters assessed. The analysis of homebase behavior across time demonstrated that even though the transitions to the homebase remained virtually unaltered, fish confined into the white cylinder travelled a shorter distance and spent less time in the homebase during the 5th and 6th minutes. These data show that animals confined into the bright environment transit more rapidly out of homebase behaviors than those confined into the dark and transparent

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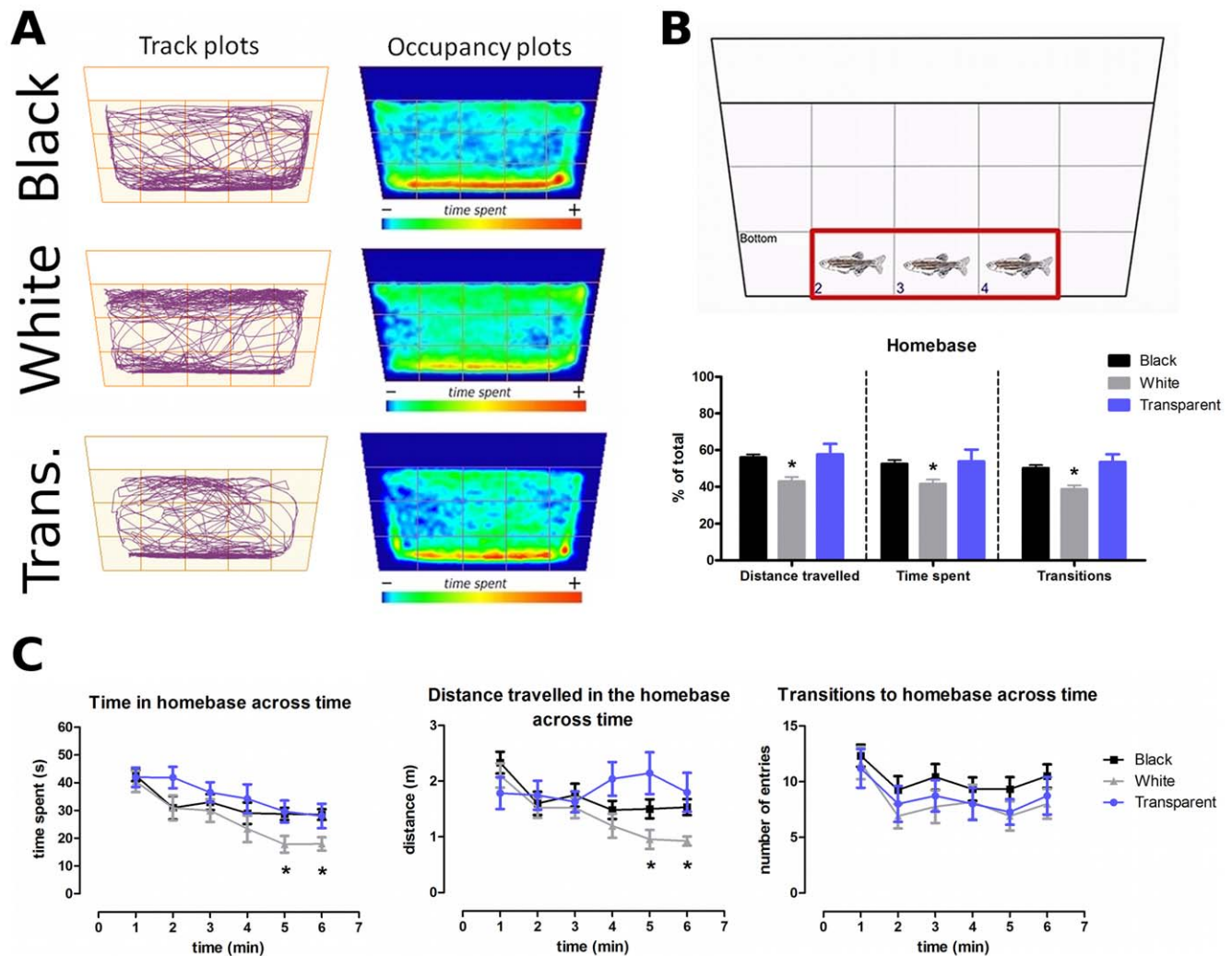
Figure 6. Overall exploratory activity and homebase formation of the experimental groups in the open tank. (A) Representative track and occupancy plots of the experimental groups obtained by video-tracking software (ANY-maze®, Stoelting CO, USA), displaying the specific patterns of their exploratory behavior during 6 min. (B) Zebrafish displays a classical homebase formation in the central sections of bottom area during the 6-min trial. Basic endpoint behaviors in the homebase were compared for black, white, and transparent cylinder-confined animals. * Significant difference between black/transparent and white cylinder-confined groups (one-way ANOVA followed by Bonferroni's test as post hoc, $p \leq 0.05$). (C) Evaluation of behavioral parameters of zebrafish in the homebase across time. * Significant difference between black/transparent and white cylinder-confined groups (repeated-measures ANOVA followed by Bonferroni's test as post hoc, $p \leq 0.05$).
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environments. It is possible that, additionally to being a reference point for the exploratory incursions, the homebase reflects a behavioral state comparable to thigmotaxis, and that confinement to the white cylinder disrupted this behavior. In this regard, Maximino et al. [29] showed that confining animals thrice in the white compartment prior to the scototaxis experiment does not alter spatio-temporal measures of preference, but decrease the frequency of burst swimming, freezing and thigmotaxis in the white compartment, suggesting that this treatment could diminish fear.

Our apparent inconsistency between predicted aversion to the white, and subsequent exploratory behavior in the novel tank may also support at dissociation between the mechanisms of black/white preference and novel tank diving behavior [43]. For example, the novel tank seems to be sensitive to diazepam, but not to chlordiazepoxide, while the scototaxis test is sensitive to other benzodiazepines as well [41]. Both behavioral paradigms also present different sensitivities to fluoxetine [23,41] and the

light/dark tank shows a lack of sensitivity for moclobemide, a MAO-A inhibitor [41]. These apparently conflicting data provided by pharmacological manipulations suggest that the two paradigms may not assess the same underlying state. Although the current study provides a detailed account of zebrafish behavioral repertoire in the open tank, further experimentations using alternative methodological approaches will be required to understand how these behaviors relate to that observed in the black/white preference task, and the neural mechanisms involved in each.

The spatio-temporal 3D reconstructions across the intra-session habituation period (first 3-min vs. last 3-min of test) showed that the white-confined fish displayed a wider distribution in the novel tank during the first 3-min of test than black and transparent-confined groups. These 3D reconstructions of behavior have several important advantages over 2D traces because they provide a more "realistic" representation of the fish swimming activity including their lateral movements. A recent study provided a
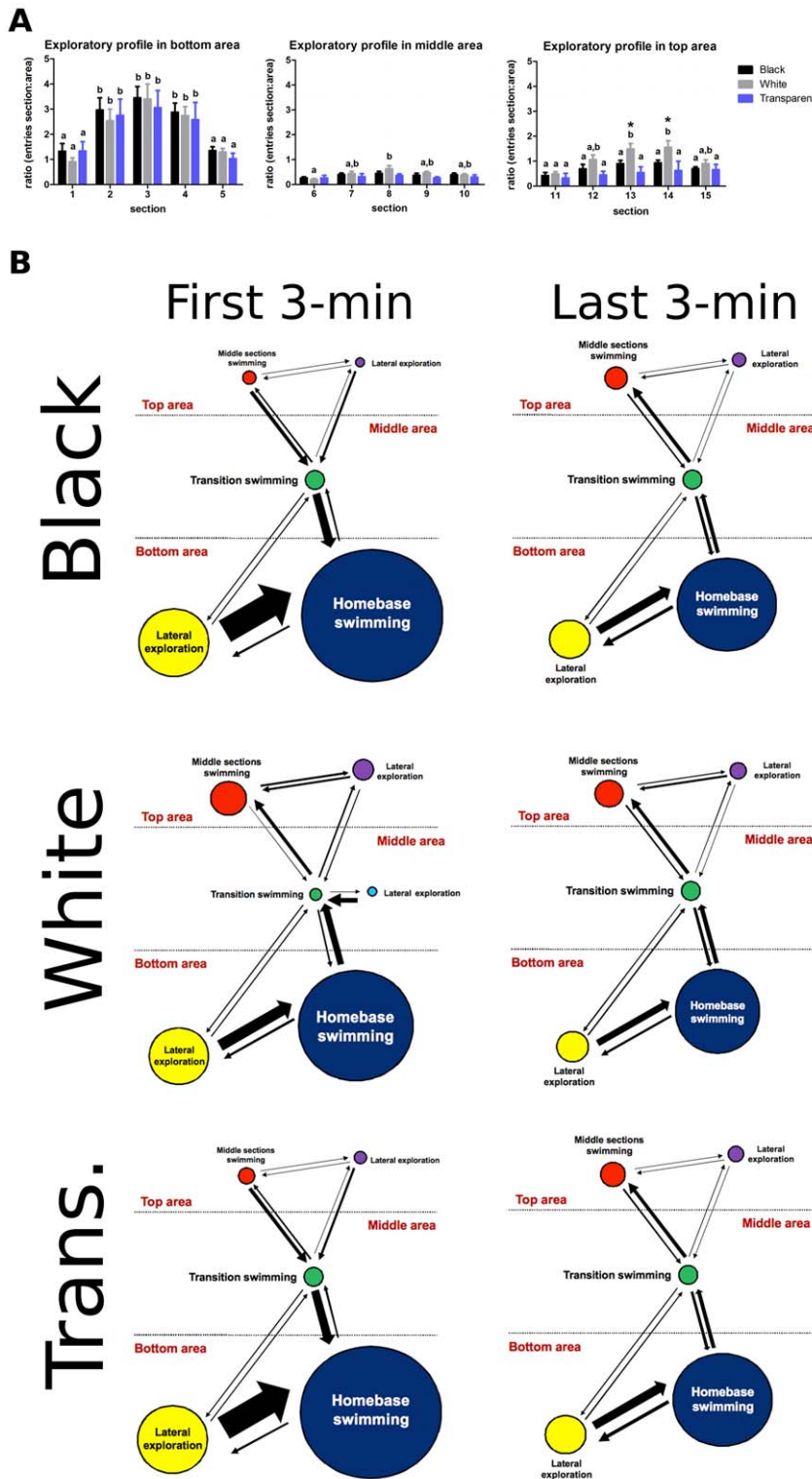
Figure 7. Behavioral profile of zebrafish in the open tank after the environmental manipulations. (A) The exploratory profile of dark, bright, and transparent-confined groups was determined by the ratio between the total transitions between sections and the number of entries in the respective area. * Significant difference between black/transparent and white cylinder-confined groups. Distinct letters mean statistically significant differences within groups (two-way ANOVA followed by Bonferroni's test as post hoc, $p \leq 0.05$). (B) Distinct behavior patterns displayed by zebrafish in the novel tank task after the short-period confinement into different environments. The ethological profiles were constructed by specifically analyzing the exploratory behaviors presented during the intra-session habituation period to the open tank. Representative ethograms were generated based on frequencies and transitions between each individual behavioral activity. The diameter of each circle corresponds to the frequency of each individual behavioral activity, whereas the arrow width and direction reflect the frequency of transitions between these behaviors.
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detailed evaluation of three-dimensional neurophenotyping of adult zebrafish behavior [44]. The authors demonstrated that the temporal reconstructions may significantly differ after pharmacological treatments, which allowed the organization of distinct behavioral clusters. This analysis of the swimming pattern has already been applied to create accurate predictive models of medaka fish movement based on high-density trajectory data sets [45,46]. In addition to the 3D data, we addressed for the first time a new insight of analysis provided by occupancy charts, taking into account not only the distribution, but also the time spent by the fish in each part of the apparatus across time. Thus, the association of both methodologies is a powerful tool which helps to characterize the exploratory profile of zebrafish after environmental manipulations into quantitative models.

Using descriptive ethological diagrams, based on mean frequency, duration and latency of every behavioral pattern, we provided an overview of the spontaneous behavioral patterns displayed by the experimental groups in the open tank. The substantial differences detected in the first 3-min vs. last 3-min of test reflect that the intra-session habituation response to the novel tank involves changes in these behaviors over the course of the test (**see video S1**). The similarities in the ethograms detected for both black and transparent-confined group strongly suggest that it truly is the confinement to white that is affecting the zebrafish behavior away from the baseline.

Perspectives of the ethological analysis of zebrafish behavior

In conclusion, this study provided detailed approaches to evaluate the spatio-temporal swimming activity and homebase formation of zebrafish during their intra-session habituation period to the novel tank test after a forced exposure to black, white, and transparent cylinders. Since naturalistic approaches may have an important place in research to better understand the biological mechanisms of the behavioral responses in vertebrates [47], the current report supports the idea that zebrafish is undoubtedly a potential animal model for translational research. It must be emphasized that future studies using different protocols could be relevant to further elucidate underlying factors that contribute to the behavioral repertoire observed. One might access the effect promoted by a large spectrum of drugs in the short-period confinement and further subject the animals to the novel tank test. However, researchers are cautioned, at this time, to interpret these data carefully, since the exact significance of the behaviors is not fully understood and little empirical evidence is available to support the validity of the behavioral measures in the open tank [28–31]. Furthermore, the current report presented a new analysis of behavioral data by occupancy plots, a quantitative

approach for determining the fish exploratory profile, and a detailed ethological analysis in the novel tank. These data help to clarify the ethological network and also bring new insights regarding the validation of spontaneous exploration models. Consequently, the analysis of the overall structure of behavior across time in the open tank task suggests that this paradigm can also be a valuable tool to analyze zebrafish behavioral responses after distinct environmental manipulations.

Supporting Information

Figure S1 Spatio-temporal analysis of the exploratory profile of dark, bright, and transparent groups. (A) Ratio between the total transitions per sections and the number of entries in the respective area during each minute of the trial. * Significant difference between black/transparent and white cylinder-confined groups. Distinct letters mean statistically significant differences within groups (two-way ANOVA followed by Bonferroni's test as post hoc, $p \leq 0.05$). (B) Representative diagrams demonstrating the transitions per minute estimated by the ratio analysis. The proportion of exploratory activity for each area (bottom, middle, top) and section (1–15) during the novel tank test (6 min) was shown for animals previously confined into dark, bright, and transparent environments.
(TIFF)

Video S1   Basic behaviors of zebrafish during the intra-session habituation period to the open tank paradigm. The video describes the protocol of the short-period confinement into dark, bright, and transparent environments (10-min period into the respective cylinder) and demonstrates the spatio-temporal behavior of the experimental groups (dark/transparent vs. bright) in the novel tank diving test. Representative movies of the first 3-min vs. last 3-min for dark/transparent and bright groups were shown (note that the exploratory profile and homebase formation are different between the groups).
(AVI)

Author Contributions

Conceived and designed the experiments: DBR RDD DLdO. Performed the experiments: DBR EPR BHMM DLdO. Analyzed the data: DBR DLdO ALP MEC CDB REB DOS. Contributed reagents/materials/analysis tools: DBR ALP DLdO. Wrote the paper: DBR MEC REB DLdO.

References

1. Walsh RN, Cummins RA (1976) The Open-Field Test: a critical review. Psychol Bull 83: 482–504.
2. Crawley JN (1985) Exploratory behavior models of anxiety in mice. Neurosci Biobehav Rev 9: 37–44.
3. Eilam D (2003) Open-field behavior withstands drastic changes in arena size. Behav Brain Res 142: 53–62.
4. Kim D, Chae S, Lee J, Yang H, Shin HS (2005) Variations in the behaviors to novel objects among five inbred strains of mice. Genes Brain Behav 4: 302–306.
5. Kalueff AV, Zimbardo PG (2007) Behavioral neuroscience, exploration, and K.C. Montgomery's legacy. Brain Res Rev 53: 328–331.
6. Burt MA, Ryan CL, Doucette TA (2008) Altered responses to novelty and drug reinforcement in adult rats treated neonatally with domoic acid. Physiol Behav 93: 327–336.
7. Lamprea MR, Cardenas FP, Setem J, Morato S (2008) Thigmotactic responses in an open-field. Braz J Med Biol Res 41: 135–140.
8. Birke LI, Archer J (1975) Open-field behaviour of oestrous and dioestrous rats: evidence against an 'emotionality' interpretation. Anim Behav 23: 509–512.
9. Lynn DA, Brown GR (2009) The ontogeny of exploratory behavior in male and female adolescent rats (*Rattus norvegicus*). Dev Psychobiol 51: 513–520.
10. O'Keefe J (1990) A computational theory of the hippocampal cognitive map. Prog Brain Res 83: 301–312.
11. Eisenstein EM, Eisenstein D (2006) A behavioral homeostasis theory of habituation and sensitization: II. Further developments and predictions. Rev Neurosci 17: 533–557.
12. Wong K, Elegante M, Bartels B, Elkhayat S, Tien D, et al. (2010) Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). Behav Brain Res 208: 450–457.
13. Eilam D, Golani I (1989) Home base behavior of rats (*Rattus norvegicus*) exploring a novel environment. Behav Brain Res 34: 199–211.
14. Mintz M, Russig H, Lacroix L, Feldon J (2005) Sharing of the home base: a social test in rats. Behav Pharmacol 16: 227–236.
15. Drai D, Kafkafi N, Benjamini Y, Elmer G, Golani I (2001) Rats and mice share common ethologically relevant parameters of exploratory behavior. Behav Brain Res 125: 133–140.

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16. Cachat J, Stewart A, Grossman L, Gaikwad S, Kadri F, et al. (2010) Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. Nat Protoc 5: 1786–1799.
17. Gerlai R (2010) High-throughput behavioral screens: the first step towards finding genes involved in vertebrate brain function using zebrafish. Molecules 15: 2609–2622.
18. Blaser R, Gerlai R (2006) Behavioral phenotyping in zebrafish: comparison of three behavioral quantification methods. Behav Res Methods 38: 456–469.
19. Gerlai R, Lee V, Blaser R (2006) Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (*Danio rerio*). Pharmacol Biochem Behav 85: 752–761.
20. Yu L, Tucci V, Kishi S, Zhdanova IV (2006) Cognitive aging in zebrafish. PLoS One 1: e14.
21. Levin ED, Bencan Z, Cerutti DT (2007) Anxiolytic effects of nicotine in zebrafish. Physiol Behav 90: 54–58.
22. Bencan Z, Sledge D, Levin ED (2009) Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. Pharmacol Biochem Behav 94: 75–80.
23. Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, et al. (2009) Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res 205: 38–44.
24. Grossman L, Utterback E, Stewart A, Gaikwad S, Chung KM, et al. (2010) Characterization of behavioral and endocrine effects of LSD on zebrafish. Behav Brain Res 214: 277–284.
25. Stewart A, Cachat J, Wong K, Gaikwad S, Gilder T, et al. (2010) Homebase behavior of zebrafish in novelty-based paradigms. Behav Processes 85: 198–203.
26. López-Patiño MA, Yu L, Cabral H, Zhdanova IV (2008) Anxiogenic effects of cocaine withdrawal in zebrafish. Physiol Behav 93: 160–171.
27. Seibt KJ, Oliveira Rda L, Zimmermann FF, Capiotti KM, Bogo MR, et al. (2010) Antipsychotic drugs prevent the motor hyperactivity induced by psychotomimetic MK-801 in zebrafish (*Danio rerio*). Behav Brain Res 214: 417–422.
28. Blaser RE, Chadwick L, McGinnis GC (2010) Behavioral measures of anxiety in zebrafish (*Danio rerio*). Behav Brain Res 208: 56–62.
29. Maximino C, de Brito TM, Colmanetti R, Pontes AA, de Castro HM, et al. (2010) Parametric analyses of anxiety in zebrafish scototaxis. Behav Brain Res 210: 1–7.
30. Maximino C, de Brito TM, da Silva Batista AW, Herculano AM, Morato S, et al. (2010) Measuring anxiety in zebrafish: a critical review. Behav Brain Res 214: 157–171.
31. Maximino C, Marques de Brito T, Dias CA, Gouveia A, Jr., Morato S (2010) Scototaxis as anxiety-like behavior in fish. Nat Protoc 5: 209–216.
32. Serra EL, Medalha CC, Mattioli R (1999) Natural preference of zebrafish (*Danio rerio*) for a dark environment. Braz J Med Biol Res 32: 1551–1553.
33. Espejo EF, Mir D (1993) Structure of the rat's behaviour in the hot plate test. Behav Brain Res 56: 171–176.
34. Clifford JJ, Usiello A, Vallone D, Kinsella A, Borrelli E, et al. (2000) Topographical evaluation of behavioural phenotype in a line of mice with targeted gene deletion of the D2 dopamine receptor. Neuropharmacology 39: 382–390.
35. Kalueff AV, Ren-Patterson RF, LaPorte JL, Murphy DL (2008) Domain interplay concept in animal models of neuropsychiatric disorders: a new strategy for high-throughput neurophenotyping research. Behav Brain Res 188: 243–249.
36. Gerlai R, Lahav M, Guo S, Rosenthal A (2000) Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. Pharmacol Biochem Behav 67: 773–782.
37. Leussis MP, Bolivar VJ (2006) Habituation in rodents: a review of behavior, neurobiology, and genetics. Neurosci Biobehav Rev 30: 1045–1064.
38. Thompson RF, Spencer WA (1966) Habituation: a model phenomenon for the study of neuronal substrates of behavior. Psychol Rev 73: 16–43.
39. Gerlai R (2003) Zebra fish: an uncharted behavior genetic model. Behav Genet 33: 461–468.
40. Maximino C, Brito TM, Moraes FD, Oliveira FV, Taccolini IB, et al. (2008) A comparative analysis of the preference for dark environments in five teleosts. Int J Comp Psychol 20: 351–367.
41. Maximino C, da Silva AW, Gouveia A, Jr., Herculano AM (2011) Pharmacological analysis of zebrafish (*Danio rerio*) scototaxis. Prog Neuro-Psychopharmacol Biol Psychiatry doi:10.1016/j.pnpbp.2011.01.006.
42. Lau BY, Mathur P, Gould GG, Guo S (2011) Identification of a brain center whose activity discriminates a choice behavior in zebrafish. Proc Natl Acad Sci U S A 108: 2581–2586.
43. Sackerman J, Donegan JJ, Cunningham CS, Nguyen NN, Lawless K, et al. (2010) Zebrafish behavior in novel environments: Effects of acute exposure to anxiolytic compounds and choice of *Danio rerio* line. Int J Comp Psychol 23: 43–61.
44. Cachat J, Stewart A, Utterback E, Hart P, Gaikwad S, et al. (2011) Three-dimensional neurophenotyping of adult zebrafish behavior. PLoS One 6(3): e17597.44.
45. Park YS, Chung NI, Choi KH, Cha EY, Lee SK, et al. (2005) Computational characterization of behavioral response of medaka (*Oryzias latipes*) treated with diazinon. Aquat Toxicol 71: 215–228.
46. Chon TS, Chung N, Kwak IS, Kim JS, Koh SC, et al. (2005) Movement behaviour of Medaka (*Oryzias latipes*) in response to sublethal treatments of diazinon and cholinesterase activity in semi-natural conditions. Environ Monit Assess 101: 1–21.
47. Gerlai R (2010) Zebrafish antipredatory responses: a future for translational research? Behav Brain Res 207: 223–231.

II.5. CAPÍTULO V

Behavioral effects of taurine pretreatment in zebrafish acutely exposed to ethanol.

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Behavioral effects of taurine pretreatment in zebrafish acutely exposed to ethanol.

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Research Highlights

1. The effects of taurine and ethanol were evaluated on zebrafish behavior.

2. Taurine pretreatment prevented ethanol-induced locomotor alterations.

3. Ethanol altered the spatio-temporal exploratory behavior of zebrafish.

4. Taurine may prevent the changes in exploratory behavior promoted by ethanol.

5. The open tank may be a suitable test for pharmacological trials.

Abstract

Taurine (TAU) is a sulfur-containing β-amino acid that has several biological functions. Protective actions against ethanol (EtOH)-mediated effects have been attributed to its modulatory role on distinct neurochemical parameters. Because zebrafish presents conserved physiological responses and a complex behavioral repertoire, mounting evidence shows its applicability for evaluating several drug abuse-related phenotypes. However, the potential preventive role of TAU against EtOH-induced changes in zebrafish behavior still remains unknown. Thus, the goal of this study was to analyze the effects of TAU pretreatment on the behavior of zebrafish acutely exposed to EtOH using the open tank test. The results demonstrated that 1% EtOH (v/v) exposure for 1 h impaired locomotor activity and increased immobility during the trial, which was prevented by 42, 150, and 400 mg/L TAU. Furthermore, EtOH significantly altered both vertical exploration and the exploratory profile, which was prevented by 42 and 400 mg/L TAU, but not by 150 mg/L TAU. Representative track and occupancy charts, as well as spatio-temporal 3D reconstructions of behavior showed that 42 and 400 mg/L TAU prevented EtOH-induced modifications in the ethogram. In conclusion, our data suggest that acute EtOH exposure induces significant changes in the spatio-temporal behavior of zebrafish and that TAU may exert a preventive role by antagonizing the effects induced by EtOH Additionally, we provide a rationale framework for the wider application of zebrafish for future translational research complementing traditional rodent models.

Key words: Taurine, ethanol, zebrafish, behavior, open tank.

1. Introduction

Taurine (TAU), 2-aminoethanesulfonic acid, is a simple β-amino acid which is not incorporated into proteins. Its biosynthesis is dependent on sequential oxidative steps of cysteine, catalyzed by cysteine dioxygenase and cysteinesulfinate decarboxylase (Banerjee et al., 2008; Vivitsky et al., 2011). It has been described that considerable amounts of TAU may be detected in distinct organs and high concentrations (millimolar range) are found in the intracellular milieu of electrically excitable tissues, like brain (Huxtable, 1992, Wu and Prentice, 2010). In contrast, extracellular concentrations of TAU are usually present in micromolar range (around 80–120 mM) (Vivitsky et al., 2011). These discrepancies observed for TAU accumulation in distinct cellular environments result mainly from an efficient mechanism of transport provided by TauT protein, which utilizes transmembrane gradients of Na^+ and Cl^- as the driving force, and from TAU biosynthesis (Kozlowski et al., 2008; Kang, 2009). Studies performed *in vitro* postulate that the biosynthesis of TAU in the central nervous system (CNS) involves an intrinsic metabolic cooperation between neuronal and glial cells (Tappaz et al., 1994; Brand et al., 1997). Several reports demonstrated that TAU plays pivotal roles for CNS homeostasis, acting on regulation of osmotic pressure, neuromodulatory processes and inhibitory neurotransmission (Wu et al., 2005; Rodríguez-Navarro et al., 2009; Wu and Prentice, 2010). Although the underlying mechanisms related to TAU actions in the CNS still remain to be elucidated, evidence suggests that it acts as an agonist of $GABA_A$ and glycine receptors, modulating Ca^{2+} influx and intracellular second messenger systems (Huxtable, 1992; El Idrissi, 2008; Junyent et al., 2010). Thus, TAU is an important molecule able to modulate glutamatergic signaling, preventing excitotoxicity and oxidative stress (Saransaari and Oja, 2010; Junyent et al., 2011). In particular, cytoprotective effects against various types of injury have been described, including alcohol abuse (Lawrence, 2007).

Ethanol (EtOH) is one of the most widely consumed drugs throughout the world. Alcohol abuse is associated with the occurrence of several pathological conditions, such as metabolic diseases, brain damage, alcoholic fetal syndrome, and various forms of cancer (Ferreira and Willoughby, 2008; Joenje, 2011). At the behavioral level, acute EtOH consumption at low concentrations induces euphoria, relaxation, and attenuates stress or anxiety (Mathur and Guo, 2011). However, higher concentrations of alcohol exert sedative and depressant effects on the CNS, which characterizes a classical inverted U-shaped response promoted by alcohol (Bhisikar et al., 2009). Moreover, the acute EtOH exposure may disrupt motor coordination, sensory perception, and cognition, affecting behavioral performance.

The zebrafish (*Danio rerio*) is as a suitable model organism for neurochemical and behavioral studies (Cachat et al., 2010; Rico et al., 2011; Rosemberg et al., 2011). Some advantages, such as the small size and the facility of maintenance in laboratory conditions, along with the presence of highly conserved genes and similar physiological responses, make zebrafish a complementary vertebrate organism with classical rodent models (Lieschke and Currie, 2007; Stewart et al., 2012). The cloning and functional analysis of the TauT transporter in zebrafish showed a high degree of similarity with the human TauT protein, suggesting that TAU may also play critical physiological roles in fish (Kozlowski e al., 2008). The feasibility for large scale screening and the easy absorption of compounds directly added to the tank water are relevant characteristics for modeling behavioral and functional parameters related to human pathogenesis and preclinical treatments, including alcohol abuse and therapeutic strategies.

Several reports have demonstrated the effects of EtOH exposure in both larval and adult zebrafish, which include anatomical malformations (Bilotta et al., 2004; Sylvain et al., 2011), changes in neurotransmitter systems (Rico et al., 2007; Chatterjee and Gerlai, 2009), transduction signaling pathways (Peng et al., 2009) and oxidative damage (Rosemberg et al., 2010a). In addition, studies have demonstrated that adult zebrafish present a complex behavioral repertoire in different tasks, like the

light/dark tank and open tank paradigm, also known as novel tank diving test (Blaser et al., 2010; Maximino et al., 2010; Rosemberg et al., 2011). The pharmacological validity of both tests have already been described in the literature, and they have been increasingly used for assessing the behavioral effects of drugs including EtOH (Levin et al., 2007; Egan et al., 2009, Cachat et al., 2010; Blaser and Peñalosa, 2011; Gebauer et al., 2011; Mathur et al., 2011a; 2011b; Maximino et al., 2011). Despite the evidence that TAU prevents neurochemical changes induced by alcohol exposure in this species (Rosemberg et al., 2010a), there is no data regarding the actions of TAU on zebrafish behavior. Therefore, the aim of the current study was to evaluate a potential preventive effect of pretreatment with TAU on behavioral parameters altered by EtOH in zebrafish using the open tank paradigm.

2. Material and Methods

2.1. Animals

Adult zebrafish (*Danio rerio*) (4-6 months-old, ~50:50 male:female ratio) were purchased from a commercial distributor (Delphis, RS, Brazil) and kept in 50-L aquariums (80-100 fish per aquarium). The animals were of heterogeneous wild-type stock (standard short-fin phenotype) and were housed for 2 weeks before the experiments in order to acclimate to the laboratory facility. All tanks were filled with unchlorinated water previously treated with 132 μL.L^{-1} AquaSafe® (Tetra, VA, USA). Fish were maintained under aerated water at 26 ± 2 ºC, pH and conductivity at 7.0 – 8.0 and 1,500 – 1,600 μS.cm^{-1}, respectively, on a 12/12 light/dark photoperiod cycle (lights on at 7:00 am) provided by ceiling-mounted fluorescent light tubes. The animals were fed twice a day until satiety with commercial flake fish food (alcon BASIC®, Alcon, Brazil). Independent experiments were performed using a total of 110 animals obtained in three separate batches. All fish used in this study were experimentally naive, healthy and free of any signs of disease. Animals were maintained in accordance to the

National Institute of Health Guide for Care and Use of Laboratory Animals. The protocols were approved by the Ethical Committee of the Federal University of Rio Grande do Sul under the number 2007950.

2.2. Experimental groups

Immediately before the behavioral tests, the animals were placed individually in 500-mL beakers for a total exposure period of 2 h, which were divided in two consecutive 1 h-periods, as follows: (a) In the first 1 h, fish were exposed to home tank water or treated with TAU (Sigma, St. Louis, MO, USA) at concentrations of 42, 150 and 400 mg/L. (b) In the last 1 h-exposure period, animals were placed in beakers containing home tank water or 1% EtOH (v/v) diluted from a 96% stock solution (Merck, Darmstadt, Germany). The experimental groups used for the tests are represented in **Figure 1A**, and comprised by (i) Control (home tank water + home tank water), (ii) EtOH (home tank water + EtOH), (iii) TAU-control group (TAU + home tank water), and (iv) TAU-pretreated group (TAU + EtOH). The experiments were performed using $n =$ 12–16 animals per treatment and TAU solutions were previously buffered to pH 7.0 using 0.1 N NaOH (Sigma, St. Louis, MO, USA).

Importantly, the same exposure duration and EtOH concentration have already been shown to produce significant alterations in distinct neurochemical parameters of this species (Gerlai et al., 2000; Dlugos and Rabin, 2003; Rico et al., 2007; Rosemberg et al., 2010a). Moreover, the blood alcohol levels achieved with it are expected to be in the range what human drinkers experience (Lockwood et al., 2004; Gerlai et al., 2008). The acute TAU treatments were performed as described by Rosemberg et al. (2010a) and the concentrations chosen were based in previous studies, varying from 0.33 to 3.2 mM (Wu et al., 2005; Kong et al., 2006; Rosemberg et al., 2010b). It is relevant to emphasize that the origin and quality of the system water and the timing of drug exposure was identical for all fish.

2.3. Behavioral experiments

The behavioral test was performed during the same time frame each day (between 10:00 am and 4:00 pm). All apparatuses were filled with water adjusted to home tanks conditions and the experimental procedures were performed on a stable surface with all environmental distractions kept to a minimum. After the exposure periods, the animals were carefully removed from their beakers and placed individually in the open tank where their behavioral activity was recorded for a single session of 6 min **(Figure 1B)**. The apparatus consisted of a trapezoidal plastic tank (23.9 cm along the bottom x 28.9 cm at the top x 15.1 cm high) filled with 1.5 L of home tank water, which was virtually divided into three equal horizontal areas (bottom, middle, and top), with five sections per area as previously reported (Rosemberg et al., 2011).

A webcam (Microsoft® LifeCam 1.1 with Auto-Focus) was placed in front of the open tank to monitor the location and swimming activity of the zebrafish. In order to ensure a uniform background for the video analysis and to avoid environmental distraction of animals subjected to the behavioral test, yellow sheets of paper (standard letter size: 21.59 cm x 27.94 cm) were placed 4.3 cm behind the tank and also in both sides of the apparatus. Two 60-watts light bulbs were placed 40 cm behind the novel tank to boost the contrast between the background and fish. The webcam was connected to a laptop for recording the videos and the behavioral parameters were automatically measured at a rate of 30 frames/s, using appropriate video-tracking software (ANY-maze®, Stoelting CO, USA).

2.4. Locomotor parameters

The locomotor activity of zebrafish was measured by endpoint behaviors, which included the total distance travelled, mean speed, absolute turn angle, meandering, time mobile, and immobility. The absolute turn angle represents the sum of all vectors angle of movements created from one position to animal's center point to the next,

considering anti-clockwise movement as negative and clockwise movement as positive (-180º to 180 ºC). The meandering was calculated by the division of the absolute turn angle by the total distance travelled. Additionally, the distance travelled, average speed, number of immobile episodes and time immobile were determined in fragments of time (each 1 min) in order to better understand these behaviors across the open tank trial.

2.5. Vertical exploration

The vertical behavior of zebrafish in the open tank represents its tendency to gradually explore upper areas when subjected to novel apparatuses, which may reflect habituation during the test (Wong et al., 2010a; Rosemberg et al., 2011). The number of transitions and time spent in each vertical area were determined, as well as the time spent in each visit. Furthermore, the latency to middle and top area transitions was evaluated and the number of entries and time spent in bottom, middle, and top areas analyzed across the time.

2.6. Exploratory profile in the open tank and homebase parameters

The exploratory profile of fish was estimated by considering both horizontal and vertical parameters according to the method described by Rosemberg et al. (2011). Briefly, the number of transitions across the horizontal sections was determined and a ratio between the number of transitions per section and number of transitions per area was calculated. The results whose values were ≤ 1 predominantly characterize vertical exploration in a section, whereas > 1 values mainly suggest a significant horizontal exploration.

The homebase formation during the open tank trial was estimated using behavioral data (basically transitions and time spent per section), which were confirmed by both track and occupancy plots (Stewart et al., 2010; Rosemberg et al.,

2011). The distance travelled, number of transitions, and time spent in the homebase were analyzed across the open tank trial.

2.7. Spatio-temporal analysis of behavior

The behavioral profile of zebrafish in the novel tank diving test was evaluated using 3D track reconstructions of the spatial coordinates across time, as previously described (Grossman et al., 2010; Cachat et al., 2011; Rosemberg et al., 2011). Briefly, the coordinates of the experimental tank were properly calibrated using the ANY-maze® software and the track data across fractions of a second for each fish was exported as raw data into separate spreadsheets. The exported spatial coordinates (x center and y center) were analyzed based on similarity to each other by two trained observers (inter-rater reliability >0.85), on a consensus basis. The middle trace was selected as representative for the group, to illustrate the spatio-temporal pattern of exploration. 3D reconstructions were represented as scatter plots, which were constructed using Graphis 3D graphing software®. The x center (horizontal distribution), y center (vertical distribution), and time were plotted on the X-, Y- and Z-axis, respectively. The main behaviors observed across time were described by representative ethograms, using a spectrum of colors (blue-red) to demonstrate the position of the animal during the test (0-360 s).

2.8. Statistics

Data were expressed as mean ± standard error of the mean (S.E.M.) and analyzed by repeated-measures analysis of variance (ANOVA) using TAU, EtOH, and time as factors (temporal measures) or by two-way ANOVA (endpoint parameters), followed by Bonferroni's test as post hoc. The significance was set at $p \leq 0.05$.

3. Results

We first evaluated the effect of EtOH and TAU pretreatment on the behavioral parameters related to locomotor activity in the open tank task **(Figure 2)**. The endpoint data demonstrated that EtOH significantly decreased the total distance travelled and the mean speed in the 6-min trial when compared to control. Consistently, the increase in the absolute turn angle observed in animals exposed to EtOH along with lower distance travelled in the apparatus resulted in a significant increase of meandering. Moreover, the acute EtOH exposure induced a significant decrease in the time mobile, which was associated with an increase in the number of immobile episodes **(Figure 2A)**. The TAU concentrations tested (42, 150, and 400 mg/L) significantly prevented the alterations in locomotor activity promoted by EtOH, maintaining endpoint values similar to those observed in control group. The temporal analysis of locomotion **(Figure 2B)** showed that all experimental groups displayed a constant distance travelled, even though animals exposed to EtOH travelled a significantly lower distance across the trial (F [5,510] = 3.887, $p < 0.01$). Our data also revealed that EtOH induced a significant decrease in the average velocity of fish (F [1,102] = 8.601, $p < 0.05$) when compared to control. Although the time immobile and the number of immobile episodes of EtOH-exposed group were higher than control (F [1,102] = 5.422, $p < 0.05$ and F [1,102] = 13.752, $p < 0.01$, respectively), both parameters present a significant decrease across test (F [5,510] = 3.18, $p < 0.05$ and F [5,510] = 3.502, $p < 0.01$, respectively). The TAU pretreatment in all concentrations tested showed a preventive effect against the locomotor changes caused by alcohol exposure.

The vertical exploratory behaviors displayed by control, EtOH, and TAU/EtOH groups are shown in **Figure 3**. Analysis by repeated measures ANOVA **(Figure 3A)** demonstrated that the duration in bottom decreased across the 6-min trial (F [5,510] = 36.983, $p < 0.0001$) and that EtOH group spent significantly more time in the bottom than controls (F [1,102] = 47.858, $p < 0.0001$). The results also showed that animals from TAU42/EtOH and TAU400/EtOH groups presented a faster drop in the time spent

in the bottom area than those exposed to EtOH alone (F [15,510] = 2.152, $p < 0.05$). Fish acutely exposed to EtOH showed a slower increase in the number of entries and time spent in both middle (F [5,510] = 3.613, $p < 0.01$ and F [5,510] = 5.307, $p < 0.005$, respectively) and top (F [5,510] = 2.971, $p < 0.05$ and F [5,510] = 5.147, $p < 0.005$, respectively) across the open tank trial. These effects on vertical exploratory activity during the test were prevented by 42 and 400 mg/L TAU (F [15,510] = 1.974, $p < 0.05$). TAU42/EtOH and TAU400/EtOH groups demonstrated a gradual increase in the time spent in top area, similar to control. Interestingly, TAU150/EtOH showed a similar exploration between the vertical areas to the EtOH-exposed group.

As demonstrated by the endpoint data filtered by vertical location **(Figure 3B)**, EtOH significantly increased the time spent in bottom and the average time spent per visit in the respective area. Furthermore, the time spent in the middle and top areas of the apparatus was decreased in the EtOH-exposed group, which presented a significantly lower number of transitions to these areas in comparison to control. The acute alcohol exposure also induced a significant increase in the latency of entry to the middle and top. Pretreatment with 42 and 400 mg/L TAU were able to prevent the vertical behavior impairments, but only 400 mg/L attenuated the effects of EtOH in the latency to enter the top area. Consistently with the results observed in the temporal analysis of behavior, TAU150/EtOH did not reveal a preventive effect in the behavioral changes related to vertical exploratory activity.

We next evaluated the influence of these pharmacological manipulations on the exploratory profile of zebrafish considering the swimming activity in both dimensions of the open tank **(Figure 4)**. The ratio of transitions between horizontal sections to transitions between vertical areas demonstrated that the EtOH group presented a significant increase in the exploratory profile in the bottom associated with a decrease in the top area. The results obtained for both TAU42/EtOH and TAU400/EtOH showed a similar exploratory profile to control. Although the pretreatment with 150 mg/L TAU prevented the changes induced by EtOH in the bottom area, the exploratory profile in

top was quite similar to that of fish exposed to alcohol **(Figure 4A)**. These data were supported by the representative track and occupancy plots, which illustrate the swimming traces and the relative time spent among the areas and sections of the tank **(Figure 4B)**. It is also relevant to emphasize that neither the locomotor variables assessed nor the vertical exploration were altered by TAU *per se* **(data not shown)**. Therefore, the distance travelled, mean speed, and overall exploratory profile of TAU-treated group remained similar to control **(Figure S1).**

To reconfirm the correlations between temporal analysis and endpoints data of behavior, we investigated the effects of the experimental manipulations on the spatio-temporal exploratory pattern. Using representative temporal 3D reconstructions, we dissected the behavioral repertoire of the fish, in which the video-tracking software revealed the spatial coordinates during the trial **(Figure 5)**. Our data demonstrated that EtOH altered locomotor parameters and the ethogram of fish subjected to the novel tank test, as observed by an increase in lateral exploration of the bottom area, a considerable increase in immobile episodes, with less frequent transitions to top and less lateral exploration in the upper area of the tank in comparison to control. The analysis of the spatio-temporal exploratory profile of TAU400/EtOH showed a preventive effect of TAU pretreatment on the main behaviors of test.

The distribution of each experimental group during the trial is represented in **Figure S2**. Remarkably, TAU42/EtOH and TAU400/EtOH prevented the effects of EtOH exposure on exploration in the top area, whereas 150 mg/L TAU revealed a similar profile in the respective area when compared to EtOH. As verified by the 3D representations, all groups frequently returned to the central sections of the bottom where they spent more time across the open tank test, establishing a characteristic homebase formation. The temporal analysis demonstrated that EtOH significantly decreased the number of transitions and the time spent in the homebase (F [1,102] = 5.312, $p < 0.05$ and F [1,102] = 8.471, $p < 0.01$, respectively) when compared to the untreated group. However, the pretreatment with TAU prevented these alterations in

homebase-related parameters across the test **(data not shown)**. The behavioral changes evaluated after acute EtOH exposure in each vertical area of the open tank are depicted in the **Figure 5**.

4. Discussion

The novel findings presented in the current report are that acute EtOH exposure induces significant changes in the spatio-temporal behavior of zebrafish and that TAU may exert a preventive effect against these modifications. In this context, EtOH altered locomotor activity, in which fish exhibited a decrease in the total distance travelled probably attributed to the slower swimming activity, the increased time of immobility, and the significant number of immobile episodes during the test. Moreover, EtOH altered vertical exploration, resulting in changes in the spatio-temporal exploratory profile. Pretreatment with 42, 150 and 400 mg/L TAU prevented the modifications on locomotion, while 150 mg/L TAU more closely resembled the EtOH-exposed group. The results obtained by the behavioral analysis support the hypothesis that TAU may antagonize the effects of EtOH in zebrafish.

Several previous studies have shown the applicability of zebrafish to assess some phenotypes associated to neuropsychiatric disorder models (Seibt et al., 2010; Wong et al., 2010b; Piato et al., 2011; Stewart et al., 2011; 2012). In this regard, the effects of different compounds, such as MK-801, buspirone, diazepam, and proline have been evaluated using the light/dark tank, group behavioral task, and/or the open tank test (Bencan et al., 2009; Seibt et al., 2010; Gebauer et al., 2011; Maximino et al., 2011; Savio et al., 2011; Sison and Gerlai, 2011). We recently proposed the description of an ethogram for the open tank paradigm, which was considered a good choice for evaluating the overall exploratory profile of animals in a single 6-min trial (Rosemberg et al., 2011). However, an in depth evaluation of the changes promoted by EtOH in spatio-temporal behavior and the potential preventive effect of pharmacological manipulations in the behavioral repertoire described previously were still unknown.

The knowledge about the biological actions of TAU has been successfully increased during the last years. It is conceivable that its pleiotropic effects of protection can be attributed basically to a neuromodulatory role, the ability to regulate Ca^{2+} homeostasis, and also to antioxidant properties (Huxtable et al., 1992; Klamt and Shacter, 2005; Wu et al., 2005; El Idrissi, 2008; Rodríguez-Navarro et al., 2009; Junyent et al., 2010; 2011). A recent report demonstrated that TAU efficiently scavenges many reactive oxygen and nitrogen species when it reacts to distinct oxidantes *in vitro* (Oliveira et al., 2010) but since TAU corresponds to the maximum oxidative state of cysteine, direct mechanisms underlying its antioxidant activity are difficult to interpret. In this context, Jong et al. (2011) proposed an indirect mechanism, in which the formation of the mitochondrial 5-taurinomethyluridine conjugate plays a pivotal role in the regulation of protein synthesis, thereby enhancing electron transport chain activity and protecting the cell against excessive superoxide generation. Although the physiological concentrations of TAU in the adult zebrafish brain have not been determined directly so far, there are a few studies describing some functional properties in this species. As in other vertebrates, TAU is the most abundant amino acid in the outer retina and may act as a signaling molecule in zebrafish cells (Marc and Cameron, 2001). Knockdown of TauT by antisense morpholino oligonucleotides in embryos leads to cell death in CNS and increased mortality, suggesting that the high TAU concentrations detected (192 pmol/embryo) are biologically relevant for zebrafish development. (Kozlovski et al., 2008). Additionally, Hammes et al. (2011) showed that TAU improves hepatic steatosis by reducing oxidative stress and increasing *sirt1* expression in zebrafish liver when co-administered with thioacetamide. We recently demonstrated that TAU prevents EtOH-induced impairments on cholinergic and oxidative stress parameters, suggesting that the potential protective effects of TAU could be better evaluated through a spatio-temporal analysis of behavior (Rosemberg et al., 2010a). Considering that TAU concentrations tested in the present study did not alter locomotor parameters and exploratory profile in the open tank **(see Figure S1)**,

we further verified whether this amino acid prevents the effects promoted by acute EtOH exposure using this behavioral paradigm.

Our results showed that 1% EtOH (v/v) exposure disrupted locomotor and motor posture patterns, probably due to its sedative/depressant effects, as verified by the lower speed and distance travelled, and the increase of meandering when compared to control. Studies performed by Gerlai et al. (2000) reported the first evidence that EtOH influences the behavior of zebrafish, including locomotion, shoaling, aggression, predator avoidance, and pigment response. During the last decade, efforts have been made to describe both neural mechanisms and motivational factors associated with alcohol consumption in zebrafish. In this regard, there is evidence that EtOH modulates second messenger systems, gene expression, enzyme activities, and also neurotransmitter parameters (Rico et al., 2007; Peng et al., 2009; Wagle et al., 2011). Similar to the effects observed in humans after alcohol consumption, acute EtOH exposure in zebrafish has been described as an inverted U-shaped response in motor function, in which low to intermediate doses result in hyperactivity compared to higher doses, which cause hypoactivity due to the depressant effects of alcohol. (Gerlai et al., 2000; Gerlai et al., 2008; Echevarria et al., 2011). Dlugos and Rabin (2003) observed differences in the behavioral effects of EtOH on three strains of zebrafish, which could not be attributed to differences in brain alcohol levels, and therefore were unlikely to be caused by strain differences in metabolism, absorption, or distribution. Furthermore, the same protocol of EtOH exposure used in our study was performed by Chatterjee and Gerlai (2009), who demonstrated an increase in the levels of dopamine, serotonin, and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the zebrafish brain. Thus, it is likely that the effects observed in behavior are a result of central effects of EtOH.

The 3D reconstruction graphs displayed the exploratory activity and the main behaviors of each experimental group during the open tank trial. Since zebrafish presents both vertical and horizontal exploration, the representative 3D traces were

important in order to reconfirm our experimental data. Spatio-temporal analysis of behavior demonstrated that EtOH altered the intra-session habituation response by decreasing the transitions and time spent in the top area across the test. Although all TAU concentrations were able to prevent the alterations in locomotor parameters induced by alcohol exposure, the exploratory profile in top of TAU150/EtOH was similar to the EtOH group, as verified by the lateral exploration in the respective area. Because EtOH interacts with many different systems in the CNS, it affects multiple receptor types, such as GABA, serotonin, and glutamate receptors (Lovinger and Crabbe, 2005). Even for a single receptor type, activation in distinct neuronal locations or even brain areas could lead to different behavioral outcomes. For example, it is likely that the effects of EtOH on $GABA_A$ contribute both to its anxiolytic effects and to its sedative/locomotor effects and that TAU may act as a $GABA_A$ agonist (Davis and Wu, 2001; Sergeeva et al., 2007). However, presumably different brain areas are responsible for this phenomenon - for example, many of the locomotor effects may stem from inhibition in the cerebellum, while the anxiolytic effects may come from the habenula (Volkman et al., 2008; Amo et al., 2010; Okamoto et al., 2011). Other important targets are the glutamate receptors, which have been associated with locomotor stimulant and depressant effects after acute EtOH consumption (Badanich et al., 2011). Since NMDA receptors are involved in mediating excitatory responses by increasing Ca^{2+} influx, it would not be surprising if TAU could counteract EtOH-mediated responses by a modulatory role on these receptors. Thus, it is possible that the TAU and EtOH could have a distinct effect on different brain structures or even neutoransmitter systems. Our data also indicate that the effects of TAU on locomotor variables are rather 'dose insensitive' suggesting that once a low dose is present, additional TAU has no further effect. In contrast, the response of the exploratory behaviors changed according to dose, suggesting that the system involved here was not saturated by the lowest concentration. Because the changes in vertical exploration cannot simply be explained by changes in locomotor activity, we hypothesize that

separate systems could be involved in these responses, which are supported by the two dissociable effects of TAU in the spatio-temporal behavior of zebrafish. Further experiments are still needed in order to clarify the aspects related to the discrepancies of locomotion/exploration showed by TAU/EtOH.

The mechanisms by which EtOH modulates biological processes have been the subject of extensive research. Nevertheless, the effects promoted by alcohol are often attributed to its metabolite, acetaldehyde, which is a highly reactive molecule that forms protein-acetaldehyde adducts, induces DNA damage, and promotes oxidative stress (Quertemont and Didone, 2006; Upadhya and Ravindranath, 2002; Yu et al., 2010). The EtOH oxidation to acetaldehyde may also occur by different reactions, such as those mediated by catalase (CAT) activity or by the mitochondrial cytochrome P-4502E1 complex (Deng and Deitrich, 2008). A previous report from our laboratory demonstrated that acute EtOH exposure for 1 h promotes a significant decrease in superoxide dismutase (SOD) and an increase in CAT activities in the zebrafish brain, a fact that could contribute to acetaldehyde formation and hence, lead to oxidative stress (Rosemberg et al., 2010a). Taking into account that the effects triggered by alcohol abuse in the CNS may lead to behavioral changes, these neurochemical alterations related to EtOH metabolism in the brain of zebrafish could contribute, at least in part, to the differences in the EtOH and TAU/EtOH ethograms.

5. Conclusion

In summary, our data reinforce the sensitivity of zebrafish to alcohol exposure and demonstrate the first evidence that TAU pretreatment prevents the EtOH-induced changes on locomotor parameters. Moreover, the preventive effect of TAU on locomotion was not directly associated with the maintenance of the exploratory profile at basal levels. These apparently dissociate data between locomotion/exploration require further investigation into the neural mechanisms which play a role in the effects of TAU and EtOH and also in motivational aspects of exploration in the open tank test.

Since the use of zebrafish for the assessment of behavioral phenotypes related to alcohol abuse and dependence has been supported by predictive and face validities, the relationship between gene, and protein expression, cellular pathways, and behavior may be tempting approaches with the advances in molecular biology techniques. Therefore, future pharmacological trials on alcohol abuse and alcoholism should consider the analysis of spatio-temporal behavior as an interesting tool for preclinical studies taking advantage of the benefits of zebrafish, like the capacity of performing tests in a fast and large-scale manner, in order to complement the existent rodent model in translational research.

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Conflict of interest

The authors have declared that no competing interests exist.

References

Amo, R., Aizawa, H., Takahoko, M., Kobayashi, M., Takahashi, R., Aoki, T., Okamoto, H., 2010. Identification of the zebrafish ventral habenula as a homolog of the mammalian lateral habenula. J Neurosci. 30(4): 1566-1574.

Badanich, K.A., Doremus-Fitzwater, T.L., Mulholland, P.J., Randall, P.K., Delpire, E., Becker, H.C., 2011. NR2B-deficient mice are more sensitive to the locomotor stimulant and depressant effects of ethanol. Genes Brain Behav. 10(7): 805-816.

Banerjee, R., Vitvitsky, V., Garg, S.K., 2008. The undertow of sulfur metabolism on glutamatergic neurotransmission. Trends Biochem Sci. 2008 33(9), 413-419.

Bencan, Z., Sledge, D., Levin, E.D., 2009. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. Pharmacol Biochem Behav. 94(1): 75-80.

Bhisikar, S.M., Kokare, D.M., Nakhate, K.T., Chopde, C.T., Subhedar, N.K., 2009. Tolerance to ethanol sedation and withdrawal hyper-excitability is mediated via neuropeptide Y Y1 and Y5 receptors. Life Sci. 85(21-22), 765-772.

Bilotta, J., Barnett, J.A., Hancock, L., Saszik, S., 2004. Ethanol exposure alters zebrafish development: a novel model of fetal alcohol syndrome. Neurotoxicol Teratol. 26(6): 737-743.

Blaser, R.E., Chadwick, L., McGinnis, G.C., 2010. Behavioral measures of anxiety in zebrafish (*Danio rerio*). Behav Brain Res. 208(1), 56-62.

Blaser, R.E., Peñalosa, Y.M., 2011. Stimuli affecting zebrafish (*Danio rerio*) behavior in the light/dark preference test. Physiol Behav. 104(5), 831-837.

Brand, A., Richter-Landsberg, C., Leibfritz, D., 1997. Metabolism of acetate in rat brain neurons, astrocytes and cocultures: metabolic interactions between neurons and glia cells, monitored by NMR spectroscopy. Cell Mol Biol. 43(5), 645-657.

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K.M., Wu, N., Wong, K., Roy, S., Suciu, C., Goodspeed, J., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Tan, J., Denmark, A., Gilder, T., Kyzar, E., Dileo, J., Frank, K., Chang, K., Utterback, E., Hart, P., Kalueff, A.V., 2010. Measuring behavioral and

endocrine responses to novelty stress in adult zebrafish. Nat Protoc. 5(11), 1786-1799.

Cachat, J., Stewart, A., Utterback, E., Hart, P., Gaikwad, S., Wong, K., Kyzar, E., Wu, N., Kalueff, A.V., 2011. Three-dimensional neurophenotyping of adult zebrafish behavior. PLoS One. 6(3), e17597.

Chatterjee, D., Gerlai, R., 2009. High precision liquid chromatography analysis of dopaminergic and serotoninergic responses to acute alcohol exposure in zebrafish. Behav Brain Res. 200(1), 208-213.

Davis, K.M., Wu, J.Y., 2001. Role of glutamatergic and GABAergic systems in alcoholism. J Biomed Sci. 8(1): 7-19.

Deng, X.S., Deitrich, R.A., 2008. Putative role of brain acetaldehyde in ethanol addiction. Curr Drug Abuse Rev. 1(1): 3-8.

Dlugos, C.A., Rabin, R.A., 2003. Ethanol effects on three strains of zebrafish: model system for genetic investigations. Pharmacol Biochem Behav. 74(2), 471-480.

Echevarria, D.J., Toms, C.N., Jouandot, D.J., 2011. Alcohol-induced behavior change in zebrafish models. Rev Neurosci. 22(1): 85-93.

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., Kalueff, A.V., 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res. 205(1), 38-44.

El Idrissi A., 2008. Taurine increases mitochondrial buffering of calcium: role in neuroprotection. Amino Acids. 34(2): 321-328.

Ferreira, M.P., Willoughby, D., 2008. Alcohol consumption: the good, the bad, and the indifferent. Appl Physiol Nutr Metab. 33(1), 12-20.

Gebauer, D.L., Pagnussat, N., Piato, A.L., Schaefer, I.C., Bonan, C.D., Lara, D.R., 2011. Effects of anxiolytics in zebrafish: similarities and differences between

benzodiazepines, buspirone and ethanol. Pharmacol Biochem Behav. 99(3), 480-486.

Gerlai, R., Lahav, M., Guo, S., Rosenthal, A., 2000. Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. Pharmacol Biochem Behav. 67(4), 773-782.

Gerlai, R., Ahmad, F., Prajapati, S., 2008. Differences in acute alcohol-induced behavioral responses among zebrafish populations. Alcohol Clin Exp Res. 32(10), 1763-1773.

Grossman, L., Utterback, E., Stewart, A., Gaikwad, S., Chung, K.M., Suciu, C., Wong, K., Elegante, M., Elkhayat, S., Tan, J., Gilder, T., Wu, N., Dileo, J., Cachat, J., Kalueff, A.V., 2010. Characterization of behavioral and endocrine effects of LSD on zebrafish. Behav Brain Res. 214(2), 277-284.

Hammes, T.O., Pedroso, G.L., Hartmann, C.R., Escobar, T.D., Fracasso, L.B., da Rosa, D.P., Marroni, N.P., Porawski, M., da Silveira, T.R., 2011. The effect of taurine on hepatic steatosis induced by thioacetamide in zebrafish (*Danio rerio*). Dig Dis Sci. doi: 10.1007/s10620-011-1931-4.

Huxtable, R.J., 1992. Physiological actions of taurine. Physiol Rev. 72(1), 101-163.

Joenje, H., 2011. Metabolism: alcohol, DNA and disease. Nature. 475(7354), 45-46.

Jong, C.J., Azuma, J., Schaffer, S., 2011. Mechanism underlying the antioxidant activity of taurine: prevention of mitochondrial oxidant production. Amino Acids. doi: 10.1007/s00726-011-0962-7.

Junyent, F., Romero, R., de Lemos, L., Utrera, J., Camins, A., Pallàs, M., Auladell, C., 2010. Taurine treatment inhibits CaMKII activity and modulates the presence of calbindin D28k, calretinin, and parvalbumin in the brain. J Neurosci Res. 88(1), 136-142.

Junyent, F., Porquet, D., de Lemos, L., Romero, R., Utrera, J., Camins, A., Pallàs, M., Auladell, C., 2011. Decrease of calbindin-d28k, calretinin, and parvalbumin by

taurine treatment does not induce a major susceptibility to kainic acid. J Neurosci Res. 89(7), 1043-1051.

Kang, Y.S., 2009. Downregulation of taurine transport by calcium blockers in osteoblast cells. Adv Exp Med Biol. 643, 513-521.

Klamt, F., Shacter, E., 2005. Taurine chloramine, an oxidant derived from neutrophils, induces apoptosis in human B lymphoma cells through mitochondrial damage. J Biol Chem. 280(22): 21346-21352.

Kong, W.X., Chen, S.W., Li, Y.L., Zhang, Y.J., Wang, R., Min, L., Mi, X., 2006. Effects of taurine on rat behaviors in three anxiety models. Pharmacol Biochem Behav. 83(2), 271-276.

Kozlowski, D.J., Chen, Z., Zhuang, L., Fei, Y.J., Navarre, S., Ganapathy, V., 2008. Molecular characterization and expression pattern of taurine transporter in zebrafish during embryogenesis. Life Sci. 82(19-20), 1004-1011.

Lawrence, A.J., 2007. Therapeutics for alcoholism: what's the future? Drug Alcohol Rev. 26(1), 3-8.

Levin, E.D., Bencan, Z., Cerutti, D.T., 2007. Anxiolytic effects of nicotine in zebrafish. Physiol Behav. 90(1), 54-58.

Lieschke, G.J., Currie, P.D., 2007. Animal models of human disease: zebrafish swim into view. Nat Rev Genet. 8(5), 353-367.

Lockwood, B., Bjerke, S., Kobayashi, K., Guo, S., 2004. Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening. Pharmacol Biochem Behav. 77(3): 647-654.

Lovinger, D.M., Crabbe, J.C., 2005. Laboratory models of alcoholism: treatment target identification and insight into mechanisms. Nat Neurosci. 8(11): 1471-1480.

Marc, R.E., Cameron, D., 2001. A molecular phenotype atlas of the zebrafish retina. J Neurocytol. 30(7): 593-654.

Mathur, P., Berberoglu, M.A., Guo, S., 2011a. Preference for ethanol in zebrafish following a single exposure. Behav Brain Res. 217(1): 128-133.

Mathur, P., Lau, B., Guo, S., 2011b. Conditioned place preference behavior in zebrafish. Nat Protoc. 6(3), 338-345.

Mathur, P., Guo, S., 2011. Differences of acute versus chronic ethanol exposure on anxiety-like behavioral responses in zebrafish. Behav Brain Res. 219(2), 234-239.

Maximino, C., de Brito, T.M., Colmanetti, R., Pontes, A.A., de Castro, H.M., de Lacerda, R.I., Morato, S., Gouveia, A. Jr., 2010. Parametric analyses of anxiety in zebrafish scototaxis. Behav Brain Res. 210(1), 1-7.

Maximino, C., da Silva, A.W., Gouveia, A. Jr., Herculano, A.M., 2011. Pharmacological analysis of zebrafish (*Danio rerio*) scototaxis. Prog Neuropsychopharmacol Biol Psychiatry. 35(2), 624-631.

Okamoto, H., Agetsuma, M., Aizawa, H., 2011. Genetic dissection of the zebrafish habenula, a possible switching board for selection of behavioral strategy to cope with fear and anxiety. Dev Neurobiol. doi: 10.1002/dneu.20913.

Oliveira, M.W., Minotto, J.B., de Oliveira, M.R., Zanotto-Filho, A., Behr, G.A., Rocha, R.F., Moreira, J.C., Klamt, F., 2010. Scavenging and antioxidant potential of physiological taurine concentrations against different reactive oxygen/nitrogen species. Pharmacol Rep. 62(1): 185-193.

Peng, J., Wagle, M., Mueller, T., Mathur, P., Lockwood, B.L., Bretaud, S., Guo, S., 2009. Ethanol-modulated camouflage response screen in zebrafish uncovers a novel role for cAMP and extracellular signal-regulated kinase signaling in behavioral sensitivity to ethanol. J Neurosci. 29(26), 8408-8418.

Piato, A.L., Capiotti, K.M., Tamborski, A.R., Oses, J.P., Barcellos, L.J., Bogo, M.R., Lara, D.R., Vianna, M.R., Bonan, C.D. 2011. Unpredictable chronic stress model in zebrafish (*Danio rerio*): behavioral and physiological responses. Prog Neuropsychopharmacol Biol Psychiatry. 35(2):561-567.

Quertemont, E., Didone, V., 2006. Role of acetaldehyde in mediating the pharmacological and behavioral effects of alcohol. Alcohol Res Health. 29(4): 258-265.

Rico, E.P., Rosemberg, D.B., Dias, R.D., Bogo, M.R., Bonan, C.D., 2007. Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain. Toxicol Lett. 174(1-3), 25-30.

Rico, E.P., Rosemberg, D.B., Seibt, K.J., Capiotti, K.M., Da Silva, R.S., Bonan, C.D., 2011. Zebrafish neurotransmitter systems as potential pharmacological and toxicological targets. Neurotoxicol Teratol. doi:10.1016/j.ntt.2011.07.007.

Rodríguez-Navarro, J.A., Gonzalo-Gobernado, R., Herranz, A.S., Gonźlez-Vigueras, J.M., Solís, J.M., 2009. High potassium induces taurine release by osmosensitive and osmoresistant mechanisms in the rat hippocampus in vivo. J Neurosci Res. 87(1), 208-217.

Rosemberg, D.B., da Rocha, R.F., Rico, E.P., Zanotto-Filho, A., Dias, R.D., Bogo, M.R., Bonan, C.D., Moreira, J.C., Klamt, F., Souza, D.O., 2010a. Taurine prevents enhancement of acetylcholinesterase activity induced by acute ethanol exposure and decreases the level of markers of oxidative stress in zebrafish brain. Neuroscience. 171(3), 683-692.

Rosemberg, D.B., Kist, L.W., Etchart, R.J., Rico, E.P., Langoni, A.S., Dias, R.D., Bogo, M.R., Bonan, C.D., Souza, D.O., 2010b. Evidence that acute taurine treatment alters extracellular AMP hydrolysis and adenosine deaminase activity in zebrafish brain membranes. Neurosci Lett. 481(2), 105-109.

Rosemberg, D.B., Rico, E.P., Mussulini, B.H., Piato, A.L., Calcagnotto, M.E., Bonan, C.D., Dias, R.D., Blaser, R.E., Souza, D.O., de Oliveira, D.L., 2011. Differences in spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments. PLoS One. 6(5), e19397.

Saransaari, P., Oja, S.S., 2010. Mechanisms of inhibitory amino acid release in the brain stem under normal and ischemic conditions. Neurochem Res. 35(12), 1948-1956.

Savio, L.E., Vuaden, F.C., Piato, A.L., Bonan, C.D., Wyse, A.T., 2011. Behavioral changes induced by long-term proline exposure are reversed by antipsychotics in

zebrafish. Prog Neuropsychopharmacol Biol Psychiatry. doi:10.1016/j.pnpbp.2011.10.002.

Seibt, K.J., Oliveira, Rda. L., Zimmermann, F.F., Capiotti, K.M., Bogo, M.R., Ghisleni, G., Bonan, C.D., 2010. Antipsychotic drugs prevent the motor hyperactivity induced by psychotomimetic MK-801 in zebrafish (*Danio rerio*). Behav Brain Res. 214(2): 417-422.

Sergeeva, O.A., Fleischer, W., Chepkova, A.N., Warskulat, U., Häussinger, D., Siebler, M., Haas, H.L., 2007. GABAA-receptor modification in taurine transporter knockout mice causes striatal disinhibition. J Physiol. 585(Pt 2): 539-548.

Sison, M., Gerlai, R., 2011. Behavioral performance altering effects of MK-801 in zebrafish (*Danio rerio*). Behav Brain Res. 220(2): 331-337.

Stewart, A., Cachat, J., Wong, K., Gaikwad, S., Gilder, T., DiLeo, J., Chang, K., Utterback, E., Kalueff, A.V., 2010. Homebase behavior of zebrafish in novelty-based paradigms. Behav Processes. 85(2), 198-203.

Stewart, A., Wong, K., Cachat, J., Gaikwad, S., Kyzar, E., Wu, N., Hart, P., Piet, V., Utterback, E., Elegante, M., Tien, D., Kalueff, A.V., 2011. Zebrafish models to study drug abuse-related phenotypes. Rev Neurosci. 22(1): 95-105.

Stewart, A., Gaikwad, S., Kyzar, E., Green, J., Roth, A., Kalueff, A.V., 2012. Modeling anxiety using adult zebrafish: A conceptual review. Neuropharmacology. 62, 135-143.

Sylvain, N.J., Brewster, D.L., Ali, D.W., 2011. Embryonic ethanol exposure alters synaptic properties at zebrafish neuromuscular junctions. Neurotoxicol Teratol. 33(2), 313-321.

Tappaz, M., Almarghini, K., Do, K., 1994. Cysteine sulfinate decarboxylase in brain: identification, characterization and immunocytochemical location in astrocytes. Adv Exp Med Biol. 359, 257-268.

Upadhya, S.C., Ravindranath, V., 2002. Detection and localization of protein-acetaldehyde adducts in rat brain after chronic ethanol treatment. Alcohol Clin Exp Res. 26(6): 856-863.

Vitvitsky, V., Garg, S.K., Banerjee, R., 2011. Taurine biosynthesis by neurons and astrocytes. J Biol Chem. 286(37), 32002-32010.

Volkmann, K., Rieger, S., Babaryka, A., Köster, R.W., 2008. The zebrafish cerebellar rhombic lip is spatially patterned in producing granule cell populations of different functional compartments. Dev Biol. 313(1): 167-180.

Wagle, M., Mathur, P., Guo, S., 2011. Corticotropin-releasing factor critical for zebrafish camouflage behavior is regulated by light and sensitive to ethanol. J Neurosci. 31(1): 214-224.

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., Goodspeed, J., Suciu, C., Tan, J., Grimes, C., Chung, A., Rosenberg, M., Gaikwad, S., Denmark, A., Jackson, A., Kadri, F., Chung, K.M., Stewart, A., Gilder, T., Beeson, E., Zapolsky, I., Wu, N., Cachat, J., Kalueff, A.V., 2010a. Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). Behav Brain Res. 208(2), 450-457.

Wong, K., Stewart, A., Gilder, T., Wu, N., Frank, K., Gaikwad, S., Suciu, C., Dileo, J., Utterback, E., Chang, K., Grossman, L., Cachat, J., Kalueff, A.V., 2010b. Modeling seizure-related behavioral and endocrine phenotypes in adult zebrafish. Brain Res. 1348: 209-215.

Wu, H., Jin, Y., Wei, J., Jin, H., Sha, D., Wu, J.Y., 2005. Mode of action of taurine as a neuroprotector. Brain Res. 1038(2), 123-131.

Wu, J.Y., Prentice, H., 2010. Role of taurine in the central nervous system. J Biomed Sci. 17 Suppl 1:S1.

Yu, H.S., Oyama, T., Isse, T., Kitagawa, K., Pham, T.T., Tanaka, M., Kawamoto, T., 2010. Formation of acetaldehyde-derived DNA adducts due to alcohol exposure. Chem Biol Interact. 188(3): 367-375.

Legend for Figures

Figure 1. Schematic representations of the methodological approach used for the evaluation of the spatio-temporal behavior of zebrafish after EtOH exposure and TAU treatments. (A) The experimental protocol consisted of a total period of 2 h divided in two 1 h exposure periods, which were characterized differently for each experimental group. After, the behavior was assessed during a single 6-min trial in the open tank. **(B)** Representative cartoon of the protocol. Animals were previously isolated in 500-mL beakers during the exposure periods and further transferred to the behavioral apparatus. The open tank was virtually divided in three vertical areas (bottom, middle, and top), with five sections per area for the evaluation of the exploratory profile of fish.

Figure 2. Prevention of TAU against the EtOH-induced changes on behavioral parameters related to zebrafish locomotor activity. (A) Effect of TAU pretreatments (42, 150, and 400 mg/L) and EtOH (1% v/v) on endpoint data. * Significant difference to control. # Significant difference to EtOH group. Data were analyzed by two-way ANOVA followed by Bonferroni's test as post hoc, considering $p \leq 0.05$ as significant. **(B)** Temporal analysis of locomotor parameters during the open tank trial (repeated-measures ANOVA followed by Bonferroni's test as post hoc, $p \leq 0.05$).

Figure 3. Vertical exploration during the behavioral analysis. (A) Analysis across time, demonstrating the intra-session habituation profile for control, EtOH, and TAU-pretreated groups. The data show the time in each vertical area (bottom, middle, and top) and the number of transitions between these areas (repeated-measures ANOVA followed by Bonferroni's test as post hoc, $p \leq 0.05$). **(B)** Endpoint parameters of vertical exploratory activity. Data were analyzed by two-way ANOVA followed by Bonferroni's

test as post hoc, considering $p \leq 0.05$ as significant. * Significant difference to control. # Significant difference to EtOH group.

Figure 4. Effect of EtOH and TAU-pretreated groups in the overall exploratory profile of zebrafish in the open tank test. (A) The exploratory profile of control, EtOH and TAU/EtOH was determined by calculating the ratio between the total transitions between sections and the number of entries in the respective area. * Significant difference to control. # Significant different to EtOH group. Data were analyzed by two-way ANOVA followed by Bonferroni's test as post hoc, considering the differences at a $p \leq 0.05$ level. **(B)** Representative track and occupancy plots of the groups. The data obtained by video-tracking system (ANY-maze®, Stoelting CO, USA) display the specific patterns of exploratory behavior affected during 6 min.

Figure 5. Ethological analysis of the spatio-temporal exploratory activity of zebrafish after EtOH and TAU manipulations. The representative 3D reconstructions of EtOH, control, and TAU400/EtOH groups during the 6 min of test were obtained by plotting animal traces (X- and Y-axis) across time (Z-axis). The segments of test (0-360s) were represented by a color scale gradient, indicating the beginning (dark blue) and the end of trial (red). The ethograms observed for the experimental groups were shown and the preventive effect of the 400 mg/L TAU pretreatment was demonstrated by the similarities of the behavioral parameters in relation to control (+).

Supporting Information

Figure S1. TAU treatments did not alter zebrafish behavior in the open tank. (A) Effect of 42, 150, and 400 mg/L TAU on basic locomotor parameters. Endpoint data were analyzed by one-way ANOVA, whereas the analysis across time was performed by repeated measures ANOVA. Post hoc comparisons were carried out using Bonferroni's test, considering $p \leq 0.05$ as significant. **(B)** Representative plots for TAU-treated groups, demonstrating no effects on the overall exploratory profile. The data obtained by video-tracking system (ANY-maze®, Stoelting CO, USA) display the patterns of exploratory behavior during 6 min.

Figure S2. Representative 3D reconstructions of control, EtOH, and TAU-pretreated groups. The figure demonstrates the horizontal and vertical swimming traces (X- and Y-axis, respectively) across time (Z-axis). The segments of test (0-360s) were represented by a color scale gradient, indicating the beginning (dark blue) and the end of trial (red). The exploratory profile in top area of each group is marked, showing the differences observed for TAU150/EtOH.
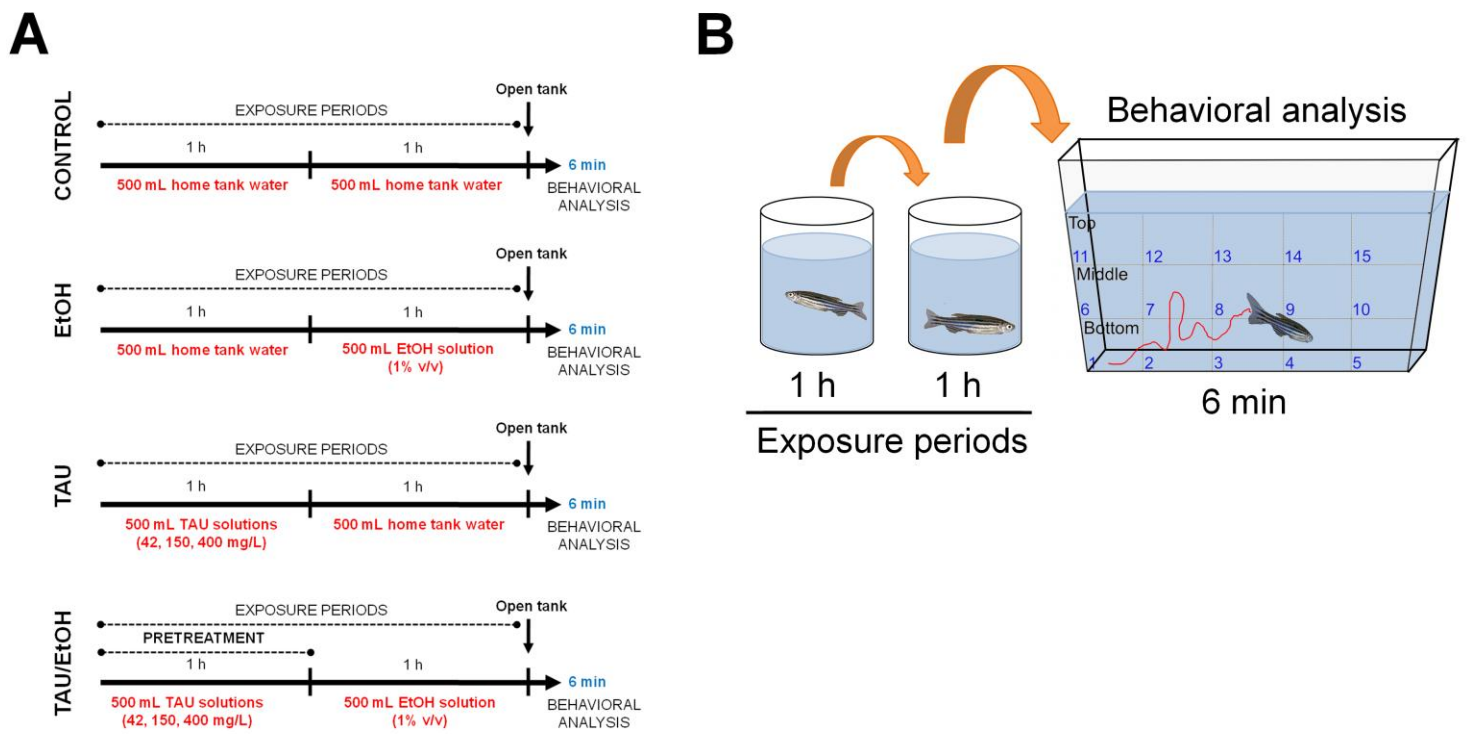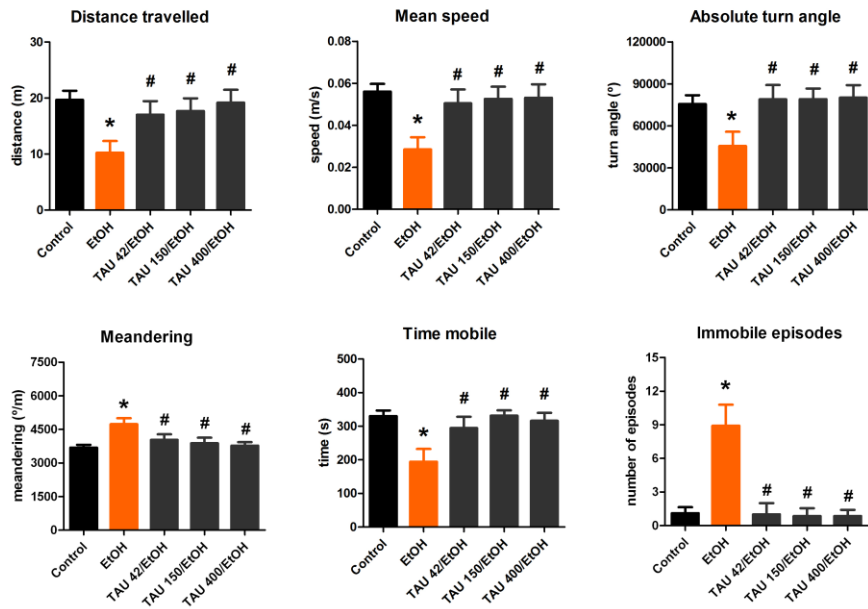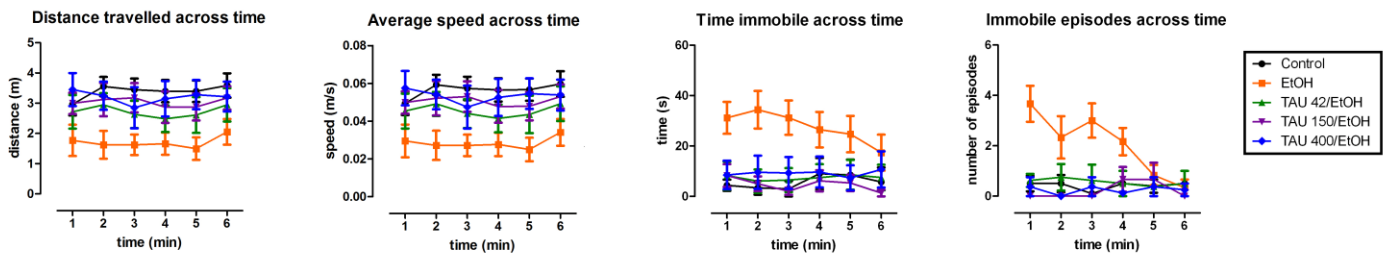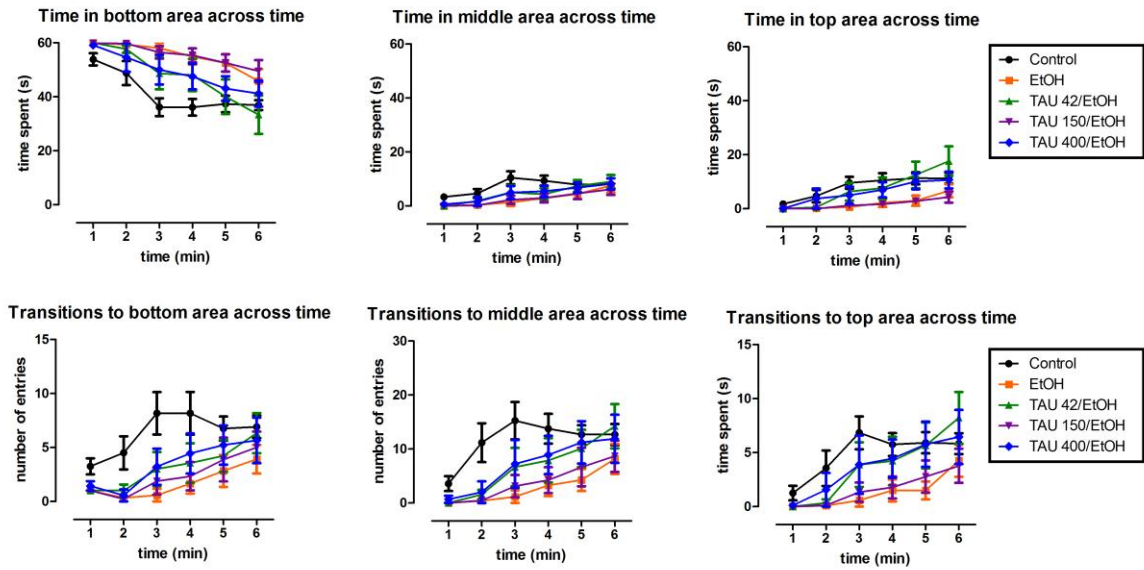
Figure 1

A

Distance travelled

Mean speed

Absolute turn angle

Meandering

Time mobile

Immobile episodes

B

Distance travelled across time

Average speed across time

Time immobile across time

Immobile episodes across time

Control
EtOH
TAU 42/EtOH
TAU 150/EtOH
TAU 400/EtOH

Figure 2

Figure 3

A

Exploratory profile in bottom area

Exploratory profile in middle area

Exploratory profile in top area

Control
EtOH
TAU 42/EtOH
TAU 150/EtOH
TAU 400/EtOH

B

TRACK PLOTS

OCCUPANCY PLOTS

Control

EtOH

TAU 42/EtOH

TAU 150/EtOH

TAU 400/EtOH

− time spent +

Figure 4

EtOH

LOCOMOTOR ACTIVITY
- ⬇ Distance travelled
- ⬆ Meandering
- ⬇ Mean speed
- ⬇ Time mobile

time (s) 0 — 360

Lateral exploration in top area

TOP AREA
- ⬇ Time spent
- ⬇ Lateral exploration
- ⬆ Latency

MIDDLE AREA
- ⬇ Transition swimming
- ⬆ Latency

Transition swimming

Lateral exploration in bottom area

BOTTOM AREA
- ⬆ Time spent
- ⬆ Immobility detection
- ⬆ Lateral exploration
- ⬇ Transitions to homebase across time
- ⬆ Time in homebase across time

Immobility detection

CONTROL

Abundant transition swimming

Significant lateral exploration in top area

Reduced immobility detection

Constant lateral exploration in bottom area

TAU400/EtOH

➕ Lateral exploration in top area

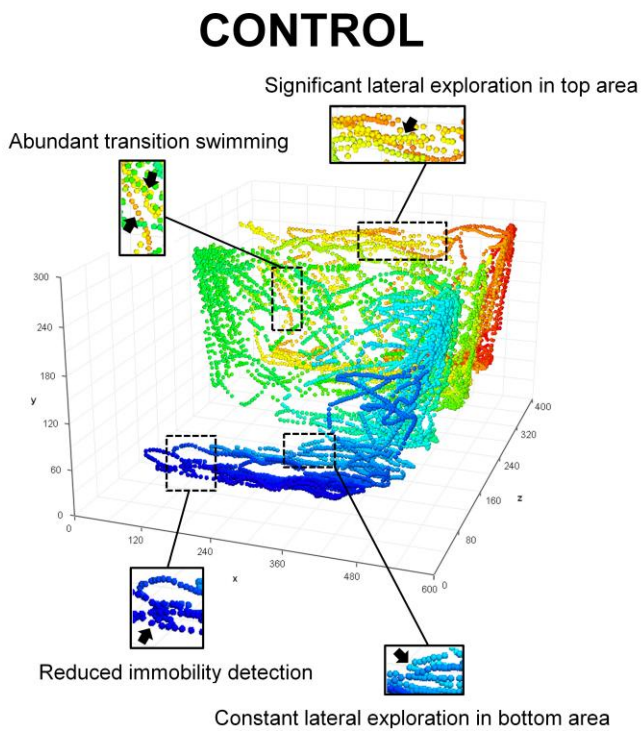➕ Transition swimming

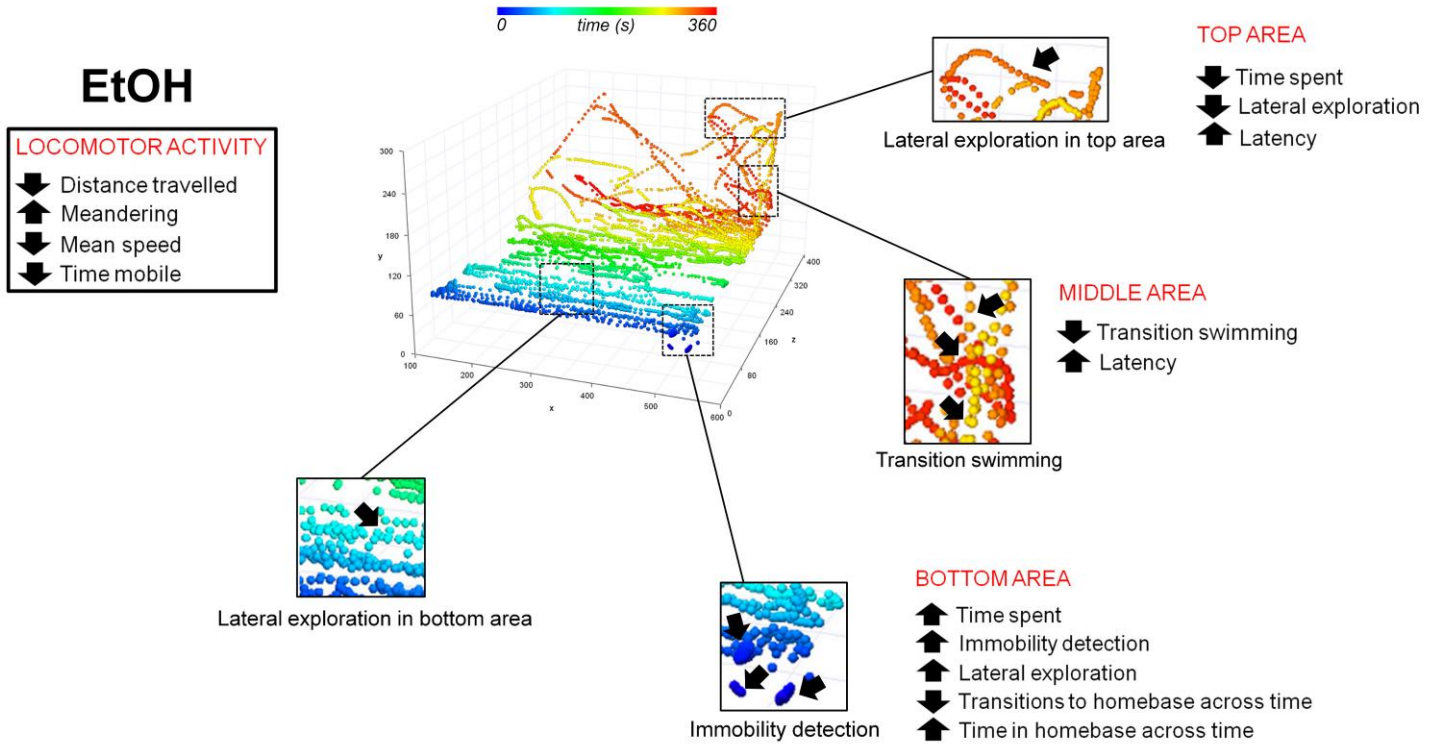➕ Immobility detection
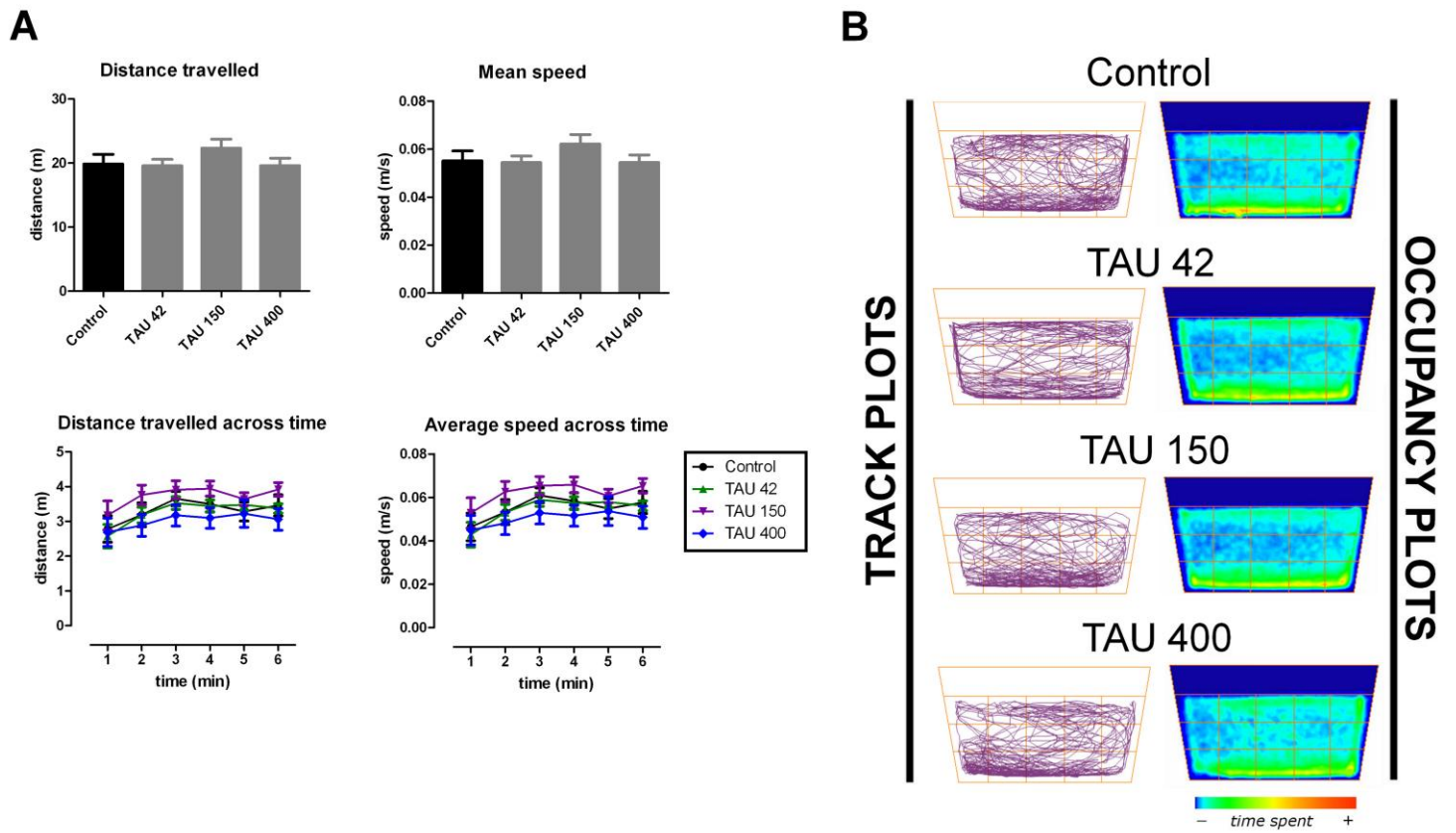
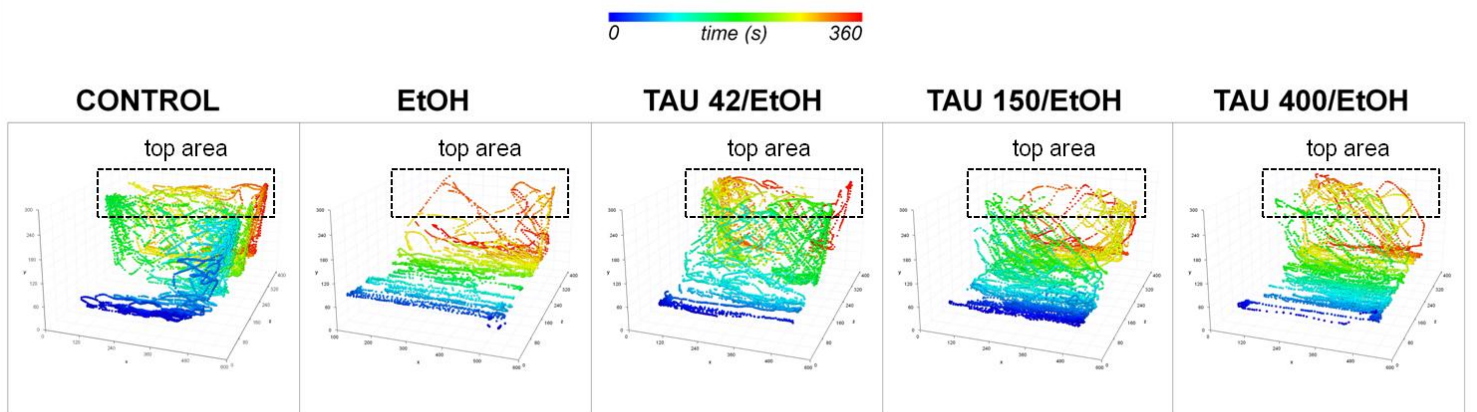➕ Lateral exploration in bottom area

Figure 5

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Figure S1

Figure S2

PARTE III

III.1. DISCUSSÃO

Os resultados apresentados nesta tese de doutorado demonstraram a utilidade do peixe-zebra na avaliação de parâmetros bioquímicos e comportamentais, com ênfase nas sinalizações purinérgica, colinérgica e nos efeitos promovidos pela taurina no modelo de exposição aguda ao etanol.

Experimentos realizados por Morales et al. (2007) e Li e Olson (2008) demonstraram a existência de uma correlação entre a sinalização mediada por ATP com o efluxo de taurina, sugerindo uma participação do sistema purinérgico com esse aminoácido na osmorregulação. Outros estudos postularam que a liberação simultânea de taurina e adenosina possui um papel chave na atenuação da excitotoxicidade. Foi demonstrado que o agonista do receptor A_1, N(6)cicloexiladenosina (CHA), potencializou a liberação basal de taurina, enquanto que o hidrocloreto de 2-p-(2-carbóxietil)fenilamino-5'-N-etilcarboxaminoadenosina (CGS 21680), um agonista do receptor A_{2A}, aumentou a liberação de taurina em estruturas cerebrais de camundongos submetidas ao protocolo de isquemia *in vitro* (Saransaari e Oja, 2003; 2010).

Considerando os estudos sobre o papel da taurina nos vertebrados, a aparente relação entra a taurina e a sinalização purinérgica e o indicativo de uma potencial importância desse aminoácido para o peixe-zebra, **o primeiro capítulo** desta tese teve por objetivo verificar se o tratamento com taurina poderia exercer um efeito sobre a atividade das ectonucleotidases e da adenosina deaminase no cérebro dessa espécie. Para isso, foram realizados tratamentos agudos com taurina nas concentrações de 42, 150 e 400 mg/L durante 1h, bem como análises *in vitro* com a adição de taurina no meio de incubação. É importante salientar que a quantificação dos níveis de taurina em

peixe-zebra demonstrou que este é o quarto aminoácido mais abundante em embriões (192 pmol/embrião), perdendo apenas para o glumatato (460 pmol/embrião), alanina (382 pmol/embrião) e aspartato (371 pmol/embrião) (Kozlowski et al., 2008). Embora as concentrações testadas variassem de 0,33 até 3,2 mM, as quais aparentam ser elevadas para o SNC da espécie até o presente momento, diversos estudos utilizando roedores ou culturas de células testam concentrações similares ou até mesmo extremamente maiores para a análise dos efeitos promovidos pela taurina (Wu et al., 2005; Kong et al., 2006; Zhang et al., 2010), pois sabe-se que em células eletricamente excitáveis o *pool* intracelular desse aminoácido pode atingir quantidades na faixa do milimolar (Huxtable, 1992).

Nossos resultados demonstraram que o tratamento agudo com 150 e 400 mg/L de taurina promoveu um aumento significativo na hidrólise de AMP e uma diminuição na atividade da adenosina deaminase em membranas cerebrais. Possivelmente, esse aumento da hidrólise do nucleotídeo monofosfatado da adenina não está diretamente relacionado com um aumento na expressão da ecto-5'-nucleotidase, visto que não ocorreram alterações significativas na hidrólise do GMP e a enzima atua sobre ambos os substratos (Senger et al., 2004; Sträter, 2006). Interessantemente, os resultados *in vitro* demonstraram que apenas a atividade da ecto-ADA foi significativamente reduzida quando 150 e 400 mg/L de taurina foram adicionadas no sistema de incubação. Os resultados sugerem que os efeitos observados no tratamento *in vivo* poderiam ocorrer através de um mecanismo de ação indireto, visto que apenas concentrações elevadas de taurina promoveram efeitos significativos *in vitro*. A diferença nos resultados observados para na atividade da ecto-5'-nucleotidase e da ecto-ADA nos ensaios *in vitro* poderia ser atribuída a um

possível efeito sobre membranas celulares. Sabe-se que ambas as enzimas possuem diferentes mecanismos de ancoramento na membrana plasmática, pois enquanto a ecto-5'-nucleotidase é ancorada através do GPI, a ecto-ADA ancora-se à superfície celular através da união com proteínas específicas, tais como receptores A_1 e A_{2B} (Ciruela et al., 1996; Herrera et al., 2001; Sträter, 2006). Embora os resultados obtidos nesse estudo não permitam afirmar se a taurina é capaz de exercer efeitos benéficos em peixe-zebra, eles descrevem a primeira evidência que o tratamento agudo com taurina é capaz de aumentar a hidrólise de AMP e diminuir a desaminação da adenosina em membranas cerebrais. Esses dados poderiam sugerir um aumento nos níveis de adenosina extracelular no SNC dessa espécie, o que poderia modular a ação do nucleosídeo nos seus respecetivos receptores.

A hidrólise de nucleotídeos tri e difosfatados determinada pela técnica de quantificação do Pi liberado poderia ser resultante da atividade conjunta de diferentes membros pertencente à família das NTPDases em peixe-zebra (Rosemberg et al., 2007b; Rico et al., 2008). Portanto, o **segundo capítulo** desta tese teve como objetivo caracterizar essa família enzimática em tecidos distintos. Dessa forma, visamos demonstrar sua importância no controle da sinalização mediada por purinas, propondo novas informações a respeito da complexidade da família das NTPDases em peixe-zebra. Os resultados descritos nesse capítulo demonstraram perfis diferenciais de hidrólise de ATP e ADP na presença de Ca^{2+} em cérebro, fígado e coração, bem como uma distinta sensibilidade a inibidores, sugerindo uma possível contribuição de diferentes membros da família das NTPDases.

Por meio de uma busca no genoma do peixe-zebra, foram identificados genes que codificam distintos membros relacionados à família das NTPDases.

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Utilizando as sequências gênicas deduzidas de outros organismos como parâmetro de busca, foram encontrados ortólogos das NTPDases1-6 e NTPDase8 em peixe-zebra, os quais apresentaram um alto grau de homologia com as sequências de outras espécies. Além disso, três membros parálogos da NTPDase2 e dois da NTPDase5 também puderam ser identificados com a estratégia adotada. A presença dessas cópias parálogas poderia ser atribuída a um evento de duplicação genômica ocorrido na infraclasse dos teleósteos (Amores et al., 1998; Postlethwait et al., 1998), sendo que os mesmos tendem a apresentar distintas cópias para uma mesma família gênica em comparação aos mamíferos (Force et al., 1999). Embora muitos genes tenham sido perdidos evolutivamente, algumas sequências gênicas se apresentam duplicadas, tendo um elevado grau de homologia com os genes de mamíferos porém, com pouca noção funcional. Estudos realizados por Low et al. (2008) demonstraram a existência de cópias parálogas para os receptores $P2X_3$, $P2X_4$ e $P2X_5$ em peixe-zebra, o que indica que a sinalização mediada por nucleotídeos e nucleosídeos na espécie pode ser mais complexa do que previamente imaginado.

A análise filogenética sugeriu que os membros das NTPDases podem ser encontrados em diferentes compartimentos celulares, além de apresentarem um elevado escore para sítios putativos de modificação pós-traducional. Dados na literatura têm demonstrado que as NTPDases de mamíferos possuem sítios de fosforilação em regiões semelhantes às preditas para o peixe-zebra (Kegel et al., 1997; Javed et al., 2007), sugerindo que as modificações nessas proteínas poderiam ser evolutivamente conservadas.

Os experimentos de RT-PCR demonstraram a existência de um perfil diferencial do padrão de expressão das *entpd* em cérebro, fígado e coração de

peixe-zebra. Uma vez que não foram apresentadas nesta tese as características específicas de cada um dos membros isoladamente, é provável que mais de uma enzima participe da atividade enzimática detectada podendo ter importâncias funcionais particulares para cada tecido, similar ao verificado em estudos utilizando outros organismos experimentais (Knowles et al., 2002; Nedeljkovic et al., 2005; Bagatini et al., 2011). Portanto, os diversos membros previamente identificados poderiam exercer um papel distinto na regulação fina da hidrólise de nucleotídeos tri e difosfatados nos tecidos de peixe-zebra. Dessa forma, a ausência de efeitos promovidos pela taurina na hidrólise de nucleotídeos promovida pela atividade das NTPDases em membranas cerebrais analisada no primeiro capítulo poderia ser resultante de uma complexa e distinta interação desse aminoácido com os diferentes membros dessa família enzimática, sendo esta uma hipótese que necessitaria futuras investigações.

O primeiro estudo relacionado aos efeitos promovidos pelo etanol em diferentes comportamentos de peixe-zebra foi realizado por Gerlai et al., (2000). Os dados forneceram importantes contribuições para a comunidade científica na descrição dos efeitos promovidos por drogas de abuso no comportamento de locomoção, agressividade, preferência claro-escuro, interação social, resposta ao predador e alteração na pigmentação. A resposta mediada pela exposição aguda ao etanol em peixe-zebra ocorre de maneira bifásica tal como visto em mamíferos, com as concentrações mais baixas induzindo um aumento na locomoção e agressividade e as mais altas diminuindo atividade locomotora, preferência no comportamento social e aumentando a intensidade de pigmentação (Gerlai, 2003; Echevarria et al., 2011). Também foi demonstrado que os efeitos desencadeados pelo etanol

podem ser distintos, sendo dependentes do tempo de exposição e da linhagem utilizada (Dlugos e Rabin, 2003; Rico et al., 2011b). Nosso grupo previamente demonstrou que a exposição ao etanol durante 1h na concentração de 1% em volume é capaz de promover um aumento na atividade da AChE em cérebro de peixe-zebra, bem como uma significativa diminuição na sua expressão gênica (Rico et al., 2007). Esses dados sugerem que os efeitos promovidos pelo etanol em peixe-zebra podem possuir uma correlação com alterações em parâmetros da sinalização colinérgica.

Estudos recentes têm atribuído à taurina funções pleiotrópicas de citoproteção devido as suas ações como uma molécula neuromoduladora inibitória e pela capacidade de prevenir o estresse oxidativo (Wu et al., 2010; Hammes et al., 2011). Foi demonstrado que os efeitos mediados pelo etanol no SNC estão correlacionados com alterações na coordenação motora, percepção sensorial e cognição em diferentes modelos experimentais, podendo ser desencadeados por estresse oxidativo, além de modificações em diferentes vias de sinalização celular e sistemas de neurotransmissores (Hanchar et al., 2005; Belmeguenai et al., 2003). No **terceiro capítulo** desta tese, foi investigado se a taurina é capaz de prevenir o aumento da atividade da AChE promovido pela exposição aguda ao etanol e se os efeitos de ambas as moléculas poderiam envolver mudanças em parâmetros relacionados ao estresse oxidativo em cérebro de peixe-zebra. Para isso, utilizamos dois protocolos: um co-tratamento com etanol e taurina durante 1h e um pré-tratamento com taurina por 1h, seguido por posterior exposição ao etanol pelo mesmo período. Os resultados demonstraram que quando os animais foram expostos concomitantemente à taurina e etanol, apenas a concentração de 400 mg/L manteve a atividade da AChE similar ao controle. Por outro lado, o pré-

tratamento com 150 e 400 mg/L de taurina preveniu o aumento da atividade da AChE induzido pela exposição aguda ao etanol. Rico et al. (2007) sugeriram que a modulação da atividade da AChE por etanol poderia ocorrer de maneira indireta, visto que experimentos *in vitro* não demonstraram alterações significativas nesse parâmetro.

Com isso, foi investigado se as alterações neuroquímicas promovidas pelo etanol e os efeitos da taurina poderiam ter alguma relação com mudanças no perfil redox em peixe-zebra. Tanto o co-tratamento quanto o pré-tratamento com taurina preveniram a diminuição e o aumento nas atividades da SOD e CAT, respectivamente, induzidos pela exposição aguda ao etanol. Apesar de parecer contraditório, os resultados observados estão de acordo com dados da literatura que avaliaram as ações exercidas pelo álcool em diferentes modelos experimentais (Ledig et al., 1981; Siler-Marsiglio, 2004). Os efeitos distintos verificados em ambas as enzimas poderiam estar relacionados com a ação do etanol sobre as mesmas, bem como do seu metabólito acetaldeído, o qual é capaz de formar aductos e modificar diversas estruturas celulares (Pastor et al., 2002; Kayani e Parry, 2010). A diminuição da atividade da SOD poderia acarretar em um aumento do ânion $O_2^{\cdot-}$, o qual seria capaz de promover efeitos nocivos *per se* ou através da formação de outras espécies reativas, tais como $NO^{\cdot}$, $ONOO^{\cdot}$ ou OH^- (Radi et al., 2002). O aumento da atividade da CAT poderia ser interpretado através de dois aspectos. Na medida em que ocorreria uma redução dos níveis de H_2O_2, poderia estar acontecendo, em paralelo, um aumento nos níveis de acetaldeído, tendo em conta que a CAT é a principal enzima que metaboliza o etanol no SNC (Cohen et al., 1980; Swift, 2003).

Embora não tenham sido detectadas mudanças significativas em parâmetros de defesa não enzimática pelas técnicas de análise do potencial

antioxidante reativo total (TRAP) e de reatividade antioxidante total (TAR), o pré-tratamento com taurina a 150 e 400 mg/L promoveu um aumento nos níveis de sulfidrilas em comparação com os grupos controle e etanol. Além disso, ambas as concentrações diminuíram os níveis basais de espécies reativas ao ácido tiobarbitúrico (TBARS), bem como preveniram a peroxidação lipídica induzida pelo álcool. Entratanto, o co-tratamento com etanol e 400 mg/L de taurina não preveniu o aumento observado na técnica de TBARS. É possível sugerir que a prevenção da peroxidação lipídica induzida pelo álcool no protocolo do pré-tratamento esteja relacionada com o aumento dos níveis de sulfidrilas, pois estudos têm demonstrado que a taurina previne estresse oxidativo e pode aumentar os níveis de GSH a partir da conversão da cisteína (Hagar, 2004; Banerjee et al., 2008). Interessantemente, um estudo recente verificou que a taurina previne o dano hepático induzido por tioacetamida em peixe-zebra através da modulação de parâmetros metabólicos e de estresse oxidativo, corroborando um papel protetor exercido pela molécula na espécie (Hammes et al., 2011). De maneira geral, os resultados desse capítulo sugerem que a taurina, principalmente quando administrada antes da exposição ao etanol, poderia exercer efeitos protetores em peixe-zebra, tanto por exercer um controle nas defesas antioxidantes enzimáticas quanto por prevenir a lipoperoxidação.

Outro objetivo proposto pela presente tese foi o de verificar o efeito promovido pelo tratamento com taurina no modelo de exposição aguda ao etanol no comportamento de peixe-zebra. Entretanto, os artigos relacionados à biologia do comportamento na espécie até o presente momento basicamente abordam a ação de diferentes drogas e fármacos nas tarefas. Qualquer modelo experimental é constantemente questionado sobre a sua validação, a qual é,

infelizmente, simplificada e reduzida ao nível farmacológico (efeito de drogas), sendo limitada em termos de referências às validações de face (correlação de efeitos entre espécies) e construto (compreensão dos mecanismos e dos parâmetros avaliados pelos testes). Com o advento das técnicas de farmacologia e biologia molecular, os estudos relacionados à biologia do comportamento em modelos animais estão se tornando disseminados e qualquer diferença observada em parâmetros comportamentais durante um tratamento é rapidamente adotada sem os devidos critérios de validação. Somente após uma extensiva análise é que tais perguntas começama a aparecer, devido à acumulação de dados inconsistentes e/ou contraditórios (Ennaceur et al., 2006). Sendo assim, por ser bastante recente em comparação aos estudos com roedores, o significado biológico dos parâmetros avaliados e o repertório comportamental espaço-temporal do peixe-zebra ainda carecem de esclarecimento e correlação entre os diferentes testes aplicados, tais como o *open tank*, conhecido como *novel tank test* e o tanque claro-escuro (Levin et al., 2007; Egan et al., 2009; Maximino et al., 2011).

Dados na literatura têm sugerido que a utilização de estímulos naturalísticos poderia ser uma ferramenta interessante para a definição do etograma da espécie em testes comportamentais (Blaser et al., 2010; Maximino et al., 2010). Com relação ao teste do *open tank*, são avaliados geralmente parâmetros de exploração vertical, uma vez que quando o peixe-zebra é colocado em um ambiente novo, a resposta inicial é de permanência no fundo do aparato, com exploração gradual da superfície à medida que o animal se habitua ao teste (Wong et al., 2010). Já a tarefa do claro-escuro é baseada no conflito existente entre permanência em um ambiente preferencial escuro

(escototaxia) e exploração a um ambiente novo de características aversivas aos animais adultos da espécie (Peng et al., 2009; Maximino et al., 2010).

No **quarto capítulo** desta tese, nós verificamos o efeito comportamental promovido pelo confinamento prévio em ambiente escuro, claro e transparente durante um curto período de tempo no paradigma do *open tank*. Os resultados obtidos através de análises automatizadas ao longo do tempo e de parâmetros *endpoint* demonstraram que, surpreendentemente, os animais confinados em ambiente claro apresentaram uma habituação intra-sessão mais rápida ao teste quando comparados aos grupos escuro e transparente (controle experimental). Embora sejam de difícil interpretação, nossos achados apontam para a necessidade de uma maior investigação sobre os mecanismos neurais envolvidos no teste do claro-escuro e *open tank*, bem como na interação entre estado motivacional e exploração vertical em peixe-zebra. É sabido que os efeitos observados após a exposição a uma mesma droga ansiolítica pode ser distinto em ambos os testes, bem como drogas ansiolíticas diferentes podem levar a comportamentos aparentemente relacionados nessas tarefas (parâmetros de permanência na região superior de um tanque e tempo em ambientes claros) (Egan et al., 2009; Maximino et al., 2011). Em corroboração aos resultados obtidos por Sackerman et al. (2010), nossos achados permitem hipotetizar que o teste do *open tank* e do claro-escuro poderiam medir diferentes níveis diferentes de construto e, portanto, futuros experimentos relacionados à correlação dos repertórios comportamentais de ambas as tarefas são imprescindíveis. Apesar dos resultados parecerem inconsistentes à primeira instância, existe a evidência de que diferentes mecanismos de sinalização neural podem estar envolvidos nos testes de campo aberto e

labirinto em cruz elevado utilizadas para roedores (Ramos, 2008), o que não seria surpreendente para tarefas comportamentais em peixe-zebra.

Além disso, foi proposta uma nova abordagem com relação ao paradigma do *open tank*, a qual consiste em uma análise tridimensional (espaço-temporal) do perfil exploratório. Através da divisão do tanque em sessões, as transições foram avaliadas ao longo do tempo, sendo que os dados obtidos sugerem que os efeitos comportamentais observados são causados por modificações no perfil exploratório, e não em parâmetros relacionados à locomoção. A construção do etograma dos grupos experimentais não só possibilitou a comparação entre os efeitos detctados entre os mesmos como, pioneiramente, demonstrou o repertório comportamental dos animais submetidos ao teste do *open tank*.

Com o conhecimento dos parâmetros relevantes que poderiam ser detectados através do teste do *open tank*, o **quinto capítulo** desta tese avaliou os efeitos comportamentais do tratamento com taurina no modelo de exposição aguda ao etanol nessa tarefa. De acordo com os resultados obtidos no terceiro capítulo, nós optamos por utilizar o pré-tratamento com taurina, visto que o mesmo foi mais eficaz na prevenção dos efeitos neuroquímicos induzidos pelo álcool. A exposição ao etanol por 1h promoveu alterações significativas em parâmetros relacionados à atividade locomotora, tais como distância percorrida e sinuosidade do nado, possivelmente devido a um efeito depressor do etanol na concentração testada (1% em volume). Esses dados poderiam ser suportados pela diminuição verificada na velocidade de nado média e diminuição do tempo móvel causada por um aumento dos episódios de imobilidade, os quais foram prevenidos pelo pré-tratamento com taurina. Na literatura, foi demonstrado que concentrações elevadas de etanol durante um

curto período de tempo acarretam em efeitos sedativos, os quais estão relacionados a diversas alterações neuroquímicas promovidas pelo álcool, inclusive em peixe-zebra (Gerlai, 2003; Boyce-Rustay et al., 2008; Chatterjee e Gerlai, 2009). Cabe enfatizar que, possivelmente, os efeitos observados em parâmetros relacionados à locomoção sejam devido aos efeitos centrais promovidos pelo álcool e não possuam relação com uma alteração na visão dos animais, uma vez que as respostas ópticocinéticas da espécie não são modificadas na presença de 1,5% de etanol (Brockerhoff et al., 1995., Wagle et al., 2011).

A análise do perfil exploratório dos peixes submetidos à tarefa do *open tank* demonstrou que a exposição aguda ao etanol aumentou a razão entre os números de entrada nas seções pelas transições na região inferior do aparato e diminuiu de forma significativa os valores obtidos para as seções da área superior. As concentrações de 42 e 400 mg/L de taurina foram capazes de prevenir as alterações promovidas pelo álcool no perfil exploratório. Esses dados foram corroborados pela análise em parâmetros de exploração vertical, *track* e *occupancy plots* representativos e por uma análise tridimensional do comportamento. Através de representações gráficas da locomoção horizontal e vertical dependente do tempo nos grupos experimentais, foi possível de identificar e relacionar os efeitos promovidos pelo etanol no etograma, bem como o efeito preventivo promovido pela taurina no modelo comportamental de exposição aguda ao etanol.

A partir dos nossos resultados, podemos sugerir que as modificações verificadas na atividade locomotora e no padrão exploratório do peixe-zebra poderiam ser relacionadas a mecanismos diferenciados ou à ação da taurina e do etanol em estruturas cerebrais distintas. Embora o pré-tratamento com 150

mg/L de taurina tenha sido capaz de manter os parâmetros de atividade locomotora em níveis basais, a mesma concentração não foi preveniu as alterações na exploração da área superior do aparato. Dessa forma, os efeitos da taurina nos parâmetros neurooquímicos avaliados na presente tese, tais como atividade de enzimas do sistema purinérgico e AChE, bem como das defesas antioxidantes enzimáticas e não enzimáticas poderiam contribuir, pelo menos em parte, para o fenótipo comportamental observado.

Apesar de demonstrarmos um efeito modulatório da taurina sobre as enzimas relacionadas à sinalização purinérgica, bem como sobre parâmetros relacionados ao estresse oxidativo, os mecanismos relacionados aos seus efeitos neuroquímicos não foram efetivamente esclarecidos nos trabalhos apresentados. Múltiplos fatores podem contribuir para esse aspecto, tais como: i) a ação pleiotrópica que a taurina é capaz de exercer em diversos sistemas biológicos; ii) a limitação do conhecimento sobre os processos que medeiam as ações da taurina em SNC de diferentes modelos experimentais; iii) a falta de informações que existe a respeito dos estudos relacionados à taurina em peixe-zebra; iv) a relação de construto do paradigma do *open tank* e os processos neurais envolvidos no mesmo ainda não estão bem elucidados; v) a falta de conhecimento adequado sobre a farmacocinética e farmacodinâmica da taurina e do etanol em *zebrafish*. Certamente, esses fatores ainda necessitam ser investigados com mais profundidade e nível de detalhamento em pesquisas futuras.

Estudos prévios têm demonstrado que a suplementação com taurina acarreta em uma diminuição dos níveis de adiponectina sérica, estresse oxidativo e expressão do fator de necrose tumoral alfa (TNFα), atenuando a esteatose hepática causada por etanol em roedores (Chen et al., 2009). Além

disso, é sabido que a produção de espécies reativas ocorre principalmente na mitocôndria, sendo essa uma organela que pode ser suscetível aos efeitos deletérios do etanol e do acetaldeído (González et al., 2007). O primeiro relato de incorporação da taurina a uma biomolécula é sua associação com tRNA mitocondrial sugerindo uma particular importância na síntese protéica, visto que modificações desse grupamento poderiam ser responsáveis pelos efeitos observados em doenças mitocondriais (Suzuki et al., 2002; 2011). Evidências têm demonstrado que muitas das ações promovidas pela taurina também podem ser relacionadas com modulação em parâmetros do sistema glutamatérgico e no metabolismo do Ca^{2+}, atenuando a excitotoxicidade em diversos modelos experimentais (Wu et al., 2005; Junyent et al., 2010; Wu and Prentice, 2010). Portanto, os efeitos comportamentais aparentemente não correlacionados com os parâmetros neuroquímicos analisados nesta tese poderiam ocorrer devido a outros fatores. Dessa maneira, não podem ser descartados efeitos no metabolismo hepático do etanol promovidos pelo pré-tratamento com taurina, os quais poderiam levar a uma distribuição diferencial dos compostos e dos metabólitos do álcool nos tecidos, bem como alterações a nível mitocondrial no SNC e em outros sistemas de sinalização e neurotransmissão.

III.2. CONCLUSÕES

A presente tese apresentou os resultados experimentais obtidos do estudo sobre os efeitos da taurina em parâmetros neuroquímicos e comportamentais de peixe-zebra, bem como descreveu a caracterização da família das NTPDases em diferentes tecidos dessa espécie e a aplicabilidade do teste do *open tank* para a avaliação de diferentes manipulações experimentais. A modulação de enzimas do sistema purinérgico pela taurina e os benefícios promovidos por esse aminoácido frente às alterações neuroquímicas e comportamentais induzidas pelo etanol puderam ser observadas.

Como importantes contribuições desta tese destacam-se a proposta de uma nova abordagem do paradigma do *open tank*, no qual as análises etológicas puderam ser empregadas para o estudo do perfil exploratório espaço-temporal dos animais. Nesse sentido, é importante ressaltar que o etograma pode apresentar variações de acordo com o tipo de manipulação (ex: estímulos naturalísticos, farmacológica) e que a taurina exerce um efeito na prevenção dos efeitos agudos mediados pelo etanol em peixe-zebra em nível neuroquímico e comportamental. Ao longo da elaboração desta tese e através da sequência dos trabalhos apresentada, podemos observar uma clara evolução no conhecimento sobre os parâmetros bioquímicos e comportamentais estudados em peixe-zebra. Nossos achados perimitirão a expansão da utilização desse vertebrado em pesquisas científicas como um organismo complementar aos roedores em estudos translacionais.

III.3. PERSPECTIVAS

Em relação aos efeitos promovidos pela taurina em peixe-zebra abordados nesta tese, futuras investigações ainda devem ser realizadas com intuito de elucidar os mecanismos relacionados à prevenção das alterações neuroquímicas e comportamentais observados no modelo de exposição aguda ao etanol. O objetivo principal é o de investir na utilização desse modelo a fim de investigar uma possível participação dos sistemas glutamatérgico e GABAérgico nos efeitos mediados pelo álcool e taurina em peixe-zebra. O modelo parece interessante também para a investigação de uma potencial ação neuroprotetora mediada pelas purinas, especialmente através dos nucleosídeos adenosina e guanosina, proposta pelo grupo em trabalhos prévios utilizando diferentes protocolos experimentais em roedores. A quantificação do álcool e dos metabólitos encontrados nos tecidos de peixe-zebra associada a um maior conhecimento do metabolismo da taurina e à determinação dos níveis de diferentes purinas nessa espécie contribuirá significativamente para a elaboração de futuros protocolos experimentais. A importância de serem testadas outras concentrações de etanol, visto que a exposição ao mesmo apresenta uma resposta bifásica em peixe-zebra similar à encontrada em mamíferos, bem como de diferentes tempos de tratamento ao álcool e taurina poderão fornecer informações relevantes para a compreensão dos efeitos prolongados promovidos ambas as substâncias. Adicionalmente, outros fármacos, sistemas de neurotransmissão, transdução de sinal e metabolismo celular a nível mitocondrial poderão ser avaliados e investigados utilizando o modelo proposto, o qual se apresenta viável, consistente e simples.

A identificação da presença de distintos membros pertencentes à família das NTPDases em peixe-zebra permitirá importantes interações com outros pesquisadores no sentido de investigar potenciais estratégicas terapêuticas abordando essa família enzimática. Especial atenção deverá ser concedida à funcionalidade dessas enzimas, bem como aos genes parálogos identificados, tais como os membros da NTPDase2 e NTPDase5, visto que, até o presente momento, ainda não existem anticorpos comerciais específicos capazes de reconhecer tais proteínas em peixe-zebra.

Também como perspectiva adicional de testar metodologias não-farmacológicas na análise comportamental de peixe-zebra, emerge a utilização de estímulos naturalísticos como uma possibilidade de estudar o repertório comportamental da espécie. O confinamento em distintos ambientes (claro, escuro e transparente) durante um curto período de tempo apresentado nesta tese possibilitou a descrição de um etograma para o paradigma do *open tank* através das análises espaço-temporais do perfil exploratório dos animais submetidos a essa tarefa. É importante salientar que os projetos iniciais com o comportamento de peixe-zebra contemplavam apenas a verificação dos efeitos promovidos por diferentes substâncias, sem a preocupação de uma avaliação global do comportamento e a compreensão do significado das variáveis analisadas. O objetivo e perspectivas traçadas com este estudo são de entender um pouco mais sobre o significado das respostas comportamentais descritas para o peixe-zebra, contribuir para a compreensão dos mecanismos neurais envolvidos em diferentes testes de comportamento, bem como fornecer suporte para a validação ao nível de construto para as tarefas comportamentais descritas na literatura. A elaboração de diferentes protocolos experimentais para análise dos comportamentos da espécie em outros tipos de aparatos,

como por exemplo, avaliação da atividade exploratória horizontal também não pode ser descartada.

Finalmente, em uma perspectiva mais ampla e de longo prazo, fica o planejamento da consolidação de uma linha consistente de pesquisa dentro de nossa Universidade, a qual vise fazer novos estudos avaliando aspectos relacionados à excitotoxicidade e neuroproteção em peixe-zebra. Dessa maneira, a elaboração de projetos em colaboração com diferentes laboratórios e grupos de pesquisa poderá contribuir para a aquisição de conhecimento sobre as técnicas relacionadas à utilização de morfolinos e ensaios de microarranjo. A implementação deste campo de pesquisa juntamente com ambas as técnicas certamente trará benefícios não apenas na utilização do peixe-zebra como um modelo alternativo para triagens pré-clínicas em larga escala, mas contribuirá para a formação de recursos humanos dentro da nossa instituição.

III.4. REFERÊNCIAS

Amores, A., Force, A., Yan, Y.L., Joly, L., Amemiya, C., Fritz, A., Ho, R.K., Langeland, J., Prince, V., Wang, Y.L., Westerfield, M., Ekker, M., Postlethwait, J.H. 1998. Zebrafish hox clusters and vertebrate genome evolution. Science. 282(5394): 1711-4.

Andersson, K.E. 2011. Muscarinic acetylcholine receptors in the urinary tract. Handb Exp Pharmacol. (202): 319-44.

Bachman, E.S., Berger-Sweeney, J., Coyle, J.T., Hohmann, C.F. 1994. Developmental regulation of adult cortical morphology and behavior: an animal model for mental retardation. Int J Dev Neurosci. 12(4): 239-53.

Bagatini, M.D., Martins, C.C., Gasparetto, D., Spanevello, R.M., Becker, L.V., Rosa, C.S., Battisti, V., Bellé, L., Gonçalves, J.F., Schetinger, M.R., Dos Santos, R.B., Oliveira, L.Z., Morsch, V.M. 2011. Enzymes that hydrolyze adenine nucleotides in patients with ischemic heart disease. Clin Chim Acta. 412(1-2): 159-64.

Banerjee, R., Vitvitsky, V., Garg, S.K. 2008. The undertow of sulfur metabolism on glutamatergic neurotransmission. Trends Biochem Sci. 33(9): 413-9.

Barbazuk, W.B., Korf, I., Kadavi, C., Heyen, J., Tate, S., Wun, E., Bedell, J.A., McPherson, J.D., Johnson, S.L. 2000. The syntenic relationship of the zebrafish and human genomes. Genome Res. 10(9): 1351-8.

Barreto-Chaves, M.L., Carneiro-Ramos, M.S., Cotomacci, G., Júnior, M.B., Sarkis, J.J. 2006. E-NTPDase 3 (ATP diphosphohydrolase) from cardiomyocytes, activity and expression are modulated by thyroid hormone. Mol Cell Endocrinol. 251(1-2): 49-55.

Beldi, G., Wu, Y., Sun, X., Imai, M., Enjyoji, K., Csizmadia, E., Candinas, D., Erb, L., Robson, S.C. 2008. Regulated catalysis of extracellular nucleotides by vascular CD39/ENTPD1 is required for liver regeneration. Gastroenterology. 135(5): 1751-60.

Belmeguenai, A., Botta, P., Weber, J.T., Carta, M., De Ruiter, M., De Zeeuw, C.I., Valenzuela, C.F., Hansel, C. 2008. Alcohol impairs long-term depression at the cerebellar parallel fiber-Purkinje cell synapse. J Neurophysiol. 100(6): 3167-74.

Bertrand, C., Chatonnet, A., Takke, C., Yan, Y.L., Postlethwait, J., Toutant, J.P., Cousin, X. 2001. Zebrafish acetylcholinesterase is encoded by a single gene localized on linkage group 7. Gene structure and polymorphism; molecular forms and expression pattern during development. J Biol Chem. 276(1): 464-74.

Bilotta, J., Barnett, J.A., Hancock, L., Saszik, S. 2004. Ethanol exposure alters zebrafish development: a novel model of fetal alcohol syndrome. Neurotoxicol Teratol. 26(6): 737-43.

Blaser, R.E., Chadwick, L., McGinnis, G.C. 2010. Behavioral measures of anxiety in zebrafish (*Danio rerio*). Behav Brain Res. 208(1): 56-62.

Blaser, R.E., Peñalosa, Y.M. 2011. Stimuli affecting zebrafish (*Danio rerio*) behavior in the light/dark preference test. Physiol Behav. 104(5): 831-7.

Bonan, C.D., Amaral, O.B., Rockenbach, I.C., Walz, R., Battastini, A.M., Izquierdo, I., Sarkis, J.J. 2000. Altered ATP hydrolysis induced by pentylenetetrazol kindling in rat brain synaptosomes. Neurochem Res. 25(6): 775-9.

Boveris, A. 1998. Biochemistry of free radicals: from electrons to tissues. Medicina (B Aires). 58(4): 350-6.

Boyce-Rustay, J.M., Palachick, B., Hefner, K., Chen, Y.C., Karlsson, R.M., Millstein, R.A., Harvey-White, J., Holmes, A. 2008. Desipramine potentiation of the acute depressant effects of ethanol: modulation by alpha2-adrenoreceptors and stress. Neuropharmacology. 55(5): 803-11.

Brockerhoff, S.E., Hurley, J.B., Janssen-Bienhold, U., Neuhauss, S.C., Driever, W., Dowling, J.E. 1995. A behavioral screen for isolating zebrafish mutants with visual system defects. Proc Natl Acad Sci U S A 92:10545-549.

Bulley, S., Shen, W. 2010. Reciprocal regulation between taurine and glutamate response via Ca^{2+}-dependent pathways in retinal third-order neurons. J Biomed Sci. 17 Suppl 1:S5.

Burnstock, G., Campbell, G., Satchell, D., Smythe, A. 1970. Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. Br J Pharmacol. 40(4): 668-88.

Burnstock, G. 1972. Purinergic nerves. Pharmacol Rev. 24(3): 509-81.

Burnstock, G. 2004. Cotransmission. Curr Opin Pharmacol. 4(1): 47-52.

Burnstock, G., Knight, G.E. 2004. Cellular distribution and functions of P2 receptor subtypes in different systems. Int Rev Cytol. 240: 31-304.

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K.M., Wu, N., Wong, K., Roy, S., Suciu, C., Goodspeed, J., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Tan, J., Denmark, A., Gilder, T., Kyzar, E., Dileo, J., Frank, K., Chang, K., Utterback, E., Hart, P., Kalueff, A.V. 2010. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. Nat Protoc. 5(11): 1786-99.

Chatterjee, D., Gerlai, R. 2009. High precision liquid chromatography analysis of dopaminergic and serotoninergic responses to acute alcohol exposure in zebrafish. Behav Brain Res. 200(1): 208-13.

Chen, X., Sebastian, B.M., Tang, H., McMullen, M.M., Axhemi, A., Jacobsen, D.W., Nagy, L.E. 2009. Taurine supplementation prevents ethanol-induced decrease in serum adiponectin and reduces hepatic steatosis in rats. Hepatology. 49(5): 1554-62.

Ciruela, F., Saura, C., Canela, E.I., Mallol, J., Lluis, C., Franco, R. 1996. Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. FEBS Lett. 380(3): 219-23.

Cohen, G., Sinet, P.M., Heikkila, R. 1980. Ethanol oxidation by rat brain in vivo. Alcohol Clin Exp Res. 4(4): 366-70.

Cunha, R.A., Ribeiro, J.A. 2000. ATP as a presynaptic modulator. Life Sci. 68(2): 119-37.

Dahm, R., Geisler, R. 2006. Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. Mar Biotechnol (NY). 8(4):329-45.

Danbolt, N.C. 2001. Glutamate uptake. Prog Neurobiol. 65(1): 1-105.

Dani, J.A, De Biasi, M. 2001. Cellular mechanisms of nicotine addiction. Pharmacol Biochem Behav. 70(4): 439-46.

David-Watine, B., Goblet, C., de Saint Jan, D., Fucile, S., Devignot, V., Bregestovski, P., Korn, H. 1999a. Cloning, expression and electrophysiological characterization of glycine receptor alpha subunit from zebrafish. Neuroscience. 90(1): 303-17.

David-Watine, B., Shorte, S.L., Fucile, S., de Saint Jan, D., Korn, H., Bregestovski, P. 1999b. Functional integrity of green fluorescent protein conjugated glycine receptor channels. Neuropharmacology. 38(6): 785-92.

Devi, S.L., Anuradha, C.V., 2010. Oxidative and nitrosative stress in experimental rat liver fibrosis: Protective effect of taurine. Environ Toxicol Pharmacol. 29(2): 104-10.

Diana, M., Brodie, M., Muntoni, A., Puddu, M.C., Pillolla, G., Steffensen, S., Spiga, S., Little, H.J. 2003. Enduring effects of chronic ethanol in the CNS: basis for alcoholism. Alcohol Clin Exp Res. 27(2): 354-61.

Dlugos, C.A., Rabin, R.A. 2003. Ethanol effects on three strains of zebrafish: model system for genetic investigations. Pharmacol Biochem Behav. 74(2): 471-80.

Echevarria, D.J., Toms, C.N., Jouandot, D.J. 2011. Alcohol-induced behavior change in zebrafish models. Rev Neurosci. 22(1): 85-93.

Edwards, J.G., Michel, W.C. 2002. Odor-stimulated glutamatergic neurotransmission in the zebrafish olfactory bulb. J Comp Neurol. 454(3): 294-309.

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., Kalueff, A.V. 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res. 205(1): 38-44.

Ennaceur, A., Michalikova, S., Chazot, P.L. 2006. Models of anxiety: responses of rats to novelty in an open space and an enclosed space. Behav Brain Res. 171(1): 26-49.

Ferreira, M.P., Willoughby, D. 2008. Alcohol consumption: the good, the bad, and the indifferent. Appl Physiol Nutr Metab. 33(1): 12-20.

Flavell, S.W., Greenberg, M.E. 2008. Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. Annu Rev Neurosci. 31: 563-90.

Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.L., Postlethwait, J. 1999. Preservation of duplicate genes by complementary, degenerative mutations. Genetics. 151(4): 1531-45.

Franco, R., Valenzuela, A., Lluis, C., Blanco, J. 1998. Enzymatic and extraenzymatic role of ecto-adenosine deaminase in lymphocytes. Immunol Rev. 161: 27-42.

Fredholm, B.B., Chen, J.F., Cunha, R.A., Svenningsson, P., Vaugeois, J.M. 2005. Adenosine and brain function. Int Rev Neurobiol. 63: 191-270.

Gebauer, D.L., Pagnussat, N., Piato, A.L., Schaefer, I.C., Bonan, C.D., Lara, D.R. 2011. Effects of anxiolytics in zebrafish: similarities and differences between benzodiazepines, buspirone and ethanol. Pharmacol Biochem Behav. 99(3): 480-6.

Gerlai, R., Lahav, M., Guo, S., Rosenthal, A. 2000. Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. Pharmacol Biochem Behav. 67(4): 773-82.

Gerlai, R. 2003. Zebra fish: an uncharted behavior genetic model. Behav Genet. 33(5): 461-8.

Goldsmith, P. 2004. Zebrafish as a pharmacological tool: the how, why and when. Curr Opin Pharmacol. 4(5): 504-12.

González, A., Pariente, J.A., Salido, G.M. 2007. Ethanol stimulates ROS generation by mitochondria through Ca2+ mobilization and increases GFAP content in rat hippocampal astrocytes. Brain Res. 1178: 28-37.

Gotti, C., Clementi, F. 2004. Neuronal nicotinic receptors: from structure to pathology. Prog Neurobiol. 74(6): 363-96.

Grunwald, D.J., Eisen, J.S. 2002. Headwaters of the zebrafish -- emergence of a new model vertebrate. Nat Rev Genet. 3(9): 717-24.

Gulati-Leekha, A., Goldman, D. 2006. A reporter-assisted mutagenesis screen using alpha 1-tubulin-GFP transgenic zebrafish uncovers missteps during neuronal development and axonogenesis. Dev Biol. 296(1): 29-47.

Guo, S. 2004. Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish? Genes Brain Behav. 3(2): 63-74.

Hagar, H.H. 2004. The protective effect of taurine against cyclosporine A-induced oxidative stress and hepatotoxicity in rats. Toxicol Lett. 151(2): 335-43.

Halliwell, B. 2006. Oxidative stress and neurodegeneration: where are we now? J Neurochem. 97(6): 1634-58.

Hammes, T.O., Pedroso, G.L., Hartmann, C.R., Escobar, T.D., Fracasso, L.B., da Rosa, D.P., Marroni, N.P., Porawski, M., da Silveira, T.R. 2011. The effect of taurine on hepatic steatosis induced by thioacetamide in zebrafish (*Danio rerio*). Dig Dis Sci. doi: 10.1007/s10620-011-1931-4.

Hanchar, H.J., Dodson, P.D., Olsen, R.W., Otis, T.S., Wallner, M. 2005. Alcohol-induced motor impairment caused by increased extrasynaptic GABA(A) receptor activity. Nat Neurosci. 8(3): 339-45.

Herrera, C., Casadó, V., Ciruela, F., Schofield, P., Mallol, J., Lluis, C., Franco, R. 2001. Adenosine A2B receptors behave as an alternative anchoring

protein for cell surface adenosine deaminase in lymphocytes and cultured cells. Mol Pharmacol. 59(1): 127-34.

Hohmann, CF. 2003. A morphogenetic role for acetylcholine in mouse cerebral neocortex. Neurosci Biobehav Rev. 27(4):351-63.

Hortopan, G.A., Dinday, M.T., Baraban, S.C. 2010. Spontaneous seizures and altered gene expression in GABA signaling pathways in a mind bomb mutant zebrafish. J Neurosci. 30(41): 13718-28.

Hughes, M.N. 1999. Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. Biochim Biophys Acta. 1411(2-3): 263-72.

Huxtable, R.J. 1992. Physiological actions of taurine. Physiol Rev. 72(1): 101-63.

Imboden, M., De Saint Jan, D., Leulier, F., Korn, H., Goblet, C., Bregestovski, P. 2001. Isolation and characterization of an alpha 2-type zebrafish glycine receptor subunit. Neuroscience. 103(3): 799-810.

Ishibashi, M., Leonard, C.S., Kohlmeier, K.A. 2009. Nicotinic activation of laterodorsal tegmental neurons: implications for addiction to nicotine. Neuropsychopharmacology. 34(12): 2529-47.

Israel, Y., Orrego, H., Carmichael, F.J. 1994. Acetate-mediated effects of ethanol. Alcohol Clin Exp Res. 18(1): 144-8.

Javed, R., Yarimizu, K., Pelletier, N., Li, C., Knowles, A.F. 2007. Mutagenesis of lysine 62, asparagine 64, and conserved region 1 reduces the activity of human ecto-ATPase (NTPDase 2). Biochemistry. 46(22): 6617-27.

Junyent, F., Romero, R., de Lemos, L., Utrera, J., Camins, A., Pallàs, M., Auladell, C. 2010. Taurine treatment inhibits CaMKII activity and modulates the presence of calbindin D28k, calretinin, and parvalbumin in the brain. J Neurosci Res. 88(1): 136-42.

Junyent, F., De Lemos, L., Utrera, J., Paco, S., Aguado, F., Camins, A., Pallàs, M., Romero, R., Auladell, C. 2011. Content and traffic of taurine in hippocampal reactive astrocytes. Hippocampus. 21(2): 185-97.

Kayani, M.A., Parry, J.M. 2010. The in vitro genotoxicity of ethanol and acetaldehyde. Toxicol In Vitro. 24(1): 56-60.

Kegel, B., Braun, N., Heine, P., Maliszewski, C.R., Zimmermann, H. 1997. An ecto-ATPase and an ecto-ATP diphosphohydrolase are expressed in rat brain. Neuropharmacology. 36(9): 1189-200.

Khan, A.U., Kovacic, D., Kolbanovskiy, A., Desai, M., Frenkel, K., Geacintov, N.E. 2000. The decomposition of peroxynitrite to nitroxyl anion (NO-) and singlet oxygen in aqueous solution. Proc Natl Acad Sci U S A. 97(7): 2984-9.

Knowles, A.F., Nagy, A.K., Strobel, R.S., Wu-Weis, M. 2002. Purification, characterization, cloning, and expression of the chicken liver ecto-ATP-diphosphohydrolase. Eur J Biochem. 269(9): 2373-82.

Knowles, A.F. 2011. The GDA1_CD39 superfamily: NTPDases with diverse functions. Purinergic Signal. 7(1): 21-45.

Köhler, D., Eckle, T., Faigle, M., Grenz, A., Mittelbronn, M., Laucher, S., Hart, M.L., Robson, S.C., Müller, C.E., Eltzschig, H.K. 2007. CD39/ectonucleoside triphosphate diphosphohydrolase 1 provides myocardial protection during cardiac ischemia/reperfusion injury. Circulation. 116(16): 1784-94.

Kong, W.X., Chen, S.W., Li, Y.L., Zhang, Y.J., Wang, R., Min, L., Mi, X. 2006. Effects of taurine on rat behaviors in three anxiety models. Pharmacol Biochem Behav. 83(2): 271-6.

Kozlowski, D.J., Chen, Z., Zhuang, L., Fei, Y.J., Navarre, S., Ganapathy, V. 2008. Molecular characterization and expression pattern of taurine transporter in zebrafish during embryogenesis. Life Sci. 82(19-20): 1004-11.

Kucenas, S., Li, Z., Cox, J.A., Egan, T.M., Voigt, M.M. 2003. Molecular characterization of the zebrafish P2X receptor subunit gene family. Neuroscience. 121(4): 935-45.

Langer, D., Hammer, K., Koszalka, P., Schrader, J., Robson, S., Zimmermann, H. 2008. Distribution of ectonucleotidases in the rodent brain revisited. Cell Tissue Res. 334(2): 199-217.

Lavoie, E.G., Kukulski, F., Lévesque, S.A., Lecka, J., Sévigny, J. 2004. Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-3. Biochem Pharmacol. 67(10): 1917-26.

Ledig, M., M'Paria, J.R., Mandel, P. 1981. Superoxide dismutase activity in rat brain during acute and chronic alcohol intoxication. Neurochem Res. 6(4):385-90.

Lee, I.S., Renno, W.M., Beitz, A.J. 1992. A quantitative light and electron microscopic analysis of taurine-like immunoreactivity in the dorsal horn of the rat spinal cord. J Comp Neurol. 321(1): 65-82.

Lele, Z., Krone, P.H. 1996. The zebrafish as a model system in developmental, toxicological and transgenic research. Biotechnol Adv. 14(1): 57-72.

Levin, E.D., Bencan, Z., Cerutti, D.T. 2007. Anxiolytic effects of nicotine in zebrafish. Physiol Behav. 90(1): 54-8.

Li, G., Olson, J.E. 2008. Purinergic activation of anion conductance and osmolyte efflux in cultured rat hippocampal neurons. Am J Physiol Cell Physiol. 295(6): C1550-60.

Lieschke, G.J., Currie, P.D. 2007. Animal models of human disease: zebrafish swim into view. Nat Rev Genet. 8(5): 353-67.

López Patiño, M.A., Yu, L., Yamamoto, B.K., Zhdanova, I.V. 2008. Gender differences in zebrafish responses to cocaine withdrawal. Physiol Behav. 95(1-2): 36-47.

Low, S.E., Kuwada, J.Y., Hume, R.I. 2008. Amino acid variations resulting in functional and nonfunctional zebrafish P2X(1) and P2X (5.1) receptors. Purinergic Signal. 4(4):383-92.

Maier, S.A., Galellis, J.R., McDermid, H.E. 2005. Phylogenetic analysis reveals a novel protein family closely related to adenosine deaminase. J Mol Evol. 61(6): 776-94.

Marc, R.E., Cameron, D. 2001. A molecular phenotype atlas of the zebrafish retina. J Neurocytol. 30(7): 593-654.

Mathur, P., Lau, B., Guo, S. 2011. Conditioned place preference behavior in zebrafish. Nat Protoc. 6(3): 338-45.

Maximino, C., Marques de Brito, T., Dias, C.A., Gouveia, A. Jr., Morato, S. 2010. Scototaxis as anxiety-like behavior in fish. Nat Protoc. 5(2): 209-16.

Maximino, C., da Silva, A.W., Gouveia, A. Jr., Herculano, A.M. 2011. Pharmacological analysis of zebrafish (*Danio rerio*) scototaxis. Prog Neuropsychopharmacol Biol Psychiatry. 35(2): 624-31.

Mody, I,, MacDonald, J.F. 1995. NMDA receptor-dependent excitotoxicity: the role of intracellular Ca^{2+} release. Trends Pharmacol Sci. 16(10): 356-9.

Morales, I., Dopico, J.G., Sabate, M., Gonzalez-Hernandez, T., Rodriguez, M. 2007. Substantia nigra osmoregulation: taurine and ATP involvement. Am J Physiol Cell Physiol. 292(5): C1934-41.

Neary, J.T., Zimmermann, H. 2009. Trophic functions of nucleotides in the central nervous system. Trends Neurosci. 32(4): 189-98.

Nedeljkovic, N., Banjac, A., Horvat, A., Stojiljkovic, M., Nikezic, G. 2005. Developmental profile of NTPDase activity in synaptic plasma membranes isolated from rat cerebral cortex. Int J Dev Neurosci. 23(1): 45-51.

North, R.A., Barnard, E.A. 1997. Nucleotide receptors. Curr Opin Neurobiol. 7(3): 346-57.

Oliveira, M.W., Minotto, J.B., de Oliveira, M.R., Zanotto-Filho, A., Behr, G.A., Rocha, R.F., Moreira, J.C., Klamt, F. 2010. Scavenging and antioxidant potential of physiological taurine concentrations against different reactive oxygen/nitrogen species. Pharmacol Rep. 62(1): 185-93.

Pastor, R., Sanchis-Segura, C., Aragon, C.M. 2002. Ethanol-stimulated behaviour in mice is modulated by brain catalase activity and H2O2 rate of production. Psychopharmacology (Berl). 165(1):51-9.

Peng, T.I., Jou, M.J. 2010. Oxidative stress caused by mitochondrial calcium overload. Ann N Y Acad Sci. 1201: 183-8.

Peng, J., Wagle, M., Mueller, T., Mathur, P., Lockwood, B.L., Bretaud, S., Guo, S. 2009. Ethanol-modulated camouflage response screen in zebrafish uncovers a novel role for cAMP and extracellular signal-regulated kinase signaling in behavioral sensitivity to ethanol. J Neurosci. 29(26): 8408-18.

Piato, A.L., Rosemberg, D.B., Capiotti, K.M., Siebel, A.M., Herrmann, A.P., Ghisleni, G., Vianna, M.R., Bogo, M.R., Lara, D.R., Bonan, C.D. 2011. Acute restraint stress in zebrafish: behavioral parameters and purinergic signaling. Neurochem Res. 36(10):1876-86.

Pivovarova, N.B., Andrews, S.B. 2010. Calcium-dependent mitochondrial function and dysfunction in neurons. FEBS J. 277(18): 3622-36.

Postlethwait, J.H., Yan, Y.L., Gates, M.A., Horne, S., Amores, A., Brownlie, A., Donovan, A., Egan, E.S., Force, A., Gong, Z., Goutel, C., Fritz, A., Kelsh, R., Knapik, E., Liao, E., Paw, B., Ransom, D., Singer, A., Thomson, M., Abduljabbar, T.S., Yelick, P., Beier, D., Joly, J.S., Larhammar, D., Rosa, F., Westerfield, M., Zon, L.I., Johnson, S.L., Talbot, W.S. 1998. Vertebrate genome evolution and the zebrafish gene map. Nat Genet. 18(4): 345-9.

Pushpakiran, G., Mahalakshmi, K., Viswanathan, P., Anuradha, C.V. 2005. Taurine prevents ethanol-induced alterations in lipids and ATPases in rat tissues. Pharmacol Rep. 57(5): 578-87.

Quertemont, E., Grant, K.A., Correa, M., Arizzi, M.N., Salamone, J.D., Tambour, S., Aragon, C.M., McBride, W.J., Rodd, Z.A., Goldstein, A., Zaffaroni, A., Li, T.K., Pisano, M., Diana, M. 2005. The role of acetaldehyde in the central effects of ethanol. Alcohol Clin Exp Res. 29(2): 221-34.

Radi, R., Cassina, A., Hodara, R., Quijano, C., Castro, L. 2002. Peroxynitrite reactions and formation in mitochondria. Free Radic Biol Med. 33(11): 1451-64.

Ralevic, V., Burnstock, G. 1998. Receptors for purines and pyrimidines. Pharmacol Rev. 50(3): 413-92.

Ramos, A. 2008. Animal models of anxiety: do I need multiple tests? Trends Pharmacol Sci. 29(10): 493-8.

Reimers, M.J., Hahn, M.E., Tanguay, R.L. 2004. Two zebrafish alcohol dehydrogenases share common ancestry with mammalian class I, II, IV, and V alcohol dehydrogenase genes but have distinct functional characteristics. J Biol Chem. 279(37): 38303-12.

Reimers, M.J., La, Du J.K., Periera, C.B., Giovanini, J., Tanguay, R.L. 2006. Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants. Neurotoxicol Teratol. 28(4): 497-508.

Ricatti, M.J., Battista, A.G., Zorrilla Zubilete, M., Faillace, M.P. 2011. Purinergic signals regulate daily S-phase cell activity in the ciliary marginal zone of the zebrafish retina. J Biol Rhythms. 26(2): 107-17.

Rico, E.P., Senger, M.R., Fauth, Mda. G., Dias, R.D., Bogo, M.R., Bonan, C.D. 2003. ATP and ADP hydrolysis in brain membranes of zebrafish (*Danio rerio*). Life Sci. 73(16): 2071-82.

Rico, E.P., Rosemberg, D.B., Dias, R.D., Bogo, M.R., Bonan, C.D. 2007. Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain. Toxicol Lett. 174(1-3): 25-30.

Rico, E.P., Rosemberg, D.B., Senger, M.R., de Bem Arizi, M., Dias, R.D., Souto, A.A., Bogo, M.R., Bonan, C.D. 2008. Ethanol and acetaldehyde alter NTPDase and 5'-nucleotidase from zebrafish brain membranes. Neurochem Int. 52(1-2): 290-6.

Rico, E.P., Rosemberg, D.B., Seibt, K.J., Capiotti, K.M., Da Silva, R.S., Bonan, C.D. 2011a. Zebrafish neurotransmitter systems as potential pharmacological and toxicological targets. Neurotoxicol Teratol. doi:10.1016/j.ntt.2011.07.007.

Rico, E.P., Rosemberg, D.B., Langoni, A.D., Souto, A.A., Dias, R.D., Bogo, M.R., Bonan, C.D., Souza, D.O. 2011b. Chronic ethanol treatment alters purine nucleotide hydrolysis and nucleotidase gene expression pattern in zebrafish brain. Neurotoxicology. doi:10.1016/j.neuro.2011.05.010.

Robson, S.C., Sévigny, J., Zimmermann, H. 2006. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signal. 2(2): 409-30.

Rodríguez-Navarro, J.A., Gonzalo-Gobernado, R., Herranz, A.S., Gonźlez-Vigueras, J.M., Solís, J.M. 2009. High potassium induces taurine release by osmosensitive and osmoresistant mechanisms in the rat hippocampus in vivo. J Neurosci Res. 87(1): 208-17.

Roesler, R., Vianna, M.R., Lara, D.R., Izquierdo, I., Schmidt, A.P., Souza, D.O. 2000. Guanosine impairs inhibitory avoidance performance in rats. Neuroreport. 11(11): 2537-40.

Rosemberg, D.B., Rico, E.P., Guidoti, M.R., Dias, R.D., Souza, D.O., Bonan, C.D., Bogo, M.R. 2007a. Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (*Danio rerio*). Life Sci. 81(21-22): 1526-34.

Rosemberg, D.B., Rico, E.P., Senger, M.R., Arizi, Mde B., Dias, R.D., Bogo, M.R., Bonan, C.D. 2007b. Acute and subchronic copper treatments alter extracellular nucleotide hydrolysis in zebrafish brain membranes. Toxicology. 236(1-2): 132-9.

Rosemberg, D.B., Rico, E.P., Senger, M.R., Dias, R.D., Bogo, M.R., Bonan, C.D., Souza, D.O. 2008. Kinetic characterization of adenosine deaminase activity in zebrafish (*Danio rerio*) brain. Comp Biochem Physiol B Biochem Mol Biol. 151(1): 96-101.

Rücker, B., Almeida, M.E., Libermann, T.A., Zerbini, L.F., Wink, M.R., Sarkis, J.J. 2008. E-NTPDases and ecto-5'-nucleotidase expression profile in rat heart left ventricle and the extracellular nucleotide hydrolysis by their nerve terminal endings. Life Sci. 82(9-10): 477-86.

Ruiz, M.A., Escriche, M., Lluis, C., Franco, R., Martín, M., Andrés, A., Ros, M. 2000. Adenosine A(1) receptor in cultured neurons from rat cerebral cortex: colocalization with adenosine deaminase. J Neurochem. 75(2): 656-64.

Sackerman, J., Donegan, J.J., Cunningham, C.S., Nguyen, N.N., Lawless, K., Long, A., Benno, R.H., Gould, G.G. 2010. Zebrafish behavior in novel environments: effects of acute exposure to anxiolytic compounds and choice of *Danio rerio* line. Int J Comp Psychol. 23(1): 43-61.

Salgado, A.I., Cunha, R.A., Ribeiro, J.A. 2000. Facilitation by P(2) receptor activation of acetylcholine release from rat motor nerve terminals: interaction with presynaptic nicotinic receptors. Brain Res. 877(2): 245-50.

Saransaari, P., Oja, S.S. 2003. Interactions of taurine and adenosine in the mouse hippocampus in normoxia and ischemia. Adv Exp Med Biol. 526: 445-51.

Saransaari, P., Oja, S.S. 2010. Mechanisms of inhibitory amino acid release in the brain stem under normal and ischemic conditions. Neurochem Res. 35(12): 1948-56.

Sarter, M., Parikh, V. 2005. Choline transporters, cholinergic transmission and cognition. Nat Rev Neurosci. 6(1): 48-56.

Schetinger, M.R., Morsch, V.M., Bonan, C.D., Wyse, A.T. 2007. NTPDase and 5'-nucleotidase activities in physiological and disease conditions: new perspectives for human health. Biofactors. 31(2): 77-98.

Schmidt, A.P., Lara, D.R., Souza, D.O. 2007. Proposal of a guanine-based purinergic system in the mammalian central nervous system. Pharmacol Ther. 116(3): 401-16.

Schousboe, A., Belhage, B., Frandsen, A. 1997. Role of Ca^{+2} and other second messengers in excitatory amino acid receptor mediated neurodegeneration: clinical perspectives. Clin Neurosci. 4(4): 194-8.

Seitz, H.K., Homann, N. 2007. The role of acetaldehyde in alcohol-associated cancer of the gastrointestinal tract. Novartis Found Symp. 285: 110-9;

Senger, M.R., Rico, E.P., Dias, R.D., Bogo, M.R., Bonan, C.D. 2004. Ecto-5'-nucleotidase activity in brain membranes of zebrafish (*Danio rerio*). Comp Biochem Physiol B Biochem Mol Biol. 139(2): 203-7.

Siler-Marsiglio, K.I., Paiva, M., Madorsky, I., Serrano, Y., Neeley, A., Heaton, M.B. 2004. Protective mechanisms of pycnogenol in ethanol-insulted cerebellar granule cells. J Neurobiol. 61(2): 267-76.

Soreq, H., Seidman, S. 2001. Acetylcholinesterase--new roles for an old actor. Nat Rev Neurosci. 2(4): 294-302.

Sprague, J., Clements, D., Conlin, T., Edwards, P., Frazer, K., Schaper, K., Segerdell, E., Song, P., Sprunger, B., Westerfield, M. 2003. The Zebrafish Information Network (ZFIN): the zebrafish model organism database. Nucleic Acids Res. 31(1): 241-3.

Stern, H.M., Zon, L.I. 2003. Cancer genetics and drug discovery in the zebrafish. Nat Rev Cancer. 3(7): 533-9.

Sträter, N. 2006. Ecto-5'-nucleotidase: Structure function relationships. Purinergic Signal. 2(2): 343-50.

Su, L.D., Sun, C.L., Shen, Y. 2010. Ethanol acutely modulates mGluR1-dependent long-term depression in cerebellum. Alcohol Clin Exp Res. 34(7): 1140-5.

Sung, D.Y., Walthall, W.W., Derby, C.D. 1996. Identification and partial characterization of putative taurine receptor proteins from the olfactory organ of the spiny lobster. Comp Biochem Physiol B Biochem Mol Biol. 115(1): 19-26.

Suzuki, T., Suzuki, T., Wada, T., Saigo, K., Watanabe, K. 2002. Taurine as a constituent of mitochondrial tRNAs: new insights into the functions of taurine and human mitochondrial diseases. EMBO J. 21(23): 6581-9.

Suzuki, T., Miyauchi, K., Suzuki, T., Yokobori, S., Shigi, N., Kondow, A., Takeuchi, N., Yamagishi, A., Watanabe, K. 2011. Taurine-containing uridine modifications in tRNA anticodons are required to decipher non-universal genetic codes in ascidian mitochondria. J Biol Chem. 286(41): 35494-8.

Swift, R. 2003. Direct measurement of alcohol and its metabolites. Addiction. 98 Suppl 2: 73-80.

Tappaz, M., Almarghini, K., Do, K. 1994. Cysteine sulfinate decarboxylase in brain: identification, characterization and immunocytochemical location in astrocytes. Adv Exp Med Biol. 359: 257-68.

Taylor, M.R., Hurley, J.B., Van Epps, H.A., Brockerhoff, S.E. 2004. A zebrafish model for pyruvate dehydrogenase deficiency: rescue of neurological dysfunction and embryonic lethality using a ketogenic diet. Proc Natl Acad Sci U S A. 101(13): 4584-9.

Thomazi, A.P., Boff, B., Pires, T.D., Godinho, G., Battú, C.E., Gottfried, C., Souza, D.O., Salbego, C., Wofchuk, S.T. 2008. Profile of glutamate uptake and cellular viability in hippocampal slices exposed to oxygen and glucose deprivation: developmental aspects and protection by guanosine. Brain Res. 1188: 233-40.

Tinsley, M.R., Quinn, J.J., Fanselow, M.S. 2004. The role of muscarinic and nicotinic cholinergic neurotransmission in aversive conditioning: comparing pavlovian fear conditioning and inhibitory avoidance. Learn Mem. 11(1): 35-42.

Vascotto, S.G., Beckham, Y., Kelly, G.M. 1997. The zebrafish's swim to fame as an experimental model in biology. Biochem Cell Biol. 75(5): 479-85.

Vinadé, E.R., Schmidt, A.P., Frizzo, M.E., Izquierdo, I., Elisabetsky, E., Souza, D.O. 2003. Chronically administered guanosine is anticonvulsant, amnesic and anxiolytic in mice. Brain Res. 977(1): 97-102.

Vitvitsky, V., Garg, S.K., Banerjee, R. 2011. Taurine biosynthesis by neurons and astrocytes. J Biol Chem. 286(37): 32002-10.

Vogel, G. 2000. Genomics. Sanger will sequence zebrafish genome. Science. 290(5497): 1671.

Vorhoff, T., Zimmermann, H., Pelletier, J., Sévigny, J., Braun, N. 2005. Cloning and characterization of the ecto-nucleotidase NTPDase3 from rat brain: Predicted secondary structure and relation to other members of the E-NTPDase family and actin. Purinergic Signal. 1(3): 259-70.

Wagle, M., Mathur, P., Guo, S. 2011. Corticotropin-releasing factor critical for zebrafish camouflage behavior is regulated by light and sensitive to ethanol. J Neurosci. 31(1): 214-24.

Wang, G.H., Jiang, Z.L., Fan, X.J., Zhang, L., Li, X., Ke, K.F. 2007. Neuroprotective effect of taurine against focal cerebral ischemia in rats possibly mediated by activation of both $GABA_A$ and glycine receptors. Neuropharmacology. 52(5): 1199-209.

Westerblad, H., Allen, D.G. 2011. Emerging roles of ROS/RNS in muscle function and fatigue. Antioxid Redox Signal. 15(9): 2487-99.

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., Goodspeed, J., Suciu, C., Tan, J., Grimes, C., Chung, A., Rosenberg, M., Gaikwad, S., Denmark, A., Jackson, A., Kadri, F., Chung, K.M., Stewart, A., Gilder, T., Beeson, E., Zapolsky, I., Wu, N., Cachat, J., Kalueff, A.V. 2010. Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). Behav Brain Res. 208(2): 450-57.

Wu, H., Jin, Y., Wei, J., Jin, H., Sha, D., Wu, J.Y. 2005. Mode of action of taurine as a neuroprotector. Brain Res. 1038(2): 123-31.

Wu, J.Y., Tang, X.W., Tsai, W.H. 1992. Taurine receptor: kinetic analysis and pharmacological studies. Adv Exp Med Biol. 315: 263-68.

Wu, J.Y., Prentice, H. 2010. Role of taurine in the central nervous system. J Biomed Sci. 17 Suppl 1:S1.

Wu, Z.Y., Xu, T.L. 2003. Taurine-evoked chloride current and its potentiation by intracellular Ca^{2+} in immature rat hippocampal CA1 neurons. Amino Acids. 24(1-2): 155-61.

Yalfani, M.S., Contreras, S., Medina, F., Sueiras, J.E. 2011. Hydrogen substitutes for the in situ generation of H2O2: an application in the Fenton reaction. J Hazard Mater. 192(1): 340-6.

Yu, H.S., Oyama, T., Isse, T., Kitagawa, K., Pham, T.T., Tanaka, M., Kawamoto, T. 2010. Formation of acetaldehyde-derived DNA adducts due to alcohol exposure. Chem Biol Interact. 188(3): 367-75.

Zhang, B., Yang, X., Gao, X. 2010. Taurine protects against bilirubin-induced neurotoxicity in vitro. Brain Res. 1320: 159-67.

Zimmermann, H. 1994. Signalling via ATP in the nervous system. Trends Neurosci. 17(10): 420-6.

Zimmermann, H. 2000. Extracellular metabolism of ATP and other nucleotides. Naunyn Schmiedebergs Arch Pharmacol. 362(4-5): 299-309.

Zimmermann, H. 2008. ATP and acetylcholine, equal brethren. Neurochem Int. 52(4-5): 634-48.

Zirger, J.M., Beattie, C.E., McKay, D.B., Boyd, R.T. 2003. Cloning and expression of zebrafish neuronal nicotinic acetylcholine receptors. Gene Expr Patterns. 3(6): 747-54.
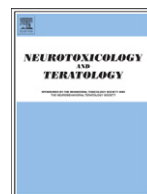
ANEXOS

A.1. Revisão sobre a utilização do peixe-zebra em testes farmacológicos e toxicológicos. *Neurotoxicology and Teratology* (no prelo).

Review article

Zebrafish neurotransmitter systems as potential pharmacological and toxicological targets

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ABSTRACT

Recent advances in neurobiology have emphasized the study of brain structure and function and its association with numerous pathological and toxicological events. Neurotransmitters are substances that relay, amplify, and modulate electrical signals between neurons and other cells. Neurotransmitter signaling mediates rapid intercellular communication by interacting with cell surface receptors, activating second messenger systems and regulating the activity of ion channels. Changes in the functional balance of neurotransmitters have been implicated in the failure of central nervous system function. In addition, abnormalities in neurotransmitter production or functioning can be induced by several toxicological compounds, many of which are found in the environment. The zebrafish has been increasingly used as an animal model for biomedical research, primarily due to its genetic tractability and ease of maintenance. These features make this species a versatile tool for pre-clinical drug discovery and toxicological investigations. Here, we present a review regarding the role of different excitatory and inhibitory neurotransmitter systems in zebrafish, such as dopaminergic, serotoninergic, cholinergic, purinergic, histaminergic, nitrergic, glutamatergic, glycinergic, and GABAergic systems, and emphasizing their features as pharmacological and toxicological targets. The increase in the global knowledge of neurotransmitter systems in zebrafish and the elucidation of their pharmacological and toxicological aspects may lead to new strategies and appropriate research priorities to offer insights for biomedical and environmental research.

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1. Introduction

Neurotransmitters are chemical messengers that initiate, amplify, and modulate signals between neurons and other cells in the body. Neuronal activity depends on the balance between the number of excitatory and inhibitory processes affecting it, which may occur individually or simultaneously (Prange et al., 2004). In addition, neurological and psychiatric disorders are associated with the abnormal production or function of neurotransmitters, and experimental approaches involving transporters, receptors, and enzymes in such conditions have been characterized (Raiteri, 2006). These assays include the analysis of various neurotransmitter characteristics, such as localization, function, and pharmacological properties (Raiteri, 2006).

In this context, there is a growing interest in biological models to investigate the basis of neurotransmission. Although many researchers study these parameters through cell culture methods, using the whole organism allows the screening of processes that are not easily replicated in vitro, such as organ development. Furthermore, drug metabolism is an important factor for the conservation of drug activity across species. Despite that in vitro studies provide speed and efficiency to screen a larger number of compounds, whole organisms offer advantages over cell lines for chemical genetic screens, which provide information regarding tissue specificity, toxicity, and biologic availability.

The zebrafish has become a promising model in many research areas, including neuroscience, developmental biology, toxicology, transgenic research, vertebrate genome evolution, and teratology (Lele and Krone, 1996; Vascotto et al., 1997; Ivetac et al., 2000; Bowman and Zon, 2010). Additionally, it has been shown that the zebrafish genome shares similarities with the human genome (Barbazuk et al., 2000). The characterization of alternative animal models, which permits embryologic and molecular screenings, contributes to a better knowledge of neurochemical mechanisms and helps in drug development and

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screening. Besides the zebrafish has been used to screen novel compounds and small molecules as a starting point for drug discovery, it has been an emergent vertebrate model for analyses of transcriptome, proteome, and metabolome (Sukardi et al., 2010).

Neurotoxicity can result from exposure to drugs used for chemotherapy, radiation treatment, and organ transplantation as well as from food additives and environmental toxins (Parng et al., 2007). The ability to examine the nervous system and visually evaluate the brain in early stages of development makes the zebrafish an exceptional model for neurotoxicity assessments. The investigation of environmental toxins (e.g., organic compounds and heavy metals) on zebrafish neurotransmission has been used to understand the biological basis of the cumulative effects of pollutants and other chemicals. Technological advancements and knowledge of the zebrafish genome have allowed for the development and improvement of sophisticated strategies such as mutant individuals, genetic and tissue manipulation, morpholinos and microarray technology. The development of new bioassays for toxic and therapeutic endpoints in this species has contributed to the understanding of mechanisms triggered by chemical toxicity (Hill et al., 2005). These advances, associated to the simplicity of evaluating the morphological, biochemical, and physiological information at all stages of early development in juveniles and adults of both sexes, make this species ideal for identifying the adverse effects of chemical exposure. This review will focus on the role of different excitatory and inhibitory neurotransmitter systems in the zebrafish, including dopaminergic, serotoninergic, cholinergic, glutamatergic, purinergic, histaminergic, nitric oxide synthase, glycinergic, and GABAergic systems (Fig. 1). Furthermore, we will emphasize their importance and potential application for pharmacological and toxicological studies.

2. Dopamine and serotonin

The aminergic neurotransmitters, including dopamine (DA) and serotonin (5HT), mediate several important brain functions. Abnormalities in their levels have been implicated in distinct human central nervous system (CNS) diseases (Belmaker, 2008; Murray et al., 2008). Although dopaminergic neurons account for less than 1% of the total neuronal population of the brain, they have important effects on brain physiology. For example, DA regulates locomotion, cognition, emotion, and reward (Goldman-Rakic, 1998; Schultz, 2002). The effects promoted by DA are mediated by a group of G-protein-coupled receptors (Nürnberger et al., 2004). In mammals, there are five dopamine receptor (DR) subtypes that are grouped into two families, D_1 and D_2, based on pharmacological profiles and sequence similarities (Callier et al., 2003; Surmeier et al., 2007). The D_1 family consists of the D_1 and D_5 receptor subtypes, and the D_2 family comprises the D_2, D_3, and D_4 receptors (Callier et al., 2003). Dysfunction in dopaminergic neurotransmission is associated with a variety of neuropathologies, such as Parkinson's disease, Tourette syndrome, and schizophrenia (Missale et al., 1998). The neurotransmitter serotonin is an important modulator of brain physiology and behavior, and it plays a fundamental role during development and plasticity in the vertebrate CNS (Daubert and Condron, 2010). The serotonergic neurons in the mammalian CNS are primarily located in the raphe nuclei, and they innervate nearly all regions of the brain (Sallinen et al., 2009). Serotonin regulates perception, aggressiveness, anxiety, sexual behavior, appetite, vascular function, and pain (Lucki, 1998; Parsey, 2010). In addition to neural communication, serotonin plays fundamental developmental roles and influences plasticity in the vertebrate CNS (Cote et al., 2007; Fricker et al., 2005; Gaspar et al., 2003). Importantly, the dysfunction of serotonergic neurons during development or adulthood has been implicated in several psychiatric diseases, including depression, drug addiction, and schizophrenia (Lucki, 1998; Sallinen et al., 2009).

Zebrafish dopaminergic and serotonergic systems share similarities to respective mammalian systems, making this species a feasible model for evaluating the general properties of both systems (Panula

et al., 2006; Flinn et al, 2008). During the last decade, the zebrafish has been suggested as a tool for the analysis of the effects of alcohol on adult brain function (Gerlai et al., 2000; Gerlai et al., 2009). Alcoholism is known to affect aminergic neurons and can lead to abnormalities in the levels of aminergic neurotransmitters, resulting in significant behavioral changes (Rodd-Henricks et al., 2000; Thielen et al., 2004). For example, intermediate doses of alcohol (0.25–0.50%, v/v), when administered acutely, were shown to increase locomotor activity and aggression (Gerlai et al., 2000). Shoaling, a form of social behavior also known as group preference, was also impaired by increasing doses of acute alcohol exposure (Gerlai et al., 2008). Moreover, behavioral responses to a predator or its computer-animated image were enhanced or impaired after acute exposure to intermediate or high alcohol doses, respectively (Gerlai et al., 2000, 2008). In addition, Chatterjee and Gerlai (2009) showed significant changes in levels of 5HT and DA and their metabolites in zebrafish after alcohol treatment (Chatterjee and Gerlai, 2009).

Dopaminergic deficiency in the zebrafish brain has been previously induced by systemic administration of catecholaminergic neurotoxins, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Anichtchik et al., 2004). The levels of dopamine and noradrenaline decreased significantly after the injection of MPTP and 6-OHDA. These drugs also caused neurotransmitter-associated behavioral changes; for example, general locomotor activity (distance moved and velocity) was markedly decreased, and the fish altered the swimming patterns (Anichtchik et al., 2004). Silver nanoparticle consumption can alter the levels of dopamine and behavior in zebrafish. A recent report demonstrated that nervous system development is disrupted in zebrafish exposed to Ag^+ (Powers et al., 2011). Ag^+ is a developmental neurotoxin that causes persistent neurobehavioral effects, reinforcing health concerns regarding the Ag^+ that is released from silver nanoparticles. In addition, early developmental exposure to Ag^+ elevated the DA and 5HT turnover in adult zebrafish (Powers et al., 2011).

Giacomini et al. (2006) investigated the effects of antipsychotics on larval zebrafish. The antipsychotics haloperidol and fluphenazine produced hypoactivity associated with erratic swimming bouts, which were rescued by coadministration with the dopamine precursor levodopa (Giacomini et al., 2006). Clozapine, an atypical antipsychotic, also induced hypoactivity. Interestingly, this effect was prevented by the D_4 receptor selective agonist ABT-724, but not by quinpirole, a D_2/D_3 agonist, which produces hyperactivity in zebrafish larvae (Boehmler et al., 2007). The acute administration of fluoxetine, a selective serotonin reuptake inhibitor, produces a hyperlocomotor effect accompanied by diminished expression of the serotonin transporter protein and the $5\text{-}HT_{1A}$ receptor in the spinal cord, but not in the zebrafish larval brain (Airhart et al., 2007). Other researchers have reported that the hallucinogenic drug lysergic acid diethylamide (LSD), a nonselective serotonin receptor agonist, produces a pattern of disorganized exploration in many models, including the novel tank diving test and the scototaxis test (Grossman et al., 2010).

These studies highlight the potential role of dopaminergic and serotonergic systems in zebrafish. Further evidence is required to clarify the impact of changes in these neurotransmitter systems on brain development in this species as well as their implications for potential pharmacological targets.

3. Acetylcholine

Acetylcholine (ACh) is a signaling molecule that elicits several actions at neuromuscular junctions and in the CNS (Panula et al., 2010). This molecule activates two classes of receptors (AChRs): the ionotropic nicotinic ACh receptors (nAChRs) and G-protein-coupled muscarinic AChRs (mAChRs). Whereas the mAChRs may be involved in neurotransmission, neuromodulation (Brown, 2010), and olfactory
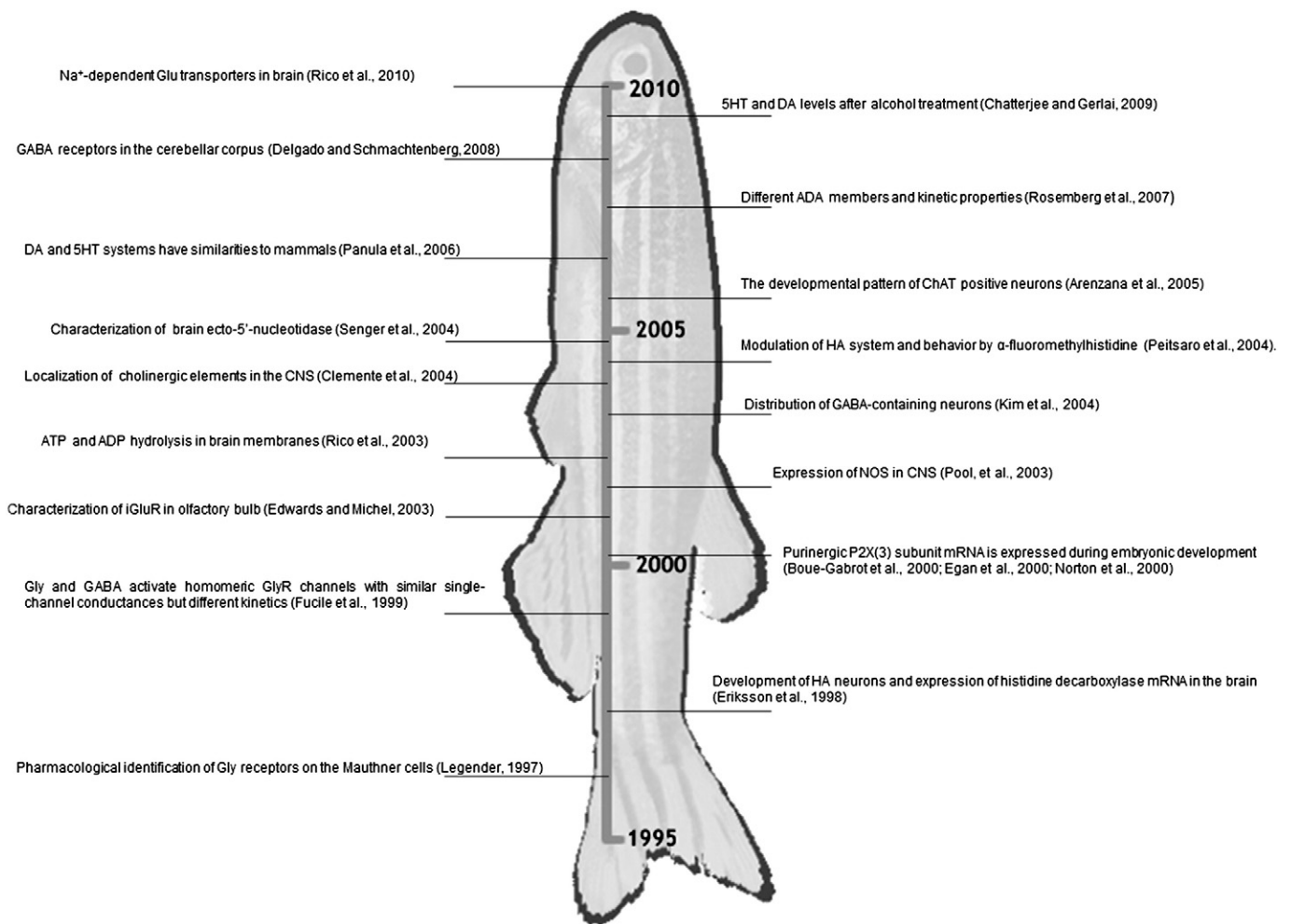
Fig. 1. Research hallmarks regarding neurotransmission in zebrafish. The timeline shows important studies related to proteins (enzymes, receptors, transporters) involved in neurotransmitter systems over the last few decades. Abbreviations: ADA, adenosine deaminase; ChAT, choline acetyltransferase; CNS, central nervous system; DA, dopamine; GABA, Gamma amino butyric acid; Gly, glycine; GlyR, glycine receptor; Glu, glutamate; HA, histamine; iGluR, ionotropic glutamate receptor; NOS, nitric oxide synthase; P2X(3), ionotropic purinergic receptor 3; 5HT, serotonin.

mechanisms (Durand et al., 1998), the nAChR plays a key role in modulating glutamate release (Alkondon et al., 1996) and memory formation (Kenney et al., 2010). Activation of nAChRs may directly depolarize cells or exert a neuromodulatory role by controlling neurotransmitter release (Vizi and Lendvai, 1999). The fine-tuned regulation of ACh-mediated signaling is performed by the activity of acetylcholinesterase (AChE, EC 3.1.1.7). This enzyme is a serine hydrolase related to the type B carboxylesterase family, which cleaves ACh into choline and acetate, effectively terminating cholinergic transmission. The de novo synthesis of ACh is dependent on choline acetyltransferase (ChAT, EC 2.3.1.6) activity, which catalyzes the reaction of acetate and choline in pre-synaptic neurons (Jamal et al., 2009).

The identification of cholinergic neurons in the zebrafish CNS has been previously reported by using specific antibodies against ChAT (Clemente et al., 2004; Kaslin et al., 2004; Mueller et al., 2004). Because of the different methodological approaches used, the anatomical identification of ChAT immunoreactive neurons differs among these studies. For example, Mueller et al. (2004) detected significant staining only in the lateral nucleus of the ventral telencephalic area, whereas Kaslin et al. (2004) observed ChAT immunoreactivity in the central, dorsal and subcommissural nuclei of the ventral telencephalic area of adults. In the diencephalon, the preoptic area, dorsal thalamus, pretectal nucleus and hypothalamus showed distinct ChAT positive staining. Prominent staining was also detected in the mesencephalon, whereas the optic tectum (OT) and

tegmentum showed immunoreactive cells (Clemente et al., 2004; Kaslin et al., 2004; Mueller et al., 2004). The primary developmental pattern of ChAT-positive neurons was described for the zebrafish (Arenzana et al., 2005). In this study, it was demonstrated that, at 60 hours post-fertilization (hpf), the tegmental ChAT positive neurons may be identified within the oculomotor, trochlear and rostral tegmental nuclei, whereas the tectal cholinergic neurons develop only at 5 days post-fertilization (dpf).

The mAChRs have been characterized in the zebrafish brain by radioligand binding techniques (Williams and Messer, 2004). These authors suggested that, similar to rodents, this species might be a useful model for evaluating the role of cholinergic systems in learning, memory, and behavior. Moreover, Steele et al. (2009) showed that the M(2) muscarinic receptor plays a role in the initiation of hypoxic bradycardia in larval zebrafish at 4 dpf. The role that nicotine plays in memory and behavioral tasks has already been reported in adult zebrafish (Levin and Chen, 2004; Levin et al., 2007). In these studies, low nicotine doses significantly improved fish memory, whereas higher doses induced memory impairment (Levin and Chen, 2004). Additionally, a nicotine-induced anxiolytic effect in zebrafish has been suggested by studies that evaluate vertical swimming in the novel tank paradigm (Levin et al., 2007), and both nicotinic α7 and α4β2 receptors can be involved in this response (Bencan and Levin, 2008). Considering the effects of organophosphate pesticides, a recent study showed that the zebrafish is sensitive to chlorpyrifos exposure during development, resulting in persisting developmental neurobehavioral

effects. A putative role for AChR was suggested to trigger these effects (Eddins et al., 2010).

There is evidence suggesting that AChE activity and expression are important for regulating zebrafish brain function. The early expression of AChE in diverse cell types suggests that it may play a role during development and may thus be a target for neurotoxicity in zebrafish (Hanneman and Westerfield, 1989). Several studies have evaluated the effects of metals (Senger et al., 2006a, 2006b; Richetti et al., 2011), typical and atypical antipsychotics (Seibt et al., 2009), methanol (Rico et al., 2006), antiepileptics (Siebel et al., 2010), and ethanol (Rico et al., 2007) on AChE activity in the zebrafish brain. These data suggest that altering the activity of this enzyme may impair ACh-mediated neurotransmission in this species, supporting the idea that cholinergic signaling may be affected by toxins and drugs of abuse. Importantly, the development of strategies to maintain and/or prevent drug-induced changes in AChE activity is an interesting approach for future translational research. A recent report demonstrated that the effects of ethanol on AChE activity may be related to changes in oxidative stress parameters, which were prevented by pretreatment with taurine (Rosemberg et al., 2010a). Although the mechanisms underlying these changes in AChE activity still remain to be elucidated, these studies highlight the need to evaluate the potential effects of distinct drugs/toxins on other neurotransmitter systems. Because ATP may be co-released at the synaptic cleft with ACh, several reports have aimed to correlate modifications in cholinergic neurotransmission with changes in purinergic signaling parameters (Senger et al., 2006b; Rico et al., 2006, 2007, 2008).

Based on these data, the evaluation of cholinergic system parameters has emerged as an important strategy to assess neurochemical, behavioral, and toxicological phenotypes in both larval and adult zebrafish. Advances in the knowledge of several behavioral paradigms (Rosemberg et al., 2011) associated with pharmacological/toxicological manipulations provide useful tools for understanding how alterations in these neurotransmitter systems correlate with changes in the behavior of this species.

4. Purine nucleotides and nucleosides

Nucleosides and nucleotides exert their actions through the activation of specific membrane purinoceptors, which are divided into the two purinergic receptor families, P1 and P2 (Burnstock, 1978). Purinergic receptors are divided based on their response to specific agonists and molecular cloning (Burnstock and Kennedy, 1985). Extracellular nucleotides exert their effects through two major receptor subfamilies: P2X receptors are ligand-gated ion channels comprising a family of seven receptors, and P2Y receptors are a group of eight G-protein coupled receptors (Khakh et al., 2001; Abbracchio et al., 2006). In mammals, there are seven known P2X receptor subtypes (P2X$_{1-7}$) and eight P2Y receptor subtypes (P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, P2Y$_{11}$, P2Y$_{12}$, P2Y$_{13}$, and P2Y$_{14}$) (Abbracchio et al., 2006). Neurotransmission through P2X receptors is considered a short-term effect and is primarily mediated through ATP binding, whereas long-term effects, such as cytotoxicity, cell proliferation, differentiation and migration, are primarily mediated through P2Y receptors, which bind both purine and pyrimidine nucleotides (Agresti et al., 2005). Considering their importance for cell signaling, the concentration of extracellular nucleotides is tightly regulated by a variety of cell surface enzymes called ectonucleotidases. These enzymes hydrolyze nucleoside triphosphates, diphosphates and monophosphates to their respective nucleosides (Zimmermann, 2001; Yegutkin, 2008). There are four major families of ectonculeotidases in mammals, namely, E-NTPDases (ectonucleoside triphosphate diphosphohydrolases), E-NPPs (ectonucleotide pyrophosphate/phosphodiesterases), alkaline phosphatases and ecto-5′-nucleotidase (Robson et al., 2006; Schetinger et al., 2007). Additionally to their role in the inactivation of purinergic signaling, ectonucleotidases have been proposed to prevent P2 receptor desensitization (Enjyoji et al., 1999) and to control the availability of ligands for nucleotide and adenosine receptors (Bonan et al., 2001). Adenosine, the product of ATP catabolism, has anticonvulsant, neuroprotective, and antinociceptive roles (Van Dycke et al, 2010; Paterniti et al, 2011). This nucleoside exerts these effects through the activation of four G-protein-coupled receptor subtypes: A$_1$, A$_{2A}$, A$_{2B}$ and A$_3$ (Burnstock, 1978).

The purinergic system has been studied in zebrafish through the characterization of P2 and P1 receptors as well as the enzymes involved in the control of nucleotide and nucleoside levels. A P2X subunit cloned from the zebrafish has been identified as an ortholog of the mammalian P2X(3) subunit (Egan et al., 2000). In addition, zebrafish P2X(3) subunit mRNA is exclusively expressed at high levels in trigeminal neurons and Rohon-Beard cells during embryonic development (Boué-Grabot et al., 2000; Norton et al., 2000). Recently, it has been shown that *p2rx3.1* in ectodermal cells is involved in purinergic signaling that is essential for proper craniofacial development and sensory circuit formation in embryonic and larval zebrafish (Kucenas et al., 2009). The cloning and characterization of the zebrafish P2X(4) and P2X(5) subunits were also performed (Diaz-Hernandez et al., 2002), and a more complete analysis of the P2X family identified nine genes. Of these, six are orthologs of mammalian genes, two are paralogs of previously described zebrafish subunits, and one remains unclassified (Kucenas et al., 2003). Specifically, *p2rx2*, *p2rx3.1*, *p2rx3.2* and *p2rx8* were expressed in the trigeminal ganglia and Rohon-Beard neuronal subsets. In contrast to mammals, *p2rx2* was not expressed in hypocretin cells (Appelbaum et al., 2007). Previous studies also provide evidence for the presence of a P2Y1 receptor in zebrafish thrombocytes (Gregory and Jagadeeswaran, 2002). Regarding P1 receptors, two zebrafish A2A (*adora2a.1 and adora2a.2*) genes and one A$_2$B (*adora2b*) adenosine receptor gene were identified in the CNS of developing embryos. Moreover, caffeine, an A$_2$A adenosine receptor antagonist, is neuroprotective against the adverse effects of MPTP in zebrafish embryos, which suggests that these receptors may serve as useful targets for testing novel therapeutic strategies for the treatment of Parkinson's disease (Boehmler et al., 2009).

The NTPDase and ecto-5′-nucleotidase activities were described in zebrafish brain membranes, and these enzymes share several kinetic properties with the enzymes previously identified in mammals (Rico et al., 2003; Senger et al., 2004). Homology-based searches identified the presence of NTPDase1-6 and NTPDase8 orthologs, and the phylogeny also grouped three NTPDase2 and two NTPDase5 paralogs (Rosemberg et al., 2010b). A distinct expression profile for *entpd1-6* and *entpd8* was observed in the brain, liver, and heart of zebrafish (Rosemberg et al., 2010b), and studies also showed that *entpd3* was expressed with *p2rx8* in the hypothalamic region (Appelbaum et al., 2007). In the zebrafish retina, NTPDases1 and 2 appear to be expressed within the germinal margin, which contains proliferative and differentiating cells (Ricatti et al., 2009). Another enzyme involved in the control of purinergic signaling is adenosine deaminase, which is responsible for cleaving the neuromodulator adenosine into inosine. Two members of the ADA subfamily, ADA1 and ADA2, were described, and the evidence showed another similar protein group, called ADAL (adenosine deaminase-like). The existence of different ADA-related genes, their distinct expression patterns and a truncated *ADA2-1* isoform suggests a high degree of complexity within the zebrafish adenosinergic system (Rosemberg et al., 2007a). The kinetic properties of these enzymes in the membrane and soluble fractions from zebrafish brains were determined, and the results indicate that the presence of ADA activity is important for regulating the adenosine/inosine levels in the zebrafish CNS (Rosemberg et al., 2008).

Several studies have demonstrated that these enzymes may be a target of the neurotoxic effects induced by pesticides, alcohols, and metals. Exposure to carbofuran and malathion for seven days significantly decreased ADP and AMP hydrolysis in zebrafish brain

membranes (Senger et al., 2005). Other organic compounds, such as methanol and ethanol, also induced significant changes in extracellular nucleotide and nucleoside levels. Methanol or ethanol exposure for one hour decreased NTPDase activity and NTPDase1 and mRNA transcript levels for three NTPDase2 genes in the zebrafish brain. However, no significant alterations in ecto-5′-nucleotidase activity were observed after exposure to methanol or ethanol (Rico et al., 2006, 2008). Exposure to lead and mercury for 24 h, 96 h or 30 days caused differential inhibitory effects on ATP, ADP and AMP hydrolysis, whereas no significant changes were found in the expression of NTPDase1 and 5′-nucleotidase following 30 days of exposure to both metals (Senger et al., 2006a). Soluble ADA activity also decreased after both acute (24 h) and subchronic (96 h) exposure to mercury, whereas enzyme activity was inhibited only after subchronic exposure in brain membranes. Semiquantitative RT-PCR analysis showed that mercury chloride did not alter ADA gene expression (Senger et al., 2010). Acute copper treatment for 24 hours decreased ATP hydrolysis; however, subchronic treatment for 96 hours inhibited both NTPDase and ecto-5′-nucleotidase activities. In contrast to the findings observed for other metals, NTPDase1, NTPDase2_mg and NTPDase2_mv transcripts were decreased after copper exposure for 24 and 96 h. Subchronic copper treatment also reduced NTPDase2_mq and ecto-5′-nucleotidase expression (Rosemberg et al., 2007b). The co-existence of several enzymes in the zebrafish CNS represents a sophisticated route for the appearance and inactivation of extracellular nucleotides on the cell surface. Therefore, the regulation of the nucleotidase pathway and, consequently, of the nucleotide levels may play a modulatory role during the evolution of neurotoxicity initiated by metals, pesticides, and organic compounds. Thus, identifying toxicant-induced changes and the mechanisms by which these enzymes regulate local nucleotide and nucleoside concentrations may represent important strategies for better understanding their role as a potential target for neurotoxins.

5. Histamine

Histamine (HA) is a biogenic amine widely distributed in the human brain (Lipinski et al., 1973), and its synthesis is induced by the enzyme L-histidine descarboxylase. HA acts through at least four types of G-protein-coupled receptors in mammals: the H1, H2, H3, and H4 receptors (Liu et al., 2001). The organization of the histaminergic system (HS) appears to be similar in all vertebrates (Panula et al., 1984). The HS is involved in several brain regulatory mechanisms, including alertness and sleep, hormone regulation, circadian rhythms, locomotor activity, consciousness, memory, and eating/drinking (Schwartz et al., 1991; Haas and Panula, 2003). Furthermore, this system may be associated with neuropsychiatric diseases such as schizophrenia (Jin et al., 2009), Alzheimer's (Panula et al., 1998) and Parkinson's disease (Anichtchik et al., 2000). In the zebrafish brain, histaminergic innervations and the molecular cloning and expression of L-histidine descarboxylase have been described (Eriksson et al., 1998). The zebrafish HS resembles that of other vertebrates; however, HA concentrations are slightly lower in zebrafish brain compared to higher vertebrates (Yamatodani et al., 1991). The HA content in the adult zebrafish brain varies with circadian rhythms, with decreased concentrations during the light period, which is similar to what is observed in rodents (Mochizuki et al., 1992).

In zebrafish embryos, the first histamine-immunoreactive neurons appear in the ventral hypothalamus at about 85 hpf. At 90 h, immunoreactive fibers can be observed terminating in the dorsal telencephalon. The HS appears during the period when the larva begins actively searching for prey, suggesting that this system may play a role in alertness (Eriksson et al., 1998). The HA-immunoreactive neurons that first appear in the larva likely belong to the same population of adult neurons because they are also located in the developing ventral hypothalamus and innervate the rostrodorsal

telencephalon, a major projection area in adults. In zebrafish, this area corresponds to the mammalian amygdala and hippocampus, which are densely innervated by histaminergic fibers (Eriksson et al., 1998; Peitsaro et al., 2003). The HS may have a similar role for alertness in the zebrafish tectum opticum (TeO). In addition to the TeO, the dorsal telencephalon and the torus semicircularis also receive dense histaminergic innervation (Northcutt, 1981; Meek, 1990).

The histamine H3-like receptor and the H3-related G-protein have been described in the zebrafish brain (Peitsaro et al., 2000). All three zebrafish HA receptors are expressed in the brain. Binding sites for H2 and H3 ligands were identified in zebrafish brains in areas that correspond to those in mammals (Peitsaro et al., 2000). In addition, the H1 receptor is expressed in the intestine, liver, and spleen, whereas the H2 receptor was found peripherally in the gills, heart, and spleen (Peitsaro et al., 2007).

Studies have shown that changes in HA levels alter behavior (Peitsaro et al., 2003). HA levels in the zebrafish brain can be reduced by α-fluoromethylhistidine administration, and this decrease is associated with changes in exploratory behavior and T-maze performance. These changes may be due to reduced anxiety and some memory-related mechanisms following HA depletion (Peitsaro et al., 2003). As demonstrated by Renier et al. (2007), histaminergic H1 antagonists produce a concentration-dependent reduction in immobility, with higher concentrations producing a state of complete unresponsiveness similar to general anesthesia. Because few studies exist that focus on behavior and pharmacology in the HS of zebrafish, further investigations are necessary to evaluate the role of this system in toxicity processes.

6. Nitric oxide

Nitric oxide (NO) is formed by endogenous NO synthase (NOS) and is involved in various normal, pathophysiological, and developmental events, which suggests that it participates in plasticity processes (Cramer et al., 1998; Moncada et al., 1998). NO is a free radical that is formed from L-arginine in biological tissues by three major NOS isoforms, including neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), by using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor (Alderton et al., 2001). In teleosts, NO plays a role in the development of the CNS during the embryonic and post-embryonic life stages (Fritsche et al., 2000). Holmqvist et al. (2000) demonstrated that nNOS mRNA-expressing cell populations are closely associated with the proliferation zones that generate new cells throughout life, which include the ventricular regions of the telencephalon, diencephalon, and mesencephalon. The expression of NOS in zebrafish embryos was detected at 16 hpf in the hypothalamus, and it was present in discrete CNS locations after 3 dpf (Pool et al., 2007). Since there are few studies about the functional role of NO in zebrafish, further investigations are necessary to evaluate changes in this signaling system induced by pharmacological approaches and toxic agents.

7. Glutamate

Glutamate is the primary excitatory neurotransmitter in the vertebrate CNS. Many biological events are affected by the modulation of glutamatergic signaling (Ozawa et al., 1998; Anderson and Swanson, 2000), such as memory and learning (Izquierdo and Medina, 1997), development and aging (Segovia et al., 2001), and adaptation to the environment (Mattson et al., 2002). However, the glutamate concentration profile at the synaptic cleft is variable and may also act as an excitotoxin at high concentrations due to receptor overstimulation (Anderson and Swanson, 2000; Danbolt, 2001; Maragakis and Rothstein, 2004). Glial cells are essential for maintaining extracellular glutamate concentrations below neurotoxic levels, and this is achieved by high-affinity sodium-dependent glutamate transporters primarily present in

astrocytes (Chen and Swnason, 2003). Glutamate toxicity has been associated with neuronal death following ischemia and trauma (Choi, 1988; Ikonomidou et al., 1989), as well as with several neurodegenerative disorders, such as Huntington's and Alzheimer's diseases (Brewer, 2000; Ingram et al., 2001; Maragakis and Rothstein, 2001, 2004; Segovia et al., 2001).

Several studies have emerged that aimed at identifying and understanding the basis of glutamatergic signaling. Glutamate uptake is tightly regulated by a group of excitatory amino acid transporters (EAATs) that belong to the solute carrier family 1 (SLC1). To date, five structurally distinct subtypes of EAATs have been identified and characterized in the mammalian brain. The presence of EAAT-related sequences has been recently described by phylogenetic analysis and mRNA expression profiling in the zebrafish CNS (Rico et al., 2010). Furthermore, the evolutionary history of EAATs was also analyzed, and these members were included in the SLC1 gene family (Gesemann et al., 2010; Neuhauss et al., 2010). After these EAAT-related genes were identified, glutamate transporter activity was investigated in the zebrafish by assessing sodium-dependent glutamate uptake in distinct brain structures (Rico et al., 2010).

Hair cells are the sensory receptors for the auditory and vestibular system in zebrafish. They detect sound and movement and transmit this information through specialized ribbon synapses, which coordinate synaptic vesicles. In one study, hair cells presented a decrease in the number of ribbon-associated synaptic vesicles in zebrafish with mutations in vesicular glutamate transporter 3 (*vglut3*), indicating the involvement of the glutamate transporter during synaptic transmission (Obholzer et al., 2008). This family of proteins mediates glutamate uptake by synaptic vesicles, which is necessary for glutamatergic transmission in the retina. Other researchers reported that the zebrafish vesicular glutamate transporter 2 (*vglut2*) is expressed in retinal ganglion cells and is partially responsible for glutamatergic transmission at the retinotectal synapse (Smear et al., 2007; Demas and Cline, 2007).

Glutamate receptors are divided into two main categories: metabotropic (mGluRs) and ionotropic receptors (iGluRs). The mGluRs trigger intracellular secondary messengers through G-proteins. In contrast, the iGluRs are ligand-gated ion channels that manage rapid changes in sodium, calcium, and potassium concentrations. The subtypes of iGluRs include N-methyl-D-aspartate (NMDA), the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and kainate (KA). The molecular characterization and embryonic expression of the family of NMDA receptor subunit genes have already been established in zebrafish (Cox et al., 2005). Moreover, behavioral and neuroanatomical studies have shown that the brain area responsible for learning in teleost fish is the telencephalon, which is analogous to the hippocampus and amygdala in the mammalian brain (Portavella et al., 2002; Rodríguez et al., 2002). Long-term potentiation (LTP) is representative of the synaptic modification that underlies the process of learning and memory. Nam et al. (2004) demonstrated NMDA receptor-dependent LTP in the telencephalon of the zebrafish. In this context, a simple inhibitory avoidance task in adult zebrafish showed that the resulting memory is robust, long-lasting and sensitive to the NMDA-receptor antagonist MK-801, which was added to the tank water immediately after training (Blank et al., 2009).

Each iGluR subtype has unique properties, including activation/deactivation kinetics, ion permeability, voltage-dependence and kinase regulation. A variation in the subunit composition of each iGluR further contributes to the unique cellular responses elicited by glutamate (Nakanishi et al., 1994). Edwards and Michel (2003) demonstrated the pharmacological characterization of iGluRs in the olfactory bulb. This group advanced the understanding of the glutamatergic system in teleosts by characterizing the distribution of functional NMDA and KA-stimulated neurons. Furthermore, Tabor and Friedrich (2008) pharmacologically investigated the iGluR function in zebrafish olfactory bulb neuronal circuits.

Studies also exist that evaluate glutamatergic signaling beyond the CNS. There is growing interest in understanding the role of signaling molecules in visual function in zebrafish retina. Previous studies on the localization of the glutamatergic system in the zebrafish outer plexiform layer (OPL) have shown glutamate-immunoreactivity in rod and cone photoreceptors (Connaughton et al., 1999). In addition to the visual system, glutamate receptors were found in the peripheral nervous system. The activation of iGluRs on the peripheral axons of primary motor neurons mediates neurotransmitter release at the zebrafish neuromuscular junction (Todd et al., 2004). Considering the wide spectrum of biological functions involving the glutamatergic system, it becomes important to evaluate this signaling system as a target of toxicological and pharmacological agents.

8. Glycine and GABA

The correct development of the spinal cord leads to the normal control of movements and the integration of signals from the periphery. To promote this integration, several types of neurons and neurotransmitter systems must work synchronously. A balance of both excitatory (glutamate) and inhibitory (glycine and GABA) neurotransmitters are involved in this process. Glycine receptors (GlyRs) and GABA receptors are members of the ligand-gated chloride channel family. As one of the predominant inhibitory neurotransmitters in the vertebrate brain stem and spinal cord, glycine is also critically important for the regulation of interneuron differentiation during the development of the central neural network (McDearmid et al., 2006). GABA and glycine-mediated neurotransmission arise relatively early in fish development, as shown by the circuitry underlying locomotor behaviors such as the escape response and rhythmic swimming, which are established soon after the patterning of the hindbrain and spinal cord (Saint-Amant and Drapeau, 2000). Two types of postsynaptic glycinergic receptors, with different subconductances and sensibilities to picrotoxin, were pharmacologically identified on Mauthner cells in zebrafish larvae (52 hpf) (Legendre, 1997). These types of postsynaptic receptors have been identified as the determinant receptors that control synaptic events in Mauthner cells (Legendre, 1998). Relative mRNA levels from glycine transporters (GlyT1 and GlyT2), NMDA receptor glycine binding subunit (NR1.1), and the *alpha 1* subunit of the glycine receptor (GlyRα1) have also been measured in Mauthner cells (Mongeon et al., 2008). Zebrafish GlyT1 mutants initially present with dysfunction in motility; however, proper swimming behavior is later recovered by a mechanism involving glycine tolerance and reduced glycine receptor expression (Mongeon et al., 2008). Mutants that are defective in glycinergic synaptic transmission due to a lack of synaptic aggregation of GlyRs exhibit simultaneous motor neuron activation on both sides, resulting in bilateral contraction of the axial muscles (Hirata et al., 2010).

GABA-containing neurons appear in the zebrafish olfactory bulb (OB), telencephalon, tectum stratum, and in the hypothalamus (Kim et al., 2004). In the cerebellar corpus and valves of the zebrafish, GABA receptors are present in the molecular layer, Purkinje cells and groups of Golgi cells in the granular layer (Delgado and Schmachtenberg, 2008).

In zebrafish, glycine and GABA activate homomeric GlyR channels with similar single-channel conductances but different kinetics (Fucile et al., 1999). The glycinergic and GABAergic inhibitory control of motor neurons and the balance between excitatory and inhibitory synapses on interneurons and motor neurons underlies the normal functioning of locomotor circuits that produce rhythmic motor output (Grillner et al., 1995; Hultborn and Nielsen, 2007). Thus, because neurological effects can be detected by movement disorders, these systems are key candidates for neurotoxicological assessment. However, few studies contribute to the study of this topic. One report using a phenylpyrazole insecticide (fipronil) on zebrafish embryos
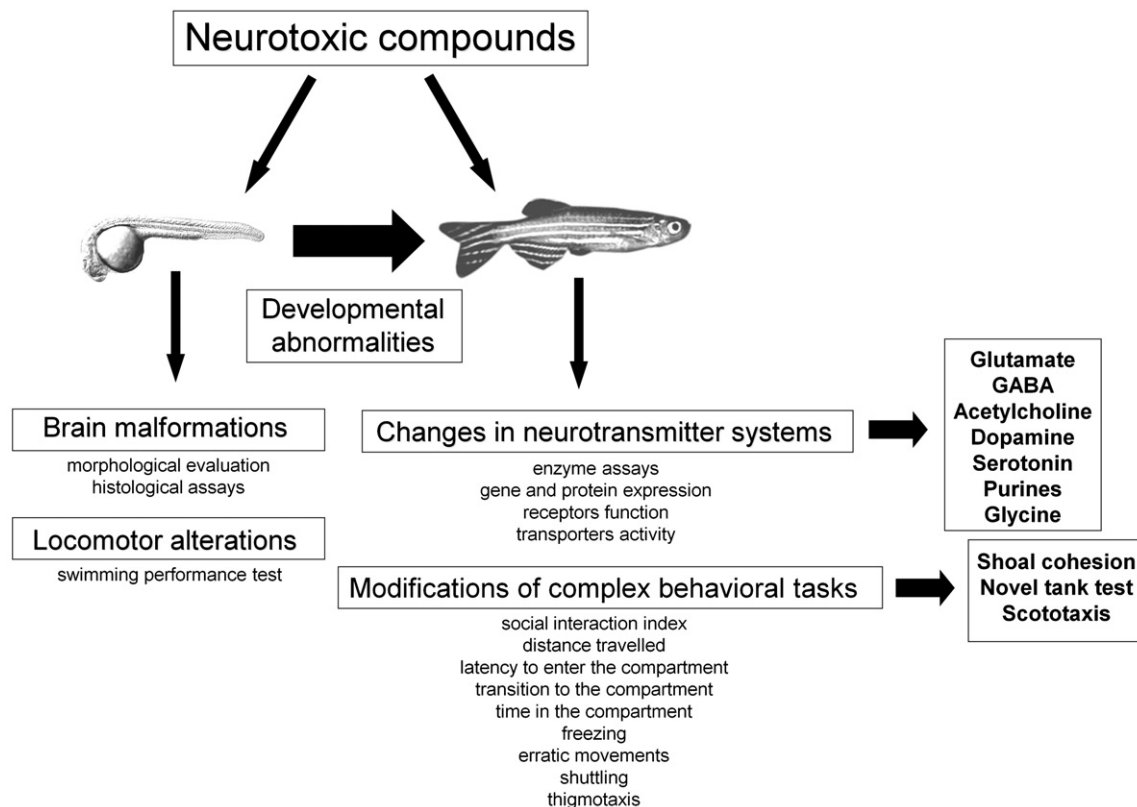
Fig. 2. The zebrafish model offers the potential to evaluate the effects of several compounds. This figure illustrates emerging approaches and perspectives for studying cellular, morphological, physiological, and behavioral aspects using larval and adult zebrafish. The strategies described are interesting tools for testing the potential neuroprotective activities of distinct compounds in a fast and large-scale manner.

shows that, although this insecticide is an inhibitor of GABA receptors, it may inhibit a structurally related GlyR subtype expressed during the development of spinal locomotor pathways in zebrafish. This inhibition of GlyR had no effect on the morphology of zebrafish embryos until 30 hpf; however, after this period, embryos began to show reduced body length, notochord degeneration, abnormal axial muscle morphology, and locomotor defects.

The use of zebrafish as a complementary vertebrate model for the evaluation of seizures induced by GABA antagonists has also emerged. Baraban et al. (2005) reported that the exposure to the common convulsant agent, pentylenetetrazole, induced changes in zebrafish behavior, neural activity and significantly increased *c-fos* expression. Moreover, it has been shown that a mind bomb mutant zebrafish presents several changes in the brain metabolism, including downregulation of several genes necessary for GABA-mediated signaling (Hortopan et al., 2010). These approaches point the zebrafish model as an interesting system to explore how many known anti-epileptic drugs (AEDs), such as carbamazepine, sodium valproate, and phenytoin, would be detected when running such a screen (Berghmans et al., 2007).

9. Conclusion

In this review, we have highlighted the different neurotransmitter systems in zebrafish and described their pharmacological and toxicological implications. These advances reinforce the benefits that zebrafish offer as a model system, and emphasizes the research efforts undertaken to understand the function of the neurotransmitter systems in this species. These efforts have used the zebrafish as a model for the induction of neurological disorders, which has contributed to the understanding of the mechanisms involved in different neuronal dysfunctions. Due to its ability to provide rapid

pharmacological and toxicological responses in different stages of development, to exhibit a complex set of behaviors and genomic similarities to mammals, it is important to reinforce that the zebrafish is a useful tool for performing preclinical assays on a large scale before pharmacological validation in rodent models. In addition, the ability to test neurotoxic compounds during early development and in adults permits the evaluation of morphological, behavioral, and neurochemical parameters and the study of the mechanisms involved in environmental and drug toxicity (Fig. 2). Therefore, the zebrafish represents an attractive organism for screening strategies used in drug discovery and neurotoxicity assays.

Conflict of interest statement

There are no competing interests.

Acknowledgments

References

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, et al. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 2006;58(3):281–341.

Agresti C, Meomartini ME, Amadio S, Ambrosini E, Serafini B, Franchini L, et al. Metabotropic P2 receptor activation regulates oligodendrocyte progenitor migration and development. Glia 2005;50(2):132–44.

Airhart MJ, Lee DH, Wilson TD, Miller BE, Miller MN, Skalko RG. Movement disorders and neurochemical changes in zebrafish larvae after bath exposure to fluoxetine (PROZAC). Neurotoxicol Teratol 2007;29(6):652–64.

Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J 2001;357:593–615.

Alkondon M, Rocha ES, Maelicke A, Albuquerque EX. Diversity of nicotinic acetylcholine receptors in rat brain. V. alpha-Bungarotoxin-sensitive nicotinic receptors in olfactory bulb neurons and presynaptic modulation of glutamate release. J Pharmacol Exp Ther 1996;278(3):1460–71.

Anderson CM, Swanson RA. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 2000;32:1–14.

Anichtchik OV, Rinne JO, Kalimo H, Panula P. An altered histaminergic innervation of the substantia nigra in Parkinson's disease. Exp Neurol 2000;163(1):20–30.

Anichtchik OV, Kaslin J, Peitsaro N, Scheinin M, Panula P. Neurochemical and behavioural changes in zebrafish Danio rerio after systemic administration of 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. J Neurochem 2004;88(2):443–53.

Appelbaum L, Skariah G, Mourrain P, Mignot E. Comparative expression of p2x receptors and ecto-nucleoside triphosphate diphosphohydrolase 3 in hypocretin and sensory neurons in zebrafish. Brain Res 2007;1174:66–75.

Arenzana FJ, Clemente D, Sánchez-González R, Porteros A, Aijón J, Arévalo R. Development of the cholinergic system in the brain and retina of the zebrafish. Brain Res Bull 2005;66(4–6):421–5.

Baraban SC, Taylor MR, Castro PA, Baier H. Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. Neuroscience 2005;131(3):759–68.

Barbazuk WB, Korf I, Kadavi C, Heyen J, Tate S, Wun E, et al. The syntenic relationship of the zebrafish and human genomes. Genome Res 2000;10:1351–8.

Belmacer RH. The future of depression psychopharmacology. CNS Spectr 2008;13:682–7.

Bencan Z, Levin ED. The role of alpha7 and alpha4beta2 nicotinic receptors in the nicotine-induced anxiolytic effect in zebrafish. Physiol Behav 2008;95(3):408–12.

Berghmans S, Hunt J, Roach A, Goldsmith P. Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. Epilepsy Res 2007;75(1):18–28.

Blank M, Guerim LD, Cordeiro RF, Vianna MR. A one-trial inhibitory avoidance task to zebrafish: rapid acquisition of an NMDA-dependent long-term memory. Neurobiol Learn Mem 2009;92(4):529–34.

Boehmler W, Carr T, Thisse C, Thisse B, Canfield V, Levenson R. D4 dopamine receptor genes of zebrafish and effects of the antipsychotic clozapine on larval swimming behaviour. Genes Brain Behav 2007;6:155–66.

Boehmler W, Petko J, Woll M, Frey C, Thisse B, Thisse C, et al. Identification of zebrafish A2 adenosine receptors and expression in developing embryos. Gene Expr Patterns 2009;9(3):144–51.

Bonan CD, Schetinger MRC, Battastini AMO, Sarkis JJF. Ectonucleotidases and synaptic plasticity: implications in physiological and pathological conditions. Drug Dev Res 2001;52:57–65.

Boué-Grabot E, Akimenko MA, Séguéla P. Unique functional properties of a sensory neuronal P2X ATP-gated channel from zebrafish. J Neurochem 2000;75(4):1600–7.

Bowman TV, Zon LI. Swimming into the future of drug discovery: in vivo chemical screens in zebrafish. ACS Chem Biol 2010;5(2):159–61.

Brewer GJ. Neuronal plasticity and stressor toxicity during aging. Exp Gerontol 2000;35(9–10):1165–83.

Brown DA. Muscarinic acetylcholine receptors (mAChRs) in the nervous system: some functions and mechanisms. J Mol Neurosci 2010;41(3):340–6.

Burnstock G. Do some sympathetic neurones synthesize and release both noradrenaline and acetylcholine? Prog Neurobiol 1978;11(3–4):205–22.

Burnstock G, Kennedy C. Is there a basis for distinguishing two types of P2-purinoceptor? Gen Pharmacol 1985;16:433–40.

Callier S, Snapyan M, Le Crom S, Prou D, Vincent JD, Vernier P. Evolution and cell biology of dopamine receptors in vertebrates. Biol Cell 2003;95(7):489–502.

Chatterjee D, Gerlai R. High precision liquid chromatography analysis of dopaminergic and serotoninergic responses to acute alcohol exposure in zebrafish. Behav Brain Res 2009;200(1):208–13.

Chen Y, Swnason RA. Astrocytes and brain injury. J Cereb Blood Flow Metab 2003;23:137–49.

Choi DW. Glutamate neurotoxicity and diseases of the nervous system. Neuron 1988;1(8):623–34.

Clemente D, Porteros A, Weruaga E, Alonso JR, Arenzana FJ, Aijón J, et al. Cholinergic elements in the zebrafish central nervous system: Histochemical and immunohistochemical analysis. J Comp Neurol 2004;474(1):75–107.

Connaughton VP, Behar TN, Liu WLS, Massey SC. Immunocytochemical localization of excitatory and inhibitory neurotransmitters in the zebrafish retina. Vis Neurosci 1999;16:483–90.

Cote F, Fligny C, Bayard E, Launay JM, Gershon MD, Mallet J, et al. Maternal serotonin is crucial for murine embryonic development. Proc Natl Acad Sci U S A 2007;104:329–34.

Cox JA, Kucenas S, Voigt MM. Molecular characterization and embryonic expression of the family of N-methyl-D-aspartate receptor subunit genes in the zebrafish. J Dev Dyn 2005;234(3):756–66.

Cramer KS, Leamey CA, Sur M. Nitric oxide as a signalling molecule in visual system development. In: Mize RR, Dawson TM, Dawson VL, Friedlander MJ, editors. Nitric oxide in brain development, plasticity, and disease. Progress Brain Res.Amsterdam: Elsevier; 1998. p. 101–14.

Danbolt NC. Glutamate uptake. Prog Neurobiol 2001;65:1–105.

Daubert EA, Condron BG. Serotonin: a regulator of neuronal morphology and circuitry. Trends Neurosci 2010;33:424–34.

Delgado L, Schmachtenberg O. Immunohistochemical localization of GABA, GAD65, and the receptor subunits GABAAalpha1 and GABAB1 in the zebrafish cerebellum. Cerebellum 2008;7(3):444–50.

Demas J, Cline HT. The blu Blur: mutation of a vesicular glutamate transporter reduces the resolution of zebrafish vision. Neuron 2007;53(1):4–6.

Diaz-Hernandez M, Cox JA, Migita K, Haines W, Egan TM, Voigt MM. Cloning and characterization of two novel zebrafish P2X receptor subunits. Biochem Biophys Res Commun 2002;295(4):849–53.

Durand C, Coronas V, Jourdan F, Quirion R. Developmental and aging aspects of the cholinergic innervation of the olfactory bulb. Int J Dev Neurosci 1998;16(7–8):777–85.

Eddins D, Cerutti D, Williams P, Linney E, Levin ED. Zebrafish provide a sensitive model of persisting neurobehavioral effects of developmental chlorpyrifos exposure: comparison with nicotine and pilocarpine effects and relationship to dopamine deficits. Neurotoxicol Teratol 2010;32(1):99–108.

Edwards JG, Michel WC. Pharmacological characterization of ionotropic glutamate receptors in the zebrafish olfactory bulb. Neuroscience 2003;122(4):1037–47.

Egan TM, Cox JA, Voigt MM. Molecular cloning and functional characterization of the zebrafish ATP-gated ionotropic receptor P2X(3) subunit. FEBS Lett 2000;475(3):287–90.

Enjyoji K, Sévigny J, Lin Y, Frenette PS, Christie PD, Esch II JS, et al. Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med 1999;5(9):1010–7.

Eriksson KS, Peitsaro N, Karlstedt K, Kaslin J, Panula P. Development of histidine decarboxylase mRNA in the zebrafish brain in the absence of all peripheral histaminergic system. Eur J Neurosci 1998;10:3799–812.

Flinn L, Bretaud S, Lo C, Ingham PW, Bandmann O. Zebrafish as a new animal model for movement disorders. Neurochemistry 2008;106:1991–7.

Fricker AD, Rios C, Devi LA, Gomes I. Serotonin receptor activation leads to neurite outgrowth and neuronal survival. Brain Res Mol Brain Res 2005;138:228–35.

Fritsche R, Schwerte T, Peitser B. Nitric oxide and vascular reactivity in development zebrafish, Danio rerio. Am J Physiol Regul Integr Comp Physiol 2000;279:2200–7.

Fucile S, Saint Jan D, David-Watine B, Korn H, Bregestovski P. Comparison of glycine and GABA actions on the zebrafish homomeric glycine receptor. J Physiol 1999;517(2):369–83.

Gaspar P, Cases O, Maroteaux L. The developmental role of serotonin: news from mouse molecular genetics. Nat Rev Neurosci 2003;4:1002–12.

Gerlai R, Lahav M, Guo S, Rosenthal A. Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. Pharmacol Biochem Behav 2000;67:773–82.

Gerlai R, Ahmad F, Prajapati S. Differences in acute alcohol-induced behavioral responses among zebrafish populations. Alcohol Clin Exp Res 2008;32:1763–73.

Gerlai R, Chatterjee D, Pereira T, Sawashima T, Krishnannair R. Acute and chronic alcohol dose: population differences in behavior and neurochemistry of zebrafish. Genes Brain Behav 2009;8(6):586–99.

Gesemann M, Lesslauer A, Maurer CM, Schönthaler HB, Neuhauss SC. Phylogenetic analysis of the vertebrate excitatory/neutral amino acid transporter (SLC1/EAAT) family reveals lineage specific subfamilies. BMC Evol Biol 2010;10:117.

Giacomini NJ, Rose B, Kobayashi K, Guo S. Antipsychotics produce locomotor impairment in larval zebrafish. Neurotoxicol Teratol 2006;28:245–50.

Goldman-Rakic PS. The cortical dopamine system: role in memory and cognition. Adv Pharmacol 1998;42:707–11.

Gregory M, Jagadeeswaran P. Selective labeling of zebrafish thrombocytes: quantitation of thrombocyte function and detection during development. Blood Cells Mol Dis 2002;28(3):418–27.

Grillner S, Deliagina T, Ekeberg O, el Manira A, Hill RH, Lansner A, et al. Neural networks that coordinate locomotion and body orientation in lamprey. Trends Neurosci 1995;18:270–9.

Grossman L, Utterback E, Stewart A, Gaikwad S, Wong K, Elegante M, et al. Characterization of behavioral and endocrine effects of LSD on zebrafish. Behav Brain Res 2010;214:277–84.

Haas H, Panula P. The role of histamine and the tuberomamillary nucleus in the nervous system. Nat Rev Neurosci 2003;4(2):121–30.

Hanneman E, Westerfield M. Early expression of acetylcholinesterase activity in functionally distinct neurons of the zebrafish. J Comp Neurol 1989;284(3):350–61.

Hill AJ, Teraoka H, Heideman W, Peterson RE. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci 2005;86(1):6–19.

Hirata H, Carta E, Yamanaka I, Harvey RJ, Kuwada JY. Defective glycinergic synaptic transmission in zebrafish motility mutants. Front Mol Neurosci 2010;2:26.

Holmqvist B, Ellingsen B, Alm P, Forsell J, Oyan MA, Goksøyr A, et al. Identification and distribution of nitric oxide synthase in the brain the zebrafish. Neurosci Lett 2000;292:119–22.

Hortopan GA, Dinday MT, Baraban SC. Spontaneous seizures and altered gene expression in GABA signaling pathways in a mind bomb mutant zebrafish. J Neurosci 2010;30(41):13718–28.

Hultborn H, Nielsen JB. Spinal control of locomotion from cat to man. Acta Physiol (Oxf) 2007;189:111–21.

Ikonomidou C, Price MT, Mosinger JL, Frierdich G, Labruyere J, Salles KS, et al. Hypobaric–ischemic conditions produce glutamate-like cytopathology in infant rat brain. J Neurosci 1989;9(5):1693–700.

Ingram EM, Wiseman JW, Tessler S, Emson PC. Reduction of glial glutamate transporters in the parietal cortex and hippocampus of the EL mouse. J Neurochem 2001;79(3):564–75.

Ivetac I, Becanovic J, Krishnapillai V. Zebrafish: genetic tools and genomics, Asia–Pacific. J Mol Biol Biotechnol 2000;8:1–11.

Izquierdo I, Medina JH. Memory formation: the sequence of biochemical events in the hippocampuss and its connection to activity in other brain structures. Neurobiol Learn Mem 1997;68:285–316.

Jamal M, Ameno K, Miki T, Wang W, Kumihashi M, Isse T, et al. Cholinergic alterations following alcohol exposure in the frontal cortex of Aldh2-deficient mice models. Brain Res 2009;1295:37–43.

Jin CY, Anichtchik O, Panula P. Altered histamine H3 receptor radioligand binding in post-mortem brain samples from subjects with psychiatric diseases. Br J Pharmacol 2009;157(1):118–29.

Kaslin J, Nystedt JM, Ostergård M, Peitsaro N, Panula P. The orexin/hypocretin system in zebrafish is connected to the aminergic and cholinergic systems. J Neurosci 2004;24(11):2678–89.

Kenney JW, Florian C, Portugal GS, Abel T, Gould TJ. Involvement of hippocampal jun-N terminal kinase pathway in the enhancement of learning and memory by nicotine. Neuropsychopharmacology 2010;35(2):483–92.

Khakh BS, Burnstock G, Kennedy C, King BF, North RA, Séguéla P, et al. International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. Pharmacol Rev 2001;53(1):107–18.

Kim YJ, Nam RH, Yoo YM, Lee CJ. Identification and functional evidence of GABAergic neurons in parts of the brain of adult zebrafish (*Danio rerio*). Neurosci Lett 2004;355(1–2):29–32.

Kucenas S, Li Z, Cox JA, Egan TM, Voigt MM. Molecular characterization of the zebrafish P2X receptor subunit gene family. Neuroscience 2003;121(4):935–45.

Kucenas S, Cox JA, Soto F, Lamora A, Voigt MM. Ectodermal P2X receptor function plays a pivotal role in craniofacial development of the zebrafish. Purinergic Signal 2009;5 (3):395–407.

Legendre P. Pharmacological evidence for two types of postsynaptic glycinergic receptors on the Mauthner cell of 52-h-old zebrafish larvae. J Neurophysiol 1997;77:2400–15.

Legendre P. A reluctant gating mode of glycine receptor channels determines the time course of inhibitory miniature synaptic events in zebrafish hindbrain neurons. J Neurosci 1998;18(8):2856–70.

Lele Z, Krone PH. The zebrafish as a model system in developmental, toxicological and transgenic research. Biotechnol Adv 1996;14(1):57–72.

Levin ED, Chen E. Nicotinic involvement in memory function in zebrafish. Neurotoxicol Teratol 2004;26(6):731–5.

Levin ED, Bencan Z, Cerutti DT. Anxiolytic effects of nicotine in zebrafish. Physiol Behav 2007;90(1):54–8.

Lipinski JF, Schaumburg HH, Baldessarini RJ. Regional distribution of histamine in human brain. Brain Res 1973;52:403–5.

Liu C, Wilson SJ, Kuei C, Lovenberg TW. Comparison of human, mouse, rat and guinea pig histamine H4 receptors reveals substantial pharmacological species variation. J Pharmacol Exp Ther 2001;299:121–30.

Lucki I. The spectrum of behaviors influenced by serotonin. Biol Psychiatry 1998;44:151–62.

Maragakis NJ, Rothstein JD. Glutamate transporters in neurologic disease. Arch Neurol 2001;58(3):365–70.

Maragakis NJ, Rothstein JD. Glutamate transporters: animal models to neurologic disease. Neurobiol Dis 2004;15:461–73.

Mattson MP, Chan SL, Duan W. Modification of brain ageing and neurodegenerative disorders by genes, diet, and behavior. Physiol Rev 2002;82:637–72.

McDearmid JR, Liao M, Drapeau P. Glycine receptors regulate interneuron differenti-ation during spinal network development. Proc Natl Acad Sci U S A 2006;103(25):9679–84.

Meek J. Tectal morphology: connections, neurons and synapses. In: Douglas RH, Djamgoz MBA, editors. The visual system of Fish. London: Chapman and Hall; 1990. p. 239–77.

Missale C, Nash SR, Robinson SW, Jaber M, Caron MG. Dopamine receptors: from structure to function. Physiol Rev 1998;78:189–225.

Mochizuki T, Yamatodani A, Okakura K, Horii A, Inagaki N, Wada H. Circadian rhythm of histamine release from the hypothalamus of freely moving rats. Physiol Behav 1992;51(2):391–4.

Moncada S, Nistico G, Bagetta G, Higgs E. Nitric oxide and the cell proliferation, differentiation and death. United Kingdon: Portland Press; 1998. p. 320.

Mongeon R, Gleason MR, Masino MA, Fetcho JR, Mandel G, Brehm P, et al. Synaptic homeostasis in a zebrafish glial glycine transporter mutant. J Neurophysiol 2008;100:1716–23.

Mueller T, Vernier P, Wullimann MF. The adult central nervous cholinergic system of a neurogenetic model animal, the zebrafish *Danio rerio*. Brain Res 2004;1011(2):156–69.

Murray RM, Lappin J, Di Forti M. Schizophrenia: from developmental deviance to dopamine dysregulation. Eur Neuropsychopharmacol 2008(Suppl 3):S129–34.

Nakanishi S, Masu M, Bessho Y, Nakajima Y, Hayashi Y, Shigemoto R. Molecular diversity of glutamate receptors and their physiological functions. Experientia Suppl 1994;71:71–80.

Nam RH, Kim W, Lee CJ. NMDA receptor-dependent long-term potentiation in the telencephalon of the zebrafish. Neurosci Lett 2004;370(2–3):248–51.

Neuhauss SC, Rico EP, Gesemann M. Nomenclature of glutamate transporters in zebrafish and other vertebrates. Brain Res Bull 2010;83(6):297.

Northcutt RG. Evolution of the optic tectum in ray-finned fishes. In: Davis RE, Northcutt RG, editors. Fish neurobiology 2: higher brain areas and functions. Ann. Arbor: University of Michigan Press; 1981. p. 1–42.

Norton WH, Rohr KB, Burnstock G. Embryonic expression of a P2X(3) receptor encoding gene in zebrafish. Mech Dev 2000;99(1–2):149–52.

Nürnberger A, Räbiger M, Mack A, Diaz J, Sokoloff P, Mühlbauer B, et al. Subapical localization of the dopamine D3 receptor in proximal tubules of them rat kidney. J Histochem Cytochem 2004;52(12):1647–55.

Obholzer N, Wolfson S, Trapani JG, Mo W, Nechiporuk A, Busch-Nentwich E, et al. Vesicular glutamate transporter 3 is required for synaptic transmission in zebrafish hair cells. J Neurosci 2008;28(9):2110–8.

Ozawa A, Kamiya H, Tsuzuki K. Glutamate receptors in the mammalian central nervous system. Prog Neurobiol 1998;54:581–618.

Panula P, Yang HY T, Costa E. Histamine-containing neurons in rat hypothalamus. Proc Natl Acad Sci U S A 1984;81:2572–6.

Panula P, Rinne J, Kuokkanen K, Eriksson KS, Sallmen T, Kalimo H, et al. Neuronal histamine deficit in Alzheimer's disease. Neuroscience 1998;82(4):993–7.

Panula P, Sallinen V, Sundvik M, Kolehmainen J, Torkko V, Tiittula A, et al. Modulatory neurotransmitter systems and behavior: towards zebrafish models of neurode-generative diseases. Zebrafish 2006;3(2):235–47.

Panula P, Chen YC, Priyadarshini M, Kudo H, Semenova S, Sundvik M, et al. The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases. Neurobiol Dis 2010;40(1):46–57.

Parng C, Roy NM, Ton C, Lin Y, McGrath P. Neurotoxicity assessment using zebrafish. J Pharmacol Toxicol Methods 2007;55(1):103–12.

Parsey RV. Serotonin receptor imaging: clinically useful? J Nucl Med 2010;51(10):1495–8.

Paterniti I, Melani A, Cipriani S, Corti F, Mello T, Mazzon E, et al. Selective adenosine A2A receptor agonists and antagonists protect against spinal cord injury through peripheral and central effects. J Neuroinflammation 2011;8(1):31.

Peitsaro N, Anichtchik OV, Panula P. Identification of a histamine H(3)-like receptor in the zebrafish (*Danio rerio*) brain. J Neurochem 2000;75:718–24.

Peitsaro N, Kaslin J, Anitchick OV, Panula P. Modulation of histaminergic system and behaviour by alpha-fluorothylhistidine in zebrafish. J Neurochem 2003;86:432–41.

Peitsaro N, Sundvik M, Anichtchik OV, Kaslin J, Panula P. Identification of zebrafish histamine H1, H2 and H3 receptors and effects of histaminergic ligands on behavior. Biochem Pharmacol 2007;73(8):1205–14.

Pool KL, Richardson M, Lan SC, Khoo HE, Korzh V. Expression pattern of neuronal nitric oxide synthase in embryonic zebrafish. Gene Expr Patterns 2007;3(4):463–6.

Portavella M, Vargas JP, Torres B, Salas C. The effects of telencephalic pallial lesions on spatial, temporal, and emotional learning in goldfish. Brain Res Bull 2002;57(3–4):397–9.

Powers CM, Levin ED, Seidler FJ, Slotkin TA. Silver exposure in developing zebrafish produces persistent synaptic and behavioral changes. Neurotoxicol Teratol 2011;33 (2):329–32.

Prange O, Wong TP, Gerrow K, Wang YT, El-Husseini A. A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. Proc Natl Acad Sci U S A 2004;101(38):13915–20.

Raiteri M. Functional pharmacology in human brain. Pharmacol Rev 2006;58(2):162–93.

Renier C, Faraco JH, Bourgin P, Motley T, Bonaventure P, Rosa F, et al. Genomic and functional conservation of sedative-hypnotic targets in the zebrafish. Pharmaco-genet Genomics 2007;17(4):237–53.

Ricatti MJ, Alfie LD, Lavoie EG, Sévigny J, Schwarzbaum PJ, Faillace MP. Immunocyto-chemical localization of NTPDases1 and 2 in the neural retina of mouse and zebrafish. Synapse 2009;63(4):291–307.

Richetti SK, Rosemberg DB, Ventura-Lima J, Monserrat JM, Bogo MR, Bonan CD. Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure. Neurotoxicology 2011;32(1):116–22.

Rico EP, Senger MR, Fauth M da G, Dias RD, Bogo MR, Bonan CD. ATP and ADP hydrolysis in brain membranes of zebrafish (Danio rerio). Life Sci 2003;73(16):2071–82.

Rico EP, Rosemberg DB, Senger MR, Arizi M de B, Bernardi GF, Dias RD, et al. Methanol alters ecto-nucleotidases and acetylcholinesterase in zebrafish brain. Neurotoxicol Teratol 2006;28(4):489–96.

Rico EP, Rosemberg DB, Dias RD, Bogo MR, Bonan CD. Ethanol alters acetylcholines-terase activity and gene expression in zebrafish brain. Toxicol Lett 2007;174(1–3):25–30.

Rico EP, Rosemberg DB, Senger MR, de Bem Arizi M, Dias RD, Souto AA, et al. Ethanol and acetaldehyde alter NTPDase and 5′-nucleotidase fromzebrafish brain mem-branes. Neurochem Int 2008;52(1–2):290–6.

Rico EP, de Oliveira DL, Rosemberg DB, Mussulini BH, Bonan CD, Dias RD, et al. Expression and functional analysis of Na(+)-dependent glutamate transporters from zebrafish brain. Brain Res Bull 2010;81(4–5):517–23.

Robson S, Sévigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal 2006;2:409–30.

Rodd-Henricks ZA, McKinzie DL, Edmundson VE, Dagon CL, Murphy JM, McBride WJ, et al. Effects of 5-HT(3) receptor antagonists on daily alcohol intake under acquisition, maintenance, and relapse conditions in alcohol-preferring (P) rats. Alcohol 2000;21(1):73–85.

Rodríguez F, López JC, Vargas JP, Broglio C, Gómez Y, Salas C. Spatial memory and hippocampal pallium through vertebrate evolution: insights from reptiles and teleost fish. Brain Res Bull 2002;57(3–4):499–503.

Rosemberg DB, Rico EP, Guidoti MR, Dias RD, Souza DO, Bonan CD, et al. Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (*Danio rerio*). Life Sci 2007a;81(21–22):1526–34.

Rosemberg DB, Rico EP, Senger MR, Arizi M de B, Dias RD, Bogo MR, et al. Acute and subchronic copper treatments alter extracellular nucleotide hydrolysis in zebrafish brain membranes. Toxicology 2007b;236(1–2):132–9.

Rosemberg DB, Rico EP, Senger MR, Dias RD, Bogo MR, Bonan CD, et al. Kinetic characterization of adenosine deaminase activity in zebrafish (*Danio rerio*) brain. Comp Biochem Physiol B Biochem Mol Biol 2008;151(1):96–101.

Rosemberg DB, da Rocha RF, Rico EP, Zanotto-Filho A, Dias RD, Bogo MR, et al. Taurine prevents enhancement of acetylcholinesterase activity induced by acute ethanol

exposure and decreases the level of markers of oxidative stress in zebrafish brain. Neuroscience 2010a;171(3):683–92.

Rosemberg DB, Rico EP, Langoni AS, Spinelli JT, Pereira TC, Dias RD, et al. NTPDase family in zebrafish: nucleotide hydrolysis, molecular identification and gene expression profiles in brain, liver and heart. Comp Biochem Physiol B Biochem Mol Biol 2010b;155(3):230–40.

Rosemberg DB, Rico EP, Mussulini BH, Piato AL, Calcagnotto ME, Bonan CD, et al. Differences in the spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments. PLoS One 2011;6(5):e19397.

Saint-Amant L, Drapeau P. Motoneuron activity patterns related to the earliest behavior of the zebrafish embryo. J Neurosci 2000;20:3964–72.

Sallinen V, Sundvik M, Renila I, Peitsaro N, Khrustalyov D, Anichtchik O, et al. Hyperserotonergic phenotype after monoamine oxidase inhibition in larval zebrafish. J Neurochem 2009;109(2):403–15.

Schetinger MR, Morsch VM, Bonan CD, Wyse AT. NTPDase and 5′-nucleotidase activities in physiological and disease conditions: new perspectives for human health. Biofactors 2007;31(2):77–98.

Schultz W. Getting formal with dopamine and reward. Neuron 2002;36:241–63.

Schwartz JC, Arrang JM, Garbarg M, Pollard H, Ruat M. Histaminergic transmission in mammalian brain. Physiol Rev 1991;71:1–51.

Segovia G, Porras A, Del Arco A, Mora F. Glutamatergic neurotransmission in aging: a critical perspective. Mech Ageing Dev 2001;122(1):1–29.

Seibt KJ, Oliveira R da L, Rico EP, Dias RD, Bogo MR, Bonan CD. Typical and atypical antipsychotics alter acetylcholinesterase activity and AChE expression in zebrafish (Danio rerio) brain. Comp Biochem Physiol C Toxicol Pharmacol 2009;150(1):10–5.

Senger MR, Rico EP, Dias RD, Bogo MR, Bonan CD. Ecto-5′-nucleotidase activity in brain membranes of zebrafish (Danio rerio). Comp Biochem Physiol B Biochem Mol Biol 2004;139(2):203–7.

Senger MR, Rico EP, de Bem Arizi M, Rosemberg DB, Dias RD, Bogo MR, et al. Carbofuran and malathion inhibit nucleotide hydrolysis in zebrafish (Danio rerio) brain membranes. Toxicology 2005;212(2–3):107–15.

Senger MR, Rico EP, de Bem Arizi M, Frazzon AP, Dias RD, Bogo MR, et al. Exposure to Hg2+ and Pb2+ changes NTPDase and ecto-5′-nucleotidase activities in central nervous system of zebrafish (Danio rerio). Toxicology 2006a;226(2–3):229–37.

Senger MR, Rosemberg DB, Rico EP, de Bem Arizi M, Dias RD, Bogo MR, et al. In vitro effect of zinc and cadmium on acetylcholinesterase and ectonucleotidase activities in zebrafish (Danio rerio) brain. Toxicol In Vitro 2006b;20(6):954–8.

Senger MR, Rosemberg DB, Seibt KJ, Dias RD, Bogo MR, Bonan CD. Influence of mercury chloride on adenosine deaminase activity and gene expression in zebrafish (Danio rerio) brain. Neurotoxicology 2010;31(3):291–6.

Siebel AM, Rico EP, Capiotti KM, Piato AL, Cusinato CT, Franco TM, et al. In vitro effects of antiepileptic drugs on acetylcholinesterase and ectonucleotidase activities in zebrafish (Danio rerio) brain. Toxicol In Vitro 2010;24(4):1279–84.

Smear MC, Tao HW, Staub W, Orger MB, Gosse NJ, Liu Y, et al. Vesicular glutamate transport at a central synapse limits the acuity of visual perception in zebrafish. Neuron 2007;53(1):65–77.

Steele SL, Lo KH, Li VW, Cheng SH, Ekker M, Perry SF. Loss of M2 muscarinic receptor function inhibits development of hypoxic bradycardia and alters cardiac beta-adrenergic sensitivity in larval zebrafish (Danio rerio). Am J Physiol Regul Integr Comp Physiol 2009;297(2):R412–20.

Sukardi H, Ung CY, Gong Z, Lam SH. Incorporating zebrafish omics into chemical biology and toxicology. Zebrafish 2010;7(1):41–52.

Surmeier DJ, Ding J, Day M, Wang Z, Shen W. D1 and D2 dopamine-receptor modulation of striatal glutamatergic signaling in striatal medium spiny neurons. Trends Neurosci 2007;30(5):228–35.

Tabor R, Friedrich RW. Pharmacological analysis of ionotropic glutamate receptor function in neuronal circuits of the zebrafish olfactory bulb. PLoS One 2008;3(1):e1416.

Thielen RJ, Engleman EA, Rodd ZA, Murphy JM, Lumeng L, Li TK, et al. Ethanol drinking and deprivation alter dopaminergic and serotonergic function in the nucleus accumbens of alcohol-preferring rats. J Pharmacol Exp Ther 2004;309(1):216–25.

Todd KJ, Slatter CA, Ali DW. Activation of ionotropic glutamate receptors on peripheral axons of primary motoneurons mediates transmitter release at the zebrafish NMJ. J Neurophysiol 2004;91(2):828–40.

Van Dycke A, Raedt R, Dauwe I, Sante T, Wyckhuys T, Meurs A, et al. Continuous local intrahippocampal delivery of adenosine reduces seizure frequency in rats with spontaneous seizures. Epilepsia 2010;51(9):1721–8.

Vascotto SG, Beckham Y, Kelly GM. The zebrafish's swim to fame as an experimental model in biology. Biochem Cell Biol 1997;75:479–85.

Vizi ES, Lendvai B. Modulatory role of presynaptic nicotinic receptors in synaptic and non-synaptic chemical communication in the central nervous system. Brain Res Brain Res Rev 1999;30(3):219–35.

Williams FE, Messer Jr WS. Muscarinic acetylcholine receptors in the brain of the zebrafish (Danio rerio) measured by radioligand binding techniques. Comp Biochem Physiol C Toxicol Pharmacol 2004;137(4):349–53.

Yamatodani A, Inagaki N, Panula P, Itowi N, Watanabe T, Wada H. Structure and functions of the histaminergic neuron system. Histamine and histamine agonists. Heidelberg: Springer; 1991. p. 243–83.

Yegutkin GG. Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. Biochim Biophys Acta 2008;1783(5):673–94.

Zimmermann H. Ectonucleotidases: some developments and a note on nomenclature. Drug Dev Res 2001;52:44–56.

A.2. Lista de artigos científicos publicados em periódicos internacionais indexados durante a realização desta tese

1. Kist, L.W., **Rosemberg, D.B.**, Pereira, T.C., de Azevedo, M.B., Richetti, S.K., de Castro Leão, J., Yunes, J.S., Bonan, C.D., Bogo, M.R. 2011. Microcystin-LR acute exposure increases AChE activity via transcriptional ache activation in zebrafish (*Danio rerio*) brain. Comp Biochem Physiol C Toxicol Pharmacol. doi: 10.1016/j.cbpc.2011.09.002.

2. Rico, E.P., **Rosemberg, D.B.**, Seibt, K.J., Capiotti, K.M., Da Silva, R.S., Bonan, C.D. 2011. Zebrafish neurotransmitter systems as potential pharmacological and toxicological targets. Neurotoxicol Teratol. doi: 10.1016/j.ntt.2011.07.007.

3. Schirmer, H., Pereira, T.C., Rico, E.P., **Rosemberg, D.B.**, Bonan, C.D., Bogo, M.R., Souto, A.A. 2011. Modulatory effect of resveratrol on SIRT1, SIRT3, SIRT4, PGC1α and NAMPT gene expression profiles in wild-type adult zebrafish liver. Mol Biol Rep. doi: 10.1007/s11033-011-1096-4.

4. Rico, E.P., **Rosemberg, D.B.**, Langoni, A.D., Souto, A.A., Dias, R.D., Bogo, M.R., Bonan, C.D., Souza, D.O. 2011. Chronic ethanol treatment alters purine nucleotide hydrolysis and nucleotidase gene expression pattern in zebrafish brain. Neurotoxicology. doi: 10.1016/j.neuro.2011.05.010.

5. Piato, A.L., **Rosemberg, D.B.**, Capiotti, K.M., Siebel, A.M., Herrmann, A.P., Ghisleni, G., Vianna, M.R., Bogo, M.R., Lara, D.R., Bonan, C.D. 2011. Acute restraint stress in zebrafish: behavioral parameters and purinergic signaling. Neurochem Res. 36(10): 1876-86.

6. Weizenmann, M., Frasson, A.P., de Barros, M.P., Vieira, Pde B., **Rosemberg, D.B.**, De Carli, G.A., Bogo, M.R., Bonan, C.D., Tasca, T. 2011. Kinetic characterization and gene expression of adenosine deaminase in intact trophozoites of Trichomonas vaginalis. FEMS Microbiol Lett. 319(2): 115-24.

7. Pereira, T.C., Rico, E.P., **Rosemberg, D.B.**, Schirmer, H., Dias, R.D., Souto, A.A., Bonan, C.D., Bogo, M.R. 2011. Zebrafish as a model organism to evaluate drugs potentially able to modulate sirtuin expression. Zebrafish. 8(1): 9-16.

8. Nery, L.R., Rodrigues, M.M., **Rosemberg, D.B.**, Bogo, M.R., De Farias, C.B., Abujamra, A.L., Schwartsmann, G., Roesler, R., Vianna, M.R. 2011. Regulation of E-cadherin expression by growth factor receptors in cancer cells. J Surg Oncol. 104(2): 220-1.

9. Giordani, R.B., Vieira, Pde B., Weizenmann, M., **Rosemberg, D.B.**, Souza, A.P., Bonorino, C., De Carli, G.A., Bogo, M.R., Zuanazzi, J.A., Tasca, T. 2011. Lycorine induces cell death in the amitochondriate parasite, *Trichomonas vaginalis*, via an alternative non-apoptotic death pathway. Phytochemistry. 72(7): 645-50.

10. Sant'Anna, M.C., Soares, V. de M., Seibt, K.J., Ghisleni, G., Rico, E.P., **Rosemberg, D.B.**, de Oliveira, J.R., Schröder, N., Bonan, C.D., Bogo, M.R. 2011. Iron exposure modifies acetylcholinesterase activity in zebrafish (*Danio rerio*) tissues: distinct susceptibility of tissues to iron overload. Fish Physiol Biochem. 37(3): 573-81.

11. Richetti, S.K., **Rosemberg, D.B.**, Ventura-Lima, J., Monserrat, J.M., Bogo, M.R., Bonan, C.D. 2011. Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure. Neurotoxicology. 32(1): 116-22.

12. Brunetto, de Farias C., **Rosemberg, D.B.**, Heinen, T.E., Koehler-Santos, P., Abujamra, A.L., Kapczinski, F., Brunetto, A.L., Ashton-Prolla, P., Meurer, L., Reis Bogo, M., Damin, D.C., Schwartsmann, G., Roesler, R. 2010. BDNF/TrkB content and interaction with gastrin-releasing peptide receptor blockade in colorectal cancer. Oncology. 79(5-6): 430-9.

13. Giordani, R.B., Vieira, P de B., Weizenmann, M., **Rosemberg, D.B.**, Souza, A.P., Bonorino, C., De Carli, G.A., Bogo, M.R., Zuanazzi, J.A., Tasca, T. 2010. Candimine-induced cell death of the amitochondriate parasite *Trichomonas vaginalis*. J Nat Prod. 73(12): 2019-23.

14. Senger, M.R., **Rosemberg, D.B.**, Seibt, K.J., Dias, R.D., Bogo, M.R., Bonan, C.D. 2010. Influence of mercury chloride on adenosine deaminase activity and gene expression in zebrafish (*Danio rerio*) brain. Neurotoxicology. 31(3): 291-6.

15. Giordani, R.B., Weizenmann, M., **Rosemberg, D.B.**, De Carli, G.A., Bogo, M.R., Zuanazzi, J.A., Tasca, T. 2010. *Trichomonas vaginalis* nucleoside triphosphate diphosphohydrolase and ecto-5'-nucleotidase activities are inhibited by lycorine and candimine. Parasitol Int. 59(2): 226-31.

16. Rückert, C., Stuepp, C dos S., Gottardi, B., Rosa, J., Cisilotto, J., Borges, F.P., **Rosemberg, D.B.**, Bogo, M.R., Tasca, T., De Carli, G.A., Bonan, C.D. 2010. *Trichomonas vaginalis*: dehydroepiandrosterone sulfate and 17beta-estradiol alter NTPDase activity and gene expression. Exp Parasitol. 125(3): 187-95.

17. Rico, E.P., de Oliveira, D.L., **Rosemberg, D.B.**, Mussulini, B.H., Bonan, C.D., Dias, R.D., Wofchuk, S., Souza, D.O., Bogo, M.R. 2010. Expression and functional analysis of Na(+)-dependent glutamate transporters from zebrafish brain. Brain Res Bull. 81(4-5): 517-23.

18. Rico, E.P., **Rosemberg, D.B.**, Senger, M.R., de Bem Arizi, M., Dias, R.D., Souto, A.A., Bogo, M.R., Bonan, C.D. 2008. Ethanol and acetaldehyde alter NTPDase and 5'-nucleotidase from zebrafish brain membranes. Neurochem Int. 52(1-2): 290-6.